Louisiana State University
LSU Digital Commons

LSU Master's Theses | Graduate School

2015

Impact of Exposure to Weathered Crude Oil and Accumulation of PAHs in Crawfish (Procambarus clarkii)

Brittany Lynn Chichester
Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses

 Part of the Environmental Sciences Commons

Recommended Citation

Chichester, Brittany Lynn, "Impact of Exposure to Weathered Crude Oil and Accumulation of PAHs in Crawfish (Procambarus clarkii)" (2015). *LSU Master's Theses*. 2226.
https://digitalcommons.lsu.edu/gradschool_theses/2226

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

IMPACT OF EXPOSURE TO WEATHERED CRUDE OIL AND ACCUMULATION OF PAHS IN CRAWFISH (*PROCAMBARUS CLARKII*)

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Environmental Sciences

by
Brittany L. Chichester
B.S., Southeastern Louisiana University, 2009
May 2016

ACKNOWLEDGEMENTS

I would like to thank the many people that have supported and encouraged me of the course of my studies. Thank you to my major advisor Dr. Vince Wilson, and my committee members Dr. Ralph Portier, Dr. M. Scott Miles, Dr. Steven Hall, and Dr. Robert Romaire. I would also like to thank the members of the RCAT lab; Buffy Meyer, Dr. Greg Olson, Megan Calvit and all of the student workers. Thanks for allowing me to the lab space and always helping me along the way.

I would like to thank my fellow graduate students Erin Saal, Matthew Vidrine, and Brendan Marsh for not only helping me directly with my research but also for their support throughout the entire process. Thank you to Scott Nesbit for allowing me to collect sediment from the Spanish Lake Restoration bank. Also thank you Gina Saizan for introducing me to the project.

Thank you to my family for support, especially Sierra Chichester for assisting with the cleanup process after each trial. Last, but certainly not least thank you to my fiancé Mark Berthelot. Without your help, support, and encouragement throughout this process I could not have accomplished this.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The number of pipelines transporting crude oil and other refined petroleum products across the U.S. has increased 15.4% from 2004 to 2013. In Louisiana, over 3,450 miles of liquid petroleum pipelines crisscross the state. In January 2013, crude oil was accidentally released from an underground pipeline into Bayou Sorrel, Louisiana. This freshwater wetland is located within the Atchafalaya River Basin which is home to large populations of wild crawfish that could be impacted by the crude oil released. This study aims to assess the potential lethal impacts that crude oil spill may have produced in adult crawfish (*P. clarkii*) and determine if the hepatopancreas will accumulate polycyclic aromatic hydrocarbons (PAHs) during exposure. Adult crawfish exposed for 96 hours to sediment contaminated with weathered crude oil concentrations of 30,000 ppm were determined to have a survival rate of no less than 87.7%. A modified QuEChERS extraction, dispersive solid-phase cleanup and a GC-MS system was used to quantitate the concentrations of PAHs in the crawfish hepatopancreas tissue. The PAH concentrations increased with increasing oil treatment level, but were not above a level of concern used by the FDA to assess PAH contamination in shrimp and crabs after the Deepwater Horizon Oil Spill event. Therefore it is unlikely that the crawfish in this study would be deemed unsafe for human consumption.

CHAPTER 1. INTRODUCTION

Louisiana is home to over 39 different species of crawfishes [1] but commercially the most important of those species are the red swamp (*Procambarus clarkii*) and white river (*P. zonangulus*) crawfishes [2]. They are both well adapted to the annual hydrological cycle of Louisiana and thrive in the swamps and rivers [2]. Many organisms, including humans, regularly consume crawfish. In 2014, over 145 million pounds of crawfish were harvested in Louisiana alone [3], consisting mainly of the species *P. clarkii* [2]. Commercial harvests favor the wild red swamp crawfish over the wild white river crawfish, and the red swamp typically dominates the harvest of commercial aquaculture ponds [2, 4], and is the focus of this study.

Bayou Sorrel, Louisiana is located within the Atchafalaya River Basin and was the site of an accidental release of crude oil from an underground pipeline in 2013 [5]. The number of pipelines transporting petroleum products across the United States is increasing [6] and Louisiana is crisscrossed with over 3,450 miles of them [7]. The Atchafalaya River Basin is home to large populations of wild crawfish [2] and therefore this release of crude oil posed a threat to the health of the crawfish as well as organisms that consume them, including humans. A number of studies have shown the accumulation of petroleum related PAHs in various crawfish species from contaminated sites around the globe [8, 9, 10, 11, 12, 13]. Generally, the accumulation rate was higher for low molecular weight polycyclic aromatic hydrocarbons (PAHs) than high molecular weight PAHs [11, 13]. The octanol-water partition coefficient (log K_{ow} value) of the PAH was also found to play a role in the accumulation rates [11, 14].

However, only one study has investigated the lethal effects of procambarid crawfish exposed to crude oil, which calculated the 96 hour lethal of fresh South Louisiana Crude (SLC) oil to be 89 mg/L for juvenile crawfish [15]. A lethal concentration for adult crawfish has not been determined and is the basis for this study. Instead of using fresh oil, the SLC oil used was allowed to weather for six days in an effort to mimic the conditions of the Bayou Sorrel spill and allow the oil to spread evenly over the sediment layer.

Quick, Easy, Cheap, Effective, Rugged, and Safe (QUECHERS) is a method first developed to extract pesticides from fruits and vegetables. It is coupled with a dispersive solid-phase cleanup to remove interferences such as sugars, lipids, proteins, pigments and excess water [16] along with analysis using either GC-MS or LC-MS. This method has since been modified for the extraction of PAHs from crawfish tissue [13] and has been employed in this study to achieve PAH extraction from the hepatopancreas of the red swamp crawfish after exposure to weathered SLC oil for 96 hours.

This study attempts to discern the lethal concentration of weathered SLC oil exposure to adult red swamp crawfish. Additionally the concentrations of 31 individual PAHs, ranging from 2-6 total rings have been determined in the hepatopancreas of the exposed crawfish. To assess the risk of concern associated with consuming these tissues, the concentrations are compared to levels of concern established by the FDA for the consumption of shrimp and crab tissues following the DWH oil spill event.

CHAPTER 2. LITERATURE REVIEW

2.1. Crawfish

2.1.1. Production and Consumption

Consumption of crawfish by humans in Louisiana dates back hundreds of years and production has steadily increased over the last 120 years. The first commercial recorded harvest of crawfish occurred in 1880 and consisted of 23,400 pounds worth $2,140 [2]. In 2014, Louisiana wild caught crawfish totaling over 17 million pounds and valued over $14 million were harvested. In the same year, aquaculture harvesters raised crawfish totaling over 127 million pounds and valued over $172 million, as calculated by the Louisiana State University Ag Center [3]. The industry growth has been fueled by the advent of improved refrigeration and transportation, more efficient nets to capture crawfish, and efficient aquaculture farms. Crawfish are produced in states other than Louisiana such as Texas, Mississippi, Alabama, Arkansas and North and South Carolina, but it's estimated that as much as 95% of harvested crawfish in the U.S. is produced in Louisiana [2].

Historically the consumption of crawfish in the U.S. is concentrated around Louisiana due to the shelf life of live crawfish; generally no more than several days [2]. In recent years the introduction of frozen tail meat and boiled whole body crawfish imported from the People's Republic of China increased consumption around the U.S. [2]. Crawfish are also harvested in smaller quantities for fish bait and recreational aquariums [2]. In addition to humans, crawfish are consumed by other mammals such as raccoons, opossums, and otters [2]. Other natural predators of crawfish include wading birds such as herons, egrets, and ibises [17]. Invertebrate predators include other crawfish

and blue crabs, and aquatic insects that feed on recently hatched crawfish [17]. Crawfish are also consumed by predatory fish including sunfishes, catfishes, bowfins and gars, as well as water snakes, eels, alligators, bullfrogs [17].

Crawfish harvested in Louisiana are primarily produced in aquaculture ponds; over the last 10 years aquaculture ponds have contributed an average of 90% of the total production of crawfish in the state (Table 1) compared to wild caught crawfish [18]. Wild caught crawfish are commercially harvested primarily from the Atchafalaya River Basin, [2] but are also caught in limited quantities in other areas of Louisiana [18]. Commercial aquaculture crawfish ponds were found in 30 different parishes in Louisiana in 2014 [18] and are typically constructed in areas with soils containing greater than 27 percent clay, and access to water [2]. Additional requirements include that locations are easily drained, and are void of contamination and flooding from outside of the pond during the summer months [2].

Table 1. Total annual crawfish production in Louisiana [18]

Crawfish Production in Louisiana						
	Wild		Aquaculture			
	Pounds	Value	Acres	Pounds	Value	% of Total
2014	17,089,396	$14,292,025	225,789	127,459,700	$172,070,595	88%
2013	6,474,131	$7,824,361	182,387	101,831,982	$137,473,176	94%
2012	8,778,399	$9,312,723	182,167	90,973,725	$152,835,858	91%
2011	14,461,753	$13,521,303	189,860	111,912,571	$195,846,999	89%
2010	16,570,465	$13,329,897	184,315	110,879,343	$168,535,866	87%
2009	15,428,094	$9,320,686	173,078	98,088,549	$115,746,943	86%
2008	14,899,387	$8,467,939	184,101	111,879,010	$121,276,847	88%
2007	1,350,792	$1,182,628	168,012	109,165,127	$84,602,973	99%
2006	14,451,173	$7,930,041	129,832	79,727,062	$95,672,474	85%
2005	8,199,331	$4,595,478	116,734	73,846,792	$40,615,736	90%
10 Year Average	11,770,292	$8,977,708	173,628	101,576,386	$128,467,747	90%

Successful aquaculture crawfish ponds mimic natural habitats of crawfish; swamps and rivers, with seasonal flooding during the late fall, winter, and spring and drying during the summer and early autumn [2]. During periods of inundation crawfish are able to feed, reproduce and grow. Dry periods during warmer temperatures reduce aquatic predators, increase aeration in sediments and promote growth of vegetation that not only protects crawfish from additional predators, but also serves as a source of food [2]. Red swamp crawfish can tolerate low oxygen levels, but dissolved oxygen levels should be maintained above 2 mg/L. Degradation of vegetation, increased water temperatures and biological activity reduce dissolved oxygen levels and will periodically necessitate water changes in aquaculture ponds [2]. Other water quality variables that can impact crawfish include pH, hardness, alkalinity, iron, hydrogen sulfide content, ammonia, nitrite and salinity. Levels should be maintained between 6.5-8.5 for pH, greater than 50 mg/L for hardness as $CaCO_3$ and alkalinity as $CaCO_3$, less than 0.1 mg/L for iron, less than 0.002 mg/L for hydrogen sulfide, less than 0.06 mg/L for ammonia, less than 0.6 mg/L for nitrite and less than 6 ppt for salinity [4]. Unlike other forms of aquaculture, crawfish are not stocked with hatchery-reared young, instead natural reproduction of crawfish not harvested in previous seasons or mature adults are used to stock ponds [2].

2.1.2. Crawfish Biology and Ecology

Louisiana is home to over 39 different species of freshwater crawfishes [1], but the red swamp crawfish (*Procambarus clarkii*) and the white river crawfish (*P. zonangulus*) make up nearly the entire annual commercial harvest [2]. The red swamp crawfish is native to the Southern Mississippi Valley and Northern Mexico [17]. It has

been introduced in other areas of North America, Europe, Africa, Asia and South America and commercial harvests have been recorded from Europe, Asia and Africa [17].

The red swamp and white river crawfishes are well adapted to the annual hydrological cycle of Louisiana's swamps and rivers, comprised of overflow in the winter and spring months followed by dry conditions in the summer and autumn, and often both species are found thriving in the same habitat [4]. Both species are similar in appearance and construct shallow burrows for reproduction and to escape dry periods [2]. Although, the white river crawfish only reproduces seasonally in fall and winter and the red swamp crawfish is able to reproduce whenever environmental conditions are favorable [4]. Despite their ecological similarities the red swamp crawfish dominates the harvest (70 – 80%) from commercial ponds which may be in-part due to the red swamp crawfish's ability to reproduce throughout the year, and is more desirable for sale at market [2, 4]. An effective way to distinguish between the two species is to check for the presence of a blue-gray pigmented line on the underside of the tail, which is only found in red swamp crawfish [2, 4]. Additional differences in appearance between the two species include adult color of the exoskeleton; the mature red swamp crawfish is generally a bright red color while the mature white river crawfish appears white or tan [4] and red swamp crawfish primary claws, also known as chelae are typically shorter and wider than those of the white river crawfish [4].

In Louisiana the life cycle of the red swamp crawfish is dependent on the annual hydrological cycle; in the spring mating occurs in the open waters, as the water levels decrease and the temperatures increase females retreat to burrows to continue the reproduction process. All crawfish, including males and juveniles eventually retreat to

burrows in the summer and autumn months to escape dewatering. During the fall and winter when water levels increase hatchlings are released from the tails of females and all crawfish begin to emerge from burrows, juveniles mature during the winter and spring while water levels are high [2]. For both the red swamp and white river crawfish the autumn months produce the most new juveniles to the population [2].

Crawfish must shed their exoskeleton to grow and are considered mature and able to reproduce after 11 complete molts, including two that occur while the hatchlings are still attached to the female's tail [17]. Given ideal environmental conditions this process can occur in as little as two months [17] and mature adults will appear in the late spring [2]. Once maturity is reached crawfish will no longer molt, their exoskeleton will appear darker in color, they will have larger claws, and hardened sexual structures [2]. The molting process can be divided into the premolt phase, molt phase, postmolt phase and intermolt phase [17]. Premolt phase involves development of the new exoskeleton along with reabsorption of minerals from the old exoskeleton. At this time, the old exoskeleton will become brittle allowing it to easily crack [17, 4]. The premolt phase is stressful for crawfish and immediately prior to molting the old exoskeleton crawfish cease feeding and seek shelter to avoid predators including other crawfish [2, 4]. Shedding of the old exoskeleton is rapid and can occur within an hour [17]. The postmolt phase begins with the hardening of the new soft exoskeleton by absorption of calcium from the surrounding water and storage in body tissues [4]. Crawfish in the intermolt phase are between molts and have a fully formed exoskeleton and are feeding regularly to increase tissue and energy storage [4]. The lifespan of both species is considered to be two years in the deep South, but can be longer at more northerly latitudes [2].

Crawfish are considered polytrophic and have been classified as omnivores, herbivores and detritivores [17, 19]. Historically they were considered to consume mainly vegetation and detritus [17], but recently it has been recognized that they have a dietary requirement of animal protein to sustain their growth which can impact aquatic ecosystems [4, 19]. Crawfish food sources vary with habitat and availability and when sources of small fish and invertebrates declines they will also consume seeds, algae, microorganisms, decomposing plant matter and living vegetation [4]. Consuming detritus when alternative food sources are low provides increased energy for crawfish compared to living plant matter alone due to the presence of small microorganisms that naturally feed on detritus that will also be ingested [4].

2.2 Crude Oil and Polycyclic Aromatic Hydrocarbons

2.2.1 Bayou Sorrel Spill

On January 9th, 2013 the U.S. Coast Guard's Marine Safety Unit in Baton Rouge, LA was notified of a ruptured underground pipeline at the Frog Lake Bayou Sorrel facility, at approximately 30° 11.67' North 91° 27.28' West (Figure 1) owned by ORB Exploration [5]. The original date of the incident is unknown, but estimated to be on January 3rd, 2013. As of January 18th, 2013 it was estimated that 5,000 gallons of crude oil were discharged into the surrounding environment before the leak was secured and of that 1,750 gallons were recovered [20, 21]. Due to the remote location and flooding which brought 4 feet of standing water over the spill site, an in-situ burn was authorized to remove the oil from the environment [21]. The nature of the spill; remote location, release from an underground pipeline and rainfall induced flooding over the site, made it difficult to determine the amount of oil spilled. Responders observed that the rising water

levels continued to bring oil to the surface long after the leak had been contained [21]. The location of the spill is located within the Atchafalaya River Basin, a known natural habitat of the crawfish *P. clarkii* and therefore that species was used a model of an oil spill for this study.

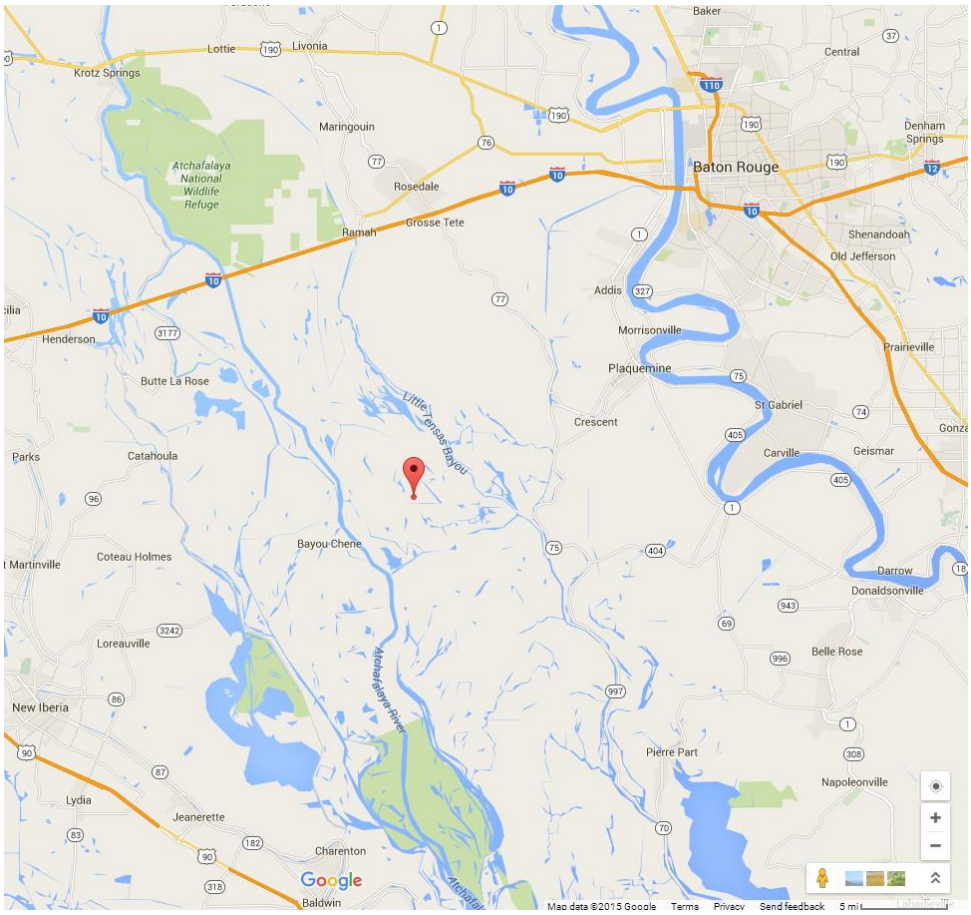


Figure 1 Location of oil spill at Frog Lake Bayou Sorrel facility in Iberville Parish, LA. [22, 23]

2.2.2. Crude Oil and PAHs: Properties and Toxicity

When oil is released into the environment there are many factors that govern the toxicological impact it will have on the plants and animals exposed, including the type of oil, volume spilled, location, environmental conditions and season of the year [24]. Oil is a complex mixture of hundreds of chemicals that varies depending on the geographical

location of origin, therefore producing variable toxicities for each type of oil [25]. Recently over 17,500 different compounds were identified in oil, making assessment of the toxicological impact of an oil spill a very complex environmental issue [24].

In general, crude oil contains 83-87% carbon, 10-14% hydrogen, 0.05-6% sulfur, 0.1-2% nitrogen and 0.05-1.5% oxygen and trace metals such as nickel, vanadium and chromium. The type of oil used in this study is South Louisiana Crude (SLC) oil which is generally described as having a moderate to high viscosity and moderate volatility. Up to one-third of the mass can be lost to evaporation in the first 24 hours of release. The toxicity can vary depending on the amount of the more volatile compounds and it is known to smother organisms when stranded [26].

Hydrocarbons make up the major constituent of oil and are classified by their structure into two main types; aliphatic hydrocarbons which may or may not be saturated with the maximum number of hydrogens and include straight-chain and branched-chain alkanes as well as cycloalkanes, and aromatic hydrocarbons which contain at least one benzene ring [24]. Aromatic hydrocarbons generally make up 0 to 15% of crude oil and the benzene rings that define their structure are made up of a six carbon ring system. Three carbon to carbon double bonds float around the ring and all of the six carbon bonds are equivalent providing stability to the ring. This allows for benzene rings to be highly persistent in the environment [24].The aromatic portion of oil includes the most toxic component known as BTEX, consisting of benzene, toluene, ethyl-benzene and xylenes. These compounds are made of a single benzene ring with differing functional groups [24]. Research has shown that BTEX as well as low molecular weight aromatics composing two to four rings induce a narcotic response in many organisms immediately

after an oil spill event [27]. However, since they are the most volatile components of oil they also rapidly evaporate and therefore are lost to the atmosphere shortly after an oil spill [27].

Polycyclic aromatic hydrocarbons (PAHs) are included in the aromatic portion of oil and are considered important due to their toxicology and persistence in the environment. PAHs are characterized as compounds containing at least two benzene rings. The U.S. Environmental Protection Agency (EPA) lists 16 PAHs as priority chemicals due to their persistence in the environment, toxicity, and potential carcinogenicity to mammals and aquatic organisms [24]. Table 2 contains the list of 16 EPA priority PAHs along with alkylated PAHs that were focused on in this study, as well as the International Agency for Research on Cancer's (IARC) designation for each compound. IARC is an agency composed of expert scientists from various disciplines that review scientific literature on given chemicals and agents to determine if exposure to the agent increases the risk of causing cancer in humans. After conducting the review they categorize the agent based on the potential of that agent to cause cancer in humans (Table 2) [28].

PAHs are an important component of this study due to their toxicity, potential carcinogenicity and persistence in the environment. They can be produced naturally by low-temperature, high-pressure reactions of organic matter (petrogenic). They are also produced by incomplete combustion of wood and petroleum products (pyrogenic). Alkylated PAHs are found in higher concentrations in crude oil than unsubstitued PAHs and make up 80 to 90 % of the total PAHs in crude oil [29]. Alkylated PAHs are used as a method for determining the source of PAHs found in the environment; either pyrogenic

or petrogenic since they can be found in such high concentrations in oil [30]. Generally PAHs are characterized as having high melting and boiling points, low vapor pressure and very low water solubility which decrease with increasing molecular weight [31]. Their solubility and vapor pressure are the most important chemical and physical factors that govern their distribution in the environment. As a rule, PAHs with low molecular weight will have higher solubilities, be more highly volatile, and less lipophilic when compared to higher molecular weight PAHs [31].

Due to their hydrophobicity and solubility the highest concentrations of PAHs can be found in the sediments and on particulate matter in aquatic environments which results in benthic organisms having higher risks of exposure to PAHs [31]. Their hydrophobicity and solubility govern their fate after uptake into organisms; increased hydrophobicity increases the likelihood of association with non-polar phases and therefore the lipid rich tissues of organisms. Invertebrates contain the highest concentration of PAHs in internal organs such as the hepatopancreas [31]. Their carcinogenic potential arises from the metabolic activation into electrophilic metabolites that readily bind with DNA and other macromolecules [31]. Recently, research has indicated that alkylated PAHs can cause more toxicity than the unsubstituted parent PAHs [32, 33, 34].

The metabolic activation of PAHs is achieved by the cytochromes P450 mixed-function oxygenases. These enzymes are found in most tissues of the body, but are in the highest concentration in the liver of mammals and the hepatopancreas of crustaceans [35]. P450 enzymes transform xenobiotics as well as compounds naturally occurring in the body into more electrophilic compounds making them more water-soluble and therefore increasing excretion from the body. The increased water solubility and

Table 2. Target PAHs and International Agency for Research on Cancer Grouping. EPA 16 Priority PAHs are highlighted in green [28] [31]

Target Compound	IARC Group	Rings
Naphthalene	2B	2
Benzothiophene	N/A	2
2-methylnaphthalene	N/A	2
Biphenyl	N/A	2
Acenaphthylene	N/A	3
Acenaphthene	3	3
Dibenzofuran	N/A	3
Fluorene	3	3
Dibenzothiophene	3	3
Phenanthrene	3	3
Anthracene	3	3
Carbozole	2B	3
4-methyldibenzothiophene	N/A	3
2-methylphenanthrene	N/A	3
2-methylanthracene	N/A	3
1-methylphenanthrene	3	3
Fluoranthene	3	5
Pyrene	3	4
Benzo(b)fluorene	3	5
1-methylpyrene	N/A	4
Benz(a)anthracene	2B	4
Chrysene	2B	4
5-methylchrysene	2B	4
Benzo(b)fluoranthene	2B	5
Benzo(k)fluoranthene	2B	5
Benzo(e)pyrene	3	5
Benzo(a)pyrene	1	5
Perylene	3	5
Dibenz(a,h)anthracene	2A	6
Benzo(g,h,i)perylene	3	6
Indeno(1,2,3-cd)pyrene	2B	6
Group 1: Carcinogenic to humans		
Group 2A: Probably Carcinogenic to humans		
Group 2B: Possibly Carcinogenic to humans		
Group 3; Not classifiable as to its carcinogencity to humans		
Group 4: Probably not carcinogenic to humans		

electrophilic nature of the transformed compounds enhances or activates the ability of otherwise unreactive compounds to bind with the nucleophilic components of macromolecules such as DNA and proteins [36]. P450s are known to activate xenobiotics such as the PAH benzo[a]pyrene (B[a]P) into the ultimate carcinogen benzo[a]pyrene-7,8-diol-9,10-epoxide which is known to form DNA adducts [35]. These DNA adducts and in some cases protein adducts can lead to DNA mutations which can lead to carcinogenesis [36]. Jewell et al. (1997) determined that B[a]P metabolism in the hepatopancreas and green gland of *P. clarkii* by low microsomal NADPH-dependent cytochrome P450 reductase activity and was only 2% compared to levels found in rat liver [35].

In addition to the pro-carcinogenic potential of PAHs and narcotic effect of low molecular weight components of oil, exposure to oil can be toxic to organisms in other ways. Oil that is accumulated on the feathers of seabirds or the fur of mammals will reduce the ability of these organisms to insulate and waterproof themselves, leading to extreme hypothermia. Preening of oiled feathers or fur can cause ingestion of oil which can lead to irritation and erosion of the mucosal lining of the mouth, throat, stomach and intestines and potentially gastric bleeding. Dermal exposure will also damage mucosal membranes. Smothering is another common way in which oil exposure can induce lethal effects on organisms and will occur with exposure to large amounts of oil. Inhalation of the highly volatile and toxic aromatic components of oil can cause damage to the respiratory system [24]. Additional toxic effects include impaired reproduction, reduced ability to escape predators and reduction in availability of suitable prey or food sources [24].

Oil toxicity has often had the greatest impacts on eggs, larvae, juveniles and other early-life stages of many organisms, especially fish and invertebrates which impairs reproduction and affects population dynamics of future generations. Blue sac disease which is characterized by edema, hemorrhaging, developmental defects and induction of cytochrome P450 enzymes commonly occurs in salmonid fishes and has been linked to exposure to crude oil and specifically alkylated PAHs [37]. Oil has also been found to have increased toxicity after exposure to U.V. light which is known as photo enhanced toxicity [38].

2.2.3. Crawfish Exposure to Crude Oil and PAHs

Spills in inland freshwater environments are more common than marine environments and usually incur smaller volumes of oil spilled. Generally, inland spills will consist of refined petroleum products while marine spills will consist of crude oil products [26]. Due to the prevalence of oil spills of larger quantities in marine environments research on potential toxicity of crude oil and other petroleum products has been somewhat limited on freshwater aquatic organisms such as crawfishes.

In 1981 research conducted on crawfish *Procambarus clarkii* and *P. acutus* (now *P. zonangulus*) exposed to a 5% water-soluble fraction of No. 2 fuel oil containing radiolabeled naphthalene for 1, 2 or 4 hours showed greater uptake in the cephalothorax compared to the tail flesh or exoskeleton. Placement in oil-free water after exposure showed a reduction of the naphthalene accumulation within the first 24 hours. The loss of naphthalene was reduced with increasing time after exposure [8].

To assess the impacts of naphthalene exposure on the hepatopancreas of the crawfish *P. clarkii* investigators [9] quantitated the mean concentration of live

hepatopancreas cells, the mean percent relative enzyme (dehydrogenase) activity and the mean weight of the hepatopancreas. Crawfish were exposed to 10 ppm of naphthalene for 1, 2, 3, 4, 7, and 15 days and two groups exposed for 15 days were transferred to clean freshwater for depuration of 7 and 15 days. Compared to control groups, the mean concentration of live hepatopancreas cells, the mean percent relative enzyme and the mean weight of the hepatopancreas of the control groups all followed a pattern of decline with increasing time of exposure to naphthalene followed by an increase towards the levels observed in the controls during depuration in clean freshwater. Researchers conclude that the results indicate that naphthalene exposure produces negative effects in the hepatopancreas, but the hepatopancreas has a strong regenerative ability [9].

Bio-monitoring studies were conducted at four sites in river Meuse, located in Western Europe, to evaluate contamination of PAHs, PCBs and heavy metals in crawfish (*Orconectus limosus*) hepatopancreas tissues and corresponding water and sediment samples. No consistent tendency could be determined between concentrations of 16 unsubstituted PAHs in water or sediment samples and PAH induced DNA adducts. The highest levels of DNA adducts were found at the site with the most contamination of PCBs in the water column and third highest concentration of PAHs in sediments. Researchers were unable to differentiate between DNA adducts induced by PAHs compared to PCBs, but did conclude that crawfish are suitable biological indicators of organic and inorganic pollution [10].

Two studies conducted at Five Mile Creek in Birmingham, Alabama, USA and Lake Erie, USA investigated PAH accumulation in crawfish *P. clarkii* and *Orconectes propinquus*, respectively, by using the biota-sediment accumulation factor (BASF) which

is defined as “the ratio of the chemical concentration in the organism on a lipid-normalized basis to the chemical concentration in the sediment on an organic carbon-normalized basis” to compare pollution in sediment to accumulation in crawfish [11, 39]. In Alabama, BASFs for *P. clarkii* were higher in comparison to higher trophic organisms like sunfish and researchers attributed this to reduced metabolism and higher gut assimilation efficiencies in crawfish [39]. Yet in Lake Erie, *O. propinquus* contained the lowest BASFs when compared to the lower trophic level organisms including mayfly larvae, dreissenid mussels and amphipods and again researchers concluded this was a result of varying rates of metabolism among the organisms investigated [11]. It is worth noting that only the 17 EPA priority PAHs were evaluated in the research from Lake Erie while additional PAHs were evaluated in the research from Alabama including alkylated PAHs and non-alkylated substituted PAHs [11, 39].

In 2005 NOAA conducted research on the uptake and elimination rates of radiolabeled PAHs and PCBs by juvenile crawfish *Pacifiastacus leniusculus* that ranged from 1-2 grams in weight. Overall it was determined that crawfish whole body uptake rates were higher for radiolabeled pyrene, chrysene and benzo[a]pyrene compared to hexachlorobiphenyl. Uptake rates for the hepatopancreas decreased with increasing log K_{ow} and were highest among individual tissues investigated which included the abdominal muscle, gills, hepatopancreas, viscera and thorax muscle [14].

The total petroleum hydrocarbon (TPH) concentration was determined for two shellfishes from the Qua Iboe Estuary in Nigeria. The estuary receives petroleum and municipal waste and an oil spill occurred in the estuary during the course of the study. The two species investigated were *Macura reptantia* and *P. clarkii* and TPH

concentrations within their tissues ranged between 3.05 and 11.30 μg/g dry weight and between 1.62 and 9.00 μg/g dry weight, respectively, and they both were correlated to the total petroleum hydrocarbons found in the sediment [12].

In 2010 after an oil spill accident in the Philippines researchers analyzed fish and shellfish for contamination of PAHs including alkylated PAHs. Analysis of the soft tissues of the shellfish species and whole body of the fish species (excluding the head and bones) reported higher concentrations of PAHs in the shellfish compared to the fish, especially the alkylated PAHs. They reported these differences to be attributed to different metabolism and uptake or elimination rates in the shellfish compared to the fish species [40].

A study conducted in the Calumet region of southwestern Lake Michigan compared the accumulation of 17 parent PAHs in six aquatic taxa including crawfish, sunfish, minnows, alewife, yellow perch, and round goby. Researchers concluded that the crawfish *Orconectes* spp. contained higher concentrations of PAHs than all other taxa at three of the four location investigated [41].

Crawfish *Astacus leptodactylus* were caged for 1 week intervals at one reference site in the Mreznica River, Croatia and three sites in the Sava River, Croatia containing various degrees of pollution from municipal and industrial wastewaters and discharge from an oil refinery. Sediment samples were monitored for 16 unsubstituted PAHs, mineral oils and heavy metals. The comet assay and micronucleus test were used to determine the amount of DNA damage in haemocytes. At two sites with the highest levels of PAHs and mineral oils in the sediment crawfish contained the highest levels of DNA damage in haemocytes, and all three of the polluted sites showed increased DNA

damage compared to the reference site. Researchers concluded that the crawfish *A. leptodactylus* is capable of being used for environmental monitoring of pollutants such as PAHs with the use of the comet assay and micronucleus test [42].

The results from the studies listed indicate that multiple crawfish species are capable of accumulating petroleum related polycyclic aromatic hydrocarbons into their tissues. This appears to be dependent on the octanol-water partition coefficient (log K_{ow} value), with rates of accumulation being highest for PAHs with the lowest log K_{ow} values. It has also been shown that given ample about of time removed from oil or PAH exposure crawfish will eliminate PAHs, but this may also be dependent on the log K_{ow} value of a given PAH. A number of studies are presented that have focused on the bioaccumulation of PAHs by crawfish; however, there are no research studies focusing on the lethal impacts that petroleum may have on adult *P. clarkii*. Therefore a need is presented to determine if petroleum products such as crude oil can induce a lethal response in adult crawfish.

The lethal dose or concentration at which 50% mortality of the test organism is observed (LD_{50} or LC_{50}) is a standard toxicological assessment. These types of studies have been well documented for many aquatic organisms, yet only one has been conducted to date on the crawfish *P. clarkii*. The study determined the LC_{50} of the exposure of approximately 3 month old juvenile crawfish *P. clarkii* to water contaminated with mineral oil, peanut oil and South Louisiana Crude oil. The crawfish averaged 3.0 cm total length and averaged 0.82 g (wet weight). From this research it was determined that the LC_{50} of SLC oil to juvenile crawfish was 89 mg/L and is attributed to poisoning from the water soluble fraction of the oil. In comparison, the LC_{50} of peanut oil

and mineral oil was 622 mg/L and 17,000 mg/L, respectively. The mortality induced by the peanut and mineral oils was considered to be caused by poisoning from water soluble aliphatic hydrocarbons as well as smothering due to the presence of oil droplets coating the gills of the crawfish exposed [15]. As previously stated juvenile and other larval stages of organisms are known to be more vulnerable to exposure to oil and therefore these results are not applicable to adult crawfish. An independent study is needed to determine the LC_{50} of SLC oil for adult crawfish.

2.3 Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) Method for Extraction of PAHs from Crawfish Tissues

The method used in this study for the extraction of PAHs from crawfish tissues is the Quick, Easy, Cheap, Effective, Rugged and Safe method known as QuEChERS and it was first developed to determine pesticide residues in produce [16]. The QuEChERS method has since been modified and proven applicable to PAH determination in tissues of various organisms. The original method uses acetonitrile as the solvent and liquid-liquid partitioning by the addition of anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl). Dispersive solid-phase cleanup is performed by the addition of anhydrous $MgSO_4$, primary secondary amine (PSA), and C18, which removes residual water, polar compounds such as organic acids, pigments, sugars, and non-polar fats, respectively. Gas chromatography coupled with mass spectrometry (GC-MS) is used to quantify pesticide residues and recoveries for the original method ranged between 85 and 101% [16].

In response to the *Deepwater Horizon* oil spill and the vast amount of tissue analysis that would be required to monitor levels of PAHs in edible seafood the FDA in

conjunction with other researchers effectively modified the QuEChERS method for quantification of PAH residues in edible seafood. Historically, tissue analysis for PAHs has been a labor intensive, time consuming and expensive process so adaptation of a rapid and cost-effective method was required. The method was successfully adapted for oyster, shrimp, crab and finfish for parent and substituted PAHs. The modified method provided spiked recoveries ranging from 78 to 128% with method detection limits in the low ppb using high-performance liquid chromatography with fluorescence detection [43].

Forsberg et al. (2011) used a modified QuEChERS extraction method to quantify PAHs in high-fat smoked salmon by using a mixture of ethyl acetate, acetone and iso-octane (2:2:1, v/v/v) in place of the original acetonitrile solvent. Using GC-MS, this modified method enhanced recoveries of 2, 3, and 5 ring PAHs 50 – 200% and overall PAHs 67% (including parent and substituted PAHs) compared to the original QuEChERS method while also delivering method detection limits in the low ppb. This provided a method for extraction of PAHs from seafood tissues with a fat content up to 11% while previous QuEChERS methods were not validated for tissues containing greater than a 3.5% fat content [44].

Forsberg et al. (2014) also used a modified QuEChERS method for extraction of parent and substituted PAHs from crawfish (*Pacifastacus leniusculus*) visceral tissues [13]. Again the mixture of ethyl acetate, acetone, and iso-octane was used in place of the traditional acetonitrile solvent. After solvent exchange to n-hexane crawfish samples were cleaned up using dispersive solid-phase extraction with primary-secondary amines, followed by vacuum elution with n-hexane and concentration before quantification using GC-MS [13]. The study collected crawfish from within and around the Portland Harbor

Superfund Mega-site in Portland, OR and compared concentrations of PAHs in their visceral tissues to concentrations of PAHs accumulated in deployed semipermeable membrane passive sampling devices (SMPDs). The median total PAH concentration in crawfish from the superfund site was 214 ng/g (wet weight) and it was significantly higher than concentrations outside of the superfund site. Additionally, crawfish tissues contained higher concentrations of high molecular weight PAHs compared to the SMPDs [13].The validation of a modified QuEChERS extraction method for PAHs in crawfish species as well as high-fat content seafood tissues provides the framework for the method used in this study.

CHAPTER 3. METHODS AND MATERIALS

3.1 Laboratory Exposure of Adult Crawfish (*Procambarus clarkii*) to Sediment Contaminated with Weathered Crude Oil

3.1.1. Objective

The objective of this study was to determine the concentration of weathered South Louisiana Crude (SLC) oil that causes acute toxicity (50% mortality) of adult crawfish (*Procambarus clarkii*) over 96 hours of exposure.

3.1.2. Chemicals and Materials

Reagent grade hexane (HEX), methanol (MeOH), and dichloromethane (DCM) were purchased from Fisher Scientific (Pittsburg, PA) or VWR International (Radnor, PA). Water quality reagents for pH, dissolved oxygen, and ammonia for the Saltwater Aquaculture Test Kit Model FF-3 were purchased from Hach (Loveland, CO). OMEGA ONE Veggie Micro Pellets and OMEGA ONE Veggie Rounds were purchased from PETCO (Baton Rouge, LA). All glassware and equipment was cleaned thoroughly with Alconox laboratory detergent (White Plains, NY) and whenever applicable solvent rinsed with methanol and dichloromethane.

South Louisiana Crude (SLC) surrogate oil distributed by BP as a surrogate research oil after the DWH oil spill was used for exposure trials. The surrogate oil originated from the Marlin Platform of the Dorado field located 36 miles northeast of the Macondo spill site and has similar hydrocarbon composition and toxicological properties as the Mississippi Canyon lease block 252 oil [45, 46].

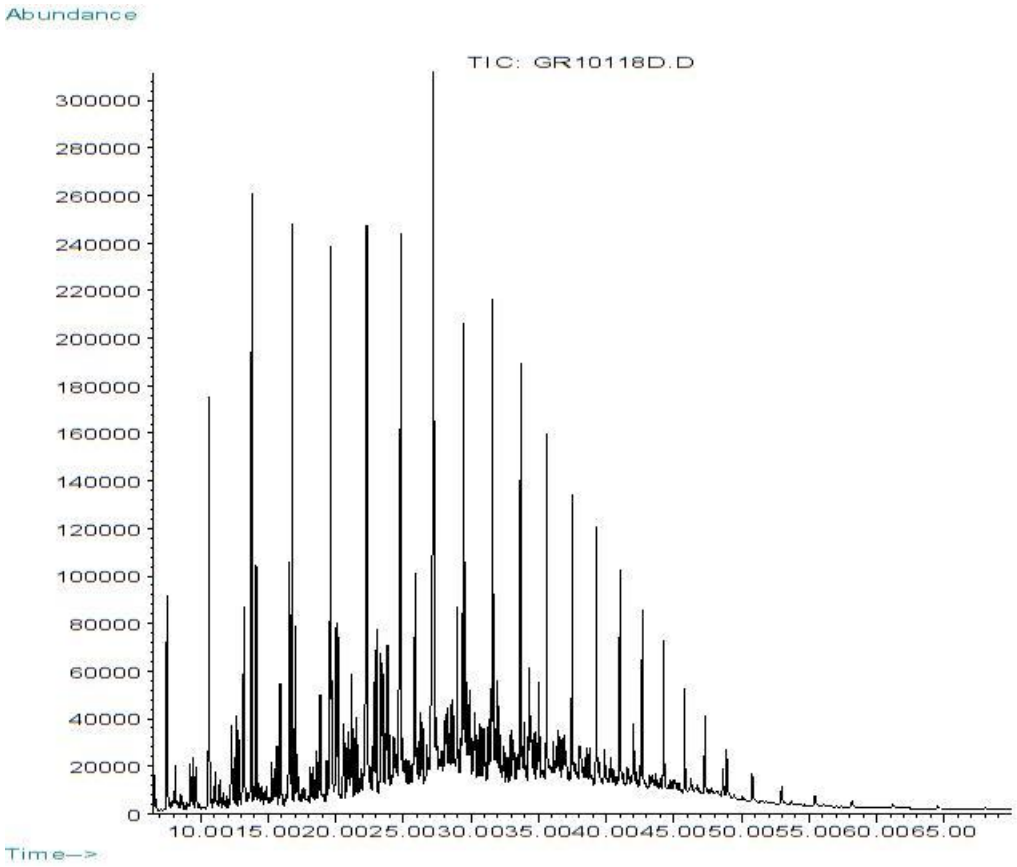


Figure 2 Chromatogram of a reference SLC oil similar to the oil used in this study, provided by LSU Response and Chemical Assessment Team (RCAT) Lab April 2010.

3.1.3. Crawfish

Adult crawfish (*Procambarus clarkii*) for definitive toxicity trials were obtained from the Louisiana State University Aquaculture Research Station (Baton Rouge, LA) between January 2015 and April 2015. Range finding studies were also conducted between June 2014 and August 2014 and crawfish for these experiments were obtained from Tony's Seafood (Baton Rouge, LA) and Louisiana Crawfish Company (Natchitoches, LA). Crawfish obtained for the range finding experiments offered little information about handling, holding environment, and crop origin (natural environment vs aquaculture raised) providing a large number of uncontrollable variables. Crawfish

obtained from the LSU Aquaculture Research Station were raised in earthen aquaculture ponds, held in large aerated tanks with substrates mimicking their natural habitat and fed regularly.

3.1.4. Acclimation Period

Upon arrival to the Energy, Coast and Environment building all specimens were weighed and placed into one of fourteen acclimation tanks. For all exposures in 2015, crawfish were also sexed and whenever possible were placed in tanks in a ratio of 1:1 male to female. Acclimation tanks were filled with approximately 4 L of chlorinated tap water that was aerated for at least 24 hours. An aeration stone was placed in the center of each tank to maximize aeration over the whole tank. Each tank contained four crawfish, segregated by plastic dividers with plastic lids placed on top to reduce fighting amongst specimen and ensure crawfish remained isolated from each other (Figure 3).

Crawfish were maintained in the laboratory for a 7-day acclimation period to ensure the health of each batch. Dead crawfish were removed immediately upon discovery, weighed and placed in a -80°C freezer. Crawfish were held on a 12-hour light cycle and fed OMEGA ONE Veggie Micro Pellets and/or OMEGA ONE Veggie Rounds as necessary; generally every other day during the acclimation period up until 48 hours before exposure. Water quality parameters were measured during the acclimation period by sampling two to four tanks every other day, each tank was sampled at least one time during the acclimation period for all parameters. Temperature (°C) of the tanks and the room, ammonia (mg/L), pH, and dissolved oxygen (mg/L) were monitored and Table 3 gives the range of measurements recorded during all of the holding periods.

Figure 3. Example of crawfish holding tanks with plastic divider and lid

With the exception of Trial 2 all crawfish selected for placement in the exposure tanks were intermolt crawfish. If the mortality percent exceed 10% during the acclimation period the experiment was terminated and a new batch of crawfish was obtained. This occurred once during a range finding test in the end of July 2014, when the availability of crawfish was limited.

Table 3. Range of water quality measurements during holding period

Water Quality Parameter	Range
Temperature (°C)	18 - 21
pH	7.5 - 8.5
Non-ionized Ammonia (mg/L)	0 - 0.31
Dissolved Oxygen (mg/L)	6.36 - 12.8

3.1.5. Preparation of Exposure Tanks

Sediment for exposure tanks was collected from Saint Gabriel, Louisiana in the Spanish Lake Restoration bank off of Shell Road. The location was selected due to the presence of crawfish as well as isolation from human influence. Approximately 4 kg of sediment was weighed into empty 10-gallon glass aquariums using a Mettler PM600 scale. A South Louisiana Crude surrogate oil was added to the exposure tanks at nominal concentrations of 0, 1,000, 7,500, 15,000, 20,000, and 30,000 ppm. To reduce the viscosity of the oil, 100 mL of hexane was added to the oil which enhanced the spread ability of the oil over the sediment surface. After allowing four days for the hexane to evaporate, approximately 3.9 L of aerated dechlorinated tap water was added to each tank and left for two days to acclimate. Preparation of tanks did not commence until after a sufficient group of crawfish was obtained and the 7-day crawfish acclimation period began.

A total of 12 tanks were prepared for each exposure trial; two for each concentration of oil including two controls and each tank contained four crawfish giving 48 crawfish per experiment. Tanks were placed randomly on one of four shelves on a metal shelving unit with three tanks on each rack (Figure 4). Shelves were lined with oil absorbing pads to reduce any possible cross contamination. Exposure tanks contained plastic dividers with plastic tops to isolate crawfish for one another and ensure crawfish remained in one of the four compartments. An aeration stone was placed in the center of the tank to maximize aeration throughout the tank. Temperature of the room and the tanks was recorded daily.

Figure 4. Example of exposure tank setup.

3.1.6. Exposure Period

At the end of the 7-day acclimation period crawfish were randomly placed in the exposure tanks with an equal number of males and females placed in each tank whenever possible. Crawfish were sexed and weighed before placement in exposure tanks and were not fed during exposures. Samples of sediment and water were taken immediately before crawfish were placed in the tanks along with initial water quality measurements. During exposures crawfish were maintained on the same 12 hour light cycle as the acclimation period. Crawfish were monitored 2-3 times per day and dead crawfish were removed immediately upon discovery, sexed and weighed and placed in -80°C freezer. A mid-point water quality sample was taken after approximately 48 hours of exposure and a final water quality sample was taken four hours prior to termination of the exposure (approximately 92 hours). After 96 hours in exposure tanks crawfish were removed, sexed and weighed again and sacrificed by freezing at -80°C.

3.1.7. Water Quality Testing

Water quality measurements were taken during the acclimation period and exposure period, using Hach Saltwater Aquaculture Test Kit, Model FF-3. Descriptions of how samples were analyzed for pH, dissolved oxygen and ammonia can be found in Appendix A. After placement of crawfish in exposure tanks they immediately began to burrow into the available sediment, suspending large amounts of sediment into the water column and preventing measurement of water quality parameters based on colorimetric changes. To obtain water quality measurements during these conditions (mid-point and end point measurements of exposure) water samples were centrifuged at 4,000 rpm for 30

minutes to obtain clear samples. Samples were centrifuged in a Sorvall SA-600 Rotor at a maximum temperature of 18°C in a Sorvall Instruments RC5C centrifuge system.

3.1.8. Statistical Analysis

Results of number of crawfish to survive the trial were analyzed using the R statistical software program. The survival rate was determined using a binomial function and calculating the 95% confidence limit. Only the survival rate for crawfish exposed to 30,000 ppm of weathered crude oil was determined due to the lack of mortality in the trials conducted.

3.2. Determination of Polycyclic Aromatic Hydrocarbons in Crawfish Hepatopancreas Tissue Using Modified QuEChERS Extraction, Dispersive Solid-Phase Cleanup and GC-MS

3.2.1. Objective

The objective of this study was to determine the amount of polycyclic aromatic hydrocarbons (PAHs) in crawfish hepatopancreas tissues after laboratory exposure to weathered South Louisiana Crude oil.

3.2.2. Chemicals and Materials

Pesticide/ Reagent grade acetone (ACE), ethyl acetate (EA), hexane (HEX), isooctane (ISO), ethanol (EtOH), methanol (MeOH), and dichloromethane (DCM) were purchased from Fisher Scientific (Pittsburg, PA) or VWR International (Radnor, Pennsylvania). Commercially available Q-sep QuEChERS AOAC 2007.01 extraction salts (magnesium sulfate and sodium acetate) and AOAC 2007.01 sample dispersive SPE tubes (150 mg of magnesium sulfate, 50 mg of primary secondary amines, and 50 mg of C18) were purchased from Restek Corporation (Bellefonte, PA). Polypropylene conical centrifuge tubes were purchased from Cole-Parmer (Vernon Hills, IL)

3.2.3. Standards

Stock solutions of 33 parent and substituted PAHs (1,000 – 4,000 µg/mL) were prepared by combining a mix of 16 EPA priority pollutant PAHs, two custom PAH mixes, three individual PAH standards, and two deuterated compounds to volume with isooctane (Table 4). An internal standard solution (200 µg/mL) was prepared by combining three deuterated compounds to volume in isooctane. A stock surrogate standard (SS) solution (100 µg/mL) was prepared by combining two isotope-labeled standards to volume in isooctane. An instrument calibration curve (10, 5, 2.5, 0.5 and 0.1 µg/mL) of the parent and substituted PAHs was prepared by dilution of the stock PAH spiking solution. All standards and spiking solutions were stored in a refrigerator at 4°C. All PAH standards were purchased from Absolute Standards, Inc. (Hamden, CT) or Accustandard (New Haven, CT).

3.2.4. Sample Preparation

Polycyclic aromatic hydrocarbons were determined in the hepatopancreas using a modified QuEChERS extraction procedure [44]. Crawfish were obtained from a local supplier LA Boilers (Baton Rouge, LA) for initial method efficacy determination, non-exposed crawfish from Louisiana Aquaculture Research Station (Baton Rouge, LA) were used for final method efficacy and method detection limits. Upon arrival to the Energy, Coast and Environment building crawfish were sexed and weighed before being sacrificed by freezing at -80°C. Whole frozen crawfish were brought to room temperature and dissected to remove the hepatopancreas; samples were not pooled for analysis.

Table 4. List of target PAHs, associated retention times, and total rings

PAH	Chromatogram #	DB5 RT (min)	SIM ions (*m/z*)		Rings
			Quant	Confirm	
Naphthalene-d8	ISTD #1	12.90	136	68	2
Naphthalene	1	12.96	128	127, 129	2
Benzothiophene	2	13.22	134	89	2
2-methylnaphthalene	3	16.19	142	141, 115	2
Biphenyl	4	18.55	154	152	2
Acenaphthylene	5	20.31	152	151, 150	3
Acenaphthene-d10	SSTD #1	21.01	164	162, 160	3
Acenaphthene	6	21.16	154	153, 152	3
Dibenzofuran	7	22.05	168	139	3
Fluorene	8	23.63	166	165, 167	3
Dibenzothiophene	9	27.42	184	139, 185	3
Phenanthrene-d10	ISTD #2	27.91	188	94, 80	3
Phenanthrene	10	28.01	178	176, 179	3
Anthracene	11	28.24	178	176, 179	3
Carbozole	12	29.53	167	139	3
4-methyldibenzothiophene	13	29.57	198	184	3
2-methylphenanthrene	14	30.53	192	191, 165	3
2-methylanthracene	15	30.72	192	191, 165	3
1-methylphenanthrene	16	31.02	192	191, 165	3
Fluoranthene	17	33.65	202	203, 200	4
Pyrene	18	34.62	202	203, 200	4
Benzo[b]fluorene	19	36.69	216	215, 217	4
1-methylpyrene	20	37.35	216	215, 217	4
Benzo[a]anthracene	21	40.37	228	226, 229	4
Chrysene-d12	SSTD #2	40.43	240	236	4
Chrysene	22	40.54	228	226, 229	4
5-methylchrysene	23	42.67	242	241, 226	4
Benzo[b]fluoranthene	24	45.16	252	253, 250	5
Benzo[k]fluoranthene	25	45.25	252	253, 250	5
Benzo[e]pyrene	26	46.20	252	253, 250	5
Benzo[a]pyrene	27	46.40	252	253, 250	5
Perylene-d12	ISTD #3	46.65	264	260, 265	5
Perylene	28	46.74	252	253, 250	5
Indeno(1,2,3-cd)pyrene	29	51.53	276	277, 274	6
Dibenz[a,h]anthracene	30	51.75	278	279, 276	5
Benzo[g,h,i]perylene	31	52.81	276	277, 274	6

3.2.5. PAH Extraction Using QuEChERS and dSPE

Approximately 1 g of crawfish hepatopancreas tissue (wet weight) was added to a clean 15 mL pre-weighed conical centrifuge tube using a Mettler PM600 scale. Tissues were fortified with 10 µL of 100 µg/ mL surrogate standard containing acenaphthene-D10 and chrysene- D12 and allowed to acclimate for 2 minutes. Samples were then spiked with 1 mL of solvent cleaned deionized water and shaken for 1 minute on a Vortex-Genie 2 mixer from Scientific Industries, Inc. (Bohemia, NY). Two milliliters of a solution of acetone, ethyl acetate and isooctane (2:2:1; v/ v/ v) was added to the sample and then shaken on a Vortex-Genie 2 for 5 minutes. Then 1.3 g of traditional QuEChERS AOAC 2007.01 salts; anhydrous magnesium sulfate and anhydrous sodium acetate were added to samples and again shaken on a Vortex-Genie 2 for 5 minutes. Next, samples were centrifuged at 1,750 g for 11 minutes on an IEC Clinical Centrifuge.

Cleanup was performed by dispersive solid-phase extraction; 1 mL aliquot of extract was transferred to Restek Q-sep QuEChERS AOAC 2007.01 dSPE 2 mL centrifuge tube and shaken on a Vortex-Genie 2 for 5 minutes. Samples were then centrifuged at 13,600 g for 5 minutes using an Eppendorf 5145C microcentrifuge (Hauppauge, NY). For analysis, 200 µL aliquots were transferred to 2-mL amber autosampler vials with small volume inserts, spiked with 10 µL of a 200 µg/ mL internal standard solution composed of naphthalene-D8, phenanthrene-D10 and perylene-D12), and stored at 4°C until GC-MS analysis.

3.2.6. GC-MS Analysis

Target PAHs were quantified using an Agilent GC-MS system equipped with a 6890A GC interfaced with a 5973 MS detector (Santa Clara, CA) with a 70-eV electron

impact ionization source. Mass spectral data was acquired utilizing selective ion monitoring (SIM) mode and capillary GC column (30 m length, 0.25 mm i.d., 0.25 µm film thickness), low bleed, fused silica 5% diphenyl/ 95% di methyl polysiloxane (Agilent J&W DB-5MS). An Agilent 7693 Auto Injector introduced the sample into the GC-MS at a maintained temperature of 280°C with a high-temperature, low thermal-bleed septum. Ultra High Purity Helium was used as the carrier gas. The method operates in the temperature program mode with an initial oven temperature of 60°C held for 3 minutes. The first temperature ramp increases the temperature to 280°C at a rate of 5°C per minute and held for 3 minutes. The second temperature ramp then increases the temperature to 300°C at a rate of 1.5°C per minute and held at 300°C for two minutes. The total run time is 65.33 minutes per sample.

3.2.7. Method Detection Limit

The Method Detection Limit (MDL) was determined following 40 CFR 136 Appendix B for target PAHs in crawfish hepatopancreas [47]. Ten replicates of crawfish hepatopancreatic tissues were spiked at 500 ppb with a PAH spiking solution and SS solution. Recovered concentrations of target PAHs were normalized to 1 g of tissue before the average and standard deviation were calculated to account for variability in crawfish hepatopancreas tissue weight. Standard deviations were then multiplied by 2.821 to determine the MDL with 99% confidence [47]. The Limit of Quantitation (LOQ) was determined by multiplying the standard deviation by 10 [47].

Table 5. QuEChERS method detection limit (MDL) and limit of quantitation (LOQ)

PAH	MDL	LOQ	PAH	MDL	LOQ
	µg/g	µg/g		µg/g	µg/g
Naphthalene	0.057	0.200	Fluoranthene	0.055	0.194
Benzothiophene	0.078	0.278	Pyrene	0.058	0.206
2-Methylnaphthalene	0.075	0.267	Benzo[b]fluorene	0.041	0.145
Biphenyl	0.082	0.290	1-Methylpyrene	0.057	0.201
Acenaphthylene	0.075	0.267	Benzo[a]anthracene	0.034	0.121
Acenaphthene-d10 SSTD #1	0.083	0.294	Chrysene-d12 SSTD #2	0.074	0.262
Acenaphthene	0.045	0.161	Chrysene	0.059	0.210
Dibenzofuran	0.056	0.200	5-Methylchrysene	0.086	0.304
Fluorene	0.052	0.186	Benzo[b]fluoranthene	0.087	0.309
Dibenzothiophene	0.055	0.196	Benzo[k]fluoranthene	0.048	0.170
Phenanthrene	0.076	0.271	Benzo[e]pyrene	0.077	0.272
Anthracene	0.064	0.229	Benzo[a]pyrene	0.070	0.249
Carbozole	0.084	0.297	Perylene	0.071	0.253
4-Methyldibenzothiophene	0.066	0.232	Indeno[1,2,3-cd]pyrene	0.076	0.271
2-Methylphenanthrene	0.056	0.199	Dibenz[a,h]anthracene	0.048	0.169
2-Methylanthracene	0.077	0.274	Benzo[g,h,i]perylene	0.040	0.140
1-Methylphenanthrene	0.069	0.244			

3.2.8. Quality Control

All sample batches contained method blanks, spiked matrix blanks, instrument blanks and continuing calibration verification standards. Two method blanks contained minor levels of naphthalene; 0.067 µg/g and 0.064 µg/g, which were not subtracted from sample concentrations. All other targeted PAHs were below method detection limit. Continuing calibration verification standards were within accepted range of values (+/- 20%). Surrogate standard recoveries ranged from 80% to 110 % for acenaphthaene-d10, and ranged from 96% to 116% for chrysene-d12. Recoveries of all targeted PAHs in spiked matrix blanks ranged from 73% to 117%. Instrument blanks did not recover any targeted PAHs above the method detection limit.

3.2.9. Lipid Analysis

Total lipid content was determined using a modified hot soxhlet extraction method [48]. The total lipid content was determined for four crawfish from Trial 1 that were not used in the exposure trial, instead they were immediately sacrificed by freezing at -80°C immediately after arrival to the Energy, Coast, and Environment Building. Hepatoapancreatic tissues were extracted from crawfish, weighed on a Mettler AE200 scale separately and dried in an Isotemp Muffle Furnace at 80°C for 24 hours. Immediately upon removal from the furnace crawfish tissues were weighed again to determine percent moisture and stored in a desiccator until analysis.

A boiling stone was placed in a 250 mL flat bottom flask and the flask was dried in an oven at 105°C for 30 minutes and cooled in a desiccator for 30 minutes before the initial weight was recorded to the nearest thousandth of a gram on a Mettler AE200 scale. Approximately 125 mL of a hexane / ethanol (3:1; v/v) solution was added to the flask. Crawfish tissue was homogenized using a glass mortar and pestle and added to a cellulose thimble containing approximately 1 g of solvent cleaned anhydrous sodium sulfate. Cellulose thimbles were packed with solvent cleaned glass wool and placed in a soxhlet extraction tube. Soxhlet tubes attached to round bottom flasks were placed on heating plates with condensing units attached at the top. Solvent was heated to a boil and the extraction was allowed to run overnight; at least 16 hours. Lipid extract in flat bottom flasks were rotary evaporated at 70°C for 20 minutes or until dry and further dried in a muffle furnace at 100°C for 7 hours and cooled in a desiccator until a constant weight was achieved. All weights were measured on a Mettler AE 200 scale to the nearest thousandth of a gram. All extractions a included deionized water blank.

Figure 5. Example of soxhlet extraction for total lipid content.

3.2.10. Statistical Analysis

Results of PAH concentrations were analyzed using SAS 9.4 (SAS Institute Inc., Carly, North Carolina) software. A one-way ANOVA with a Tukey adjustment was conducted on the total PAH, individual PAH, two and three ring PAH, and four and five ring PAH concentrations extracted from the hepatopancreas tissue of crawfish from trial 1 in oil treatments of 7,500 ppm, 20,000 ppm, 30,000 ppm and the control (0 ppm). The results were used to determine if significant differences were present between oil treatment levels and the selected PAH concentrations. A significant difference was

produced when a p-value of less than 0.05 was generated. The tukey adjustment selected a letter for each oil treatment and treatments with the same letter are not significantly different from each other. This provides a clear analysis of the effect of each oil treatment on the selected concentrations of PAH in crawfish hepatopancreas tissues.

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Lethal Concentration Determination

To determine the sediment concentration of weathered crude oil at which 50% of mortality of the crawfish is observed three separate exposure trials were conducted between January 2015 and May 2015. The results are summarized in Table 6.

Table 6. Summary results of crawfish 96 hour exposure to weathered crude oil.

Exposure Concentration	Trial					
	1		2		3	
	Alive	Dead	Alive	Dead	Alive	Dead
Control	8	0	8*	0	7	1
1,000 ppm	8	0	8*	0	8	0
7,500 ppm	8	0	7*	1	8	0
15,000 ppm	8	0	8*	0	8	0
20,000 ppm	7	1	8*	0	8	0
30,000 ppm	8	0	8*	0	8	0

*Total includes one crawfish entered exposure 48 hours after other crawfish in concentration group.

4.1.1. Lethal Concentration Determination Results of Trial 1

Trial 1 was conducted from January 2015 to February 2015. After 48 hours of exposure one crawfish was found dead in the 20,000 ppm oil treatment level. The crawfish was found dead underneath a portion of the divider and removal from the exposure tank revealed a fracture on the dorsal side of carapace in the cephalothorax region of the organism which can be seen in Figure 6. This exposed the internal organs of the crawfish to surrounding environment. Therefore, the death should be attributed to physical damage of the crawfish caused by the plastic dividers used to separate the crawfish from each other and not due to exposure to weathered SLC oil alone. All

remaining crawfish survived the duration of the exposure trial. At the termination of the exposure period surviving crawfish were sacrificed by freezing at -80°C.

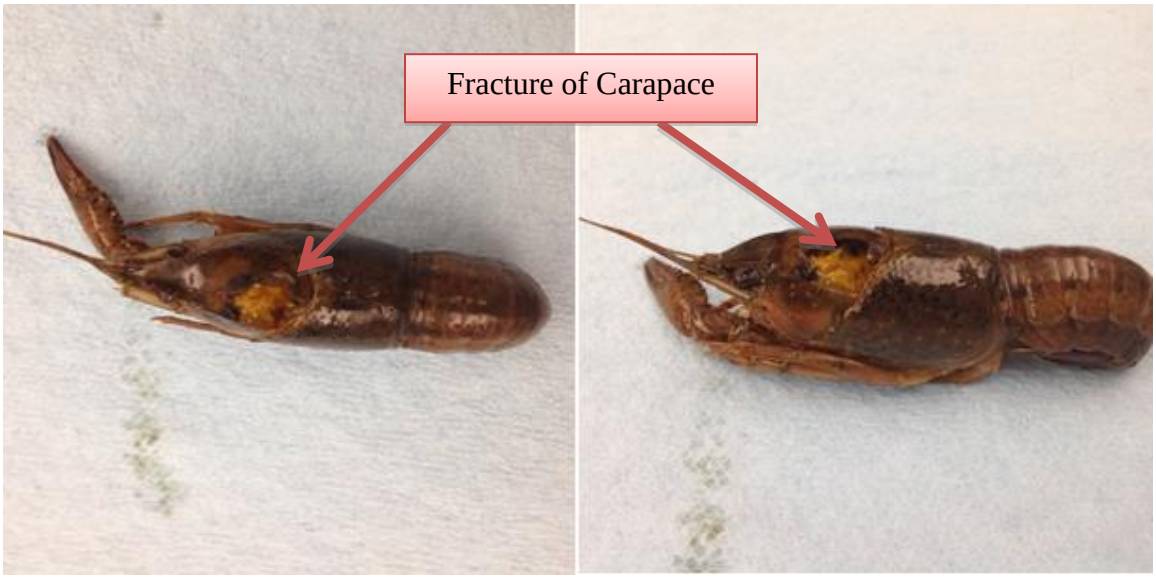


Figure 6. Fracture on the dorsal side of the carapace in the cephalothorax region exposing internal organs including the hepatopancreas.

4.1.2. Lethal Concentration Determination Results of Trial 2

Trial 2 was conducted in March 2015. In all other trials crawfish selected for placement in exposure tanks were intermolt crawfish. This was done for reproducibility and to limit the amount of stress the crawfish were under. In trial 1 and 3, crawfish were excluded from the exposure tanks if they had molted within 72 hours prior to the exposure period. During the 7-day acclimation period a total of 56 crawfish were held to ensure the health of the batch. Of the 56 crawfish, 48 are selected for the exposure tanks which provide a buffer for any potential deaths or molting immediately prior to exposure. However during this trial, 3 crawfish died during the acclimation period and 9 crawfish molted within 72 hours prior to the exposure. This resulted in not having enough healthy intermolt crawfish available for placement in the exposure tanks. To accommodate this issue only seven crawfish for each concentration were placed in exposure tanks. After

two more acclimation days for the crawfish that recently molted six additional crawfish were placed in exposure tanks; one at each concentration level to bring the total number of exposed crawfish to 48. These six crawfish remained in the exposure tanks for an additional 2 days (48 hours) to account for the delay at the beginning of their exposure and ensure they were exposed for the full 96 hours.

The results from trial 2 were similar to trial 1; one crawfish was observed dead in the 7,500 ppm concentration. This crawfish began to molt after 48 hours of exposure and was found with the old exoskeleton attached 64 hours into the exposure period. The crawfish was so lethargic it appeared to be dead after 64 hours, but after removal from the exposure tanks and prodding with a metal spatula movement was observed. After this discovery the crawfish was placed back in the exposure tank and monitored, it remained lethargic, but still alive until the 94^{th} hour of the exposure period. Therefore it died in the last two hours of the 96 hour study. The crawfish never completely removed itself from the old exoskeleton, which can be seen in Figure 7. When a crawfish is in the late premolt stage it experiences high levels of stress [4] which may have increased the vulnerability of this crawfish to the exposure of crude oil.

This trial witnessed several crawfish molt during the exposure period and the acclimation period. This is likely because all of the crawfish came from the same pond and experienced the same environmental conditions; both before and after capture, and are likely close in age. In addition to the crawfish that died while molting, four other crawfish molted while in the exposure tanks during this trial. The first crawfish molt occurred after 40.5 hours of exposure by a crawfish also from the 7,500 ppm oil treatment level. The second crawfish molt came from the 20,000 ppm treatment level and

molted after 49.5 hours of exposure. The last two crawfish began to molt just prior to the termination of the exposure period (96 hours) and both were in the control tanks. No other crawfish were observed to be lethargic after molting. All other crawfish survived the duration of the trial and were sacrificed by freezing at -80°C at the termination of the trial.

Figure 7. Crawfish died while molting in 7,500 ppm concentration with part of old exoskeleton still attached.

4.1.3. Lethal Concentration Determination Results of Trial 3

Trial 3 was conducted between April 2015 and May 2015. Similar to trial 1 and 2, only one crawfish died during the exposure period. The crawfish was found dead after 87 hours of exposure in the control tanks. Although measures were taken to keep the crawfish separated from each other to reduce aggression and avoid accidental deaths

caused by fighting, the crawfish that died was found in a compartment with another crawfish. Figure 8 shows the physical damage experienced by the crawfish that was found dead. Both of the second pair of walking legs and one of the third pair of walking legs appear to be torn off which could have caused the crawfish to bleed to death. All other crawfish survived the duration of the trial and were sacrificed by freezing at -80°C at the termination of the trial

Figure 8. Dead crawfish from control tanks with three walking legs missing.

4.1.4. Discussion of Lethal Concentration Results

A total of three trials exposing crawfish to weathered crude oil concentrations of 0, 1,000, 7,500, 15,000, 20,000, 30,000 ppm were conducted. Only three deaths were reported from the trials out of 144 oil exposed crawfish including control tanks. All of the crawfish deaths occurred in different concentrations and two of the three should be

attributed to physical damage and not induced by oil exposure. The death reported in trial 2 may have been the result of exposure to oil, but was likely enhanced by the physiological stress of molting. While it appears molting may have increased the impact of oil exposure on crawfish, it is difficult to assess since two other crawfish also molted during exposure to oil and survived to the termination of the trial. One of these crawfish was exposed to a higher concentration (20,000 ppm) than the crawfish that died (7,500 ppm) while the other one was exposed to the same concentration. Also, these two crawfish molted slightly sooner in the exposure period than the crawfish that died.

No crawfish were reported dead after exposure to 30,000 ppm of crude oil across the three trials. Statistical analysis was performed using a binomial function to quantitate the survival rate and determine the 95% confidence limit for the 30,000 ppm 96 hour exposure to weathered crude oil. The survival rate was determined to be no less than 88.3%. Even if one crawfish from trial 2 is not included because it was exposed two days later, potentially enhanced the weathering processes, the 95% confidence limit determines that the survival rate is still no less than 87.7%. Statistical analysis was only performed on crawfish exposed to the 30,000 ppm oil treatment concentration. This is because it was the highest level of weathered crude oil and the three deaths that did occur in the lower concentrations are not considered a result of exposure to oil since evidence of physical damage and uncontrolled stress involved in molting are strongly considered the cause of death in these organisms.

Barbee et al. (2010) reported a 96 hour lethal concentration of fresh South Louisiana Crude oil to juvenile crawfish (*P. clarkii*) of 89 mg/ L (ppm) [15]. This value is more than two orders of magnitude below the highest concentration (30,000 ppm) of

weathered crude oil the adult crawfish in this study survived exposure to. It has been established in other studies that juvenile or early-life stage organisms are generally more susceptible to the exposure of petroleum [49]. Tatem et al. (1978) determined the 96 hour LC_{50} values of three life stages of the grass shrimp (*Palaemonetes pugio*) exposed to the water-soluble fraction of refined No. 2 fuel oil based on the total petroleum hydrocarbon (TPH) content. The value determined for adult shrimp was 3.5 ppm TPH, 2.5 ppm TPH for post larvae shrimp, and 1.2 ppm TPH for larvae shrimp [50]. This severe difference in survival could therefore be partially accounted for by the difference in age of the crawfish between the two studies. However, in the present study it is likely that allowing the oil to weather for six days also contributed greatly to the lack of toxicological response. Weathering alters the composition of oil and therefore the toxicity; it begins immediately after the oil is released to the environment at which time the rate is usually the highest [51]. The weathering rate is considered to be slow in sediments and is dependent on temperature, the type of oil released, and additional environmental factors. The density of the oil plays a large role in the rate of weathering with heavier oils experiencing more resistance to weathering compared to lighter oils. Light oils have higher concentrations of low molecular weight hydrocarbons that readily evaporate, including BTEX along with two and three ring PAHs [51]. Therefore the most acutely toxic components of crude oil likely evaporated before the crawfish were placed in the tanks.

The study was designed in such a manner to reflect the conditions of the oil spill event that occurred in Bayou Sorrel, Louisiana, which released crude oil from an underground pipeline. As of 2013, there are over 190,000 miles of liquid pipelines delivering crude oil as well as other petroleum products in the U.S., and over 60,000

miles of those pipelines are devoted to transport crude oil alone [6]. Within those pipelines over 15 billion gallons of petroleum products were transported, over 8.3 billion of that being crude oil [6]. Over the last 10 years the mileage of petroleum pipelines has increased 15.4% [6]. Crude oil pipelines experienced the greatest increase of mileage, up 23.6% compared to liquid natural gas, up 21.1% and refined petroleum, up 1.8%. [6]. In Louisiana alone there are over 3,450 miles of pipelines that transport crude oil and refined oil products [7]. Therefore there is a substantial amount of risk for an oil spill event like the one at Bayou Sorrel to occur in many areas of the country, especially in Louisiana, impacting the population of crawfish.

4.2. Analysis of Crawfish Hepatopancreas Total Lipid Content

The average percent of total lipids for the hepatopancreas tissues was 70.93 ± 4.66, reported as a percent of dry weight. This high lipid content of the hepatopancreas required selection of an alternative solvent or solvents from the typical acetonitrile solvent used in the original QuEChERS method. Forsberg et al. (2011) demonstrated increased recoveries of high molecular weight PAHs using a modified QuEChERS method with a solvent combination of ethyl acetate, acetone, and isooctane from salmon tissues [44]. Forsberg et al. (2014) also used a similar modified QuEChERS method utilizing the ethyl acetate, acetone and iso-octane solvent combination to quantify PAHs in crawfish tissues [13], therefore this solvent combination was employed in this study.

4.3. Accumulation of PAHs in Hepatopancreas Tissue

4.3.1. Selection of Crawfish and PAHs for Analysis

The hepatopancreas serves as the liver and pancreas of the crawfish and is the main digestive organ [2]. PAHs concentrations are usually the highest in the

hepatopancreas of invertebrates compared to other internal tissues [31]. Therefore the hepatopancreas was selected for PAH analysis due to the high percent total lipid value of the tissue and the known association of PAHs with lipid rich tissues [31]. Crawfish from Trial 1 were analyzed using the previously described QuEChERS method. All crawfish in the control group as well as the oil treatments levels of 7,500 ppm, 20,000 ppm, and 30,000 ppm were analyzed for the 31 targeted PAHs. Statistical analysis was conducted on the average total concentration of all targeted PAHs, the average total of all targeted two and three ring PAHs, the average total of all targeted four and five ring PAHs, and the average individual targeted PAHs. All targeted six ring PAHs were below detection limits in all samples and therefore were not statistically analyzed.

4.3.2. Concentration of Total PAHs

The total concentration of PAHs in crawfish hepatopancreas tissue increased with increasing exposure to weathered crude oil. An ANOVA ($F_{(3, 4)} = 68.97$, $p < 0.0007$) determined that treatment with weathered crude oil had a statistically significant effect on the total PAH concentration in hepatopancreas tissue. The average total concentration of PAHs in hepatopancreas tissues were 0.243, 49.236, 86.094, and 115.600 μg/g (ppm) wet weight for the oil treatment levels 0, 7,500, 20,000, and 30,000 ppm, respectively. Evaluation of each oil treatment level on the total PAH concentration in hepatopancreas tissue revealed significant effects for 7,500 ppm ($p=0.0012$), 20,000 ppm ($p=0.0001$), and 30,000 ppm ($p < 0.0001$) while no significant effects were found for the control ($p=0.9696$).

A tukey post-hoc test revealed there were no significant differences between oil treatment levels 20,000 ppm and 30,000 ppm ($p=0.0812$), but there were significant

differences between 7,500 ppm and 30,000 ppm (p=0.005), 7,500 ppm and 20,000 ppm (p=0.0403), 7,500 ppm and control (p=0.0151), control and 20,000 ppm (p=0.0019), and control and 30,000 ppm (0.0006). Figure 9 shows these results including the tukey letter assigned to each oil treatment indicating significant differences.

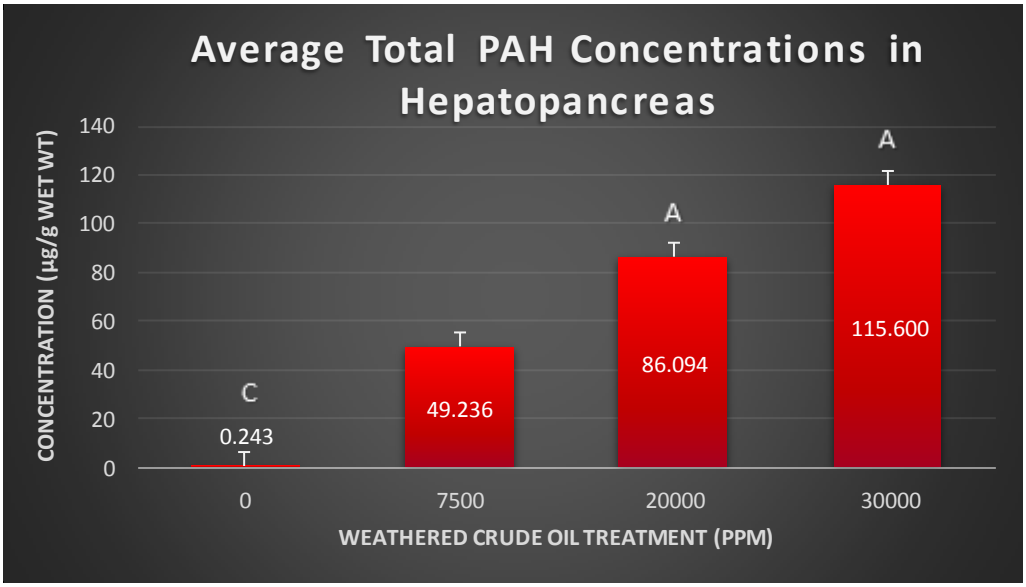


Figure 9. Average concentration of the total PAH in hepatopancreas tissue. Tukey assigned letters show significant differences in amongst groups.

4.3.3. Concentration of Two and Three Ring PAHs

Total two and three ring PAH concentrations in hepatopancreas tissue followed a trend similar to total PAHs; increasing with increasing oil treatment levels. An ANOVA ($F_{(3, 4)} = 67.4$, $p < 0.0007$) determined that treatment with weathered crude oil had a statistically significant effect on the average total two and three ring PAH concentration in hepatopancreas tissue. Figure 10 shows the average total concentration of two and three ring PAHs in hepatopancreas tissues were 0.243, 48.645, 84.947, and 114.160 µg/g (ppm) wet weight for the oil treatment levels 0, 7,500, 20,000, and 30,000 ppm, respectively. Evaluation of each oil treatment on the total two and three ring PAH

concentration in hepatopancreas tissue revealed significant effects for 7,500 ppm ($p=0.0012$), 20,000 ppm ($p=0.0001$), and 30,000 ppm ($p < 0.0001$), however significant effects were not found for the control ($p=0.9696$).

A tukey post-hoc test revealed there were no significant differences between oil treatment levels 20,000 ppm and 30,000 ppm ($p=0.0833$), but there were significant differences between 7,500 ppm and 30,000 ppm ($p=0.0052$), 7,500 ppm and 20,000 ppm ($p=0.0422$), 7,500 ppm and control ($p=0.0157$), control and 20,000 ppm ($p=0.002$), and control and 30,000 ppm (0.0006). The letters assigned by the tukey post-hoc adjustment which represents statistically significant groups are shown in Figure 10.

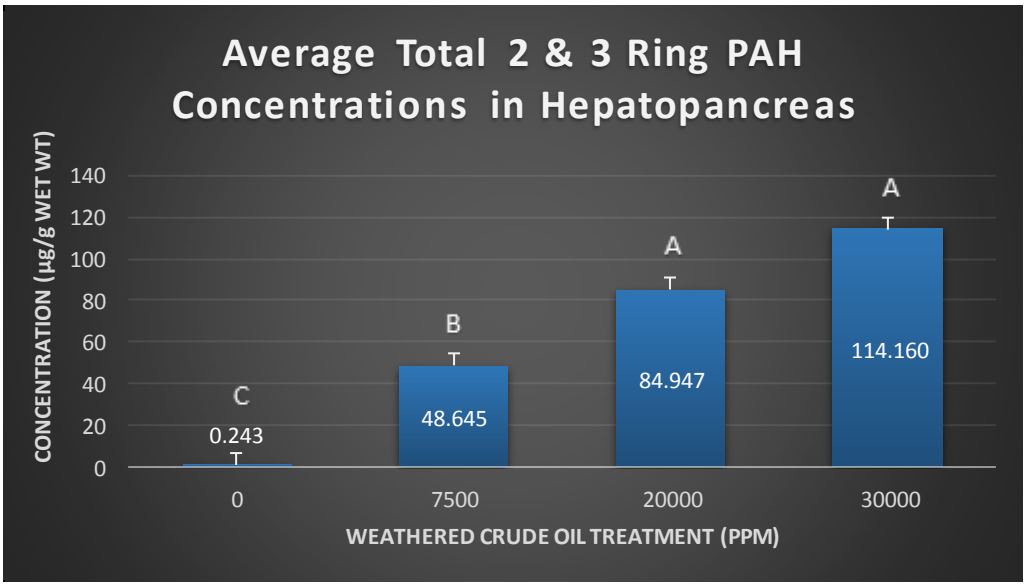


Figure 10. Average total concentration of two and three ring PAH in hepatopancreas tissue. Tukey assigned letters show significant differences in amongst groups

4.3.4. Concentration of Four and Five Ring PAHs

Total four and five ring PAH concentrations in hepatopancreas tissue again followed a similar trend as the total PAH concentrations; increasing with increasing oil treatment levels, however these totals are considerably less than the concentration of two

and three ring PAHs. An ANOVA ($F_{(3, 4)} = 46.06$, $p < 0.0015$) determined that treatment with weathered crude oil had a statistically significant effect on the average total four and five ring PAH concentration in hepatopancreas tissue. The average total four and five ring PAH concentration were 0.000, 0.591, 1.147, and 1.436 µg/g (ppm) wet weight for the oil treatment levels 0, 7,500, 20,000, and 30,000 ppm, respectively and can be seen in Figure 11. The control group is listed as zero because all of the concentrations of PAHs were below the method detection limit. Evaluation of each oil treatment on the total concentration of four and five ring PAHs in hepatopancreas tissue revealed significant effects for 7,500 ppm (p=0.0032), 20,000 ppm (p=0.0003), and 30,000 ppm (p =0.0001), however significant effects were not found for the control (p=1).

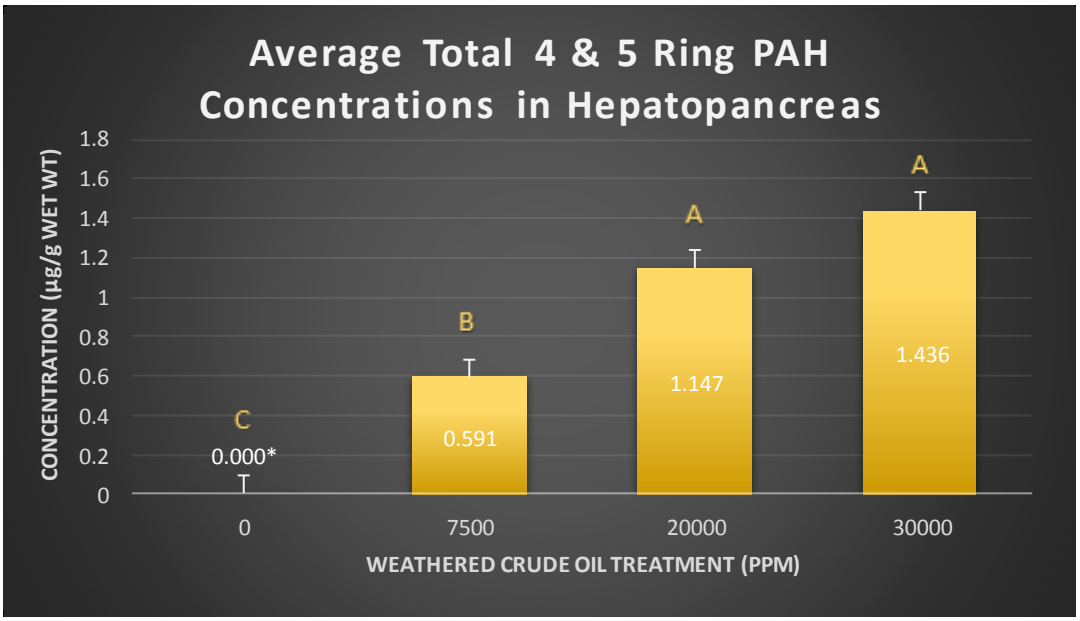


Figure 11. Average total concentration of 4 & 5 ring PAH in hepatopancreas tissue. Tukey assigned letters show significant differences in amongst groups. *Indicates concentration was below method detection limit.

A tukey post-hoc test revealed there were no significant differences between oil treatment levels 20,000 ppm and 30,000 ppm (p=0.2682), but there were significant

differences between 7,500 ppm and 30,000 ppm (p=0.0106), 7,500 ppm and 20,000 ppm (p=0.0452), 7,500 ppm and control (p=0.0368), control and 20,000 ppm (p=0.0034), and control and 30,000 ppm (0.0014). The letters assigned by the tukey post-hoc adjustment which determines groups that are statistically significant from each other are included in Figure 11.

4.3.5. Concentration of Individual Target PAHs

The average individual concentrations of all targeted PAHs (Figure 12) followed a trend similar to the total and selected PAH concentration groupings; increasing PAH concentration in hepatopancreas with increasing oil treatment concentration. Figures 13 and 14 show the average individual PAH concentrations separated into two and three ring PAHs and four and five PAHs, respectively. Figure 14 highlights the increasing trend in concentration that is observed in the four and five ring PAHs, but is not clearly emphasized in the chart that contains all of the individual PAHs (Figure 12) due to the extremely reduced rate of accumulation in the four and five ring PAHs.

Figure 15 provides the PAH profile of a South Louisiana Crude oil sample which is comparable to the SLC oil used in this study. As can be seen from the table the PAHs in the greatest concentration are the low molecular weight compounds. This includes naphthalene, fluorene, dibenzothiophene, and phenanthrene and their associated alkylated homologs. There is a strong decrease in concentrations of higher molecular weight PAHs including anthracene, fluoranthene, pyrene, chrysene, benzo[b]fluoranthene, benzo[k] fluoranthene, benzo[a]pyrene, benzo[e]pyrene, perylene, indeno[1,2,3 – cd]pyrene,

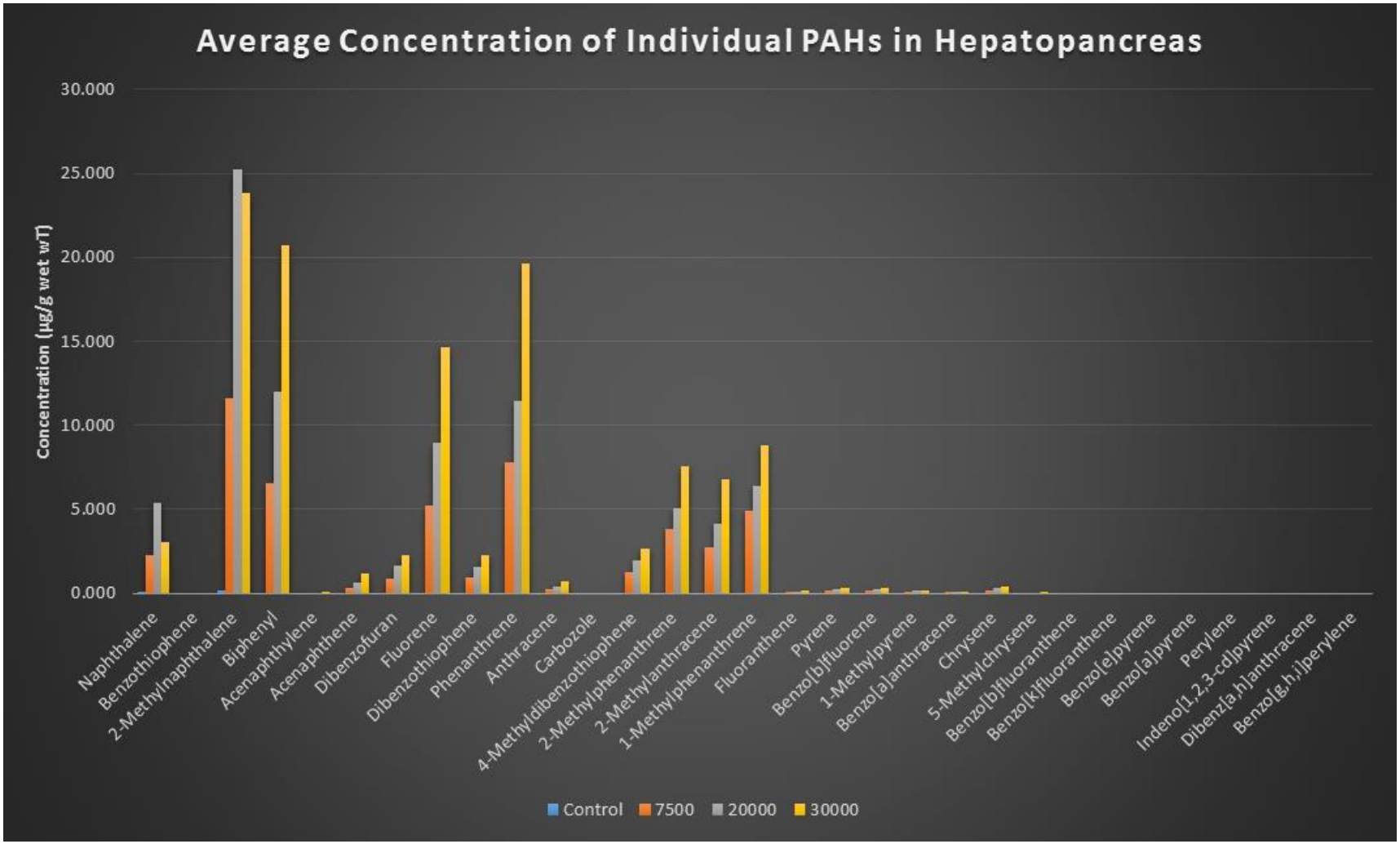


Figure 12. Average of individual PAH concentrations ppm (wet weight) in hepatopancreas tissue

dibenzo[a,h]anthracene, and benzo[g,h,i]perylene and all of their associated alkylated homologs. This pattern is clearly reflected in the hepatopancreas (Figure 12) and provides an explanation for the reduction of accumulation of the four and five ring PAHs.

The only individual PAHs that didn't follow this increasing trend are naphthalene, 2-methylnaphthalene and 1-methylpyrene which had higher average concentrations in the hepatopancreas of crawfish from 20,000 ppm treatment level than from the 30,000 ppm treatment level. The average concentrations of naphthalene were 5.416 ppm in the 20,000 ppm oil treatment and 3.021 ppm in the 30,000 ppm oil treatment. The average concentrations of 2-methylnaphthalene were 25.271 ppm in the 20,000 ppm oil treatment and 23.858 ppm in the 30,000 ppm oil treatment. The average concentrations of 1-methylpyrene were 0.144 ppm in the 20,000 ppm oil treatment and 0.130 ppm in the 30,000 ppm oil treatment. The average naphthalene concentration found in the crawfish exposed to the 20,000 ppm oil treatment was significantly different ($p = 0.0294$) from the average concentration found in the crawfish from the 30,000 ppm oil treatment level, but there were no statistical differences between the average concentrations for the 2-methylnaphthalene ($p = 0.9687$) or the 1-methylpyrene ($p = 0.9865$) found in the hepatopancreas of crawfish from the two oil treatment groups.

Significant differences across all treatment groups were reported for biphenyl, acenaphthene, and 4-methyldibenzothiopene. Statistical data including p-values for all individual PAHs can be found in Appendix B. The remaining individual PAH concentrations were not significantly different from all other treatment groups and

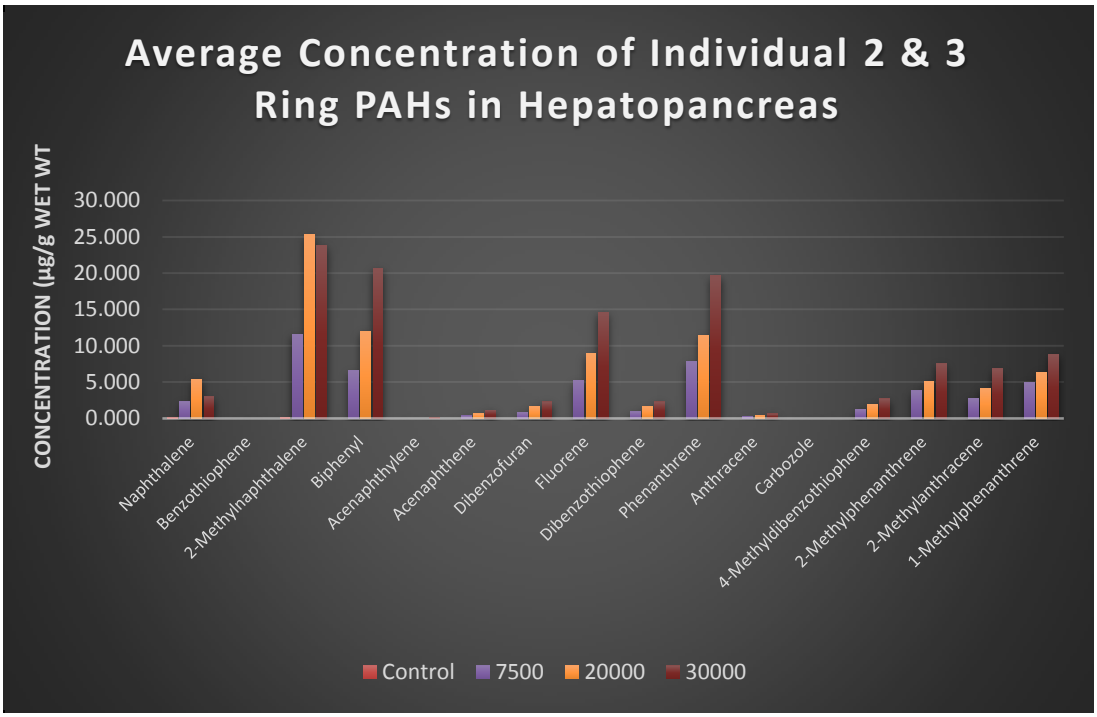


Figure 13. Average concentration of individual two and three ring PAHs in hepatopancreas

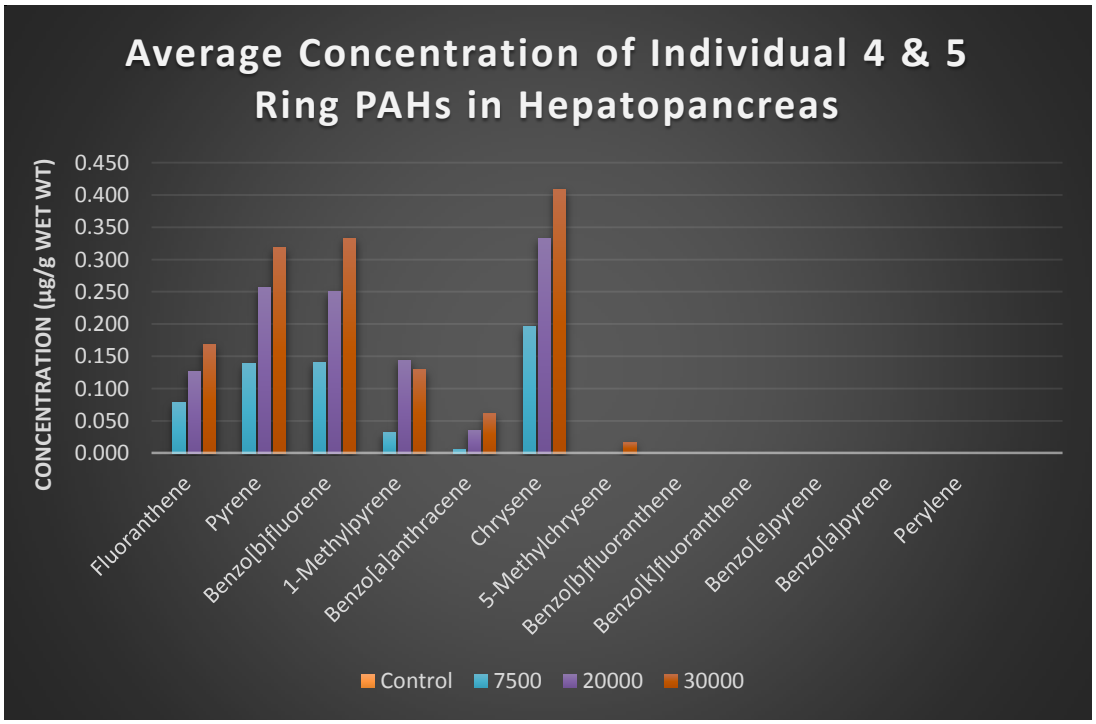


Figure 14. Average concentration of individual four and five ring PAHs in hepatopancreas

LSU ID#: Lab Reference Oil
South LA Crude Oil
Sample Weight: 500 mg
Final Extracted Volume: 20 mL

Aromatic Analyte:	Concentration (ng/mg)
Naphthalene	670
C1-Naphthalenes	1000
C2-Naphthalenes	1300
C3-Naphthalenes	1100
C4-Naphthalenes	600
Fluorene	110
C1-Fluorenes	260
C2-Fluorenes	290
C3- Fluorenes	260
Dibenzothiophene	59
C1-Dibenzothiophenes	220
C2-Dibenzothiophenes	280
C3- Dibenzothiophenes	230
Phenanthrene	200
C1-Phenanthrenes	340
C2-Phenanthrenes	340
C3-Phenanthrenes	200
C4-Phenanthrenes	68
Anthracene	5.7
Fluoranthene	4.5
Pyrene	7.6
C1- Pyrenes	44
C2- Pyrenes	38
C3- Pyrenes	28
C4- Pyrenes	18
Naphthobenzothiophene	7.6
C-1 Naphthobenzothiophenes	33
C-2 Naphthobenzothiophenes	30
C-3 Naphthobenzothiophenes	29
Benzo (a) Anthracene	5.3
Chrysene	13
C1- Chrysenes	24
C2- Chrysenes	26
C3- Chrysenes	17
C4- Chrysenes	5.9
Benzo (b) Fluoranthene	1.7
Benzo (k) Fluoranthene	1.3
Benzo (e) Pyrene	1.0
Benzo (a) Pyrene	1.5
Perylene	1.4
Indeno (1,2,3 - cd) Pyrene	1.0
Dibenzo (a,h) anthracene	0.63
Benzo (g,h,i) perylene	1.3
Total Aromatics	***7873***

Figure 15. GC/MS quantitative analysis of SLC reference oil provided by LSU RCAT, May 2010

typically significant differences were not seen for oil treatments of 7,500 ppm and 20,000 ppm, 20,000 ppm and 30,000 ppm and between the control and 7,500 ppm. Significant differences between the control and 30,000 ppm were reported for all recovered PAH concentrations above the method detection limit except 1-methylpyrene. All recovered PAH concentrations above the method detection limit were significantly different between 20,000 ppm and the control except 1-methylpyrene and benzo[a]anthracene. All targeted 6 ring PAHs were below method detection limit

4.3.6. Discussion of Results

The results show the exposure of crawfish to sediment contaminated with weathered crude oil will cause accumulation of PAHs into the hepatopancreas of crawfish. The PAH concentration in the hepatopancreas increased with increasing oil treatment level. The rate of accumulation was not consistent among the PAHs investigated in this study. This is likely attributed to the fact that crude oil is a complex mixture of thousands of compounds including many different PAHs at varying concentrations; therefore exposure to crude oil will not consist of exposure to the same concentration of all the targeted PAHs in this study. Additionally, the fate of PAHs in the environment differs based on their solubility and volatility and therefore will influence their presence in sediment, water and accumulation rates into tissue [31].

These factors also influenced other studies investigating the accumulation of PAHs into crawfish tissues. Figure 16 shows the concentration of total PAHs separated into two and three ring compounds and four and five ring compounds. This figure highlights the increase in accumulation of two and three ring PAHs in crawfish hepatopancreas compared to the four and five ring PAHs. As indicated in Figure 15, the

oil used in this study is composed of greater concentrations of two and three ring PAHs compared to four, five and six ring PAHs and may provide a reason for this decrease in concentration of higher molecular weight PAHs. It is worth noting that the four and five ring PAHs increased 43% from the 7,500 ppm to the 30,000 ppm oil treatment level compared to only a 35% increase for the two and three ring PAHs. This could be important since the four and five ring PAHs usually possess a greater toxicological risk.

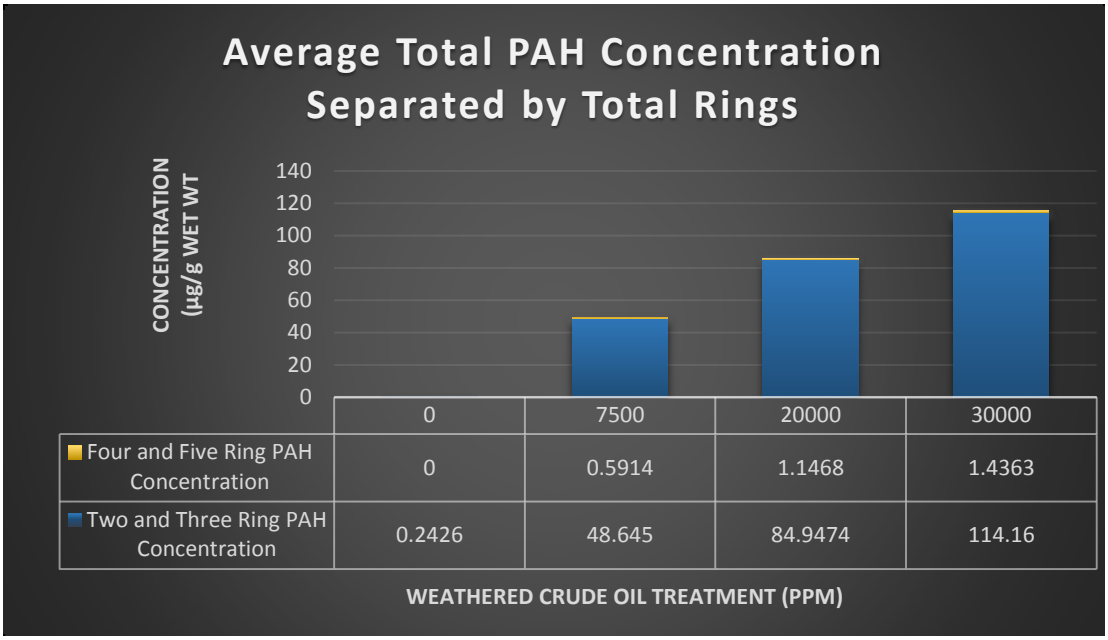


Figure 16. Stacked bar graph showing concentrations of two and three ring PAHs and four and five ring PAHs

The greater accumulation of lower molecular weight PAHs is also consistent with results from Forberg et al. (2014); which found higher levels of two and three ring PAHs than four and five ring PAHs in crawfish visceral tissues, although the reduction of four and five ring PAHs was not as extreme as reported in this study [13]. Gewurtz et al. (2000) also showed greater levels of PAHs in crawfish tissues with low molecular weights and low log octanol-water partition coefficient (K_{ow}) values, which are related to a compound's solubility, compared to PAHs with higher molecular weights and higher

log K_{ow} values [11, 13]. They attributed this trend to be the result of reduced metabolism of PAHs in crawfish and possibly reduced exposure to the less water-soluble, generally higher log K_{ow} values PAHs. It is possible that this is also the reason for the results reported in this study.

Gossiaux and Landrum (2005) observed the hepatopancreas of crawfish (*Pacifiastacus leniusculus*) had uptake rate coefficients decrease with increasing log K_{ow} after exposure to chrysene, pyrene, benoz[a]pyrene, and hexachlorobiphenyl. Elimination rate coefficients experienced a similar trend and decreased with increasing log K_{ow} [14]. As shown in Table 7, the log K_{ow} generally increases with increasing molecular weight and number of rings. Therefore the increased accumulation rates of two and three ring PAHs observed in this study is consistent with the results from Gossiaux and Landrum (2005), Gewurtz et al (2000), and Forsberg et al (2014) [11, 14, 13]. While this study does not include information on elimination rates by removal of crawfish from crude oil exposure, based on other research it is likely that the accumulation in the hepatopancreas tissues would have decreased with a depuration period and the largest decreases may have occurred in the PAHs with the lower log K_{ow} values [8, 9, 14].

The concentrations of all individual PAHs increased with increasing oil treatment level except naphthalene, 2-methylnaphthalene and 1-methylpyrene. These PAHs recorded higher average concentrations in the crawfish exposed to the 20,000 ppm oil treatment than the crawfish exposed to the 30,000 ppm oil treatment, but only the naphthalene concentration was statistically significantly higher in the crawfish exposed to the 20,000 ppm oil treatment which may be in part due to contamination within the lab. Low levels of naphthalene were found in two of the method blanks; 0.064 ppm and 0.067

Table 7 Target PAHs with total rings and log K_{ow} values

PAH	Chromatogram #	Rings	Log K_{ow} [a]
Naphthalene-d8	ISTD #1	2	N/A
Naphthalene	1	2	3.3
Benzothiophene	2	2	3.1
2-methylnaphthalene	3	2	3.9
Biphenyl	4	2	4.0
Acenaphthylene	5	3	3.7
Acenaphthene-d10	SSTD #1	3	N/A
Acenaphthene	6	3	3.9
Dibenzofuran	7	3	4.1
Fluorene	8	3	4.2
Dibenzothiophene	9	3	4.4
Phenanthrene-d10	ISTD #2	3	N/A
Phenanthrene	10	3	4.5
Anthracene	11	3	4.4
Carbozole	12	3	3.7
4-methyldibenzothiophene	13	3	4.7
2-methylphenanthrene	14	3	5.2
2-methylanthracene	15	3	5.1
1-methylphenanthrene	16	3	5.1
Fluoranthene	17	4	5.2
Pyrene	18	4	4.9
Benzo[b]fluorene	19	4	5.8
1-methylpyrene	20	4	5.4
Benzo[a]anthracene	21	4	5.8
Chrysene	22	4	5.7
5-methylchrysene	23	4	6.0
Benzo[b]fluoranthene	24	5	6.4
Benzo[k]fluoranthene	25	5	6.4
Benzo[e]pyrene	26	5	6.4
Benzo[a]pyrene	27	5	6.0
Perylene-d12	ISTD #3	5	N/A
Perylene	28	5	5.8
Indeno(1,2,3-cd)pyrene	29	6	7.0
Dibenz[a,h]anthracene	30	5	6.5
Benzo[g,h,i]perylene	31	6	6.6

[a] Log Kow values were obtained from PubChem Substance and Compound database

ppm, but it is not likely that a concentration this small would be entirely responsible for these results. Gewurtz et al. (2000) found that naphthalene was the strongest contributor to the PAH concentration recorded in crawfish [11] so it is possible that crawfish preferentially accumulate naphthalene or possibly rapidly metabolize higher molecular weight PAHs into naphthalene leading to these inconsistent results. It is also possible that there was uncontrolled variability in the amount of naphthalene that crawfish in the 30,000 ppm oil treatment were exposed to since the oil and the sediment was not homogenized prior to exposure. Another possible explanation could be that the crawfish in the 30,000 ppm oil treatment remained submerged in the sediment longer, reducing their exposure to the more water-soluble PAHs such as naphthalene.

The U. S. Food and Drug Administration (FDA) is responsible for monitoring the safety of all fish and fishery products and protecting consumers from adulterated seafood following a chemical or oil spill. Once a fishery is closed due to suspicion of oil contamination the FDA uses two ways to determine if the seafood is contaminated with oil; olfaction or taint and chemical analysis to determine levels of PAHs in seafood tissues. Seafood must pass the olfaction test and the chemical analysis before it can be deemed safe for human consumption. If the levels of PAHs exceed the FDA's levels of concern (LOC) it is considered not safe for human consumption and the fishery will remain closed [52].

Table 8 provides the total ring structures, the FDA levels of concern used for shrimp and crabs and the highest average concentration recorded for the targeted PAHs in this study. The levels of concern listed were used after the Deepwater Horizon Oil Spill event to evaluate whether the levels of PAHs in shrimp and crab tissues were safe for

human consumption. Currently the FDA has not issued levels of concern for crawfish and therefore the LOCs for shrimp and crabs are used for comparison since they are the closest related organism that data is available for. The table indicates that the levels of PAHs recorded in this study are well below the levels of concern established by the FDA and would not pose a risk to humans by consumption. However, the crawfish in the 20,000 ppm and 30,000 ppm oil treatment groups may not have passed an olfaction test because the tissue possessed a petroleum smell. When determining the presence of a petroleum odor and taste after cooking, the FDA requires 6 sub-samples for each seafood type to be analyzed by a panel of 7 expert assessors for the presence of a petroleum odor in both a raw and cooked state as well as petroleum flavor [52]. To pass this test, 5 out of 7 (70%) of the expert assessors must find no detectable petroleum odor or flavor [52]. The petroleum odor was observed when isolating the hepatopancreas for PAH analysis and was determined by the researcher alone, therefore it is unclear if it would have passed or failed the FDA's more thorough testing

Although the concentrations of PAHs recorded for this study are below the FDA's levels of concern it is important to state that the FDA developed these thresholds specifically for the Deepwater Horizon Oil Spill event and recommends that the values be determined independently for subsequent oil spills. The FDA calculated the levels of concern based on the carcinogenic activity relative to benzo[a]pyrene using a toxicity equivalency factor (TEF), compounds possessing potential carcinogenic activity include chrysene, benzo[k]fluoranthene, benzo[a]anthracene, indeno[1,2,3- cd]pyrene, benzo[b]fluoranthene, and dibenz[a,h]anthracene and their TEFs are 0.001, 0.01, 0.1, 0.1, 0.1, and 1, respectively. Other factors used to calculate the LOCs include Risk Level,

Table 8. Target PAHs with total rings, FDA levels of concern in shrimp and crabs [52], and highest average concentration found in the crawfish hepatopancreas

PAH	Rings	FDA LOC (ppm) Crabs & Shrimp 13g/ day	Highest Average Concentration in Crawfish (ppm)
Naphthalene	2	123[a]	5.416[c]
Benzothiophene	2	N/A	<MDL
2-methylnaphthalene	2	N/A[a]	25.271[c]
Biphenyl	2	N/A	20.689
Acenaphthylene	3	N/A	0.077
Acenaphthene	3	N/A	1.153
Dibenzofuran	3	N/A	2.272
Fluorene	3	246	14.632
Dibenzothiophene	3	N/A	2.273
Phenanthrene	3	1846[b]	19.659
Anthracene	3	N/A[b]	0.675
Carbozole	3	N/A	<MDL
4-methyldibenzothiophene	3	N/A	2.689
2-methylphenanthrene	3	N/A[a]	7.529
2-methylanthracene	3	N/A[a]	6.821
1-methylphenanthrene	3	N/A[a]	8.810
Fluoranthene	4	246	0.168
Pyrene	4	185	0.319
Benzo[b]fluorene	4	N/A	0.333
1-methylpyrene	4	N/A	0.144[c]
Benzo[a]anthracene	4	1.32	0.061
Chrysene	4	132	0.409
5-methylchrysene	4	N/A	<MDL
Benzo[k]fluoranthene	5	13.2	<MDL
Benzo[e]pyrene	5	N/A	<MDL
Benzo[a]pyrene	5	0.132	<MDL
Perylene	5	N/A	<MDL
Indeno(1,2,3-cd)pyrene	6	1.32	<MDL
Dibenz[a,h]anthracene	5	0.132	<MDL
Benzo[g,h,i]perylene	6	N/A	<MDL

Body Weight of the average consumer, Averaging Time or life expectancy, a Conversion Factor for unit conversion, Cancer Slope Factor which is the upper-bound probability that exposure to a given carcinogenic (B[a]P) will induce cancer, Consumption Rate which used the 90^{th} percentile meal size of the average consumer, and the Exposure Duration which estimates the potential retention period of DWH oil contaminants in Gulf seafood [52].

The levels of concern for naphthalene, phenanthrene, anthracene, fluoranthene, fluorine, and pyrene were calculated using a different set of parameters since they lack a carcinogenic potential. Instead, a Reference Dose was used which is established by the EPA's Integrated Risk Information Service and is the exposure concentration that will not cause significant risk of adverse health effects during a lifetime. The Body Weight, Conversion Factor and Consumption Factor used for the carcinogenic PAHs were also used in this calculation.

The levels recorded in this study of for the PAHs monitored by the FDA are reduced by an order of magnitude or more compared to the levels of concern listed. Naphthalene combined with 2-methylnaphthalene is slightly closer to the level of concern, but still only about one quarter of the level of concern for naphthalene. Therefore there is no potential risk for cancer or other adverse health effects from the consumption of the crawfish exposed to weathered crude oil in this study.

While the carcinogenic ability and other toxic effects of the targeted PAHs will remain constant, the other factors used to determine the levels of concern listed would likely change for subsequent oil spill such as the oil spill focused on in this study. It is also likely that the consumption rates will differ for crawfish from those calculated for

shrimp and crabs. Another important note is that the crawfish hepatopancreas is regularly consumed when eating boiled crawfish, especially in Louisiana. Therefore a level of concern to evaluate the safety of crawfish for human consumption after an oil spill event would need to account for PAH contamination in both the abdominal muscle and hepatopancreas tissue. Also, it is likely that the PAH concentrations observed in the hepatopancreas are much higher than the concentrations that would be found in the abdominal muscle. This is because PAHs more closely associate with non-polar matrices such as the lipid rich hepatopancreas than tissues of lower lipid content such as the abdominal muscle.

CHAPTER 5. SUMMARY AND CONCLUSIONS

5.1. Conclusions of Lethal Concentration Trials

The results from the three oil exposure trials indicate that the crawfish have at least an 87.7 % chance of survival for at least 96 hours in sediment contaminated with 30,000 ppm of weathered crude oil. The results of this study are unable to determine how long crawfish would be able to survive in weathered crude oil and therefore further investigation is required. In these exposure trials the SLC oil was weathered for six days resulting in evaporation of the lighter molecular weight, more volatile hydrocarbons, such as BTEX, and lower weight PAHs like naphthalene. These components of crude oil are known to induce narcosis in organisms and if fresh crude oil was used in this study, which would have contained higher levels of these compounds, an increase in mortality may have occurred [27].

From this study it is evident that adult crawfish are resilient to weathered crude oil exposure for 96 hours. Their ability to survive these conditions indicates that they would be poor bio indicators of short-term exposure to weathered crude oil. The over 3,450 miles of pipelines transporting petroleum products in Louisiana generate a risk for the exposure of crawfish to crude oil and refined oil products. Although this study didn't attempt to investigate whether exposure to weathered crude oil would induce mortality of crawfish in the molting stage, it appears that molting could enhance crawfish sensitivity to it.

The perseverance of crawfish in heavily oiled conditions provides the potential for them to accumulate petroleum related compounds such as PAHs. Crawfish are regularly consumed by a variety of organisms, including humans. In 2014, Louisiana

harvested over 144 million pounds of crawfish alone [3]. Therefore, as indicated by the results of this study, there could potentially be a risk of consuming crawfish that have survived an oil spill event and accumulated PAHs into their tissues.

5.2. Conclusions of PAH Accumulation in Hepatopancreas

This study demonstrates the ability of adult crawfish to accumulate polycyclic aromatic hydrocarbons into the hepatopancreas tissue after exposure to weathered crude oil. Statistical analysis of the accumulation of average total PAH concentrations, average total two and three ring PAH concentrations, and average total four and five ring PAH concentrations all indicated that treatment with weathered crude oil significantly affected the concentration in the hepatopancreas. There were no significant differences found for the average concentrations of any of the three groupings of PAHs (total PAHs, two and three ring PAHs, and four and five ring PAHs) recorded in the control crawfish. All oil treatment levels (7,500 ppm, 20,000 ppm, and 30,000 ppm) had a significant effect on the concentration of all three grouping of PAHs found in crawfish hepatopancreas tissues. The concentration of PAHs in hepatopancreas tissue increased with increased oil treatment levels. The total PAH, two and three ring PAH, and four and five ring PAH concentrations were significantly different between all treatment levels except between the 20,000 ppm and the 30,000 ppm oil treatment levels.

Accumulation in the hepatopancreas tissue consisted of mainly two and three ring PAHs. This is consistent with results of other studies conducted on the accumulation of PAHs by various crawfish species [11, 13]. There was a significant reduction in the accumulation of four and five ring PAHs compared to the accumulation of two and three ring PAHs. Gewurtz et al. (2000) attributed a similar trend to reduced exposure to the

less-soluble higher molecular weight PAHs [11]. These compounds generally have higher log K_{ow} values compared to the two and three ring PAHs indicating they have more of a tendency to stay in the organic phase which would correspond to the layer of sediment. It is possible these compounds stayed bound in the sediment layer and the suspended solids, making them less available for accumulation than the more water-soluble PAHs were. This would agree with the known distribution of PAHs in the environment, which is based on their physical and chemical properties, most importantly their solubility and vapor pressure [31]. In the environment, water is dominated by two and three ringed PAH species, while sediment is dominated by four, five and six ringed species [31], therefore the sediment may have acted like a sink for the higher molecular weight PAHs. Additionally, this difference could be attributed to greater concentrations of two and three ring PAHs than four, five, and six ring PAHs in the oil used in this study. This would provide more availability for the two and three ring PAHs and should result in higher concentrations in the hepatopancreas tissue.

All individual PAHs concentrations also increased with increasing oil treatment level except naphthalene, 2-methylnaphthalene, and 1-methylpyrene. The 20,000 ppm oil treatment levels contained the highest concentration of these three compounds, although only the naphthalene concentration was significantly higher than the naphthalene concentration in the 30,000 ppm oil treatment level. This discrepancy in the increasing trend is possibly due to contamination in the lab, variability in the accumulation of naphthalene in crawfish, and reduced exposure to naphthalene by variation in time spent burrowed in the sediment layer by individual crawfish. Naphthalene has the lowest log K_{ow} value of the PAHs recovered in hepatopancreas tissue, therefore it would have a

higher tendency to remain in the aqueous phase and crawfish that stay buried in the sediment could possibly have a reduction in exposure to it.

The highest average concentration of all individual PAHs recovered in this study were below the levels of concern generated by the FDA for seafood with possible oil contamination. All of the PAHs that are selected for monitoring by the FDA except the naphthalenes were reduced by an order of magnitude compared to the FDA's levels of concern. Therefore consumption of the crawfish wouldn't present a risk of cancer or other adverse health effects. The levels of concern used for comparison are for shrimp and crabs since there are no published values for crawfish. However, the values can't be truly compared since they were calculated for different species and a different oil spill event.

5.3 Future Research

The ability of the adult crawfish (*Procambarus clarkii*) to survive in the weathered crude oil for 96 hours generates the need for future research to determine the amount of time crawfish can survive in weathered crude oil before any consistent trends in mortality could be discerned. To determine the LC_{50} value an increase in the concentration of weathered crude oil that the crawfish are being exposed to would be needed, however for safety reasons it is recommended that the researcher uses proper protective equipment if the concentrations are significantly increased. A comparison study to determine the 96 hour LC_{50} value for fresh crude oil exposure to adult crawfish would provide information on the lethal impacts of the more volatile components of crude oil that readily evaporate. Further investigation is needed to determine if crawfish possess an increased susceptibility to crude oil exposure while molting.

This study highlights the ability of the crawfish to accumulate PAHs as a result of short-term exposure to weathered crude oil. Analysis of all of the crawfish used in this study may help to statistically differentiate between the concentrations of PAHs in the hepatopancreas tissue and all of the oil treatment levels. Correlation of these results with the sediment and water concentration of PAHs would further validate the results and offer explanations for the trends observed in the crawfish tissues. Research should also be conducted to determine how crawfish accumulate PAHs over a long-term period and it should include a period of depuration which other researchers have shown reduces the accumulation of PAHs in crawfish tissues [14]. This would also provide useful information about the tendency of certain PAHs to remain in crawfish tissues for longer periods of time compared to others and may therefore be present in higher concentrations within the tissues after chronic exposures. Further research using lower concentrations of weathered crude oil would also provide useful information about the threshold at which crawfish begin accumulating PAHs. Investigations into the accumulation rates of petroleum related PAHs from exposure to fresh crude oil might show even greater quantities of lower molecular weight PAHs. Future studies should also include a period of depuration after PAH exposure to assess the elimination rate of individual PAHs from the hepatopancreas. Monitoring of PAHs levels in crawfish tissues following an oil spill event may also be helpful to prevent exposure of humans as well as other consumers of crawfish from potentially harmful concentrations of polycyclic aromatic hydrocarbons.

REFERENCES

[1] J. G. Walls, Crawfishes of Louisiana, Baton Rouge: Louisiana State University Press, 2009, p. 240.

[2] W. R. McClain, R. P. Romaire, C. G. Lutz and M. G. Shirley, "Louisiana Crawfish Production Manual," Baton Rouge, 2007.

[3] Louisiana State University Agricultural Center, "Louisiana Summary Agricultural and Natrual Resources 2014 Data," Baton Rouge, 2014.

[4] W. R. McClain and R. P. Romaire, "Procambarid Crawfish: Life History and Biology," Southern Regional Aquaculture Center, 2007.

[5] NOAA Incident News, "ORB Exploration, Bayou Sorrel 2013-Jan-15," 15 January 2013. [Online]. Available: http://www.incidentnews.noaa.gov/incident/8556. [Accessed 19 October 2015].

[6] Association of Oil Pipelines and American Petroleum Institute, "U.S. Liquids Pipeline Usage & Mileage Report," American Petroleum Institute, 2014.

[7] Louisiana Geological Survey, "Louisiana Petroleum Industry Facts," Louisiana Geological Survey, Baton Rouge, 2000.

[8] I. B. Tarshis, "Uptake and Depuration of Petroleum Hydrocarbons by Crayfish," *Archives of Environmental Contamination and Toxicology*, vol. 10, no. 1, pp. 79-86, 1981.

[9] R. Sarojini, et al., "Naphthalene-Induced Cytotoxicity on the Hepatopancreatic Cells of the Red Swamp Crayfish, Procambarus clarkii," *Bulletin of Environmental Contamination and Toxicology*, vol. 51, no. 5, pp. 689-695, 1993.

[10] P. Schilderman, et al., "Use of Crayfish in Biomonitoring Studies of Environmental Pollution of the River Meuse," *Ecotoxicology and Environmental Safety,* vol. 44, pp. 241-252, 1999.

[11] S. B. Gewurtz, R. Lazar and G. D. Haffner, "Comparison of Polycyclic Aromatic Hydrocarbon and Polychlorinated Biphenyl Dynamics in Benthic Invertebrates of Lake Erie, USA," *Environmental Toxicology and Chemistry,* vol. 19, no. 12, pp. 2943-2950, 2000.

[12] N. Benson, et al., "Petroleum hydrocarbons and limiting nutrients in Macura reptantia, Procambarus clarkii and benthic sediment from Qua Iboe Estuary, Nigeria," *Environmentalist,* vol. 28, pp. 275-282, 2008.

[13] N. Forsberg, et al., "Predicting Polycyclic Aromatic Hydrocarbons Concentations in Resident Aquatic Organisms Using Passive Samplers and Partial Least-Squares Calibration," *Environmental Science and Technology,* vol. 48, no. 11, pp. 6291-6299, 2014.

[14] D. C. Gossiaux and P. F. Landrum, "Toxicokinetics and Tissue Distribution of Non-polar Contaminants from Aqueous and Dietary Exposures for the Crayfish Pacifastacus Leniusculus," U.S. Department of Commerce, NOAA, Great Lakes Environmental Research Laboratory, Ann Arbor, 2005.

[15] G. C. Barbee, et al., "Acute Toxicity (LC50) of South Louisiana Crude Oil, Mineral Oil, and Peanut Oil to the Red Swamp Crayfish Procambarus clarkii," *Freshwater Crayfish,* vol. 17, pp. 227-233, 2010.

[16] M. Anastassiades, et al., "Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/ Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce," *Journal of AOAC International,* vol. 86, no. 2, pp. 412-431, 2003.

[17] J. Huner and J. Barr, Red Swamp Crawfish: Biology and Exploitation, Baton Rouge, LA: Louisiana Sea Grant College Program, 1991.

[18] Louisiana State University College of Agriculture, "Louisiana State University Ag Center, Louisiana Summary Agricultural and Natural Resources 2014 Data," 2014. [Online]. Available: http://www.lsuagcenter.com/agsummary/Data.html. [Accessed 14 October 2015].

[19] W. T. Momot, "Redefining the role of crayfish in aquatic ecosystems," *Reviews in Fisheries Science,* vol. 3, no. 1, pp. 33-63, 1995.

[20] U.S. Coast Guard Newsroom, "News Release, Coast Guard Oversees Controlled Burn at Frog Lake Bayou Sorrel Facility," U.S. Coast Guard Marine Safety Unit, Baton Rouge, 2013.

[21] NOAA Office of Response and Restoration, "When Setting Fire to an Oil Spill in a Flooded Swamp is a Good Thing," 12 February 2013. [Online]. Available: http://response.restoration.noaa.gov/about/media/when-setting-fire-oil-spill-flooded-louisiana-swamp-good-thing.html. [Accessed 19 October 2015].

[22] Google, "Google Maps," 19 October 2015. [Online]. Available: https://www.google.com/maps/place/30%C2%B010'51.0%22N+91%C2%B027'16.0%22W/@30.2345423,-91.5341696,10.5z/data=!4m2!3m1!1s0x0:0x0. [Accessed 19 October 2015].

[23] National Response Center, "Incident Report #1035166," 2013.

[24] M. Fingas, Handbook of Oil Spill Science and Technology, Hoboken: John Wiley & Sons, 2014, pp. 52-77.

[25] NOAA Office of Response and Restoration, "Alaska North Slope Crude Blends," 21 October 2015. [Online]. Available: http://response.restoration.noaa.gov/oil-and-chemical-spills/oil-spills/resources/alaska-north-slope-crude-blends.html. [Accessed 2015 21 October].

[26] NOAA Hazardous Materials Response & Assessment Division and American Petroleum Institute, "Options for Minimizing Environmental Impacts of Freshwater Spill Response," 1994.

[27] D. P. French-McCay, "Development and Application of an OIl Toxicity and Exposure Model, OILTOXEX," *Environmental Toxicology and Chemistry,* vol. 21, no. 10, pp. 2080-2094, 2002.

[28] International Agency for Research on Cancer, "Agents Classified by the IARC Monographs, Volumes 1-112," IARC, Lyon, 2015.

[29] D. Wu, et al., "Comparative Toxicity of Four Chemically Dispersed and Undispersed Crude Oils to Rainbow Trout Embryos," *Environmental Toxicology and Chemistry,* vol. 31, no. 4, pp. 754-765, 2012.

[30] J. Wickliffe, et al., "Evaluation of Polycyclic Aromatic Hydrocarbons Using Analytical Methods, Toxicology, and Risk Assessment Resarch: Seafood Safety after a Petroleum Spill as an Example," *Environmental Health Perspectives,* vol. 122, no. 1, pp. 6-9, 2014.

[31] P. E. Douben, PAHs: An Ecotoxicological Perspective, Chichester: Wiley, 2003.

[32] S. Rhodes, et al., "The effects of dimethylated and alkylated polycylic aromatic hydrocarbons on the embryonic development of the Japanese medaka," *Ecotoxicology and Envrionmental Safety,* vol. 60, no. 3, pp. 247-258, 2005.

[33] Y. Moon, et al., "Toxicity and Bioaccumulation of Petroleum Mixtures with Alkyl PAHs in Earthworms," *Human and Ecological Risk Assessment: An International*

Journal, vol. 19, no. 3, pp. 819-835, 2013.

[34] F. S. Ott, R. P. Harris and S. C. O'Hara, "Acute and sublethal toxicity of naphthalene and three methylated derivatives to the estuarine copepod, Eurytemora affinis," *Marine Environmental Research,* vol. 1, no. 1, pp. 49-58, 1978.

[35] C. S. Jewell, M. H. Mayeaux and G. W. Winston, "Benzo[a]pyrene Metabolism by the Hepatopancreas and Green Gland of the Red Swamp Crayfish, Procambarus clarkii, In Vitro," *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* vol. 118, no. 3, pp. 369-374, 1997.

[36] K. Skupinska, I. Misiewicz and T. Kasprzycka-Guttman, "Polycyclic Aromatic Hydrocarbons: Physicochemical Properties, Envrionmental Appearance and Impact on Living Organisms," *ACTA POLONIAE PHARMACEUTICA,* vol. 61, no. 3, pp. 233-240, 2004.

[37] P. Hodson, et al., "What Compounds in Crude Oil Cause Chronic Toxicity to Larval Fish?," in *Oil Spill Response: A Global Perspective,* Dorderecht, Springer ebooks, 2008, pp. 193-194.

[38] I. N. E. Onwurah, et al., "Crude Oil Spills in the Environment, Effects and Some Innovative Clean-up Biotechnologies," *International Journal of Environmental Research,* vol. 1, no. 4, pp. 307-320, 2007.

[39] R. V. Thomann and J. Komlos, "Model of Biota-Sediment Accumulation Factor for Polycyclic Aromatic Hydrocarbons," *Environmental Toxicology and Chemistry,* vol. 18, no. 5, pp. 1060-1068, 1999.

[40] S. Uno, et al., "Monitoring of PAHs and alkylated PAHs in aquatic organisms after 1 month from the Solar I oil spill of the coast of Guimaras Island, Philippines," *Environmental Monitoring and Assessment,* vol. 165, no. 1, pp. 501-515, 2010.

[41] J. M. Levengood and D. J. Schaeffer, "Polycyclic aromatic hydrocarbons in fish and crayfish from the Calumet region of southwestern Lake Michigan," *Ecotoxicology,* vol. 20, no. 6, pp. 1411-1421, 2011.

[42] G. I. V. Klobucar, et al., "Genotoxicity monitoring of freshwater environments using caged crayfish (Astacus leptodactylus)," *Chemosphere,* vol. 87, pp. 62-67, 2012.

[43] S. Gratz, et al., "Screening and Determination of Polycyclic Aromatic Hydrocarbons in Seafoods Using QuEChERS-based Extraction and High-Performance Liquid Chromatography with Fluorescence Detection," *International Journal of AOAC,* vol. 94, no. 5, pp. 1601-1616, 2011.

[44] N. D. Forsberg, G. R. Wilson and K. A. Anderson, "Determination of Parent and Substituted Polycyclic Aromatic Hydrocarbons in High-Fat Salmon Using Modified QuEChERS Extraction, Dispersive SPE and GC-MS," *Journal of Agricultural and Food Chemistry,* vol. 59, no. 15, pp. 8108-8116, 2011.

[45] BP Exploration & Production Inc. and BP Gulf Coast Restroation Organization, "Data Publication Summary Report: Reference Oil Characterization. Refeence No. O-04v01-02," 2014.

[46] O. Pelz, et al., "Selection of a Surrogate MC252 Oil as a Reference Material for Future Aquatic Toxicity Tests and Other Studies," in *SETAC*, Boston, 2011.

[47] U.S. Code of Federal Regulations, "Title 40-Protection of Envrionment, Part 136 Appendix B, Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11".

[48] P. Manirakiza, A. Covaci and P. Schepens, "Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods," *Journal of Food Composition and Analysis*, vol. 14, no. 1, pp. 93-100, 2001.

[49] A. Dupuis and F. Ucan-Marin, "A literature review on the aquatic toxicology of petroleum oil: An overview of oil properties and effects to aquatic biota.," DFO Can. Sci. Advis. Sec. Res. Doc.2015/007, 2015.

[50] H. E. Tatem, B. A. Cox and J. W. Anderson, "Toxicity of Oil and Petroleum Hydrocarbons to Estuarine Crustaceans," *Estuarine and Coastal Marine Science*, vol. 6, pp. 365-373, 1978.

[51] NRC, "Oil in the Sea III: Inputs, Fates, and Effects.," The National Academies Press, Washington, D. C., 2003.

[52] U.S. Food and Drug Administration, "Protocol for Interpretation and Use of Sensory Testing and Analytical Chemistry Results for Re-Opening Oil-Impacted Areas Closed to Seafood Harvesting Due to The Deepwater Horizon Oil Spill," U.S. FDA, Silver Spring, 2010.

[53] M. J. Hemmer, M. G. Barron and R. M. Greene, "Comparative toxicity of eight oil dispersants, Louisiana sweet crude oil (LSC), and chemically dispersed LSC to two aquatic test species," *Environmental Toxicology and Chemistry*, vol. 30, no. 10, pp. 2244-22552, 2011.

APPENDIX

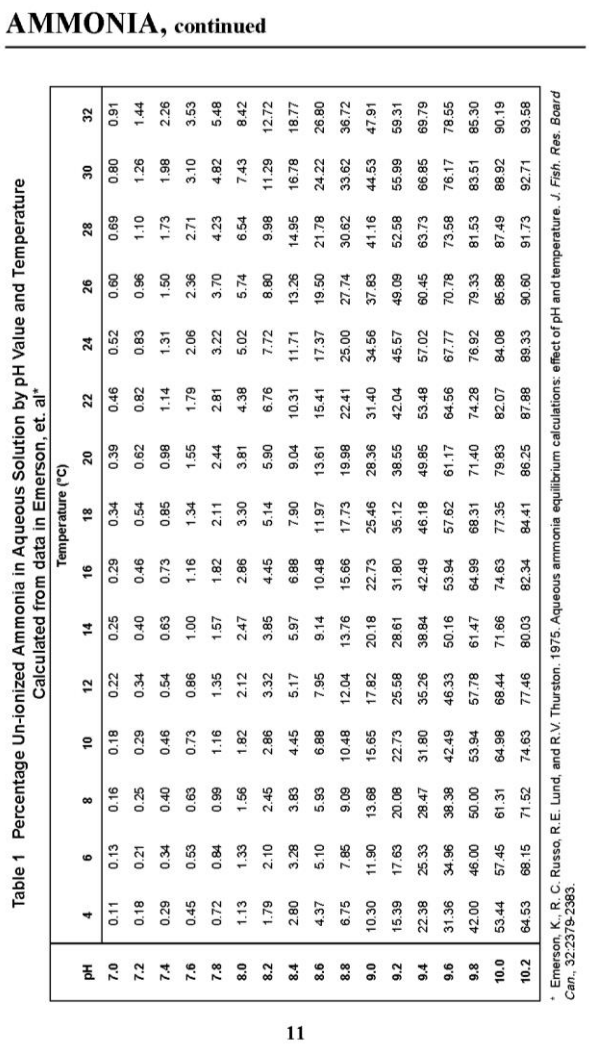
Appendix A. Description of Water Quality Analysis

Water quality analysis was performed using the Hach Saltwater Aquaculture Test Kit, Model FF-3. To measure pH two 5 mL samples of water from the selected tank were placed in two color viewing plastic tubes and six drops of Wide Range 4 pH Indicator Solution was added to one tube and shaken vigorously. The tubes were placed in the Color Comparator and held up to the light while rotating the Wide Range pH Color Disc to match the color and give a pH measurement.

To measure ammonia two 5 mL samples of water from the selected tanks were placed into two color viewing plastic tubes and one Ammonia Salicylate Reagent Powder Pillow was added to one tube and vigorously shaken until dissolved. After at least three minutes one Ammonia Cyanurate Reagent Powder Pillow was added to the same tube and shaken vigorously until dissolved, fifteen minutes was allowed to pass before both tubes were placed in the Color Comparator and held up to the light. The measurement of ammonia nitrogen in mg/L (N) was obtained by placing the Ammonia Salicylate Color Disc into the Color Comparator and rotated until the color matched. To calculate the amount of toxic ammonia the following formula was used:

$$\left(\frac{\frac{mg}{L}NH_3\ as\ N\ \times value\ from\ HACH\ Saltwater\ Aquaculture\ Manual\ Table\ 1}{100}\right)\times 1.2 = \frac{mg}{L}NH_3$$

Note: See Figure 17 below for Table 1 values.



AMMONIA, continued

Table 1 Percentage Un-ionized Ammonia in Aqueous Solution by pH Value and Temperature
Calculated from data in Emerson, et. al*

pH	Temperature (°C)																			
	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32
7.0	0.11	0.13	0.16	0.18	0.22	0.25	0.29	0.34	0.39	0.46	0.52	0.60	0.69	0.80	0.91
7.2	0.18	0.21	0.25	0.29	0.34	0.40	0.46	0.54	0.62	0.82	0.83	0.96	1.10	1.26	1.44
7.4	0.29	0.34	0.40	0.46	0.54	0.63	0.73	0.85	0.98	1.14	1.31	1.50	1.73	1.98	2.26
7.6	0.45	0.53	0.63	0.73	0.86	1.00	1.16	1.34	1.55	1.79	2.06	2.36	2.71	3.10	3.53
7.8	0.72	0.84	0.99	1.16	1.35	1.57	1.82	2.11	2.44	2.81	3.22	3.70	4.23	4.82	5.48
8.0	1.13	1.33	1.56	1.82	2.12	2.47	2.86	3.30	3.81	4.38	5.02	5.74	6.54	7.43	8.42
8.2	1.79	2.10	2.45	2.86	3.32	3.85	4.45	5.14	5.90	6.76	7.72	8.80	9.98	11.29	12.72
8.4	2.80	3.28	3.83	4.45	5.17	5.97	6.88	7.90	9.04	10.31	11.71	13.26	14.95	16.78	18.77
8.6	4.37	5.10	5.93	6.88	7.95	9.14	10.48	11.97	13.61	15.41	17.37	19.50	21.78	24.22	26.80
8.8	6.75	7.85	9.09	10.48	12.04	13.76	15.66	17.73	19.98	22.41	25.00	27.74	30.62	33.62	36.72
9.0	10.30	11.90	13.68	15.65	17.82	20.18	22.73	25.46	28.36	31.40	34.56	37.83	41.16	44.53	47.91
9.2	15.39	17.63	20.08	22.73	25.58	28.61	31.80	35.12	38.55	42.04	45.57	49.09	52.58	55.99	59.31
9.4	22.38	25.33	28.47	31.80	35.26	38.84	42.49	46.18	49.85	53.48	57.02	60.45	63.73	66.85	69.79
9.6	31.36	34.96	38.38	42.49	46.33	50.16	53.94	57.62	61.17	64.56	67.77	70.78	73.58	76.17	78.55
9.8	42.00	46.00	50.00	53.94	57.78	61.47	64.99	68.31	71.40	74.28	76.92	79.33	81.53	83.51	85.30
10.0	53.44	57.45	61.31	64.98	68.44	71.66	74.63	77.35	79.83	82.07	84.08	85.88	87.49	88.92	90.19
10.2	64.53	68.15	71.52	74.63	77.46	80.03	82.34	84.41	86.25	87.88	89.33	90.60	91.73	92.71	93.58

* Emerson, K., R. C. Russo, R.E. Lund, and R.V. Thurston. 1975. Aqueous ammonia equilibrium calculations: effect of pH and temperature. *J. Fish. Res. Board Can.*, 32:2379-2383.

11

Figure 17. Table 1 from Hach Saltwater Aquaculture Test Kit, Model FF-3, pg 11

Dissolved oxygen was measured by placing a sample of water from the selected tank into a 60 mL glass-stoppered BOD bottle, until the titration step the stopper was placed specifically to ensure no air bubbles were present in the bottle. To the sample one Dissolved Oxygen 1 Powder Pillow and Dissolved Oxygen 2 Powder Pillow were added. The bottle was inverted 10 times and the floc created was allowed to settle to the bottom half of the sample, this step is repeated twice. One Dissolved Oxygen 3 Powder Pillow was added to the bottle and inverted 10 times and then 50 mL of the sample was poured

off using a graduated cylinder. The remaining 10 mL of sample was titrated to a pale yellow color using 0.0250 N Sodium Thiosulfate in digital titrator. Two drops of Starch Indicator Solution were added and the bottle was swirled developing a dark blue color. Titration continued until the sample changed to a colorless solution. Dividing the reading on the digital titrator by 40 provided the amount of dissolved oxygen in mg/L.

Appendix B. Statistical Analysis Output from SAS 9.4

Total PAH Concentration

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	68.97	0.0007

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0.2426	5.9915	4	0.04	0.9696
trt	7500	49.2364	5.9915	4	8.22	0.0012
trt	20000	86.0938	5.9915	4	14.37	0.0001
trt	30000	115.6	5.9915	4	19.29	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-48.9938	8.4733	4	-5.78	0.0044	Tukey	0.0151
trt	0	20000	-85.8511	8.4733	4	-10.13	0.0005	Tukey	0.0019
trt	0	30000	-115.35	8.4733	4	-13.61	0.0002	Tukey	0.0006
trt	7500	20000	-36.8574	8.4733	4	-4.35	0.0122	Tukey	0.0403
trt	7500	30000	-66.3589	8.4733	4	-7.83	0.0014	Tukey	0.005
trt	20000	30000	-29.5015	8.4733	4	-3.48	0.0253	Tukey	0.0812

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	115.6	5.9915	A
2	20000	86.0938	5.9915	A
3	7500	49.2364	5.9915	B
4	0	0.2426	5.9915	C

Two and Three Ring PAH Concentration

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	67.4	0.0007

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0.2426	5.9834	4	0.04	0.9696
trt	7500	48.645	5.9834	4	8.13	0.0012
trt	20000	84.9474	5.9834	4	14.2	0.0001
trt	30000	114.16	5.9834	4	19.08	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-48.4024	8.4618	4	-5.72	0.0046	Tukey	0.0157
trt	0	20000	-84.7048	8.4618	4	-10.01	0.0006	Tukey	0.002
trt	0	30000	-113.92	8.4618	4	-13.46	0.0002	Tukey	0.0006
trt	7500	20000	-36.3024	8.4618	4	-4.29	0.0127	Tukey	0.0422
trt	7500	30000	-65.514	8.4618	4	-7.74	0.0015	Tukey	0.0052
trt	20000	30000	-29.2116	8.4618	4	-3.45	0.026	Tukey	0.0833

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	114.16	5.9834	A
2	20000	84.9474	5.9834	A
3	7500	48.645	5.9834	B
4	0	0.2426	5.9834	C

Four and Five Ring PAH Concentration

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	3	4	46.06	0.0015

Least Squares Means						
Effect	trt	Estimate	Standard Error	DF	t Value	Pr > |t|
trt	0	0	0.09351	4	0	1
trt	7500	0.5914	0.09351	4	6.32	0.0032
trt	20000	1.1468	0.09351	4	12.26	0.0003
trt	30000	1.4363	0.09351	4	15.36	0.0001

Differences of Least Squares Means									
Effect	trt	_trt	Estimate	Standard Error	DF	t Value	Pr > |t|	Adjustment	Adj P
trt	0	7500	-0.5914	0.1322	4	-4.47	0.0111	Tukey	0.0368
trt	0	20000	-1.1468	0.1322	4	-8.67	0.001	Tukey	0.0034
trt	0	30000	-1.4363	0.1322	4	-10.86	0.0004	Tukey	0.0014
trt	7500	20000	-0.5554	0.1322	4	-4.2	0.0137	Tukey	0.0452
trt	7500	30000	-0.8449	0.1322	4	-6.39	0.0031	Tukey	0.0106
trt	20000	30000	-0.2895	0.1322	4	-2.19	0.0938	Tukey	0.2682

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	trt	Estimate	StdErr	MSGROUP
1	30000	1.4363	0.09351	A
2	20000	1.1468	0.09351	A
3	7500	0.5914	0.09351	B
4	0	0	0.09351	C

Acenaphthene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	250.19	<.0001

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.03099	4	0	1
trt	7500	0.3396	0.03099	4	10.96	0.0004
trt	20000	0.652	0.03099	4	21.04	<.0001
trt	30000	1.1535	0.03099	4	37.23	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.3396	0.04382	4	-7.75	0.0015	Tukey	0.0052
trt	0	20000	-0.652	0.04382	4	-14.88	0.0001	Tukey	0.0004
trt	0	30000	-1.1535	0.04382	4	-26.32	<.0001	Tukey	<.0001
trt	7500	20000	-0.3124	0.04382	4	-7.13	0.002	Tukey	0.0071
trt	7500	30000	-0.8139	0.04382	4	-18.57	<.0001	Tukey	0.0002
trt	20000	30000	-0.5015	0.04382	4	-11.44	0.0003	Tukey	0.0012

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	trt	Estimate	StdErr	MSGROUP
1	30000	1.1535	0.03099	A
2	20000	0.652	0.03099	B
3	7500	0.3396	0.03099	C
4	0	0	0.03099	D

Anthracene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	54.95	0.001

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.03834	4	0	1
trt	7500	0.247	0.03834	4	6.44	0.003
trt	20000	0.4165	0.03834	4	10.86	0.0004
trt	30000	0.6751	0.03834	4	17.61	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.247	0.05422	4	-4.56	0.0104	Tukey	0.0346
trt	0	20000	-0.4165	0.05422	4	-7.68	0.0015	Tukey	0.0053
trt	0	30000	-0.6751	0.05422	4	-12.45	0.0002	Tukey	0.0008
trt	7500	20000	-0.1695	0.05422	4	-3.13	0.0353	Tukey	0.111
trt	7500	30000	-0.4281	0.05422	4	-7.9	0.0014	Tukey	0.0048
trt	20000	30000	-0.2586	0.05422	4	-4.77	0.0088	Tukey	0.0296

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	0.6751	0.03834	A
2	20000	0.4165	0.03834	B
3	7500	0.247	0.03834	B
4	0	0	0.03834	C

Benzo[a]anthracene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	11.33	0.02

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.008417	4	0	1
trt	7500	0.00525	0.008417	4	0.62	0.5666
trt	20000	0.03575	0.008417	4	4.25	0.0132
trt	30000	0.06075	0.008417	4	7.22	0.002

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.00525	0.0119	4	-0.44	0.682	Tukey	0.968
trt	0	20000	-0.03575	0.0119	4	-3	0.0398	Tukey	0.1241
trt	0	30000	-0.06075	0.0119	4	-5.1	0.007	Tukey	0.0235
trt	7500	20000	-0.0305	0.0119	4	-2.56	0.0625	Tukey	0.1873
trt	7500	30000	-0.0555	0.0119	4	-4.66	0.0096	Tukey	0.032
trt	20000	30000	-0.025	0.0119	4	-2.1	0.1036	Tukey	0.2922

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	0.06075	0.008417	A
2	20000	0.03575	0.008417	AB
3	7500	0.00525	0.008417	B
4	0	0	0.008417	B

Benzo[b]fluorene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	26.67	0.0042

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.0279	4	0	1
trt	7500	0.1414	0.0279	4	5.07	0.0071
trt	20000	0.2505	0.0279	4	8.98	0.0009
trt	30000	0.3331	0.0279	4	11.94	0.0003

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.1414	0.03946	4	-3.58	0.0231	Tukey	0.0745
trt	0	20000	-0.2505	0.03946	4	-6.35	0.0032	Tukey	0.0108
trt	0	30000	-0.3331	0.03946	4	-8.44	0.0011	Tukey	0.0037
trt	7500	20000	-0.1091	0.03946	4	-2.77	0.0506	Tukey	0.1546
trt	7500	30000	-0.1918	0.03946	4	-4.86	0.0083	Tukey	0.0278
trt	20000	30000	-0.08263	0.03946	4	-2.09	0.1044	Tukey	0.2941

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	0.3331	0.0279	A
2	20000	0.2505	0.0279	AB
3	7500	0.1414	0.0279	BC
4	0	0	0.0279	C

Biphenyl

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	153.27	0.0001

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.7072	4	0	1
trt	7500	6.554	0.7072	4	9.27	0.0008
trt	20000	11.9948	0.7072	4	16.96	<.0001
trt	30000	20.6893	0.7072	4	29.26	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-6.554	1.0001	4	-6.55	0.0028	Tukey	0.0096
trt	0	20000	-11.9948	1.0001	4	-11.99	0.0003	Tukey	0.001
trt	0	30000	-20.6893	1.0001	4	-20.69	<.0001	Tukey	0.0001
trt	7500	20000	-5.4408	1.0001	4	-5.44	0.0055	Tukey	0.0188
trt	7500	30000	-14.1353	1.0001	4	-14.13	0.0001	Tukey	0.0005
trt	20000	30000	-8.6945	1.0001	4	-8.69	0.001	Tukey	0.0034

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	20.6893	0.7072	A
2	20000	11.9948	0.7072	B
3	7500	6.554	0.7072	C
4	0	0	0.7072	D

Chrysene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	74.47	0.0006

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.02078	4	0	1
trt	7500	0.1959	0.02078	4	9.43	0.0007
trt	20000	0.3322	0.02078	4	15.99	<.0001
trt	30000	0.4089	0.02078	4	19.68	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.1959	0.02938	4	-6.67	0.0026	Tukey	0.009
trt	0	20000	-0.3322	0.02938	4	-11.31	0.0003	Tukey	0.0012
trt	0	30000	-0.4089	0.02938	4	-13.92	0.0002	Tukey	0.0005
trt	7500	20000	-0.1364	0.02938	4	-4.64	0.0097	Tukey	0.0325
trt	7500	30000	-0.213	0.02938	4	-7.25	0.0019	Tukey	0.0066
trt	20000	30000	-0.07663	0.02938	4	-2.61	0.0596	Tukey	0.1794

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	0.4089	0.02078	A
2	20000	0.3322	0.02078	A
3	7500	0.1959	0.02078	B
4	0	0	0.02078	C

Dibenzofuran

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	19.39	0.0076

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.2233	4	0	1
trt	7500	0.8526	0.2233	4	3.82	0.0188
trt	20000	1.6363	0.2233	4	7.33	0.0018
trt	30000	2.2722	0.2233	4	10.17	0.0005

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.8526	0.3158	4	-2.7	0.0541	Tukey	0.1644
trt	0	20000	-1.6363	0.3158	4	-5.18	0.0066	Tukey	0.0223
trt	0	30000	-2.2722	0.3158	4	-7.19	0.002	Tukey	0.0068
trt	7500	20000	-0.7836	0.3158	4	-2.48	0.0681	Tukey	0.2024
trt	7500	30000	-1.4196	0.3158	4	-4.5	0.0109	Tukey	0.0362
trt	20000	30000	-0.636	0.3158	4	-2.01	0.1143	Tukey	0.3177

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	2.2722	0.2233	A
2	20000	1.6363	0.2233	AB
3	7500	0.8526	0.2233	BC
4	0	0	0.2233	C

Dibenzothiophene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	36.3	0.0023

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.16	4	0	1
trt	7500	0.9614	0.16	4	6.01	0.0039
trt	20000	1.5734	0.16	4	9.84	0.0006
trt	30000	2.2725	0.16	4	14.21	0.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.9614	0.2262	4	-4.25	0.0132	Tukey	0.0435
trt	0	20000	-1.5734	0.2262	4	-6.96	0.0022	Tukey	0.0077
trt	0	30000	-2.2725	0.2262	4	-10.05	0.0006	Tukey	0.0019
trt	7500	20000	-0.612	0.2262	4	-2.71	0.0538	Tukey	0.1635
trt	7500	30000	-1.3111	0.2262	4	-5.8	0.0044	Tukey	0.015
trt	20000	30000	-0.6991	0.2262	4	-3.09	0.0366	Tukey	0.1146

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	2.2725	0.16	A
2	20000	1.5734	0.16	AB
3	7500	0.9614	0.16	B
4	0	0	0.16	C

Fluoranthene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	39.74	0.002

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.01147	4	0	1
trt	7500	0.07825	0.01147	4	6.82	0.0024
trt	20000	0.1274	0.01147	4	11.11	0.0004
trt	30000	0.168	0.01147	4	14.65	0.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.07825	0.01622	4	-4.83	0.0085	Tukey	0.0285
trt	0	20000	-0.1274	0.01622	4	-7.86	0.0014	Tukey	0.0049
trt	0	30000	-0.168	0.01622	4	-10.36	0.0005	Tukey	0.0017
trt	7500	20000	-0.04912	0.01622	4	-3.03	0.0388	Tukey	0.1211
trt	7500	30000	-0.08975	0.01622	4	-5.53	0.0052	Tukey	0.0177
trt	20000	30000	-0.04063	0.01622	4	-2.51	0.0664	Tukey	0.1978

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	0.168	0.01147	A
2	20000	0.1274	0.01147	AB
3	7500	0.07825	0.01147	B
4	0	0	0.01147	C

Fluorene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	86.93	0.0004

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.661	4	0	1
trt	7500	5.2316	0.661	4	7.91	0.0014
trt	20000	8.9375	0.661	4	13.52	0.0002
trt	30000	14.6319	0.661	4	22.13	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-5.2316	0.9349	4	-5.6	0.005	Tukey	0.017
trt	0	20000	-8.9375	0.9349	4	-9.56	0.0007	Tukey	0.0023
trt	0	30000	-14.6319	0.9349	4	-15.65	<.0001	Tukey	0.0003
trt	7500	20000	-3.7059	0.9349	4	-3.96	0.0166	Tukey	0.0544
trt	7500	30000	-9.4002	0.9349	4	-10.06	0.0006	Tukey	0.0019
trt	20000	30000	-5.6944	0.9349	4	-6.09	0.0037	Tukey	0.0126

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	14.6319	0.661	A
2	20000	8.9375	0.661	B
3	7500	5.2316	0.661	B
4	0	0	0.661	C

4-Methyldibenzothiophene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	114.65	0.0002

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.1069	4	0	1
trt	7500	1.28	0.1069	4	11.98	0.0003
trt	20000	1.9664	0.1069	4	18.4	<.0001
trt	30000	2.6893	0.1069	4	25.16	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-1.28	0.1512	4	-8.47	0.0011	Tukey	0.0037
trt	0	20000	-1.9664	0.1512	4	-13.01	0.0002	Tukey	0.0007
trt	0	30000	-2.6893	0.1512	4	-17.79	<.0001	Tukey	0.0002
trt	7500	20000	-0.6864	0.1512	4	-4.54	0.0105	Tukey	0.035
trt	7500	30000	-1.4093	0.1512	4	-9.32	0.0007	Tukey	0.0026
trt	20000	30000	-0.7229	0.1512	4	-4.78	0.0088	Tukey	0.0294

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	2.6893	0.1069	A
2	20000	1.9664	0.1069	B
3	7500	1.28	0.1069	C
4	0	0	0.1069	D

Naphthalene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	38.37	0.0021

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0.09025	0.3543	4	0.25	0.8115
trt	7500	2.2976	0.3543	4	6.49	0.0029
trt	20000	5.4156	0.3543	4	15.29	0.0001
trt	30000	3.0208	0.3543	4	8.53	0.001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-2.2074	0.501	4	-4.41	0.0116	Tukey	0.0386
trt	0	20000	-5.3254	0.501	4	-10.63	0.0004	Tukey	0.0016
trt	0	30000	-2.9305	0.501	4	-5.85	0.0043	Tukey	0.0145
trt	7500	20000	-3.118	0.501	4	-6.22	0.0034	Tukey	0.0116
trt	7500	30000	-0.7231	0.501	4	-1.44	0.2224	Tukey	0.5392
trt	20000	30000	2.3949	0.501	4	4.78	0.0088	Tukey	0.0294

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	20000	5.4156	0.3543	A
2	30000	3.0208	0.3543	B
3	7500	2.2976	0.3543	B
4	0	0.09025	0.3543	C

1-Methylphenanthrene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	87.72	0.0004

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.3965	4	0	1
trt	7500	4.9373	0.3965	4	12.45	0.0002
trt	20000	6.3619	0.3965	4	16.04	<.0001
trt	30000	8.8104	0.3965	4	22.22	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-4.9373	0.5607	4	-8.8	0.0009	Tukey	0.0032
trt	0	20000	-6.3619	0.5607	4	-11.35	0.0003	Tukey	0.0012
trt	0	30000	-8.8104	0.5607	4	-15.71	<.0001	Tukey	0.0003
trt	7500	20000	-1.4246	0.5607	4	-2.54	0.0639	Tukey	0.1912
trt	7500	30000	-3.8731	0.5607	4	-6.91	0.0023	Tukey	0.0079
trt	20000	30000	-2.4485	0.5607	4	-4.37	0.012	Tukey	0.0398

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	8.8104	0.3965	A
2	20000	6.3619	0.3965	B
3	7500	4.9373	0.3965	B
4	0	0	0.3965	C

1-Methylpyrene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	5.37	0.0691

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.03079	4	0	1
trt	7500	0.03175	0.03079	4	1.03	0.3607
trt	20000	0.144	0.03079	4	4.68	0.0095
trt	30000	0.1299	0.03079	4	4.22	0.0135

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.03175	0.04354	4	-0.73	0.5063	Tukey	0.881
trt	0	20000	-0.144	0.04354	4	-3.31	0.0297	Tukey	0.0945
trt	0	30000	-0.1299	0.04354	4	-2.98	0.0406	Tukey	0.1264
trt	7500	20000	-0.1123	0.04354	4	-2.58	0.0614	Tukey	0.1845
trt	7500	30000	-0.09813	0.04354	4	-2.25	0.0873	Tukey	0.252
trt	20000	30000	0.01412	0.04354	4	0.32	0.7619	Tukey	0.9865

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	20000	0.144	0.03079	A
2	30000	0.1299	0.03079	A
3	7500	0.03175	0.03079	A
4	0	0	0.03079	A

Phenanthrene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	36.43	0.0023

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	1.353	4	0	1
trt	7500	7.784	1.353	4	5.75	0.0045
trt	20000	11.4672	1.353	4	8.48	0.0011
trt	30000	19.659	1.353	4	14.53	0.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-7.784	1.9134	4	-4.07	0.0152	Tukey	0.0501
trt	0	20000	-11.4672	1.9134	4	-5.99	0.0039	Tukey	0.0133
trt	0	30000	-19.659	1.9134	4	-10.27	0.0005	Tukey	0.0018
trt	7500	20000	-3.6833	1.9134	4	-1.93	0.1265	Tukey	0.3459
trt	7500	30000	-11.875	1.9134	4	-6.21	0.0034	Tukey	0.0117
trt	20000	30000	-8.1918	1.9134	4	-4.28	0.0128	Tukey	0.0425

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	19.659	1.353	A
2	20000	11.4672	1.353	B
3	7500	7.784	1.353	BC
4	0	0	1.353	C

Pyrene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	70.83	0.0006

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	-5.55E-17	0.0167	4	0	1
trt	7500	0.139	0.0167	4	8.32	0.0011
trt	20000	0.2571	0.0167	4	15.4	0.0001
trt	30000	0.3188	0.0167	4	19.09	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-0.139	0.02362	4	-5.88	0.0042	Tukey	0.0142
trt	0	20000	-0.2571	0.02362	4	-10.89	0.0004	Tukey	0.0014
trt	0	30000	-0.3188	0.02362	4	-13.5	0.0002	Tukey	0.0006
trt	7500	20000	-0.1181	0.02362	4	-5	0.0075	Tukey	0.0252
trt	7500	30000	-0.1798	0.02362	4	-7.61	0.0016	Tukey	0.0055
trt	20000	30000	-0.06163	0.02362	4	-2.61	0.0595	Tukey	0.1791

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	0.3188	0.0167	A
2	20000	0.2571	0.0167	A
3	7500	0.139	0.0167	B
4	0	-5.55E-17	0.0167	C

2-Methylanthracene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	22.86	0.0056

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0	0.5945	4	0	1
trt	7500	2.7564	0.5945	4	4.64	0.0098
trt	20000	4.1474	0.5945	4	6.98	0.0022
trt	30000	6.8213	0.5945	4	11.47	0.0003

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-2.7564	0.8408	4	-3.28	0.0305	Tukey	0.0969
trt	0	20000	-4.1474	0.8408	4	-4.93	0.0079	Tukey	0.0264
trt	0	30000	-6.8213	0.8408	4	-8.11	0.0013	Tukey	0.0044
trt	7500	20000	-1.391	0.8408	4	-1.65	0.1734	Tukey	0.4463
trt	7500	30000	-4.0649	0.8408	4	-4.83	0.0084	Tukey	0.0283
trt	20000	30000	-2.6739	0.8408	4	-3.18	0.0335	Tukey	0.1057

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	30000	6.8213	0.5945	A
2	20000	4.1474	0.5945	AB
3	7500	2.7564	0.5945	BC
4	0	0	0.5945	C

2-Methylnaphthalene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	26.59	0.0042

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	0.1525	2.2829	4	0.07	0.9499
trt	7500	11.6146	2.2829	4	5.09	0.007
trt	20000	25.2706	2.2829	4	11.07	0.0004
trt	30000	23.8581	2.2829	4	10.45	0.0005

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-11.4621	3.2285	4	-3.55	0.0238	Tukey	0.0766
trt	0	20000	-25.1181	3.2285	4	-7.78	0.0015	Tukey	0.0051
trt	0	30000	-23.7056	3.2285	4	-7.34	0.0018	Tukey	0.0063
trt	7500	20000	-13.656	3.2285	4	-4.23	0.0134	Tukey	0.0442
trt	7500	30000	-12.2435	3.2285	4	-3.79	0.0192	Tukey	0.0626
trt	20000	30000	1.4125	3.2285	4	0.44	0.6843	Tukey	0.9687

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	**trt**	**Estimate**	**StdErr**	**MSGROUP**
1	20000	25.2706	2.2829	A
2	30000	23.8581	2.2829	AB
3	7500	11.6146	2.2829	BC
4	0	0.1525	2.2829	C

2-Methylphenanthrene

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
trt	3	4	48	0.0014

Least Squares Means						
Effect	**trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
trt	0	-8.88E-16	0.454	4	0	1
trt	7500	3.7892	0.454	4	8.35	0.0011
trt	20000	5.1076	0.454	4	11.25	0.0004
trt	30000	7.5291	0.454	4	16.58	<.0001

Differences of Least Squares Means									
Effect	**trt**	**_trt**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adjustment**	**Adj P**
trt	0	7500	-3.7893	0.642	4	-5.9	0.0041	Tukey	0.0141
trt	0	20000	-5.1076	0.642	4	-7.96	0.0014	Tukey	0.0047
trt	0	30000	-7.5291	0.642	4	-11.73	0.0003	Tukey	0.0011
trt	7500	20000	-1.3184	0.642	4	-2.05	0.1093	Tukey	0.3057
trt	7500	30000	-3.7399	0.642	4	-5.83	0.0043	Tukey	0.0147
trt	20000	30000	-2.4215	0.642	4	-3.77	0.0196	Tukey	0.0636

Effect=trt ADJUSTMENT=Tukey(P<0.05) bygroup=1

Obs	trt	Estimate	StdErr	MSGROUP
1	30000	7.5291	0.454	A
2	20000	5.1076	0.454	AB
3	7500	3.7892	0.454	B
4	0	-8.88E-16	0.454	C

Appendix C. Data Tables of Recovered Concentrations of PAHs

Control

	LSU Sample ID	GT15274C.D	GT15274D.D	GT15274E.D	GT15274F.D	GT15274G.D	GT15274H.D	GT15274I.D	GT15274J.D
	Field ID	4701	4702	4703	4704	4705	4706	4707	4708
	Exposure Concentration	Control	Control	Control	Control	Control	Control	Control	Control
	Tank	1	1	1	1	2	2	2	2
	Number in Tank	1	2	3	4	1	2	3	4
	Weight (g)	0.57	1.09	0.62	0.94	0.92	0.91	0.97	1.07
Rings	Name	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)
2	Naphthalene	0.110	0.078	0.100	0.082	0.112	0.076	0.095	0.069
2	Benzothiophene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	2-Methylnaphthalene	0.105	0.146	0.187	0.111	0.278	0.120	0.141	0.132
2	Biphenyl	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Acenaphthylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Acenaphthene-d10 SSTD #1	0.871	0.501	0.767	0.530	0.575	0.573	0.477	0.447
3	Acenaphthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Dibenzofuran	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Fluorene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Dibenzothiophene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Phenanthrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Carbozole	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	4-Methyldibenzothiophene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	2-Methylphenanthrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	2-Methylanthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	1-Methylphenanthrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	Fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	Pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	Benzo(b)fluorene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	1-Methylpyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	Benzo(a)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	Chrysene-d12 SSTD #2	0.961	0.528	0.932	0.614	0.622	0.613	0.591	0.488
4	Chrysene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	5-Methylchrysene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(b)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(k)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(e)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Indeno(1,2,3-cd)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Dibenz(a,h)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Benzo(g,h,i)perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Surrogate Recovery								
	Acenaphthene-d10 SSTD #1	99.280	109.186	95.168	99.561	105.878	104.241	92.450	95.741
	Chrysene-d12 SSTD #2	109.591	115.097	115.573	115.412	114.362	111.648	114.623	104.381
	Total Aromatics (µg/g)	0.215	0.224	0.287	0.193	0.389	0.196	0.236	0.201
	Total 2 & 3 Ring (µg/g)	0.215	0.224	0.287	0.193	0.389	0.196	0.236	0.201
	Total 4 & 5 Ring (µg/g)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

7,500 PPM

	LSU Sample ID	GT15289C.D	GT15289D.D	GT15289E.D	GT15289F.D	GT15289G.D	GT15289H.D	GT15289I.D	GT15289J.D
	Field ID	4717	4718	4719	4720	4721	4722	4723	4724
	Exposure Concentration	7500 ppm	7500 ppm	7500 ppm	7500 ppm	7500 ppm	7500 ppm	7500 ppm	7500 ppm
	Tank	5	5	5	5	6	6	6	6
	Number within tank	1	2	3	4	1	2	3	4
	Weight (g)	0.67	0.63	0.64	0.6	0.92	0.69	0.71	0.82
Rings	Name	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)
2	Naphthalene	1.260	2.144	1.850	2.530	1.578	0.446	5.084	3.489
2	Benzothiophene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	2-Methylnaphthalene	8.389	11.798	11.311	15.117	8.167	3.559	20.293	14.283
2	Biphenyl	6.320	6.377	7.242	8.868	5.034	4.223	7.697	6.671
3	Acenaphthylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Acenaphthene-d10 SSTD #1	0.711	0.758	0.777	0.779	0.529	0.790	0.650	0.596
3	Acenaphthene	0.378	0.336	0.377	0.423	0.262	0.258	0.360	0.323
3	Dibenzofuran	0.845	0.882	0.947	1.131	0.686	0.518	0.961	0.851
3	Fluorene	5.664	5.160	5.788	7.329	3.977	3.722	5.326	4.887
3	Dibenzothiophene	1.060	0.998	1.137	1.365	0.665	0.699	0.926	0.841
3	Phenanthrene	9.299	7.944	9.232	11.196	5.009	5.549	7.300	6.743
3	Anthracene	0.299	0.262	0.320	0.303	0.132	0.200	0.244	0.216
3	Carbozole	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	4-Methyldibenzothiophene	1.581	1.222	1.392	1.585	0.954	1.335	1.045	1.126
3	2-Methylphenanthrene	5.176	3.312	4.478	5.197	2.316	3.791	3.034	3.010
3	2-Methylanthracene	4.818	2.100	3.622	3.959	1.227	2.383	2.077	1.865
3	1-Methylphenanthrene	6.312	4.593	5.490	6.188	3.578	5.497	3.939	3.901
4	Fluoranthene	0.074	0.074	0.090	0.118	0.064	0.075	0.060	0.071
4	Pyrene	0.147	0.133	0.149	0.201	0.097	0.137	0.109	0.139
4	Benzo(b)fluorene	0.205	0.105	0.181	0.208	0.081	0.148	0.086	0.117
4	1-Methylpyrene	0.063	0.000	0.057	0.075	0.000	0.059	0.000	0.000
4	Benzo(a)anthracene	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4	Chrysene-d12 SSTD #2	0.771	0.847	0.860	0.916	0.606	0.811	0.741	0.662
4	Chrysene	0.226	0.175	0.218	0.285	0.127	0.197	0.150	0.189
4	5-Methylchrysene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(b)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(k)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(e)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Indeno(1,2,3-cd)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Dibenz(a,h)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Benzo(g,h,i)perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Surrogate Recovery								
	Acenaphthene-d10 SSTD #1	95.275	95.530	99.500	93.484	97.413	109.001	92.269	97.794
	Chrysene-d12 SSTD #2	103.366	106.735	110.132	109.902	111.442	111.859	105.206	108.517
	Total Aromatics (µg/g)	52.156	47.614	53.883	66.077	33.952	32.797	58.690	48.722
	Total 2 & 3 Ring (µg/g)	51.400	47.127	53.187	65.190	33.583	32.181	58.286	48.206
	Total 4 & 5 Ring (µg/g)	0.756	0.487	0.696	0.887	0.369	0.616	0.404	0.516

20,000 PPM

	LSU Sample ID	GT15281C.D	GT15281D.D	GT15281E.D	GT15281F.D	GT15281G.D	GT15281H.D	GT15281I.D	GT15281J.D
	Field ID	4733	4734	4735	4736	4737	4738	4739	4540
	Exposure Concentration	20000 ppm	20000 ppm	20000 ppm	20000 ppm	20000 ppm	20000 ppm	20000 ppm	20000 ppm
	Tank	9	9	9	9	10	10	10	10
	Number in Tank	1	2	3	4	1	2	3	4
	Weight (g)	0.61	1.19	0.88	0.99	0.67	0.62	0.51	0.47
Rings	Name	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)
2	Naphthalene	5.043	8.171	4.404	5.925	4.115	2.520	1.969	11.178
2	Benzothiophene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	2-Methylnaphthalene	30.009	36.868	21.765	28.337	21.887	15.833	14.528	32.938
2	Biphenyl	13.964	14.936	12.034	11.732	12.873	12.046	10.600	7.773
3	Acenaphthylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Acenaphthene-d10 SSTD #1	0.742	0.345	0.459	0.461	0.746	0.843	0.988	1.080
3	Acenaphthene	0.827	0.882	0.560	0.532	0.748	0.648	0.664	0.355
3	Dibenzofuran	2.130	2.410	1.610	1.553	1.586	1.366	1.307	1.128
3	Fluorene	11.816	12.367	7.911	7.974	9.306	8.480	8.402	5.244
3	Dibenzothiophene	2.277	2.197	1.394	1.477	1.426	1.470	1.458	0.888
3	Phenanthrene	16.630	16.461	10.890	10.319	10.092	10.103	10.970	6.273
3	Anthracene	0.617	0.640	0.316	0.294	0.409	0.402	0.434	0.220
3	Carbozole	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	4-Methyldibenzothiophene	2.421	2.276	1.598	1.454	2.071	2.380	2.820	0.711
3	2-Methylphenanthrene	7.048	6.407	4.231	4.162	4.635	5.690	6.782	1.906
3	2-Methylanthracene	6.249	6.480	3.192	3.346	3.269	3.530	5.284	1.829
3	1-Methylphenanthrene	8.230	6.751	5.093	4.814	6.842	7.913	9.165	2.087
4	Fluoranthene	0.127	0.205	0.133	0.104	0.155	0.141	0.154	0.000
4	Pyrene	0.240	0.391	0.258	0.179	0.307	0.283	0.335	0.064
4	Benzo(b)fluorene	0.242	0.398	0.210	0.203	0.280	0.251	0.354	0.066
4	1-Methylpyrene	0.103	0.138	0.099	0.000	0.258	0.240	0.314	0.000
4	Benzo(a)anthracene	0.055	0.078	0.037	0.000	0.000	0.041	0.075	0.000
4	Chrysene-d12 SSTD #2	0.861	0.487	0.653	0.589	0.851	0.911	1.133	1.197
4	Chrysene	0.369	0.456	0.312	0.207	0.364	0.363	0.500	0.087
4	5-Methylchrysene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(b)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(k)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(e)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Indeno(1,2,3-cd)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Dibenz(a,h)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Benzo(g,h,i)perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Surrogate Recovery								
	Acenaphthene-d10 SSTD #1	90.506	82.033	80.811	91.185	99.951	104.508	100.776	101.538
	Chrysene-d12 SSTD #2	105.062	115.847	114.880	116.589	114.077	113.019	115.594	112.548
	Total Aromatics (µg/g)	108.395	118.510	76.048	82.613	80.624	73.700	76.113	72.747
	Total 2 & 3 Ring (µg/g)	107.260	116.846	74.999	81.919	79.260	72.382	74.383	72.530
	Total 4 & 5 Ring (µg/g)	1.136	1.665	1.049	0.694	1.364	1.318	1.731	0.217

30,000 PPM

	LSU Sample ID	GT15268C.D	GT15268D.D	GT15268E.D	GT15268F.D	GT15268G.D	GT15268H.D	GT15268I.D	GT15268J.D
	Field ID	4741	4742	4743	4744	4745	4746	4747	4748
	Exposure Concentration	30000ppm	30000ppm	30000ppm	30000ppm	30000ppm	30000ppm	30000ppm	30000ppm
	Tank	11	11	11	11	12	12	12	12
	Number in Tank	1	2	3	4	1	2	3	4
	Weight (g)	0.68	0.47	0.48	0.93	0.47	0.76	0.5	0.39
Rings	Name	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)	Conc. (ug/g)
2	Naphthalene	0.661	3.065	4.246	2.524	4.356	3.295	2.862	3.157
2	Benzothiophene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	2-Methylnaphthalene	8.101	24.146	29.088	25.108	37.460	22.258	21.031	23.673
2	Biphenyl	17.695	18.752	20.181	24.305	24.059	17.218	22.634	20.670
3	Acenaphthylene	0.614	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	Acenaphthene-d10 SSTD #1	0.767	1.135	1.100	0.540	1.082	0.642	1.033	1.420
3	Acenaphthene	0.869	1.100	1.065	1.576	1.458	0.809	1.096	1.255
3	Dibenzofuran	0.185	2.073	2.347	3.182	3.107	2.018	2.616	2.650
3	Fluorene	13.161	12.828	13.494	19.529	17.635	10.660	14.634	15.114
3	Dibenzothiophene	1.833	1.902	2.092	3.117	2.887	1.676	2.293	2.380
3	Phenanthrene	16.488	15.491	17.280	27.543	24.490	14.129	20.615	21.236
3	Anthracene	0.696	0.670	0.709	0.749	0.694	0.483	0.649	0.751
3	Carbozole	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	4-Methyldibenzothiophene	2.170	2.408	2.442	4.268	3.084	1.709	2.727	2.706
3	2-Methylphenanthrene	6.885	6.568	6.336	11.792	8.574	4.585	7.654	7.839
3	2-Methylanthracene	5.496	5.653	5.481	12.495	7.986	3.877	6.657	6.925
3	1-Methylphenanthrene	7.786	7.965	7.647	13.154	10.120	5.440	8.643	9.728
4	Fluoranthene	0.112	0.153	0.150	0.312	0.198	0.100	0.175	0.144
4	Pyrene	0.181	0.271	0.268	0.659	0.390	0.175	0.354	0.252
4	Benzo(b)fluorene	0.290	0.278	0.258	0.678	0.376	0.177	0.330	0.278
4	1-Methylpyrene	0.063	0.118	0.110	0.247	0.123	0.000	0.126	0.252
4	Benzo(a)anthracene	0.054	0.057	0.049	0.141	0.071	0.000	0.061	0.053
4	Chrysene-d12 SSTD #2	0.823	1.129	1.109	0.602	1.182	0.673	1.090	1.430
4	Chrysene	0.250	0.365	0.349	0.785	0.459	0.221	0.449	0.393
4	5-Methylchrysene	0.000	0.000	0.000	0.134	0.000	0.000	0.000	0.000
5	Benzo(b)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(k)fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(e)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Indeno(1,2,3-cd)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	Dibenz(a,h)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	Benzo(g,h,i)perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Surrogate Recovery								
	Acenaphthene-d10 SSTD #1	104.266	106.673	105.568	100.404	101.673	97.615	103.301	110.778
	Chrysene-d12 SSTD #2	111.877	106.087	106.450	111.978	111.093	102.247	109.003	111.543
	Total Aromatics (µg/g)	83.591	103.864	113.592	152.297	147.526	88.831	115.605	119.456
	Total 2 & 3 Ring (µg/g)	82.640	102.622	112.408	149.341	145.909	88.158	114.110	118.084
	Total 4 & 5 Ring (µg/g)	0.951	1.242	1.184	2.956	1.617	0.673	1.495	1.372

VITA

Brittany Lynn Chichester a native of Schoharie, New York received her bachelor's degree at Southeastern Louisiana University in Hammond, Louisiana in 2009. Thereafter, she began pursuit of her master's degree in Baton Rouge, Louisiana at Louisiana State University in the School of the Coast and the Environment in August of 2013. She will receive her master's degree in May 2016