INTEGRATION OF POLLUTANT BODY-BURDENS WITH FATTY ACID BIOMARKERS OF TROPHIC ECOLOGY IN FISH FROM SABINE LAKE, TEXAS

A Thesis

by

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ABSTRACT

The hepatic body-burdens of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were integrated with fatty acid levels in four fish species from Sabine Lake (TX), namely: bull shark (*Carcharhinus leucas*), alligator gar (*Atractosteus spatula*), red drum (*Sciaenops ocellatus*) and gafftopsail catfish (*Bagre marinus*). Fatty acids were used as tracers of dietary preferences, and as PAHs and PCBs bio-accumulate, it was anticipated that dietary preference may delineate the source of pollutant exposure. Results showed unique PAH and PCB profiles in fish. Specifically, PAH profiles indicated mainly petrogenic sources of exposure. The multivariate analysis of pollutant hepatic body-burdens indicated bull sharks to have a distinct profile relative to the other fish species. This trend was confirmed by the observation that bull sharks also exhibited a significantly higher body-burden of PCBs relative to the other fish species. The risk assessment of the high PCB body-burden in bull sharks was also reflected in a high toxic equivalent (TEQ) value. The TEQ level in bull sharks was above the upper limits of toxicity thresholds reported for fish and aquatic mammals. The size range of bull sharks indicated young-of-the-year (YoY) life stages. Therefore, a unique exposure history for YoY bull sharks is postulated, and implicates a role of maternal transfer. The analysis of fatty acid profiles in fish showed elevated levels (45x higher) of nervonic acid (24:1n9) (indicator of calanoid copepods and soil filamentous fungi), in gafftopsail catfish and red drum vs. alligator gar and bull shark. This indicated that the feeding ecology of catfish and red drum was ‘closer’ to the base of the marine food web. The correlational analysis of pollutant body-burdens with fatty acids indicated no clear distinction or preference of pollutant correlation with any particular type of fatty acid. My results suggest that pollutant bioaccumulation is

unlikely to be associated with the specific fatty acid composition of lipids. Finally, my results showed that while only 24% of PCBs were highly correlated with fatty acids, 57% of these were dioxin-like PCBs. Therefore, future efforts may aim to understand whether the intrinsic pollutant body-burdens in fish from Sabine Lake are capable of exerting quantifiable toxicity effects.

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Contributors

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CHAPTER I

INTRODUCTION

Background and objective

The Gulf of Mexico is a heavily prospected region for offshore oil and gas production, with crude oil production at an estimated 1.7 million barrels/day in January 2017 (U.S. Energy Information Administration, 2017). Offshore exploration and production contributes to 30% of U.S. oil and 10% of U.S. natural gas production (Diffley et al., 2010). The collective revenue generated by such activities in the Gulf of Mexico was ~$3.8 billion in 2017 (U.S. Department of the Interior, 2017).

However, such a high level of petrochemical industrialization has contributed to the pollution of various aquatic ecosystems along the Gulf of Mexico by oil-derived polycyclic aromatic hydrocarbons (PAHs), and 'legacy' industrial pollutants, such as polychlorinated biphenyls (PCBs) (Howell et al., 2008; Katner et al., 2010; Lakshmanan et al., 2010; Oziolor et al., 2018; Qian et al., 2001; Santschi et al., 2001; Willett et al., 1997). For example in Galveston Bay, which comprises a major route for oil tanker traffic (as it connects the northern Gulf of Mexico with the Houston Ship Channel), and whose coastline harbors major oil refineries, an estimated average of 275 oil spills/year with average amount of oil spilt ranging from 1 – 100 gallons per spill have occurred during a period encompassing from 1998 – 2014 (HARC, 2014).

There are exceptions to such estimates, case in point being the recent petrochemical fire and leak of oil storage tanks from March 17th to 20th, 2019 at the International Terminals Company (ITC) in Deer Park (Houston, TX). This event was soon followed by a barge collision which spilled gasoline (May 10th, 2019) into the upper Galveston Bay. At present, the extent of

oil leak into the surrounding waters is not fully known. The Deer Park fire is estimated to have released ~696,990 gallons of oil-contaminated water and ~1.5 million gallons of flame retardants (Rice, 2019). Whereas, the barge spill is estimated to have released ~378,000 gallons of gasoline into Galveston Bay (Trevizo, 2019). The magnitude of these disturbances is suspected to have a significant impact on the local and national economy due to a partial closure of adjacent waterways of the Houston Ship Channel, and estimates of economic impacts ranging from $0.5 – $1 billion (Leinfelder and Blum, 2019; Trevizo, 2019).

Furthermore, the propensity of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) to bio-concentrate in organisms, bio-accumulate across food webs, and exert toxicity has led to environmental monitoring efforts to quantify their levels in various ecosystems (Biddinger and Gloss, 1984; El-Shahawi et al., 2010; Jensen et al., 1969; Livingstone, 1998; Suedel et al., 1994). Ultimately, sediments and biota act as reservoirs and refuges for these pollutants in the environment. Such sequestration ensures their long-term persistence, contributing to chronic toxicity in exposed organisms (Bejarano and Michel, 2010; Peterson et al., 2003; Randolph et al., 1998).

The sources of PAHs in the marine environment are mainly from pyrogenic (combustion of petroleum-derived hydrocarbons) or petrogenic (petroleum-derived) sources (Hylland, 2006). These hydrocarbons comprise a mixture of various alkyl (short or long chain), cyclic (naphthenes or cycloalkanes) and aryl (polycyclic aromatic ≥ 2 aromatic rings) hydrocarbons, some of which also contain sulfur (ethanethiol) and nitrogen atoms (carbazole) (Eneh, 2011). Of these, lower molecular weight hydrocarbons (LMWs, ≤3 aromatic rings) are volatile and are easily degraded in the environment, while high molecular weight hydrocarbons (HMWs, ≥4 aromatic rings) are more persistent and toxic. For example, increasing molecular weights (and

'ring' numbers) of polycyclic aromatic hydrocarbons positively correlate with bioaccumulation potential and toxicity (Black et al., 1983; Southworth et al., 1978). The spectrum of adverse health effects in exposed organism includes metabolic (energy loss), hemolytic (anemia), endocrine, osmoregulatory and teratogenic effects (Vandermeulen, 1987).

In contrast, PCBs constitute 'legacy' or persistent organic pollutants (POPs), and comprise a diverse family of compounds that possess from 1-10 chlorine atoms distributed across two fused benzene rings (Safe et al., 1985). PCBs were originally used as dielectrics and coolants in electrical devises until they were banned in the late 1970s due to environmental concerns (Boyle and Highland, 1979). PCBs are still detected in various aquatic ecosystems due to their persistence, long half-lives and low biodegradation in exposed biota and the environment (Porta and Zumeta).

At present, pollution monitoring studies mainly focus attention on the Galveston Bay watershed. This is mainly as Galveston Bay comprises a catchment area for major industrial, agricultural, municipal point- and non-point sources of nutrient and pollutant discharges (Houston is the fourth most populous city in the U.S.). As a result, Galveston Bay and its surrounding areas (such as the Houston Ship Channel) have been a key focus for environmental health monitoring studies which have quantified elevated levels of PAHs, PCBs, dioxins, and metals in resident biota, waters and sediment samples (Howell et al., 2008; Lakshmanan et al., 2010; Oziolor et al., 2018; Qian et al., 2001; Suarez et al., 2006; Willett et al., 1997).

This project focusses environmental monitoring efforts on Sabine Lake, a large coastal estuary on the Texas-Louisiana border in the northwestern Gulf of Mexico, constitutes mainly salt marsh habitats with a myriad of channels and bayous. Sabine Lake drains directly into the northwestern Gulf of Mexico through a narrow opening called Sabine Pass (Kane, 1959). This

lake occupies 93 square miles of the southernmost border separating the Texas and Louisiana coast. It serves as a catchment to approximately 500,000 square miles of Texas and Louisiana aquatic runoff, including the Neches and Sabine rivers (Ropicki et al., 2016). In this region of southeast Texas the economy is dominated by the chemical refining industry. Currently, vast networks of interconnected chemical refining industrial complexes line the banks of Sabine Lake, the Neches River, and the Sabine River (Hughes and Leifeste, 1967).

Since 1901, increasing industrial expansion in the area has led to the widespread replacement of natural habitats once serving as river bank, and shoreline estuarine ecosystems. As industrial expansion continued, economic expansion followed, leading to increases in local populations, housing development, and subsequent anthropogenic encroachment, further exacerbating the loss of functional ecosystem area. Close proximity to these anthropogenic influences has subjected Sabine Lake and surrounding waterways to many ecological perturbations including; repeated dredging, major industrial, agricultural, municipal point- and non-point sources of nutrient and pollutant discharges (Ravichandran et al., 1995).

The overall objective of this project is to assess whether the trophic ecology of fish are associative indicators of pollutant exposure. The fish species chosen for study include various commercially and recreationally important fish in Sabine Lake (Ropicki et al., 2016), these include: bull shark (*Carcharhinus leucas*), alligator gar (*Atractosteus spatula*), red drum (*Sciaenops ocellatus*) and gafftopsail catfish (*Bagre marinus*). These fish are expected to exhibit unique feeding ecologies and occupy different tropic positions in the coastal/estuarine ecosystems of Sabine Lake. In this study, pollutants constituting oil-derived hydrocarbons (polycyclic aromatic hydrocarbons or PAHs) and persistent organic pollutants (polychlorinated biphenyls or PCBs) have been chosen for analysis. The trophic ecology of these fish will be

determined by using fatty acids as tracers to indicate dietary preferences. In addition, data on the gut contents of these fish will also be presented. The results of fish gut content analysis have been kindly provided by Dr. Philip Matich (Texas A&M University at Galveston). As both PAHs and PCBs bio-accumulate in aquatic biota and exhibit the propensity to biomagnify across food webs (and trophic levels) (Nfon et al., 2008; Suedel et al., 1994), it is anticipated that dietary preferences will determine the extent of pollutant exposure.

All fish were sampled in Sabine Lake, TX from April 10th to May 15th 2018 (by Dr. Philip Matich and his team). Body-burdens of sixteen U.S. EPA priority PAHs and twenty-nine individual PCB congeners were quantified in fish livers. All pollutants were measured using gas chromatography and mass spectrometry (GCMS). The liver was chosen as a target tissue for analysis as it is the major metabolically active organ involved with metabolizing dietary foods, regulating various physiological functions, and contains elevated lipid reserves which can serve as a storage depot for various persistent pollutants (Sivarajah et al., 1978). In addition, thirty-seven fatty acids were also measured in the livers of fish (as fatty acid methyl esters or FAMEs). The fatty acids measured include saturated and un-saturated FAMEs spanning from those containing four carbon atoms (4:0, butyric acid) to twenty-four carbon atoms (24:0, lignoceric acid), and included those commonly used as markers for dietary sources to delineate trophic positions of marine animals (Parrish et al., 2000). All fatty acids were also quantified using GCMS by derivatization fatty acids to FAMEs.

Hypotheses

The integrative approach taken in this project will provide unique insights into the ecological factors (such as dietary choice and trophic position) that affect exposure and bio-

accumulation of oil- or combustion-derived hydrocarbons (PAHs) and persistent organic pollutants (PCBs) in fish.

The central hypothesis of this thesis is that the dietary preference and trophic position of fish will influence PAH and PCB body-burdens. The stated central hypothesis will be addressed by determining the following two specific hypotheses.

Hypothesis 1

Elevated pollutant body-burdens in fish will be associative with trophic position.

Hypothesis 2

Dietary preference will reflect the trophic position of fish (as determined by gut content and FAME analysis) and will be associative with the levels of bio-accumulated pollutants.

CHAPTER II

POLLUTANT BODY-BURDENS

Sources of PAHs into the environment

The major sources of PAHs into the environment include combustion-related pyrogenic and oil-derived petrogenic sources (Qian et al., 2001; Wang et al., 2014). Combustion-related sources are mainly associated with the incomplete burning of organic matter, such as coal, petroleum, wood, garbage incineration and forest fires. Whereas, petrogenic sources are mainly associated with petroleum, crude oil and its refined products, such as: kerosene, gasoline, diesel fuel, lubricating oils etc. (Wolska et al., 2012).

Typically, the concentration ratios of various low vs. high molecular weight PAHs are used to distinguish between petrogenic vs. pyrogenic sources. The increasing aromaticity of PAHs allows the delineation of molecules formed at relatively low temperatures, such as 100-300^{o}C, relative to those formed at higher temperatures, such as 400-700^{o}C. For example, low molecular weight (LMW) PAHs comprising $\leq$ 3 aromatic rings are typically representative of petrogenic sources formed at relatively moderate temperatures (i.e. 100-300^{o}C). Whereas, high molecular weight (HMW) PAHs comprising $\geq$4 aromatic rings are typically representative of pyrogenic sources formed at higher (combustion related) temperatures (i.e. 400-700^{o}C) (Budzinski et al., 1997; Wolska et al., 2012).

The analysis of LMW to HMW PAH ratios in biota and sediments are commonly used to indicate sources of exposure (Wolska et al., 2012). Using such ratios, studies in the northern Gulf of Mexico show near-shore (salt marshes) environments to be dominated by pyrogenic or combustion-related HMW PAHs, whereas offshore environments (continental shelf sediments)

appear to be enriched with LMW petrogenic (or uncombusted) PAHs (Wang et al., 2014). Similarly, the analysis of PAH levels in oysters and sediments from Galveston Bay show a predominance of HMW PAHs relative to LMW PAHs, indicating pyrogenic sources of input in near-shore environments. Interestingly, HMW:LMW ratios were higher in biota (oysters) relative to sediments, indicating a preferential bioaccumulation of 'heavier' HMW PAHs in biota (Qian et al., 2001).

The molecular weight of PAHs also influences their transport in the environment. LMW PAHs typically partition into the gaseous phase, while HMW PAHs are emitted in the particulate phase. Therefore, overall input and exposure to the environment is in both the gaseous and particulate phases (Lee and V Vu, 2010). Volatilized PAHs (mostly two to four ringed compounds) in the gaseous phase appear to be highly reactive to hydroxyl (·OH) and nitrate (NO_3) radicals, and ozone (O_3). However, despite such reactivity in the atmosphere, the particle phase partitoning of PAHs leads to wide ranging atmospheric transport and deposition (Keyte et al., 2013).

Concomitant with increasing molecular weights of PAHs, their hydrophobicity as represented by the logarithm of the octanol-water partition coefficient or log K_{ow}, also increases. Such increasing hydrophobicity further favors the partioning of PAHs into non-polar matrices, such as the organic carbon of particulate materials (Yang et al., 2011), and the lipid fraction of an organism (Bond et al., 1985; Meador et al., 1995). It is this sequestration of PAHs in the lipid fraction of an organism that is a cause for concern as it enables PAH bioconcentration (from the surrounding aqueous environment) or bioaccumulation (through consumption of contaminated foodstuffs), which may ultimately attain levels that can be toxic (Hylland, 2006; Meador et al., 1995).

While the overall hydrophobicity assists with the sequestration of PAHs into lipids, their bioavailability is not necessarily a function of such increasing hydrophobicity. In some cases an inverse relationship has been shown for PAH bioaccumulation while comparing PAH levels in biota relative to concentrations in the surrounding environment (water or sediment). Baumard et al., (1998). measured the preferential bioaccumulation of the moderately water soluble PAH phenanthrene (1.3 mg/L, (Mackay and Shiu, 1977)) relative to the poorly water soluble anthracene (~0.1 mg/L, (Mackay and Shiu, 1977)), in various benthic invertebrates (shrimps, crabs) from polluted sites across the Mediterranean sea. The preferential bioaccumulation of phenanthrene versus anthracene was demonstrated as the ratio of the two PAHs was relatively equivalent in surrounding sediments (Baumard et al., 1998).

Such preferential bioaccumulation of LMW (and moderately hydrophilic) PAHs can also influence toxicity, as these PAHs constitute more readily bioavailable pollutants. For example, laboratory toxicity tests using the invertebrate soil-dwelling springtail, *Folsomia fimetaria*, showed highly lipophilic (poorly water soluble) PAHs comprising: benzo[a]anthracene, chrysene, benzo[b]fluoranthene, perylene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene and dibenz[a,h]anthracene) (log K_{ow} > 5.6, water solubility = 0.6 - 14 µg/L); to not be as toxic as moderately hydrophobic (moderately water soluble) PAHs comprising: naphthalene, acenaphthylene, acenaphthene, fluorine, anthracene, phenanthrene, pyrene and fluoranthene (log K_{ow} 3.3 – 5.2, water solubility = 0.3 - 32 mg/L). In some cases the LC_{50} values (i.e. concentration required to cause mortality in 50% of exposed organisms) for moderately hydrophobic chemicals ranged ~10x lower (i.e. more toxic) than highly hydrophobic chemicals (Sverdrup et al., 2002).

The eco-toxicity of PAHs in the environment

Regardless of differences in water solubility, the overall sum total bioaccumulation of PAHs is mainly in the lipid fraction of exposed biota (Neely et al., 1974; Porte and Albaiges, 1994). As a result, a common environmental monitoring strategy to assess hydrocarbon pollution involves the quantifications of PAH body burdens in exposed organisms (DeLeon et al., 1988; Porte and Albaiges, 1994). Controlled studies in which mussels (*Mytilus edulis* and *Mytilus californianus*) were grown in clean waters, and then transferred to oil polluted sites demonstrated the uptake and bioaccumulation of various hydrocarbon pollutants (including aromatic hydrocarbons) after ~3-4 months exposure. When these organisms were transferred from polluted sites back to clean sites, on average a $\leq$90% loss of hydrocarbon body-burdens was seen over a 5-10 week period (DiSalvo et al., 1975). This rate of loss is similar to those reported for oysters (*Crassostrea virginica*) exposed to hydrocarbon mixtures for 50 days under controlled laboratory conditions, i.e. ~90% loss of body-burdens after 2 weeks of transfer to clean water conditions (Stegeman and Teal, 1973).

While the studies by DiSalvo et al., (1975) and Stegeman and Teal (1973) focused attention on hydrocarbon uptake and depuration in bivalve molluscs, Neff et al., (1976) compared uptake and release of petroleum-derived aromatic hydrocarbons in marine molluscs, shrimp and fish. Their study showed variable rates of bioaccumulation across all species. Overall, their study observed a rapid rate of uptake and slower depuration of the HMW (and highly lipophilic) PAH, benzo[a]pyrene, versus the LMW (and moderately lipophilic) PAH, naphthalene, which was both rapidly accumulated and depurated. Interestingly, the depuration of hydrocarbons was more rapid in shrimp and fish, relative to clams and oysters. These differences in depuration rates were postulated to be due to species-specific differences in detoxication (or

pollutant biotransformation) capabilities (Neff et al., 1976). Regardless, such accumulation of PAHs in organisms enables their availability for trophic transfer across food chains and webs (DeLeon et al., 1988).

The assessment of PAH body-burdens in organisms comprising different trophic levels shows some evidence of 'trophic dilution' of PAH levels (Wan et al., 2007). Such trends are evident when comparing between invertebrate (occupying lower trophic positions) vs. vertebrate species (occupying higher trophic positions) (Nakata et al., 2003; Porte and Albaiges, 1994), and even within species (such as invertebrates) comprising various trophic positions (Gewurtz et al., 2000). Comparisons of the hepatic metabolism of benzo[a]pyrene in rat vs. piscine species (mullet, *Mugil cephalus*) shows up to an order of magnitude higher activity in the mammal (for the formation of a diol metabolite) (Tan and Melius, 1986). Similarly, the metabolism of benzo[a]pyrene has been shown to be ~3x faster in rats vs. fish (starry flounder (*Platichthys stellatus*) and English sole (*Parophrys vetulus*)). While overall benzo[a]pyrene biotransformation to diol, quinone and hydroxylated metabolites was equivalent between the two fish species, rats showed ~3-31x higher productions for these metabolites (with exception of 7,8-diol, whose production was ~2x higher in English sole vs. rat) (Varanasi et al., 1986).

Studies in mammals have shown the metabolic biotransformation of PAHs to create highly reactive metabolites that are capable of binding to DNA, forming adducts with nucleobases (Brookes and Lawley, 1964). Specifically, the formation of a diol-epoxide metabolite of benzo[a]pyrene is known to act as a potent mutagen capable of forming PAH-DNA adducts, which can lead to mutations in oncogenes and/or tumor-suppressor genes, in-turn initiating the first stages of carcinogenesis (i.e. unabated cell proliferation) and subsequent tumorigenesis (Basu, 2018; Ewa and Danuta, 2017). The likely link between human PAH

exposures and the initiation of carcinogenesis has led to considerable regulatory oversight and exposure advisories (ATSDR, 1995; Moorthy et al., 2015).

A similar mechanism of PAH-DNA adduct formation is suspected between PAH exposure and the formations of liver lesions or neoplasms in fish inhabiting PAH polluted environments (Baumann, 1992; Baumann and Harshbarger, 1998). The hepatic metabolism of PAHs to reactive metabolites such diol-epoxides is also seen in fish due to the presence of conserved enzyme systems as seen in mammals (such as cytochrome P450 mixed function oxygenases) (Lewis et al., 1999; Nelson et al.; Stegeman and Lech, 1991; Varanasi et al., 1987). Furthermore, DNA adduct formation in PAH exposed fish is also demonstrated, confirming a conserved mechanism for tumorigenesis in fish as seen in mammals (Balk et al., 2011; Stein et al., 1990). From a perspective of environmental monitoring, the correlation of elevated PAH body-burdens and activities of hepatic biotansformation enzymes has led to the use of both, PAH bioaccumulation and measures of biotransformation enzyme induction, to be used as biomarkers (or biological-response-markers) of pollution and stress in wildlife (van der Oost et al., 2003; Van der Oost et al., 1991).

Sources of PCBs into the environment

While PAHs can be of both anthropogenic (i.e. petrogenic spills and pyrolysis of hydrocarbons and or organic matter) and natural origins (i.e. forest fires, oil seeps), PCBs are exclusively of anthropogenic origin. The largest anthropogenic use of PCBs is associated with the electrical industry where they were used as heat absorbing and electrical insulating materials in transformers and capacitors (which are used in electrical systems to control voltage output) (Delzell et al., 1994). Other minor sources also include production in chlorination processes involved with pulp and paper mill industries (EPA, 1977). PCBs are discharged into the aquatic

environment with effluents (such as for pulp in paper mills), or as a result of leakages of transformer or capacitor oils (Wolska et al., 2012).

While PAHs are composed of multiple aromatic rings (constituting ≥2 rings), PCBs consist of only two benzene rings joined by carbon-carbon bonds with varying numbers of chlorine atoms (between 1 and 10 chlorine atoms) that substitute for hydrogen atoms at various ring positions. In total, the various numbers and positions of the chlorine atoms can yield 209 isomers of PCBs, with congeners ranging from three mono-chlorinated (trichlorobiphenyl) isomers to a fully chlorinated decachlorobiphenyl isomer (i.e. all five positions on each biphenyls is chlorinated) (Delzell et al., 1994). These structural configurations of chlorine atoms on PCB molecules lends to their environmental persistence. Typically, an increase in the numbers of chlorine atoms on PCB molecules, decreases their rate of degradation in the environment while increasing their environmental persistence (Delzell et al., 1994).

In the environment, the microbial biodegradation of PCBs is mainly via aerobic oxidation or anaerobic reduction (Abramowicz, 1995). Anaerobic dechlorination of PCBs proceeds from the preferential removal of chlorine atoms (in highly chlorinated PCB congeners) from the *meta* and *para* positions, resulting in an increase in lower chlorinated *ortho*-substituted PCB congeners (Abramowicz, 1995; Tiedje et al., 1993). Such partial dechlorination of PCBs to monochloro- and dichloro-PCBs is expected to result in risk reduction as they demonstrate low or no toxicity (using undeclared bioassays). The lowered risk of toxicity is mainly associated with the dechlorination of chlorine atoms in the *meta* and *para* positions of the PCBs, as the presence of chlorine atoms on these positions yields structures similar to the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 2,3,7,8-TCDD (Tiedje et al., 1993). Some of the most toxic PCB congeners are those with chlorine atoms at both *para*, and at two or more *meta* positions. These

include 3,4,4',5-tetra- (PCB-81), 3,3',4,4'-tetra- (PCB-77), 3,3',4,4',5-penta- (PCB-126) and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB-169). The chlorine atom substitutions on these four PCBs results in a coplanar structure (i.e. all atoms lie in the same geometric plane), which is similar to 2,3,7,8-TCDD, and thus are capable of inducing a similar mode of toxicity (Safe et al., 1985).

Regardless of such biodegradative fate in the environment, PCBs exhibit very lipophilic (or hydrophobic) $\log K_{ow}$ values, ranging from ~4.5 – 8.2 (Hawker and Connell, 1988; IARC, 2016; Walters et al., 2011). Such lipophilicity ensures the bioaccumulation of PCBs in fatty tissues of exposed organisms (Beyer and Biziuk, 2009). In turn, such bioaccumulation within an organism contributes to biomagnification of PCBs 'up' trophic levels and across food webs (Walters et al., 2011).

The eco-toxicity of PCBs in the environment

The environmental persistence and high bioaccumulation potential of PCBs results in their elevated body-burdens in exposed wildlife and humans (McFarland and Clarke, 1989; Tanabe et al., 1987). The consumption of PCB contaminated fish was implicated in the declines of bald eagle (*Haliaeetus leucocephalus*) populations in North America (Bowerman et al., 1995), and more recently has been postulated as a causal factor in declining cetacean populations (Hall et al., 2018). The co-exposure of wildlife and humans to PCBs is also implicated in a wider suite of subtle endocrine disorders, which include immunological, neurological and metabolic effects (Birnbaum, 1994; Crinnion, 2011).

The most biologically active PCBs are substituted (with a chlorine atom) at the *para* and at least *meta* position of both phenyl rings. Furthermore, the PCB must not contain any *ortho*-chloro substitutes. Such configuration exhibits a co-planar structure (all atoms lie in the same

plane) that approaches a relatively flat structure similar to the polychlorinated dibenzo-p-dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD). Therefore, the most toxic PCBs are PCB-77 (3,3',4,4'-tetrachlorobiphenyl), PCB-126 (3,3'4,4',5-pentachlorobiphenyl), and PCB-169 (3,3',4,4',5.5'-hexachlorobiphenyl) (Safe et al., 1985). These PCBs are potent inducers of the aryl hydrocarbon receptor (AhR) signaling system.

Almost all invertebrate and vertebrate taxa (including fish and humans) metabolize or detoxify planar aromatic compounds (such as PAHs and PCBs) using a superfamily of enzymes that include the cytochrome P450 (cyp450) mixed function oxygenases (or monooxygenases) (Moorthy et al., 2015; Safe et al., 1985). The specificity of cyp450 induction is controlled by a cell receptor signaling pathway called the aryl hydrocarbon receptor (AhR) signaling system (Lo and Matthews, 2012). Much like cyp450 enzymes, the AhR signaling system is also highly conserved (or shared) amongst invertebrate and vertebrate taxa (Beischlag et al., 2008). AhR is activated by planar aromatic compounds, such as PAHs and PCBs, and the biological consequence of exposure to these compounds is in large part AhR-mediated (Denison et al., 2002; Safe et al., 1985). Once activated, AhR regulates various detoxification enzyme genes, some of which include cyp450s (Beischlag et al., 2008; Rowlands et al., 1996).

PCB metabolism via cyp450 monooxygenase reactions leads to the productions of hydroxylated intermediate metabolites, which are typically conjugated and eliminated from the body (Morck et al., 2002). However, hydroxylated PCB metabolites can also be further oxidized to form electrophilic intermediates such as epoxides (arene oxides) and (semi) quinones that can react with various biological structures such as proteins, DNA and lipids (Grimm et al., 2015; Pereg et al., 2001). Such disruptions of biological systems are expected to causally underlie

various physiological effects, such as disruptions of immune, neurological and endocrine functions (Crinnion, 2011).

Taken together, the highly lipophilic properties of PCBs (that contribute to their bio-accumulation potential) (Walters et al., 2011), their environmental persistence (Delzell et al., 1994), and toxicity (Crinnion, 2011), has led to their categorization as persistent organic pollutants (or POPs). And despite a ban on their use in the late 1970's (Boyle and Highland, 1979), these compounds continue to constitute environmental pollutants of high concern.

Risk assessment of dioxin-like PCBs in fish from Sabine Lake

A standard risk assessment method to determine the likely toxicity of exposure to mixtures of PCBs involves the toxicity equivalent quotient (TEQ) approach. The toxicity effects considered in mammals include the activation of cyp450 enzymes (indicating the induction of AhR signaling) or immunospression (Kannan et al., 2000; Van den Berg et al., 1998). For piscine species, the toxicity endpoint of fish mortality is considered (Steevens et al., 2005).

The typical TEQ approach summates the standardized toxic potencies (also called toxicity equivalent factors or TEFs) of individual dioxin-like PCB congeners to a standard (or reference) compound, the dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (Van den Berg et al., 1998). This relationship (TEQ = toxic equivalents, PCB_i = concentration of dioxin-like PCB congener i as measured in fish, TEF_i = toxic equivalency factor for dioxin-like PCB congener i in fish) is mathematically represented in the equation below (Van den Berg et al., 1998):

$$TEQ = \sum (PCB_i * TEF_i)$$

It is important to note that the existing TEQ risk assessment approach assumes an 'additive' toxicity for the sum total exposure to dioxin-like PCB congeners. This premise is

based upon the observation that structurally related compounds exhibit a similar mode of toxicity (i.e. AhR activation and cyp450 enzyme induction), therefore together a mixture of such compounds will exert toxicity that is equal to the sum of its constituents. Such additive toxicity has been demonstrated using select *in vitro* or *in vivo* bioassays (Sawyer et al., 1984; Schmitz et al., 1995). While a reasonable generality, this assumption is challenged by additional studies that show non-additive toxicities, whereby some PCB congeners exhibit antagonistic activities (Aarts et al., 1995; Biegel et al., 1989; Billiard et al., 2008). Therefore, such findings have cast doubt on whether the TEQ approach is adequately protective of wildlife and human health (Safe, 1997; van den Berg et al., 2000). Nevertheless, the TEQ approach presents (at present) a reasonable means with which to assess whether dioxin-like PCB body-burdens are capable of eliciting a toxicity response or adverse effect.

In this Chapter, the levels of sixteen EPA priority PAHs and twenty-nine PCBs were quantified in the hepatic tissue of fish from Sabine Lake. The fish selected for study included: bull shark (*Carcharhinus leucas*), alligator gar (*Atractosteus spatula*), red drum (*Sciaenops ocellatus*) and gafftopsail catfish (*Bagre marinus*). This Chapter aims to investigate whether pollutant (PAH and PCB) bioaccumulation was reflective of trophic position, i.e. lower levels of pollutants in fish occupying lower trophic position vs. those at higher trophic position. Amongst the twenty-nine PCB congeners, there were twelve dioxin-like PCBs. Therefore, a TEQ-based risk assessment was also performed to assess whether the body-burdens of dioxin-like PCBs were at sufficiently high levels to illicit toxicity or adverse effects.

PAH and PCB methods

Sample collection

Bull shark (*Carcharhinus leucas*) (n=8), alligator gar (*Atractosteus spatula*) (n=6), red drum (*Sciaenops ocellatus*) (n=8), and gafftopsail catfish (*Bagre marinus*) (n=7) were sampled in Sabine Lake between April 25th – May 8th 2018. Fish were collected by mid depth trawling with a gill net. Upon capture, standard/pre-caudal length (mm), total length (mm) and fish weight (gram) was recorded, and each specimen was immediately frozen until dissection. Frozen fish samples were defrosted in a refrigerator over the night prior to dissection of liver samples. The excised samples were stored at -80°C for further analysis.

Sample extraction and clean-up

A ~1 gram sub-sample was excised in duplicate from each tissue (i.e. muscle or liver). Each sub-sample was homogenized in a 7-mL polypropylene tube containing ceramic beads (Fisher Scientific) and filled with 3 mL of 1:1 (v/v) hexane:ethyl acetate. Tubes were placed in a Fisherbrand^{TM} Bead Mill 4 Homogenizer (Fisher Scientific) and homogenized at a processing power of 150 *g* for 2 minutes. Homogenate was transferred to an acid -washed 50-mL glass tube. A 5 µL aliquot of 100 ppm benzo[a]pyrene-d_{12} (Sigma-Aldrich) and 100 ppm PCB 65-d_5 (CDN Isotopes) was spiked to each sample as internal standards (2.5 ppm at final volume). After spiking tissue homogenates, glass tubes were placed in a Branson Ultrasonics^{TM} M2800 Ultrasonic Bath (Fisher Scientific) for 30 minutes to further extract PAHs and PCBs into 1:1 (v/v) hexane:ethyl acetate solvent. Phase separation of the solvent from the tissue matrix was assisted by centrifugation at 2000 *g* for 10 minutes. The supernatant was pipetted into pre-weighed 20-mL glass vial and dried under N_2 gas for 30 minutes. Following extraction, lipid content of each sample was determined gravimetrically. The remaining residue was rinsed with 1

mL of acetonitrile (ACN) and pipetted into a 2-mL amber vial. All samples were then dried in a Savant™ SPD121P SpeedVac™ Concentrator (Thermo Scientific) and reconstituted into 200 μL ACN before transferring to a glass insert. Following (Yang et al., 2004), sample freezing was conducted at -20°C for one hour to precipitate lipids out of solution. Afterwards, a clean 50 μL sub-aliquot was removed, dried (SpeedVac™) and reconstituted into 50 μL dichloromethane (DCM) prior to gas chromatography and mass spectrometry (GCMS) analysis.

PAH and PCB analysis

Concentrations of the U.S. EPA's 16 priority PAHs and 29 individual PCB congeners were quantified in fish liver samples. All PAHs with $\leq$ 3 aromatic rings (naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, phenanthrene) were classified as low molecular weight (LMW) PAHs, while congeners $\geq$ 4 aromatic rings (fluoranthene, chrysene, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene) were classified as high molecular weight (HMW) PAHs. Of the 29 PCB congeners, 12 were dioxin-like (DL-PCBs): PCBs 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189. Analytical grade standards were obtained from the following sources: acenaphthene (ACE), acenaphthylene (ACY), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF), benzo[g,h,i]perylene (BghiP), fluoranthene (FLT), pyrene (PYR), and PCBs 1, 18, 52, 101, 138, and 180 from Sigma-Aldrich; anthracene (ANT), chrysene (CHR), benzo[a]anthracene (BaA), benzo[k]fluoranthene (BkF), dibenz[a,h]anthracene (DahA), fluorene (FLU), indeno[1,2,3-cd]pyrene (IcdP), phenanthrene (PHE), and naphthalene (NAP) from Supelco; PCBs 28, 33, 77, 81, 95, 105, 114, 118, 123, 126, 128, 149, 153, 156, 157, 167, 169, 170, 171, 177, 183, 187, and 189 from Ultra Scientific. All PCBs are identified according to the IUPAC numbering system.

Samples were analyzed for the 45 individual PAH/PCB congeners by GCMS. This analysis was conducted on a Hewlett Packard HP-6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer. Samples were injected in splitless mode (2 μL) equipped with a DB-5MS (J&W Scientific) capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness). Helium was the carrier gas at a flow rate of 1.0 mL/min. Temperatures at the front inlet and the MS interface were set at 250 and 280°C, respectively. Following injection of the sample, the GC oven was programmed at 40°C and held for 1 min, then ramped up to 180°C at 20°C/min, and finally ramped up to 300°C at 5°C/min and then held for 10 min. The MS was operated in electron impact (EI) mode at an electron energy of 70 eV while the MS source temperature was maintained at 230°C. Selected ion monitoring (SIM) mode was used for identification and quantification of all 45 analytes. Quantification of all PAH and PCB congeners were performed against a linear 13-point calibration curve ($r^2 > 0.97$) using serially diluted standards that were prepared in DCM (2.5 to 10,000 ng/mL). Sample quality assurance and quality control measures were conducted by running a solvent blank and a mixed standard prior to analysis. The limit of detection (LOD) was quantified by the signal-to-noise ratio of 5:1 for the lowest detectable calibration point. Blanks showed no signs of external contamination above the LOD.

Dioxin-like PCB body-burdens and toxic equivalents (TEQ) based risk assessment

The toxicity potential of the hepatic body-burdens of twelve dioxin-like PCBs was assessed using the toxic equivalents (TEQ) approach. The TEQs were calculated for PCBs 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189, by multiplying the lipid normalized hepatic body-burden of each dioxin-like PCB (as ng/gram lipid wet weight), with its respective toxic equivalent factor (or TEF) as determined for fish by (Van den Berg et al., 1998). The resulting TEQ values for each fish species were summed and compared against values reported

for mammalian and piscine toxicities (Kannan et al., 2000; Steevens et al., 2005). All TEQ values were $\log_{10}$ transformed prior to comparisons.

Statistical analysis

All statistical analyses were performed using the Python programing language (v2.7.15) and associated data frame handling (pandas) and statistical (scikit) libraries. The normal distribution of datasets was tested using the Shapiro-Wilks test, with homogeneity of variance tested using Levene test. Parametric testing was done using one-way analysis of variance (ANOVA), followed by Tukey's posthoc test to compare for differences.

Non-parametric testing was done using the Kruskal-Wallis test, followed by the Dunn's posthoc test to compare for differences. For non-parametric datasets conforming to a two-factor 'block' design, i.e. datasets in which there is a primary factor (e.g. pollutant concentration in fish) and a blocking factor (e.g. pollutant type) that is a source of variability, a friedman's rank sum test was firstly done to test for a significant difference. If significance was detected, a nemenyi-friedman posthoc test was conducted to perform pair-wise comparisons between groups in order to identify significantly different groups. Any pollutant values that were below detection were given an arbitrarily selected low value of 0.01. This was done to avoid null (or zero) values while performing statistical tests, and the value chosen was two orders of magnitude lower than detectable pollutant levels. Significance for all tests was tested at $\alpha=0.05$. Finally, multivariate analyses of PAH and PCB body-burdens across the four fish species was performed using principal component analysis. All graphs were plotted using the python Matplotlib plotting library.

PAH and PCB results

Analysis of fish lengths and weights

Precaudal length (in mm) exhibited a majority normal distribution with homogeniety of variance. One-way ANOVA indicated significant differences in precaudal lengths. Tukeys posthoc comparisons showed only catfish and red drum as not being significantly different from one another, whereas all other species were significantly different from each another (**Figure 1**(a)). Similarly, the analysis of fish weights (grams) also exhibited a majority normal distribution with homogeneity of variance. One-way ANOVA indicated significant differences in weights, with Tukeys posthoc analysis indicating catfish and red drum, gar and red drum, and bull shark and gar as not being significantly different from one another. Whereas gar was significantly different from catfish, and bull sharks were significantly different from catfish and red drum (**Figure 1**(b)).

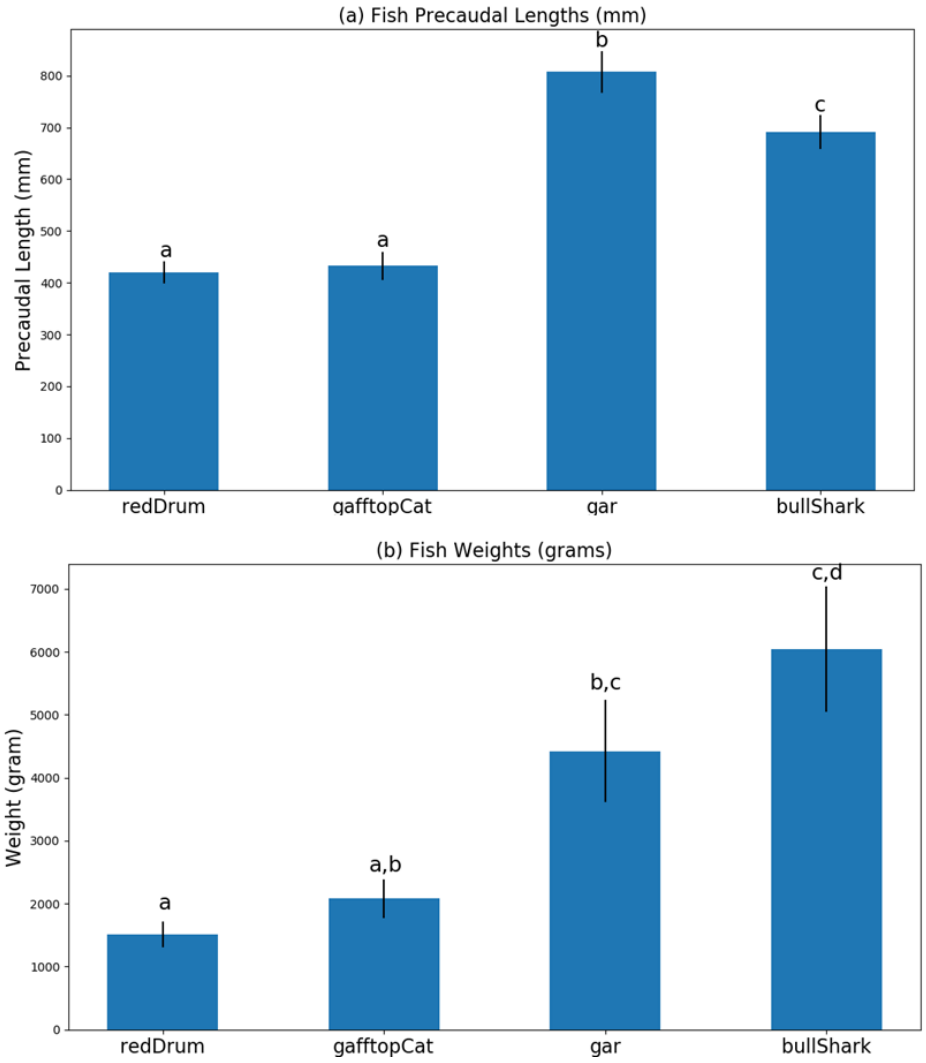

Figure 1 Bar graphs showing fish (a) precaudal lengths (mm), and (b) weights (grams). Gafftopsail catfish (gafftopCat), red drum (redDrum), alligator gar (gar) and bull shark (bullShark) were sampled from Sabine Lake. Statistically significant groups are shown by different letters above each box plot. Groups that are not statistically different from one another are shown with same letter.

Analysis of PAH and PCB body burdens in fish

All PAH and PCB concentrations in liver tissue of fish (ng/gram tissue wet weight) were normalized relative to the sum total of congener concentrations (**Figure 2**). Gafftopsail catfish, red drum and alligator gar exhibited high levels of low molecular weight (LMW) PAHs, such as naphthalene (NAP, 25-40%), and anthracene (ANT, 9-19%). In contrast, levels of these LMW

PAHs were lower in bull sharks (NAP=6%, ANT=5%). The only high molecular weight (HMW) PAH to show comparably high levels in catfish and red drum was dibenzo[a,h]anthracene (DahA, 15% and 12% respectively). In bull sharks, the HMW PAHs of dibenzo[a,h]anthracene (DahA, 32%), and indeno[1,2,3-cd]pyrene (IcdP, 11%) showed highest levels relative to all other PAH congeners (**Figure 2**(a)). The overall predominance of LMW PAHs in catfish red drum and gar indicates the mainly petrogenic (or uncombusted) PAH exposure (Wang et al., 2014). Non-parametric Friedman's chi-square analysis of PAH levels showed significant differences amongst fish ($p \leq 0.05$). Subsequent posthoc analysis using Nemenyi-Friedman's pair-wise comparisons showed bull shark and alligator gar as not being significantly different from one another, whereas the remainder species were significantly different from one another.

The analysis of PCB levels showed PCB-18 to be the most highly detected congener across all species (22-66% of normalized sum total levels). The remaining congeners contributed to the overall levels to varying degrees in each species. Notable exceptions include the prevalence of PCB-156 (16%) in alligator gar, and PCB-169 (16%) in red drum (**Figure 2**(b)). Friedman's chi-square analysis of PCB levels showed significant differences amongst fish ($p \leq 0.05$). Posthoc analysis using Nemenyi-Friedman's pair-wise comparisons showed only gafftopsail catfish and alligator gar as not being significantly different from one another, while all other species were significantly different from one another.

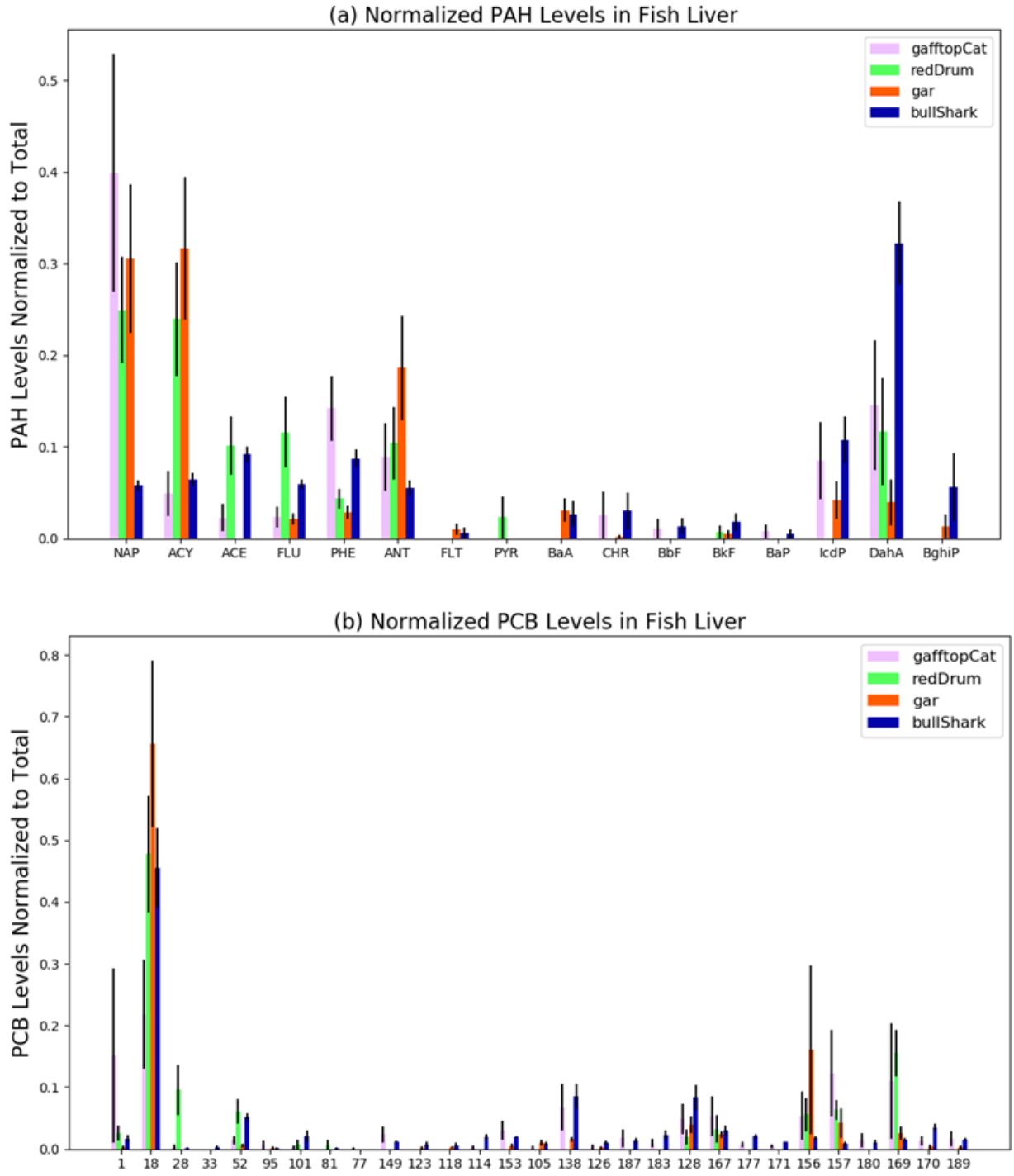

Figure 2 Grouped bar graphs showing the profiles of individual (a) PAHs, and (b) PCB congeners in the livers of fish. All levels are normalized to sum total PAH and PCB concentrations as ng/gram liver wet weight.

In contrast to the analysis of specific PAHs and PCB congeners, the assessment of sum total PAH levels in fish from Sabine Lake showed alligator gar to have significantly higher levels than the remainder of fish species (wich were also not significantly different from one another) (**Figure 3**). Whereas the analysis of sum total PCB levels showed overall significantly higher levels of PCBs in alligator gar and bull sharks (**Figure 3**).

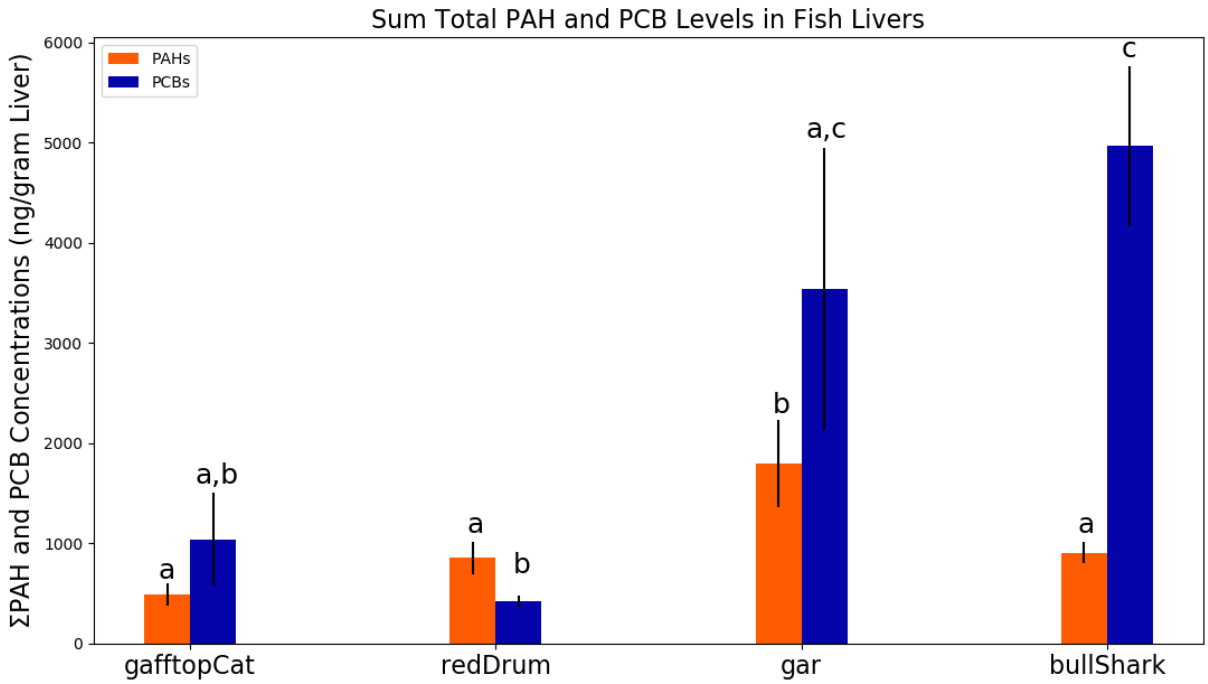

Figure 3 Sum total PAH and PCB levels in hepatic tissue of fish from Sabine Lake (ng/gram liver wet weight). Levels sharing similar letters are not significantly different from one another, whereas dissimilar letters indicate significant differences.

Multivariate analysis

Principal component analysis (PCA) of PAH and PCB body burdens was used to look for characteristic associations or differences amongst the four fish species (**Figure 4**). The first coordinate explained 32% of the variance, while the second coordinate explains 11% of the variance. The spread of species along the first coordinate reveals catfish, red drum and alligator

gar to be relatively closely clustered together, whereas bull sharks comprised a distinctive cluster with little overlap with the other fish species. This analysis alludes to a relatively distinctive pollutant body-burden of sharks relative to the other fish species.

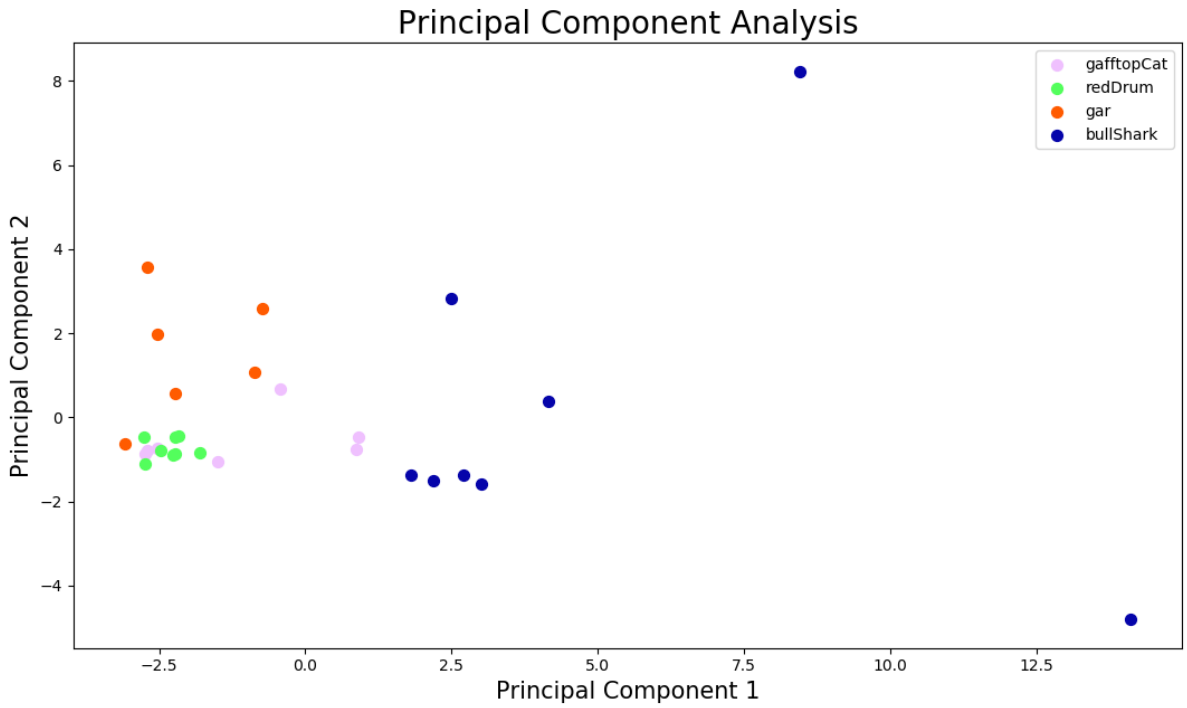

Figure 4 Principal component analysis (PCA) of PAH and PCB body-burdens.

TEQ-based risk assessment

The TEQ analysis of dioxin-like PCB body-burdens in fish from Sabine Lake showed red drum, gafftopsail catfish, and alligator gar to have relatively similar TEQ values. The median TEQ values for these fish were all above the lower threshold established for fish early life-stage toxicity (early life-stage mortality) (Steevens et al., 2005), and were either at or below the lower threshold set for mammalian (aquatic mammal) toxicity (immunosuppression in pinnipeds, cetaceans, and mustelids) (Kannan et al., 2000) (**Figure 5**). In contrast, bull sharks exhibited a median TEQ value that exceeded the upper threshold limit for aquatic mammal toxicity (and also

the upper limit set for fish early life-stage toxicity). Overall, the elevated TEQ levels in bull sharks reflects the high hepatic body-burden of sum total PCBs quantified in this species as seen below in (**Figure 5**).

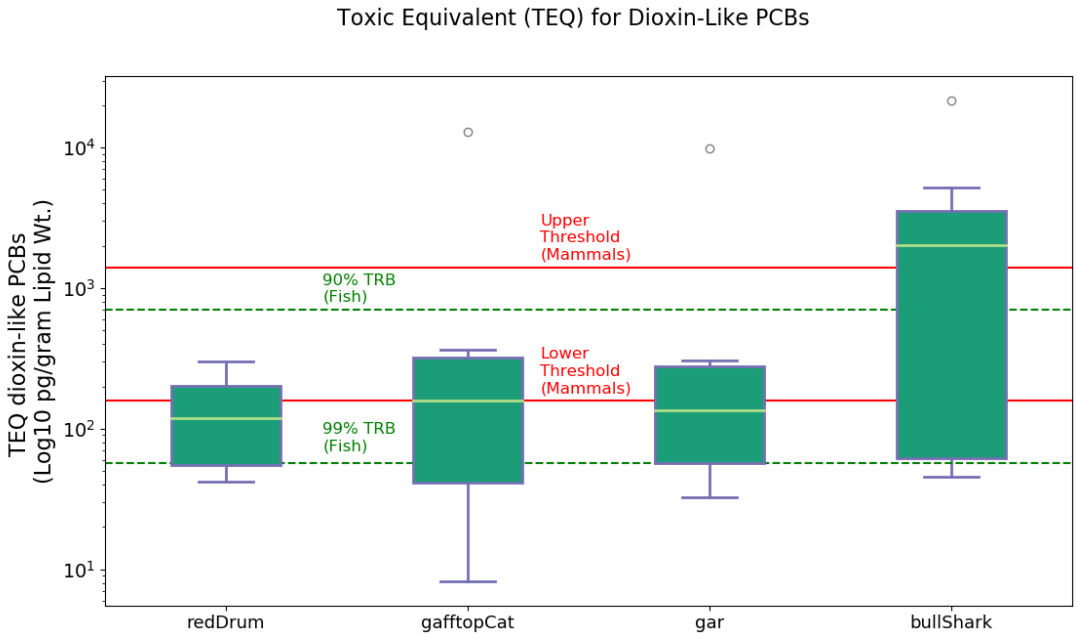

Figure 5 Boxplots showing the toxic equivalent (TEQ) risk assessment of dioxin-like PCBs in fish from Sabine Lake. A comparison with established thresholds is also shown for early life-stage fish (as tissue residue-based toxicity benchmarks or TRBs), and lower/upper thresholds for toxicity effects in aquatic mammals.

PAH and PCB discussion

Fish morphometric parameters

The analysis of fish weight and lengths demarcates significant differences between the gafftopsail catfish and red drum versus alligator gar and bull sharks (**Figure 1**). While the weight and size ranges for catfish and red drum indicate adult life-stages, gar and bull shark represent juvenile and young-of-year (i.e. ≤1 year old) respectively (Cruz-Martínez et al., 2004; TPWD, 2019).

PAH levels in fish from Sabine Lake

The analysis of PAH levels in fish showed a predominance of LMW PAHs (≤ 3 aromatic rings) in gafftopsail catfish, red drum and alligator gar (**Figure 2**(a)). Naphthalene (NAP, 25-40% of total PAHs) and anthracene (ANT, 9-19%) constituted the highest levels detected in these fish as compared to all other congeners. Bull sharks exhibited the lowest levels for NAP (6%) and ANT (5%), while exhibiting the highest levels for the high molecular weight (HMW) PAHs (≥ 4 aromatic rings) of indeno[1,2,3-cd]pyrene (IcdP, 11%) and dibenzo[a,h]anthracene (DahA, 32%). In contrast, catfish, red drum, and alligator gar showed the lowest levels for IcdP (≤8%) and DahA (4-15%). Bull sharks and alligator gar were also the only two species to exhibit detectable levels of the HMW PAH benzo[ghi]perylene (BghiP), with concentrations constituting 1 and 6% (relative to sum total PAH body burdens) respectively for gar and bull shark (**Figure 2**(a)). Furthermore, the statistical analysis of individual PAH levels across the four fish species showed alligator gar and bull shark to not be significantly different from one another, whereas all other species were significantly different from one another.

The analysis of sum total PAH levels showed the alligator gar to have significantly higher levels of sum total PAHs relative to all other fish species sampled from Sabine Lake. While bull sharks were not significantly different from gafftopsail catfish and red drum, nevertheless the sum total PAH levels in bull shark were 1.1x and 1.9x higher than red drum and catfish respectively (**Figure 3**).

The trend of greater accumulation of LMW in fish from Sabine Lake agrees with the higher bioavailability of these PAHs due to their greater water solubility (Baumard et al., 1998; Djomo et al., 1996). The overall predominance of LMW PAHs also suggests exposure to petrogenic PAHs in Sabine Lake (Budzinski et al., 1997; Wolska et al., 2012), and reflects the

high level of petrochemical industrialization along the Sabine-Neches waterway, with refineries processing approximately 13% of the Nations daily fuel consumption needs and housing ~55% of strategic oil reserves (SNND, 2019).

The PAH profiles quantified in fish from Sabine Lake also contrasted to those measured in other biota from the northern Gulf of Mexico. For example, analysis of PAHs in oysters (*Crassostrea virginica*) from Galveston Bay has shown the predominance of HMW ($\geq$ 4 rings) PAHs of pyrogenic origins (Qian et al., 2001). Similarly, the analysis of PAHs in ampeliscid amphipods (*Ampelisca mississippiana*) sampled from the Houston Ship Channel also show a predominance of HMW relative LMW PAHs (also confirming pyrogenic sources) (Soliman and Wade, 2008). A more recent study by Cullen et al., (2019) measured the same profile of PAHs (as reported in this study) in juvenile bull sharks opportunistically sampled from around Galveston Bay and other nearshore sites. Their analysis also indicated a predominance of HMW PAHs such as dibenzo[a,h]anthracene (DahA, 5 rings) and indeno[123-cd]pyrene (IcdP, 6 rings) in bull shark livers, relative to all other PAHs. This observation was also shown to be consistent for two other shark species sampled, namely blacktip (*Carcharhinus limbatus*) and bonnethead (*Sphyrna tiburo*) sharks (Cullen et al., 2019). The contrast of the reported measurements (i.e. a predominance of HMW PAHs) versus those observed at Sabine Lake (i.e. predominance of LMW PAHs), hints at distinctive PAH exposure at Sabine Lake. This proposition is strengthened by the fact that HMW PAHs appear to dominate across the various trophic levels of Galveston Bay and surrounding areas (i.e. HMW PAHs dominate in invertebrates and vertebrates).

Comparing total PAH levels measured in fish from Sabine Lake with those in fish from other Gulf of Mexico ecosystems show overall comparable levels as measured in this study. For example, Willett et al., (1997) report sum total PAH levels spanning from 393 – 1,520 ng/gram

fish liver wet weight in hardhead catfish (*Arius felis*) and Atlantic croaker (*Micropogon undulates*) from Galveston Bay. Similarly, Cullen et al., (2019) report sum total PAH levels of 1,560 ng/gram in the livers of juvenile bull sharks caught from Galveston Bay and northwestern Gulf of Mexico. These levels are within the range of sum total PAH levels detected in the livers of gafftopsail catfish (487 $\pm$ 110 ng/gram liver wet weight), red drum (852 $\pm$ 160 ng/gram), alligator gar (1792 $\pm$ 436 ng/gram), and bull sharks (904 $\pm$ 109 ng/gram) from Sabine Lake. Therefore, while there may be differences in PAH profiles at different sites, the overall PAH body-burden appears to be comparable.

It is uncertain whether the PAH concentrations detected in young-of-the-year (YoY) bull sharks are reflective of environmental exposure, maternal 'offloading', or some combination thereof (Lyons and Lowe, 2015; Lyons et al., 2013). Bull sharks do appear demarcated by exhibiting higher levels of HMW PAHs (relative to LMW PAHs that dominate in the remainder of fish species). Furthermore, bull sharks also exhibit significantly higher sum total PCB levels as compared to the other fish species (as described in greater detail in the section below). These trends are reflected in the PCA analysis, which shows bull sharks to have relatively distinctive pollutant (hepatic) body-burden as compared to the other fish species (**Figure 4**). These observations suggest a more unique exposure history for YoY bull sharks, potentially implicating the role of maternal transfer for these persistent pollutants (Lyons et al., 2013; Mull et al., 2013).

PCB levels in fish from Sabine Lake and TEQ risk assessment of dioxin-like PCBs

The analysis of PCB congeners in fish showed a lack of significant differences between gafftopsail catfish and alligator gar, whereas the remainder of fish species were significantly different from one another (**Figure 2**(b)). A comparison of congener profiles showed considerable variabilities in PCB levels between the four fish species. An exception however was

seen for PCB-18, which showed the highest and overall most comparable levels across all four fish species sampled from Sabine Lake (22 – 66% of total PCBs). The analysis of sum total PCB levels showed bull sharks and alligator gar to have the highest levels of PCBs (**Figure 3**).

Despite their persistence and toxicity (McFarland and Clarke, 1989), more is known of PCB congeners in coastal/marine sediments than in aquatic biota. The environmental degradation of PCBs favors de-chlorination of highly chlorinated congeners to low chlorinated congeners (≤tetrachloro-PCBs) (Abramowicz, 1995; Tiedje et al., 1993). As a result, studies from various sites in the Gulf of Mexico show tri- and tetrachloro-PCBs to be amongst the most abundant PCB congeners measured in sediments (Mohrherr et al., 2012; Oziolor et al., 2018; Santschi et al., 2001). However, it is unclear whether PCB-18 (trichloro-PCB) constitutes a prominent congener in the sediments from Sabine Lake. A detailed analysis of PCB congener profiles in sediments from various sites around Galveston Bay quantified detectable levels of PCB-18, however the study noted a more prominent presence of highly chlorinated PCBs, such as PCB-153 (six chlorines) and 170 (seven chlorines) (NOAA, 2003).

A direct comparison of the congener profiles measured in YoY bull sharks in our study, can be made with those measured in juvenile bull sharks as reported by Cullen et al., (2019). For example, Cullen et al., (2019) report a predominance of highly chlorinated PCBs, such as PCB-153 (six chlorine atoms), PCB-138 (six chlorine atoms), and PCB-187 (seven chlorine atoms) in YoY sharks sampled from Galveston Bay and the northwestern Gulf of Mexico. In contrast, the bull sharks sampled from Sabine Lake exhibited ≤9% for these PCBs (relative to sum total PCBs), and with PCB-18 constituting the highest congener concentration (46%) of total PCBs.

The mean PCB levels measured in gafftopsail catfish, red drum and alligator gar from Sabine Lake are at most two-orders of magnitude higher than those measured by the Texas

Department of State Health Services (DSHS) environmental survey. While the DSHS survey reported mean values in the edible skin-off fillets of muscle to be: 0.06, 0.01 and 0.03 mg/Kg in gafftopsail catfish, red drum, and alligator gar respectively (DSHS, 2011); we measured: 1.0 $\pm$ 0.5 mg/Kg in gafftopsail catfish (mean $\pm$ standard error), 0.4 $\pm$ 0.1 mg/Kg in red drum, and 3.5 $\pm$ 1.4 mg/Kg in alligator gar in hepatic tissue (and the highest level of 5.0 $\pm$ 0.8 mg/Kg in bull sharks). The levels measured in hepatic tissue are expected to be higher than those in muscle. Cullen et al., (2019) report hepatic levels of total PCBs in bull, blacktip, and bonnethead sharks to be 13x, 5x, and 4x higher in liver relative to muscle in each species of shark. While PCBs in muscle tissue was not measured in the fish from Sabine Lake in our study, we can anticipate levels in muscle to be equivalent to, or above those measured by the DSHS survey in 2011.

Finally, the TEQ risk assessment of the hepatic levels of dioxin-like PCBs shows bull sharks to have the highest median value, which was in excess of the upper toxicity thresholds for fish (early life-stage) mortality (Steevens et al., 2005), and for immunosuppressive effects in aquatic mammals (pinnipeds, cetaceans, mustelids) (Kannan et al., 2000). Steevens et al., (2005) establish an upper limit of 699 pg TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)/gram lipid or TEQ equivalent as being protective of 90% of fish species (i.e. with 10% adversely affected). This limit was derived from a survey of toxicity studies with early life-stage cyprinid fish (trout, catfish, minnows). The median TEQ value calculated for bull sharks in our study (2024 pg TEQs/gram lipid weight) is approximately 3x higher than the 90% protective threshold (699 pg TCDD/gram lipid) calculated by Steevens et al., (2005). Assuming that the linearity of the toxicity benchmark calculated by Steevens et al., (2005) extends below 90%, and that there is effective ‘read-across’ (or similarity of toxicity effects) between cyprinid and elasmobranchs, a simple linear extrapolation of the median value measured in our study yields a much lower

protective threshold of ~30%. Thus, indicating the likelihood that 70% of bull sharks may be adversely affected by their elevated dioxin-like PCB body-burdens.

In addition to the comparison of likely adverse effects with fish, the median TEQ level in bull sharks (2024 pg TEQs/gram lipid weight) is within range (~1.4x) of the upper threshold shown to cause immunosuppressive effects in aquatic mammals (1400 pg TEQ/gram lipid weight). The adverse effects reported at such a high level include the suppression of lymphocyte (natural killer or NK) cell proliferation, and lowered plasma concentrations of Vitamin A and thyroid hormone (Kannan et al., 2000). Whether such adverse effects are induced in the fish sampled at Sabine Lake is not certain, and constitutes an avenue for further research.

CHAPTER III

TROPHIC ECOLOGY AND FATTY ACIDS

Trophic ecology and the use of biomarkers to study food webs

Trophic ecology encompasses the study of the 'interactions' amongst organisms occupying an ecosystem, whereby the 'linkages' amongst organisms allows for the exchange of energy and materials (Garvey and Whiles, 2017a). Specifically as a sub-set, autotroph-heterotroph-decomposer interactions yields an interconnected network or food web, whose properties represent the structure and diversity of the ecosystem under study (Pimm et al., 1991).

The terms 'linkages' and 'interactions' refer to the dietary niches of organisms whereby nourishment (energy and materials) are derived from prey items by predation or consumption. Organisms with similar energetic and nutritional needs and with relatively equivalent modes of acquiring (or capturing) such needs, co-occupy a similar trophic position (Garvey and Whiles, 2017a). However, an organism's trophic position cannot be viewed as a stringently limiting constraint on its dietary (or prey) preferences, as organisms are capable of exhibiting a 'dietary niche width', whereby nourishment capture strategies can vary between dietary generalist vs. dietary specialist strategies, i.e. reflecting the diversity of prey species that can be consumed by an individual occupying any given trophic position (Hayden et al., 2019).

The core of trophic ecology is the food web, which describes the complex interrelationships by which organisms consume one-another (McKinney and Schoch, 1998). The linkages amongst organisms and their respective trophic positions are typically established by observational studies (Choy et al., 2017; Paine, 1966), analysis of stomach or gut contents (Sagar

et al., 2019), use of stable isotopes (Boecklen et al., 2011), or the analysis of fatty acids in tissues (Iverson et al., 2002).

Establishing predator-prey relations in food webs is essential for understanding the functional intricacies of any ecological system (Hutchinson, 1959). For example, Paine's (1966; Paine, 1980) observations of feeding relationships in various rocky intertidal ecosystems discerned the changing dietary dependence of predatory-to-prey species and associated energy (or calorie) transfers given dietary flexibility, and the numbers of prey items consumed (inferred as proportional to the abundance of prey item). More recently, advances in technology, such as the use of satellite tags, remote sensing and computing have allowed the extension of traditional observational studies to a more global reach to study large-scale ecological systems (Sagarin and Pauchard, 2010).

In organisms that are difficult to directly observe (such as pelagic fish), gut content analysis can provide detailed information on predatory-prey interactions and help to establish food web linkages (i.e. who is eating whom) (Baker et al., 2014; Hyslop, 1980). Dance et al., (2018) used gut content analysis to elucidate differences in prey item choice over ontogeny, and niche specialization in two reef fish species from the northwest Gulf of Mexico, gray triggerfish (*Balistes capriscus*) and red snapper (*Lutjanus campechanus*). Similarly, gut content analysis helped to distinguish prey choice and respective feeding ecologies (i.e. hunting habitats) for three species of coastal sharks that occupied relatively similar trophic position (Plumlee and Wells, 2016).

The use of stable isotope analysis (SIA) has been shown to be a versatile tool to study various terrestrial and aquatic food webs (Middelburg, 2014; Pitt et al., 2009; Wang et al., 2004). The enrichment of stable 'heavy' isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) relative to

elemental carbon and nitrogen in diet is used to delineate food sources and trophic position (DeNiro and Epstein, 1978; Deniro and Epstein, 1981). Increasing ^{15}N isotope enrichment is seen across trophic levels, i.e. primary producers (phytoplankton) have lower average $\delta^{15}N$ relative to consumers (zooplankton, fish) (Minagawa and Wada, 1984). Whereas changes in $\delta^{13}C$ enrichment can be used to distinguish between the sources of carbon at the base of food webs, i.e. in aquatic ecosystems distinctive differences in ^{13}C enrichment in eelgrass vs. phytoplankton were also reflected in their consumers (bivalves, shrimp, fish) (McConnaughey and McRoy, 1979).

Fatty acids can also be used as dietary tracers to study trophic position and food web dynamics (Iverson, 2009). Studies with prototypic aquatic food chains comprising primary producers, zooplankton, and fish show the trophic transfer, accumulation and endogenous modifications of dietary fatty acids (Kayama et al., 1963; Kirsch et al., 1998; St. John and Lund, 1996). The extension of such work to study the trophic ecology of coastal/marine ecosystems confirms the efficacy of fatty acids as tracers of diet and trophic position. For example, the feeding ecology of pacific harbor seals (*Phoca vitulina richardsi*) was clearly discerned by comparing the fatty acid profiles in prey species (invertebrates and fish) versus those detected in the blubber of seals (Iverson et al., 1997). A similar approach was also used by Hebert et al., (2006) to study food web changes and associated shifts in the diet consumption of Great Lakes herring gulls (*Larus argentatus*).

Fatty acids as food web biomarkers in marine ecosystems

Fatty acids (FAs) are ubiquitous constituents of all living organisms and provide integral building blocks for the formation of phospholipids (precursors for biomembranes) and triglycerides (constituents of lipids) (Lodish et al., 2016). Fatty acids comprise long hydrocarbon

chains with varying numbers of carbon atoms, and constitute a methyl carbon at the distal end (also called the ω or omega carbon) of the hydrocarbon chain which starts with a carboxylic acid at the carbon 1 terminus position. The carbon atoms in fatty acids are numbered starting at the carboxyl carbon terminus end, with the subsequent carbon atoms 2 and 3 often referred to as α and β respectively (Berg et al., 2015b).

Some fatty acids have variable degrees of unsaturation (i.e. presence of one or more double bonds), whereas fatty acids with no double bonds are saturated. The position or carbon atom on which the double bond is located is represented by the Δ symbol followed by a superscript number. Therefore, *cis*-Δ^9 indicates that there is a *cis* double bond between carbon atoms 9 and 10 (i.e. functional groups are on the same side), whereas *trans*-Δ^2 indicates that there is a *trans* double bond between carbon atoms 2 and 3 (i.e. functional groups are on opposing sides) (Berg et al., 2015b). An alternative naming nomenclature involves indicating the position of the double bond from the distal ω carbon (or methyl carbon) atom, and also reporting the number of double bonds. A shorthand notation of the form $A{:}\,B\omega X$ can be used, where A represents the number of carbon atoms in the hydrocarbon chain comprising the fatty acid; B gives the number of double bonds, and X gives the position of the double bond closest to the ω distal methyl group (Berg et al., 2015b; Parrish et al., 2000). For example, the ω-3 fatty acid docosahexaenoic acid (DHA) can be written in shorthand as 22:6ω3, indicating a 22 carbon-long unsaturated fatty acid with 6 double bonds, and the first double bond being on the third carbon atom from the distal methyl group. In this thesis we represent the ω notation as 'n', i.e. 22:6ω3 is represented as 22:6n3. In contrast to unsaturated fatty acids, saturated fatty acids, such as palmitic acid can be written as 16:0, to indicate that it comprises 16 carbon atoms with no double bonds being present. Finally, based on carbon chain length, fatty acids can be categorized as

either short-chain (comprising <6 carbons), medium-chain (6-12 carbons), long-chain (13-21 carbons), or very long-chain (>21 carbons) (Garvey and Whiles, 2017b).

The two key characteristics of fatty acids that make them valuable tracers for trophic ecology studies include: 1) their ability to distinguish phylogenetic order due to species differences in biosynthesis and modification of fatty acids; and 2) the dietary acquisition and subsequent storage of fatty acids as lipids enables them to bioaccumulate through food sources, allowing them to be traced through a specific food web (Iverson, 2009).

The synthesis of fatty acids

Fatty acid synthesis is a multi-step process that initiates with the condensation of an activated acyl group (acetyl-ACP; ACP or acyl carrier protein) with a malonyl unit (malonyl-ACP) (catalyzed by a ketoacyl synthase). The condensation reaction results in the formation of acetoacetyl-ACP with the maolonyl group contributing two carbons to acetoacetyl-ACP (with the third carbon from the malonyl group lost as CO_2, and loss of one ACP). The condensation reaction is followed by a reduction reaction (catalyzed by a ketoacyl reductase, using NADPH as cofactor), which yields a 3-hydroxybutyryl-ACP. The reduction reaction is followed by a dehydration reaction (catalyzed by 3-hydroxyacyl dehydratase) which yields an unsaturated or *trans*-Δ^2-enoyl-ACP (along with loss of oxygen atom as H_2O). This dehydration reaction is followed by an additional reduction reaction (catalyzed by enoyl reductase, using NADPH as cofactor) which yields a saturated acyl-ACP (butyryl-ACP). The four carbon chain length of butyryl-ACP can be lengthened by the addition of two carbon atoms from malonyl-ACP via a renewed rounds of condensation reaction, followed by reduction, dehydration and reduction; depending on chain length (Berg et al., 2015a).

During the assembly of fatty acids, additional dehydration or desaturation reactions as catalyzed by desaturase enzymes can produce unsaturated fatty acids with variable numbers of double bonds. Phylogenetic differences in desaturase enzyme activities can produce a variety of characteristic polyunsaturated fatty acids (PUFAs) (Hashimoto et al., 2008; Sperling et al., 2003). For example in marine ecosystems, primary producers such as dinoflagellates characteristically produce long-chain ω-3 PUFAs such as 22:6ω-3 (docosahexaenoic acid, DHA), 18:4ω-3 and 20:5ω-3 (eicosapentaenoic acid, EPA) respectively, while some diatoms also predominantly produce 16:4ω-1 (Iverson, 2009; Jónasdóttir, 2019; Parrish et al., 2000). Zooplankton (such as copepods) feeding primarily on phytoplankton primary producers can also accumulate elevated levels of ω-3 PUFAs (Sargent and Falk-Petersen, 1988). In contrast to primary producers, animals predominantly produce fewer and simpler fatty acids, such as 14:0, 16:0 and 18:0 saturated fatty acids, and their mono-unsaturated isomers 14:1ω-5, 16:1ω-7 and 18:1ω-9 (Iverson, 2009)

In this Chapter, both gut content analysis and fatty acid analysis in livers of fish from Sabine Lake was analyzed to establish their trophic position. In addition, the interrelation between fatty acid composition and pollutant body-burdens for PAHs and PCBs was also analyzed. Given that fatty acid profiles can be reflective of dietary source, this Chapter explores whether the sources of pollutant exposure could relate to the type of diet consumed by the fish sampled from Sabine Lake.

Methods FAME

Fish sampling

The methods used for fish sampling are described in greater detail in the *Methods* section of *Chapter 1*. For brevity, bull shark (*Carcharhinus leucas*), alligator gar (*Atractosteus spatula*),

red drum (*Sciaenops ocellatus*), and gafftopsail catfish (*Bagre marinus*) were sampled in Sabine Lake between April 25th – May 8th 2018. Fish were collected by mid depth trawling with a gill net. Fish were stored on ice prior to freezing upon return to the lab. Frozen fish samples were defrosted in a refrigerator overnight prior to the dissection of liver samples, which were then stored at -80°C until further analysis.

Fatty acid extraction and clean-up

All glassware used for fatty acid extraction and analysis was previously combusted in a Thermolyne (Thermo Scientific) furnace at 450^{o}C for 8hrs. Approximately 1 gram of hepatic tissue was prepared for fatty acid analysis. Lipid extraction was carried out using a modified version of the Folch et al., (1957) method. Each sample of liver tissue was homogenized in a 20 mL combusted glass vial using a PT 1300 D Polytron (Kinematic AG) handheld homogenizer, in a solution containing 2 mL chloroform, 1 mL methanol and 0.5 mL MilliQ-water. After homogenization, N_2 gas was added to displace the air in the headspace of the vial (to prevent the oxidation of fatty acids), the vial was immediately capped, vortexed for 30 seconds and sonicated by placing in a Branson Ultrasonics^{TM} M2800 Ultrasonic Bath (Fisher Scientific) containing ice for 4 minutes, to further extract fatty acids into solvent. Following sonication, the phase-separation of the solvent from the aqueous tissue homogenate was assisted by centrifugation at 125 *g* for 3 minutes. The resulting solvent or chloroform layer (at the bottom of vial) was recovered with any residual moisture removed by filtering the recovered solvent through sodium sulfate. The resulting eluate was placed in a pre-weighed 20 mL glass vial and evaporated under N_2 gas until a lipid residue remained. Following evaporation, the vial was weighed to gravimetrically obtain the lipid weight.

After weighing, the lipid residue was transesterified (to form fatty acid methyl esters or FAMEs) by adding 1.5 mL of Boron trifluoride-methanol (BF_3/CH_3OH) solution (Sigma-Aldrich CAS# 373-57-9) and 0.5 mL hexane. Once again, N_2 gas was added to each vial, the vial was capped and vortexed for 30 seconds before incubating in an oven at 85^{o}C for 60 min. Half-way through the incubation duration, vials were removed from the oven, vortexed again for 30 seconds and returned to the oven at 85^{o}C for the remainder incubation time. After transesterification, vials were removed from the oven and FAMEs were extracted into the hexane layer by adding an additional 2 mL of hexane and 0.5 mL MilliQ-water to the vial, this solution was spiked with 10 µL of internal standard (10,000 µg/mL of d33-Heptadecanoic acid) and vortexed for 30 seconds. The presence of any moisture from the hexane layer was once again removed by filtering hexane through sodium sulfate. The recovered hexane was evaporated in a speedvac concentrator (Thermo Scientific). The FAMEs from the resulting residue were extracted by reconstituting the residue with 0.5 mL MilliQ-water and 0.5 mL dichloromethane (DCM). The solution was vortexed and centrifuged at 2000 g for 1 minute. The DCM layer was recovered and any moisture residues removed using sodium sulfate. The recovered DCM eluate was dried in a speedvac and reconstituted to 1 mL with DCM and stored at -20^{o}C until gas chromatography and mass spectrometry (GCMS) analysis.

GCMS analysis of FAME's

A total of thirty seven fatty acid methyl esters were analyzed using gas chromatography mass spectrometer (GCMS). This analysis was conducted using a Hewlett Packard HP-6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer. Samples were injected in splitless mode (1µL) equipped with a CP-Sil 88 (J&W Scientific) capillary column (100 m x 0.25 mm i.d.; 0.20 µm film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL/min.

Temperatures at the front inlet and the MS interface were set at 250 and 260°C, respectively. Following injection of the sample, the GC oven was programmed at 100°C and held for 5 min, then ramped up to 180°C at 8°C/min, held for 9 min, and finally ramped up to 230°C at 1°C/min and then held for 15 min. The total run time was 80 minutes.

The MS was operated in electron impact (EI) mode at an electron energy of 70 eV, while the MS source temperature was maintained at 230°C. Selected ion monitoring (SIM) mode was used for identification and quantification of all 37 FAMEs. Quantification of all FAMEs was performed against a linear 10-point calibration curve ($r^2 > 0.97$) using serially diluted standards that were (200 ppm to 0.391 ppm). Sample quality assurance and quality control measures was performed by running a solvent blank and a mixed standard prior to analysis, as well as running sample blanks throughout analysis (by using MilliQ-water as surrogate for the tissue homogenate). The limit of detection (LOD) was established as 5x the average of the background signal for each compound. Any FAME concentrations that were below the LOD were given an arbitrarily low value of 0.01 µg/mL in order to avoid a null value. All blanks showed no signs of external contamination above the LOD. All FAMEs were identified according to the IUPAC nomenclature followed by their respective abbreviated notations, chromatographic retention times and quantifying/qualifying ions are listed in the table below.

Table 1 All 37 FAMEs, according to the IUPAC nomenclature. Column headings are; abbreviated notations, elution order, elution time, chromatographic retention times and quantifying ions.

Compound	**Notation**	**Elution Order**	**Elution Time**	**Quantifying Ion**	**RT Range**	
Butyric_acid	C4:0	1	10.71	74	10.66	10.76
Caproic_acid	C6:0	2	12.46	74	12.40	12.52
Caprylic_acid	C8:0	3	14.99	74	14.92	15.06
Capric_acid	C10:0	4	17.74	74	17.65	17.83
Undecanoic_acid	C11:0	5	19.21	74	19.11	19.31

Table 1 Continued

Compound	Notation	Elution Order	Elution Time	Quantifying Ion	RT Range	
Lauric_acid	C12:0	6	20.70	74	20.60	20.80
Tridecanoic_acid	C13:0	7	22.29	74	22.18	22.40
Myristic_acid	C14:0	8	24.02	74	23.90	24.14
Myristoleic_acid	C14:1	9	25.79	55	25.66	25.92
Pentadecanoic_acid	C15:0	10	26	74	25.87	26.13
cis-10-pentadecenoic_acid	C15:1	11	28.27	55	28.13	28.41
Palmitic_acid	C16:0	12	28.48	74	28.34	28.62
Palmitoleic_acid	C16:1n7	13	30.14	55	29.99	30.29
Heptadecanoic_acid	C17:0	14	30.85	74	30.70	31.00
cis-10-heptadecenoic_acid	C17:1	15	33.62	55	33.45	33.79
Stearic_acid	C18:0	16	33.83	74	33.66	34.00
Elaidic_acid	C18:1n9 (trans)	17	35.35	55	35.17	35.53
Oleic_acid	C18:1n9 (cis)	18	36.21	55	36.03	36.39
Linoelaidic_acid	C18:2n6 (trans)	19	38.54	67	38.35	38.73
Linoleic_acid	C18:2n6 (cis)	20	39.26	67	39.06	39.46
Arachidic_acid	C20:0	21	41.81	74	41.60	42.02
gamma-linolenic_acid	C18:3n6	22	42.85	79	42.64	43.06
cis-11-Eicosenoic_acid	C20:1n9	23	43.63	55	43.41	43.85
α-Linolenic_acid	C18:3n3	24	44.44	79	44.22	44.66
Henicosanoic_acid	C21:0	25	44.77	74	44.55	44.99
cis-11,14-Eicosadienoic_acid	C20:2	26	47.13	67	46.89	47.37
Behenic_acid	C22:0	27	50.09	74	49.84	50.34
cis-8,11,14-Eicosatrienoic_acid	C20:3n6	28	50.04	79	49.79	50.29
Erucic_acid	C22:1n9	29	52.70	55	52.44	52.96
cis-11,14,17-Eicosatrienoic_acid	C20:3n3	30	52.90	79	52.64	53.16
Arachidonic_acid	C20:4n6 (ARA)	31	52.34	79	52.08	52.60
Tricosanoic_acid	C23:0	32	53.32	74	53.05	53.59
cis-13,16-Docosadienoic_acid	C22:2n6	33	56.90	67	56.62	57.18
cis-5,8,11,14,17-Eicosapentaenoic_acid	C20:5n3 (EPA)	34	57.39	79	57.10	57.68
Lignoceric _acid	C24:0	35	57.8	74	57.51	58.09
Nervonic_acid	C24:1	36	61.66	55	61.35	61.97
cis-4,7,10,13,16,19-Docosahexaenoic_acid	C22:6n3 (DHA)	37	70.35	79	70.00	70.70

Stomach content analysis

Fish gut content analysis was performed by Dr. Philip Matich. Following capture and storage, fish were moved from -80°C and allowed to defrost for 24hrs at -20°C. Defrosted fish were removed from the cooler for dissection, an incision was made laterally along the ventral side from the cloaca to the pectoral fin. The stomach was removed from each fish and another incision was made along the length of the excised stomach. The contents of the stomach were inspected and recognizable prey items were recorded.

Statistical analysis

All statistical analyses were performed using the Python programing language (v2.7.15), along with associated data handling (Pandas), graphing (matplotlib), and statistical (Scikit) libraries. The normal distribution of data was tested using the Shapiro-Wilks test, with homogeneity of variance tested using the Levene test. Parametric testing was done using one-way analysis of variance (ANOVA), followed by Tukey's posthoc test to compare for pair-wise differences. Non-parametric testing was done using the Kruskal-Wallis test, followed by the Dunn's posthoc test to compare for differences. For non-parametric datasets conforming to a two-factor 'block' design, i.e. datasets with a primary factor (e.g. FAME concentration in fish), and a blocking factor (e.g. FAME type) that could be a source of variability, a Friedman's rank sum test was firstly done to test for a significant effect. If significance was detected, a Nemenyi-Friedman posthoc test was conducted to perform pair-wise comparisons between groups. Significance for all analyses was tested at $\alpha = 0.05$.

Multivariate analyses of hepatic FAME concentrations and pollutant (PAHs, PCBs) levels was performed using multidimensional scaling (MDS) analysis on the pairwise cosine

distances of $\log_{10}(n+1)$ transformed concentrations. While MDS allowed visualization of the similarity between all data points, the FAME and pollutants exhibiting the closest correspondences (≥ 0.8) were parsed to represent fatty acids and associated pollutants. This final analysis allowed assessment of which FAMEs strongly corresponded with PAH and PCB pollutants.

Results FAME

Fish gut content analysis

Gut content of each fish was identified to the lowest taxon (or species) level. The diet composition of all fish was compared with a diet composition matrix (**Figure 6**). The values (shown as heat map colors) along the main diagonal of the heat map matrix represent the numbers of separate taxon identified in each species of fish from Sabine Lake. Whereas off-diagonal numbers represent shared taxon commonly found in multiple fish species. The gut content analysis showed a clear distinction between gafftopsail catfish and red drum vs. alligator gar and bull sharks. Additionally, bull shark gut content analysis also showed the presence of red drum in its diet.

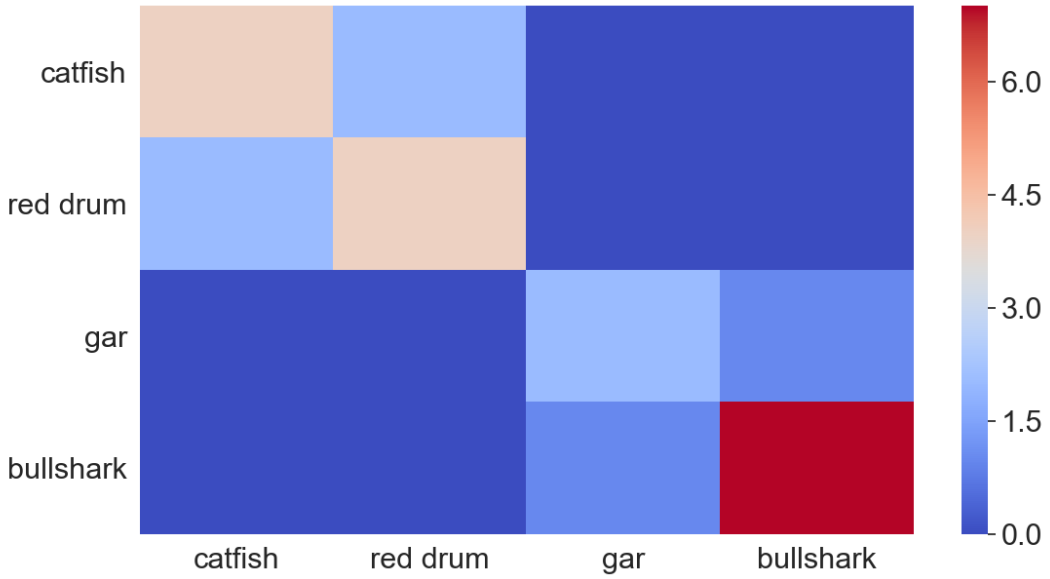

Figure 6 A diet composition heat map showing distinct and shared taxon identified in the gut contents of fish from Sabine Lake.

Fatty acid methyl ester (FAME) levels in fish from Sabine Lake

The concentrations of FAMEs are reported as µg/gram liver wet weight. Fatty acid levels were not normalized to lipid weights as there were no statistically significant inter-species differences in lipid weights (one-way ANOVA, $p \geq 0.05$) (data not shown), intra-species coefficients of variation ranged from 48-87% (data not shown). Such large difference in lipid levels were contrasted to the relatively constant ~1 gram of hepatic tissue that was sampled from each fish, with overall coefficients of variation ranging from 3-23%, and with also no statistically significant differences in liver weights (one-way ANOVA, $p \geq 0.05$) (data not shown). Therefore, all FAME concentrations were expressed as µg/gram liver wet weight.

The analysis of individual FAMEs normalized to sum total FAME concentrations showed notable levels of long- (13-21 carbons) and very long-chain (>21 carbons) fatty acids (Garvey and Whiles, 2017b). These fatty acids included: myristic acid (FAME#8 in (**Figure 7**), 14:0, 2-

5% of sum total FAMEs), palmitic acid (FAME#12, 16:0, 20-23%), palmitoleic acid (FAME#13, c16:1n7, 8-17%), stearic acid (FAME#16, c18:0, 4-8%), oleic acid (FAME#18, 18:1n9, 7-18%), linoleic acid (FAME#20, 18:2n6, 1-3%), erucic acid (FAME#29, 22:1n9, 2-4%), arachidonic acid or ARA (FAME#31, 20:4n6, 5-7%), eicosapentaenoic acid or EPA (FAME#34, 20:5n3, 4-9%), nervonic acid (FAME#36, c24:1, 0-11%), and docosahexaenoic acid or DHA (FAME#37, 22:6n3, 12-21%). The remainder fatty acids contributed to <1% to the levels measured in fish.

The statistical analysis of all thirty seven FAME congeners in fish from Sabine Lake showed statistically significant differences between the four fish species (Friedman's rank sum test, $p \leq 0.05$) (**Figure 7**), significantly different groups are not shown). The subsequent Nemenyi-Friedman posthoc test of pair-wise comparisons showed alligator gar and bull sharks as not being significantly different from one another, while all other species differed significantly from each other. While the 'blocked design' of the Nemenyi-Friedman posthoc test allowed consideration of individual FAME congeners, the comparison of sum total FAMEs across the four fish species showed no significant differences between the fish (one-way ANOVA, $p \geq 0.05$) (data not shown).

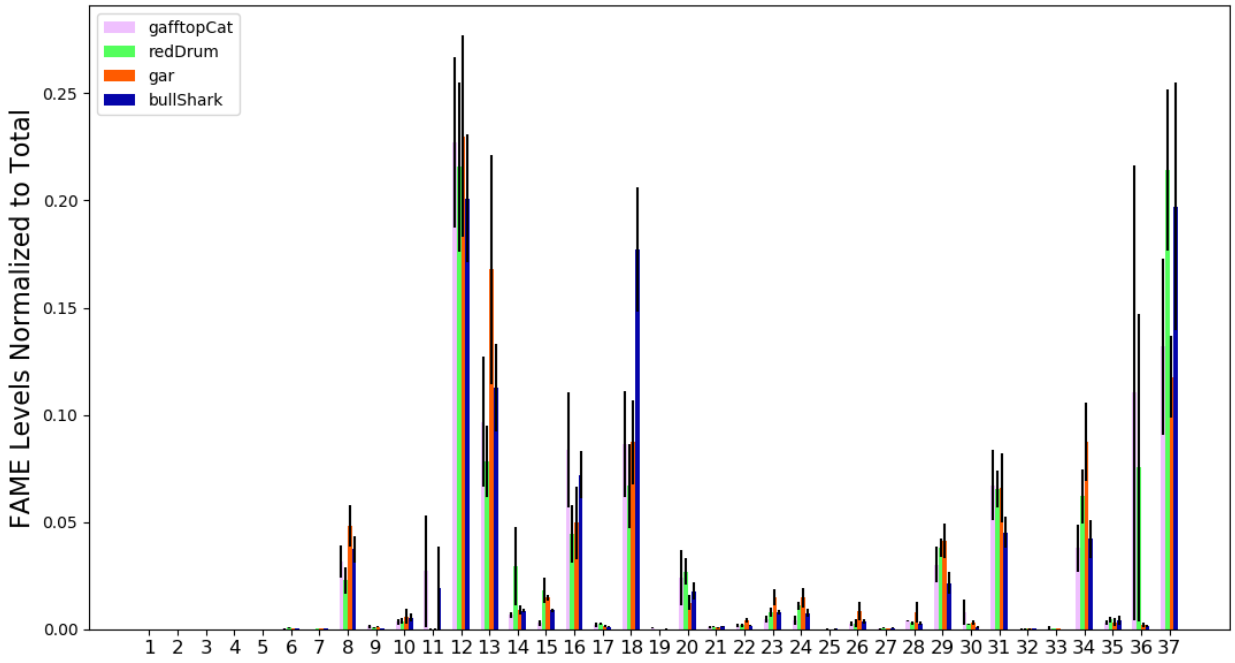

Figure 7 Levels of thirty seven fatty acid methyl esters (FAMEs). FAMEs were measured in gafftopsail catfish (*Bagre marinus*), red drum (*Sciaenops ocellatus*), alligator gar (*Atractosteus spatula*), and bull shark (*Carcharhinus leucas*). FAME numbering is as per listed in (**Table**).

Multivariate correspondence analysis of FAMEs and pollutant body-burdens

Multidimensional scaling analysis (MDS) allowed for a visual assessment of the pairwise cosine similarities between hepatic fatty acids, PAHs and PCBs (**Figure 8**). The cosine similarity matrix generated during this analysis was exported to excel, and FAMEs exhibiting correlational coefficients $\geq$ 0.8 with PAHs or PCBs were parsed out. The numbers of PAHs and PCBs corresponding to these parsed FAMEs were visualized as a bar graph (**Figure 9**). This analysis aimed to discern whether there were any characteristic groupings (or correlations) between hepatic fatty acids and associated body-burdens of pollutants.

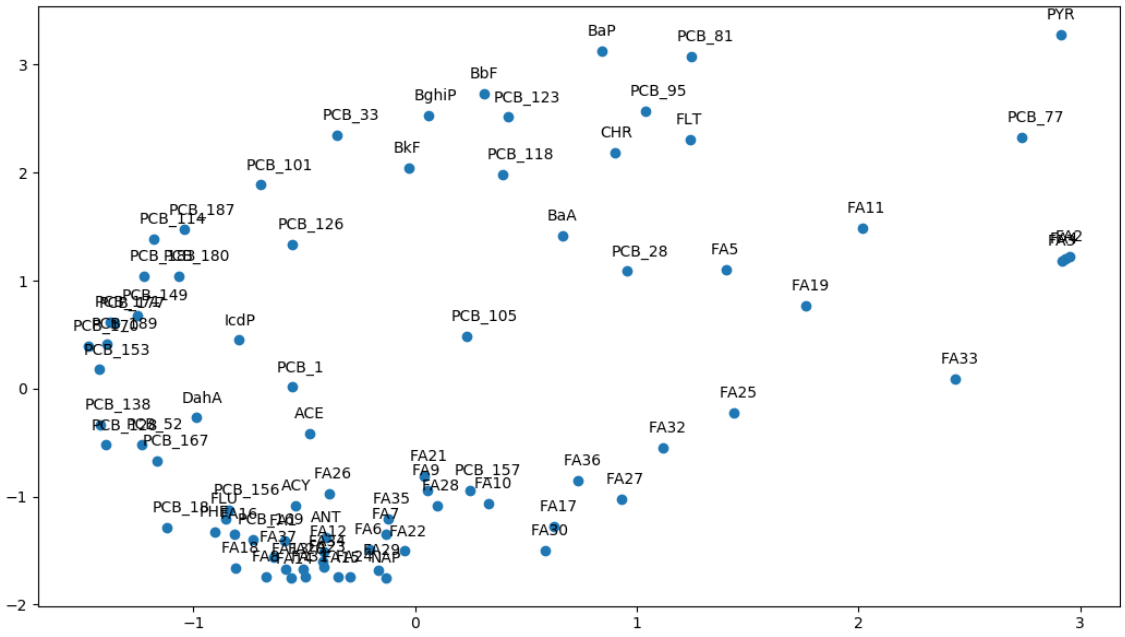

Figure 8 Multidimensional scaling (MDS) analysis of the pairwise cosine similarities between hepatic levels of FAMEs, PAHs and PCBs.

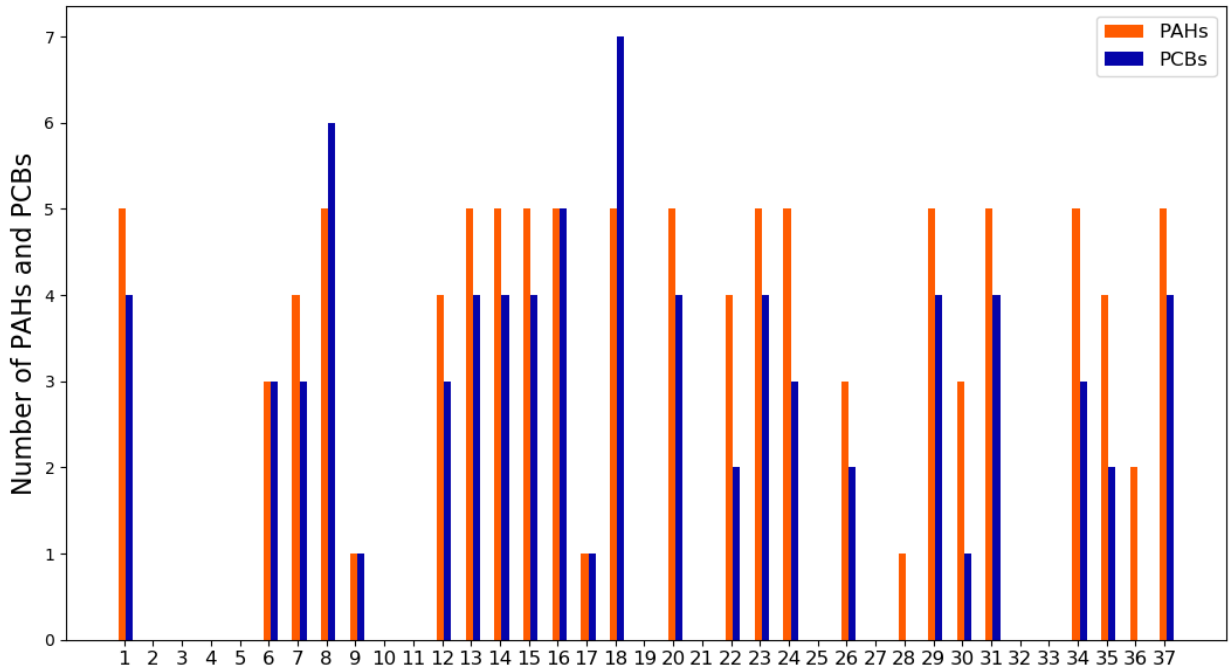

Figure 9 Graph showing the numbers of PAHs and PCBs that were highly correlated with FAMEs in fish livers.

Discussion FAME

Fatty acids and the trophic ecology of fish from Sabine Lake

The trophic transfer of fatty acids from prey to predator species makes them effective markers for trophic ecology analysis (Iverson, 2009; Kayama et al., 1963; Kirsch et al., 1998; St. John and Lund, 1996). While the gut content analysis of prey items in the digestive tracts of animals provides a snapshot of recently acquired (or preferred) prey items, fatty acid profiles can be expected to provide a more integrative assessment of prey choice, and hence the breadth of an organisms feeding ecology.

In our study, the gut content analysis of fish from Sabine Lake clearly showed a demarcation between catfish and red drum vs. alligator gar and bull shark (**Figure 6**). The gut content analysis of catfish and red drum was clearly distinguished from that of alligator gar and bull shark by the exclusive presence of invertebrate taxa (crabs and shrimp) in catfish and gar. Whereas, alligator gar and bull shark gut contents exclusively comprised teleost fish. The morphometric analysis of fish weight and lengths presented in Chapter 1 indicated adult life-stages for gafftopsail catfish and red drum, versus juvenile and young-of-year ($\leq$1 year old) life-stages for gar and bull shark respectively (Cruz-Martínez et al., 2004; TPWD, 2019). As these size classes were sampled together at the same sampling locations (i.e. on Sabine Lake), a shared niche utilization was expected, but does not appear to be the case. Using gut-content analysis alone it is clear that the catfish and red drum mainly oblige to benthic feeding habits as evidenced from the predominance of crustacean prey items in their gut.

FAME analyses showed that alligator gar and bull sharks were not significantly different from one another, while all other species were significantly different from each other. The uniqueness of the FAME profiles of catfish as compared to gar and bull sharks is explainable by

the complete absence of overlap in prey items identified in their respective gut-contents. However, there is a 50% overlap of gut-contents (mainly for crabs) between catfish and red drum, making it harder to explain differences in FAMEs between these two fish species. A possible explanation for the differences in FAME profiles between catfish and red drum could be related to the proportional differences in the abundances of prey items comprising gut contents. As the fatty acid compositions of prey items were not identified in this study, the proportional differences in their abundances may attribute to distinctive fatty acid profiles of the predators that predate upon them. Such quantification of prey fatty acid signatures are necessary for the accurate allocation of predator trophic positions (Iverson et al., 2004).

In marine ecosystems, photoautotrophic bacteria, diatoms and dinoflagellates provide the primary fatty acids that are found across marine food webs. Fatty acid biosynthesis in photoautotrophs comprising two-carbon chain elongation and desaturation reactions are capable of generating almost all long/very-long chain saturated and unsaturated fatty acids (Berge and Barnathan, 2005). Furthermore, various ratios of fatty acids are typically used to indicate their predominant sources in food webs, such as from terrestrial plants, diatoms or dinoflagellates (Berge and Barnathan, 2005; Parrish et al., 2000).

Overall in our study, the analysis of such ratios confirms the near-shore coastal habitats from which the fish were sampled. For example, terrestrial plants are attributed to produce elevated levels of 18:2n-6 and 18:3n-3, with ratios >2.5 indicating the inputs of plant-derived lipids (Budge and Parrish, 1998). Our study reports ratios ranging from 2.5 – 4.5 across the four fish species sampled from Sabine Lake. Interestingly, comparisons of *cis* vs. *trans* isomers of the mono- and polyunsaturated (marine algae derived) fatty acids of elaidic acid (*trans*-18:1n-9) vs. oleic acid (*cis*-18:1n-9), and linolelaidic acid (*trans*-18:2n-6) vs. linoleic acid (*cis*-18:2n-6),

showed a predominance of the *cis* over the *trans* isoforms (66x and 159x respectively). This indicates the two-carbon chain elongation of palmitic acid (16:0) to stearic acid (18:0), was followed by desaturation to predominantly to *cis*-18:1n-9 and *cis*-18:2n-6 (Berge and Barnathan, 2005). Palmitic acid, which itself is a good tracer for algal fatty acids (Kelly and Scheibling, 2012), is able to serve as an adequate precursor to these fatty acids as its levels were ~4x that of stearic, oleic and linoleic acids. Such a predominance of *cis* isoforms may improve cell membrane fluidity and viability (de Carvalho and Caramujo, 2018). High ratios (>1) of the *ω*-3 PUFAs 22:6n-3/20:5n-3 was also observed, indicating the presence of dinoflagellate-derived fatty acids (Budge and Parrish, 1998). Finally, the analysis of essential long-chain PUFAs showed the presence of ratios that are required for the maintenance of normal fish growth and development. For example, we find optimal dietary ratios of 22:6n-3 (docosahexaenoic acid, DHA): 20:5n-3 (eicosapentaenoic acid, EPA) of >2:1, and 20:5n-3 (EPA): 20:4n-6 (arachidonic acid, ARA) of ~1:1 (Sargent et al., 1999). The near equivalence of these ratios across all four fish species indicates prominent dietary sources from primary producers (dinoflagellates) and zooplankton (calanoid copepods) (Berge and Barnathan, 2005).

At present, a review of the published literature suggests that our study is amongst a very few that have profiled FAMEs in the studied fish species. We can compare some of the FAME profiles measured in our study with those measured and presented in a comprehensive report on FAME levels in various Gulf of Mexico fish by Lytle and Lytle (1992). However, such comparisons can only be made for red drum and gafftopsail catfish, as these were the only two species from our study that were also included and studied by Lytle and Lytle. Overall, Lytle and Lytle (1992) report n-3/n-6 PUFA ratios of 1.5-3.0 for red drum, and 3.0-4.5 for the gafftopsail catfish. Our study was within range for the red drum (n-3/n-6 ratio of 3.0), but was lower for the

gafftopsail catfish (ratio of 1.9). Finally, Lytle and Lytle (1992) also report a DHA/EPA ratio of ~4 for the red drum, which is similar to the ratio of 3.5 found in our study.

In summary, our study suggests that overall there are similar dietary sources and an equivalent trophic position, for the fish inhabiting Sabine Lake. The coastal near-shore (saltwater/estuarine) habitat use of the fish sampled strongly indicates the presence of a diversity of primary producers that include macroalgae and terrestrial plants. While there is overall similarity in fatty acid composition across the four species, there are differences in their relative amounts amongst these species. For example, nervonic acid (24:1(n-9)) was abundant in catfish and red drum, and near-absent in alligator gar and bull sharks (**Figure 7**). Given that its production is mainly in calanoid copepods (Berge and Barnathan, 2005) and soil filamentous fungi (such as *Mortierella capitata*, (Umemoto et al., 2014)), the presence of nervonic acid in catfish and red drum places their feeding ecology towards the 'base' of the marine food web. While nervonic acid levels were highly contrasted between catfish/red drum vs. gar/bull shark (45x higher in the former), most other fatty acid congeners varied by up to 2x amongst the fish species (**Figure 7**), these remainder differences are likely due to various compositions of prey items and unaccounted for inter-species differences in the deposition and metabolism of fatty acids.

Integrating fatty acid content with pollutant (PAH and PCB) body-burdens

The trophic transfer of both fatty acids and persistent pollutants through food webs is likely to be intricately linked with the trophic ecology of exposed species (Kainz and Fisk, 2009). However, pollutant bioaccumulation in an organism can also be dependent on its physicochemical properties, such as its hydrophobicity (Bond et al., 1985; Hawker and Connell, 1988). The moderate to high hydrophobicity of PAHs (log K_{ow} ~3.3 to >5.6) (Sverdrup et al.,

2002), and high hydrophobicity of PCBs (log K_{ow} 4.5 to > 8.2) (Hawker and Connell, 1988; IARC, 2016; Walters et al., 2011), ensures their bioaccumulation in the lipid fraction and fatty tissues of exposed organisms (Beyer and Biziuk, 2009; Neely et al., 1974). Subsequently, such bioaccumulation of PAHs and PCBs in an individual organism lends to their trophic transfer (or trophic 'magnification') across food webs (Nakata et al., 2003; Porte and Albaiges, 1994; Walters et al., 2011; Wan et al., 2007).

Our study suggests there is 2 distinct trophic positions for the fish sampled from Sabine Lake. Gafftopsail catfish and red drum could be considered closer to the 'base' of the marine food web (relative to alligator gar and bull sharks), due to exclusively elevated nervonic acid levels likely acquired from zooplankton and soil filamentous fungi (as discussed in the previous section). The analysis of pollutant body-burdens in hepatic tissues of fish showed sum total PCB levels to be significantly higher in bull sharks. The size class of the bull sharks sampled in this study identifies them as young-of-year (i.e. $\leq$1 year old) fish (Cruz-Martínez et al., 2004). Given that diet may not be a contributory factor in influencing the higher PCB body-burden in bull sharks, we posit a role of maternal transfer to explain such high body-burdens (Lyons et al., 2013; Mull et al., 2013).

A closer examination of the interrelation between highly correlated (cosine similarity distance $\geq$0.8) fatty acids and pollutant body burdens showed 68% of fatty acids to be highly correlated with PAHs and PCBs (**Figure 9**). In amongst these fatty acids we do not see a clear preference for pollutant bioaccumulation. For example, there is near equivalent representation of some of the highest numbers of PAHs and PCBs in a short-chain fatty acid (butyric acid (4:0)), a medium-chain fatty acid (lauric acid (12:0)), some long-chain fatty acids (tridecanoic acid (13:0),

eicosapentaenoic acid (20:5n3), etc.), and a very long-chain fatty acid (docosahexaenoic acid (22:6n3)).

Furthermore, the analysis of PAHs that were highly correlated with these fatty acids showed a predominance of low molecular (LMW) weight ($\leq$ 3 aromatic rings) compounds such naphthalene (NAP), phenanthrene (PHE), anthracene (ANT), acenaphthylene (ACY), and fluorene (FLU). This observation agrees with the overall high abundance (and likely higher bioavailability) of LMW PAHs in fish from Sabine Lake, and also indicated a mainly petrogenic source of PAH exposure (Baumard et al., 1998; Budzinski et al., 1997; Djomo et al., 1996; Wolska et al., 2012) Despite dibenzo[a,h]anthracene (DahA) comprising the largest fraction of high molecular weight (HMW) PAHs ($\geq$4 aromatic rings) across all fish species from Sabine Lake (4-32% of normalized sum total PAHs), it did not strongly correlate with any fatty acid.

While only five PAHs correlated strongly with fatty acids (representing only 31% of the total numbers of PAHs quantified), seven PCBs were found to strongly correlate with fatty acids (representing 24% of the total numbers of PCBs quantified). Much like the PAHs, the highly correlated PCBs (with fatty acids) were also amongst the most abundant congeners quantified, which included PCB's 18, 128, 138, 156, 157, 167, and 169. Of these PCBs that were highly correlated with fatty acids, 57% were dioxin-like PCBs, which included PCB's 156, 157, 167, and 169.

Therefore in this study, some of the most highly quantified PAHs and PCBs were also found to be strongly correlated with a broad suite of fatty acids, with carbon numbers ranging from 4:0 (butyric acid) to 22:6n3 (docosahexaenoic acid). Our data suggests that there is no preferential accumulation of pollutants with a specific type of fatty acid (i.e. based upon carbon number or saturated vs. unsaturated fatty acid). This observation agrees with the expectation that

under physicochemical conditions, the total lipid solubility of a pollutant (as represented by its log K_{ow} value) is unlikely to be related to the biochemical composition of lipids.

However, some exceptions can be found when considering the ecological fate and behavior of pollutants across trophic levels (Kainz and Fisk, 2009). Kainz et al., (2006) showed methyl-mercury bioaccumulation in macrozooplankton (Daphnia spp., calanoid copepods, etc.) to be significantly correlated with essential fatty acids such as eicosapentaenoic acid or EPA (20:5n3), arachidonic acid or ARA (20:4n6), and linoleic acid (18:2n6). In the same study, the authors also measured higher bioaccumulation of methyl-mercury in fish (rainbow trout, *Oncorhynchus mykiss*) consuming macrozooplankton, with high methyl-mercury:essential fatty acid ratio measured for docosahexaenoic acid or DHA (22:6n3) (Kainz et al., 2006). A more recent study by Le Crozier et al., (2016) also showed significant correlations between the bioaccumulation of various metal pollutants and essential fatty acids, such as observed for iron (Fe), manganese (Mn), lead (Pb) and zinc (Zn) levels with ARA (20:4n6). Finally, Countway et al., (2003) report significant correlations between PAH levels (phenanthrene and perylene) and long-chain PUFAs (including palmitoleic acid, 16:1n7) in autochthonous organic matter (phytoplankton) from riverine surface waters (York River estuary, VA).

Regardless, a key observation from this study suggests that pollutant bioaccumulation is more likely to reflect the unique exposure history of an organism and its overall lipid reservoir, than any particular fatty acid type. As FAMEs were measured in the total lipid fraction from liver tissue of fish, our data appears to reflect upon the capacity of this fraction, as a whole, to bioaccumulate PAHs and PCBs. As a closing note, it is interesting to consider that while only 24% of PCBs were found to be highly correlated with fatty acids, 57% of these were dioxin-like PCBs. Therefore, while we cannot ascribe a particular fatty acid as being responsible for

‘carrying’ the greatest pollutant burden (and by extension preventing the designation of any specific dietary source of such a fatty acid), future efforts may be better expended in understanding whether an organisms intrinsic pollutant body-burden is exerting quantifiable toxicity.

CHAPTER IV
CONCLUSIONS

Pollutant body-burdens of fish from Sabine Lake

The results of this thesis show, the levels of sixteen PAH and twenty-nine PCB pollutants measured in the hepatic tissue of four fish species from Sabine Lake, namely: gafftopsail catfish (*Bagre marinus*), red drum (*Sciaenops ocellatus*), alligator gar (*Atractosteus spatula*) and bull shark (*Carcharhinus leucas*). The analyses presented show relatively unique profiles for PAHs and PCBs in the fish from Sabine Lake. Specifically, PAH profiles indicated mainly petrogenic sources of exposure. The multivariate analysis of pollutant (hepatic) body-burdens indicated bull sharks to have a distinct pollutant profile relative to that other fish species. This trend was confirmed by the observation that bull sharks also exhibited a significantly higher body-burden of PCBs relative to the other fish species. Furthermore, the high PCB body-burden for bull sharks was also reflected in the high TEQ values measured for this species. Bull sharks exhibited the highest TEQ values amongst the four fish species sampled at Sabine Lake, with levels above the upper limits of toxicity thresholds for fish and aquatic mammals. The size range of bull sharks indicated YoY life stages. Therefore, a unique exposure history for YoY bull sharks is postulated, and implicates maternal transfer as having a role in influencing PAH and PCB pollutant body-burdens.

Body-burdens relation to pelagic trophic ecology in Sabine Lake

In this thesis trophic ecology was assessed using thirty-seven fatty acids measured in the hepatic tissue of four fish species from Sabine Lake, namely: gafftopsail catfish (*Bagre marinus*), red drum (*Sciaenops ocellatus*), alligator gar (*Atractosteus spatula*) and bull shark (*Carcharhinus leucas*). The analyses presented showed two distinct levels of trophic ecology for

the fish from Sabine Lake. The elevated levels (45x higher) of nervonic acid (24:1n9) (which is a fatty acid indicator of calanoid copepods and soil filamentous fungi), in gafftopsail catfish and red drum vs. alligator gar and bull shark, strongly indicates the feeding ecology of catfish and red drum to be closer to the 'base' of the marine food web. The correlational analysis of pollutant body-burdens of PAHs and PCBs with fatty acids indicated that the most abundantly detected pollutants were correlated with a wide variety of fatty acids. No clear distinction was apparent for a preference of pollutant correlation with any particular type of fatty acid (i.e. unsaturated vs. saturated, short-chain vs. very-long chain). Our results suggest that pollutant bioaccumulation is unlikely to be associated with the specific fatty acid composition of lipids.

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