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Three Year Assessment of Nearshore Crude Oil Contamination in the Gulf of Mexico Using Gulf Menhaden (Brevoortia Patronus) as an Indicator Species: Menhaden Watch

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THREE YEAR ASSESSMENT OF NEARSHORE CRUDE OIL CONTAMINATION IN THE GULF OF MEXICO USING GULF MENHADEN (*BREVOORTIA PATRONUS)* AS AN INDICATOR SPECIES: MENHADEN WATCH

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To my family and friends; especially my mother Carmen, my wife Helena, and my late grandfather James. Without the support structure that you provided I would not have made it this far.

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ABSTRACT

Approximately 4.9 million barrels of crude oil along with natural gas were released into the Gulf of Mexico (GoM) from April to July 2010 (Deepwater Horizon, DWH, spill). Impacts of this magnitude seldom occur in the GoM (Ixtoc I was the last spill close to this magnitude occurring in 1979), and one cannot predict when they will happen. Major constituents of concern found in crude oil are Polycyclic Aromatic Hydrocarbons (PAHs), which often have low volatility that allows for prolonged existence in the environment. PAHs are compounds of concern according to the United States Environmental Protection Agency (USEPA), with one characteristic being that they have the potential to accumulate within adipose tissue. Several PAHs are listed as mutagenic and carcinogenic, making their presence in commercial fishery populations of major environmental concern. Gulf menhaden fishery was chosen for use as an indicator for impact of crude oil exposure in the years following the spill event. Total whole body PAH concentrations along with both benzo[a]pyrene, toxic and mutagenic equivalents (BaP-TEQ and BaP-MEQ respectively), were used to determine overall impact on the species. Proposed standard weight equations and length categories for Gulf menhaden were developed to assess morphological changes in the species. Lipid content was also used as a metric for determining overall health of the Gulf menhaden. Results are outlined in each chapter conspectus.

CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

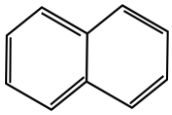
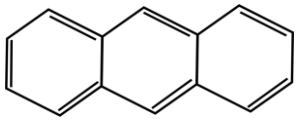
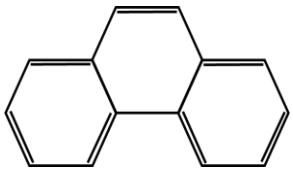
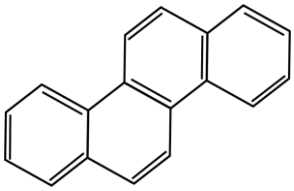
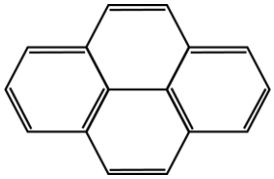
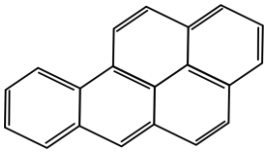
1.1. Introduction

The release of large quantities of crude oil into the Gulf of Mexico (GoM) in 2010 raised concerns over the possible contamination of marine organisms based on the prolonged time of the continuous oiling event (April - September 2010) (Weber, 2010). As can be seen in figure

Figure 1.1.1 NASA's Terra Satellite Sees Deepwater Horizon Spill on May 24, 2010 (Credit, NASA)

1.1.1, the extent of the Deepwater horizon spill was quite large, with an estimated total spill of 4.9 million barrels of oil (approx. 210 million US gallons). One major constituent of concern that can be found in crude oil is hydrocarbons with low volatilization properties that can remain in the environment for extended time periods. Polycyclic Aromatic Hydrocarbons (PAHs) are part of this class of crude oil constituents and are characterized by multiple conjoined ring structures, with naphthalene and its alkylated forms being the smallest (molecular mass of 128.17 g/mol) (Haritash & Kaushik, 2009). Higher molar mass PAHs volatilize less, allowing the compounds to remain in nature longer than the lighter constituents of crude oil (Feng, et al., 2009).

Table 1.1.1: Examples of PAH Ring Structures

Aromatic Rings	Name	Structure
2	Naphthalene	
3	Anthracene	
	Phenanthrene	
4	Chrysene	
	Pyrene	
5	Benzo[a]pyrene	

This leads to the possibility of bioaccumulation within the adipose fraction of marine organisms and possible biomagnification within the trophic structure of the GoM. PAHs are considered compounds of concern according to the United States Environmental Protection Agency (USEPA) due to their ability to accumulate within adipose tissue (USEPA, 2008). There are

several PAHs listed as mutagenic and carcinogenic, making their possible presence in commercial fishery populations a major environmental concern (USEPA, 2008) (Durant, et al., 1996) (Nisbet & LaGoy, 1992).

Table 1.1.2: USEPA Priority PAHs and Chemical Abstract Service (CAS) Registry Numbers

Compound Name	CAS Registry Number
Naphthalene	91-20-3
Acenaphthylene	208-96-8
Acenapthene	83-32-9
Fluorene	86-73-7
Phenanthrene	85-01-8
Anthracene	120-12-7
Fluoranthene	206-44-0
Pyrene	129-00-0
Benzo(b)fluoranthrene	205-99-2
Benzo(k)fluoranthrene	207-08-9
Benzo(a)pyrene	50-32-8
Dibenz(a,h)anthracene	215-24-2
Benzo(g,h,i)perylene	191-24-2
Indeno(1,2,3-cd)pyrene	193-39-5

Programs exist for the continual monitoring of coastal waters using invertebrate, filter feeding mollusks (Mussel Watch) that have helped elucidate nearshore impact dynamics (NOAA: CCMA, 2012); however, there are no such programs for assessing near and off shore impact dynamics using a vertebrate species of similar characteristics. The current project presents new information on the concentrations of PAHs within a commercially valuable fish harvested in great quantities from the GoM. Gulf menhaden (*Brevoortia patronus*) was identified using government assessments as the second largest commercial harvest from United States waters and the largest from the GoM from 2005 to 2010 (Van Voorhees & Lother, 2011). Population harvests along with several other factors presented gulf menhaden as a principal candidate for this study. The organism selection was further supported by the fact that menhaden

are harvested due to the amount of fats and oils that are extracted and refined for consumer use and, as such is of particular interest in evaluating the fat or lipid soluble constituents found it in crude oil (Franklin, 2007) (Van Voorhees & Lother, 2011). Menhaden are also significant due to their position in the GoM food web as obligate filter feeders (also the same feeding mechanism employed by mollusks). This particular mode of feeding increases interaction with possible

Table 1.1.3: Major US Species Landed in 2010 Ranked by Quantity (adapted from Van Voorhees & Lother, 2011)

Rank	Species	Pound
1	Pollock	1,958,936
2	Menhaden	1,471,80
3	Salmon	787,740
4	Flatfish	624,358
5	Cod	557,349
6	Hakes	378,277
7	Crabs	349,604
8	Squid	337,223
9	Shrimp	258,972
10	Herring (sea)	253,381

surface and subsurface oil through dermal contact and direct ingestion, and based on the primary diet of phytoplankton, positions menhaden as the main link between producers and secondary consumers (Franklin, 2007) (Van Voorhees & Lother, 2011) (Vaughan, et al., 2007). The lifespan of menhaden is approximately three years, allowing for whole life assessment every three years as well as pre-, during-, and post-event temporal assessment for future oiling events. Commercial fishing grounds in the Gulf of Mexico stretch from Eastern Florida in the Florida Keys to the bay of Campeche in Mexico to the west. From roughly April to October each year,

the fish form large schools and are harvested for industrial refining of their fats and oils (Franklin, 2007) (Vaughan, et al., 2007). Menhaden oil is used in a variety of commercial

Figure 1.1.2 Gulf Menhaden (*Brevoortia patronus*) Caught in Vermilion Bay, Louisiana (Credit, Gregory Olson)

products ranging from makeup to over-the-counter health supplements (Franklin, 2007). As mentioned earlier PAHs are lipophilic and can accumulate within the adipose tissue of an organism (Larsen, et al., 2002). Menhaden are fatty fish that can accumulate PAHs in their tissue, leading to the possible magnification of the toxic compounds through trophic transfer due to predator/prey consumption interactions. Menhaden are a principal forage food for other fish, birds, and marine mammals. They represent the primary connection between producers and secondary consumers within the GoM (Franklin, 2007) (Vaughan, et al., 2007). Gulf menhaden do not undergo major longitudinal migrations, as the fish remain in coastal waters seasonally and spend the first year of their life cycle in estuarine waters (Vaughan, et al., 2007). As a result, Gulf menhaden develop solely in the Gulf throughout the duration of their life, moving between deep (roughly 80 km off shore) and coastal waters (Vaughan, et al., 2007). Gulf menhaden

spawn between October and March, with peak spawning between December and January; April to October is the optimal harvest season ((Raynie & Shaw, 1994). The spatial distribution, feeding patterns, and abundance of the organism within the desired region of study are all major factors contributing to the importance of the Gulf menhaden as an indicator species to continually assess the health of the GoM.

1.1.1 Rational

The GoM is projected to produce upwards of 1.7 million barrels of oil per day (MMBOPD) and 8 billion cubic feet per day (BCFPD) of natural gas by 2016 (Karl, et al., 2007). The GoM is a significant petrochemical exploration and development region of the United States. It has and will continue to be a major source of crude oil and natural gas. The GoM is also one of the most productive marine ecosystems in the United States, accounting for an average of 18% of the total U.S. domestic commercial fish landings from 2009 to 2010 (Van Voorhees & Lother, 2011). The GoM will continually be affected by petroleum exploration for the immediate future. Because of the connection to the petrochemical industry, commercial and sport fishing in this region will always have the potential to be affected; therefore the GoM should be monitored continually in order to assess overall health as well as specific temporal and spatial events impacting this region.

1.2. Gulf Menhaden (*Brevoortia patronus*)

Gulf menhaden (*Brevoortia patronus*) are considered smaller than Atlantic menhaden, with fork lengths of no more than 22 cm, and also have a shorter life cycle (Franklin, 2007). As stated earlier menhaden are obligate filter feeders that consume anything collected within their gill rakers as they school through the water (Vaughan, et al., 2007). Their commercial fishing grounds are as far east as the southern tip of Florida and stretch westward to the Yucatan

peninsula in Mexico. From roughly April to October each year, the fish form large schools that are harvested for industrial refining of their fats and oils along with their proteinaceous meal (Franklin, 2007) (Vaughan, et al., 2007). Menhaden oil is used in a variety of commercial products ranging from makeup to over-the-counter health supplements (Franklin, 2007). As stated before, the primary concern with menhaden contamination depends on the refined lipid fraction used by consumers from this fish. A more basic concern, however, is bioaccumulation and magnification throughout the trophic structure of the Gulf (USEPA, 2008). PAHs are lipophilic and can accumulate within the adipose tissue of an organism (Larsen, et al., 2002). Menhaden are fatty fish that have the ability to accumulate PAHs in their adipose tissue, leading to the possible magnification of the toxic compounds through trophic transfer due to prey consumption. Menhaden are considered a standard forage food for other fish, birds, and marine mammals and also represent the primary connection between producers and secondary consumers within the GoM (Franklin, 2007) (Vaughan, et al., 2007). Gulf menhaden do not undergo major longitudinal migrations, as the fish remain in coastal waters seasonally and spend the first year of their life cycle in estuarine waters (Vaughan, et al., 2007). As a result, Gulf menhaden spawn in the principal areas affected by the DWH oil spill for the duration of their life, moving between deep GoM waters (roughly 80 km offshore) and GoM coastal waters (Vaughan, et al., 2007). Spawning occurs between October and March, with peak spawning taking place between December and January; April to October is the optimal harvest season and was used as the season of harvest for this study (Raynie & Shaw, 1994).

1.3. GoM Sublittoral Current Systems and Distribution Patterns

Currents in the GoM impacted the distribution of oil released from the DWH spill, with the greatest contributors being the Loop Current (LC) and the Eddy Franklin (EF) (Hamilton, et

al., 2011) (Fig. 1.3.1 Copyright 2011 American Geophysical Union, Reproduced/modified by permission of American Geophysical Union). The LC moves between the latitudes of 24–28°N on varying timescales (0.5–18.5 months) after entering the Yucatan Channel. During its maximum penetration, the LC turns anticyclonically and exits through the Florida Straight. The LC is comprised of salty (36.7–36.8), warm water (25–26°C) and has a baroclinic flow structure (Hamilton, et al., 2011) (Vukovich, 2007). The majority of the flow is above 800 m with the habitual non-chaotic northward branching of the LC contributing to the upper level mesoscale variability among marine species of the eastern Gulf. The LC enters the Gulf at 23–27 Sverdrups, typically shedding an eddy at its northernmost position (Hamilton, et al., 2011). The eddy contributes to

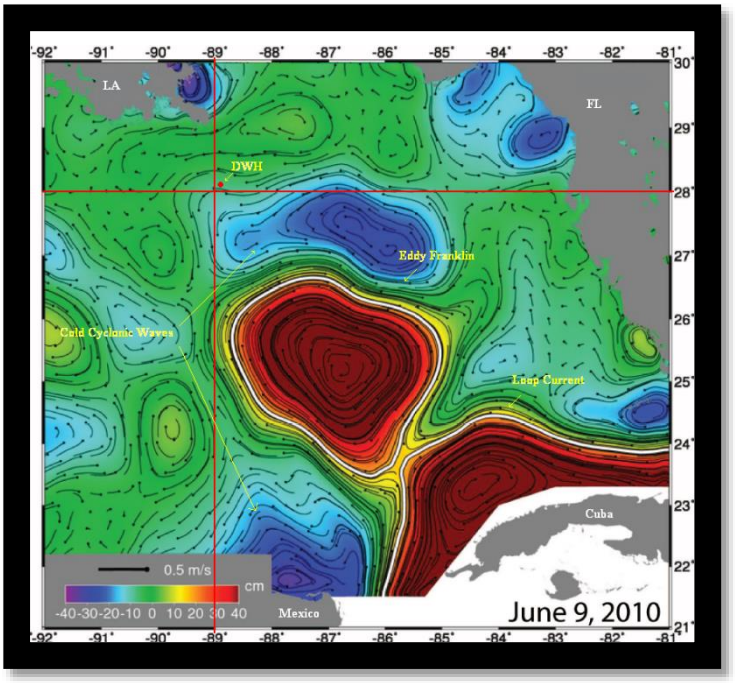


Figure 1.3.1 Lagrangian particle path computed from geostrophic flow fields derived from sea surface height (SSH) maps for the selected LC intrusion event associated with the separation of Eddy Franklin in 2010. This shows Eddy Franklin at the time of the first observed detachment from the LC. This event exhibited a deep intrusion into the northern Gulf, a large LC eddy detachment, and a significant retreat of the LC northern boundary after eddy detachment to well south of 25°N. Labeled within the map is the location of the Deep Water Horizon oil platform (DWH), the Loop Current, Eddy Franklin, and the Cold Cyclonic Wave systems surrounding both EF and the LC. (Adapted from Hamilton et al. 2011)

the upper layer mesoscale distribution of the water column, as well as the transfer of mass, heat, momentum, and salt from the eastern to western Gulf basins (Hamilton, et al., 2011) (Vukovich, 2007). Frontal or cold cyclonic waves, located along the edge of the LC and the fringes of EF, are additional phenomena that create movement in the GoM. (Walker, et al., 2003).The anterior eddies fluctuate systematically around the LC, becoming largest at the northern edge, intermediate on the eastern side, and smallest on the southern edge (Vukovich, 2007) (Walker, et al., 2009). In May and June of 2010, measurable particulates freely suspended in the water showed that EF was displaying a closed anticyclonic flow, with intense southwestward currents between the Campeche bank and the west Florida slope (Hamilton, et al., 2011). The LC and EF, as well as the smaller currents surrounding the DWH platform, were major contributors to the movement of Gulf water and influenced the fate and distribution of the oil.

1.4. Properties and Characteristics of PAHs

PAHs are found naturally occurring in crude and coal, resulting from conversion of natural compounds to aromatic hydrocarbons (Roy, 1995). PAHs are lipophilic and do not easily solubilize in water; rather they adsorb onto the organic matter of the substrate on which they are located. In soils, PAHs generally do not penetrate beyond the organic fraction, limiting leaching into groundwater (Larsen, et al., 2002). Although PAHs of a lesser molecular mass are semi-volatile, most of the PAHs found in the environment are heavier and preferentially react with particulate material (>2.5 μm in diameter). This mechanism is the standard route of atmospheric deposition of PAHs (Edwards, 1983) (Nielsen, et al., 1996). Two- and three-ringed PAHs are almost entirely found in vapor form, with four-ring PAHs transitioning between vapor and solid state. Five or greater ringed PAHs are predominantly solid state and found adsorbed to other materials; these particulates can settle out of the atmosphere into fresh and/or marine water

(Nielsen, et al., 1996). The PAH-particulate complex can be a vector if consumed by organisms or if it settles to the benthic layer of a body of water (Larsen, et al., 2002).

It is important to understand basic routes of exposure to PAHs in order to quantify the impact of the DWH oil spill on the PAH concentrations found in the GoM. PAHs are a component of air pollution and released through the incomplete combustion of fossil fuels such as coal, oil, gasoline, as well as via incineration processes (Larsen, et al., 2002). PAHs are found in wood preservatives composed of tar and/or creosote and also enter the atmosphere as a result of natural events such as volcanic eruptions and forest fires (Hites, et al., 1980). PAHs contaminate the environment through mechanisms that are necessary to enumerate when identifying the impact of PAH concentrations in a particular species. PAHs are found in air, soil, and water through the processes of deposition and transference. Atmospheric PAHs are deposited onto soil or water after adsorption to organic and particulate matter; PAHs in the soil are transferred to water through weathering, and the surface water can be contaminated by atmospheric deposition and soil transfer of PAHs, regardless of origin (Larsen, et al., 2002).

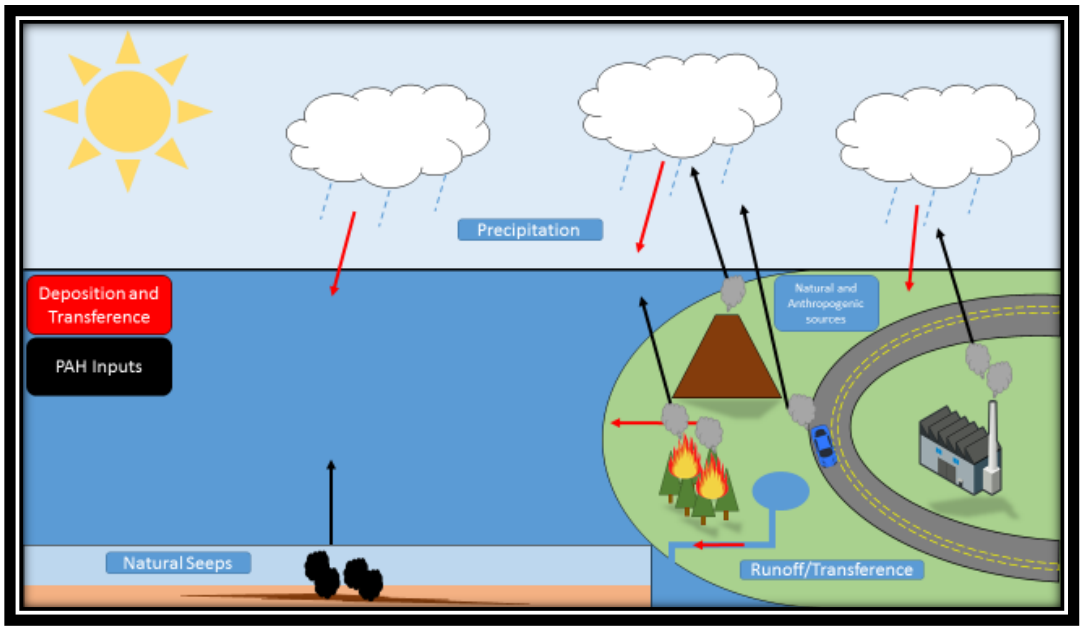


Figure 1.4.1 PAH deposition and transference/ fate of PAHs in the environment

Background levels of PAHs exist in Gulf menhaden, and the background concentrations were identified through the use of a control to assign meaning to the PAH values quantified in the current study.

PAHs are inherently stable and not readily broken down through hydrolysis. However, the compounds can undergo photodissociation and oxidation (Howard, et al., 1991). The reaction kinetics are largely controlled by the location of the PAH in the environment. Non-adsorbed PAHs have half-lives of hours to days, depending on mass and structure, but in substrates such as soil, the half-lives may be months or years (Park, et al., 1990). The abiotic degradation of PAHs can produce oxidized derivatives that are as dangerous as the parent compounds (Larsen, et al., 2002). Biologically, PAHs are metabolized in two phases by the cytochrome p-450 super family of enzymes, and the biotransformation efficiency is directly related to the cytochrome p-450 dependent oxidative function of specific organisms (James, 1989). It has been reported that the initial transformation of PAHs is slower in invertebrates than vertebrates, and the elimination of the resulting metabolites is also slower (IPCS, 1998). Alkylated PAHs do not necessarily behave the same as their non-alkylated counterparts during the processes of abiotic and biotic degradation.

1.5. Matrix Solid Phase Dispersion of Tissues

The Matrix Solid Phase Dispersion (MSPD) extraction method used in this study is characterized by the total disruption of the sample through the use of an appropriate bonded phase or other solid support material. Octadecylsilyl (ODS)-derivatized silica (C-18 silica) was ground with the tissue sample and packed into a container suitable for a series of elutions with the desired solvent. A new phase consisting of the sample and bonded phase material was created and used for distinctive sample fractionation (Barker, 2007) (García-López, et al., 2008). A

lipophilic bonding phase of C-18 silica was used in the current study, however; C-8 silica could have been used as an alternative for binding lipids (Barker, 2007). Gravity filtration followed by vacuum extraction was used to facilitate the extraction of the C-18/tissue matrix. The eluate collected from this process was "clean" enough to run on analytical instruments. However, additional cleanup measures can be conducted, such as co-column cleanup (Barker, 2007). In the case of the eluate collected from menhaden, the only secondary cleanup method employed was a standard settling period after the extraction process. This allowed any material large enough to pass through the glass microfiber filter time to settle out. This method, along with a solvent exchange/dilution with hexane, was sufficient to create a sample that did not cause damage to the GC/MS column (Olson, et al., 2014).

1.6. Standard Weight Equations and Length Categories for Fishes

Length and weight data are frequently used by those who manage fisheries to evaluate the condition of individual fish (Wege & Anderson, 1978) (Anderson, 1980) (Murphy, et al., 1990) (Anderson & Neumann, 1996). Conversely, suitable assessment can be confounded by discrepancies in weight among fish of similar lengths within and among populations, and by allometric growth rates (American Fisheries Society, 1996). Relative weight (W_r), the ratio of a given fish's weight compared with the standard weight (W_s) of a rapidly growing fish of the same length, was created as a predictor of condition to normalize evaluations across varying length classes (American Fisheries Society, 1996). Standard weight equations have to initially be developed for managers. It was therefore possible to calculate W_r for the species of interest. Wege and Anderson (1978) first developed W_s equations by fitting a curve to the 75^{th} percentile of weights of largemouth bass, *Micropterus salmoides* (Wege & Anderson, 1978). Murphy et al. (1990) refined the methods of Wege and Anderson (1978) to reduce length-related biases,

developing the regression-line percentile (RLP) technique using regression equations for each population in their study rather than pooled length/weight data (Murphy, et al., 1990) (Murphy & Willis, 1992). Ranney et al. (2010) found that equations derived from the RLP performed equally well with regard to length bias compared to other techniques and concluded that those methods are equivalent in terms of their significance to stock management (Ranney, et al., 2010). A common practice in fish assessment for sport fish is to develop a quality index based on length. Several of these indices have been developed for various marine and freshwater fish (Neumann, et al., 2012) (Raymond, et al., 1998).

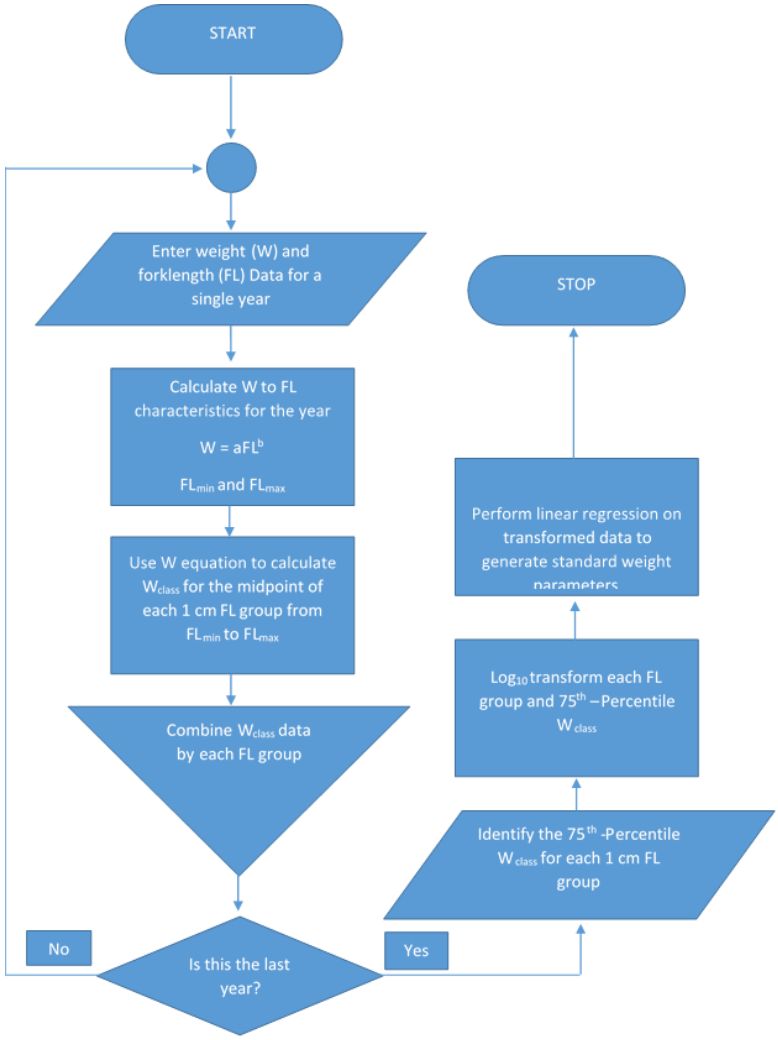


Figure1.6.1 Flowchart outlining the RLP method used to determine the standard weight equation (W_s) for Gulf menhaden condition assessment. (Adapted from Murphy et al., 1990)

Gabelhouse (1984) proposed a standard length categorization technique based on percentage of world record length (Gabelhouse, 1984). Using the Proportional Size Distribution (PSD) calculation, PSD-X (where X represents a specified category of quality) and the PSD X-Y, (where X-Y represents the incremental difference between categories) (Neumann, et al., 2012) an attempt to categorize Gulf menhaden based on length was conducted.

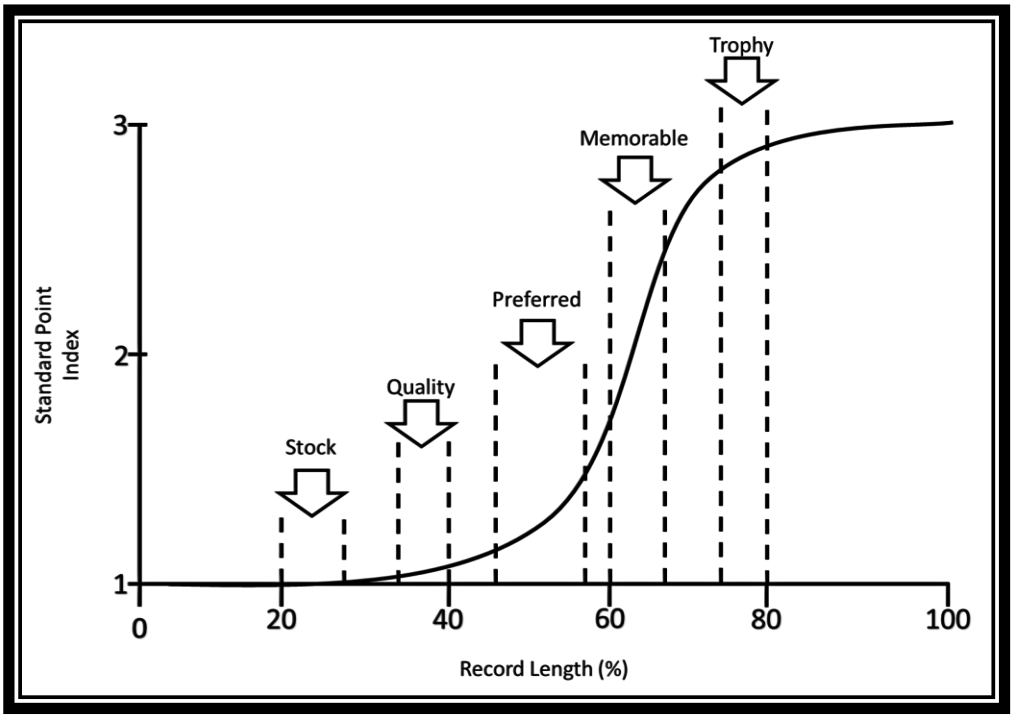


Figure1.6.2 Gabelhouse's adoption of Weithman's (1978) fish quality index to identify length ranges from which (or near to which) minimum stock, quality, preferred, memorable, and trophy lengths were selected (adapted from Neuman et al., 2012)

PSD is a numerical descriptor of length-frequency data and can either be categorical or incremental in nature. Each method was therefore employed in attempting to develop an appropriate measure of menhaden. The standard quality index of "Stock", "Quality", "Preferred", "Memorable", and "Trophy" do not apply. I am thus proposing the adoption of a new length category system for use with fish that conform to the same profile as menhaden.

Categories were based on possible end use of fish harvested, “Bait”, “Commercial”, “Quality Commercial”, and “Exceptional Commercial”.

1.7. Lipid Quantitation Using Soxhlet Extraction Techniques

Lipids are classified as an assorted group of natural substances comprised chiefly of non-polar compounds (triglycerides, diglycerides, monoglycerides, and sterols) as well as polar compounds (free fatty acids, phospholipids, and sphingolipids) (Christie, 1993). Lipids join covalently to carbohydrates and proteins to form glycolipids and lipoproteins. Solvents used for lipid extraction generally should have a high solubility for all lipid compounds and be sufficiently polar to remove them from their binding sites with cell membranes, lipoproteins and glycolipids (Smedes & Askland, 1999). This holds true for complete lipid analysis. However, it is suggested that based on the targets for analysis (i.e. non-polar lipid soluble chemicals), the use of non-polar and polar solvents might be unnecessary. The knowledge of lipid content in food or other tissues is important for several reasons, one of which is determining concentrations of persistent organic contaminants (dioxins, PCBs, organochlorine pesticides, PAHs) in tissue (De Boer, 1988). If these contaminants can be removed from the sample matrix along with the non-polar fraction of lipids, a standard non-polar lipid total should be quantified for method analysis. Several methods have been developed for total lipid extraction (Folch, et al., 1957); (Bligh & Dyer, 1959); (Gardner, et al., 1985); (De Boer, 1988); (Booij & van den Burg, 1994); (Smedes, 1999). Heated solvent extraction through a Soxhlet apparatus was used to extract lipids from sample menhaden tissue along with controls. Traditionally, chloroform-methanol (2:1), hexane-ethanol (3:1), and several other solvent mixtures have been used to determine total lipid concentrations for various substrates (Nelson, 1975). Several papers have discussed the harmful nature of chloroform and have cited its toxicity and low volatility as reasons to move away from

its use (Cequier-Sanchez, et al., 2008) (Drouillard, et al., 2004). There have been attempts to determine a suitable analogue to which dichloromethane has been suggested (Cequier-Sanchez, et al., 2008) (Drouillard, et al., 2004).

Figure 1.7.1 Soxhlet extraction apparatus used to obtain lipids from Gulf menhaden tissue. (Credit, Gregory Olson)

Based on several methods of analysis for lipid concentration, it was determined that the use of dichloromethane (DCM) as a singular non-polar solvent to extract total non-polar lipids (TNPLs) from tissues that will be analyzed further for non-polar compounds of concern (i.e. PAHs) would be appropriate. The single-solvent method was compared to hexane-ethanol (3:1) for total lipid recovery as well to show the % lost through incomplete lipid extraction.

1.8. References

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CHAPTER 2: ADAPTATION OF SONICATION-ASSISTED MATRIX SOLID PHASE DISPERSION OF TISSUES FOR THE SUBSEQUENT EXTRACTION OF POLYCYCLIC ATOMATIC HYDROCARBONS FROM GULF MENHADEN (*BREVOORTIA PATRONUS*)

2.1. Conspectus

A new adaptation based on matrix solid phase dispersion (MSPD) of tissue for the subsequent isolation of polycyclic aromatic hydrocarbons was developed and used for extractions of Gulf Menhaden caught during the summer of 2011. Many MSPD methods require specific cartridges and other clean up materials in order to achieve proper extraction. For this study, the tissues were lyophilized prior to applying the adapted MSPD method, allowing for a much more complete homogenization with the C18 silica. The tissue was spiked with phenanthrene d_{10} as a surrogate as a measure of PAH recovery prior to the lyophilisation process to determine if any target compounds were lost and prior to sonication as per the finalized adaptation procedure to determine method efficiency. This technique used C18 silica in a 1:1 ratio as the primary homogenizing material for the menhaden tissue matrix. This new matrix was eluted with dichloromethane (DCM) until visibly clear. The overall study mean recovery was 88% ± 5%, with method detection limits between 0.4 ng/g and 4.4 ng/g tissue dry weight. This adapted protocol has been used exclusively on the analysis of high-lipid-content fish stocks affected by dispersed and weathered oil from the BP Horizon incident.

2.2. Introduction

The release of large quantities of crude oil into the Gulf of Mexico during the 2010 BP Deepwater Horizon incident has raised concerns based on contamination of marine organisms with constituents of weathered crude oil. One major group of compounds found in crude oil that is of major concern are the Polycyclic Aromatic Hydrocarbons (PAHs) (Haritash & Kaushik, 2009). This group of compounds can be characterized by multiple conjoined ring structures, with

naphthalene and its alkylated forms being the smallest (molecular mass of 128.17 g/mol) (Albero, et al., 2003) (Ling, et al., 1994). The higher molar mass of theses PAHs results in less volatilization, which in turn allows those compounds to remain in nature far longer than other constituents of oil (Feng, et al., 2009). This leads to the possibility of bioaccumulation within the adipose fraction of marine organisms and possible biomagnification within the trophic structure of the Gulf of Mexico (Pensado, et al., 2005).

PAHs are considered compounds of concern according to the U.S. Environmental Protection Agency due to their ability to accumulate within adipose tissue (USEPA, 2008). Several PAHs are considered mutagenic as well as carcinogenic, making their possible presence in a commercially important fish such as menhaden a major concern (USEPA, 2008). Gulf Menhaden (*Brevoortia patronus*) was identified as the fish that accounts for the largest commercial harvest from the Gulf of Mexico and subsequently selected as the principal organism to study by our group (Van Voorhees & Lother, 2011).

Menhaden were collected due to the amount of fats and oils that can be extracted from them and refined for consumer use, which is important because of the lipophilic nature of PAHs (Franklin, 2007) (Vaughan, et al., 2007). This fish also plays a key role in the trophic structure of the Gulf of Mexico, acting as the main forage fish for many species of fish, dolphins, and water-fowl (Franklin, 2007).

This obligate filter feeder has two very important factors contributing to its selection as a sentinel species: 1) menhaden are in contact with surface and subsurface oil through dermal exposure and direct ingestion; and 2) due to sheer fish stock volumes and trophic predation, menhaden are the main link between producers and secondary consumers (Franklin, 2007) (Van Voorhees & Lother, 2011) (Vaughan, et al., 2007).

The matrix solid phase dispersion (MSPD) method is an extraction method characterized by the total disruption of the sample through the use of an appropriate bonded phase or other solid support material such as octadecylsilyl (ODS)-derivatized silica (C18 Silica), which is ground with the sample (Barker, 2007). This creates a new phase consisting of the sample and bonded phase material and allows for distinctive sample fractionation (Barker, 2007) (García-López, et al., 2008). For this experiment a lipophilic bonding phase of C-18 silica was used; however, the use of C-8 silica is considered a possible alternative (Barker, 2007). The form of MSPD extraction used in the study can be described as vacuum-assisted because of the vacuum applied to the apparatus after gravity filtration has been completed. Generally, the eluent collected from this process is sufficiently "clean" for direct injection into analytical instruments. However, additional clean-up measures can be conducted such as co-column clean-up, where the addition of other support materials are added to the bottom of the container (Barker, 2007).

The goal of this study was to determine if the outlined adaptations (Lyophilization and Ultrasonication) to MSPD extraction techniques would result in valid and quantifiable data for use in monitoring waters impacted by oil spill events.

2.3. Materials and Methods

2.3.1. Solvents, Reagents, and Chemicals

Pesticide reagent grade solvents were used in all standard preparations, sample analysis, and rinsing procedures. The dichloromethane (DCM), hexane (Mallinckrodt Chemicals), and RediSep C18 silica (40–60 µm, Teledyne Isco) were used for tissue extraction. Sodium sulfate (anhydrous, 10–60 mesh, Fisher Scientific) was used for final sample preparation.

2.3.2. Gulf Menhaden

Menhaden were sampled at locations around Grand Isle, Louisiana (GI) and Vermilion Bay, Louisiana (VB) which based on SCAT mapping showed a considerable difference in shoreline oil exposure with GI being more heavily exposed to shoreline oil from the DWH spill event. They were harvested between June and September of 2011.The samples were collected using a standard five-panel gill net. This net was approximately 200 m in length with 5 distinct plastic mesh panels. The menhaden were separated by length, bagged in plastic freezer bags, and placed on ice until frozen to -4°C in a laboratory setting. Pre-spill (July 2009) menhaden tissue control samples were created from processed menhaden donated by a menhaden processing company located in Louisiana (Non-Disclosure Agreement). Fish oil and meal were combined in a ratio consistent with oil yields reported in this study for size-appropriate tissue concentrations.

2.3.3. Calibration Standards

A commercially prepared crude oil analysis standard (Oil Analysis Standard, Part #90311, Absolute Standards) was used to prepare the five-point calibration standards (0.5 ppm. 1.0 ppm, 5.0 ppm, 10.0 ppm, and 25.0 ppm) using the internal standard method for determining concentrations. Calibration standard solutions were stored in amber vials with PTFE-lined caps. The calibration standards were checked frequently for signs of degradation or evaporation and replaced if necessary during laboratory quality control checks. A continuing calibration standard (one point of the initial five-point calibration standard) was analyzed in each batch of extracted tissue samples or during each 12-hour period during which analyses were performed. The acceptance criterion for each compound in the calibration standard was ±20% of the mean relative response factor calculated from the initial five-point curve. If the acceptance criterion was not met, all analyses were discontinued until the instrument was re-aligned to meet optimal

operations criteria. With instrument maintenance or troubleshooting, a new five-point calibration curve was generated as per good laboratory practices.

Calibration standards are used to ensure consistency in the instrument response when identifying compounds. Each time the instrument source was adjusted or the column was clipped or altogether changed, all five calibration concentrations were analyzed and used as a means to determine instrument quality. The continuing calibration that accompanies all sample batches was one concentration of the five initial calibration standards and was run to ensure accurate measurement of the detector (EPA SW-846 method 8000B) (USEPA, 2012). The mean response factor for each analyte was also calculated during the process of the initial five-point calibration and was used to determine analyte concentration that can be seen in the equation found in sub-section Internal Standard Solutions.

2.3.4. Internal Standard Solutions

Internal standards were naphthalene-d_8 (Part # Z-014J-4), acenaphthene-d_{10} (Part # Z-014J-1), chrysene-d_{12} (Part # Z-014J-2), and perylene-d_{12} (Part # Z-014J-5), all purchased from AccuStandard Inc., New Haven, CT and stored individually until combined to make 4 mL of the internal standard injecting solution. Each internal standard was used to determine the concentrations of analytes with similar molecular weights. This was done by spiking each GC vial with 10 µl of the prepared internal standard solution (10 µl in 1 mL of sample) and then standardizing each target response to the known concentrations of the four standards. Once this was complete, the analyte target response could then be converted to a concentration using the appropriate equation (1).

Analyte Concentration= ((Target Response)×(Internal Standard Concentration)×(Final Volume)×(Dilution Factor))/((Response of Internal Standard)×(Analyte Mean Response Factor)×(Volume Injected)×(Dry Mass)) (1)

Figure 2.3.4.1 Equation used to quantify analyte concentration based on internal standard recovery

2.3.5. Reference Oil Standard

The usual laboratory reference oil established by USEPA has been Alaska North Slope Crude Oil (ANSCO); however, the reference oil standard used for these analyses was Macondo 252 (MC 252) collected directly from the riser of the Deepwater Horizon oil rig. Reference oil standards were prepared by extracting 1 gram of pure oil in 40 mL of solvent (or equivalent ratio of 1 g:40 mL, e.g. 0.50 g:20 mL). The laboratory reference oil was analyzed in each sample batch as an additional QA/QC sample, i.e., a laboratory control sample.

2.3.6. Surrogate Spiking Standards

The surrogate spiking standards were 5-alpha androstane (Part # GRH-IS-10X, AccuStandard) and 10 mg of phenanthrene-d_{10} neat (Part # 364622, Sigma-Aldrich, St. Louis, MO) combined with 500 mL of DCM to make the needed concentration. The extraction efficiency for each sample was based on percent recovery of surrogate standard with an acceptable percent recovery range of 70–120% (Meyer, et al., 2010).

2.3.7. Preparation of the Sample Extracts

Frozen menhaden were weighed, and their fork lengths were taken. Triplicate composite samples of menhaden with fork lengths of 16 cm or less (small) were selected from each field location and then chopped into small cubes approximately 12 mm × 24 mm × 24 mm. These

pieces were then placed into pre-cleaned/solvent-rinsed 200-mL beakers. The cubed tissue was then compressed into the base of the beaker with a clean glass pestle, placed in a –86°C freezer and allowed to freeze. The surrogate spiking solution was added prior to freeze-drying in 7 individuals to determine if lyophilization affected recovery. Frozen samples were then freeze-dried for 24 hours (VirTis, Model Freezemobile 6). This process was repeated for menhaden with fork lengths greater than 16 cm (large) from each field location. Dried samples were placed in a desiccator prior to solvent extraction. It is important to note that this step is performed with no less than 18 samples. Batch lyophilisation is crucial in reducing overall extraction time.

Desiccated fish tissue was pulverized to a fine powder, and a 10-g subsample (as little as 2.5 g can be used) was removed and amended at a 1:1 ratio with C-18 silica. Sodium sulfate in excess of 2–5 g was added and mixed in with a spatula to bind up excess moisture. Samples were then spiked with 1 mL of the surrogate spiking solution. Samples were then filled with 50 mL of DCM and sonicated (Branson 2210) for 30 minutes. After the sonication process, each sample was gravity filtered through a Fisherbrand filter (09-801-G, 24 cm diameter) covered with a 10-g layer of sodium sulfate. The container used to lyophilize and sonicate the sample was rinsed three times with DCM into the homogenized sample to ensure complete transfer of all materials. The funnel (Corning, 6120-6) was attached to a side-arm flask (Corning, 5340-250) affixed to a vacuum manifold. After gravity filtration stopped, a slight vacuum (vacuum-assisted solvent extraction) was applied to finish the removal of all DCM. The resulting eluent was then moved to a flat-bottom Florence flask (Corning, 4060-250), using the triple DCM rinsing technique, and rotary evaporated (Rotavap™ Buchi Laboratory Equipment) until all excess DCM was removed. Figure 1 illustrates the general apparatus used for this study. The residual material in the flat-bottom Florence flask was then reconstituted in hexane and transferred to a solvent-rinsed glass

graduated cylinder. An appropriate amount of hexane was then used to dilute the resulting material to a whole number volume in mL (this amount is not set, enough hexane is used to dilute the sample to sufficient clarity deemed by the GC/MS operator, but was usually between 15–25ml final volume). The solution was aspirated and homogenized with a Pasteur pipette to sufficiently mix the sample. A 10-mL aliquot was collected from the graduated cylinder in a volatile organic analysis (VOA) vial for long-term storage. In the case of the eluent collected from menhaden, the only secondary cleanup method employed was a settling period after the extraction process. This allowed any material large enough to pass through the filter time to precipitate out of solution. Multiple 1-mL aliquots were collected for GC/MS analysis. Samples were placed in appropriate amber auto-sampler vials, spiked with 10 µl of the prepared internal standard, capped, and placed in a refrigerator prior to GC/MS analysis.

2.3.8. Preparation of Menhaden Controls

Control menhaden facsimile tissue was formulated using meal and oil collected during June 2009 from a commercial source. Determining the appropriate oil/meal ratio for both "small" menhaden as well as "large" menhaden allowed for the creation of these facsimile controls. Datasets were generated using a Soxhlet extraction method. Ten grams of homogenized tissue were extracted using DCM for 12–18 hours. Final material was evaporated to completion, and the mass of the extracted "raw" oil was recorded. Once the oil/meal ratios for each size category were determined, control facsimiles were generated. Using the calculated means of "small" (0.13 g menhaden oil/g dry tissue) and "large" (0.39 g menhaden oil/g dry tissue) menhaden oil/meal ratios, controls were created in a 150-mL beaker. The controls were subjected to the extraction procedures as outlined above.

2.3.9. Preparation of Method Detection Limits Analysis

The Method Detection Limit (MDL) procedure involved spiking 0.1 mL of oil analysis calibration standard at 25 ppm into 3 g of prepared menhaden tissue controls created as described in section 2.8. This was repeated six more times for a total of seven replicates. One milliliter (1-mL) of surrogate standard at 20 ppm was added to each of the seven replicates prior to extraction. The samples were then extracted using the previously described adapted MSPD technique and quantified using ChemStation E.02.01.1177.

2.4. Analytical Apparatus

2.4.1. Gas Chromatograph

All GC/MS analyses used an Agilent 5890 GC system configured with a 5% diphenyl/95% dimethyl polysiloxane high-resolution capillary column (30 meter, 0.25 mm ID, 0.25 micron film) directly interfaced to an Agilent 5972 mass selective detector system. An Agilent 6890 series Auto Injector was used for sample introduction into the GC/MS system. The GC flow rates were optimized to provide a required degree of separation, particularly n-C_{17} and pristane (baseline resolved), and n-C_{18} and phytane (baseline resolved). The injection (split) temperature was set at 250°C and only high-temperature, a low thermal-bleed septum (gasket between injection and inlet) was used in the GC inlet. The GC was operated in temperature program mode with an initial column temperature of 60°C for 3 minutes, increased to 280°C at a rate of 5°C/minute, and finally held for 3 minutes. The oven was then heated from 280°C to 300°C at a rate of 1.5°C/min and held at 300°C for two minutes. Injection volume was set to 1 µl. Total run time was 65.33 minutes per sample. The interface to the MS was maintained at 280°C. Ultra High Purity (UHP) helium was the carrier gas for the GC/MS system, with a flow rate of 1 mL per minute.

2.4.2. Mass Spectrometer

The MS was operated in Selective Ion Monitoring (SIM) mode to maximize the detection of several trace target constituents unique to crude oil. Ionization was achieved with electron impact at 70 eV. Selected ions for each acquisition window were scanned at a rate greater >1.5 scans/sec with a dwell time of 60 milliseconds. At the start of each analysis period or every twelve hours, the MS was tuned to PFTBA, an internal instrument standard. Laboratory reference standards such as reference oil and a continuing calibration standard were also analyzed prior to the analysis of tissue/oil sample extracts. This standard operating procedure ensured quality assurance/quality control of the instrument conditions prior to sample analysis.

2.4.3. Data Analysis

The analytical method used in the study utilized MSD ChemStation E.02.01.1177 and identified 71 key constituents of crude oil, with 43 components classified as aromatic (Table 2.4.3.1). This method relies on both NIST and Wiley MS databases to identify selected PAHs in the sample matrix. The significant extraction portion of this study focused on the total aromatic concentrations found within each menhaden sampling group. Samples were individually integrated and compared to the known peaks of the 71 key constituents used to identify crude oil. From the resulting integrations and retention times the analyte concentrations in ng/g of dry wt. tissue for whole menhaden were calculated.

Limits of detection (MDL) were calculated from the GC/MS-SIM analysis of the oil analysis calibration standard along with the tissue controls created in the previous section to determine limits of quantitation were estimated from the oil analysis calibration standard at a concentration of 10 ppb. The analysis of the 10-ppb oil analysis calibration standard resulted in detection of 10-pg peaks with signal-to-noise ratios above 5. Therefore, assuming a 10-gram

sample size and injection of 1 ul out of a total extract volume of 1000 µl, this translates to a detection limit of 1 ppb for the target analytes with a specific range of 0.4–4.4 ppb. The limits of quantitation (LOQ) were then derived by multiplying an approximate value of 5 ppb by a factor of 5, resulting in a LOQ of 25 ppb for all analytes.

Table 2.4.3.1: Analytes of interest and the selected (SIM) ions that are associated with each compound

Analyte	SIM Ion (m/z)	Retention Time	Analyte	SIM Ion (m/z)	Retention Time
Naphthalene - d_8	**136**	**13.06**	C2- Pyrenes	230	38.29
Naphthalene	127	12.86	C3- Pyrenes	244	40.72
C1-Naphthalenes	142	16.01	C4- Pyrenes	258	42.40
C2-Naphthalenes	156	19.35	Naphthobenzothiophene	234	38.94
C3-Naphthalenes	170	22.14	C-1 Naphthobenzothiophenes	248	40.66
C4-Naphthalenes	184	25.41	C-2 Naphthobenzothiophenes	262	42.52
Acenaphthene - d_{10}	**164**	**21.52**	C-3 Naphthobenzothiophenes	276	44.70
Fluorene	166	23.37	Benzo (a) Anthracene	228	40.09
C1-Fluorenes	180	26.17	Chrysene	228	40.24
C2-Fluorenes	194	28.81	C1- Chrysenes	242	42.09
C3- Fluorenes	208	31.04	C2- Chrysenes	256	43.88
Dibenzothiophene	184	27.19	C3- Chrysenes	270	46.16
C1-Dibenzothiophenes	198	29.31	C4- Chrysenes	284	47.68
C2-Dibenzothiophenes	212	31.33	**Perylene - d_{12}**	**264**	**48.64**
C3- Dibenzothiophenes	226	33.54	Benzo (b) Fluoranthene	252	45.25
Phenanthrene	178	27.77	Benzo (k) Fluoranthene	252	45.30
C1-Phenanthrenes	192	30.56	Benzo (e) Pyrene	252	45.89
C2-Phenanthrenes	206	32.87	Benzo (a) Pyrene	252	46.07

Bold = Internal Standard

Table 2.4.3.1 Continued

C3-Phenanthrenes	220	35.10	Perylene	252	46.56
C4-Phenanthrenes	234	37.74	Indeno (1,2,3 - cd) Pyrene	276	51.50
Anthracene	178	27.98	Dibenzo (a,h) anthracene	278	51.23
Chrysene - d_{12}	**240**	**41.95**	Benzo (g,h,i) perylene	276	52.22
Fluoranthene	202	33.87			
Pyrene	202	34.32			
C1- Pyrenes	216	36.09			

BOLD = Internal Standard

2.5. Results and Discussion

2.5.1. Method Evaluation using Phenanthrene d_{10} Recovery

The spiking surrogate solution containing phenanthrene d_{10} was administered at two different times of the study in order to show method validity. The samples spiked prior to lyophilizing had a mean recovery of 87% of the phenanthrene d_{10}. The samples spiked after the subsample was taken had a mean recovery of 89%. The standard deviation of the samples spiked prior to the lyophilizing process was 8%, and those spiked after the lyophilizing process had a

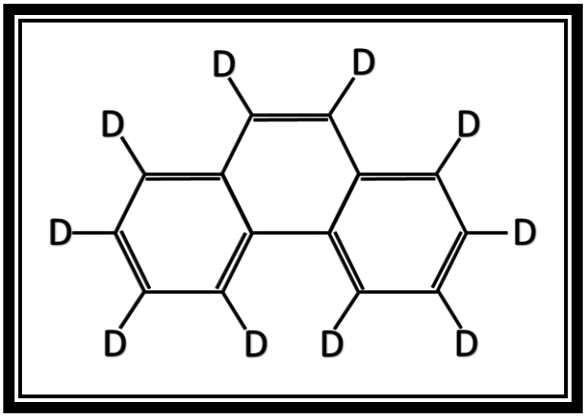

Figure 2.5.1.1 Structure of Phenanthrene D10 (surrogate spiking standard used to assess aromatic recovery).

standard deviation of 4%. There was no significant loss in phenanthrene d_{10} recovery in the lyophilizing process. The adaptation of a sonication-assisted MSPD extraction yielded recoveries greater than 90%. Overall study summary statistics were 1) a recovery mean of 88%, ±5% and 2) a range from 75–96% with a total sample size of N=36 (Table 2.5.1.1). The lowest recorded

Table 2.5.1.1: Assessment of phenanthrene d_{10} recovery using a modified MSPD protocol

Treatment	Whole Fish (mean dry wt. in g)	Mean Fork Length (cm)	% Recovery (Mean ± Std Dev.)	Corrected Mean Total PAHs[a] (ng/g)	Sample (n)
Spiked Before Freeze Drying	40.16	18.25	87% (±8%)	8415	7
Spiked After Freeze Drying	36.94	15.83	89% (±3%)	6485	29
Mean/Total of Whole Study	37.56	16.30	88% (±5%)	6860	36
Controls	N/A	N/A	87% (±1%)	3501	6

a = corrected for recovery of phenanthrene d_{10}

recovery among the samples spiked after the lyophilizing process was 80%, with the lowest recorded recovery among the samples spiked before the lyophilizing process being 75%. Highest recorded recovery among the samples spiked after the lyophilizing process was 96%, with the highest recorded recovery among samples spiked before the lyophilizing process being 93%. Size as a controlling factor for PAH recoveries indicated minimal variation that could be linked to life cycle. Monthly variations were also not significant for phenanthrene d_{10} recoveries. The only month with a noticeable difference in recoveries versus size and location was July 2011. However, this difference was not significant between Vermilion Bay (VB) and Grand Isle (GI) (Figure 2.5.1.2). Similar responses were seen for small menhaden from both VB and GI. (Figure 2.5.1.3) and all fish harvested, independent of sample site (Figure 2.5.1.4). The disparity noted in July between location (Figures 2.5.1.2 and 2.5.1.3) as well as size (Figure 2.5.1.4), indicated that the difference in recoveries stemmed from human error. These inefficiencies still resulted in an

81% recovery when comparing phenanthrene d_{10} by site and an 86% recovery when comparing phenanthrene d_{10} by size, i.e., well within the acceptable method range of 70%–120% (Meyer, et al., 2010). Statistical analysis of the recoveries using a one-way analysis of variance with a *p* of 0.05 showed that all sample means, including pre-lyophilisation, post-lyophilisation, and control (Table 2.5.1.1), demonstrated no significant difference (*p*-value = 0.57). Total ion

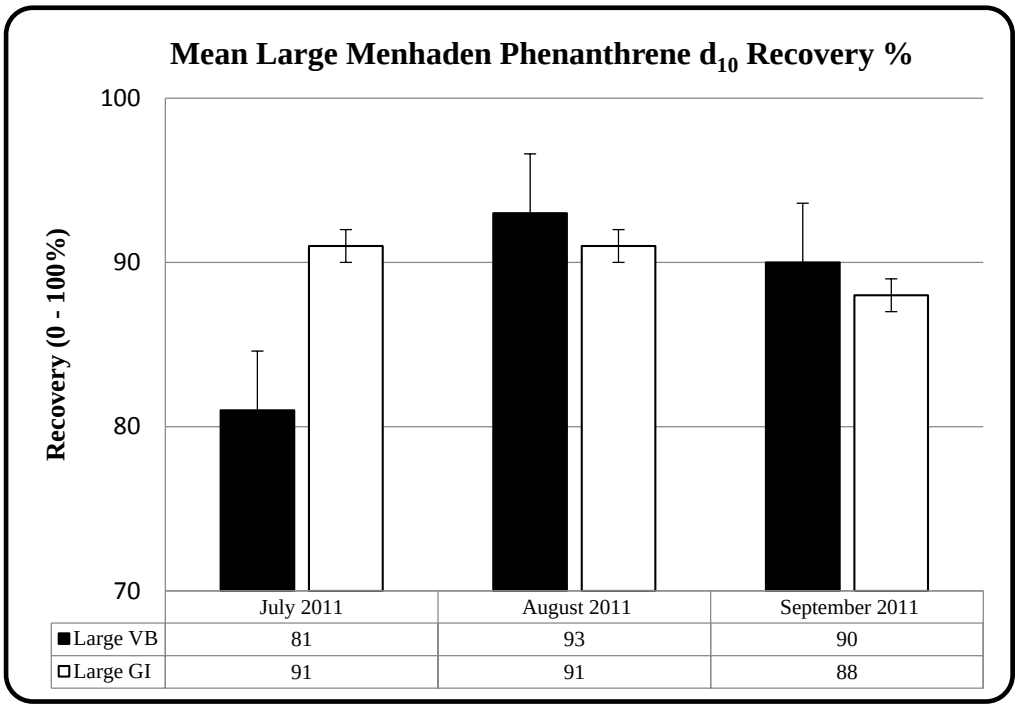

Figure 2.5.1.2 Mean "large" menhaden phenanthrene d10 recoveries based on site and month of harvest: Summer 2011 (recoveries by month were not significantly different $p > 0.05$).

chromatograms (TIC) are shown of representative samples to indicate separation on column as well as general relative abundance versus acquisition method time. Figures 2.5.1.5a and 2.5.1.5b are labelled to identify internal and spiking standards as being within the selectedsample matrix. Method blanks were analyzed throughout each batch and were all free of contamination, as can be seen in Figure 2.5.1.4c. The continuing calibration standards were all within 20% RSD (relative standard deviation) of the five-point calibration mean, showing consistency and

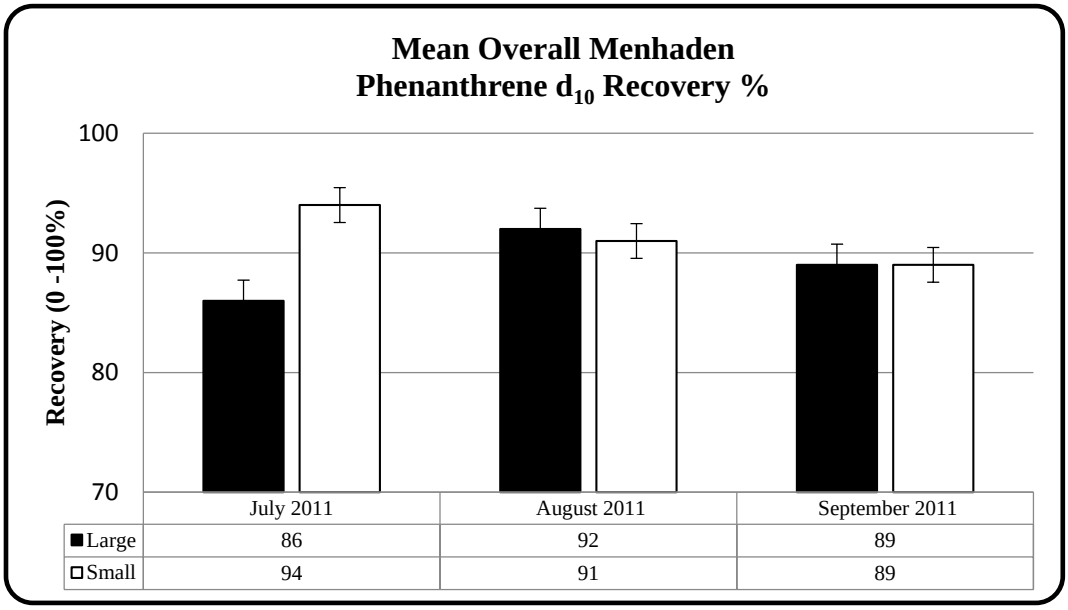

Figure 2.5.1.3 Overall mean phenanthrene d10 recoveries based on size and month: Summer 2011(recoveries by month were not significantly different at $p > 0.05$).

precision: a representative TIC can be seen in Figure 2.5.1.4d. All samples followed the basic progression outlined in Figure 2.5.1.4. Based on the general uniformity of each sample, representative chromatograms were used for clarification. Each sub-figure is labelled accordingly, and all standards are marked and labelled.

It is important to realize that each individual chromatogram had variations on the expression of various PAHs, however these variations were not outside of the expected normalization of the sample chromatograms. As such chromatograms with a representative peak pattern were used to outline the various total ion patterns seen in the majority of the samples analyzed.

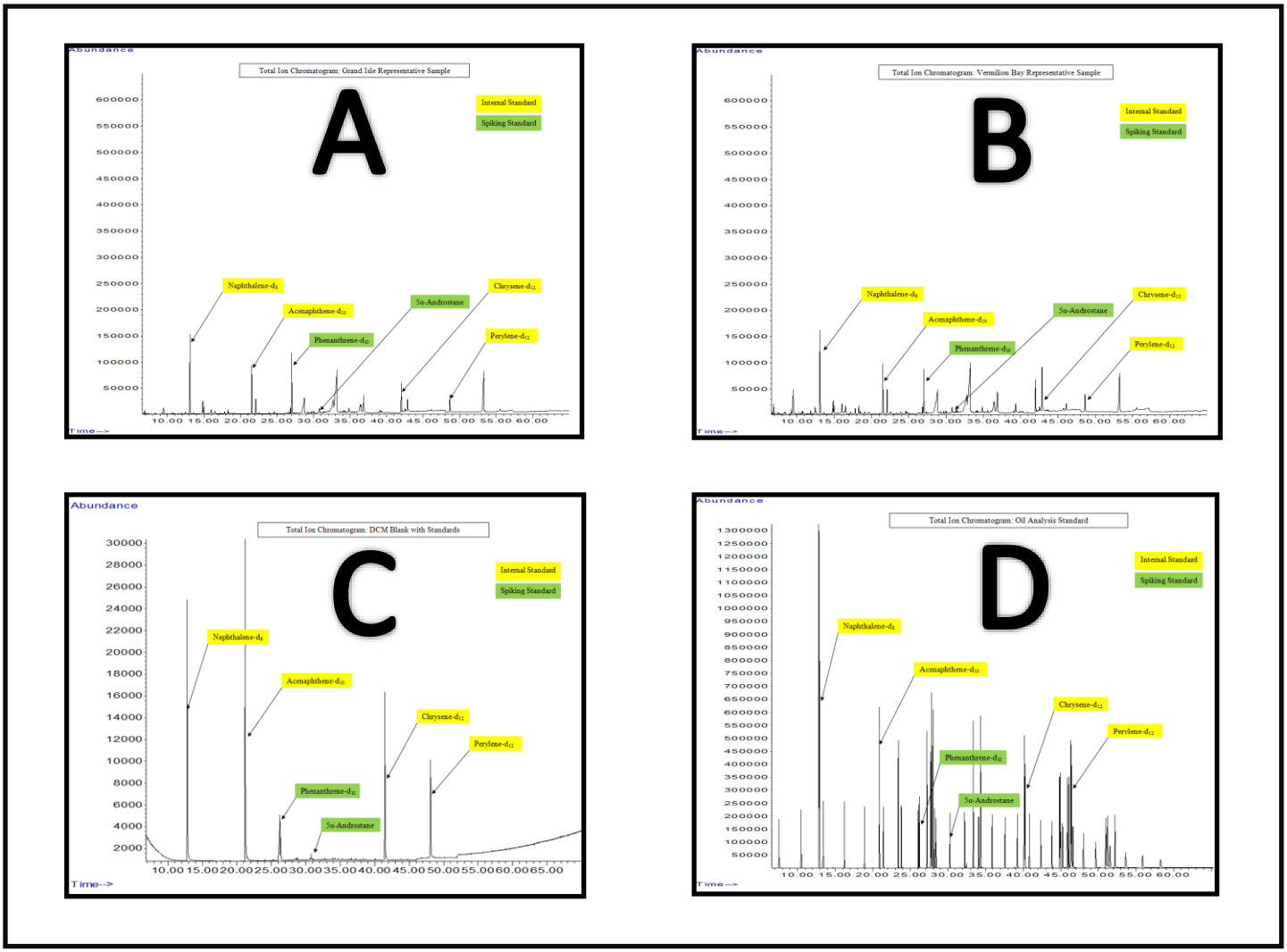

Figure 2.5.1.4 Total Ion Chromatograms of A) Representative Grand Isle Sample, B) Representative Vermilion Bay Sample, C) Method Blank, D) Oil Analysis Calibration Standard @ 5.0 ppm

2.6. Conclusions

The adaptations of lyophilisation and sonication made to the gravity- and then vacuum-assisted MSPD extraction method improved minimum recovery by approximately 18%. The overall standard deviation of ± 5% from an average recovery of 88% demonstrated minimal variation in individual sample recovery, and overall recoveries were consistent. Extractions used as little as 2.5 g of sample, with minimal amounts (<50 mL) of DCM needed to elute fish tissue

to completion. Minimal amounts of hexane were needed to reconstitute the residual menhaden material. Control extractions maintained a mean recovery of 87% ±1%. GC/MS analysis time required to separate all key oil constituents outlined in Table 1 was approximately 65 min per sample, resulting in a relatively fast and simple method for extraction that provided next-day results. Overall the adapted MSPD method was faster than more traditional methods such as Soxhlet extraction and more cost efficient than supercritical fluid or microwave extraction. The modified MSPD was reliable, with an overall recovery of 88% ± 5%. This paper outlines a reliable and consistent method for adapting the MSPD extraction technique for the quick assessment of tissue samples during a marine pollution event

2.7. Acknowledgments

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CHAPTER 3: ASSESSMENT OF THE TOXIC POTENTIAL OF POLYCYCLIC AROMATIC HYDROCARBONS AFFECTING GULF MENHADEN (*BREVOORTIA PATRONUS*) HARVESTED FROM WATERS IMPACTED BY THE BP HORIZON SPILL

3.1. Conspectus

Approximately 4.9 million barrels of crude oil and gas were released into the Gulf of Mexico (GoM) from April to July 2010 during the Deepwater Horizon (DWH) spill. This resulted in the possible contamination of marine organisms with polycyclic aromatic hydrocarbons (PAHs), USEPA-identified constituents of concern. To determine the impact of the DWH oil spill, Gulf menhaden (*Brevoortia patronus*), a commercially harvested and significant trophic grazing species, was sampled from two Louisiana coastal regions from 2011 and 2013. Tissue extraction and GC/MS analysis demonstrated measurable concentrations of PAH within menhaden. Analysis yielded total PAHs, carcinogenic equivalents (BaP-TEQ), and mutagenic equivalents (BaP-MEQ), which provided an initial toxic potential assessment of this GoM Fishery. Gulf menhaden contained less total PAH concentrations in 2012 and significantly less in 2013 as compared to 2011($p<0.05$). Carcinogenic and mutagenic PAHs were also significantly reduced ($p<0.05$) over the three-year period. The reduction of total PAH concentrations and the reduction of B[a]P-TEQs and MEQs between 2011 and 2013 indicate a diminished input of new source PAHs along with a reduction of carcinogenic and mutagenic PAHs in menhaden populations.

3.2. Introduction

The release of large quantities of crude oil into the Gulf of Mexico (GoM) in 2010 raised concerns over the possible contamination of marine organisms due to the scale of the continuous oiling event (Weber, 2010)(Figure 3.2.1). One major constituent of concern in crude oil is the

hydrocarbon fraction with properties of low volatilization that remains in the environment. These compounds, known as Polycyclic Aromatic Hydrocarbons (PAHs), are characterized by multiple

Figure 3.2.1 Platform supply vessels battle the blazing remnants of the off shore oil rig Deepwater Horizon (Credit, US Coast Guard)

conjoined ring structures, with naphthalene and its alkylated forms being the smallest (molecular mass of 128.17 g/mol) (Haritash & Kaushik, 2009). Higher-molar-mass PAHs volatilize less, allowing the compounds to remain in nature longer than the lighter constituents of crude oil (Feng, et al., 2009). PAHs are nonpolar and have a strong affinity to other nonpolar materials such as natural oils and fats (USEPA, 2008). This leads to the possibility of bioaccumulation within the adipose fraction of marine organisms and possible biomagnification through the trophic structure of the GoM.

PAHs are considered compounds of concern according to the United States Environmental Protection Agency (USEPA) due to their ability to accumulate within adipose tissue (USEPA, 2008). There are several PAHs listed as mutagenic and carcinogenic, making their possible presence in commercial fishery populations a major concern for the GoM (Durant, et al., 1996) (Nisbet & LaGoy, 1992) (USEPA, 2008). (Table 3.2.1) The current study attempts

to understand how the concentrations of PAHs within a commercially valuable fish harvested in great quantities from the GoM had affected the fisherys. In order to quantify PAH concentrations found in the commercial fishery of the GoM, an initial phase assessment of the

Table 3.2.1: 15 mutagenic and carcinogenic PAHs as identified by Nisbet & Lagoy, 1992 and Durant et al., 1996

Compound
Dibenz[a,h]Anthracene
Benzo[a]Pyrene
Indeno[1,2,3 - cd]Pyrene
Pyrene
Benzo[b]Fluoranthene
Benzo[k]Fluoranthene
Benzo[g,h,i]Perylene
Fluoranthene
Benzo[a]Anthracene
Chrysene
Anthracene
[1]Acenaphthene
[1]Acenaphthylene
Fluorene
[1]2-Methylnaphthalene
Naphthalene
Phenanthrene

1 = Compound was not an analyte of interest and was therefore not quantified for this study

fishery was conducted. The Gulf menhaden (*Brevoortia patronus*) accounted for the second largest commercial harvest from United States waters and the largest from the GoM from 2005 to 2010 and was chosen as the principal study organism (Van Voorhees & Lother, 2011). The organism selection was further supported by the fact that menhaden are harvested because of the amount of their fats and oils that are extracted and refined for consumer use (Franklin, 2007)

(Vaughan, et al., 2007). Menhaden are also a significant GoM species due to their position in the food web as obligate filter feeders. The mode of feeding increases interactions with surface and subsurface oil through dermal contact and direct ingestion, and positions menhaden as the main link between producers and secondary consumers (Franklin, 2007) (Van Voorhees & Lother, 2011) (Vaughan, et al., 2007).

The current research is critical for understanding trophic level transfer of PAHs within marine ecosystems like that of the GoM. Examining trophic transfer is also significant for the Atlantic menhaden (*Brevoortia tyrannus*) fishery along the eastern coast of the United States of America due to its proximity to industrialized areas. Sport fishermen use the GoM as a recreational fishing area, and the current research may indirectly shape their fishing habits. If the principal prey for sport fish caught in the GoM accumulates PAHs, there is the opportunity for those compounds to accumulate within the recreational catch as well.

3.2.1. Gulf Menhaden (*Brevoortia patronus*)

Gulf menhaden (*Brevoortia patronus*) are considered smaller than Atlantic menhaden, with fork lengths of no more than 22 cm, and also have a shorter life cycle (Franklin, 2007).

Figure 3.2.1.1 Menhaden fishing vessel collecting a single load of menhaden for use in the commercial market (Credit, Dave Harp)

Menhaden are obligate filter feeders that consume anything collected within their gill rakers as they school through the water (Vaughan, et al., 2007). Their commercial fishing grounds are as far east as the southern tip of Florida and stretch westward to the Yucatan peninsula in Mexico. From roughly April to October each year, the fish form large schools that are harvested for industrial refining of their fats and oils along with their proteinaceous meal (Franklin, 2007) (Vaughan, et al., 2007) (Figure 3.2.1.1). Menhaden oil is used in a variety of commercial products ranging from makeup to over-the-counter health supplements (Franklin, 2007). As stated before, the primary concern with menhaden contamination depends on the refined lipid fraction used by consumers from this fish. However, a more basic concern is bioaccumulation and magnification throughout the trophic structure of the Gulf (USEPA, 2008). PAHs are lipophilic and can accumulate within the adipose tissue of an organism (Larsen, et al., 2002).

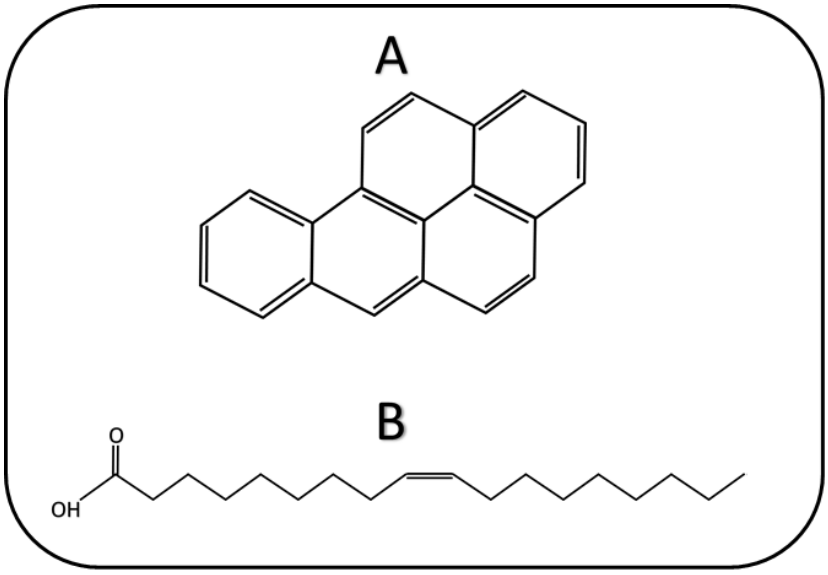

Figure 3.2.1.2 Structure of a common PAH (benzo[a]pyrene) "A" as compared to a common fatty acid found in menhaden oil (oleic acid) "B"

Menhaden are fatty fish that have the ability to accumulate PAHs in their adipose tissue, which can lead to the possible magnification of toxic compounds through trophic transfer due to prey consumption. Menhaden are considered a standard forage food for other fish, birds, and marine

mammals and also represent the primary connection between producers and secondary consumers within the GoM (Franklin, 2007) (Vaughan, et al., 2007). Gulf menhaden do not undergo major longitudinal migrations, as the fish remain in coastal waters seasonally and spend the first year of their life cycle in estuarine waters (Vaughan, et al., 2007). As a result, Gulf menhaden develop in the principal areas affected by the DWH oil spill for the duration of their life, moving between deep GoM waters (roughly 80 km offshore) and GoM coastal waters (Vaughan, et al., 2007). Spawning occurs between October and March, with peak spawning taking place between December and January; April to October is the optimal harvest season and was used as the season of harvest for this study (Raynie & Shaw, 1994).

3.2.2. Physical and Chemical Properties of PAHs

PAHs occur naturally in crude oil and coal as a result of the conversion of natural compounds, such as sterols, to aromatic hydrocarbons (Roy, 1995) (Feng, et al., 2009). PAHs are lipophilic and do not easily solubilize in water; rather they adsorb onto the organic matter of the substrate on which they are located. In soils, PAHs generally do not penetrate beyond the organic fraction, limiting leaching into groundwater (Larsen, et al., 2002). Although PAHs of a lesser molecular mass are semi-volatile, most of the PAHs found in the environment are heavier and preferentially react with particulate material (>2.5 μm in diameter). This mechanism is the standard route of atmospheric deposition of PAHs (Edwards, 1983) (Nielsen, et al., 1996). Two- and three-ringed PAHs are almost entirely found in vapor form, with four-ring PAHs transitioning between vapor and solid state. Five- or greater ringed PAHs are predominantly solid state and found adsorbed to other materials; these particulates can settle out of the atmosphere into fresh and/or marine water (Nielsen, et al., 1996). The PAH-particulate complex is consumed by organisms or eventually settles to the benthic layer (Larsen, et al., 2002).

3.2.3. Routes of Exposure

PAHs are mainly a constituent of air pollution, released through the incomplete burning of fossil fuels (such as coal, oil, and gasoline) as well as via incineration processes used for waste (Larsen, et al., 2002). PAHs are also found in wood additives composed of tar and/or creosote. They also enter the atmosphere as a result of natural events such as volcanic eruptions and forest fires (Hites, et al., 1980). PAHs pollute the environment through mechanisms that are essential to discuss when ascertaining the impact of PAH concentrations in a particular marine species. PAHs can be found in air, soil, and water through the processes of deposition and transference. Atmospheric PAHs are deposited onto soil or water after adsorption to organic and particulate matter; PAHs in the soil are transferred to water through weathering, and the surface water can be contaminated by atmospheric deposition and soil transfer of PAHs, regardless of origin (Larsen, et al., 2002). Background levels of PAHs exist in Gulf menhaden, and the

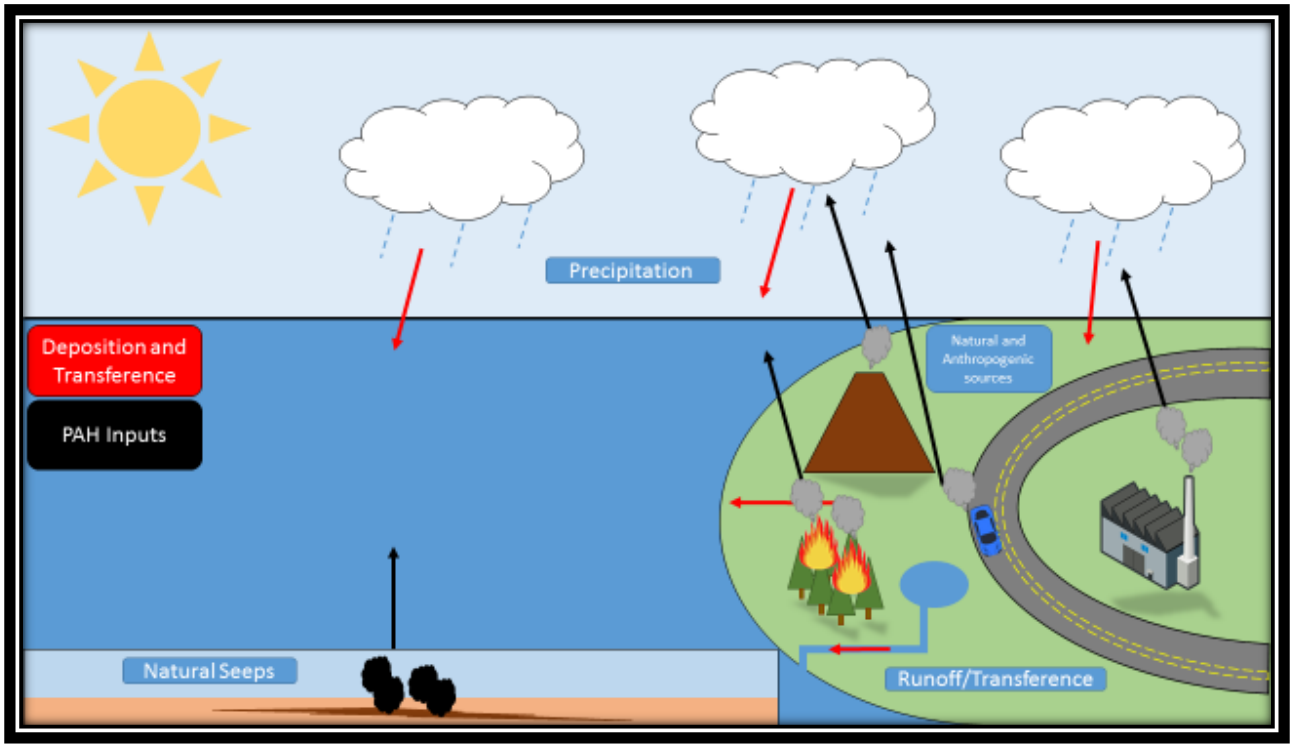

Figure 3.2.3.1 PAH deposition and transference/ fate of PAHs in the environment

background concentrations were identified through the use of a control to assign meaning to the PAH values quantified in the current study.

3.2.4. Abiotic and Biotic Degradation of PAHs

PAHs are inherently stable and not readily broken down through hydrolysis. However, the compounds can undergo photo-dissociation and oxidation (Howard, et al., 1991). The reaction kinetics are largely controlled by the location of the PAH in the environment. Non-adsorbed PAHs have half-lives of hours to days, depending on mass and structure, but in substrates such as soil, the half-lives may be months or years (Park, et al., 1990). The abiotic degradation of PAHs can produce oxidized derivatives that are as dangerous as the parent compounds (Larsen, et al., 2002). Biologically, PAHs are metabolized in two phases by the cytochrome p-450 super family of enzymes, and the biotransformation efficiency is directly related to the cytochrome p-450-dependent oxidative function of specific organisms (James, 1989) (Figure3.2.4.1). It has been reported that the initial transformation of PAHs is slower in invertebrates than vertebrates, and the elimination of the resulting metabolites is also slower (IPCS, 1998). Alkylated PAHs do not necessarily behave in the same way as their non-alkylated counterparts during the processes of abiotic and biotic degradation.

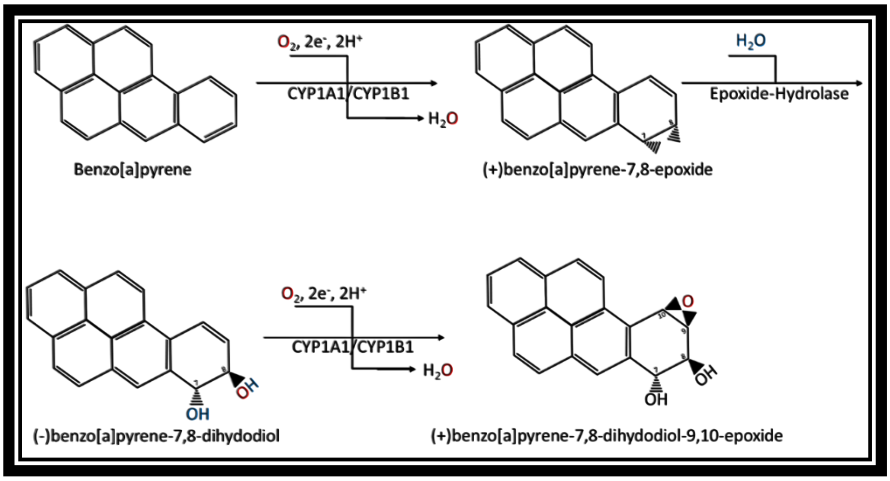

Figure 3.2.4.1 Metabolism of benzo[a]pyrene, a commonly studied PAH, yielding carcinogenic benzo[a]pyren-7, 8-diol-9, 10-epoxide

3.2.5. Chromatographic Approach to PAH Analysis

The Matrix Solid Phase Dispersion (MSPD) extraction method used in this study is characterized by the total disruption of the sample through the use of an appropriate bonded phase or other solid support material. Octadecylsilyl (ODS)-derivatized silica (C-18 Silica) was ground with the tissue sample and packed into a container suitable for a series of elutions with the desired solvent (Olson, et al., 2014). The samples were also subjected to ultrasonication techniques outlined in the USEPA 8000 series methods (USEPA, 2012). A new phase consisting

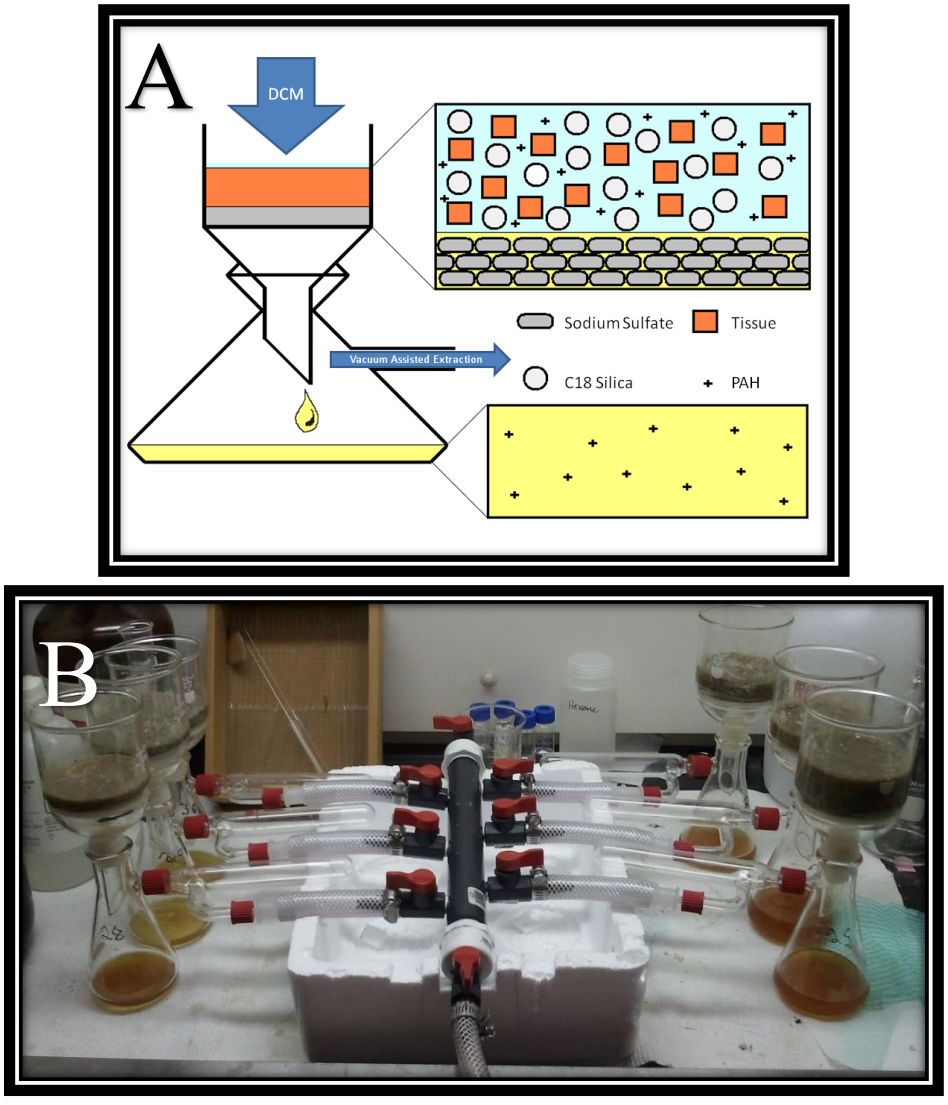

Figure 3.2.5.1 A) Process flow diagram used to perform the B)MSPD extraction of gulf menhaden for the purpose if this study (adapted from Olson et al, 2014).

of the sample and bonded phase material was created to allow for distinctive sample fractionation (Barker, 2007) (García-López, et al., 2008). A lipophilic bonding phase of C-18 silica was used in the current study, however; C-8 silica could have been used as an alternative for binding lipids (Barker, 2007). The addition of a slight vacuum during the last elution with DCM helped to assist in additional analyte recovery (Olson, et al., 2014). The eluate collected from this process was sufficiently "clean" to run on analytical instruments. However, additional cleanup measures can be conducted, such as co-column cleanup (Barker, 2007). In the case of the eluate collected from menhaden, the only secondary cleanup method employed was a standard settling period after the extraction process. This allowed any material large enough to pass through the glass microfiber filter time to settle out. This method along with a dilution with hexane was sufficient to create a sample that would not cause damage to the GC/MS columns.

3.3 Materials and Methods

3.3.1 Adaptation of Sonication-Assisted Matrix Solid Phase Dispersion of Tissues

A cursory materials and methods section will follow. However, it should be noted that for full method procedures, please reference Chapter 2 of this dissertation. All primary methods used for *this* specific study will be discussed in greater detail below.

3.3.2. Solvents, Reagents, and Chemicals

Pesticide/reagent grade dichloromethane (DCM) and hexane were obtained through the university supply (originally from Mallinckrodt Chemicals, St. Louis, MO), and the RediSep C-18 silica (40–60 µm) was obtained though Teledyne Isco, Inc (Lincoln, NE). The A.C.S. certified sodium sulfate (anhydrous, 10–60 mesh) was purchased through the university supply store (originally from Fisher Scientific, Waltham, MA).

3.3.3. Standards

A commercially prepared oil analysis standard (from Absolute Standards, Hamden, CN) was used to prepare a five-point calibration standard, preformed quarterly. A continuing calibration standard, one point of the initial five-point calibration standard, was analyzed in each batch of samples or during each 12-hour period when analyses were being performed. The acceptance criterion for the continuing calibration standard was ± 20% of the average relative response factor calculated from the initial five-point curve. The internal standards consisted of naphthalene-d_8, acenaphthene-d_{10}, chrysene-d_{10}, and perylene-d_{12} (AccuStandard Inc., New Haven, CT). The internal standards were stored individually until they were combined into the internal standard solution. Macondo 252 (MC 252) source oil collected directly from the riser of the DWH oil rig was the reference oil standard used for all analyses. The reference oil standard was prepared by extracting 1 g of pure oil in 40 mL of solvent (or equivalent ratio of 1g:40 mL, e.g. 0.50 g:20 mL). The laboratory reference oil was analyzed in each sample batch as an additional QA/QC sample. The surrogate standards included 5α-androstane (alkanes) and phenanthrene-d_{10} (aromatics) and were also stored individually until the standard solution was made (AccuStandard Inc., New Haven, CT). The extraction efficiency for each sample was evaluated based on the percent recovery of the surrogate standard with an acceptable percent recovery range of 70–120% (Meyer, et al., 2010).

3.3.4. Sample Collection

Menhaden were sampled at locations near Grand Isle (GI) and Vermilion Bay (VB), Louisiana. The samples were collected using a five-panel gill net approximately 700 ft. long with distinct plastic mesh panels. Sampling events took place under the supervision of the Louisiana Department of Wildlife and Fisheries (LDWF), and all sampling protocols were dictated by the

LDWF agents. However, a detailed Institutional Animal Care and Use Committee approved Animal Care and Use Protocol was developed for the harvesting of Gulf menhaden (see appendix). After collection, the menhaden were separated by length, bagged in plastic freezer bags, and placed on ice until they were frozen in a -4°C freezer.

Figure 3.3.4.1 Five-panel gill net used to capture Gulf menhaden as per the LDWF collection protocol. (Credit, Gregory Olson)

Menhaden were collected during the normal harvest season between April and November for 2011, 2012, and 2013. Over the course of the collection, several months overlapped from season to season. Not all sampling events were successful. The months of July, August, and September were the most congruent between seasons and for this reason they were used as the standard months for quantitation.

3.3.5. Protocol for Preparing Menhaden for Tissue Extraction

Six samples of menhaden with fork lengths of 16 cm or less (designated “small” menhaden) and six samples of menhaden with fork lengths of greater than 16 cm (designated “large” menhaden) were frozen for each location and sampling event. The menhaden were

segmented into smaller pieces based on initial size and placed in glass beakers. All glassware was washed and rinsed with DCM prior to contact with the menhaden samples. The pieces of menhaden were compressed, covered, and placed in a –86°C freezer until frozen solid. After freezing, the samples were lyophilized for 24–36 hours and then removed to a desiccator for 24 hours.

3.3.6. Protocol for Extracting PAHs from Tissue

Lyophilized menhaden were prepared for a modified matrix solid phase dispersion extraction by homogenization. Each fully homogenized sample was then sub-sampled and

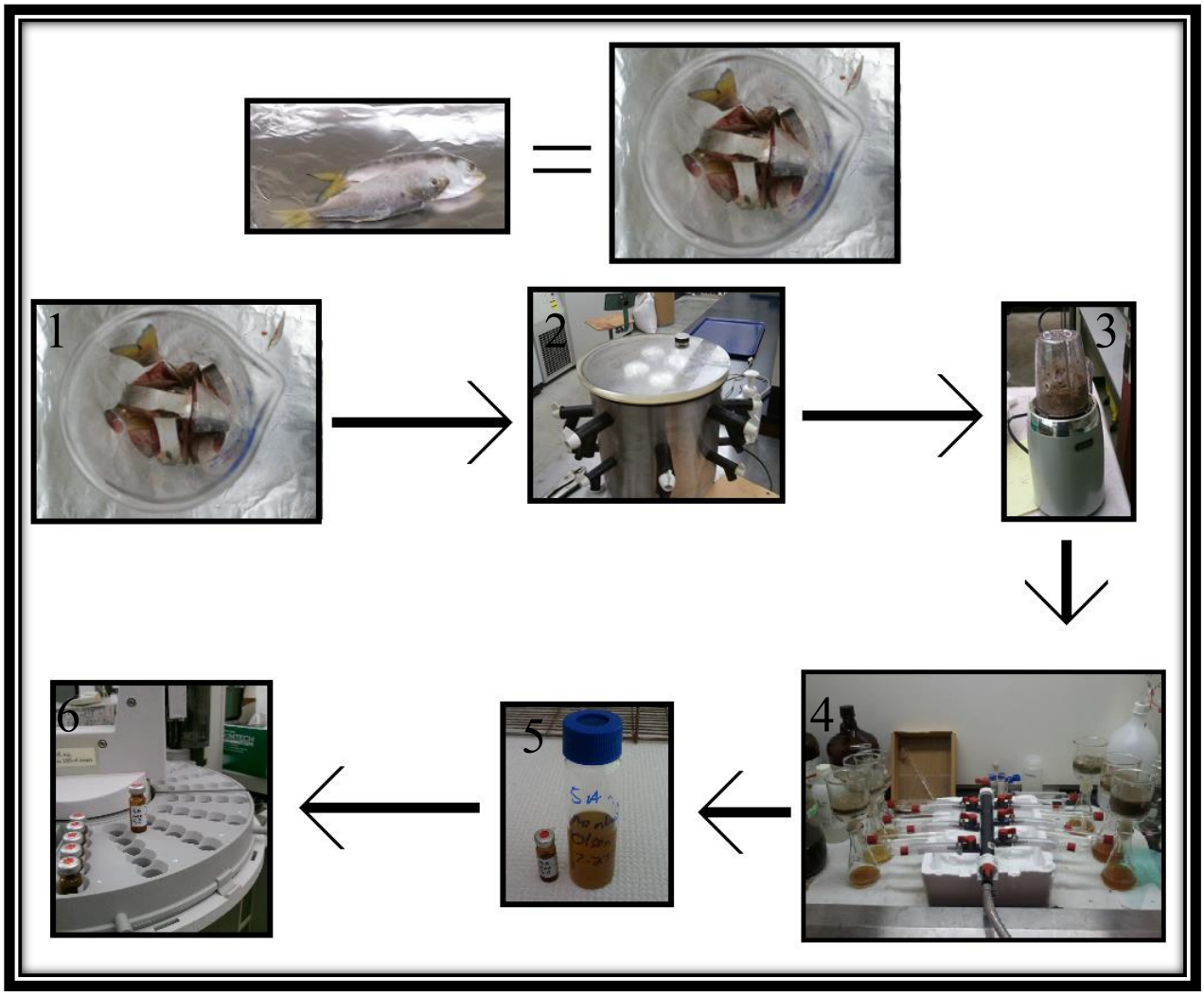

Figure 3.3.6.1 Complete menhaden extraction process (Credit, Gregory Olson)

blended with a 1:1 ratio of C-18 Silica. The new matrix was then spiked with surrogate standard and sonicated with DCM for 30 minutes. The final slurry was then removed to a side-arm flask with a large funnel fixed to the opening. The funnel was filled with sodium sulfate supported by filter paper. The slurry was rinsed three times with DCM into the funnel and allowed to gravity filter. After all gravity filtration was complete, a slight vacuum was applied to the side-arm flask to remove the excess DCM in an attempt to increase recovery. The resulting material was then amended with 5 mL of hexane and rotary evaporated to approximately 1 mL. The concentrate was then diluted to sufficient clarity for analysis (15 mL) and placed in a VOA bottle. From this bottle 1 mL of sample was transferred to an amber GC autosample vial and either analyzed immediately or stored in a freezer until analysis could be performed.

3.3.7. Protocol for GC/MS Analysis

All GC/MS analyses were conducted using a Hewlett Packard 5890 GC system (Agilent Technologies, Santa Clara, CA) configured with a 5% diphenyl/95% dimethyl polysiloxane high-resolution capillary column (30 meter, 0.25 mm ID, 0.25 micron film) directly interfaced to an Agilent 5972 mass selective detector system (Agilent Technologies, Santa Clara, CA). An Agilent 6890 series Auto Injector (Agilent Technologies, Santa Clara, CA) was used for sample introduction into the GC/MS system. The MS was operated in Selective Ion Monitoring (SIM) mode to maximize the detection of several trace target constituents unique to crude oil. Selected ions for each acquisition window were scanned at a rate greater >1.5 scans/sec with a dwell time of 60 milliseconds. At the start of each analysis period or every 12 hours, the MS was tuned to PFTBA, an internal instrument standard.

A calibration standard and a MC 252 source oil standard were analyzed with each sample. The analytical method identified 71 key constituents of crude oil, including 43 aromatic

compounds. Two surrogates standards, 5α-androstane and phenanthrene d_{10}, were used to identify recovery for the alkane and aromatic portions of the sample, respectively. The samples were integrated using the chromatographic peaks of the known 71 constituents identified in the MC252 reference oil. From the retention times, integrations, and target response factors, the PAH concentration in ng/g of dry weight tissue was quantified.

3.3.8. Protocol for Data Analysis

Data analysis was performed using SAS, MatLab, and Microsoft Excel. The datasets were determined to be inconsistent with the assumptions of parametric analysis and required the use of two specific sets of nonparametric tests in order to determine significance. Initial analysis was accomplished using the Kruskal-Wallis test for significance. Based on the raw data, several anomalies were noted in the significance reported for BaP-TEQs and BaP-MEQs. It was determined that a specific Chi Squared test for significance was required to accommodate a shift in data based on population changes. All analyses were performed using a type one error rate (α) of 5% as the measure for significance.

3.4. Results and Discussion

3.4.1. Whole Fish Total PAH Concentration

The total PAH (ΣPAH:WF_{43} Table 3.4.1.1) whole fish concentration was calculated by summing all of the compounds calculated using Table 3.4.1.1, excluding the bold-face internal standards. A significant contributor to total PAH was C3-phenanthrene. It is unknown how many isomers of this particular homologue were present with current analytical limitations. Total PAH concentrations were shown to decrease from years 2011 to 2013. This decrease was shown to be significant based on a Kruskal–Wallis test for significance at $p < 0.05$. Figure 3.4.1.1 shows the difference between years to be roughly 1500ng/g dry weight tissue, indicating a reduction of

total PAH concentration between each year with a significant decrease from the initial year to the final year.

Table 3.4.1.1: Analytes of interest and the ions that are associated with each compound. **Bold** indicates Internal Standard

Analyte	SIM Ion (m/z)	Retention Time	Analyte	SIM Ion (m/z)	Retention Time
Naphthalene - d_8	**136**	**13.06**	C2- Pyrenes	230	38.29
Naphthalene	127	12.86	C3- Pyrenes	244	40.72
C1-Naphthalenes	142	16.01	C4- Pyrenes	258	42.40
C2-Naphthalenes	156	19.35	Naphthobenzothiophene	234	38.94
C3-Naphthalenes	170	22.14	C-1 Naphthobenzothiophenes	248	40.66
C4-Naphthalenes	184	25.41	C-2 Naphthobenzothiophenes	262	42.52
Acenaphthene - d_{10}	**164**	**21.52**	C-3 Naphthobenzothiophenes	276	44.70
Fluorene	166	23.37	Benzo (a) Anthracene	228	40.09
C1-Fluorenes	180	26.17	Chrysene	228	40.24
C2-Fluorenes	194	28.81	C1- Chrysenes	242	42.09
C3- Fluorenes	208	31.04	C2- Chrysenes	256	43.88
Dibenzothiophene	184	27.19	C3- Chrysenes	270	46.16
C1-Dibenzothiophenes	198	29.31	C4- Chrysenes	284	47.68
C2-Dibenzothiophenes	212	31.33	**Perylene - d_{12}**	**264**	**48.64**
C3- Dibenzothiophenes	226	33.54	Benzo (b) Fluoranthene	252	45.25
Phenanthrene	178	27.77	Benzo (k) Fluoranthene	252	45.30
C1-Phenanthrenes	192	30.56	Benzo (e) Pyrene	252	45.89
C2-Phenanthrenes	206	32.87	Benzo (a) Pyrene	252	46.07
C3-Phenanthrenes	220	35.10	Perylene	252	46.56
C4-Phenanthrenes	234	37.74	Indeno (1,2,3 - cd) Pyrene	276	51.50
Anthracene	178	27.98	Dibenzo (a,h) anthracene	278	51.23
Chrysene - d_{12}	**240**	**41.95**	Benzo (g,h,i) perylene	276	52.22
Fluoranthene	202	33.87			
Pyrene	202	34.32			
C1- Pyrenes	216	36.09			

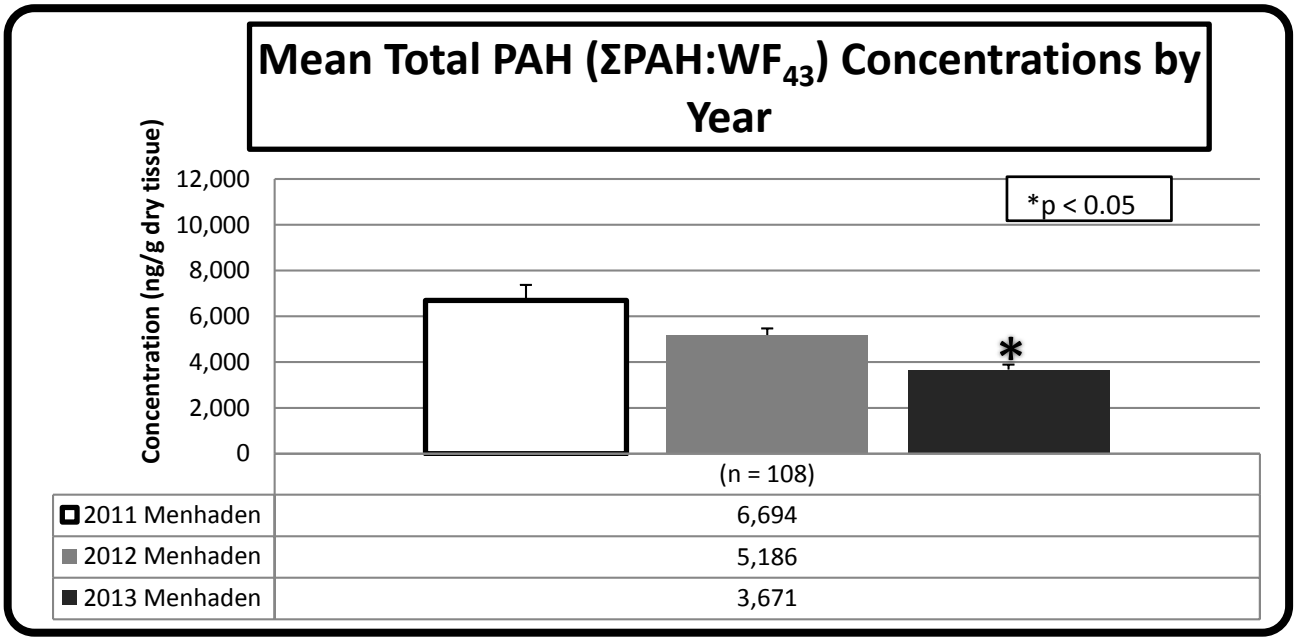

Figure 3.4.1.1 Mean ΣPAH:WF_{43} concentrations for all sampled menhaden 2011-2013. * indicates significant difference from 2011.

Figure 3.4.1.2 shows the total PAH breakdown by sampling location. This graph indicates that 2011 numbers were statistically similar between locations. Overall, total PAH concentrations did not differ between locations, but they decreased significantly from 2011 to 2013. Total PAH concentrations were slightly higher in the VB area, which is consistent with reported "mini-spills" from pipelines witnessed during the 2011 sampling season by the reduced input

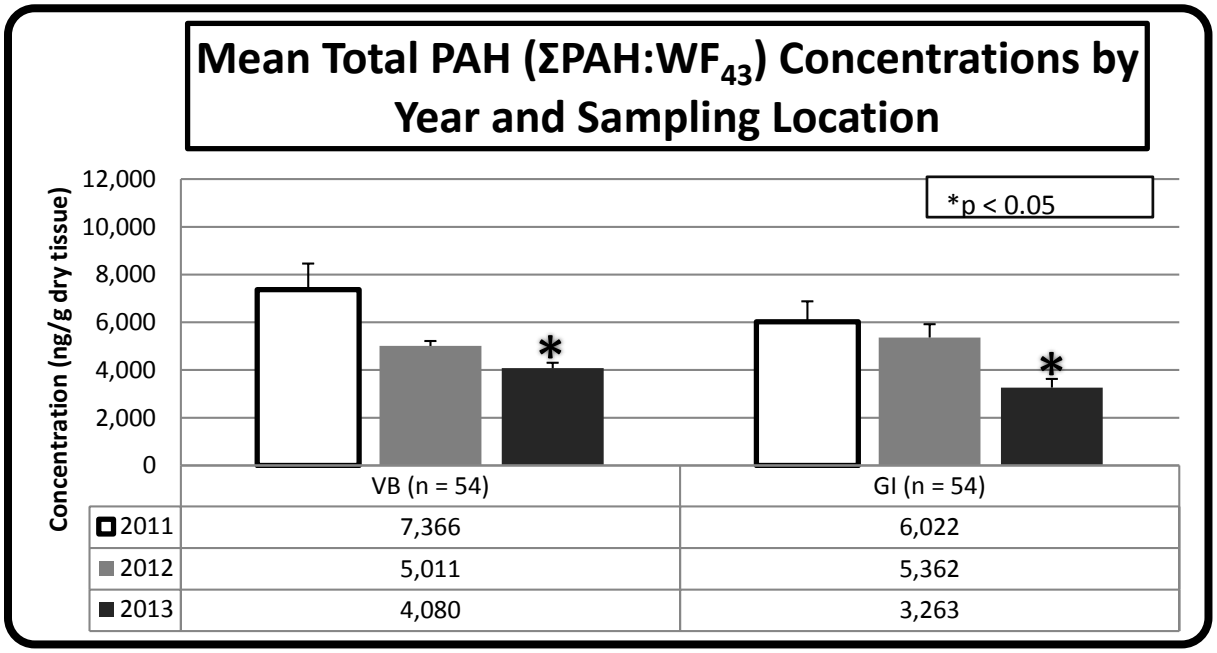

Figure 3.4.1.2 Mean ΣPAH:WF_{43} concentrations by year and sampling location. * indicates significant difference from 2011

of PAHs from a new source and will be characterized as a reduction of "new source" PAHs impacting the sampling species.

3.4.2. Benzo[a]pyrene Toxic Equivalents (TEQs) and Mutagenic Equivalents (MEQs)

The characterization of whole fish total PAH concentration alone does not provide a complete picture of possible exposure and uptake of weathered oil. Benzo[a]pyrene toxic equivalents (BaP-TEQs) of known PAHs isolated from menhaden were calculated using toxic equivalent factors (TEF) first proposed by Nisbet and LaGoy in 1992 (Table 3.4.2.1). The

Benzo[a]pyrene mutagenic equivalents (BaP-MEQs) were determined using the minimum mutagenic concentrations (MMCs) found in Durant *et al.* 1996 (Table 3.4.2.1). Only the

Table 3.4.2.1: Benzo[a]pyrene mutagenic and toxic equivalents factors

Compound	TEF*	MEF**	Rings
Dibenz[*a,h*]Anthracene	5	0.29	5
Benzo[*a*]Pyrene	1	1	5
Indeno[*1,2,3 - cd*]Pyrene	0.1	0.31	5
Benzo[*b*]Fluoranthene	0.1	0.25	4
Benzo[*k*]Fluoranthene	0.1	0.11	4
Benzo[*a*]Anthracene	0.1	0.082	4
Benzo[*g,h,i*]Perylene	0.01	0.19	5
Chrysene	0.01	0.017	4
Anthracene	0.01	[2]na	3
Pyrene	0.001	0.000	4
Fluoranthene	0.001	0.000	3
Phenanthrene	0.001	[2]na	3
[1]**Acenaphthene**	0.001	0	2
[1]**Acenaphthylene**	0.001	0.00056	2
Fluorene	0.001	[2]na	2
[1]**2-Methylnaphthalene**	0.001	[2]na	2
Naphthalene	0.001	[2]na	2

*TEF = Toxic Equivalency Factor (see Nisbet and LaGoy, 1992)
**MEF = Mutagenic Equivalency Factor (Minimum Mutagenic Concentration [B(a)P]/MMC [Selected PAH] where [ng/mL] see Durant et al., 1996)
1 = Compound was not an analyte of interest and was therefore not quantified for this study.
2= Compound was not analyzed in Durant et al., 1996

concentrations of parent compounds were used to calculate BaP-TEQ and BaP-MEQ values. The PAH concentrations were multiplied by their respective TEFs to give individual concentrations per menhaden and summed to give a total BaP-TEQ in ng/g dry weight tissue. By dividing the MMC of Benzo[a]pyrene by the MMC of the selected PAH, mutagenic equivalent factors (MEFs) were generated and applied to the previously quantified concentration of the selected PAH in menhaden tissue.

The use of these separate indices was employed to help better resolve raw PAH data. Both carcinogenicity and mutagenicity are characteristics commonly attributed to heavier PAHs. The more environmentally persistent (heavier) the PAH, the greater the impact it will have, both in nature as well as within tissue. The total PAH concentrations identified previously are representative of materials that are both fresh as well as weathered. TEQs and MEQs will help to establish the impact of weathered oil in the water column. The heavier, more persistent compounds in theory should be in greater concentration if PAHs bioaccumulate within the indicator species, regardless of total PAH concentrations. A reduced amount of TEQ and MEQ over time would either indicate biological remediation and sequestration outside of the food chain or decreased living organisms with high levels of exposure. A decreased concentration could show recovery in the affected organism. However, menhaden are a prey species, and any negative effect on mobility and/or health could translate to reduced TEQs and MEQs in the sampled population. These indices apply an equivalent multiplicative factor to each compound, based on the literature. Figure 3.4.2.1 represents mean TEQ values from 2011 to 2013 that have been log adjusted for scale. There was a significant decrease of TEQs over time between 2011 and 2012 as well as between 2011 and 2013. Figure 3.4.2.2 shows decreased TEQ values with respect to sampling location. That concentrations of carcinogenic PAHs were decreasing at both

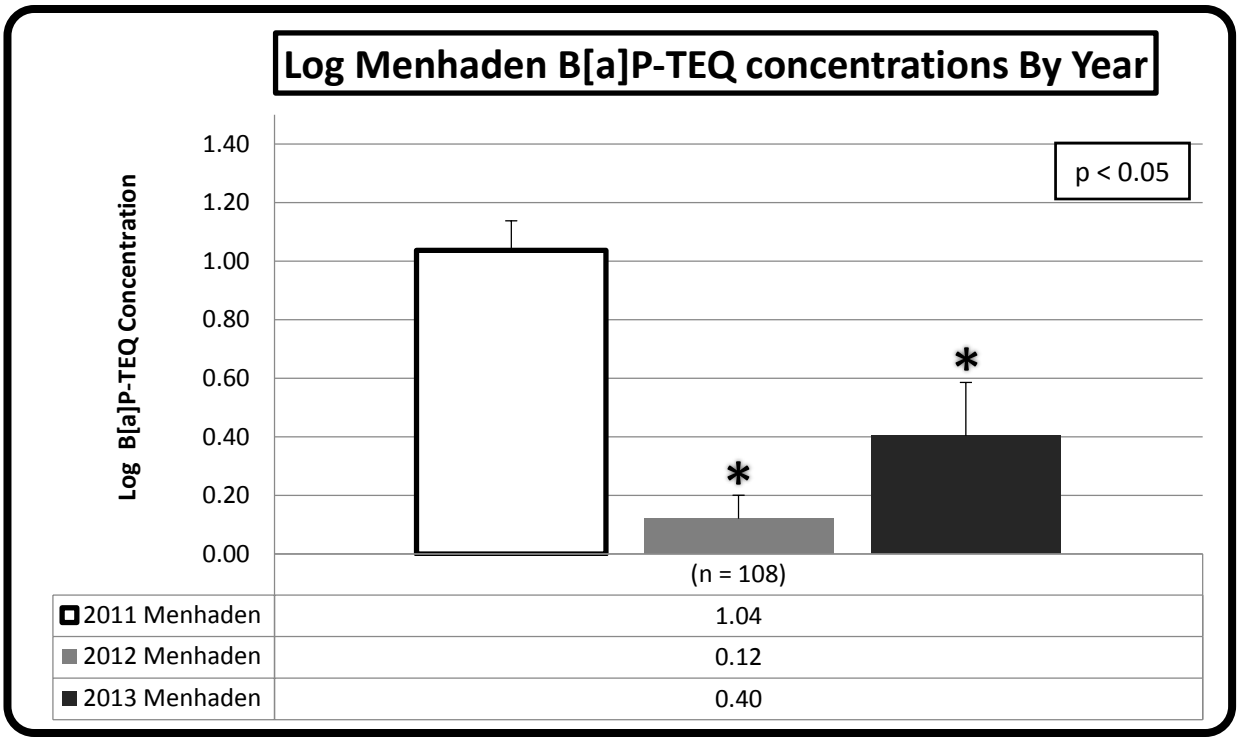

Figure 3.4.2.1 Menhaden BaP-TEQ concentrations by harvest year. * indicates significantly different from 2011

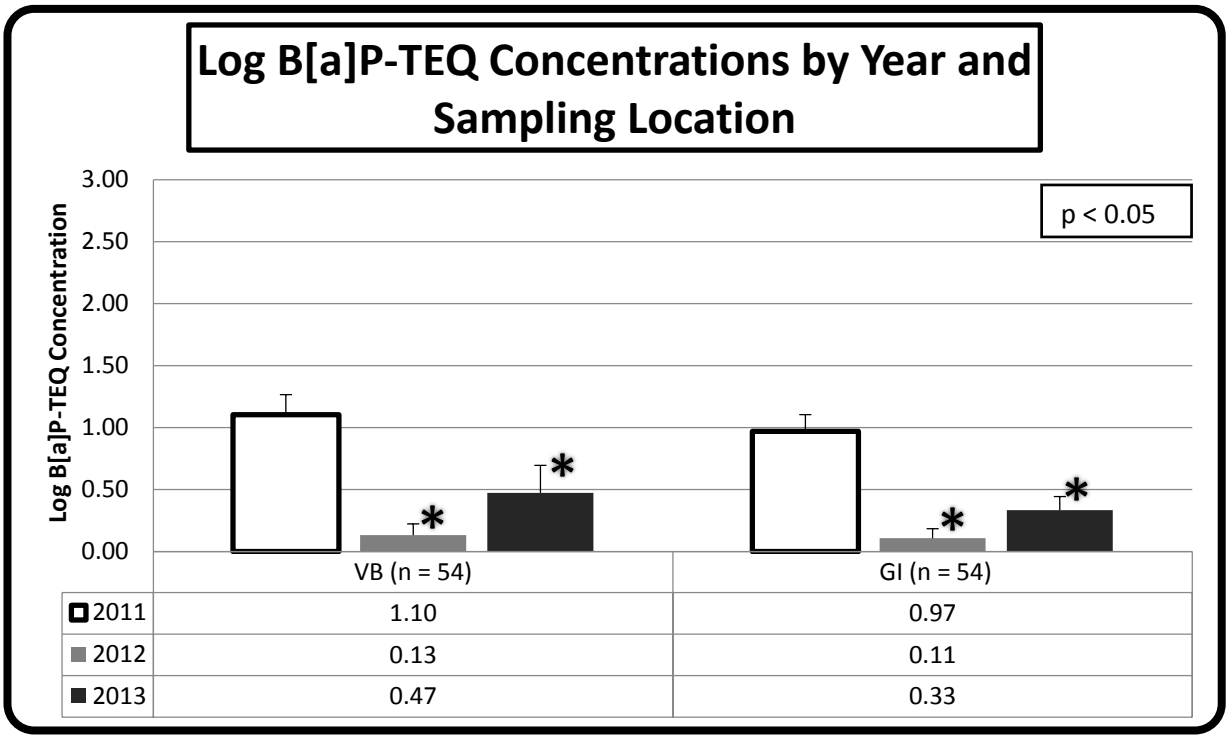

Figure 3.4.2.2 Menhaden BaP-TEQ concentrations by harvest year and sampling location
* indicates significantly different from 2011

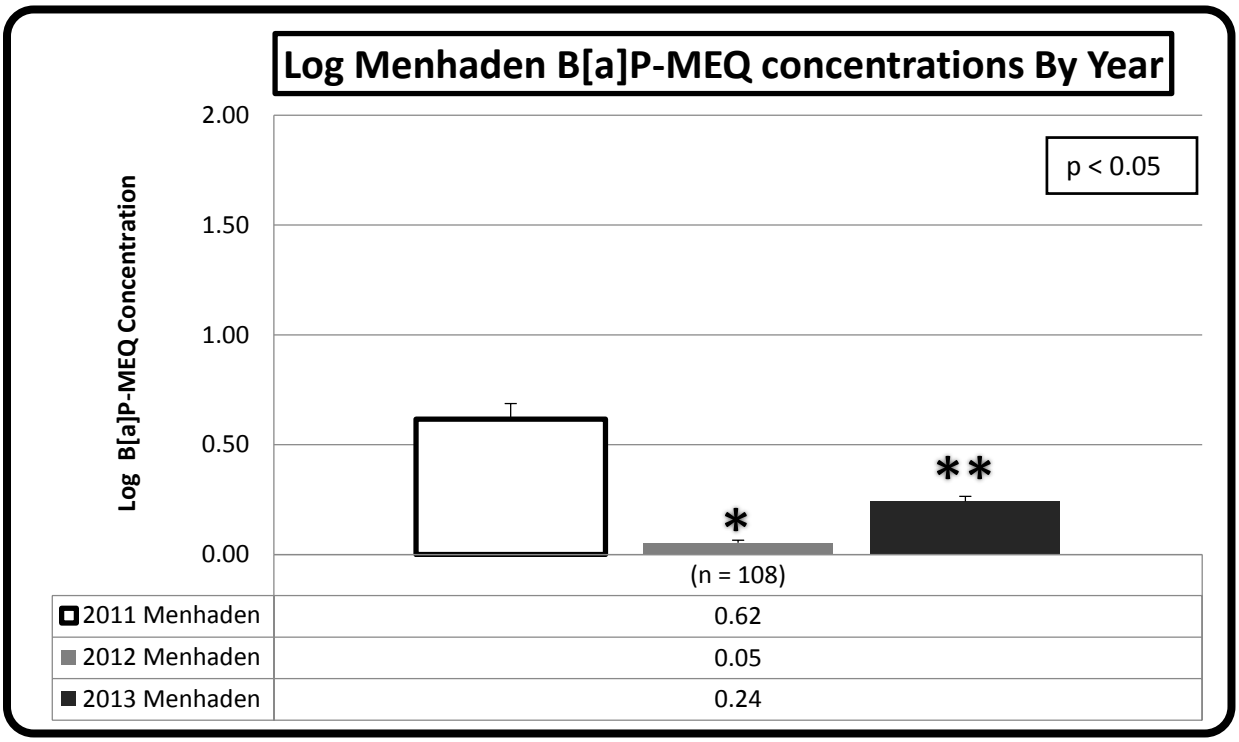

Figure 3.4.2.3 Menhaden BaP-MEQ concentrations by harvest year. * indicates significantly different from 2011. ** indicates significantly different from 2011 and 2012

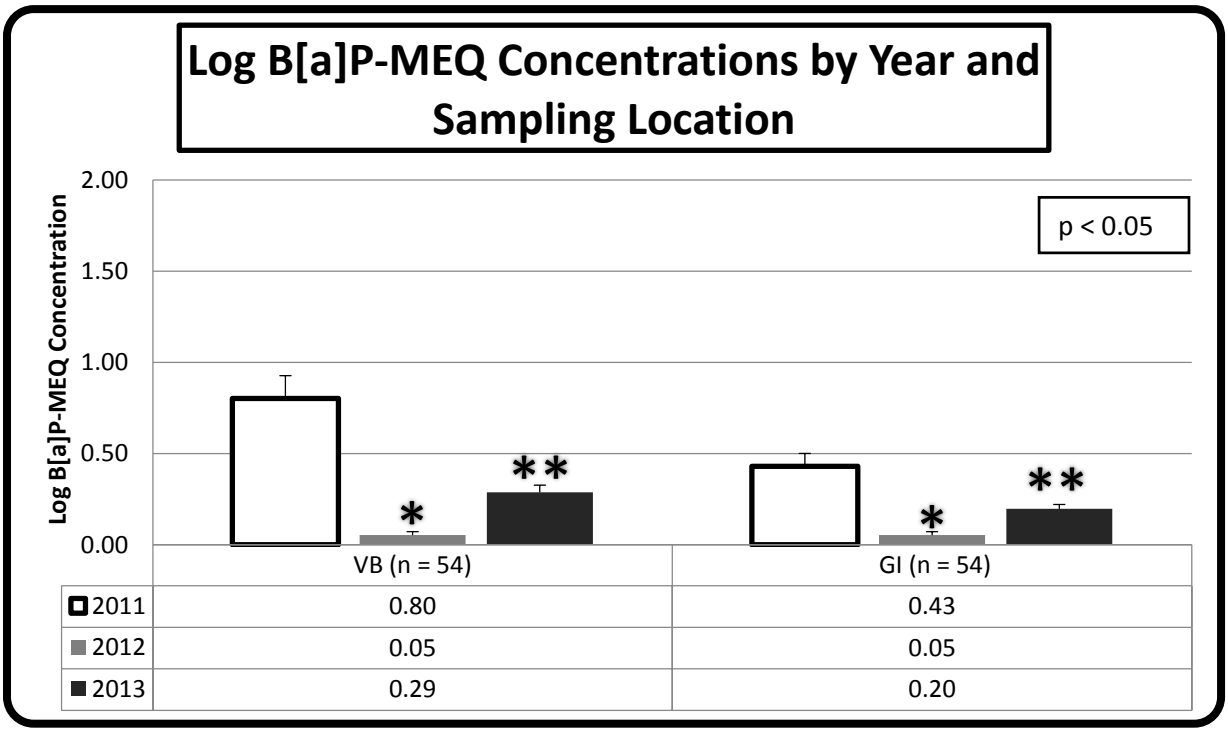

Figure 3.4.2.4 Menhaden BaP-TEQ concentrations by harvest year and sampling location
* indicates significantly different from 2011. ** Indicates significantly different from 2011 and 2012

locations can be attributed to the factors discussed in the above paragraph. Figure 3.4.2.3 represents the concentration of mutagenic PAHs (BaP-MEQs). The decrease seen here is almost visually identical to the decrease first realized in the TEQ graph (Figure 3.4.2.1) and shows that concentrations of mutagenic PAHs were significantly decreasing in menhaden in the three years after the Deepwater Horizon Oil Spill. The major difference here is that there was also a significant increase between 2012 and 2013. This suggests some form of re-suspension event. The lack of significant increase between total PAHs for this sampling period suggests that the increase was not due to a "new source" event. Despite the increase between 2012 and 2013, the final year was still significantly less than the first year. This pattern is the same when the concentrations are separated by sampling location in Figure 3.4.2.4. There was not a significant difference between VB and GI.

Table 3.4.2.2 Mean BaP-TEQs for other fish as compared to Gulf menhaden

Fish (n)	Mean TEQ (ng/g)	Extraction Method	Detection Apparatus	Solvent(s)	Tissue
Farmed Salmon (n=4)[a]	0.010	Soxhlet	GC/HRMS	DCM/Pentane	Muscle Homogenate
Wild Salmon (n=4)[a]	0.013	Soxhlet	GC/HRMS	DCM/Pentane	Muscle Homogenate
Eel (n=15) [b]	1.8	Ultrasonication	RP/HPLC	Hexane/Acetone	Muscle Homogenate
Bleak (n=15)[c]	15.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Carp (n=1)[c]	38.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Tench (n=2)[c]	3.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Alose (n=3)[c]	10.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Chub (n=4)[c]	6.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Pike (n=3)[c]	5.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Perch (n=3)[c]	5.0	Soxhlet	HPLC	DCM/Cyclohexane	Muscle Homogenate
Brown Ray (n=11)[d]	2.27*	Saponification/ Column Cleanup	GC/MS	n-Hexane/DCM	Muscle Homogenate
Megrim (n=8)[d]	2.02*	Saponification/ Column Cleanup	GC/MS	n-Hexane/DCM	Muscle Homogenate
Angler (n=12)[d]	2.35*	Saponification/ Column Cleanup	GC/MS	n-Hexane/DCM	Muscle Homogenate
Gulf Menhaden					
2011 (n=36)	75.35	MSPD	GC/MS	DCM/Hexanes	Whole Body Homogenate
2012 (n=36)	0.34	MSPD	GC/MS	DCM/Hexanes	Whole Body Homogenate
2013 (n=36)	1.73	MSPD	GC/MS	DCM/Hexanes	Whole Body Homogenate

a - (Easton, et al., 2002)
b - (Patrolecco, et al., 2010)
c - (Binelli & Provini, 2004)
d - (Storelli, et al., 2013)
* Indicates only 10 PAH were used for BaP-TEQ Calculation

Looking at several other studies that quantified BaP-TEQs, it is clear that the year directly after the DWH spill shows elevated concertations of these specific PAHs. In Table 3.4.2.2, the greatest BaP-TEQ recorded, other than that of the menhaden in 2011, was in a single carp from Binelli & Provini, 2004 (with 38ng/g BaP-TEQ). Years 2012 and 2013 fall into the range of BaP-TEQ concentrations idenified in these four studies (approx. 0-40 ng/g BaP-TEQ).

3.4.3. Vanishing Bi-Modal Distribution

The initial Kruskal-Wallis test for significance did not account well for the bi-modal distribution that was apparent in the first year but not in the rest of the sampling years. The raw data suggested that a specific portion of the population containing higher concentrations of BaP-

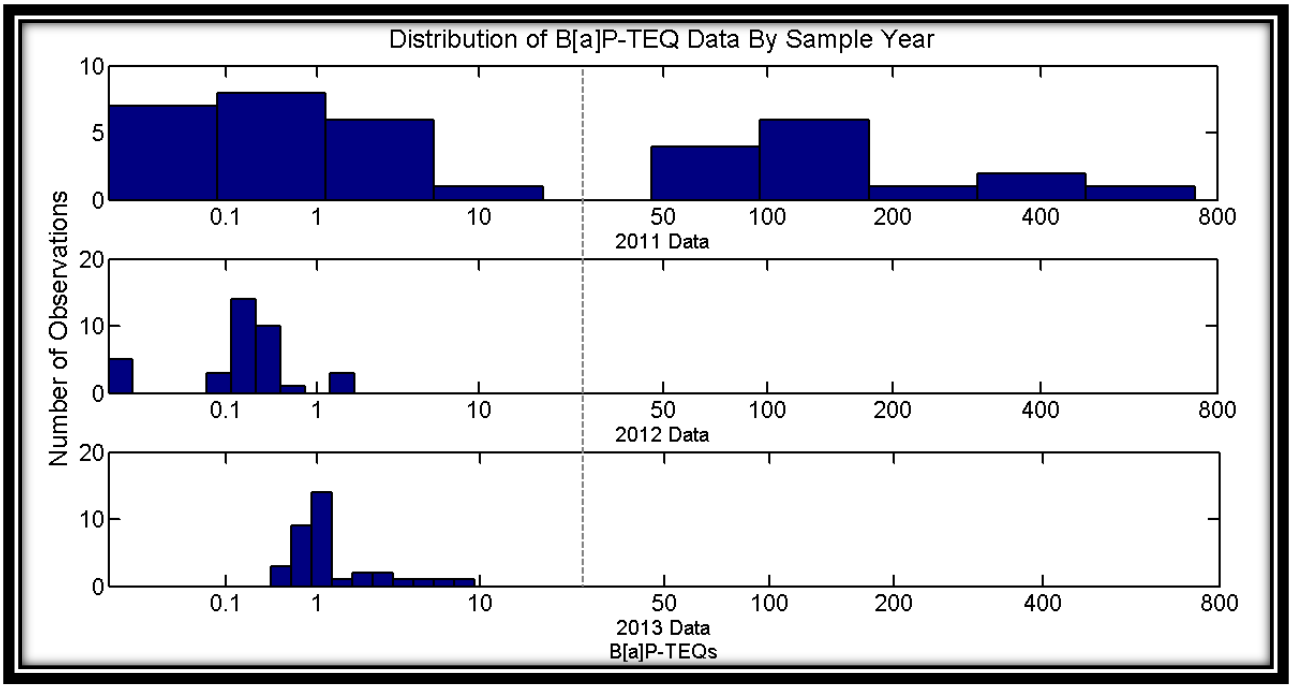

Figure 3.4.3.1 Distribution of BaP-TEQ data by sampling year. Dotted line @ 20ng/g dry weight.

TEQs and MEQs were no longer available for sampling (Figures 3.4.3.1 & 3.4.3.2). Assuming the sampling distribution was the same between years (all efforts were made to maintain valid

sampling events), there was a missing portion of the population containing heavier PAHs. The modified Chi Squared test for significance was able to show the actual changes between years in a more appropriate fashion. However, the fact that this test was needed shows how greatly changes in the characteristics of the probability distribution function from year to year can skew results. When analyzing data it is important to look closely at the raw data before applying specific statistical analyses.

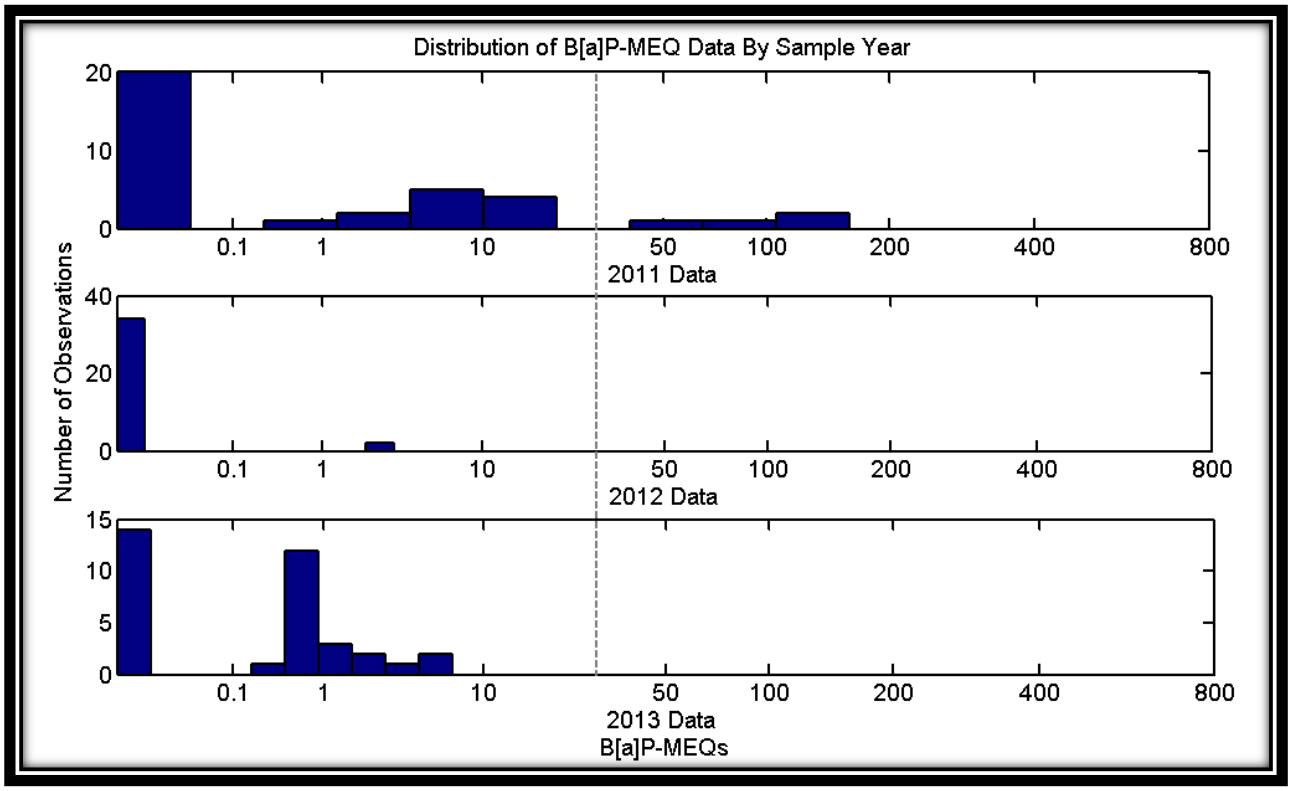

Figure 3.4.3.2 Distribution of BaP-MEQ data by sampling year. Dotted line indicates 20ng/g dry weight.

The disappearance of individuals in the high mode of the 2011 bimodal distribution in subsequent years suggests that the concentrations greater than 20ng/g BaP-TEQs and BaP-MEQs affected the individuals to the point of death or weakened the individuals to the point of predatory culling. It is also possible that the individuals were able to depurate the PAHs back

down to lower levels. Either way, there was a portion of the population containing greater than 20ng/g BaP-TEQs and BaP-MEQs in the year after the Deepwater Horizon Spill that was no longer there in the second and third years after the spill.

3.5. Conclusions

The decrease in "new source" PAH concentrations over the course of this study are indicative of reduced external oiling events in the Gulf of Mexico. The decrease in BaP-TEQ and MEQ values are indicative of a reduction of environmentally persistent PAHs with heavier molecular weights; however, the loss of menhaden with elevated concentrations of BaP-TEQs and MEQs from 2011 through 2013 suggests that the decrease in carcinogenic and mutagenic PAHs is a result of either depuration to lower levels or fish mortality (based on the vanishing bi-modlas distribution). It was also noted that the concentration of BaP-TEQs in year 2011 was an order of magnitude greater when compared to other TEQ values obtained from other fish studies. However, these values returned to a more reasonable number in 2012 and 2013.

The use of Gulf menhaden as an indicator species for assessing basic PAH exposure after a significant oiling event was successful in that PAHs were present in concentrations great enough to be consistently and reliably measured. The menhaden species itself is ubiquitous throughout the water body. The menhaden species, however, is not so robust that a major event has minimal impact on the population, and the commercial value of the species assures annual harvest events for continual monitoring of the GoM. The use of Gulf menhaden can help elucidate acute impact events as well as generate a continuous 3–4 year cycle of fish health, giving researchers the ability to assess impacts over longer time periods. Germline exposure may also be possible with long term assessment of the population. This is an important "next step" is assessing this important filter feeding fish species.Gulf menhaden provide a specific lens with

which to assess the GoM and should be used as a sentinel species for future evaluation of pollution events.

3.6. Acknowledgments

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CHAPTER 4: STANDARD WEIGHT EQUATIONS AND STANDARD LENGTH CATEGORIES USED FOR GULF MENAHDEN PHYSIOLOGICAL ASSESSMENT

4.1. Conspectus

Gulf menhaden fork length and mass data were solicited from the NOAA Southeast Fisheries Science Center, Beaufort Laboratory, North Carolina in order to generate a standard weight (W_s) equation for use in assessing menhaden physiological state (condition) (n≈76,000). Using an 11-year dataset spanning 2000–2010, the regression-line percentile technique was used to develop the standard weight equation for Gulf menhaden (*Brevoortia patronus*). It was then validated with an independent dataset composed of three additional years of sampling data (n≈1,400). The equation was $Log_{10}W_s = –1.7884 + 3.0793(Log_{10}FL)$, where W_s is standard weight in grams and FL is forklength in millimeters. Forklength was used in place of total length based on traditional menhaden sampling techniques. The equation is valid for Gulf Menhaden 10 mm in FL and longer. Relative weight (W_r) values computed with this equation did not exhibit any systematic length bias. The gulf menhaden quality index was developed using principles set forth by the sport fishing quality index as described by Gablehouse (1984) with adjustments made for a commercially harvested non-sport fish. Length categories were denoted as Bait, Quality Bait, Commercial, Quality Commercial, and Exceptional Commercial. A sigmoid function describing the relationship of fork length to record fork length was developed for future use in Gulf menhaden quality index assessment development.

4.2. Introduction

The release of large quantities of crude oil into the Gulf of Mexico (GoM) in 2010 raised concerns over the possible contamination of marine organisms due to the scale of the continuous oiling event (Weber, 2010). The complete impact of this event is still unclear, just as Prince

William Sound continues to be studied today, so will the Deepwater Horizon spill. Because of this, several methods were developed to determine the impact of this event on the heath of the Gulf (Thibodeaux, et al., 2011). It was proposed by our group that the large commercial fishery of Gulf menhaden should be looked at using metrics for heath analysis. The use of a common stock assessment was proposed as a means to determine baseline heath and growth in menhaden. Relative weight (W_r) is the ratio of a fish's weight, W, to the weight of a ''standard'' fish of the same length, (W_s) and is a commonly used fish condition index (Blackwell, et al., 2000). To estimate the condition of individual fish, relative weight is calculated as follows: $W_r = (W/W_s) \times 100$, where W is fish body weight (g) and W_s is the standard weight determined on the basis of the predetermined standard weight equation (Wege & Anderson, 1978) (Pompei, et al., 2011). Standard weight (W_s) equations have not yet been developed for Gulf menhaden, as it is a brackish/marine species; however, there are several factors that support the use of this assessment for the fishery along with a few examples of this analysis in the literature (ASMFC, 2006) (Kopf, et al., 2005) (Cooney & Kwak, 2010). In order to appropriately assess this species, the population distribution must be known. According to Anderson (2006), there is little genetic diversity across the distribution of menhaden in the GoM, suggesting that the population distribution is uniform (Anderson, 2006). Menhaden are harvested annually because of their commercial importance and can provide yearly stock data from simple subsampling of the commercial catch, allowing for minimal effort in collecting raw data (Van Voorhees & Lother, 2011) (Franklin, 2007). Menhaden are a principal forage food source for much of the secondary and tertiary consumers in the Gulf (Vaughan, et al., 2007) (Franklin, 2007). The specific role that Gulf menhaden play in the trophic structure of the Gulf allows them to be a direct indicator for overall heath (Franklin, 2007). The quality of the Gulf menhaden fishery can be an indicator of

the quality of the consumers that rely on it as a principal forage food. As an obligate filter feeding organism, any adverse conditions affecting the water column will literally pass through the Gulf menhaden fishery. Menhaden filter Gulf water for phytoplankton and zooplankton. However, suspended solids, dissolved organics, and various other non-desirable materials can make their way into the fish (Vaughan, et al., 2011). Gulf menhaden act as the kidneys of the Gulf, sequestering materials suspended in the water column for ingestion, effectively dosing themselves with any material present.

Length and weight data are frequently used by those who manage fisheries to evaluate the condition of individual fish (Wege & Anderson, 1978) (Anderson, 1980) (Murphy, et al., 1990) (Anderson & Neumann, 1996). Conversely, suitable assessment can be mired by discrepancies in weight among fish of similar lengths within and among populations, and by discrepancies in allometric growth rates (American Fisheries Society, 1996). Relative weight (W_r), the ratio of a given fish's weight compared with the standard weight (W_s) of a rapidly growing fish of the same length, was created as a predictor of condition to normalize evaluations across varying length classes (American Fisheries Society, 1996). Standard weight equations have to initially be developed for managers so it is possible to calculate W_r for the species of interest. Wege and Anderson (1978) first developed W_s equations by fitting a curve to the 75^{th} percentile of weights of largemouth bass, *Micropterus salmoides* (Wege & Anderson, 1978). Murphy et al. (1990) refined the methods of Wege and Anderson (1978) to reduce length-related biases, developing the regression-line percentile (RLP) technique using regression equations for each population in their study rather than pooled length/weight data (Murphy, et al., 1990) (Murphy & Willis, 1992). Ranney et al. (2010) found that equations derived from the RLP performed equally well

with regard to length bias compared to other techniques and concluded that those methods are equivalent in terms of their significance to stock management (Ranney, et al., 2010).

4.3. Methods

4.3.1. Determination of Minimum Fork Length

The RLP technique depends upon the calculation of the minimum total length (TL) to be used in the W_s equation development (Kolander, et al., 1993) (Bister, et al., 2000) (Muoneke & Pope, 1999). For our purposes, the fork length (FL) was used based on standardization of methods between the Louisiana Department of Wildlife and Fisheries (LDWF) collection and our own measuring protocol. The minimum fork length was then developed so as to avoid erroneous or inaccurate weights for smaller fish (Murphy, et al., 1990) (Muoneke & Pope, 1999). We calculated an error estimate (variance to mean ratio) for mean $\log_{10}$weight by centimeter FL group (Murphy, et al., 1990). The minimum FL was selected where the variance: mean error reached the inflection point in the plot for Gulf menhaden (Murphy, et al., 1990).

4.3.2. Assessment of Sampling Year Congruency

We calculated a $\log_{10}$weight/$\log_{10}$FL regression equation for each year in the data set using only fish longer than the minimum FL selected in the previous step. All years retained an r^2 value of greater than 0.95, indicating a strong correlation for the plotted data after removing menhaden with minimum FL, and as such each year was comparable to the next. This allowed for a valid ANCOVA between years prior to assessment.

4.3.4. Application of RLP and Subsequent Development of the W_s

The individuals remaining after minimum FL determination were then subjected to the RLP process. Using SAS, all values were separated by 1-cm increments from the minimum FL to the maximum observed result. The values that represented the top 25^{th} % of individuals in

each length category were then removed, leaving the 75th % percentile individuals to be used for the development of the W_s. From this point, all remaining data points for each centimeter category were then used to create mean weights for each centimeter group and their log/log relationships were analyzed using an ANCOVA program. The years were compared, and based on a type one error rate of 5% were determined to be similar or different. This was to ascertain the possible effect of the DWH spill on menhaden condition (we do not want to build a condition index with results from unhealthy individuals). The slopes were evaluated and compared to a standard growth rate slope of 3 (Morey, et al., 2003) and used to compare growth between years.

All data from statistically similar slopes (p>0.05) were then combined to form a standard equation (2) for the specific purpose of assessing each year based on the raw data contained in the study. The development of a post-spill W_s equation for use in determining a return to pre-spill conditions was realized for this specific study but should not be considered universally applicable for determining heath in the long term. More datasets should be incorporated, despite all slope values being greater than or equal to 3.

$$\log_{10}(y) = m(\log_{10}(x)) + b \qquad (2)$$

where:

y = Mass
m = Slope
x = Fork Length
b = Intercept

4.3.5. Development of Proportional Size Distribution (PSD) and Standard Length Categories

A common practice in fish assessment for sport fish is to develop a quality index based on length. Several of these indices have been developed for various marine and freshwater fish (Neumann, et al., 2012) (Raymond, et al., 1998). Gabelhouse (1984) proposed a standard length

categorization technique based on percentage of world record length (Gabelhouse, 1984). Using the Proportional Size Distribution (PSD) calculation (3), PSD-X (where X represents a specified category of quality), and the PSD X-Y (where X-Y represents the incremental difference between categories) (4) (Neumann, et al., 2012) an attempt to categorize Gulf menhaden based on length was conducted. PSD is a numerical descriptor of length-frequency data and can either be categorical or incremental in nature, so each method was employed in attempting to develop an appropriate measure of menhaden.

$$\text{PSD} = \left(\frac{\text{Number of Fish } \geq \text{Quality Length}}{\text{Number of Fish } \geq \text{Minimum Stock length}}\right) \times 100 \tag{3}$$

$$\begin{aligned} \text{PSD} - \text{X} &= \left(\frac{\text{Number of Fish } \geq \text{Category X}}{\text{Number of Fish } \geq \text{Minimum Stock legth}}\right) \times 100 \\ \text{PSD (X} - \text{Y)} &= \left(\frac{\text{Category X} \leq \text{Number of Fish } \leq \text{Category Y}}{\text{Number of Fish } \geq \text{Minimum Stock legth}}\right) \times 100 \end{aligned} \tag{4}$$

Additionally, the development of size categories appropriate for a non-sport commercial fish must be complete in order to utilize the above calculations. The SEDAR 27 Stock assessment of menhaden along with the approximately 1400 individuals sampled for this study were used to create the standard length categories and minimum stock length for Gulf menhaden (Vaughan, et al., 2011). Once this process was competed, a sigmoid function was developed to express the PSD along with all of the proposed length categories as it applies to the 1400 samples collected over the past three years (5).

$$f(x) = L_l + \left(\frac{L_u - L_l}{1 + e^{-a(x-c)}}\right) \quad (5)$$

where:
f(x) = Standard point index (L_l - L_u)
L_l = Lower limit
L_u = Upper limit
e = Euler's number
a = Desired slope at median
c = Median of data set (%)
x = % Study fork length record

4.4. Results and Discussion

4.4.1. Determination of Minimum Fork Length

First, minimum individual length data was determined by plotting the variance to mean ratio of $\log_{10}$(mass) by the specific size categories common for menhaden (6 cm to 23 cm) (Murphy, et al., 1990) (Vaughan, et al., 2011). As per the literature, the measure at which the data increase sharply was used as the cutoff minimum fork length used for data calculating the standard weight equation. This can be seen in Figure 4.4.1.1 as a variance: mean plot against fork

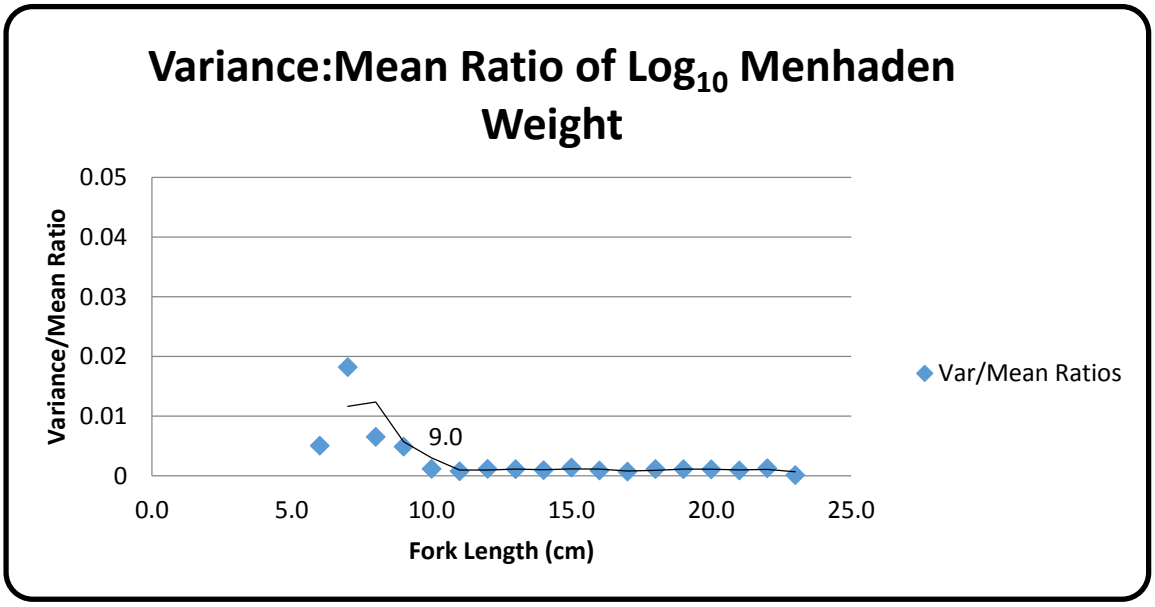

Figure 4.4.1.1 Variance: mean ratio of Log_{10} menhaden weight plotted against fork length

length (Murphy, et al., 1990) (Kolander & D.W., 1993). The inflection point was determined to be located at a fork length of 9 cm. All data points that fell outside of this minimum were discarded prior to the RLP determination of the W_s equations.

4.4.2. Development W_s Equation

The RLP standard weight equation was based on 19,153 computerized records (derived from the initial 76,000 fish harvested between the years 2000–2010) and tested with an independent dataset from 1,400 records (2011, 2012, and 2013). The proposed equation for gulf

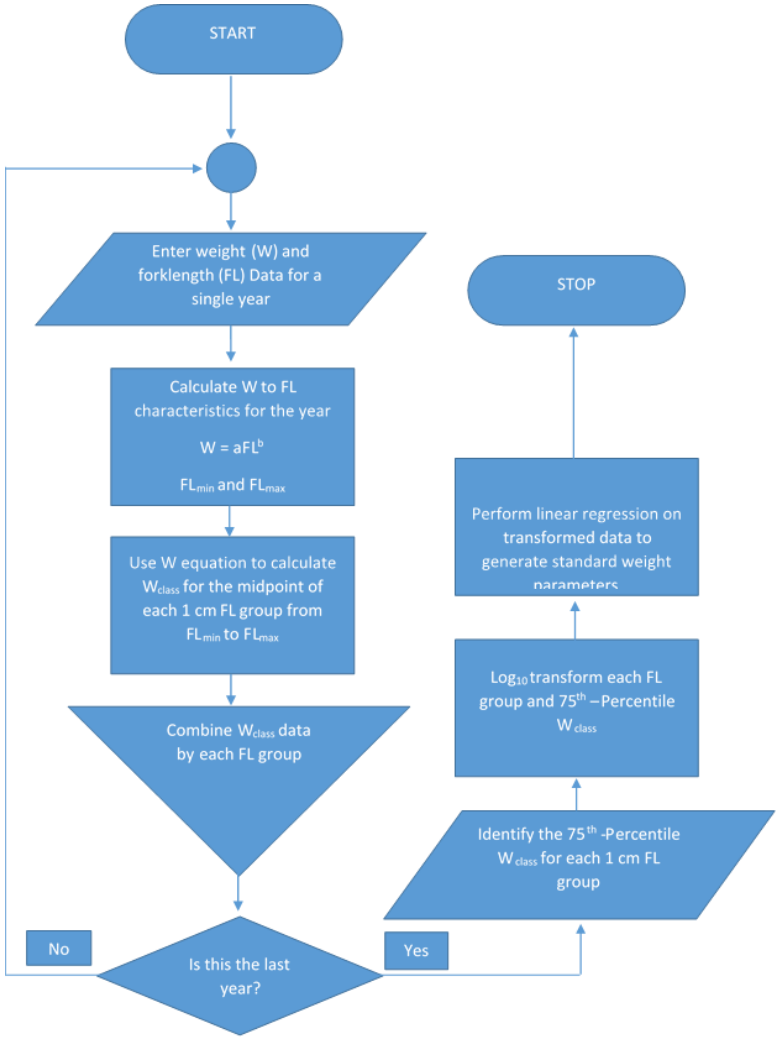

Figure 4.4.2.1 RLP Flowchart

menhaden is $Log_{10}W_s = –1.7884 + 3.0793(Log_{10}FL)$, where W_s is standard weight in grams and FL is fork length in millimeters. (Figure 4.4.2.1)(6) The minimum applicable length for the equation is 10 mm FL. Significant W_t–FL relationships were evident in 2 of the 3 test years. Chi-square analysis indicated no significant difference in the frequency of occurrence of both positive and negative slopes. The W_s equation derived in this study is recommended for use in gulf menhaden condition assessments.

2000-2010 Regression Line Equation:

$$\log_{10}(Ws) = 3.0793(\log_{10}(FL)) - 1.7884 \quad (6)$$

Figure 4.4.2.2 Standard weight equation (W_s) developed for Gulf menhaden

The 2011–2012 sampling period produced a similar regression line for comparing Gulf menhaden condition. This can be used as a "Post-Spill" condition assessment of Gulf menhaden

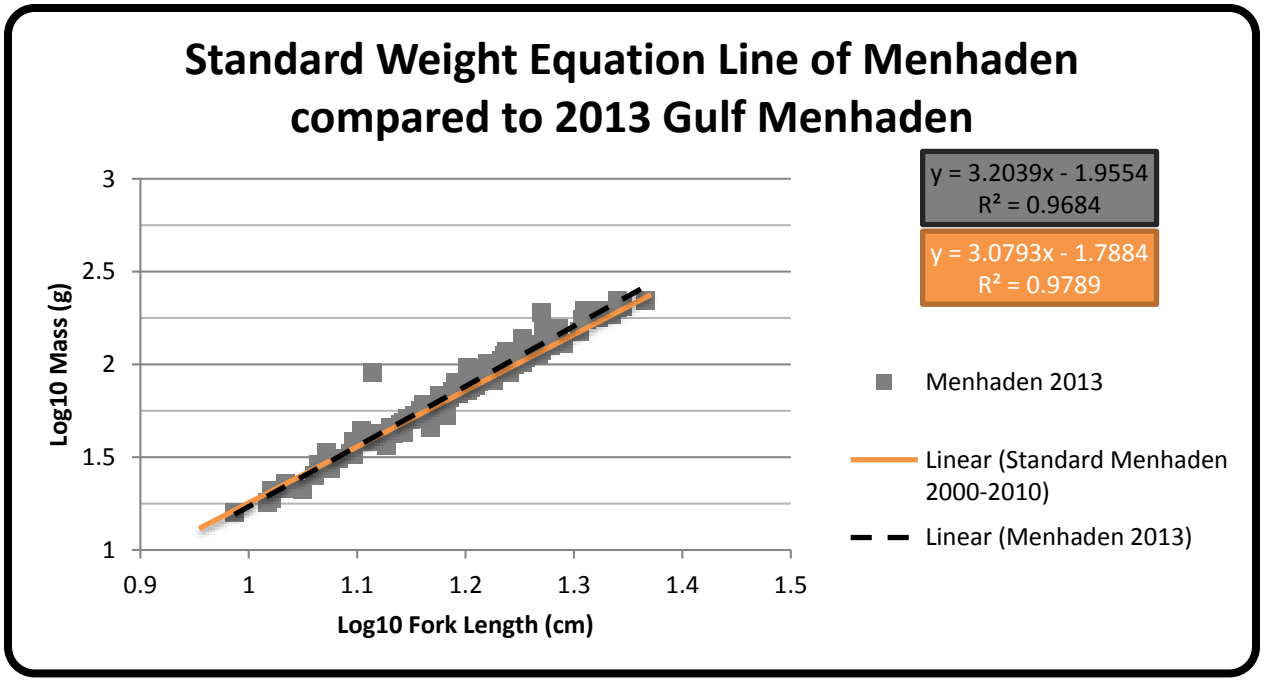

Figure 4.4.2.3 Menhaden condition compared to the standard weight equation (solid line)

to determine the condition of the species as compared to the W_s developed previously. The 2013 results suggest a strengthening of condition as compared to the 2011–2012 regression data as well as the W_s equation. The increase in condition is a good sign that the fishery is returning to baseline conditions.

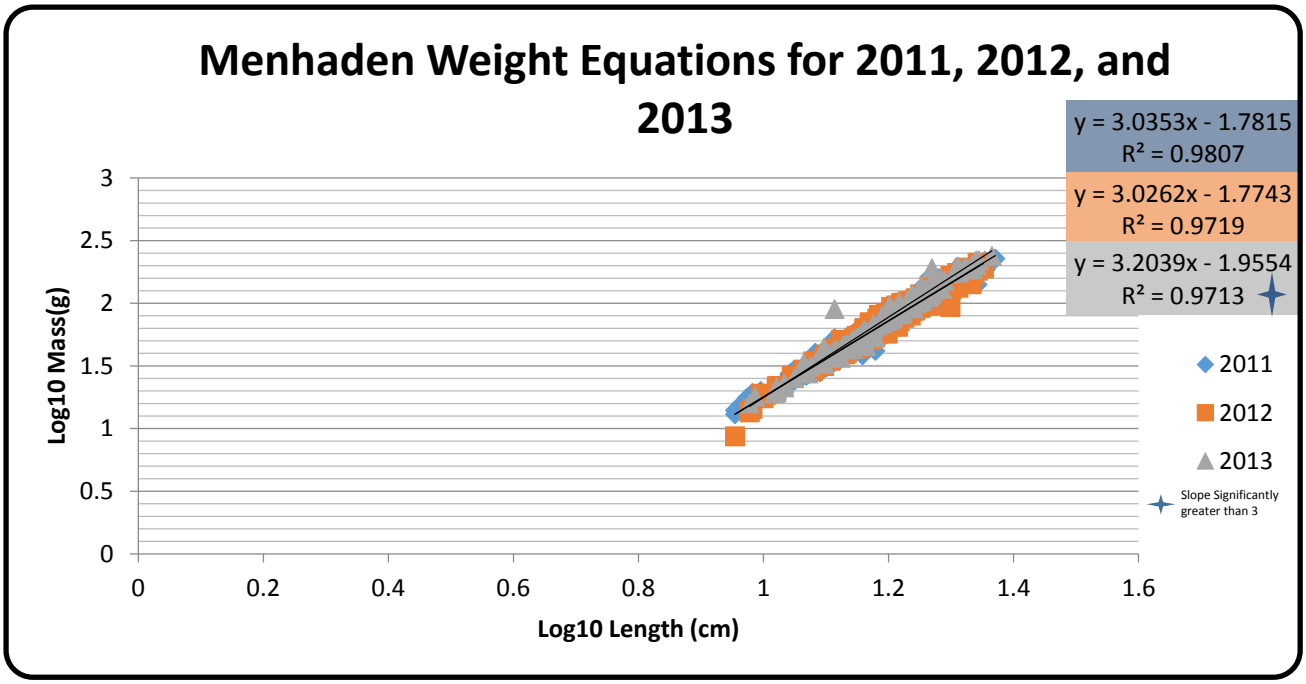

Figure 4.4.2.4 Menhaden condition based on weight equations produced from data collected 2011, 2012, and 2013

4.4.3 Development of Proportional Size Distribution and Standard Length Categories

Menhaden are a commercially harvested, non-sport fish to which the standard quality index of "Stock", "Quality", "Preferred", "Memorable", and "Trophy" do not apply. One does not harvest a menhaden and consider it in the context of the current length category names. We are proposing the adoption of a new length category system for use with fish that conform to the same profile as menhaden. Categories were based on possible end use of fish harvested, "Bait", "Commercial", "Quality Commercial", and "Exceptional Commercial". Gulf Menhaden were

compared, and the record FL and minimum stock FL were rounded to the nearest whole number (6–24 cm), and a percentage for each individual was calculated. The general size categories and distribution compared nicely with those found in the SEDAR 27 Stock Assessment of Gulf Menhaden (Minimum FL was 5.5 cm, while maximum FL was 34 cm. However, Vaughn et al. suggest using the 99th percentile, which is approximately 22 cm) (Vaughan, et al., 2011). Based on the size distribution for menhaden largely used for bait vs. commercial purposes, these categories were created using percentages of the sample population collected. In both this study and the SEDAR assessment, the majority of the population distribution was found between 27 and 59.9% of record FL or approximately 6–14 cm. Using this as the principal group (Bait) to calculate the PSD, the remaining categories were then determined. PSD was calculated at 56, and used as the basis for the numerator. This can be seen in the equation below (7).

$$\text{PSD} = \left(\frac{\text{Number of Fish } \geq \text{ Commercial Fork Length}}{\text{Number of Fish } \geq \text{ Minimum Stock Fork length}}\right) \times 100 \qquad (7)$$

Figure 4.4.3.1 Calculation of the PSD

From the PSD calculation, the remaining categories were determined both using Incremental and Traditional PSD: *Incremental* = Commercial 60–71.9%, Quality Commercial 72–84.9%, Exceptional Commercial 85–100% *Traditional* = Commercial 60%, Quality Commercial 72%, and Exceptional Commercial 85% (8). Table 4.4.3.1 shows each size category along with the

$$\text{PSD} - \text{X} = \left(\frac{\text{Number of Fish} \geq \text{Category X}}{\text{Number of Fish} \geq \text{Minimum Stock legth}}\right) \times 100$$
$$\text{PSD (X} - \text{Y)} = \left(\frac{\text{Category X} \leq \text{Number of Fish} \leq \text{Category Y}}{\text{Number of Fish} \geq \text{Minimum Stock legth}}\right) \times 100 \qquad (8)$$

Figure 4.4.3.2 Calculation of Traditional PSD vs Incremental PSD

calculated PSD values. As you can see, there is direct agreement with the PSD and the sum of the categories, which accounts for 100% of the proportional size distribution. Next a sigmoid function was determined to represent the PSD categories identified by this study.

Table 4.4.3.1: Incremental and Traditional Proportional Size Distribution (PSD) values calculated for Gulf menhaden 2011-2013

% of Record Length	PSD Quality	PSD Value*	Minimum Category Length	Maximum Category Length	# Fish
27-59.9%	PSD B-C	44	6.5	14.4	635
60-71.9%	PSD C-QC	31	14.5	17.3	438
72-84.9%	PSD QC-EC	20	17.4	20.4	287
85-100%	PSD EC	5	20.5	24.0	70
60%	PSD	56	14.5	N/A	795
72%	PSD-QC	25	17.4	N/A	357
85%	PSD-EC	5	20.5	N/A	70

NOTE*
(PSD C-QC) + (PSD QC-EC) + (PSD-EC)
= 31 + 20 + 5 = 56 = PSD
(PSD B-C) + (PSD C-QC) + (PSD QC-EC) + (PSD EC)
= 44 + 31 + 20 + 5 = 100%

Using this function, an individual can be plotted against the record % fork length for the species distributed by quality category, as can be seen in Figure 4.4.3.3. This function was designed with menhaden in mind; however, it can be applied to any non-sport stock fish data, assuming the median % of record fork length is known for the species (9). It can also be adjusted for a desired

$$f(x) = 1 + \left(\frac{3-1}{1+e^{-0.2(\%\ of\ record\ FL\ of\ a\ given\ individual - 61.67)}}\right) \quad (9)$$

Figure 4.4.3.3 Sigmoidal function used to generate the proposed menhaden quality index standard point index utilizing the numerator for this particular dimension. In this case the standard point index used reflects that provided in Gablehouse, 1984. Economic value, catch quality, lipid quantity, etc can be described using this metric (Figure 4.4.3.4).

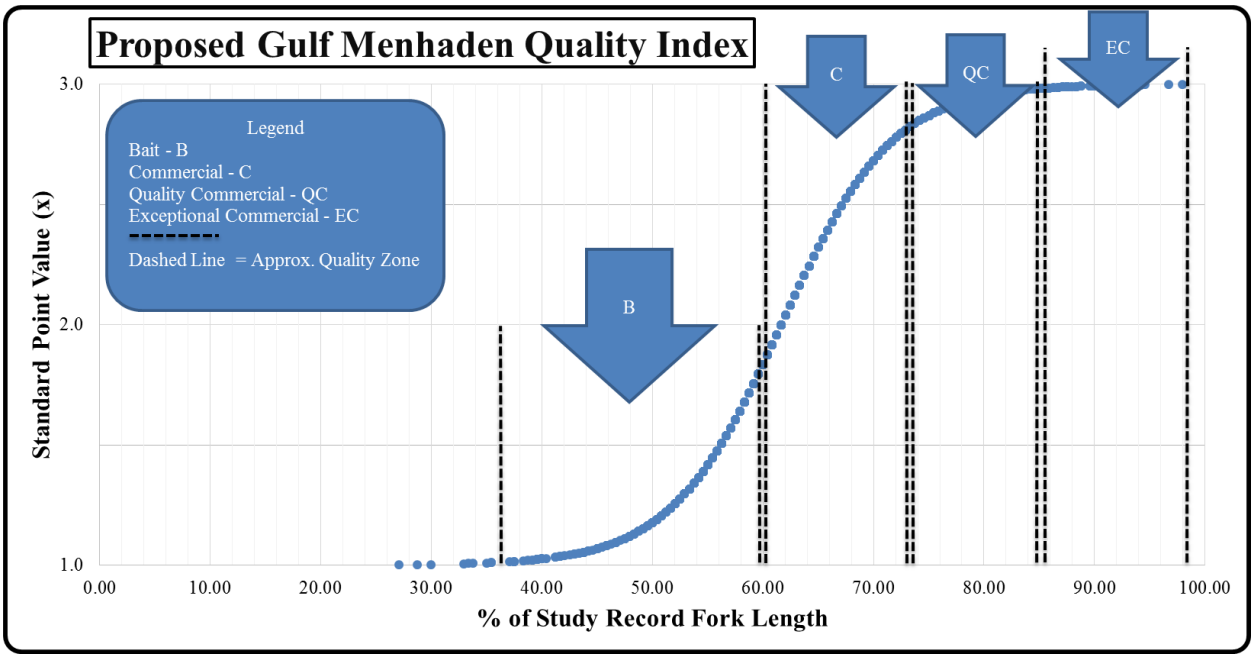

Figure 4.4.3.4 Proposed Gulf menhaden quality index based on approx. 1400 menhaden collected between 2011 and 2013

4.5. Conclusions

The development of a W_s equation for menhaden was successful based on 11 years of previous menhaden length and mass data provided by Mr. Joseph Smith of NOAA: National Marine Fisheries Service located in Beaufort, North Carolina. The 2013 increase in condition implies a return to the base menhaden condition and bodes well for the future creation of a menhaden W_s equation. At this stage, the regression lines determined for 2011–2012 data should be used to assess condition of menhaden directly after the spill.

The development of standard length categories for a non-sport, commercially harvested fish was successful using the determined PSD calculations from known length data along with study length data. A sigmoid function was developed to express the population distribution. This function can be used for several different purposes, including but not limited to a standard point

quality index (1–3), predicting harvest distribution, attributing economic/monetary value to menhaden landings, possible quality grading for consumer products, etc.

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CHAPTER 5: EXTRACTION OF MENHADEN OIL FROM WHOLE BODY HOMOGENATE USING DICHLOROMETHANE: A COMPARISON WITH HEXANE-ETHANOL

5.1. Conspectus

Solubility in adipose tissues constitutes a major concern for bioaccumulation of many non-polar compounds. Isolation of these compounds from target organisms is important when assessing environmental impacts of various pollution and exposure events. Standard lipid extraction techniques utilize multiple solvents to ensure a complete extraction of both non-polar (triglycerides, diglycerides, monoglycerides and sterols) and polar compounds (free fatty acids, phospholipids and sphingolipids) that comprise the standard lipid fraction contained in various fatty tissues as well as foods. It was proposed that the use of a single non-polar solvent can be used in place of solvent mixtures for lipid extractions performed for the analysis of non-polar compounds sequestered in adipose tissues, specifically polycyclic aromatic hydrocarbons. Gulf menhaden were identified as a commercial species primarily harvested for its high lipid content and used as the primary lipid source for this study. It was also proposed that the amount of lipids removed from Gulf menhaden using a single non-polar solvent would be comparable to the extracted oil obtained from a commercial source. The use of a single solvent (DCM) proved useful for the assessment of menhaden oil as compared to commercially harvested oils. It was noted that menhaden raw oil yields were significantly different in 2013 from the previous two years, with a higher oil yield in sampled fish.

5.2. Introduction

Lipids are classified as an assorted group of natural substances comprised chiefly of non-polar compounds (triglycerides, diglycerides, monoglycerides and sterols) as well as polar compounds (free fatty acids, phospholipids, and sphingolipids) (Christie, 1993). Lipids join

covalently to carbohydrates and proteins to form glycolipids and lipoproteins. Solvents used for lipid extraction generally should have a high solubility for all lipid compounds and be sufficiently polar to remove them from their binding sites with cell membranes, lipoproteins, and glycolipids (Smedes & Askland, 1999). This holds true for complete lipid analysis. However, we have suggested that based on the targets for analysis (i.e. non-polar, lipid-soluble chemicals) the use of non-polar and polar solvents might be unnecessary. The knowledge of lipid content in food or other tissues is important for several reasons, one of which is determining concentrations of persistent organic contaminants (dioxins, PCBs, organochlorine pesticides, PAHs) in tissue (Booij & van den Burg, 1994). If these contaminants can be removed from the sample matrix along with the non-polar fraction of lipids, a standard non-polar lipid total should be quantified for method analysis. Several methods have been developed for total lipid extraction (Folch, et al., 1957); (Bligh & Dyer, 1959); (Gardner, et al., 1985); (De Boer, 1988); (Booij & van den Burg, 1994); (Smedes, 1999). This study proposes the use of the total non-polar lipids (TNPL) fraction as an alternative for measuring lipids in conjunction with specific contaminants. Heated solvent extraction through a Soxhlet apparatus was used to extract lipids from sample menhaden tissue along with controls.

Traditionally, chloroform-methanol (2:1), hexane-ethanol (3:1), and several other solvent mixtures have been used to determine total lipid concentrations for various substrates (Nelson, 1975). There have been several papers that discuss the harmful nature of chloroform, citing its toxicity and low volatility as reasons to move away from its use (Drouillard, et al., 2004). There have been attempts to determine a suitable analogue, for which dichloromethane has been suggested (Drouillard, et al., 2004); (Cequier-Sanchez, et al., 2008). Based on several methods of analysis for lipid concentration, it was determined that the use of DCM as a single non-polar

solvent to extract TNPLs from tissues that will be analyzed further for non-polar compounds of concern (i.e. PAHs) would be appropriate (Carlson, 1985) (Drouillard, et al., 2004) (Cequier-Sanchez, et al., 2008). The use of a Soxhlet extraction apparatus was employed as a standard method for the extraction of lipids (Manirakiza, et al., 2001). The single solvent method was compared to hexane-ethanol (3:1) for total lipid recovery as well to show the % lost through incomplete lipid extraction. Additionally, this method was tested against commercially supplied menhaden oil to determine if the TNPL concentration can be used to characterize the commercially viable fraction of lipids in menhaden oil

5.3. Materials and Methods

5.3.1. Chemicals

Pesticide reagent grade solvents were used in all standard preparations, sample analysis, and rinsing procedures. Dichloromethane (DCM), hexane, and methanol (Mallinckrodt Chemicals) were used for tissue extraction of lipids. Sodium sulfate (anhydrous, 10–60 mesh, Fisher Scientific) was used for control preparation.

5.3.2. Gulf Menhaden

Menhaden were sampled at locations around Grand Isle, Louisiana (GI). These samples were harvested using a standard five-panel gill net. This net was approximately 200 m in length with five distinct plastic mesh panels. The menhaden were separated by length, bagged in plastic freezer bags, and placed on ice until frozen to –4°C in a laboratory setting. Menhaden tissue control samples were created from processed menhaden donated by a prominent menhaden processing company located in Louisiana. Fish oil and meal were combined in a ratio consistent with oil yields reported in this study for size-appropriate tissue concentrations.

5.3.3. Preparation of Menhaden

Frozen menhaden were massed, and their fork lengths were taken. Triplicate samples of menhaden with fork lengths of 16 cm or less (small) were selected and then chopped into small cubes approximately 12 mm × 24 mm × 24 mm. These pieces were then placed into pre-cleaned and solvent-rinsed 200-mL beakers. The cubed tissue was then compressed into the base of the beaker with a clean glass pestle, placed in a –86°C freezer and allowed to freeze. Frozen samples

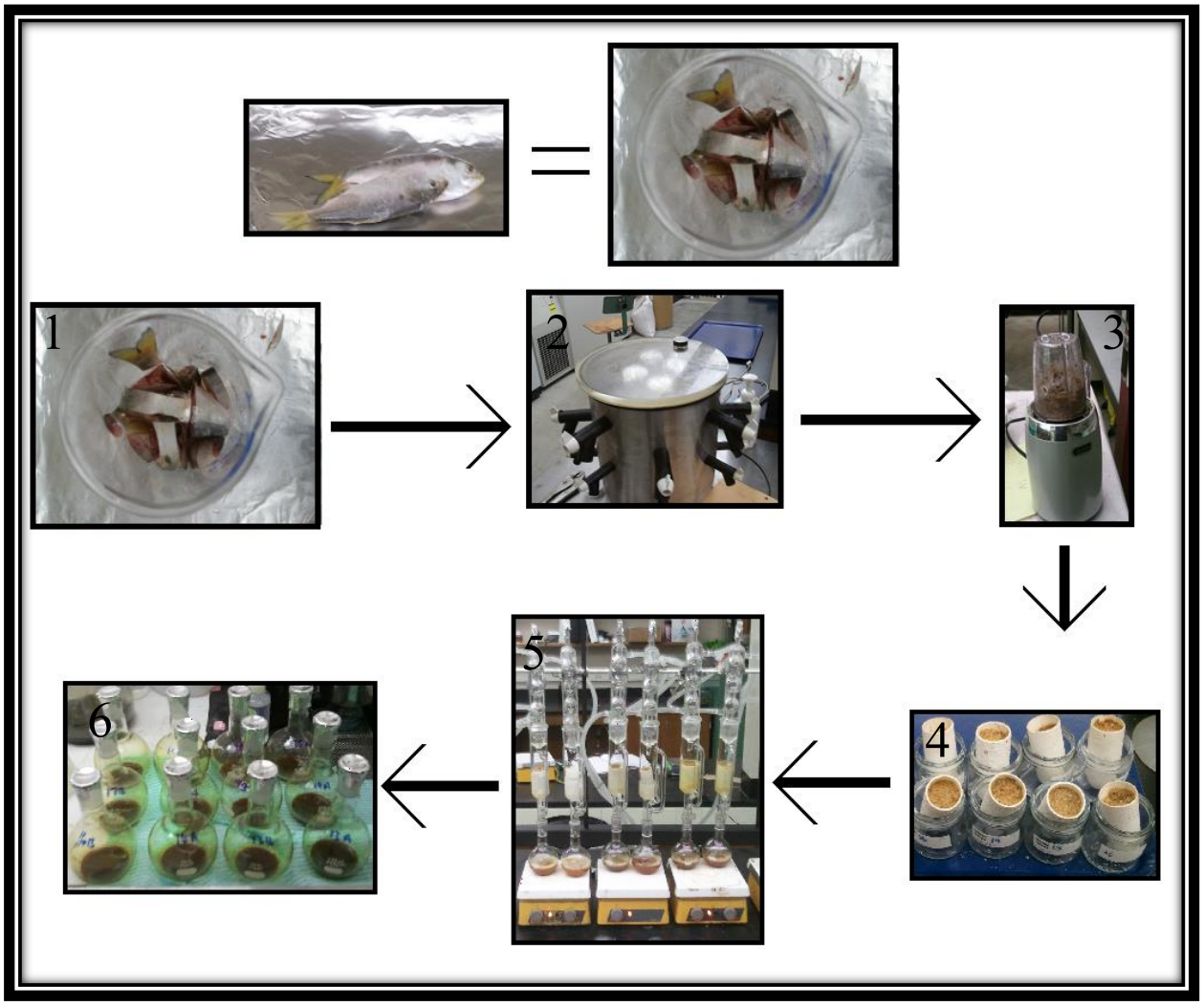


Figure 5.3.3.1 Complete lipid extraction process (Credit, Gregory Olson)

were then freeze-dried for 24 hours (VirTis, Model Freezemobile 6). This process was repeated for menhaden with fork lengths greater than 16 cm (large). Dried samples were placed in a desiccator prior to solvent extraction.

The whole menhaden was then homogenized, and a 10 g subsample was taken and mixed with 5 g of sodium sulfate to bind up any moisture possibly present within the lyophilized fish. The sample was packed into a cellulose extraction thimble and placed into a Soxhlet extraction column. An aliquot of roughly 100 mL of desired solvent/solvent mixture was placed into a tared, flat-bottom Florence flask resting on a hot plate. The column was connected to the flat-bottom Florence flask and the solvent/solvent mixture was heated to a boil. The resulting process ran for 16–18 hours (overnight) in an attempt to extract the lipid fraction within the menhaden. The flask was then removed to a rotary evaporator and all excess solvent evaporated. The extract was then placed in a drying oven at 80°C over night and allowed to cool in a desiccator. The resulting material was weighed within the flask, and the initial mass of the flask was subtracted from the total. This provided the total amount of lipid material extracted from the menhaden in grams.

5.3.4. Preparation of Commercial Menhaden Oil Controls

Approximately 5 g of sodium sulfate was spiked with 0.5 g of commercial menhaden oil. This was performed in triplicate three times for each treatment, resulting in 18 total controls (9 for DCM and 9 for Hex:EtOH). The sodium sulfate/menhaden oil mixtures were then removed to cellulose extraction thimbles and placed in a Soxhlet-heated solvent extraction apparatus. The resulting process ran for 16–18 hours (overnight) in an attempt to extract lipids from the menhaden oil controls. The flat-bottom Florence flask that housed the extracted oil was removed to a rotary evaporator, and all excess solvent evaporated. The resulting material was weighed within the flask and the initial mass of the flask was subtracted from the total. This provided the total amount of lipid material extracted from the menhaden oil controls.

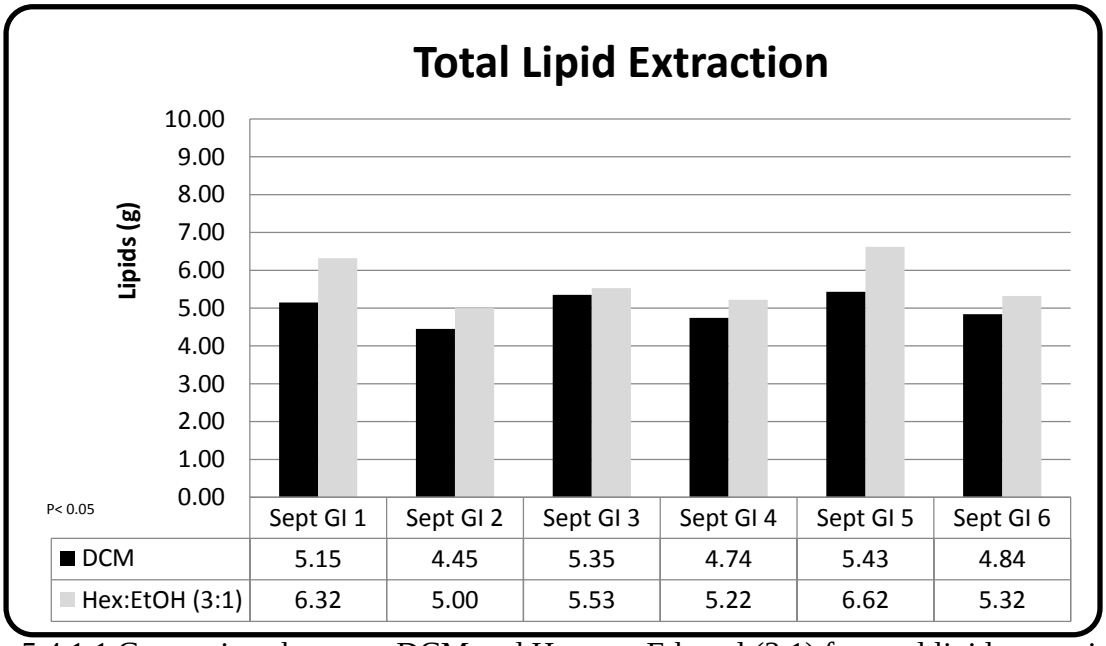
Figure 5.3.4.1 Commercial menhaden meal and oil collected June of 2009 used to create controls (Credit, Gregory Olson)

5.4. Results and Discussion

5.4.1 Gulf Menhaden

A sample set of large (Fork-length > 16 cm) and small (Fork-length < 16 cm) from Grand Isle, Louisiana was prepared and extracted using both the DCM and Hexane:Ethanol extraction solvents. Each sample was divided evenly into two 10-g subsamples to be extracted using the soxhlet extraction method. The samples were then extracted alongside one another to determine the efficiency of each solvent for extracting lipids. A tissue blank with a known lipid amount was also extracted with each solvent to determine extraction efficiency. These menhaden averaged 4.99 g (DCM) and 5.67 g (Hex:EtOH) of extracted lipids, respectively. Using a paired *t*-test for two-sample means, the difference between treatments (n = 12) was significant with a *p* value = 0.010, with the Hex:EtOH extraction method recovering more material than the single solvent DCM method (Figure 5.4.1.1). The tissue blanks for each extraction method bolstered these results, with the Hex:EtOH extraction method recovering 5.25 g of lipids (control = 5.0 g), and the DCM recovering 3.10 g of lipids (38% error vs 5% error for the Hex:EtOH method) (Figure

5.4.1.2). Assuming the Hex:EtOH results to be the "true" representation of total lipids, we can then state that the percent error incurred by using only DCM as the solvent for lipid assessment is roughly twelve percent (11.91%). This leads to the next question, "Do commercially harvested fish oils contain the same fraction of lipids that DCM will not extract?"



	Sept GI 1	Sept GI 2	Sept GI 3	Sept GI 4	Sept GI 5	Sept GI 6
DCM	5.15	4.45	5.35	4.74	5.43	4.84
Hex:EtOH (3:1)	6.32	5.00	5.53	5.22	6.62	5.32

Figure 5.4.1.1 Comparison between DCM and Hexane: Ethanol (3:1) for total lipid extraction of Gulf menhaden. Note (g) represents lipids per 20g lyophilized menhaden tissue.

Figure 5.4.1.2 Lipid recovery from tissue controls. Note (g) represents lipids per 30g control tissue.

5.4.2. Commercial Menhaden Oil Controls

Commercially harvested pre-refined menhaden oil was used to test each solvent's extraction efficiency as it applies to commercially extracted menhaden oil. Eighteen 5-g samples of $NaSO_4$ were spiked with approximately 0.5 g of menhaden oil. Half of these samples were extracted using DCM, and the other half were extracted using a Hex:EtOH (3:1) solvent mixture. Using a paired *t*-test for two sample means, it was determined that the recovered means were not significantly different from each other ($p = 0.802$) (Figure 5.4.2.1). It was determined that the

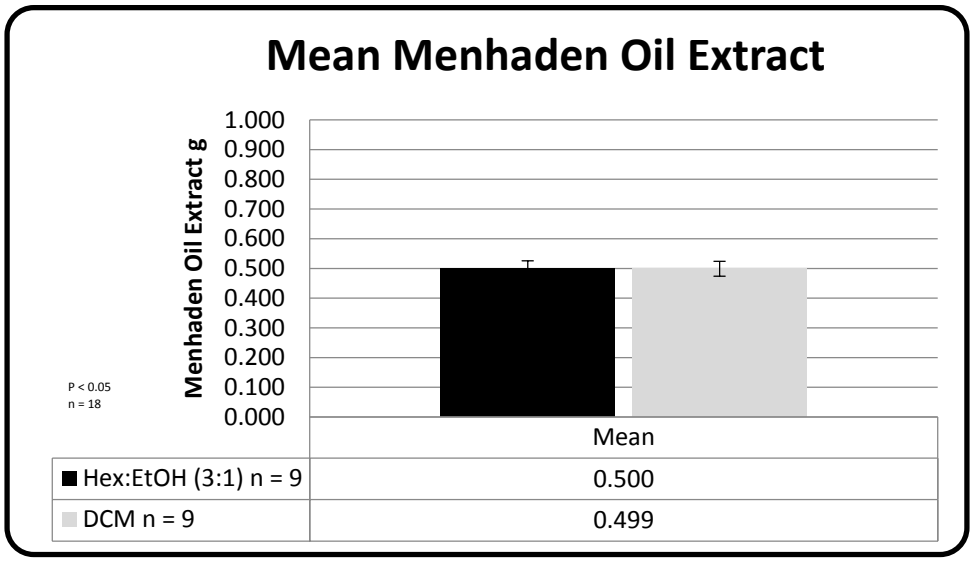


Figure 5.4.2.1 Mean menhaden control raw oil recovery by extraction method.

extraction technique used to retrieve the commercial menhaden oil for refining does not show a difference in oil recovery between DCM and Hexane:Ethanol (3:1). Based on this result, it can be assumed that the use of a single, non-blended solvent such as DCM will provide sufficient extraction of the TNPL fraction of the menhaden tissue and will recover the same lipids (by gravimetric analysis) as a more specialized solvent blend such as Hexane:Ethanol (3:1). As can be seen from the previous section, gulf menhaden contain more complex fats that *can be* obtained using the Hex:EtOH extraction solvent. However, this method is not applicable to the actual oil extracted for commercial use. So for the sake of chemical analysis, menhaden tissue can be extracted with DCM for both TNPL analysis as well as PAH analysis, of which the latter does not require a polar solvent such as ethanol to successfully extract all lipids from tissues.

5.4.3 Assessment of Gulf Menhaden Oil recovery after the DWH Spill Event.

The overall concertation of commercially valuable menhaden oil (TNPLs) was markedly higher in 2013 as compared to 2011 and 2012 (Figure 5.4.3.1). This increase suggests that the menhaden has a greater ability to store its energy as fats and oils. This can mean that the fishery itself is becoming healthier. When compared to the results of chapter 4, it can be stated that this increase in lipid content is a result of elevated fish condition and suggests that overall menhaden oil yields will return to pre-spill volumes.

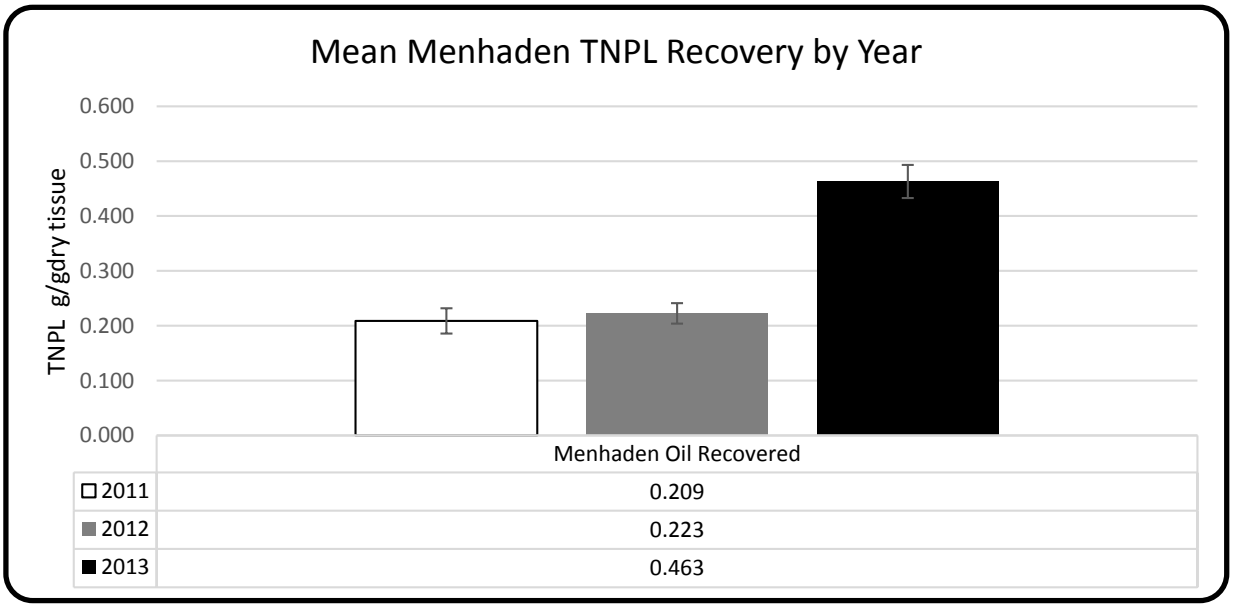


Figure 5.4.3.1 Mean Menhaden raw Total Non-Polar Lipid recoveries by year. * indicates significantly different from 2011 and 2012.

5.5. Conclusions

It can be concluded that the use of a blend of non-polar and polar solvents (i.e. Hex:EtOH) proves more efficient at extracting lipids from tissue than the use of a single non-

polar solvent (DCM). These lipids represent the complete extraction of complex lipid and lipid-like structures from a given tissue. As such, DCM is not a viable replacement for a blend of solvents when attempting to extract all lipids from a tissue. However, when the desired fraction of lipids to be extracted is the non-polar fraction (TNPL), then a single non-polar solvent can be used. It was additionally concluded that for the purpose of extracting lipids from Gulf menhaden, the DCM extraction technique provided the same yield as the Hex:EtOH blend when applied to unrefined, raw menhaden oil as provided by a commercial source. For the sake of ease and analytical consistency, the use of a single non-polar solvent such as DCM can be used to extract the commercially viable lipid fraction from Gulf menhaden, as well as any possible non-polar contaminants such as PAHs that may become bound through bioaccumulation within the fish tissue. Additionally, it can be concluded that the resulting oil yields from menhaden collected in years 2011, 2012, and 2013 show an increase, with 2013 having a significant increase over both 2011 and 2012.

5.6. Acknowledgments

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CHAPTER 6: SUMMATION AND EVALUATION

6.1. Conspectus

The use of several metrics was employed to determine if Gulf menhaden health can be linked to the overall health of the Gulf of Mexico. Based on a three-year assessment from 2011 to 2013, total PAHs, benzo[a]pyrene toxic and mutagenic equivalents, standard weight with population distribution, and total non-polar lipid recoveries were calculated and applied to the Gulf Menhaden fishery. All items suggested a return to a healthier fishery by the year 2013. Significant decreases in persistent PAHs found in fish tissue along with significant increases in both condition and total non-polar lipids support the increased health of the Gulf menhaden fishery in years subsequent to the DWH spill event. It was noted, however, that these returns are more than likely a result of acute oil exposure that caused the susceptible portion of the Gulf menhaden population to either die or become easier targets for predation.

6.2. Introduction

Brevoortia patronus, more colloquially known as the pogy in south Louisiana, is an important species that plays a central role in the Gulf of Mexico. Filtering tremendous amounts of water, these fish feed on the plankton floating in the water column as they school in the waters of the GoM. The obligate filter feeding nature of these fish places them in a unique position in the trophic structure of the GoM. Primarily, these fish turn phytoplankton into usable protein for the rest of the marine environment to enjoy. They are the primary forage food for almost all Gulf consumers. This places them directly in line for biomagnification through the trophic pyramid of the Gulf. For this reason, the nature of how they feed takes on a more important role. Filtering the water column for plankton will also filter out any other materials that happen to be present. In the case of the Deepwater Horizon Oil Spill, this meant dispersed crude oil. So we have the largest commercial catch by weight, which happens to filter the water column *and* is a primary

forage fish for almost all sports fish and water fowl in the Gulf; we have an opportunity to glean much information from this interaction.

6.3. Menhaden Watch

The idea of using a filter-feeding organism to assess the overall condition of a specific coastline/marine environment is not new. There have been several programs that use mollusks throughout the years, the most notable being the "Mussel Watch" overseen by NOAA's National Centers for Coastal Ocean Science. This program has been running since 1986 and is a massive and truly extensive dataset that is used to determine the health of coastal areas as well as the health of the Great Lakes here in the United States. Menhaden watch is an attempt to establish a similar assessment of coastal waters in the Gulf of Mexico (as well as the Atlantic – Atlantic Menhaden). The initial phase study (three-year assessment) was completed using the DWH spill as an impact event that could show the usefulness of knowing some basic parameters associated with menhaden. Looking at key persistent compounds of concern (CoCs) (in this case PAHs, but could easily be any CoCs identified by the EPA), using additional analytical indices for these CoCs, determining fishery condition as applied to pre-spill condition, and measuring the commercial oil yield from these fish were all used to assess menhaden health and the impact the spill had on the fishery. To continue these analyses and to maintain this dataset will increase our ability for impact assessment as well as help in our understanding of baseline health as it applies to years without incidents.

6.3.1. The Fish

Gulf menhaden were used to determine the overall effects of the Deepwater Horizon Oil spill that left approximately 4.9 million barrels of oil circulating through the Gulf of Mexico. It was the first vertebrate filter-feeder used in this capacity. Initially, tissue samples were isolated

and extracted in an attempt to identify key persistent components of crude oil known as polycyclic aromatic hydrocarbons (PAHs). These compounds were identified in fish tissue, and a subsequent evaluation of the mutagenicity and carcinogenicity was carried out through the use of a standardization processes using benzo[a]pyrene. Not only were there significant decreases in total PAHs ($\sum PAH_{43}$, See Table 3.4.1.1) from 2011 to 2013, but there were also significant decreases in the Benzo[a]pyrene toxic equivalents as well as mutagenic equivalents (BaP-$TEQ_{\sum PAH14}$ and BaP-$MEQ_{\sum PAH10}$, respectively, see Table 3.4.2.1). It was also determined that the portion of the menhaden population that originally contained higher levels of heavier PAHs (the more persistent PAHs) did not seem to be in the samples taken in 2012 and 2013, the suggestion being that they might not have lived to reach those harvesting seasons. It was also determined that the overall total non-polar lipids increased dramatically for the 2013 harvest season, the suggestion being that there was an increase in menhaden health. This was supported by the increase in condition seen by applying the standard weight calculation to the years after the spill. The 2013 harvest season had a slope (3.2039) significantly greater than 3.0793 (the slope calculated by determining the standard weight equation for menhaden collected from 2000–2010).

6.3.2. The Spill

Approximately 4.9 million barrels of crude oil and gas were released into the Gulf of Mexico (GoM) from April to July 2010 during the Deepwater Horizon (DWH) spill. Impacts of this magnitude seldom occur in the GoM, and we cannot predict when these types of events will happen. Major constituents of concern (CoC) in crude oil are Polycyclic Aromatic Hydrocarbons, which often have low volatility that allows them to persist (persistent organic pollutants, POPs) in the environment. It is proposed that PAHs be used to perform a continual

toxic potential assessment of oil contamination in the GoM. PAHs are considered compounds of concern according to the United States Environmental Protection Agency (USEPA) due to their ability to accumulate within adipose tissue (USEPA, 2008). There are several PAHs listed as mutagenic and carcinogenic, making their possible presence in commercial fishery populations a major environmental concern (Durant et al., 1996; Nisbet and LaGoy, 1992; USEPA, 2008).

The GoM is projected to produce upwards of 1.7 million barrels of oil per day (MMBOPD) and 8 billion cubic feet per day (BCFPD) of natural gas by 2016 (Karl et al., 2007). The GoM is a significant petrochemical exploration and development region of the United States. It has and will continue to be a major source of crude oil and natural gas. The GoM is also one of the most productive marine ecosystems in the United States, accounting for an average of 18% of the total U.S. domestic commercial fish landings during 2009–2010(Van Voorhees and Lother, 2011). The GoM will continually be affected by petroleum exploration for the immediate future. Because of the connection to the petrochemical industry, commercial and sport fishing in this region will always have the potential to be affected; therefore the GoM should be monitored continually in order to assess overall health as well as specific temporal and spatial events impacting this region.

6.4. Moving Forward

It is impossible for a program like this to work without funding or a driving force. I have already written a grant proposal to continue research on Gulf menhaden for the purpose of generating a long-term dataset that I hope will one day become as strong as those generated for the Mussel Watch program. One of the key elements for this research is the commercial harvesting of these fish. This allows for a continuous harvest dataset that can be collected as part of everyday fishing. This will require less manpower and helps to streamline the overall process.

I firmly believe that these assessments will not only help us to understand the health of the fishery and the Gulf, but will also become useful to the industry itself.

6.4.1. Future Impact Assessment

Below is a list of proposed research interests that will be associated with the "Menhaden Watch" program that I envision. Several key factors can be identified as areas of need in the research proposed for this program, such as additional CoCs that should be added to the list of contaminants that are screened.

1. Determine total PAH concentrations and apply BaP-TEQ and BaP-MEQ indices to menhaden harvested at specific nearshore and offshore locations around the Gulf of Mexico from April to October each year, creating a continuous contaminant monitoring program based on analysis of chemical contaminant trends in tissue collected from menhaden, compared spatially and by size.

2. Determine average whole life body accumulation using three-year interval analysis of total PAH concentrations as well as BaP-TEQ and BaP-MEQ indices of harvested menhaden compared spatially and by size.

3. Assess Gulf menhaden health based on yearly condition as applied to the standard weight equation generated by this research.

4. Assess Gulf menhaden health based on yearly mean raw menhaden oil concentrations compared spatially and by size.

5. Generate baseline datasets that can be used in conjunction with annual data collection to determine overall heath as well as specific temporal and spatial impacts on the GoM.

6. Develop similar studies as indicated in objectives 1–4. Isolate metals and various other CoCs associated with contamination as identified in the NCCOS Mussel Watch program.

7. Assess Gulf menhaden health-based histopathological analysis compared spatially and by size.

6.4.2. Strengths and Flaws

There are several strengths that can be attributed to the use of Gulf menhaden as the first vertebrate filter-feeding indicator species to assess overall GoM health. First, the menhaden is found in vast quantities in the GoM and is harvested commercially by season (April to November). This means that there will always be samples to collect, and those samples can be obtained relatively easily with a simple working relationship with both the commercial industry as well as the Louisiana Department of Wildlife and Fisheries (LDWF) or the analogous wildlife and fisheries department in any participating state. Second, menhaden are filter feeders, which places them in direct contact (not only through dermal and pulmonary contact, but also through ingestion) with CoCs in the water column. This "triple" exposure ensures an easily detected response within the system, allowing for a more identifiable change. Third, Gulf menhaden are endemic to the GoM. This means that exposure and impacts measured within the fishery can be easily attributed to events in the GoM. Fourth, menhaden live for three to four years, so an acute assessment along with germline assessment is easily achieved. Last, the species is not so robust that an event will have little to no impact on them. They will, based on the finding of this study, respond to an event in a measureable and timely way.

As with anything, there are several flaws that can be attributed to this study. First, menhaden as a fishery has been on the decline since the early 1900s, especially in the Atlantic fishery. This can cause some conflicts of interest when working with the commercial industries for sample collections. Second, menhaden live for only three to four years, meaning that a chronic impact assessment will be difficult to achieve. Third, there are no menhaden species located on the Pacific coast, so an analogous species would have to be determined for those waters. There are species in Mussel Watch that are uniquely "Pacific", anchovy or sardine may

be an acceptable lipid intense fish species amendable to petroleum and chlorinated hydrocarbon assimilation. Fourth, only those areas sampled by commercial vessels as well as by state departments of wildlife and fisheries will be assessed (this is not too bad of an issue, seeing as menhaden are not sessile). Last, the species is a vertebrate, making it more difficult to get proper permitting for use in research. However, it should be noted that those procedures were followed to complete this research, so the permits are not unattainable.

6.5. Conclusions

- Matrix Solid Phase Dispersion of menhaden tissue for the subsequent extraction of PAH compounds was a practical extraction method to determine PAH concentrations in menhaden tissue, and the addition of lyophilization increased tissue disruption for better extraction results.

- Using the adapted MSPD extraction method total PAHs ($\sum_{PAH:WF}$), BaP-TEQs, and BaP-MEQs were quantified for Gulf menhaden in years 2011, 2012, and 2013. Total PAHs showed a significant decrease ($p<0.05$) by year 2013. BaP-TEQs showed a significant decrease ($p<0.05$) by 2013. BaP-MEQs showed a significant decrease ($p<0.05$) by 2013. However, the 2013 mean was also significantly higher ($p<0.05$) than the 2012 mean. This trend was noted in the BaP-TEQs, despite the difference not being significant ($p>0.05$). This suggests that "new source" PAHs are not being introduced to the Gulf. However, resuspension of persistent PAHs is impacting the fishery. It was noted that the levels of BaP-TEQs and BaP-MEQs measured in menhaden in 2013 are significantly lower than the levels measured in 2011, despite the elevated levels when compared to 2012.

- Stock assessments from approximately 76,000 Gulf menhaden (provided by NOAA Southeast Fisheries Science Center, Beaufort Laboratory, North Carolina) were used to generate a W_s equation to be used to measure menhaden condition. Linear regressions of each sample year were then compared to this W_s to determine menhaden condition in the years after the DWH spill. It was noted that 2011 and 2012 were not significantly different ($p>0.05$) from the W_s. Year 2013 was significantly different ($p<0.05$) from the W_s, indicating an improved condition from standard menhaden of the same fork length.

- Commercially harvested menhaden oil was extracted to determine efficiency of Hexane:EtOH (3:1) vs Dichloromethane. It was determined that commercially harvested menhaden oil contains compounds that are extracted with equal efficiency using either solvent. Based on this, DCM was used to extract "Total Non-polar Lipids" from menhaden samples harvested in years 2011, 2012, and 2013. Menhaden oil yields were significantly greater ($p<0.05$) in 2013 than in 2011 and 2012. Higher menhaden oil yield

is an indication of a healthier population based on the fact that the calories are transferred to lipid reserves as opposed to being used entirely for energy.

- In conclusion, the decrease in PAHs (Total, BaP-TEQs and MEQs), the increase in condition, and the increase in commercial menhaden oil recovery show an overall increase in menhaden health by three years after the DWH spill. This suggests that Gulf menhaden are returning to their pre-spill levels.

As a final conclusion, the use of Gulf menhaden has been shown to generate useable results when assessing the impact of an event. The continued monitoring of this fishery will provide pertinent, real-time assessment to any impact on the Gulf. However, Gulf menhaden have too short a lifespan to assess the overall health of the Gulf for extended periods. This can be seen by the return to a stronger fishery only three years after the Deepwater Horizon event. The measure of persistent (carcinogenic and mutagenic) contaminants stemming from the incident was only seen in the year after the spill (2011), with those individuals no longer represented in the sampled data after that. Menhaden condition as well as TNPL increased by 2013, showing a strengthening of the fishery as a whole and indicating a return to baseline in these areas but not indicating a return to baseline in PAH exposure. PAH exposure can be seen as a "new source" vs. baseline exposure. With the understanding that menhaden with heavier PAHs do not live beyond the first year after exposure, we can assume that no "new source" exposure happened during the study period 2011–2013. The significant decrease in overall PAHs can be seen as a return to baseline PAH concentrations in menhaden tissue. However, we cannot say that these current concentrations are within baseline concentrations. Continued monitoring will allow for a definitive answer as to when Gulf menhaden return to background PAH exposure.

Think back to the major collapse of the Atlantic menhaden fishery. Having the knowledge and manpower to assess Atlantic menhaden prior to this collapse could have prevented the harm caused by treating the menhaden fishery as a limitless resource supply. We

now have the means and the skillset to monitor the menhaden fishery as a whole which will help to prevent future coastal declines as well as generate immediate assessment for future impact events. Application of the assessments proposed during the course of this study are essential for maintaining a robust dataset that will give us, as scientists, a window into coastal water health.

6.6. Acknowledgements

The authors would like to extend their appreciation to: The Louisiana Department of Wildlife and Fisheries for project funding especially Secretary Mr. Robert Barham and Assistant Secretary Mr. Randy Pausina. Paul Cook, Jeff Marx, Damien Barque, and Carolina Monteiro from the LDWF New Iberia Station Keith Ibos, Bill Hano, John Pituch, Chris Baker, Joan Pravatiner, Russell Hutzler, Lindsey Green, Sean Jackson, Kevin Borne, Trot Sehlinger, Michelle Malouf, Heather Helfrich , Brittany Dephilips, Natasha Breaux, Kelly McBride, Jason Hughes, and Nicole Hughes from the LDWF New Orleans Office Brian Hardcastle, Schuyler Dartez, Clint Edds, Robert Boothe, and Matthew Boasso from the LDWF Grand Isle Research Station Personnel at Louisiana State University for continued support. Dr. Edward Overton, Dr. Scott Miles , Buffy Meyer, Dr. Heng Gao, Kayla Carter, Laura Basirico-Newell, Dr. Carey Gelpi, Courtney Drayton, Hannah Rockett, and Garrett Miles.

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APPENDICIES

A: Chapter 1

Loop Current Observations – Letter of Permission

Gregory Olson <golson2@tigers.lsu.edu>

Loop Current Observations

Michael Connolly <MConnolly@agu.org> Fri, May 4, 2012 at 7:04 AM
To: Gregory Olson <golson2@tigers.lsu.edu>

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B: Chapter 2

Data Tables and Graphs

Raw Menhaden Data

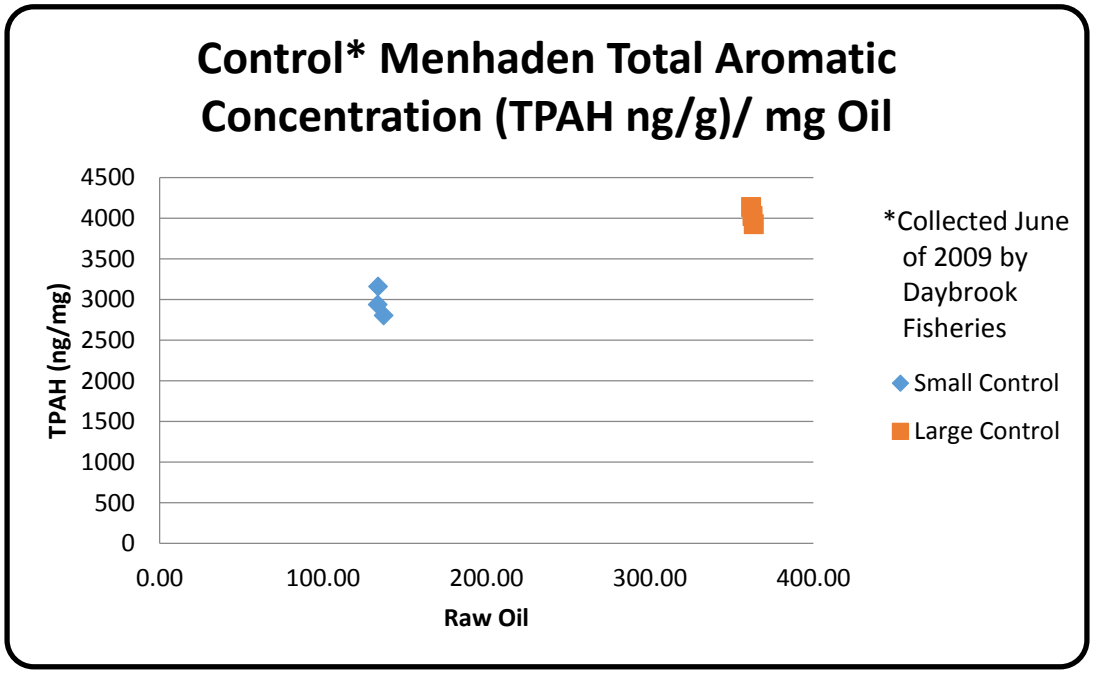
Dry Mass (g)	Fork Length (cm)	Phenanthrene D10 Recovery %	Corrected TPAH (ng/g)	C3-Phenanthrenes Adjusted TPAH (ng/g)
30.8	14.9	90	6458.00	3966.00
26.6	14.3	90	13041.00	10186.00
18.3	12.7	89	10132.00	7739.00
17.1	12.6	92	7227.00	5419.00
18.2	12.9	89	7007.00	5279.00
31.7	17.2	91	2517.00	669.00
45.2	18.6	92	2422.00	495.00
64.3	19.6	95	3413.00	285.00
50.6	18.7	91	3229.00	264.00
39.0	18.0	92	1892.00	262.00
50.2	18.4	90	2666.00	352.00
40.2	13.6	89	2860.00	1118.00
45.0	13.6	89	4429.00	570.00
49.1	13.7	91	2982.00	302.00
47.9	13.5	88	3672.00	374.00
49.6	27.2	88	4838.00	459.00
46.9	13.4	91	5877.00	582.00
44.6	18.4	91	13128.00	1729.00
45.8	18.4	90	20523.00	3018.00
30.6	16.5	90	13038.00	2260.00
37.1	18.9	86	8438.00	1915.00
49.4	18.1	85	10216.00	1539.00
50.0	18.8	92	12907.00	1519.00
28.1	13.6	83	6728.00	769.00
28.9	13.3	83	5685.00	260.00
27.7	13.3	84	6326.00	329.00
18.9	12.3	86	2306.00	94.00
20.5	12.8	80	1890.00	98.00
18.9	12.3	85	2210.00	72.00

Mean Phenanthrene d10 Recovery by Size, Date, and Location

Mean % Phenanthrene d10 recovery	Date	Size
81	Jul-11	Large VB
91	Jul-11	Large GI
86	Jul-11	Large Avg
94	Jul-11	Small VB
94	Jul-11	Small GI
94	Jul-11	Small Avg
93	Aug-11	Large VB
91	Aug-11	Large GI
92	Aug-11	Large Avg
91	Aug-11	Small VB
90	Aug-11	Small GI
91	Aug-11	Small Avg
90	Sep-11	Large VB
88	Sep-11	Large GI
89	Sep-11	Large Avg
89	Sep-11	Small VB
90	Sep-11	Small GI
89	Sep-11	Small Avg

TPAH and Recovery data for Menahden Controls

Date	ID	Control for	Phenanthrene d-10 Surrogate Recovery	Corrected TPAH (ng/g)	Adusted TPAH (ng/g) for C3-Phenanthrenes	% C3-Phenanthrens
1/23/2012	C1	small	0.88	2806	47	98.33%
1/23/2012	C2	small	0.88	2940	47	98.40%
1/23/2012	C3	small	0.88	3162	46	98.55%
1/23/2012	C4	large	0.87	4030	47	98.83%
1/23/2012	C5	large	0.88	4140	47	98.86%
1/23/2012	C6	large	0.87	3929	47	98.80%

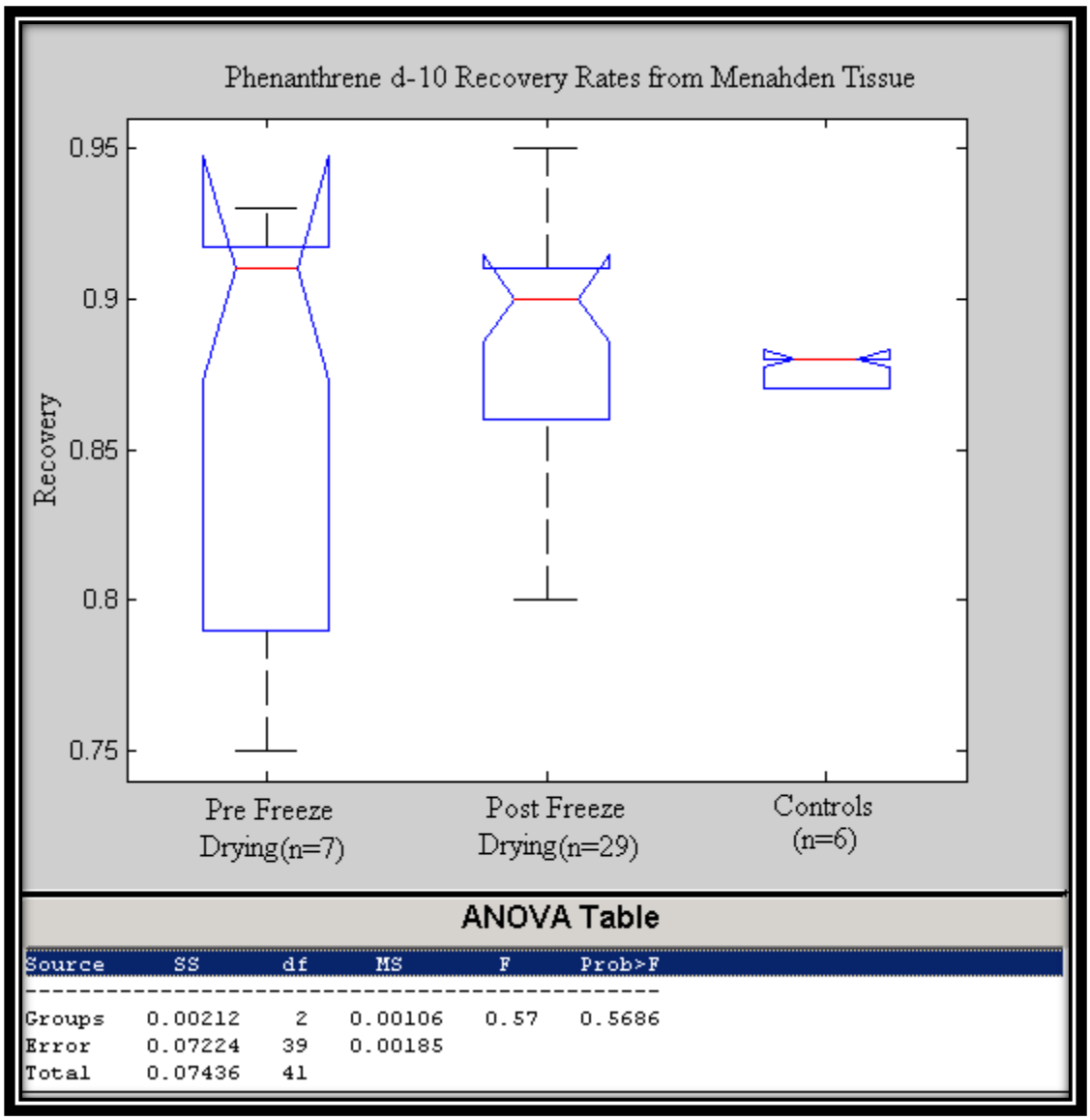


Date	ID	Control for	Mass (g)	Total PAHs (ng/g)	FVOL (mL)	Phenanthrene d-10 Surrogate Recovery	Corrected Total PAHs (ng/g)	Corrected C3-Phenanthrenes	% C3-Phenanthrens
1/23/2012	C1	small	2.870	2461	20	0.88	2806	2759	98.33%
1/23/2012	C2	small	2.849	2580	35	0.88	2940	2893	98.40%
1/23/2012	C3	small	2.858	2792	25	0.88	3162	3116	98.55%
1/23/2012	C4	large	3.470	3502	25	0.87	4030	3983	98.83%
1/23/2012	C5	large	3.460	3655	25	0.88	4140	4093	98.86%
1/23/2012	C6	large	3.434	3407	25	0.87	3929	3882	98.80%

Raw Sampling Menhaden Data

Small								
Date	ID	# of Fish	Wet Mass (g)	Dry Mass (g)	Fork Length (cm)	FVOL (mL)	Sub Sample (g)	Phenanthrene d-10 Surrogate Recovery
7/6/2011	VB88	2	82.3	27.9	12.5/13.1	40	10.0	0.92
7/6/2011	VB89	2	80.5	26.1	12.8/13.4	35	10.0	0.96
7/6/2011	VB90	2	74.3	21.8	12.3/12.6	40	10.0	0.95
7/28/2011	GI91	2	84.1	24.9	13.6/13.0	40	10.0	0.95
7/28/2011	GI92	2	69.8	18.1	12.1/12.0	35	10.0	0.95
7/28/2011	GI93	2	79.1	21.9	13.2/12.5	35	10.0	0.93
8/23/2011	VB25	2	118.3	35.6	14.5/15.0	45.0	35.6	0.93
8/23/2011	VB26	2	111.9	30.8	15.0/14.8	30.0	10.0	0.90
8/23/2011	VB27	2	98.6	26.6	14.5/14.1	25.0	10.0	0.90
8/24/2011	GI28	2	65.5	18.3	12.5/12.9	25.0	10.0	0.89
8/24/2011	GI29	2	67.2	17.1	12.8/12.4	30.0	10.0	0.92
8/24/2011	GI30	2	70.1	18.2	12.9/12.8	35.0	10.0	0.89
9/21/2011	VB46	2	91.9	47.9	13.8/13.1	20.0	10.0	0.88
9/21/2011	VB47	2	93.4	49.6	13.7/13.5	25.0	10.0	0.88
9/21/2011	VB48	2	88.6	46.9	13.4/13.4	20.0	10.0	0.91
9/13/2011	GI43	2	83.1	40.2	13.5/13.6	20.0	10.0	0.89
9/13/2011	GI44	2	86.4	45.0	14.1/13.0	20.0	10.0	0.89
9/13/2011	GI45	2	93.2	49.1	14.1/13.2	25.0	10.0	0.91

Large								
Date	ID	# of Fish	Wet Mass (g)	Dry Mass (g)	Fork Length (cm)	FVOL (mL)	Sub Sample (g)	Phenanthrene d-10 Surrogate Recovery
7/6/2011	VB19	1	127.6	34.2	19.5	50.0	34.2	0.75
7/6/2011	VB20	1	140.6	50.7	19.0	55.0	50.7	0.75
7/6/2011	VB21	1	131.4	48.5	19.6	50.0	48.5	0.92
7/28/2011	GI22	1	107.9	37.0	17.8	80.0	37.0	0.91
7/28/2011	GI23	1	118.0	37.7	18.8	80.0	37.7	0.91
7/28/2011	GI24	1	113.0	37.4	18.3	80.0	37.4	0.91
8/23/2011	VB31	1	92.4	31.7	17.2	30.0	10.0	0.91
8/23/2011	VB32	1	116.8	45.2	18.6	40.0	10.0	0.92
8/23/2011	VB33	1	147.8	64.3	19.6	33.0	10.0	0.95
8/24/2011	GI34	1	124.8	50.6	18.7	25.0	10.0	0.91
8/24/2011	GI35	1	99.8	39.0	18.0	30.0	10.0	0.92
8/24/2011	GI36	1	122.0	50.2	18.4	30.0	10.0	0.90
9/21/2011	VB61	1	125.5	44.6	18.4	50	10.0	0.91
9/21/2011	VB62	1	122.2	45.8	18.4	50	10.0	0.90
9/21/2011	VB63	1	95.7	30.6	16.5	50	10.0	0.90
9/13/2011	GI64	1	117.9	37.1	18.9	40	10.0	0.86
9/13/2011	GI65	1	120.3	49.4	18.1	35	10.0	0.85
9/13/2011	GI66	1	125.7	50.0	18.8	30	10.0	0.92

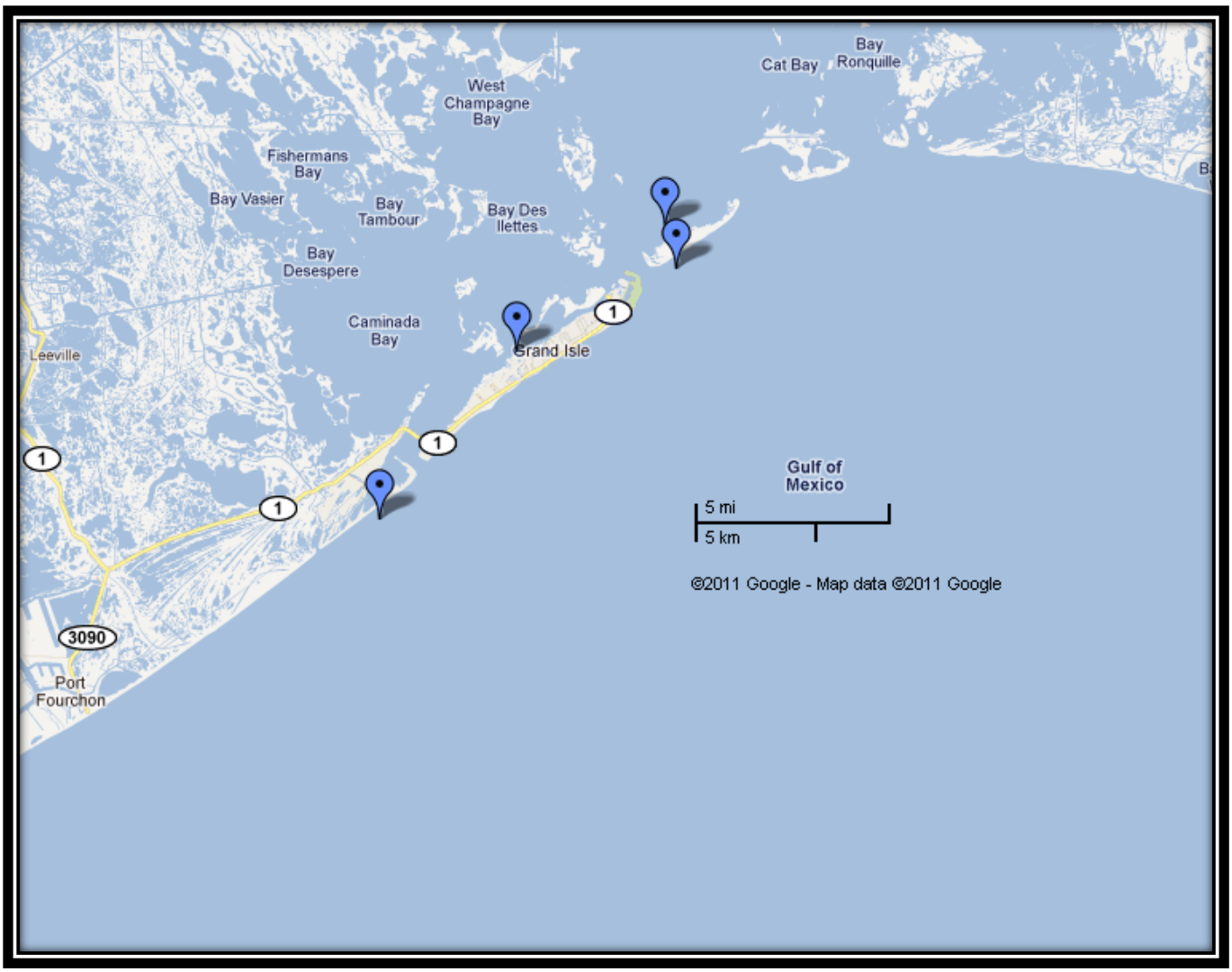


ANOVA Table

Source	SS	df	MS	F	Prob>F
Groups	0.00212	2	0.00106	0.57	0.5686
Error	0.07224	39	0.00185		
Total	0.07436	41			

Sampling Locations and Maps

General Sampling locations for Menahden						
Vermilion Bay				Grand Isle		
vb	29° 33' 30.64"N	92° 1' 1.63"W		gi	29° 17' 48.12"N	89° 41' 47.01"W
vb	29° 34' 54.00"N	92° 5' 36.00"W		gi	29°15'58.27"N	89°56'34.31"W
vb	29° 28' 20.93"N	91° 49' 57.77"W		gi	29° 10' 35.74"N	90° 3' 41.34"W


Bay
Ronquille
Cat Bay
West
Champagne
Bay
Fishermans
Bay
Bay Vasier
Bay
Tambour
Bay Des
Ilettes
Bay
Desespere
Caminada
Bay
Grand Isle
Leeville
1
1
1
1
Gulf of
Mexico
5 mi
5 km
©2011 Google - Map data ©2011 Google
3090
Port
Fourchon

MatLab Code for Statistical Analysis

Phenenthrene d_{10} Recovery

```
pre=[0.75	0.75	0.92	0.91	0.91	0.91	0.93];

post=[0.9	0.9	0.89	0.92	0.89	0.91	0.92	0.95	0.91	0.92
0.9	0.89	0.89	0.91	0.88	0.88	0.91	0.91	0.9	0.9	0.86
0.85	0.92	0.83	0.83	0.84	0.86	0.8	0.85];

control=[0.88	0.88	0.88	0.87	0.88	0.87];

all=[0.75	0.75	0.92	0.91	0.91	0.91	0.93	0.9	0.9	0.89
0.92	0.89	0.91	0.92	0.95	0.91	0.92	0.9	0.89	0.89	0.91
0.88	0.88	0.91	0.91	0.9	0.9	0.86	0.85	0.92	0.83	0.83
0.84	0.86	0.8	0.85	0.88	0.88	0.88	0.87	0.88	0.87];

p=anova1(all,[1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3]);
```

Detailed Protocols

All size menhaden will be extracted in this fashion through the duration of this study. All extractions done with any previous methods have been redone using this protocol.

Purpose

The purpose of this method is to extract Polycyclic Aromatic Hydrocarbons (PAHs) from menhaden tissue.

****

Where applicable all materials including chemicals should be solvent rinsed and then dried before use (Not the C-18 silica). All Plastic components should be only solvent rinsed with Methanol.

****

C-18 Silica Extraction Process

Materials

150-200 ml Beakers
400-600 ml Beakers
100 ml Glass Graduated Cylinder
Spatula

Sonicator
Rotary Evaporator
Büchner flask (250 ml)
Büchner funnel with a sintered glass disc (350 ml)
Filter paper
Blender/Coffee Mill (Sunbeam Heritage Series Kitchen Assistant 2774 or similar item)
500 ml Beaker
Glass Funnel
1 ml Gas Tight Syringe
25 µl Gas Tight Syringe
GC Bottles and Caps
Capping Device

Chemicals

Dichloromethane (DCM)
Hexane
Methanol
C-18 Silica
Sodium Sulfate

Washing

All materials should be washed using the following method.

Soak in hot soapy water over night.
Wash with hot soapy water again.
Rinse with hot water 3 times (if the item holds liquids fill to the top 3 times)
Rinse with DI water 3 times (if the item holds liquids fill to the top 3 times)
Rinse with methanol to remove water.
Rinse with DCM and allow the item to flash in a hood.
Bake in a vented oven until completely dry.
Cover any open areas with aluminum foil until use.

The glass wool and the sodium sulfate need to be solvent rinsed. Do this by placing an amount of the wool or sodium sulfate in a beaker (250 ml is fine, but this is up to you) then rinse with DCM. Allow this to flash in the hood overnight and then remove to a vented oven and bake until dry. Cover with aluminum foil and store until needed.

Pasture Pipettes and the Graduated Syringes should be rinsed as per step 9A and 9B below.

Procedure

1. Take frozen menhaden and cut them into pieces. Arrange them into a labeled beaker and using a glass pestle gently compress the menhaden pieces into the beaker. Make a note as to how many organisms were used for the sample. Cover with foil and puncture two to three holes into the top.

2. Cool sample to -60°C or lower, then place in a freeze dryer for 36-48 hours. Remove and store in a dessicator until ready for step 3.

3. Homogenize the freeze dried material until all of the tissue has been evenly distributed. Remove a 10 g subsample of the tissue back into the grinder and add a 1:1 ratio dry weight C-18 Silica. Homogenize further until the material is powdery and well broken down. Add an amount of sodium sulfate to cover the top of the contents in the beaker.Do not blend the sodium sulfate, simply mix with a spatula until evenly dispersed. (Between samples rinse the blender/coffee mill with water, then apply methanol to a cloth or paper towel and wipe the blades and container and then allow it to dry under the hood.

4. Fill the beaker with DCM until the tissue is covered, then using the solvent rinsed spatula mix thoroughly. Sonicate for 20 minutes. After sonication, use the spatula to again mix thoroughly.

5. Using a Büchner flask (attached to a vacuum) with a Büchner funnel (with a sintered glass disc) filter the tissue extract into the flask. Once the flask is full remove the contents to a labeled flat bottom flask.

6. Evaporate the extract in a rotary evaporator until there is no DCM left. Transfer the extract to a graduated cylinder using hexanes and rinse the flat bottom flask with hexanes into the graduated cylinder for final volume measurement three times (This allows for the hexane exchange). It may be necessary to reconstitute the material with Hexane first before the contents are transferred.

A. If the material within the flat bottom flask is minimal then the contents should be transferred to a 15 ml graduated concentrator tube for final volume measurement (7A).

7. Using Hexane, dilute the recovered extract to a whole number volume. Mix thoroughly with a solvent rinsed pasture pipette. Make a final volume measurement and record in ml. Transfer 20 ml of the resulting material using a pasture pipette to a volatile organic analysis (VOA) bottle for storage. Allow to settle for 24 hours.

A. Using the graduated concentrator tube:

Attach a Snyder column to the concentrator tube and heat in a water bath until you are left with 1 ml of extract. If the material seems to be rather dark in coloration you will need to dilute with pure hexane (this will always be the case with menhaden).

i. <u>Dilute the sample:</u>

Use the graduated concentrator tube to measure the volume of added hexane. Fill the tube to 10 ml using pure hexanes and then transfer to the previously used VOA bottle associated with this sample. Remember to rinse this VOA bottle with hexane 3 times before making the transfer. Fill the graduated concentrator tube

back to the 10 ml mark again and then transfer this amount to the VOA bottle. There is now a final volume of 20 ml which can then be sampled to run on the GC/MS. This final volume will be required to interpret the data.

step 8 is only followed if the sample is relatively clear, otherwise follow Step 9 for dilution and simply remove 1 ml as needed to be analyzed on the GC/MS

8. It is important to concentrate to 1 ml or less (this is for samples that will not be diluted). If you use the 1 ml syringe to remove all the liquid and there is more than 1 ml in the syringe place the liquid back in the concentrator tube and continue to evaporate until there is 1 ml or less. If there is exactly 1 ml, then transfer to a GC bottle and then add the internal standard (this will usually be done with the graduated 25µl syringe). If the volume is less than 1 ml, pull an amount of clean hexane into the syringe that will give a final volume of 1 ml.

9. If you are pulling from a diluted sample simply remove 1 ml from the 20 ml dilution and place in the GC bottle. Once this is completed, cap the GC bottle and store at 4°C until step 10.

A. Cleaning the syringe and adding "clean" hexane:

It is important to have two VOA bottles marked clean hexanes and waste. Fill the VOA bottle labeled clean hexane with clean hexanes. Any time that you need to add hexanes to a sample as mentioned above use this volume of hexanes. Remember to rinse the needle of the syringe with hexane before placing it into the "clean" hexanes bottle. If you simply want to clean the syringe, rinse the needle and then draw in a full amount of hexanes. Expel this into the VOA bottle marked waste. Repeat this process 3 times. This can be done when working with DCM as well. Simply follow the steps, but instead of hexanes use DCM.

B. Preparing the syringe:

Once the syringe has been cleaned as stated above (9A) it is important to remove the possible dilution factor of residual Hexane or DCM left in the syringe. This can be done by simply drawing in a small amount of the liquid to be transferred with the syringe and then drawing that liquid back and forth into the syringe several times. Discard the amount of liquid drawn into the syringe in the usual manner. Repeat as needed.

10. When ready run the samples on a GC/MS.

Surrogates and Standards

There will be three sets of standards used during the process of tissue extraction and analysis. The primary standard is the **surrogate spike solution** added to the tissue at the beginning of the extraction process. This is simply a deuterated PAH solution of known concentration added to the initial processes of both protocols. These standards can be obtained from Supelco pre mixed. They can then be further diluted to fit within the analytical range of the GC being used. The secondary standard will be the **GC/MS internal standard solution**. This again is a mixture of deuterated PAHs at varying molecular weights used to maintain the validity of the instrument. The last standard will be the **calibration curve standards** passed through the MS to verify that the MS is in a fully functional state.

To address the non-tissue experimental designs (fish oil):
The surrogate spike solution will be added to the portions within the round bottom centrifuge tube and then macerated and homogenized with the meat and/or skin. This will take the place of the addition of the surrogate at the beginning of the tissue extraction processes discussed previously.

Surrogate Spike Solution:

1. 1.0 ml of 5-alpha Androstane at 10 mg/ml (dissolved in DCM) is added to 500 ml DCM in a 500 ml volumetric flask.

2. Mass 0.0100 g (10 mg) of Phenanthrene - d10 (neat) and add to the 500 ml DCM.

3. Allow time for the Phenanthrene - d10 to dissolve.

Final Volume = 500 ml
Final Concentration = 20 mg/ml

Store in aliquots determined by need using amber glass.

This surrogate is added to each sample at 1 ml. per sample extracted.

GC/MS Internal Standard Solution:

1. Add 1 ml of the following to a 5 ml amber vial
Napthalene - d8 at 4.0 mg/ml in DCM
Acenapthene - d10 at 4.0 mg/ml in DCM
Chrysene - d12 at 4.0 mg/ml in DCM
Perylene - d12 at 4.0 mg/ml in DCM

Final Volume = 4.0 ml
Final Concentration = 1000 mg/ml

This internal standard is added at 10µl to each GC bottle.

Calibration Curve Standard:

- Surrogate Spike for Calibration Standards
Add 3.0 ml of DCM to an 8 ml amber vial.
Add 1.0 ml of 5-alpha Androstane at 1000 µg/ml in DCM to the 8 ml amber vial.
Add 1.0 ml of Phenanthrene - d10 at 1000 µg/ml in DCM to the 8 ml amber vial.

Final Volume = 5.0 ml
Final Concentration = 200 µg/ml

- Oil Analysis Standard (44 oil constituents) 100µg/ml in Hexane/DCM (9:1)
Order from http://www.absolutestandards.com/ Absolute Standards part # 90311

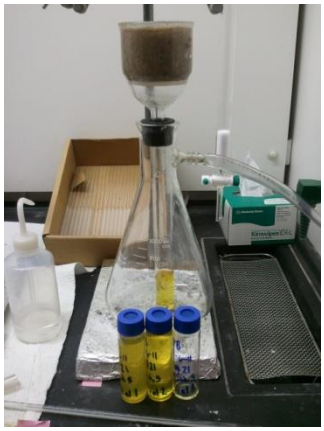
0.5 ppm = 10.0µl Oil Analysis Standard 5.0µl Surrogate Spike for Calibration Standard	in 1.985 ml DCM Final Volume = 2.0 ml
1.0 ppm = 20.0µl Oil Analysis Standard 10.0µl Surrogate Spike for Calibration Standard	in 1.97 ml DCM Final Volume = 2.0 ml
5.0 ppm = 100µl Oil Analysis Standard 50µl Surrogate Spike for Calibration Standard	in 1.85 ml DCM Final Volume = 2.0 ml
10.0 ppm = 200µl Oil Analysis Standard 100µl Surrogate Spike for Calibration Standard	in 1.70 ml DCM Final Volume = 2.0 ml
25.0 ppm = 500µl Oil Analysis Standard 250µl Surrogate Spike for Calibration Standard	in 1.25 ml DCM Final Volume = 2.0 ml

These are placed in a GC bottle that has been adapted to hold 0.2 ml.

The final ppm will depend on the range you set the GC/MS for the sample. Any of these will be fine as long as you make a note as to which one used for that particular sample run.

The Calibration Standard is placed in the GC and is processed along with the extracted samples.

****



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C: Chapter 3

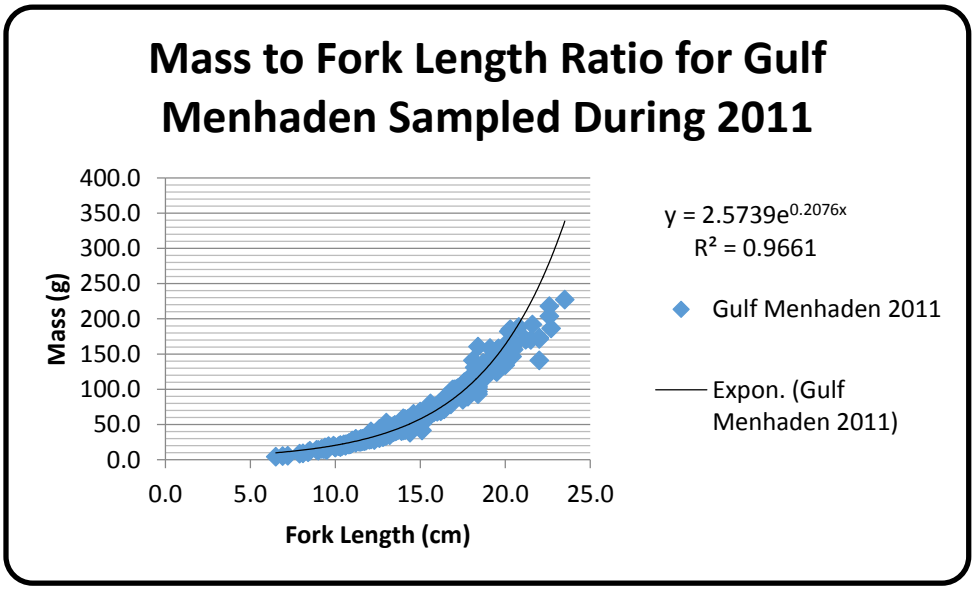
Data Tables and Graphs

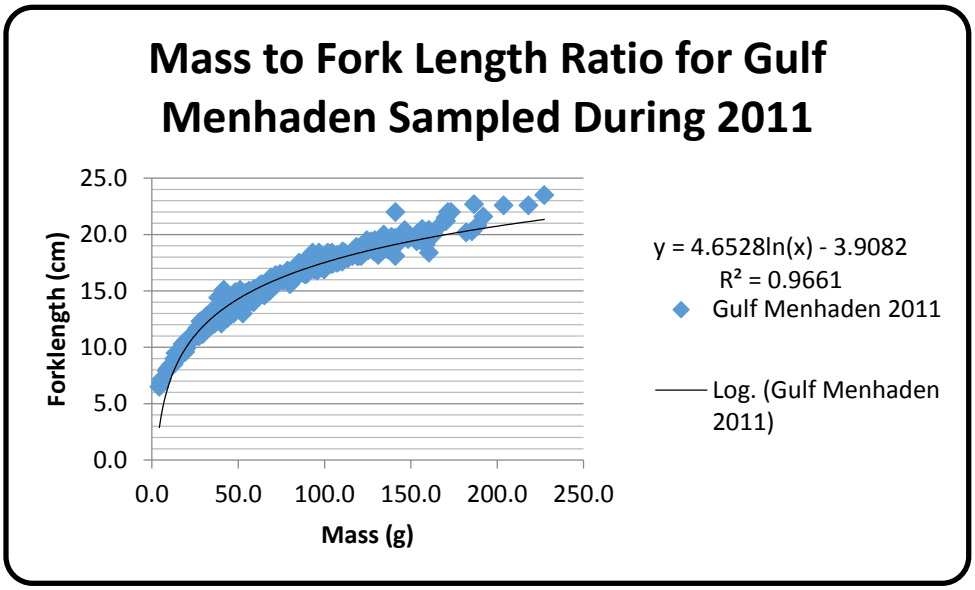
Raw Menhaden Sampling Data

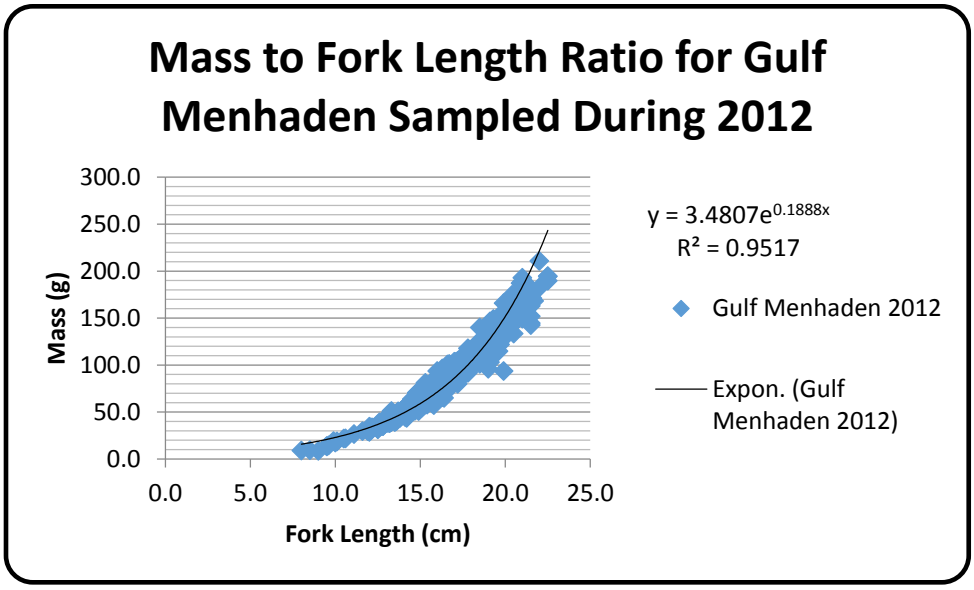
Sample	Site	Size	Wet Mass(g)	Forklength(cm)	Dry Mass(g)	Moisture%	Fat(mg)/Dry Tissue(g)	Fat(mg)/Wet Tissue(g)	Fat % whole fish	PAH(ng)/Dry Tissue(g)	TEQ(ng)/Dry Tissue(g)	MEQ(ng)/Dry Tissue(g)	Month	Year
VB19	vb	lg	127.6	19.5	34.2	73.2	352.17	127.36	12.74	11438	2.8	3.9	jul	2011
VB20	vb	lg	140.6	19.0	50.7	63.9	395.91	157.20	15.72	6233	2.1	3.3	jul	2011
VB21	vb	lg	131.4	19.6	48.5	63.1	494.60	197.07	19.71	7525	76.5	17.3	jul	2011
GI22	gi	lg	107.9	17.8	37.0	65.7	388.21	156.05	15.60	8988	129.2	14.9	jul	2011
GI23	gi	lg	118.0	18.8	37.7	68.1	454.92	186.55	18.66	9320	3.7	4.9	jul	2011
GI 24	gi	lg	113.0	18.3	37.4	66.9	438.26	168.53	16.85	9154	66.4	9.9	jul	2011
VB25	vb	sm	118.3	14.5/15.0	35.6	69.9	97.22	33.73	3.37	6245	2.8	0.0	aug	2011
VB26	vb	sm	111.9	15.0/14.8	30.8	72.5	26.32	8.93	0.89	6458	2.1	0.5	aug	2011
VB27	vb	sm	98.6	14.5/14.1	26.6	73.0	70.06	24.55	2.46	13041	76.5	0.0	aug	2011
GI28	gi	sm	65.5	12.5/12.9	18.3	72.1	27.97	9.88	0.99	10132	129.2	0.0	aug	2011
GI29	gi	sm	67.2	12.8/12.4	17.1	74.6	45.98	16.19	1.62	7227	3.7	0.0	aug	2011
GI30	gi	sm	70.1	12.9/12.8	18.2	74.0	58.39	19.80	1.98	7007	66.4	0.0	aug	2011
VB31	vb	lg	92.4	17.2	31.7	65.7	198.76	54.51	5.45	2517	468.1	155.7	aug	2011
VB32	vb	lg	116.8	18.6	45.2	61.3	198.86	54.69	5.47	2422	369.7	103.6	aug	2011
VB33	vb	lg	147.8	19.6	64.3	56.5	167.66	45.45	4.55	3413	252.5	44.2	aug	2011
GI34	gi	lg	124.8	18.7	50.6	59.5	198.68	57.69	5.77	3229	137.0	13.5	aug	2011
GI35	gi	lg	99.8	18.0	39.0	60.9	136.36	35.29	3.53	1892	164.7	9.5	aug	2011
GI36	gi	lg	122.0	18.4	50.2	58.9	76.92	18.60	1.86	2666	155.1	9.0	aug	2011
GI43	gi	sm	83.1	13.5/13.6	40.2	51.6	277.51	125.68	12.57	2860	104.0	6.0	sept	2011
GI44	gi	sm	86.4	14.1/13.0	45.0	47.9	327.09	162.85	16.28	4429	0.2	0.0	sept	2011
GI45	gi	sm	93.2	14.1/13.2	49.1	47.3	232.67	107.18	10.72	2982	0.2	0.0	sept	2011
VB46	vb	sm	91.9	13.8/13.1	47.9	47.9	362.04	160.73	16.07	3672	0.1	0.0	sept	2011
VB47	vb	sm	93.4	13.7/13.5	49.6	46.9	382.81	188.28	18.83	4838	0.0	0.0	sept	2011
VB48	vb	sm	88.6	13.4/13.4	46.9	47.1	374.27	158.22	15.82	5877	0.2	0.0	sept	2011
VB61	vb	lg	125.5	18.4	44.6	64.5	116.79	28.88	2.89	13128	737.7	161.9	sept	2011
VB62	vb	lg	122.2	18.4	45.8	62.5	300.00	91.28	9.13	20523	7.6	13.6	sept	2011
VB63	vb	lg	95.7	16.5	30.6	68.0	198.41	50.30	5.03	13038	0.2	0.0	sept	2011
GI64	gi	lg	117.9	18.9	37.1	68.5	133.33	34.31	3.43	8438	0.3	0.0	sept	2011
GI65	gi	lg	120.3	18.1	49.4	58.9	128.44	31.67	3.17	10216	0.4	0.0	sept	2011
GI66	gi	lg	125.7	18.8	50.0	60.2	188.03	50.23	5.02	12907	0.2	0.0	sept	2011
VB73	vb	sm	86.2	13.8/13.4	28.1	67.4	418.99	159.30	15.93	6728	0.2	0.0	oct	2011
VB74	vb	sm	84.1	13.4/13.2	28.9	65.6	387.28	141.05	14.11	5685	0.3	0.0	oct	2011
VB75	vb	sm	84.0	13.3/13.2	27.7	67.0	294.85	107.39	10.74	6326	0.3	0.0	oct	2011
GI76	gi	sm	68.4	12.4/12.1	18.9	72.4	434.67	151.77	15.18	2306	0.1	0.0	oct	2011
GI77	gi	sm	72.7	12.7/12.8	20.5	71.8	498.80	189.95	19.00	1890	0.1	0.0	oct	2011
GI78	gi	sm	67.4	12.3/12.3	18.9	72.0	444.44	159.29	15.93	2210	0.1	0.0	oct	2011
VB88	vb	sm	82.3	12.5/13.1	27.9	66.1	200.00	78.52	7.85	5283	0.0	0.0	jul	2011
VB89	vb	sm	80.5	12.8/13.4	26.1	67.6	209.30	75.31	7.53	1901	0.0	0.0	jul	2011
VB90	vb	sm	74.3	12.3/12.6	21.8	70.7	31.01	9.59	0.96	5040	0.0	0.0	jul	2011
GI91	gi	sm	84.1	13.6/13.0	24.9	70.4	71.75	27.83	2.78	2952	0.0	0.0	jul	2011
GI92	gi	sm	69.8	12.1/12.0	18.1	74.1	76.92	29.30	2.93	1916	0.0	0.0	jul	2011
[illegible]														
1	vb	sm	75.6	15.7	22.0	70.9	0.280	0.081	8.148	5084	0.27	0.00	Sept	2012
2	vb	sm	43.7	14.2	11.1	74.6	0.182	0.046	4.618	6711	0.55	0.00	Sept	2012
3	vb	sm	82.1	15.9	24.8	69.8	0.320	0.097	9.686	5162	0.35	0.00	Sept	2012
4	gi	sm	72.5	15.5	20.9	71.2	0.222	0.064	6.406	4173	0.27	0.00	Sept	2012
5	gi	sm	75.5	15.7	21.8	71.1	0.229	0.066	6.617	4520	0.21	0.00	Sept	2012
6	gi	sm	75.4	15.2	22.5	70.2	0.273	0.081	8.138	6565	0.22	0.00	Sept	2012
7	vb	lg	139.9	18.5	47.2	66.3	0.423	0.143	14.274	3969	0.15	0.00	Sept	2012
8	vb	lg	113.5	18.5	39.8	64.9	0.404	0.142	14.163	6674	0.12	0.00	Sept	2012
9	vb	lg	125.3	19.1	38.6	69.2	0.301	0.093	9.272	4800	0.17	0.00	Sept	2012
10	gi	lg	117.4	18.7	37.3	68.2	0.220	0.070	6.990	3757	0.10	0.00	Sept	2012
11	gi	lg	130.7	19.3	44.0	66.3	0.363	0.122	12.212	4816	0.13	0.00	Sept	2012
12	gi	lg	174.8	20.4	63.2	63.8	0.416	0.150	15.035	4054	0.22	0.00	Sept	2012
13	vb	lg	156.3	20.0	48.2	69.2	0.270	0.083	8.326	7795	0.42	0.00	Aug	2012
14	vb	lg	109.3	17.6	34.8	68.2	0.300	0.096	9.552	5881	0.00	0.00	Aug	2012
15	vb	lg	95.5	17.1	30.1	68.5	0.290	0.091	9.140	6175	1.93	3.28	Aug	2012
16	gi	lg	65.2	16.0	22.2	66.0	0.320	0.109	10.896	9990	0.30	0.00	Aug	2012
17	gi	lg	66.4	16.1	21.2	68.1	0.250	0.080	7.982	7189	0.00	0.00	Aug	2012
18	gi	lg	65.3	16.0	21.6	66.9	0.320	0.106	10.585	9371	1.93	3.27	Aug	2012
19	vb	sm	48.9	14.1	11.9	75.7	0.020	0.005	0.487	3986	0.15	0.00	Aug	2012
20	vb	sm	66.6	15.2	19.3	71.0	0.160	0.046	4.637	4935	0.23	0.00	Aug	2012
21	vb	sm	52.9	14.5	14.9	71.8	0.200	0.056	5.633	4643	0.00	0.00	Aug	2012
22	gi	sm	53.0	14.7	17.0	67.9	0.300	0.096	9.623	3586	0.00	0.00	Aug	2012
23	gi	sm	57.7	14.9	19.0	67.1	0.330	0.109	10.867	4153	0.16	0.00	Aug	2012
24	gi	sm	49.5	14.0	16.4	66.9	0.300	0.099	9.939	3494	0.12	0.00	Aug	2012
25	vb	sm	46.1	13.8	11.7	74.6	0.050	0.013	1.269	7800	1.58	0.00	Jul	2012
26	vb	sm	59.6	15.0	13.6	77.2	0.030	0.007	0.685	5416	0.27	0.00	Jul	2012
27	vb	sm	51.7	14.7	11.6	77.6	0.020	0.004	0.449	4467	0.25	0.00	Jul	2012
28	gi	sm	55.7	14.8	17.4	68.8	0.130	0.041	4.061	7766	0.37	0.00	Jul	2012
29	gi	sm	53.3	14.6	15.2	71.5	0.100	0.029	2.852	8763	0.44	0.00	Jul	2012
30	gi	sm	65.7	15.4	20.8	68.3	0.150	0.047	4.749	9966	0.44	0.00	Jul	2012
31	vb	lg	158.3	21.0	42.9	72.9	0.080	0.022	2.168	3470	0.16	0.00	Jul	2012
32	vb	lg	109.3	18.6	32.0	70.7	0.150	0.044	4.392	4378	0.30	0.00	Jul	2012
33	vb	lg	101.3	18.5	31.0	69.4	0.130	0.040	3.978	5360	0.00	0.00	Jul	2012
34	gi	lg	170.5	20.9	55.4	67.5	0.140	0.045	4.549	4020	0.17	0.00	Jul	2012
35	gi	lg	130.7	19.5	39.3	69.9	0.150	0.045	4.510	3825	0.16	0.00	Jul	2012
36	gi	lg	124.2	18.4	42.7	65.6	0.190	0.065	6.532	4350	0.19	0.00	Jul	2012

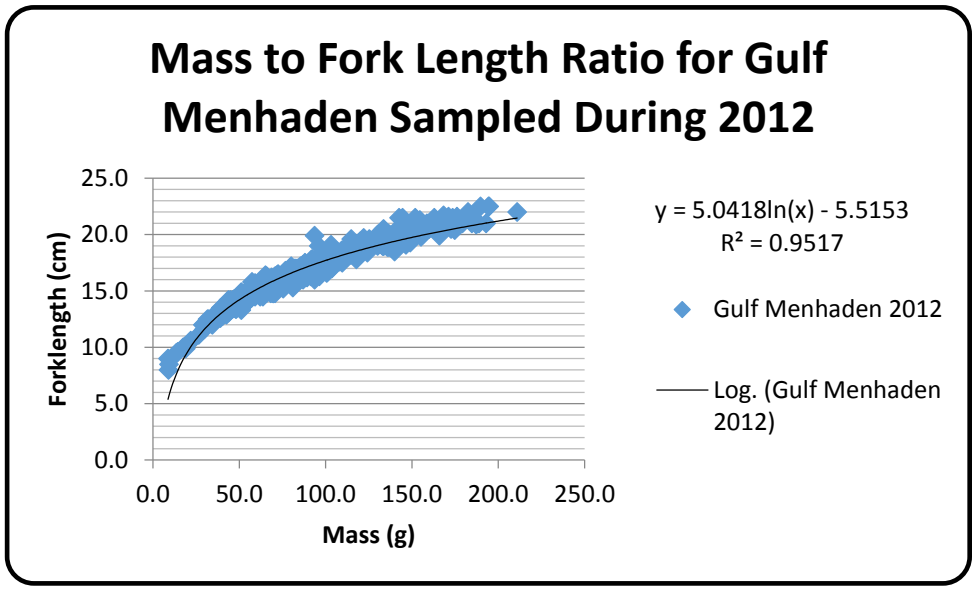
Sample	Site	Size	Wet Mass(g)	Forklength(cm)	Dry Mass(g)	Moisture%	Fat(g)/Dry Tissue(g)	Fat(g)/Wet Tissue(g)	Fat % whole fish	PAH(ng)/Dry Tissue(g)	TEQ(ng)/Dry Tissue(g)	MEQ(ng)/Dry Tissue(g)	Month	Year
VB13Jul1	vb	lg	126.4	17.9	49.0	61.2	0.470	0.182	18.220	6,231	0.68	0.00	jul	2013
VB13Jul2	vb	lg	130.3	19.3	48.1	63.1	0.529	0.195	19.528	5,740	1.21	0.00	jul	2013
VB13Jul3	vb	lg	97.5	17.1	36.2	62.9	0.517	0.192	19.195	4,258	1.49	0.00	jul	2013
VB13Jul4	vb	sm	45.6	14.6	11.0	75.9	0.082	0.020	1.978	3,698	0.37	0.00	jul	2013
VB13Jul5	vb	sm	95.8	15.9	33.4	65.1	0.437	0.152	15.236	5,900	0.88	0.00	jul	2013
VB13Jul6	vb	sm	40.1	13.2	11.9	70.3	0.230	0.068	6.825	5,198	0.87	0.00	jul	2013
GI13Jul1	gi	lg	157.0	19.2	68.3	56.5	0.572	0.249	24.884	4,506	0.52	0.00	jul	2013
GI13Jul2	gi	lg	101.1	17.4	37.4	63.0	0.504	0.186	18.645	5,631	0.76	0.00	jul	2013
GI13Jul3	gi	lg	117.9	17.3	48.8	58.6	0.542	0.224	22.434	5,270	0.85	0.00	jul	2013
GI13Jul4	gi	sm	73.8	15.6	24.0	67.5	0.385	0.125	12.520	5,477	0.71	0.00	jul	2013
GI13Jul5	gi	sm	38.7	13.0	9.6	75.2	0.100	0.025	2.481	5,692	0.83	0.00	jul	2013
GI13Jul6	gi	sm	31.3	11.9	9.2	70.6	0.212	0.062	6.231	4,253	0.37	0.00	jul	2013
VB1Aug2013	vb	lg	195.6	20.4	83.3	57.4	0.592	0.252	25.211	3,346	0.74	0.00	aug	2013
VB2Aug2013	vb	lg	179.0	20.6	74.5	58.4	0.630	0.262	26.221	4,192	1.02	0.00	aug	2013
VB3Aug2013	vb	lg	196.3	20.5	85.0	56.7	0.614	0.266	26.587	3,228	1.12	0.86	aug	2013
VB4Aug2013	vb	sm	138.7	17.9	56.8	59.0	0.591	0.242	24.202	3,152	1.04	0.45	aug	2013
VB5Aug2013	vb	sm	100.8	16.5	34.9	65.4	0.771	0.267	26.694	2,874	1.02	0.61	aug	2013
VB6Aug2013	vb	sm	88.6	16.3	32.6	63.2	0.979	0.360	36.022	3,479	6.46	5.26	aug	2013
GI1Aug2013	gi	lg	83.6	16.1	28.1	66.4	0.447	0.150	15.025	2,252	1.10	0.82	aug	2013
GI2Aug2013	gi	lg	78.2	16.0	26.0	66.8	0.414	0.138	13.765	3,581	1.14	0.66	aug	2013
GI3Aug2013	gi	lg	86.3	16.2	29.1	66.3	0.423	0.143	14.263	3,186	1.23	0.67	aug	2013
GI4Aug2013	gi	sm	70.6	15.4	22.1	68.7	0.382	0.120	11.958	2,038	0.99	0.39	aug	2013
GI5Aug2013	gi	sm	53.0	14.2	14.3	73.0	0.220	0.059	5.936	2,896	1.11	0.86	aug	2013
GI6Aug2013	gi	sm	72.4	15.8	21.4	70.4	0.496	0.147	14.661	3,358	1.30	0.83	aug	2013
vbsept1	vb	lg	186.7	21.6	78.3	58.1	0.427	0.179	17.908	2,874	389.72	84.92	sept	2013
vbsept2	vb	lg	177.6	20.3	82.3	53.7	0.479	0.222	22.197	2,859	2.61	1.30	sept	2013
vbsept3	vb	lg	151.7	18.6	71.1	53.1	0.574	0.269	26.903	3,437	3.34	2.27	sept	2013
vbsept4	vb	sm	95.0	16.4	37.5	60.5	0.640	0.253	25.263	4,463	4.03	2.58	sept	2013
vbsept5	vb	sm	106.8	17.4	43.6	59.2	0.540	0.220	22.045	3,626	2.52	1.67	sept	2013
vbsept6	vb	sm	129.8	17.9	58.3	55.1	0.528	0.237	23.715	4,880	9.40	7.05	sept	2013
gisept1	gi	lg	175.4	20.2	78.8	55.1	0.505	0.227	22.688	1,596	0.99	0.69	sept	2013
gisept2	gi	lg	118.1	17.6	47.3	59.9	0.481	0.193	19.264	1,456	1.01	0.78	sept	2013
gisept3	gi	lg	115.6	18.4	48.8	57.8	0.533	0.225	22.500	1,976	4.96	3.84	sept	2013
gisept4	gi	sm	75.1	15.5	23.6	68.6	0.341	0.107	10.716	1,916	2.13	1.41	sept	2013
gisept5	gi	sm	54.7	14.5	17.0	68.9	0.346	0.108	10.753	1,595	1.10	0.78	sept	2013
gisept6	gi	sm	39.4	12.7	10.3	73.9	0.134	0.035	3.503	2,053	0.79	0.50	sept	2013

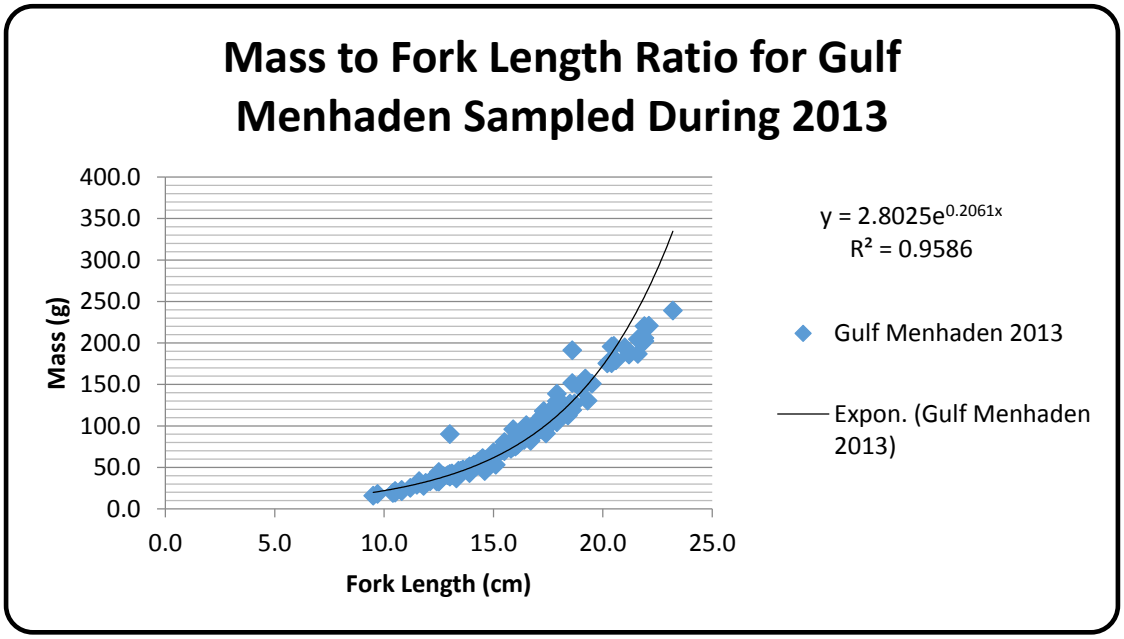
Menhaden Population Dynamics

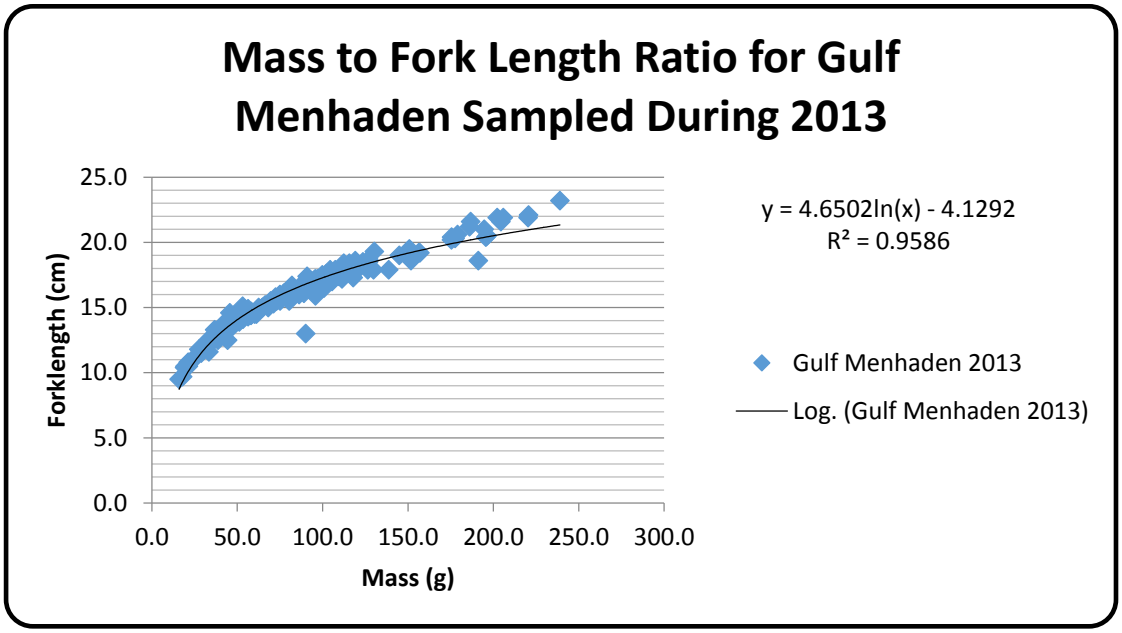



Mass to Fork Length Ratio for Gulf Menhaden Sampled During 2011
Forklength (cm)
25.0
20.0
15.0
10.0
5.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
Mass (g)
y = 4.6528ln(x) - 3.9082
R² = 0.9661
Gulf Menhaden 2011
Log. (Gulf Menhaden 2011)


Mass to Fork Length Ratio for Gulf Menhaden Sampled During 2012
Mass (g)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
Fork Length (cm)
y = 3.4807e0.1888x
R² = 0.9517
Gulf Menhaden 2012
Expon. (Gulf Menhaden 2012)


Mass to Fork Length Ratio for Gulf Menhaden Sampled During 2012
Forklength (cm)
25.0
20.0
15.0
10.0
5.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
Mass (g)
y = 5.0418ln(x) - 5.5153
R² = 0.9517
Gulf Menhaden 2012
Log. (Gulf Menhaden 2012)


Mass to Fork Length Ratio for Gulf Menhaden Sampled During 2013
Mass (g)
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
Fork Length (cm)
y = 2.8025e0.2061x
R² = 0.9586
Gulf Menhaden 2013
Expon. (Gulf Menhaden 2013)



SAS Code for Statistical Analysis

PDMIX800:

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/*************************************************************************
PDMIX800     08/08/2003  slice correction, handles groups with one mean;
             03/26/2002  error in by processing;
             10/18/2001  printing changed again, turned off log notes;
             06/08/2001  bug in slice and printing modified;
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************************************************************/

**** PDMIX800, for SAS Version 8 ******;

/*
ORIGINAL REFERENCE:
Saxton, A.M.  1998.  A macro for converting mean separation output to letter
groupings in Proc Mixed.  In Proc. 23rd SAS Users Group Intl., SAS Institute,
Cary, NC, pp1243-1246.

PURPOSE:
This macro takes two data sets from Proc MIXED (Version 8), created by the
 DIFFS option on the LSMEANS statement. If an ADJUST= option is used,
the pdiffs from this are used, not the unadjusted defaults.
The pdiffs are converted to groups, labeled by numbers, and this
is merged onto the lsmeans data set.
The numbers are converted to letters, and for cases where more than
26 letters are needed, sections of letters are coded.  For example,
3 means might have the letters A, (2)A, and (3)A.  These 3 means
are all different, because although all have the letter A, each A
belongs to a different section, identified by (#).
CAUTIONS!!!!!!!
 Depends on computer using ASCII characters, with 32=blank and capital
 letters following this.
 Requires temporary SAS datasets MSGRPZZ, LSDVALZZ, PDTEMPZZ, PDTEMPZZZ,
PDTEMPMZZ,
   so any existing SAS dataset with these names will be destroyed.
 There may be an IML limit of 90 total characters in the group
  letter labels, but space for 200 are hardcoded.
 Since SAS/IML is used, this must be installed on the computer, along
  with BASE and STAT.

Parameters.
 -First required parameter must name a dataset created by
  ODS OUTPUT DIFFS in proc mixed;
```

-Second required parameter must name a dataset created by
ODS OUTPUT LSMEANS in proc mixed;
-Optional parameters, given in any order, case insensitive.
SORT=YES - printing of means is in order of least squares mean
value. Any value other than YES leaves means in
the proc mixed sort order.
ALPHA=.05 - critical probability value for deciding if means
differ or not. The default is .05, and values must
be between 0 and 1.
WORKSIZE=1 - number of Kb of memory for IML to use. This should
only be needed in very extreme circumstances as IML
dynamically increases memory as needed.
TEST0=YES - this requests that 3 variables (df, t, p) be
included in the printing. Any value other than NO
prints all variables produced by the lsmeans.
MIXFMT=NO - this removes the formatting assigned by proc mixed,
which helps compress the page width of the output.
This also will result in the means and std. errors
being rounded, which usually is desirable. Any value
besides NO retains the proc mixed formatting.
NUMLET=200 - This specifies maximum number of letters that will
be permitted. Many means may possibly require many
letters, but memory requirements get excessive. The
default of 200 should fail only in unusual cases. If
failure occurs (error message in log), rerun with this
option set higher.
SLICE=variables Effects containing all the slice variables will
be subdivided, and mean separation reporting done within
slice levels. Note that all comparisons are made, just
reporting of comparisons across slice levels is suppressed.
This is useful to reduce the complexity of letter groupings.

Example of use.
Assume the file pdmix800.sas, containing the macro code,
is on the a: drive. Then the code below will run MIXED, and run
pdmix800 on the lsmeans. MIXED is told not to print the means and
pdiffs, using the ODS exclude statement, as
pdmix800 does the printing in the more desirable format.
Also shown are two optional parameters.

```
proc mixed;
 class block a b;
 model y = a b a*b;
 random block;
 lsmeans a b a*b/pdiff;
 ods output diffs=ppp lsmeans=mmm;
```

```
 ods listing exclude diffs lsmeans;
run;
%include 'a:pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.01,sort=yes);

*************************************************************/




*************************************************************************;
%macro pdmix800(pname,lname,sort=NO,alpha=.05,worksize=1,test0=NO,
            mixfmt=YES,numlet=200,slice=);
/*************************************************************
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*************************************************************/
%let printdebug=0; **this does not turn on debug printing within IML;

*** check arguments;
%global bylistzz slicezz varlistzz;   **put out for possible use by backtrans;
%let slicezz=&slice;
%local dsid chk3 error1 error neweffectlength lastslicevar var adjust bylist
      printdebug;
  %let error=0;
```

```
 %if %length(&lname)=0 %then %let error=1;
 %if  %sysfunc(exist(&lname)) %then %do;
   %let dsid=%sysfunc(open(&lname,I));
  %let chk3=%sysfunc(varnum(&dsid,ESTIMATE));
  %if &chk3=0 %then %let error=2;
  %let chk3=%sysfunc(varnum(&dsid,EFFECT));
  %if &chk3=0 %then %let error=2;
  %let dsid=%sysfunc(close(&dsid));
 %end;
 %else %let error=1;

 %if &error>0 %then %do;
  %if &error=1 %then %put WARNING: Dataset &lname does not exist.;
  %if &error=2 %then %put WARNING: Dataset &lname was not made by proc mixed.;
 %end;
 %let error1=&error;

 %let error=0;
 %if %length(&pname)=0 %then %let error=1;
 %if %sysfunc(exist(&pname)) %then %do;
  %let dsid=%sysfunc(open(&pname,I));
  %let chk3=%sysfunc(varnum(&dsid,ESTIMATE));
  %if &chk3=0 %then %let error=3;
  %let chk3=%sysfunc(attrn(&dsid,nobs));
  %if &chk3=0 %then %let error=2;
  %let dsid=%sysfunc(close(&dsid));
 %end;
 %else %let error=1;

 %if &error>0 %then %do;
  %if &error=1 %then %put WARNING: Dataset &pname does not exist.;
  %if &error=2 %then %put WARNING: There are no observations in dataset &pname.;
  %if &error=3 %then %put WARNING: Dataset &pname was not made by proc mixed.;
 %end;
 %if (&error or &error1) %then %do;
  %put NOTE: PDMIX800 terminated due to errors in input values.;
  %goto skip;
 %end;

%if &error %then %do;
  %put PDMIX800 terminated due to errors in input values.;
  %if &error=3 %then %put Alpha can only have values between 0 and 1.;
  %if &error=4 %then %put ADJUST=Dunnett output not supported.;
  %goto skip;
%end;
** save setting of notes option;
```

```
%let notesval=notes;
options nonotes;
%put PDMIX800 08.08.2003 processing;

****need list of variable names, either sliced or not;
data _null_;
 *** First get unique list of all names used in BY statements;
 *** these come before the variable EFFECT, but include EFFECT in list;
 dsid=open("&lname",'i');
 length namlist $ 512;
 ii=1;
 value=varname(dsid,ii);
 do while (value ^= 'Effect') ;
   if ii=1 then namlist=value;
   else namlist=trim(namlist)||' '||value;
   ii=ii+1;
   value=varname(dsid,ii);
 end;
 call symput('bylistzz',compbl(namlist)); **list without effect;
 if namlist='' then namlist=value;
 else namlist=trim(namlist)||' '||value;
 namlist=trim(namlist);
 call symput('bylist',namlist);   **list with effect;
*****************************************************;
*** Now get list of all class variables (always between effect and estimate);
 length list list1 list2 $ 3200;
 start=varnum(dsid,"EFFECT") +1;
 ii=1;jj=start;
 slicein=upcase("&slice");
 do while(ii);
  name=varname(dsid,jj);
  name1=upcase(name); **case sensitive names are returned by varname;
  type=vartype(dsid,jj);
  if name1 ^= 'ESTIMATE' then do;
    kk=indexw(slicein,name1);
    if kk=0 then do; list=compress(list||'='||name);
             if type='N' then
              list2= trim(list2)||' left('||trim(name)||left('')= '_' and") ;
             else list2= trim(list2)||' left('||trim(name)||left('')=" and") ;
    end;
    else do;
      if type='N' then
         list1= trim(list1)||' left('||trim(name)||left('')='_' or") ;
      else list1= trim(list1)||' left('||trim(name)||left('')=" or") ;
           end;
    jj=jj+1;
```

```
  end;
  else ii=0;
 end;
 list=substr(list,2);
 jj=length(list1); if jj>2 then list1=substr(list1,1,jj-2);
 list2=substr(list2,1,length(list2)-3);
 call symput('slice1',trim(list1));
 call symput('varlist1',trim(list2));
 list=translate(list,' ','=');
call symput ('varlistzz',trim(list));
run;
%if &printdebug=1 %then %do;
  %put bylist      &bylist;
  %put bylistzz    &bylistzz;
  %put varlistzz   &varlistzz;
  %put varlist1    &varlist1;
  %put slice1    &slice1;
%end;

********** add variables to datasets ********************************;
data pdtempzz; set &pname; by &bylist  notsorted;
** if adjusted probs are not there, an LSD was used;
 if ADJP=. then do; ADJP=PROBT; ADJUSTMENT='LSD    '; end;
 length _mstech_ $ 30;
 if ADJUSTMENT ='' then _mstech_=compress('LSD(P<'||"&alpha"||')');
  else do;
    _mstech_=compress(ADJUSTMENT||'(P<'||"&alpha"||')' );
   if substr(ADJUSTMENT,1,7)='Dunnett' then call symput('error','4');
  end;
 *** numerical value check only possible in data step;
 if &alpha < 0.0 or &alpha > 1.0 then call symput('error','3');
run;
data pdtempmzz; set &lname; by &bylist notsorted;
  *** add bygroup variable to means dataset;
  retain bygroup 0;
  if first.effect then bygroup+1;
 if first.EFFECT and last.EFFECT then  df0=1;
 else df0=0;
 dothiseffectzz=0;
run;
***means and diffs data may have different effects, due to 0 df,
   so copy bygroup over to diffs;
data pdtempzzz; set pdtempmzz; by bygroup notsorted;
 if first.bygroup;
 keep &bylist bygroup effect;
run;
```

```
** use bylist for merging;
proc sort data=pdtempzz; by &bylist ;
proc sort data=pdtempzzz; by &bylist ;
data pdtempzz; merge pdtempzz (in=have) pdtempzzz; by &bylist;
 if have;
run;
***this sort is required to give IML data by slice;
proc sort data=pdtempzz; by bygroup &slice; run;

%if %length(&slice) ne 0 %then %do;
*****************************************************************;
*****************************************************************;
*** sort, edit, relabel diff and mean data for the slice option ***;
*** this works by redefining effects that are being sliced ***;
*** Example:  In a 2*2 factorial, slicing the A*B interaction by A
***  means only 2 comparisons are needed of the 4*3/2=6 possible.
***  These are A1B1-A1B2  and  A2B1-A2B2;

%if %length(&varlistzz)=0 %then %put ERROR: No variables left after slicing.;
%else %do;
%let lastslicevar=%scan(&slice,-1);
*** identify sliced effects;
*** use pdtempzzz created above, with one record per effect;
proc sort data=pdtempmzz; by bygroup ;
data pdtempmzz ;   set pdtempmzz;
  dothiseffectzz=0;
  *****test if effect should be sliced;
  if not(&slice1) then do; **no slice vars missing;
          if not(&varlist1)  then dothiseffectzz=1;
  end;
run;

*** now fix up diffs dataset;
data pdtempzzz; set pdtempmzz; by  bygroup;
 if first.bygroup;
 keep dothiseffectzz bygroup;
run;
proc sort data=pdtempzz ; by  bygroup ;
data pdtempzz; merge pdtempzz (in=have) pdtempzzz;
   by bygroup ;
   if have;
 ***Delete any pdiffs information that compares across slices;
 ***compared factor levels must match on all slice variables;
  discardzz=0;
  if dothiseffectzz then do;
   %let ii=1;
```

```
   %let var=%scan(&slice,1);
   %do %while(%length(&var) ne 0);
      %let var2=_&var;
      %if %length(&var2)>32 %then %let var2=%substr(&var2,1,32);
      if &var ne &var2 then discardzz=1;
     %let ii=%eval (&ii+1);
     %let var=%scan(&slice,&ii);
   %end;
   if discardzz then delete;
  end;
 drop discardzz ;
run;
%end;

**** if means data set has single means (eg 0 df)
     then sort these to the bottom so they do not
     merge with the msgrp letter output;
proc sort data=pdtempmzz; by &bylist &slice;
data pdtempmzz; set pdtempmzz; by &bylist &slice ;
 **slicing is being done, so may have slice groups with just one level;
 if dothiseffectzz >0 and first.&lastslicevar and last.&lastslicevar then  df0=1;
run;
%end;
***sort single means to bottom, and get data back to original bygroup order;
proc sort data=pdtempmzz; by df0 bygroup ;

%if &printdebug=1 %then %do;
   proc print data=pdtempmzz; title3 'Means data set ready'; run;
   proc print data=pdtempzz; title3 'Diffs data set ready for IML'; run;
   title3 ;
%end;


*************************************************************;
*** ready to process for differences within each effect ***;
proc iml worksize=&worksize; reset nolog fw=7;  printdebug=0;
 alpha=&alpha;
 use pdtempmzz;  **for reading later;
 **** create mean separation output dataset with length 200;
 temp=j(1,&numlet,'0'); msgroup=rowcatc(temp);
 ADJUSTMENT='                              ';
 create msgrpzz var{msgroup bygroup lsmrank ADJUSTMENT};

 **** create indexes of effect and by group locations;
 *** For all useful variable names, read in levels;
 test='a'; ii=1;
```

```
 use pdtempzz;
 varlist= "&bylistzz &slice &varlistzz";
 value='a'; ii=1;
 do while (value ^= '') ;
  value=scan(varlist,ii);
  if value ^= '' then do;
    *** the BY variables are not guaranteed to be character,
    *** so convert them if necessary;
     read all var value into hold;
     if type(hold)='N' then level=level||char(hold);
     else level=level||hold;
     free hold;
  end;
  ii=ii+1;
 end;
if printdebug=1 then print  varlist level;
 if ncol(level)=0 then do;
   file log;
   put "NOTE: No variables found for use in &pname.";
   dataerr=1;
 end;
 else dataerr=0;
 if dataerr ^= 1 then do;
   call change(level,',','-');
   level=rowcatc(level);
   idx=1;
   dim=nrow(level);
if printdebug=1 then print dim level;
 ***search down for number of comparisons in each section;
 ***read number of rows involving first mean to get number of means,
   then calculate number of comparisons;
  byby=0;
  do jj=1 to dim;
    first=level[jj,1];
    byby=byby+1;
    **go to end of comparisons with mean 1;
    kk=jj; flag=1;
    do while(flag=1);
      kk=kk+1;
      if(kk > dim) then flag=0;
      else if (level[kk,1] ^= first) then flag=0;
    end;
    num=kk-jj+1;
    idx=idx || idx[1,byby] + num;
    jj=jj-1+num*(num-1)/2;  ** skip to next section;
   end;
```

```
  free level;
 end;
if printdebug=1 then print idx byby;
 ** BIG BB loop through rows of prob data
 ** subsetting out block dealing with each effect;
 pptr=1;  **points to where probs start for current means;
 do bygroup = 1 to byby;

  dim= idx[1,bygroup+1]-idx[1,bygroup];
  nn= dim*(dim-1)/2;

  ************************************************************;
  **for sorting letters need descending order, and antiranks;
  setin pdtempmzz;
  range=idx[1,bygroup] : idx[1,bygroup+1]-1 ;
  read point range var {ESTIMATE} into lsmcur;

  **stupid rank function fails on missing values;
  **so must temporarily make them non missing;
  test=lsmcur[><,]-1.e-30;
  locmiss=loc(lsmcur=.); kk=ncol(locmiss);
  if kk>0 then lsmcur[locmiss,]=test;
  lsmrnk=dim+1-rank(lsmcur);
  if kk>0 then lsmcur[locmiss,]=.;
  lsmarnk=lsmrnk;
  lsmarnk[lsmrnk,]=(1:(dim))`;
if printdebug=1 then print pptr nn;
************************************************************;
**** get prob file data for these means.
  _adjp_ contains the probs, no matter what adjust method;
  setin pdtempzz;
  range=pptr:pptr+nn-1;
  read point pptr var {_mstech_} into ADJUSTMENT;
  read point range var {ADJP} into data;
  pptr=pptr+nn;
if printdebug=1 then print data;
  *** put p values into matrix;
  p = j(dim,dim,0);
  kk=1; do ii=1 to dim-1; do jj=ii+1 to dim;
    if data[kk,1]=. then  p[jj,ii]=1;
    else  p[jj,ii] = data[kk,1];
    p[ii,jj]=p[jj,ii];  **fill in upper triangle for next sort;
    kk=kk+1;
 end;end;

  *** sort matrix by lsm value, so high mean gets first letter;
```

```
  temp=p;
  p[,lsmrnk]=temp;
  temp[lsmrnk,]=p;
  p=temp; free temp;
  if nn>&numlet then maxlet=&numlet; **memory use limit;
  else maxlet=nn+1;
  group = j(dim, maxlet, 0);
  members=j(dim,1,0);
if printdebug=1 then print p dim data;
  gcode=1; ngroup=1;
  do ii=1 to dim;
    kk=0;
    flag=0;
    do jj=ii+1 to dim;  * go down row, find group members ;
      if p[jj,ii] > alpha then do;   * jj and ii are the same ;
        * check jj against members ;
        do mm=1 to kk ;
          ll=members[mm,1];
          if jj>ll then test1=p[jj,ll];
          else    test1=p[ll,jj];
          if test1<0 then test1=-test1;
          if(test1 < alpha) then goto jmp0; * need new group ;
        end;
        jmp0:
        if mm=kk+1 then do;
          do mm=ii+1 to dim;
            if mm=jj then mm=mm+1; *skip jj (on diagonal);
            if mm>dim then go to jmp2;
            if jj>mm then test1=p[jj,mm];
            else    test1=p[mm,jj];
            if test1 > alpha && -p[mm,ii] > alpha then do;
            * previous grouped mean mm may belong in this group ;
            * so check if already in and current members;
            * dont conflict ;
              do ll=1 to kk;
                nn=members[ll,1];
                if nn=mm then goto jmp1;
                if nn<mm then test1=p[mm,nn];
                else      test1=p[nn,mm];
                if(test1<0.0) then test1=-test1;
                if(test1<alpha) then goto jmp1;
              end;
              jmp1: if(ll=kk+1)then do;
                group[mm,ngroup]=gcode;
                kk=kk+1; members[ll,1]=mm;
              end;
```

```
            end;
          end;
     jmp2:  p[jj,ii]=-p[jj,ii];  * set so not put in next group ;
          do mm=1 to kk;
            ll=members[mm,1];
            * set so not used again ;
            if ll<jj then do;
              if p[jj,ll]>0 then  p[jj,ll]=-p[jj,ll]; end;
            else do;
            if p[ll,jj]>0 then p[ll,jj]=-p[ll,jj]; end;
          end;
          group[jj,ngroup]=gcode;
          kk=kk+1;  members[kk,1]=jj;
        end;
        else flag=1;
      end;
    end;
    if(kk=0) then do;  * no members ;
      do jj=1 to ngroup until (group[ii,jj] ^= 0) ; end;
      * not in a group yet, so set flag ;
      if(jj=ngroup+1) then   kk=kk+1;
    end;
    if(kk^=0) then do;   * need to set current mean ;
      group[ii,ngroup]=gcode;
      ngroup=ngroup+1; gcode=gcode+1;
      if ngroup > &numlet then do;
        ** number of letters needed exceeded maximum;
        jj=dim; ii=dim; **stop loops this way to avoid warnings;
        bygroup=byby; dataerr=1;
        call symput('error','1');
      end;
    end;
    if(flag^=0) then ii=ii-1; * need another group for this mean;
  end;
  if dataerr=0 then do; **skip below if error;
  ngroup=ngroup-1;
  group=group[,1:ngroup];

****** this section just takes the groups identified by numbers
       above and converts numbers to letters.  This depends on
       the ASCII character definitions, eg. 64 value below is what
       gets capital letters;

    *** write out letters;
    kk=nrow(group);
    do ii=1 to kk;
```

```
      gc='';nsect=1;
      do jj=1 to ngroup;
        mm=group[ii,jj];
        if mm > 0 then do; ** blanks are 0, do not do them;
          sect=floor((mm-1)/26);  *** 26 letters in alphabet;
          offset=mm-sect*26;
          sect=sect+1;
          if sect > nsect then do;
            nsect=sect;
            gc=gc||"("||char(sect)||")";
          end;
          gc=gc||byte(64+offset);
        end;
      end;
      lsmrank=lsmarnk[ii,1];
      msgroup=rowcatc(gc);
      ** save letters, by group and sort info;
      append var {msgroup bygroup lsmrank ADJUSTMENT};
    end;
  end; **dataerr;

end;  ** for the big bb loop over effect sections;
quit;

%if &error=1 %then %do;
  %put ERROR: PDMIX800 terminated due to exceeding NUMLET limit.;
%end;

**** put group letters back in original lsm order;
**** they were sorted so largest mean gets letter A;
proc sort data=msgrpzz; by bygroup lsmrank;
%if &printdebug=1 %then %do; proc print data=msgrpzz; run; %end;


**** merge letters with means and print ****;
data msgrpzz; merge pdtempmzz msgrpzz;
 label msgroup='Letter Group';
 if ESTIMATE=. then do;
   **do not print for missing means;
   msgroup='';
 end;
 %if %upcase(&mixfmt)=NO %then %do; format _all_; %end;
run;
proc sort; by &bylistzz bygroup effect; run;

*****************************************************************;
```

```
**** before printing, add the lsdvalues;

proc means noprint data=pdtempzz; by &bylist &slice notsorted;
 id df adjustment;
 var STDERR ;
 output out=lsdvalzz n=numcomp mean=meanse max=maxse min=minse;
run;
data lsdvalzz; set lsdvalzz;
 if upcase(substr(adjustment,1,3))='LSD' then critt=tinv( (1-&alpha/2),DF);
 if upcase(substr(adjustment,1,3))='BON' then critt=tinv( 1-&alpha/(2*numcomp), DF);
 if upcase(adjustment)='SIDAK' then do;
       prob=exp( log(1-&alpha/2) /numcomp );
       critt=tinv( prob  , DF);
 end;
 if upcase(adjustment)='SCHEFFE' then do;
      numdf=-1+(sqrt(1+8*numcomp)+1)/2;
      critt=sqrt(numdf*finv(1-&alpha,numdf,DF));
 end;
 if upcase(substr(adjustment,1,5))='TUKEY' then do;
      numdf=(sqrt(1+8*numcomp)+1)/2;  ** number of treatments;
      critt=probmc('RANGE', . , 1-&alpha,DF,numdf);
put critt;
      critt=critt/sqrt(2);  **adjust for tukey needing sd of mean, not diff;
 end;
 AvgSigDiff=meanse*critt;
 MaxSigDiff=maxse*critt;
 MinSigDiff=minse*critt;
 keep &bylist &slice avgsigdiff maxsigdiff minsigdiff;
 format minsigdiff maxsigdiff avgsigdiff best7. ;
 put adjustment ' values for ' &bylist &slice ' are ' avgsigdiff ' (avg) ' minsigdiff ' (min) '
maxsigdiff  ' (max).' ;
run;

******** print mean separation ***********;
proc sort data=msgrpzz; by &bylist  &slice;
proc sort data=msgrpzz; by ADJUSTMENT bygroup EFFECT;
%if %upcase(&sort)=YES %then %do;
 proc sort data=msgrpzz; by ADJUSTMENT bygroup EFFECT descending ESTIMATE;
%end;
 %if %upcase(&test0)=NO  %then %do;
  data msgrpzz; set msgrpzz;
     drop tvalue probt df;
  run;
%end;
data msgrpzz; set msgrpzz;
 ** drop working variables before printing;
```

```
 drop df0 dothiseffectzz lsmrank;
run;
proc print data=msgrpzz label ;
 by  effect adjustment bygroup notsorted;
 label bygroup='  Set'
       adjustment='  Method';
run;
%skip:
*** restore notes option;
options &notesval;
%mend;
```

GregMen:

```
dm 'output; clear;log; clear';
/*ods rtf file='C:\Users\golson2\Desktop';
options nodate pageno=1;
%let NAME = Gregory ;
footnote "menahden";
footnote2 "&NAME";*/
PROC IMPORT OUT= WORK.menpah
                DATAFILE= "C:\Users\golson2\Desktop\sasthing.xlsx"
          DBMS=EXCEL2000 REPLACE;
                        scantext=yes;
          GETNAMES=YES;
                range = "a1:f73";
RUN;

data fish;
set WORK.menpah;
*lConc = log10(Conc + 1);
run;
*proc print;
quit;

proc mixed data=fish;
class place size month year;
model pahngg=place|size|month|year;
repeated/group=place;
lsmeans place|size|month|year / adjust=tukey;
ods output diffs=ppp lsmeans=mmm;
run;
%include 'C:\Users\golson2\Desktop\pdmix800.sas';
%pdmix800 (ppp,mmm,alpha=0.05,sort=yes);
run;
quit;
```

GregMenTEQ

```
PROC IMPORT OUT= WORK.menpah
            DATAFILE= "C:\Users\golson2\Desktop\sasthingteq.xlsx"
      DBMS=EXCEL2000 REPLACE;
                  scantext=yes;
      GETNAMES=YES;
            range = "a1:f73";
RUN;

data fish;
set WORK.menpah;
*lConc = log10(Conc + 1);
run;
*proc print;
quit;


proc mixed data=fish;
class place size month year;
model teqngg=place|size|month|year;
repeated/group=place;
lsmeans place|size|month|year / adjust=tukey;
ods output diffs=ppp lsmeans=mmm;
run;
%include 'C:\Users\golson2\Desktop\pdmix800.sas';
%pdmix800 (ppp,mmm,alpha=0.05,sort=yes);
run;
quit;
```

GregMenMEQ

```
PROC IMPORT OUT= WORK.menpah
            DATAFILE= "C:\Users\golson2\Desktop\sasthingmeq.xlsx"
      DBMS=EXCEL2000 REPLACE;
                  scantext=yes;
      GETNAMES=YES;
            range = "a1:f73";
RUN;

data fish;
set WORK.menpah;
*lConc = log10(Conc + 1);
```

```
run;
*proc print;
quit;



proc mixed data=fish;
class place size month year;
model meqngg=place|size|month|year;
repeated/group=place;
lsmeans place|size|month|year / adjust=tukey;
ods output diffs=ppp lsmeans=mmm;
run;
%include 'C:\Users\golson2\Desktop\pdmix800.sas';
%pdmix800 (ppp,mmm,alpha=0.05,sort=yes);
run;
quit;
```

Fish	Place	Size	Month	Year	pahngg	teqngg	meqngg
1	vb	lg	jul	2011	11438	2.8	3.9
2	vb	lg	jul	2011	6233	2.1	3.3
3	vb	lg	jul	2011	7525	76.5	17.3
4	gi	lg	jul	2011	8988	129.2	14.9
5	gi	lg	jul	2011	9320	3.7	4.9
6	gi	lg	jul	2011	9154	66.4	9.9
7	vb	sm	aug	2011	6245	2.8	0.0
8	vb	sm	aug	2011	6458	2.1	0.5
9	vb	sm	aug	2011	13041	76.5	0.0
10	gi	sm	aug	2011	10132	129.2	0.0
11	gi	sm	aug	2011	7227	3.7	0.0
12	gi	sm	aug	2011	7007	66.4	0.0
13	vb	lg	aug	2011	2517	468.1	155.7
14	vb	lg	aug	2011	2422	369.7	103.6
15	vb	lg	aug	2011	3413	252.5	44.2
16	gi	lg	aug	2011	3229	137.0	13.5
17	gi	lg	aug	2011	1892	164.7	9.5
18	gi	lg	aug	2011	2666	155.1	9.0
19	gi	sm	sept	2011	2860	104.0	6.0
20	gi	sm	sept	2011	4429	0.2	0.0
21	gi	sm	sept	2011	2982	0.2	0.0
22	vb	sm	sept	2011	3672	0.1	0.0
23	vb	sm	sept	2011	4838	0.0	0.0
24	vb	sm	sept	2011	5877	0.2	0.0
25	vb	lg	sept	2011	13128	737.7	161.9
26	vb	lg	sept	2011	20523	7.6	13.6

27	vb	lg	sept	2011	13038	0.2	0.0
28	gi	lg	sept	2011	8438	0.3	0.0
29	gi	lg	sept	2011	10216	0.4	0.0
30	gi	lg	sept	2011	12907	0.2	0.0
31	vb	sm	jul	2011	5283	0.0	0.0
32	vb	sm	jul	2011	1901	0.0	0.0
33	vb	sm	jul	2011	5040	0.0	0.0
34	gi	sm	jul	2011	2952	0.0	0.0
35	gi	sm	jul	2011	1916	0.0	0.0
36	gi	sm	jul	2011	2073	0.0	0.0
37	vb	sm	Sept	2012	5084	0.27	0.00
38	vb	sm	Sept	2012	6711	0.55	0.00
39	vb	sm	Sept	2012	5162	0.35	0.00
40	gi	sm	Sept	2012	4173	0.27	0.00
41	gi	sm	Sept	2012	4520	0.21	0.00
42	gi	sm	Sept	2012	6565	0.22	0.00
43	vb	lg	Sept	2012	3969	0.15	0.00
44	vb	lg	Sept	2012	6674	0.12	0.00
45	vb	lg	Sept	2012	4800	0.17	0.00
46	gi	lg	Sept	2012	3757	0.10	0.00
47	gi	lg	Sept	2012	4816	0.13	0.00
48	gi	lg	Sept	2012	4054	0.22	0.00
49	vb	lg	Aug	2012	7795	0.42	0.00
50	vb	lg	Aug	2012	5881	0.00	0.00
51	vb	lg	Aug	2012	6175	1.93	3.28
52	gi	lg	Aug	2012	9990	0.30	0.00
53	gi	lg	Aug	2012	7189	0.00	0.00
54	gi	lg	Aug	2012	9371	1.93	3.27
55	vb	sm	Aug	2012	3986	0.15	0.00
56	vb	sm	Aug	2012	4935	0.23	0.00
57	vb	sm	Aug	2012	4643	0.00	0.00
58	gi	sm	Aug	2012	3586	0.00	0.00
59	gi	sm	Aug	2012	4153	0.16	0.00
60	gi	sm	Aug	2012	3494	0.12	0.00
61	vb	sm	Jul	2012	7800	1.58	0.00
62	vb	sm	Jul	2012	5416	0.27	0.00
63	vb	sm	Jul	2012	4467	0.25	0.00
64	gi	sm	Jul	2012	7766	0.37	0.00
65	gi	sm	Jul	2012	8763	0.44	0.00
66	gi	sm	Jul	2012	9966	0.44	0.00
67	vb	lg	Jul	2012	3470	0.16	0.00
68	vb	lg	Jul	2012	4378	0.30	0.00
69	vb	lg	Jul	2012	5360	0.00	0.00
70	gi	lg	Jul	2012	4020	0.17	0.00
71	gi	lg	Jul	2012	3825	0.16	0.00
72	gi	lg	Jul	2012	4350	0.19	0.00

73	vb	lg	jul	2013	6,231	0.68	0.00
74	vb	lg	jul	2013	5,740	1.21	0.00
75	vb	lg	jul	2013	4,258	1.49	0.00
76	vb	sm	jul	2013	3,698	0.37	0.00
77	vb	sm	jul	2013	5,900	0.88	0.00
78	vb	sm	jul	2013	5,198	0.87	0.00
79	gi	lg	jul	2013	4,506	0.52	0.00
80	gi	lg	jul	2013	5,631	0.76	0.00
81	gi	lg	jul	2013	5,270	0.85	0.00
82	gi	sm	jul	2013	5,477	0.71	0.00
83	gi	sm	jul	2013	5,692	0.83	0.00
84	gi	sm	jul	2013	4,253	0.37	0.00
85	vb	lg	aug	2013	3,346	0.74	0.00
86	vb	lg	aug	2013	4,192	1.02	0.00
87	vb	lg	aug	2013	3,228	1.12	0.86
88	vb	sm	aug	2013	3,152	1.04	0.45
89	vb	sm	aug	2013	2,874	1.02	0.61
90	vb	sm	aug	2013	3,479	6.46	5.26
91	gi	lg	aug	2013	2,252	1.10	0.82
92	gi	lg	aug	2013	3,581	1.14	0.66
93	gi	lg	aug	2013	3,186	1.23	0.67
94	gi	sm	aug	2013	2,038	0.99	0.39
95	gi	sm	aug	2013	2,896	1.11	0.86
96	gi	sm	aug	2013	3,358	1.30	0.83
97	vb	lg	sept	2013	2,874	389.72	84.92
98	vb	lg	sept	2013	2,859	2.61	1.30
99	vb	lg	sept	2013	3,437	3.34	2.27
100	vb	sm	sept	2013	4,463	4.03	2.58
101	vb	sm	sept	2013	3,626	2.52	1.67
102	vb	sm	sept	2013	4,880	9.40	7.05
103	gi	lg	sept	2013	1,596	0.99	0.69
104	gi	lg	sept	2013	1,456	1.01	0.78
105	gi	lg	sept	2013	1,976	4.96	3.84
106	gi	sm	sept	2013	1,916	2.13	1.41
107	gi	sm	sept	2013	1,595	1.10	0.78
108	gi	sm	sept	2013	2,053	0.79	0.50

Menhaden PAH Statistics

Summary SAS Printouts

Model Information	
Data Set	WORK.FISH
Dependent Variable	pahngg
Covariance Structure	Variance Components
Group Effect	place
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information		
Class	Levels	Values
place	2	gi vb
size	2	lg sm
month	3	aug jul sept
year	3	first second third

Dimensions	
Covariance Parameters	2
Columns in X	144
Columns in Z	0
Subjects	108
Max Obs per Subject	1

Summary SAS Printouts (Cont.)

Number of Observations	
Number of Observations Read	108
Number of Observations Used	108
Number of Observations Not Used	0

Iteration History			
Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	1290.55757951	
1	1	1278.34963295	0.00000000

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
place	1	72	4.39	0.0396
size	1	72	11.64	0.0011
place*size	1	72	0.05	0.8265
month	2	72	2.90	0.0617
place*month	2	72	6.36	0.0029
size*month	2	72	10.40	0.0001
place*size*month	2	72	1.18	0.3118
year	2	72	40.90	<.0001
place*year	2	72	3.61	0.0320
size*year	2	72	13.24	<.0001
place*size*year	2	72	0.06	0.9428
month*year	4	72	12.08	<.0001
place*month*year	4	72	0.25	0.9087
size*month*year	4	72	46.10	<.0001
plac*size*month*year	4	72	3.12	0.0199

Summary SAS Printouts (Cont.)

Convergence criteria met.

Covariance Parameter Estimates		
Cov Parm	Group	Estimate
Residual	place gi	954320
Residual	place vb	3161608

Fit Statistics	
-2 Res Log Likelihood	1278.3
AIC (Smaller is Better)	1282.3
AICC (Smaller is Better)	1282.5
BIC (Smaller is Better)	1287.7

Null Model Likelihood Ratio Test		
DF	Chi-Square	Pr > ChiSq
1	12.21	0.0005

Effect=size Method=Tukey(P<0.05) Set=2

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
1		lg			5787.81	195.22	A
2		sm			4845.77	195.22	B

Summary SAS Printouts (Cont.)

Effect=month Method=Tukey(P<0.05) Set=4

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
3			jul		5646.11	239.09	A
4			sept		5442.36	239.09	A
5			aug		4861.90	239.09	A

Effect=size*month Method=Tukey(P<0.05) Set=6

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
6		lg	sept		6695.47	338.13	A
7		lg	jul		6094.35	338.13	AB
8		sm	jul		5197.88	338.13	BC
9		sm	aug		5150.19	338.13	BC
10		lg	aug		4573.61	338.13	C
11		sm	sept		4189.25	338.13	C

Summary SAS Printouts (Cont.)

Effect=year Method=Tukey(P<0.05) Set=8

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
12				first	6693.89	239.09	A
13				second	5585.10	239.09	B
14				third	3671.38	239.09	C

Effect=size*year Method=Tukey(P<0.05) Set=10

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
15		lg		first	8169.28	338.13	A
16		sm		second	5621.61	338.13	B
17		lg		second	5548.60	338.13	B
18		sm		first	5218.50	338.13	B
19		sm		third	3697.21	338.13	C
20		lg		third	3645.56	338.13	C

Summary SAS Printouts (Cont.)

Effect=month*year Method=Tukey(P<0.05) Set=12

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
21			sept	first	8575.67	414.12	A
22			jul	first	5985.25	414.12	B
23			aug	second	5933.04	414.12	B
24			jul	second	5798.48	414.12	B
25			aug	first	5520.75	414.12	B
26			jul	third	5154.61	414.12	B
27			sept	second	5023.79	414.12	B
28			aug	third	3131.91	414.12	C
29			sept	third	2727.63	414.12	C

Effect=size*month*year Method=Tukey(P<0.05) Set=14

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
30		lg	sept	first	13042	585.66	A
31		lg	jul	first	8776.33	585.66	B
32		sm	aug	first	8351.67	585.66	BC
33		lg	aug	second	7733.44	585.66	BCD
34		sm	jul	second	7363.00	585.66	BCDE
35		sm	sept	second	5369.19	585.66	CDEF
36		lg	jul	third	5272.76	585.66	DEFG
37		sm	jul	third	5036.46	585.66	DEFG
38		lg	sept	second	4678.39	585.66	EFG
39		lg	jul	second	4233.96	585.66	FG
40		sm	aug	second	4132.64	585.66	FG
41		sm	sept	first	4109.67	585.66	FG
42		lg	aug	third	3297.55	585.66	FG
43		sm	jul	first	3194.17	585.66	FG
44		sm	sept	third	3088.90	585.66	FG
45		sm	aug	third	2966.27	585.66	FG
46		lg	aug	first	2689.83	585.66	FG
47		lg	sept	third	2366.36	585.66	G

Summary SAS Printouts (Cont.)

Effect=place Method=Tukey-Kramer(P<0.05) Set=1

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
48	vb				5606.17	241.97	A
49	gi				5027.41	132.94	B

Effect=place*size Method=Tukey-Kramer(P<0.05) Set=3

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
50	vb	lg			6107.56	342.19	A
51	gi	lg			5468.07	188.00	A
52	vb	sm			5104.79	342.19	AB
53	gi	sm			4586.76	188.00	B

Effect=place*month Method=Tukey-Kramer(P<0.05) Set=5

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
54	vb		sept		6423.09	419.10	A
55	gi		jul		5773.52	230.26	AB
56	vb		jul		5518.71	419.10	ABC
57	vb		aug		4876.72	419.10	ABC
58	gi		aug		4847.08	230.26	BC
59	gi		sept		4461.64	230.26	C

Summary SAS Printouts (Cont.)

Effect=place*size*month Method=Tukey-Kramer(P<0.05) Set=7

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
60	vb	lg	sept		7922.46	592.70	A
61	gi	lg	jul		6118.38	325.63	AB
62	vb	lg	jul		6070.32	592.70	AB
63	gi	lg	sept		5468.48	325.63	B
64	gi	sm	jul		5428.65	325.63	B
65	vb	sm	aug		5423.56	592.70	ABC
66	vb	sm	jul		4967.10	592.70	BC
67	vb	sm	sept		4923.71	592.70	BC
68	gi	sm	aug		4876.82	325.63	BC
69	gi	lg	aug		4817.33	325.63	BC
70	vb	lg	aug		4329.89	592.70	BC
71	gi	sm	sept		3454.79	325.63	C

Effect=place*year Method=Tukey-Kramer(P<0.05) Set=9

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
72	vb			first	7366.22	419.10	A
73	gi			first	6021.56	230.26	AB
74	gi			second	5797.73	230.26	B
75	vb			second	5372.48	419.10	BC
76	vb			third	4079.82	419.10	CD
77	gi			third	3262.94	230.26	D

Summary SAS Printouts (Cont.)

Effect=place*size*year Method=Tukey-Kramer(P<0.05) Set=11

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
78	vb	lg		first	8915.22	592.70	A
79	gi	lg		first	7423.33	325.63	AB
80	gi	sm		second	5887.33	325.63	BC
81	vb	sm		first	5817.22	592.70	BC
82	gi	lg		second	5708.13	325.63	C
83	vb	lg		second	5389.06	592.70	BCD
84	vb	sm		second	5355.89	592.70	BCD
85	gi	sm		first	4619.78	325.63	CD
86	vb	sm		third	4141.26	592.70	CD
87	vb	lg		third	4018.38	592.70	CD
88	gi	lg		third	3272.73	325.63	D
89	gi	sm		third	3253.16	325.63	D

Effect=place*month*year Method=Tukey-Kramer(P<0.05) Set=13

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
90	vb		sept	first	10179	725.90	A
91	gi		sept	first	6972.00	398.81	B
92	gi		jul	second	6448.50	398.81	BC
93	gi		aug	second	6297.08	398.81	BCD
94	vb		jul	first	6236.67	725.90	BCD
95	gi		jul	first	5733.83	398.81	BCD
96	vb		aug	first	5682.67	725.90	BCDE
97	vb		aug	second	5569.00	725.90	BCDE
98	vb		sept	second	5399.96	725.90	BCDE
99	gi		aug	first	5358.83	398.81	BCD
100	vb		jul	third	5171.00	725.90	BCDE
101	vb		jul	second	5148.46	725.90	BCDE
102	gi		jul	third	5138.22	398.81	BCD
103	gi		sept	second	4647.62	398.81	CDE
104	vb		sept	third	3689.96	725.90	CDEF
105	vb		aug	third	3378.51	725.90	DEF
106	gi		aug	third	2885.32	398.81	EF
107	gi		sept	third	1765.30	398.81	F

Summary SAS Printouts (Cont.)

Effect=plac*size*month*year Method=Tukey-Kramer(P<0.05) Set=15

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
108	vb	lg	sept	first	15563	1026.58	A
109	gi	lg	sept	first	10520	564.01	B
110	gi	lg	jul	first	9154.00	564.01	BC
111	gi	lg	aug	second	8849.87	564.01	BCD
112	gi	sm	jul	second	8831.67	564.01	BCD
113	vb	sm	aug	first	8581.33	1026.58	BCDEF
114	vb	lg	jul	first	8398.67	1026.58	BCDEFG
115	gi	sm	aug	first	8122.00	564.01	BCDE
116	vb	lg	aug	second	6617.00	1026.58	BCDEFGH
117	vb	sm	jul	second	5894.33	1026.58	BCDEFGHI
118	vb	sm	sept	second	5652.34	1026.58	CDEFGHI
119	vb	lg	jul	third	5409.69	1026.58	CDEFGHI
120	vb	lg	sept	second	5147.59	1026.58	CDEFGHI
121	gi	sm	jul	third	5140.62	564.01	EFGH
122	gi	lg	jul	third	5135.82	564.01	EFGH
123	gi	sm	sept	second	5086.04	564.01	EFGH
124	vb	sm	jul	third	4932.31	1026.58	CDEFGHI
125	vb	sm	sept	first	4795.67	1026.58	CDEFGHI
126	vb	sm	aug	second	4521.00	1026.58	CDEFGHI
127	vb	lg	jul	second	4402.59	1026.58	DEFGHI
128	vb	sm	sept	third	4323.13	1026.58	DEFGHI
129	gi	lg	sept	second	4209.20	564.01	FGHI
130	vb	sm	jul	first	4074.67	1026.58	EFGHI
131	gi	lg	jul	second	4065.33	564.01	FGHI
132	gi	sm	aug	second	3744.28	564.01	GHI
133	vb	lg	aug	third	3588.66	1026.58	EFGHI
134	gi	sm	sept	first	3423.67	564.01	HI
135	vb	sm	aug	third	3168.35	1026.58	FGHI
136	vb	lg	sept	third	3056.80	1026.58	FGHI
137	gi	lg	aug	third	3006.44	564.01	HI
138	vb	lg	aug	first	2784.00	1026.58	FGHI
139	gi	sm	aug	third	2764.19	564.01	HI
140	gi	lg	aug	first	2595.67	564.01	HI
141	gi	sm	jul	first	2313.67	564.01	HI
142	gi	sm	sept	third	1854.67	564.01	I
143	gi	lg	sept	third	1675.92	564.01	I

Menhaden TEQ Statistics

Summary SAS Printouts

The Mixed Procedure

Model Information	
Data Set	WORK.FISH
Dependent Variable	teqngg
Covariance Structure	Variance Components
Group Effect	place
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
place	2	gi vb
size	2	lg sm
month	3	aug jul sept
year	3	first second third

Summary SAS Printouts (Cont.)

Dimensions	
Covariance Parameters	2
Columns in X	144
Columns in Z	0
Subjects	108
Max Obs per Subject	1

Number of Observations	
Number of Observations Read	108
Number of Observations Used	108
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	882.58307901	
1	1	819.61156185	0.00000000

Summary SAS Printouts (Cont.)

Convergence criteria met.

Covariance Parameter Estimates		
Cov Parm	Group	Estimate
Residual	place gi	648.84
Residual	place vb	13595

Fit Statistics	
-2 Res Log Likelihood	819.6
AIC (Smaller is Better)	823.6
AICC (Smaller is Better)	823.8
BIC (Smaller is Better)	829.0

Null Model Likelihood Ratio Test		
DF	Chi-Square	Pr > ChiSq
1	62.97	<.0001

Summary SAS Printouts (Cont.)

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
place	1	72	2.72	0.1035
size	1	72	8.57	0.0046
place*size	1	72	4.46	0.0381
month	2	72	2.42	0.0964
place*month	2	72	1.31	0.2761
size*month	2	72	1.03	0.3629
place*size*month	2	72	1.81	0.1717
year	2	72	9.86	0.0002
place*year	2	72	1.06	0.3501
size*year	2	72	5.34	0.0069
place*size*year	2	72	2.32	0.1053
month*year	4	72	2.70	0.0373
place*month*year	4	72	0.62	0.6491
size*month*year	4	72	1.23	0.3060
plac*size*month*year	4	72	1.03	0.3955

Effect=size Method=Tukey(P<0.05) Set=2

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
1		lg			55.4663	11.4841	A
2		sm			7.9115	11.4841	B

Effect=month Method=Tukey(P<0.05) Set=4

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
3			aug		51.4255	14.0651	A
4			sept		35.4563	14.0651	A
5			jul		8.1848	14.0651	A

Summary SAS Printouts (Cont.)

Effect=size*month Method=Tukey(P<0.05) Set=6

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
6		lg	aug		86.5550	19.8911	A
7		lg	sept		63.8877	19.8911	AB
8		sm	aug		16.2959	19.8911	AB
9		lg	jul		15.9562	19.8911	AB
10		sm	sept		7.0250	19.8911	AB
11		sm	jul		0.4134	19.8911	B

Effect=year Method=Tukey(P<0.05) Set=8

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
12				first	82.2131	14.0651	A
13				third	12.5117	14.0651	B
14				second	0.3419	14.0651	B

Effect=size*year Method=Tukey(P<0.05) Set=10

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
15		lg		first	143.02	19.8911	A
16		lg		third	23.0271	19.8911	B
17		sm		first	21.4111	19.8911	B
18		sm		third	1.9963	19.8911	B
19		lg		second	0.3568	19.8911	B
20		sm		second	0.3270	19.8911	B

Effect=month*year Method=Tukey(P<0.05) Set=12

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
21			aug	first	152.32	24.3615	A
22			sept	first	70.9239	24.3615	AB
23			sept	third	35.2163	24.3615	B
24			jul	first	23.3985	24.3615	B
25			aug	third	1.5228	24.3615	B
26			jul	third	0.7959	24.3615	B
27			aug	second	0.4368	24.3615	B
28			jul	second	0.3600	24.3615	B
29			sept	second	0.2289	24.3615	B

Effect=size*month*year Method=Tukey(P<0.05) Set=14

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
30		lg	aug	first	257.85	34.4524	A
31		lg	sept	first	124.41	34.4524	AB
32		lg	sept	third	67.1053	34.4524	B
33		sm	aug	first	46.7886	34.4524	B
34		lg	jul	first	46.7886	34.4524	B
35		sm	sept	first	17.4363	34.4524	B
36		sm	sept	third	3.3273	34.4524	B
37		sm	aug	third	1.9877	34.4524	B
38		lg	aug	third	1.0580	34.4524	B
39		lg	jul	third	0.9181	34.4524	B
40		lg	aug	second	0.7620	34.4524	B
41		sm	jul	third	0.6738	34.4524	B
42		sm	jul	second	0.5580	34.4524	B
43		sm	sept	second	0.3114	34.4524	B
44		lg	jul	second	0.1620	34.4524	B
45		lg	sept	second	0.1463	34.4524	B
46		sm	aug	second	0.1116	34.4524	B
47		sm	jul	first	0.008354	34.4524	B

Summary SAS Printouts (Cont.)

Effect=place Method=Tukey-Kramer(P<0.05) Set=1

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
48	vb				45.0816	15.8668	A
49	gi				18.2962	3.4663	A

Effect=place*size Method=Tukey-Kramer(P<0.05) Set=3

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
50	vb	lg			86.0156	22.4390	A
51	gi	lg			24.9171	4.9021	B
52	gi	sm			11.6753	4.9021	B
53	vb	sm			4.1476	22.4390	AB

Effect=place*month Method=Tukey-Kramer(P<0.05) Set=5

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
54	vb		aug		65.8801	27.4821	AB
55	vb		sept		64.3926	27.4821	AB
56	gi		aug		36.9709	6.0039	A
57	gi		jul		11.3974	6.0039	B
58	gi		sept		6.5201	6.0039	B
59	vb		jul		4.9722	27.4821	AB

Effect=place*size*month Method=Tukey-Kramer(P<0.05) Set=7

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
60	vb	lg	sept		126.85	38.8655	AB
61	vb	lg	aug		121.72	38.8655	AB
62	gi	lg	aug		51.3853	8.4908	A
63	gi	sm	aug		22.5565	8.4908	AB
64	gi	lg	jul		22.4400	8.4908	AB
65	gi	sm	sept		12.1145	8.4908	AB
66	vb	sm	aug		10.0354	38.8655	AB
67	vb	lg	jul		9.4724	38.8655	AB
68	vb	sm	sept		1.9356	38.8655	AB
69	gi	lg	sept		0.9258	8.4908	B
70	vb	sm	jul		0.4719	38.8655	AB
71	gi	sm	jul		0.3548	8.4908	B

Summary SAS Printouts (Cont.)

Effect=place*year Method=Tukey-Kramer(P<0.05) Set=9

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
72	vb			first	111.06	27.4821	A
73	gi			first	53.3706	6.0039	A
74	vb			third	23.8067	27.4821	AB
75	gi			third	1.2167	6.0039	B
76	vb			second	0.3826	27.4821	AB
77	gi			second	0.3011	6.0039	B

Effect=place*size*year Method=Tukey-Kramer(P<0.05) Set=11

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
78	vb	lg		first	213.03	38.8655	A
79	gi	lg		first	73.0010	8.4908	B
80	vb	lg		third	44.6582	38.8655	ABC
81	gi	sm		first	33.7403	8.4908	BC
82	vb	sm		first	9.0819	38.8655	BC
83	vb	sm		third	2.9552	38.8655	BC
84	gi	lg		third	1.3960	8.4908	C
85	gi	sm		third	1.0374	8.4908	C
86	vb	sm		second	0.4059	38.8655	BC
87	vb	lg		second	0.3594	38.8655	BC
88	gi	lg		second	0.3541	8.4908	C
89	gi	sm		second	0.2481	8.4908	C

Summary SAS Printouts (Cont.)

Effect=place*month*year Method=Tukey-Kramer(P<0.05) Set=13

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
90	vb		aug	first	195.29	47.6003	AB
91	vb		sept	first	124.31	47.6003	ABC
92	gi		aug	first	109.35	10.3990	A
93	vb		sept	third	68.6025	47.6003	ABC
94	gi		jul	first	33.2255	10.3990	BC
95	gi		sept	first	17.5392	10.3990	C
96	vb		jul	first	13.5714	47.6003	ABC
97	vb		aug	third	1.8992	47.6003	ABC
98	gi		sept	third	1.8300	10.3990	C
99	gi		aug	third	1.1465	10.3990	C
100	vb		jul	third	0.9183	47.6003	ABC
101	gi		jul	third	0.6736	10.3990	C
102	vb		aug	second	0.4545	47.6003	ABC
103	vb		jul	second	0.4268	47.6003	ABC
104	gi		aug	second	0.4190	10.3990	C
105	gi		jul	second	0.2932	10.3990	C
106	vb		sept	second	0.2666	47.6003	ABC
107	gi		sept	second	0.1911	10.3990	C

Summary SAS Printouts (Cont.)

Effect=plac*size*month*year Method=Tukey-Kramer(P<0.05) Set=15

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
108	vb	lg	aug	first	383.43	67.3170	A
109	vb	lg	sept	first	248.51	67.3170	AB
110	gi	lg	aug	first	152.26	14.7064	A
111	vb	lg	sept	third	131.89	67.3170	AB
112	gi	lg	jul	first	66.4392	14.7064	B
113	gi	sm	aug	first	66.4392	14.7064	B
114	gi	sm	sept	first	34.7698	14.7064	B
115	vb	sm	aug	first	27.1380	67.3170	AB
116	vb	lg	jul	first	27.1380	67.3170	AB
117	vb	sm	sept	third	5.3144	67.3170	AB
118	vb	sm	aug	third	2.8407	67.3170	AB
119	gi	lg	sept	third	2.3198	14.7064	B
120	gi	sm	sept	third	1.3402	14.7064	B
121	gi	lg	aug	third	1.1584	14.7064	B
122	gi	sm	aug	third	1.1347	14.7064	B
123	vb	lg	jul	third	1.1263	67.3170	AB
124	vb	lg	aug	third	0.9576	67.3170	AB
125	vb	lg	aug	second	0.7815	67.3170	AB
126	gi	lg	aug	second	0.7424	14.7064	B
127	vb	sm	jul	third	0.7104	67.3170	AB
128	gi	lg	jul	third	0.7099	14.7064	B
129	vb	sm	jul	second	0.7006	67.3170	AB
130	gi	sm	jul	third	0.6373	14.7064	B
131	gi	sm	jul	second	0.4154	14.7064	B
132	vb	sm	sept	second	0.3895	67.3170	AB
133	gi	lg	sept	first	0.3087	14.7064	B
134	gi	sm	sept	second	0.2334	14.7064	B
135	gi	lg	jul	second	0.1711	14.7064	B
136	vb	lg	jul	second	0.1530	67.3170	AB
137	gi	lg	sept	second	0.1489	14.7064	B
138	vb	lg	sept	second	0.1438	67.3170	AB
139	vb	sm	aug	second	0.1275	67.3170	AB
140	vb	sm	sept	first	0.1029	67.3170	AB
141	gi	sm	aug	second	0.09557	14.7064	B
142	gi	sm	jul	first	0.01188	14.7064	B
143	vb	sm	jul	first	0.004826	67.3170	AB

Menhaden MEQ Statistics

Summary SAS Printouts

Model Information	
Data Set	WORK.FISH
Dependent Variable	meqngg
Covariance Structure	Variance Components
Group Effect	place
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information		
Class	Levels	Values
place	2	gi vb
size	2	lg sm
month	3	aug jul sept
year	3	first second third

Dimensions	
Covariance Parameters	2
Columns in X	144
Columns in Z	0
Subjects	108
Max Obs per Subject	1

Number of Observations	
Number of Observations Read	108
Number of Observations Used	108
Number of Observations Not Used	0

Summary SAS Printouts (Cont.)

Iteration History			
Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	671.24645624	
1	1	519.47827382	0.00000000

Convergence criteria met.

Covariance Parameter Estimates		
Cov Parm	Group	Estimate
Residual	place gi	2.8024
Residual	place vb	753.80

Fit Statistics	
-2 Res Log Likelihood	519.5
AIC (Smaller is Better)	523.5
AICC (Smaller is Better)	523.7
BIC (Smaller is Better)	528.8

Null Model Likelihood Ratio Test		
DF	Chi-Square	Pr > ChiSq
1	151.77	<.0001

Summary SAS Printouts (Cont.)

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
place	1	72	6.90	0.0105
size	1	72	10.01	0.0023
place*size	1	72	6.53	0.0127
month	2	72	1.83	0.1679
place*month	2	72	1.83	0.1676
size*month	2	72	1.53	0.2229
place*size*month	2	72	1.73	0.1840
year	2	72	6.57	0.0024
place*year	2	72	3.87	0.0254
size*year	2	72	6.66	0.0022
place*size*year	2	72	4.26	0.0178
month*year	4	72	1.67	0.1668
place*month*year	4	72	1.55	0.1979
size*month*year	4	72	1.68	0.1635
plac*size*month*year	4	72	1.54	0.1996

Effect=size Method=Tukey(P<0.05) Set=2

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
1		lg			12.3795	2.6468	A
2		sm			0.5355	2.6468	B

Effect=month Method=Tukey(P<0.05) Set=4

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
3			aug		9.8321	3.2417	A
4			sept		8.0382	3.2417	A
5			jul		1.5022	3.2417	A

Summary SAS Printouts (Cont.)

Effect=size*month Method=Tukey(P<0.05) Set=6

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
6		lg	aug		19.1700	4.5844	A
7		lg	sept		14.9641	4.5844	AB
8		lg	jul		3.0044	4.5844	AB
9		sm	sept		1.1122	4.5844	AB
10		sm	aug		0.4943	4.5844	AB
11		sm	jul		-904E-15	4.5844	B

Effect=year Method=Tukey(P<0.05) Set=8

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
12				first	15.8795	3.2417	A
13				third	3.3111	3.2417	B
14				second	0.1819	3.2417	B

Effect=size*year Method=Tukey(P<0.05) Set=10

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
15		lg		first	31.3967	4.5844	A
16		lg		third	5.3780	4.5844	B
17		sm		third	1.2442	4.5844	B
18		lg		second	0.3639	4.5844	B
19		sm		first	0.3622	4.5844	B
20		sm		second	-917E-15	4.5844	B

Summary SAS Printouts (Cont.)

Effect=month*year Method=Tukey(P<0.05) Set=12

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
21			aug	first	27.9996	5.6147	A
22			sept	first	15.1322	5.6147	AB
23			sept	third	8.9823	5.6147	AB
24			jul	first	4.5067	5.6147	AB
25			aug	third	0.9510	5.6147	B
26			aug	second	0.5458	5.6147	B
27			jul	second	-158E-15	5.6147	B
28			jul	third	-549E-15	5.6147	B
29			sept	second	-121E-14	5.6147	B

Effect=size*month*year Method=Tukey(P<0.05) Set=14

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
30		lg	aug	first	55.9173	7.9404	A
31		lg	sept	first	29.2595	7.9404	AB
32		lg	sept	third	15.6329	7.9404	AB
33		lg	jul	first	9.0133	7.9404	B
34		sm	sept	third	2.3317	7.9404	B
35		sm	aug	third	1.4009	7.9404	B
36		lg	aug	second	1.0916	7.9404	B
37		sm	sept	first	1.0048	7.9404	B
38		lg	aug	third	0.5011	7.9404	B
39		sm	aug	first	0.08187	7.9404	B
40		lg	sept	second	3.27E-12	7.9404	B
41		sm	aug	second	1.62E-12	7.9404	B
42		sm	jul	second	1.31E-12	7.9404	B
43		sm	jul	third	-815E-16	7.9404	B
44		lg	jul	third	-102E-14	7.9404	B
45		lg	jul	second	-162E-14	7.9404	B
46		sm	jul	first	-394E-14	7.9404	B
47		sm	sept	second	-568E-14	7.9404	B

Summary SAS Printouts (Cont.)

Effect=place Method=Tukey-Kramer(P<0.05) Set=1

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
48	vb				11.3749	3.7362	A
49	gi				1.5401	0.2278	B

Effect=place*size Method=Tukey-Kramer(P<0.05) Set=3

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
50	vb	lg			22.0791	5.2838	A
51	gi	lg			2.6800	0.3222	B
52	vb	sm			0.6707	5.2838	BC
53	gi	sm			0.4002	0.3222	C

Effect=place*month Method=Tukey-Kramer(P<0.05) Set=5

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
54	vb		aug		17.4689	6.4713	A
55	vb		sept		15.2969	6.4713	A
56	gi		aug		2.1954	0.3946	A
57	gi		jul		1.6456	0.3946	A
58	vb		jul		1.3589	6.4713	A
59	gi		sept		0.7794	0.3946	A

Summary SAS Printouts (Cont.)

Effect=place*size*month Method=Tukey-Kramer(P<0.05) Set=7

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
60	vb	lg	aug		34.1809	9.1518	A
61	vb	lg	sept		29.3386	9.1518	ABC
62	gi	lg	aug		4.1591	0.5580	A
63	gi	lg	jul		3.2911	0.5580	AB
64	vb	lg	jul		2.7177	9.1518	ABC
65	vb	sm	sept		1.2552	9.1518	ABC
66	gi	sm	sept		0.9691	0.5580	BC
67	vb	sm	aug		0.7569	9.1518	ABC
68	gi	lg	sept		0.5897	0.5580	C
69	gi	sm	aug		0.2316	0.5580	C
70	gi	sm	jul		1.78E-15	0.5580	C
71	vb	sm	jul		-181E-14	9.1518	ABC

Effect=place*year Method=Tukey-Kramer(P<0.05) Set=9

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
72	vb			first	28.0002	6.4713	A
73	vb			third	5.9425	6.4713	ABC
74	gi			first	3.7587	0.3946	B
75	gi			third	0.6797	0.3946	C
76	vb			second	0.1819	6.4713	BC
77	gi			second	0.1819	0.3946	C

Summary SAS Printouts (Cont.)

Effect=place*size*year Method=Tukey-Kramer(P<0.05) Set=11

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
78	vb	lg		first	55.9459	9.1518	A
79	vb	lg		third	9.9275	9.1518	BC
80	gi	lg		first	6.8476	0.5580	B
81	vb	sm		third	1.9576	9.1518	BC
82	gi	lg		third	0.8285	0.5580	C
83	gi	sm		first	0.6699	0.5580	C
84	gi	sm		third	0.5308	0.5580	C
85	vb	lg		second	0.3639	9.1518	BC
86	gi	lg		second	0.3639	0.5580	C
87	vb	sm		first	0.05458	9.1518	BC
88	gi	sm		second	0	0.5580	C
89	vb	sm		second	-184E-14	9.1518	BC

Effect=place*month*year Method=Tukey-Kramer(P<0.05) Set=13

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
90	vb		aug	first	50.6646	11.2086	A
91	vb		sept	first	29.2595	11.2086	ABC
92	vb		sept	third	16.6312	11.2086	ABC
93	gi		aug	first	5.3346	0.6834	B
94	gi		jul	first	4.9367	0.6834	B
95	vb		jul	first	4.0766	11.2086	ABC
96	gi		sept	third	1.3334	0.6834	C
97	vb		aug	third	1.1964	11.2086	ABC
98	gi		sept	first	1.0048	0.6834	C
99	gi		aug	third	0.7056	0.6834	C
100	vb		aug	second	0.5458	11.2086	ABC
101	gi		aug	second	0.5458	0.6834	C
102	gi		jul	third	1.02E-14	0.6834	C
103	gi		sept	second	8.88E-15	0.6834	C
104	gi		jul	second	-178E-17	0.6834	C
105	vb		jul	second	-311E-15	11.2086	ABC
106	vb		jul	third	-111E-14	11.2086	ABC
107	vb		sept	second	-242E-14	11.2086	ABC

Summary SAS Printouts (Cont.)

Effect=plac*size*month*year Method=Tukey-Kramer(P<0.05) Set=15

Obs	place	size	month	year	Estimate	Standard Error	Letter Group
108	vb	lg	aug	first	101.17	15.8513	A
109	vb	lg	sept	first	58.5191	15.8513	ABC
110	vb	lg	sept	third	29.4967	15.8513	ABC
111	gi	lg	aug	first	10.6692	0.9665	B
112	gi	lg	jul	first	9.8734	0.9665	B
113	vb	lg	jul	first	8.1532	15.8513	BC
114	vb	sm	sept	third	3.7657	15.8513	BC
115	vb	sm	aug	third	2.1070	15.8513	BC
116	gi	sm	sept	first	2.0096	0.9665	C
117	gi	lg	sept	third	1.7691	0.9665	C
118	vb	lg	aug	second	1.0917	15.8513	BC
119	gi	lg	aug	second	1.0916	0.9665	C
120	gi	sm	sept	third	0.8977	0.9665	C
121	gi	lg	aug	third	0.7165	0.9665	C
122	gi	sm	aug	third	0.6948	0.9665	C
123	vb	lg	aug	third	0.2857	15.8513	BC
124	vb	sm	aug	first	0.1637	15.8513	BC
125	vb	lg	sept	second	6.54E-12	15.8513	BC
126	vb	sm	sept	first	3.84E-12	15.8513	BC
127	vb	sm	aug	second	3.25E-12	15.8513	BC
128	vb	sm	jul	second	2.63E-12	15.8513	BC
129	gi	sm	sept	second	2.31E-14	0.9665	C
130	gi	lg	sept	first	1.91E-14	0.9665	C
131	gi	lg	jul	third	1.73E-14	0.9665	C
132	gi	sm	jul	first	1.55E-14	0.9665	C
133	gi	sm	aug	first	1.2E-14	0.9665	C
134	gi	lg	jul	second	7.11E-15	0.9665	C
135	gi	sm	jul	third	8.88E-16	0.9665	C
136	gi	lg	sept	second	-355E-17	0.9665	C
137	gi	sm	aug	second	-107E-16	0.9665	C
138	gi	sm	jul	second	-129E-16	0.9665	C
139	vb	sm	jul	third	-164E-15	15.8513	BC
140	vb	lg	jul	third	-205E-14	15.8513	BC
141	vb	lg	jul	second	-325E-14	15.8513	BC
142	vb	sm	jul	first	-789E-14	15.8513	BC
143	vb	sm	sept	second	-114E-13	15.8513	BC

Disappearing Bi-Modal Distribution of Raw Data Calculation Exception

Benzo[a]Pyrene-Toxic Equivalents (Carcinogenicity)

The kruskal-wallis test for significance was being shifted by a specific group present in only one year of sampling. In order to account for this shift an appropriate chi squared test for significance was completed to supplement the kruskal-wallis test.

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-TEQs. No measure (x = 0), Greater than zero but less than twenty (0<x<20), and greater than or equal to twenty (x>=20). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.1121 0.7570 0.1308

We then categorized each year based on total samples collected as "S"

S =

36 36 35

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)=

4.0374 27.2523 4.7103
4.0374 27.2523 4.7103
3.9252 26.4953 4.5794

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<20, and the right column being x>=20.

T(x)=

7 15 14
5 31 0
0 35 0

We then calculated the Chi Squared test statistic "s" using the E(x) and T(x).

s =

42.6936

This was then used in the Chi Squared CDF operator along with DF = 4 ((Columns-1)*(Rows-1)) and subtracted from 1 to determine our p value.

p =

1.1979e-08

This shows a <u>significant</u> difference between the three sampling years. We must now apply this logic to the following arrangements.

2011 vs 2012
2011 vs 2013
2012 vs 2013

This will allow for a more appropriate test of significant difference than the Kruskal-Wallis test allows for taking into consideration the lack of an upper group in 2 out of the 3 sampling years ($x >= 20$).

<u>2011 vs 2012</u>

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-TEQs. No measure ($x = 0$), Greater than zero but less than twenty ($0<x<20$), and greater than or equal to twenty ($x>=20$). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.1667 0.6389 0.1944

We then categorized each year based on total samples collected as "S"

S =

36 36

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)=

6 23 7
6 23 7

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<20, and the right column being x>=20.

T(x)=

7	15	14
5	31	0

We then calculated the Chi Squared test statistic "s" using the E(x) and T(x).

s =

19.8986

This was then used in the Chi Squared CDF operator along with DF = 2 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

p =

4.7762e-05

This shows a <u>significant</u> difference between 2011 and 2012

<u>2011 vs 2013</u>

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-TEQs. No measure (x = 0), Greater than zero but less than twenty (0<x<20), and greater than or equal to twenty (x>=20). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.0986	0.7042	0.1972

We then categorized each year based on total samples collected as "S"

S =

36	35

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)=

3.5493	25.3521	7.0986
3.4507	24.6479	6.9014

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<20, and the right column being x>=20.

T(x)=

7	15	14
0	35	0

We then calculated the Chi Squared test statistic "s" using the E(x) and T(x).

s =

28.9917

This was then used in the Chi Squared CDF operator along with DF = 2 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

p =

5.0645e-07

This shows a <u>significant</u> difference between 2011 and 2013

<u>2012 vs 2013</u>

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-TEQs. No measure (x = 0), Greater than zero but less than twenty (0<x<20), and greater than or equal to twenty (x>=20). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.0704	0.9296	0

We then categorized each year based on total samples collected as “S”

S =

36 35

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)=

2.5352	33.4648	0
2.4648	32.5352	0

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<20, and the right column being x>=20.

T(x)=

5	31	0
0	35	0

We then calculated the Chi Squared test statistic “s” using the E(x) and T(x).

s =

5.2294

This was then used in the Chi Squared CDF operator along with DF = 2 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

p =

0.0732

This shows <u>no significant</u> difference between 2012 and 2013

Benzo[a]Pyrene-Mutagenic Equivalents (Mutagenicity)

The kruskal-wallis test for significance was being shifted by a specific group present in only one year of sampling. In order to account for this shift an appropriate chi squared test for significance was completed to supplement the kruskal-wallis test.

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-MEQs. No measure (x = 0), Greater than zero but less than eight (0<x<10), and greater than or equal to eight (x>=10). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.6355 0.2897 0.0748

We then categorized each year based on total samples collected as "S"

S =

36 36 35

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)

22.8785	10.4299	2.6916
22.8785	10.4299	2.6916
22.2430	10.1402	2.6168

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<10, and the right column being x>=10.

T(x)

20	8	8
34	2	0
14	21	0

We then calculated the Chi Squared test statistic "s" using the E(x) and T(x).

s =

43.6110

This was then used in the Chi Squared CDF operator along with DF = 4 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

p =

7.7273e-09

This shows a <u>significant</u> difference between the three sampling years. We must now apply this logic to the following arrangements.

2011 vs 2012
2011 vs 2013
2012 vs 2013

This will allow for a more appropriate test of significant difference than the Kruskal-Wallis test allows for taking into consideration the lack of an upper group in 2 out of the 3 sampling years ($x >= 10$).

<u>2011 vs 2012</u>

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-MEQs. No measure ($x = 0$), Greater than zero but less than eight ($0<x<10$), and greater than or equal to eight ($x>=10$). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.7500 0.1389 0.1111

We then categorized each year based on total samples collected as "S"

S =

36 36

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)

27 5 4
27 5 4

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<10, and the right column being x>=10.

T(x)

20	8	8
34	2	0

We then calculated the Chi Squared test statistic "s" using the E(x) and T(x).

s =

15.2296

This was then used in the Chi Squared CDF operator along with DF = 2 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

p =

4.9309e-04

This shows a <u>significant</u> difference between the 2011 and 2012.

<u>2011 vs 2013</u>

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-MEQs. No measure (x = 0), Greater than zero but less than eight (0<x<10), and greater than or equal to eight (x>=10). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.4789	0.4085	0.1127

We then categorized each year based on total samples collected as "S"

S =

36	35

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)

17.2394	14.7042	4.0563
16.7606	14.2958	3.9437

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<10, and the right column being x>=10.

T(x)

20	8	8
14	21	0

We then calculated the Chi Squared test statistic "s" using the E(x) and T(x).

s =

14.8753

This was then used in the Chi Squared CDF operator along with DF = 2 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

p =

5.8867e-04

This shows a <u>significant</u> difference between the 2011 and 2013.

<u>2012 vs 2013</u>

First we needed to determine where our groups are located. We chose three levels of concentration in terms of B[a]P-MEQs. No measure (x = 0), Greater than zero but less than eight (0<x<10), and greater than or equal to eight (x>=10). We then determined the probability of each outcome based on the data. This is listed as "s"

s =

0.6761	0.3239	0

We then categorized each year based on total samples collected as “S”

S =

36 35

From this we were able to determine the expected value of x (E(x)) where E(x) = the number of samples expected in each category based on total sample size.

E(x)

24.3380	11.6620	0
23.6620	11.3380	0

The true values of x (T(x)) were then arranged by category with the left column being x=0, the middle column being 0<x<10, and the right column being x>=10.

T(x)

34	2	0
14	21	0

We then calculated the Chi Squared test statistic “s” using the E(x) and T(x).

s =

24.0197

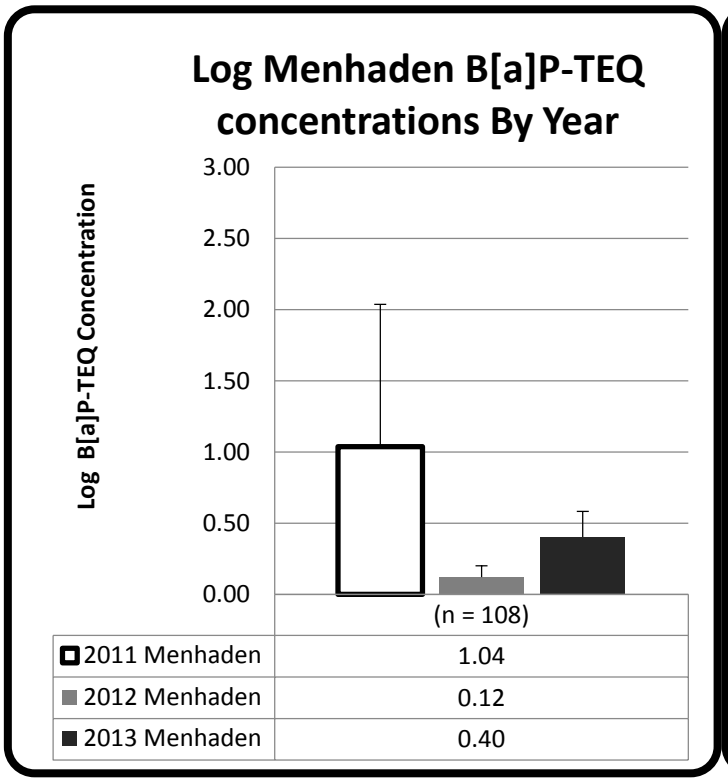
This was then used in the Chi Squared CDF operator along with DF = 2 ((C-1)*(R-1)) and subtracted from 1 to determine our p value.

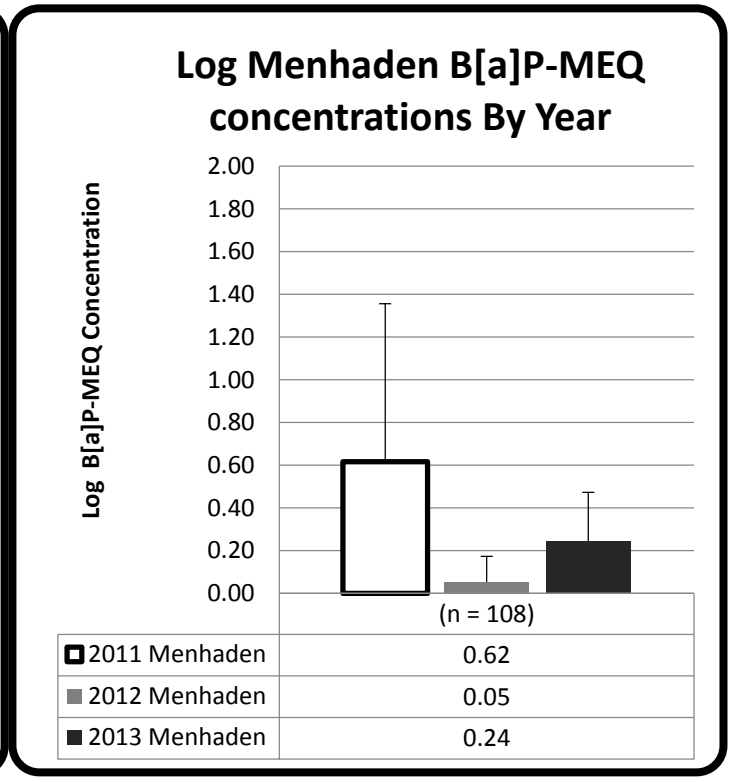
p =

6.0841e-06

This shows a <u>significant</u> difference between the 2012 and 2013.

		Significantly Different	p-value
B[a]P-TEQ		Yes	1.20E-08
	2011 vs 2012	Yes	4.78E-05
	2011 vs 2013	Yes	5.06E-07
	2012 vs 2013	No	0.0732
B[a]P-MEQ		Yes	7.73E-09
	2011 vs 2012	Yes	4.93E-04
	2011 vs 2013	Yes	5.89E-04
	2012 vs 2013	Yes	6.08E-06





MatLab Code for Statistical Analysis

TEQ Chi Squared

```
clear;

teq2013=[0.68 1.21 1.49 0.37 0.88 0.87 0.52 0.76 0.85
0.71 0.83 0.37
0.74 1.02 1.12 1.04 1.02 6.46 1.10 1.14 1.23
0.99 1.11 1.30 2.61
3.34 4.03 2.52 9.40 0.99 1.01 4.96 2.13 1.10
0.79];
teq2012=[0.27 0.55 0.35 0.27 0.21 0.22 0.15 0.12 0.17
0.10 0.13 0.22
0.42 0.00 1.93 0.30 0.00 1.93 0.15 0.23 0.00
0.00 0.16 0.12 1.58
0.27 0.25 0.37 0.44 0.44 0.16 0.30 0.00 0.17
0.16 0.19];
teq2011=[2.8 2.1 76.5 129.2 3.7 66.4 2.8 2.1 76.5
129.2 3.7 66.4
468.1 369.7 252.5 137.0 164.7 155.1 104.0 0.2 0.2
0.1 0.0 0.2 737.7
7.6 0.2 0.3 0.4 0.2 0.0 0.0 0.0 0.0
0.0 0.0];



no=0;


I=find (teq2011==0);
m(1,1)=length(I);
I=find (teq2011>0 & teq2011<20);
m(1,2)=length(I);
I=find (teq2011>=20);
m(1,3)=length(I);

if no==1;
I=find (teq2012==0);
m(1,1)=length(I);
I=find (teq2012>0 & teq2012<20);
m(1,2)=length(I);
I=find (teq2012>=20);
m(1,3)=length(I);
end
```

```
%move the if no statement to remove a set of data from the analysis
%make sure the matrix elements agree with a 2x3 matrix ex m(1,1)


I=find (teq2013==0);
m(2,1)=length(I);
I=find (teq2013>0 & teq2013<20);
m(2,2)=length(I);
I=find (teq2013>=20);
m(2,3)=length(I);




%disp(m);
s=sum(m);

s=s/sum(s)

m=m';
S=sum(m)

for k=1:2;
    e(k,:)=S(k)*s;



end;
disp(e);
m=m';
disp(m);

%m=m(:,1:2);
%e=e(:,1:2);


z=((m-e).^2)./e;
s=sum(sum(z))

p=1-chi2cdf(s,2)
```

Meq Chi Squared

```
clear;

meq2013=[0.00   0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
         0.00   0.00  0.00
0.00     0.00   0.86  0.45  0.61  5.26  0.82  0.66  0.67
         0.39   0.86  0.83 1.30
2.27     2.58   1.67  7.05  0.69  0.78  3.84  1.41  0.78
         0.50];
meq2012=[0.00   0.00  0.00  0.00  0.00  0.00  0.00  0.00
         0.00   0.00  0.00
0.00     0.00   3.28  0.00  0.00  3.27  0.00  0.00  0.00
         0.00   0.00  0.00  0.00
0.00     0.00   0.00  0.00  0.00  0.00  0.00  0.00  0.00
         0.00   0.00];
meq2011=[3.9    3.3   17.3  14.9  4.9   9.9   0.0   0.5   0.0
         0.0    0.0   0.0
155.7    103.6  44.2  13.5  9.5   9.0   6.0   0.0   0.0
         0.0    0.0   0.0   161.9
         13.6   0.0   0.0   0.0   0.0   0.0   0.0   0.0
         0.0    0.0   0.0];



no=0;

if no==1;
I=find (meq2011==0);
m(1,1)=length(I);
I=find (meq2011>0 & meq2011<10);
m(1,2)=length(I);
I=find (meq2011>=10);
m(1,3)=length(I);
end

I=find (meq2012==0);
m(1,1)=length(I);
I=find (meq2012>0 & meq2012<10);
m(1,2)=length(I);
I=find (meq2012>=10);
m(1,3)=length(I);
```

```
%move the if no statement to remove a set of data from the analysis
%make sure the matrix elements agree with a 2x3 matrix ex m(1,1)


I=find (meq2013==0);
m(2,1)=length(I);
I=find (meq2013>0 & meq2013<10);
m(2,2)=length(I);
I=find (meq2013>=10);
m(2,3)=length(I);




%disp(m);
s=sum(m);

s=s/sum(s)

m=m';
S=sum(m)

for k=1:2;
    e(k,:)=S(k)*s;



end;
disp(e);
m=m';
disp(m);

m=m(:,1:2);
e=e(:,1:2);


z=((m-e).^2)./e;
s=sum(sum(z))

p=1-chi2cdf(s,2)
```

PAH Analysis

```
pah2013=[6231 5740 4258 3698 5900 5198 4506 5631 5270
5477 5692 4253 3346
4192 3228 3152 2874 3479 2252 3581 3186 2038
2896 3358 2874 2859 3437
4463 3626 4880 1596 1456 1976 1916 1595 2053];
pah2012=[5084 6711 5162 4173 4520 6565 3969 6674 4800
3757 4816 4054 7795
5881 6175 9990 7189 9371 3986 4935 4643 3586
4153 3494 7800 5416 4467
7766 8763 9966 3470 4378 5360 4020 3825 4350];
pah2011=[11438 6233 7525 8988 9320 9154 6245 6458 13041
10132 7227 7007 2517
2422 3413 3229 1892 2666 2860 4429 2982 3672
4838 5877 13128 20523 13038
8438 10216 12907 5283 1901 5040 2952 1916 2073];


vbgi2011=[1 11 2 2 2 1 1 1 2
22 1
1 12 2 2 2 2 2 1 1
11 1 1
2 22 1 1 1 2 2 2];
vbgi2012=[1 11 2 2 2 1 1 1 2
22 1
1 12 2 2 1 1 1 2 2
21 1 1
2 22 1 1 1 2 2 2];
vbgi2013=[1 11 1 1 1 2 2 2 2
22 1
1 11 1 1 2 2 2 2 2
21 1 1
1 11 2 2 2 2 2 2];
% 1 = Vermillion Bay
% 2 = Grand Isle

n=1/8;
pah2011=pah2011.^n;
pah2012=pah2012.^n;
pah2013=pah2013.^n;

subplot(3,1,1);
hist(pah2011);
set(gca,'xlim', [1000 21000].^n);
```

```
set(gca,'xtick', [1000 5000 10000 15000 20000].^n,'xticklabel',{'1000' '5000' '10000' '15000'
'20000'});
xlabel('2011 Data')
subplot(3,1,2);
hist(pah2012);
set(gca,'xlim', [1000 21000].^n);
set(gca,'xtick', [1000 5000 10000 15000 20000].^n,'xticklabel',{'1000' '5000' '10000' '15000'
'20000'});
xlabel('2012 Data')
subplot(3,1,3);
hist(pah2013);
set(gca,'xlim', [1000 21000].^n);
set(gca,'xtick', [1000 5000 10000 15000 20000].^n,'xticklabel',{'1000' '5000' '10000' '15000'
'20000'});
xlabel('2013 Data')
[p,anovatab,stats]=kruskalwallis([pah2011 pah2012 pah2013],[ones(1,length(pah2011))
2*ones(1,length(pah2012)) 3*ones(1,length(pah2013))]);
pause;
compare=multcompare(stats);
```

TEQ Analysis

```
teq2013=[0.68   1.21   1.49   0.37   0.88   0.87   0.52   0.76   0.85
         0.71   0.83   0.37   0.74
         1.02   1.12   1.04   1.02   6.46   1.10   1.14   1.23
         0.99   1.11   1.30 2.61     3.34
         4.03   2.52   9.40   0.99   1.01   4.96   2.13   1.10
         0.79];
teq2012=[0.27   0.55   0.35   0.27   0.21   0.22   0.15   0.12   0.17
         0.10   0.13   0.22   0.42
         0.00   1.93   0.30   0.00   1.93   0.15   0.23   0.00
         0.00   0.16   0.12   1.58   0.27
         0.25   0.37   0.44   0.44   0.16   0.30   0.00   0.17
         0.16   0.19];
teq2011=[2.8    2.1    76.5   129.2  3.7    66.4   2.8    2.1    76.5
         129.2  3.7    66.4   468.1
         369.7  252.5  137.0  164.7  155.1  104.0  0.2    0.2
         0.1    0.0    0.2    737.7  7.6
         0.2    0.3    0.4    0.2    0.0    0.0    0.0    0.0
         0.0    0.0];

vbgi2011=[1     11     2      2      2      1      1      1      2
         22     1
1        12     2      2      2      2      2      1      1
         11     1      1
```

```
2                       22      1       1       1       2       2       2];
vbgi2012=[1             11      2       2       2       1       1       1       2
                        22      1
1                       12      2       2       2       2       2       1       1
                        11      1       1
2                       22      1       1       1       2       2       2];
vbgi2013=[1             11      1       1       1       2       2       2       2
                        22      1
1                       11      1       1       2       2       2       2       2
                        21      1       1
1                       11      2       2       2       2       2       2];

% 1 = Vermillion Bay
% 2 = Grand Isle

n=1;
teq2011=teq2011.^n;
teq2012=teq2012.^n;
teq2013=teq2013.^n;

subplot(3,1,1);
hist(teq2011);
set(gca,'xlim', [0 800].^n);
set(gca,'xtick', [0.1 1 10 100 400 800].^n,'xticklabel',{'0.1' '1' '10' '100' '400' '800'});
xlabel('2011 Data')
subplot(3,1,2);
hist(teq2012);
set(gca,'xlim', [0 800].^n);
set(gca,'xtick', [0.1 1 10 100 400 800].^n,'xticklabel',{'0.1' '1' '10' '100' '400' '800'});
xlabel('2012 Data')
subplot(3,1,3);
hist(teq2013);
set(gca,'xlim', [0 800].^n);
set(gca,'xtick', [0.1 1 10 100 400 800].^n,'xticklabel',{'0.1' '1' '10' '100' '400' '800'});
xlabel('2013 Data')


no=0;


I=find (teq2011==0);
m(1,1)=length(I);
I=find (teq2011>0 & teq2011<20);
m(1,2)=length(I);
I=find (teq2011>=20);
m(1,3)=length(I);
```

```
I=find (teq2012==0);
m(2,1)=length(I);
I=find (teq2012>0 & teq2012<20);
m(2,2)=length(I);
I=find (teq2012>=20);
m(2,3)=length(I);

I=find (teq2013==0);
m(3,1)=length(I);
I=find (teq2013>0 & teq2013<20);
m(3,2)=length(I);
I=find (teq2013>=20);
m(3,3)=length(I);

%disp(m);
s=sum(m);

s=s/sum(s)

m=m';
S=sum(m)

for k=1:3;
   e(k,:)=S(k)*s;



end;
disp(e);
m=m';
disp(m);

z=((m-e).^2)./e;
s=sum(sum(z))

p=1-chi2cdf(s,4)


[p,anovatab,stats]=kruskalwallis([teq2011 teq2012 teq2013],[ones(1,length(teq2011))
2*ones(1,length(teq2012)) 3*ones(1,length(teq2013))]);
```

Meq Analysis

```
meq2013=[0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
0.00 0.00 0.86 0.45 0.61 5.26 0.82 0.66 0.67
0.39 0.86 0.83 84.92
1.30 2.27 2.58 1.67 7.05 0.69 0.78 3.84 1.41
0.78 0.50];
meq2012=[0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
0.00 0.00 3.28 0.00 0.00 3.27 0.00 0.00 0.00
0.00 0.00 0.00 0.00
0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00
0.00 0.00];
meq2011=[3.9 3.3 17.3 14.9 4.9 9.9 0.0 0.5 0.0
0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0
155.7 103.6 44.2 13.5 9.5 9.0 6.0 0.0 0.0
0.0 0.0 0.0 161.9
13.6 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0
0.0 0.0];
```

```
vbgi2011=[1 11 2 2 2 1 1 1 2
22
1 11 2 2 2 2 2 2 1
11 1
1 12 2 2 1 1 1 2 2
2];
vbgi2012=[1 11 2 2 2 1 1 1 2
22
1 11 2 2 2 2 2 2 1
11 1
1 12 2 2 1 1 1 2 2
2];
vbgi2013=[1 11 1 1 1 2 2 2 2
22
1 11 1 1 1 2 2 2 2
22 1
1 11 1 1 2 2 2 2 2
2];
```

% 1 = Vermillion Bay
% 2 = Grand Isle

```
I=find(vbgi2011==1);
vb2011=meq2011(I);
I=find(vbgi2011==2);
gi2011=meq2011(I);


I=find(vbgi2012==1);
vb2012=meq2012(I);
I=find(vbgi2012==2);
gi2012=meq2012(I);

I=find(vbgi2013==1);
vb2013=meq2013(I);
I=find(vbgi2013==2);
gi2013=meq2013(I);

[p,anovatab,stats]=kruskalwallis([vb2011 vb2012 vb2013 gi2011 gi2012
gi2013],[ones(1,length(vb2011)) 2*ones(1,length(vb2012)) 3*ones(1,length(vb2013))
4*ones(1,length(gi2011)) 5*ones(1,length(gi2012)) 6*ones(1,length(gi2013))]);
pause;
compare=multcompare(stats);
pause;




n=1/4;
meq2011=meq2011.^n;
meq2012=meq2012.^n;
meq2013=meq2013.^n;




subplot(3,1,1);
hist(meq2011);
xlabel('2011 MEQ');
set(gca,'xlim',[0 800].^n,'xtick',[0.1 1 10 50 100 200 400 800].^n,'xticklabel',{'0.1' '1' '10' '50'
'100' '200' '400' '800'});
subplot(3,1,2);
hist(meq2012);
xlabel('2012 MEQ');
```

```
set(gca,'xlim',[0 800].^n,'xtick',[0.1 1 10 50 100 200 400 800].^n,'xticklabel',{'0.1' '1' '10' '50'
'100' '200' '400' '800'});
subplot(3,1,3);
hist(meq2013);
xlabel('2013 MEQ');
set(gca,'xlim',[0 800].^n,'xtick',[0.1 1 10 50 100 200 400 800].^n,'xticklabel',{'0.1' '1' '10' '50'
'100' '200' '400' '800'});
```

IACUC ACUP

PROTOCOL NUMBER: _____ **APPROVAL DATE: _____**

LSU PROTOCOL FOR ANIMAL CARE AND USE

SECTION 1: Principal Investigator

Name: Dr. Ralph J. Portier	Department: Environmental Sciences
Office Phone: 225-578-4287 Home Phone: 225-921-1518	E-mail Address: rportie@lsu.edu

SECTION 2:

A. Project Title (Enter the name of your project/course number below.)

A comparative and correlative study of PAH accumulation within Gulf and Atlantic menhaden populations versus Gulf coastal and Atlantic coastal oyster populations.

B. Anticipated Project Start Date

Summer 2011

SECTION 3:

A. Animal Species

Species (common name): *Brevoortia patronus* and *Brevoortia tyrannus* (Gulf and Atlantic Menhaden)	Strain:

Number of animals needed: Year 1:___**876**__ Year 2:_____ Year 3:_____	Maximum number needed at one time: 51	Number of animals to be placed in each group: 17

TOTAL: 876		

Yes:	No:	Are you using wild, invasive, or non-native species for which permits are necessary? (ATTACH COPY OF PERMIT) Note: a copy of the permit(s) must be received before animal work begins.
	X	

B. Source of Animals

	Order through DLAM
X	Other (list source): Natural capture off the Louisiana, Florida, and New Jersey coasts as well as tanks located at LUMCON in Cocodrie, LA. Other possible locations of tanks: Key West, Florida and in New Jersey
	Transfer from Approved Protocol (list protocol number):

C. Location of Animal Housing

	DLAM Vivarium
	Life Sciences Vivarium
	SVM Barns (list site):
	SVM Fish Building
	Research Herd
	LAES (list site):
X	Other (list site): LUMCON (Louisiana Universities Marine Consortium) Marine Center Cocodrie, LA
	Field Study (Do not complete D and E)

Animal housing and veterinary care have been coordinated with DLAM office or LSU Agricultural Center Unit.

Yes: _____
No: ____**X**____

Name of Animal Housing Representative Contacted (typed):

Signature (required): _____

Mr. Michael Keowen's signature is also required below if you plan to use animals from the EHSP Herd:

_____

D. Special Husbandry Requirements

Do your animals have special needs to be address by DLAM?

xX	Housing under the direct care of DLAM is not required. (e.g. SVM fish building)
	NO. Animals will be cared for according to standard operating procedures of DLAM.
	YES (complete table below)

TEMPERATURE RANGE	(F)	Humidity:	(%)	
LIGHT CYCLE	Hours light:	Hours dark:		
CAGING	Type:	Size:	ABSL2:	ABSL3:
BEDDING/LITTER	Type:	Autoclaved:	Changes/week:	
WATER	Sterile:	De-ionized:	Acidified:	Tap: Other:
DIET	List Special Feeding Requirements:			
OTHER SPECIAL NEEDS	List:			

E. Animal Management

Individual (or groups of) animals are identified by:

	Tag
	Tattoo
X	Cage, Tank, or Stall Card
	Other. List type of identification:

Check all applicable below:

CARE OF SICK ANIMALS		DISPOSAL OF DEAD ANIMALS		PEST CONTROL	
X	Call Investigator	X	Call Investigator		Call Investigator
	Clinician to Treat		Necropsy		Pesticides OK
	Euthanasia		Disposal. List any special requirements:	X	No Pesticides

F. Disposition of Animals

What will be done with any animals at the conclusion of the project? Mark all that apply.

xX	Animals will be euthanized.
	DLAM/LAES has permission to REASSIGN animals to another IACUC-approved protocol.
	TRANSFER animals to the following IACUC-approved protocol(s). List Protocol Number(s):
	Catch and release (applies to field studies).
	Return to owner/supplier.
	Other (please state):

	TRANSFER animals to another institution (please state):

SECTION 4: Layman's Summary of Research/Teaching

Provide a brief (100 word maximum), non-scientific (i.e., no jargon) explanation of the purpose, materials, and methods in the block below for the benefit of reviewers and animal handlers who need to understand the research project.

Menhaden will be caught in naturally occurring waters and packed in ice. Once they are in the lab, they will be placed in an ultra cold freezer (-80 degrees Celsius) and then freeze dried. They will be blended up and a tissue extraction will be done to identify PAH (Polycyclic Aromatic Hydrocarbons which are a constituent of oil) concentrations in the organism.

For those menhaden from LUMCON, they will be fed a clean diet to control tissue concentrations for 1 month. The above procedure will be carried out on them in order to determine the natural amount of PAHs in the body of menhaden.

SECTION 5: Investigator's Statement. Assurances for the Humane Care and Use of Vertebrate Animals.

By signing this form, we agree to abide by the Policy for the Care and Use of Animals of Louisiana State University. This project will be in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (except as explained in the accompanying protocol), and the Louisiana State University Animal Welfare Assurance on file with the U.S. Public Health Service.

We further assure the Committee that: 1) We will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; and 3) the fewest number of animals required to produce valid results are being used in this study. (Add additional rows as needed)

Principal Investigator Signature:	Principal Investigator Name (Typed): Ralph J. Portier	Title/Rank: Dr./Full Professor	Date:
Co-Investigator Signature:	Co-Investigator Name (Typed): Gregory M. Olson	Title/Rank: Mr./Graduate Student	Date:
Surgeon Signature:	Surgeon Name (Typed):	Title/Rank:	Date:

SECTION 6: Hazardous Materials

Will zoonotic or recombinant, radioactive, or hazardous chemical agents be **PRESENT IN THE ANIMAL ROOM?**

If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the IBRDS Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Final approval will not be granted until IBRDS approval is received by the IACUC. Similarly, if hazardous chemicals are to be used in the animal room, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE** health and safety measures for animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and **attach a copy of your SOP(s) to this protocol proposal.**

Will Zoonotic Agents be used? ___ YES __X__ NO

List agents:_____

Has request for use of agents been submitted to the Institutional Biological Recombinant DNA Safety (IBRDS) Committee? ___ YES __X__ NO

If not, please contact either Dr. Greg Hayes, Biological Safety Manager, at (225) 578-4658 / ghayes@lsu.edu in the Office of Occupational and Environmental Safety; or Dr. Gregg Pettis, Chair of the IBRDS, at (225) 578-2798 / gpettis@lsu.edu in the Department of Biological Sciences.

Also note that a Door Posting Form for the Animal Room is required when using zoonotic agents. Please submit this form to the IBRDS along with your request for use of agents. This form must be signed by either Dr. Hayes or Dr. Pettis. (Blank form is attached at end of protocol. It can also be obtained from Dr. Hayes.)

Will Recombinant DNA and/or Virus Vectors be used? ___ YES __X__ NO

List:_____

Has request for use been submitted to the IBRDS Committee? ___ YES __X__ NO

If not, please contact either Dr. Greg Hayes, Biological Safety Manager at (225) 578-4658 / ghayes@lsu.edu in the Office of Occupational and Environmental Safety; or Dr. Gregg Pettis, Chair of the IBRDS, at (225) 578-2798 / gpettis@lus.edu in the Department of Biological Sciences.

Will radioisotopes be used? ___ YES _X_ NO

List isotope(s):_____

Are you certified by the Radiation Safety Committee? _X_ YES ___ NO

Will hazardous chemicals be used? ___ YES _X_ NO

List compound(s):_____

Please note that approval from the Mr. Jerry Steward, Chemical Safety Manager, is required when using hazardous chemicals in the animal facilities. You can contact him at (225) 578-5640 / jsteward@lsu.edu regarding a list of hazardous chemicals, and approval of these chemicals.

SECTION 7: Type of Project and Narrative Statement

	TYPE B – Animals being bred, conditioned, or held for use in teaching or research but not yet used for such purposes. (e.g. a breeding colony of mice which will transfer individuals to experimental protocols.)
xX	**TYPE C -** Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to the procedures that induce pain or distress.
	TYPE D - Pain or distress will be relieved by appropriate therapy, e.g. sedatives, analgesics, anesthetics, or euthanasia.
	TYPE E - Drug intervention for pain or distress would interfere with the protocol. **(If this block is checked, specific justification MUST be provided here.)**

Federal regulations mandate that you provide **written, narrative statements** for all projects.

1. You must state that "the proposed activities do not unnecessarily duplicate previous experiments". In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc.) If an electronic database was used, include database, years and words searched, and date of search.

The proposed activities do not unnecessarily duplicate previous experiments based on a lack of similar research within these expansive databases listed below. The hyperlinks below are linked to the exact search terms used, databases included, and years searched that were performed.

http://www.lib.lsu.edu/apps/onoffcampus.php?url=http://search.ebscohost.com/login.aspx?direct=true&db=a9h&db=syh&db=fzh&bquery=(XX+%22menhaden%22%5b100%5d+AND+(XX+%22polycyclic

%22%5b76%5d+OR+XX+%22hydrocarbon%22%5b64%5d+OR+XX+%22aromatic%22%5b58%5d+OR+XX+%22accumulation%22%5b45%5d))&type=1&site=ehost-live&scope=site

http://www.lib.lsu.edu/apps/onoffcampus.php?url=http://search.ebscohost.com/login.aspx?direct=true&db=a9h&db=syh&db=fzh&bquery=(XX+%22menhaden%22%5b100%5d+AND+(XX+%22pah%22%5b79%5d+OR+XX+%22accumulation%22%5b45%5d))&type=1&site=ehost-live&scope=site

Database used: Academic Search Complete, Science & Technology Collection, Wildlife & Ecology Studies Worldwide

Years searched: 1976 – 2011

Words searched: PAH accumulation in menhaden, Polycyclic Aromatic Hydrocarbon accumulation in menhaden

Date of search: May 18, 2011

Note: Address the following items only if you indicated project **Type D or E**.

2. You must indicate that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Describe any alternatives available and why they are not appropriate.

3. Describe the methods you used to determine that alternatives to such procedures were not available (Databases, years and words searched, date of search etc.). Put your statements in the block below.

Database used:_____
Years Searched:_____
Words Searched:_____
Date of Search:_____

SECTION 8: Animal Treatment Checklist

Check "Yes" or "No" to each of the following questions. Provide an explanation in Section 9 for any "yes" answers.

Q#	YYES	NNO		
11		xX	Will animals be restrained? *(Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)*	Not applicable
22		xX	Will animals be fasted?	Not applicable

23		xX	Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? Include drug, dose, route and frequency, and how animals will be monitored in Section 9.	**Who will administer?** **_____**
44		xX	Are neuromuscular blocking agents to be used? Include drug, dose, route and frequency, and how animals will be monitored in Section 9.	**Who will administer?** **_____**
55		xX	Will surgical procedures be employed? Check all that apply! Are they: Survival_____ Multiple-Major Survival _____ Multiple-Minor Survival _____ *Major survival surgery= Any procedure which penetrates **and** exposes a body cavity or alters function. Terminal_____ In addition to describing surgical procedures in Sec. 9, you must indicate the time frame between multiple procedures. **Note**: *Survival mammalian surgeries must be conducted aseptically, and major surgical procedures performed on non-rodent species must be conducted in a dedicated surgical facility.*	**Who** will perform surgery? **_____** If survival: **1) Who** will be responsible for recovery of the animals? _____ **2) Who** will maintain post-operative records? _____ **3) Where** will records be maintained? _____ **4) Who** will provide post-operative analgesics? _____
66		xX	Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)?	Not applicable.
77		xX	Is death an endpoint in your experimental procedure? **Note**: *Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.*	Not applicable.

88		xX	Are there emergency treatments by the DLAM veterinary staff that would not be allowed?	Not applicable.
99	xX		Will animals be euthanized during or at the close of the study?	**Who will perform euthanasia?** **Gregory Olson and/or Dr. Ralph Portier__**
010		xX	Will animals be used for antibody production?	Not applicable.
111		xX	Will Complete Freund's Adjuvant be used? **Must be scientifically justified in Section 9.**	Not applicable.
112		xX	Will other adjuvants be used?	**If yes, please specify here:** **_____**
113		xX	Will blood be collected? **Note**: *Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.*	**How often? _____** **Volume? _____** **Who will collect blood? _____**
114		xX	Will live animals be taken from approved housing facilities for procedures followed by their return later that day? **Note:** *Animals may not be housed outside of the Vivarium (e.g. in a laboratory) overnight.*	**If yes, please specify to which building and room/rooms the animals will be taken:** **Note:** *This room(s) must be approved for use before the animals can be brought there. Contact IACUC coordinator for list of approved rooms.*
115		xX	Will live animals be brought onto campus for demonstration, teaching, euthanasia, etc. for which no housing is required?	**If yes, please specify to which building and room/rooms the animals will be taken:** **Note:** *This room(s) must be approved for use before the animals can be brought there. Contact IACUC coordinator for list of approved rooms.*

SECTION 9: Summary of Procedures

Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a scientific discipline unrelated to yours. Do not use jargon. **Please answer each statement in its own expanding box.**

1 a: What is the rationale for using animals?
Menhaden are filter feeding fish. They are the entry points for PAHs in the food chain as well as possible vectors of PAH transfer to humans. (Menhaden are used for fish meal as well as any commodity that has fish oil in it such as cosmetics and fish oil supplements)

1 b: Why should this study be done?
To asses PAH accumulation after the prolonged oiling of the Gulf of Mexico in juvenile menhaden. Menhaden are also an extremely important commercial species and this study will help understand the impact of the Gulf oil spill on the health of this species. Additionally they are a commercially harvested fish with potential consumer product impacts.

1 c: What hypothesis will be tested?
Hypothesis – the addition of oil due the spill has increased PAH accumulation in filter feeding marine organisms.

2. Explain how and/or why the particular animal species was selected?
It is a near shore filter feeding organism that can be compared to PAH accumulation in oysters (an immobile filter feeding organism located near shore as well)

3. Explain how you arrived at the number of animals to be used (e.g., power analysis in comparison studies, permitted animal limits in field studies, etc).

Menhaden will be collected from 2 sites here in Louisiana over the period of 6 sampling events. Menhaden will also be collected twice from a location in Florida and twice from a location in New Jersey. This is a total of 16 separate sampling events. For each location and event I will need a maximum of 51 menhaden. There will also be a collection of menhaden as a control from the facilities at LUMCON. This makes the total maximum number of menhaden 876 (17 * 51). We will be collecting fish that range from 3 to 8 inches. This means that the total number of menhaden required will vary greatly and the numbers presented here are estimates based on the maximum number needed for a 90% power. My numbers are over estimated because I am attempting to account for size and weight variation. The statistical analysis assumes uniform individuals. The power analysis was performed using GPower 3.1.2

[1] *-- Monday, May 23, 2011 -- 10:37:43*

t tests – Correlation: Point biserial model

Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	One (Do not need to know if the PAH level is too small)
	Effect size |ρ|	=	0.1 (Want to measure a small effect change in PAH concentration)
	α err prob	=	0.05 (Will accept a possible 5% type 1 error rate)
	Power (1−β err prob)	=	0.90 (Will accept a power of 90%)
Output:	Noncentrality parameter δ	=	2.9301636
	Critical t	=	1.6466525
	Df	=	848
	Total sample size	=	850
	Actual power	=	0.9002490

4. Provide a complete description of the proposed use of the animals. Describe the experimental design of the study. Include a list of any physical, chemical or biological agents (name, dose, volume, route, frequency) that may be administered. If animals are being transported between facilities, describe conditions of transport. If multiple surgical procedures are planned you must include the time frame between those procedures. If food or fluid restriction and/or restraint are used you must

include the duration of each. Use tables and outlines to indicate group assignments and study progression.

The menhaden will be used to determine PAH accumulation within filter feeding vertebrates. They will be captured via a cast net and then bagged into groups of 8-17 in a 10% TMS, MS 222 solution (based on size) and then placed on ice until they are placed in the freezer. Once the menhaden are cooled to a temperature of -80 degrees Celsius they will be placed in a freeze dryer for 24 hours to remove all moisture. They will then be homogenized with sodium sulfate to create more surface area (the sodium sulfate will remove any remaining water). The mixture will then go through the process of Ultrasonic Extraction EPA Method 3550C. They will then go through a modified cleanup technique that combines EPA Method 3630C and 3611B. The remaining liquid will then be evaporated down to 1 ml and be analyzed through Gas Chromatography. Any menhaden that may be transported will be placed in tanks of water collected from the location of their collection. No menhaden will be transferred any more than 3 hours.

1) Collection site (includes collection from 1 time lab site)
    a) Bagged in a group of 8-17
        i) Placed in 10% TMS,MS 222 solution
        ii) Placed on ice
    b) Bagged in a group of 8-17
        i) Placed in 10% TMS,MS 222 solution
        ii) Placed on ice
    c) Bagged in a group of 8-17
        i) Placed in 10% TMS,MS 222 solution
        ii) Placed on ice
2) Lab site (transported from collection site on ice)
    a) Ultra Cold Freezer
        i) Temp down to -80 degrees Celsius
    b) Freeze Dryer
        i) Removes water
    c) Ultrasonic Extraction
        i) GC analysis of total PAHs within the composites

5 a: Describe expected adverse effects.
The menhaden will be caught via a cast net in the same manner recreational fishermen catch them. The menhaden will be euthanized prior to any adverse effects.

5 b: What is the likelihood of these effects (high, low, unknown)?
Every menhaden will be caught in a net and then euthanized.

6. Describe procedures designed to assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. For anesthesia and survival surgeries, include a description of post-procedural care and monitoring. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. Include any conditions where veterinary treatment would not be allowed. Specify which treatments would not be allowed, and include a scientific justification. It is advisable that you obtain input from LSU's Attending Veterinarian (Dr. David Baker) or from another veterinarian familiar with the species to be used**.**

Appropriate netting will be use to catch fish. Live fish will be transported in a manner that maximizes their survival rate. The menhaden will be placed in a 10% solution of TMS, MS 222 and water before being placed on ice. This will alleviate any stress the fish would have felt as the Dissolved Oxygen levels go down and the temperature decreased.

7. Describe any euthanasia method to be used. Even if euthanasia is not planned please provide a "What If" scenario in the event of unforeseen circumstances. Justify any deviation from AVMA Guidelines on Euthanasia, 2007. Text, viewable at http://avma.org/resources/euthanasia.pdf.

The menhaden will be placed in a 10% solution of TMS, MS 222 and water before being placed on ice.

SECTION 10: Investigator Training

In accordance with IACUC policy, all personnel conducting animal-based research must attend a Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques they are responsible for.

List all persons involved in animal care and use for this study below. Add additional lines as needed.

***Exemption from wet lab training for specific procedures needed for the protocol may be obtained by written request to the IACUC. Training wet labs will be scheduled on an 'as needed' basis. Please contact Ms. Dawn Best-Desjardins at 578-9643 or dbest@vetmed.lsu.edu to sign up for these courses.**

Name	Online Investigator Training Course Attended? (Indicate Yes or No)	Date Attended	Species Wet Lab Taken? (Indicate Yes or No)*	Date Attended or Exempted	Training or Experience? (Indicate Yes or No)**
Dr. Ralph J. Portier	Yes	June 22, 2011	No		Yes
Gregory M. Olson	Yes	May 19, 2011	No		Yes
Dr. John R. Sowa	Yes	June 29, 2011	No		Yes
Dr.Carolyn Bentivegna			No		Yes

****The person named has training/experience in assigned procedures for this protocol.**

Who will train individuals for participation in protocol procedures? Answer in the block below.

Dr. Ralph J. Portier

Personnel participating in the project must complete the online investigator training course once every three years. Those who have not attended the online course or the applicable Species Wet Lab, will have **six (6) months** from the approval date of the project to complete them.

The online investigator training course is offered through the AALAS Learning Library www.aalaslearninglibrary.org . Training wet labs will be scheduled on an **'as needed'** basis. Please contact Ms. Best-Desjardins at 578-9643 or dbest@vetmed.lsu.edu to sign up for these courses.

SECTION 11: Occupational Health and Safety

It is the responsibility of the principal investigator to conduct a hazard analysis and risk assessment to determine if personnel involved in the proposed study should participate in the Occupational Health and Safety Program administered through DLAM and the Student Health Center. Currently, there is no direct cost for participation in the program**. All persons listed in Section 10 must read the following and indicate level of participation with their signature. Add additional rows in the table as needed.**

The Division of Laboratory Animal Medicine operates an Occupation Heath Program (OHP). Participation is voluntary, and is open to all personnel with direct or indirect contact with animals used in teaching and research, their bodily products, or materials to which they may be exposed, as described in this protocol. Eligible persons include facility services personnel, animal caretakers, principal investigators, technical staff, graduate and other student workers, and post-

doctoral and visiting scientists. All medical information is kept confidential, and is retained by the Student Health Center. You have the right to refuse any and all procedures recommended.

To determine the extent of your participation in the OHP, discuss with the principal investigator named on this protocol, and/or your health professional, any potential physical, chemical, or infectious hazards to which you may be exposed while working on the project. Whether or not you participate, questions related to health risks should be directed to Dr. Tim Honigman, Campus Physician, at the Student Health Center.

If you are at increased risk of illness or injury due to drug-related immune suppression, HIV infection, pregnancy, concurrent illness, musculoskeletal problems, etc., you are advised to discuss your risks with Dr. Honigman, your physician, or another health professional.

To participate in the OHP, contact Ms. Dawn Best-Desjardins at 578-9643 or dbest@vetmed.lsu.edu for information.

Printed Name: Dr. Ralph J. Portier	Signature:	____I choose to participate __X__I choose NOT to participate
Printed Name: Gregory M. Olson	Signature:	____I choose to participate __X__I choose NOT to participate
Printed Name: Dr. John R. Sowa	Signature:	____I choose to participate __X__I choose NOT to participate
Printed Name: Dr. Carolyn Bentivegna	Signature:	____I choose to participate __X__I choose NOT to participate

Exemption Letter

Institutional Animal Care and Use Committee June 20, 2011
Division of Laboratory Animal Medicine
LSU School of Veterinary Medicine
Skip Bertman Drive
Baton Rouge, LA 70803

To whom it may concern:

My name is Dr. Ralph Portier of the Department of Environmental Sciences. I am completing the required protocol for using menhaden (an estuarine fish) in a study that our laboratory is conducting and it requires a wet lab training session for our proposed work. I am asking that we are granted an exemption from this wet lab training on the grounds that I have 34 years of experience with microcosms and small scale natural habitats including aquarium habitats designed for estuarine fish. All designs and decisions will be under my direct supervision and all other co-PIs will be trained to handle the aquarium systems if we actually need these laboratory scale microcosms. The need for us to maintain an estuarine system is virtually nonexistent due to our partnership with Dr. Edward Chesney at LUMCON and his lab's ability to house menhaden.

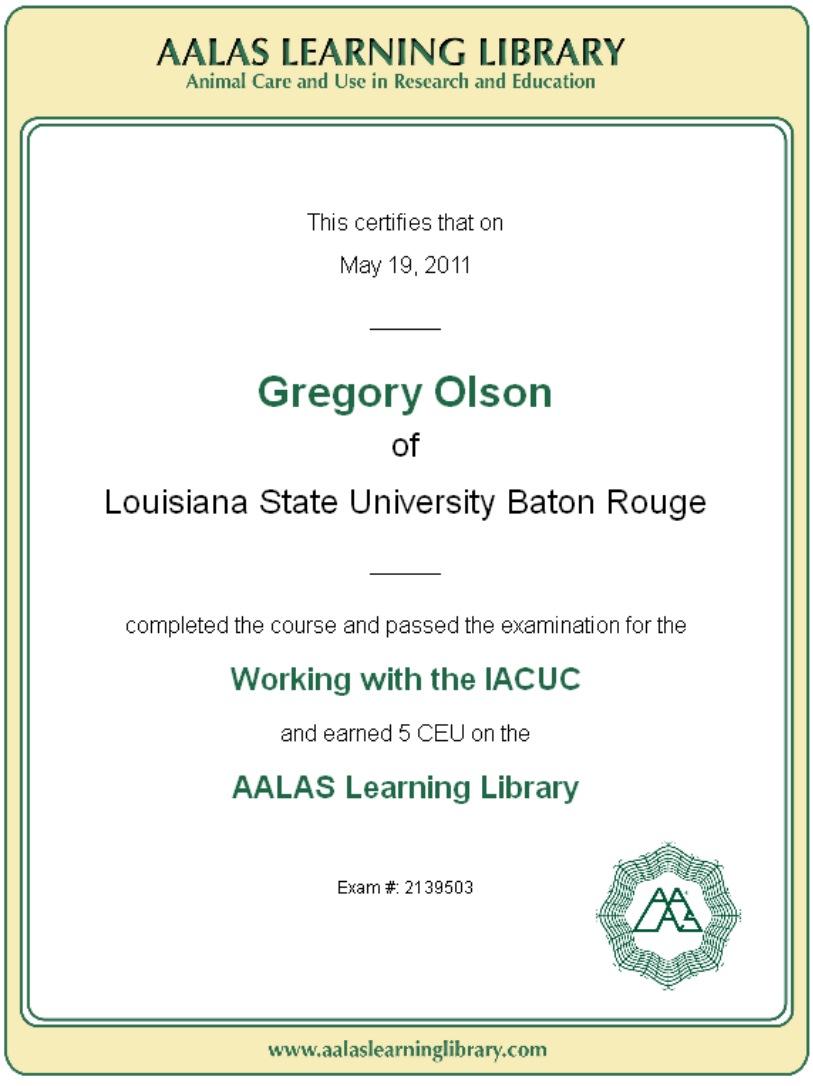
I am asking on behalf of myself and all other co-PIs for exemption on the grounds of my experience with small scale habitat systems as well as the fact that we will not be housing any live animal in our direct care.

Sincerely,

Dr. Ralph J. Portier
Professor of Environmental Sciences
School of the Coast & Environment
Louisiana State University
1165 EC&E Bldg
Baton Rouge, LA 70803

Office: (225)-578-4287
Cell: (225)-921-1518
Fax: (225)-578-4286
Email: rportie@lsu.edu

IACUC Training Certificate



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May 19, 2011

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Louisiana State University Baton Rouge

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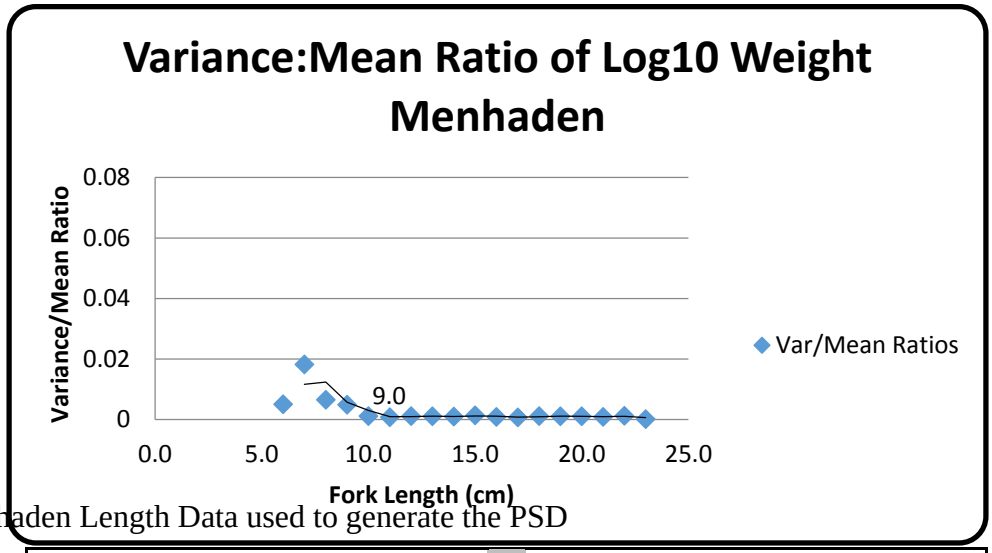
D: Chapter 4

Data Tables and Graphs

Variance: Mean Ratio for Gulf Menhaden Length Categories

6.0	Mean	0.67
	variance	0.003406004
	Var/mean	0.005047907
7.0	Mean	0.85
	Variance	0.015504066
	Var/mean	0.01820851
8.0	Mean	1.03
	Variance	0.006714249
	Var/mean	0.006526123
9.0	Mean	1.19
	Variance	0.005817815
	Var/mean	0.004883759
10.0	Mean	1.31
	Variance	0.00151635
	Var/mean	0.001153526
11.0	Mean	1.45
	Variance	0.001109882
	Var/mean	0.000765884
12.0	Mean	1.54
	Variance	0.001774881
	Var/mean	0.001149107
13.0	Mean	1.64
	Variance	0.001808396
	Var/mean	0.001102144
14.0	Mean	1.73
	Variance	0.001649
	Var/mean	0.000954463
15.0	Mean	1.82
	Variance	0.002509732

	Var/mean	0.001380975
16.0	Mean 1.92	
	Variance	0.001710095
	Var/mean	0.000890954
17.0	Mean 2.00	
	Variance	0.001404444
	Var/mean	0.000702043
18.0	Mean 2.07	
	Variance	0.002345547
	Var/mean	0.001134341
19.0	Mean 2.12	
	Variance	0.002345547
	Var/mean	0.001103938
20.0	Mean 2.20	
	Variance	0.002384834
	Var/mean	0.001082344
21.0	Mean 2.24	
	Variance	0.001997316
	Var/mean	0.00089059
22.0	Mean 2.28	
	Variance	0.002871135
	Var/mean	0.001260837
23.0	Mean 2.37	
	Variance	0.000237587
	Var/mean	0.000100353



Raw Menhaden Length Data used to generate the PSD

length	mass	Log_{10} of Length	% of Study Record Length		length	mass	Log_{10} of Length	% of Study Record Length
6.5	4.3	0.81	27		15.0	54.5	1.18	63
6.9	5.2	0.84	29		15.0	59.6	1.18	63
7.2	5.8	0.86	30		15.0	58.9	1.18	63
7.9	8.7	0.90	33		15.0	62.1	1.18	63
8.0	9.0	0.90	33		15.0	59.0	1.18	63
8.1	9.2	0.91	34		15.0	59.8	1.18	63
8.4	10.8	0.92	35		15.0	64.4	1.18	63
8.5	9.3	0.93	35		15.0	68.2	1.18	63
8.5	12.7	0.93	35		15.0	56.3	1.18	63
8.9	14.1	0.95	37		15.0	62.4	1.18	63
9.0	8.7	0.95	38		15.0	56.6	1.18	63
9.0	13.0	0.95	38		15.0	60.5	1.18	63
9.0	14.0	0.95	38		15.0	59.8	1.18	63
9.2	15.6	0.96	38		15.0	64.8	1.18	63
9.3	15.2	0.97	39		15.0	62.5	1.18	63
9.4	15.3	0.97	39		15.0	64.8	1.18	63
9.4	17.5	0.97	39		15.0	68.1	1.18	63
9.5	13.5	0.98	40		15.0	62.1	1.18	63
9.5	13.8	0.98	40		15.0	62.7	1.18	63
9.5	15.9	0.98	40		15.0	56.1	1.18	63
9.5	13.8	0.98	40		15.0	56.6	1.18	63
9.5	16.2	0.98	40		15.0	59.2	1.18	63
9.5	16.3	0.98	40		15.0	59.8	1.18	63
9.5	16.4	0.98	40		15.0	61.6	1.18	63
9.5	16.5	0.98	40		15.0	68.3	1.18	63
9.5	17.1	0.98	40		15.1	58.5	1.18	63

9.6	14.4	0.98	40	15.1	59.5	1.18	63
9.6	16.9	0.98	40	15.1	60.1	1.18	63
9.6	19.3	0.98	40	15.1	54.9	1.18	63
9.7	18.0	0.99	40	15.1	53.1	1.18	63
9.9	19.0	1.00	41	15.1	51.1	1.18	63
9.9	20.0	1.00	41	15.1	61.5	1.18	63
10.0	17.5	1.00	42	15.1	41.7	1.18	63
10.0	17.6	1.00	42	15.1	58.9	1.18	63
10.0	17.6	1.00	42	15.1	59.3	1.18	63
10.1	18.7	1.00	42	15.1	60.8	1.18	63
10.2	19.4	1.01	43	15.2	66.6	1.18	63
10.3	17.9	1.01	43	15.2	65.9	1.18	63
10.3	20.5	1.01	43	15.2	62.4	1.18	63
10.3	20.7	1.01	43	15.2	56.7	1.18	63
10.4	19.1	1.02	43	15.2	75.4	1.18	63
10.4	19.6	1.02	43	15.2	60.1	1.18	63
10.5	21.8	1.02	44	15.2	66.4	1.18	63
10.5	21.4	1.02	44	15.2	59.4	1.18	63
10.5	20.9	1.02	44	15.3	63.5	1.18	64
10.5	19.4	1.02	44	15.3	58.0	1.18	64
10.5	20.2	1.02	44	15.3	60.7	1.18	64
10.5	21.8	1.02	44	15.3	71.4	1.18	64
10.6	21.9	1.03	44	15.3	81.0	1.18	64
10.6	19.7	1.03	44	15.3	69.5	1.18	64
10.6	21.8	1.03	44	15.3	66.9	1.18	64
10.7	22.3	1.03	45	15.3	59.8	1.18	64
10.8	22.8	1.03	45	15.3	68.0	1.18	64
10.8	21.3	1.03	45	15.3	71.2	1.18	64
10.8	23.4	1.03	45	15.3	65.2	1.18	64
10.8	21.6	1.03	45	15.4	59.6	1.19	64
10.8	22.1	1.03	45	15.4	58.9	1.19	64
10.8	23.5	1.03	45	15.4	65.7	1.19	64
10.9	22.0	1.04	45	15.4	57.8	1.19	64
10.9	23.4	1.04	45	15.4	59.5	1.19	64
11.0	24.5	1.04	46	15.4	70.6	1.19	64
11.0	23.6	1.04	46	15.4	65.6	1.19	64
11.0	24.0	1.04	46	15.4	65.7	1.19	64
11.0	26.4	1.04	46	15.4	62.7	1.19	64
11.0	27.4	1.04	46	15.5	63.0	1.19	65
11.1	26.5	1.05	46	15.5	65.9	1.19	65
11.2	25.2	1.05	47	15.5	63.4	1.19	65
11.2	25.0	1.05	47	15.5	66.9	1.19	65
11.2	27.9	1.05	47	15.5	72.2	1.19	65
11.2	28.4	1.05	47	15.5	62.4	1.19	65
11.2	24.1	1.05	47	15.5	61.5	1.19	65

11.2	29.4	1.05	47	15.5	68.3	1.19	65
11.2	29.5	1.05	47	15.5	65.3	1.19	65
11.3	26.9	1.05	47	15.5	72.5	1.19	65
11.4	25.4	1.06	48	15.5	67.5	1.19	65
11.4	26.7	1.06	48	15.5	69.5	1.19	65
11.4	28.1	1.06	48	15.5	75.1	1.19	65
11.4	26.6	1.06	48	15.5	73.0	1.19	65
11.5	28.9	1.06	48	15.5	80.4	1.19	65
11.5	25.6	1.06	48	15.5	63.4	1.19	65
11.5	28.7	1.06	48	15.5	66.5	1.19	65
11.5	29.5	1.06	48	15.5	70.1	1.19	65
11.5	28.3	1.06	48	15.5	70.9	1.19	65
11.6	29.6	1.06	48	15.5	71.5	1.19	65
11.6	33.4	1.06	48	15.5	62.5	1.19	65
11.6	27.1	1.06	48	15.5	64.1	1.19	65
11.6	29.3	1.06	48	15.5	66.1	1.19	65
11.6	26.7	1.06	48	15.6	70.6	1.19	65
11.6	29.9	1.06	48	15.6	69.5	1.19	65
11.7	26.3	1.07	49	15.6	73.8	1.19	65
11.7	28.1	1.07	49	15.6	80.0	1.19	65
11.7	28.3	1.07	49	15.6	63.4	1.19	65
11.7	28.8	1.07	49	15.6	67.3	1.19	65
11.7	29.9	1.07	49	15.7	66.6	1.20	65
11.7	29.2	1.07	49	15.7	75.6	1.20	65
11.8	27.5	1.07	49	15.7	66.3	1.20	65
11.8	27.2	1.07	49	15.7	72.3	1.20	65
11.8	28.1	1.07	49	15.7	59.1	1.20	65
11.8	28.9	1.07	49	15.7	82.7	1.20	65
11.8	27.2	1.07	49	15.7	75.5	1.20	65
11.8	27.9	1.07	49	15.7	68.3	1.20	65
11.8	30.6	1.07	49	15.7	67.4	1.20	65
11.8	30.7	1.07	49	15.7	71.5	1.20	65
11.9	29.8	1.08	50	15.7	71.8	1.20	65
11.9	31.3	1.08	50	15.8	57.4	1.20	66
11.9	28.9	1.08	50	15.8	71.5	1.20	66
11.9	29.5	1.08	50	15.8	65.1	1.20	66
11.9	30.2	1.08	50	15.8	65.4	1.20	66
11.9	30.7	1.08	50	15.8	83.5	1.20	66
11.9	31.5	1.08	50	15.8	78.0	1.20	66
11.9	31.8	1.08	50	15.8	72.4	1.20	66
11.9	29.5	1.08	50	15.8	67.2	1.20	66
11.9	30.6	1.08	50	15.9	72.1	1.20	66
12.0	28.9	1.08	50	15.9	80.9	1.20	66
12.0	29.3	1.08	50	15.9	70.8	1.20	66
12.0	34.4	1.08	50	15.9	67.2	1.20	66

12.0	28.5	1.08	50	15.9	66.4	1.20	66
12.0	30.7	1.08	50	15.9	76.9	1.20	66
12.0	30.8	1.08	50	15.9	95.8	1.20	66
12.0	31.2	1.08	50	15.9	74.8	1.20	66
12.0	31.4	1.08	50	15.9	76.9	1.20	66
12.0	31.5	1.08	50	15.9	83.0	1.20	66
12.0	32.1	1.08	50	15.9	69.6	1.20	66
12.0	32.4	1.08	50	15.9	76.0	1.20	66
12.0	35.3	1.08	50	16.0	76.6	1.20	67
12.0	30.4	1.08	50	16.0	71.7	1.20	67
12.0	30.5	1.08	50	16.0	74.5	1.20	67
12.0	31.3	1.08	50	16.0	93.6	1.20	67
12.0	32.0	1.08	50	16.0	82.1	1.20	67
12.0	32.2	1.08	50	16.0	68.8	1.20	67
12.0	33.6	1.08	50	16.0	71.5	1.20	67
12.1	30.4	1.08	50	16.0	72.0	1.20	67
12.1	33.7	1.08	50	16.0	74.6	1.20	67
12.1	33.2	1.08	50	16.0	65.2	1.20	67
12.1	29.0	1.08	50	16.0	86.9	1.20	67
12.1	30.1	1.08	50	16.0	70.0	1.20	67
12.1	30.5	1.08	50	16.0	78.2	1.20	67
12.1	30.6	1.08	50	16.0	75.0	1.20	67
12.1	30.6	1.08	50	16.0	86.2	1.20	67
12.1	32.6	1.08	50	16.0	76.1	1.20	67
12.1	33.0	1.08	50	16.0	68.4	1.20	67
12.1	34.3	1.08	50	16.0	68.5	1.20	67
12.1	35.0	1.08	50	16.1	79.2	1.21	67
12.1	36.3	1.08	50	16.1	75.4	1.21	67
12.1	40.3	1.08	50	16.1	85.2	1.21	67
12.1	30.7	1.08	50	16.1	84.3	1.21	67
12.1	31.6	1.08	50	16.1	83.6	1.21	67
12.2	33.6	1.09	51	16.1	89.0	1.21	67
12.2	30.9	1.09	51	16.1	77.2	1.21	67
12.2	31.4	1.09	51	16.1	72.5	1.21	67
12.2	31.8	1.09	51	16.1	79.8	1.21	67
12.2	33.0	1.09	51	16.2	77.2	1.21	68
12.2	34.1	1.09	51	16.2	83.1	1.21	68
12.2	34.4	1.09	51	16.2	80.5	1.21	68
12.2	35.9	1.09	51	16.2	84.9	1.21	68
12.2	33.5	1.09	51	16.2	89.2	1.21	68
12.2	36.7	1.09	51	16.2	85.9	1.21	68
12.3	28.1	1.09	51	16.2	84.9	1.21	68
12.3	30.1	1.09	51	16.2	68.7	1.21	68
12.3	31.5	1.09	51	16.2	86.3	1.21	68
12.3	32.7	1.09	51	16.2	81.4	1.21	68

12.3	33.2	1.09	51	16.2	68.9	1.21	68
12.3	33.4	1.09	51	16.3	65.9	1.21	68
12.3	34.3	1.09	51	16.3	83.4	1.21	68
12.3	34.8	1.09	51	16.3	84.9	1.21	68
12.4	33.0	1.09	52	16.3	71.0	1.21	68
12.4	31.3	1.09	52	16.3	96.4	1.21	68
12.4	32.6	1.09	52	16.3	91.4	1.21	68
12.4	32.8	1.09	52	16.3	81.0	1.21	68
12.4	33.4	1.09	52	16.3	75.3	1.21	68
12.4	34.3	1.09	52	16.3	79.2	1.21	68
12.4	34.8	1.09	52	16.3	70.6	1.21	68
12.4	35.5	1.09	52	16.3	87.6	1.21	68
12.4	36.1	1.09	52	16.3	88.2	1.21	68
12.4	36.8	1.09	52	16.3	88.6	1.21	68
12.4	38.4	1.09	52	16.3	88.1	1.21	68
12.4	34.6	1.09	52	16.3	74.9	1.21	68
12.5	31.6	1.10	52	16.3	75.6	1.21	68
12.5	36.7	1.10	52	16.3	76.2	1.21	68
12.5	35.3	1.10	52	16.3	77.2	1.21	68
12.5	32.1	1.10	52	16.4	74.9	1.21	68
12.5	38.7	1.10	52	16.4	65.1	1.21	68
12.5	44.3	1.10	52	16.4	79.7	1.21	68
12.5	33.7	1.10	52	16.4	71.7	1.21	68
12.5	32.8	1.10	52	16.4	85.8	1.21	68
12.5	31.9	1.10	52	16.4	81.4	1.21	68
12.5	33.0	1.10	52	16.4	79.9	1.21	68
12.5	33.5	1.10	52	16.4	74.5	1.21	68
12.5	34.1	1.10	52	16.4	80.5	1.21	68
12.5	34.7	1.10	52	16.4	82.5	1.21	68
12.5	36.5	1.10	52	16.4	81.5	1.21	68
12.5	37.8	1.10	52	16.4	88.1	1.21	68
12.5	37.9	1.10	52	16.4	91.3	1.21	68
12.5	37.9	1.10	52	16.4	78.6	1.21	68
12.5	34.4	1.10	52	16.4	74.8	1.21	68
12.5	37.0	1.10	52	16.4	79.3	1.21	68
12.5	37.3	1.10	52	16.4	79.0	1.21	68
12.5	40.6	1.10	52	16.4	85.3	1.21	68
12.6	35.5	1.10	53	16.4	95.0	1.21	68
12.6	39.0	1.10	53	16.4	82.9	1.21	68
12.6	37.9	1.10	53	16.4	88.3	1.21	68
12.6	30.7	1.10	53	16.4	79.7	1.21	68
12.6	32.0	1.10	53	16.4	84.9	1.21	68
12.6	32.3	1.10	53	16.4	71.7	1.21	68
12.6	32.4	1.10	53	16.4	75.1	1.21	68
12.6	33.9	1.10	53	16.4	75.3	1.21	68

12.6	34.4	1.10	53	16.4	80.4	1.21	68
12.6	34.8	1.10	53	16.5	78.7	1.22	69
12.6	35.8	1.10	53	16.5	82.9	1.22	69
12.6	35.9	1.10	53	16.5	81.7	1.22	69
12.6	36.7	1.10	53	16.5	72.5	1.22	69
12.6	38.2	1.10	53	16.5	93.9	1.22	69
12.6	38.4	1.10	53	16.5	87.5	1.22	69
12.6	43.9	1.10	53	16.5	87.9	1.22	69
12.6	36.0	1.10	53	16.5	84.6	1.22	69
12.6	37.2	1.10	53	16.5	80.4	1.22	69
12.6	38.4	1.10	53	16.5	80.7	1.22	69
12.6	40.0	1.10	53	16.5	77.9	1.22	69
12.6	40.9	1.10	53	16.5	85.6	1.22	69
12.7	35.4	1.10	53	16.5	86.2	1.22	69
12.7	35.3	1.10	53	16.5	90.7	1.22	69
12.7	39.4	1.10	53	16.5	92.0	1.22	69
12.7	34.0	1.10	53	16.5	82.8	1.22	69
12.7	35.6	1.10	53	16.5	83.5	1.22	69
12.7	35.7	1.10	53	16.5	82.5	1.22	69
12.7	36.5	1.10	53	16.5	100.8	1.22	69
12.7	37.7	1.10	53	16.5	83.5	1.22	69
12.7	38.9	1.10	53	16.5	74.3	1.22	69
12.7	34.2	1.10	53	16.5	88.4	1.22	69
12.7	34.9	1.10	53	16.5	88.8	1.22	69
12.7	38.9	1.10	53	16.5	89.7	1.22	69
12.7	41.4	1.10	53	16.6	85.3	1.22	69
12.8	35.1	1.11	53	16.6	82.5	1.22	69
12.8	35.0	1.11	53	16.6	84.6	1.22	69
12.8	40.4	1.11	53	16.6	95.8	1.22	69
12.8	37.4	1.11	53	16.6	100.6	1.22	69
12.8	32.0	1.11	53	16.6	89.9	1.22	69
12.8	33.6	1.11	53	16.6	86.9	1.22	69
12.8	37.1	1.11	53	16.6	87.6	1.22	69
12.8	37.4	1.11	53	16.6	80.8	1.22	69
12.8	37.5	1.11	53	16.6	81.5	1.22	69
12.8	38.8	1.11	53	16.7	80.9	1.22	70
12.8	40.0	1.11	53	16.7	86.4	1.22	70
12.8	40.8	1.11	53	16.7	90.6	1.22	70
12.8	35.7	1.11	53	16.7	92.4	1.22	70
12.8	36.9	1.11	53	16.7	88.5	1.22	70
12.8	39.1	1.11	53	16.7	77.9	1.22	70
12.8	39.3	1.11	53	16.7	91.0	1.22	70
12.8	40.8	1.11	53	16.7	88.3	1.22	70
12.8	40.9	1.11	53	16.7	89.8	1.22	70
12.9	37.1	1.11	54	16.7	101.1	1.22	70

12.9	42.5	1.11	54	16.7	89.1	1.22	70
12.9	37.7	1.11	54	16.7	82.0	1.22	70
12.9	40.6	1.11	54	16.7	87.2	1.22	70
12.9	33.7	1.11	54	16.8	83.9	1.23	70
12.9	33.8	1.11	54	16.8	81.1	1.23	70
12.9	34.0	1.11	54	16.8	88.7	1.23	70
12.9	34.1	1.11	54	16.8	81.6	1.23	70
12.9	35.8	1.11	54	16.8	76.7	1.23	70
12.9	35.8	1.11	54	16.8	90.6	1.23	70
12.9	36.9	1.11	54	16.8	82.5	1.23	70
12.9	37.2	1.11	54	16.8	95.1	1.23	70
12.9	37.8	1.11	54	16.8	86.6	1.23	70
12.9	38.5	1.11	54	16.8	98.4	1.23	70
12.9	38.9	1.11	54	16.8	94.4	1.23	70
12.9	42.7	1.11	54	16.8	86.8	1.23	70
12.9	34.8	1.11	54	16.8	95.8	1.23	70
12.9	37.9	1.11	54	16.8	78.6	1.23	70
12.9	39.9	1.11	54	16.9	83.2	1.23	70
12.9	41.7	1.11	54	16.9	89.2	1.23	70
12.9	43.9	1.11	54	16.9	89.0	1.23	70
13.0	42.0	1.11	54	16.9	92.9	1.23	70
13.0	42.8	1.11	54	16.9	88.3	1.23	70
13.0	40.2	1.11	54	16.9	86.9	1.23	70
13.0	39.7	1.11	54	16.9	84.0	1.23	70
13.0	40.9	1.11	54	16.9	91.0	1.23	70
13.0	39.3	1.11	54	16.9	86.9	1.23	70
13.0	38.7	1.11	54	16.9	86.6	1.23	70
13.0	90.1	1.11	54	16.9	100.8	1.23	70
13.0	39.7	1.11	54	16.9	98.4	1.23	70
13.0	42.0	1.11	54	16.9	97.4	1.23	70
13.0	35.2	1.11	54	16.9	89.7	1.23	70
13.0	35.2	1.11	54	16.9	85.0	1.23	70
13.0	35.3	1.11	54	16.9	87.1	1.23	70
13.0	36.4	1.11	54	16.9	93.8	1.23	70
13.0	36.4	1.11	54	16.9	99.8	1.23	70
13.0	37.2	1.11	54	16.9	90.7	1.23	70
13.0	37.3	1.11	54	17.0	82.1	1.23	71
13.0	38.1	1.11	54	17.0	94.2	1.23	71
13.0	38.3	1.11	54	17.0	82.8	1.23	71
13.0	39.3	1.11	54	17.0	83.9	1.23	71
13.0	41.3	1.11	54	17.0	93.9	1.23	71
13.0	42.6	1.11	54	17.0	94.6	1.23	71
13.0	34.1	1.11	54	17.0	103.6	1.23	71
13.0	38.7	1.11	54	17.0	99.6	1.23	71
13.0	39.2	1.11	54	17.0	105.5	1.23	71

13.0	39.6	1.11	54	17.0	96.6	1.23	71
13.0	39.6	1.11	54	17.0	93.8	1.23	71
13.0	39.7	1.11	54	17.0	90.5	1.23	71
13.0	40.2	1.11	54	17.0	93.7	1.23	71
13.0	41.0	1.11	54	17.0	96.0	1.23	71
13.0	42.8	1.11	54	17.0	100.0	1.23	71
13.0	47.3	1.11	54	17.0	88.3	1.23	71
13.0	52.7	1.11	54	17.1	95.5	1.23	71
13.1	41.4	1.12	55	17.1	96.8	1.23	71
13.1	38.6	1.12	55	17.1	86.6	1.23	71
13.1	40.7	1.12	55	17.1	103.3	1.23	71
13.1	38.5	1.12	55	17.1	97.1	1.23	71
13.1	43.8	1.12	55	17.1	99.6	1.23	71
13.1	42.7	1.12	55	17.1	90.5	1.23	71
13.1	40.9	1.12	55	17.1	97.5	1.23	71
13.1	36.9	1.12	55	17.1	98.0	1.23	71
13.1	37.3	1.12	55	17.1	95.1	1.23	71
13.1	42.1	1.12	55	17.1	100.0	1.23	71
13.1	42.9	1.12	55	17.1	92.0	1.23	71
13.1	45.4	1.12	55	17.1	94.6	1.23	71
13.1	38.6	1.12	55	17.2	80.1	1.24	72
13.1	39.2	1.12	55	17.2	101.1	1.24	72
13.1	40.0	1.12	55	17.2	104.3	1.24	72
13.1	40.3	1.12	55	17.2	101.1	1.24	72
13.1	40.4	1.12	55	17.2	85.4	1.24	72
13.1	41.7	1.12	55	17.2	97.8	1.24	72
13.1	42.0	1.12	55	17.2	96.9	1.24	72
13.1	42.5	1.12	55	17.2	111.3	1.24	72
13.1	45.5	1.12	55	17.2	95.7	1.24	72
13.1	45.9	1.12	55	17.2	101.3	1.24	72
13.2	38.1	1.12	55	17.2	101.4	1.24	72
13.2	38.9	1.12	55	17.2	90.8	1.24	72
13.2	42.3	1.12	55	17.2	93.1	1.24	72
13.2	41.3	1.12	55	17.3	94.7	1.24	72
13.2	39.9	1.12	55	17.3	86.8	1.24	72
13.2	44.4	1.12	55	17.3	117.9	1.24	72
13.2	43.2	1.12	55	17.3	106.4	1.24	72
13.2	40.0	1.12	55	17.3	101.4	1.24	72
13.2	40.1	1.12	55	17.3	94.8	1.24	72
13.2	39.1	1.12	55	17.3	101.8	1.24	72
13.2	39.8	1.12	55	17.3	101.2	1.24	72
13.2	42.0	1.12	55	17.4	88.0	1.24	73
13.2	42.6	1.12	55	17.4	93.7	1.24	73
13.2	43.1	1.12	55	17.4	96.5	1.24	73
13.2	47.1	1.12	55	17.4	98.6	1.24	73

13.2	47.8	1.12	55	17.4	94.6	1.24	73
13.2	34.7	1.12	55	17.4	106.8	1.24	73
13.2	39.7	1.12	55	17.4	106.1	1.24	73
13.2	40.3	1.12	55	17.4	101.1	1.24	73
13.2	41.5	1.12	55	17.4	90.9	1.24	73
13.2	41.6	1.12	55	17.4	109.1	1.24	73
13.2	42.6	1.12	55	17.4	102.2	1.24	73
13.2	44.0	1.12	55	17.4	101.6	1.24	73
13.2	44.9	1.12	55	17.4	102.8	1.24	73
13.2	47.2	1.12	55	17.4	103.7	1.24	73
13.3	42.9	1.12	55	17.4	94.1	1.24	73
13.3	51.1	1.12	55	17.4	95.7	1.24	73
13.3	39.3	1.12	55	17.4	102.3	1.24	73
13.3	43.3	1.12	55	17.4	104.7	1.24	73
13.3	44.0	1.12	55	17.4	101.0	1.24	73
13.3	40.2	1.12	55	17.5	95.7	1.24	73
13.3	36.7	1.12	55	17.5	92.9	1.24	73
13.3	41.8	1.12	55	17.5	95.5	1.24	73
13.3	44.3	1.12	55	17.5	101.0	1.24	73
13.3	39.3	1.12	55	17.5	100.4	1.24	73
13.3	41.6	1.12	55	17.5	109.6	1.24	73
13.4	43.6	1.13	56	17.5	88.0	1.24	73
13.4	41.7	1.13	56	17.5	101.5	1.24	73
13.4	48.2	1.13	56	17.5	100.1	1.24	73
13.4	41.2	1.13	56	17.5	104.8	1.24	73
13.4	44.1	1.13	56	17.5	111.2	1.24	73
13.4	46.0	1.13	56	17.5	99.8	1.24	73
13.4	47.9	1.13	56	17.5	100.3	1.24	73
13.4	45.9	1.13	56	17.5	101.6	1.24	73
13.4	41.3	1.13	56	17.5	103.3	1.24	73
13.4	41.5	1.13	56	17.5	103.4	1.24	73
13.4	42.4	1.13	56	17.5	104.1	1.24	73
13.4	43.5	1.13	56	17.5	85.3	1.24	73
13.4	44.5	1.13	56	17.5	107.3	1.24	73
13.4	46.6	1.13	56	17.6	92.9	1.25	73
13.4	47.1	1.13	56	17.6	109.3	1.25	73
13.4	39.2	1.13	56	17.6	101.4	1.25	73
13.4	39.7	1.13	56	17.6	100.7	1.25	73
13.4	42.1	1.13	56	17.6	100.7	1.25	73
13.4	42.9	1.13	56	17.6	108.1	1.25	73
13.4	44.1	1.13	56	17.6	118.1	1.25	73
13.4	44.7	1.13	56	17.6	102.2	1.25	73
13.4	45.0	1.13	56	17.6	96.3	1.25	73
13.4	45.2	1.13	56	17.6	100.3	1.25	73
13.4	45.3	1.13	56	17.6	101.5	1.25	73

13.4	47.2	1.13	56	17.6	106.1	1.25	73
13.5	43.8	1.13	56	17.6	108.4	1.25	73
13.5	42.7	1.13	56	17.6	110.2	1.25	73
13.5	44.9	1.13	56	17.6	89.6	1.25	73
13.5	41.8	1.13	56	17.6	96.8	1.25	73
13.5	43.1	1.13	56	17.7	102.0	1.25	74
13.5	39.3	1.13	56	17.7	89.7	1.25	74
13.5	45.9	1.13	56	17.7	92.8	1.25	74
13.5	41.8	1.13	56	17.8	92.3	1.25	74
13.5	45.7	1.13	56	17.8	105.3	1.25	74
13.5	47.3	1.13	56	17.8	96.2	1.25	74
13.5	45.4	1.13	56	17.8	91.8	1.25	74
13.5	42.8	1.13	56	17.8	111.0	1.25	74
13.5	45.1	1.13	56	17.8	107.5	1.25	74
13.5	47.4	1.13	56	17.8	117.8	1.25	74
13.5	42.7	1.13	56	17.8	104.0	1.25	74
13.5	45.9	1.13	56	17.8	110.2	1.25	74
13.5	44.3	1.13	56	17.8	89.5	1.25	74
13.5	44.6	1.13	56	17.8	97.3	1.25	74
13.5	41.2	1.13	56	17.8	98.4	1.25	74
13.5	41.4	1.13	56	17.9	98.5	1.25	75
13.5	41.8	1.13	56	17.9	99.8	1.25	75
13.5	42.0	1.13	56	17.9	108.0	1.25	75
13.5	42.2	1.13	56	17.9	100.0	1.25	75
13.5	44.8	1.13	56	17.9	95.1	1.25	75
13.5	40.1	1.13	56	17.9	100.4	1.25	75
13.5	42.6	1.13	56	17.9	104.7	1.25	75
13.5	43.0	1.13	56	17.9	110.3	1.25	75
13.5	43.4	1.13	56	17.9	107.3	1.25	75
13.5	44.5	1.13	56	17.9	102.7	1.25	75
13.5	44.8	1.13	56	17.9	126.4	1.25	75
13.5	45.5	1.13	56	17.9	138.7	1.25	75
13.5	45.9	1.13	56	17.9	129.8	1.25	75
13.5	46.4	1.13	56	17.9	111.8	1.25	75
13.5	46.9	1.13	56	17.9	104.4	1.25	75
13.5	48.8	1.13	56	17.9	109.6	1.25	75
13.5	49.4	1.13	56	17.9	111.1	1.25	75
13.6	44.1	1.13	57	17.9	107.4	1.25	75
13.6	40.4	1.13	57	17.9	115.2	1.25	75
13.6	43.8	1.13	57	17.9	113.6	1.25	75
13.6	47.0	1.13	57	17.9	104.3	1.25	75
13.6	45.8	1.13	57	17.9	111.6	1.25	75
13.6	44.8	1.13	57	18.0	109.4	1.26	75
13.6	43.3	1.13	57	18.0	101.8	1.26	75
13.6	48.0	1.13	57	18.0	103.6	1.26	75

13.6	46.1	1.13	57	18.0	104.8	1.26	75
13.6	47.5	1.13	57	18.0	112.1	1.26	75
13.6	46.4	1.13	57	18.0	117.6	1.26	75
13.6	47.4	1.13	57	18.0	100.2	1.26	75
13.6	40.9	1.13	57	18.0	101.5	1.26	75
13.6	45.1	1.13	57	18.0	102.8	1.26	75
13.6	46.5	1.13	57	18.0	104.4	1.26	75
13.6	47.7	1.13	57	18.0	109.9	1.26	75
13.6	41.3	1.13	57	18.0	113.4	1.26	75
13.6	44.1	1.13	57	18.0	107.4	1.26	75
13.6	44.2	1.13	57	18.0	108.7	1.26	75
13.6	44.6	1.13	57	18.1	128.0	1.26	75
13.6	44.8	1.13	57	18.1	104.3	1.26	75
13.6	45.5	1.13	57	18.1	109.5	1.26	75
13.6	46.0	1.13	57	18.1	111.5	1.26	75
13.6	47.1	1.13	57	18.1	112.6	1.26	75
13.7	44.9	1.14	57	18.1	113.1	1.26	75
13.7	46.4	1.14	57	18.1	116.2	1.26	75
13.7	44.2	1.14	57	18.1	119.0	1.26	75
13.7	45.2	1.14	57	18.1	119.7	1.26	75
13.7	50.0	1.14	57	18.1	121.3	1.26	75
13.7	48.9	1.14	57	18.1	141.0	1.26	75
13.7	50.8	1.14	57	18.1	111.3	1.26	75
13.7	47.1	1.14	57	18.2	107.4	1.26	76
13.7	47.2	1.14	57	18.2	109.3	1.26	76
13.7	42.3	1.14	57	18.2	105.1	1.26	76
13.7	46.0	1.14	57	18.2	114.6	1.26	76
13.7	46.7	1.14	57	18.2	109.3	1.26	76
13.7	46.7	1.14	57	18.2	116.7	1.26	76
13.7	48.2	1.14	57	18.2	103.9	1.26	76
13.7	49.2	1.14	57	18.2	110.3	1.26	76
13.8	46.1	1.14	58	18.2	112.2	1.26	76
13.8	46.6	1.14	58	18.2	131.1	1.26	76
13.8	44.2	1.14	58	18.2	107.5	1.26	76
13.8	46.4	1.14	58	18.2	116.5	1.26	76
13.8	43.8	1.14	58	18.3	112.5	1.26	76
13.8	48.4	1.14	58	18.3	105.3	1.26	76
13.8	47.8	1.14	58	18.3	119.5	1.26	76
13.8	49.4	1.14	58	18.3	103.9	1.26	76
13.8	49.2	1.14	58	18.3	109.8	1.26	76
13.8	48.8	1.14	58	18.3	116.1	1.26	76
13.8	46.4	1.14	58	18.3	120.7	1.26	76
13.8	46.8	1.14	58	18.3	118.4	1.26	76
13.8	48.1	1.14	58	18.4	107.0	1.26	77
13.8	44.0	1.14	58	18.4	124.2	1.26	77

13.8	44.7	1.14	58	18.4	115.6	1.26	77
13.8	45.9	1.14	58	18.4	112.4	1.26	77
13.8	46.5	1.14	58	18.4	101.9	1.26	77
13.8	46.8	1.14	58	18.4	104.3	1.26	77
13.9	45.9	1.14	58	18.4	111.7	1.26	77
13.9	46.6	1.14	58	18.4	122.0	1.26	77
13.9	47.1	1.14	58	18.4	122.3	1.26	77
13.9	47.4	1.14	58	18.4	93.1	1.26	77
13.9	51.7	1.14	58	18.4	96.8	1.26	77
13.9	51.1	1.14	58	18.4	160.5	1.26	77
13.9	48.8	1.14	58	18.5	106.5	1.27	77
13.9	46.2	1.14	58	18.5	101.3	1.27	77
13.9	48.8	1.14	58	18.5	113.5	1.27	77
13.9	47.9	1.14	58	18.5	139.9	1.27	77
13.9	43.0	1.14	58	18.5	123.6	1.27	77
13.9	51.2	1.14	58	18.5	126.9	1.27	77
13.9	46.6	1.14	58	18.5	126.1	1.27	77
13.9	47.6	1.14	58	18.5	110.5	1.27	77
13.9	48.4	1.14	58	18.5	128.7	1.27	77
13.9	52.4	1.14	58	18.5	118.5	1.27	77
13.9	40.7	1.14	58	18.6	109.3	1.27	78
13.9	42.5	1.14	58	18.6	122.3	1.27	78
13.9	42.9	1.14	58	18.6	112.8	1.27	78
13.9	44.0	1.14	58	18.6	151.7	1.27	78
13.9	45.3	1.14	58	18.6	191.2	1.27	78
13.9	47.2	1.14	58	18.6	119.1	1.27	78
13.9	49.6	1.14	58	18.6	121.7	1.27	78
13.9	50.8	1.14	58	18.6	125.3	1.27	78
13.9	54.3	1.14	58	18.6	133.7	1.27	78
14.0	48.3	1.15	58	18.6	117.5	1.27	78
14.0	50.5	1.15	58	18.7	120.4	1.27	78
14.0	49.3	1.15	58	18.7	117.4	1.27	78
14.0	52.5	1.15	58	18.7	118.1	1.27	78
14.0	49.3	1.15	58	18.7	127.0	1.27	78
14.0	52.9	1.15	58	18.7	121.4	1.27	78
14.0	53.4	1.15	58	18.7	127.4	1.27	78
14.0	52.6	1.15	58	18.7	136.2	1.27	78
14.0	47.8	1.15	58	18.7	137.4	1.27	78
14.0	51.9	1.15	58	18.8	122.0	1.27	78
14.0	49.7	1.15	58	18.8	119.5	1.27	78
14.0	50.8	1.15	58	18.8	126.4	1.27	78
14.0	51.1	1.15	58	18.9	138.6	1.28	79
14.0	49.5	1.15	58	18.9	135.8	1.28	79
14.0	51.1	1.15	58	18.9	136.6	1.28	79
14.0	50.3	1.15	58	18.9	118.5	1.28	79

14.0	49.2	1.15	58	18.9	127.7	1.28	79
14.0	49.9	1.15	58	19.0	122.3	1.28	79
14.0	50.1	1.15	58	19.0	125.9	1.28	79
14.0	51.4	1.15	58	19.0	133.6	1.28	79
14.0	51.8	1.15	58	19.0	126.5	1.28	79
14.0	54.4	1.15	58	19.0	133.0	1.28	79
14.0	41.6	1.15	58	19.0	133.5	1.28	79
14.0	42.7	1.15	58	19.0	96.0	1.28	79
14.0	44.5	1.15	58	19.0	123.3	1.28	79
14.0	44.6	1.15	58	19.0	133.3	1.28	79
14.0	47.5	1.15	58	19.0	137.4	1.28	79
14.0	49.8	1.15	58	19.0	130.1	1.28	79
14.0	49.8	1.15	58	19.0	144.9	1.28	79
14.0	49.9	1.15	58	19.0	150.1	1.28	79
14.0	50.9	1.15	58	19.0	128.5	1.28	79
14.0	58.9	1.15	58	19.0	130.8	1.28	79
14.1	48.9	1.15	59	19.0	123.3	1.28	79
14.1	51.5	1.15	59	19.0	141.2	1.28	79
14.1	52.5	1.15	59	19.1	103.1	1.28	80
14.1	51.4	1.15	59	19.1	113.9	1.28	80
14.1	55.1	1.15	59	19.1	115.6	1.28	80
14.1	48.7	1.15	59	19.1	146.5	1.28	80
14.1	51.9	1.15	59	19.1	125.3	1.28	80
14.1	51.2	1.15	59	19.1	119.7	1.28	80
14.1	50.8	1.15	59	19.1	124.0	1.28	80
14.1	47.7	1.15	59	19.1	113.4	1.28	80
14.1	51.9	1.15	59	19.1	113.1	1.28	80
14.1	44.7	1.15	59	19.1	132.7	1.28	80
14.1	53.3	1.15	59	19.1	125.0	1.28	80
14.1	50.8	1.15	59	19.1	158.6	1.28	80
14.1	51.0	1.15	59	19.1	127.5	1.28	80
14.1	52.0	1.15	59	19.2	123.1	1.28	80
14.1	57.1	1.15	59	19.2	128.8	1.28	80
14.1	57.2	1.15	59	19.2	127.4	1.28	80
14.1	57.8	1.15	59	19.2	156.2	1.28	80
14.1	42.1	1.15	59	19.2	157.0	1.28	80
14.1	46.7	1.15	59	19.2	136.0	1.28	80
14.1	47.0	1.15	59	19.3	130.7	1.29	80
14.1	47.3	1.15	59	19.3	149.0	1.29	80
14.1	47.5	1.15	59	19.3	130.3	1.29	80
14.1	47.9	1.15	59	19.4	130.6	1.29	81
14.1	49.0	1.15	59	19.4	120.0	1.29	81
14.1	51.8	1.15	59	19.4	127.0	1.29	81
14.1	51.9	1.15	59	19.4	132.0	1.29	81
14.1	53.2	1.15	59	19.4	132.5	1.29	81

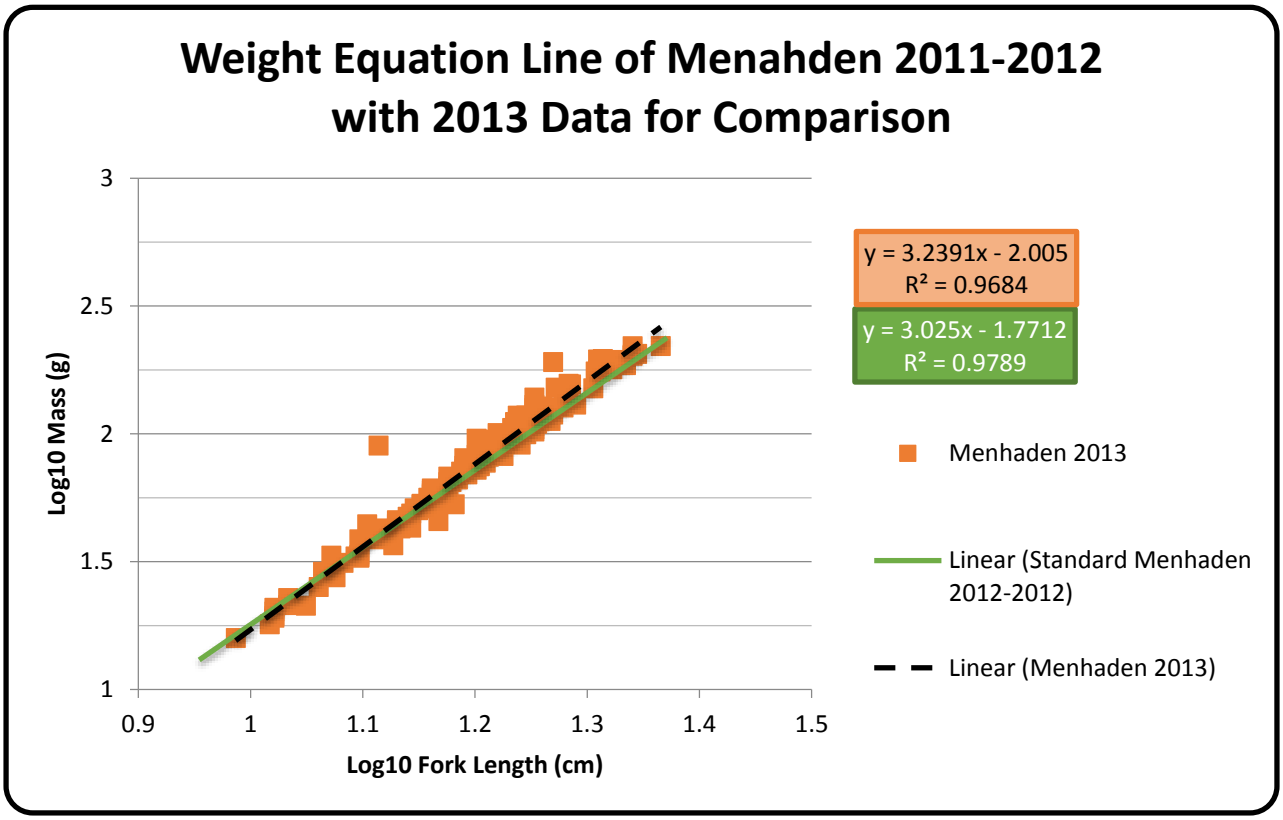
14.2	43.7	1.15	59	19.4	137.2	1.29	81
14.2	51.8	1.15	59	19.4	153.4	1.29	81
14.2	52.1	1.15	59	19.5	146.8	1.29	81
14.2	49.2	1.15	59	19.5	130.2	1.29	81
14.2	49.7	1.15	59	19.5	146.3	1.29	81
14.2	50.2	1.15	59	19.5	149.3	1.29	81
14.2	44.8	1.15	59	19.5	121.5	1.29	81
14.2	46.0	1.15	59	19.5	130.7	1.29	81
14.2	53.1	1.15	59	19.5	137.3	1.29	81
14.2	49.0	1.15	59	19.5	150.8	1.29	81
14.2	47.3	1.15	59	19.5	142.2	1.29	81
14.2	51.5	1.15	59	19.5	153.3	1.29	81
14.2	52.3	1.15	59	19.5	124.5	1.29	81
14.2	55.0	1.15	59	19.5	129.1	1.29	81
14.2	53.6	1.15	59	19.5	137.1	1.29	81
14.2	55.1	1.15	59	19.5	155.6	1.29	81
14.2	54.6	1.15	59	19.6	125.3	1.29	82
14.2	51.1	1.15	59	19.6	142.9	1.29	82
14.2	53.0	1.15	59	19.6	114.8	1.29	82
14.2	45.6	1.15	59	19.6	133.7	1.29	82
14.2	55.0	1.15	59	19.6	133.8	1.29	82
14.2	55.5	1.15	59	19.6	158.1	1.29	82
14.2	49.8	1.15	59	19.6	148.4	1.29	82
14.2	49.9	1.15	59	19.7	122.0	1.29	82
14.2	50.7	1.15	59	19.7	140.8	1.29	82
14.2	50.8	1.15	59	19.7	144.3	1.29	82
14.2	56.1	1.15	59	19.8	155.2	1.30	83
14.2	56.5	1.15	59	19.8	138.8	1.30	83
14.3	48.4	1.16	60	19.9	136.9	1.30	83
14.3	47.2	1.16	60	19.9	165.9	1.30	83
14.3	52.2	1.16	60	19.9	93.6	1.30	83
14.3	56.2	1.16	60	19.9	156.4	1.30	83
14.3	53.0	1.16	60	19.9	154.2	1.30	83
14.3	49.2	1.16	60	20.0	132.4	1.30	83
14.3	53.5	1.16	60	20.0	156.3	1.30	83
14.3	47.9	1.16	60	20.0	130.2	1.30	83
14.3	55.7	1.16	60	20.0	137.0	1.30	83
14.3	50.9	1.16	60	20.0	132.2	1.30	83
14.3	50.8	1.16	60	20.0	155.4	1.30	83
14.3	51.8	1.16	60	20.0	144.6	1.30	83
14.3	51.2	1.16	60	20.0	145.8	1.30	83
14.3	51.5	1.16	60	20.0	163.3	1.30	83
14.3	51.4	1.16	60	20.0	134.3	1.30	83
14.3	49.1	1.16	60	20.1	136.1	1.30	84
14.3	56.3	1.16	60	20.1	153.8	1.30	84

14.3	52.6	1.16	60	20.2	153.2	1.31	84
14.3	58.2	1.16	60	20.2	175.4	1.31	84
14.3	58.5	1.16	60	20.2	182.1	1.31	84
14.3	47.9	1.16	60	20.3	140.4	1.31	85
14.4	50.9	1.16	60	20.3	177.6	1.31	85
14.4	52.4	1.16	60	20.3	185.4	1.31	85
14.4	53.0	1.16	60	20.4	143.3	1.31	85
14.4	52.7	1.16	60	20.4	174.8	1.31	85
14.4	51.7	1.16	60	20.4	195.6	1.31	85
14.4	55.5	1.16	60	20.4	175.5	1.31	85
14.4	53.0	1.16	60	20.4	146.5	1.31	85
14.4	57.1	1.16	60	20.4	160.4	1.31	85
14.4	51.6	1.16	60	20.4	160.5	1.31	85
14.4	53.5	1.16	60	20.5	148.9	1.31	85
14.4	54.7	1.16	60	20.5	159.9	1.31	85
14.4	58.0	1.16	60	20.5	133.5	1.31	85
14.4	51.4	1.16	60	20.5	172.6	1.31	85
14.4	52.0	1.16	60	20.5	144.9	1.31	85
14.4	52.9	1.16	60	20.5	196.3	1.31	85
14.4	58.3	1.16	60	20.5	156.6	1.31	85
14.4	38.4	1.16	60	20.6	166.3	1.31	86
14.4	49.5	1.16	60	20.6	179.0	1.31	86
14.4	49.8	1.16	60	20.6	165.4	1.31	86
14.4	53.1	1.16	60	20.7	169.4	1.32	86
14.5	52.9	1.16	60	20.7	168.1	1.32	86
14.5	52.9	1.16	60	20.8	167.7	1.32	87
14.5	62.1	1.16	60	20.8	172.7	1.32	87
14.5	63.7	1.16	60	20.8	157.9	1.32	87
14.5	59.1	1.16	60	20.8	188.6	1.32	87
14.5	50.0	1.16	60	20.9	169.9	1.32	87
14.5	52.4	1.16	60	20.9	170.5	1.32	87
14.5	56.1	1.16	60	20.9	187.2	1.32	87
14.5	55.0	1.16	60	20.9	164.8	1.32	87
14.5	54.4	1.16	60	21.0	158.6	1.32	88
14.5	54.8	1.16	60	21.0	184.7	1.32	88
14.5	54.6	1.16	60	21.0	188.9	1.32	88
14.5	53.2	1.16	60	21.0	188.1	1.32	88
14.5	52.6	1.16	60	21.0	192.9	1.32	88
14.5	52.7	1.16	60	21.0	149.4	1.32	88
14.5	54.3	1.16	60	21.0	179.2	1.32	88
14.5	51.3	1.16	60	21.0	167.7	1.32	88
14.5	54.7	1.16	60	21.0	161.1	1.32	88
14.5	60.2	1.16	60	21.0	194.6	1.32	88
14.5	51.7	1.16	60	21.1	155.3	1.32	88
14.5	54.7	1.16	60	21.1	179.6	1.32	88

14.5	58.7	1.16	60	21.2	162.1	1.33	88
14.5	61.2	1.16	60	21.2	187.2	1.33	88
14.5	55.7	1.16	60	21.2	186.0	1.33	88
14.5	53.0	1.16	60	21.2	170.4	1.33	88
14.5	53.3	1.16	60	21.3	154.6	1.33	89
14.5	55.6	1.16	60	21.3	170.5	1.33	89
14.5	49.6	1.16	60	21.3	179.8	1.33	89
14.5	52.2	1.16	60	21.3	176.1	1.33	89
14.5	56.5	1.16	60	21.5	151.9	1.33	90
14.5	59.0	1.16	60	21.5	144.6	1.33	90
14.5	61.4	1.16	60	21.5	173.6	1.33	90
14.6	59.0	1.16	61	21.5	162.9	1.33	90
14.6	51.0	1.16	61	21.5	167.9	1.33	90
14.6	53.3	1.16	61	21.5	142.5	1.33	90
14.6	56.4	1.16	61	21.5	170.2	1.33	90
14.6	54.8	1.16	61	21.6	171.2	1.33	90
14.6	54.2	1.16	61	21.6	176.1	1.33	90
14.6	57.2	1.16	61	21.6	186.7	1.33	90
14.6	55.6	1.16	61	21.6	204.4	1.33	90
14.6	51.4	1.16	61	21.6	192.0	1.33	90
14.6	53.0	1.16	61	21.7	168.3	1.34	90
14.6	57.4	1.16	61	21.9	202.2	1.34	91
14.6	54.0	1.16	61	21.9	220.3	1.34	91
14.6	45.6	1.16	61	21.9	205.8	1.34	91
14.6	58.7	1.16	61	22.0	182.5	1.34	92
14.6	59.2	1.16	61	22.0	210.9	1.34	92
14.6	65.2	1.16	61	22.0	141.1	1.34	92
14.6	55.8	1.16	61	22.0	173.3	1.34	92
14.6	61.0	1.16	61	22.0	171.5	1.34	92
14.7	51.7	1.17	61	22.1	220.6	1.34	92
14.7	56.7	1.17	61	22.5	194.3	1.35	94
14.7	58.1	1.17	61	22.5	189.7	1.35	94
14.7	55.3	1.17	61	22.5	194.7	1.35	94
14.7	61.9	1.17	61	22.6	203.7	1.35	94
14.7	53.2	1.17	61	22.6	218.1	1.35	94
14.7	51.6	1.17	61	22.7	186.6	1.36	95
14.7	53.0	1.17	61	23.2	239.0	1.37	97
14.7	53.4	1.17	61	23.5	227.3	1.37	98
14.7	57.1	1.17	61				
14.7	58.2	1.17	61				
14.7	52.7	1.17	61				
14.8	57.8	1.17	62				
14.8	58.2	1.17	62				
14.8	53.4	1.17	62				
14.8	65.6	1.17	62				

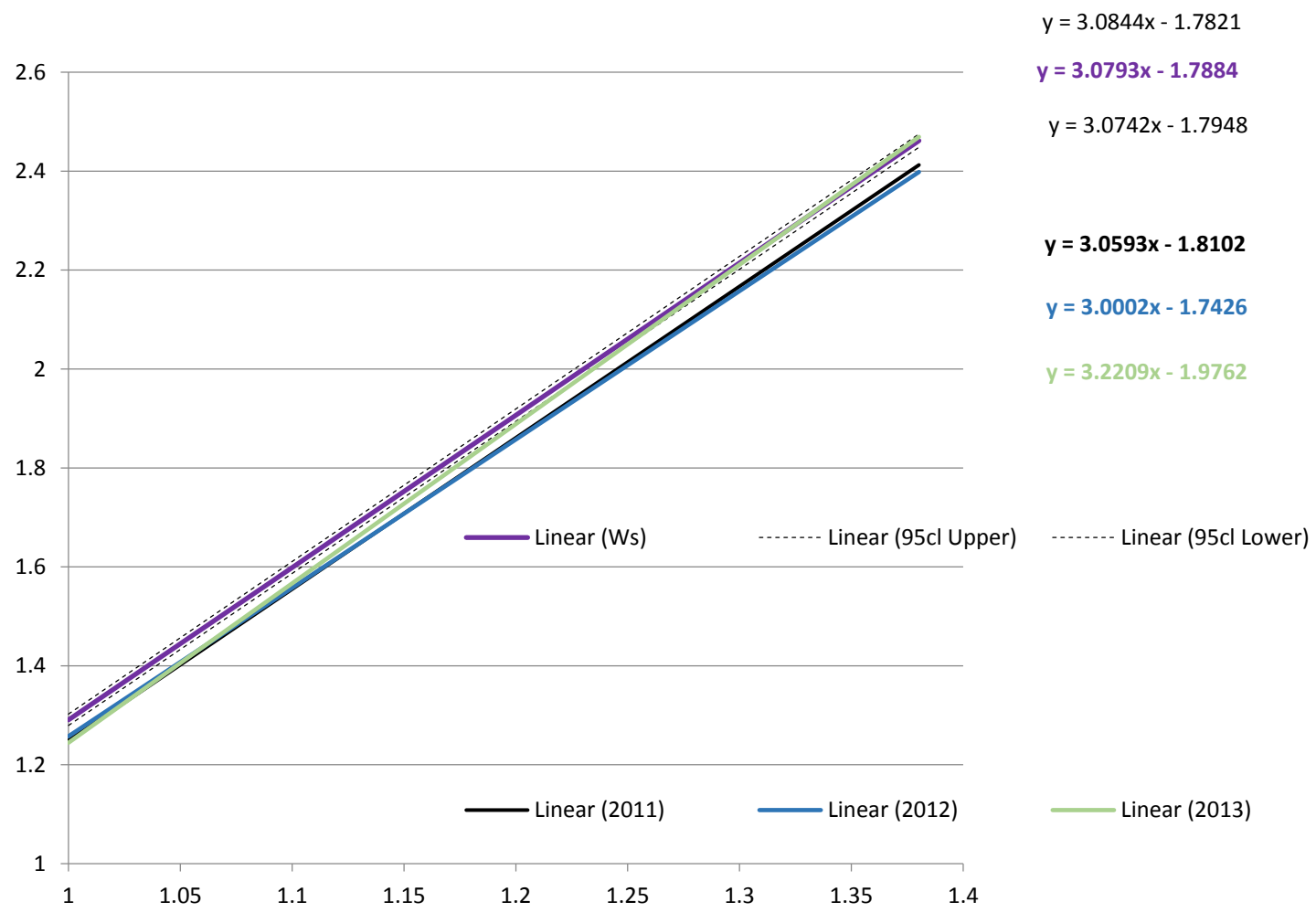
14.8	68.2	1.17	62
14.8	69.5	1.17	62
14.8	69.8	1.17	62
14.8	70.7	1.17	62
14.8	69.5	1.17	62
14.8	59.6	1.17	62
14.8	58.6	1.17	62
14.8	58.7	1.17	62
14.8	56.0	1.17	62
14.8	54.8	1.17	62
14.8	56.3	1.17	62
14.8	56.6	1.17	62
14.8	54.7	1.17	62
14.8	54.4	1.17	62
14.8	55.0	1.17	62
14.8	54.1	1.17	62
14.8	56.7	1.17	62
14.8	61.3	1.17	62
14.9	51.1	1.17	62
14.9	56.6	1.17	62
14.9	61.6	1.17	62
14.9	65.0	1.17	62
14.9	61.0	1.17	62
14.9	64.1	1.17	62
14.9	56.5	1.17	62
14.9	57.6	1.17	62
14.9	56.5	1.17	62
14.9	61.2	1.17	62
14.9	57.7	1.17	62
14.9	58.5	1.17	62
14.9	54.4	1.17	62
14.9	56.3	1.17	62
14.9	48.4	1.17	62
14.9	65.4	1.17	62
14.9	62.7	1.17	62

Weight equations for menhaden harvest years



Year	Actual difference from the 3rd Quartile$_{(2000\text{-}2010)}$ Intercept (±)	Standard Error	Significant difference from the 3rd Quartile$_{(2000\text{-}2010)}$ Intercept
2000	0.2384	0.01172	<.0001
2001	0.0534	0.01607	0.0009
2002	-0.05763	0.01265	<.0001
2003	0.006016	0.01056	0.5689
2004	-0.1544	0.01045	<.0001
2005	-0.01083	0.01291	0.4016
2006	-0.03996	0.01357	0.0032
2007	-0.1323	0.01434	<.0001
2008	-0.237	0.01556	<.0001
2009	0.027	0.01652	0.1023
2010	0.03416	0.01323	0.0098
2011	-0.02175	0.02141	0.3099
2012	0.04583	0.02597	0.0776
2013	-0.1878	0.04187	<.0001

Year	Actual difference from the 3rd Quartile$_{(2000\text{-}2010)}$ Slope (±)	Standard Error	Significant difference from the 3rd Quartile$_{(2000\text{-}2010)}$ Slope	Significant Difference in Condition?
2000	-0.2234	0.009399	<.0001	Y
2001	-0.02889	0.01272	0.0232	Y
2002	0.03335	0.01013	0.001	Y
2003	-0.02297	0.008565	0.0073	Y
2004	0.1254	0.008549	<.0001	Y
2005	0.008017	0.0105	0.4454	N
2006	0.02567	0.01105	0.0202	Y
2007	0.1104	0.01175	<.0001	Y
2008	0.1936	0.0125	<.0001	Y
2009	-0.03434	0.01312	0.0089	Y
2010	-0.03357	0.01077	0.0018	Y
2011	-0.01994	0.01833	0.2767	N
2012	-0.07907	0.02154	0.0002	Y
2013	0.1416	0.03481	<.0001	Y



SAS Code for Statistical Analysis

Regression Line Percentile Manipulation of the Data

```
dm 'log; clear';
ods html close;
ods html;
ods listing;


libname greg "E:\EXST 7083 Consulting\Greg";

*ods rtf;

/*
Imported each excel file for years 2000-2010
*/


proc import out=work.y2000 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2000.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2001 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2001.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2002 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2002.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2003 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2003.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2004 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2004.xlsx'
```

```
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2005 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2005.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2006 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2006.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2007 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2007.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2008 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2008.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2009 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2009.xlsx'
        dbms=excel replace;
        getnames=yes;
run;

proc import out=work.y2010 (keep=FL        Mass logfl logmass year)
        file='C:\Users\tearle.LSU\Desktop\Fish Workbooks\2010.xlsx'
        dbms=excel replace;
        getnames=yes;
run;




/*
concatenated (stacked) all data form years 2000-2010 into one sas data set called "work.allyears"
*/
```

```
data work.allyears;
	set work.y2000 work.y2001 work.y2002 work.y2003 work.y2004
		work.y2005 work.y2006 work.y2007 work.y2008 work.y2009
		work.y2010;
	keep FL	Mass logfl logmass year;
run;



/*
Took sas data set and gruouped by FL and removed those less than 10 and greater than 24 cm.
New sas data sets for each FL integer vale were created for the values between 10-24
sas data sets were denoted as FL(interger of FL)
*/


data FL10 FL11 FL12 FL13 FL14 FL15 FL16 FL17 FL18 FL19 FL20 FL21 FL22 FL23;
	set work.allyears;
	if fl<10 then delete;
	else if 10<=fl<11 then output FL10;
	else if 11<=fl<12 then output FL11;
	else if 12<=fl<13 then output FL12;
	else if 13<=fl<14 then output FL13;
	else if 14<=fl<15 then output FL14;
	else if 15<=fl<16 then output FL15;
	else if 16<=fl<17 then output FL16;
	else if 17<=fl<18 then output FL17;
	else if 18<=fl<19 then output FL18;
	else if 19<=fl<20 then output FL19;
	else if 20<=fl<21 then output FL20;
	else if 21<=fl<22 then output FL21;
	else if 22<=fl<23 then output FL22;
	else if 23<=fl<24 then output FL23;
	else if fl>24 then delete;
run;



/*
Each asa data set was then sorted by Mass and ouput as "work.sortedfl(integerfl)"
*/


proc sort data=work.fl10
```

```
        out=work.sortedfl10;
        by mass;
run;

proc sort data=work.fl11
        out=work.sortedfl11;
        by mass;
run;

proc sort data=work.fl12
        out=work.sortedfl12;
        by mass;
run;

proc sort data=work.fl13
        out=work.sortedfl13;
        by mass;
run;

proc sort data=work.fl14
        out=work.sortedfl14;
        by mass;
run;

proc sort data=work.fl15
        out=work.sortedfl15;
        by mass;
run;

proc sort data=work.fl16
        out=work.sortedfl16;
        by mass;
run;

proc sort data=work.fl17
        out=work.sortedfl17;
        by mass;
run;

proc sort data=work.fl18
        out=work.sortedfl18;
        by mass;
run;

proc sort data=work.fl19
        out=work.sortedfl19;
```

```
        by mass;
run;

proc sort data=work.fl20
        out=work.sortedfl20;
        by mass;
run;

proc sort data=work.fl21
        out=work.sortedfl21;
        by mass;
run;

proc sort data=work.fl22
        out=work.sortedfl22;
        by mass;
run;

proc sort data=work.fl23
        out=work.sortedfl23;
        by mass;
run;




/*
looked at the sas log and identified the total observations for each FL and calculated the 50th and
75th
50th was rounded up and 75th was rounded down to the nearest integer. These values are found
in first obs
and obs respectively
*/


data quartilefl10;
        set sortedfl10 (firstobs=46 obs=68);
run;

data quartilefl11;
        set sortedfl11 (firstobs=108 obs=161);
run;

data quartilefl12;
        set sortedfl12 (firstobs=375 obs=562);
run;
```

```
data quartilefl13;
	set sortedfl13 (firstobs=1115 obs=1671);
run;

data quartilefl14;
	set sortedfl14 (firstobs=2267 obs=3400);
run;

data quartilefl15;
	set sortedfl15 (firstobs=4041 obs=6060);
run;

data quartilefl16;
	set sortedfl16 (firstobs=6157 obs=9234);
run;

data quartilefl17;
	set sortedfl17 (firstobs=8112 obs=12168);
run;

data quartilefl18;
	set sortedfl18 (firstobs=8273 obs=12408);
run;

data quartilefl19;
	set sortedfl19 (firstobs=5020 obs=7530);
run;

data quartilefl20;
	set sortedfl20 (firstobs=2148 obs=3222);
run;

data quartilefl21;
	set sortedfl21 (firstobs=541 obs=811);
run;

data quartilefl22;
	set sortedfl22 (firstobs=85 obs=127);
run;

data quartilefl23;
	set sortedfl23 (firstobs=13 obs=18);
run;

/*
```

```
printed each third quartile for each fork integer length to verify appropriate values
*/

proc print data=quartilefl10;
run;

proc print data=quartilefl11;
run;

proc print data=quartilefl12;
run;

proc print data=quartilefl13;
run;

proc print data=quartilefl14;
run;

proc print data=quartilefl15;
run;

proc print data=quartilefl16;
run;

proc print data=quartilefl17;
run;

proc print data=quartilefl18;
run;

proc print data=quartilefl19;
run;

proc print data=quartilefl20;
run;

proc print data=quartilefl21;
run;

proc print data=quartilefl22;
run;

proc print data=quartilefl23;
run;
/*
Concateinated the quartile years into a single sas data set called "work.quantileyears"
```

```
*/
data work.quantileyears;
	set quartilefl10 quartilefl11 quartilefl12 quartilefl13 quartilefl14 quartilefl15
		quartilefl16 quartilefl17 quartilefl18 quartilefl19 quartilefl20 quartilefl21
		quartilefl22 quartilefl23;
	if year <=2010 then year=2014;
run;

/*
Printed to verify sas data set "work.quantileyears"
*/

proc print;
run;

/*
Ran the baseline model (regression outlining the Ws equation for menahden 2000-2010)
*/

proc glm data=quantileyears PLOTS(MAXPOINTS=19153);
	model logmass=logfl;
run;

quit;

/*
Imported each excel file for years 2011-2013 (my study data)
*/

proc import out=work.y2011 (keep=FL	Mass logfl logmass year)
	file='E:\EXST 7083 Consulting\Greg\Greg Data\GregOlson2011.xlsx'
	dbms=excel replace;
	getnames=yes;
run;

proc import out=work.y2012 (keep=FL	Mass logfl logmass year)
	file='E:\EXST 7083 Consulting\Greg\Greg Data\GregOlson2012.xlsx'
	dbms=excel replace;
	getnames=yes;
run;

proc import out=work.y2013 (keep=FL	Mass logfl logmass year)
	file='E:\EXST 7083 Consulting\Greg\Greg Data\GregOlson2013.xlsx'
	dbms=excel replace;
	getnames=yes;
```

```
/*
concateinating into new sas data set called "work.gregyears" and added work.allyears to the
concatination
and deleted FL less than 10 and greater than 24 as per protocol
*/

data work.GregYears;
	set work.y2011 work.y2012 work.y2013 work.allyears;
	if fl<10 then delete;
	else if fl>24 then delete;
run;

/*
printed to verify new sas data set
*/

proc print;
run;

/*
baseline model for my 2011-2013 sample year. gives me means for each year,
main effects by year, and logfl as well as interaction between logfl and year
*/

proc glm data=GregYears Plots (MAXPOINTS=1398);
	class year;
	model logmass=logfl|year;
	lsmeans year;
run;

/*
concatenating quantileyears with gregyears into new sas data set called "work.combineyears"
*/

data work.CombineYears;
	set work.GregYears work.quantileyears;
run;

/*
sorted work.combineyears by year so that we can eventually use contrasts but we did
not use contrasts
*/

proc sort data=work.CombineYears;
	 by year;
run;
```

```
/*
printing to verify sorted combineyears
*/

proc print data=work.CombineYears;
run;

/*
 turned on graphics. ran proc mixed on all years which gives us comparisons between years
*/

ods graphics on / ANTIALIASMAX=97200;
proc mixed data=work.CombineYears;
	class year;
	model logmass=logfl|year / solution outp=pred;
run;
/*
 plotted the predicted slopes between years
*/
proc sgplot data=pred;
	series y=pred x=logfl / group=Year;
run;

/*
this model was designed to show the actual slopes and intercepts but the comparisons are not
to be used for comparision
*/
proc glm data=work.combineyears;
	class year;
	model logmass=year logfl*year / noint solution;
run;

ods graphics off;



/*
Performed "contrasts" on comparisons of 2000-2010 logfl to those of 2011, 2012, 2013
respectively,
and contrasts between every pair of 2011, 2012, 2013

Used a Tukey adjustment which is more conservative (harder to reject the null)
and gived pairwise comparisons of the years
```

using the contrast function requires the summ of all constituents to equal zero. the mean of the means for each
year between 2000-2010 contrasted to the mean desired year.
*/

```
/*
proc glm data=work.CombineYears;
        class year;
        model logmass=logfl|year / solution;
        lsmeans year / adjust=tukey pdiff;
        contrast 'Years 2000-2010 & 2011' year 1 1 1 1 1 1 1 1 1 1 -11   0   0;
        contrast 'Years 2000-2010 & 2012' year 1 1 1 1 1 1 1 1 1 1   0 -11   0;
        contrast 'Years 2000-2010 & 2013' year 1 1 1 1 1 1 1 1 1 1   0   0 -11;
        contrast 'Years 2011 & 2013' year 0 0 0 0 0 0 0 0 0 0 1 0 -1;
        contrast 'Years 2012 & 2013' year 0 0 0 0 0 0 0 0 0 0 0 1 -1;
        contrast 'Years 2011 & 2012' year 0 0 0 0 0 0 0 0 0 0 1 -1 0;
run;
/*
```

*ods rtf close;

Menhaden Standard Weight Statistics

Model Information	
Data Set	WORK.COMBINEYEARS
Dependent Variable	logmass
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	Levels	Values
year	15	2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014

2014 is a dummy variable that represents 2000-2010 data sets

Dimensions	
Covariance Parameters	1
Columns in X	32
Columns in Z	0
Subjects	1
Max Obs per Subject	97152

Number of Observations	
Number of Observations Read	97152
Number of Observations Used	97152
Number of Observations Not Used	0

Covariance Parameter Estimates	
Cov Parm	Estimate
Residual	0.001107

Fit Statistics	
-2 Res Log Likelihood	-385204
AIC (Smaller is Better)	-385202
AICC (Smaller is Better)	-385202
BIC (Smaller is Better)	-385192

Table represents all years compared to the intercept ("year") of the 2000-2010 data (represented by 2014) and the slope (logfl*year) as compared to 2000-2010 (represented by 2014)

Solution for Fixed Effects

Effect	year	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept		-1.7884	0.006340	97E3	-282.10	<.0001
logfl		3.0793	0.005113	97E3	602.27	<.0001
year	2000	0.2384	0.01172	97E3	20.35	<.0001
year	2001	0.05340	0.01607	97E3	3.32	0.0009
year	2002	-0.05763	0.01265	97E3	-4.56	<.0001
year	2003	0.006016	0.01056	97E3	0.57	0.5689
year	2004	-0.1544	0.01046	97E3	-14.77	<.0001
year	2005	-0.01083	0.01291	97E3	-0.84	0.4016
year	2006	-0.03996	0.01357	97E3	-2.95	0.0032
year	2007	-0.1323	0.01434	97E3	-9.23	<.0001
year	2008	-0.2370	0.01556	97E3	-15.23	<.0001
year	2009	0.02700	0.01652	97E3	1.63	0.1023
year	2010	0.03416	0.01323	97E3	2.58	0.0098
year	2011	-0.02175	0.02141	97E3	-1.02	0.3099
year	2012	0.04583	0.02597	97E3	1.76	0.0776
year	2013	-0.1878	0.04187	97E3	-4.48	<.0001
year	2014	0	.	.	.	.
logfl*year	2000	-0.2234	0.009399	97E3	-23.77	<.0001
logfl*year	2001	-0.02889	0.01272	97E3	-2.27	0.0232
logfl*year	2002	0.03335	0.01013	97E3	3.29	0.0010
logfl*year	2003	-0.02297	0.008565	97E3	-2.68	0.0073
logfl*year	2004	0.1254	0.008549	97E3	14.67	<.0001
logfl*year	2005	0.008017	0.01050	97E3	0.76	0.4454
logfl*year	2006	0.02567	0.01105	97E3	2.32	0.0202
logfl*year	2007	0.1104	0.01175	97E3	9.39	<.0001
logfl*year	2008	0.1936	0.01250	97E3	15.49	<.0001
logfl*year	2009	-0.03434	0.01312	97E3	-2.62	0.0089
logfl*year	2010	-0.03357	0.01077	97E3	-3.12	0.0018
logfl*year	2011	-0.01994	0.01833	97E3	-1.09	0.2767
logfl*year	2012	-0.07907	0.02154	97E3	-3.67	0.0002
logfl*year	2013	0.1416	0.03481	97E3	4.07	<.0001
logfl*year	2014	0	.	.	.	.

These values are not to be compared to 2014 as they comprise 2014 (2000-2010)

These values are not to be compared to 2014 as they comprise 2014 (2000-2010)

This table actually gives the intercept ("year ####") and slope (logfl*year ####) for each year. The comparison (pvalue) is a dummy value not to be used for any statistical analysis. 2014 represents the 2000-2010 regression

Parameter	Estimate	Standard Error	t Value	Pr > |t|
year 2000	-1.550004938	0.00985668	-157.25	<.0001
year 2001	-1.735044244	0.01476787	-117.49	<.0001
year 2002	-1.846072929	0.01094539	-168.66	<.0001
year 2003	-1.782423656	0.00844691	-211.01	<.0001
year 2004	-1.942839143	0.00830982	-233.80	<.0001
year 2005	-1.799269662	0.01124896	-159.95	<.0001
year 2006	-1.828402820	0.01199678	-152.41	<.0001
year 2007	-1.920772374	0.01286736	-149.27	<.0001
year 2008	-2.025441963	0.01421443	-142.49	<.0001
year 2009	-1.761444257	0.01525709	-115.45	<.0001
year 2010	-1.754277784	0.01161763	-151.00	<.0001
year 2011	-1.810184936	0.02045454	-88.50	<.0001
year 2012	-1.742606432	0.02518877	-69.18	<.0001
year 2013	-1.976198820	0.04138717	-47.75	<.0001
year 2014	-1.788439532	0.00633969	-282.10	<.0001
logfl*year 2000	2.855891152	0.00788648	362.12	<.0001
logfl*year 2001	3.050382788	0.01165137	261.80	<.0001
logfl*year 2002	3.112623221	0.00874868	355.78	<.0001
logfl*year 2003	3.056301795	0.00687193	444.75	<.0001
logfl*year 2004	3.204650083	0.00685187	467.70	<.0001
logfl*year 2005	3.087289146	0.00917601	336.45	<.0001
logfl*year 2006	3.104938567	0.00980099	316.80	<.0001
logfl*year 2007	3.189690390	0.01058407	301.37	<.0001
logfl*year 2008	3.272873797	0.01140919	286.86	<.0001
logfl*year 2009	3.044936482	0.01208608	251.94	<.0001
logfl*year 2010	3.045701438	0.00947470	321.46	<.0001
logfl*year 2011	3.059333263	0.01760366	173.79	<.0001
logfl*year 2012	3.000197661	0.02092869	143.35	<.0001
logfl*year 2013	3.220866770	0.03443203	93.54	<.0001
logfl*year 2014	3.079272525	0.00511274	602.27	<.0001

MatLab Code for Statistical Analysis

Minimum Forklength

```
x=[Insert data set here];
y=x(2,:);
x=x(1,:);
y=log10(y);

s=6:23;

for k=1:length(s);
   I=find(x>s(k) & x<=s(k)+1);
   v(k)=var(y(I));
   m(k)=mean(y(k));
end;
plot (s, v./m,'o');
set(gca,'ylim',[0 0.08]);
```

Ws Equation Matlab

```
years=[Insert the years you wish to compare here];

xc=[];
yc=[];
data=[];
rlp=0.875;



%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
test=years-2000;


x=[Insert dataset here]

if(prod(test)==0);
s=[9:24];
Xc=[];
Yc=[];

y=x(2,:);
x=x(1,:);




m=0;

for k=1:length(s);
    I=find(x>s(k) & x<=s(k)+1);
    X=x(I);
    Y=log10(y(I));

  YM=mean(Y);
  YS=std(Y);
  t=tinv(rlp,length(Y)-1);
  YU=YM+t*YS;
  YL=YM-t*YS;

  J=find(Y>=YL & Y<=YU);
  yc=[yc mean(Y(J))];
  xc=[xc mean(X(J))];
  Xc=[Xc mean(X(J))];
```

```
  Yc=[Yc mean(Y(J))];
  m=m+length(X(J));



end
disp(m);
p=polyfit(log10(Xc),Yc,1)
data=[data m];

end;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

results=ancovaclass(log10(xc),(yc),data)
```

Ancovaclass

```
function p=ancovaclass(x,y,data);


n=length(data); % n is the number of datasets
xm=mean(x);
ym=mean(y);
sxx=sum((x-xm).^2);
sxy=sum((x-xm).*(y-ym));
B=sxy/sxx;

bmat=B;

A=ym-B*xm;
yc=A+B*x;
ss3=sum((y-yc).^2); % one line to all the data
df3=sum(data)-2;

nstart=1;
for k=1:n;
   nend=nstart+data(k)-1;
   m=nstart:nend;
   X=x(m);
   Y=y(m);
   xm=mean(X);
```

```
ym=mean(Y);
sxx=sum((X-xm).^2);
SXX(k)=sxx;
sxy=sum((X-xm).*(Y-ym));
B(k)=sxy/sxx;

bmat=[bmat B(k)];

A=ym-B(k)*xm;
yc=A+B(k)*X;
ss(k)=sum((Y-yc).^2);
nstart=nstart+data(k);
end;
ss1=sum(ss); % one line to each dataset
df1=sum(data)-2*n;

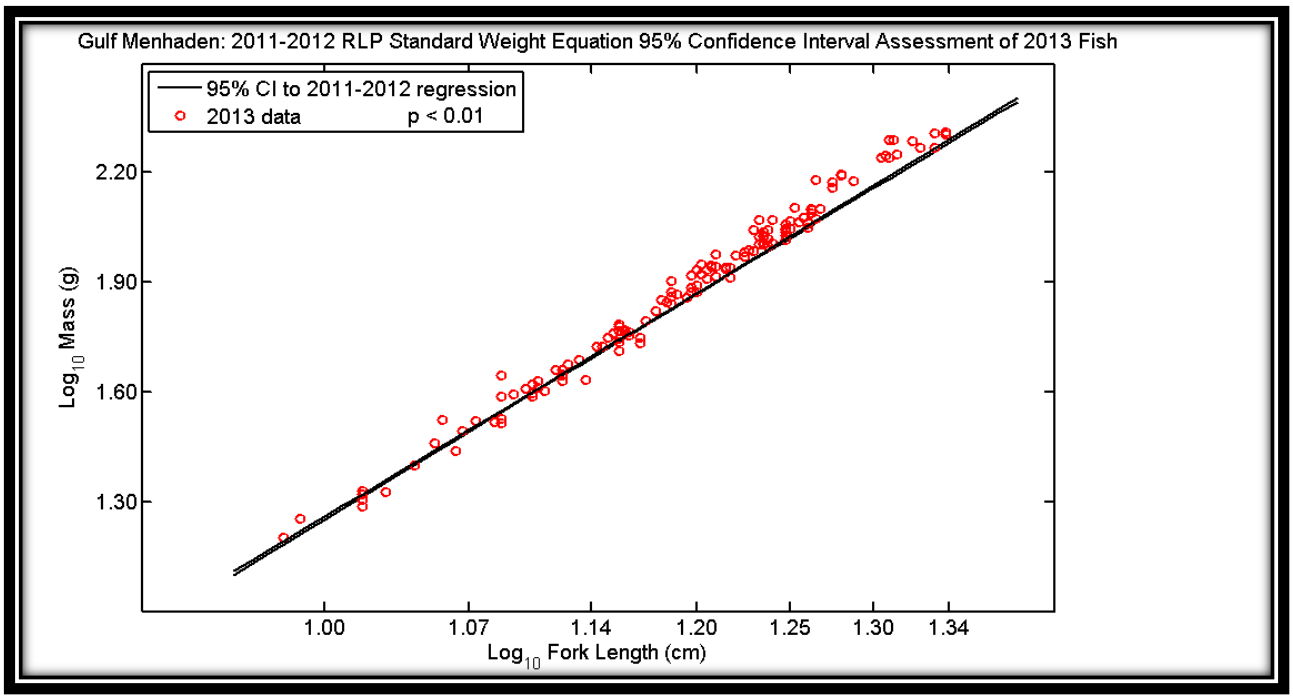
w=SXX/sum(SXX);
bp=sum(B.*w);

bmat=[bmat bp];

nstart=1;
for k=1:n;
   nend=nstart+data(k)-1;
   m=nstart:nend;
   X=x(m);
   Y=y(m);
   xm=mean(X);
ym=mean(Y);
A=ym-bp*xm;
yc=A+bp*X;
ss(k)=sum((Y-yc).^2);
nstart=nstart+data(k);
end;
ss2=sum(ss); % parallel lines to each dataset
df2=sum(data)-n-1;
F23=(ss3-ss2)/(df3-df2)/(ss2/df2); % test for different elevations
F12=(ss2-ss1)/(df2-df1)/(ss1/df1); % test for different slopes
p12=1-fcdf(F12,df2-df1,df1); % probability of F12 being this large or larger
p23=1-fcdf(F23,df3-df2,df2); % % probability of F23 being this large or larger

format short g;
p=[F12 p12;F23 p23];
```

Matlab Graphic depicting 2011-2012 linear regression of menhaden data to 2013



E: Chapter 5

Data Tables and Graphs

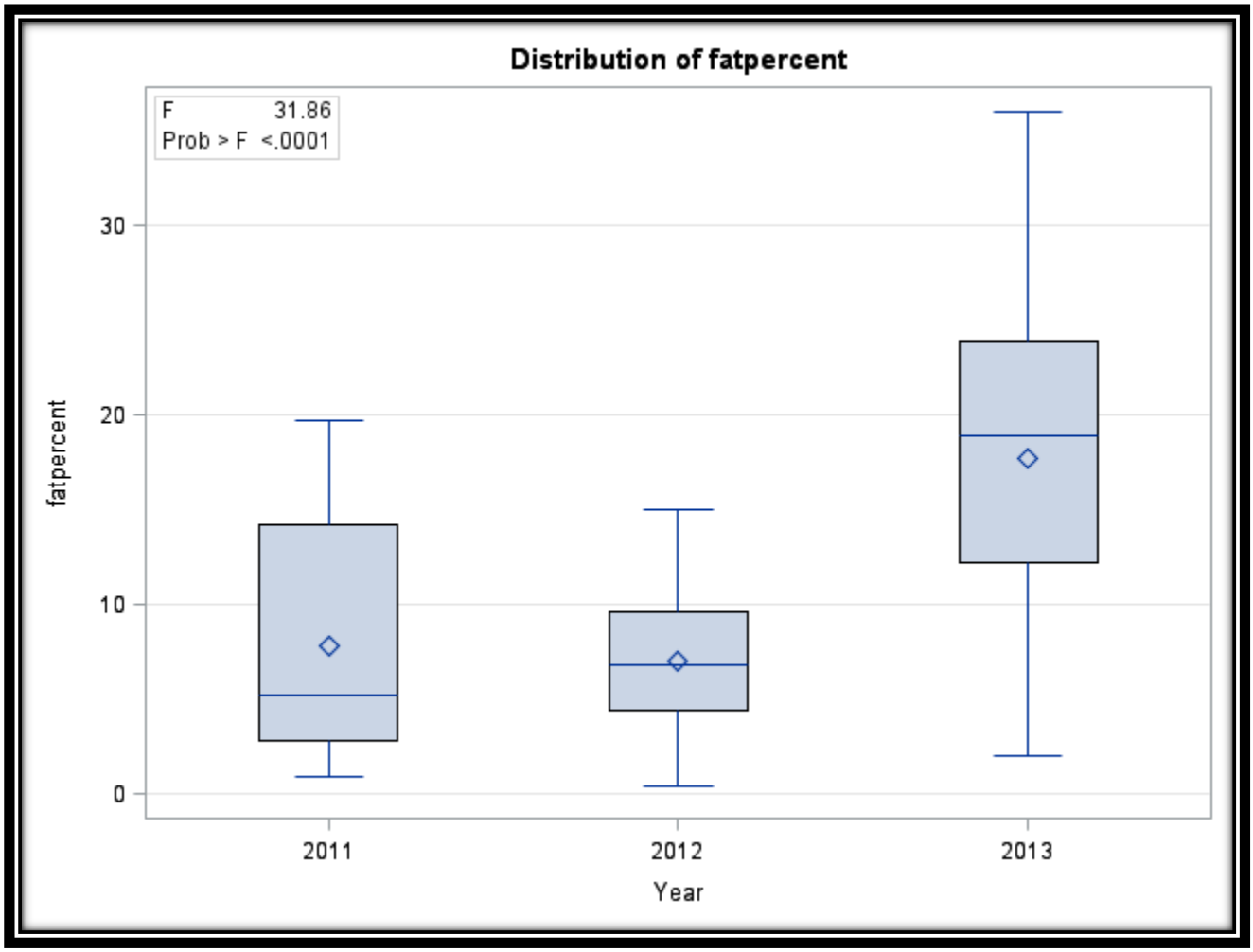
Raw Menahden Fat Data

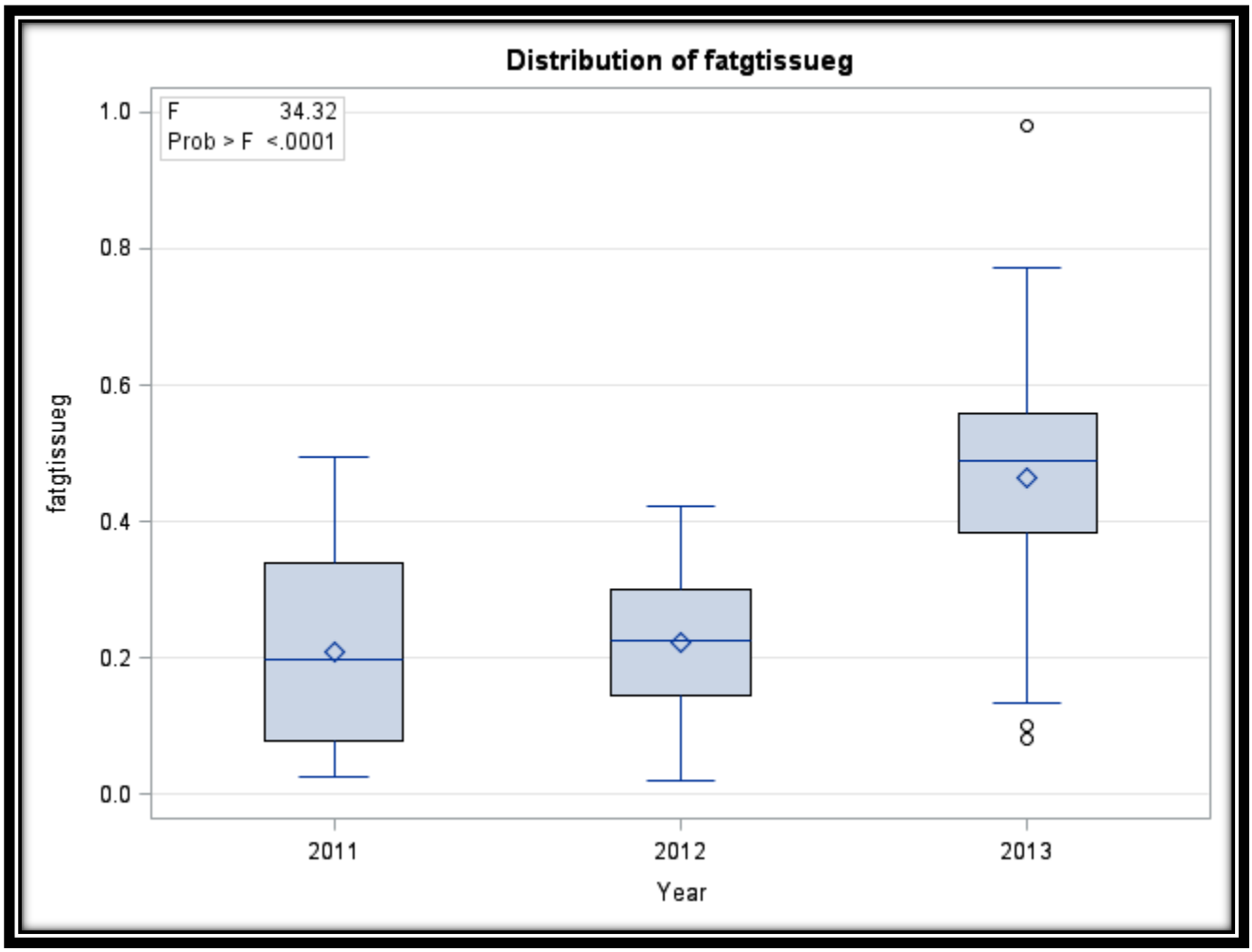
fatpercent	Month	Year	Site	Size	fatgtissueg
12.736	jul	2011	vb	lg	0.352
15.720	jul	2011	vb	lg	0.396
19.707	jul	2011	vb	lg	0.495
15.605	jul	2011	gi	lg	0.388
18.655	jul	2011	gi	lg	0.455
16.853	jul	2011	gi	lg	0.438
3.373	aug	2011	vb	sm	0.097
0.893	aug	2011	vb	sm	0.026
2.455	aug	2011	vb	sm	0.070
0.988	aug	2011	gi	sm	0.028
1.619	aug	2011	gi	sm	0.046
1.980	aug	2011	gi	sm	0.058
5.451	aug	2011	vb	lg	0.199
5.469	aug	2011	vb	lg	0.199
4.545	aug	2011	vb	lg	0.168
5.769	aug	2011	gi	lg	0.199
3.529	aug	2011	gi	lg	0.136
1.860	aug	2011	gi	lg	0.077
12.568	sept	2011	gi	sm	0.278
16.285	sept	2011	gi	sm	0.327
10.718	sept	2011	gi	sm	0.233
16.073	sept	2011	vb	sm	0.362
18.828	sept	2011	vb	sm	0.383
15.822	sept	2011	vb	sm	0.374
2.888	sept	2011	vb	lg	0.117
9.128	sept	2011	vb	lg	0.300
5.030	sept	2011	vb	lg	0.198
3.431	sept	2011	gi	lg	0.133
3.167	sept	2011	gi	lg	0.128
5.023	sept	2011	gi	lg	0.188
7.852	jul	2011	vb	sm	0.200
7.531	jul	2011	vb	sm	0.209
0.959	jul	2011	vb	sm	0.031
2.783	jul	2011	gi	sm	0.072
2.930	jul	2011	gi	sm	0.077
2.698	jul	2011	gi	sm	0.075
8.148	sept	2012	vb	sm	0.280

4.618	sept	2012	vb	sm	0.182
9.666	sept	2012	vb	sm	0.320
6.406	sept	2012	gi	sm	0.222
6.617	sept	2012	gi	sm	0.229
8.138	sept	2012	gi	sm	0.273
14.274	sept	2012	vb	lg	0.423
14.163	sept	2012	vb	lg	0.404
9.272	sept	2012	vb	lg	0.301
6.990	sept	2012	gi	lg	0.220
12.212	sept	2012	gi	lg	0.363
15.035	sept	2012	gi	lg	0.416
8.326	aug	2012	vb	lg	0.270
9.552	aug	2012	vb	lg	0.300
9.140	aug	2012	vb	lg	0.290
10.896	aug	2012	gi	lg	0.320
7.982	aug	2012	gi	lg	0.250
10.585	aug	2012	gi	lg	0.320
0.487	aug	2012	vb	sm	0.020
4.637	aug	2012	vb	sm	0.160
5.633	aug	2012	vb	sm	0.200
9.623	aug	2012	gi	sm	0.300
10.867	aug	2012	gi	sm	0.330
9.939	aug	2012	gi	sm	0.300
1.269	jul	2012	vb	sm	0.050
0.685	jul	2012	vb	sm	0.030
0.449	jul	2012	vb	sm	0.020
4.061	jul	2012	gi	sm	0.130
2.852	jul	2012	gi	sm	0.100
4.749	jul	2012	gi	sm	0.150
2.168	jul	2012	vb	lg	0.080
4.392	jul	2012	vb	lg	0.150
3.978	jul	2012	vb	lg	0.130
4.549	jul	2012	gi	lg	0.140
4.510	jul	2012	gi	lg	0.150
6.532	jul	2012	gi	lg	0.190
18.220	jul	2013	vb	lg	0.470
19.528	jul	2013	vb	lg	0.529
19.195	jul	2013	vb	lg	0.517
1.978	jul	2013	vb	sm	0.082
15.236	jul	2013	vb	sm	0.437
6.825	jul	2013	vb	sm	0.230
24.884	jul	2013	gi	lg	0.572
18.645	jul	2013	gi	lg	0.504
22.434	jul	2013	gi	lg	0.542
12.520	jul	2013	gi	sm	0.385
2.481	jul	2013	gi	sm	0.100

6.231	jul	2013	gi	sm	0.212
25.211	aug	2013	vb	lg	0.592
26.221	aug	2013	vb	lg	0.630
26.587	aug	2013	vb	lg	0.614
24.202	aug	2013	vb	sm	0.591
26.694	aug	2013	vb	sm	0.771
36.022	aug	2013	vb	sm	0.979
15.025	aug	2013	gi	lg	0.447
13.765	aug	2013	gi	lg	0.414
14.263	aug	2013	gi	lg	0.423
11.958	aug	2013	gi	sm	0.382
5.936	aug	2013	gi	sm	0.220
14.661	aug	2013	gi	sm	0.496
17.908	sept	2013	vb	lg	0.427
22.197	sept	2013	vb	lg	0.479
26.903	sept	2013	vb	lg	0.574
25.263	sept	2013	vb	sm	0.640
22.045	sept	2013	vb	sm	0.540
23.715	sept	2013	vb	sm	0.528
22.688	sept	2013	gi	lg	0.505
19.264	sept	2013	gi	lg	0.481
22.500	sept	2013	gi	lg	0.533
10.716	sept	2013	gi	sm	0.341
10.753	sept	2013	gi	sm	0.346
3.503	sept	2013	gi	sm	0.134

Distribution of Fat in both Percent and Grams



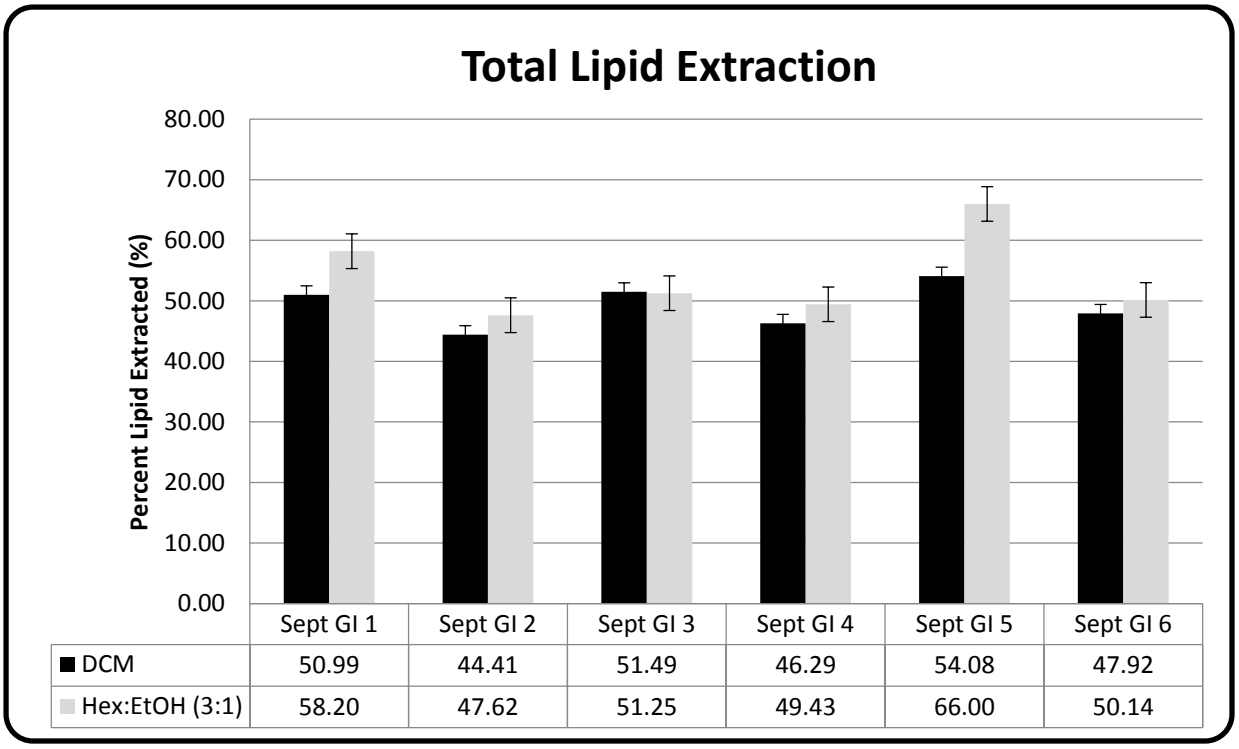

Distribution of fatgtissueg
F 34.32
Prob > F <.0001
1.0
0.8
0.6
0.4
0.2
0.0
fatgtissueg
2011
2012
2013
Year

Commercial Oil Recovery Hex:EtOH vs DCM

Sample ID	Oil Extract Added (g)	Solvent Used	Extract Yield (g)	Abs(Difference (g))	% Error
OilExtract - 1	0.512	Hex:EtOH (3:1)	0.498	0.014	2.73
OilExtract - 2	0.506	Hex:EtOH (3:1)	0.492	0.014	2.77
OilExtract - 3	0.506	Hex:EtOH (3:1)	0.501	0.005	0.99
OilExtract - 4	0.503	Hex:EtOH (3:1)	0.505	0.002	0.40
OilExtract - 5	0.504	Hex:EtOH (3:1)	0.488	0.016	3.17
OilExtract - 6	0.521	Hex:EtOH (3:1)	0.504	0.017	3.26
OilExtract - 7	0.511	Hex:EtOH (3:1)	0.496	0.015	2.94
OilExtract - 8	0.517	Hex:EtOH (3:1)	0.505	0.012	2.32
OilExtract - 9	0.513	Hex:EtOH (3:1)	0.515	0.002	0.39
Mean	0.510		0.500	0.011	1.94
Stdev	0.006		0.008	0.006	
%RSD	1.200		1.608	52.990	
OilExtract - 10	0.509	DCM	0.504	0.005	0.98
OilExtract - 11	0.514	DCM	0.510	0.004	0.78
OilExtract - 12	0.505	DCM	0.492	0.013	2.57
OilExtract - 13	0.509	DCM	0.494	0.015	2.95
OilExtract - 14	0.512	DCM	0.502	0.01	1.95
OilExtract - 15	0.512	DCM	0.514	0.002	0.39
OilExtract - 16	0.505	DCM	0.501	0.004	0.79
OilExtract - 17	0.501	DCM	0.486	0.015	2.99
OilExtract - 18	0.506	DCM	0.489	0.017	3.36
Mean	0.508		0.499	0.009	1.77
Stdev	0.004		0.009	0.005	
%RSD	0.826		1.801	57.587	

DCM vs Hex:EtOH extraction comparison between realworld menhaden samples

Sample ID	Solvent Used	Sub-Sample (g)	Lipids (g)	% Fat Recovered
Sept GI 1	DCM	10.10	5.15	50.99
Sept GI 2	DCM	10.02	4.45	44.41
Sept GI 3	DCM	10.39	5.35	51.49
Sept GI 4	DCM	10.24	4.74	46.29
Sept GI 5	DCM	10.04	5.43	54.08
Sept GI 6	DCM	10.10	4.84	47.92
Spet GI 1	Hex:EtOH (3:1)	10.86	6.32	58.20
Sept GI 2	Hex:EtOH (3:1)	10.50	5.00	47.62
Sept GI 3	Hex:EtOH (3:1)	10.79	5.53	51.25
Spet GI 4	Hex:EtOH (3:1)	10.56	5.22	49.43
Sept GI 5	Hex:EtOH (3:1)	10.03	6.62	66.00
Sept GI 6	Hex:EtOH (3:1)	10.61	5.32	50.14



	Sept GI 1	Sept GI 2	Sept GI 3	Sept GI 4	Sept GI 5	Sept GI 6
DCM	50.99	44.41	51.49	46.29	54.08	47.92
Hex:EtOH (3:1)	58.20	47.62	51.25	49.43	66.00	50.14

SAS Code for Statistical Analysis

Fat Analysis of Gulf Menhaden Sampled from 2011-2013

```
dm 'log;clear;outout;clear';
options nodate nocenter pageno = 1 ls=78 ps=53;
title1 'Menhaden Fat Analysis';
ods html file = 'C:\Users\golson2\Desktop\fatsas.html';

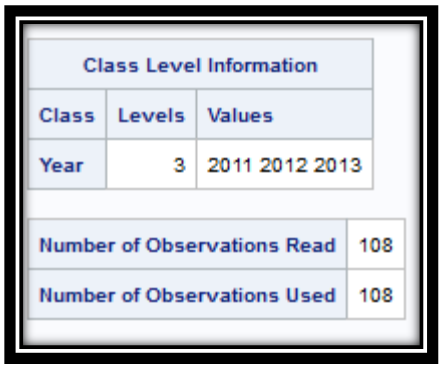

proc import out=work.fatsas (keep=obs fatpercent pahnngMonth Year Site Size)
        file='C:\Users\golson2\Desktop\fatsas.xlsx'
        dbms=excel replace;
        getnames=yes;
run;


data fat;
set work.fatsas;
run;
```

```
proc glm data=fat;
class year;
model fatpercent = year;
means year / lsd tukey hovtest=bartlett;
output out=outdata p=pred residual=resid;
run;

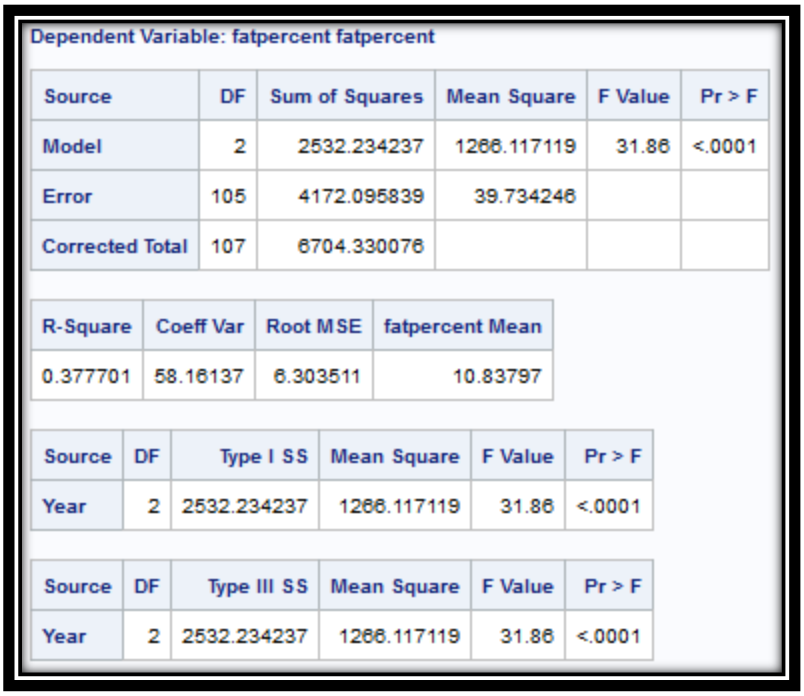
proc univariate data=outdata normal plot;
        var resid;
run;
proc plot data=outdata;
        plot resid*pred;
run;
```

SAS Summary Statistics



Class Level Information		
Class	Levels	Values
Year	3	2011 2012 2013

Number of Observations Read	108
Number of Observations Used	108



Dependent Variable: fatpercent fatpercent

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	2532.234237	1266.117119	31.86	<.0001
Error	105	4172.095839	39.734246		
Corrected Total	107	6704.330076			

R-Square	Coeff Var	Root MSE	fatpercent Mean
0.377701	58.16137	6.303511	10.83797

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Year	2	2532.234237	1266.117119	31.86	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Year	2	2532.234237	1266.117119	31.86	<.0001

SAS Summary Statistics (Cont.)

t Tests (LSD) for fatpercent

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	105
Error Mean Square	39.73425
Critical Value of t	1.98282
Least Significant Difference	2.946

Means with the same letter are not significantly different.

t Grouping	Mean	N	Year
A	17.672	36	2013
B	7.803	36	2011
B	7.039	36	2012

Tukey's Studentized Range (HSD) Test for fatpercent

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	105
Error Mean Square	39.73425
Critical Value of Studentized Range	3.36216
Minimum Significant Difference	3.5322

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Year
A	17.672	36	2013
B	7.803	36	2011
B	7.039	36	2012

Excel Tables for Statistical Analysis

t-Test: Paired Two Sample for Means Commercial Oil Extraction

	Hex:EtOH	*DCM*
Mean	0.500444444	0.499111111
Variance	6.47778E-05	9.08611E-05
Observations	9	9
	-	
Pearson Correlation	0.536772375	
Hypothesized Mean Difference	0	
df	8	
t Stat	0.259281489	
P(T<=t) one-tail	0.400982634	
t Critical one-tail	1.859548033	
P(T<=t) two-tail	0.801965268	
t Critical two-tail	2.306004133	

t-Test: Paired Two Sample for Means DCM-HexEtOH mass Test

	DCM	*Hex:EtOH*
Mean	4.993333333	5.668333333
Variance	0.145066667	0.423696667
Observations	6	6
Pearson Correlation	0.805448409	
Hypothesized Mean Difference	0	
df	5	
	-	
t Stat	4.017311556	
P(T<=t) one-tail	0.005073879	
t Critical one-tail	2.015048372	
P(T<=t) two-tail	0.010147758	
t Critical two-tail	2.570581835	

t-Test: Paired Two Sample for Means DCM-HexEtOH Test

	DCM	*Hex:EtOH*
Mean	49.19776927	53.77343436
Variance	13.06995918	49.07392344
Observations	6	6
Pearson Correlation	0.85771644	
Hypothesized Mean Difference	0	
df	5	
	-	
t Stat	2.591897867	
P(T<=t) one-tail	0.024362387	
t Critical one-tail	2.015048372	
P(T<=t) two-tail	0.048724775	
t Critical two-tail	2.570581835	

F: Chapter 6

Grant Proposal

Continual Assessment of Near and Off-Shore Contamination in the Gulf of Mexico Using Brevoortia patronus as an Indicator Species

"Menhaden Watch"

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SUMMARY

The primary focus of "menhaden watch" represents an initial phase program assessment of crude oil contamination concentrating on Louisiana coastal and off-shore waters. Once completed, continual assessment of the Gulf of Mexico should be extended to each state surrounding the Gulf, creating a complete continual assessment of near and off-shore waters similar to the National Center for Coastal Ocean Science (NCCOS) Mussel Watch program.

PROBLEM STATEMENT AND NEEDS ASSESSMENT

Approximately 4.9 million barrels of crude oil and gas were released into the Gulf of Mexico (GoM) from April to July 2010 during the Deepwater Horizon (DWH) spill. Impacts of this magnitude do not always occur in the GoM, however we cannot predict when these types of events will happen. Major constituents of concern (CoC) in crude oil are Polycyclic Aromatic Hydrocarbons, which often have low volatility allowing for elongated existence in the environment. It is proposed that PAHs are used to perform a continual toxic potential assessment of oil contamination in the GoM. PAHs are considered compounds of concern according to the United States Environmental Protection Agency (USEPA) due to their ability to accumulate within adipose tissue (USEPA, 2008). There are several PAHs listed as mutagenic and carcinogenic, making their possible presence in commercial fishery populations a major environmental concern (Durant et al., 1996; Nisbet and LaGoy, 1992; USEPA, 2008).

Programs exist for the continual monitoring of coastal waters using invertebrate, filter feeding mollusks (Mussel Watch) which have helped elucidate near shore impact dynamics(NOAA, 2012); however there are no such programs for assessing near and off shore impact dynamics using a vertebrate species of similar characteristics. The current proposal attempts to understand the concentrations of PAHs within a commercially viable fish harvested in great quantities from the GoM. In order to quantify PAH concentrations found in the commercial fishery of the GoM, an initial phase assessment of the fishery was conducted. The Gulf menhaden (*Brevoortia patronus*) was identified as the second largest commercial harvest from United States waters and the largest from the GoM from 2005 to 2010 and chosen as the principle proposed study organism (Van Voorhees and Lother, 2011). The organism selection was further supported as menhaden are harvested due to the amount of fats and oils that are extracted and refined for consumer use (Franklin, 2007; Vaughan et al., 2007). Menhaden are also significant due to their position in the GoM food web as obligate filter feeders (also the same feeding mechanism employed by mollusks). This particular mode of feeding increases interaction with possible surface and subsurface oil through dermal contact and direct ingestion, and based on the primary diet of phytoplankton, positions menhaden as the main link between producers and secondary consumers (Franklin, 2007; Van Voorhees and Lother, 2011; Vaughan et al., 2007). Menhaden

whole life cycle turnover is approximately three years, allowing for whole life assessment every three years as well as pre, during, and post event temporal assessment for future oiling events.

Commercial fishing grounds are as far east as Florida and stretch west to Mexico. From roughly April to October each year, the fish form large schools and are harvested for industrial refining of their fats and oils (Franklin, 2007; Vaughan et al., 2007). Menhaden oil is used in a variety of commercial products ranging from makeup to over-the-counter health supplements (Franklin, 2007). PAHs are lipophilic and can accumulate within the adipose tissue of an organism (Larsen et al., 2002). Menhaden are fatty fish that can accumulate PAHs in their tissue leading to the possible magnification of the toxic compounds through trophic transfer due to predator/prey consumption interactions. Menhaden are a standard forage food for other fish, birds, and marine mammals. They represent the primary connection between producers and secondary consumers within the GoM (Franklin, 2007; Vaughan et al., 2007). Gulf menhaden do not undergo major longitudinal migrations as the fish remain in coastal waters seasonally and spend the first year of their life cycle in estuarine waters (Vaughan et al., 2007). As a result, Gulf menhaden develop solely in the Gulf throughout the duration of their life, moving between deep (roughly 80 km off shore) and coastal waters (Vaughan et al., 2007). Gulf menhaden spawn between October and March with peak spawning between December and January; April to October is the optimal harvest season (Raynie and Shaw, 1994). The spatial distribution, feeding patterns, and abundance of the organism within the desired region of study are all major factors contributing to the importance of the Gulf menhaden as a continual indicator species to assess the health of the GoM.

RELEVANCE

The GoM is projected to produce upwards of 1.7 million barrels of oil per day (MMBOPD) and 8 billion cubic feet per day (BCFPD) of natural gas by 2016 (Karl et al., 2007). The GoM is a significant petrochemical exploration and development region of the United States. It has and will continue to be a major source of crude oil and natural gas. The GoM is also one of the most productive marine ecosystems in the United States, accounting for an average of 18% of the total U.S. domestic commercial fish landings from 2009 - 2010(Van Voorhees and Lother, 2011). The GoM will continually be affected by petroleum exploration for the immediate future. Because of the connection to the petrochemical industry, commercial and sport fishing in this region will always have the potential to be affected; therefore the GoM should be monitored continually in order to assess overall health as well as specific temporal and spatial events impacting this region.

This proposal is focusing on a particular facet of toxic potential assessment, specifically total PAH concentrations, carcinogenic equivalents (BaP-TEQ), and mutagenic equivalents (BaP-MEQ). The work will be done in collaboration with cross discipline research involving histology, metals analysis (specifically heavy metals associated with oil exploration), total

polychlorinated biphenyls (PCBs), dioxin, DDT, and TBT analyses as outlined in the established "Mussel Watch" program.

PROPOSED STUDIES

1. Determine total PAH concentrations and apply BaP-TEQ and BaP-MEQ indices to menhaden harvested at specific near shore and off shore locations around the Gulf of Mexico from April to October each year, creating a continuous contaminant monitoring program based on analysis for chemical contaminant trends in menhaden tissue collected. Compared spatially and by size.

2. Determine average whole life body accumulation using three year interval analysis of total PAH concentrations as well as BaP-TEQ and BaP-MEQ indices of harvested menhaden compared spatially and by size.

3. Assess Gulf menhaden health based on yearly mean raw menhaden oil concentrations compared spatially and by size.

4. Generate baseline data sets that can be used in conjunction with annual data collection to determine overall heath as well as specific temporal and spatial impacts on the GoM.

5. Develop similar studies as labeled 1 - 4, isolating metals and various other CoCs associated with contamination as identified in the NCCOS Mussel Watch program.

6. Assess Gulf menhaden health based histopathological analysis compared spatially and by size.

MATERIALS & METHODS

Sample Collection

Menhaden will be sampled at locations near Grand Isle (GI), Vermilion Bay (VB), and Calcasieu Lake (CL) across the coast of Louisiana. The samples will be collected using a five-panel gill net approximately 750 ft long with distinct plastic mesh panels. Sampling events will either take place under the supervision of Louisiana Department of Wildlife and Fisheries (LDWF) biologists or by the LDWF biologists. All sampling protocols will be congruent with LDWF protocols along with the appropriate International Animal Care and Use Committee (IACUC) approved Animal Care and Use Protocol (ACUP). After collection, the menhaden will be separated by length, bagged in plastic freezer bags, and placed on ice until histopathology sample preparation and / or storage at -4°C.

Sample Preparation - Histopathology

Approximately 20 - 30 menhaden will be fixed in formalin from each sampling event. The operculum will be removed from both sides and an incision will be made from the anus to the region below the pectoral fin using a scalpel or sharp filet knife. Using scissors, vertical cuts will be made from the anus towards the dorsal fin and from the region under the pectoral fin to the dorsal fin so as to create a panel that can be raised to expose the internal organs of the menhaden. Samples will then be placed in 5% formalin and stored until analysis.

Sample Preparation - Tissue Analysis (Total lipids, PAH, Alkanes, Metals, and Various CoCs)

Menhaden will be removed from -4°C storage and segmented into 2.5cm x 2.5cm x 2.5cm sections (approximate size). These sections will be placed in a lyophilization safe container and cooled to -86°C using an ultra-cold freezer. The samples will then be lyophilized for 24 to 36 hours and removed to a dessicator. The samples will them be homogenized and stored in laboratory quality resealable zipper bags.

Solvents, Reagents, and Chemicals

Pesticide reagent grade solvents will be used in all standard preparations, sample analysis, and rinsing procedures. Dichloromethane (DCM) and hexane (Mallinckrodt Chemicals) will be used for sample extraction. Sodium sulfate (anhydrous, 10-60 mesh, Fisher Scientific) will also be used for sample preparation.

Calibration Standards

A commercially-prepared crude oil analysis standard (Oil Analysis Standard, Absolute Standards), will be used to prepare the five-point calibration standards. Calibration standard solutions are stored in amber vials with PTFE-lined caps. The calibration standards are checked frequently for signs of degradation or evaporation and replaced if indicated in laboratory quality control checks. A continuing calibration standard (one point of the initial five-point calibration standard) will be analyzed in each batch of extracted tissue samples or during each 12-hour period during which analyses will be performed. Acceptance criterion for the continuing calibration standard is ±20% of the mean relative response factor calculated from the initial five-point curve. If the acceptance criterion is not met, all analyses will be discontinued until the instrument is re-aligned to meet optimal operations criteria. With instrument maintenance or troubleshooting, a new five-point calibration curve will be generated as per good laboratory practices.

Internal Standard Solutions

Internal standards are naphthalene-d_8, acenaphthene-d_{10}, chrysene-d_{10}, and perylene-d_{12} (AccuStandard Inc.) and each are stored individually until mixed with DCM to make the internal standard injecting solution. Each internal standard is used to determine the concentrations of

analytes with similar molecular weight. This is done by spiking each GC vial with 10 µl of the prepared internal standard and then standardizing each target response to the known concentrations of the four standards. Once this is complete, the analyte target response can then be converted to a concentration using the formula below:

$$Analyte\ Concentration = \frac{(Target\ Response) \times (Internal\ Standard\ Concentration) \times (Final\ Volume) \times (Dilution\ Factor)}{(Response\ of\ Internal\ Standard) \times (Analyte\ Mean\ Response\ Factor) \times (Volume\ Injected) \times (Dry\ Mass)}$$

Reference Oil Standard

The usual laboratory reference oil established by USEPA has been Alaska North Slope Crude Oil (ANSCO); however, the reference oil standard used for these analyses will be Macondo 252 (MC 252) collected directly from the riser of the Deepwater Horizon oil rig. Reference oil standards are prepared by extracting 1 gram of pure oil in 40-mL of solvent (or equivalent ratio of 1g: 40mL, e.g. 0.50g: 20mL). The laboratory reference oil is analyzed in each sample batch as an additional QA/QC sample, i.e., a laboratory control sample.

Surrogate Standards

The surrogate standards are 5-alpha androstane (alkanes) and phenanthrene-d_{10} (aromatics) (AccuStandard) and each are stored individually until mixed with DCM to make the needed concentration of surrogate standard. The extraction efficiency for each sample is based on percent recovery of surrogate standard with an acceptable percent recovery range of 60 – 120% (USEPA, 2012).

Sample Extraction - Alkanes and PAHs Using Matrix Solid Phase Dispersion and GC/MS Analysis

A two gram subsample will be removed and homogenized to a consistency of roughly 200 mesh with a 1:1 ratio of C-18 silica. Approximately 2 g of sodium sulfate will be added to remove excess moisture. The sample will then be spiked with 1 ml of a surrogate spiking solution containing alkane and aromatic standards. The sample will be covered with DCM and sonicated for 30 minutes. After the sonication process each sample will be gravity filtered through a Fisherbrand filter (09-801-G, 24 cm diameter) covered with a 10 g layer of sodium sulfate. The container used to lyophilize and sonicate the sample will be rinsed three times with DCM into the homogenized sample to ensure complete transfer of all materials. The funnel (Corning, 6120-6) will be attached to a side-arm flask (Corning, 5340-250) affixed to a vacuum manifold. After gravity filtration stops a slight vacuum (vacuum-assisted solvent extraction) will be applied to finish the removal of all DCM and possible analytes. The resulting eluent will be moved to a flat bottom Florence flask (Corning, 4060-250), rinsing 3 times with DCM, and rotary evaporated (Rotavap™ Buchi Laboratory Equipment) until all excess DCM is removed. The residue will then be reconstituted in hexane and transferred to a solvent rinsed glass graduated cylinder (Corning, 3024-500). An appropriate amount of hexane will then be used to dilute the resulting material to a whole number volume in ml (25ml - 50ml). Final volumes will be recorded and the solution will be aspirated and homogenized with a Pasture pipette before a 20 ml aliquot is collected from the graduated cylinder in a volatile organic analysis (VOA) vial (Cole Parmer, T-

99536-12). Multiple 1 ml aliquots will be used for GC/MS analysis. Samples will be placed in appropriate amber auto-sampler vials (Wheaton, W225172-01), spiked with 10 µl of the prepared internal standard, capped and placed in refrigeration prior to GC/MS analysis.

Sample Extraction - Total Lipid Analysis Using Soxhlet

Menhaden tissue (10 g) will be homogenized and mixed with approximately 5g of sodium sulfate to bind up any moisture present in the lyophilized fish. The sample will then be packed into a cellulose extraction thimble (Advantec grade 84, ID33mm OD37mm L80mm), spiked with 1 ml of surrogate solution, and placed into a Soxhlet extraction column. 100 ml of DCM is then placed into a tared, flat-bottom Florence flask on a hotplate; a Soxhlet column (filled with 50ml of DCM) is connected to the flat-bottom flask and the flask is heated to a boil. The column will continuously extract for 16 to 18 hours to removing the lipid and oil fraction from the menhaden sample. Excess DCM is removed through rotary evaporation (Rotavap™ Buchi Laboratory Equipment, New Castle, DE) and the resulting material will be weighed to yield the total amount of oil extracted from the menhaden. This value can then be compared to the total mass of the wet menhaden to generate an oil content percentage. This procedure will be done for each sampling location and for both small and large menhaden. Mean oil percentage per fish and mean mass of oil per gram of menhaden will be determined for each size category.

Sample Analysis - Metal Analysis Using ICP-OES

One gram of dry fish tissue is massed and placed in a 55 ml glass digestion tube (Pyrex, AKM-2100-0242). Next, 5 mL of concentrated trace-metal-grade nitric acid will be added to the tubes and allowed to digest for 12 hours. Samples are placed in a digestion block for 8 hours at 120˚C. After complete digested, the mixture is evaporated down approximately 1.5 ml. The mixture is cooled and diluted to 50 ml with deionized water. It will then be covered with Parafilm (Cole Parmer, EW-06720-34) and vigorously shaken. Samples will be allowed to settle for a minimum of 14 hours until all residual material has precipitated to the bottom of the tube. 14 to 15 ml of sample solution is transferred to 15mL glass ICP tubes. Digestion and ICP tubes are washed in a 5% nitric acid bath for 14 hours and rinsed six times with deionized water before use.

TIMELINE

Year 1	Menhaden Season											
	M	J	J	A	S	O	N	D	J	F	M	A
Data Collection	X	X	X	X	X	X						
PAH, BaP-TEQ, BaP-MEQ						X	X	X				
PCBs						X	X	X				
Total Lipids									X	X		
Metals									X	X		
Histology								X	X	X	X	
Annual Report												X

Year 2	Menhaden Season											
	M	J	J	A	S	O	N	D	J	F	M	A
Data Collection	X	X	X	X	X	X						
PAH, BaP-TEQ, BaP-MEQ						X	X	X				
PCBs						X	X	X				
Total Lipids									X	X		
Metals									X	X		
Histology								X	X	X	X	
Annual Report												X

Year 3	Menhaden Season											
	M	J	J	A	S	O	N	D	J	F	M	A
Data Collection	X	X	X	X	X	X						
PAH, BaP-TEQ, BaP-MEQ						X	X	X				
PCBs						X	X	X				
Total Lipids									X	X		
Metals									X	X		
Histology								X	X	X	X	
Three Year Report												X

This timeline repeats every three years

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THREE YEAR BUDGET:

BUDGERT REMOVED FOR PRIVACY

BUDGET JUSTIFICATION

A. Senior Personnel Costs:

Ralph Portier, PI: (1 month of salary). Dr. Portier will coordinate research efforts and assess toxicological indices for all data collected.
Gregory Olson, Research Associate and Project Lead: (12 months of salary). If funded Mr. Olson will be hired on as a research associate with an anticipated yearly salary of $40,000. Mr. Olson will be responsible for overall project management, sediment and tissue toxicological analysis, total fat analysis, conduct all research trips, report preparation, and presentation of scientific findings.
Buffy Meyer, Research Associate: (1 month of salary). Mrs. Meyer will coordinate the QA/QC relating to GC/MS instrumentation maintenance and all data analysis.

B. Other Personnel Salary and Wages:

Graduate Assistant, (12 months of salary) graduate student with experience in ICP-OES analysis will be hired to conduct all metal analysis and assist in report preparation.

Student Worker, (360 - 540 hours per 9 months) Funds are requested in support of one student worker at a cost of approximately $9 an hour for no more than 15 hours a week per academic year. He/she will help senior scientists in sample processing and extraction, basic laboratory maintenance (cleaning glassware, ordering supplies, etc.) and total fat analysis.

D. Fringe Benefits:
LSU's current fringe benefits rate of 36% is applied to all personnel except student worker positions.

G. Travel:
Funds are requested ($3000) to attend to attend 1 regional or national meeting per year in order to present research findings.

H. Supplies:
Funding for supplies in requested for 3 tasks. 1) Funds are requested for supplies to be used onboard the research vessel during sampling trips. Supplies include coolers to transport samples,

formalin, fixatives, sampling vials, etc. at a cost of $2,000 per year. 2) Funds are necessary for the laboratory supplies for ICP-OES sample analysis including reagents, glassware, equipment, etc. at a cost of $2,000 per year. 3) Funds are requested for supplies for toxicological analysis of samples including, solvents (dichloromethane, hexane), glassware (250 ml Florence flasks, 350 ml funnels, side-arm flasks), gas chromatography vials, volatile organic analysis vials, C-18 silica, etc. at a cost of $5,500. Truck fuel is budgeted for sampling trips $500.

I. Operating Services:
In order to meet LDWF biologists with equipment and supplies, one truck will be rented from the SC&E field support shop at a rate of $35 per day for four days a month for six months out of the year costing a total of $840. Trace and organo metals will be analyzed in tissue and sediment for a cost of $4,000 per year by Dr. Robert Gambrell in the Department of Oceanography at LSU WBIAS service center.

J. Professional services:
GC/MS annual maintenance contract of $12,000 per year as well as analytical instrument maintenance must be maintained at a cost of $5,000 per year.

L. Equipment:
Aqueous and sediment/tissue extraction instrument valued at approximately $90,000 during the first year.

LSU's federally negotiated research rate of 24% MTDC is applied.

INVESTIGATORS

Ralph J. Portier, Ph.D.,
Distinguished Professor of Louisiana Environmental Science
School of the Coast & Environment, Department of Environmental Sciences
Louisiana State University, Baton Rouge, LA 70803 USA
Director, Aquatic/Industrial Toxicology Laboratory
Adjunct Professor, Department of Oceanography and Coastal Sciences
Adjunct Professor, Department of Food Sciences

Education

B.S., (Science/Math Education) Nicholls State University (1974)
M.S., (Marine Sciences) Louisiana State University (1979)
Major: Marine Microbiology, Minor: Food Science/Technology
Ph.D. (Marine Sciences) Louisiana State University (1982)
Major: Marine Microbiology, Minor: Marine Toxicology/Food Science

Major Areas of Research Interests

Microbiology/microbial physiology of extreme marine environments; environmental toxicology of impacted coastal wetland microenvironments from oil spills; remediation approaches for contaminated water and soils/sediments; risk assessment of toxic chemicals on detrital-based food systems and dependent fisheries.
Dr. Portier's research has focused on alleviating the problems associated with industrial and oil production activity in coastal estuarine environments. Current research areas include evaluation of fate and effect of potential carcinogens in aquatic and marine environments, the evaluation of microorganisms for detoxification of contaminated soils and sediments and the development of new technologies using immobilized bacteria for the continuous detoxification of trace contaminants in typical coastal industrial effluents. He has also worked extensively in the area of seafood microbiology focusing on wastewater treatment and waste food grade product usage.

Awards

George W. Goethals Medal, 1988 Recipient, The Society of American Military Engineers.
American Forest & Paper Association Environmental Achievement Award. May 1996.
Tiger Athletic Foundation Excellence in Teaching Award. May, 2007.
LSU Distinguished Faculty Award. April, 2010.

Recent Patent

U.S. Patent # 7109300. "Extraction of collagen from calcified tissues" J. Losso, M. Ogawa, R. Portier, and M. Schexnayder. Awarded September 19, 2006. A method is disclosed for extracting collagen from calcified tissue without a prior decalcification step.

Recent Publications (refereed)

Portier, R.J., L.M. Basirico, G.P. Curole, R.M. Conger and C. Metosh-Dickey. 2010. In situ bioremediation of an aniline spill in an industrial setting. Remediation 20(4) 105-117.

Parker, K.A., D.P. Dickey, C.A. Metosh-Dickey and R.J. **Portier**. 2008. Application of current risk-based remediation criteria for the post-closure assessment of a former National Priorities List (NPL) site. International Journal of Environmental and Waste Management 3(2) 292-308.

Igbal, J.I., C.A. Metosh-Dickey and R.J. **Portier**. 2007. Temperature effects on bioremediation of PAHs and PCP contaminated South Louisiana soils: A laboratory mesocosm study. Journal of Soils and Sediments 7(3) 153-158.

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Portier, R.J., J.I. Iqbal, C.A. Metosh-Dickey and T.M. Davis. 2007. Managing bioremediation of a creosote-contaminated Superfund site by optimizing moisture and temperature in a biopile. Remediation 18(1) 73-82.

Conger, R.M. and R.J. **Portier**, 2007. Before-after control-impact paired modeling of groundwater bentazon treatment at a phytoremediation site. Remediation 17(1) 81-96.
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Goktepe, I. and R.J. **Portier**, 2004. Ecological risk assessment of Neem-based pesticides. Journal of Environmental Science and Health. 29(2) 311-320.

Millward, R.N., K.R. Carman, J.W. Fleeger, R.P. Gambrell, R.J. **Portier**. 2004. Mixtures of metals and hydrocarbons elicit complex responses by a benthic invertebrate community. Journal of Experimental Marine Biology and Ecology 310 115-130.

Gregory M. Olson
School of the Coast & Environment, Department of Environmental Sciences
Louisiana State University, Baton Rouge, LA 70803 USA
Graduate Research Assistant
PhD Candidate

Education
B.S., (Environmental Science Education) McNeese State University (2009)
Minor: Chemistry & History
M.S., (Environmental Science) Louisiana State University (2012)
Concentration: Environmental Toxicology

Major Areas of Research Interests:

Past projects include a product screening designed to determine effectiveness of various proprietary oil cleaning products that have been submitted for use during the Deepwater Horizon oil spill remediation process, determining the best method to create an energy source from a waste stream created by a local soda canning factory through the use of various bacteria and fungi fermentation processes. Mr. Olson is currently involved with the collection and analysis of menhaden from the Gulf Coast for use in a joint project with Rutgers and Seton Hall University. The focus of his research is the assessment of toxic potential of polycyclic aromatic hydrocarbons (PAHs) affecting Gulf menhaden (*Brevoortia patronus*) harvested from waters impacted by the BP Deepwater Horizon spill.

Awards:
Kuniko Fujisaki Honor Award - Outstanding work as a Graduate/Research Associate

VITA

Gregory Michael Olson was born to Carmen Olson during the summer of 1984 in Fort Sam Houston, Texas just outside of San Antonio. He Moved to Pickering, Louisiana in 1987 with his mother and from here he attended Pickering Elementary and High School. He graduated in 2003 and began his undergraduate degree at McNeese State University in Lake Charles, Louisiana. He graduated in 2009 and taught in the public school system. He wanted to do more with his life so he pursued a graduate assistantship at Louisiana State University in Baton Rouge, Louisiana and complete a Masters in Environmental Science concentrating in biophysical systems and environmental toxicology. He is currently completing his PhD in environmental science with a minor in food science while he works for the LSU Response and Chemical Assessment Team directed by Dr. Ed Overton. During his time as a research associate he has had the opportunity to teach the LSU general education course ENVS 1126 during summer and regular semesters.