FINAL PROJECT REPORT

TITLE: Trophic Transfer of Oil Contaminants from Menhaden Fish: Will the Gulf Oil Spill Effect NJ?

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TABLES

FIGURES

I. SUMMARY OF FINDINGS

The DeepWater Horizon (DWH) oil spill in the Gulf of Mexico, 2010, is an ongoing environmental catastrophe that is likely to have widespread and chronic effects on fisheries, with potential of affecting human health. The release of petroleum from the BP's Macondo 252 continued for 87 days until the well was capped on July 15, 2010. This research project was initiated late in September of 2010. A major goal was to establish background levels of oil contaminants, particularly polycyclic aromatic hydrocarbons (PAH), in Atlantic coast menhaden and levels of PAHs in crude oil exposed menhaden from the Gulf of Mexico (GOM). Menhaden is an oily, prey species of marine fish. It has been commercially important for the bait and reduction industries in both the mid-Atlantic and Gulf coast states. Due to their oily nature, menhaden will likely accumulate oil contaminants and potentially spread it to predatory fish such as bluefish and striped bass, thereby affecting marine ecosystems as well as the sport fishing industry. It is also possible that by consuming bluefish and striped bass and/or contaminated omega-3 from menhaden, humans may be exposed to oil contaminates such as PAHs.

For this project, adult menhaden were collected from Barataria Bay near Grand Isle, Louisiana (BBLA), James River, Virginia (JRVA), and Delaware Bay, New Jersey (DBNJ), and juvenile menhaden were collected from the Delaware River, NJ and Barataria Bay, LA. Barataria Bay was chosen because significant amounts of DWH oil came ashore there. The James River in VA was chosen because two of the Gulf species of menhaden, *Brevoortia smithi* (yellowfin menhaden) and *B. patronus* (gulf menhaden), are known to migrate from LA to VA, and future studies might indicate if the contamination in the Gulf could spread to the Atlantic coast by prey species. Atlantic menhaden, *Bevoortia tyrannus*, have also been found in the VA area as well as further up the coast in the Delaware Bay, NJ area. Testing menhaden along the Atlantic coast in 2010 established "background" levels of PAHs prior to migration of fish from the GOM or to a major oil spill event along the Atlantic coast. In addition, the James River and Delaware Bay have been affected by high urbanization. They thereby served as a type of control for urban (pyrogenic) versus oil spill (petrogenic) PAH contamination- information that could prove useful in the future for assessing natural resource damage.

Scientific questions addressed by this research were as follows. 1) Is there a distinctive chemical "fingerprint" for the DWH oil that can be detected in contaminated menhaden fish oil? If there is, can the fingerprint be used to detect menhaden contaminated in LA that have migrated to VA. This type of information would support the use of fish oil as a way of tracking the spatial impact of future oil spills. 2) What is the concentration of PAHs in contaminated fish oil and PAH metabolites in fish liver? Do the concentrations in recently exposed LA fish exceed background levels in menhaden from urbanized areas such as the James River in VA and the lower Delaware River in NJ? This information would indicate the ability of oily fish to concentrate and retain contaminants from major oil spills and allow comparisons between fish recently exposed to a major oil spill to those exposed to low levels of chronic pollution. It would also indicate whether oil spills have the potential to affect the omega-3 fishing industry. 3) Is there a relationship between histopathological effects and tissue levels of oil contaminants? This information would demonstrate whether or not oil spill contamination has a detrimental effect on an important prey species. 4) Are the contaminants in oily prey fish transferred to predatory fish? Trophic transfer of PAHs would suggest ecosystem level effects of oil spills and potential human exposure.

Several types of chemical analyses were used to address the question as to whether or not there was a distinctive "fingerprint" in fish oil of DWH exposed fish. Fish oil was analyzed by headspace solid-phase microextraction (HS-SPME) in combination with gas chromatography-mass spectroscopy (GCMS). HS-SPME GCMS is a relatively new method and can extract substances from "dirty" samples such as fish oil

with high sensitivity. Concentrations of parent PAHs in all fish oils were low overall ranging from 10-185 µg/L (ppb). PAHs detected were naphthalene, fluorene, fluoranthene, pyrene and anthracene. Naphthalene and fluorene were too low to quantify. Comparison of LA and two separate samples of NJ fish showed no major differences in total PAHs- 342, 384 and 300 ppb, respectively. Since there is no information on background levels of PAHs in menhaden, it was not known if the levels found in LA fish were increased due to the DWH oil spill. The low number of PAHs detected using this technique did not allow a PAH 'fingerprint" to be developed. The GCMS chromatographs did show more similarities between NJ fish oil samples than LA ones. Comparison of additional menhaden samples in the future should help determine whether the peaks are consistent between locations and in abundance.

Fixed emission fluorescence spectroscopy (FEFS) was also used to analyze fish oil samples as well as fish liver in adult menhaden. A method was developed specifically for the purposes of detecting multiple PAHs and distinguishing them from biological compounds such as vitamins A & E and degraded chlorophyll (pheophytin a). This involved holding the emission wavelength fixed at Em350 for 2-3 ring PAHs or Em450 for 4-6 ring PAHs and scanning for excitation peaks. A major advantage of FEFS was the large number of samples that could be handled in a short period of time and the ability to detect substituted and hydroxylated PAHs; however, the overlapping excitation peaks did not allow specific PAHs to be quantified. Analyses resulted in naphthol (HNP), phenanthrol (HPN) and hydroxypyrene-like (HPY) PAHs being quantified. Results showed that fish oil and fish liver had similar spectra with a single major peak at Em350/Ex280 for HNP-like PAHs. While the peak did not match any of the PAH standards, it was in the region of naphthol and fluorene, which were both detected by HS-SPME GCMS. It was also near Em350/Ex290, which represented vitamin E, and Em350/Ex280, which represented vitamin A. The fluorescence intensities of vitamins A& E were lower than those of 2-3 ring PAHs, approximately 1000x and 10x, respectively. Their interference with measuring these PAHs was difficult to determine. For the larger PAHs detected using Em450, spectra indicated the presence of fluoranthene and pyrene, both of these PAHs were found using HS-SPME GCMS. In addition, vitamin A was detected in fish oil using Em450. The presence of vitamin A at Em450/Ex320 was easy to distinguish from 4-6 ring PAHs which typically had peaks at Em450/Ex250 and Em450/Ex350. Altogether, results indicated that HS-SPME GCMS and FEFS were detecting similar PAHs.

Comparison of PAH concentrations in fish liver and fish oil showed differences in tissue accumulation and sources of exposure. HNP-like PAHs were approximately 100x higher in liver than fish oil. HPY-like PAHs were approximately 10x higher in liver than oil. These results indicated higher elimination and/or lower circulation of 2-3 ring PAHs than 4-6 ring PAHs. HNP-like PAHs were similar between BBLA and DBNJ fish, while HPY-like PAHs were usually higher in DBNJ than BBLA fish. JRVA menhaden had the highest concentration of HPY-like PAHs in both fish oil and liver. The high HNP to HPY ratio in BBLA fish indicated recent petrogenic exposure, while the low ratio in NJ and VA fish indicated pyrogenic exposure. This was consistent with our hypothesis. Total PAHs in NJ fish were typically higher than in LA fish; however, concentrations greatly varied between NJ samples suggesting different levels of exposures. Not enough samples from different fishing areas in NJ were taken to determine possible sources. Monitoring menhaden using FEFS could be useful in the future for detecting sources of exposure even if it is unlikely to distinguish a unique PAH profile for a particular type of crude oil. It was interesting that NJ and VA menhaden had higher PAH levels than LA fish faced with a major oil spill.

Juvenile menhaden, also known as young of the year (YOY), were collected from Barataria Bay, LA and Delaware Bay, NJ in order to determine if there was a relationship between histopathological effects and tissue levels of oil contamination. However, the YOY menhaden were too small for sufficient fish oil preparation. Instead tissue concentrations of PAHs were determined by analyzing whole body tissue

using MSPD C-18 Silica Extraction Process (MSEP) followed by GCMS and by analyzing gastrointestinal tissues (GI track and liver) using FEFS. Gastrointestinal tissues were used because the liver was too small and integrated into the GI track for complete separation. In addition, a sample of crude oil collected from the pipeline of the DWH in April 2010 was also analyzed by GCMS using EPA method 8270.

Chemical analysis of the DWH oil found numerous PAH constituents including high concentrations of alkylated napththalenes, fluorenes, and phenanthrenes. Major high MW PAHs included alkylated pyrenes and chrysenes. Analysis of YOY menhaden, however, found very limited types of PAHs in whole body tissue. Both DBNJ and BBLA fish had alkylated phenanthrenes. Concentrations in DBNJ fish were approximately 3x higher than those in BBLA fish. High MW PAHs, such as benzofluoranthene and benzopyrene were found in BBLA but not DBNJ fish. This may have been due to crude oil exposure; however, the BBLA menhaden were significantly larger than the DBNJ fish. Their larger size may have increased the detection of these PAHs. FEFS results showed that the DBNJ and BBLA had very similar fluorescence spectra despite their considerable geographic distance from one another. HNP-like PAH concentrations were similar in fish from both sites; however, HPY-like PAHs were significantly higher in NJ fish. This resulted in a higher HNP/HPY ratio in BBLA, which is thought to indicate exposure to petroleum products. This was similar to the findings for adult menhaden fish oil and liver. Even though the ratio indicated petroleum exposure, the total PAH concentrations were not as different for BBLA and DBNJ fish as might have been expected following such a large oil spill in the GOM.

Histopathological analysis was used to assess the physiological condition of YOY menhaden. Results showed considerably more damage to BBLA than DBNJ fish. In particular, there was extensive damage to gill as identified by epithelial cell hyperplasia, fusion of lamellae and waxy lamellae. Menhaden may have been more affected than other types of fish as their 2nd lamellae are used for filter feeding. Gill tissue showed some repair but also that chronic irritation was still occurring. BBLA fish also showed more necrotic foci in liver and bile duct proliferation and dilation than DBNJ fish. These changes were cholangioma like lesions and required time to be manifested. This suggested that the initial damage may have been caused from the oil spill during the summer. Overall, damage in BBLA fish was much more extensive than in DBNJ fish and typical of the types of damage associated with crude oil exposure. Histological findings and PAH tissue concentrations did not coincide. DBNJ and BBLA had similar concentrations of PAHs even though tissue damage was much more extensive in BBLA fish. This indicated that damaged caused by crude oil exposure could remain even while representative contaminants, such as PAHs, were eliminated from tissues.

Trophic studies provided strong evidence of absorption and distribution of PAHs that were incorporated into menhaden fish oil, DWH crude oil and menhaden fishmeal. This occurred over a short period of time, either 24 hours for the oils or 96 hours for the fishmeal. The predatory fish used in trophic studies were silver perch and bluefish. Data were complicated by the low number of test organisms per group, individual fish variation, what appeared to be pre-existing PAHs in test organisms, and natural compounds such as vitamin A and E. In the three trophic studies, FEFS analyses showed PAH-like compounds in all tissues tested (GI track, liver, gill, spleen and gall bladder contents). Liver consistently had higher concentrations of PAH-like compounds than other tissues; although, there were some tissue and species related differences. Concentrations of HPT-like PAHs were usually higher than HNP-like PAHs which were higher than HPY-like PAHs. HPT-like PAH spectra were found in fish treated with DWH crude oil and fish oil spiked with naphthalene and phenanthrene; however, they were seldom found in control fish or those fed only fish oil. This showed that PAHs could be absorbed from diet. The exception to this was gill. HPT-like PAHs were found in gill of silver perch and bluefish regardless of treatment and suggested that this type of PAH was there prior to testing. Both silver perch and bluefish

were collected from Tuckerton, NJ about the same time. Comparing fluorescence spectra of different tissues in the same fish showed transport of PAHs from the GI track into liver, spleen and gall bladder. Spectra often showed matching major peaks in fish fed spiked fish oil, DWH crude oil and JRVA fish meal. These findings demonstrated the ability of PAHs to be absorbed from a predator's diet and distributed throughout its tissues. They also emphasized the importance of looking for the entire spectrum of a PAH standard as opposed to a specific wavelength, which is usually how FEFS data is reported in the literature.

This project provided evidence that PAHs accumulate in menhaden fish oil and liver and that they can be transferred to predators through diet. Tissue concentrations of PAHs in menhaden may not coincide with tissue damage especially if the exposure occurred months ago. As with the predator fish tested here, it is likely that tissue concentrations of PAHs in menhaden will reflect GI track contents and therefore, levels of contamination in areas where they have been recently feeding. However, this requires further testing. Menhaden is a very important fish in our coastal ecosystems. This research has laid a foundation for using menhaden as a model organism in future studies and provided background data on PAH concentrations in Atlantic coast menhaden.

II. INTRODUCTION

The DeepWater Horizon (DWH) oil spill in the Gulf of Mexico, 2010, was an environmental catastrophe that is likely to have widespread and chronic effects on fisheries, with potential of affecting human health. The release of petroleum from the BP's Macondo 252 ruptured well started on April 20, 2010 and continued for 87 days until the well was capped on July 15, 2010. This research project was initiated late in September of 2010. A major goal was to establish background levels of oil contaminants, particularly polycyclic aromatic hydrocarbons (PAH), in Atlantic coast and Gulf of Mexico menhaden. This was in anticipation that Gulf coast menhaden, exposed to the DWH oil spill, might migrate up the Atlantic coast carrying the oil contamination with them. Menhaden is an oily, prey species of marine fish. It has been commercially important for the bait and reduction industries in both the mid-Atlantic and Gulf coast states. Due to their oily nature, menhaden will likely accumulate oil contaminants and potentially spread it to predatory fish such as bluefish and striped bass, thereby affecting marine ecosystems as well as the sport fishing industry. It is also possible that by consuming bluefish and striped bass and/or contaminated omega-3 from menhaden, humans may be exposed to oil contaminates such as PAHs. Some PAHs are known carcinogens.

For this project, adult menhaden were collected from Grand Isle, Louisiana (LA), James River, Virginia (VA) and the Delaware Bay region, New Jersey (NJ), and juvenile menhaden were collected from the Delaware River, NJ and Grand Isle, LA. Grand Isle, LA was chosen because significant amounts of DWH oil came ashore there (Ramsey et al. 2011). The James River in VA was chosen because two of the Gulf species of menhaden, *Brevoortia smithi* (yellowfin menhaden) and *B. patronus* (gulf menhaden) are known to migrate from LA to VA. It was therefore thought possible that DWH contamination could reach VA in 2011. Yellowfin and gulf menhaden mix with another species of menhaden in VA, *B. tyrannus* or Atlantic menhaden. *B. tyrannus* spawns in VA and migrates along the Atlantic coastline (Ahrenholtz, 1981). It also spawns off the NJ shore and migrates into estuaries such as the Delaware Bay (Light and Able, 2003). Menhaden are an important prey species where ever they are found. They are heavily fished in LA, VA and NJ and therefore commercially important in all sampling locations. Recently, new regulations on Atlantic menhaden landings were imposed in order to improve and protect their population (ASMFC, 2011). Comparing these sites can provide insights into existing levels and profiles of PAHs in menhaden and possibly distinguish urban signals from those of oil spills.

Scientific questions addressed by this research are as follows. 1) Is there a distinctive chemical "fingerprint" for the DWH oil that can be detected in contaminated menhaden fish oil? If there is, can the fingerprint be used to detect menhaden contaminated in LA that have migrated to VA or NJ. This type of information would support the use of fish oil as a way of tracking the spatial impact of future oil spills. 2) What is the concentration of PAHs in contaminated fish oil and PAH metabolites in fish liver? Do the concentrations in recently exposed LA fish exceed background levels in menhaden from urbanized areas such as the James River in VA and the lower Delaware River in NJ? This information would indicate the ability of oily fish to concentrate and retain contaminants from major oil spills and allow comparisons between fish recently exposed to a major oil spill to those exposed to low levels of chronic pollution. It would also indicate whether oil spills have the potential to affect the omega-3 fishing industry. 3) Is there a relationship between histopathological effects and tissue levels of oil contamination? This information would demonstrate whether or not oil spill contamination has a detrimental effect on an important prey species. 4) Are the contaminants in oily prey fish transferred to predatory fish? Trophic transfer of PAHs would suggest ecosystem level effects of oil spills and potential human exposure. While the focus of this project is on the impact of the Gulf oil spill, the information

learned is highly transferable. As long as we continue to use refined crude oil as a primary energy source, major oil spills are a constant threat.

Primary objectives for this research projected are shown below. The work on Corexit® was not performed due to the inability to obtain the compound from the manufacturer without compromising publication rights. Data was generated to meet all other objectives.

1. Determine PAH concentrations and identify metabolites of Corexit® in fish oil prepared from menhaden collected in NJ, VA and LA this year, 2010.
2. Establish PAH and Corexit® metabolite "finger-prints" in the fish oil prepared from the different menhaden populations.
3. Evaluate the current health of NJ and LA menhaden through histopathological examination.
4. Assess the levels of trophic transfer of PAHs from menhaden fish oil to YOY bluefish.

The experimental design overview as proposed is shown below. Modifications to the design included 1) conducting two types of chemical analyses on fish oil of large menhaden- headspace solid-phase microextraction (HS-SPME) with gas chromatography-mass spectrometry (GC-MS) as well as fixed emission fluorescence spectroscopy (FWFS), 2) using fish oil from large menhaden for trophic studies (insufficient fish oil was obtained from small menhaden), 3) measuring PAHs in liver of large menhaden and DWH crude oil using FWFS, 4) conducting two types of chemical analyses on small menhaden- GC-MS on whole body tissue and FWFS on gastro intestinal track tissue, 5) measuring PAHs in DWH crude oil using GC-MS and 6) conducting trophic studies in both silver perch and bluefish.

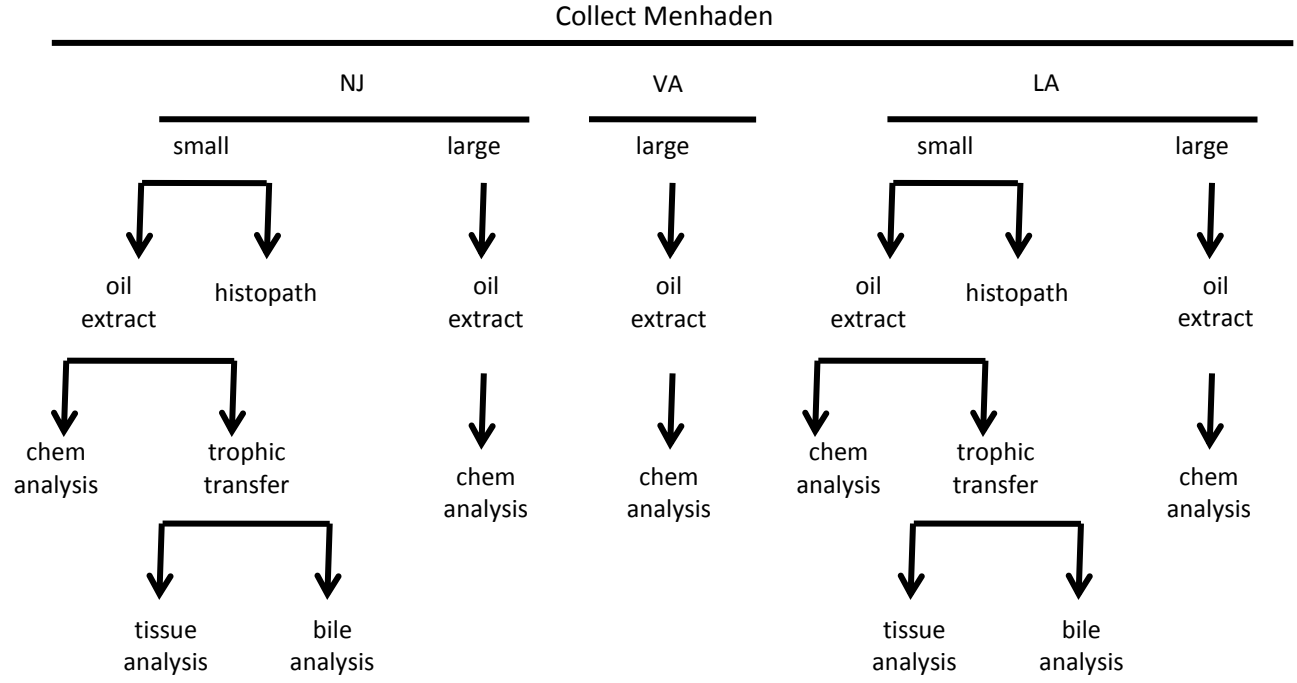

Figure 2.1 Experimental Study Design: Fish oil will be extracted from small (10-15 cm) menhaden collected in NJ and LA and used for trophic transfer, histology (histopath) and chemical analyses (chem analysis). Fish oil from large (20-25 cm) menhaden collected in NJ, VA and LA will be used for chemical analysis only.

III. COLLECTION AND MEASUREMENT OF FISH

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In fall 2010, small menhaden (7-12 cm) were collected from the Delaware River around Pennsville and New Castle, NJ. Approximately 200 fish were collected on September 2nd and on September 21st with the help of the NJDEP Division of Fish and Wildlife. Small menhaden (70-120mm) were also collected along the bulkhead of the marina at the Department of Wildlife and Fisheries facility in Grand Isle, LA on October 30, 2010. This location was within Barataria Bay, LA. Approximately 30 of these fish were collected for histopathology and 20 for chemical analyses. The collection of the menhaden from Barataria Bay was facilitated by the Louisiana Department of Wildlife. (More information on the size and condition of the fall 2010 fish is provided in the gross morphology/histopathology section of this report.)

Fish oil was most successfully extracted from the larger menhaden (12-29 cm), so the larger fish were used primarily for oil preparation while the smaller fish were used for histopathology. In fall 2010, large menhaden were collected from NJ, VA and LA. Three collections were made of NJ fish. They were collected from different ships seining along the NJ shore (areas 394 & 397) or off shore near the five fathom bank area (area 621) (picture 1). One collection was made of LA and VA fish. The LA fish were caught off of Grande Terre Beach in Grand Isle. The Louisiana Department of Wildlife assisted with collecting the fish. The VA fish were caught in the lower part of the James River near the mouth of the Chesapeake Bay. Dr. Michael Newman of Marine Science College of William & Mary Virginia Institute of Marine Science (VIMS) arranged for the fish collection. Dr. Xiaoyu Xu and his survey team at VIMS collected the fish and shipped them to us. In summer 2011, we continued to collect fish. We increased the number of locations in the Gulf of Mexico, adding collections from Key West, FL and from Vermillion Bay, LA. This work was supported by the Louisiana Department of Wildlife and findings will be reported to that agency.

Figure3. 1 and 3.2 show where the 2010 fish were caught. Tables 3.1 and 3.2 show when they were caught and their average length (cm) and weight (g). A photograph of each fish was taken prior to dissection, so that the species could be identified. For the large menhaden collected, the NJ fish were larger than the LA fish (Table 3.1). This was primarily because the species collected in NJ were Atlantic menhaden, *B. tyrannus,* and those collected in LA were gulf menhaden, *B. patronus*. Atlantic menhaden are known to be the larger of the two species (Nelson and Ahrenholz, 1986). Menhaden from VA were a mix of these two species. For the young of the year (YOY), fish caught in NJ were generally smaller than those caught in LA (Table 3.2). This indicated that the NJ YOY were younger fish and probably not in the estuary as long as the LA fish. Menhaden migrate into the ocean in the fall (Ahrenholtz, 1991). Given the limited time available for collecting fish in both NJ and LA, fall 2010, it was not possible to obtain fish of similar size. Acronyms for the collections were based on collection location or ship: Barataria Bay, LA- BBLA, James River, VA- JRVA, Enterprise, NJ- EPNJ, Mt. Vernon, NJ- MVNJ, and Sea Huntress, NJ-SHNJ. Two collections of large menhaden were obtained from the ships, Enterprise and Mt Vernon, and are referred to as EPNJ1/MVNJ1 and EPNJ2/MVNJ2.

Table 3.1 Collection of large menhaden. Table shows the state, site and more specific location of where large menhaden were collected. The date(s) of collection, number of fish and species are given. Species include *B. patronus* (P) and *B. tyrannus* (T). Average length (cm) and weight (g) of fish from a particular collection are shown with their standard deviation (SD). NJ fish are categorized by the seining ship from which they were obtained. Areas in which those ships were fishing are shown in Figure 3.1 (see below). These fish were used to generate fish oil for trophic studies and PAH chemical analyses. PAHs in their livers were also analyzed.

State	Site	Location	Date	Amount	Species	Length Ave(SD)	Weight Ave(SD)
LA	Barataria Bay	Grande Terre Beach	10/30/2010	38	P	17.9 (3.3)	143 (57)
VA	James River	Hampton, VA	10/1-21/2010	8	P	19.0 (2.7)	120(43)
VA	James River	Hampton, VA	10/1-21/2010	10	T	22.0 (4.4)	177 (94)

State	Site	Area	Date	Amount	Species	Length Ave(SD)	Weight Ave(SD)
NJ	Enterprise1	621	9/8/2010	12	T	26.2 (1.7)	351 (50)
NJ	Enterprise2	621	9/21/2010	10	T	24.7 (1.8)	295 (50)
NJ	Mt Vernon1	397	9/7/2010	11	T	26.4 (9.9)	324 (41)
NJ	Mt Vernon2	397	9/23/2010	8	T	26.4 (10.3)	355 (53)
NJ	Sea Huntress	394	10/18/2010	18	T	24.0 (2.2)	279 (75)

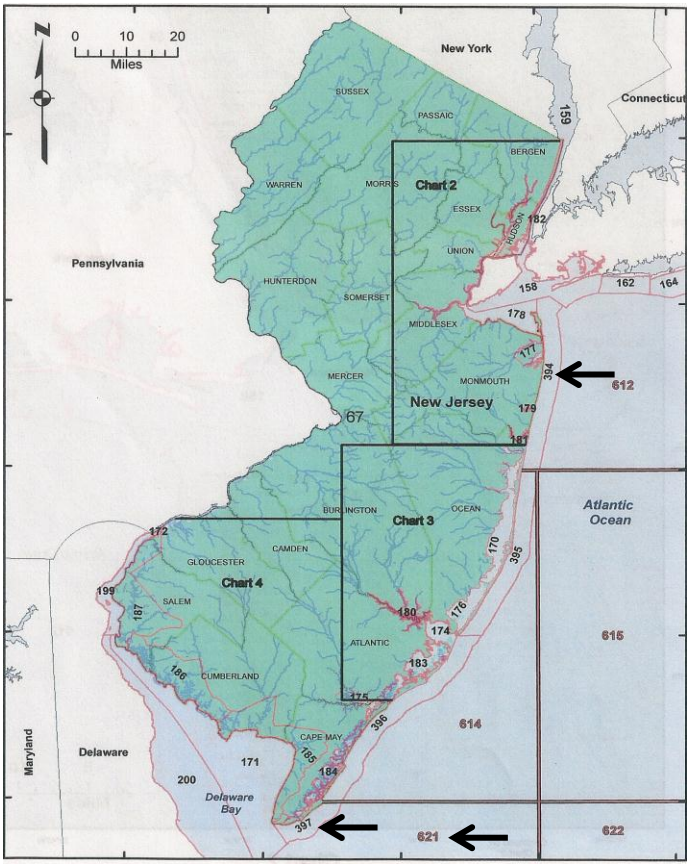

Figure 3.1 Fishing locations for NJ menhaden. Fish from the ship, Enterprise were collected further off shore on Sept 8 and 21, 2010 in area 621. Fish from the ship Mt Vernon were collected closer to shore on Sept 7 and 23, 2010 in area 397. Fish from the ship Sea Huntress were collected further north along the shore on October 18.

Table 3.2 Characteristics of small menhaden from Barataria Bay, LA and Delaware Bay, NJ. Species of menhaden included *B. patronus* (P) from LA and *B. tyrannus* (T) from NJ. Data applied to fish used in a particular analysis- fixed emission fluorescence spectroscopy (FEFS), gas chromatography- mass spectroscopy (GCMS) and histopathology (Histo). Characteristics included weight (g) and length (cm). n= number of fish used in each analysis. Areas where the fish were caught are shown in Figure 3.2 (see below).

Location/Collection Date	n	species	analysis	Length Ave(SD)	Weight Ave(SD)
Barataria Bay, LA	13	P	FEFS	10.0 (4.5)	15.8(1.8)
10/25/2010	6	P	GCMS	11.3(0.4)	22.3(1.9)
	18	P	Histo	10.2(1.6)	21.6(1.0)
Delaware Bay, NJ	13	T	FEFS	8.0(0.9)	4.1 (1.0)
9/21/2010	6	T	GCMS	8.2(0.2)	8.3(0.9)
	16	T	Histo	5.9(4.0)	16.9(4.0)

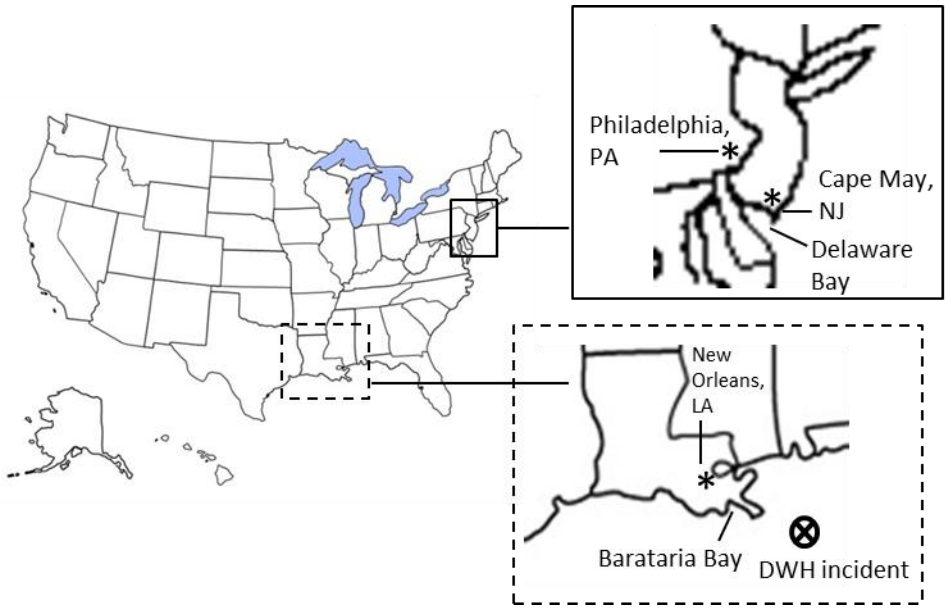

Figure 3.2 Location of field sites along the Atlantic and Gulf coasts. YOY Atlantic menhaden were collected in the Delaware Bay, NJ, near the Delaware Memorial Bridge, on September 21, 2010. YOY Gulf menhaden were collected in the Barataria Bay near Grand Isle, LA, on October 25, 2010.

IV. ANALYSES ASSOICATED WITH LARGE MENHADEN

IV.1 Procedure for Preparing Menhaden Fish Oil

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The procedure for fish oil preparation was standardized. After catching the menhaden, the fish were place on ice immediately and frozen as soon as possible upon returning to shore. (Fish collected for histopathology were fixed in 10% formalin on site). Frozen fish were shipped to Seton Hall University and remained frozen until dissection. Prior to dissection, fish were quickly thawed in warm water and cataloged by species, weight, length, date, and location of capture. Each fish was also photographed. Groups of five fish, from the same date, location and approximately the same size were used to prepare an oil aliquot. These fish were dissected by removing the internal organs, including the liver, heart, stomach and digestive tract. These organs were refrozen for future study. Pieces of the liver were collected for fluorescent spectrometry. Then the head and tail were cut off, and the fish was filleted and de-boned. The filets were cut into smaller pieces and pounded into meal using a glass test tube inside a round-bottom centrifuge tube.

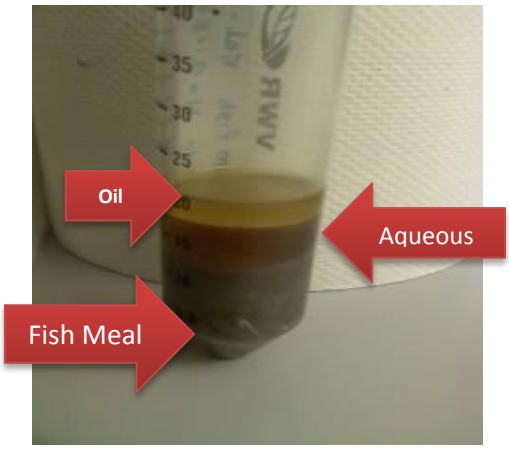

Figure 4.1.1. Layers generated by centrifugation of dissected fish material.

The next steps involved centrifuging the round bottom tubes for six hours at 10,000 rpm with a rotational gravitational force of 12,376.26 N. Once the centrifugation was complete, the tubes were removed and the top two layers, one oil and one aqueous (See picture 2), were decanted into a new, clean conical bottom centrifuge tube. The samples were allowed to settle. Then an 18 gauge needle was used to pierce the bottom of the conical. Once the needle was removed, the aqueous layer was free to drip through the hole that was formed. Once the aqueous layer was removed, the oil layer was collected in a clean glass vial. Oil from each aliquot was combined resulting in a single oil sample for a particular site/ship and collection date. Nitrogen gas was blown over the vial, and the oil sample was frozen until analysis and/or trophic studies.

Analyses of the fish oil procedure showed that the most oil was collected from NJ fish, 3.47 grams of oil per fish, and the least from VA fish, 0.35 grams of oil per fish. The amount of oil from LA fish was similar to that for NJ fish.

Table 4.1.1. Fish oil extraction data. Examples of information from aliquots of fish oil prepared from large NJ, LA and VA menhaden are shown. Oil was prepared from 5 fish collected from a particular location/ship. Weight (g) is the combined weight of the 5 fish and the total amount of oil (g) from those 5 fish. Also provided is the % of oil from the 5 fish and amount (g) and % of oil per fish.

Location	Weight of Fish	Amount of Oil	Total % Oil	Oil per Fish	% Oil per Fish
NJ	1723	17.34	1.01	3.47	0.010
LA	826	6.35	0.77	1.22	0.007
VA	1191	1.729	0.15	0.35	0.001

IV.2 Measuring PAHs in Fish Oil from Large Menhaden using Headspace Solid-Phase Microextraction

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Headspace solid-phase microextraction (HS-SPME) was used in combination with gas chromatography-mass spectrometry (GC-MS) to screen laboratory prepared EPA standards of polycyclic aromatic hydrocarbons (PAH) at part per billion levels and to detect and quantify PAHs in three fish oil samples from menhaden collected in 2010. HS-SPME allowed analysis of contaminants without extensive sample preparation. The process and analysis of menhaden fish oils were established using EPA PAH standards. PAH standards were prepared by spiking commercial fish oil (Nature's Bounty fish oil: commercial product) with 10 µl of EPA 610 PAH mix (in 500 µl methanol and 500 µl dichloromethane). The procedure, in brief, was as follows. Aliquots (1 gram) of standard PAHs in fish oil or menhaden fish oil were weighed and sealed in a 20 mL headspace vial. The vial was loaded into a Combi-PAL autosampler, and heated to 150 ^{o}C for 15 minutes. A 100 µm thick polydimethylsiloxane SPME fiber was exposed to the headspace of the heated vial for 30 minutes. The fiber was then transferred to the inlet of the GC-MS for a 2 minute desorption period , injection and separation of the extracted components on a 15m x 0.25mm x 0.25 mm DB-5MS (5% phenyl polydimethyl siloxane) capillary column. The temperature program was 50 °C held for 1 minute. Temperature was ramped up at a rate of 20 °C/minute before being held at 325 °C for 2 minutes. The flow rate of helium carrier gas was 2.4 mL/minute. The mass spectrometer was operated in selected ion monitoring mode, with a total of 15 PAHs monitored. Examples of the selected ions monitored were naphthalene (n=undeuterated and d_8N=deuterated) at 128 (n) and 136 (d_8 N) mass charge ratio (m/z) and benzo(a)pyrene (undeuterated) at 252 m/z. The fiber was baked at 360 ^{o}C for 15 minutes to eliminate carry over before its next use. The baked fiber was tested before the run of each sample resulting in the "blank". Standard curves were generated for PAHs and the limit of quantification (LOQ) and limit of detection (LOD) were determined.

Results for HS-SPME are shown below. Concentrations and m/z of PAHs in the 610 mix are shown in Table 4.2.1. Selected ion chromatograms at m/z 128 and 136 are shown for naphthalene (figure 4.2.1) and at m/z 252 for benzo(a) pyrene (figure 4.2.2). Note that benzo(a) pyrene, a relatively large PAH, can be successfully extracted from fish oil using a headspace technique. Table 4.2.2 shows the LOQ and LOD for several PAHs. Note that detection and precision are in the parts per billion. Full scan MS chromatograms are shown for EPA 610 mix (figure 4.2.3), NJ menhaden fish oil (collected fall 2010 from the ship, Mount Vernon: MVNJ) (figure 4.2.4) and LA menhaden fish oil (collected fall 2010 from Grand Isle) (figure 4.2.5). Table 4.2.3 shows the concentrations of PAHs detected in the menhaden fish oils that were analyzed. Note that the levels of PAHs in the fish oils, both NJ and LA, are low, i.e. in the parts per billion (µg/L). A chromatogram that compares the selected ion for anthracene is shown in figure 4.2.6. It compares menhaden oil from BBLA and MVNJ with the EPA standard.

Comparisons of PAHs in fish oil show some similarities as well as differences between samples. First it should be noted that the overall levels of PAHs were low in all samples. For example, concentrations of anthracene ranged from 91 to 133 µg/L, while those for naphthalene were detected but below quantification in all samples tested. Of the PAHs detected, fluoranthrene concentrations were similar in LA and both NJ samples, ranging from 182 to 185 µg/L. Anthracene, which is a persistence PAH, was highest in NJ fish collected near shore, 133 µg /L (MVNJ) and lower in NJ fish collected off shore, 106 µg/L (EPNJ). Anthracene was lowest in LA fish collected from Barataria Bay, 91 µg/L (BBLA). Pyrene was similar for NJ fish collected near shore, 71 µg/L, and LA fish which were also collected near shore- that is

off of Grande Terre beach, 69 µg/L. NJ fish collected off shore had the lowest level of pyrene, 10 µg/L. It is too soon to draw conclusions regarding the relative amounts of PAHs and collection site.

Comparison of chromatograms was interesting. They showed similar peaks in NJ fish between retention times (RT) of 4.0 to 5.2 minutes. These peaks were absent or much lower in GILA fish. Peaks with 6.1 to 9.4 RT were similar for all samples. One exception was a group of peaks between 6.6 and 7.0- these were higher in NJ fish than LA fish. LA fish had a peak at 10.5 RT that was higher than found in either NJ sample. GILA and MVNJ fish both had a group of high peaks between 11.4 and 11.6, which was much lower in EPNJ fish. Comparisons to additional menhaden samples should help determine whether these finger-prints are consistent between locations and in abundance.

Table 4.2.1 PAH standards in EPA 610 mix: concentrations and major masses. PAHs are fragmented into major masses following gas chromatography (GC). The major fragments are then detected by mass spectroscopy (MS) using their mass to charge ratio (m/z).

PAH's	Conc.(ng/ml)	Major Masses
Napthalene	1000	128, 102
Biphenylene	2000	152, 126, 76
Acenapthene	1000	154, 153, 76
Fluorene	200	166, 139, 82
Anthracene	100	178, 152, 89
Phenanthrene	100	178 ,152 ,89
Fluoranthene	200	202,174,150,101
Pyrene	100	202,174,150,101
Benz(a)anthracene	100	228,200,114
Chrysene	100	228,200,114
benzo[k]fluoranthene	100	252 ,224 ,126
Perylene	100	252 ,224 ,126
Benzo[a]pyrene	100	252,224,126,113
Benzo[b]fluoranthene	100	226,198,174
Dibenz(a,h)anthracene	100	278,139,125

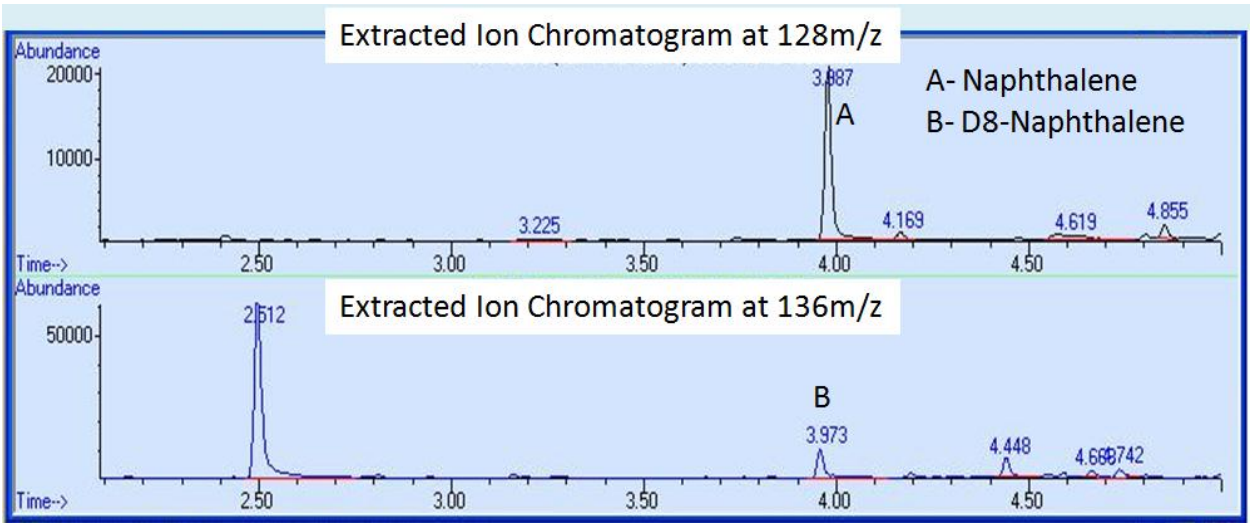

Figure 4.2.1 Ion chromatogram for naphthalene. This figure shows select ion monitoring of undeuterated (A) and deuterated (B) forms of naphthalene.

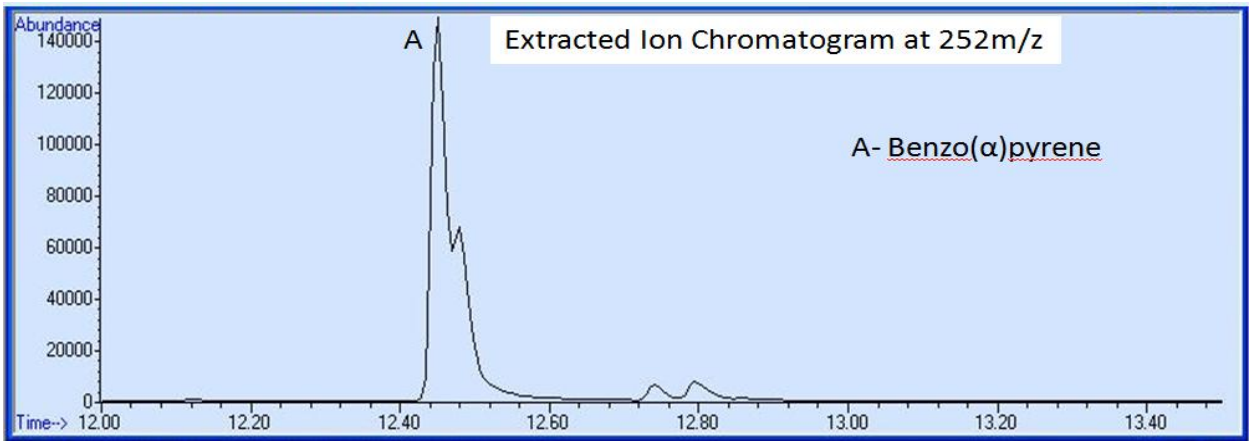

Figure 4.2.2 Ion chromatogram for benzo(a)pyrene. This figure shows select ion monitoring of undeuterated BaP (A).

Table 4.2.2. Limits of Detection (LOD) and Limits of Quantification (LOQ) for several standard PAHs. LOD is the lowest concentration of an analyte that an analytical process can reliably detect (S/N=2). LOQ is the lowest level of analyte that can be accurately and precisely measured (S/N=10).

PAH	LOD (ppb)	LOQ (ppb)
Napthalene	0.0781	1.25
Fluorene	0.015	0.025
Acenapthene	1.2500	50
Biphenylene	2.5000	20
Phenanthrene	0.10	12.5

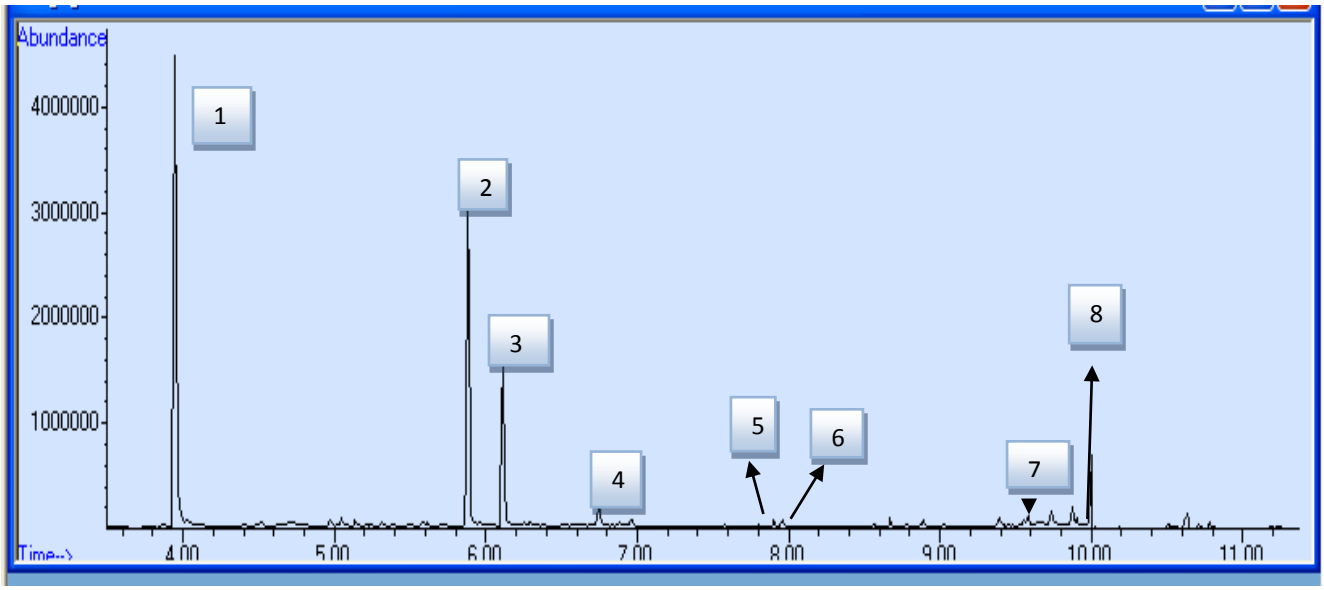

Figure 4.2.3 GC-MS chromatogram for PAHs from the EPA 610 mix spiked into commercial fish oil. Scan shows retention times from 4 to 11 minutes, which includes the following PAHs: 1 Naphthalene, 2 Biphenylene, 3 Acenapthene, 4 Fluorene, 5 Anthracene, 6 Phenanthrene, 7 Fluroanthene and 8 Pyrene.

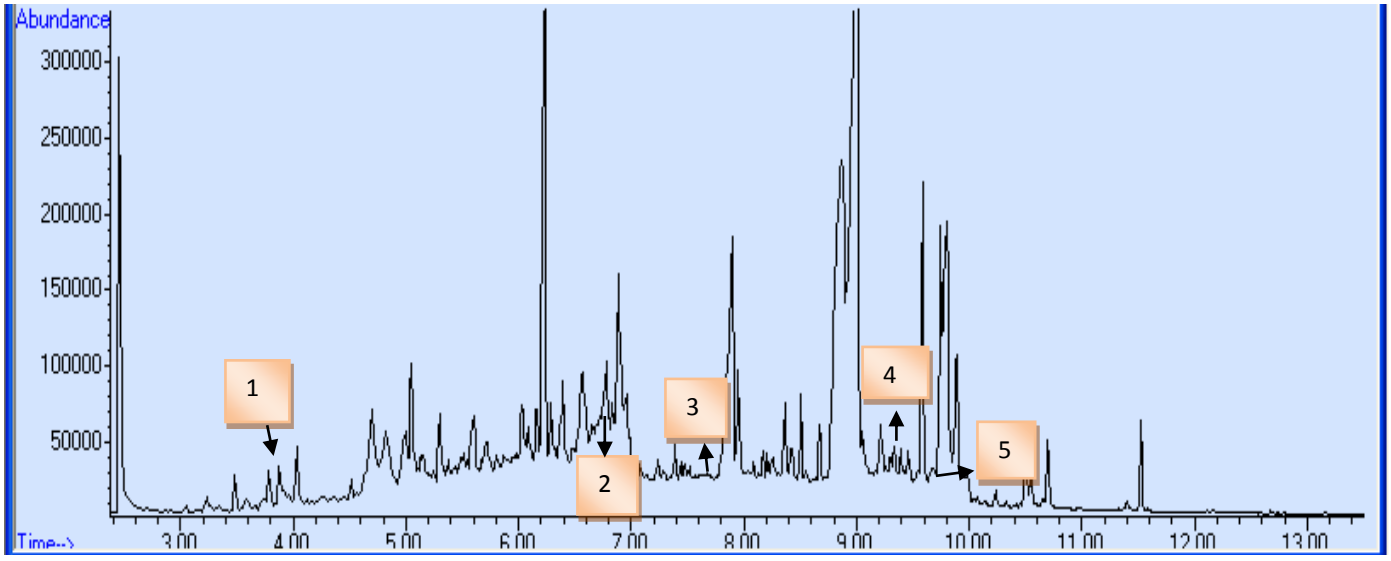

Figure 4.2.4. GC-MS chromatogram for PAHs in fish oil from MVNJ1 menhaden. The menhaden were collected in fall 2010 from the ship, Mt Vernon, seining for menhaden in the Delaware Bay area. The PAHs detected were: 1 Naphthalene, 2 Fluorene, 3 Anthracene, 4 Fluroanthene and 5 Pyrene.

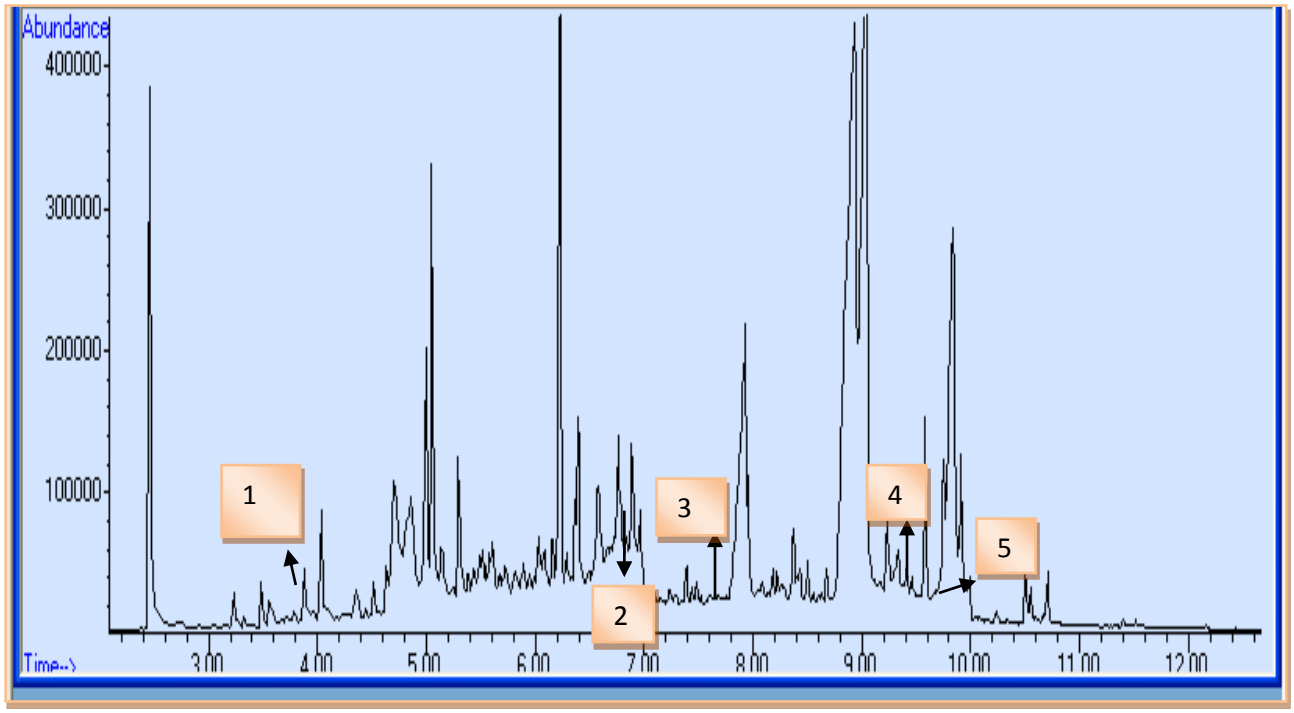

Figure 4.2.5. GC-MS chromatogram for PAHs in fish oil from EPNJ menhaden. The menhaden were collected in fall 2010 from the ship, Enterprise, seining for menhaden in the Delaware Bay area. The PAHs detected were: 1 Naphthalene, 2 Fluorene, 3 Anthracene, 4 Fluoranthene and 5 Pyrene.

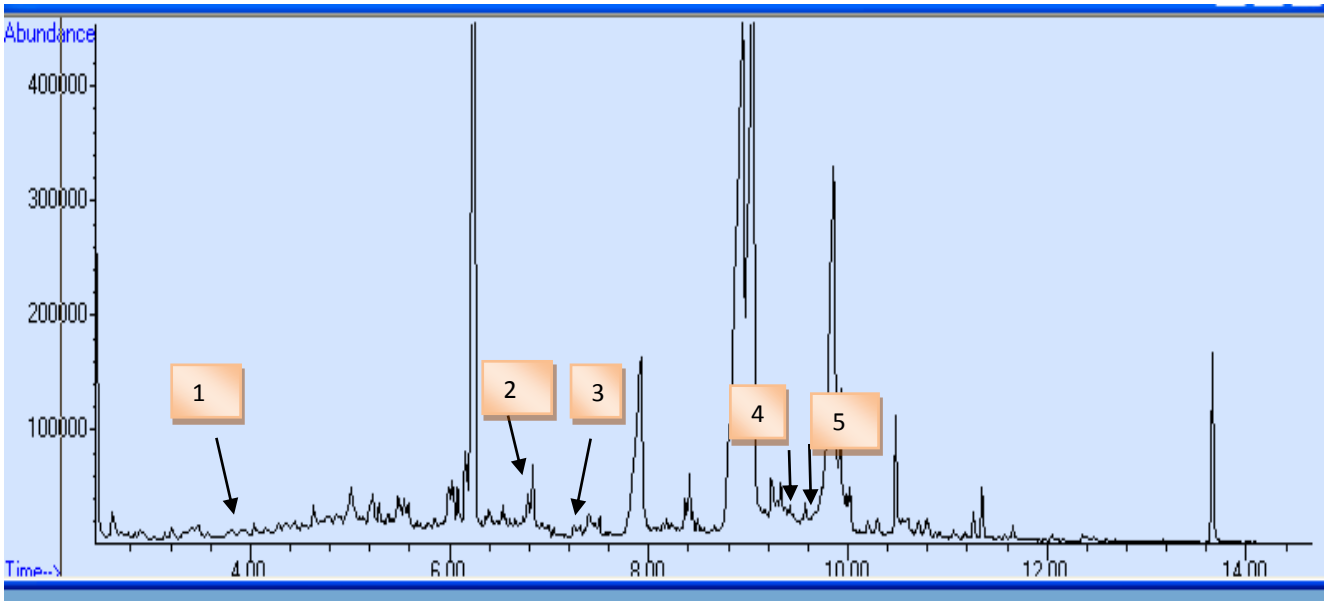

Figure 4.2.6 GC-MS chromatogram for PAHs in fish oil from BBLA menhaden. The menhaden were collected in fall 2010 from the Barataria Bay near Grand Terre beach, LA. The PAHs detected were: 1 Naphthalene, 2 Fluorene, 3 Anthracene, 4 Fluoranthene and 5 Pyrene.

Table 4.2.3 PAHs in menhaden fish oil collected in fall 2010. Samples were from Barataria Bay, LA (BBLA) off of Grande Terre beach, and Delaware Bay, NJ from the ships, Mt Vernon (MVNJ1) and Enterprise (EPNJ1). See figures 3.1 and 3.2 for maps of where fish were collected.

PAH	BBLA (ppb)	MVNJ1 (ppb)	EPNJ1 (ppb)
Naphthalene	NQ	NQ	NQ
Fluorene	NQ	NQ	NQ
Acenapthene	ND	ND	ND
Biphenylene	ND	ND	ND
Fluoranthene	182	185	184
Pyrene	69	71	10
Anthracene	91	133	106
Phenanthrene	ND	ND	ND
Benz(a)anthracene	ND	ND	ND
Chrysene	ND	ND	ND
Benzo(a)pyrene	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND

ND = NOT detected means the value is below LOD
NQ= NOT quantified means the value is below LOQ

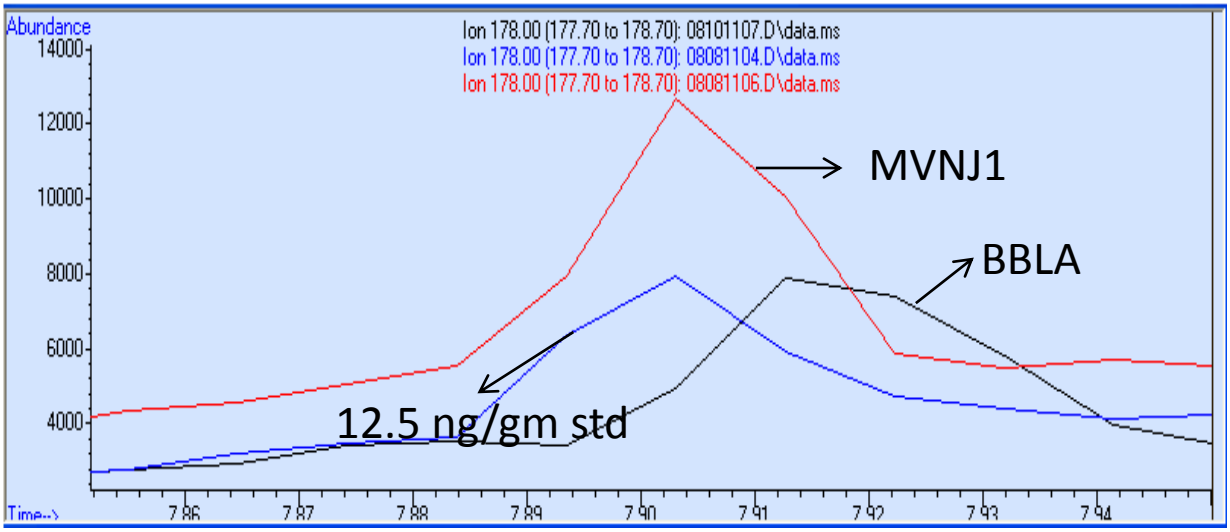

Figure 4.2.7 Select ion chromatogram of anthracene from menhaden oil of MVNJ1 and BBLA. The EPA standard is shown in blue at 12.5 ng/g or ppb.

IV.3 PAHs in Fish Oil and Liver Tissue of Large Menhaden as Determined by Fixed Emission Fluorescence Spectroscopy

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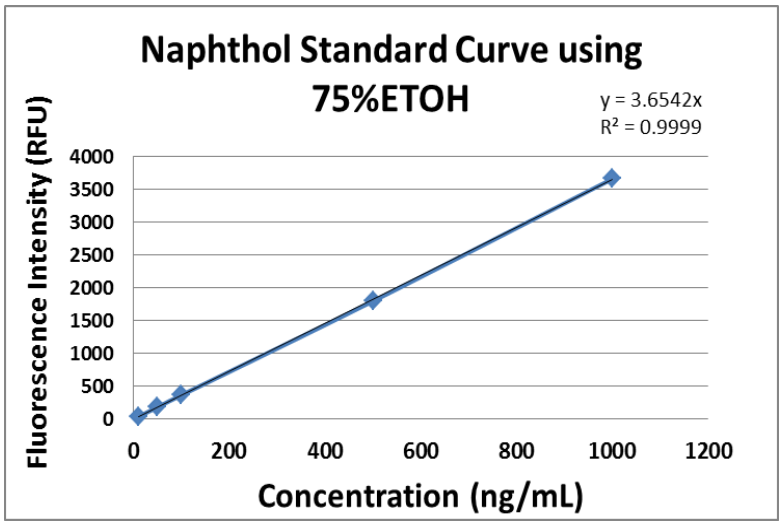
IV.3.1 Overview

In order to evaluate the bioaccumulation of petroleum contaminants by menhaden, fish body oil and liver tissue were analyzed for PAHs by Fixed Emission Fluorescence Spectroscopy (FEFS). Liver tissue was obtained by dissecting it from large menhaden and freezing it at -20^{o}C until analysis. Fish oil was prepared as described above. The technique used for analyzing PAHs by FEFS was developed for the purpose of this project. It was based on fluorescence techniques for fish bile (Aas et al, 2000). Development of the technique involved optimizing extraction and detection of 2-3 ring and 4-5 ring PAHs using two PAH metabolite standards, 2-naphthol (HNP) and 1-hydroxyprene (HPY), respectively. The best way to extract PAHs from liver and fish oil was investigated by testing several solvents- ethanol, isopropanol and hexane. Different concentrations of each solvent were generated by adding water (50, 60, 70, 75, 80, 90 and 100% solvent). HNP and HPY were added to fish oil or liver homogenate and then extracted into the different concentrations of solvents. The success of the extraction was based on percent recovery of the two PAHs. Results of these experiments indicated that 75% ethanol (ETOH) provided sufficient extraction of HNP and HPY simultaneously for their detection in fish oil and liver tissue (data not shown). Samples were analyzed for fluorescent compounds using two settings on a SpectraMax® M5/M5 scanning fluorometer (Molecular Dynamics). This was a unique approach to PAH analysis. The first setting involved holding the emission wavelength (Em) at 350 nm and scanning for excitation wavelengths (Ex) from 250 to 330 nm. This setting was best for aromatic hydrocarbons with one, two or three aromatic rings such as vitamin A and E (1 ring), naphthol (2 rings) and hydroxyfluorene (three rings). The second setting involved holding the Em at 450 nm and scanning for Ex from 250 to 430 nm. This setting was best for PAHs with 4 and 5 rings such as hydroxypyrene and benzo(a)pyrene, respectively. Scanning for excitation as opposed to emission was also unique. Preliminary work measuring absorbance (fluorescent chemical excitation) showed more overlap for excitation than emission wavelengths of vitamins and PAHs. Since fish liver and fish oil were expected to contain vitamins A&E, it was decided to hold emission and not excitation fixed (data not shown). Emission wavelengths of 350 and 450 nm were chosen not because of their sensitivity but because their excitation spectra best discriminated between the different PAHs as well as vitamins A&E. Other than establishing the solvent for extraction and settings for spectral analyses, method development included 1) establishing standard curves for HNP and HPY, 2) establishing fluorescence spectra for multiple PAH standards, vitamins A&E and pheophytin (a breakdown product of chlorophyll), 3) developing the extraction procedure for fish oil and fish tissues and 4) determining % recovery of HNP and HPY in DWH crude oil, fish oil and fish liver. It was discovered that fish oil and liver samples produced two major types of spectra, one of which might have been due to artifacts. Some work was done to study this as well.

IV.3.2 Standard curves and fluorescence spectra for chemical standards

Standard curves for HNP and HPY were generated by dissolving different amounts of each chemical in 75% ETOH- 10, 50, 100, 500, and 1000 ng/ml. The major peak for HNP was Em350/Ex270 nm and for HPY, Em450/Ex340 nm. Fluorescence values (RFU) at each chemical's major peak were used to generate its standard curve (Figures 4.3.1). The line equation for HNP was $y = 3.6542x$ with $R^2 = 0.9999$. The line equation for HPY was $y = 2.7137x$ with $R^2 = 0.9992$. The detection limit was established as 2x the blank-75% ETOH, 54 ng/ml for HNP and 5.9 ng/ml for HPY.



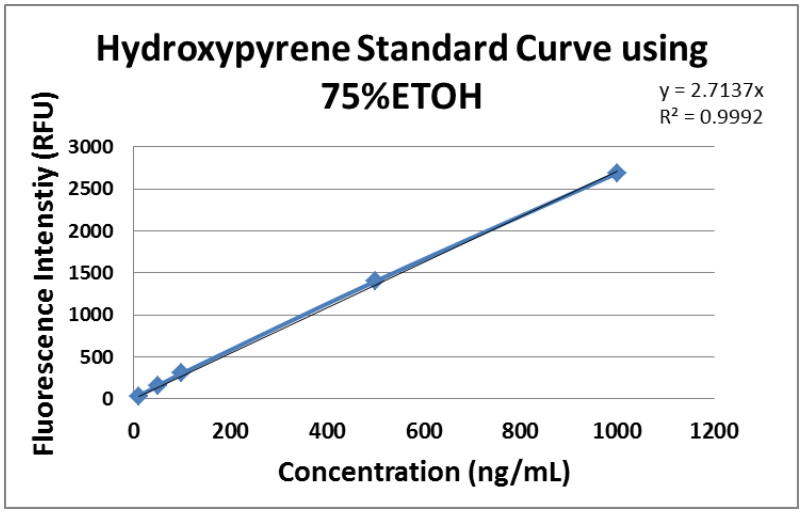


Figure 4.3.1 Standard Curves for PAH metabolites 2- naphthol (HNP) and 1-hydroxypyrene (HPY). Fluorescence Intensities were based on the major peak for each standard: Em350/Ex270 nm for HNP and Em450/Ex340 for HPY.

Spectra for chemical standards were generated by dissolving PAHs into 100% isopropanol at 1 mg/ml. This stock was diluted to 25 µg/ml in 75% ETOH, and then 50 µl of this was added to 1.15 ml of 75% ETOH to achieve a final concentration of 1250 ng/ml. This solution was run at the established spectral settings. All chemicals were of ACS grade or better and purchased from Sigma-Aldrich. Fluorescence intensities of major and minor peaks using the fixed emission wavelengths of 350 or 450 nm resulted in unique spectra for each chemical tested (Figure 4.3.2 A-F). For example, hydroxyfluorine (HFL) and naphthol (HNP) both had a major Ex peak at 270 nm, but HFL also had a minor peak at 310 nm while HNP had one at 320 nm using Em350. Fluoranthene (FAN) and pyrene (PYR) both had two major peaks using EM450; however, those for FAN were EX280 and EX350 while those for PYR were EX270 and EX340. The overlapping of peaks made it impossible to distinguish concentrations of particular aromatic compounds in fish and crude oil samples. It did allow approximate concentrations of naphthol-like and hydroxypyrene-like PAHs to be calculated. This has advantages in that environmental samples, including crude oil, are a mixture of compounds to start with and are further metabolized by organisms resulting

in an unknown mixture of chemicals. This technique did not require that each component be identified in order to quantify them.

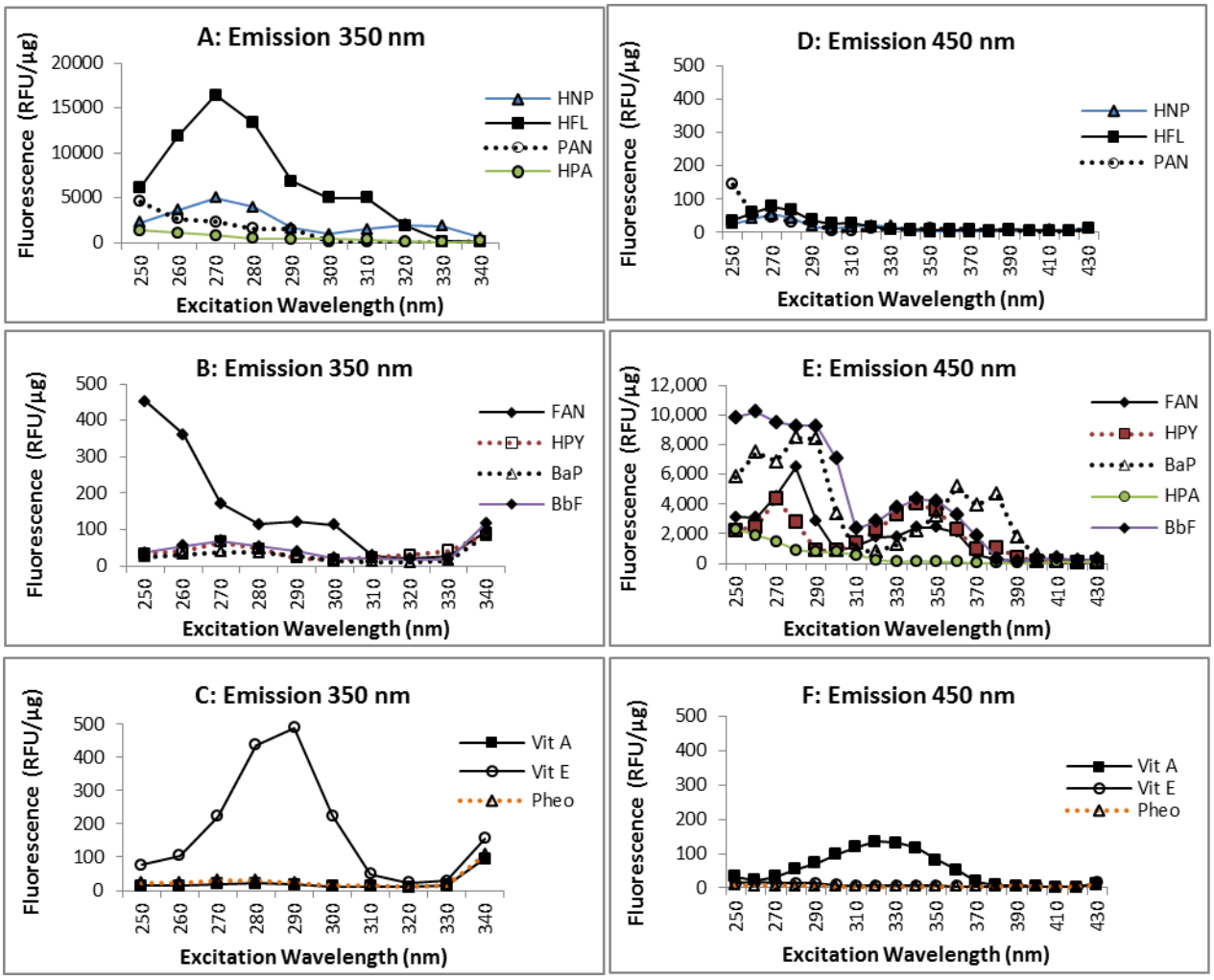


Figure 4.3.2 Fixed emission wavelength (nm) spectra for PAHs, vitamins E and A, and pheophytin A. Two types of spectra are shown: Em350 fixed with Ex250-340 sweep and Em450 fixed with Ex250-430 sweep. PAHs with 2-3 rings are shown in A and D, except HPA, which is shown in A and E. PAHs with 4-5 rings are shown in B and E. Vitamins and pheophytin A are shown in C and F. Units are fluorescence (RFU) per µg sample. HNP= naphthol, HFL= 9-hydroxyfluorene, PAN= phenanthrene, HPA=9-phenanthrol, FAN= fluoranthene, HYP= 1-hydroxypyrene, BaP= benzo(a)pyrene, BbF=benzo(b)fluoranthene, Vit A= vitamin A, Vit E= vitamin E, and pheo =pheophytin A. The PAHs were spiked directly into 75% ETOH.

IV.3.3 Procedure for extracting PAHs from fish oil, crude oil and fish liver tissue and determination of percent recovery

Development of this FEFS technique required the establishment of % recoveries for PAHs from the samples tested. It was assumed that the samples would have different matrixes that would retain the PAHs and effect their extraction. By establishing % recoveries, concentrations of PAHs in the solvent could be adjusted to account for the total amount in the sample. Determination of percent recovery was done as follows. Three types of samples were generated. The first consisted of sample- fish oil, crude oil or fish liver tissue alone, the second of sample plus PAH standard and the third the PAH standard alone. Two PAH standards were tested, HNP and HPY. This allowed recovery to be determined for 2-3 ring and 4-5 ring PAHs. Percent recovery was calculated by 1) subtracting RFU values of the unspiked (fish sample) from those of the spiked fish samples, 2) dividing that difference by the RFU value of the standard and 3) multiplying the quotient by 100.

The procedure for extracting PAHs from fish oil was as follows. Fish oil samples remained frozen until analysis. Nitrogen was blown over stored samples. To extract PAHs, the oil was thawed and mixed by vortexing. In a 1.5 ml microcentrifuge tube, 50 µl of fish oil and 1.15 ml of 75% ethanol (ETOH) were combined. The mixture was vortexed continuously for 1 minute and then the oil was separated from the ETOH by centrifuging for 20 minutes at 13,000 rpm. The oil went to the bottom of the tube. One milliliter of the ETOH was removed and placed in a quartz cuvette. Samples were analyzed for fluorescent compounds using two settings on a SpectraMax® M5/M5 scanning fluorometer as described above (IV.3.1).

For fish oil percent recovery, the fish oil alone sample was generated as above, with 50 µl of fish oil extracted into a final volume of 1200 µl 75% ETOH. The fish oil plus PAH was generated by adding 25 µl of 1 mg/ml PAH stock in 100% isopropanol to 975 µl fish oil for a PAH concentration of 25 µg/ml in fish oil. This was vortex and then 50 µl of the spiked fish oil was extracted into a final volume of 1200 µl 75% ETOH as described. The final amount of PAH standard in the fish oil was 1250 ng, and if completed extracted into the ETOH the final concentration would be 1042 ng/ml. The PAH standard alone was prepared by adding 50 µl of a 25 µg/ml stock to 1150 µl of 75% ETOH for a final concentration of 1042 ng/ml.

The PAHs in crude oil was much more concentrated than in fish oil. Samples for establishing fluorescence spectra and percent recovery were as follows. The crude oil alone samples was generated by adding 2 µl of crude oil to 10 ml of 75% ETOH in a 50 ml polypropylene centrifuge tube. The mixture was vortexed for 1 minute and allowed to settle for one hour. Then one milliliter was withdrawn, carefully avoiding any oil on the surface, and place in the fluorescence cuvette for analysis. The spiked sample was generated by adding 500 µl of 25 µg/ml PAH to 9.5 ml 75% ETOH for a final concentration of 1250 ng/ml. To this 2 µl of crude oil was added and processed as described. The PAH alone sample was prepared by adding 500 µl of 25 µg/ml PAH to 9.5 ml 75% without the oil added and processing as described.

PAHs in liver tissue from large menhaden were analyzed in addition to their fish oil. The procedure involved homogenizing 0.1 g of wet weight liver in 1 ml of 75% ETOH using a 2 ml tissue grinder. The homogenate was centrifuged for 20 minutes at 13,000 rpm. The resulting supernatant was then diluted by adding 100 µl of it to 1.1 ml of 75% ETOH. This diluted sample was then analyzed on the fluorometer as described above. The remaining homogenate and supernatant were frozen at -20 ^{o}C in case of later analysis. Evaluating percent recovery involved spiking a separate homogenate of the same fish liver

with 50 µl of 25 µg/ml solution of HNP or HPY (final concentration 1250 ng/ml) and proceeding as described for the unspiked sampled.

Results for fish oil showed similar percent recoveries for HNP and HPY (Figure 4.3.3). Two samples of fish oil were tested, one from fish collected in the Delaware Bay, NJ area on September 7, 2010 (MVNJ) and one from fish collected in the Barataria Bay, LA area on October 30, 2010 (BBLA). The fish from NJ were *B. tyrannus* and those from LA *B. patronus*. Percent recovery of HNP from MVNJ and BBLA were 43.9 and 45.8%, respectively, with an average of 44.9%. Percent recovery of HPY from MVNJ and BBLA were more different, 55.9 and 40.0%, respectively, with an average of 47.9%. Overall it seemed that species did not affect recovery. Results for DWH crude oil had different percent recoveries for HNP and HPY (Figure 4.3.4). Percent recovery for HNP was similar to that of fish oil, 44.4%. However, that for HPY was higher, 92.3%. The reason for this was unknown. It was important to note that while the concentrations of standards were similar, much less crude oil matrix (2 µl into 10 ml ETOH) was added to the extraction than fish oil matrix (50 µl into 1.15 ml ETOH). Why less HNP was recovered than HPY was unknown. Results for liver homogenate showed even less % recovery (Figure 4.3.5). Percent recovery for HNP and HPY were 25 and 35%, respectively. Again, recovery of HPY was greater than for HNP. It was likely that PAH standards were binding to tissue particles as with the oil particles, thereby reducing their recovery. This indicated that we need to work on our extraction procedure. However, the fluorescent compounds recovered were sufficient for detection at the ng/ml level.

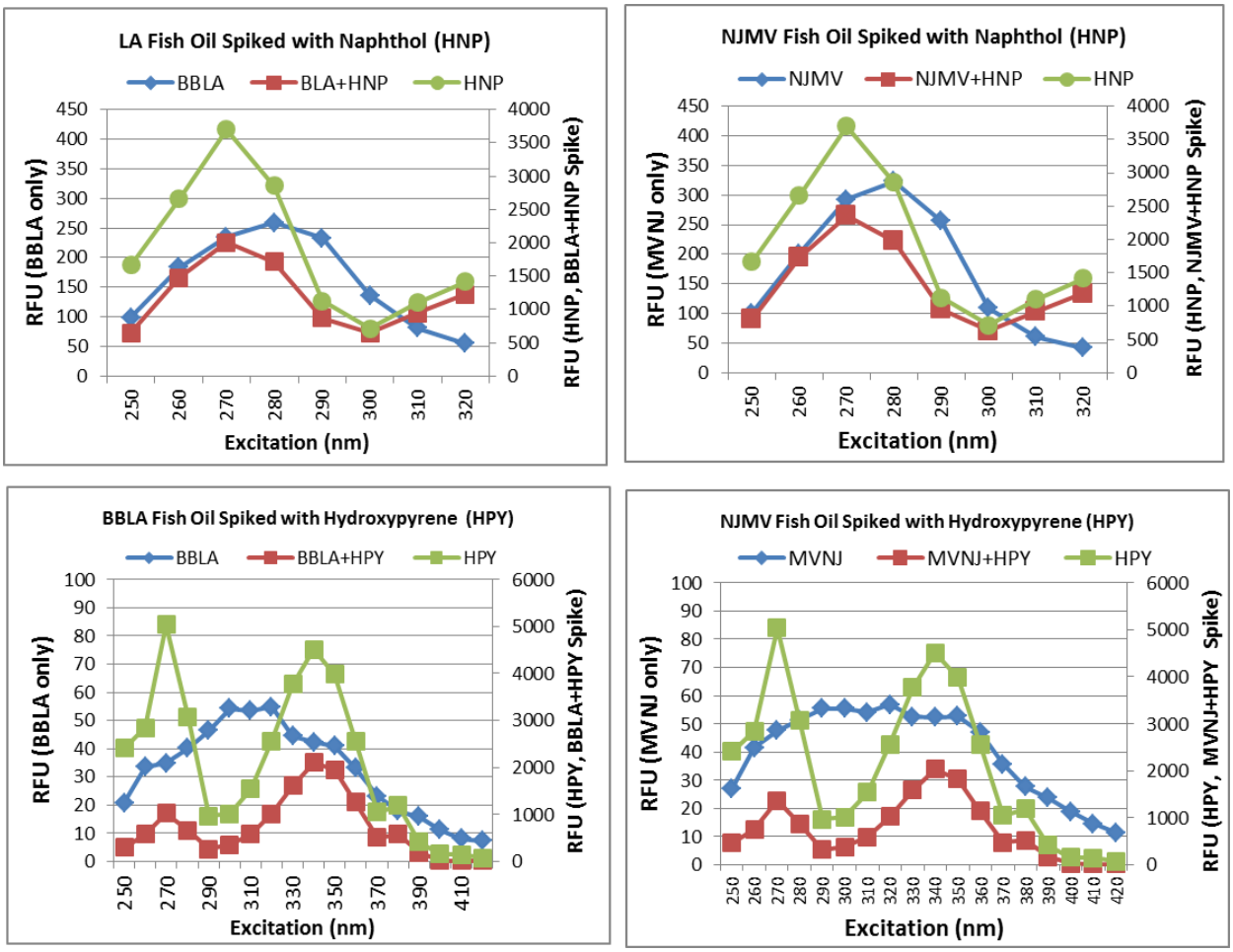


Figure 4.3.3 Percent recovery of naphthol (HNP) and hydroxypyrene (HPY) in fish oil. Two fish oil samples were used. One from fish collected from the Mount Vernon ship in NJ on 9/7/2010 and one from fish collected from Barataria Bay, LA on 10/30/2010. RFU represents fluorescence intensity. The concentration of both PAH standards alone was 1042 ng/ml. Average percent recovery was 47.9 for HNP and 44.9 for HPY using peaks at Em350/EX270 and Em450/Ex340, respectively. Note the differences in spectra for the two fish oils as determined by number and intensity of peaks.

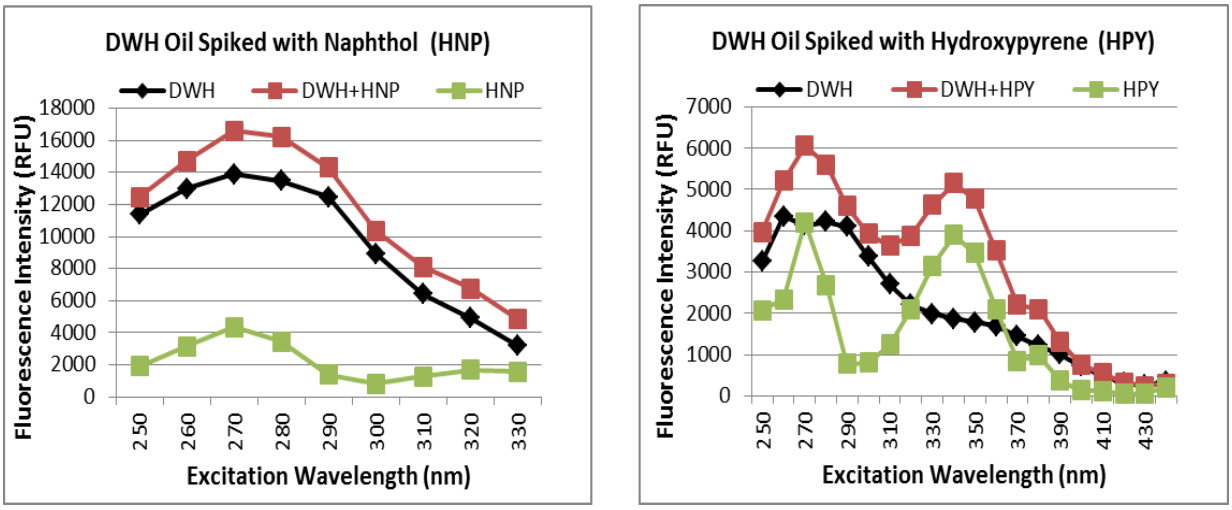


Figure 4.3.4 Percent recovery of naphthol (HNP) and hydroxypyrene (HPY) in DWH crude oil. The crude oil sample was collected from the riser pipeline on April 24, 2010, four days after the oil rig explosion. The concentration of both PAH standards alone was 1250 ng/ml. Average percent recovery was 44.4 for HNP and 92.3 for HPY using peaks at Em350/EX270 and Em450/Ex340, respectively.

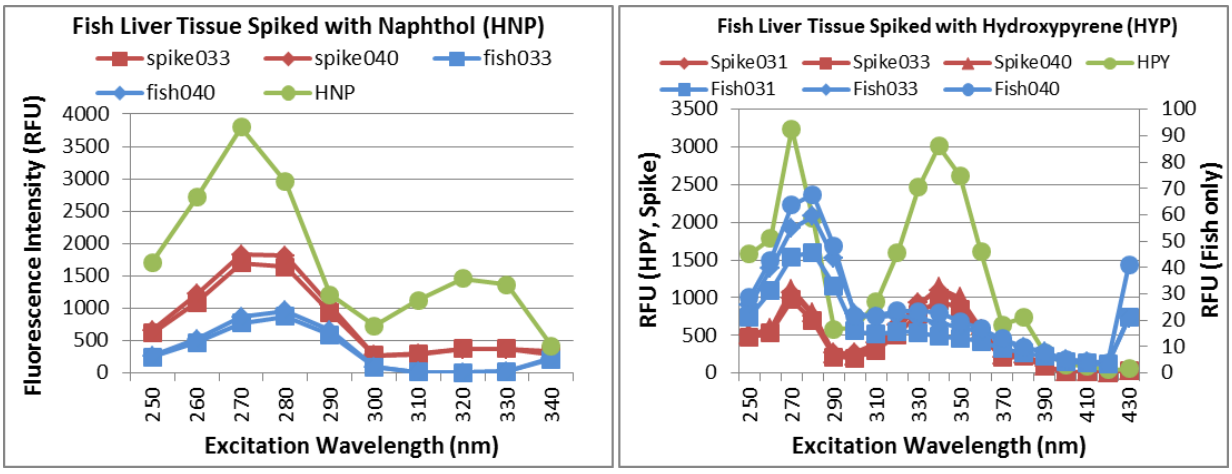


Figure 4.3.5. Percent recovery of naphthol (HNP) and hydroxypyrene (HPY) in fish liver homogenate. The fish were collected from Delaware Bay, NJ and each fish was designated by a different number (i.e. fish033 or fish040). PAH standards were spiked into separate homogenates of the same fish livers (spike). The concentration of both PAH standards alone was 1250 ng/ml. Average percent recovery was 25 and 35%, respectively. Note secondary vertical scale for fish samples in the HPY experiment.

IV.3.4 Investigation into the cause of two different fluorescence spectra for the same sample

Repeated testing of samples over time revealed changes in some of them associated with modified spectra at both the Em350 and Em450 fixed wavelengths. For fish oil, some became cloudy over time even though they were covered with nitrogen and stored at -20°C. The cloudiness appeared related to white particles that could be removed by brief centrifugation (5 min, 13,000 rpm). Centrifugation produced a white-yellow pellet and a clear, light yellow supernatant. Experiments with several fish oils found that the change in excitation spectrum for Em350 was seen as an increased fluorescence at Ex280. This occurred when testing the same fish oil sample two months after the first analysis (7/27/2011 then 9/19/2011). When the same oils were tested a third time (10/26/2011), the resulting spectra match the second one (Figure 4.3.6). Changes in the spectra over time were also found using Em450. The excitation peak at 350 nm increased each time the sample was retested, while the peaks at 260 and 310 nm were lost. Several of the 4-5 ring PAHs had major excitation peaks at 350 nm, so the increased fluorescence at this wavelength could have been due to more release of PAHs from the oil matrix overtime.

Liver tissue homogenates also showed changes in their spectra over time as well as two major types of spectra for fish collected and tested at the same time (Figure 4.3.7). The most notable differences occurred using a fixed emission wavelength of 450 nm. Some fish showed two major peaks of similar fluorescence intensity, one at Ex280 and one at Ex350. This was called a type I spectrum (MVNJ023). Other fish collected and tested at the same time showed one major peak at Ex350, which had much greater intensity than found for the type I spectrum (MVNJ026). This was called a type II spectrum. Changes in spectra included an increase of the type I spectrum over time (MVNJ022), a switch from a type I to type II spectrum at a later date (MVNJ023) or no major changes in the type II spectrum over time (MVNJ026). Since concentrations of HPY-like PAHs were determined using EM450/EX350, this presented a dilemma.

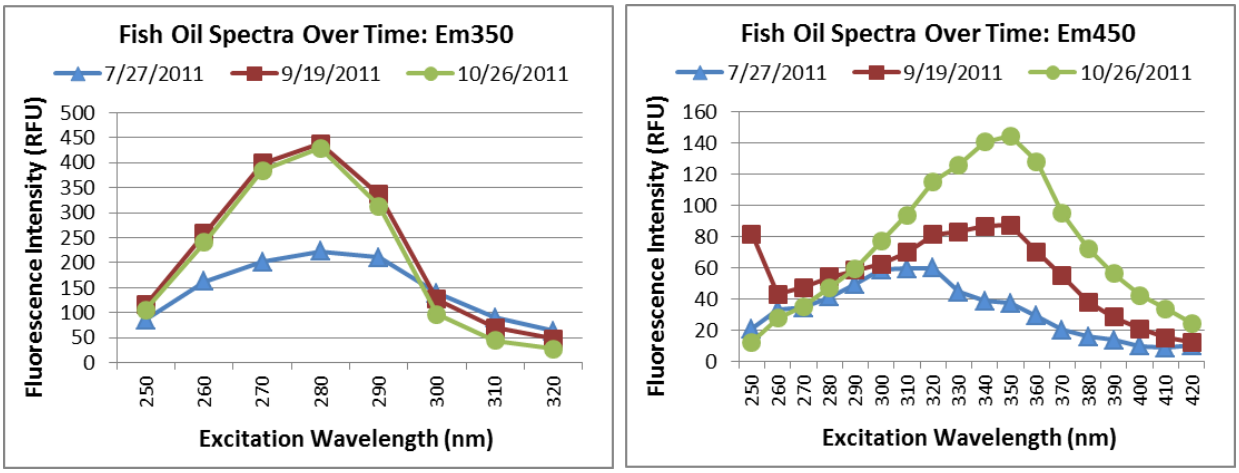


Figure 4.3.6 Change in fish oil spectra over time. The same fish oil sample (EPNJ1) was repeatedly sampled at three dates- 7/27/2011, 9/19/2011, and 10/26/2011. Spectra for which emission was fixed at 350 nm or 450 nm are shown. Data showed increased fluorescence intensity for the Ex280 peak and Ex350 peak for Em350 and Em450, respectively.

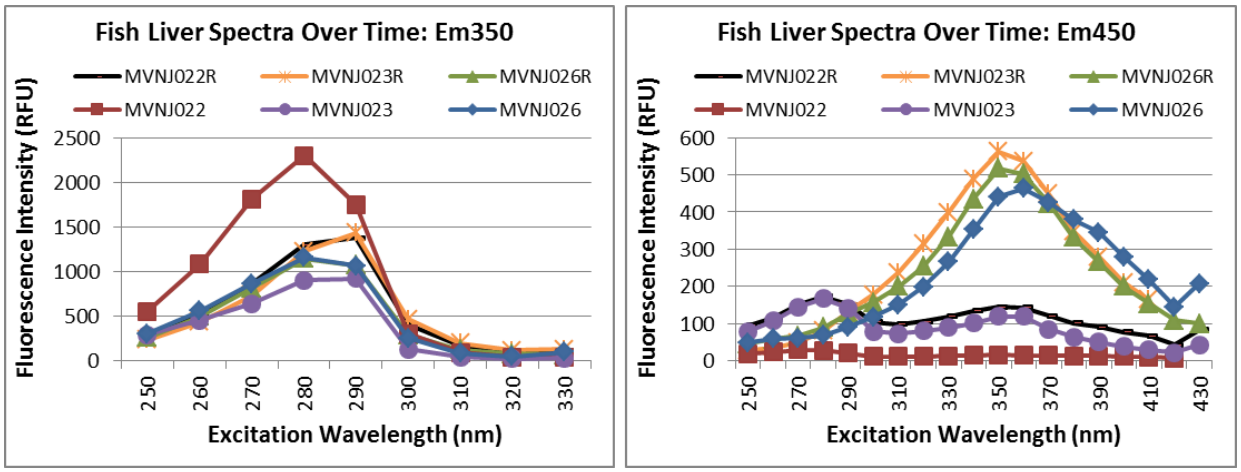


Figure 4.3.7 Change in liver tissue spectra over time. The same samples of liver homogenates were tested at two different dates, 11/11/2011 and 5/16/2011. Each sample was prepared from an individual fish (represented by a unique number) collected from the Delaware Bay area by the ship, Mount Vernon (MVNJ). Homogenates were kept frozen between analyses, -20 ^{o}C. Spectra for which emission was fixed at 350 nm or 450 nm are shown. One sample (MVNJ023) showed increased fluorescence intensity for both the EM350/Ex280 and Em450/Ex350 peak as seen in fish oil. R=sample analysis repeated at the later date.

Experiments were performed in order to investigate possible reasons for the change in spectra over time as well as the two different types of spectra found in fish liver tissue. In both cases, there was as increase in the Em450/Ex350 peak. One experiment involved testing extraction volume (Figure 4.3.8). Crude oil from the DWH (2 µl) was extracted into 1 or 10 ml of 75% ETOH. Results showed that extracting into a small volume created different spectra than extracting into a large one. Of particular interest was the occurrence of the high intensity Ex350 peak when using the Em450 setting. This was the same spectra as found in aged fish oil and some fish liver samples.

A second experiment involved testing a cloudy, aged fish oil sample before and after centrifugation (EPNJ1). Centrifuging produced a white-yellow pellet and a clear supernatant. The clear supernatant and uncentrifuged samples were extracted into 75% ETOH in accordance with the standard procedure, final volume 1.2 ml. Results showed a type II spectrum for uncentrifuged oil and a spectrum similar to type I for centrifuged oil (Figure 4.3.9). This indicated that the deteriorating particles in aged oil were releasing more fluorescent compounds into the extract. The two experiments suggested that an insufficient extraction volume was creating an unusual spectrum, possible due to high levels of aggregated fluorescent compounds in the extract. This requires more testing.

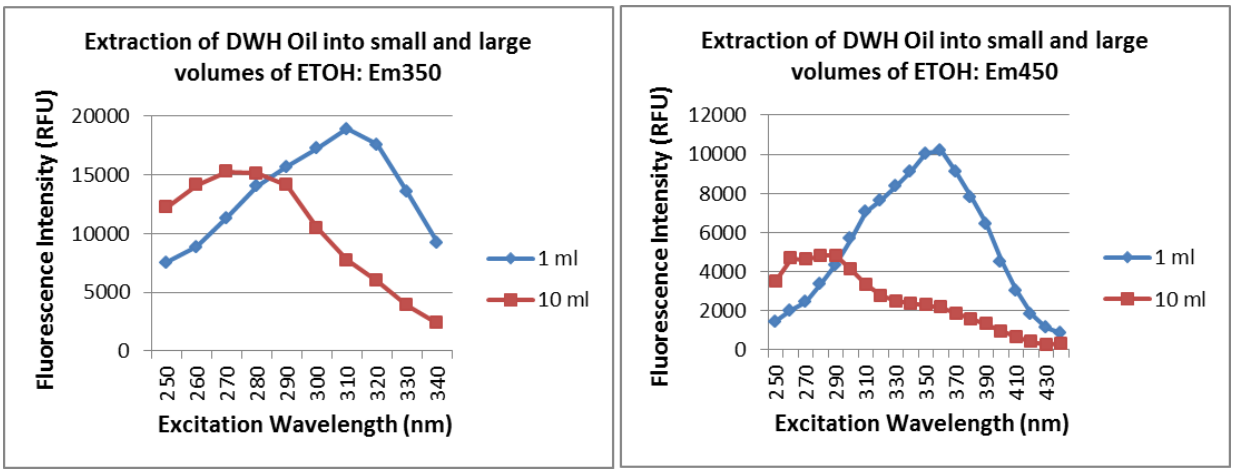


Figure 4.3.8 Effect of extraction volume on FEFS. The same volume of DWH oil (2µl) was extracted into 1 or 10 ml of 75% ETOH. Note changes in both the EM350 and EM450 spectra.

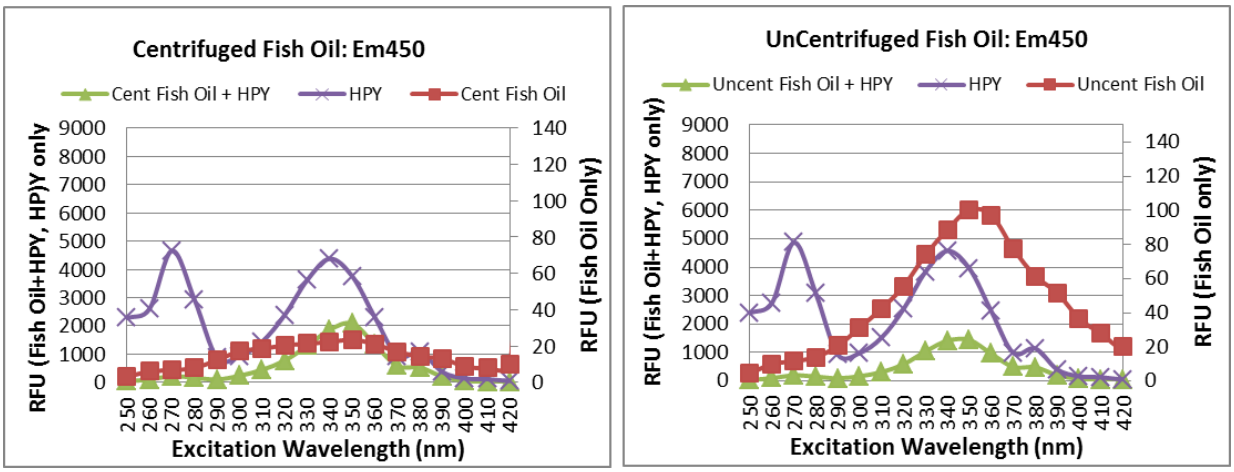


Figure 4.3.9 Effect of aging fish oil on FEFS. Samples of EPNJ1 were analyzed before and after centrifugation of particles suspended in age oil. Presence of the particles was associated with greater extraction of fluorescent particles.

IV.3.5 Comparison of fish oil samples from the Gulf and Atlantic coast in 2010

Fish oil from Barataria Bay, LA (impacted by the DWH oil spill), James River, VA and Delaware Bay, NJ were analyzed and compared. It was expected that the large menhaden from VA and NJ had not recently been exposed to a large oil spill and that PAHs in their body oil were primarily due to pyrogenic not petrogenic sources. Both locations have been heavily exposed to urbanization. Fish oil samples were prepared from the skins and fillets of 5-10 fish as described (IV.1 Procedure for Preparing Menhaden Fish Oil). Each sample was extracted and analyzed as described (IV.3.3 Procedure for extracting PAHs from fish oil, crude oil and fish liver tissue and determination of percent recovery). Data were determined by first generating standard curves for 2-naphthol (HNP) and 3-hydroxypyrene (HPY) using RFU values at their major peaks, Em350/Ex270 and Em450/Ex340, respectively. Concentrations (ng/mL) of PAHs in fish oil were based on RFU values at their major peaks, Em350/Ex280 for NP-like PAHs and Em450/Ex350 for HP-like PAHs. The standard curves were used to convert RFU values of sample extracts to ng PAH/mL ETOH. This value was adjusted for percent recovery and dilution factor and divided by the weight of the original amount of oil used (50 µl). Final units were ng PAH/mg fish oil. The % recoveries were 44.9 % for NP and 47.9 % for HP.

Results for fish oil were broken down into type I and type II spectra so that they could be more accurately compared. Some samples did not have a type I (JRVA, MVNJ1) or type II spectrum (SHNJ). Spectra for HNP-like PAHs were from the same fish oil extract used to generate HPY-like PAHs spectra. In results for type I spectra, all fish oil samples showed a major excitation peak at 280 nm using Em350, i.e. settings for HNP-like PAHs (Figure 4.3.10). This peak, Em350/Ex280, did not match any of the standard PAHs tested (see Figure 4.3.2). It was similar to the vitamin E spectrum, which had one major peak at Em350/Ex290 and to the vitamin A spectrum, which had one major peak at Em350/Ex280. However, vitamin A (50 RFU/ µg) had a much lower fluorescence intensity than vitamin E (500 RFU/ µg) and 2-3 ring PAHs (5000-15,000 RFU/ µg). So if the Em350/Ex280 was either of these vitamins, their concentrations had to be very high. The spectrum for DWH crude oil showed a broad peak between Ex260-290. This region encompassed the Ex280 peak found in fish oil indicating that this peak could have been generated by unidentified PAHs in crude oil. There were different levels of fluorescence at Em350/Ex280 such that BBLA>> EPNJ1 ≈EPNJ2>SHNJ (Table 4.3.1). Statistical differences could not be determined between locations as there was only one fish oil sample from LA. However, BBLA fish oil (4.68±0.17 ng/mg) clearly had higher levels of HNP-like PAHs than NJ samples (0.74±0.08 to 1.09±0.05 ng/mg).

Using settings for HPY-like PAHs, all type I spectra had a major excitation peak at 350 nm (Figure 4.3.11). This peak, Em450/Ex350, coincided with major peaks for several PAH standards (see Figure 4.3.2). Two minor peaks were also seen, one between 260-270 nm and another at 320 nm. The Ex260-270 peak together with the Ex350 peak was indicative of hydroxypyrene and/or fluoranthene. The Ex320 peak might have represented vitamin A in the fish oil. The spectrum for DWH crude oil showed a large, broad peak between Ex260-290 and a minor one at Ex350. This matched the spectrum for fluoranthene the best. Comparison of HPY-like PAHs in fish oils showed different levels of fluorescence: EPNJ1>SHNJ>BBLA> EPNJ2 (Table 4.3.1). This order differed from that for HNP-like PAHs indicating that two of three NJ samples had higher HPY-like PAHs than the LA sample. This also showed that concentrations of HNP-like and HPY-like PAHs in a sample were independent and not necessarily proportional to each other. Comparing the relative concentrations of these two types of PAHs showed that BBLA fish oil had the highest HNP to HPY ratio (4.2) compared to other samples (0.47 to 1.52) using type I spectra (Figure 4.3.11). A high ratio of HNP to HPY-like PAHs was also found in DWH oil (12.29). Therefore, a high ratio of HNP to HPY in fish oil could have indicated recent crude oil exposure.

Data for type II spectra were similar to that found for type I. Concentrations of HNP-like PAHs were high in BBLA fish oil (10.16±1.23 ng/mg) compared to two of the three NJ samples (0.79 and 0.83 ng/mg) and VA sample (2.37±0.20 ng/ml) (Table 4.3.2). The third NJ sample, MVNJ1, also had high HNP-like PAH concentrations (21.24±4.35). The major peak was at Em350/Ex280 as for the type I spectrum (Figure 4.3.13). For MVNJ and BBLA, the HNP-like PAH concentrations were about twice that found using type I spectra. This increase was not consistent as levels of HNP-like PAHs were somewhat lower for EPNJ1 and EPNJ2 samples. This suggested that type II spectra were associated with better extraction of HNP-like PAHs for some samples. The order of concentrations for HNP-like PAHs using data from type II spectra were MVNJ1>BBLA>JRVA>EPNJ1≈EPNJ2. This compared to BBLA>> EPNJ1 ≈EPNJ2>SHNJ for type I spectra. Therefore, the relative concentrations of HNP-like PAHs in fish oil were the same regardless of the spectra used to calculate them.

Concentrations of HPY-like PAHs were higher for all fish oil samples when calculated using type II spectra (Table 4.3.2). There was one major peak at Em450/Ex350 indicating that the peak at Em450/Ex320 (vitamin A) found with type I spectra had been lost due to the higher concentration of compounds fluorescing at Ex350 (Figure 4.3.14). The order of HPY-like PAHs using data from type II spectra was JRVA>MVNJ>BBLA>EPNJ1≈EPNJ2. This was a different order than for HNP-like PAHs, again showing independent concentrations for these two types of PAHs in a particular fish oil sample. The order was somewhat different for HPY-like PAHs calculated using type I spectra: MVN1J> EPNJ1>BBLA> EPNJ2. Therefore, better extraction was achieved for BBLA samples than EPNJ1 samples using the aged fish oil. The ratio of HNP to HPY-like PAHs was high for both BBLA and MVNJ1 samples (Figure 4.3.15). This was consistent with the data found using type I spectra and again indicated recent exposure to crude oil. Interestingly, JRVA had a very low concentration of HNP-like PAHs compared to HPY-like PAHs. This might have indicated exposure to legacy PAHs. Collection of menhaden in MVNJ1 occurred closer to shore than for EPNJ samples. This may have accounted for the higher ratio.

Table 4.3.1 PAHs in DWH crude oil and in fish oil (ng/mg) from large menhaden of the Atlantic and Gulf coasts as determined from type I spectra. Collection location and date are provided. BBLA= Barataria Bay, LA. SHNJ, EPNJ and MVNJ represent different seining ships collecting fish from Delaware Bay, NJ. Two separate collections of fish were made from these ships. The fish used to make up an oil sample composite are given. HNP-like PAHs were represented by the Em350/Ex280 peak in fish oil and the Em350/Ex270 peak in DeepWater Horizon (DWH) crude oil. HPY-like PAHs were represented by the Em450/Ex350 peak in both fish oil and DWH. R= number of times the samples was analyzed. Data were average±SD. JRVA and MVNJ1 samples had no type I spectra.

Location	Collection Date	Fish in Composite	R	HNP-like (ng/mg)	HPY-like(ng/mg)	Ratio HNP/HPY
JRVA	10/31/2010	001-018	0	ND	ND	ND
BBLA	10/30/2010	002,010-014,020-026,028-033	2	4.68±0.17	1.11±0.01	4.20
SHNJ	10/25/2010	001-018	2	0.74±0.08	1.57±0.26	0.47
EPNJ1	9/8/2010	002,004-009,011,018	2	1.09±0.05	2.33±0.23	0.47
EPNJ2	9/21/2010	020-031	1	1.20	0.78	1.52
MVNJ1	9/7/2010	001-005,007-010	0	ND	ND	ND
DWH	4/21/2010		2	798169±24621	64969±542	12.29

Table 4.3.2 PAHs in DWH crude oil and in fish oil (ng/mg) from large menhaden of the Atlantic and Gulf coasts as determined from type II spectra. Collection location and date are provided. BBLA= Barataria Bay, LA. JRVA= James River, VA. EPNJ and MVNJ represent different seining ships collecting fish from Delaware Bay, NJ. Two separate collections of fish were made from these ships. The fish used to make up an oil sample composite are given. HNP-like PAHs were represented by the Em350/Ex280 peak in fish oil and the Em350/Ex270 peak in DeepWater Horizon (DWH) crude oil. HPY-like PAHs were represented by the Em450/Ex350 peak in both fish oil and DWH. R= number of times the samples was analyzed. Data were average±SD. SHNJ samples had no type II spectra.

Location	Collection Date	Fish Composite	R	HNP-like (ng/mg)	HPY-like (ng/mg)	Ratio HNP/HPY
JRVA	10/31/2010	001-018	3	2.37±0.20	11.78±1.14	0.20
BBLA	10/30/2010	002,010-014,020-026,028-033	2	10.16±1.23	4.65±0.17	2.18
SHNJ	10/25/2010	001-018	0	ND	ND	ND
EPNJ1	9/8/2010	002,004-009,011,018	1	0.79	2.88	0.27
EPNJ2	9/21/2010	020-031	1	0.83	2.70	0.31
MVNJ1	9/7/2010	001-005,007-010	3	21.24±4.35	6.50±0.44	3.27
DWH	4/21/2010		2	798169±24621	64969±542	12.29

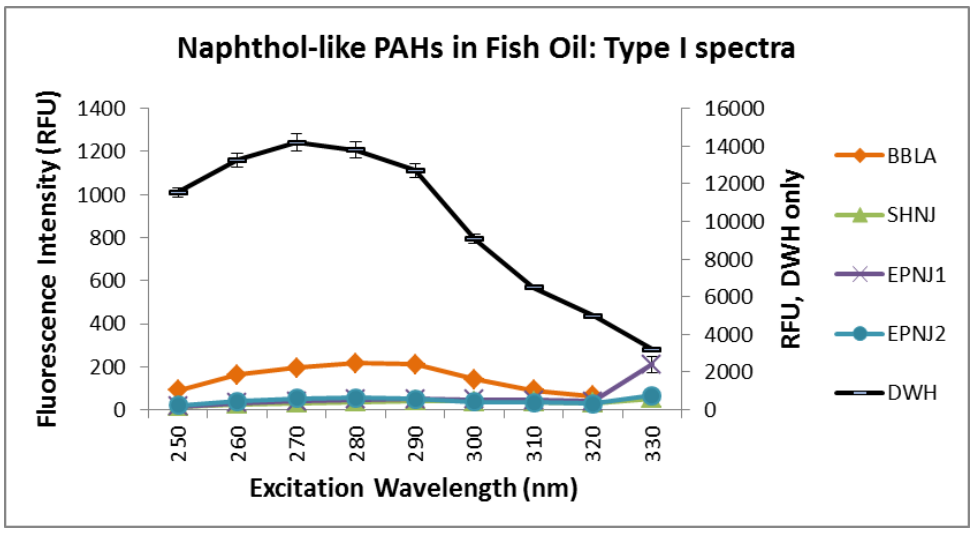


Figure 4.3.10 Comparison of type I spectra for naphthol-like (HNP) from different fish oils and DWH crude oil using FEFS. Oil was prepare from fish collected in fall 2010. Fish from LA were collected from Barataria Bay (BBLA). Fish collected from Delaware Bay, NJ were named according to the seining ship from which they were obtained: SHNJ= Sea Huntress, EPNJ1 and 2= two separate collections from Enterprise, and MVNJ= Mount Vernon. DWH= pipiline oil from DeepWater Horizon oil rig after the blowout. Replicate analyses were performed on some sample- average ± SD. N= 2, 2, 2, 1, 2 for BBLA, SHNJ, EPNJ1, EPNJ2, and DWH, respectively.

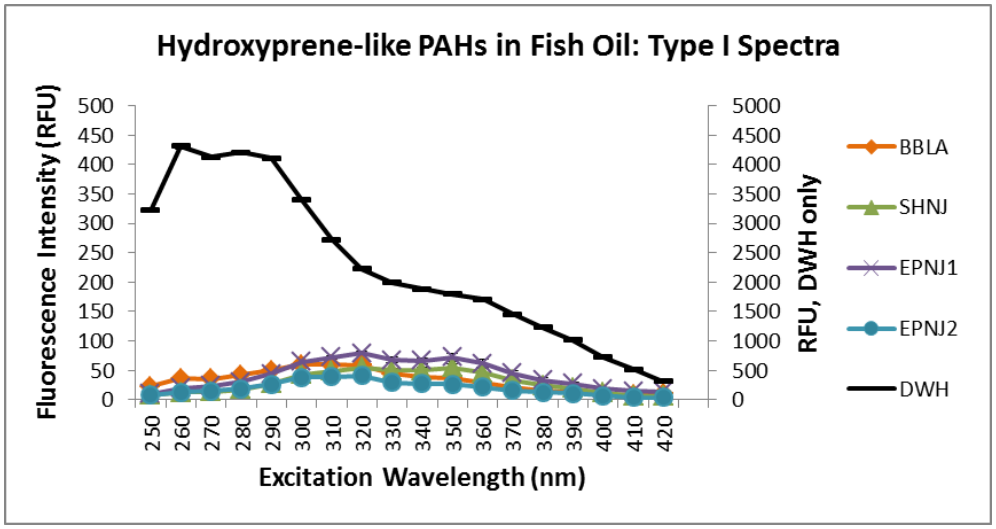


Figure 4.3.11 Comparison of type I spectra for hydroxypyrene (HPY) PAHs from different fish oils and DWH crude oil using FEFS. Oil was prepare from fish collected in fall 2010. Fish from LA were collected from Barataria Bay (BBLA). Fish collected from Delaware Bay, NJ were named according to the seining ship from which they were obtained: SHNJ= Sea Huntress, EPNJ1 and 2= two separate collections from Enterprise, and MVNJ= Mount Vernon. DWH= pipiline oil from DeepWater Horizon oil rig after the blowout. Replicate analyses were performed on some sample- average ± SD. N= 2, 2, 2, 1, 2 for BBLA, SHNJ, EPNJ1, EPNJ2, and DWH, respectively.

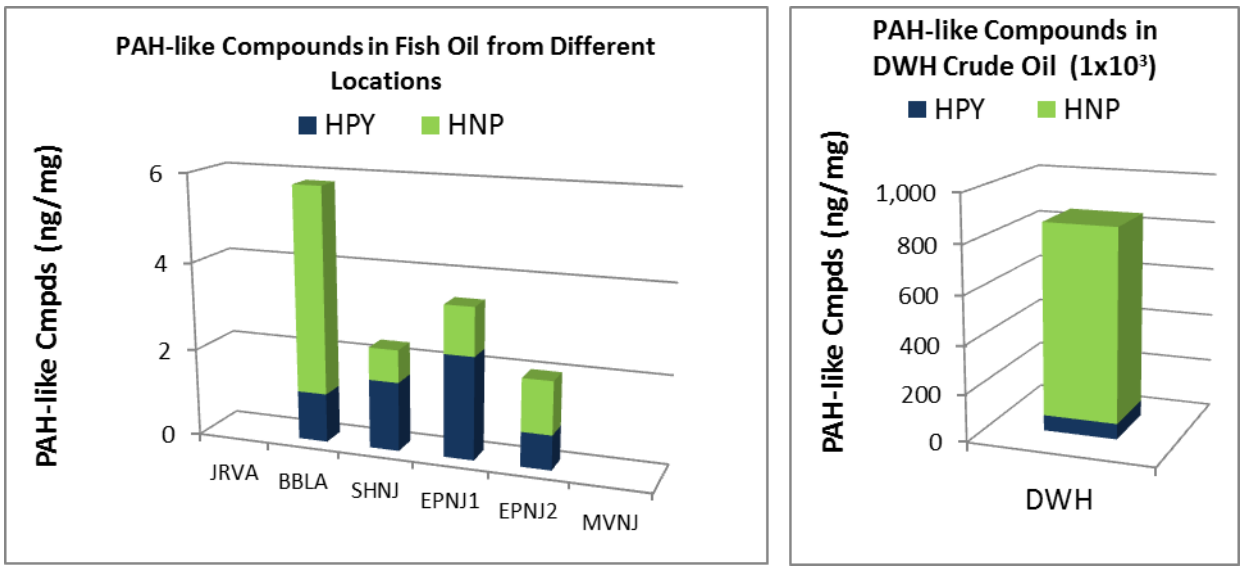


Figure 4.3.12 Relative concentrations of PAH-like compound in fish oil and DWH crude oil based on type I spectra. Data are from Table 4.1.1. Note that BBLA has a higher proportion of HNP-like PAHs as did DWH oil. This indicated recent exposure of fish to crude oil at these two sites.

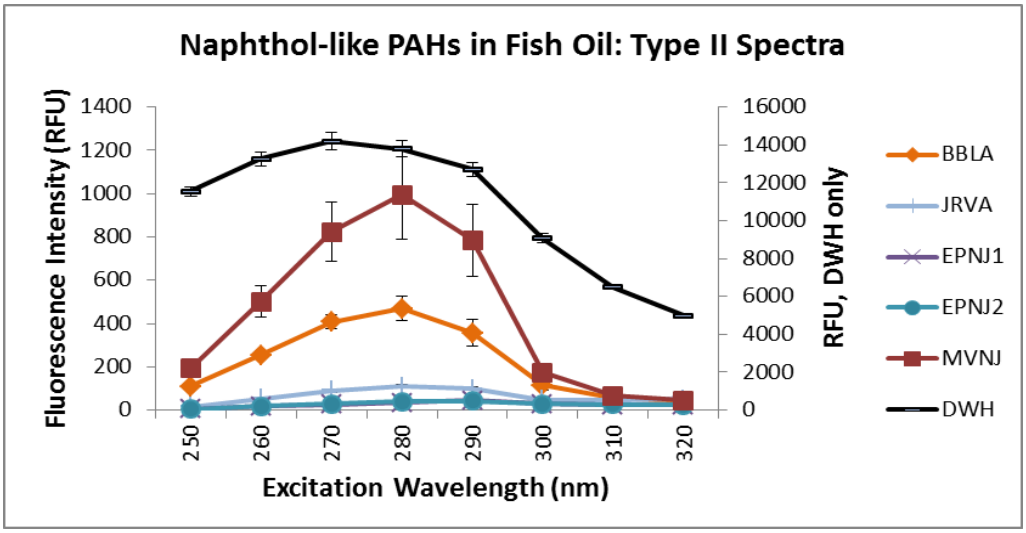


Figure 4.3.13 Comparison of type II spectra for naphthol-like (HNP) from different fish oils and DWH crude oil using FEFS. Oil was prepare from fish collected in fall 2010. Fish from LA were collected from Barataria Bay (BBLA). Fish collected from Delaware Bay, NJ were named according to the seining ship from which they were obtained: SHNJ= Sea Huntress, EPNJ1 and 2= two separate collections from Enterprise, and MVNJ1= Mount Vernon, collection 9/7/2010. DWH= pipiline oil from DeepWater Horizon oil rig after the blowout. Replicate analyses were performed on some sample- average ± SD. N= 2, 3, 2, 1, 1, 3, and 2 for BBLA, JRVA, EPNJ1, EPNJ2, MVNJ and DWH, respectively.

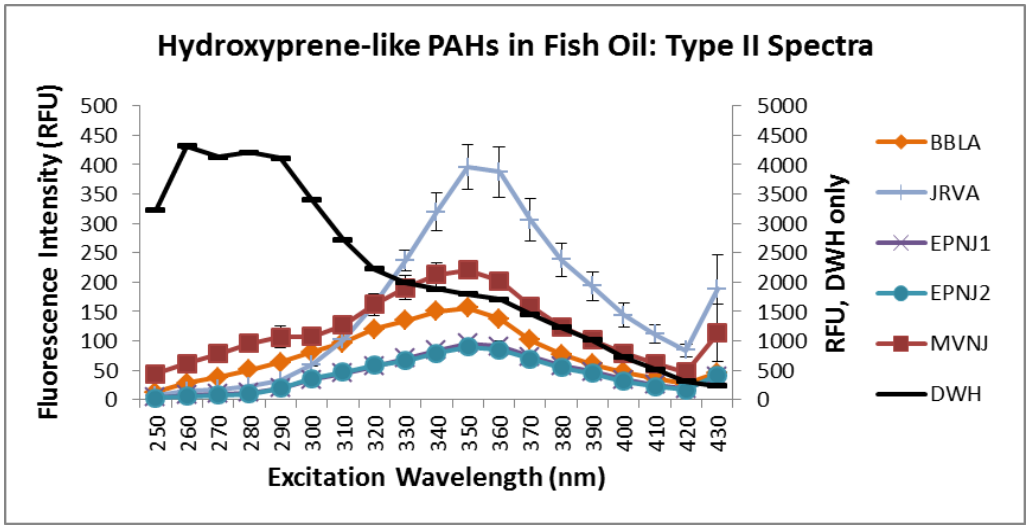


Figure 4.3.14 Comparison of type II spectra for hydroxypyrene (HPY) PAHs from different fish oils and DWH crude oil using FEFS. Oil was prepare from fish collected in fall 2010. Fish from LA were collected from Barataria Bay (BBLA). Fish collected from Delaware Bay, NJ were named according to the seining ship from which they were obtained: SHNJ= Sea Huntress, EPNJ1 and 2= two separate collections from Enterprise, and MVNJ1= Mount Vernon, collection 9/7/2010. DWH= pipiline oil from DeepWater Horizon oil rig after the blowout. Replicate analyses were performed on some sample- average ± SD. N= 2, 3, 2, 2, 1, 1, and 2 for BBLA, JRVA, SHNJ, EPNJ1, EPNJ2, MVNJ and DWH, respectively.

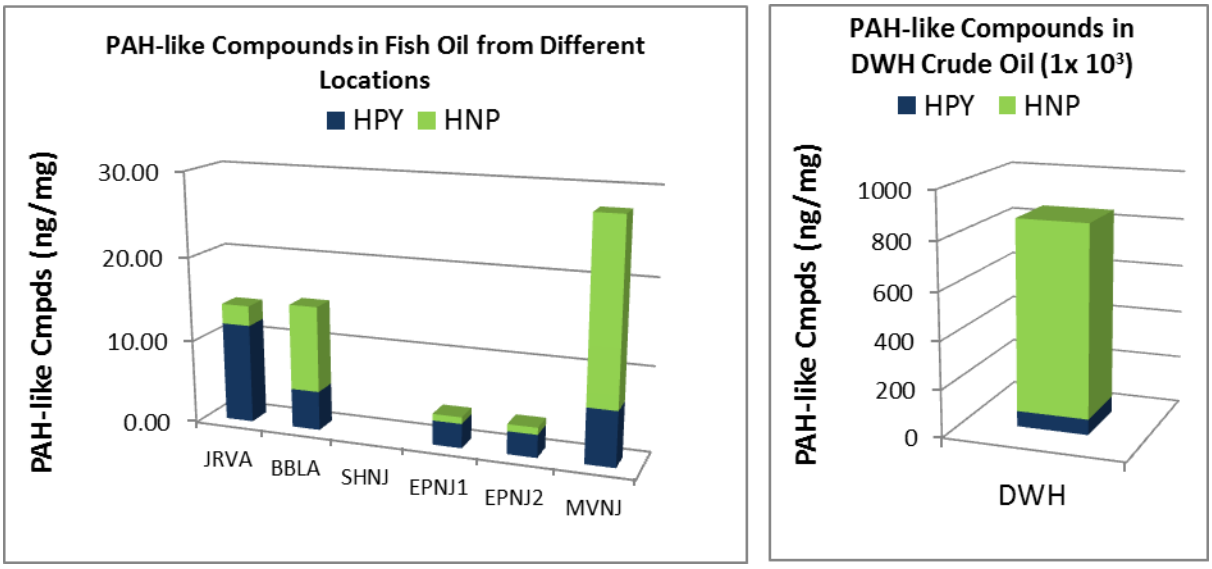


Figure 4.3.15 Relative concentrations of PAH-like compound in fish oil and DWH crude oil based on type II spectra. Data are from Table 4.1.2. Note that as with type I spectra BBLA and MVNJ1 have a higher proportion of HNP-like PAHs as did DWH oil. This indicated recent exposure of fish to crude oil at these two sites and also that similar findings were found with both types of spectra.

IV.3.6 Comparison of fish liver samples from the Gulf and Atlantic coast in 2010

Fish liver tissue from Barataria Bay, LA (impacted by the DWH oil spill), James River, VA and Delaware Bay, NJ were analyzed and compared. As with fish oil, it was expected that the large menhaden from VA and NJ had not recently been exposed to a large oil spill and that PAHs in their liver tissues were primarily due to pyrogenic and not petrogenic PAHs. Each fish liver sample was generated from one individual. Samples were extracted and analyzed as described (IV.3.3 Procedure for extracting PAHs from fish oil, crude oil and fish liver tissue and determination of percent recovery). Results were analyzed using one way ANOVA followed by Tukey post hoc test: $p>0.05$ were not considered statistically different.

FEFS results for fish liver found the same two types of spectrum as shown with fish oil. Therefore, data were separated based on menhaden species and spectra type. Fish from Barataria Bay, LA (BBLA) were all *B. patronu*s and all had type I spectra. Fish from James River, VA (JRVA) were either *B. tyrannus* or *B. patronus*. All of the JRVA *tyrannus* had type I spectra (9 of 9), while most of the *patronus* had type II spectra (5 of 6). Since the BBLA *patronus* had type I spectra, the finding of type II spectra in JRVA *patronus* was not just due to species. Fish from Delaware Bay, NJ were all *B. tyrannus* and all collections had type I and II spectra. The influence of species could not be tested as the only site besides BBLA with *patronus* was VA and most of the *patronus* from VA had type II spectra while those from BBLA had type I. However, since the type II spectrum was found to be related to high levels of HPY-like PAHs, one can deduce that VA fish had higher levels of HPY-like PAHs than BBLA (see Figures 4.3.6-4.3.8).

Results found for type I spectra are shown in Table 4.3.3. Using this spectrum type, HNP-like PAHs ranged from 717±130 to 267±105 ng/mg. They were highest in EPNJ2>JRVA>MVNJ2>SHNJ>>BBLA≈MVNJ1 (Figure 4.3.16). Concentrations for BBLA and MVNJ1 were significantly lower than the other samples. This data showed that concentrations of HNP-like PAHs varied among catches in NJ fish and that BBLA fish liver contained relatively low HNP levels compared to VA and NJ fish. HPY-like PAH concentrations ranged from 79±43 to 19±11 ng/mg. They were highest in MVNJ1>MVNJ2>SHNJ≈JRVA≈EPNJ2>>BBLA. No significant differences in HPY-like PAHs were found for NJ and VA fish. Concentrations in fish from LA were significantly lower. Ratios of HNP to HPY-like PAHs ranged from 15±11 to 4±3. They were highest in BBLA≈JRVA≈EPNJ2>SHNJ≈MVNJ2>>MVNJ1. As with fish oil data, HNP-like and HPY-like PAH concentrations were independent of one another. Even though BBLA had unexpectedly low levels of PAHs, the proportion of HNP to HPY in liver was as high as that found in locations with urban exposures using type I spectra.

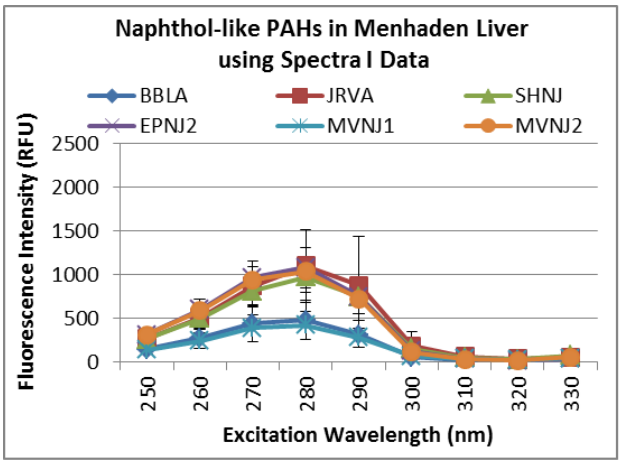
Results found for type II spectra are shown in Table 4.3.4. Using this spectrum type, HNP-like PAHs ranged from 932±307 to 198±5 ng/mg. They were highest in JRVA>MVNJ2>SHNJ>EPNJ2>>MVNJ1 (Figure 4.3.17). Levels of HNP-like PAHs were significantly lower in MVNJ1 than in other samples. This was similar to what was found for the type I spectrum. HPY-like PAH concentrations as determined using type II spectra ranged from 338±60 to 170±57. Levels of HPY-like PAHs were significantly lower in SHNJ samples as compared to JRVA and MVNJ2. This was different than found for spectra I. Using that data, HPY-like concentrations for SHNJ were similar to those of VA and other NJ samples. The ratios of HNP-like to HPY-like PAHs ranged from 4.3±1.3 and 0.8±0.01. These values were lower overall than those found for spectra I. This appeared due to better extraction of HPY-like PAH as mentioned above. Another consistency between type I and type II spectra was that MVNJ1 had a low HNP/HPY ratio indicating high exposure to pyrogenic PAHs as opposed to petrogenic PAHs. This contradicted findings with fish oil, which showed a relatively high ratio. The reason for this was unknown.

Table 4.3.3 Concentrations (ng/mg) of naphthol-like (HNP-like) and hydroxypyrene-like (HPY-like) PAHs in liver of large menhaden as determined using type I spectra. Collection location and date are provided. BBLA= Barataria Bay, LA. JRVA= James River, VA. EPNJ and MVNJ represent different seining ships collecting fish from Delaware Bay, NJ. Two separate collections of fish were made from MVNJ represented as MVNJ1 and MVNJ2. HNP-like PAHs and HPY-like PAHs were represented by the Em350/Ex280 peak and Em450/Ex350 peak, respectively. n= number of individual fish with a type I spectrum per collection. Data were average ± SD and separated by the two types of species found. Shared letters were not statistically different, p>0.05.

Location	Collection Date	Species	spectra	n	HNP-like	HPY-like	Ratio HNP/HPY
JRVA	10/31/2010	B. tyrannus	I	9	703±265[a]	57±20[a]	15±11[a]
		B. patronus	I	1	563	51	11
BBLA	10/30/2010	B. tyrannus	I	0			
		B. patronus	I	9-10	303±144[b]	19±11[b]	15±5[a]
SHNJ	10/25/2010	B. tyrannus	I	9	603±110[a]	59±13[a]	10±1[ab]
		B. patronus	I	0			
EPNJ2	9/21/2010	B. tyrannus	I	9	717±130[a]	55±20[a]	14±4[a]
		B. patronus	I	0			
MVNJ1	9/7/2010	B. tyrannus	I	9	267±105[b]	79±43[a]	4±3[b]
		B. patronus	I	0			
MVNJ2	9/23/2010	B. tyrannus	I	1	664	66	10
		B. patronus	I	0			

Table 4.3.4 Concentrations (ng/mg) of naphthol-like (HNP-like) and hydroxypyrene-like (HPY-like) PAHs in liver of large menhaden as determined using type II spectra. Collection location and date are provided. BBLA= Barataria Bay, LA. JRVA= James River, VA. EPNJ and MVNJ represent different seining ships collecting fish from Delaware Bay, NJ. Two separate collections of fish were made from MVNJ represented as MVNJ1 and MVNJ2. HNP-like PAHs and HPY-like PAHs were represented by the Em350/Ex280 peak and Em450/Ex350 peak, respectively. n= number of individual fish with a type II spectrum per collection. Data were average ± SD and separated by the two types of species found. Shared letters were not statistically different, p>0.05.

Location	Collection Date	Species	spectra	n	HNP-like	HPY-like	Ratio HNP/HPY
JRVA	10/31/2010	B. tyrannus	II	0			
		B. patronus	II	5	932±307[a]	338±60[a]	2.9±1.3[ab]
BBLA	10/30/2010	B. tyrannus	II	0			
		B. patronus	II	0			
SHNJ	10/25/2010	B. tyrannus	II	9	719±209[a]	170±57[b]	4.3±1.3[a]
		B. patronus	II	0			
EPNJ2	9/21/2010	B. tyrannus	II	1	578	252	2.3
		B. patronus	II	0			
MVNJ1	9/7/2010	B. tyrannus	II	2	198±5[b]	256±9[ab]	0.8±0.01[b]
		B. patronus	II	0			
MVNJ2	9/23/2010	B. tyrannus	II	7	767±130[a]	299±48[a]	2.5±0.72[b]
		B. patronus	II	0			



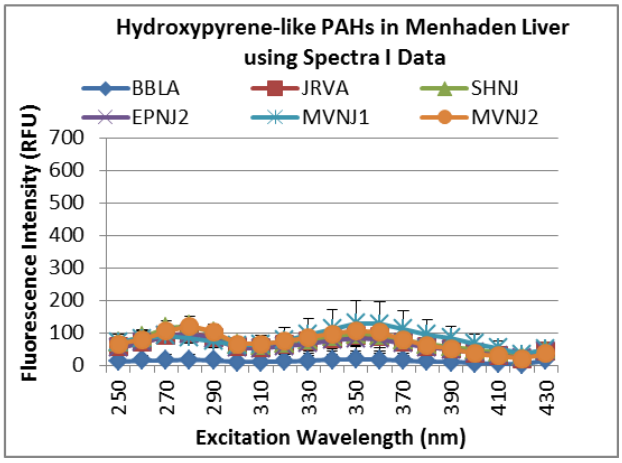
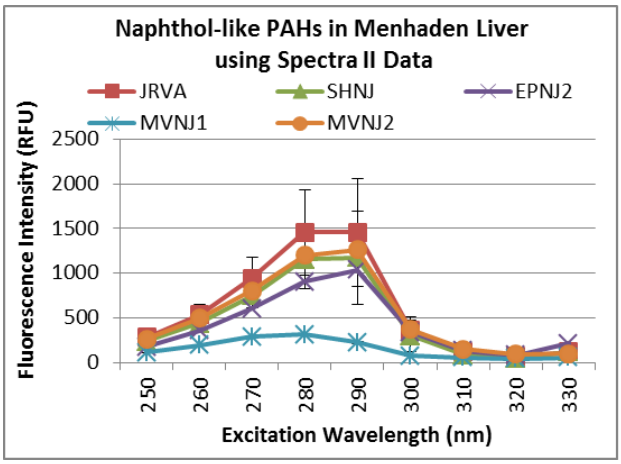


Figure 4.3.16 Naphthol-like and hydroxypyrene-like PAHs in large menhaden liver as determined using type I spectrum data in FEFS. BBLA= Fish collected from Barataria Bay, LA. JRVA= Fish collected from James River, VA. Fish collected from Delaware Bay, NJ were named according to the seining ship from which they were obtained: SHNJ= Sea Huntress, EPNJ= Enterprise and MVNJ= Mount Vernon. Two separate collections were made from MVNJ designated as MVNJ1 and MVNJ2. Data are average ± SD for spectra and were not separated out by species. Only data for JRVA contained both species. See Table 4.3.3 for species, n values and collection dates.



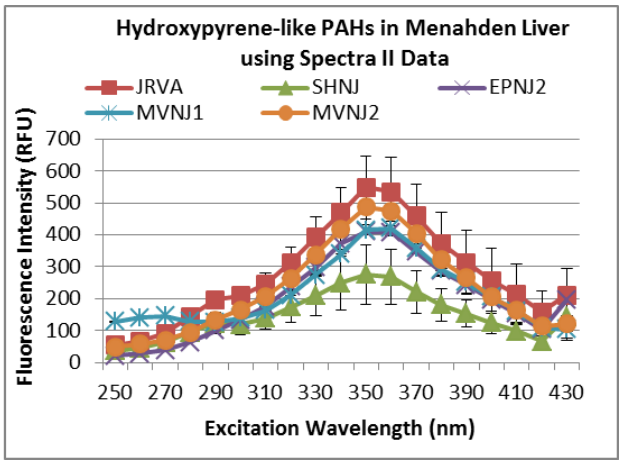


Figure 4.3.17 Naphthol-like and hydroxypyrene-like PAHs in liver of large menhaden as determined using type II spectrum data in FEFS. JRVA= Fish collected from James River, VA. Fish collected from Delaware Bay, NJ were named according to the seining ship from which they were obtained: SHNJ= Sea Huntress, EPNJ= Enterprise and MVNJ= Mount Vernon. Two separate collections were made from MVNJ designated as MVNJ1 and MVNJ2. Data are average ± SD. See Table 4.3.4 for species, n values and collection dates. BBLA had no menhaden with type II spectra.

IV.3.7 Summary of Findings in Large Menhaden

Findings for large menhaden involved determining types and concentrations of PAHs in DWH crude oil, fish oil and fish liver. Fish oil was analyzed using headspace solid-phase microextraction in combination with gas chromatography-mass spectrometry (HS-SPME-GCMS). DWH crude oil, fish oil and fish liver were analyzed using fixed emission fluorescence spectroscopy (FEFS). The major scientific question addressed was whether the types and/or concentrations of PAHs found in menhaden could distinguish exposure to a recent crude oil spill (petrogenic source) from exposure to legacy PAHs from urban contamination (pyrogenic source). More specifically, is there a distinctive chemical "fingerprint" for the DWH oil that can be detected in contaminated menhaden fish oil and liver and what is the concentration of PAHs in contaminated fish oil and PAH metabolites in fish liver?

Data from HS-SPME-GCMS found a limited number of PAHs in fish oil. The analysis targeted only the 15 PAHs found in EPA 610 mix of PAH standards. Three fish oil samples were analyzed using this technique- two NJ samples, MVNJ1 and EPNJ1 and the one LA sample, BBLA. PAHs detected were naphthalene (2 ring), fluorene (3 ring), anthracene (3 ring), fluoranthene (4 ring) and pyrene (4 ring). Concentrations of naphthalene and fluorene were too low to quantify. Fluoranthene had the highest concentration and similar levels were found in all three samples (182-185 ng/ml fish oil). Pyrene concentrations were similar for BBLA and MVNJ1 (69 and 71 ng/ml, respectively) but lower in EPNJ1 (10 ng/ml). Anthracene was highest in MVNJ1>EPNJ1>BBLA (133, 106 and 91 ng/ml, respectively). Based on these findings, there was no consistent difference in types and concentrations of PAHs that distinguished petroleum exposed fish (BBLA) from those exposed to background urbanization (MVNJ1, EPNJ1). Results did show variation in levels of PAHs for fish caught in NJ, such that MVNJ1>EPNJ1 for all PAHs found, and suggested that fluoranthene is accumulated in fish oil regardless of PAH source.

The FEFS technique allowed more samples to be analyzed. Types of PAHs were grouped according to emission wavelength such that 2-3 PAHs fluoresced best at Em350 (naphthol-like PAHs) while 4-5 ring PAHs fluoresced at Em450 (hydroxypyrene-like PAHs). Testing of multiple PAH standards and vitamins A and E showed overlapping peaks (Figure 4.3.2). Therefore, it was not possible to quantify specific PAHs. While this was a limitation of the technique, it did allow for alkylated and hydroxylated PAHs to be detected. This could be considered advantageous as environmental sources of PAHs, particularly crude oil, are often alkylated and because biological samples are likely to contained metabolized PAHs not the pure parent compounds traditionally detected by GCMS. Results showed that fish oil and fish liver had similar spectra with a single major peak at Em350/Ex280 for HNP-like PAHs. This peak did not match any standard but was close to that of naphthol/fluorine but also vitamin E, Em350/Ex290. The HS-SPME-GCMS technique did detect naphthol and fluorine even though the levels were not quantifiable. Fish oil and liver also had similar Em450 peaks with the major one being at Ex350. The complete spectra best matched fluoranthene and pyrene, which were also found with HS-SPME-GCMS. Therefore, these different techniques detected some of the same PAHs. The spectrum for anthracene could not be distinguished (Em450/Ex250 and Ex350). In the type I spectrum using Em450, vitamin A appeared to be present in fish oil and not fish liver.

Comparison of PAH concentrations in fish liver and fish oil showed differences in tissue accumulation and sources of exposure. PAH concentrations were much higher in liver than fish oil. For example, HNP-like PAHs in BBLA (type I) were 4.68±0.17 ng/mg in fish oil and 303±144 ng/mg in fish liver (Figure 4.3.18). This was also true for HPY-like PAHs- 1.11±0.01 and 19±11 ng/mg, respectively (Figure 4.3.19). HPY-like PAH concentrations in BBLA liver samples were relatively low compared to NJ and VA samples. This indicated recent exposure to pyrogenic PAHs in Atlantic coast fish. The ratio of HNP to HPY has

been used to differentiate between petrogenic and pyrogenic sources of PAHs. Relatively high levels of naphthalene and phenanthrene have been associated with recent exposure to petroleum products (Krahn et al, 1993, Aas et al, 2000), while relatively high levels of pyrene and/or benzo(a)pyrene (BAP) have been associated with pyrogenic sources (Aas et al, 2000, Trisciani et al, 2011) or legacy contamination (Jewetta et al, 2002, Ferreira et al, 2006). The results showed that fish liver had a larger HNP/HPY ratio than fish oil (Table 4.3.1 and 4.3.3). For example, the ratio for EPNJ2 was 14±4 in liver and 1.52 in fish oil. This suggested that while PAHs were accumulating in fish oil, HNP-like PAHs were being eliminated more rapidly and less were accumulating in fish oil. Interesting, the ratio was high for BBLA in both liver and fish oil indicating recent exposure to crude oil. Therefore, monitoring menhaden using FEFS could be useful in the future for detecting crude oil exposure even if it is unlikely to distinguish a unique PAH profile for a particular type of crude oil.

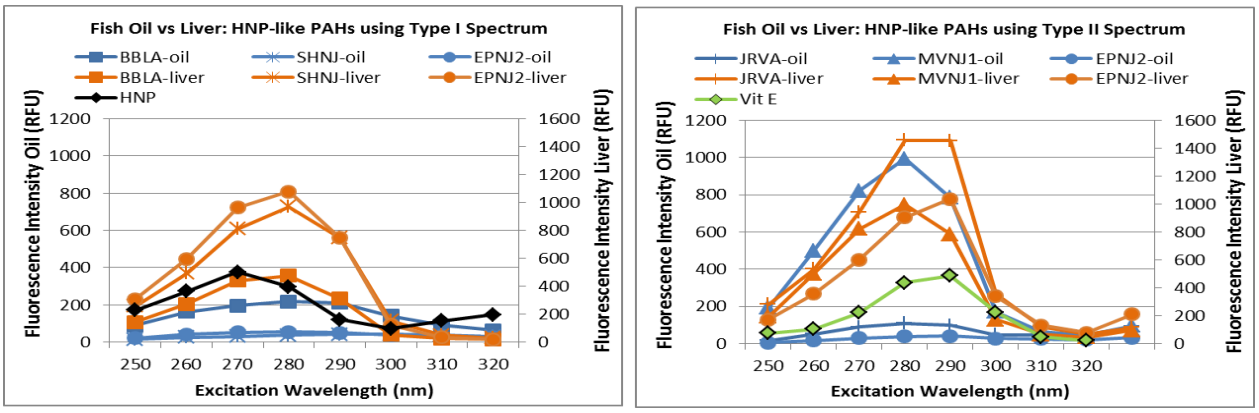


Figure 4.3.18 Comparison of HNP-like spectra for fish oil and fish liver. Scans for both type I and II are shown. Naphthol (HNP) and vitamin E (Vit E) standards are included. Note that the major peak in both fish liver and oil was as Em350/Ex280-290. Note that units are in RFU and not normalized to sample weight, i.e. peak height is not directly related to PAH concentrations in liver and oil. Data are the average for individual fish (liver) or repeated analysis of one sample (fish oil).

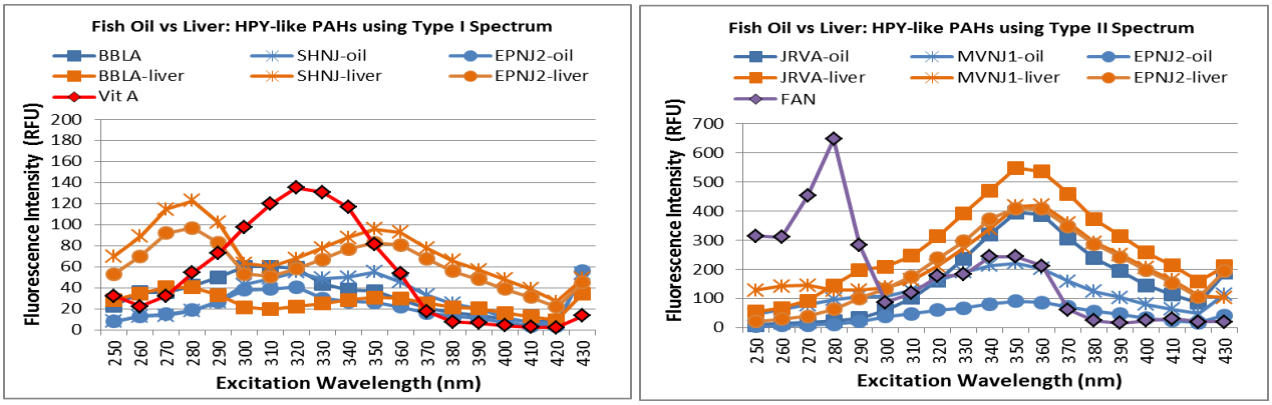


Figure 4.3.19 Comparison of HPY-like spectra for fish oil and fish liver. Scans for both type I and II are shown. Fluoranthene(FAN) and vitamin A (Vit A) standards are included. Note that increased extraction of PAHs (type II) was associated with a shift toward a single large peak at Em450/Ex350. Note that units are RFU and not normalized to sample weight, i.e. peak height is not directly related to PAH

concentrations in liver and oil. Note that in type I spectra, fish oil and not liver appeared to contain vitamin A. Data are the average for individual fish (liver) or repeated analysis of one sample (fish oil).

V. ANALYSIS ASSOCIATED WITH SMALL MENHADEN

V.1. Overview

Small menhaden (8-14 cm) were collected in 2010 from Delaware River, NJ, and Barataria Bay near Grande Isle, LA. As with the large menhaden, it was expected that the fish from LA would have been exposed to the crude oil from DWH while those from NJ were not exposed to a recent oil spill. Histopathological analysis was performed in order to determine what if any detrimental effects had occurred due to crude oil exposure. The Delaware Bay fish were essentially a control, but were also expected to have been effected by living in an urbanized estuary. Analysis of PAHs in fish oil of young-of-the-year menhaden was originally planned. However, we were unable to obtain enough fish oil from them for analyses. Alternatively, the whole body tissue was analyzed by GCMS and gastrointestinal tissue including liver tissue was analyzed by FEFS.

V.2 Histopathological Effects of Crude Oil Exposure on Small Menhaden

Keith R. Cooper, PhD, Department of Biochemistry, Rutgers, The State University of New Jersey

V.2.1 Fish collection and histological methods

The fish were collected by seine net and placed on ice until the boat reached shore. Those fish intended for histopathology were fixed in 10% buffered formalin and those for tissue analyses were frozen.

The fish for histopathology were processed as follows. Once the fixed samples reached the laboratory, they were assigned accession numbers, placed in 70 % ethanol and embedded into paraffin blocks. The blocks were sectioned and the slides were stained with hematoxylin and eosin for histological examination. The length and weight of processed fish were recorded. The tissues that have been processed include gill, GI track and liver. The fish were too young to process gonadal tissue.

Fish used for histology were selected in a similar size range. Total Length for NJ was 9.8 ± 0.9 cm (X ±SD), n=16. Total Length for LA was 9.4 ± 0.5, n=18. Total Weight selected was 21 g or above. The sizes for all of the fish collected for histology showed that menhaden from LA were significantly larger than those from NJ (Table 5.2.1). That led to the need to select fish for histology from those caught and thereby compare fish of similar size and age.

Table 5.2.1 Size for all of the fish collected for histopathology. The NJ collection had a whole bunch of very small fish that were not used for histology to allow comparison between similar size classes. See text for sizes of NJ fish used for histology.

Location	Number of Fish	Total Weight (X + SD)	Total Length (X + SD)
Delaware Bay, NJ	56	16.91 ± 4.0 g (Min 12.00 Max 24.00)	5.9 ± 4.0 cm (Min 15 Max 148 cm)
Barataria Bay, LA	21	21.60 ± 1.0 g (Min 20.00 Max 24.00)	10.2 ± 1.6 cm (Min 86 Max 139 cm)
		Mann Whitney Rank Significant $p < 0.001$	Mann Whitney Rank Significant $p < 0.001$

V.2.2 Histopathology Evaluation

Gills were compared from representative fish from N.J. waters and those collected from Gulf Coast waters impacted by the DWH oil spill (Figure 5.2.1). The NJ fish did have some minor clubbing and fusion of the primary lamellae, but the fish from the Louisiana Gulf Coast had moderate to extensive areas of where the primary lamellae were fused and there was permanent damage to the gill structure. The oil spilled in the Gulf of Mexico was a light sweet crude oil which would have had higher levels of lower molecular weight PAHs and less higher molecular weight PAHs in the original oil. The epithelial cells lining the lamellae in the Gulf Coast fish were increased in number (hyperplasia -H) and resulted in fusion of multiple lamellae which reduces the gas exchange and causes stress to the fish. There were areas where the entire secondary lamellae were eroded away, and had not begun to repair (erosion - E) (Figure 5.2.2). The fish from the Gulf Coast also displayed secondary lamellae that were elongated and wavy in appearance (W) which also indicated damage from an exposure to compounds that disrupted normal growth. Although it cannot be proven that the exposure to the DWH oil caused these lesions, it is consistent with previous literature that documented adverse effects from oil coming into contact with gill structures (Pal et al, 2011, Simonato et al, 2008). Since the gills filter large amounts of water for gas exchange, they are also the most likely organ to show effects from irritants and caustic compounds. Parasites and parasitic crustaceans can cause focal damage to the gill that results in localized damage but not the widespread damage observed in the Gulf of Mexico fish. If an oil spill was to occur in New Jersey waters and menhaden came into contact with that oil, similar lesions would be expected to be observed. Acute responses from massive exposure would involve hemorrhaging and a larger amount of an inflammatory response. Based on the extent of the lesions observed and the hyperplasia, erosion and dismorphic lamellae, the exposure to the gills indicates some repair but with some chronic irritation still occurring.

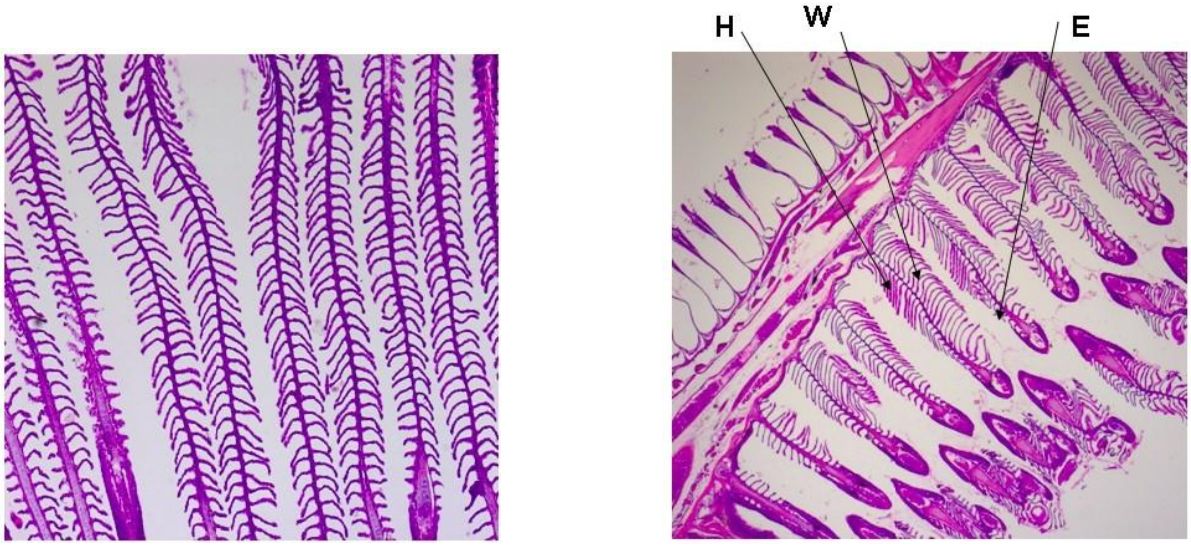


Figure 5.2.1 Gill lamellae showing little or no clubbing and other lesions in New Jersey menhaden compared to moderate lesions in the oil spill exposed menhaden. Note: No clubbing, lamellar fusion, erosion or wavy secondary lamellae in NJ menhaden. Note: gill hyperplasia (H), and wavy appearance (W) and erosion (E) in LA menhaden.

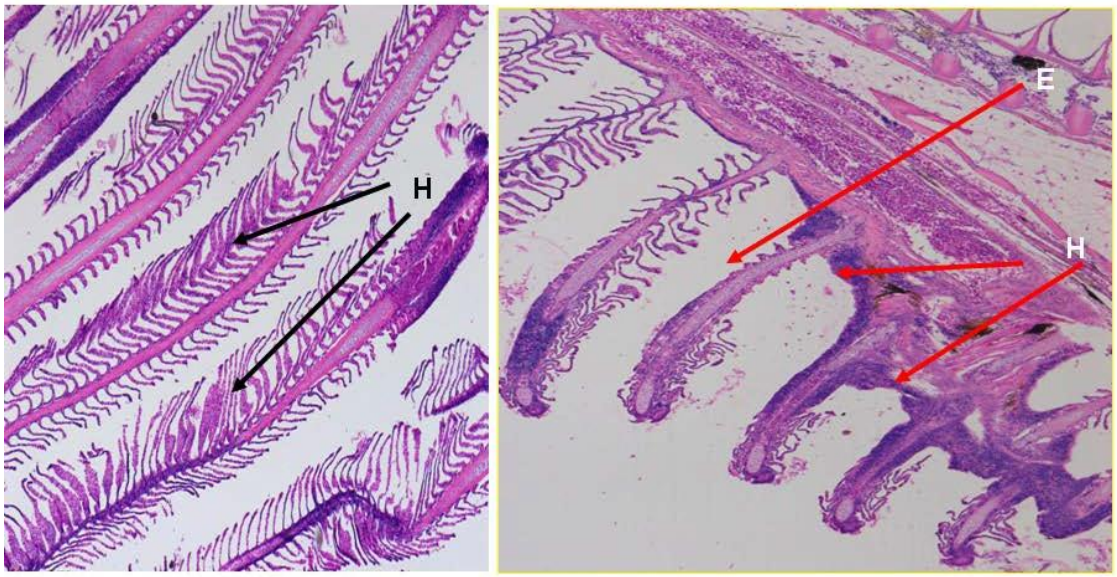


Figure 5.2.2 Higher magnification (40x) of hyperplasia (H) and erosion (E) of gill lamellae

Menhaden are filter feeders so their gills, digestive track and associated organs are quite different from other commonly studied omnivores. The secondary lamellae of the gills are more densely packed and, along with the gill rakers, help in sorting of algae that is passed through the esophagus into the stomach. Because algae have a high surface to area ratio and also have silica and cell walls that the oil would adhere to this serves as another means by which these fish are highly sensitive to oil spills. The algae are digested in the stomach and out pocketing pyloric ceca associated with the stomach. The intestine is a coiled intestine with the liver interspersed among the coils. In the fish from the Louisiana Gulf Coast, our preliminary assessment of the stomach in several fish (Figure 5.2 3) showed a large consolidation of the stomach muscle mass that was not observed in any fish from the NJ fish sampled. This lesion may have been directly associated with the oil entering the stomach and producing chronic irritation, which subsequently caused consolidation of the muscle tissue with connective tissue. In the pyloric ceca and lower intestine, food and pancreatic tissue were observed interspersed on the outside of the intestine (Figure 5.2.4). Monogenetic trematodes were present sometimes in high numbers but with little or no tissue response in these regions of the gastrointestinal track in fish from both NJ and LA Gulf Coast (Figure 5.2.5). There was some evidence of a minor vacuolization of the pancreatic tissue in the Gulf of Mexico fish, but this was minor and several fish from NJ also showed similar vacuolization.

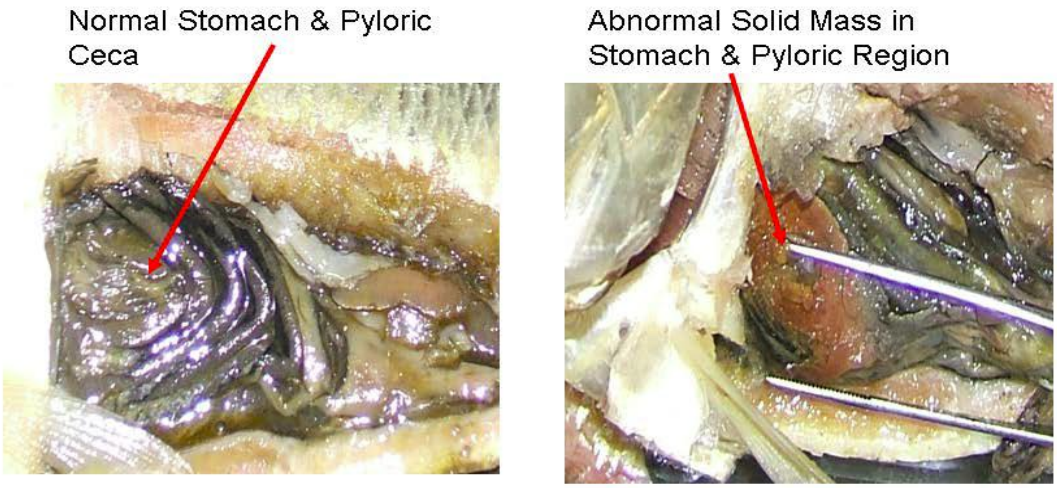


Figure 5.2.3 Large solid mass involving the stomach and liver only seen in LA menhaden.

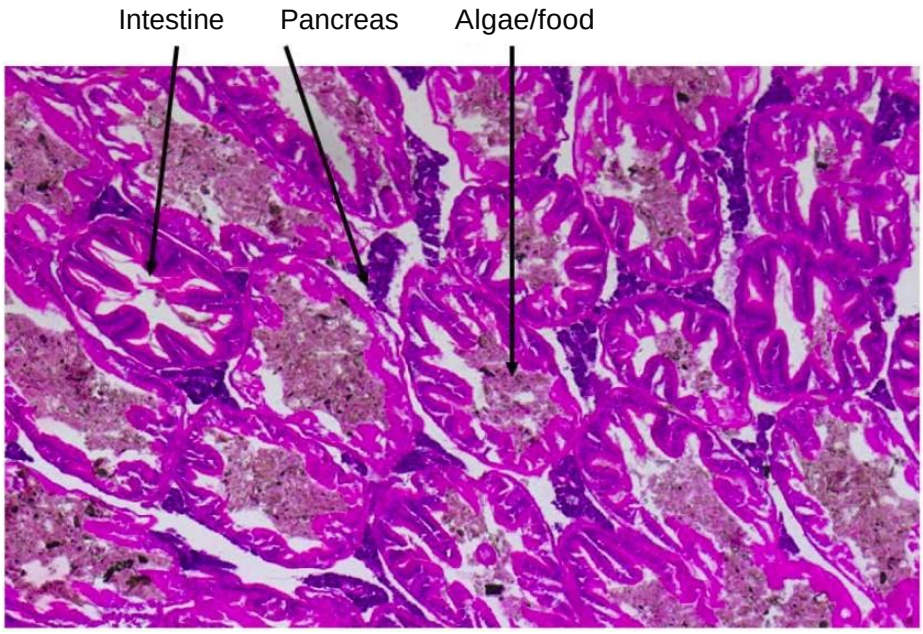


Figure 5.2.4 Normal intestine, pyloric ceca and pancreas

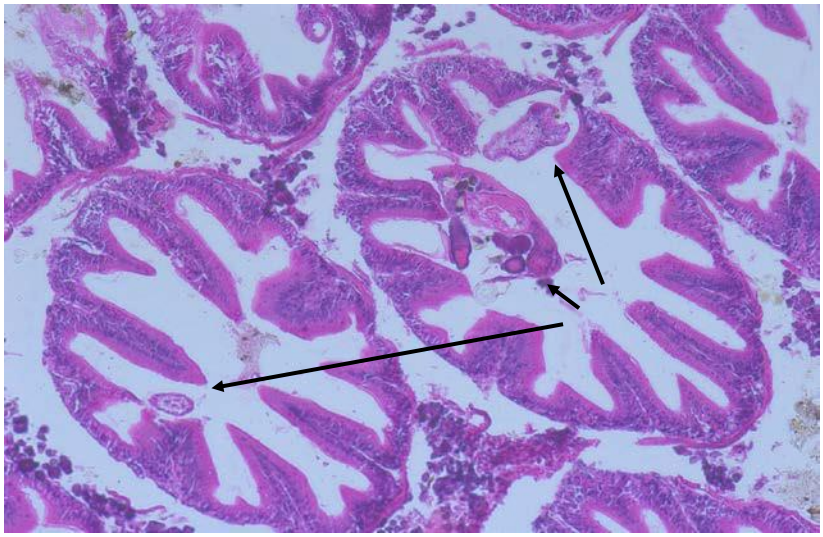


Figure 5.2.5 Monogenetic trematode (MT) in pyloric ceca present in all

Although the liver in menhaden is not as well defined as in other omnivore and carnivores, it does have a similar structure with the hepatocytes forming chord like structures and having a distinct collecting duct system leading to the gall bladder (Figure 5.2.6). If the liver is damaged, the hepatocytes often show altered staining characteristics and accumulation of vacuoles within the cytoplasm. The gall bladder is also an area that can be damaged both by parasites causing obstruction of the ducts or by chemicals that are eliminated through the gall bladder. As with the gill, acute high dose exposures can result in hemorrhages and necrosis of the hepatocytes with inflammatory cells invading the areas. In the case of the gall bladder, lesions are usually caused by chronic irritation that results in a thickening of the gall bladder and the ducts. The back-up of the bile acids can further exacerbate the problem and cause damage to the hepatocytes.

In Figure 5.2.7 is shown extensive damage to the gallbladder with ductal dilation, liver necrosis and hemosiderin deposits. Although there was some minor involvement of the gallbladder in the NJ fish, none were as extensive as seen in the LA Gulf of Mexico fish. Because of the ductal proliferation, this could be classified as a benign neoplasm (Cholangioma). This is a chronic condition that would have manifested itself over a long period of time. In Figure 5.2.8 you can see the normal liver tissue adjacent to the necrotic hepatocytes. In Figure 5.2.9 there is severe congestion (SC) of the central veins along with hepatocyte vacuolization (V) and dilation (D). These types of lesions were only observed in the LA Gulf of Mexico menhaden.

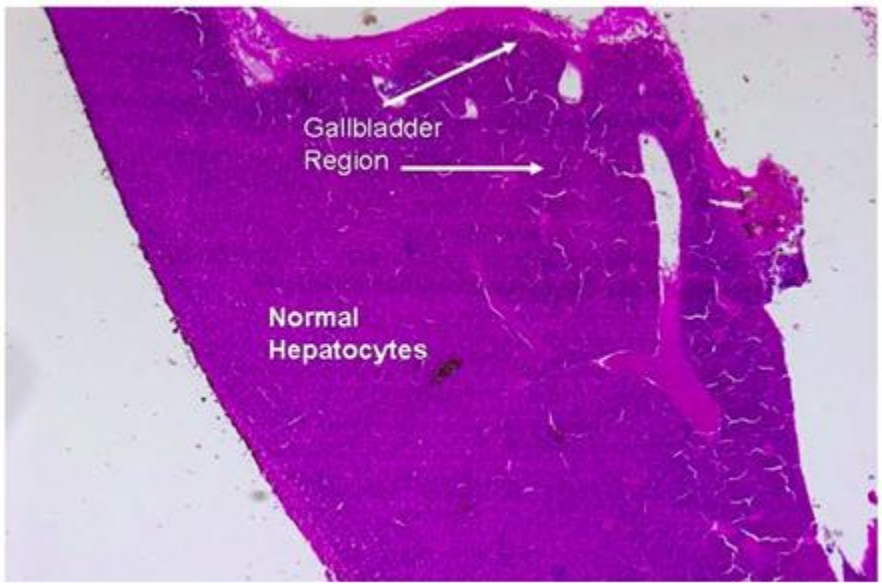


Figure 5.2.6 Normal liver and gallbladder from NJ menhaden

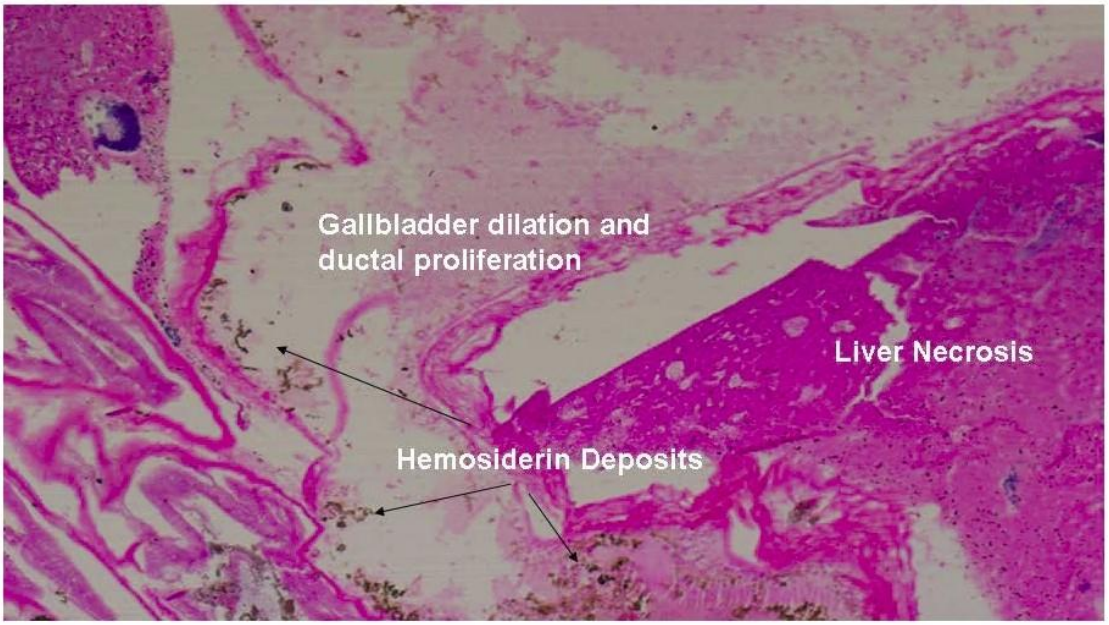


Figure 5.2.7 Gall bladder and liver effects ("Cholangioma Like Lesion") and liver necrosis in LA menhaden.

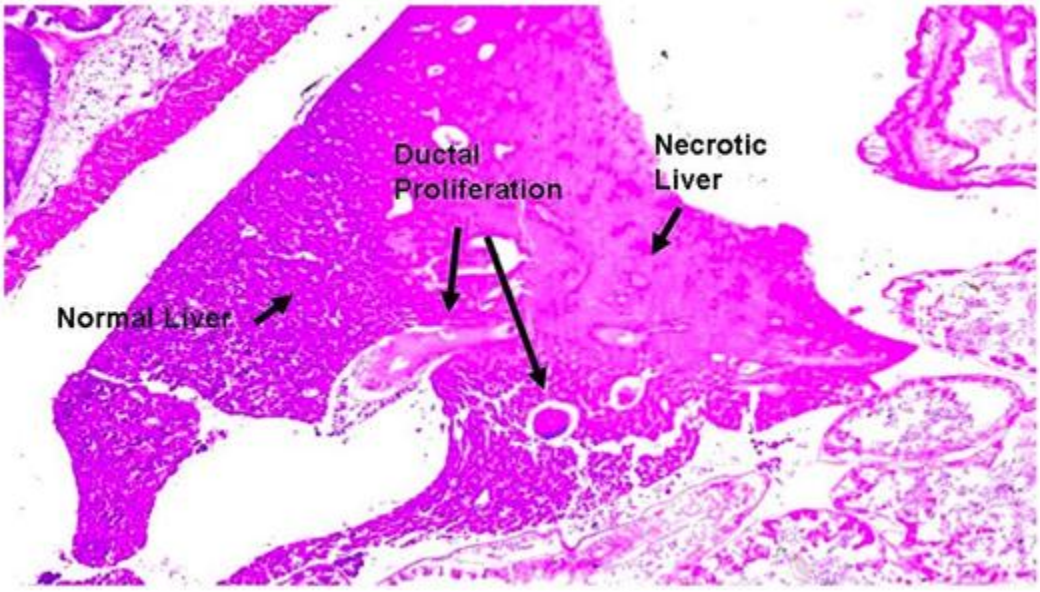


Figure 5.2.8 Liver necrosis and cholangioma like lesion.

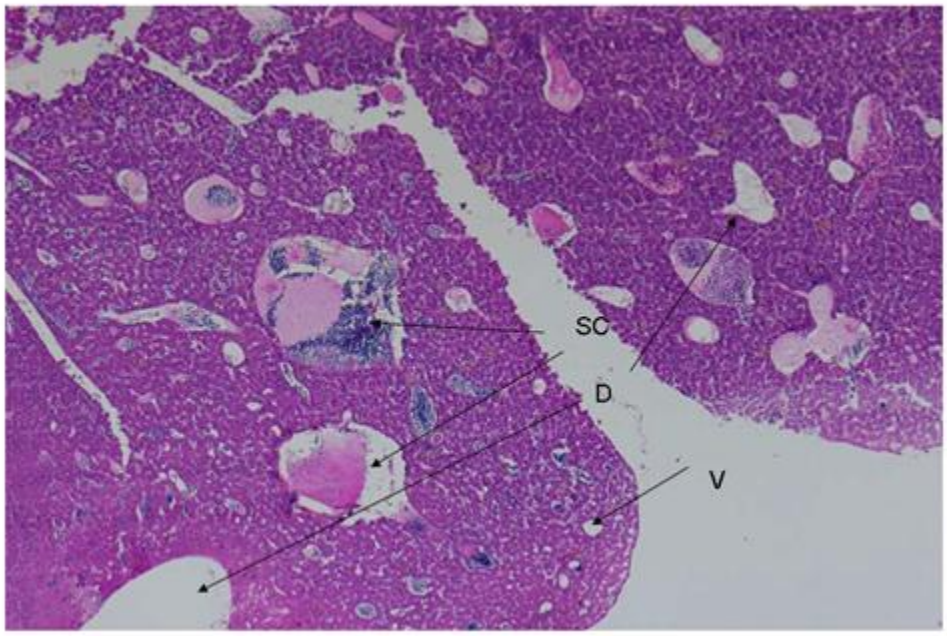


Figure 5.2.9 Hepatocyte vacuolization (V) and Dilation (D) and severe congestion (SC) of vessels.

The fish collected from both NJ and LA Gulf of Mexico had similar lesions that were apparent on the external portion of the fish. There were some fish with minor fin erosion and small hemorrhages. Both these lesions are likely due to predation and the damage done in collecting the fish. The menhaden collected from LA did have less muscle density and minor hyperplasia along the epithelium of the scales (Figure 5.2.10).

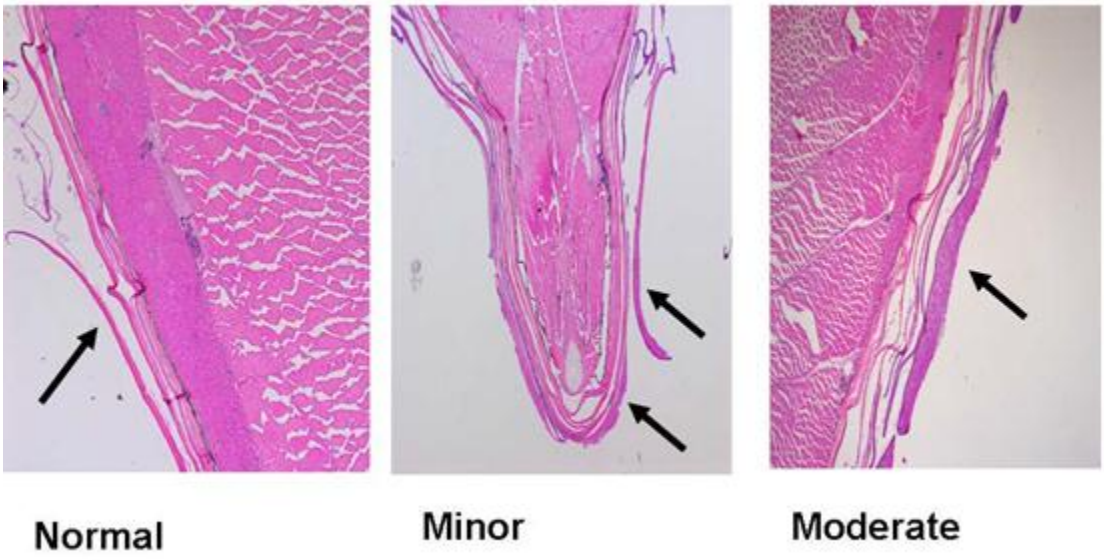


Figure 5.2.10 Minor hyperplasia of epidermal scales.

The kidney and the head kidney (bone marrow equivalent) of menhaden from both NJ and LA Gulf Coast did show accumulation of hemosiderin and macrophage centers in this tissue. The number of macrophage centers appeared (qualitatively) to be greater in the LA Gulf Coast fish but they were not quantified. The accumulation of these deposits (Figure 5.2.11) is associated with urban environments and stress to fish.

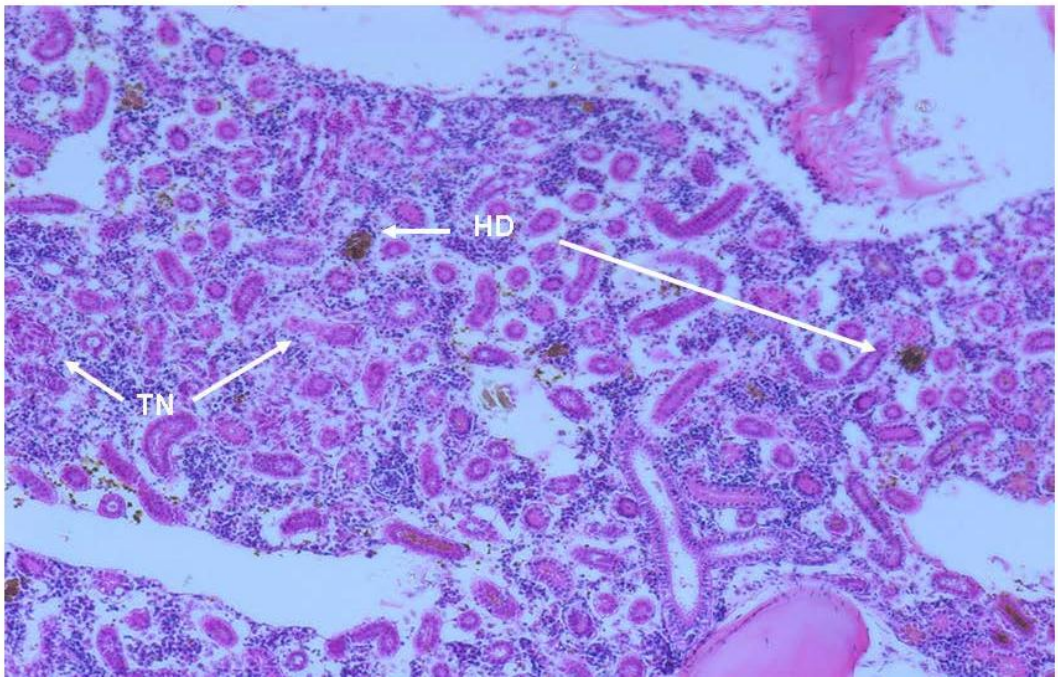


Figure 5.2.11 Head kidney and kidney with hemosiderin deposits (HD) and focal tubular necrosis (TN) in LA menhaden.

The observations made between the populations collected from NJ and those from the LA Gulf of Mexico fish did show striking differences in the damage associated with a number of tissues. This preliminary work will allow for comparison of impacts of a potential oil spill on menhaden if it were to occur in New Jersey waters. The lesions observed are consistent with what has been observed from other oil spills on fish populations (Marty et al, 2003, Marigomez et al, 2006). There were several lesions observed in the menhaden that appeared to be unique to this class of filter feeding fish that could be of great interest for resource managers. Within Table 5.2.2 below is shown the comparison for relative occurrence of the different lesions discussed above between the two groups of fish. The menhaden collected from NJ waters were in much better health than those collected from the oil impacted area of the Gulf of Mexico. The lesions highlighted in red appear to be associated with the population of menhaden collected from the Gulf of Mexico. More extensive studies and comparisons between menhaden populations may give a better feel for the overall health of the standing fishery. This species is a critical prey species and yet there is very little information on the health or responses of this class of fish to oil spills or other contaminants.

Table 5.2.2 Summary of Histological Lesions. Red text indicates effects particular evident in LA peanut menhaden.

Organ – Lesion	NJ Sites	**LA Site**	Comment
Gill Hyperplasia	+	++ to +++	Response to continuous exposure
Gill Lamellar Fusion	±	++ to +++	Response to past extensive exposure Permanent change
Gill Wavy 2nd Lamellae	--	++ to +++	Response to past extensive exposure Permanent change
Liver & Gallbladder	+	++	Focal necrotic areas occurred in both locations but some LA fish had extensive necrosis Gallbladder involvement was present and ranged from dilation to Cholangioma like lesions
Epithelial Hyperplasia	--	+	Minor
Kidney Hemosiderin	+	*+/*	More was evident in the oiled area fish, but was present in both populations
Parasitism	+	+	Minor no real difference

V.3 PAH Tissue Concentrations in Small Menhaden using Gas Chromatography Mass Spectroscopy

V.3.1 Materials and Methods

V.3.1.1 Sample Materials
The weathered oil samples used were obtained on May 11, 2010 from the ruptured wellhead site (28° 44.20′ N, 88° 23.23′ W). See attached GC/MS data on this oil below.

The fish were obtained from Barataria Bay, near Grand Isle, LA on 10/25/2010 and from Delaware Bay, near New Castle, NJ on 9/21/2010. They were from the same collection used for histopathological analysis. The average ± SD for LA was 11.3 ± 0.4 cm length and 22.3 ±1.9 g weight. The average ± SD for NJ was 8.2 ±0.2 cm length and 8.3 ± 0.9 g weight. Therefore, the LA fish were larger than the NJ fish, which might have influenced the concentrations of pollutants.

V.3.1.2 Source Oil GC/MS Methods
Extraction of PAHs and alkanes from source oil recovered from the DWH Horizon crude oil followed methods outlined in EPA Method 8270 series. Triplicate flasks (1.0 g source oil in 100 ml site seawater) were sacrificed for oil extraction. The flasks were rinsed with DCM to ensure the complete solubilization of all oil into the final, extractable liquid fraction. Approximately 80 ml of water was poured into a 250-ml separatory funnel and adjusted to a pH of 7. A 30-ml aliquot of dichloromethane (DCM) was added to the separatory funnel and spiked with a known amount of standard surrogate. The funnel was capped and shaken for approximately 3 minutes, venting occasionally to remove solvent pressure. The solvent and water were allowed to separate and the solvent was drained through an anhydrous sodium sulfate funnel into a 250-ml flat-bottom flask. The solvent addition and draining step were repeated two more times. The sodium sulfate funnel was rinsed with DCM and allowed to drain completely. The flat-bottom flask was then placed on a rotary evaporation system and concentrated to a volume of 5-10 ml DCM and placed in a calibrated extraction thimble. If concentrating was necessary, the extract volume was placed under a nitrogen blow down concentrator and reduced to a volume of 1.0 ml. The DCM extract was exchanged to hexane using approximately 4-5 ml of hexane. A micro distillation column was added to the extraction thimble and placed in a hot water bath. The DCM was evaporated off and the remaining hexane extract was reduced to a volume of 1-2 ml. The hexane extract was placed beneath a nitrogen blow down device and reduced to a final volume of 1.0 ml hexane.

The following method was used for GC/MS instrument analyses. After addition of internal standards, samples were analyzed using an Agilent 7890A GC fitted with a 0.25 mm ID × 30 m HP-5MS column and an Agilent 7683B autosampler. The injector was set to 250°C and the detector to 280°C. Detection of analytes involved the utilization of a HP 5975C Inert XL Series Mass Selective Detector operating in the Selected Ion Monitoring mode. The column was held at 60°C for 1 min and then ramped at 25°C/min to 160°C followed by 3°C/min to 268°C and 12°C/min to 300°C, where it was held for 8 min. Concentrations of parent PAHs were calculated based on calibrations using a five-point curve which were checked for each batch of samples analyzed. Concentrations were reported on a dry weight basis. Approximate alkylated PAH concentrations were calculated assuming the same response factors for each parent and corresponding alkylated analogues. For alkylated phenanthrene/anthracenes, the

results were reported as pairs to incorporate the uncertainty of the measurements and quantification based on the average response factor of the individual parent PAHs.

Table 5.3.1 Compounds (77) quantified by GC/MS analysis in each of the 210 test flasks over the 5 designated time intervals.

Internal Standard	n-Alkanes	n-Alkanes	PAHs
Napthalene-d8	nC-10 Decane	nC-22 Docosane	Naphthalene
Acenaphthen-d10	nC-11 undecane	nC-23 Tricosane	Fluorene
Chrysene-d12	nC-12 Dodecane	nC-24 Tetracosane	Dibenzothiophene
Perylene-d12	nC-13 Tridecane	nC-25 Pentacosane	Phenanthrene
Surrogate Standard	nC-14 Tetradecane	nC-26 Hexacosane	Anthracene
Phenanthrene-d10	nC-15 Pentadecane	nC-27 Heptacosane	Fluoranthene
Androstane	nC-16 Hexadecane	nC-28 Octacosane	NBT
	nC-17 Heptadecane	nC-29 Nonacosane	Benzo (a) Antracene
	Pristane	nC-30 Triacontane	Chrysene
	nC-18 Octadecane	nC-31 Hentriacontane	Benzo (b) Fluoranthene
	Phytane	nC-32 Dotriacontane	Benzo (k) Fouoranthene
	nC-19 Nonadecane	nC-33 Tritriacontane	Benzo (e) Pyrene
	nC-20 Eicosane	nC-34 Tetratriacontance	Benzo (a) Pyrene
	nC-21 Heneicosane	nC-35 Pentatriacontane	Perylene
			Indeno (1,2,3-cd) Pyrene
			Dibeno (a,h) anthracene
			Benzo (g,h,i) perylene
			Pyrene

V.3.1.3 Whole Body PAH Analysis of Small Menhaden using MSPC C-18 Extraction and GCMS Analysis

The MSPD C-18 Silica Extraction Process is an extraction method identified by the total disruption of the sample through the use of an appropriate bonded phased or other solid support material such as octadecylsilyl (ODS)-derivatized silica (C-18 Silica) being ground with the sample. Once this step took place the material was packed into a container suitable for a series of elutions with the desired solvent. This created a new phase consisting of the sample and bonded phase material and allowed for distinctive sample fractionation (Barker, 2007; García-López et al., 2008). For menhaden, a lipophilic bonding phase of C-18 silica was used. Within the procedure used for MSPD extraction, a negative pressure was applied at the receiving end of the process. This form of MSPD extraction is known as pressurized-liquid extraction (PLE) or accelerated solvent extraction (ASE) (Barker, 2007). Generally, the elution collected from this process was sufficiently "clean" enough to run on analytical instruments; however, with menhaden, a secondary clean up method was employed. This involved a standard settling period of approximately 24 h after the extraction process. This allowed any material large enough to pass through the glass microfiber filter time to settle out.

The procedure for extracting PAHs from whole body tissue menhaden was as follows. Frozen menhaden were cut into pieces. They were arranged in a labeled beaker, and using a glass pestle, the menhaden pieces were gently compressed in the beaker. Tissues from 2 menhaden were combined. The beaker was covered with foil, and the top of the foil was punctured with two to three holes. Samples were cooled to -60°C and then placed in a freeze dryer for 36-48 hours. The freeze dried material was homogenized using a blender (Sunbeam Heritage Series Kitchen Assistant 2274) until all of the tissue was evenly distributed. Then a 5 g subsample of tissue was removed and placed into a beaker. A 1:1 ratio dry weight C-18 Silica was added, and the mixture was homogenized using a glass pestle until the material was powdery and well broken down. Sodium sulfate was added to cover the top of the contents. It was blended into the mixture using a spatula until evenly dispersed. (Between samples, the blender/coffee mill was rinsed with water, then wiped with methanol on a cloth or paper towel and allowed to dry under the hood.) The beaker was filled with dichloromethane (DCM) until the tissue was covered, then using a solvent rinsed spatula, the contents were mix thoroughly and sonicated for 20 minutes. After sonication, a spatula was used again to mix thoroughly. The tissue extract was filtered using a Büchner flask (attached to a vacuum) with a Büchner funnel (with a sintered glass disc) into a flask. Once the flask was full, the contents were added to a labeled flat bottom flask. The extract was evaporated until there was no DCM left using a rotary evaporator. The contents were transferred into a graduated concentrator tube. A Snyder column was attached to the concentrator tube, and it was heated in a water bath until 1 ml of extract remained. This extract was diluted using hexane to a final volume of 25-30 ml and analyzed by GCMS using conditions provided above for DWH oil.

V.3.2 Results

GCMS analysis of DWH crude oil showed relatively high levels of 2-3 ring PAHs compared to 4-6 ring PAHs (Table 5.3.2). HNP/HPY-like PAH ratio was 20. The concentration of C-2 naphthalenes was highest for 2-3 ring PAHs (1900 ±72 ng/mg) and anthracene the lowest (8.5 ±0.20 ng/mg). The higher concentrations of alkylated PAHs to parent compound supported the need for tracking these PAHs when biomonitoring PAHs in fish. C-1 and C-2 chrysenes were highest for 4-6 ring PAHs, about 120 ng/mg, and indeno (1,2,3 - cd) pyrene the lowest, 0.52 ±0.04 ng/mg. Again the alkylated PAHs were higher than parent compounds.

Table 5.3.2 Concentrations (ng/mg) of PAHs in DWH crude oil as detected by GCMS.

Naphthalene	840±23	Fluoranthene	3.6 ±0.21	Benzo (a) Pyrene	0.93±0.05
C-1 Naphthalenes	1800±102	Pyrene	8.4±0.59	Perylene	0.75±0.02
C-2 Naphthalenes	1900±72	C-1 Pyrenes	68±4.0	Indeno (1,2,3 - cd) Pyrene	0.52±0.04
C-3 Naphthalenes	1200±70	C-2 Pyrenes	85±5.1	Dibenzo (a,h) anthracene	1.3±0.10
C-4 Naphthalenes	460±28	C-3 Pyrenes	84±4.9	Benzo (g,h,i) perylene	1.8±0.13
Fluorene	170±12	C-4 Pyrenes	56±2.9		
C-1 Fluorenes	360±30	Napthobenzothiophene	18±1.1		
C-2 Fluorenes	430±27	C-1 NBTs	58±5.7		
C-3 Fluorenes	280±14	C-2 NBTs	55±3.8		
Dibenzothiophene	68±4.6	C-3 NBTs	40±3.1		
C-1 Dibenzothiophenes	190±13	Benzo(a)Anthracene	4.6±0.27		
C-2 Dibenzothiophenes	260±20	Chrysene	48±3.6		
C-3 Dibenzothiophenes	180±11	C-1 Chrysenes	120±12		
Phenanthrene	410±32	C-2 Chrysenes	120±9.1		
C-1 Phenanthrenes	900±86	C-3 Chrysenes	70±3.4		
C-2 Phenanthrenes	860±81	C-4 Chrysenes	35±2.1		
C-3 Phenanthrenes	540±50	Benzo (b) Fluoranthene	3.5±0.23		
C-4 Phenanthrenes	230±19	Benzo (k) Fluoranthene	1.4±0.09		
Anthracene	8.5±0.20	Benzo (e) Pyrene	8.5±0.61		
Total 2-3 PAHs	11,821	Total 4-6 ring PAHs	589	Ratio HNP/HPY-like PAHs	20

GCMS analyses were done on the whole body tissue of fish where two fish of similar size were combined to make one sample, n= three samples per location. The same suite of parent and alkylated PAHs were analyzed in fish as for DWH crude oil (Table 5.3.3). The following PAHs were below detection limits in both DBNJ and BBLA fish: naphthalene, C1-C4 naphthalenes, fluorene, C1-C4 fluorenes, dibenzothiophene, anthracene, fluoranthene, pyrene, C1-C4 pyrenes, naphthobenzothiophene, C1-C4 naphthobenzothiophenes, benzo(a)anthracene, chrysene, C-1-C-4 chrysenes, perylene, indeno(1,2,3 - cd)pyrene, and benzo (g,h,i) perylene. In DBNJ fish, only C-2 and C-3 phenanthrenes were detected. Their combined concentrations were 6.52, 3.50 and 2.94 ng/mg for each of three fish samples. No other PAHs were detected in DBNJ fish using this method. In BBLA fish, phenanthrene was detected in two of three samples and C-3 phenanthrene was detected in all three samples. Their combined concentrations were 1.22, 1.48, and 1.23 ng/mg. These concentrations were lower than found for DBNJ fish. Larger 4-6 ring PAHs were only found in BBLA fish (Figure 3B). These included benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), and dibenz[a,h]anthracene (DBA). One BBFL sample had all five PAHs with a combined concentration of 0.34 ng/mg. A second sample contained only B(b)F and B(k)F for a combined concentration of 0.06 ng/mg. None of these large MW PAHs were detected in the third sample. The ratio of HNP/HPY-like PAHs in

fish was highly variable, 3.6 to 24.7, most likely due to the inconsistent levels of high MW PAHs. The ratio for DWH oil, using only the PAHs detected in fish was quite high, 174, compared to that using all PAHs, 20 (Table 5.3.2). This indicated that many of the HPY-like PAHs in DWH oil were not detectable in whole body fish tissue possibly due to low technique sensitivity and/or poor bioaccumulation. In general the concentration of PAHs in whole body fish tissue was 1000x less than in the crude oil.

Table 5.3.3 Concentration of PAHs (ng/mg) in whole body fish tissue using GCMS. Each sample (S1-3) was a composite of two fish from Barataria Bay, LA (BBLA) or Delaware Bay, NJ (DBNJ). Data from DWH source oil is also shown. U= undetected.

		BBLA			DBNJ			DWH
PAH		S1 ng/mg	S2 ng/mg	S3 ng/mg	S1 ng/mg	S2 ng/mg	S3 ng/mg	ng/mg
Phenanthrene	PHN	0.031	0.026	0.0	U	U	U	410
C1-Phenanthrenes	C1-PHN	U	U	U	U	U	U	900
C2-Phenanthrenes	C2-PHN	U	U	U	2.29	1.43	1.18	860
C3-Phenanthrenes	C3-PHN	1.20	1.45	1.22	3.71	3.43	3.51	540
C4-Phenanthrenes	C4-PHN	U	U	U	U	U	U	230
Total 2-3 ring PAHs		1.23	1.48	1.22	6.00	4.86	4.68	2940
Benzo (b) Fluoranthene	B(b)F	0.072	0.035	0.0	U	U	U	3.5
Benzo (k) Fluoranthene	B(k)F	0.072	0.029	0.0	U	U	U	1.4
Benzo (e) Pyrene	B(e)P	0.037	0.00	0.0	U	U	U	8.5
Benzo (a) Pyrene	B(a)P	0.062	0.0	0.0	U	U	U	0.93
Perylene	PRL	U	U	U	U	U	U	0.75
Indeno (1,2,3 - cd) Pyrene	IDP	U	U	U	U	U	U	0.52
Dibenzo (a,h) anthracene	DBA	0.094	0.0	0.0	U	U	U	1.3
Total 4-6 ring PAHs		0.34	0.06	0.00	0.00	0.00	0.00	16.9
Combined Total All PAHs		1.57	1.54	1.22	6.00	4.86	4.68	2957
Ratio HNP/HPY-like PAHs		3.6	24.7					174

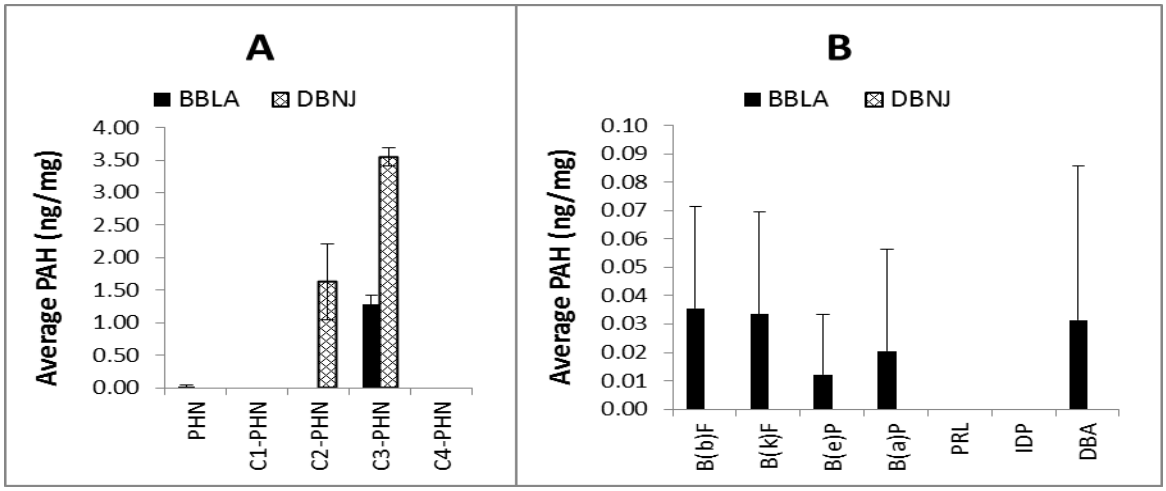


Figure 5.3.1. PAHs detected by GCMS in whole body tissues. Data represent the average and SD of fish samples from Barataria Bay, LA (BBLA) and Delaware Bay, NJ (DBNJ). Only select PAHs are shown: phenanthrene (PHN) with 1 to 4 methyl groups (C)- A, and PAHs with 5 aromatic rings including benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), and dibenz[a,h]anthracene (DBA)- B. Other PAHs that were not detected are given in the text. N=3 for DBNJ and BBLA. The larger fish used for GCMS analysis from BBLA may have allowed for detection of high molecular weight PAHs.

V.4 PAH Tissue Concentrations in Small Menhaden using Fixed Emission Fluorescence Spectroscopy

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V.4.1 Materials and Methods

Fish collected for histopathology and GCMS analyses were also used for FEFS. A portion of the upper gastro intestinal tract (GI) including liver tissue was dissected from the fish. In the small young of the year fish, a distinct liver was not found; therefore, the glandular tissue and GI tract in the most anterior part of the fish was used. Approximately 0.1 g of the tissue (wet weight) was homogenized in 75% ethanol (ETOH). Tissue was homogenized using a 2 ml glass tissue grinder. A 100 µl aliquot of the homogenate was diluted to 1 ml using 75% ETOH. This suspension was vortexed for 30 sec in order to extract the solutes into the solvent. It was then centrifuged for 20 min at 13,000 rpm in a microcentrifuge at room temperature. The resulting supernatant was place into a fluorescent cuvette for analyses. The resulting pellet was dried and weighed in order to normalize tissue weight for each homogenate. Studies presented above established two types of fluorescence scans for small and large MW PAHs using SpectraMax® M5/M5. The one for 2-3 aromatic rings held emission at 350 nm and scanned for excitation from 250-340 nm. The one for 3-5 aromatic rings held emission at 450 nm and scanned for excitation from 250-440 nm. Of concern in these experiments was interfering spectra of vitamins A and E found in fish liver and in phytoplankton which make up the menhaden's diet. Analyses of multiple PAH standards and vitamins A and E resulted in establishing two types of fluorescent scans.

Samples were also analyzed for pheophytin A, which is degraded chlorophyll and likely came from gut contents. Pheophytin was generated by acidifying chlorophyll a and the concentration of pheophytin was determined using a fixed emission of 670 nm and fixed excitation of 410 nm (USEPA Method 445.0, Revision 1.2).

Fluorescent data was analyzed as follows. Fluorescent intensity (RFU) of one major peak in the spectrum was converted to ng/ml using standard curves for 2-naphthol (Em350/Ex280), 1-hydroxypyrene (Em450/Ex340) and pheophytin A (Em670/Ex400). Data were adjusted for % recovery and dilution. The final ng/ml was then converted to ng/mg by dividing by tissue pellet dry weight. Significant differences between treatments were determined by independent student T-test or one-way ANOVA and Tukey posthoc test, $p \leq 0.05$. Percent recovery for 2-naphthol, 1-hydroxypyrene and pheophytin a were 39, 76 and 35, respectively. Obviously, the biological compounds in the samples were attracting PAHs and pheophytin and reducing their extraction.

V.4.2 Results

Spectra for fish samples and source oil were compared to standards (Figure 5.4.1). Using Em350, fish from Delaware Bay, NJ (DBNJ) had highly similar spectra to those from Barataria Bay, LA (BBLA) having one major peak of similar intensity at 280 nm, 7.54±2.09 and 7.53±1.76 RFU/µg tissue, respectively. This peak did not match any of the standards tested. Source oil had a different spectrum from those of the fish having one, broad major peak ranging from 260 to 280 nm with a maximum at 270 nm (39.37 RFU/ µg oil). The peak at 270 nm matched best with spectra of HNP and HFL. Using Em450, fish from DBNJ and BBLA also had similar spectra; however, fluorescence intensity for DBNJ was higher than for BBLA. The major peak for both occurred at 280 nm that had RFU/ µg values of 0.73±0.19 and 0.57±0.19, respectively. Both also had a broad minor peak at from 340-360 nm. The RFU/µg values at its highest point, 340 nm, were 0.50±0.09 and 0.36±0.11, respectively. The spectra for fish best matched that for fluoranthene.

Fish digestive tissue and source oil samples were compared for HNP and HPY-like PAHs as well as levels of pheophytin a (Pheo)(Table 5.4.1). Because the major peaks for PAHs overlapped, concentrations of specific PAHs could not be determined. Therefore, results for HNP-like PAHs were determined using fluorescence at the major peak in fish, Em350/Ex280, those for HPY-like PAHs were determined using fluorescence at the minor peak in fish, Em450/Ex350. Using the minor peak eliminated overlap with 9-phenanthrol (HPA) fluorescence at Em450/Ex250 and 280. Results showed that HNP-like PAHs in DBNJ and BBLA were very similar, approximately 5800 ng/mg tissue dry weight. On the other hand, HPY-like PAHs were significantly higher in DBNJ fish compared to BBLA, 49±14 and 31±5 ng/mg, respectively. The ratios of HNP and HPY-like PAHs were calculated as an indicator of PAH source. Higher levels of HNP versus HPY have been considered indicative of petrogenic as opposed to pyrogenic PAH exposure as crude oil contains a high proportion of 2-3 ring compared to 4-6 ring PAHs (Krahn et al, 1993, Aas et al, 2000, Trisciani et al, 2011). The ratio of HNP/HPY was significantly higher in BBLA (49±14) compared to DBNJ (31±5) fish (Figure 5.4.2). This was primarily due to higher levels of HPY-like PAHs in NJ fish. The ratio for source oil (12) was lower than for both fish samples, which was unexpected given the literature. It suggested selective accumulation and retention of high MW PAHs in menhaden. Pheo concentrations indicated the presence of degraded phytoplankton in gut tissues of menhaden. Pheo was approximately

3x higher in BBLA than DBNJ fish. This was understandable given the lower latitude and associated greater solar energy in LA versus NJ during the fall. The ratio of HNP-like PAHs to Pheo was also calculated. Results showed approximately 3x more HNP than Pheo in DBNJ than BBLA (Figure 5.4.2). This suggested that fish from NJ were accumulating HNP from a source other than phytoplankton. Literature has shown that menhaden can accumulate toxicants for detritus in addition to plankton (Deegan et al, 1990).

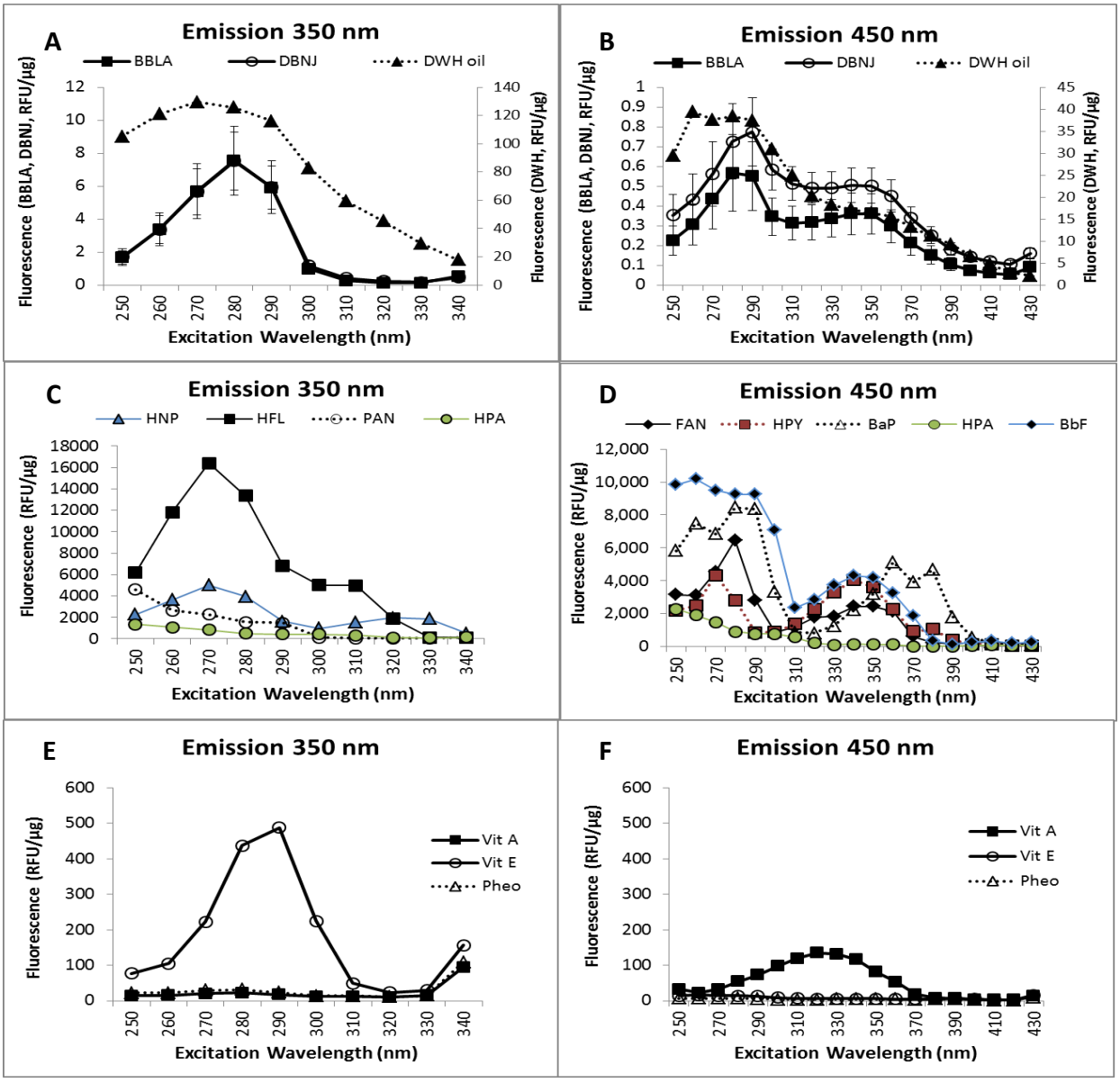

Figure 5.4.1 Comparison of PAHs in digestive track tissues of peanut fish from different locations and with DWH oil, vitamins, pheophytin A and PAH standards. Two types of spectra are shown: Em350 fixed with Ex250-340 sweep and Em450 fixed with Ex250-430 sweep. Naphthol-like PAHs in fish and DWH oil are shown in A, those with hydroxypyrene-like PAHs are shown in B. PAH standards with 2-3 rings are shown in C, except HPA, which is shown in D. PAHs standards with 4-5 rings are shown in D. Vitamins and pheophytin A are shown in E and F. Units are fluorescence (RFU) per µg sample. HNP= naphthol, HFL= 9-hydroxyfluorene, PAN= phenanthrene, HPA=9-phenanthrol, FAN= fluoranthene, HYP= 1-hydroxypyrene, BaP= benzo(a)pyrene, BbF=benzo(b)fluoranthene, Vit A= vitamin A, Vit E= vitamin E, and pheo =pheophytin A. The PAH standards were spiked directly into 75% ETOH.

Table 5.4.1 Concentrations of naphthol and hydroxypyrene-like PAHs and pheophytin A in digestive track tissue of menhaden and DWH pipe oil. Em/Ex values represented the emission/excitation wavelengths used to calculate concentrations (ng/mg). HNP= 1-naphthol, HPY= 2-hydroxypyrene, Pheo A= pheophytin A, DBNJ and BBLA= menhaden from Delaware Bay, NJ and Barataria Bay, LA, respectively. DWH oil= crude oil collected from the DeepWater (DWH) pipe line in 2010. ND= no data. Data are average±SD.

Sample (ng/mg)	n	HNP Em350/Ex280	HPY Em450/Ex340	Pheo A Em680/Ex400	HNP/HPY Ratio	HNP/Pheo A Ratio
DBNJ	13	5855±1626	186±32	2008±512	31±5	3.01±0.90
BBLA	13	5821±1449	129±38*	6054±2177*	49±14*	1.16±0.49*
DWH oil		$80x10^4$±$2x10^4$	$6x10^4$±$0.05x10^4$	ND	12.29	ND

*= statistically different from DBNJ, Independent Samples T-test, p≤ 0.05

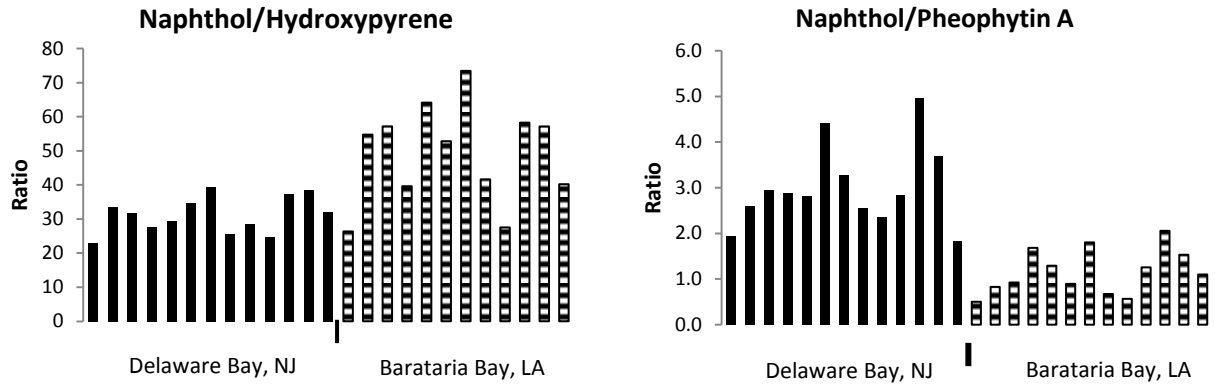

Figure 5.4.2 Ratios of naphthol (HNP) to hydroxypyrene (HPY) and HNP to pheophytin A (Pheo A) in digestive track tissue of individual menhaden from Delaware Bay, NJ and Barataria Bay, LA. Results showed that peanuts from LA had relatively higher levels of HNP than HPY compared to fish from NJ. This could indicate that NJ fish showed an urban signature associated with pyrogenic PAHs (4-5 ring PAHs) as opposed to petrogenic PAHs (2-3 ring PAHs). The higher HNP to Pheo A ratio in NJ fish indicated that they had a source of HNP other than phytoplankton.

V.5 Summary of Results in Small Menhaden

Histopathological analysis was used to assess the physiological condition of young-of-the-year (small) menhaden from BBLA and DBNJ. Results showed considerably more damage to LA than NJ fish. In particular, there was extensive damage to the gill as identified by epithelial cell hyperplasia, fusion of lamellae and waxy lamellae. This damage was consistent with exposure to an oil spill. Menhaden may have been more affected than other types of fish as their 2nd lamellae are used for filtering feeding. Gill tissue showed some repair but also that chronic irritation was still occurring. The organization of the GI track and accessory organs were unique to the filter feeding lifestyle. The liver tissue was wrapped around the upper GI track, while in the pyloric ceca and lower intestine, food and pancreatic tissue were

observed interspersed on the outside of the intestine. LA fish as compared to NJ fish showed more necrotic foci in liver and bile duct proliferation and dilation. These changes were cholangioma like lesions and required time to be manifested (Figure 5.2.8). This suggested that the initial damage may have been caused from the oil spill during the summer and continued to manifest itself into the fall. Taken together, damage in LA fish was much more extensive than in NJ fish and indicated exposure to crude oil in the recent past and continuing irritation.

The YOY menhaden were too small to collect sufficient samples of fish oil, so whole body tissues were analyzed instead by MSPD C-18 Silica Extraction Process followed by GCMS. Analyses showed only C-2 and C-3 alkylated phenanthrenes in DBNJ fish (Table 5.3.3). C-3 phenanthrene was the major 2-3 ring PAH found in BBLA fish as well. This finding showed that alkylated 2-3 ring PAHs were being preferentially bioaccumulated compared to parent PAHs by fish at both locations. It indicated that these types of PAHs need to be monitored. Concentrations of alkylated phenanthrenes were lower in LA, 1.22 to 1.48 ng/mg, than in NJ fish, 2.94 to 6.52 ng/mg. This was surprising given the histopathological analyses. It may be that accumulation of this type of PAH was not associated with the tissue damage. BBLA fish contained higher MW PAHs not found in DBNJ fish. The PAHs most consistently found were benzo[b]fluoranthene (B[b]F) and benzo[k]fluoranthene (B[k]F). Their combined concentrations ranged from 0.064 to 0.144 ng/mg. The presence of high MW PAHs in BBLA might have been due to the oil spill, or it could have been due to the larger size of LA fish used in this analysis. Larger, or older fish, would have had more time to accumulate the 5-6 ring PAHs. It is also possible that using larger amounts of tissue increased their detectability. Interestingly, neither pyrenes nor chrysenes were detected using the GCMS method. These PAHs had the highest concentrations among the 4-6 ring PAHs found in DWH crude oil (Table 5.3.2).

The GI track and associated tissues were also analyzed for PAHs; however, this was done using FEFS instead of GCMS. Since most of the liver tissue surrounded the GI track, the two tissues could not be sufficiently separated and were therefore analyzed together. Results showed very similar fluorescence spectra for both NJ and LA fish (Figure 5.4.1). This indicated that the types of fluorescent compounds in their GI tissues were very similar despite their considerable geographic distance from one another and differences in crude oil exposure. The concentrations of HNP-like PAHs were very similar, about 5800 ng/ml, while those for HPY-like PAHs were significantly higher in DBNJ fish than BBLA fish, 186±32 and 129±38 ng/ml, respectively. This resulted in a higher HNP/HPY ratio in BBLA fish than DBNJ fish, 49±14 and 31±5, respectively and indicated exposure of BBLA fish to petrogenic PAHs and DBNJ fish to pyrogenic PAHs. Again, there did not seem to be a relationship between PAH concentrations in the GI track tissues and histological damage. Concentrations in NJ and LA fish were generally similar. This indicated that the levels of PAHs found in tissues in fall 2010 were not associated with the severe damage found in LA fish. It suggested that the levels of PAHs causing the damage had been reduced likely through metabolism and elimination. It is possible that the PAHs found in the GI track were continuing to cause irritation in LA fish.

The high HNP/HPY ratio found in LA versus NJ menhaden indicated that they were exposed to different sources of PAHs, petrogenic and pyrogenic, respectively. A similar result was found for fish oil from large menhaden. The ratio for BBLA fish oil was 4.2 compared to 0.47-1.52 for DBNJ using the type I spectrum (Table 4.3.1). Results for liver of large menhaden were more ambiguous. The ratio for BBLA

was still high (15) but more similar to that found in DBNJ fish (4-15). All of the FEFS data supported a high proportion of HNP-like PAHs in BBLA fish and thereby indicated exposure to petrogenic PAHs. However, biological source and age of fish seemed to make a difference. Fish oil had approximately 10x less PAHs than fish liver in large menhaden. It did have a relativity higher level of HPY (i.e. lower ratio). This may have been due to easier elimination of 2-3 ring PAHs. Small menhaden had approximately 10x more PAHs in their GI track tissues than liver of large menhaden. This could have been due to differences in combined gut tissues versus liver, but it could also have been due to small menhaden having greater exposure to PAHs in estuaries versus coastal waters.

FEFS spectra of GI track tissues could not be used to clearly distinguish between different types of PAHs, and the PAHs detected were different than those found in whole body tissue using GCMS. PAHs detected in GI track using FEFS best matched naphthol and hydroxyfluorene for low MW PAHs and pyrene and fluoranthene for high MW PAHs. Interestingly, none of these PAHs were detected in whole body tissues using GCMS (Table 5.3.3). Phenanthrene was the only 2-3 ring PAH detected using GCMS. Larger and usually more abundant PAHs, such as pyrene and fluoranthene, were not detected using GCMS. Strangely, even larger PAHs with 5-6 rings were detected including benzo(b)fluoranthene and benzo(e)pyrene. The reason why FEFS and GCMS did not find the same type of PAHs was unknown and could best be attributed to differences in the tissues analyzed. Analysis of fish oil from large menhaden using HS-SPME with GCMS did find fluoranthene and some pyrene (Table 4.2.3) as was found using FEFS. So perhaps PAHs in fish oil and GI track tissues were more similar than those PAHs detectable in whole body tissue. This was supported by FEFS data for liver of large menhaden. The fluorescence type I spectra for HNP-like and HPY-like PAHs of large menhaden (Figures 4.3.18 and 4.3.19) were very similar to spectra of small menhaden. This indicated similar types of PAHs in their tissues. The inconsistency between PAH analysis of whole body tissue and fish oil/liver has been found by other researchers. Kreitsberg and coworkers found that fish exposed to an oil spill in the Baltic Sea had low PAH concentrations in muscle tissue (major tissue in whole body) which were not representative of exposure. Samples of liver, bile and urine in the same fish were exposure dependent (Kreitsberg et al, 2010).

Comparison of the different analytical techniques showed the high sensitivity of FEFS as well as the ease of sample preparation compared to the GCMS methods. However, the FEFS method was complicated by artifacts (type II spectra) and the presence of other fluorescence compounds such as vitamins A and E. Some of these difficulties could be resolved with further method development. None of the analytical methods used could find a distinctive “finger print” indicative of DWH oil. It may be that the fish were collected too long after the event.

VI. TROPHIC TRANSFER STUDY

VI.1 Overview

Trophic transfer studies were conducted in order to assess whether PAHs in menhaden fish oil and/or body tissues could be bioaccumulated by predators if ingested. It was also of interest to determine where the PAHs might distribute if absorbed by the gut. Three experiments were performed. In the first and second experiments, predatory fish received fish oil, which was prepared as described for chemical analyses, or DWH crude oil. Treatments were pipeted directly into the stomachs of the predatory fish. This was done to increase the likelihood that all fish would receive the same dose. Silver perch (*Bidyanus bidyanus*) was used in the first experiment and bluefish (*Pomatomus saltatri*) in the second one. In the third experiment, bluefish were fed fishmeal made from chopped menhaden skin and fillet. They were fed once a day for 4 days. Fish oil was prepared from the same fishmeal used in this study. Tissues collected for analyses included GI track, liver, gill and gall bladder (for bile analysis) as well as spleen in the second and third experiment. PAH tissue concentrations were analyzed by FEFS as described above.

VI.2 Materials and Methods

First experiment: Perch were collected on 10/4/2011 from Tuckerton, NJ using a seining net. The experiment was initiated on 10/11/2011. Prior to the experiment, perch were fed blue mussels collected from Canada and purchased from the grocery store. They were not fed during the experiment. Six perch were used per treatment. Tanks were set up with salt water from The Institute of Marine and Coastal Sciences at Rutgers University, New Burnswick, NJ. Each treatment group was housed in a separate glass tank, 10x20x12 inches. Salt water was 22 ppt and the temperature was 18 ^{o}C. Perch were dosed twice on day 0, once at approximately 12:00 PM and a second time at approximately 4:00 PM. For dosing, individual fish were transferred to a clean aquarium and placed in MS222 until asleep. The fish was then gavaged with the treatment (see below for volumes) and placed into another tank with a stream of air until recovered. It was then returned to its treatment tank. The exposure was concluded by anesthetizing fish with MS222 at approximately 12:00 PM on 10/12/2011. This resulted in a 24 h exposure. They were weighed and measured. Their tissues were removed and weighed. The tissues were frozen, -20 ^{o}C, and stored separately in labeled glass containers until chemical analysis. Tissues included gall bladder for bile, liver, GI track, gill and fillet (data for fillet are not presented). All fish survived treatment.

Treatments for silver perch and 1st bluefish experiments were as follows:

- Control: Phosphate Buffer Solution (PBS)- 100uL
- Daybrook (Daybrook): fish oil from menhaden collected from the Gulf of Mexico in 2008 and commercially prepared by Daybrook Fisheries, Inc- 20uL
- EPNJ: fish oil from menhaden collected from Delaware Bay, NJ in 2010- 100uL
- Spiked EPNJ: EPNJ fish oil was spiked with deuterated (d-)naphthalene and d- phenanthrene (25 µg/mL)-100uL
- DWH crude oil: crude oil collected from the DeepWater Horizon pipeline on April 21, 2010- 0.66g crude oil plus 0.66g of Daybrook fish oil was mixed and given -20uL. The crude oil and DB fish oil were mixed in order to reduce the viscosity of the crude oil and make it easier to pipet.

Fluorescence spectra for the fish oils and DWH crude oil used as treatments are shown in Figure 6.2.1. For reference, the spectra for vitamin A and some of the PAH standards are also shown.

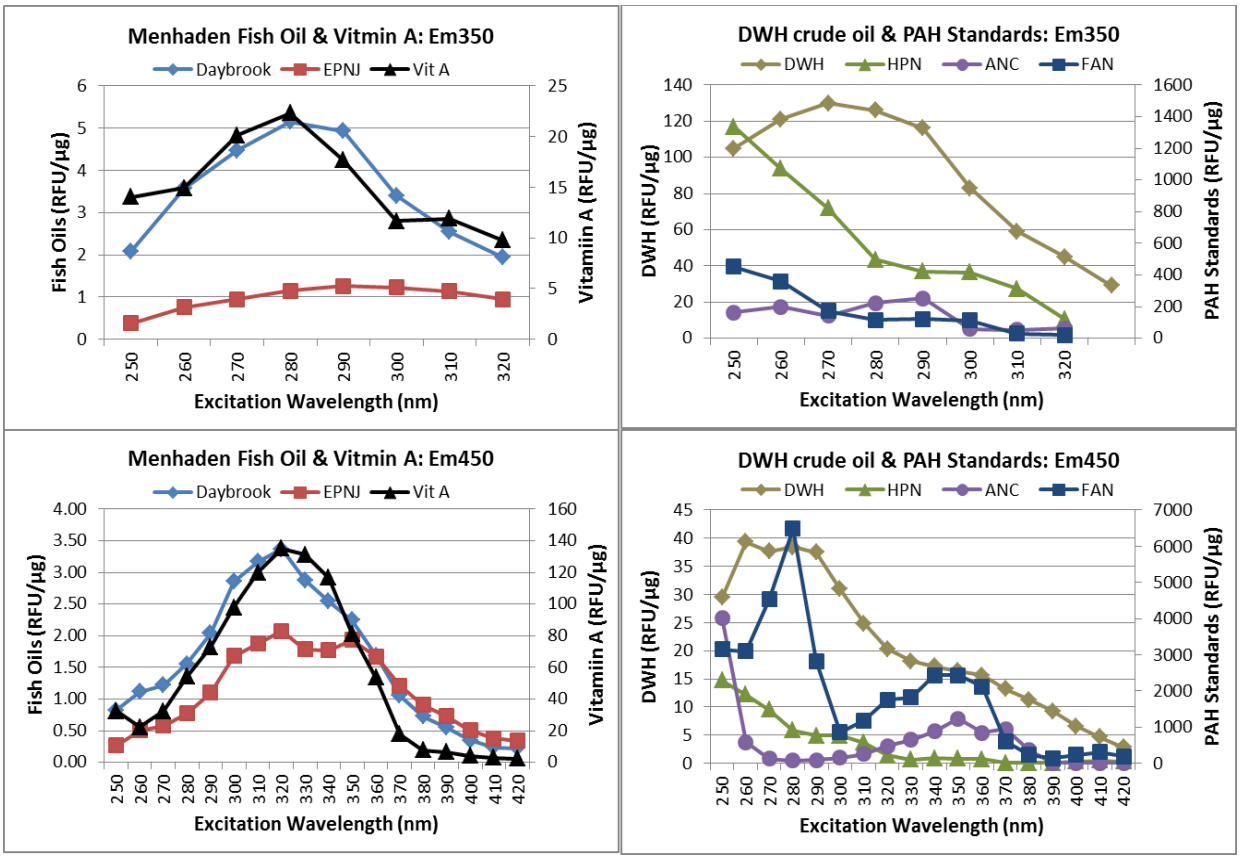

Figure 6.2.1 Fluorescence spectra for fish oils and DWH crude oil used in trophic studies as well as some standards. The top two graphs are for FEFS with fixed Em350, the bottom two with fixed Em450. The Daybrook and EPNJ fish oils were used as treatments. The Daybrook fish oil was mixed with DWH crude oil in order to make it easier to administer- see text. Also shown are spectra for vitamin A and PAHs phenanthrol (HPN), anthracene (ANC) and fluoranthene (FAN). Units are fluorescence intensity (RFU) per µg.

Second experiment: Bluefish were collected on 10/13/2011 from Tuckerton, NJ (same location as for perch). They were fed frozen silversides supplied by San Francisco Bay Brand, Inc. (Newark, CA). The experiment was initiated on 10/21/2011. Housing of fish and dosing technique for fish oils or DWH crude oil was as described above. There were 3 fish per treatment. Salinity in treatment tanks was 22-24 ppt. The temperature was 20 ^{o}C. Fish were dosed twice on day 0 once at approximately 11:00 AM and again at 2:45 PM. They were given 200 µl each time for a total of 400 µl. At 8:00 PM of day 0, two fish were found dead in the EPNJ treatment. Tissues from these fish were dissected and frozen for analysis. All other fish survived until the end of the experiment. The experiment concluded on

10/22/2011 at approximately 11:00 AM for a 24 h exposure. Bluefish were weighed and measured. Their tissues were removed and weighed. The tissues were frozen, -20 ^{o}C, and stored separately in labeled glass containers until chemical analysis. Tissues collected included gall bladder (for bile), liver, GI track, gill and spleen. It was noted that the bluefish contained gill parasites (crustaceans) and parasites external to their GI tracks. They were black segmented ribbon-like worms. Most of the fish contained them.

The treatments were the same as for the first experiment except the bluefish were given a total of 400 µl of fish oil or DB/crude oil mixture and the spike concentration was increased from 25 to 100 µg/ml D-naphthalene and D-phenanthrene in EPNJ fish oil.

Third experiment: The bluefish collected for the second experiment were also used in the third one. The third experiment was initiated on 10/28/2011. The bluefish were housed as described for the first and second experiment. They were dosed differently. In this experiment, bluefish were fed chopped fish meal (fillet and skin) from menhaden. This was tissue from the same fish used to make the fish oil samples and from which livers were collected (see section IV.3). There were five fish per treatment except control which had three. Fish were fed at 24, 48, 72 and 96 h. They were fed approximately 4 g per day for a total of 20 g. Exposure was concluded at 96 h. Fish were weighed and measured and tissues collected and stored as described.

Treatments for bluefish-fishmeal experiment were as follows:

Control: fed blue mussels
BBLA: fish meal from large menhaden collected from Barataria Bay, LA on 10/30/2010.
EPNJ: fish meal from large menhaden collected from Delaware Bay, NJ on 9/8/2010.
JRVA: fish meal from large menhaden collected from James River, VA from October 1 to 21, 2010.

Some deaths occurred during the experiment. One EPNJ fish died on day 1. One control fish and one BBLA fish died on day 4.

Tissues from all experiments were analyzed as follows. Approximately 0.1 g of wet tissue was weighed out. It was sonicated in 500 µl of 75% ETOH. After sonication, it was diluted to 1 ml with 75% ETOH. Aliquots of this homogenate (200 µl) were added to 0.8 ml of 75% ETOH and extracted by vortexing for 1 minute. This mixture was then centrifuged for 20 minutes at 13,000 rpm. The supernatant was analyzed by FEFS for 2-3 ring PAHs (emission fixed at 350 nm and excitation scanned from 250 to 340 nm) and for 4-6 ring PAHs (emission fixed at 450 nm and excitation scanned from 250 to 430 nm). The pellets were dried and weighed. Standard curves and percent recoveries were determined as described. Concentrations of naphthol-like (HNP) PAHs were determined at Em350/Ex280, phenanthrol-like (HPT) PAHs at Em450/Ex250 and hydroxypyrene-like (HPY) PAHs at Em450/Ex350. Percent recoveries were 59, 75 and 52 for HNP, HPT and HPY, respectively. Final units were ng PAH/mg tissue dry weight for liver, GI track, gill and spleen and ng PAH/gall bladder for sonicated gall bladder and its contents (bile).

VI.3. Results

VI.3.1 Size of fish used in trophic studies

The average weights and lengths of fish used for all three experiments are shown in Table 6.3.1.1. Average weights for silver perch used in the first trophic study ranged from 6.18 to 9.43 g, and average lengths ranged from 6.40 to 7.60 cm. Weights and lengths between treatment groups were similar. These perch were of similar length but lighter than the bluefish used in the second and third trophic studies. Bluefish used in the second trophic study had average weights ranging from 16.70 to 18.73 g and average lengths ranging from 7.00 to 9.29 cm. This size of bluefish was similar to those bluefish used in the third trophic study: average weights ranged from 17.00 to 18.45 g and average lengths ranged from 6.03 to 7.64 cm.

Table 6.3.1.1 Average weights (g) and lengths (cm) of predatory fish used in trophic studies.

Experiment	Fish Species	Collection Date	PAH Source	Treatment	Weight (SD)	Length (SD)
1st	silver perch	10/4/2012	Fish oil	Control	9.43 (3.95)	7.60 (1.32)
				EPNJ	8.83 (2.58)	7.35 (0.53)
				EPNJ-spike	7.57 (1.23)	7.23 (0.45)
				DayBrook	6.18 (1.96)	6.40 (0.86)
				DWH crude oil	7.70 (1.40)	7.20 (0.61)
2nd	bluefish	10/13/2012	Fish oil	Control	16.70 (1.06)	7.03 (1.31)
				EPNJ	18.43 (1.57)	9.18 (1.96)
				EPNJ-spike	17.37 (0.25)	7.00 (0.77)
				DayBrook	18.73 (0.66)	8.45 (1.07)
				DWH crude oil	18.27 (1.55)	9.29 (2.12)
3rd	bluefish	10/13/2012	Fishmeal	Control	17.00 (0.50)	6.62 (1.03)
				BBLA	18.45 (1.69)	7.64 (1.56)
				MVNJ	17.50 (1.04)	6.03 (0.88)
				JRVA	17.46 (0.81)	6.06 (1.10)

VI.3.2 Trophic study in silver perch using menhaden fish oil and DWH crude oil

In the first trophic study silver perch were gavaged with two sources of menhaden fish oil, fish oil spiked with d-naphthalene and d-phenanthrene or DWH crude oil. The purpose of the experiment was to assess the extent to which PAHs in fish oil matrix or in DWH crude oil could be absorbed from the diet, i.e. GI track. One source of fish oil was from menhaden collected on 9/8/2010 from the ship "Enterprise" seining for adult menhaden in the Delaware Bay, NJ area (EPNJ). (See Table 3.1 for size information.) The other source was fish oil commercially prepared by Daybrook Fisheries, Inc. (http://www.daybrook.com). This fish oil (Daybrook) was prepared from adult menhaden caught by seining in the Gulf of Mexico, LA area in 2008 (prior to the DWH 2010 oil spill). The control was PBS.

FEFS analysis of tissues and gall bladder content of silver perch showed differences in PAH absorption (Table 6.3.2.1). PAH-like compounds were found in all tissues and gall bladder contents. When comparing tissues regardless of treatment, GI track had significantly higher levels of HNP-like PAHs than liver ($p=0.013$), which was significantly higher than gill ($p=0.003$). Liver of EPNJ-spiked fish contained the highest average concentration of HNP-like PAHs (21.55 ± 38.25 µg/mg) compared to other treatments (ranged from 3.28 to 4.41 µg/mg). However, this was largely due to an unusually high concentration in one of five fish. Results also showed that liver had significantly higher concentrations of HPT and HPY-like PAHs than GI track and gill. For these PAHs, gill and GI track were not significantly different, $p > 0.05$. Overall, both gill and gall bladder had approximately 5 times more HNP-like PAHs than HPT and HPY-like PAHs. However, GI track and liver had 8 to 10 times more HPT-like than HNP-like PAHs. This showed that different tissues preferentially accumulated different types of PAHs.

Due to high variability within treatment groups, there were no significant treatment related effects. However, EPNJ-spike had the highest liver concentrations of HPT-like PAHs. This demonstrated that absorption of PAHs from the fish oil matrix was possible. Concentrations of PAHs in unspiked fish oils were similar to control. This showed that the fish oils did not contain higher levels of PAHs than what was already present in the silver perch prior to treatment. This indicated that the silver perch from Tuckerton, NJ were contaminated with PAHs. The liver concentrations of HPT-like PAHs were higher in EPNJ-spike than DWH treated fish, 58.68 ± 33.26 and 29.25 ± 8.52 µg/mg, respectively. However, their GI track concentrations were similar, 16.09 ± 14.98 and 16.16 ± 5.00 µg/mg, respectively, and higher than other treatments (ranged from 8.61 to 10.79 µg/mg). This indicated that PAHs were better absorbed from fish oil matrix than crude oil in silver perch. It was assumed that the GI track would contain unabsorbed and/or eliminated PAHs.

Examination of spectra for individual fish showed that some appeared to accumulate PAHs while others within the same treatment group did not. This led to high data variability. For example, fish 4 in the EPNJ-spike group had 10 times more HNP-like PAHs (Em350) than found for the other individuals in its treatment group and control (Figure 6.3.2.1). Data for higher molecular weight PAHs (Em450) showed that only three of the six fish in the spiked treatment had high HPT-like (Em450/Ex250) and HPY-like (Em450/Ex350) PAHs. Their spectra were different from the other three fish in the spiked treatment and all six control fish. The change in spectra clearly showed absorption of the PAHs. This finding showed the importance of looking at the complete spectrum for each individual fish as opposed to the

average and SD for a fixed Em/Ex wavelength. It was not known why some fish did not absorb the PAHs. It might have been due to the dosing technique and/or short time of exposure.

Table 6.3.2.1 PAH concentrations (ng/mg) in tissues of silver perch gavaged with fish oil or DWH crude oil. HNP-like PAHs were determined at Em350/Ex280, HPT-like PAHs at Em450/Ex250 and HPY-like PAHs at Em450/Ex350. N = number of fish per treatment group. Data are average (SD) µg PAH per mg of tissue dry weight for GI track, gill and liver. Spleen was not collected. For gall bladder, data are average (SD) for ng PAH per gall bladder and cannot be directly compared to other tissues.

PAH	Treatment	n	GI Track µg/mg	Spleen µg/mg	Gill µg/mg	Liver µg/mg	n	Gall Bladder µg/GB
HNP-like	Control	5-6	5.14 (4.50)	ND	1.56 (0.69)	3280 (1307)	3	1.32 (0.72)
	Daybrook	5-6	3.37 (2.35)	ND	1.59 (0.36)	3790 (2096)	3	0.99 (0.32)
	EPNJ	5-6	5.59 (4.09)	ND	1.93 (0.41)	4410 (2071)	3	1.72 (1.99)
	EPNJ-spike	5-6	6.96 (4.40)	ND	1.36 (0.35)	21551 (38252)	3	2.13 (1.32)
	DWH	5-6	6.78 (4.27)	ND	1.60 0.(60)	3666 (1131)	3	2.20 (1.77)
HPT-like	Control	5-6	8.61 (2.99)	ND	0.30 (0.09)	25123 (8881)	3	0.40 (0.11)
	Daybrook	5-6	10.79 (6.75)	ND	0.33 (0.07)	43003 (18549)	3	0.35 (0.24)
	EPNJ	5-6	9.93 (4.20)	ND	0.28 (0.08)	38811 (24968)	3	0.35 (0.29)
	EPNJ-spike	5-6	16.09 (14.98)	ND	0.29 (0.06)	58675 (33256)	3	0.45 (0.27)
	DWH	5-6	16.16 (5.00)	ND	0.29 (0.02)	29249 (8520)	3	0.46 (0.36)
HPY-like	Control	5-6	0.20 (0.10)	ND	0.30 (0.09)	0.51 (0.30)	3	0.23 (0.13)
	Daybrook	5-6	0.31 (0.15)	ND	0.33 (0.07)	0.49 (0.20)	3	0.12 (0.05)
	EPNJ	5-6	0.27 (0.14)	ND	0.29 (0.09)	0.75 (0.51)	3	0.19 (0.15)
	EPNJ-spike	5-6	0.30 (0.22)	ND	0.27 (0.06)	0.78 (0.37)	3	0.27 (0.15)
	DWH	5-6	0.26 (0.13)	ND	0.29 (0.15)	0.36 (0.06)	3	0.34 (0.26)

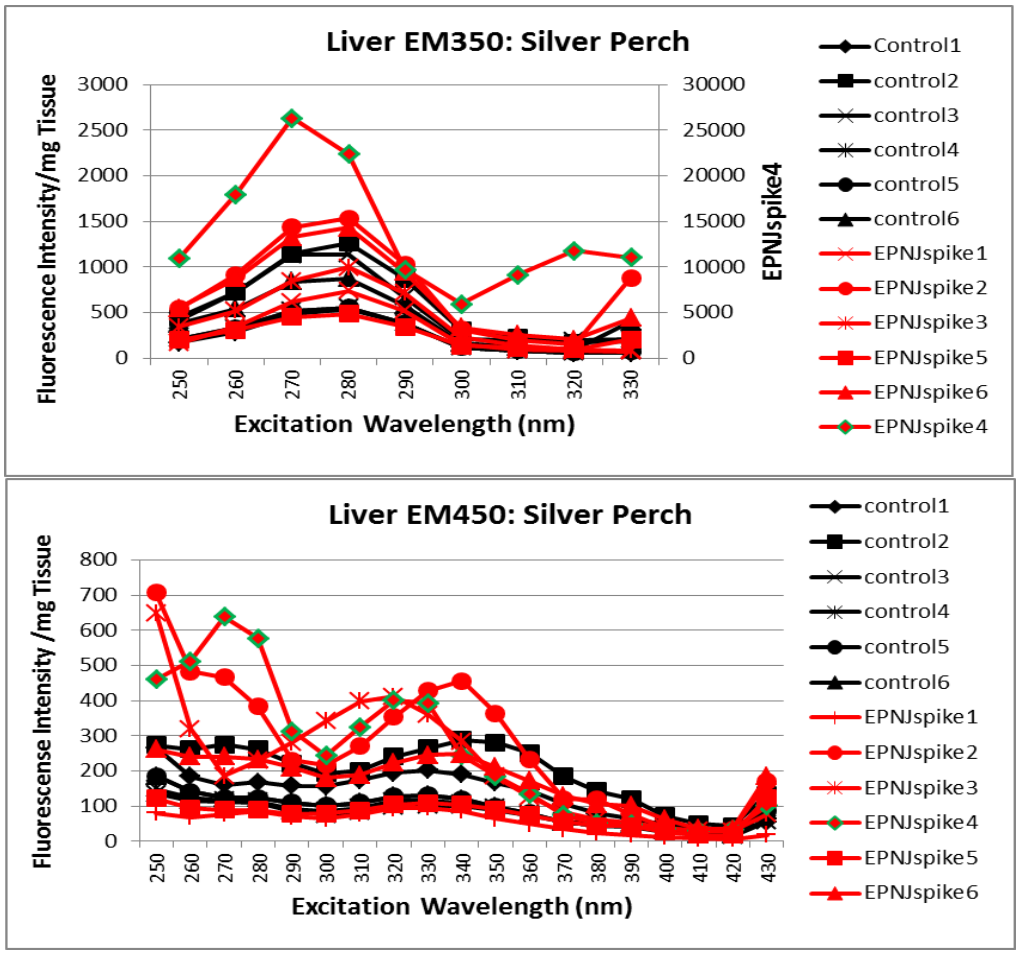

Figure 6.3.2.1 Spectra for liver tissue from silver perch. Treatment groups were control and EPNJ fish oil spiked with d-naphthalene and d-phenanthrene, 20 µg/ml each. Data for each individual fish are shown. The top figure is for HNP-like PAHs, and the bottom one for HPY and HPT-like PAHs. Note that fish 4 had very high level of HNP-like PAHs (Em350/Ex270) and the spectrum is shown on a secondary Em350 axis.

Comparison of spectra of selected fish in control and DWH groups showed some interesting findings (Figure 6.3.2.2). The spectral profiles at Em280 showed higher levels of HNP-like PAHs in DWH treated fish. However, these spectra might have represented vitamin A, vitamin E and/or PAHs. The spectral profiles at Em450 were similar between control and DWH fish in GI track even though PAHs were higher in DWH than control. Their spectra resembled that of fluoranthene. This indicated that the controls had PAH contamination in their GI track. The source was likely due to their diet prior to capture. Spectral patterns for liver were somewhat different between groups. Both had a peak at Ex250 and 280; however, control had another peak at Ex340 while that for DWH was at Ex320. This difference in spectra might have indicated some PAH absorption from DWH crude oil even though Em450/Ex250 averages for DWH and control were similar. This stressed the value of looking at the spectra.

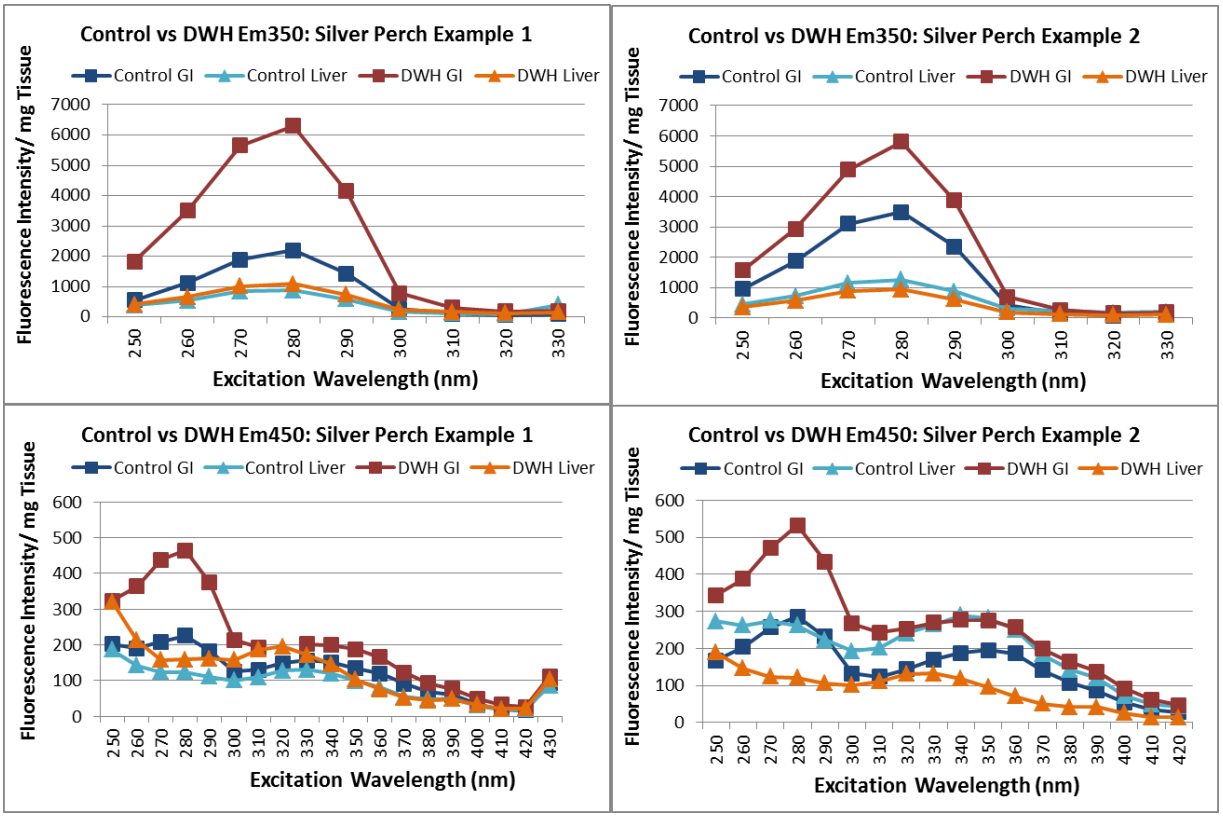

Figure 6.3.2.2 Comparison of spectra of individual fish from DWH and control treatments. Upper spectra are for fixed Em350 and lower spectra are for fixed Em450. Spectra for GI track and liver are compared. Example 1 shows control fish 6 and DWH fish 4. Example 2 shows control fish 2 and DWH fish 5. Note the presence of PAHs in control fish and higher levels of PAHs in GI track versus liver in DWH fish at Em450. Also note that the two spectra for DWH liver differed from those of control as well as GI track of DWH and control. One major peak shifted from Ex340 to Ex320.

Comparison of spectra for different tissues showed characteristic profiles and evidence of PAH distribution throughout the fish following absorption from the GI track (Figure 6.3.2.3). Examples of fish from control and EPNJ-spike showed slightly different major peaks for each tissue. Gill had a peak at Em450/Ex250 and Em450/Ex320-330. Liver tissue typically had a peak at Em450/Ex330-340. This was likely due to vitamin A (see Figure 6.3.1.1). Gall bladder had a characteristic peak at Em450/Ex320, and GI track had a major peak at Em450/Ex270-280 and Em450/Ex340. These profiles likely reflected a combination of natural fluorescent compounds as well as PAHs and other contaminants. However, spectra for tissues in the EPNJ-spike fish were clearly different from control particularly at Em450/EX250. This provided evidence of PAH uptake from the GI track and PAH distribution to liver as well as gill in this fish.

The gill showed similar spectra across treatments for most fish (Figure 6.3.2.4). There was a major peak at Em450/Ex250 and another one at approximately Em450/Ex320. The peak at Ex250 suggested

phenanthrene contamination; however, the similarity in spectra and concentrations indicated that the PAHs did not come from the GI track gavages with fish oil/DWH treatments. They suggested that the PAHs were taken up from the water possibly prior to their capture.

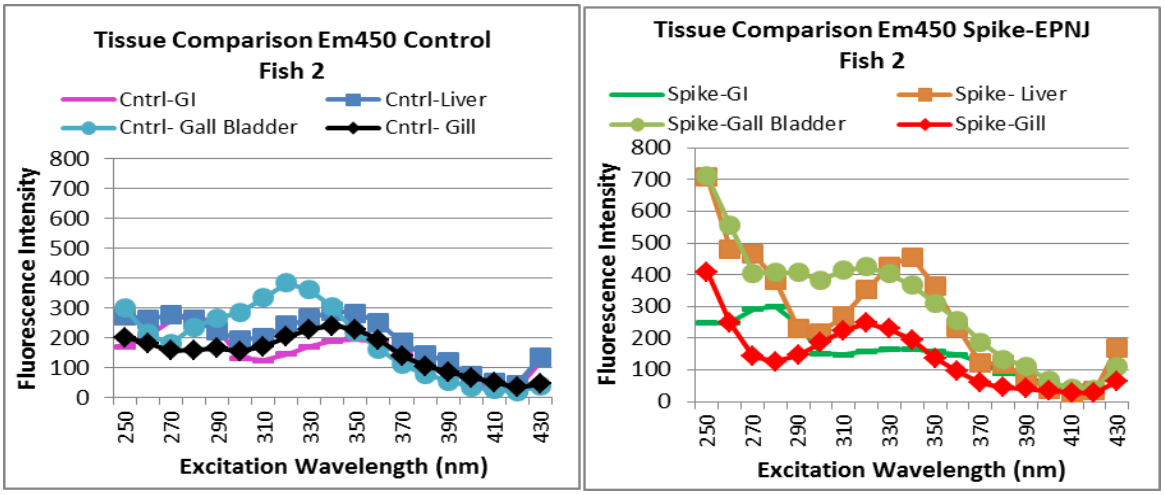

Figure 6.3.2.3 Comparison of PAHs in different tissues of the same fish. Spectra for fish 2 in control and EPNJ-spike treatments are provided. Note the increased fluorescence intensity at Ex250 for liver, gill and gall bladder of spiked fish. Also note that the spectrum for a particular tissue had characteristics peaks seen for both control and spiked fish, i.e. liver had an Ex330-340 peak and gall bladder a Ex320 peak. Spectra were not normalized to tissue weight.

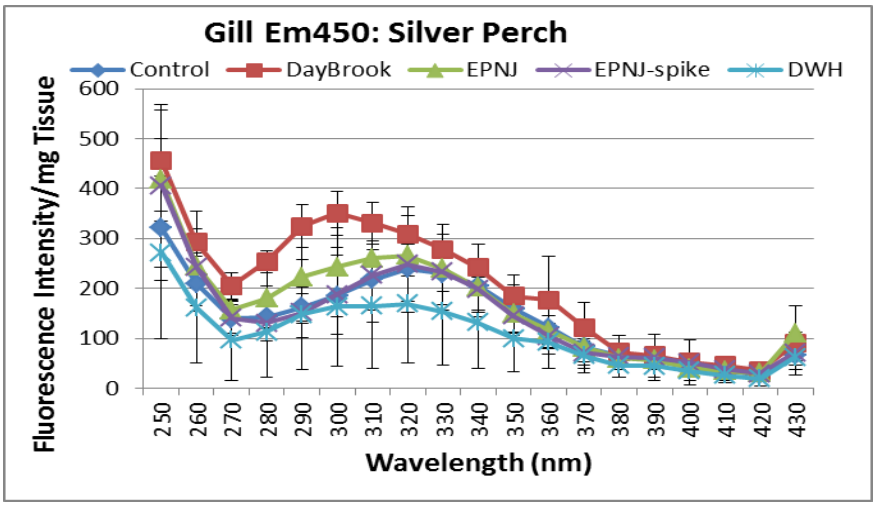

Figure 6.3.2.4 Spectra of high molecular weight PAHs (Em450) in gill of silver perch. The treatment average for each excitation wavelength in the spectral scan is shown. Error bars represent SD. Note the similar spectra profile and PAH concentrations as represented by fluorescence intensity per mg tissue dry weight. This indicated a source of PAH other than diet.

VI.3.3 Trophic study in bluefish using menhaden fish oil and DWH crude oil

Transfer of PAHs in fish oil and DWH crude oil from the GI track to other tissues was also tested in YOY bluefish. Bluefish were gavaged twice over an 8 hour period and sacrificed 24 h later. PAHs were measured in GI track, liver, spleen and gill with units of μg PAH/mg tissue dry weight and in gall bladder with units of μg PAH/gall bladder. Because the units for gall bladder were different, results could not be directly compared to those of other tissues. Results showed that all tissues contained PAHs. Tissues and gall bladder generally had concentrations of HPT-like PAHs (Em450/Ex250) that were higher than those of HNP-like PAHs (Em350/Ex280), which in turn were higher than those of HPY-like (Em450/Ex350) PAHs; however, results for HPT and HNP varied by tissue and individual (Table 6.3.3.1). This was similar to results in silver perch (Table 6.3.2.1).

Data for individual bluefish within a treatment group were variable, which can be seen by high standard deviations. However, when tissues were compared as a group, without regard to treatment, HNP-like PAHs were significantly higher in liver than in other tissues, $p \leq 0.008$. This was different than in silver perch where GI track > liver > gill. The spectra at Em350/Ex280 may have reflected vitamin A and E and not PAHs. This suggested that silver perch had higher levels of HNP-like PAHs and/or vitamin A&E in their GI than did bluefish. Concentrations of HPT-like PAHs were similar in liver and gill of bluefish and generally higher than in spleen and GI track. However, variability between individuals prevented statistical differences between tissues, $p > 0.05$. HPY-like PAHs followed the same pattern and were also higher (~3x) in liver and gill of bluefish compared to spleen and GI track. However, this time concentrations in liver and gill were significantly higher than in spleen and GI track, $p < 0.001$. These results differed somewhat from silver perch. HPT-like and HPY-like PAHs did follow the same pattern; however, PAH concentrations in liver of silver perch were significantly higher than those in gill and GI track, $p < 0.001$. These results suggested differences in PAH absorption and/or distribution in bluefish versus silver perch. Overall, these results showed that 1) PAHs were detected in all treatments groups including control bluefish, 2) HPT-like PAHs were higher in all tissues as well as gall bladder than HNP and HPY-like PAHs, and 3) liver and gill had the highest levels of high MW PAHs, while liver alone had the highest levels of low MW PAHs and/or vitamin A&E.

Statistical analyses of PAH concentrations showed no significant differences between treatments for any of the tissues tested. However, comparison of spectra definitely showed treatment effects associated with absorption and distribution of PAHs. The lack of statistical differences could be attributed to variability between individuals within the same treatment as well as preexisting PAH contamination in test organisms. Evidence of treatment differences and PAH absorption was found when comparing control and DWH treatments (Figure 6.3.3.1). In spectra for high MW PAHs (fixed Em450), 2 of 3 DWH fish had a GI track spectrum different from that of all three control fish and 1 DWH fish. The spectrum showed peaks such that Ex250>Ex280>Ex320. Control and 1 of the 3 DWH fish showed a different spectrum such that Ex250≈Ex280>Ex330. The change in spectra indicated the presence of different PAHs in the GI track of 2 of 3 DWH fish than in control fish. Also, the same spectrum found in GI track of DWH fish was found in liver of DWH treated fish such that Ex250>Ex280>Ex320. This occurred in 2 of 3 fish and the spectrum was different from that of all three control fish livers. The spectrum for control liver was Ex250<Ex280≤Ex320. This result indicated that PAHs in liver of DWH fish were absorbed from the GI track. The peak at Em450/Ex320 as seen in control has previously been associated with the

presence of vitamin A. The Em450:Ex250>>Ex280>Ex320 best resembled the spectrum for phenanthrene. An unexpected finding was the presence of an intense BaP spectrum in the liver of one DWH fish. This result was repeatable for that liver homogenate; however, it was not found in any other fish in the experiment and may therefore represent a technical error. No treatment related effects were found for low MW PAHs (Em350). It was possible that the vitamin A&E signal masked evidence of PAH absorption.

Evidence of PAH absorption and distribution was found in gill (Figure 6.3.3.2). All three DWH fish had a similar Em450 spectrum such that peaks at Ex250>>Ex320. This spectrum differed from control which showed two types of spectra. Control fish 2 and 3 had peaks at Ex250>Ex330 and control fish 1 had Ex250≈Ex280<Ex350. The different spectrum in gill of DWH fish indicated a treatment related effect. In addition, the high E250 peak was also seen in DWH GI track and liver, thereby indicating not only absorption but distribution to the gill. For EPNJ, two of the three fish had a spectrum similar to control fish 1, while the other (EPNJ fish 3) was more similar to the DWH fish. However, further comparison of the gill from this fish to its other tissues indicated this was unlikely to be a treatment related effect because the other tissues did not show a high Ex250 peak (data not shown). For EPNJ-spike, two of the three fish had a gill spectrum similar to DWH fish while the other fish had a spectrum resembling that of control 2 and 3. The EPNJ-spike fish received d-naphthalene and d-phenanthrene in EPNJ fish oil. The Em450 spectrum with peaks of Ex250>>Ex320 was consistent with the presence of phenanthrene but also anthracene. These results showed variable spectra for gill. Some associated with treatment and others not. This indicated absorption and distribution of PAHs from diet but also the possibility of pre-existing PAHs in gill not associated with diet but perhaps water exposures prior to testing. Interestingly, the spectrum found in bluefish control and some treated fish, Em450:Ex250>Ex330, was also seen in silver perch (Figure 6.3.2.4). Therefore, this spectrum appeared to be intrinsic to gill. The fluorescence might have been due to compounds other than PAHs (such as seen for vitamin A in liver) or accumulated from the environment. The bluefish and perch were collected from the same location in Tuckerton, NJ. Treatment effects could best be described as a relatively higher peak at Ex250 and a shift from Ex330 to Ex320 in some DWH and EPNJ-spike fish.

Another interesting finding in this trophic study was the relationship between PAH spectra and tissues. The spectra for GI track closely followed that of spleen in this study (Figure 6.3.3.3). The data presented compare PAH spectra of GI track and spleen for two control fish and two DWH fish. Even though the two control fish have slightly different peaks for GI track, the peaks for spleen in the same fish closely matched. This was also true for DWH fish where one fish with high levels of contamination and one with levels similar to control are presented. This finding clearly showed absorption of GI content into the circulatory system. The presence of PAH-like compounds in the spleen was likely due to its high perfusion with blood. Findings also suggested a pathway by which contaminants in diet might affect the immune system in that B-cells mature in spleen.

Comparisons across all tissues as well as gall bladder content showed a similar phenomenon (Figure 6.3.3.4). For example, the different tissues and gall bladder from a DWH fish shared the same Em450 spectrum: Ex250>Ex280>Ex320-330. The one exception was gill, which did not show the Ex280 peak but did show a similarly high Ex250 peak. An EPNJ-spike fish affected by treatment showed a similar result. Its tissues and gall bladder had a similar Em450 spectrum with Ex250>Ex280<Ex320-330. Again, gill did

not match entirely but it did resemble that of the DWH fish. The similarity in spectra across tissues and gall bladder for DWH and EPNJ-spike fish provided strong evidence of absorption and distribution of PAHs due to treatment. This was further supported by comparing tissues and gall bladder in control fish. In control, GI track, spleen and gall bladder showed a similar Em450 spectrum: Ex250<Ex280≈Ex350. However, the spectrum for liver, Ex250≈Ex280≈Ex320, was quite different as was the spectrum for gill, Ex250>Ex280<Ex340. This showed some relationship between GI content and other organs, but also that gill and liver had their own spectra, i.e. they were not contaminated by compounds in the blood. Data for Daybrook fish oil was mixed. The GI track, liver and spleen spectra resemble those of control. However, gill had a higher Ex250 than control, and the gall bladder had a much higher Ex250 and Ex320 than control. The high Ex320 in gall bladder may have reflected metabolism of vitamin A absorbed from Daybrook fish oil. The reason for the unusually high Ex250 in gall bladder and gill was unknown as Daybrook fish oil did not show a high Ex250 value. Again, this may have been due to preexisting contamination in control. Overall, results showed that similar spectra for different tissues of the same fish indicated absorption of PAHs from diet and their distribution to fish organs.

Table 6.3.3.1 PAH concentrations (μg/mg) in tissues of bluefish gavaged with fish oil or DWH crude oil. HNP-like PAHs were determined at Em350/Ex280, HPT-like PAHs at Em450/Ex250 and HPY-like PAHs at Em450/Ex350. N = number of fish per treatment group. Data are average (SD) μg PAH per mg of tissue dry weight for GI track, spleen, gill and liver. For gall bladder, data are average (SD) for μg PAH per gall bladder and cannot be directly compared to other tissues. Significant differences are provided in the text.

			GI Track	Spleen	Gill	Liver		Gall Bladder
PAH	Treatment	n	μg/mg	μg/mg	μg/mg	μg/mg	n	μg/GB
HNP-like	Control	3	1.93 (0.91)	1.96 (1.73)	0.86 (0.42)	6.79 (1.38)	3	4.45 (3.18)
	Daybrook	3	2.79 (1.00)	4.71 (2.58)	2.85 (1.38)	5.32 (0.45)	3	5.36 (3.31)
	EPNJ	3	6.81 (6.12)	7.16 (5.96)	2.86 (1.18)	4.50 (4.02)	3	7.67 (6.02)
	EPNJ-spike	3	1.90 (1.19)	4.06 (0.63)	1.30 (0.287)	4.10 (0.97)	3	7.73 (2.78)
	DWH	3	3.20 (0.74)	1.84 (1.08)	2.70 (1.56)	6.35 (2.07)	3	1.12 (0.52)
HPT-like	Control	3	6.39 (3.31)	5.77 (3.35)	16.06 (3.08)	27.09 (10.65)	3	33.39 (25.41)
	Daybrook	3	8.47 (0.83)	9.08 (3.98)	36.01 (8.97)	20.60 (2.92)	3	133.86 (89.75)
	EPNJ	3	6.00 (3.37)	7.36 (5.49)	21.23 (25.12)	14.40 (11.80)	3	34.87 (30.00)
	EPNJ-spike	3	8.16 (5.71)	10.52 (8.39)	38.12 (13.52)	20.38 (12.75)	3	125.12 (40.77)
	DWH	3	12.46 (6.34)	12.59 (7.68)	46.93 (3.60)	131.42 (177.83)	3	72.86 (37.26)
HPY-like	Control	3	0.17 (0.04)	0.08 (0.05)	0.43 (0.27)	0.46 (0.04)	3	0.57 (0.30)
	Daybrook	3	0.18 (0.02)	0.15 (0.05)	0.53 (0.07)	0.60 (0.15)	3	0.99 (0.30)
	EPNJ	3	0.23 (0.10)	0.10 (0.04)	0.51 (0.29)	0.34 (0.11)	3	0.80 (0.51)
	EPNJ-spike	3	0.14 (0.03)	0.09 (0.03)	0.54 (0.05)	0.29 (0.10)	3	1.13 (0.45)
	DWH	3	0.19 (0.05)	0.08 (0.03)	0.55 (0.09)	1.89 (2.61)	3	0.71 (0.01)

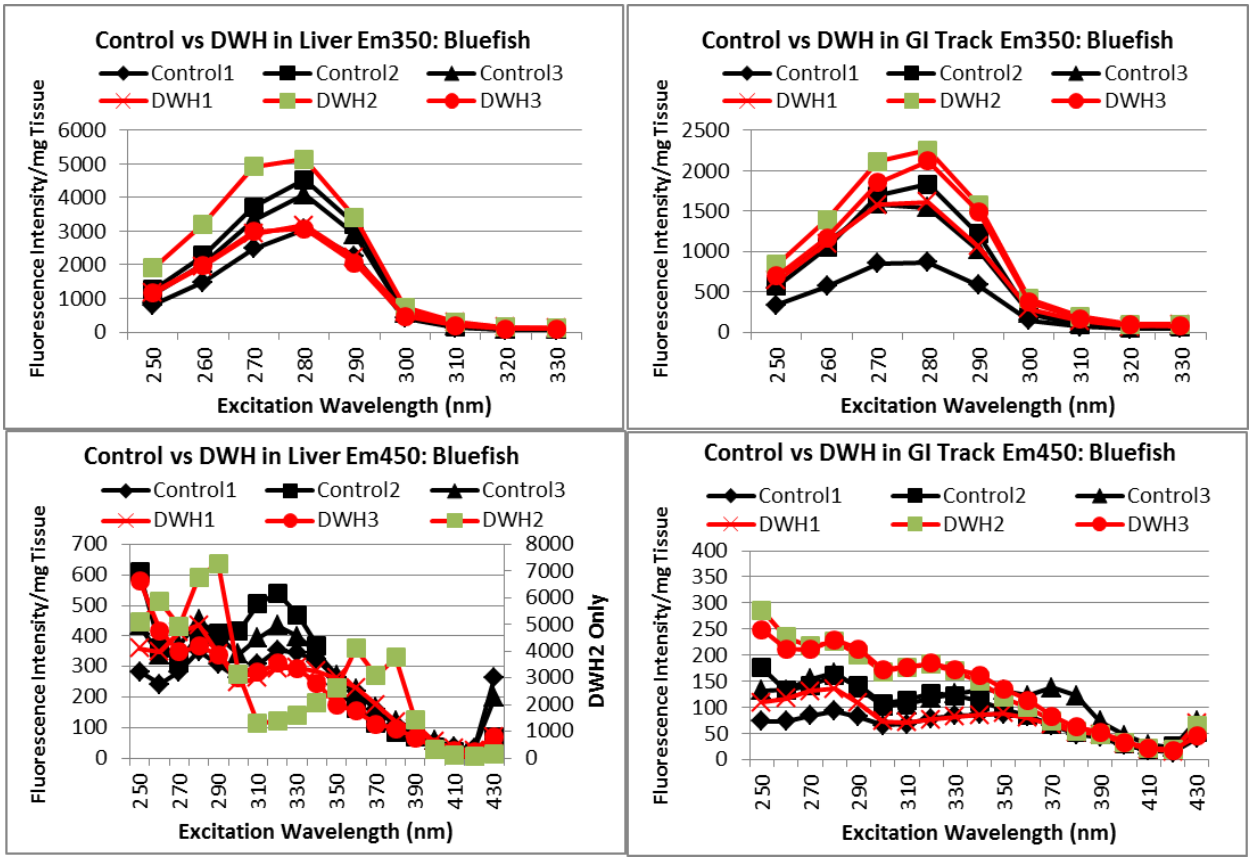

Figure 6.3.3.1 Comparison of spectra of GI track and liver from DWH and control in bluefish. Upper spectra are for fixed Em350 and lower spectra are for fixed Em450. Data are in fluorescence intensity (RFU) per mg tissue. Spectra for each fish in a treatment are shown (n=3). For example, DWH1 represents bluefish number 1 of 3 in the treatment group. Note that HNP-like PAHs (Em350/Ex280) were similar. Note the different spectra for DWH2 and 3 in GI track compared to control fish and DWH1. This indicated the presence of different PAHs in GI track of DWH exposed fish. Note high signal of BaP-like PAHs in liver of DWH2 at Em450 (2nd vertical axis). Note similarity of Em450 spectra in liver and GI track for DWH3. This indicated absorption of PAHs from DWH crude oil in diet.

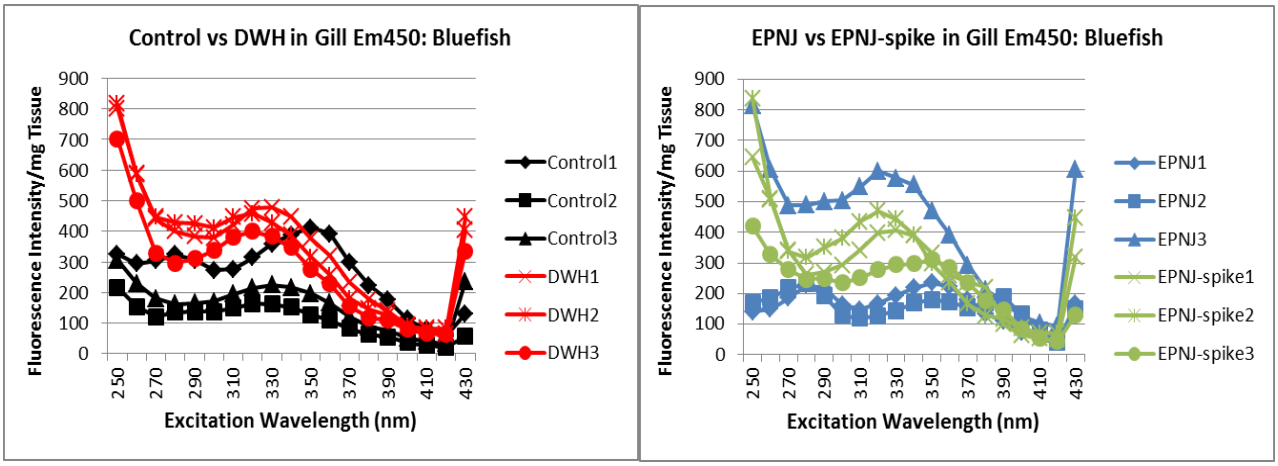

Figure 6.3.3.2 Comparison of Em450 spectra for gill in bluefish. Spectra are shown for control versus DWH and for EPNJ versus ENPNJ-spike. Data are in fluorescence intensity (RFU) per mg tissue. Spectra for all fish per treatment are shown (n=3). Note difference in DWH spectra compared to control: Ex250 was higher and second major peak shifted from Ex330 to Ex320. This indicated PAHs in gill were a result of exposure to DWH crude oil. Therefore, PAHs were circulated from GI track to gill. Note similar change in EPNJ-spike 1&2 and EPNJ 3.

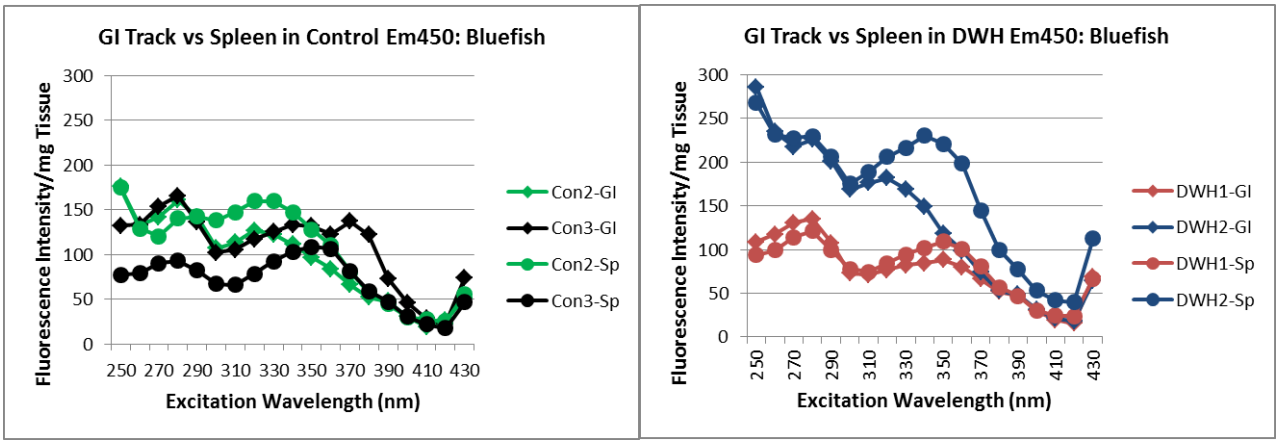

Figure 6.3.3.3 Comparison of spectra of GI track (GI) and spleen (Sp) from DWH and control in bluefish. Spectra for fixed Em450 are shown. Spectra from two of three fish are shown to improve clarity. Data are in fluorescence intensity (RFU) per mg tissue. Note how the spectral peaks of spleen matched those of GI track for a particular fish. This indicated absorption of fluorescent compounds in diet and their circulation to highly perfused organs. Also note the spectrum of DWH2 which indicated absorption and circulation of phenanthrene-like PAHs.

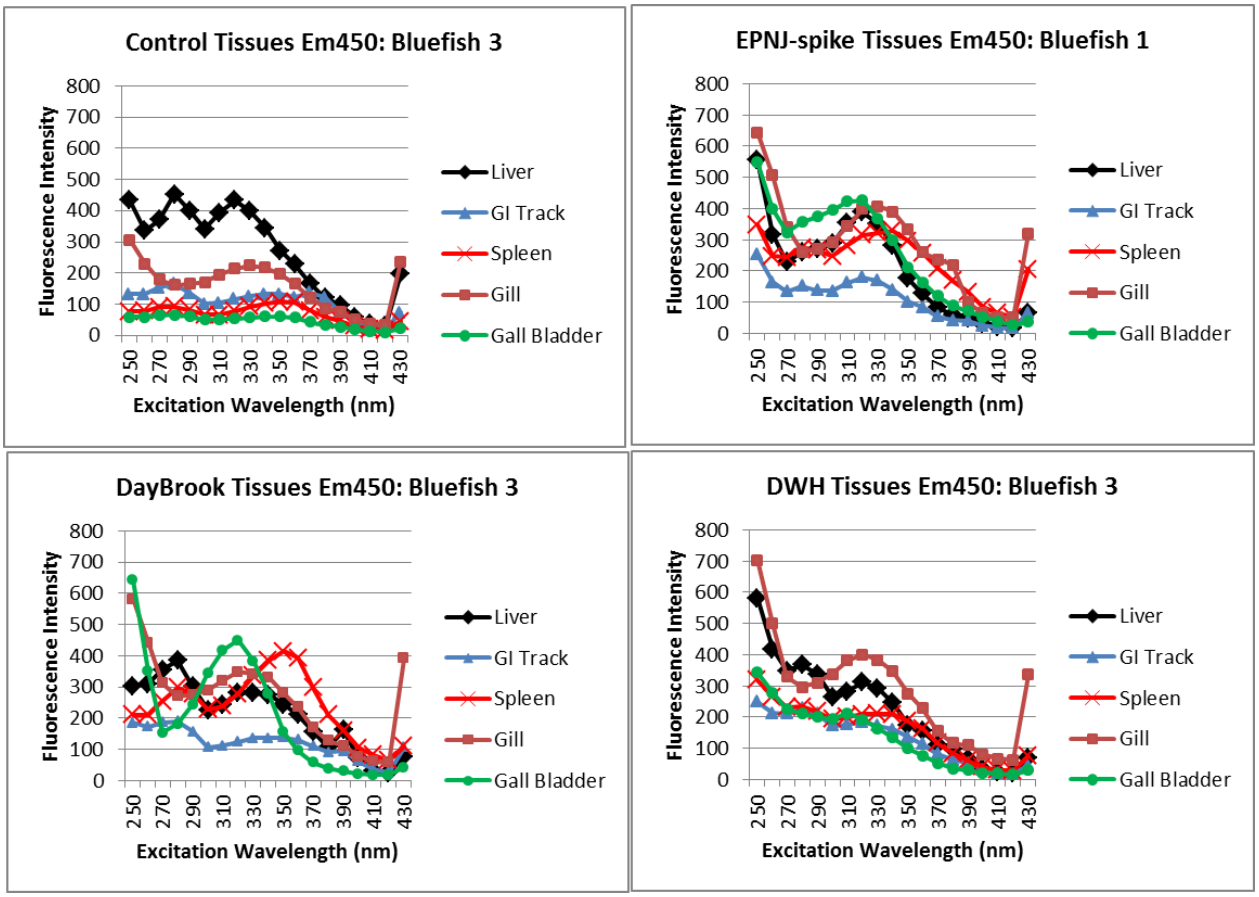

Figure 6.3.3.4 Comparison of spectra of different tissues in bluefish. Spectra for all tissues from a particular fish are shown for control, Daybrook, EPNJ-spike and DWH using fixed Em450. Fluorescence intensity is not normalized for tissue weight. Note consistent increase at Ex250 for EPNJ-spike. This indicated absorption of phenanthrene in diet (GI track) and circulation to other tissues. Note similar spectra for all DWH tissues compared to control and Daybrook. Recall that DWH was mixed into Daybrook for dosing. This indicated that PAHs absorbed from DWH crude oil were different than those in control and Daybrook fish oil.

VI.3.4 Trophic study in bluefish using menhaden fishmeal

Transfer of PAHs in menhaden fishmeal from the GI track to other tissues was tested in YOY bluefish. These bluefish were captured at the same time as those used in the previous study. They were kept in the laboratory one additional week. During this time they were fed frozen silversides. Fishmeal was prepared from the chopped skin and fillet of menhaden. These tissues were also used to make fish oil. The fishmeal was prepared from menhaden collected from three different sites. One collection site was the commercial ship named "Mount Vernon" which was seining off shore in the Delaware Bay, NJ (MVNJ) area on 9/23/2010. The other collection site was seining performed in the Hampton Roads area at the mouth of the James River, VA (JRVA) on 10/1-21/2010. The third collection site was gill netting performed in the Grand Isle area of Barataria Bay, LA (BBLA) on 10/30/2010. Bluefish were separated into four groups (n=3-5): control (fed silversides), MVNJ, JRVA and BBLA. Bluefish fed for 4 days. They were sacrificed on day 5. PAHs were measured in GI track, liver, spleen and gill with units of µg PAH/mg tissue dry weight and in gall bladder with units of µg PAH/gall bladder. Because the units for gall bladder were different, results could not be directly compared to those of other tissues. Results showed that all tissues contained PAHs (Table 6.3.4.1). Tissues and gall bladder generally had concentrations of HPT-like PAHs (Em450/Ex250) that were higher than those of HNP-like PAHs (Em350/Ex280), which in turn were higher than those of HPY-like PAHs (Em450/Ex350). The only exception was GI track, in which levels of HPT-like PAHs were similar to those of HNP and both were higher than HPY. These results were similar to results in silver perch (Table 6.3.2.1) and the 1st bluefish study (Table 6.3.3.1).

Data for individual bluefish within a treatment group were variable, as was seen in the other two studies. However, when tissues were compared as a group, without regard to treatment, HNP-like PAHs were significantly higher in liver than in other tissues, $p \leq 0.001$. This was the same as for the other bluefish study. Concentrations of HPT-like PAHs were also significantly higher in liver than the other tissues, $p<0.001$. This differed from the first bluefish study where liver and gill had similar levels of HPT-like PAHs. HPY-like PAHs followed the same pattern and were significantly higher in liver than other tissues, $p < 0.001$, with one exception. Spleen had levels of HPY-like PAHs that were similar to those in liver, and spleen concentrations were significantly higher than those in gill and GI track, $p \leq 0.012$. The high levels of HPY in spleen differed from the 1st bluefish and silver perch studies. Comparing the three types of PAHs measured in tissues found that HPT > HNP > HPY, $p \leq 0.003$. One exception was spleen, in which HPT ≈ HNP > HPY, $p < 0.001$. Comparing this experiment with the other two found overall that 1) liver contained the highest levels of PAHs compared to gill, spleen and GI track, 2) HPT-like PAHs were usually higher than HNP-like PAHs which were higher than HPY-like PAHs and 3) PAHs were detected in all treatments groups including control bluefish.

Comparison of PAH concentrations as defined by specific Em/Ex wavelengths found no significant differences between treatment groups, $p > 0.05$. This was largely due to high standard deviations within treatment groups and the low number of individuals within a treatment group. Differences in food consumption might have accounted for some of the variability. However, some results did demonstrate differences between treatments and tissues when the entire spectrum was considered. For example, gill showed a distinctive Em450 spectrum with three major excitation peaks: 250, 280 and 340 (Figure 6.3.4.1). There were no significant treatment effects, but the consistent spectrum was unique to gill. The other two experiments showed a similar pattern with Ex250 and 330 peaks; however, the addition

of the Ex280 peak was unique. Gill from bluefish in the first experiment did show an alternative Em450 spectrum with major peaks at Ex250, 280 and 350. Perhaps the gill spectrum in the current bluefish study was a composite of the two types of spectra found in the previous one. In any case, results indicated pre-existing PAHs or some other fluorescent compound in gill of both bluefish and silver perch.

Comparison of liver did show treatment related effects. Results for fixed Em450 showed 3 of 5 fish in JRVA differed from control (Figure 6.3.4.2). These three fish had major peaks such that Ex250 > Ex280 ≤ Ex320. The spectrum for control and the other two fish in JRVA had major peaks such that Ex250 < Ex280 > Ex320. The "control" spectrum was also seen in all fish of BBLA and 3 of 4 fish in MVNJ. One fish in MVNJ had the same spectra as effected fish in JRVA. The unique spectra in liver of 3 of 5 JRVA taken together with the consistent "control" spectrum seen in fish from other treatments indicated that bluefish were absorbing different PAH-like compounds from JRVA fishmeal than fish fed one of the other treatments. Likewise, it appeared that MVNJ fishmeal contained some PAHs, but the response of only one fish makes this questionable. Spectra for fixed Em350 were similar between treatments indicating no treatment related effects for HNP-like PAHs. As in silver perch and the 1st bluefish study, liver spectra continued to show a major peak at Em450/Ex320. This likely represented vitamin A and has been a common peak found in liver samples.

Stronger evidence of absorption and distribution of PAH-like compounds from fishmeal was found by comparing different tissues and gall bladder of effected fish (Figure 6.3.4.3). The data presented compares different tissues of the same fish exposed to one of the four treatments. For example, Em450 spectra of gall bladder, liver, GI track and spleen are shown for bluefish five in the JRVA treatment. Comparison of its tissues show a common spectra for GI track, liver, and spleen such that Ex250 > Ex280 ≤ Ex320. The gall bladder spectrum for this fish stands out from those of the other fish as well. The spectrum was not the same as for other tissues; however, the broad peak between Ex280 and Ex330 appeared to encompass the Ex280 and Ex320 peak seen in the other tissues. Having a common spectrum among GI track and other tissues strongly supports the uptake of PAH-like compounds from the JRVA fish meal and their distribution into the liver and spleen through the circulatory system. This finding was also supported by results showing that JRVA fish oil contained the highest levels of PAH-like compounds compared to the other fish oils (Figure 4.3.14). Tissues for the one MVNJ fish (MVNJ3) showing a similar liver spectrum as effected JRVA fish did not show a common spectra among the other tissues. Its spectra for gall bladder, GI track and spleen resembled the common spectra found in control3 and BBLA3. The reason for the different spectrum in liver was unknown but did not support the absorption and distribution of PAH-like compounds from MVNJ fishmeal. Overall, the trophic study indicated that PAH-like compounds in fishmeal could be absorbed and distributed in a predator fish after feeding for 96 hours, and that BBLA and MVNJ fishmeal did not transfer detectable levels of these compounds however JRVA fishmeal did.

Table 6.3.4.1 PAH concentrations (μg/mg) in tissues of bluefish fed menhaden fishmeal. Menhaden were collected in fall 2010 from Barataria Bay, LA (BBLA), Delaware Bay, NJ (MVNJ) and from James River, VA (JRVA). HNP-like PAHs were determined at Em350/Ex280, HPT-like PAHs at Em450/Ex250 and HPY-like PAHs at Em450/Ex350. N = number of fish per treatment group. Data are average (SD) μg PAH per mg of tissue dry weight for GI track, spleen, gill or liver. For gall bladder, data are average (SD) for μg PAH per gall bladder and cannot be directly compared to other tissues. Significant differences are provided in the text.

			GI Track	Spleen	Gill	Liver	Gall Bladder
PAH	Treatment	n	μg/mg	μg/mg	μg/mg	μg/mg	μg/GB
HNP-like	Control	2-3	1.56 (0.21)	5.55 (2.29)	2.73 (0.77)	11.64 (1.13)	6.58 (0.31)
	BBLA	3-4	4.04 (1.97)	11.64 (7.75)	2.53 (1.05)	15.88 (9.28)	5.41 (4.06)
	MVNJ	3-4	2.34 (2.01)	7.04 (2.30)	3.25 (0.98)	17.25 (1.50)	6.98 (2.24)
	JRVA	5	3.20 (2.03)	3.51 (2.63)	2.42 (1.45)	16.04 (4.40)	5.91 (4.26)
HPT-like	Control	2-3	4.10 (2.19)	7.91 (3.83)	14.68 (1.84)	27.75 (7.13)	59.82 (3.34)
	BBLA	3-4	8.35 (5.55)	7.53 (3.18)	12.76 (1.25)	23.86 (10.97)	29.64 (15.43)
	MVNJ	3-4	4.45 (3.98)	6.92 (1.81)	13.06 (3.11)	32.11 (21.14)	55.47 (22.04)
	JRVA	5	5.96 (3.88)	9.49 (3.97)	14.23 (5.22)	44.97 (29.01)	39.24 (24.56)
HPY-like	Control	2-3	0.09 (0.03)	0.57 (0.22)	0.27 (0.06)	0.37 (0.19)	0.66 (0.04)
	BBLA	3-4	0.14 (0.09)	0.36 (0.31)	0.24 (0.02)	0.47 (0.09)	0.48 (0.37)
	MVNJ	3-4	0.09 (0.06)	0.41 (0.15)	0.21 (0.05)	0.47 (0.14)	0.78 (0.38)
	JRVA	5	0.11 (0.03)	0.31 (0.17)	0.25 (0.07)	0.50 (0.24)	0.56 (0.35)

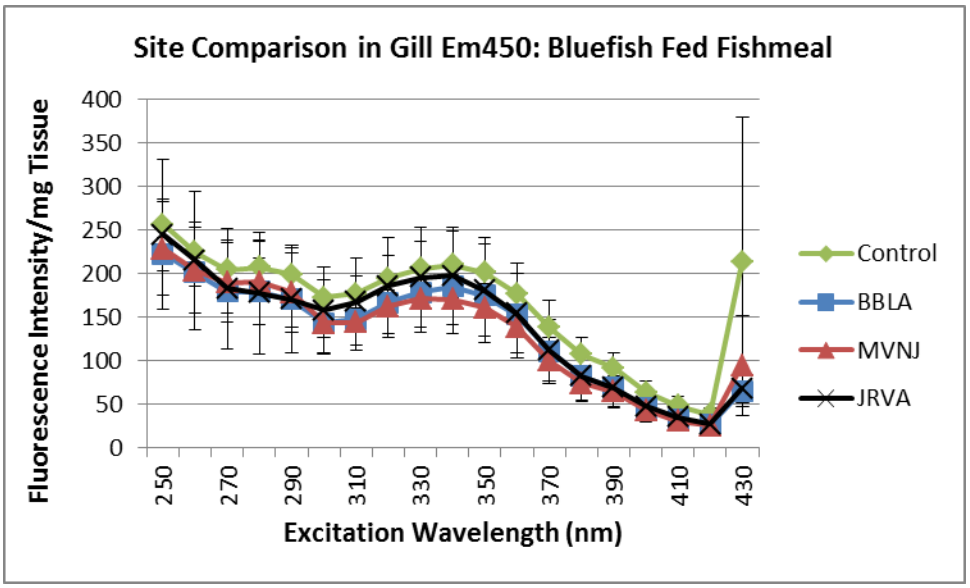

Figure 6.3.4.1 Comparison of Em450 spectra for gill in bluefish fed menhaden fishmeal. Spectra are shown for Control, Barataria Bay, LA (BBLA), Delaware Bay, NJ (MVNJ) and James River, VA (JRVA). Data are average fluorescence intensity (RFU) per mg tissue ± SD. N=2-5 bluefish per treatment (see Table 6.3.4.1). Note similarity between sites indicating no treatment-related effects. Note that this spectrum had similarities to that for control bluefish used in the fish oil experiment (Figure 6.3.3.2). This suggested that the bluefish had pre-existing levels of PAHs and/or were taking up PAHs or some other fluorescent compound while being held in the laboratory.

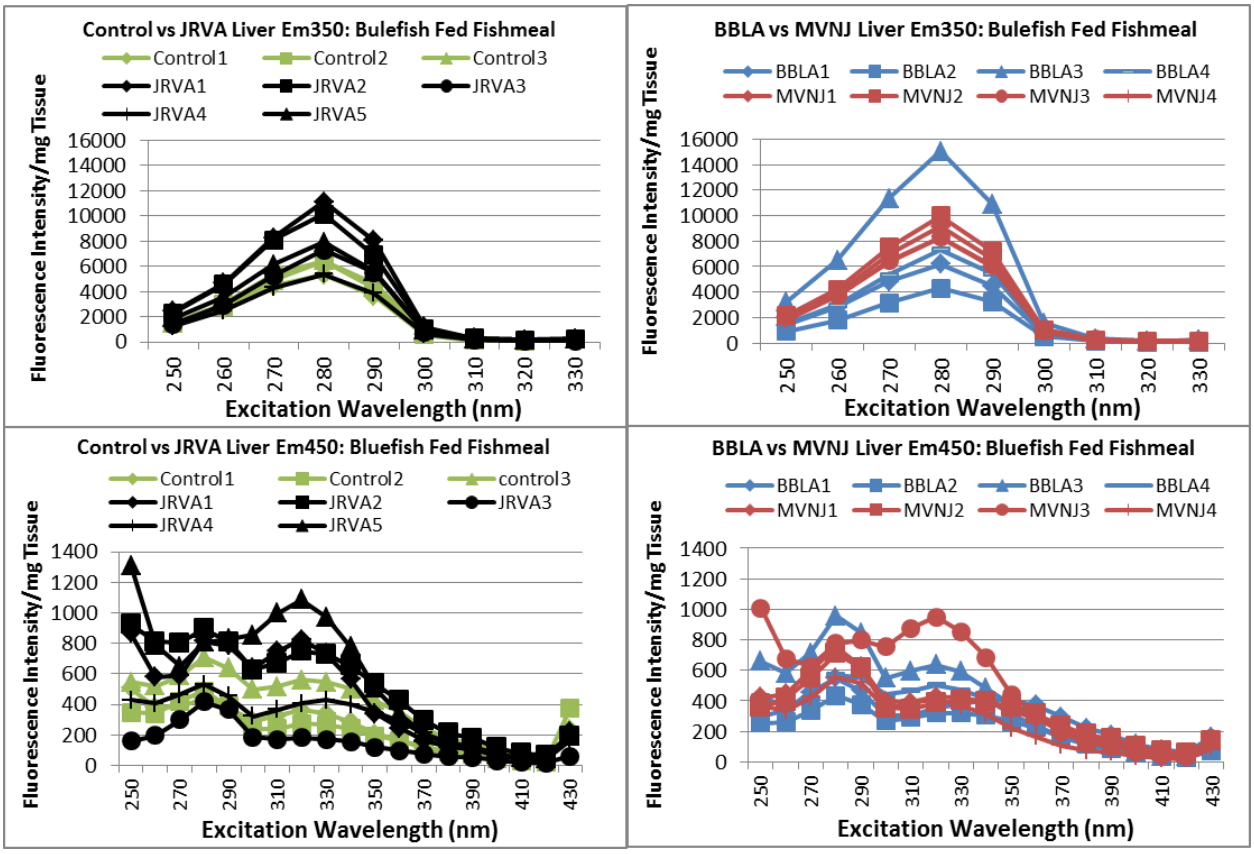

Figure 6.3.4.2 Comparison of sites using Em450 spectra in liver of bluefish fed menhaden fishmeal. Three sites from which menhaden were collected and control are shown. Each spectrum represents a single bluefish in its treatment group, n=3-5. The left two graphs compare control and menhaden from James River, VA (JRVA). The right two graphs compare menhaden from Barataria Bay, LA (BBLA) and Delaware River, NJ (MVNJ). The top two graphs show spectra generated by fixing the Em at 350 nm (HNP-like PAHs). The bottom two graphs show spectra generated by fixing the Em at 450 nm (HPT and HPY-like PAHs). Note the similar spectra at Em350 regardless of treatment. Note that three of five fish in JRVA show higher levels of fluorescence and higher peaks at Ex250 relative to other major peaks: this indicated up take of high MW PAHs from diet. This also occurred for one of four fish in MVNJ.

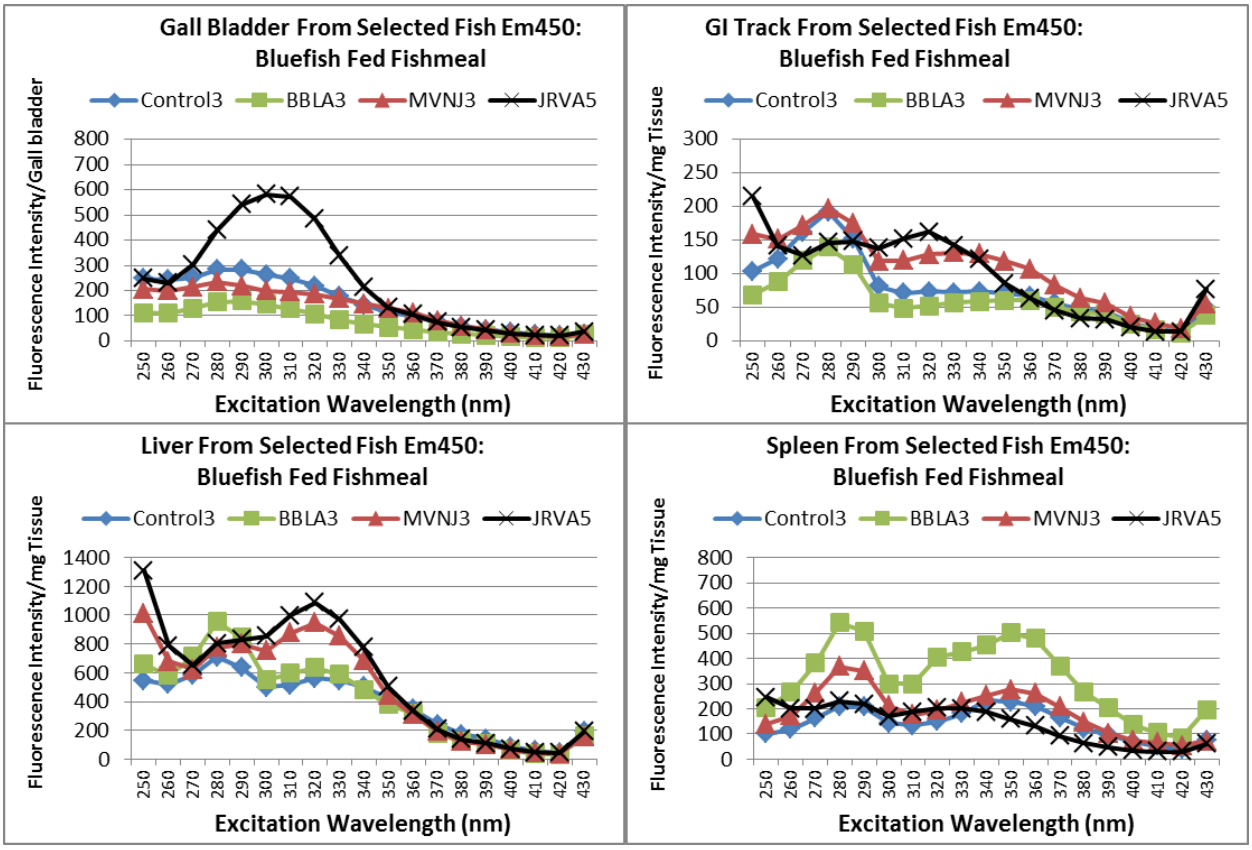

Figure 6.3.4.3. Comparison of tissues from bluefish fed fishmeal made from menhaden collected at different sites. Each graph represents one of four tissues: gall bladder, liver, GI track and spleen. Each spectrum within a graph was generated at fixed Em450 and represents one fish from a particular treatment. Treatments included control and menhaden collected in fall 2010 from Barataria, Bay, LA, Delaware Bay, NJ or James River, VA. For example, MVNJ3= bluefish 3 (n=4) treated with menhaden collected from Delaware Bay, NJ. Different tissues for a particular treatment were from the same fish. Note how spectra for liver, GI track and spleen of JRVA differ from those of other treatments, except MVNJ3 liver. The similar spectrum in all three tissues of JRVA indicated uptake of PAHs from diet and distribution to other tissues. The large peak at Ex310 in gall bladder of JRVA seemed to encompass peaks Ex280 and 320 and so also follow the trend.

VI.3.5 Summary of Trophic Studies

Trophic studies provided strong evidence of absorption and distribution of PAHs that were incorporated into menhaden fish oil, DWH crude oil and menhaden fishmeal. This occurred over a short period of time, either 24 or 96 hours. However, data were complicated by the low number of test organisms per group, what appeared to be pre-existing PAHs in test organisms, and natural compounds such as vitamin A and E that fluorescence in the same wavelength ranges as PAHs.
In the three trophic studies, FEFS analyses showed PAH-like compounds in all tissues tested (GI track, liver, gill, spleen and gall bladder contents). Liver consistently had high concentrations of PAH-like compounds; although, there were some tissue and species related differences. In silver perch, GI track had the highest levels of HNP-like PAHs while liver had the highest concentrations of HPT and HPY-like PAHs. Both gill and gall bladder had more HNP-like than HPT and HPY-like PAHs. The reason for these differences was unknown; however, they demonstrated tissue specific differences in PAH burdens. In bluefish, liver had the highest levels of HNP and HPT/HPY-like PAHs. The 1st bluefish study also showed high levels of HPT/HPY-like PAHs in gill compared to GI track and spleen. This differed from the 2nd bluefish study where liver had significantly higher levels than gill of all three types of PAHs. The two bluefish experiments were consistent in that liver always had high PAH levels and spleen and GI track had relatively low levels. Comparing tissue concentrations of different PAHs showed that HPT-like PAHs were usually higher than HNP-like PAHs which were higher than HPY-like PAHs. The only exception was GI track in which concentrations of HNP-like and HPT-like PAHs were similar. This relationship among PAH concentrations was found in all three studies. While more work is needed here, data suggested that, while similar levels of HNP and HPT-like PAHs were in GI track, less HNP-like PAHs were absorbed and/or were more quickly eliminated. This resulted in proportionally lower concentrations of HNP than HPT-like PAHs in tissues. More rapid metabolism and elimination of 2-3 versus 4-6 ring PAHs would be consistent with the literature (Pesonen et al, 1987, Kreitsberg et al, 2010). Also, more HNP has been found in bile than urine (Kreitsberg et al, 2010). From the gall bladder, HNP would have been released back into the GI track.

Treatment related effects were assessed by analyzing specific wavelengths for three major types of PAHs as well as examining and comparing fluorescence spectra of tissue samples. A major peak was chosen for each type of PAH, and it was used to convert fluorescence intensity into µg PAH/mg of tissue dry weight. The major peaks were Em350/Ex280, Em450/Ex250 and Em450/Ex350 for HNP, HPT and HPY-like PAHs, respectively. This data showed significant differences between tissue concentrations of PAHs and types of PAHs as presented above; however, there was too much variability among individuals within a group to show statistically significant treatment related effects. Our first evidence of PAH absorption was found by comparing spectra of individual silver perch and not by comparing treatment group averages of specific wavelengths. Comparison of Em450 spectra showed that 3 of 6 perch fed spiked fish oil (EPNJ-spike) had modified liver spectra. The modified spectra included more intense peaks and/or major peaks at excitation wavelengths different than control (Figure 6.3.2.1). The modified spectra in 3 of 6 EPNJ-spike fish as well as similar spectra for the other 3 treated fish and control indicated that something different was being absorbed by the fish with modified spectra. Data for fish treated with DWH crude oil also showed modified excitation peaks in liver of some fish using fixed Em450 (Figure 6.3.2.2). This indicated that both EPNJ-spike and DWH crude oil had sufficient levels of PAHs to be detected in liver. However, none of the perch given fish oil only showed spectra different

than control. This indicated insufficient levels of PAHs or exposure time for trophic transfer using the fish oils tested.

Support for the absorption and distribution of PAH-like compounds was found by comparing spectra in the different tissues of the same fish (Figure 6.3.2.3). In silver perch treated with EPNJ-spike, those with modified liver spectra also had similarly modified spectra for gill and gall bladder. In particular, liver, gill and gall bladder all showed a higher peak at Em450/Ex250 compared to control and unaffected fish. These results in silver perch indicated that PAHs could be absorbed from the fish oil matrix and crude oil. Similar findings occurred in bluefish studies. Comparison of liver from control and fish gavaged with DWH crude oil showed modified spectra in all three DWH fish (Figure 6.3.3.1). In their spectra, Ex250 and 280 became more intense peaks while Ex330 became less intense when compared to control (Figure 6.3.3.1). Two of the DWH fish (fish 1 and 3) had liver spectra that matched their GI track spectra. Fish 2 showed a GI track spectrum similar to fish 3; however, its liver spectrum was very different, it indicated high levels of BaP. This BaP spectrum was not seen in any other fish, and the reason for it was unknown. An interesting finding in bluefish studies was the similarity between GI track and spleen spectra for a particular fish (Figure 6.3.3.3). Repeated comparisons showed that the number of peaks, types of peaks and their intensity matched. This provided strong evidence that PAHs in diet could be absorbed and circulated through the blood to other tissues. The similarity between GI track and spleen was thought to be due to high perfusion of spleen with blood.

The lack of detection of PAHs in unspiked fish oil as well as the high individual variation within treatment groups led to testing PAH transfer from menhaden fishmeal over 96 hours. This experiment was done in bluefish. Fishmeal from MVNJ, JRVA and BBLA were used. Fish oil from MVNJ and JRVA had shown high levels of PAH-like compounds using FEFS while levels in BBLA fish oil were lower. Results were strikingly similar to those using fish oil. Meaning, gill typically had a similar spectrum for all treatments that suggested the presence of pre-existing PAH-like compounds (Figure 6.3.4.1). Liver typically had a major peak at Em450/Ex320 indicative of vitamin A. In terms of treatment related effects, 3 of 5 fish fed JRVA fishmeal showed modified spectra compared to control and to most of the fish in other treatment groups (Figure 6.3.4.2). An increased intensity of the Em450/Ex250 peak characterized the absorption of PAHs from the diet into liver. Also, similar spectra in the different tissues of the same fish again supported the uptake of PAHs from diet and their distribution through blood to the other tissues (Figure 6.3.4.3). Taken together, results showed that comparing spectra instead of individual wavelengths and comparing different tissues was necessary in order to detect trophic transfer of PAHs in these studies.

VII. Project Conclusions

This project was designed to address the following scientific questions: 1) Is there a distinctive chemical "fingerprint" for the DWH oil that can be detected in contaminated menhaden fish oil? 2) What is the concentration of PAHs in contaminated fish oil and PAH metabolites in fish liver? 3) Is there a relationship between histopathological effects and tissue levels of oil contamination? 4) Are the contaminants in oily prey fish transferred to predatory fish?

Several types of experiments were conducted using menhaden collected from different sites- Delaware Bay, NJ, James River, VA and Barataria Bay, LA. No major crude oil spills had been report in NJ or VA for 2010 in the areas where fish were collected; however, it was anticipated that these fish were exposed to background levels of PAHs due to urbanization. The rationale was that studying these populations would help distinguish urban signals of PAHs from those of crude oil as well as provide pre-oil spill data for NJ and VA menhaden. Barataria Bay was a site in LA where significant amounts of crude oil from the DWH came ashore in 2010. The fish were collected in October whereas the broken oil pipe was capped in July. Oil sheens attributed to the DWH spill were seen in August and September in the area, but there was some time for the fish to eliminate oil related compounds prior to capture.

The answers to our scientific questions in brief are as follows. None of the analytical methods performed could discriminate a distinctive fingerprint associated with DWH oil; however, a ratio of low MW PAHs to high MW PAHs did appear to detect crude oil exposure in LA fish. PAHs were detected in fish oil and fish liver of all fish tested. Fish liver had at least 10 times higher levels of PAHs than found in fish oil: concentrations were in the parts per million using FEFS and GC-MS and in the parts per billion using HS-SPME GC-MS. Small LA menhaden present during the oil spill showed significant histological tissue damage; however, this was not related to chemical body burden as total PAHs were similar to those found in NJ menhaden which showed no significant tissue damage. It was likely that the level of PAHs that caused the damage had been eliminated while the damage remained. Trophic transfer studies showed that PAHs can be absorbed by predatory fish such as silver perch and bluefish. Although there was a lot of individual variability within treatments, absorption and distribution could be tracked by changes in fluorescence spectra. PAHs found in DWH crude oil, PAH spiked fish oil and menhaden fishmeal transferred from the diet to organs as far away as the spleen and gill. Below is a summary table including the experiments performed, test organism and/or biological material tested and major findings.

Table 7.1 Summary of major findings by experiment

Experiment	Test Organism/Material	Major Findings
Fish oil preparation	Large menhaden	Similar amounts of fish oil per fish were obtained from NJ and LA menhaden even though they were different species. Approximately 1/10 of that amount was obtained from VA fish.
HS-SPME GC-MS	Large menhaden fish oil	PAHs in fish oil were low at all sites, µg/L levels. Fluoranthrene and pyrene concentrations were similar in NJ and LA fish, while anthracene was higher in VA fish and in NJ fish caught near shore. Chromatograms showed similarities and differences between fish oils but not enough samples were tested to establish a petroleum-related finger print.
FEFS	Large menhaden fish oil	A standard method was developed for FEFS. Two major types of spectra were found that were not related to species or site but rather PAH concentration and sampling processes. More refinement is needed. Fish oil from LA had a higher level of naphthol-like PAHs (HNP) and similar or lower levels of hydroxypyrene-like PAHs (HPY) than other sites. This resulted in a high HNP/HPY ratio which is indicative of petroleum exposure. Therefore, while sources of petroleum could not be determined using FEFS, ratios of HNP/HPY proved a promising biomarker for petroleum exposure.
FEFS	Large menhaden liver	FEFS analyses showed the same two major types of spectra found in fish oil. PAHs were much higher in liver than fish oil. In liver, LA fish had lower levels of PAHs than found in NJ and VA fish. VA fish appeared highly contaminated. HNP/HPY ratios in liver were similar between sites but much higher than found in fish oil. This suggested that HNP-like PAHs were being eliminated from liver so that less of them were accumulating in fish oil.
Histopathology	Small menhaden	Small menhaden from NJ and LA were strikingly different in terms of tissue damage. Notable damage occurred in gill of LA fish which was considered a permanent change. This indicated that crude oil could affect the filter feeding ability of exposed menhaden. Liver necrosis, bile duct dilation and Cholangioma like lesions were also found in LA fish. The types of damage indicated exposure to crude oil in the recent past and continuing irritation. Small NJ menhaden appeared in good health overall.
GC-MS	Small whole body menhaden	PAH concentrations found in whole body samples were low, ng/mg, for both LA and NJ fish. The majority of low MW PAHs detected were alkylated phenanthrenes. These PAHs were higher in NJ than LA fish (Phenanthrene was not detected in fish oil analyzed by HPS-SPME GC-MS.). High MW PAHs were only

		detected in LA fish. The larger size of LA fish may have accounted for the detection of high MW PAHs only in them.
FEFS	Small menhaden GI track tissues	GI track tissues were analyzed in small menhaden as insufficient amounts of fish oil could be generated and liver tissue could not be cleanly dissected from other GI tissues. FEFS could not detect specific PAHs. Spectra best matched naphthol and hydroxyfluorene for low MW PAHs and pyrene and fluoranthene for high MW PAHs. Spectra for NJ and LA fish looked similar; therefore, FEFS could not be used to finger print a particular source of crude oil. Concentrations of HNP-like PAHs were similar between LA and NJ fish; however, NJ fish had higher levels of HPY-like PAHs. This resulted in a high HNP/HPY ratio for LA fish, which is associated with petroleum exposure, and similar to what was found in fish oil. Total PAHs were similar between NJ and LA fish and not consistent with histological findings. This indicated that the damage to LA fish remained after the toxic levels of PAHs had been eliminated.
Trophic Transfer detected by FEFS	Silver Perch fed fish oil, 24 h exposure	PAHs were detected in all tissues and individuals including controls. Silver perch accumulated PAHs when fed DWH crude oil or PAH spiked fish oil. Concentrations in fish oil alone were too low for detection of transfer; however, the fish oils tested in this experiment were found to have relatively low PAHs compared to other fish oil samples. Comparison of tissues showed different tissues preferentially accumulated different PAHs: HNP was high in GI track compared to liver and gill, HPY and phenanthrol (HPN- a metabolite of phenanthrene) was highest in liver. Statistically significant differences among treatments were not detected due to high variability between individuals. This was likely due to difficulties with dosing and possibly PAH accumulation prior to treatment (The silver perch were wild fish collected in Tuckerton, NJ). Transfer of PAHs from spiked fish oil (spiked with naphthalene and phenanthrene) and from DWH crude oil to perch could be detected in some of the treated individuals. The transfer was detected by specific changes in their fluorescence spectrum. Most notably an increase at Emission450/Excitation250 (associated with phenanthrene exposure) compared to other peaks. Changes to the spectrum of liver also occurred in gall bladder and gill. This showed distribution of PAHs administered in diet to other organs in the fish.
	Bluefish fed fish oil, 24 h exposure	As with silver perch, PAHs were detected in all bluefish tissues and individuals including controls, indicating pretreatment exposure. The relative concentrations of PAHs in bluefish

		tissues were somewhat different than for silver perch suggesting species differences in metabolism and distribution. HPT had the highest concentration in all tissues measured. Statistical differences were not found between treatments again due to high variability among individuals within a treatment. However, comparison of spectra definitely showed treatment effects associated with absorption and distribution of PAHs in bluefish fed DWH crude oil and spiked fish oil. The types of changes in the spectra indicated absorption and distribution of phenanthrene. These PAHs were absorbed from the GI track and distributed through the circulatory system to spleen and gill. Feeding fish oil alone usually generated spectra similar to control, indicating insufficient amounts or time for PAHs to transfer from fish oil to ōfʤẐŦɬăK tissues.
	Bluefish fed fish meal, 96 h exposure	Bluefish fed menhaden fishmeal instead of fish oil did show evidence of PAH absorption and distribution from unspiked biological material. PAHs were detected in all bluefish tissues including controls. As before, liver contained the highest levels of PAHs, and HPT was the dominant PAH detected in most tissues. Statistical analyses of specific wavelengths for PAHs did not show significant treatment effects. However, as before, comparison of spectra in control and some treated fish showed changes associated with absorption of PAHs from diet. PAHs were absorbed by 3 of 5 bluefish fed fishmeal of VA menhaden and 1 of 4 bluefish fed fishmeal of NJ menhaden. Bluefish fed fishmeal of LA menhaden were all similar to control. These results were support by chemical analyses of fish oil in which the NJ (MVNJ) and VA (JRVA) samples were found to contained high levels of PAHs. Some of the variability within a treatment might be attributed to different levels of fishmeal consumption by individuals housed together. Distribution of the PAHs could be tracked as spectral changes in one tissue of an effected fish were similar to those in other tissues.

VIII. REFERENCES

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