Spring 5-2018

Correlation of Environmental Exposure to Polycyclic Aromatic Hydrocarbons and Polymorphisms in the Proto-oncogene v-Rel using Wild Atlantic Menhaden

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By:

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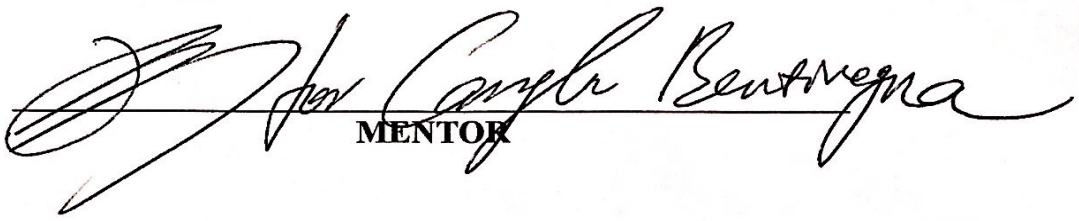
Submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology from the Department of Biological Sciences of Seton Hall University
May 2018

Approved By



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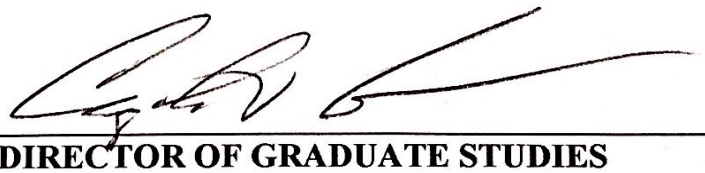

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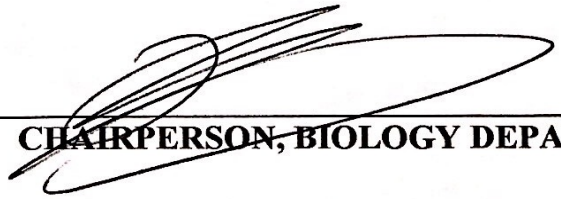

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Acknowledgements

This work is part of the US Geological Survey's response to Hurricane Sandy funded by Disaster Relief Appropriations Act of 2013 (PL 113-2). Special acknowledgement is given to the staff of NJ Fish and Wildlife for assistance with fish collection. Thank you specifically to the Department of Biological Sciences for making this research possible and allowing me to accomplish both a Bachelor and Master of Science degrees at Seton Hall University.

I would like to express my appreciation and thanks to my mentor and PI, Dr. Carolyn Bentivegna, for guidance, patience, and welcoming me into her laboratory with a wonderful project. Her dedication and encouragement allowed me to fully experience environmental toxicology. To this day I cannot thank her enough for guiding and helping me as a brilliant mentor, teacher, advisor, and friend. We all will miss her tremendously and hope that she is honored and remembered for all the lives that she has influenced and touched.

Thank you to Dr. Wyatt Murphy from the Department of Chemistry and Biochemistry for mentoring me as an undergraduate and exposing me to polycyclic aromatic hydrocarbon research. Also, I would like to acknowledge you for collaborating with this project and helping to fund our summer 2015 publication. I appreciate your help in assisting me with my final corrections and edits as a co-mentor.

Much thanks to my lab partners for assisting me with advice with various protocols for optimization of results: Frank Zadlock IV, Satshil Rana, Junyoung Kim, Gilbert Sharp, Allison Nadler, and Lauren Clark. To all my classmates: thank you for endless friendships, laboratory advice, laughs, and wonderful memories completing my years at SHU.

Thank you to Dr. Heping Zhou, Dr. Wyatt Murphy, and Dr. Allan Blake for representing as my thesis committee to help me throughout this process with their wisdom, support, and guidance.

Special thanks and love goes to all my family and friends for their constant support, motivation, and belief in me.

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Abstract:

Hurricane Sandy critically damaged the Atlantic coast of New Jersey in the fall of 2012. This was recorded as the largest storm to hit the Atlantic Ocean, which was manifested by its destruction on the homes and communities of those along the coastline. Contamination of surrounding waters due to this natural disaster was of great interest. The re-suspension of land-based pollution is proposed to make these contaminants more bioavailable to coastal species, such as Atlantic menhaden (*Brevoortia tyrannus*), which are a crucial biomonitoring species through their ecological and economic uses. A proposed increase of body burdens in Atlantic menhaden could affect human health through toxicity in the aquatic food chain via consumption of other marine species.

Atlantic menhaden were caught off the shores of the Atlantic coast surrounding New Jersey and were dissected for their raw fish oil, which was extracted from the oily-natured skin and studied with fluorescence spectroscopy to identify the presence of possible contaminants, such as polycyclic aromatic hydrocarbons (PAHs). Different levels of PAH-like substances were identified in catches from 2011, 2012, and 2013. Genomic analysis was used to study the effects of PAH-like substances on cancer-related genes, specifically proto-oncogene v-Rel (*V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog*). The presence of polymorphisms in v-Rel was studied in the previously caught Atlantic menhaden exposed to PAH pollution along the coastline of New Jersey.

Analysis of the results indicated that there was no statistical difference in PAH-like compound concentrations or fluorescence patterns of raw fish oils collected before and after Hurricane Sandy. Excitation-Emission Matrix Scanning (EEMS) scans of raw fish oil from catches exposed to high amounts of PAH-like substances showed strong fluorescent signals yet

few isomers and high genetic relatedness via gel electrophoresis analysis. EEM scans of raw fish oil with low and decreased fluorescence signals showed prominent bands with extreme variability, resulting in evident polymorphisms. It was found that high PAH concentration samples were associated together with low v-Rel variability and low PAH concentration samples were associated together with high v-Rel variability.

Introduction:

Polycyclic Aromatic Hydrocarbons are ubiquitous organic compounds that are released into the environment through a variety of processes, such as the burning of fossil fuels and crude oil spills (Rengarajan, 2015). Hydrocarbons are characterized by the presence of multiple aromatic rings containing the elements carbon and hydrogen. Their physical and chemical behaviors depend on their molecular weights and various means of environmental distribution. PAHs in the environment are a concern largely due to their carcinogenicity. Four-, five-, and six-ring PAHs appear to be more carcinogenic than those with smaller or larger ring systems. Highly angular configurations tend to be more carcinogenic than linear ring systems. Degrees of carcinogenicity are related to the structure and reactivity of the major metabolites produced (Neff 1985). PAHs act as stable aquatic pollutants. Owing to their lipophilic nature, they can easily penetrate the biological membranes and accumulate in organisms. These dangerous substances can affect bodies through molecular deformation and mutagenesis (Tuvikene 1995).

PAHs are abundantly found in substances such as coal, crude oil, tobacco, pesticides, and vehicle exhaust. Highly urbanized areas are major locations for mutagenic PAHs due to surrounding hazardous waste sites and trash incineration facilities (Bentivegna, 2016). The ingestion and respiration of such compounds for long periods of time can predictably cause adverse health effects and cancer. PAHs are also a major toxicity concern for aquatic wildlife. After entering water, these PAHs quickly become adsorbed onto organic and inorganic particulate matter and are mostly deposited in bottom sediments; this allows them to be readily accessible to most marine life for consumption. These PAHs that enter aquatic environments are localized in rivers,

estuaries and coastal waters (Tuvikene 1995). New Jersey, having coastal Atlantic waters and areas near city harbors, is a prime example of an industrial region of interest at risk for PAH contamination. Previous research has shown PAH-like substances in Atlantic menhaden fish oil (Pena et. al, 2015).

Atlantic menhaden are a North American species of fish that migrate along the Atlantic coast from Nova Scotia to Northern Florida. They are efficient filter feeders that act as important components of the food chain, providing a link between primary production and higher organisms by consuming plankton and providing forage for species such as striped bass, bluefish, and weakfish (ASMFC 2016). Atlantic menhaden play an integral part in the aquatic food web and industrial economy. They are used as common ingredients in many products such as pharmaceutical fish oils, fertilizers, and animal feed (Bentivegna 2016). Menhaden are colloquially known as bunker fish, and act as a staple food for many commercially important predator fish – which are harvested along the coast of different states. Ospreys, loons, and seagulls scoop menhaden from the top of the water column. As a result of their diet of tiny plankton and algae, menhaden are full of nutrient-rich oils (Schleifer 2014).

Atlantic menhaden are known for their oily nature and may therefore retain PAHs due to their feeding style and the lipophilic characteristics of PAHs. Menhaden feed largely within several meters of the ocean surface, so they are likely to come into direct contact with dispersed oil droplets. Menhaden may accumulate petroleum oil contaminants and potentially spread them to predatory fish when ingested (Pena et. al, 2015). The hydrophobic nature of PAHs and high body oil content of menhaden, may allow these fish to act as a primary biomonitoring species.

This project specifically focused on menhaden found at the Atlantic coast locations of Point Pleasant, Atlantic City, and Delaware Bay. Coastal species such as Atlantic menhaden are readily exposed to anthropogenic PAHs from urban areas. Recent studies have shown accumulation of PAHs in Atlantic menhaden collected along the New Jersey coast in 2010 and 2011 (Pena et. al 2015). Severely destructive weather, such as tropical storm events, may have an influence on increasing PAH exposure to aquatic wildlife. Superstorm Sandy, also known as "Hurricane Sandy," destructively damaged the Atlantic coast of New Jersey on October 29, 2012 (Crawford et. al 1994). The major hypothesis of this project was that Hurricane Sandy increased PAH exposure to menhaden.

Hurricane Sandy was the largest storm ever recorded in the Atlantic Ocean (Manuel 2013). It reached more than 1,000 miles in diameter and affected states from Florida to Maine. In terms of immediate impact, the greatest health threat came from the storm surge that swept into densely populated communities along the New Jersey shore, Long Island, and Lower Manhattan (Manuel 2013). Along with extensive damage of homes and communities, water pollution was a major concern during the aftermath. The storm knocked out power or damaged some 80 sewage treatment systems in that state, including the Passaic Valley Sewerage Commission, one of the largest sewage treatment plants in the country, estimating that as much as 2.75 billion gallons of untreated waste flowed from the plant into the nearby bay during the five days the plant was out of commission. Raw sewage spillage is one of the several ways PAHs may have entered and contaminated the waters (Manuel 2013). Damaging winds and rising waters could hypothetically re-suspend land-based contaminants, making them more bioavailable to

coastal species such as Atlantic menhaden, as indicated by increased body burdens of accumulated toxins. If body burdens increased, these fish could spread accumulated PAHs to predacious fish, which may result in the chance of affecting human health through consumption of marine species.

The potential impact of Hurricane Sandy on PAH exposure can be compared to impacts of the *Deepwater Horizon* (DWH) oil spill, occurring in April 2010 near the Gulf of Mexico. Following the DWH oil spill, PAH exposure was linked to effects in fish characterized by alterations in genome expression, that were predictive of exposure to hydrocarbon-like chemicals and indicative of physiological and reproductive impairment. (Whitehead et al 2011). In this study, adult Gulf killifish (*Fundulus grandis*), which is also a consumed species by other predatory fish, were used as a model species to study the harmful effects of PAH presence. Tissue morphology and expression of CYP1A protein, a common biomarker for exposure to select PAHs, were characterized for gills, the organ that provides the greatest surface area in direct contact with the surrounding aquatic environment. Results for gill morphology and nucleic acids showed evidence of PAH exposure and damaged tissues. In the present investigation, menhaden may have had a greater susceptibility than killifish to PAHs due to their oily nature and exposure of gill during filter feeding. In contrast to Gulf killifish, Atlantic killifish prey on zooplankton but are not filter feeders.

To detect the presence and quantity of PAH-like substances, fluorescence spectroscopy was used due to its rapid efficiency and various means of characterizing data. One type of fluorescence spectroscopy is excitation-emission matrix scanning, which generates three-dimensional data of a selected target (PerkinElmer 2000).

Fluorescence spectroscopy allows a beam of light to travel through a sample holder and detector at specified excitation and emission wavelengths. Compounds in solution can be identified based on which wavelengths they absorb light and emit fluorescence (PerkinElmer 2000). By comparing the 3D scans to standards, the types and quantities of compounds can be elucidated. However, many excitation and emission wavelengths of PAHs and other fluorescent compounds overlap, so resulting products are referred to as PAH-like (Davidson 2015). When measuring the fluorescence of Atlantic menhaden fish oil, previously established PAH standard wavelengths were used to determine the presence of PAH-like substances.

The presence of PAHs has been reported in the marine environment: sea water, sediments, and plankton, seaweed, and filter feeding organisms. The application of fluorescence techniques is important to establish simple, sensitive, and reliable methods for the determination of these compounds in marine wildlife and samples. (Dossier 2000). Although EEMS has been previously used to detect PAH-like presence in Atlantic menhaden fish oil, there are several other forms of fluorescence spectroscopy to establish PAH detection. Conventional fluorescence spectroscopy, synchronous fluorescence spectroscopy, and high-performance liquid chromatography (HPLC) with fluorescence detection are additional simple analytical techniques to determine PAHs. These techniques are essential in identification and quantification of environmental pollutants, which has resulted in important advances for the analysis of PAHs in the marine environment (Dossier 2000). They can be grouped as extreme sensitive techniques due to the direct measurement of light emission, while allowing the correction and removal of background interference.

Conventional fluorescence spectroscopy generates an emission spectrum by scanning the emission wavelength of a sample, which is illuminated at a specific single excitation wavelength. To obtain the excitation spectrum, the excitation wavelength is scanned while the emission signal is recorded at a specific single wavelength (Dossier 2000). This method is simple, has low detection limits, and allows the use of conventional instruments to determine PAHs in marine samples. When multiple compounds with high structural similarity need to be determined, conventional fluorescence spectroscopy is much too limited. Synchronous fluorescence spectroscopy allows the simultaneous scanning of both the excitation and emission wavelengths with a constant wavelength interval between them. The importance of synchronous scanning lies within the improvement of spectral resolution and the correct analysis of fluorophore overlap.

Although the technique has been successful with the analysis of polycyclic aromatic hydrocarbons in forensic cases, its application in the biology research has been limited (Rao 1991). A study was conducted at the University of Kelaniya in Sri Lanka using conventional and synchronous fluorescent spectroscopy to evaluate bile fluorescence patterns of Feral Nile tilapia as biomarkers of PAH exposure in contaminated tropical waters (Hemachandra 2011). The combination of both fluorescence techniques allowed detection of naphthalene-, phenanthrene-, pyrene-, and benzo(a)pyrene- type metabolites in the waters of Weras Ganga (Hemachandra 2011).

Aside from luminescence spectroscopy, fluorescence detection can also be paired with liquid chromatography. Liquid chromatography using fluorescence detection and gas chromatography coupled to mass spectroscopy (GC/MS) are the most powerful

techniques for monitoring PAHs. Liquid fluorescence chromatography (LC) can measure some PAH isomers that cannot be easily quantified by GC/MS due to the absence of elution. Because of its excellent separation and detection performance, LC has been specified as the method of choice by the U.S. EPA for the analyses of aqueous effluents for the determination of PAHs (Dossier 2000). High performance liquid chromatography (HPLC) separates, identifies trace concentrations, and quantifies components dissolved in a liquid solvent with a high analytical resolution by a moving gas stream of helium or nitrogen (Linde 2017).

The expression of PAHs and PAH-like compounds issues an investigation of their effects on cancer-related genes, specifically proto-oncogenes. Some PAHs are mutagenic; therefore, they could have a detrimental effect on the genome. After exposure and during metabolism, PAHs can form reactive epoxides that can covalently bind to DNA. These PAH-DNA adducts are established markers of cancer risk. PAH exposure has been associated with epigenetic alterations, including genomic cytosine methylation. Both global hypomethylation and hypermethylation of specific genes have been associated with cancer (Herbstman 2012).

A study was conducted at The Columbia Children's Center for Environmental Health (CCCEH) in New York, New York, to determine whether prenatal exposure to PAHs is associated with altered global methylation in human umbilical cord white blood cells and to determine whether methylation is associated with the presence of detectable PAH-DNA adducts (Herbstman 2012). PAH metabolite levels were measured in prenatal urine and DNA was isolated from umbilical cord leukocytes to be analyzed for global DNA methylation. It was found that newborns with detectable benzo(a)pyrene (BaP) –

DNA adducts had higher levels of genomic methylation than those with non-detectable adducts. The study had concluded that environmental airborne chemicals had the ability to form such adducts and alter methylation, which further research and study could help develop strategies to reduce health risks.

The linkage between DNA adducts and mutations in oncogenes due to carcinogenic environmental PAHs has also been studied in strain A/J mice at the Health Effects Research Laboratory in North Carolina (Nesnow 1995). A/J inbred mice are commonly used as cancer models for carcinogenic testing due to their high susceptibility to carcinogen-induced tumors (Jackson Laboratory 2017). The 6-8 week old male strain A/J mice each received single doses of varying five PAHs: benzo(a)pyrene, benzo(b)fluoranthene, dibenz[a,h]anthracene, 5-methylchrysene, and cyclopenta[cd]pyrene. After 8 months, tumors were excised from lung tissue and used for DNA analysis. Responses ranged from 0.6 lung adenomas per mouse to 97.7 lung adenomas per mouse, depending on the potency of each PAH dose-response relationship. Specific DNA adducts were identified, benzo(a)pyrene being the most significant. Seventy-four to 90% of the tumors examined exhibited mutations in Ki-ras oncogene, which was performed by PCR and dideoxy sequencing methods. Highly significant levels of GGT → CGT mutations from PAH-induced tumors were found (Nesnow 1995). Consequently, the environmental PAHs used in study had the ability to mutate specific oncogene Ki-ras and drive unpredictable levels of tumor-genesis.

A proto-oncogene is a gene coding for proteins that regulate cell growth, stimulate cell division, and control apoptosis. They have the ability to transform into an oncogene when a mutation in DNA sequence occurs due to cancer-promoting agents such

as UV light, chemicals, or varying carcinogens. Several cancer syndromes are caused by inherited mutations of proto-oncogenes that cause oncogene activations, but most cancer-causing mutations involving oncogenes are acquired, not inherited (American Cancer Society 2017). Oncogenes are generally activated by chromosome rearrangements which allows one gene to activate the other, and gene duplication, which can result in the excess production of a certain protein (American Cancer Society 2017).

The majority of cancers can be attributed to acquired mutations, meaning that these mutations only occur in the tissue that is affected by cancer and are not passed on to offspring. These changes occur at the cellular level after birth as a result of environmental exposures, lifestyle behaviors, or random change alone (Stanford Health Care 2017). Highly oncogenic retroviruses are retrovirus vectors that contain inserted sequences called oncogenes, which cause transformation of infected target cells. Highly oncogenic retroviruses cause a transformation of infected target cells shortly after infection as a result. In contrast, the normal expression of the related proto-oncogene sequences in situ in normal cells does not cause transformation (Wilhelmsen et al 1984).

Tobacco smoke carcinogens are one of the most established and evident types of PAH exposure. It is estimated that cigarette smoking kills 1,000,000 people each year by causing lung cancer as well as many other neoplasms (Pfeifer 2002). P53 mutations are frequently found in tobacco-related cancers, with a mutation load much higher in smoker-associated cancer, rather than nonsmoker-associated cancer (Pfeifer 2002). The p53 gene is a tumor-suppressor gene. Normal p53 protein monitors DNA by destroying cells that have irreparable damage to their DNA. Abnormal p53 protein fails to halt cell division when necessary to repair damaged DNA. As these damaged cells proliferate, cancer

develops (Reece 2014). The p53 mutational patterns are different between smokers and nonsmokers due to an excess of Guanine to Thymine transversions in smoking-associated cancers. Studies have indicated that there is a strong coincidence of G to T transversion hotspots in lung cancers and sites of preferential formation of PAH adducts along the p53 gene (Pfeifer 2002). PAH accumulation, specifically similar to tobacco-smoke carcinogen, has been proven to effect mutations in the p53 tumor-suppressant gene. In additional studies, the most commonly studied PAH is BaP, which is transformed in vivo into BP-7,8-epoxide by CYP1A1 gene via the CYP/EH pathway, and is further oxidized to form BP-7,8-dihydrodiol-9,10-epoxide (BPDE), the ultimate carcinogen (Beresford 1993). BPDE reacts with DNA to produce adducts that have been identified in lung tissues of smokers and may cause mutations that are observed in p53 tumor suppressor gene and KRAS oncogene (Pfeifer 2002).

Avian Reticuloendotheliosis Viral (v-Rel) *Oncogene Homolog* is an example of a proto-oncogene whose mutations or amplifications are associated with B-cell lymphomas, directly affecting white blood cells and the immune system (NCBI Gene ID: REL). Proto-oncogene v-Rel has developed as a valuable tool to aid in studying cancer-related factors and mechanisms. Activation of transcription factors Rel/NF-$_k$B allows for the regulation of the inflammatory responses of the immune system and cell proliferation. This is critical for cancer in human models, specifically hematopoietic tumors. Aggressive leukemia and lymphomas are also induced by v-Rel in animal models (Ravet 2003).

Prior studies by the Center for Advanced Biotechnology and Medicine at UMDNJ-Robert Wood Johnson Medical School in Piscataway, New Jersey, have

identified a serine-rich domain in v-Rel that is essential for biological activity. Their findings emphasized the important role of v-Rel- mediated transactivation for its oncogenic potential and suggest that the death program that v-Rel must suppress to transform lymphoid cells is inhibited by death antagonists Bcl-xL and Bcl-2 (Rayet 2003).

Considering that mutations in serine-rich areas of v-Rel can affect biological activity, it is important to analyze polymorphisms, or changes in genetic code. Polymorphisms in oncogenes may occur due to the genotoxicity of environmental PAHs and theoretically link PAH exposure to an increased potential for cancer in wild fish. The rationale for focusing the presented study on v-Rel is to identify a mutagenic effect from PAHs on a cancer-related gene.

The objective of this project were three fold: 1) to investigate the effect of Hurricane Sandy on PAH body burdens, 2) to investigate the presence of v-Rel polymorphisms in a wild population of Atlantic menhaden, and 3) to determine if there is a relationship between amounts of V-Rel polymorphisms in Atlantic menhaden and their body burdens of PAH. The fish were collected prior to and after Hurricane Sandy, and the presence and quantities of PAHs were determined using EEMS. DNA was isolated from muscle tissue of individual fish, amplified by polymerase chain reaction (PCR) and passed through agarose gels in order to detect polymorphisms associated with amplicons of various sizes. Relatedness of PAH body burdens and v-Rel polymorphisms were determined through Cluster analysis. The end result was to determine the relationships between PAH-like compound concentration in raw fish oil and positions of PCR on agarose gel.

Table 1

PAH Compound	Molecular Formal	Carcinogen Classification	Human Carcinogenicity
Anthracene	$C_{14}H_{10}$	Environmental, Food, Smoke	Not classifiable
Benzo(a)anthracene	$C_{18}H_{12}$	Environmental, Food, Smoke	Probable
Benzo(b)fluoranthene	$C_{20}H_{12}$	Environmental, Food	Possible
Benzo(a)pyrene	$C_{20}H_{12}$	Environmental, Food, Smoke	Probable
Chrysene	$C_{18}H_{12}$	Environmental, Food, Smoke	Not classifiable
Ideno(1,2,3-cd)pyrene	$C_{22}H_{12}$	Environmental, Food	Possible
Fluoranthene	$C_{16}H_{10}$	Environmental, Food, Smoke	Not classifiable
Fluorene	$C_{13}H_{10}$	Environmental, Food, Smoke	Not classifiable
Phenanthrene	$C_{14}H_{10}$	Environmental, Food, Smoke	Not classifiable
Pyrene	$C_{16}H_{10}$	Environmental, Food, Smoke	Not classifiable
Napthalene	$C_{10}H_{8}$	Environmental	Probable

Table 1: List of primary PAH pollutants considered by the Environmental Protection Agency (EPA) and the Agency for Toxic Substances & Disease Registry (ATSDR), their molecular formulas, and carcinogen classification (ATSDR, 2009).

Materials and Methods

Fish Collection Sites

Adult Atlantic menhaden were collected off the coast of NJ in 2011, 2012, and 2013 (Figure 1), focusing on the three main locations of Atlantic City, Point Pleasant, and Delaware Bay. These collections were made using boats of the Purse-Seine Industry by the staff of NJ Fish and Wildlife. Ships included Evening Star (ESNJ) and Eva Marie (EMNJ). Fish were also collected by gill net and trawling. The presence of any contaminants would represent their respective region, due to the fact that menhaden are migratory fish. After collection, all fish were frozen and sent to Seton Hall University (South Orange, New Jersey) to remain frozen until further use for fish oil preparation. When possible, two raw fish oil samples were prepared from each collection. Replicates were also made from the same catch on the same date. Tissues from three to five fish were combined to make each sample.

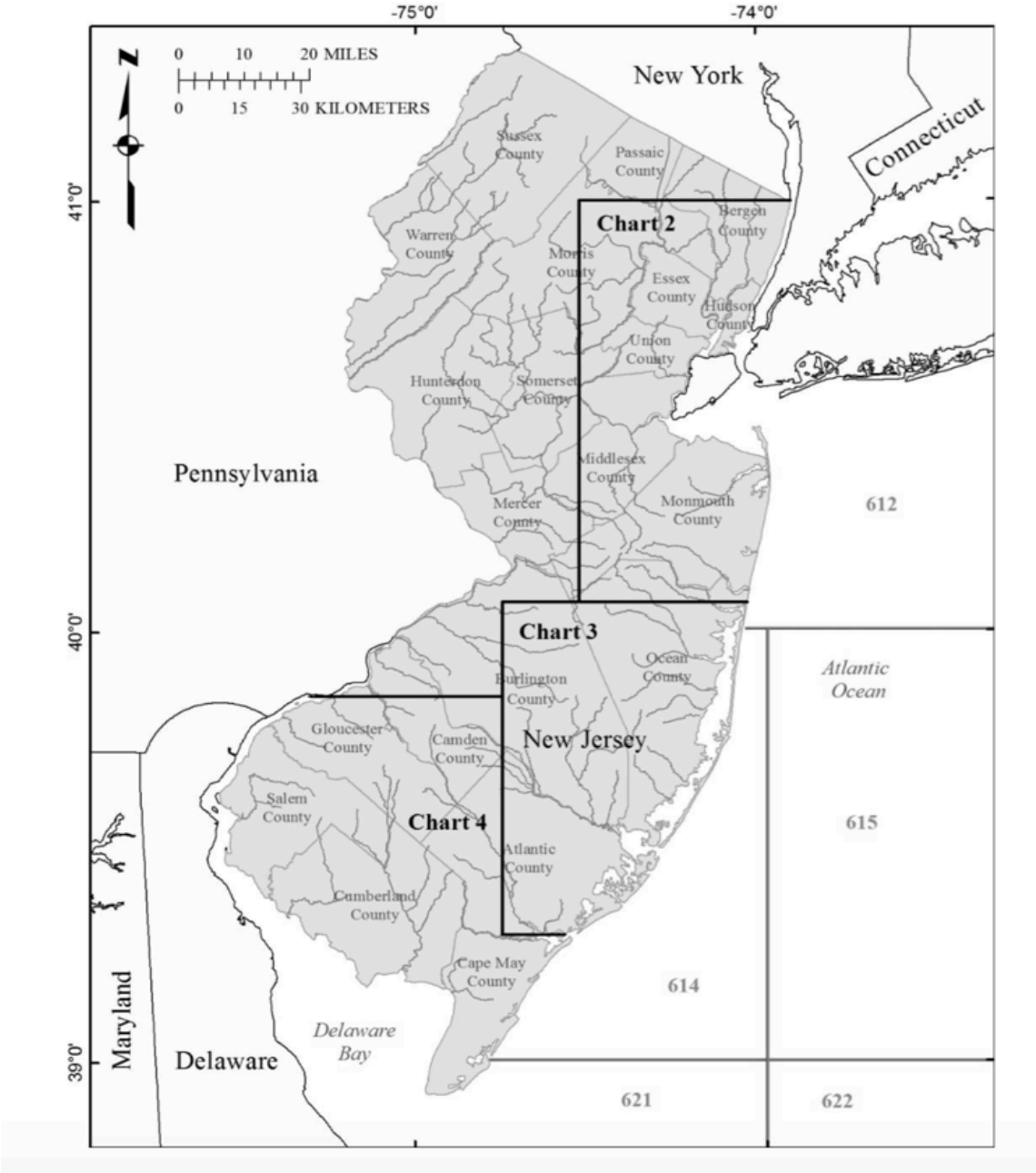


Figure 1: Map provided by NJ Fish and Wildlife showing reported areas of Atlantic menhaden collection, represented by numbers.
(Bentivegna, 2016).

Fish Oil Preparation

Prior to fish oil preparation, each fish was given a unique tracking number based on catch and ship location. Each fish was thawed and dissected by removing the skin and muscle fillet, from which the raw oil was prepared. Tissues were cut into small pieces and pounded into fishmeal by using a long glass tube into a 40 mL plastic round bottom centrifuge tube. The pounding similarly mimicked a mortar and pestle technique to achieve crushed materials. Each tube contained 16 g skin and 16 g fillet. This mixture was centrifuged for 5 hr at 10,000 rpm at 4°C (Sorvall, RC5C). Two layers appeared post centrifugation – one raw fish oil upper layer and one lower aqueous layer. A bottom meal pellet formed on the bottom of the tube. The two layers were poured off gently into a clean 50 mL centrifuge tube and were allowed to settle for 15 min, having the layers form again. An 18 gauge pre-heated needle was used to puncture the bottom of the tube. The lower layer was drained off and discarded while the top layer was collected for further use. This top layer is distinguished as raw fish oil and was stored under nitrogen gas at -20°C.

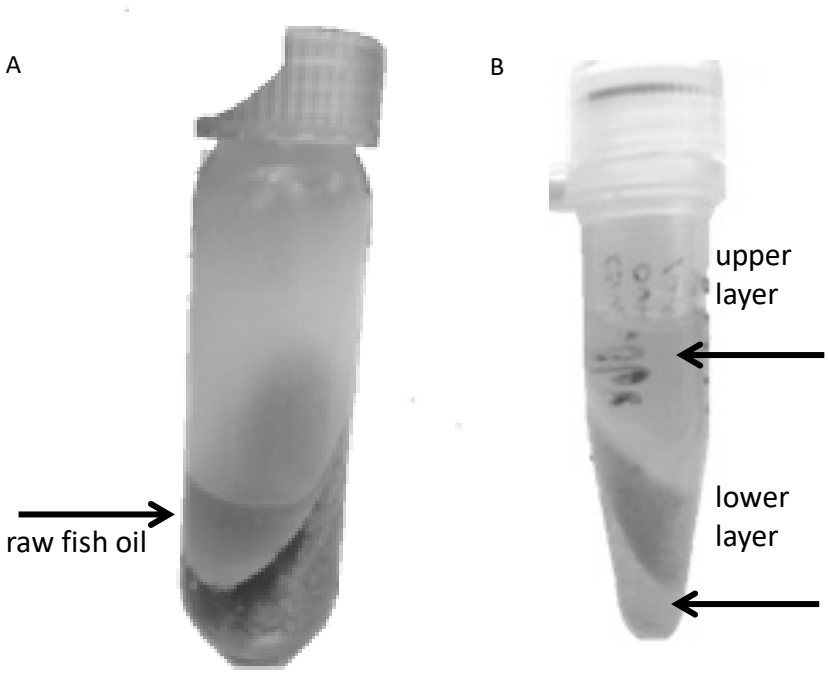


Figure 2: (A) Layers of fish material generated by centrifugation. The top layer represents raw fish oil and the bottom layer represents fish meal. (B) Layers within raw fish oil (top layer as seen in A) generated by centrifugation (Bentivegna, 2016).

PAH Extraction and Fluorescence Analysis

Previously stored raw fish oil was thawed under room temperature conditions. An aliquot of 50 µL of oil was added to 950 µL of 75% ethanol. This mixture was vortexed for 1 min and then centrifuged at 12,000 rpm for 20 min. The supernatant was used for fluorescence analysis to detect the extraction of PAHs from raw fish oil. Two replicates were used for each sample during excitation-emission matrix scanning (EEMS). A Horiba (Fluorolog 3) spectrofluorimeter was used for EEMS. Semi-micro fused silica cuvettes (Starna) with a 1 cm excitation path length were used for spectrofluorimetry. They were rinsed in between each sample with 75% ethanol. Ethanol was diluted with 18 Ohm water from Milli-Q Integral 5 via EMD Millipore Inc (Pena et al, 2015). Each sample was analyzed under PAH standard wavelengths with an excitation of 260-400 nm and emission of 320-480 nm. The following sample standards of polycyclic aromatic hydrocarbons were used for the purpose of comparison for EEMS: 100 ng/mL 1-Hydroxypyrene (HPY) and 2-Naphthol (HNP).

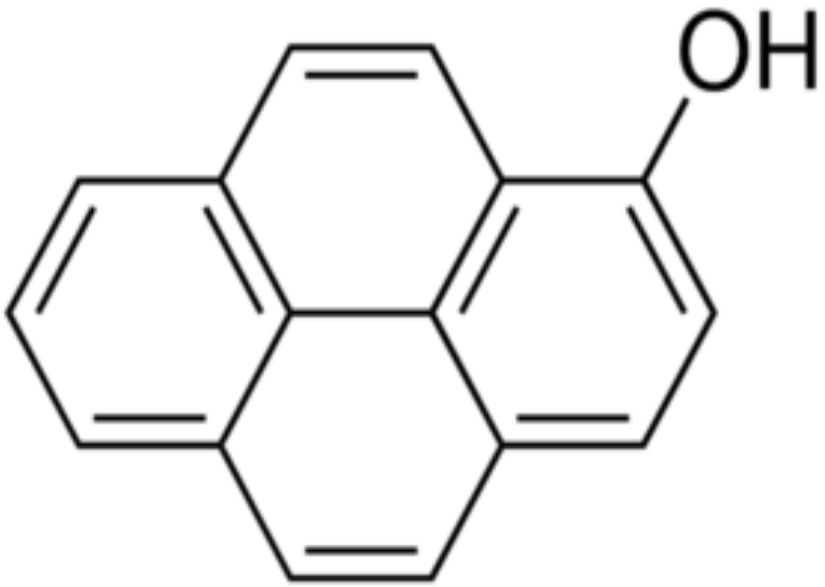


Figure 3: Molecular Structure of 1-Hydroxypyrene (HPY)
(Sigma-Aldrich, 2018).

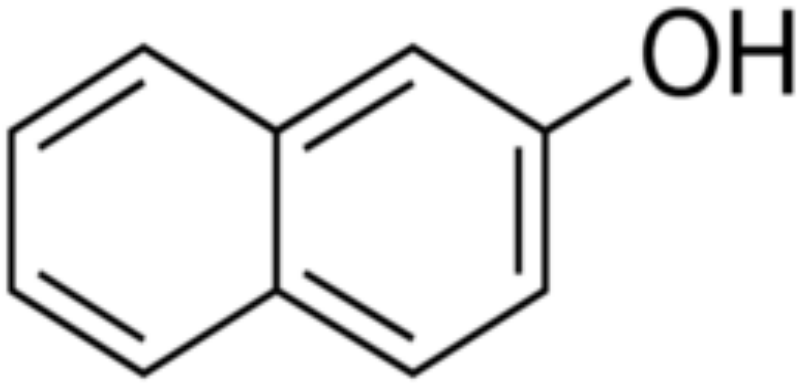


Figure 4: Molecular structure of 2-Naphthol (HNP)
(Sigma-Aldrich, 2018).

DNA Extraction/Isolation

Approximately 0.05 g of Atlantic Menhaden muscle tissue from each sample was manually homogenized in 1 mL of Tri Reagent (Sigma-Aldrich). This mixture was incubated at room temperature for 5 min. Next, 200 µL of chloroform was added. Each sample was inverted and shaken for 15 s, and then allowed to incubate for 15 min at room temperature. The supernatant was discarded and 300 µL of 100% Ethanol was added. Each sample was mixed by inversion and incubated for an additional 5 min to allow DNA precipitation. Each sample was centrifuged at 12,000 rpm for 5 min at 4°C (PrismR). The supernatant was discarded and pellet was washed in 1 mL of 0.1 Trisodium Citrate 10% Ethanol Solution. Incubation then occurred for 30 min with occasional mixing. The mixture was centrifuged at 12,000 rpm for 5 min at 4°C. The wash, incubation, and centrifugation were repeated. The pellet was resuspended in 1.5 mL of 75% Ethanol and incubated at room temperature for 10-20 min. The pellet was left to dry for 5-10 min and then dissolved into 300 µL of 8 mM NaOH. This mixture was centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to a new tube. To prepare each product for spectrophotomer readings, 60 µL of newly isolated DNA and 540 µL of UltraPure DNase/RNase-Free Distilled Watrer (Invitrogen) were combined. The quality and quantity of each product was determined by spectroscopy using the DU730 Life Science UV/Vis Spectrophotometer (Beckman Coulter). For storage, 86 µL of 0.1 M HEPES buffer was added to each product and placed into the freezer.

Polymerase Chain Reaction and Gel Electrophoresis

Primers for beta-actin housekeeping gene and v-Rel were designed from an Atlantic menhaden transcriptome courtesy of Frank Zadlock IV (Zadlock IV et. al, 2017). and Satshil Rana (S. Rana, personal communication, 2015). The PCR products were then subjected to complete gel electrophoresis. A 2% agarose gel with 2 μL of ethidium bromide was made with 2 g of agarose powder and 100 mL of 0.5% TBE. Each well contained 6 μL of each respective product, 4 μL of UltraPure DNase/RNase-Free Distilled Water (ThermoFisher Scientific), and 2 μL of TrackIt Cyan/Yellow loading buffer (ThermoFisher Scientific). A 1:10 diluted 100 base pair (bp) ladder marker was used to allow comparison of band lengths. The gel was run at 128 mV for 30 min and reviewed under a UVP Chromato-Vue UV Transilluminator TM36 (UVP Inc).

Polymerase Chain Reaction Settings

	V-REL Primers	**ARNT/Beta-Actin Primers**
Pre-Denaturation	94°C – 3 min	94°C – 3 min
Denaturation	94°C – 1 min	94°C – 15 s
Annealing	62°C – 1 min	60°C – 15 s
Elongation	72°C – 1 min	72°C – 15 s
Delay	72°C – 7 mi	72°C – 7 min
Soak	4°C	4°C
Cycle Count	45	35

Table 2: Polymerase chain reaction settings used with V-REL, ARNT, and Beta-Actin Primers on previously extracted DNA from Atlantic menhaden samples (DeFelice, 2018).

Cloning and Sequencing

The PCR product was cloned using TOPO Isomerase Kit (Invitrogen) and One Shot Chemical Transformation Protocol (ThermoFisher Scientific) by Junyoung Kim (J.Kim, personal communication, 2015). Gel purification was done by using QIAquick Gel Extraction Kit (QIAGEN ID: 28706). The cloned product containing 5 µL of 5 µM M13 Reverse Primer, 5 µL of 5 µM V-Rel Reverse Primer, and 10 µL of 2 ng/µL of template was sequenced commercially by Genewiz laboratories in South Plainfield, NJ.

Hierarchical Cluster Analysis

A Hierarchical Cluster Analysis was used to determine relationships between PAH-like compound concentrations in raw fish oil and polymorphisms linked with amplicons of different sized via gel positioning in individual fish samples using Cluster Analysis Software (NCSS).

Results:

Fish Identification, Preparation, and Organization

Atlantic menhaden were caught off the shore of New Jersey in locations that varied among Atlantic City, Point Pleasant, and Delaware Bay (as seen in Figure 1). Collections were made via gathering by purse-seine vessels, trawling by Virginia Institute of Marine Science, and gill net by NJ Fish and Wildlife Staff. The collection dates ranged from August 31, 2011 – October 3, 2013. Fish weight, length, and location were recorded. The locations were designated by ship name and collection method. Fish were frozen upon capture and thawed gently before dissection. Heads and organs were removed and discarded. The fish were skinned, which allowed the muscle fillets and skin to be separated and stored for oil extraction. When possible, two replicates were made from each catch. The number of fish per replicate was approximately ~5 fish with few exceptions. The tissues from the number of fish per replicate were combined to make each oil sample. The raw fish oil extracted from each replicate was stored under nitrogen gas at 20°C until further analysis.

Table 3

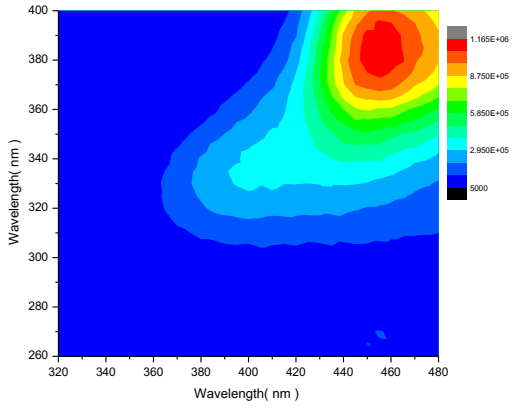
Collection Date	Ship Name (Collection Method)	Replicate	Number of fish per replicate	Fish Weight (g)	Fish Length (mm)	Oil Extracted Per Fish (mL)
2011						
8/31/2011	ESNJ (PS)	1 2	5 3	325±44 387±55	261±12 271±10	1.0 3.3
8/31/2011	EMNJ (PS)	1 2	5 5	312±73 315±15	268±10 277±18	0.9 1.2
10/8/2011	ESNJ (PS)	1 2	5 5	447±54 403±92	265±11 268±19	1.6 2.0
10/8/2011	EMNJ (PS)	1 2	5 3	357±33 375±71	254±12 255±18	1.0 4.6
2012						
9/4/2012	ESNJ (PS)	1	10	325±30	257±9	0.5
9/7/2012	BLNJ (PS)	1 2	5 5	290±33 299±47	246±12 255±18	2.0 0.5
9/12/2012	EMNJ (PS)	1 2	8 4	364±22 390±31	271±6 280±11	1.3 0.6
10/12/2012	VIMS/ACNJ (Trawl)	1 2	5 4	395±42 379±26	274±14 263±4	1.0 1.3
2013						
8/20/2013	SHNJ (PS)	1	6	335±63	265±12	0.8
8/21/2013	BLNJ (PS)	1 2	4 4	316±17 298±16	255±3 247±14	1.2 0.3
10/3/2013	GNNJ (Gill Net)	1	6	399±55	274±11	0.7

Table 3: Atlantic menhaden collection dates, ship names/collection methods, replicate number, number of fish per replicate, fish weight and length, and the amount of oil extracted per fish from 2011-2013 catches. Different fish were used to make each sample of oil from the same collection, which can be identified by the replicate number. Purse-seine vessels collected those indicated by PS; Trawl were collected by trawling off shore by a vessel out of Virginia Institute of Marine Science (VIMS); Gill Net were collected using a gill net by NJ Fish & Wildlife staff (DeFelice, 2018).

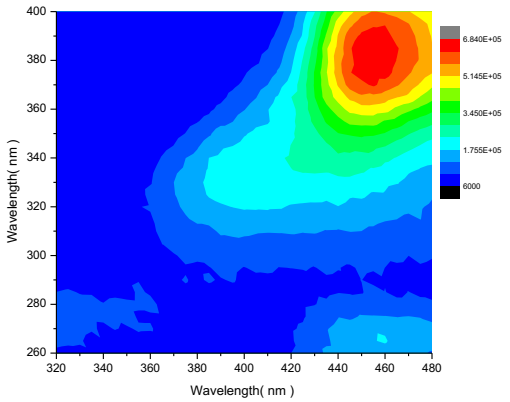
Fish Oil Preparation and Contamination Analysis

Specific numbers were assigned to each fish. Fish tissues of equal skin and muscle fillet amount (approximately 16 g each) were sliced into small pieces and pounded into fishmeal, which centrifuged for 5 hr at 10,000 rpm at 4°C in a Sorvall RC5C centrifuge. The upper raw fish oil layer was used for contamination analysis whilst the lower aqueous layer and meal pellet were discarded. The upper raw fish oil layer was centrifuged a secondary time in a microcentrifuge tube, which resulted in an additional splitting of this oil. The top layer was more viscous with the consistency of oil than the lower layer, which was more aqueous and less viscous in nature. In between the layers was an unidentified whitish matter, resembling a solidified lipid material (Figure 2B). This middle layer, which is frequently observed, is likely an emulsion of the oils and ethanol. Emulsifiers are characterized by a hydrophilic-lipophilic balance (HLB), which is the degree to which it is hydrophilic or lipophilic. Other factors that affect the stability of emulsions include the viscosity of the continuous phase, droplet size, and difference in densities between the two phases. Favorable conditions include a higher phase, smaller droplet size, and smaller differences between phases (Clark, 2013). A mixture of PAH-like substances results in a range of hydrophobicities, which ultimately affects their representation of locations in fluorescent excitation-emission scans. Aliquots of each layer (top and bottom) were separated and added independently to 75% ethanol. These mixtures were vortexed and the supernatants were used for fluorescence analysis to detect for the presence of PAH-like compounds. Contamination locations in raw fish oil layers are identified in Tables 4 and 5. PAH-like signals were observed in both top and bottom

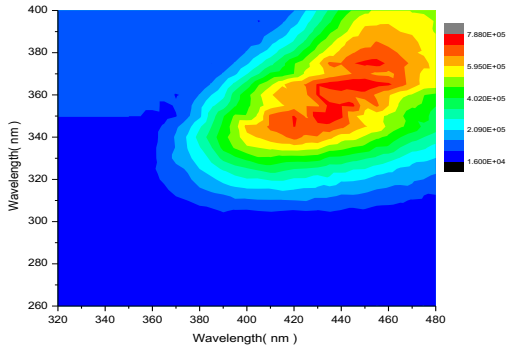
layers of the raw fish oil, with higher concentrations predominantly recovered in the lower layer due to the possibility of larger sized PAHs:



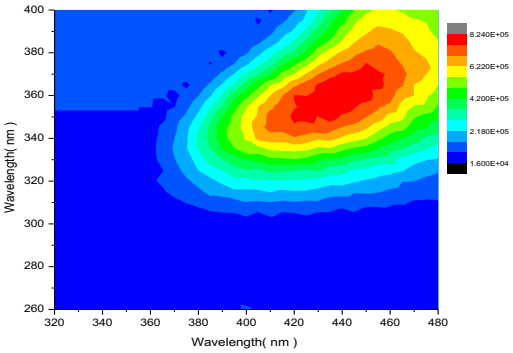
(A): EMNJ 031-035 (2011) Top



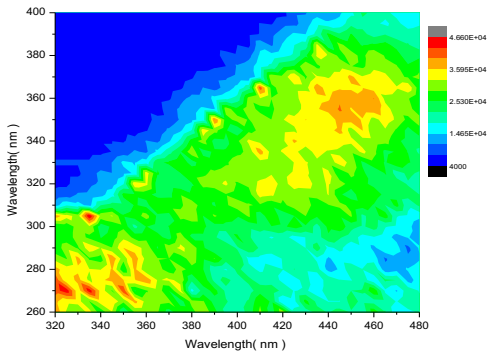
(B): EMNJ 031-035 (2011) Bottom



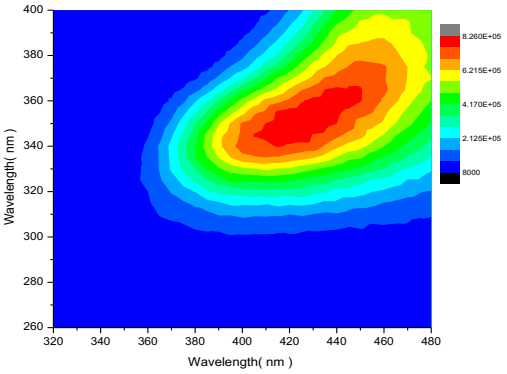
(C): EMNJ 038-041 (2012) Top



(D): EMNJ 038-041 (2012) Bottom



(E): GNNJ 001-006 (2013) Top



(F): GNNJ 001-006 (2013) Bottom

Figures 5 (A-F): Examples of EEMS scans using 75% ethanol representing the individual top and bottom layers found within the centrifuged raw fish oil, respective from year of catch. Catch acronym/name, fish numbers, layer location, and year of catch are provided (DeFelice, 2018).

Ethanol was used as the solvent in this extraction method due to its ability to dissolve polar and non-polar constituents. The hydroxyl functional group end allows the attraction of polar molecules while the ethyl functional group end allows the attraction of non-polar molecules, such as PAHs. Ethanol concentration was an important factor in the method development of this assay because it can extract different amounts of a compound based on its hydrophobicity as well as affect the fluorescence intensity of compounds. In previous investigations, spectra for PAHs spiked at a high of Em450/Ex270 when extracting with 75% EtOH, and decreased intensity with 100% EtOH (Pena et al, 2015). Ethanol also acts as a cost-effective alternate with lower toxicity, as opposed to popular lipid extractors such as chloroform and hexanes. Hexanes success relies with its ability to only extract polar solutes. Thus, chloroform is a more versatile, yet relatively more expensive solvent (Siek, 1978). Chloroform is less stable and must be stored within dark colored bottles to avoid oxidation by light.

Table 4

Collection Date	Ship Name (Collection Method)	Replicate	Fish Numbers	Separated Oil	Contamination Location
8/31/2011	ESNJ (PS)	1 2	032-036 037,038, 040	Yes Yes	Top & Bottom Top & Bottom
8/31/2011	EMNJ (PS)	1 2	030-035 036-040	Yes Yes	Top & Bottom Top & Bottom
10/8/2011	ESNJ (PS)	1 2	101-105 106-110	Yes Yes	Yes Most Bottom
10/8/2011	EMNJ (PS)	1 2	061-065 066-068	Yes Yes	Yes Most bottom
9/4/2012	ESNJ (PS)	1	001-010	Yes	Top & Bottom
9/7/2012	BLNJ (PS)	1 2	013-017 025-031	Yes Yes	Yes Most Bottom
9/12/2012	EMNJ (PS)	1 2	030-037 038-041	Yes No	Top & Bottom Top & Bottom
10/12/2012	VIMS/ACNJ (Trawl)	1 2	001-005 006-009	No No	Bottom Only Bottom Only
8/20/2013	SHNJ (PS)	1	001-006	No	Top & Bottom
8/21/2013	BLNJ (PS)	1 2	050-061 050-061	Yes Yes	Most bottom Most bottom
10/3/2013	GNNJ (Gill Net)	1	001-006	No	Bottom Only Bottom Only

Table 4: Information on Atlantic menhaden raw fish oil samples: Atlantic menhaden collection dates, ship name/method of collection, replicate number, fish number, oil separation analysis, and contamination location within raw fish oil layers (ie: top, bottom, or exact location undistinguishable represented by “yes”) (DeFelice, 2018).

Table 5

			Upper Layer			Lower layer			Combination
Sample	**Replicate**	**Ex/Em**	**ng**	**ml**	**ng/ml**	**ng**	**mL**	**ng/mL**	**ng/mL**
ESNJ 8/31/2011	1	370/450	32	0.50	316	761	0.50	7607	7923
	2	370/450	68	0.40	546	378	0.60	4536	5082
EMNJ 8/31/2011	1	385/455	882	0.15	2645	350	0.85	5944	8590
	2	385/455	731	0.50	7306	1312	0.50	13116	20421
ESNJ 10/8/2011	1	360/430	7	0.99	131	203	0.01	203	333
	2	360/430	0	0.99	0	173	0.01	173	173
EMNJ 10/8/2011	1	370/450	0	0.98	0	316	0.02	316	316
	2	370/450	0	0.75	0	292	0.25	1459	1459
ESNJ 9/4/2012	1	370/450	575	0.15	1726	273	0.85	4645	6371
BLNJ 9/7/2012	1	370/450	186	0.15	559	196	0.85	3326	3885
	2	360/430	15	0.5	151	260	0.50	2597	2748
EMNJ 9/12/2012	1	370/450	1579	0.10	3159	211	0.90	3797	6955
	2	370/450	568	0.25	2839	379	0.75	5683	8521
VIMS-ACNJ 10/12/2012	1	360/430	519	0.30	3116	347	0.70	4861	7976
	2	360/430	644	0.10	1287	431	0.90	7751	9038
SHNJ 8/20/2013	1	360/430	0	1.00	5	0	0.00	0	5
BLNJ 8/21/2013	1	345/415	0	0.50	0	426	0.50	4257	4257
	2	345/415	0	0.40	0	409	0.60	4912	4912
GNNJ 10/3/2013	1	360/430	4	0.50	39	397	0.50	3971	4010

Table 5: Contamination information on Atlantic menhaden fish oil samples: Sample name and date, replicate number, excitation/emission positioning in nanometers (nm), concentration in nanograms of contaminant per milliliter (ng/mL) of raw fish oil in respective upper and lower layers, and concentration (ng/mL) of contaminant combined in upper and lower layers (DeFelice, 2018).

Fluorescence Analysis of Raw Fish Oil

Each supernatant previously extracted was used for fluorescence analysis by EEMS. Two replicates were used for each sample. Each sample was analyzed under PAH standard wavelengths with an excitation range of 260-400 nm and an emission range of 320-480 nm. Both layers exhibited the presence of PAH-like compounds and the spectra were combined. The fluorescence intensities of the EEMS scans were adjusted to 1×10^6 counts per second (cps) or less. The fluorescence intensities and locations of fluorophores varied between scans, which indicated different levels and types of PAH-like compounds. Replicates had similar ranges of intensity. Major peaks were characterized by red shifts above Ex350/Em420, corresponding to a strong PAH-like signal. The EEMS scans were not consistent for a particular collection year pre or post-Hurricane Sandy. August 2011 samples exhibited strong fluorescent signals, indicating the presence of PAH-like compounds. October 2011 samples displayed weaker fluorescence signals in similar regions. September 2012 samples varied among intensities, while October 2012 showed strong fluorescent signals. August and October of 2013 exhibited lowest fluorescent signals.

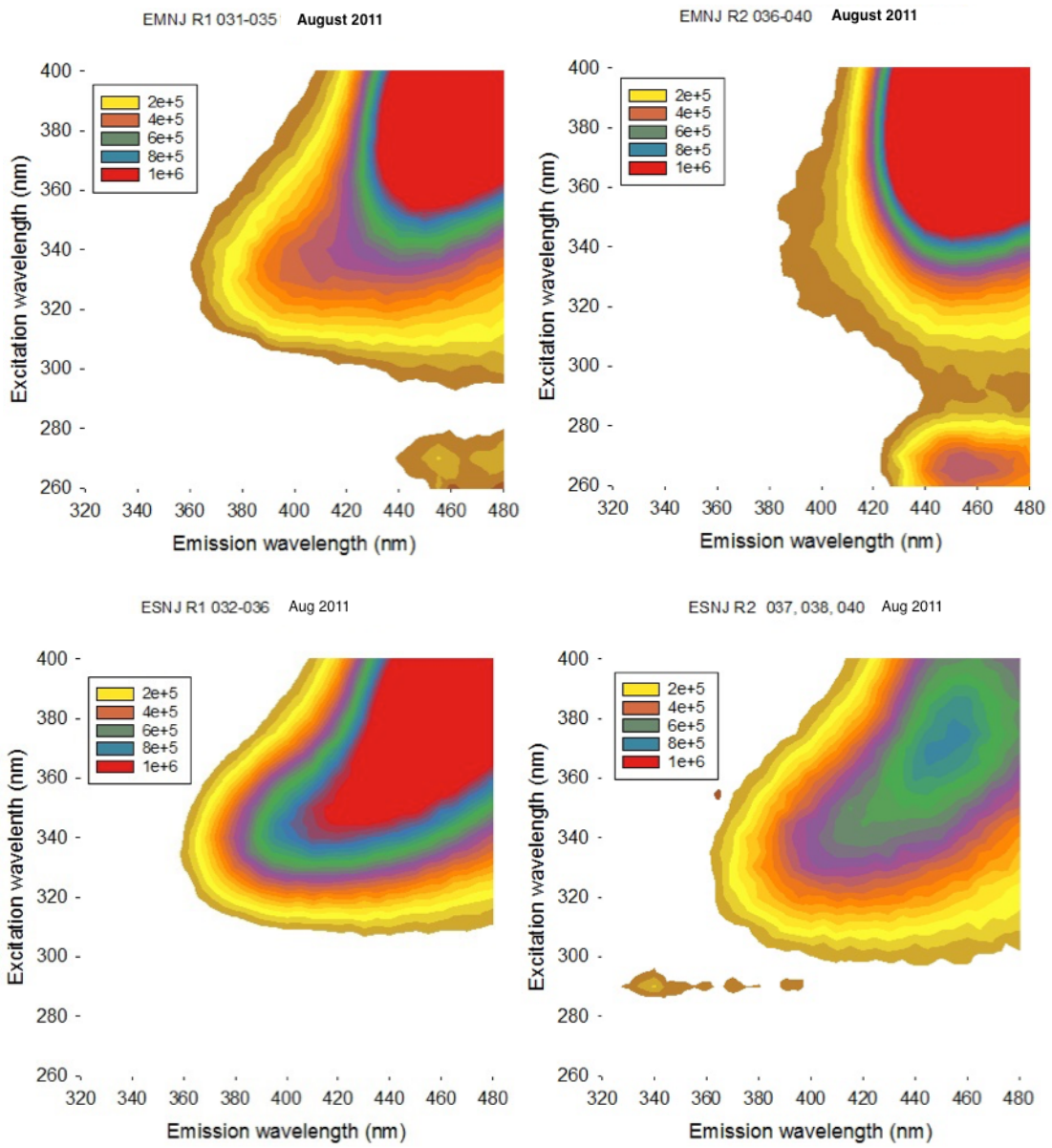


Figure 6: EEMS scans of raw fish oil collected pre-Hurricane Sandy in 2011 on August 31st. Catch name, replicate number, and fish numbers are provided. EMNJ and ESNJ represent the catch/ship names as follows: Eva Maria (EMNJ) and Evening Star (ESNJ) (DeFelice, 2018).

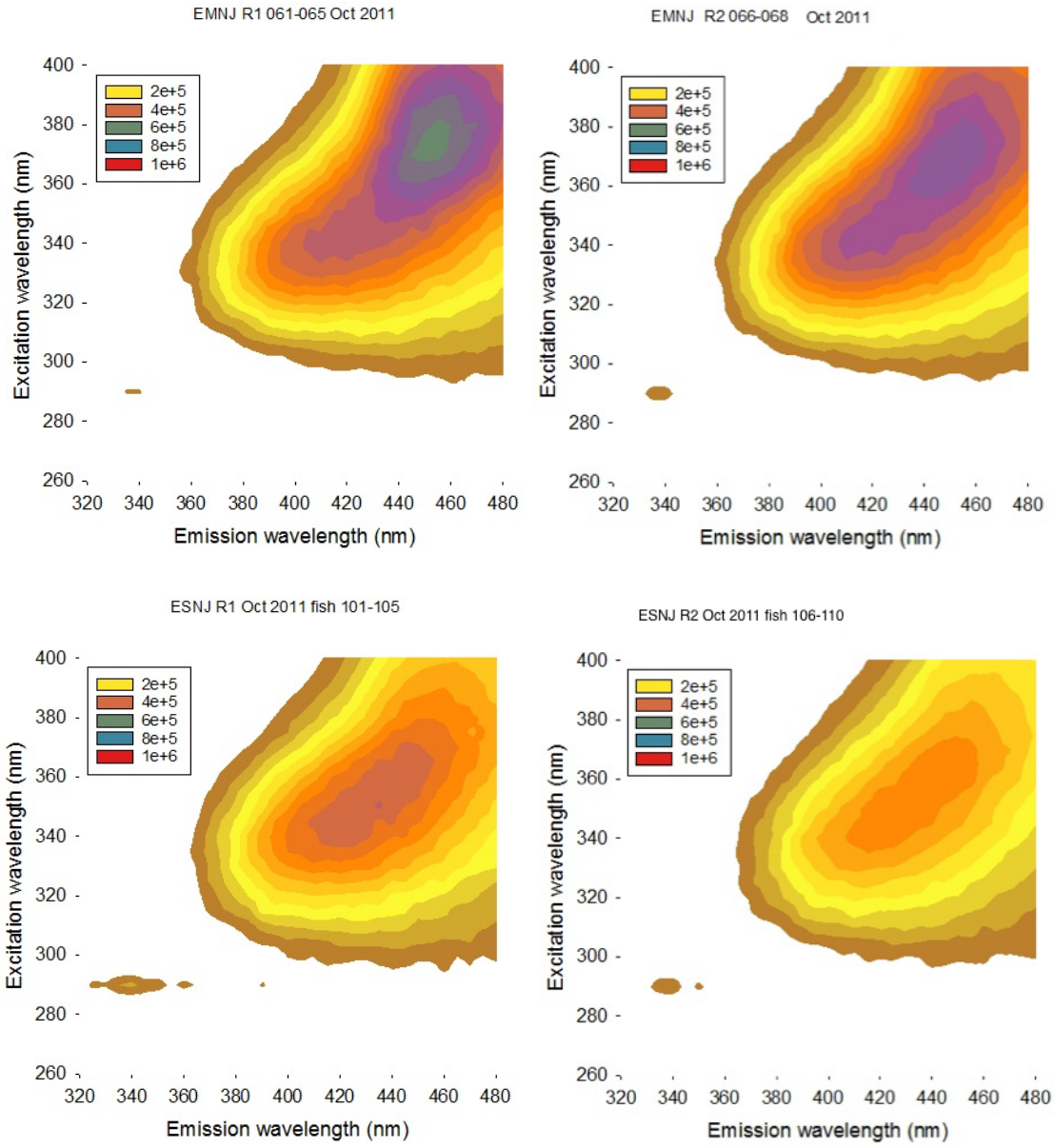


Figure 7: EEMS scans of raw fish oil collected pre-Hurricane Sandy in 2011 on October 8th. Catch name, replicate number, and fish numbers are provided (DeFelice, 2018).

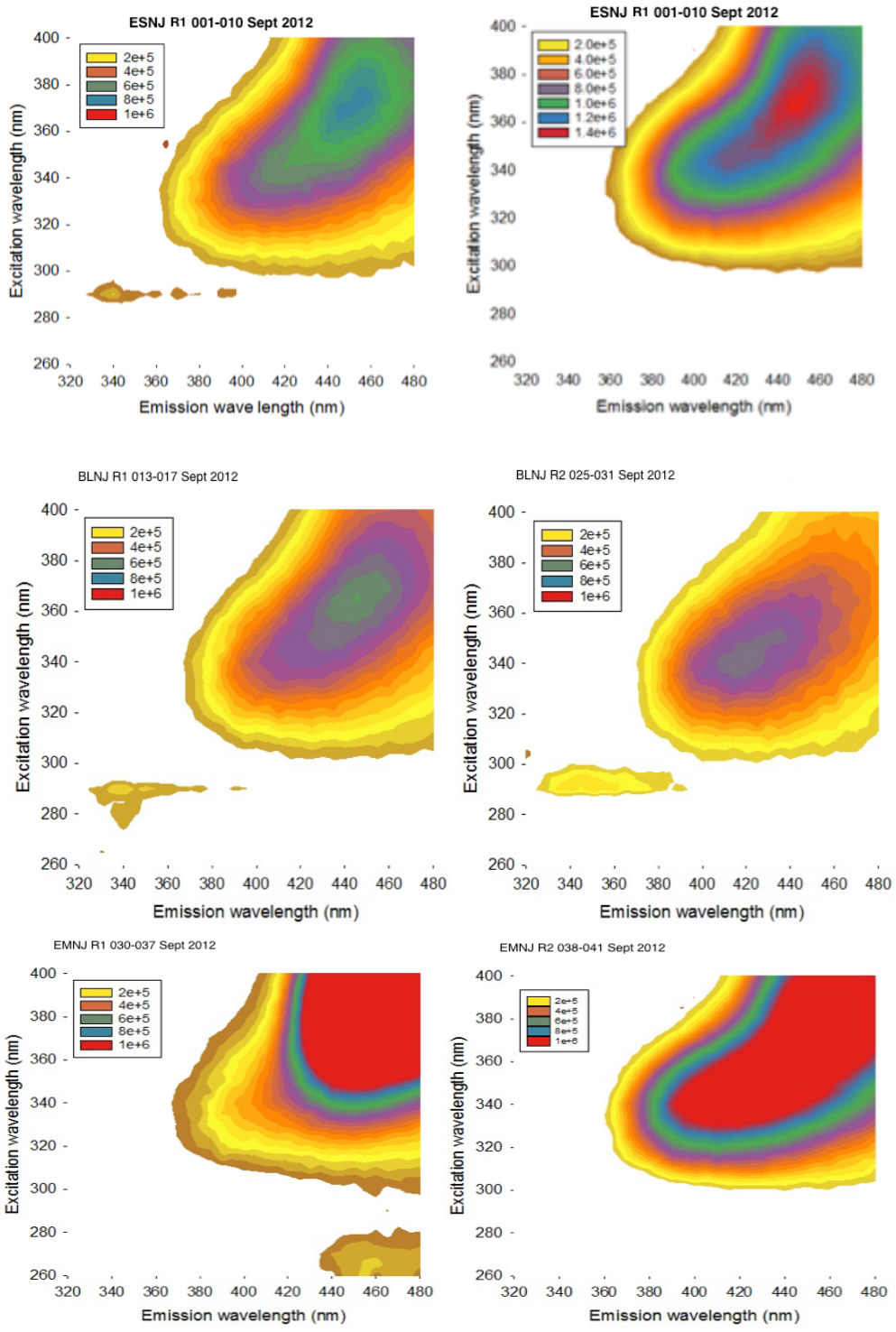


Figure 8: EEMS scans of raw fish oil collected pre-Hurricane Sandy in 2012 on September 4th, 7th, and 12th. Catch name, replicate number, and fish numbers are provided (DeFelice, 2018).

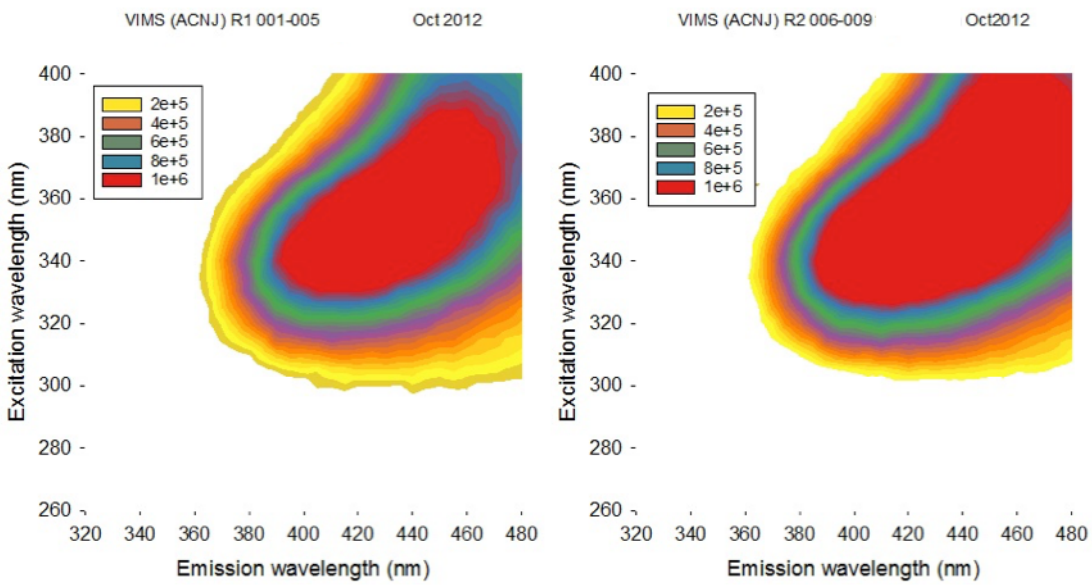


Figure 9: EEMS scans of raw fish oil collected pre-Hurricane Sandy on October 12, 2012. Catch name, replicate number, and fish numbers are provided. VIMS (ACNJ) represents the catch/ship names as follows: Virginia Institute of Marine Science (DeFelice, 2018).

Year 2013 Post Hurricane Sandy

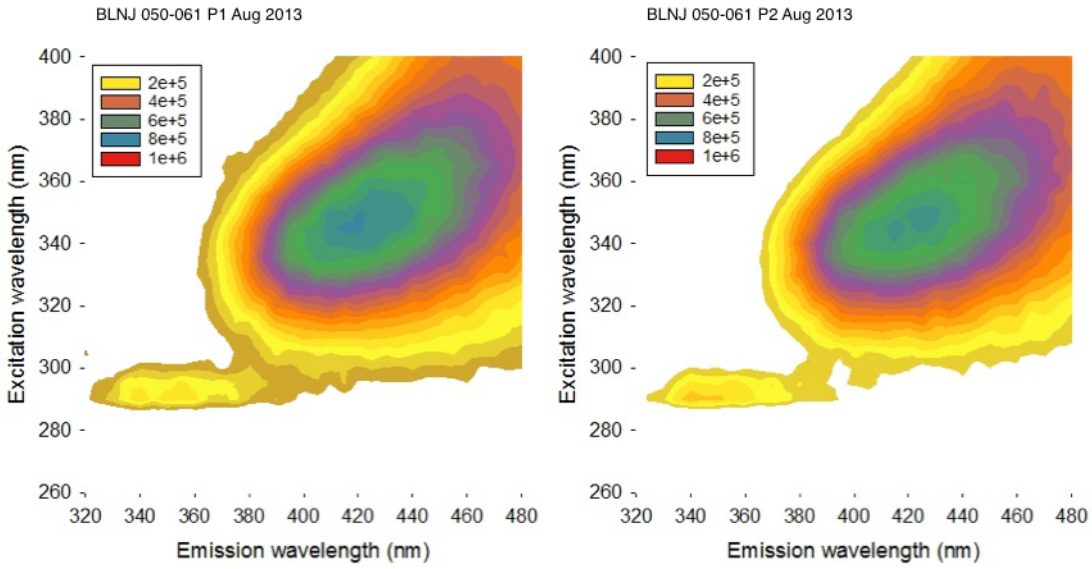


Figure 10: EEMS scans of raw fish oil collected 10 months post-Hurricane Sandy on August 21, 2013. Catch name, replicate number, and fish numbers are provided. BLNJ represents the catch/ship name as follows: Briana Louise (DeFelice, 2018).

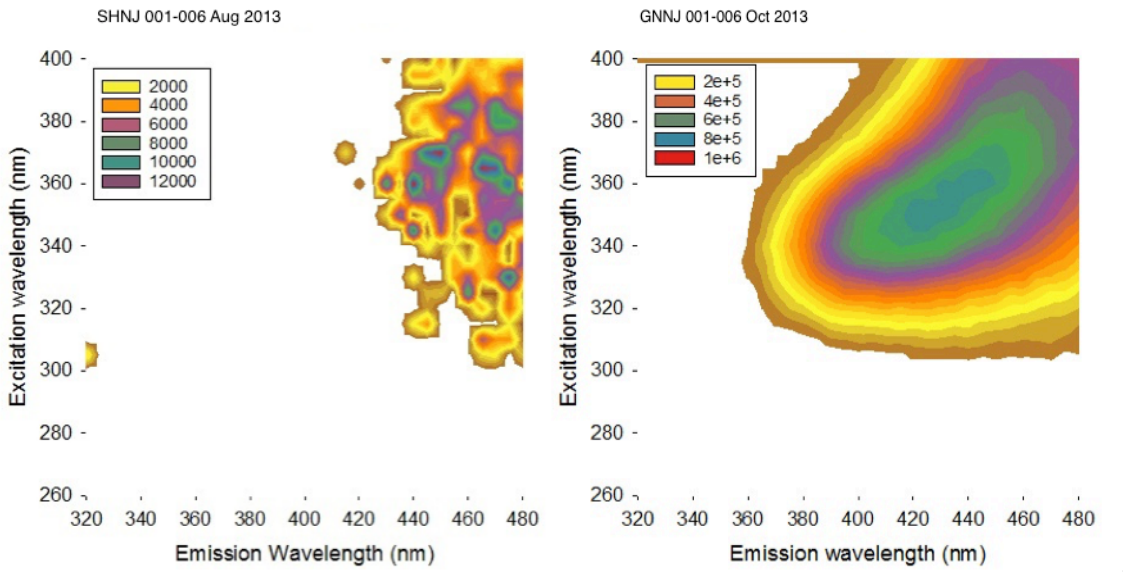


Figure 11: EEMS scans of raw fish oil collected approximately one year post-Hurricane Sandy on October 3, 2013. Catch name, replicate number, and fish numbers are provided. SHNJ and GNNJ represent the catch/ship names as follows: Sea Huntress and Gill Net (DeFelice, 2018).

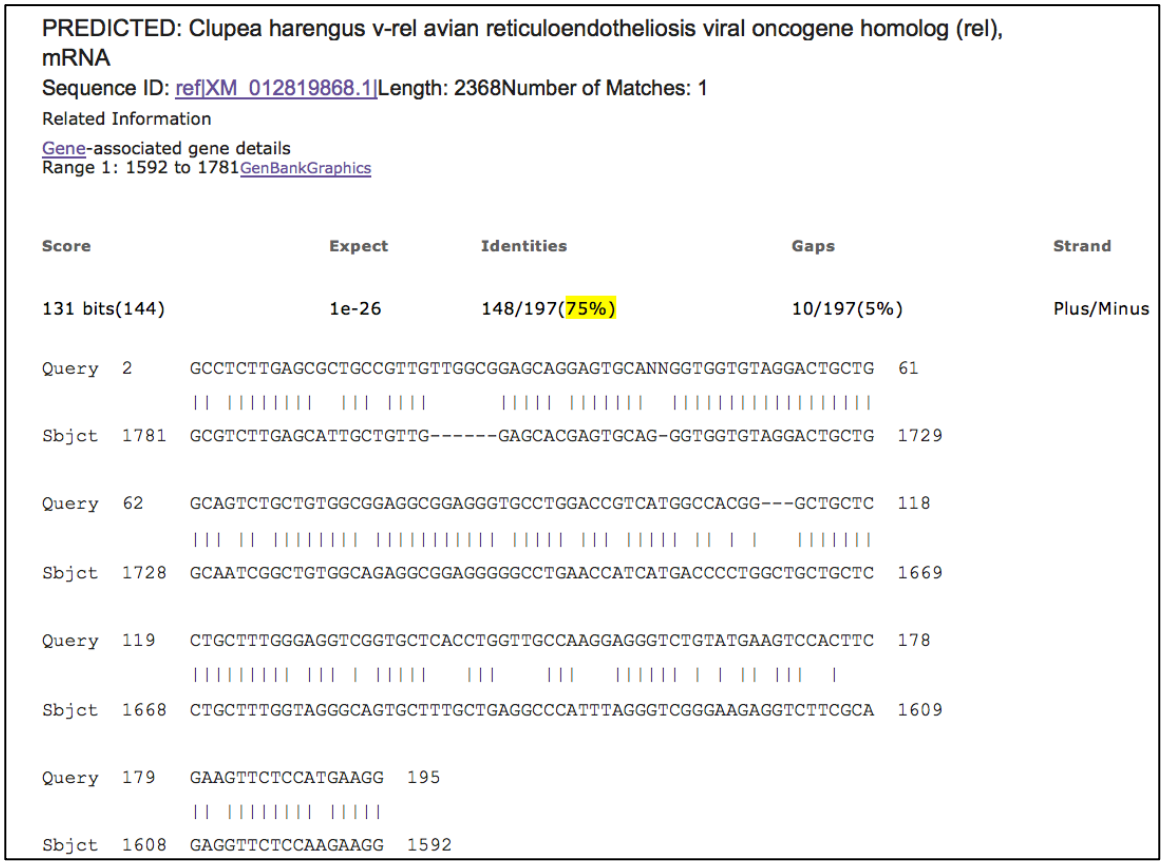
Genomic Analysis of Extracted DNA from Atlantic Menhaden Muscle Tissue

DNA was isolated from the muscle tissue of each individual fish using the DNAzol Reagent procedure from Thermo Fisher Scientific. The quality and quantity of isolated DNA was determined by spectroscopy. To determine the best possible quality of DNA, the absorbance read at 260 nm was divided by the reading at 280 nm (A_{260}/A_{280}). Samples found within the ratio of 1.5-2.0 were considered at best quality and subjected to PCR to be amplified for further use. Primers used during PCR were designed from an Atlantic menhaden transcriptome. Beta-actin antibody primers were used as a loading control to check the protein degradation and v-Rel primers were used to copy a specific segment of DNA matching with the proto-oncogene to further study an absence or presence of mutations. Samples were run on a 2% agarose gel with ethidium bromide (EtBr) and imaged under UVP GelDoc-It^e Imagine System. The PCR product cloned via TOPO Isomerase Kit (Invitrogen) and One Shot Chemical Transformation Protocol (ThermoFisher Scientific) was sequenced commercially by GeneWiz laboratories in South Plainfield, NJ. A relationship between PAH-like compound concentrations was correlated to the positions of bands in individual fish samples. The relatedness of individuals was determined using Cluster analysis. Sequenced information was compared via Basic Local Alignment Search Tool (BLAST), which predicted a 75% match to *Clupea harengus* (Atlantic herring) *avian reticuloendotheliosis viral oncogene homolog* (v-rel), mRNA. Atlantic herring and Atlantic menhaden are closely genetically related with identical taxonomic hierarchies until the level of subfamily.

Table 6

Taxonomic Hierarchy	**Atlantic herring**	**Atlantic menhaden**
Kingdom	Animalia	Animalia
Subkingdom	Bilateria	Bilateria
Infrakingdom	Deuterostomia	Deuterostomia
Phylum	Chordata	Chordata
Subphylum	Vertebrata	Vertebrata
Infraphylum	Gnathostomata	Gnathostomata
Superclass	Actinopterygii	Actinopterygii
Class	Teleostei	Teleostei
Superorder	Clupeomorpha	Clupeomorpha
Order	Clupeiformes	Clupeiformes
Suborder	Clupeoidei	Clupeoidei
Family	Clupeidae	Clupeidae
Subfamily	Clupeinae	Alosinae
Genus	Clupea	Brevoortia
Species	Clupea harengus	Brevoortia tyrannus

Table 6: Comparison of taxonomic hierarchies between Atlantic herring and Atlantic menhaden updated by the Integrated Taxonomic Information System (ITIS) based on the latest scientific consensus available (ITIS, 2018).



PREDICTED: Clupea harengus v-rel avian reticuloendotheliosis viral oncogene homolog (rel), mRNA

Sequence ID: ref|XM_012819868.1|Length: 2368Number of Matches: 1

Related Information

Gene-associated gene details

Range 1: 1592 to 1781GenBankGraphics

Score	Expect	Identities	Gaps	Strand
131 bits(144)	1e-26	148/197(75%)	10/197(5%)	Plus/Minus

```
Query  2     GCCTCTTGAGCGCTGCCGTTGTTGGCGGAGCAGGAGTGCANNGGTGGTGTAGGACTGCTG  61
             || ||||||||  ||| ||||      ||||| |||||||  ||||||||||||||||||
Sbjct  1781  GCGTCTTGAGCATTGCTGTTG------GAGCACGAGTGCAG-GGTGGTGTAGGACTGCTG  1729

Query  62    GCAGTCTGCTGTGGCGGAGGCGGAGGGTGCCTGGACCGTCATGGCCACGG---GCTGCTC  118
             ||| || |||||||| |||||||||| ||||| ||| ||||| || | |   |||||||
Sbjct  1728  GCAATCGGCTGTGGCAGAGGCGGAGGGGGCCTGAACCATCATGACCCCTGGCTGCTGCTC  1669

Query  119   CTGCTTTGGGAGGTCGGTGCTCACCTGGTTGCCAAGGAGGGTCTGTATGAAGTCCACTTC  178
             ||||||||| ||| | |||||   |||    |||   |||||| | | || |||  |
Sbjct  1668  CTGCTTTGGTAGGGCAGTGCTTTGCTGAGGCCCATTTAGGGTCGGGAAGAGGTCTTCGCA  1609

Query  179   GAAGTTCTCCATGAAGG  195
             || |||||||| |||||
Sbjct  1608  GAGGTTCTCCAAGAAGG  1592
```

Figure 12: BLAST nucleotide alignment of cloned PCR product containing: 5 µL of 5 µM M13 Reverse Primer, 5 µL of 5 µM v-Rel Reverse Primer, and 10 µL of 2 ng/µL of template. One result matched as predicted *Clupea harengus* (Atlantic herring) *avian reticuloendotheliosis viral oncogene homolog* (v-rel), mRNA (DeFelice, 2018).

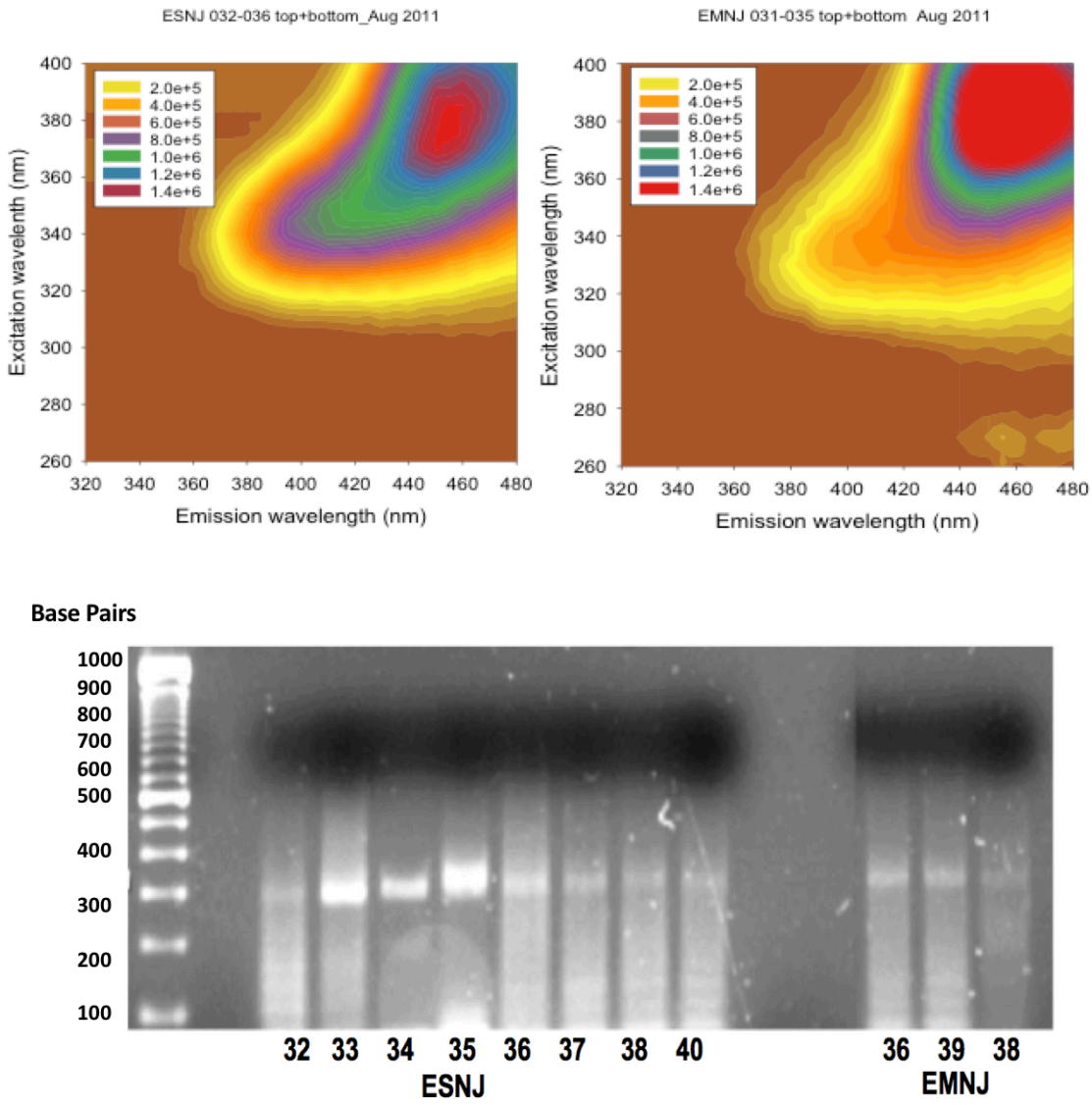


Figure 13: EEMS combined scans of raw fish oil from August 2011 and gel electrophoresis image of August 2011 DNA with V-REL associated primers. The EEMS scans of raw fish oils collected in August of 2011 emit a strong fluorescence signal, corresponding to high PAH concentration, which is evident in each replicate. PCR products were compared to a 100 bp ladder mark, showing consistent faint bands located at 300 bp; showing few isomers and high genetic relatedness among individuals exposed to high PAH-like compounds in each of these catches (DeFelice, 2018).

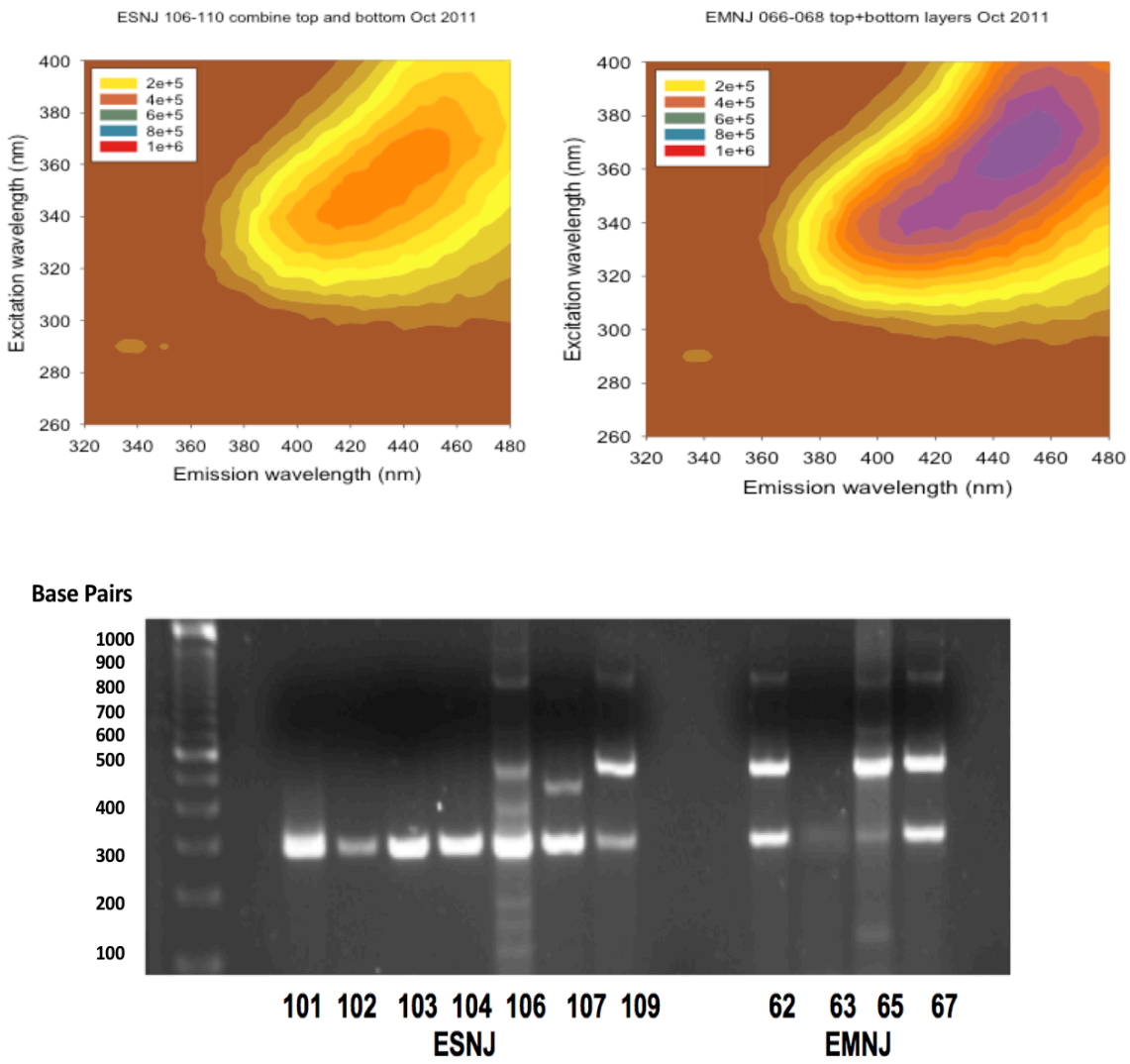


Figure 14: EEMS scans of combined raw fish oil from October 2011 and gel electrophoresis image of October 2011 DNA with V-REL associated primers. The EEMS scans of raw fish oils collected in October of 2011 emit a decreased fluorescence signal, as compared to August 2011. The intensity of PAH-like substances has decreased significantly. PCR products were compared to a 100 bp ladder mark, showing consistently prominent bands at the 300bp range. Variability is evident through the appearance of a second band at 600bp and occasional faint in-between bands at 400 bp and 500 bp. The differential display is an example of polymorphisms (DeFelice, 2018).

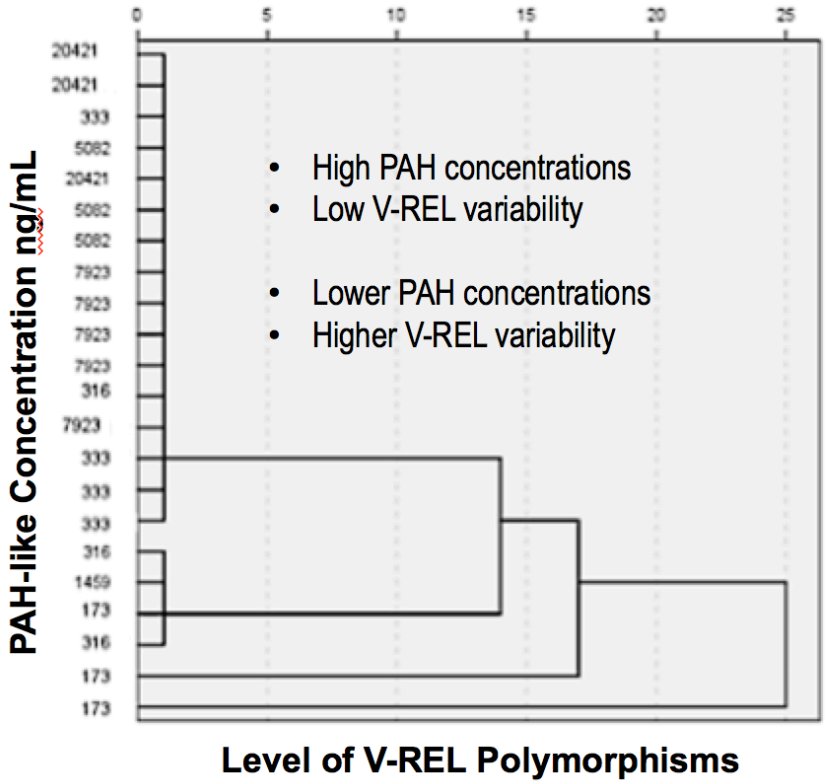


Figure 15: Rescaled distance cluster combined analysis of v-Rel polymorphisms and PAH-like concentrations in ng/mL. Higher PAH-like concentration samples are clustered together with low v-Rel variability while lower PAH-like concentration samples are clustered together with higher v-Rel variability (DeFelice, 2018).

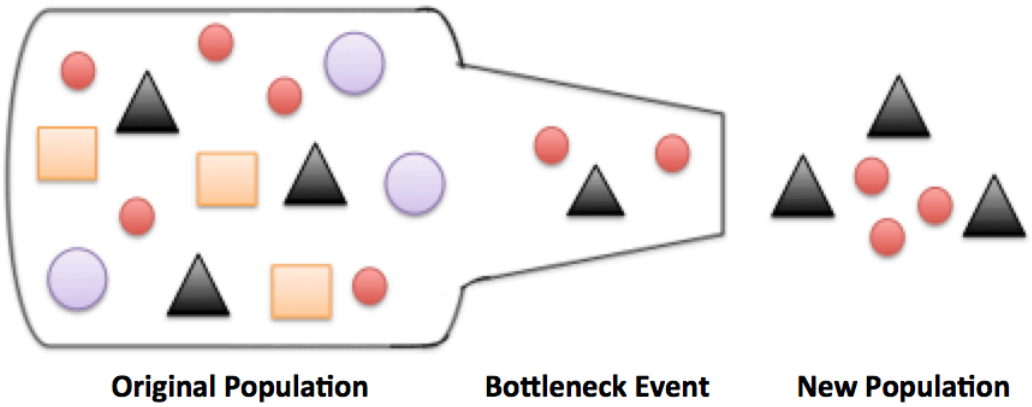


Figure 16: Graphical representation of bottleneck theoretical events. An original population with high variability experiences a drastic reduction in population through a "bottleneck" event resulting in a new surviving population of reduced variability and higher genetic relatedness [Graphic] (DeFelice, 2018).

Discussion:

All New Jersey residents and ecosystems have been and continue to be exposed to PAHs, however the degree and risks of exposure varies from region to region with higher levels predominantly in urban areas (NJDEP 2015). Lifestyle choices including but not limited to smoking and breathing in tobacco fumes, working daily in highly polluted regions such as coal and tar manufacturing, and consumption of burnt charbroiled food can greatly impact one's individual body burden. In order to become carcinogenic, PAHs must be acted upon by the body's metabolic processes. There may be an increased risk in children and adolescents due to higher rates of metabolism (NJDEP 2015).

Although there may be insufficient exposure data available to quantify the number of illnesses in New Jersey, the ecological impacts are evident. Tests on earthworms, fish, and benthic macroinvertebrates, whom are bottom-dwelling animals that are a food source for fish and others, have shown toxicity. Benthic macroinvertebrates in urban and industrial areas or adjacent to PAH-contaminated sites are at increased risk, as are plant and animal communities near these sites. This has been shown to reduce plant health and reproduction (NJDEP 2015).

The purpose of this project was to investigate the presence and amount of PAH-like compounds before and after Hurricane Sandy at various regions on the New Jersey coastline. By using fluorescence analysis, results showed crude oil-like PAHs in most, but not all collections of Atlantic menhaden. PAH presence was not associated with the size of the fish, ship collection, nor year of collection. PAH-like presence was determined by comparisons to EEMS scans of various catches of raw fish oil spiked with hydroxypyrene (HPY), mimicking intensities of major peaks and calculating similarities

using a HPY standard curve. The variations among peaks were due to differences in compositions of these substances. PAH-like substances were measure in ng/mL per 1mL of raw fish oil collected in combining both upper and lower layers. The combination of both layers resulted for the total concentration of the PAH-like substances. There was no significant statistical difference in PAH-like compound concentrations or in EEMS patterns of raw menhaden fish oil after Hurricane Sandy, thus proving the original hypothesis of increased contamination/PAH-like substance amount wrong.

Although Hurricane Sandy may not have had a statistical impact on this study's original purpose, the focus was quickly shifted toward studying the effect of PAHs on proto-oncogenes. The amplification of v-Rel, associated with B-cell lymphomas, was studied to detect a possibility of increased carcinogenesis in wild fish. Polymorphisms of v-Rel were hypothesized to increase in fish previously exposed to PAH pollution along the coastline of New Jersey. Through the processes of DNA isolation, PCR, and gel electrophoresis, the relationship between PAH-like compound concentration in raw fish oil and the positions of bands in individual fish samples were determined using v-Rel primers.

The genomic analysis showed one consistent band at 300 bp in all fish samples but variability was evident with the location of other bands in different regions of 400-600 bp. Confirmation of multiple bands in varying regions disregarded the need to further investigate single-strand conformation polymorphisms. The Rel locus is frequently altered via amplification, mutation, and rearrangement of genetic coding. Rel has an optimal DNA-binding site preference for a target sequence that is slightly different from other NF-kB subunit homodimers. Proto-oncogene c-Rel, a member of the NF-kB

transcription factor family, has certain amino acids in its specific DNA-binding sequences that are also mutated in v-Rel, which is the viral oncogenic version of avian c-Rel (Gilmore 2011). The hypothesized existent polymorphisms may have been due to varying chemical arrangements of REL isomers.

Through this report, it was established that the DNA polymorphisms found in raw fish oil had occurred less frequently in those having higher concentrations of PAH-like compounds. Higher PAH-like concentration samples were classified with low variability of the proto-oncogene v-Rel while lower PAH-like concentration samples were classified with high variability of v-Rel. While this does not fit the standard premise that there would be an increase in v-Rel polymorphisms in fish exposed to PAH pollution, one could further suggest that these results exemplify the theory of population bottleneck. The bottleneck theory was suggested after completing a cluster hierarchical analysis of PAH-like concentration with level of v-Rel polymorphisms. Cluster analysis is commonly used to compare and contrast a variety of experimental data. The agglomerative Information Bottleneck (aIB) algorithm employs a clustering technique to find a hierarchical clustering tree in a *bottom-up* fashion. In several works it has been shown to be useful for a variety of real-world problems, including supervised and unsupervised text classification, gene expression analysis, and image clustering (Slonim 2002).

The theory of population bottleneck, was proposed in 1942 by evolutionary biologist Ernst Mayr. Mayr proposed an idea of genetic revolution, where a population goes through a “bottleneck” by which it is greatly reduced through the mere change of a genetic environment, which may change the selective value of a gene; allowing genetic

relatedness and reducing variability (Provine 2004). Genetic bottlenecks are the most common consequences of genetic drift, which allow ecologists to redefine the concept of population size and strength. Population bottlenecks are defined when at least one generation faces a sudden decrease in allele frequency, having a drastic effect in proportion to the size of the population while the founder effect is easier to recognize with the presence of genetic diseases in subsequent generations (Anand 2011). This theory is prominent in closed communities of migratory species, which further suits this case of Atlantic menhaden, a seasonal migratory marine species.

Consequently having been affected by some environmental effect, such as excessive exposure to PAH-like compounds, these specific catches of Atlantic menhaden seem to have masked the predicted result of high variability in the v-Rel proto-oncogene. Events causing such a drastic reduction may include floods, hurricanes/extreme storms, bacterial exposure, and pollution. Bottlenecks can be the results of global climate changes, asteroid impacts, volcanism, or any other catastrophic event with massive environmental repercussions that result in the decimation of a species or a genetic line. They also reduce a specific population's genetic variation enough to prevent the population from adapting to its newly created environment (Jones 2015). Reducing v-Rel variability and increasing genetic relatedness is projected to aid the health and survival rate of Atlantic menhaden. This sharp reduction is proposed to halt the amount of or variability of polymorphisms in oncogenic genes, which could further assist the occurrence of carcinogenesis in coastal fish.

Future studies to further explore this topic should increase the number of raw fish oil and DNA samples of Atlantic menhaden from similar regions of interest. Dates of

catches should vary seasonally and annually due to the migratory patterns of this specific marine species to see if there would be a noticeable change in PAH-like compound concentration amount. Oil should be extracted individually from each fish rather than collectively combined as a group to further consider contamination analysis. Gas chromatography mass spectrometry would help further provide insight by identifying the molecular components of discovered PAH-like compounds and further connect them to a specific PAH. All consistent bands of same amplicon size viewed after gel electrophoresis may be nonspecific and should be confirmed via sequencing. Analysis of polymorphisms should not only be focused on v-rel, but other proto-oncogenes, especially those homologs of the Rel/NF-kB subunit family.

Conclusion:

To study the presence of PAH-like compounds in Atlantic menhaden, the raw fish oil was gathered from the skin and muscle fillet of New Jersey coastal catches of *Brevoortia tyrannus*. Extraction of PAHs was done via a 75% EtOH centrifugation method, which allowed a supernatant collection for fluorescence analysis. Fluorescence analysis of raw fish oil samples were read on a Horiba Fluorolog 3 using Excitation-Emission Matrix Scanning. PAH standard wavelengths of excitation: 260-400 nm and emission: 320-480 nm were used to identify contaminants in the proper fluorescent range. Results showed crude oil-like PAHs in most but not all collections of Atlantic menhaden. There was no statistical significant difference in PAH-like compound concentrations or in fluorescence patterns after Hurricane Sandy.

To study the effects of mutagenic PAHS on cancer related genes, genomic analysis was completed to study the effects of PAH pollution on proto-oncogene v-Rel. DNA was isolated, qualified, and quantified from the previously used Atlantic menhaden muscle tissue. Primers that were designed off an Atlantic menhaden transcriptome for v-Rel were used during PCR. PCR products were used during gel electrophoresis, which proved the presence of polymorphisms in the proto-oncogenic sequence of v-Rel. One consistent band was found in all fish samples at 300 bp. High PAH exposure from August 2011 resulted in high genetic relatedness, few isomers, and low variability. Low PAH exposure from October 2011 resulted in high variability due to the possibility of Rel isomers. DNA polymorphism occurred less frequently in fish having high concentrations of PAH-like compounds.

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