Differential Effect of Pollutants on Hatchling Success of Leatherback Sea Turtles

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

Molecular and Environmental Toxicology Center

at the

UNIVERSITY OF WISCONSIN-MADISON

2016

Date of final oral examination: 01/11/2016

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Dedicated to my Mother

Maria Magdalena Guadalupe Garcia Y Vera

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3 Abbreviations and Notations

Description	Abbreviation
Automatic Sample Extractor	ASE
Caretta caretta (Loggerhead Sea Turtle)	CC
Chelonia mydas (Green Sea Turtle)	CM
Dermochelys coriacea (Leatherback Sea Turtle)	DC
Fish and Wildlife Research Institute	FWRI
Florida Fish and Wildlife Conservation Commission	FWC
Gas Chromatography/Electron Capture Detector	GC/ECD
Gas Chromatography/Mass Spectrometry	GC/MS
Gel Permeation Chromatography)	GPC
High Water Line	HWL
Internal Review Board	IRB
Juno Beach - Southern section of nesting area	JB
Jupiter Carlin - Northern section of nesting area	JC
Limit of Detection	LOD
Limit of Quantitation	LOQ
Liquid Chromatography	LC
Loggerhead Marinelife Center	LMC
Mass Spectrometry	MS
National Marine Fisheries Service	NMFS
Polyaromatic Hydrocarbons or Polycyclic aromatic Hydrocarbons	PAHs
Retention Time	RT
Sea Surface Temperature	SST
Toe of Dune	TOD
Upper limit of linearity	ULL
Wisconsin State Laboratory of Hygiene	WSLH

4 Introduction

4.1 Aims

In this work we attempted to identify the conditions that affect hatching success of sea turtles. The work presented is divided in to two areas. First, the chemical analysis and secondly the statistical evaluation of the nest contents and the nesting conditions at the nesting site.

The Leatherback sea turtle (*Dermochelys coriacea*) was used as a biomonitor for the presence and impact of pollutants. We hypothesized that man-made toxic chemicals differentially bioaccumulate in the mothers and are transferred to eggs, thereby reducing hatchling success. Loggerhead sea turtle (*Caretta caretta*) samples were also used as a comparison species since they nest at the same beaches. Compounds studied were polyaromatic hydrocarbons and chlorinated pesticides.

We investigated the nesting conditions that are best suitable for the nesting success of the species. The nesting conditions were analyzed against air temperature, precipitation and sea surface temperature (SST). We investigated the location and date of nesting. Changing climate conditions are expected to modify the nesting sites for the populations studied. We include the analysis of the time of the start of the nesting season, the mean for the nesting season and the overall location of nesting sites.

The areas studied are presented as three separate manuscripts as they are submitted for publication. The chapter on chlorinated compounds is presented as an appendix at the end of the document as proof of concept for the extraction method.

4.2 Research Description

Leatherbacks were used to biomonitor for the presence and effects of pollutants. Leatherbacks present a unique opportunity, as an indicator species, because they are the largest living reptiles, are extremely long-lived and travel vast distances (NOAA). In addition, because Leatherbacks return to their natal beaches we have the opportunity to track the toxicant concentrations over multiple nesting seasons. Despite having larger

clutches, bigger eggs and higher reproductive output their hatching success is lower than comparable species [1]. It has been postulated that this low rate is caused by embryonic mortality or infertility [2].

Hatchling success in the Pacific population is undergoing a sharp decline, in comparison with the more stable Atlantic population, presenting a clear need to investigate the differences between these populations. Variations in the hatchling success can be as low as 0% and as high as 100%. These rates may be attributed to differences in the location of the feeding grounds and the ingestion of toxic materials. Atlantic leatherbacks stay closer to shore to feed and have a greater number of hatchlings than those in the Pacific. While Pacific populations forage in the convergence zones - such as the North Pacific Subtropical Convergence Zone - which concentrate marine debris, leading to large consumption of foreign materials. Sea turtles have been identified in these convergence zones and it has been shown that these animals are preferentially foraging in the areas that concentrate marine debris [3]. Plastic garbage has become ubiquitous in the world oceans and constitutes upwards of 86% of the total oceanic debris. Moreover, as it is similar enough in appearance, to the Leatherback's preferred food, jellyfish, it is commonly ingested in error. This is consistent with the fact that more than 60% of stranded sea turtles had plastic debris in their digestive tracts.

Polyaromatic Hydrocarbons (PAHs) and plasticizers are known endocrine disruptors and immunosuppressants that may contribute to the decline in hatchling success. Little is known about their toxic load and its effects on the overall health of the individuals. Some testudines, i.e. snapping turtles (*C. serpentina*), green sea turtle and loggerhead sea turtle have been monitored for the persistence of organic pollutants and heavy metals. Significant relationships have been found between the toxicant concentration in maternal blood and eggs [4]. Many toxicants have been associated with deformities, low fertility and low reproductive success. In a recent study, the authors reported the presence of four different PAH's in 17 of the 20 nest sites studied [5]. Whereas only one study has thus far investigated the presence of PAHs, plasticizers have not been studied at all. The efforts of this investigation will be concentrated on chlorinated

compounds and PAHs.

4.3 Research Design

Leatherback unborn eggs and hatchlings that died during the nesting cycle, will be used to assess the concentration of pollutants. The first set of samples has arrived from Jeanette Wyneken, PhD, Florida Atlantic University. We are also collaborating with Todd Jones, PhD, University of Hawaii, a renowned expert in Leatherbacks who has worked with this species at the University of British Columbia (UBC).

The samples will be tested for toxicants paying special attention to the presence of PAH's and organochlorines since they have been identified in sea turtles. The samples will be analyzed by: Gas chromatography (GC) with electron capture detection for chlorinated compounds and GC with mass spectrometry (MS).

We will then review the data for indication of change in hatchling success as it relates to the nesting parameter and in relation to the concentration of the chemicals in the eggs, and the dead offspring, to assess overall viability of the population studied.

We must also consider whether nesting site contamination contributes to, or is the primary source of, toxicant levels in the offspring. If any toxicants are encountered in the samples will investigate concentrations of such pollutants in sand and water at the nesting site. These findings could help identify the beaches that are not suitable for nesting, allowing for egg relocation.

5 Nesting Site Location

IRB approval was obtained from the University of Wisconsin-Madison in collaboration with Florida Atlantic University and Florida Fish and Wildlife Conservation Commission. Samples were collected under Consent Permit, Marine Turtle Permits #060 and 157 from the Florida Fish and Wildlife Conservation Commission.

In the Northern hemisphere, nesting of leatherbacks occurs at more southern latitudes. Prior to 1952, reports indicated that Leatherback nesting occurred in the Florida Keys and other islands in the Caribbean [6]. The first definite record of nesting in the Continental United States occurred near Flagler Beach, Flagler County, Florida on June 6th, 1947 [6]. Currently the main nesting sites for leatherbacks in the Continental United States are on the Atlantic Coast of Florida, with over 90% of the nests deposited in the southwest part of the state from Brevard County down to Dade County [7].

The site of sample collection in Juno Beach and Jupiter Carlin in Palm Beach County, Florida on the Atlantic Coast of the United States of America (USA). All samples were collected in collaboration with Loggerhead Marinelife Center (LMC). This area is approximately 12.2 Km of beach in the Eastern central part of the state. This location has the only nesting population of Leatherback sea turtles in the continental USA. This site is also a major nesting site for loggerhead and green sea turtles.

Loggerhead Marinelife Center (LMC) located in Juno Beach; Palm Beach County, Florida (Figure 5.1) has the longest monitoring program for leatherback sea turtles in the United States. The nesting survey program started in 1989 and included Juno Beach in Palm Beach County. Since then it has expanded to include Jupiter Beach, Jupiter/Carlin, Tequesta Beach, Jupiter Inlet, and Coral Cove Park. During the nesting season, LMC research team monitors a 12.2 Km beach with a south boundary at John D. MacArthur Beach State Park and north up to Jupiter Island. Nesting season runs from March 1 - October 31. Each year, in excess of 10,000 nests, from three species loggerhead, green sea turtle and leatherback, are laid on these beaches.

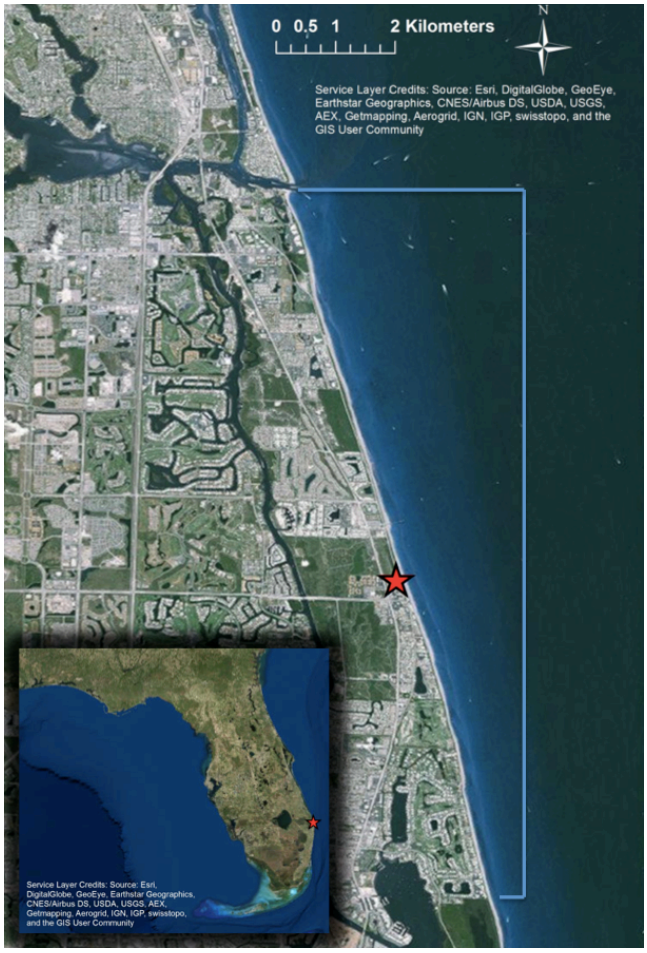

Figure 5.1 - Location Of Loggerhead Marinelife Center In Florida

Loggerhead Marinelife Center (LMC) is located on the Atlantic Coast of Florida and monitors 12.2 Km of beach from John D. MacArthur Beach State Park to Jupiter Island. This is the location of the nesting beaches were we collected the data for the nests in this study. The location is near the east most point on the Florida's east coast, and has little variation on the longitude.

Researchers at LMC developed a long-term program called 'The Leatherback Project' to study the nesting population of leatherbacks in the area. This area hosts the second highest leatherback nest counts of the counties in Florida, accounting for 38.7% of nesting in the state [8]. Although high inter-annual variability has been observed, there was an average of 86 leatherback nests per year from 2001 - 2005. The average

increased to 208 nests per year from 2009 - 2013. In 2014 and 2015 there were 236 and 177 nests respectively. As of 2013, a total of 503 individual females have been identified, 50% of which are re-migrants to North Palm Beach County. Along the study beach, 126 leatherbacks have been documented nesting during three or more seasons (unpublished data). Using the data collected from the remigrants, LMC assesses morphometric trends, nesting site selection, and reproductive success.

5.1 Sample Collection

- Nightly survey of beach during nesting season
- Nesting site location (GPS)
- Identification and Tag of mother (Leatherback)
- Daily morning survey at 60 + days until hatch
- Wait 3 days post hatch to allow for late emergence
- Excavate nest and survey

Survey

- Depth (top/bottom of chamber)
- Hatched
- Unhatched
- Live in nest
- Dead in nest
- Pipped live*
- Pipped dead**
- Spacers§

* the hatchling was broken the egg but not exited the egg - hatchling is live.

** the hatchling was broken the egg but not exited the egg - hatchling has perished.

§ eggs with no yolk - these are infertile eggs.

Since the measurements are taken by USA based staff they are done in standard units. All units were converted to the metric system. When relevant the units were converted back to standard units as applicable.

5.2 Nesting Season 2010

First nest: 2010, March 5; Last nest: 2010, June 26; Samples collected:

- 16 leatherback nest contents
- 4 sets of samples (4 each) for MeHg, Hg and heavy metals
- 3 samples of water at low (2) and high tide for metal analysis

5.3 Nesting Season 2011

First nest: 2011, March 11; Last nest: 2011, July 25; Samples collected:

- 20 leatherback nest contents
- 20 loggerhead nest contents
- 10 sets samples of water and sand at nesting site

5.4 Nesting Season 2012

First nest: 2012, March 13; Last nest: 2012, July 21; Samples collected:

- 20 leatherback nest contents
- 20 loggerhead nest contents
- 4 sand at nesting site (2 samples of old and new sand)

5.5 Nesting Season 2013

First nest: 2013, February 27; Last nest: 2013, July 2; Samples collected:

- 20 leatherback nest contents
- 20 loggerhead nest contents
- 3 sand at nesting site

6 Extraction of PAHs from Sea Turtle Eggs

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Relevant abbreviations and definitions used in the manuscript:

ASE (Accelerated Solvent Extractor)

DC (*Dermochelys coriacea*)

CC (*Caretta caretta*)

GC/MS (Gas Chromatography/Mass Spectrometry)

GPC (Gel Permeation Chromatography)

PAHs (Polyaromatic Hydrocarbons or Polycyclic aromatic Hydrocarbons)

6.1 Background

Polycyclic Aromatic Hydrocarbons (PAHs) are a widespread class of xenobiotics, found as common pollutants in air, soils and water. The majority of PAHs are introduced into the environment as a direct result of incomplete combustion of organic substances such as wood, meat, tobacco or any other carbon based substance. They are also naturally occurring as components of fossil fuels and can be released into the environment during the refinement of oil [9]. Certain locations, such as the Gulf of Mexico, have also been

shown to develop significant concentrations of PAHs solely by release from naturally occurring oil reserves [10]. PAHs have been demonstrated to have carcinogenic, mutagenic, teratogenic properties and are known endocrine disruptors [11-15].

Leatherback and loggerhead sea turtle eggs and hatchlings were used as a monitor for the presence of certain PAHs. The main objective was to identify the presence of PAHs on the eggs and dead hatchlings, and if identified, study the effect of PAHs on the hatching success of the turtles caused by these pollutants. Leatherbacks present a unique opportunity for a multi year study since they are known to return to known beaches to lay their eggs. They have significantly lower hatching success compared to other species, despite having the largest clutches, biggest eggs and highest reproductive output [1]. It has been postulated that the low rate is caused by embryonic mortality or infertility [2] due to the feeding ground locations and ingestion of toxic materials. Hatchling success can be as low as 23% and as high as 81% [2]. Loggerheads on the other hand are one of the most studied species of sea turtles and nest in the same beach as the leatherbacks in our study allowing for an interspecies comparison. Both species are known to spend some time in the oceanic convergence zones, which concentrate marine debris, thus leading to a potentially large consumption of foreign materials. Sea turtles have been identified in these convergence zones and it was concluded that these animals were preferentially foraging in the areas that concentrate marine debris [3]. More than 60% of stranded green sea turtles had plastic debris in their digestive tracts [16]. Leatherback sea turtles are known to ingest plastic debris while feeding since plastic objects have been identified in their digestive tracts [16, 17]. Plastic garbage has become ubiquitous in the world's oceans and constitutes upwards of 86% of the total oceanic debris [18, 19]. It is estimated that crude oil seepage from the processing of petroleum accounts for 53% of the total crude oil entering the marine environment [20]. The residues from petroleum derived residues is most commonly found near the coast and in estuaries near urban and industrial centers [21]. Sea turtles commonly ingest plastic debris and tar pellets since they are similar enough in appearance, to one of the sea turtle's preferred food, jellyfish. Little is known about their toxic load and its effects on the overall health of the individuals.

Some testudines, i.e. Snapping Turtles (*C. serpentina*), Green Sea Turtle (*C. mydas*) and Loggerhead Sea Turtle (*C. caretta*) have been used to monitor persistence of organic pollutants and heavy metals. Significant relationships have been found between the toxicant concentration in maternal blood and eggs [4]. Many toxicants have been associated with deformities, low fertility and low reproductive success. We focused on PAHs because they are known endocrine disruptors. Only one study has thus far investigated the presence of PAHs in sea turtles. The authors reported the presence of four different PAH's in 17 of the 20 nest sites studied [22].

Maternal transfer of some toxicants has been documented in sea turtles [23]. Maternal transport is the hypothesized mode of transmittance of PAHs to the offspring [1]. The concentration of toxicants found in the eggs and the deceased hatchlings should have been passed on from the mother if there are not external contaminants at the nesting site. It is expected that the concentration of PAHs will determine the effects on the fitness of the leatherback sea turtle.

Little is known about the toxic load of sea turtles and its effects on marine reptiles and there is limited information on the overall health of the individuals. Only one attempt has been made to correlate toxicants with other relevant chemistry blood values to assess the individuals' health [24]. Of special importance are the effects on the immune system since toxicants have been associated with immune deficiencies and pathology in multiple animal species [25].

The extraction and quantification of PAHs has been undertaken and defined in a multitude of media, including but not limited to tissue samples, blood plasma, soils and emissions [26-32]. Of most interest to us however were specifically the studies done into the determination of PAH concentration in marine organisms [26, 33-38]. PAHs' concentrations have been quantified in the majority of tissue types across several marine species. The overall process of homogenization, extraction and measurement remains constant through most of these studies, with the exact methods used to accomplish these specific tasks varying slightly between methods. The concentrations found in the different samples varied by orders of magnitude from .1 to 100 ng/g [31, 32, 39]. Loggerhead sea turtle eggs were found to have concentrations on the order of 10-

100 ng/g when detectable. Limits of detection, however, only went down to around 70 ng/g average for most PAHs tested so the data was quite limited, since the concentration was below the limit for concentration for the majority of samples. While the exact method used in the study is not defined, the NOAA Status and Trends Methods is cited as the basis for their process [40]. The NOAA guidelines are non-specific and only outline a generalized method for extraction and detection of various environmental toxicants from unspecified matrices. To our knowledge there is no published study defining a method specifically for the detection of PAHs in an sea turtle egg matrix, where the presence of many lipids can cause difficulty in the extraction and clean up steps. Therefor a reliable method for the extraction of PAHs in biological samples within the expected ng/g levels was necessary.

6.2 Methods

The method was adapted from the one used at the Wisconsin State Laboratory of Hygiene (WSLH Annex). Originally the method called for the use of Soxhlet reflux extraction that caused the loss of some of the low molecular weight, highly volatile PAHs. This method was modified to use an ASE extraction to improve recovery and reduce extraction time.

6.2.1 Reagents

All reagents used were Pesticide, HPLC grade or better.

- Ethyl Alcohol 190 Proof- Pharmaco-AAPER, Brookfield, CT, USA.
- Cyclohexane and Methanol - Honeywell, Burdick & Jackson, Muskegon, MI, USA.
- Dichloromethane - Honeywell, Burdick & Jackson, Muskegon, MI, USA.
- Hexanes and Sodium Sulfate (Anhydrous Granular) - Fisher Scientific, Fair Lawn, NJ, USA.

6.2.2 Standards

Standards were prepared by dilution of each analyte into a mixture at the appropriate concentration in Dichloromethane.

- 2,7-Dimethylnaphthalene - 10 mg, North Kingstown, RI ·USA.
- Benzo (e) pyrene; EPA 8270 Base/Neutrals Surrogate Spike Mix HC; Polynuclear Aromatic Hydrocarbon Mix HC - Supelco, Bellefonte, PA, USA.
- DFTPP (2,3,4,5,6,2′,3′,4′,5′,6′ - Decafluorotriphenylphosphine, Bis (pentafluorophenyl) phenylphosphine), Supelco, Bellefonte, PA, USA, (Branch of Sigma Aldrich, St. Louis, Missouri, USA).
- Coronene - 10 mg, North Kingstown, RI ·USA.
- Expanded PAH Mix, Accustandard, Inc. New Haven, CT USA.

6.2.3 Equipment

- Waring Commercial 7011S 2-Speed Food Blender with Stainless Steel Container, 32-Ounce (1000 mL); Model WF2211214, Torrington, CT USA.
- Furnace - Thermolyne 30400.
- ASE - Dionex ASE 200 Accelerated Solvent Extractor, Dionex Corporation, Sunnyvale, California. With 33 mL sample cells and 60 mL amber glass collection vials.
- ASE Filters for sample processing - Dionex Corporation, size 19.8 mm, Cat. No. 047017 Rev, 05.
- Büchi Rotovapor (R-114 or R-210), with Heating Bath (B-490 or B-491); Büchi Labortechnik AG, Switzerland.
- GPC - Samples were run through size exclusion chromatography on a Gilson GX-271 Liquid Handler with a 402 syringe pump and a 307 pump.
- GPC (Gel Permeation Chromatography) glass column (65 cm x 2.8 cm I.D) packed with 45 cm of 200-400 mesh beads (O-I-Analytical Envirobeads® S-X3 Select, Part No. 091-203, College Station Texas, USA). Fitted with two adjustable end plungers (Glenco Scientific).
- GC/MS - Agilent Technologies 6890N Network GC System, 7683B Series Injector, 5973 Network Mass Selective Detector, with MSD ChemStation D.02.00.275 software.

- GC column - Agilent Technologies, Inc., HP-5MS, 30 m x 0.250 mm, 0.25 micron, Cat No. 19091S-433.
- Temperature Controlled Water Bath Evaporator - 10 position, OA-SYS, Heating System, Organomation Associates, Inc. Berlin, MA, USA.
- Boiling Chips - Hengar Granules, For Smooth Boiling, Granules, Plain, Hengar Co., Division of Henry Troemner, LLC., Thorofare, NJ, USA.
- Other:
    - Analytical balance
    - Micro syringes
    - Volumetric flasks, graduated cylinders – 10, 25, 100 or 500 mL
    - 250 & 500 mL boiling flasks
    - Beakers, 100, 250, and 400 mL
    - 1.0mL or greater volumetric pipettes
    - Spatulas
    - Glass Wool - Heated to 450 degrees Celsius for > four hours for removing possible impurities

6.3 Sample Collection

Loggerhead Marinelife Center conducted a nightly patrol of an approximately 12.2 Km stretch of beach of the Atlantic Coast of central Florida. The beach has boundaries north at the Jupiter inlet and south at John D. MacArthur Beach State Park. The surveys for leatherback sea turtles begin early March and continue to late June. The patrol is performed by all-terrain vehicles nightly from 2100 to 0600 hours and covers the majority of the nests occurring in this location. Nesting turtles are approached during egg deposition and checked for tags. If no tags are identified the turtles are tagged accordingly with PIT (Passive Integrated Transponder) tags, plastic and/or metal tags. The surveyors encounter other nesting species as they survey for leatherbacks. This beach is also one of the main nesting sites for green and loggerhead sea turtles in Florida. Over 10,000 nests can occur in a season and nests are selected and monitored through October for all species.

Once a nest has been selected, it is monitored on a daily basis for the emergence of the hatchlings. Hatchlings normally emerge en masse and move out of the nest as a group. After the hatching event the nest is then monitored for 3 days to document any late emergence. The 3rd day after the documentation of the emergence the nests are excavated and a survey is taken of the nest contents. If the hatchlings do not emerge after 90 days the nest considered a “no hatch”. Eggs cannot survive > 90 days in the nest. Every leatherback nest and selected loggerhead nests are excavated as required by the Florida Wild Life Conservation Commission (FWLCC). FWLCC has specific standards and precautions that must be taken in order to work with turtle’s nests. In brief, GPS notation of nest location must be documented. Excavation can only be done by hand. No tools (e.g. shovels, buckets) can be used since they could hurt any live hatchlings trapped in the nest attempting to emerge. Surveyors must document the parameters of the nest, (e.g. location on the beach, depth of chamber). The survey also includes number of eggs hatched, number of eggs unhatched, number of live hatchlings, number of dead hatchlings, number of pipped hatchlings (live or dead in a partially opened egg) and the number of spacer eggs i.e. unfertile eggs with no yolk. All nest contents minus the research samples must be returned to the nest after the survey and the nest chamber must be refilled with sand.

A total of 20 samples from various nest contents were collected for analysis (n_{max} = 20 each species/year/species). The contents of each nest were collected and stored in stainless steel cans and placed in a -80 °C freezer until analysis. Samples included spacer eggs (n = 5 per nest or as available), unviable eggs (n = 5 per nest or as available) as well as sand and water samples that were collected and used to assess the concentration of PAHs at the nest site.

6.4 Sample Analysis

Preparation and Homogenization of Samples

All equipment and glassware was washed with both hexanes and dichloromethane (DCM) or ashed at 450 °C for > 4 hours prior to being used to prevent contamination. Homogenizing equipment was also rinsed with ethanol.

In brief, of 6-10 specimens were defrosted per batch. Each sample batch included a minimum of 2 controls (chicken eggs), one negative and one positive, and 1 system blank (consisting of pure evaporated dry ice). After defrosting, each specimen was removed from the shell and placed in a clean 250 mL beaker. Homogenization of samples was accomplished by using an industrial blender. Each batch of dry ice was homogenized until the consistency was that of a light powder and discarded. The blender was then rinsed with ethyl alcohol in between samples. When partially developed embryos were found in the egg the tissues were cut into smaller pieces prior to homogenization. After homogenization samples were covered with aluminum foil and placed at -20 °C freezer to allow the dry ice to sublimate. The following day, approximately 10.0 g of tissue powder was mixed with 60.0 g of anhydrous sodium sulfate. This mixture was stirred occasionally with a clean spatula for 30 min to allow for full absorption of water in the samples. Dry samples were stored -8 °C until analysis.

6.4.1 Option 1 - Reflux Extraction

This is the original extraction method from the WSLH - ESS ORG METHOD 1461, Polynuclear Aromatic Hydrocarbons in Fish Tissue by GC/MS– SW846 Method 8270D - Revision 4, February 2007, Matrix: Tissue.

Method 1461 is used to determine the concentration of certain PAHs in tissue. The method requires a Gas Chromatograph/Mass Spectrometer for the detection of ppb levels of certain PAHs. A measured mass of sample (~10 g) is Soxhlet extracted with dichloromethane. The resulting extract is dried, concentrated and a gel-permeation cleanup is performed. The extract is concentrated to 1.0 mL by evaporation and analyzed by GC/MS. Details of this extraction to follow:

1. Homogenized samples were retrieved and allowed to reach room temperature.
2. Add 20-40 µL of the surrogate standard spiking solution onto each sample.
3. Add 1.0 mL of the matrix spiking solution onto sample chosen for spiking.
4. Place the tissue/sodium sulfate mixture in a Soxhlet extractor with a glass wool plug.
5. Add a second glass wool plug on top of the tissue/sodium sulfate mixture. Pour 300 mL of dichloromethane into Soxhlet and let cycle to the attached 500 mL boiling

flask containing solvent rinsed boiling chips. Attach the flask and Soxhlet extraction tube to the Soxhlet bank and extract for 24 hours at 4-6 cycles per hour.

6. After extraction, allow the extract to cool and dry it by passing it through a drying column containing about 10 cm of anhydrous sodium sulfate. The eluate should be collected in a 500 mL boiling flask. After sample addition, rinse the drying column with 30 mL of dichloromethane.

6.4.2 Option 2 - ASE Extraction

1. Follow the procedure for homogenization of samples.
2. Add 20-40 µL of the surrogate standard spiking solution onto each sample.
3. Add 1.0 mL of the matrix spiking solution onto sample chosen for spiking.
4. Load the dried sample into appropriate ASE stainless steel cylinders. Fill remaining space with anhydrous sodium sulphate.
5. After extraction, allow the extract to cool and dry it by passing it through a drying column containing about 10 cm of anhydrous sodium sulfate. The eluate should be collected in a 500 mL boiling flask. After sample addition, rinse the drying column with 30 mL of dichloromethane.

Table 1 - ASE Parameters

Parameter		Units
Preheat	0	Min
Heat	5	Min
Static	5	Min
Flush %	60	%
Purge	120	Sec
Cycles	2	Cycles
Pressure	1500	Psi
Temperature	100	Celsius
Solvent	DCM	-

Samples from both extraction methods were then concentrated utilizing a Rotovapor (water temperature 45 °C) to approximately 5 mL and transfer to 10 mL volumetric flask. Bring the samples to 10 mL with dichloromethane.

6.4.3 Fat Percent Calculation

Pipette a 2.0 mL aliquot of the final extract and transfer it to an aluminum weighing dish tared to the nearest 0.1 milligram. Determine fat content of each sample by the following equation:

% fat = (residue + dish weight - tare) x 100/sample weight.

CF = Concentration Factor (10 mL total volume/2 mL used) = 5

6.4.4 GPC extraction

Most of the specimen samples had large amounts of high molecular weight lipids. These lipids are not amenable for the GC/MS and will clog the injector and column. Because of this a Gel Permeation Chromatography (GPC) column fractionation was performed. A 5 mL aliquot of each sample was analyzed by an automated GPC. The exact volume eluted for each fraction was determined from time settings on the GPC control unit. The times were determined and periodically adjusted by "calibrating" the gel resin column with GPC control standard spiked into the solvent (Figure 6.1). The GPC system was calibrated for the best window of collection of samples according to the retention time (RT) of the analytes present per the WSLH method in Annex 12.1.1. The flow was measured to be within 10 % of 5 mL/min. A 2.0 ug/mL RT standard was run through the system and fractions were collected at 3 min intervals beginning at 20 min and ending when all the compounds in question had eluted through the column (Table 2). From this data it became clear that the fraction window for the proper acquisition time of all the compounds was between 29 and 41 min. We therefore discarded the first fraction with anything earlier than 25 min.

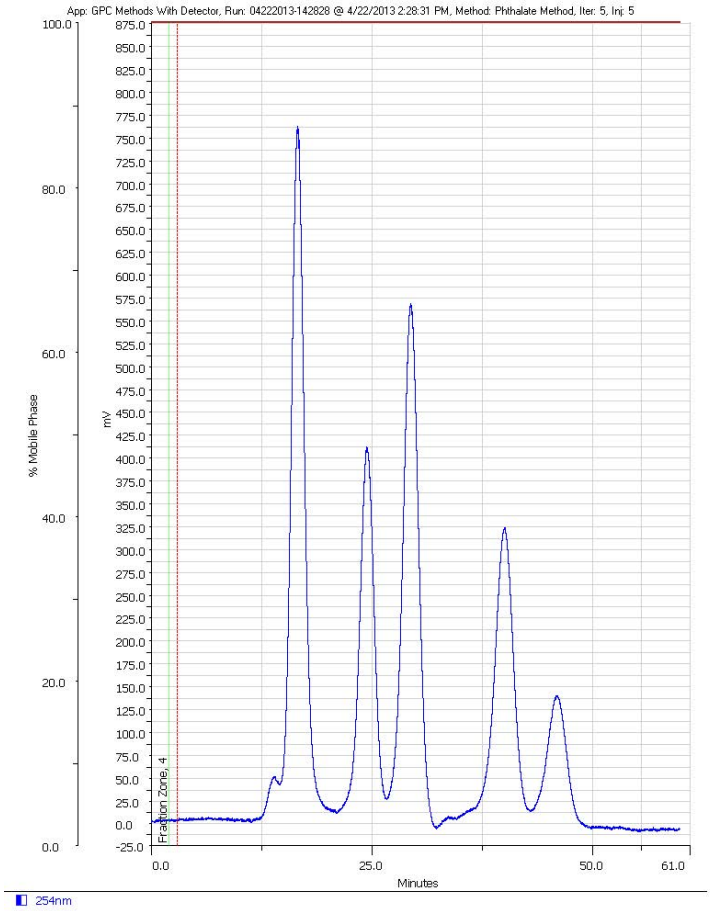


Figure 6.1 - Control used to test the GPC system for functionality.

The peak at ~ 14 minutes is the lipid front. The PAHs elute at approximately 28 minutes. Elution order: Corn oil, Bis (2-Ethyl hexyl) Phthalate, Methoxychlor, Perylene, Sulfur per method listed in Annex 12.1.1

The first 100 to 140 mL (consistent with the first 25 min fraction) was discarded. This fraction only contained the sample lipids. The second fraction (80-100 mL) was collected in 250 ml sample collection tubes. Samples were transferred to a 500 mL boiling flask with boiling chips. The GPC extracts were evaporated using the Rotovapor, at 45°C to ~ 3 mL. Then extracts were transferred to a calibrated 5 mL

centrifuge tube, rinsed dichloromethane and concentrated to 1 mL on a temperature controlled water bath evaporator.

Table 2 - Collection fractions at 5 mL/min and presence or absence of compounds

Component	20-23 min	23-26 min	26-29 min	29-32 min	32-35 min	35-38 min	38-41 min	>41 min
Naphthalene				PRE	PRE	PRE		
2-Methylmaphthalene				PRE	PRE	PRE		
1-Methylmaphthalene				PRE	PRE			
2,7-Dimethylmaphthalene				PRE	PRE			
Acenaphthylene				PRE	PRE	PRE		
Acenaphthene				PRE	PRE	PRE		
Fluorene				PRE	PRE			
Phenanthrene				PRE	PRE	PRE		
Anthracene					PRE	PRE		
Fluoranthene					PRE	PRE	PRE	
Pyrene					PRE	PRE		
Benzo(a)anthracene				PRE	PRE	PRE		
Chrysene				PRE	PRE	PRE		
Retene								
Benzo(b)fluoranthene					PRE	PRE		
Benzo(k)fluoranthene					PRE	PRE		
Benzo(e)pyrene						PRE	PRE	
Benzo(a)pyrene						PRE	PRE	
Indeno(1,2,3-cd)pyrene						PRE		
Benzo(g,h,i)perylene						PRE	PRE	
Dibenzo(a,h)anthracene					PRE	PRE		
Coronene								

Retene and Coronene were in the spike 2.0 ug/mL solution used. These compounds were added to the ASE method after the acquisition window was established (PRE = Present in fraction collected).

6.4.5 GC/MS Parameters

Interferences extracted from the samples varied considerably from sample to sample. However we did not encounter any major interferences that eluted with the peaks in question.

Extracts were analyzed with the following instrument conditions:

- Full acquisition mode with scan time: ~ 2.9 scans/sec
- Mass range: 50-550 amu
- Oven:
- Start at 50^{o}C, hold 10 min
- Ramp at 10^{o}C/min to 300^{o}C
- Hold 15.5 min at 300^{o}C
- Carrier gas: Helium
- Injector: 280^{o}C
- Transfer line: 320^{o}C
- Injection: Split/Splitless, Volume: 2 µL

These parameters were taken from EPA Method 525 and although followed while performing this method, they are considered advisory. Specific ranges for each instrument used should be established due to equipment inter-variability.

6.4.6 Standards

- Commercially available stock standards were used. These were dissolved in GC/MS quality dichloromethane, diluted to volume in a 100 mL volumetric flask. Standards were stored in Teflon-sealed screw-cap bottles in a freezer and protected from light.
- A second source standard was used to verify correct spiking of standards. Supelco offers a certified solution designated as “second source” with accompanying certification. Prepare in 100 mL volumetric flask and transfer to 125 mL Teflon-sealed screw-cap amber bottle

- A “Surrogate Standard” was used to verify extraction of each sample. This certified deuterated solution was purchased and diluted with dichloromethane to 50 mL.
- An Internal standard solution was made from certified deuterated solutions and diluted to a concentration appropriate for comparable response with the linearity of the assay.

6.4.7 Quality Control

- Before a batch of samples can be run, a method blank was analyzed to verify that each target compound's background concentration is below its LOD. If these criteria were not met samples were re-extracted and reanalyzed.
- During each batch of samples, a laboratory control was analyzed. The sample was spiked with all of the target analytes near the mid-point of the calibration range, usually 1.0 ug/mL.
- Surrogate recovery was used to determine if the extraction efficiency was acceptable. The acceptable range was 70-130% from the expected response. If surrogate recovery was not within limits, the samples were reanalyzed.

6.4.8 Method Calibration - Internal Standard Procedure

- The GC/MS was initially autotuned according to the manufacturer specifications and had to pass all criteria before any samples were analyzed
- GC/MS tuning standard: Decafluorotriphenylphosphine (DFTPP) assessed the system’s capability of fracturing and accurately detecting analytes over a wide mass range. Analysis did not proceed unless the DFTPP passed. A stock 50 ng/µL solution was prepared and diluted to 5.0 ng/µl. This was the working standard. Total mass injected did not exceed 50 ng. Injecting less helped for sensitive MS systems. The DFTPP was assessed by reasonably spanning both sides of the apex. The criteria for passing tune parameters are listed under Table 3. Within the run, the analyst injected the DFTPP at 12 h intervals. If the tune passed, the proceeding data was considered valid. Instrument maintenance and/or source cleaning were needed if the DFTPP failed. All DFTPP reports

were included in the batch folder. When not in use, stock and working standards were stored at -8 °C (Table 3).

Table 3 - GC/MS DFTTP tuning parameters

Target Mass	Rel. to Mass	Lower Limit%	Upper Limit%
51	198	10	80
68	69	0.00	2
70	69	0.00	2
127	198	10	80
197	198	0	1
198	442	50	100
199	198	5	9
275	198	10	60
365	198	1	100
441	443	0.01	100
442	442	100	100
443	442	15	24

- Calibration standards: A minimum of five calibration standards at different concentrations, were used. Each calibration standard, plus solvent blank, contained 1.0 mL of internal standard solution. The correlation coefficients (R^2) had to be ≥ 0.995.
- 1.0 mL of the internal standard solution was added to all samples. The internal standards used were those with close molecular weight to the compounds being quantitated. The individual IS and accompanying target analytes are listed below:

Table 4 - Molecular Weight of Compound Analyzed

No.	Compound Name	MW (g/mol)
1	Naphthalene	128.17
2	2-Methylnaphthalene	142.20
3	1-Methylnaphthalene	142.20
4	2,7-Dimethylnaphthalene	156.23
5	Acenaphthylene	152.20
6	Acenaphthene	154.21
7	Fluorene	166.22
8	Phenanthrene	178.23
9	Anthracene	178.23
10	Fluoranthene	202.26
11	Pyrene	202.25
12	Benzo(a)anthracene	228.29
13	Chrysene	228.29
14	Benzo(b)fluoranthene	252.31
15	Benzo(k)fluoranthene	252.32
16	Benzo(e)pyrene	252.32
17	Benzo(a)pyrene	252.32
18	Indeno(1,2,3-cd)pyrene	276.33
19	Benzo(g,h,i)perylene	276.33
20	Dibenzo(a,h)anthracene	278.35
21	Coronene*	300.36

Coronene that showed reduced linear range. This was the last eluting compound in the sequence due to its high molecular weight and therefor the largest RT. This lengthy RT on the column made the peak have a low response and broader chromatographic peak shape that made the quantitation more troublesome. It was also observed that there was a tendency to overload the column on the higher end of the linear range.

Table 5 - Internal Standards

Deuterated Internal Standards	Target compounds
Naphthalene-d_8	Naphthalene 2-methylnaphthalene 1-methylnaphthalene.
Acenaphthene-d_{10}	2,7-dimethylnaphthalene Acenaphthylene Acenaphthene Fluorene.
Phenanthrene-d_{10}	Phenanthrene Anthracene Fluoranthene.
Chrysene-d_{12}	Pyrene p-terphenyl-d_{14} (surrogate) Benzo(a) anthracene Chrysene.
Perylene-d_{12}	Benzo(b)fluoranthene Benzo(k)fluoranthene Benzo(a)pyrene Benzo(e)pyrene Indeno(1,2,3-cd)pyrene Benzo(g,h,i)perylene Dibenzo(a,h)anthracene.

- Quantitation was based on primary ions extracted from the total ion scan. The secondary ions were used to confirm target analytes. The calibration curve was set to linear (vs. exponential).
- When assessing the original calibration curve and correlation coefficients, it was apparent that low-level quantitation was suspect due to high concentration responses adversely affecting low calibration response near the origin. To remedy this, a “low level” calibration was employed with more points around the detection limit and ranging only to the mid-point calibration concentration.
- The calibration points were constructed by calculating an amount ratio and a response ratio for each level of a particular peak in the calibration table.

- The amount ratio is the amount of the compound divided by the amount of the internal standard at this level.
- The response ratio was the abundance of the compound divided by the abundance of the internal standard at this level.
- An equation for the curve through the calibration points was calculated using the linear type of curve fit.

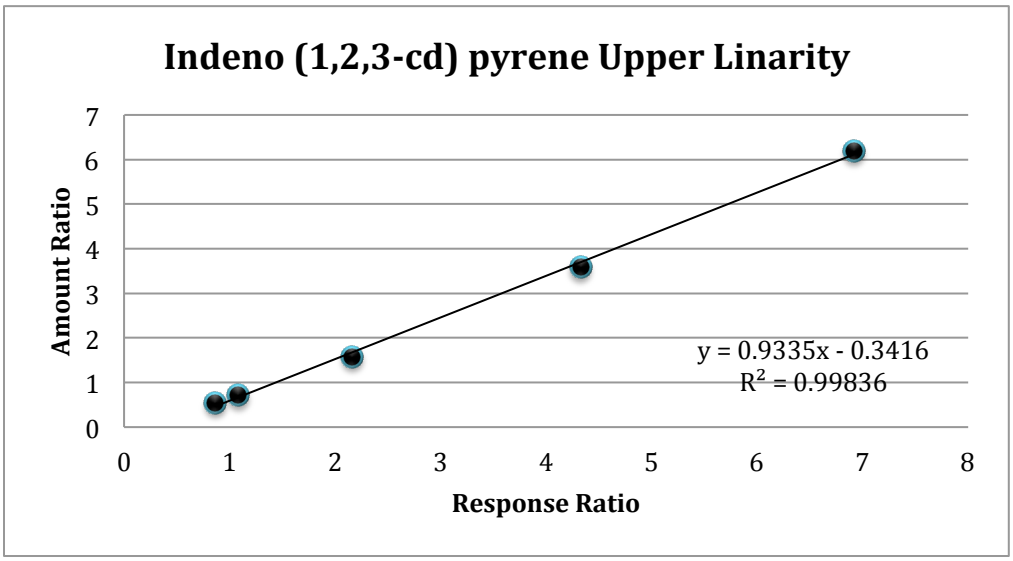
Equation 1 - Response Factor Calculation

$$RFx = \frac{\text{Amount Ratio}}{\text{Response Ratio}}$$

- Results were used to plot a calibration curve of response vs. amount ratio.
- ChemStation software calculated the above ratios. Each calibration table had at least five levels (Figure 6.2 A).

When a peak was identified the ChemStation software was used to verify the mass spectrum to the library for each compound. Not only did the quantitation ions and the secondary ion had to present and in the correct ratio, but the scan for the compound would have to match the software library (>90%) for a complete identification of the compound.

A



B

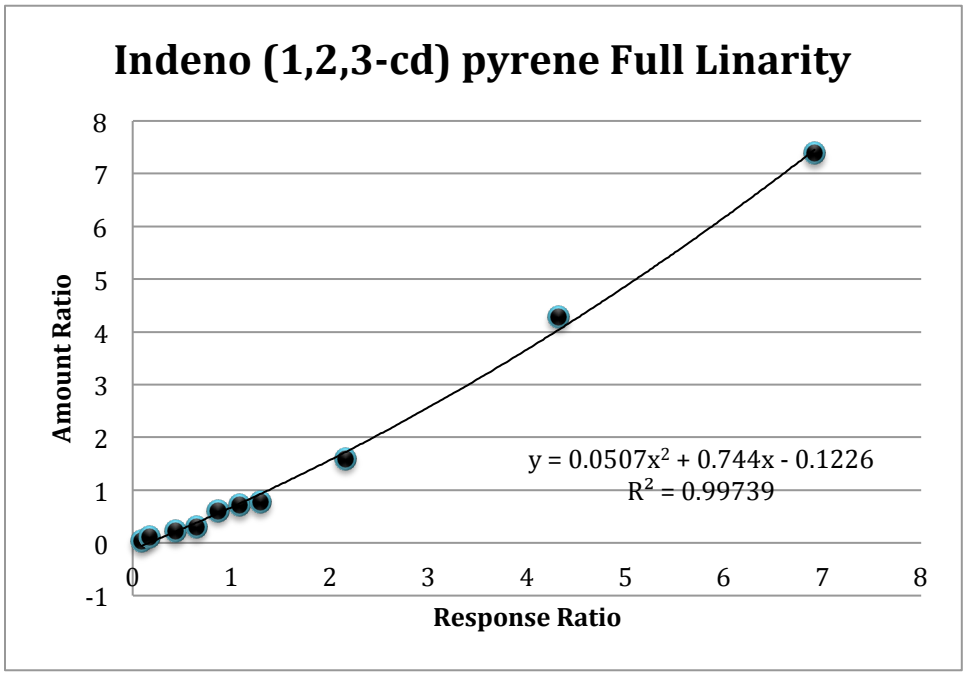


Figure 6.2 - Linearity

Sample linearity report for Indeno (1,2,3-cd) pyrene.

(A) Linear 5 point calibration, not forced through origin. In this example we have the

high curve calibrators 0.8, 1.0, 2.0, 4.0 and 6.4 µg/mL.

(B) Exponential 10 point calibration, not forced through origin. In this example we have the total curve calibrators 0.08, 0.16, 0.40, 0.60, 0.8, 1.0, 2.0, 4.0 and 6.4 µg/mL.

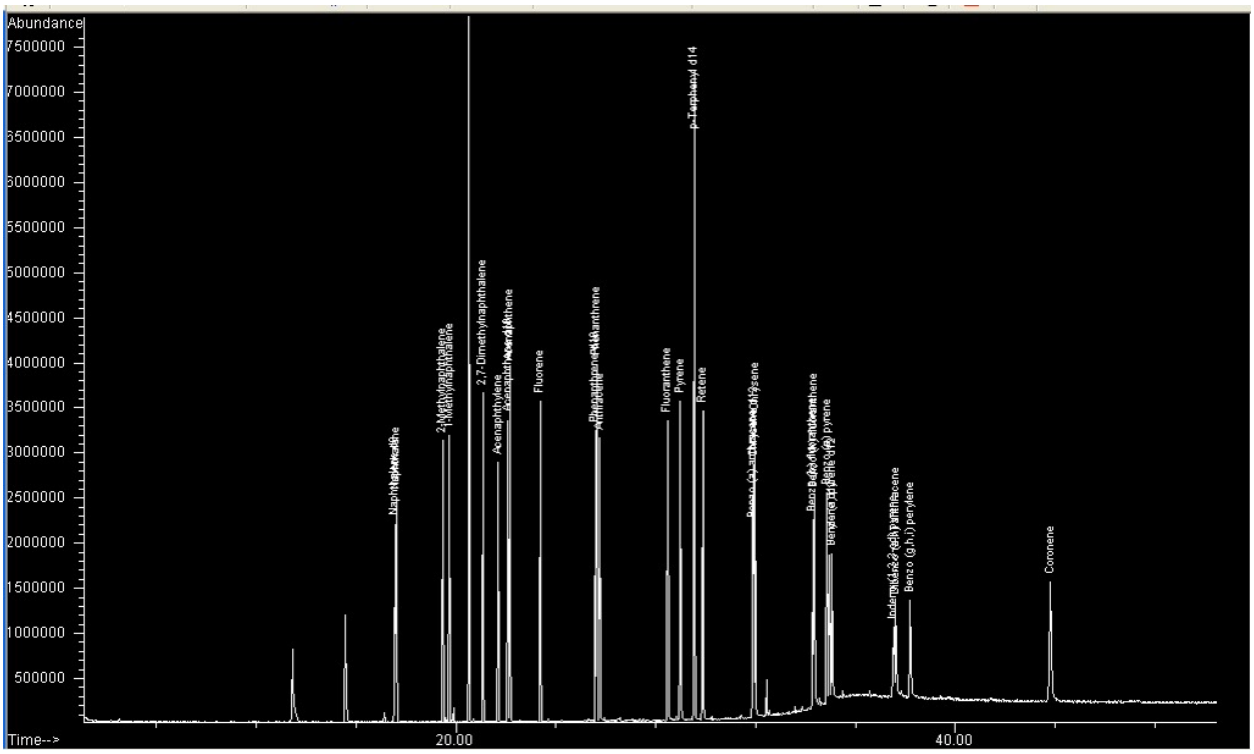


Figure 6.3 - Total Ion Chromatograph.

Total ion chromatograph (TIC) of the 1.0 ug/mL injection control used to test the GC system for suitability . The conditions used on the GC/MS system allow for the separation of all compounds analyzed.

Table 6 - Primary and Secondary Ions Select PAHs

Internal Standard	RT	Quantitation Ion	Secondary Ion	Secondary Ion Ratio %
Naphthalene d8	17.557	136.00	137.00	10.80
Acenaphthene d10	22.085	164.00	163.00	18.70
Phenanthrene d10	25.591	188.00	189.00	14.90
Fluoranthene d10	28.281	212.00	106.00	16.70
Chrysene d12	31.916	240.00	120.00	10.30
Perylene d12	35.062	264.00	265.00	21.20
p-Terphenyl d14	29.543	244.00	245.00	18.30

Analyte	RT	Quantitation Ion	Secondary Ion	Secondary Ion Ratio %
Naphthalene	17.608	128.00	129.00	10.80
2-Methylmaphthalene	19.473	142.00	115.00	27.10
1-Methylmaphthalene	19.737	142.00	115.00	29.10
2,7-Dimethylmaphthalene	21.082	156.00	141.00	62.10
Acenaphthylene	21.698	152.00	76.00	9.60
Acenaphthene	22.159	154.00	76.000	19.70
Fluorene	23.281	166.00	139.00	7.00
Phenanthrene	25.642	178.10	152.10	8.00
Anthracene	25.747	178.10	152.10	6.70
Fluoranthene	28.485	202.00	101.00	11.40
Pyrene	28.992	202.10	101.00	13.70
Benzo(a)anthracene	31.883	228.10	114.10	11.10
Chrysene	31.971	228.10	114.10	9.90
Retene	29.880	219.00	234.00	72.70
Benzo(b)fluoranthene	34.299	252.10	126.10	13.00
Benzo(k)fluoranthene	34.349	252.10	126.10	13.40
Benzo(e)pyrene	34.847	252.10	126.10	12.10
Benzo(a)pyrene	34.943	252.10	126.10	11.50
Indeno(1,2,3-cd)pyrene	37.515	276.10	138.00	18.70
Benzo(g,h,i)perylene	38.190	276.10	138.10	19.80
Dibenzo(a,h)anthracene	37.604	278.10	139.10	13.50
Coronene	43.805	300.00	150.00	27.10

Table 7 - List of Calibrators

Compound	CAL 0.08 Conc. µg/mL	CAL 0.16 Conc. µg/mL	CAL 0.40 Conc. µg/mL	CAL 0.60 Conc. µg/mL	CAL 0.80 Conc. µg/mL	CAL 1.00 Conc. µg/mL	CAL 1.20 Conc. µg/mL	CAL 2.00 Conc. µg/mL	CAL 4.00 Conc. µg/mL	CAL 6.40 Conc. µg/mL
Naphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
2-Methylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
1-Methylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
2,7-Dimethylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Acenaphthylene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Acenaphthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Fluorene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Phenanthrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Anthracene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Fluoranthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(a)anthracene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Chrysene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Retene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(b)fluoranthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(k)fluoranthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(e)pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(a)pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Indeno(1,2,3-c,d)pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(g,h,i)perylene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Dibenz(a,h)anthracene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Coronene	0.24*	0.48*	0.80*	1.2*	1.9*	2.7*	3.8*	5.4*	7.6*	10.9*
p-Terphenyl-d14	0.25*	0.50*	0.96*	1.4*	1.7*	2.0*	3.2*	3.0*	4.4*	6.0*

* Concentrations are not concordant with other compounds.

Table 8 - PAHs Low concentration calibration curve

Compound	CAL 0.08 µg/mL	CAL 0.16 µg/mL	CAL 0.40 µg/mL	CAL 0.60 µg/mL	CAL 0.80 µg/mL	R^2
Naphthalene	0.08	0.16	0.40	0.60	0.80	1.000
2-Methylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.000
1-Methylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.000
2,7-Dimethylnaphthalene	0.08	0.16	0.40	0.60	0.80	0.999
Acenaphthylene	0.08	0.16	0.40	0.60	0.80	0.998
Acenaphthene	0.08	0.16	0.40	0.60	0.80	1.000
Fluorene	0.08	0.16	0.40	0.60	0.80	0.998
Phenanthrene	0.08	0.16	0.40	0.60	0.80	0.999
Anthracene	0.08	0.16	0.40	0.60	0.80	0.999
Fluoranthene	0.08	0.16	0.40	0.60	0.80	0.998
Pyrene	0.08	0.16	0.40	0.60	0.80	1.000
Benzo(a)anthracene	0.08	0.16	0.40	0.60	0.80	0.999
Chrysene	0.08	0.16	0.40	0.60	0.80	0.999
Retene	0.08	0.16	0.40	0.60	0.80	0.999
Benzo(b)fluoranthene	0.08	0.16	0.40	0.60	0.80	1.000
Benzo(k)fluoranthene	0.08	0.16	0.40	0.60	0.80	0.999
Benzo(e)pyrene	0.08	0.16	0.40	0.60	0.80	1.000
Benzo(a)pyrene	0.08	0.16	0.40	0.60	0.80	1.000
Indeno(1,2,3-c,d)pyrene	0.08	0.16	0.40	0.60	0.80	1.000
Benzo(g,h,i)perylene	0.08	0.16	0.40	0.60	0.80	0.999
Dibenz(a,h)anthracene	0.08	0.16	0.40	0.60	0.80	0.999
Coronene	0.24*	0.48*	0.80*	1.2*	1.9*	0.999
p-Terphenyl-d14	0.25*	0.50*	0.96*	1.4*	1.7*	1.000

* Concentrations are not concordant with other compounds.

Table 9 - PAHs High concentration calibration curve

Compound	CAL 0.80 µg/mL	CAL 1.00 µg/mL	CAL 2.00 µg/mL	CAL 4.00 µg/mL	CAL 6.40 µg/mL	R^2
Naphthalene	0.80	1.0	2.0	4.0	6.4	1.000
2-Methylnaphthalene	0.80	1.0	2.0	4.0	6.4	1.000
1-Methylnaphthalene	0.80	1.0	2.0	4.0	6.4	1.000
2,7-Dimethylnaphthalene	0.80	1.0	2.0	4.0	6.4	1.000
Acenaphthylene	0.80	1.0	2.0	4.0	6.4	1.000
Acenaphthene	0.80	1.0	2.0	4.0	6.4	1.000
Fluorene	0.80	1.0	2.0	4.0	6.4	1.000
Phenanthrene	0.80	1.0	2.0	4.0	6.4	1.000
Anthracene	0.80	1.0	2.0	4.0	6.4	1.000
Fluoranthene	0.80	1.0	2.0	4.0	6.4	1.000
Pyrene	0.80	1.0	2.0	4.0	6.4	1.000
Benzo(a)anthracene	0.80	1.0	2.0	4.0	6.4	0.999
Chrysene	0.80	1.0	2.0	4.0	6.4	1.000
Retene	0.80	1.0	2.0	4.0	6.4	1.000
Benzo(b)fluoranthene	0.80	1.0	2.0	4.0	6.4	0.998
Benzo(k)fluoranthene	0.80	1.0	2.0	4.0	6.4	1.000
Benzo(e)pyrene	0.80	1.0	2.0	4.0	6.4	0.998
Benzo(a)pyrene	0.80	1.0	2.0	4.0	6.4	0.998
Indeno(1,2,3-c,d)pyrene	0.80	1.0	2.0	4.0	6.4	0.998
Benzo(g,h,i)perylene	0.80	1.0	2.0	4.0	6.4	1.000
Dibenz(a,h)anthracene	0.80	1.0	2.0	4.0	6.4	0.999
Coronene	1.9*	2.7*	5.4*	7.6*	10.9*	0.998
p-Terphenyl-d14	1.7*	2.0*	3.0*	4.4*	6.0*	0.999

* Concentrations are not concordant with other compounds.

6.5 Results

The ASE method allows for the quantitation of 21 different PAHs in egg and tissue samples. A full calibration using all standards from 0.08 to 6.40 ug/mL (with the exception of coronene - 1.0 to 10.9 ug/mL, and -Terphenyl-d14 - 1.7 - 6.0 ug/mL) was performed on the GC/MS. From this calibration it was determined that 4 compounds did not behave in a linear fashion. These were: Benzo (a) anthracene, Benzo (a) Pyrene, Indeno (1,2,3-c, d) Pyrene and Coronene. For these four compounds an exponential regression curve had to be used, even though linearity was obtained with acceptable R^2 values (Figure 6.2).

The WSLH can only use either linear or exponential curves for all compound for each run, because of this, the calibration was separated into a LOW and HIGH set of calibrators. The LOW calibration curve was used the 0.08, 0.16, 0.40, 0.60 and 0.80 calibrators. The HIGH calibration curve used 0.80, 1.00, 2.00, 4.00 and the 6.40 calibrators. By doing this, two separate linear curves can be used to quantitate results within the total linear range.

The linearity for each run was plotted using Microsoft Excel. Each dilution was compared to the others to determine the concentration in which the limits of quantitation (LOQ), the limits of detection (LOD) and the upper limit of linearity (ULL) become unreliable. For each sample extract the total fat content as well as the GC/MS full spectrum was collected.

Reproducibility was verified by extracting and analyzing 5 different batches (Table 11). The mean % error for most compounds was <10% of target with the exception of Benzo(e)pyrene, which had 11% error.

If there was peak within the RT window of acceptance of the assay the peak was first evaluated to see if the qualifier ion was in the correct % response from the quantitating ion. If there was match then the full scan was matched to the full scan from the mass spectral library to verify the correct compound was being identified.

Samples with an analyte below the LOD of the assay were evaporated to 200 uL and reshot so quantitative values could be obtained. The LOQ at the 200 uL concentration is listed in Table 12 - Minimum Concentration LOQ With 200 uL Samples.

Table 10 - PAHs Linearity in µg/mL and ng/g

Compound	LOD µg/mL	LOQ µg/mL	ULL µg/mL	LOD ng/g	LOQ ng/g	ULL ng/g
Naphthalene	0.01	0.01	6.40	2.0	2.0	1280.0
2-Methylnaphthalene	0.01	0.04	6.40	2.0	8.0	1280.0
1-Methylnaphthalene	0.01	0.01	6.40	2.0	2.0	1280.0
2,7-Dimethylnaphthalene	0.02	0.08	6.40	4.0	16.0	1280.0
Acenaphthylene	0.01	0.08	6.40	2.0	16.0	1280.0
Acenaphthene	0.02	0.08	6.40	4.0	16.0	1280.0
Fluorene	0.04	0.08	6.40	8.0	16.0	1280.0
Phenanthrene	0.04	0.08	6.40	8.0	16.0	1280.0
Anthracene	0.04	0.08	6.40	8.0	16.0	1280.0
Fluoranthene	0.02	0.08	6.40	4.0	16.0	1280.0
Pyrene	0.02	0.08	6.40	4.0	16.0	1280.0
Benzo(a)anthracene	0.08	0.08	6.40	16.0	16.0	1280.0
Chrysene	0.01	0.01	6.40	2.0	2.0	1280.0
Retene	0.01	0.08	6.40	2.0	16.0	1280.0
Benzo(b)fluoranthene	0.08	0.08	6.40	16.0	16.0	1280.0
Benzo(k)fluoranthene	0.04	0.08	6.40	8.0	8.0	1280.0
Benzo(e)pyrene	0.08	0.08	6.40	16.0	16.0	1280.0
Benzo(a)pyrene	0.08	0.08	6.40	16.0	16.0	1280.0
Indeno(1,2,3-c,d)pyrene	0.08	0.08	6.40	16.0	16.0	1280.0
Benzo(g,h,i)perylene	0.08	0.08	6.40	16.0	16.0	1280.0
Dibenz(a,h)anthracene	0.08	0.08	6.40	16.0	16.0	1280.0
Coronene	0.190	0.190	1.90	38.0	38.0	380.0
p-Terphenyl-d14	0.05	0.10	6.40	10.0	20.0	1280.0

The 1.0 ug/mL standard was analyzed on 5 different days to assess the reproducibility of the assay.

Table 11 - Reproducibility

Compound	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	%
Naphthalene	1.01	1.03	1.04	1.03	1.01	1.024	1.02
2-Methylnaphthalene	1.03	1.03	1.03	1.01	1.03	1.026	1.03
1-Methylnaphthalene	1.01	1.02	1.03	1.02	1.02	1.020	1.02
2,7-Dimethylnaphthalene	1.02	1.05	1.03	1.05	1.01	1.032	1.03
Acenaphthylene	0.96	0.93	0.92	0.81	1.05	0.934	0.93
Acenaphthene	1.04	1.06	1.05	1.07	0.95	1.034	1.03
Fluorene	1.01	1.02	1.00	1.00	1.03	1.012	1.01
Phenanthrene	1.08	1.12	1.10	1.15	0.92	1.074	1.07
Anthracene	0.95	0.94	0.95	0.86	0.97	0.934	0.93
Fluoranthene	0.89	0.89	0.91	0.85	1.07	0.922	0.92
Pyrene	1.16	1.17	1.10	1.01	0.92	1.072	1.07
Benzo(a)anthracene	1.00	0.94	0.94	0.81	1.09	0.956	0.96
Chrysene	0.98	1.01	1.01	1.09	1.02	1.022	1.02
Retene	1.19	1.15	1.04	0.94	1.08	1.080	1.08
Benzo(b)fluoranthene	1.11	1.15	1.15	1.13	0.95	1.098	1.10
Benzo(k)fluoranthene	1.02	1.07	1.04	1.07	0.92	1.024	1.02
Benzo(e)pyrene	1.18	1.18	1.12	1.19	0.88	1.109	1.11
Benzo(a)pyrene	1.00	1.00	1.00	0.95	1.03	0.996	1.00
Indeno(1,2,3-c,d)pyrene	1.13	1.11	1.02	0.98	1.03	1.054	1.05
Benzo(g,h,i)perylene	1.06	1.13	1.00	1.09	0.98	1.052	1.05
Dibenz(a,h)anthracene	1.05	1.11	0.96	1.05	1.01	1.036	1.04
Coronene	3.48	3.81	2.89	3.10	2.60	3.176	1.18
p-Terphenyl-d14	2.28	2.36	2.18	2.18	1.96	2.192	1.10

Table 12 - Minimum Concentration LOQ With 200 uL Samples

Compound	LOQ ng/g
Naphthalene	0.4
2-Methylnaphthalene	1.6
1-Methylnaphthalene	0.4
2,7-Dimethylnaphthalene	3.2
Acenaphthylene	3.2
Acenaphthene	3.2
Fluorene	3.2
Phenanthrene	3.2
Anthracene	3.2
Fluoranthene	3.2
Pyrene	3.2
Benzo(a)anthracene	3.2
Chrysene	0.4
Retene	3.2
Benzo(b)fluoranthene	3.2
Benzo(k)fluoranthene	1.6
Benzo(e)pyrene	3.2
Benzo(a)pyrene	3.2
Indeno(1,2,3-c,d)pyrene	3.2
Benzo(g,h,i)perylene	3.2
Dibenz(a,h)anthracene	3.2
Coronene	7.6
p-Terphenyl-d14	4.0

6.6 Sea Turtle Sample Results

We analyzed a total of 48 sea turtle egg and tissue samples for the presence of PAHs. For each of the two species studied we analyzed 8 samples from each collection year

(2011, 2012 and 2013). All samples analyzed were negative down to the LOQ and also no analytes were found at the LOD.

6.7 Discussion

The first part of this study was to determine the LOQ, LOD and ULL of the ASE, ESS Org Method 1461 for the Wisconsin State Laboratory of Hygiene (Annex 12.1.1). The use of the ASE with the calculated linearity with the use of low and high concentration curves would greatly improve the function and usage of ESS Org Method 1461, which is currently being used in multiple studies through universities around the state. The second purpose is to quantitate the PAHs present in sea turtle eggs and then determining the effects of varying concentrations of PAHs on fitness and survival rate of sea turtle hatchlings.

This study provides a reliable and sensitive method for analyzing PAHs in egg and tissue samples. The original method utilizing the Soxhlet tubes had two issues that needed to be addressed. The first was the lengthy setup and extraction procedure. The samples needed to reflux for a minimum of 12 hours and preferentially 24 hours in Soxhlet extraction tubes. The second issue was the loss of low molecular weight compounds. Figure 6.4 represents the deviation from target for (A) Soxhlet extraction method and (B) ASE extraction method. Both this issues were addressed by doing the ASE extraction. This updated procedure allowed for a faster extraction and prevented the evaporation of low molecular weight compounds. The extraction time for the ASE is about 15 minutes per sample tube. The maximum number of tubes is 24 samples. So the total run time would be a maximum of 6 hours for a full run. As for the reduction in evaporation of low molecular weight compounds, there is a clear reduction percent lost when recovery is calculated. Utilizing the Soxhlet method there is a maximum loss of > 25% for Naphthalene, the lowest molecular weight compound. This was reduced to < 3% using the ASE method. Two compounds had > 20% error (naphthalene and 1-Methylnaphthalene) and two had > 10% error (2-Methylnaphthalene and Acenaphthylene) for the Soxhlet method. All compounds had < 10% error for the ASE method.

There was only one compound, coronene, which showed reduced linear range. This was the compound with the longest RT and the highest molecular weight. The increased RT made the peak have broader chromatographic peak shape and a reduced response, which reduced the linear range. It was also observed that there was a tendency to overload the column with the compound on the upper part of the linear range.

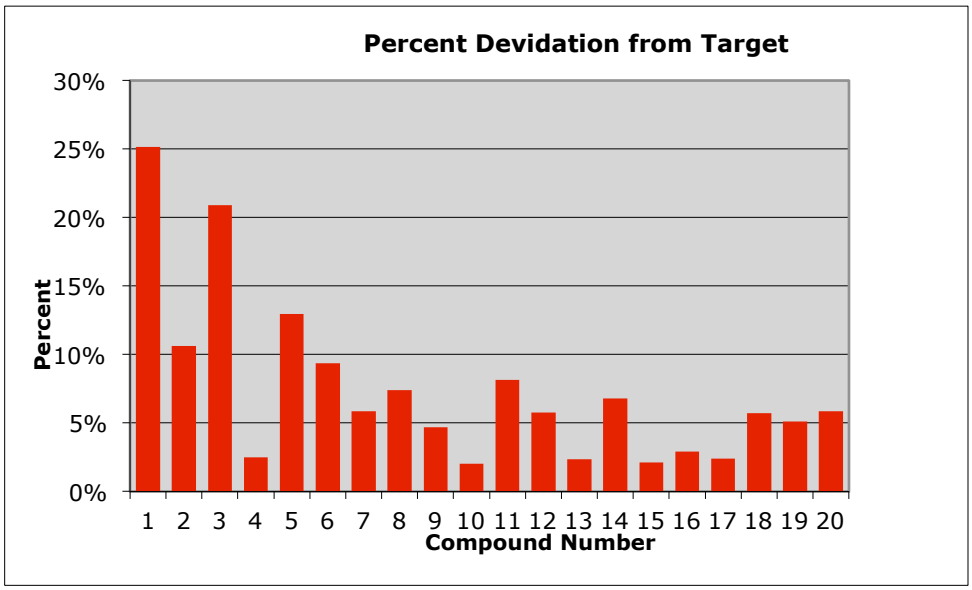
Overall the ASE method performed better than the Soxhlet methodology. Utilizing this updated technique will allow technicians to reduce the extraction time and be able to reduce the percent error for the low molecular weight compounds.

Table 13 - List of PAHs

No.	Compound Name
1	Naphthalene
2	2-Methylnaphthalene
3	1-Methylnaphthalene
4	2,7-Dimethylnaphthalene
5	Acenaphthylene
6	Acenaphthene
7	Fluorene
8	Phenanthrene
9	Anthracene
10	Fluoranthene
11	Pyrene
12	Benzo (a) anthracene
13	Chrysene
14	Benzo (b) fluoranthene
15	Benzo (k) fluoranthene
16	Benzo (e) pyrene
17	Benzo (a) pyrene
18	Indeno (1,2,3-cd) pyrene
19	Benzo (g, h, i) perylene
20	Dibenzo (a, h) anthracene
	Coronene*

* Coronene was not evaluated via the Soxhlet extraction method.

Deviation by Soxhlet method



Deviation by ASE method

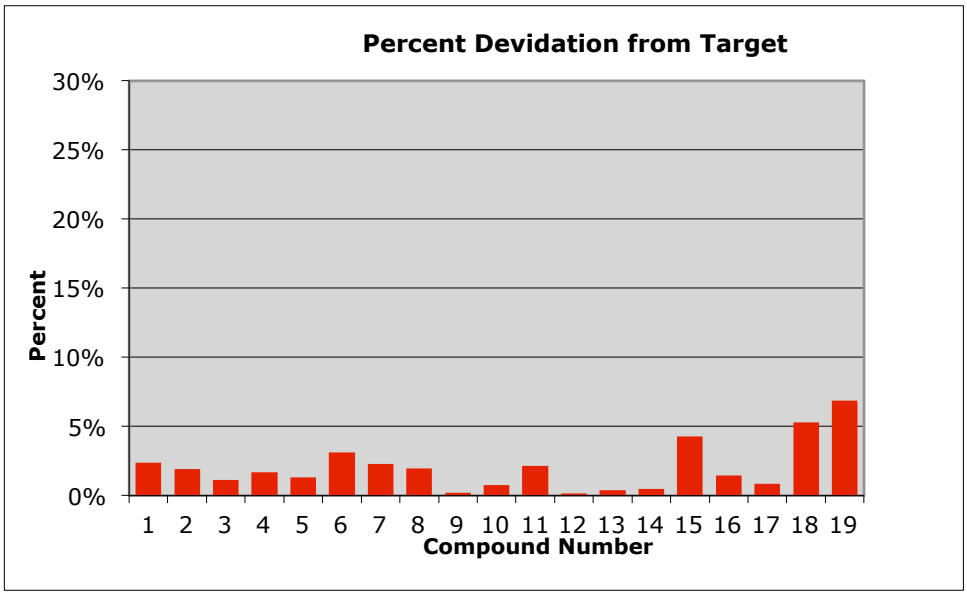


Figure 6.4 - Percent Deviation for each method

See Table 13 for peak reference identification

For the second part of the study we analyzed 48 sea turtle egg samples. The exploratory runs were done with leatherback samples collected in (data not presented). All these samples were negative for PAHs. Since PAHs have been reported in loggerhead sea turtles [22] on the West Coast of Florida we collected samples from loggerhead sea turtles on subsequent years. We had the expectation that loggerhead sea turtle samples would have PAHs present. When we analyzed the loggerhead samples from 2011, 2012 and 2013 all were negative for PAHs. At first this was an intriguing set of results. However after further evaluation it was understandable why turtles that inhabit the Atlantic coast of the USA did not contain PAHs. There are no known natural sources of PAHs in the Atlantic coast of Florida as there are in the Gulf of Mexico. This indicated that naturally occurring PAHs in the Gulf of Mexico and petrochemical industry in this area are the major source of PAHs in the loggerhead sea turtles. In the case of the leatherback sea turtles, they are pelagic organisms, which inhabit a pelagic environment where there is little expectation of contact with petrochemicals seeping from the ocean floor. There is also no expectation of encountering oil rigs or other sources of petrochemicals in the open ocean. Because of the lack of presence of the petrochemicals studied in the biological samples the water and sand were not analyzed.

Internal References - Available at the WSLH

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2. "Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater" EPA/600/4-82-057.
3. "Soxhlet Extraction", EPA Method 3540C (Revision 3, December, 1996).
4. "Determinative Chromatographic Separations", EPA Method 8000B (Revision 2, December, 1996).

7 Evaluation of Nesting Parameters vs. Nesting Success of Leatherback Sea Turtles in North Palm Beach County, Florida

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Relevant abbreviations and definitions used in the manuscript:

DC (*Dermochelys coriacea*)

CC (*Caretta caretta*)

EWMA (Exponentially Weighted Moving Average)

TOD (Toe of Dune)

HWL (High Water Line)

Top = distance from the sand surface to the top of the nest chamber where the first egg was located

Bottom = distance from the sand surface to the bottom of the nest where the farthest down egg was located

Δ Depth (nest chamber dimension) = the difference between the top and bottom of the nest

7.1 Abstract

Nest success in sea turtles is affected by many biological and physical parameters associated with the nesting site. In this study we attempt to identify the physical nesting site parameters that affect the nesting success of Leatherback sea turtles (*Dermochelys coriacea*) on the coast of north Palm Beach County, on Florida's Atlantic coast. The statistical analysis of the multi-year data set allows for the identification of the best nest location parameters for increased hatching success. We evaluated data collected from 2010 to 2013 during the nesting season that runs from late February/early March until the end of July/early August. The data was collected as part of the 'The Leatherback Project' in collaboration with Loggerhead Marinelife Center (LMC), which monitors the beaches at Juno and Jupiter/Carlin. The beaches that were monitored account for 13.3% of the total leatherback sea turtle nests in the state of Florida. Nesting success was measured as the total number of hatchlings that were able to leave the nest without human intervention. The nesting dimensions and location were evaluated against precipitation and air temperature. Exponentially weighted moving averages (EWMA) of precipitation, as well as low and high temperature, were tested versus percent hatched for highest correlation. We determined that the length of time from the time of nesting to hatching event and the nest depth and size and temperature in conjunction with the location on the beach to be important parameters in the success of a nest. It is expected that this information on the nesting success, as it relates to nest parameters, will benefit the conservation efforts and increase hatching success of relocated nests.

7.2 Background

Leatherback sea turtles (*Dermochelys coriacea*) are currently listed as endangered by the Endangered Species Act [41] and are also listed under the CITES, Appendices I, as a species that is the most endangered and threatened with extinction [42]. The world population has seen drastic decreases over the last several decades and most populations are still in decline [43-45]. The last sizable nesting leatherback population in the Pacific Ocean nests on Bird's Head Peninsula in Papua Barat, Indonesia. This population accounts for 75% of total leatherback nesting in the Western Pacific. The

population has been considerably reduced in size and continues to decline. Tapilatu et al studied the population at the two main nesting sites (Jamursba Medi and Wermon) and found that the estimated annual number of nests at Jamursba Medi has declined 78.3% over the past 27 years (5.5% annual rate of decline). Nesting at Wermon has declined 62.8% (11.6% annual rate of decline) from 2,994 nests in 2002 to 1,096 in 2011. The populations in the Atlantic appear to be more stable and in some cases increasing [8, 46]. The largest population in the Atlantic Ocean nest of the coast of Gabon [47]. The population of the Atlantic Coast of the United States being studied appears to be increasing (Figure 5).

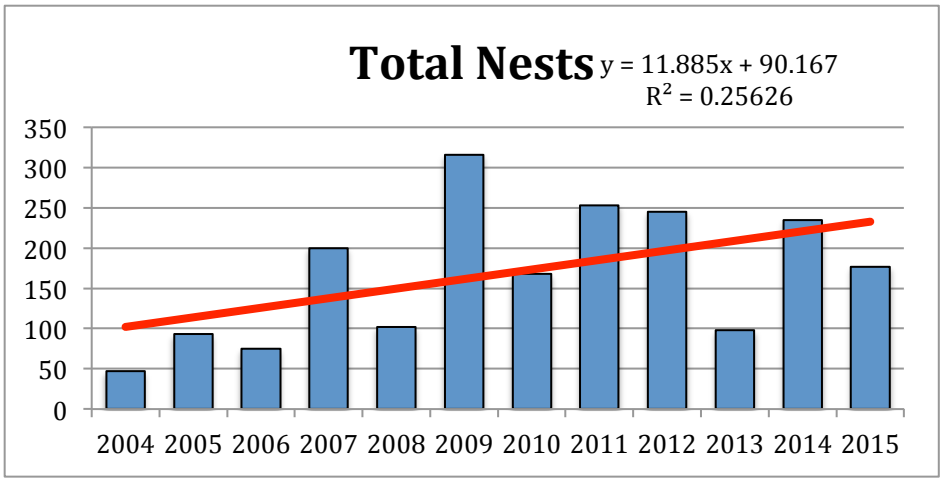


Figure 5 Total Nest Count Per Year

This graph is for total for all the nests each year form 2004 to 2015 in Juno and Jupiter beaches, the area of study. Nesting data evaluated in this paper was form 2010 to 2013.

Conservation efforts need to address all the life stages of endangered species [43, 45]. Efforts to save the juvenile or adult individuals would be ineffective if the offspring are not viable. Perils at sea decrease the adult population [45], but efforts need to be placed in producing higher numbers of viable hatchlings to replace loses at sea. It is well documented that harvesting of eggs for human consumption puts undue pressure on the reproductive viability of sea turtle populations [43, 48, 49]. In the case of

leatherbacks, one of the main reasons for the population decline has been attributed to the harvesting of eggs [43, 44, 48]. The best-documented collapse occurred in the beaches of Rantau Abang, Terengganu, in Malaysia in the early 1950s [43]. The population was estimated at > 10,000 yearly nesting females [49]. There was a complete collapse of the population mainly caused by human consumption of eggs, such that by the early 2000's few if any females nested there. When conservation efforts were fully implemented, the government purchased all the harvested eggs and relocated them to hatcheries, but from 2001 to 2011 none of the nests have hatched [50].

Relocation of sea turtle eggs has been used as a conservation effort in an attempt to increase the number of viable offspring being released into the wild [43, 51-53]. From their inception, the establishment of hatcheries as part of the conservation effort has been controversial, with many questions regarding the success of the programs [49, 53-55]. Relocation is not only necessary to prevent predation or human damage to the nests, but under some circumstances it is necessary to relocate nests due to inadequate nesting conditions and poor site selection by the nesting female, such as a nest that is too close to the HWL or in areas where humans could damage the site [51]. If the eggs are relocated, the nesting conditions at the new nesting location must be evaluated for proper nesting parameters. If this is not done, the survival of the hatchlings and sex ratios of the turtles could be affected [49, 56]. In some cases, the nests are relocated to a new section of the beach or to a hatchery [43, 49, 51]. When placed in a hatchery, the nests need to be monitored to ensure proper nesting conditions and good hatching success. If proper conditions are not maintained, hatching success could be reduced.

Biological and physical parameters associated with the nesting site are known to affect nesting success. Increased microbial and fungal abundance in the nest have been associated with a reduction in the partial pressure of oxygen which has a negative correlation with hatching success [57]. Temperature and precipitation have also been shown to affect hatching success [58, 59]. Success is also dependent on location with respect to the distance from the vegetation, toe of dune (TOD) and high water line

(HWL) [51]. Of key importance is to maintain the natural ratio of females to males in the population, since sea turtles have temperature-dependent sex determination [60]. Some of the first attempts to establish hatcheries as a conservation effort occurred in Ranatu Abang in 1961 before the temperature-dependent sex determination was identified in sea turtles [43]. The lack of temperature control lead to the production of mostly females [43] or mostly males [55].

Nesting site selection relative to the HWL appears to have a within-individual consistency, however, there is a large within-individual variation and the nesting site pattern could not be predicted from previous nesting choices for any particular nesting female [61]. If this were true, it would allow for females to have a certain degree of heritability that could be advantageous in the location of new nesting sites. There is selective pressure against nests that are laid too close to the HWL since nesting beaches are known to be dynamic and erosion causes the loss of nests close to the HWL [51]. There also appears to be a strong selection force for nesting closer to the HWL, and away from the TOD, since hatchlings are unable to move to the ocean in vegetated areas [61]. By relocating nests that are too close to the water line the selective pressure that controls the site selection for females nesting outside of acceptable beach zones would be reduced. It has been noted that there appears to be a learning period for nesting females; in the case of loggerhead sea turtles first-time breeders select significantly more unsuccessful nesting sites as related to the HWL [62].

In the northern hemisphere nesting of leatherbacks occurs at more southern latitudes. Prior to 1952 reports indicated that Leatherback nesting occurred in the Florida Keys and other islands in the Caribbean [6]. The first definite record of nesting in the Continental United States occurred near Flagler Beach, Flagler County, Florida on June 6th, 1947 [6]. Currently the main nesting sites for leatherbacks in the Continental United States are on the Atlantic Coast of Florida, with over 90% of the nests in the southwest part of the state from Brevard County down to Dade County [7] (Table 15 and Figure 6). Loggerhead Marinelife Center located in Juno Beach, Palm Beach, Florida has the longest monitoring program for leatherback sea turtles in the United States. The LMC research team monitors 12.2 Km of beach from John D. MacArthur Beach

State Park (Figure 5.1), north to Jupiter Island during sea turtle nesting season. Nesting season runs from March 1 - October 31 on Florida's east coast. Each year, in excess of 10,000 nests from three species loggerhead (*Caretta caretta*), green sea turtle (*Chelonia mydas*) and leatherback (*Dermochelys coriacea*) come to nest in these beaches. Leatherback nest numbers in northern Palm Beach County have been increasing since the late 1990's. In 2001, LMC researchers developed a long-term program (The Leatherback Project) to study the nesting population of leatherbacks in the area. Each night during nesting season, staff members patrol the beach. Each leatherback encountered is measured and tagged for identification. The program was designed to identify nesting individuals and understand the size and status of the population. The program has since expanded to include many research projects studying migration, health, reproductive behavior, contaminants, threats, and genetics.

This population presents a unique opportunity for studying naturally occurring nesting conditions since very few of the nests have to be relocated. This is mainly because there is a coordinated effort to maintain by the Palm Beach County, Shoreline Enhancement & Restoration Program. In this program, beach erosion is prevented by continuously maintaining the beach line and addressing any naturally caused erosion. Because of this there were a only a total of 12 nests that had to be relocated during the study period. The main reason for relocation was due to human activities in the area, specifically the shoreline protection program in which sand is added to eroded parts of the beach or dredging. Only 4 nests had to be relocated due to the nesting female paling the nest in an area that was not suitable for nesting; adjacent to a scarp (3), nesting below the HWL (1).

In this work, a logistic model was developed for the evaluation hatching success of leatherback nests. The physical parameters were evaluated to ascertain what nesting parameters lead to the highest rate of hatching success as measured by the number of viable offspring that leave the nest. The data was analyzed to identify if the nest size, number of eggs, depth, and location on the beach had an effect. As part of the analysis, air temperature and precipitation were included as an exponentially weighted moving average (EWMA) for the duration of nesting season.

Table 14 - Relocated Nests 2010 - 2013

Date of Nest	% Success	Reason for relocation
03/21/10	48.39%	Shoreline Protection project/Dredging project
03/29/10	65.71%	Shoreline Protection project/Dredging project
04/01/10	27.78%	Shoreline Protection project/Dredging project
04/08/10	49.28%	Shoreline Protection project/Dredging project
04/11/10	40.00%	Shoreline Protection project/Dredging project
04/12/10	0.00%	Shoreline Protection project/Dredging project
04/14/10	25.76%	Shoreline Protection project/Dredging project
04/26/10	50.77%	Nested below beach scarp
05/02/10	2.30%	Nested below the spring high tide line
03/24/10	24.00%	Nested above beach scarp
04/02/10	41.46%	Shoreline Protection project/Dredging project
04/30/13	62.32%	Nested below beach scarp

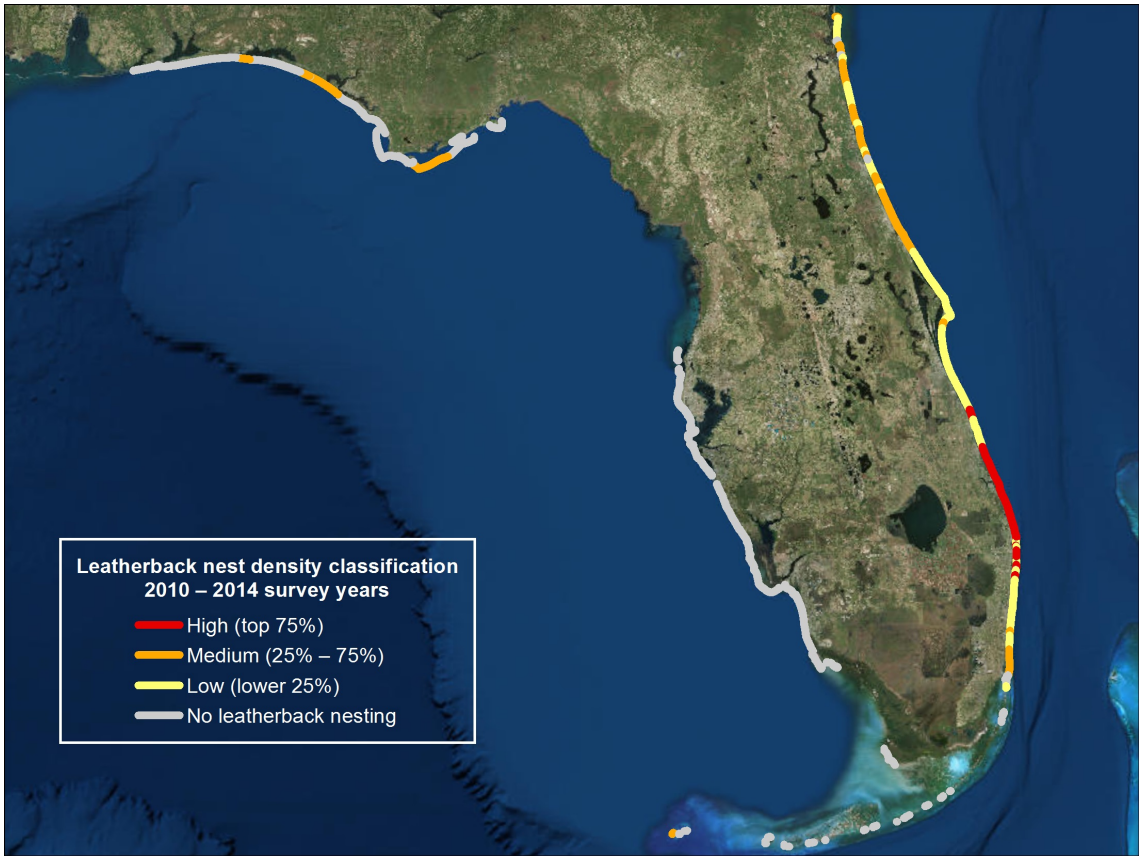


Figure 6 - Leatherback Nest Density

The map shows the location of the main nesting beaches of the east coast of the state. The majority of the nests occur in an area located in between Martin and Palm Beach County. This area accounts for 70.82 percent of the nesting population in the state during the 2010 through 2014 seasons.

http://myfwc.com/media/2988405/leatherbacknestdensity2014.jpg, Accessed 12/12/15

Table 15 - Nesting density of leatherback sea turtle in Florida 2010 - 2013

County	2010	2011	2012	2013
Nassau	1	3	7	0
Duval	2	3	6	3
St. Johns	4	23	13	16
Flagler	0	13	7	6
Volusia	15	22	23	18
Brevard	77	102	91	76
Indian River	87	61	66	56
St. Lucie	203	254	189	94
Martin	561	649	627	352
Palm Beach	**368**	**517**	**622**	**253**
Broward	14	5	46	18
Miami-Dade	2	0	11	3
Monroe	0	0	0	1
Collier	0	0	0	0
Lee	0	0	0	0
Charlotte	0	0	0	0
Sarasota	0	0	0	0
Manatee	0	0	0	0
Hillsborough	0	0	0	0
Pinellas	0	0	0	0
Franklin	0	1	0	0
Gulf	0	0	0	0
Bay	0	0	2	0
Walton	0	0	1	0
Okaloosa	0	0	1	0
Santa Rosa	0	0	0	0
Escambia	0	0	0	0
	1,334	1,653	1,712	896

http://myfwc.com/media/2988405/leatherbacknestdensity2014.jpg, Accessed 12/12/15, Source: FWC/FWRI Statewide Nesting Beach Survey Program Database as of 20 February 2015

7.3 Methods

The nesting season for leatherbacks is from late early March until early July (Table 16). During the nesting season, each nesting event is recorded. To be able to identify each nesting event, the beach is patrolled every night during the nesting season. When a nesting female is encountered, the nest's information is recorded and each nesting female is identified and tagged. The Global Positioning System (GPS) coordinates of the nest are recorded and the location is verified by excavating the nest chamber until the eggs are found. The nest is then marked with a stake at the nest location. A second stake is placed at the toe of dune for location purposes. The nests are then monitored on a daily basis. Morning patrols check the nest for tracks indicating the hatchlings have emerged. Once the nest is hatched, the beach patrol staff wait 3 days to allow for the emergence of all possible hatchlings. If the nest has not hatched after 80 days, the nest is excavated to assess the fate of the hatchlings. For earlier nests, or nests that deposited when the weather is cold earlier in the season, the wait time may be increased to 90 days. This continues until the end of the nesting season. Once the nest is excavated, the parameters of the nest are documented. The data is then entered into a database at LMC.

Table 16 - Dates Of Nesting Events And Total Counts Per Year

Year	Date of Nesting Event	Nest Count
2010	March 05 − June 24	168
2011	March 11 − June 30	252
2012	March 13 – July 7	246
2013	March 17 − June 26	98

Every leatherback nest is excavated in this area as required by the Florida Wild Life Conservation Commission (FWLCC). FWLCC has specific standards and precautions that must be taken in order to work with turtle nests. In brief, GPS notation of location of nest must be documented. The nests are monitored on a daily basis for the emergence

of the hatchlings. Hatchlings normally emerge en masse and leave the nest as a group. After the hatching event occurs, the nest is monitored for 3 days to document any late emergence. The 3rd day after documentation of the emergence, the nests are excavated and a survey is taken of the nest contents. If the hatchlings do not emerge after 90 days, the nest considered a “no hatch”. It is assumed that eggs cannot survive >90 days in the nest. Excavation can only be done by hand. No tools (i.e. shovels) can be use since they could hurt any live hatchlings trapped in the nest. Surveyors must document the parameters of the nest, e.g. depth to top of chamber. The survey also includes number of eggs hatched, number of eggs unhatched, number of live hatchlings, number of dead hatchlings, number of pipped (live or dead hatchlings still in a partially opened egg) and the number of spacer eggs, i.e. unfertile eggs with no yolk. All nest contents minus the research samples must be returned to the nest after the survey and the nest chamber must be refilled with sand. Under some circumstances a nest that is originally accounted for at the time of nesting is subsequently lost. When a nest is not located the GPS coordinates are reviewed and a pit with a radius and depth of 4 feet must be created, making the best attempt possible to locate the clutch.

In the 2010 season, 18 nests were surveyed. At the time of excavation, 11 of 18 nests had live hatchlings in the nest (n=48), 13 nests had dead hatchlings in the nest (n=68) and only 3 nests had pipped eggs (n=5). The live individuals found in the nests are normally in a weakened state since the have been in the nest for multiple days after hatching, are dehydrated, and do not have the necessary energy to excavate themselves out of the nest. It must be noted that at this time the contents of the nest have begun or are in the process of decomposition, by which point the hatchlings are not considered capable of emerging on their own. Dead hatchlings in the nest are normally partially or fully decomposed. The live individuals found in the egg chamber were not considered viable for the statistical analysis. All live hatchlings found in the nest or nest chamber are washed with ocean water to remove any putrid material they might be covered in and then placed in cooler with moist sand before being taken back to LMC for release the next day at sun rise.

7.4 Statistical Analysis

Statistical analysis was performed using SAS version 9.4, 2013 (Statistical Analysis System), SAS Institute, Cary, North Carolina. Definitions: For this description top is defined as the distance from the sand surface to the top of the nest chamber where the first egg was located. The bottom is defined as the distance from the sand surface to the bottom of the nest where the farthest-down egg was located. Delta depth (Δ depth - nest chamber dimension) is defined as the difference between the top and bottom of the nest.

Nesting success was measured as the number of hatchlings that hatched and emerged from the nest without human intervention as a proportion of the total eggs laid. For these calculations, spacer eggs were not included in the count of total eggs in the nest, i.e. only eggs with a yolk were counted. An inventory of the nest was conducted 3 days after evidence of hatching had been observed and in the case when there was not evidence of a hatching event, the nest was surveyed after 80 days post nesting. At the time the nest was surveyed, measurements of the top and the bottom of the nest chamber were also recorded (Table 17 and Table 18).

Table 17 - Nest Dimensions As Top And Bottom Of Nest

Nest Dimensions	**2010**	**2011**	**2012**	**2013**	**All**
Mean Top of Nest	51.5	62.3	60.3	63.4	59.2 (σ = 14.47)
Low (cm)	12.7	22.3	30.5	35.6	12.72
High (cm)	73.7	121.9	109.2	121.9	121.92
Mean Bottom of Nest	72.5	83.1	81.3	84.1	80.2 (σ = 13.65)
Low (cm)	43.2	53.3	53.3	58.4	43.18
High (cm)	104.1	147.3	129.5	137.2	147.32

Table 18 - Nest Dimensions as Difference in Top to Bottom (Δ Depth)

Nest Δ Depth	2010	2011	2012	2013	All
Mean (cm)	21.0	20.9	21.0	20.7	20.9 2 (σ = 7.71)
Low (cm)	7.62	7.62	7.62	10.16	7.62
High (cm)	50.8	48.26	53.34	45.72	53.34
Mean (in)	8.3	8.2	8.3	8.1	8.2
Low (in)	3	3	3	4	3
High (in)	20	19	21	18	21

Each year, a certain number of nests are lost even though they are originally found and flagged at the time the nesting female lays the eggs. Any nests that encountered a naturally occurring disturbance were documented. Each nest was given a descriptive code and the reasons for the disturbance were documented (Table 19 and Table 20 for Code Identifiers). Nests that were predated, scavenged, lost due to erosion, or damaged did not have complete data since the hatching success could no be properly calculated. These nests were not included on the nest count for calculations to prevent miscounts that would bias the calculations.

Table 19 - Nest Categories Used For The Statistical Calculations

Descriptor	2010	2011	2012	2013	Totals	Percent
Total Nests	168	252	246	98	764	100.00%
OK	116	163	160	53	492	64.40%
HNO	35	42	36	14	127	16.62%
PD	2	1	2	4	9	1.18%
SCV	2	1	3	1	7	0.92%
CNL	9	22	16	17	64	8.38%
L	1	9	9	2	21	2.75%
TS	2	0	0	0	2	0.26%
WO	1	12	6	7	26	3.40%
NR	0	2	14	0	16	2.09%

Complete nests	151	205	196	67	619
Percent Complete	89.88%	81.35%	79.67%	68.37%	81.02%

In 2011, one nest (0.5%) and 2010, two nests (1.3%) were missing TOD observations. These were most probably omissions in the data collection and/or data entry. Code of definitions is in Table 20.

Table 20 - Codes Used To Identify The Nests

Codes	Meaning	Description
OK	Undamaged	Nest hatched, eggs were found
HNO	Hatch Not Observed	Nest hatched, emergence was not observed, eggs were found
PD	Predated	Nest was predated, the number of eggs lost is in the comments
PR	Protected	Nest was marked solely for protection of the clutch, no evaluation of the nest was conducted
PV	Poached	Nest was poached
WO	Wash Out	Nest completely or partially washed out prior to anticipated or actual emergence
L	Lost	Nest not evaluated due to erosion after anticipated or actual emergence, proximity to a viable nest, stakes removed and no GPS.
CNL	Clutch Not Located	Eggs were not able to be located
SCV	Scavenged	Nest was predated after the emergence. Number of eggs lost should be noted in the comments
TS	Turtle Scattered	Eggs were scattered/damaged by a nesting female
NR	No Record	Data not recorded, missing data

Nesting success or percent 'out of nest' was measured as the fraction of individuals that emerged from the nest without human assistance e.g. percentage of viable hatchlings from the total number of eggs with no yolk in the nest chamber. This calculation eliminated the hatchlings that were found dead in the nest or any individuals that were found live in the nest at the time the nest was excavated. Any live individuals found in the nest at the time the nest was excavated were not counted as viable since they would have most certainly perished in the nest. Nesting success (N_S) was calculated as follows:

H_L = Number of live hatchlings in the nest

H_D = Number of dead hatchlings

N_H = Total number of eggs that hatched

N_U = number of eggs

P_L = Number of piped live hatchlings

P_D = Number of piped dead hatchlings

Equation 2 - Nesting Success

$$N_S = N_H - (H_L + H_D + P_L + P_D) / N_T$$

The total number of eggs in the nest was calculated by adding all the components in the nest at the time of excavation. This calculation does not include the hatchlings found in the nest, $H_L + H_D$.

Equation 3 - Nesting Total

$$N_T = N_H + N_U + P_L + P_D$$

N_T = Total number of eggs in the nest, does not include spacer eggs

For air temperature and precipitation, the weight of rho (ρ) was modified to account for each component individual variation. In the case of precipitation, rho was set as a positive value to account for the accumulation of rain over the period studied ($\rho \leq 1$) (Figure 8).

Equation 4 - Exponentially Weighted Moving Average

$$X_E = \rho X_t + (1-\rho) X_E - 1$$

X_E = EWMA (ρ) X_t = X at date t

A value of ρ was used to better describe the temperature and precipitation at the hatching event. Value for ρ was kept low for the cumulative effect of temperature is expected to be slower, reflecting the slower process of heat transfer to the deep sand. In the case of precipitation the value of ρ was increased to account the “spiky” nature of rain events wince the effect is felt fully over just a few days. In this calculation, the factor $(1-\rho)\ X_E\ -1$ decreases in weight as temporal values decrease. A value of $\rho = 0.03$ was used for temperature since this value gives a smoother moving average over time (Figure 8). Nest temperature has been shown to have a limited variation in temperature with varying air temperature [60]. This is more consistent with the confined changes in temperature that would occur at the depth of the nests since the change is no immediate. In the case of precipitation the value used was $\rho = 0.5$. Precipitation occurs in a limited amount over a shorter period of time. This is followed by evaporation of moisture; therefore, the effect should have a shorter duration than the changes in temperature (Figure 8).

A B

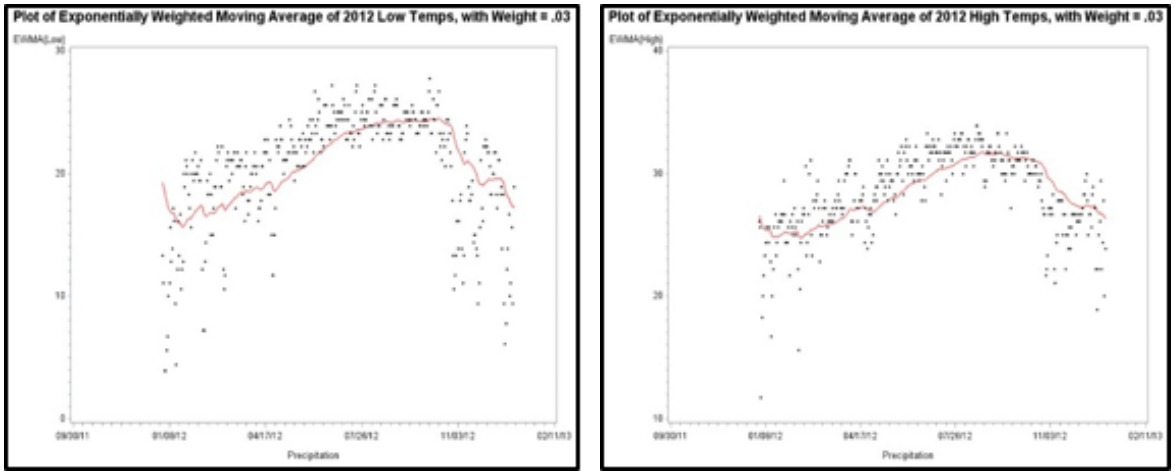


Figure 8 - EWMA Low and High Temperature

The cumulative effect of temperature is much slower, reflecting the slower process of heat transfer to the deep sand. The weight ($\rho = 0.03$) was kept low since temperature at depth does not have an immediate change with air temperature. (A) EWMA Low Temperatures, (B) EWMA High Temperatures.

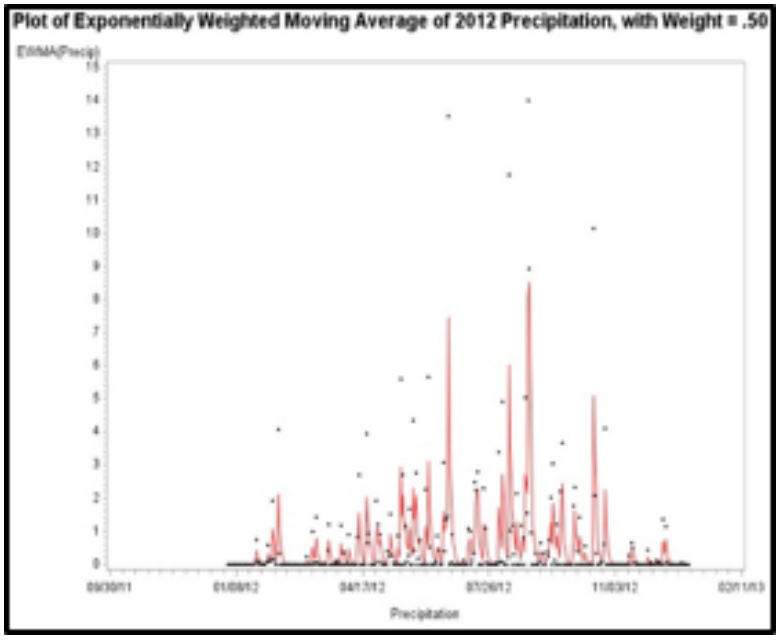


Figure 8 - Sample EWMA For Precipitation For 2012

Example of the precipitation moving average, which preserves the “spiky” nature of rain events felt fully over just a few days. The weight (ρ = 0.5) was kept high to allow for rapid changes in moisture. Black points represent total precipitation on each day. Red line is the EWMA.

7.5 Results

The study beach is located just south of the main nesting area for leatherback sea turtles (Figure 6). In the state of Florida, there were a total of 5595 nests from 2010 - 2013, with a yearly breakdown of 1334 nests in 2010, 1653 nests in 2011, 1712 nests in 2012 and 896 nests in 2013 in all the beaches. Of the total population, 30.9% (1760 nests) nest in Palm Beach County. From this population, 13.3% (764 nests) nest in the beaches in the study area (43.1% of the population nesting in the county) (Table 15).

In total there were 764 nests recorded during the nesting seasons from 2010 through 2013. The yearly nesting was in 168 nests in 2010, 252 nests in 2011, 246 nests in 2012 and in 98 nests in 2013 (Table 16). From this total 619 nests were used for the evaluation. There were 145 (18.92%) nests that had incomplete information and were

not used for the statistical analysis. These were nests that were lost, scavenged, or predated before of after hatching event, they were destroyed by erosion or sand shifting, or the data was not properly collected. Four of the nests were also scattered or damaged by a secondary female nesting at the same location (Table 19).

In many cases (16.62% of the total nests encountered), there is no evidence of the hatching event. This could be due to rain, human activity or any other event that would erase the tracks of the hatchlings as they make their way to the sea. Once the nests are excavated, the hatching numbers are documented. If there is no evidence of predation, scavenging, or any other event that could affect the nesting counts the nests are counted as compete. The total number of nests that had complete data was 619 (Table 19). This number accounted for 81.02% of the nests deposited from 2010 - 2013.

Nest size, measure as the total number of eggs found in the nest chamber (Table 21), was evaluated in the model and in relation with hatching success of the nest. However for practical reasons the nest size is not applicable. When an egg clutch is relocated the total number of eggs deposited are moved as a unit. There is no practical way to change the number of eggs in a clutch.

Table 21 - Nest Size As Total Eggs In Nest

Nest Total Eggs	2010	2011	2012	2013	All
Mean	76.9	72.7	74.9	82.2	75.5
Low Count	25	13	23	23	13
High Count	132	123	122	121	132

7.6 Logistic Model for Hatch Success Rate

Our model included all the nesting parameters collected during the nesting season. The final analysis includes the main effects, but there is no indication of significant interactions among these variables, nor any sign of non-linear effects (Table 22).

Negative estimates indicate effects leading to reduction in hatch success rates and become odds ratios that are less than one.

The nesting dimensions and location were evaluated against precipitation and air temperature. Exponentially weighted moving averages (EWMA) of precipitation, low and high temperature, were tested versus percent hatched for highest correlation.

Variation was observed when comparing each year to the 'base' of 2013, indicating that conditions are not stable through the study period. In Table 23 - Odds Ratio Estimates, one can identify the parameters that have stronger effects on nesting success. The farther a value is from one the stronger the effect. The 95% interval for each of the components is listed in Table 23.

Table 22 - Analysis Of Maximum Likelihood Estimates

Parameter	df	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	23.2135	0.6784	1170.7242	<.0001
2010	1	-0.5527	0.0193	819.689	<.0001
2011	1	0.6433	0.0243	703.2355	<.0001
2012	1	-0.1903	0.0212	80.5474	<.0001
Latitude (l)	1	-0.0957	0.0184	26.9105	<.0001
Latitude^2 (l_2)	1	-0.4144	0.0376	121.1889	<.0001
Depth	1	-0.0348	0.00129	722.8929	<.0001
Emerge Days	1	-0.0354	0.00232	232.5981	<.0001
Average Temp	1	0.0825	0.0113	53.7494	<.0001
Δ Temp	1	0.1172	0.011	114.2745	<.0001
EWMA (Prec.)	1	-0.0701	0.0104	45.2892	<.0001
EWMA (High Temp)	1	-0.648	0.0183	1248.5163	<.0001

Table 23 - Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
Year 2010 vs. 2013	0.521	0.482	0.562
Year 2011 vs. 2013	1.722	1.578	1.879
Year 2012 vs. 2013	0.748	0.7	0.8
Latitude (l)	0.909	0.876	0.942
Latitude^2 (l_2)	0.661	0.614	0.711
Depth	0.966	0.963	0.968
Emerge Days	0.965	0.961	0.97
Average Temp	1.086	1.062	1.11
Δ Temp	1.124	1.1	1.149
EWMA (Prec.)	0.932	0.913	0.952
EWMA (High Temp)	0.523	0.505	0.542

7.6.1 Model Estimates

- Latitude predicted there is a reduction in nesting success as the population moves northward. This was more significant when the latitude^2 was used.
- Δ Depth (Depth) predicted that when the size as measured from the top of the nest to the bottom of the nest increases or decreases in dimension, the nest success decreases.
- Emergence Days predicted that nests that hatch early or later would have reduced hatching success.
- Temperature predicted that increases in temperature (Average Temp) and the mean between the high and the low temperature (Δ Temp) for the day increased hatching success. However, the EWMA (High Temp) predicted that when

temperatures were at the extremes there was a strong effect toward decreasing hatching success.

- Precipitation predicted there was a reduction in hatching success with increases in precipitation.

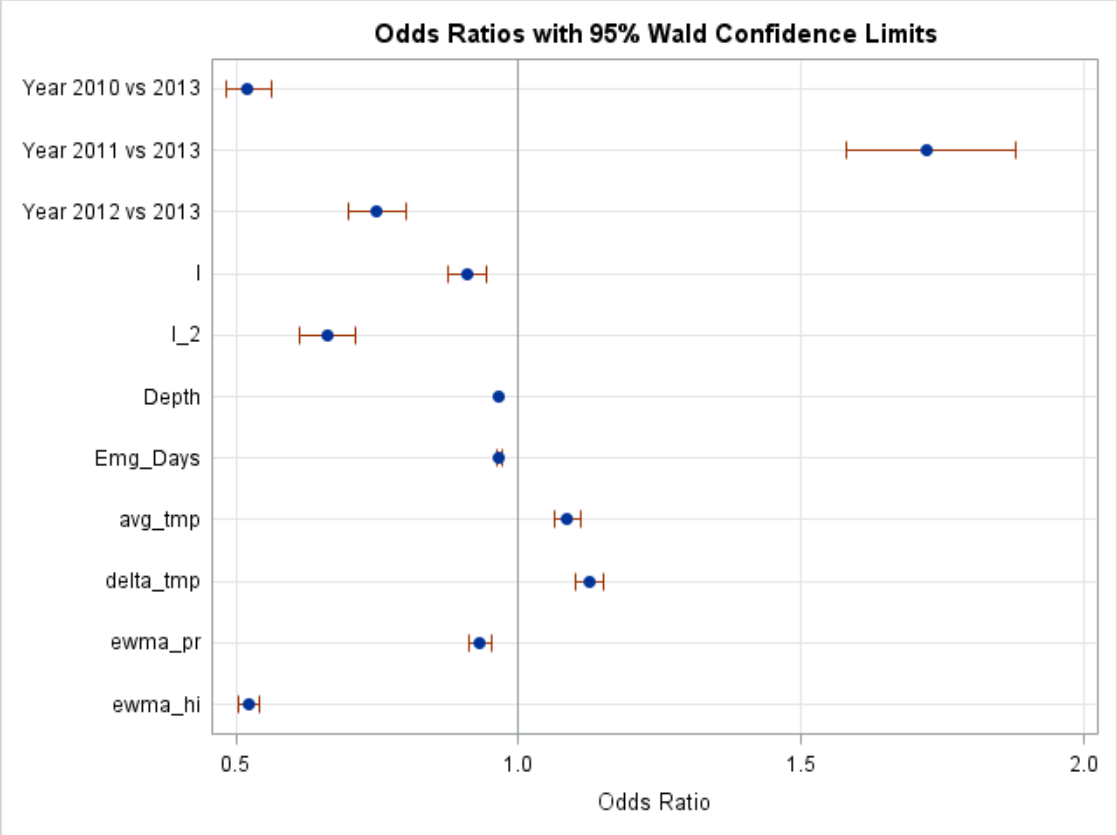


Figure 10 - Odds Ratios (Blue) With 95% Wald Intervals (Red)

Larger variation from 1.0 indicates a greater effect on hatching success from the model.

7.6.2 Nest Success vs. Nest Depth (Δ Depth)

Hatchlings have to move from the nest chamber to the top of the nest after hatching. Very deep nests were expected to have a reduced success since the hatchlings would have to travel a larger distance to the surface. The nest success was compared to the

top of the nest and the Δ depth. This was done by relating the measurement from the sand surface to the top of the nest and the bottom of the nest (Table 17). The average nest top was 59.2 cm (σ = 14.47) with a min = 12.72 cm and a max = 121.92 cm. The bottom of the nest was 80.2 cm (σ = 13.65) with a min = 43.18 cm and a max = 147.32 cm. The Δ depth was 20.9 2 cm (σ = 7.71) with a min = 7.52 cm and max = 53.34 cm.

The Δ depth of the nest was especially significant when looking at nests that were wider than 33.02 cm (13 in). This would indicate that nests that are scattered at the time of nesting would have a reduced hatching success. This indicates that it is advantageous for the nesting female to have a more compact nest chamber at the time of deposition.

When the two parameters (Δ depth vs. nest top) were evaluated against each other, it was observed that there were few nests deeper than 91.44 cm (36 in) and they had low success rates (Figure 11). However, a deep nest with a low Δ depth is as successful as a shallow nest with a higher Δ depth. This indicates that as the nest size increases (Δ depth) the nest can be located deeper in the sand without affecting nesting success.

A B

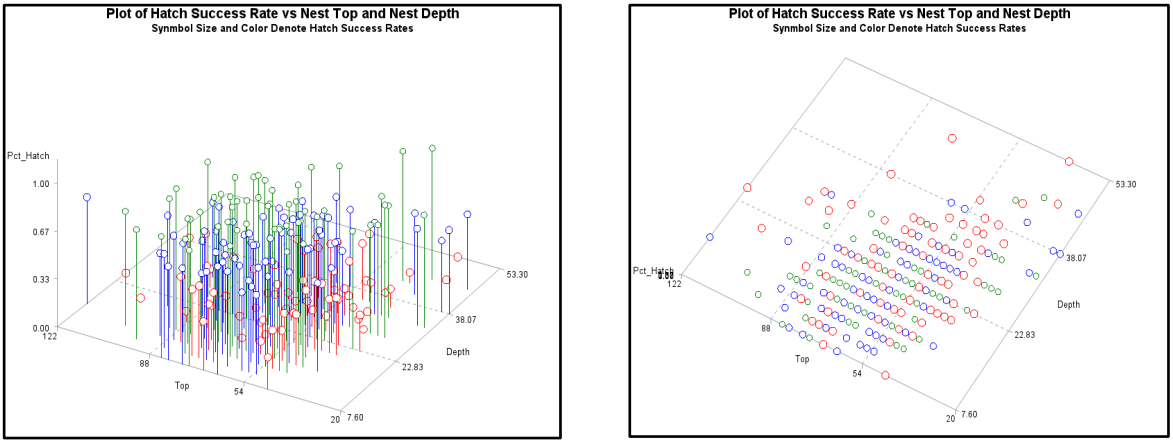


Figure 11 - Hatch Success vs. Nest Depth

Figure A - Hatch success plotted as dependence on nest top and nest Δ depth. Where nest top is the distance from the surface to the top of the nest and nest Δ depth is the distance from the top of the nest to the bottom of the nest, essentially the 'size of the nest'. The red are hatching rates between 0 and 25%, blue is 25% to 75% and green is > 75%.

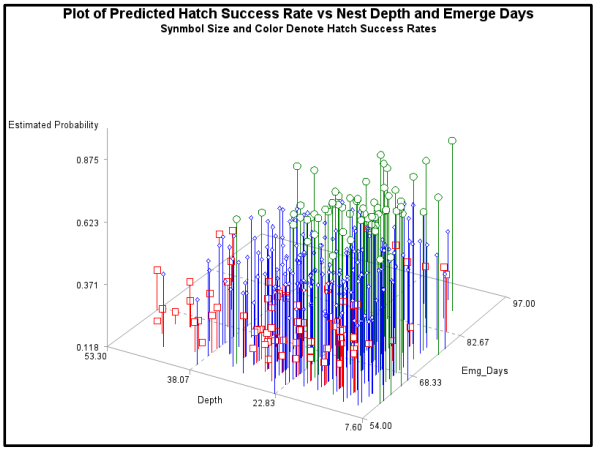
Figure B - Top view to prevent the high success nest to obscure the view of the area where the nest is deep and the Δ depth is greatest..

7.6.3 Nest Success vs. Date of Emergence

The model identified the date of emergence as having a statistically significant effect on reducing the hatching success. Date of emergence was compared to the Δ depth to ascertain if there was an association with the nest location and it became clear that there is a clustering on the best nesting success (Figure 12). The boundaries of the appropriate nesting time for the turtles appear to be between 59 and 75 days with a nest Δ depth < 30.48 cm (12 in). Nests that were less than 59 days had a clear reduction in nesting success . Hatchlings do not necessarily exit the egg at the same time, but they are known to emerge from the nest en masse. This would indicate that if there is too

much space in between the hatchings at the time of emergence there is a reduction in the number of hatchlings that can emerge from the nest.

A



B

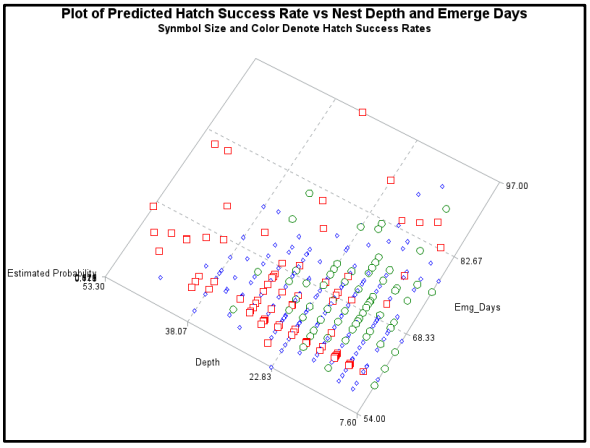


Figure 12 - Hatch success vs. Date of Emergence and Δ Depth

Figure A - Hatch success plotted as on the date of emergence and the depth of the nest (Δ Depth). The red are hatching rates between 0 and 25%, blue is 25% to 75% and green is > 75%.

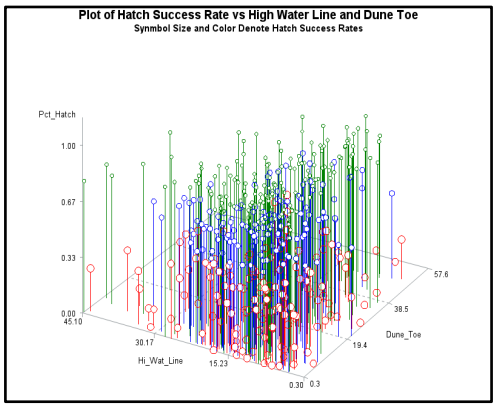
Figure B - Top view - This plot has a reduction of mid success rate (blue) markers to identify the areas were the nests have the lowest and highest success rates.

7.6.4 Nest Success vs. Location on the Beach

The model did not identify the TOD and the HWL as being significant parameters that would affect the nesting success of a nest. However, it has been established that viability of a nest is directly affected by location on the beach. If the nest is located too close to the waterline it could be lost with the changes in tide or it could be eroded with the shifting sand [51]. On the other hand, if the nest is too close to the TOD the hatchlings can become disoriented and have a lesser chance to making it to sea once they have emerged [61].

On the contour plot there appear to be three regions of the plot that have the highest success rates (Figure 13). The first location is far from the TOD. The second location is far from the HWL. And finally, the middle of the nesting area (or distances at approximate equidistant from the TOD and HWL) appears to have the best hatching success. However, there is no specific location on the beach at which the nest could be consistently located that would yield consistently high hatchling success rates.

A



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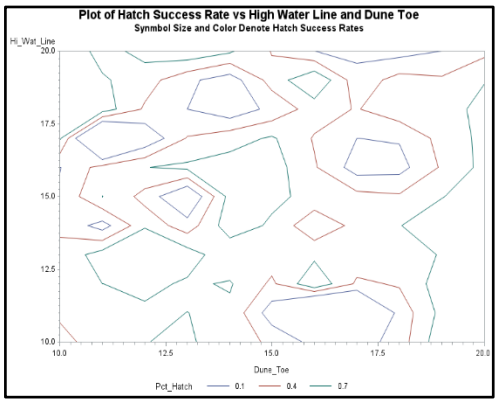

Figure 13 - Hatch success vs. Location on the beach (TOD/HWL)

Figure A - Hatch success plotted as dependence on TOD and HWL. Where TOD is the perpendicular distance from the vegetation line to the nest and HWL is the perpendicular distance from the high tide line to the nest. The red are hatching rates between 0 and 25%, blue is 25% to 75% and green is > 75%.

Figure B - Contour plot of the hatch success with relation of the depth of the nest. Blue is <10%, Red is between 10% and 50%, blue is between 50% and 70%, yellow is 70% to 90% and green is >90%.

7.6.5 Temperature

The EWMA for temperature (estimate = -.0701, p <0.001) indicated that the temperature had an important effect on the hatching success through the nesting season. Nests that are deposited earlier, at lower temperatures, and later in the season, at higher

temperatures, had a lower hatching success than those in the middle of the season (Figure 14). This would indicate a selective pressure for nesting during the mid-spring.

This relation is significant since leatherback sea turtles have temperature-dependent sex determination in which males are produced at lower temperature and females are produced at high temperatures [63, 64]. Nesting early in the season would produce mostly males and if nesting continued father into the summer months, as temperatures increase, the majority of the hatchlings would be expected to be female [65]. The sex ratios are relevant since infertility leading to low hatchling success has been proposed for a decrease in numbers of some populations [49]. Changes in nesting would lead to lack of males or females in the population leading to a population decline.

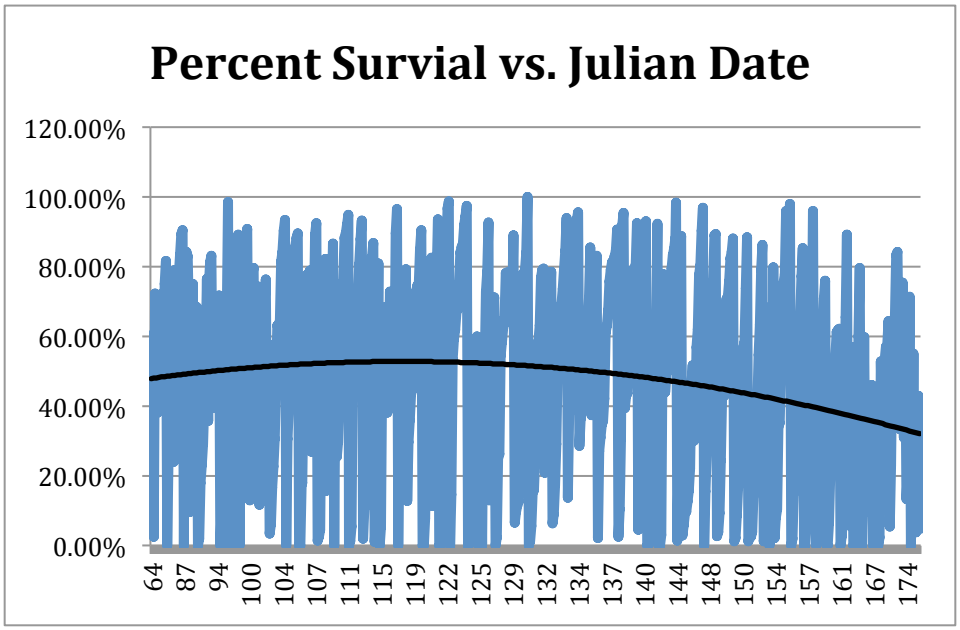

Figure 14 - Plot of Percent Survival vs. Julian Date

In order to combine the effect of all years studied the dates were converted to Julian dates and plotted. The line is a first order quadratic line representing the changes in nesting success over time.

7.7 Conclusions

In this study a logistics model is used on the nesting conditions for a naturally occurring population of leatherbacks on the Atlantic coast of Florida. It was determined that the length of time the nest takes to hatch, the location on the beach as measured by the latitude, and the depth of the nest have the statistically significant effect on hatching success.

The data was analyzed against precipitation since there is evidence that this has an effect on hatching success. Santidrián Tomillo et al. reported that in areas with dry climatic conditions, such as Playa Grande, the Pacific Ocean, and Sandy Point, Caribbean Sea, hatchling output increased with long-term precipitation [59]. On the other hand, areas with temperate climate such Maputaland, Indian Ocean did not see an effect. Areas with high precipitation saw a varied effect. Our model showed that temperature and precipitation also have a significant effect, but cannot readily be controlled in the case of a relocated nest.

When a nest needs to be relocated, the site of the relocation must be optimized for hatching success. Morreale et al. recommended the use of a beach hatchery were the nests are protected from predators and humans. This hatchery should mimic the naturally occurring conditions to try to prevent the sex bias of populations hatched in Styrofoam ™ boxes [60]. If a leatherback nests too close to the TOD and the hatchlings find themselves emerging in a vegetated area they have difficulty moving seaward [61]. Nests that are laid below to the HWL need to be relocated to prevent being washed out by erosion of the beach [51]. Nests that are laid too close the HWL will perish with changes in the water table or if they are in an area that is erosion-prone. In some areas, nest relocation ranges from 25% to 68% (43% average) [46]. During the nesting period 2010-2013 there were only 26 nests (3.4%) lost due to erosion. This could be attributed the sand stability of the beach during the time frame of the study.

It has been noted that in some locations leatherback sea turtles appear to select nesting locations that minimize the possibility of erosion [51]. This study does not directly target selective pressure of the nest location of the population since conservation efforts were

assumed to be more inclined to increase the total population of the species. It must be noted that relocation of eggs, as a conservation effort, is only part of the solution. Modeling has shown that relocation of eggs would be unsuccessful if adult mortality was over a 20% threshold [48]. Conservation efforts need to continue with all phases of the life cycle of the species.

In this study the sex of the hatchlings was not taken into account. Because of the nature of the study there was no way to identify the sex of the hatchlings being produced. The assumption was made that the best nesting location would produce the same optimal ratio of female to males as the normal nesting conditions for the wild populations.

There was a lot of variation in nesting success from one year to the next. This would indicate that there are multiple environmental as well as maternal effects in play. From one year to the next, many of the same females nest at this site due to the long re-migration interval observed in leatherbacks in this population. Remigration interval has been documented at 2.7 (+/- 1 yr.) with a range of 1 to 5.5 years [66]. For this analysis, only the environmental effects were included. The maternal effects were not considered.

It is hoped that this study will help in the selection of better nesting sites for relocation of leatherback sea turtle eggs under natural conditions in the ongoing efforts to help the conservation of the endangered Leatherback sea turtle.

7.8 AKNOWLEDGMENTS

Thanks to the Florida Fish Wild Life Conservation Commission for making the some of the relevant data available to the public. We would also like to thank all the volunteers and staff at the LMC that have helped collect the data over the years. Without their help and continued enthusiasm on the preservation of sea turtles this project would have not been possible. I specially thank my Mother’s unconditional support.

8 Spatial And Temporal Changes In Nest Location For Leatherback Sea Turtle In North Palm Beach, Florida

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Relevant abbreviations and definitions used in the manuscript:

Leatherback sea turtles (*Dermochelys coriacea*)

Florida Fish and Wildlife Conservation Commission (FWC)

Fish and Wildlife Research Institute (FWRI)

Sea Surface Temperature (SST)

8.1 Abstract

Nesting date and nest location influence sea turtle hatching success. Non-ideal nesting conditions could result in a reduction in hatching success or changes in sex ratios. Anthropogenic production of greenhouse gases is expected to create worldwide climatic changes that will influence nesting beaches. Sea turtles are known to be philopatric, but in order to adapt to these climatic changes, they will need to modify their nesting behavior. The study presented identified changes in location that appear to be associated with sea surface temperature (SST). From early March into late July, leatherback sea turtles (*Dermochelys coriacea*) nest on the Atlantic coast of Florida.

Each year, Loggerhead Marinelife Center (LMC) in Juno Beach, Palm Beach County, Florida examines 12.2 Km of beach from John D. MacArthur Beach State Park to Jupiter Island as part of The Leatherback Project, the longest program aimed at studying the Florida leatherback nesting population in the United States. Using data obtained by this project, the nesting location and nest start dates were evaluated for this local population. From 2004 to 2015 there has been a progressive northward spatial change as demonstrated by changes in mean nest latitude (R^2 = 0.549, p = 0.0035) while the timing of nesting as the annual mean nest dates have remained stable (R^2 = -0.0539, p = 0.55). Over this same period, there has been an increase in SST during the onset of spring. The mean nest location has moved 1.422 Km northward, a change that correlates with increased May SST. There are multiple factors that can affect nesting, however our results suggest that leatherback nesting location may be affected by the increase in SST.

8.2 Background

Changes in climate patterns are expected to alter temperature and precipitation across the globe [67-70]. There continues to be an increase in the amount of literature showing changes in behavior and distribution of multiple species [71-75]. These changes, in part, are explained by global warming. For instance, the habitat boundaries of British birds moved north 18.9 Km over a 20 year period [71]. In the case of butterflies in the Eastern United States, "…the population trajectories indicate increases of many species near their northern range limits and declines in nearly all species (17 of 21) near their southern range limits" [73]. In addition, earlier nesting dates have been correlated with warming sea surface temperature (SST) for loggerhead sea turtles in the Mediterranean [75].

Sea turtles nest in tropical and subtropical beaches throughout the world and will have to adapt to observed global climatic changes. They are known to be philopatric, but they are able to change beach location in response to changes in beach conditions over time [8, 76, 77]. Sea turtles are also known to have temperature-dependent sex determination [56, 60, 63, 78]. At nesting temperatures above the transitional temperature range, the hatchlings are mostly female [63]. As climate change increases

the temperature at the nesting beaches, the turtles will have to change their nesting behavior by nesting earlier or later in the season or by moving to locations in which the temperature is more amenable to proper ratios of females to males. If this is not done the female to male ratios could be altered and lack of males could lead to infertility in the females [63], threatening the viability of the population.

Sea turtle species have already been reported to exhibit behaviors that follow changes in SST. In the Gulf of Mexico, the time spent by Kemp's Ridley (*Lepidochelys kempii*) at specific locations at sea can be predicted in part by SST [79]. Loggerhead sea turtles in the Mediterranean have shown a trend toward earlier nesting, decreased clutch size, and increased hatching success with an increase in spring SST [80]. On the beaches of the east coast in central Florida, the median nesting dates for loggerheads and green turtles (*Chelonia mydas*) have been shown to be significantly earlier with higher May SST; interestingly, a higher average daily SST was related to a shorter nesting season for loggerheads and a longer nesting season for green turtles [81]. For leatherback sea turtles, modeling has shown that changes in nesting location could offer more relief for warming temperatures than changes in nest date at the nesting beaches [82].

Leatherback sea turtles are currently classified as endangered by the Endangered Species Act and have been so since 1970 [41]. They are also listed under the CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora), which is the international agreement between governments aimed to ensure that international trade in specimens of wild animals and plants does not threaten their survival, under Appendix I, as a species that is the most endangered and threatened with extinction [42]. The overall populations worldwide have seen drastic decreases from the numbers that were previously reported [43, 45, 48]. Populations in the Pacific have seen a drastic decrease in number mostly due to human activities such as long line fishing and egg poaching [43, 48, 83-85]. On the other hand, the populations in the Atlantic Ocean remain stable and, in some cases, as with the population on the east coast of Florida, appear to be increasing [8, 46, 86].

The main nesting location for leatherbacks in the United States is on the Atlantic Coast of Florida. Climatic changes are expected to increase SST and result in changes in

patterns of precipitation at nesting beaches in these locations [67]. Leatherbacks are known to be philopatric to these beaches [8], but there is a certain amount of variation and scatter related to nest site location during re-migration. Kamel & Mrosovsky reported that when a nesting female nests at least three times in a season, there appear to be no significant correlations between distance from the HWL of the first and second nests (R^2 = 0.04, p = 0.26) and the first and last (R^2 = 0.08, p = 0.08) [61]. This would indicate that there does not seem to be a pattern of predictability for individual nesting females. However, the idea that individuals have a certain amount of variation with regards to nesting site would allow for the movement of the nesting site selection over time as sea turtles vary in the nest location, this would lead the population to adapt to changing nesting conditions.

Currently, over 90% of the nests are deposited in the southeast part of the state of Florida from Brevard County to Dade County as well as in the Florida Keys and Dry Tortugas [7] (Figure 6 and Table 24). There is little nesting activity outside of Florida, with only 53 nests recorded in three other states, Georgia, South Carolina and North Carolina from 2009 to 2015 (Table 25).

Table 24 - Nesting by county in all Florida beaches from 2010 -2014

County	2010	2011	2012	2013	2014
Nassau	1	3	7	0	3
Duval	2	3	6	3	1
St. Johns	4	23	13	16	6
Flagler	0	13	7	6	3
Volusia	15	22	23	18	17
Brevard	77	102	91	76	122
Indian River	87	61	66	56	54
St. Lucie	203	254	189	94	173
Martin	561	649	627	352	667
Palm Beach	**368**	**517**	**622**	**253**	**511**
Broward	14	5	46	18	39
Miami-Dade	2	0	11	3	4
Monroe	0	0	0	1	0
Collier	0	0	0	0	0
Lee	0	0	0	0	0
Charlotte	0	0	0	0	0
Sarasota	0	0	0	0	0
Manatee	0	0	0	0	0
Hillsborough	0	0	0	0	0
Pinellas	0	0	0	0	0
Franklin	0	1	0	0	4
Gulf	0	0	0	0	0
Bay	0	0	2	0	0
Walton	0	0	1	0	0
Okaloosa	0	0	1	0	0
Santa Rosa	0	0	0	0	0
Escambia	0	0	0	0	0
	1,334	1,653	1,712	896	1,604

This table includes the last 5 years of nesting data from the FWC.

FWC Fish and Wildlife Research Institute Statewide Nesting Beach Survey Program

Source: FWC/FWRI Statewide Nesting Beach Survey Program Database as of 20 February 2015

Table 25 - Leatherback nesting in the U.S. Atlantic Coast outside of Florida

State/Location	2009	2010	2011	2012	2013	2014	2015
Georgia							
Cumberland	2	0	5	0	0	0	3
Jekyll	2	1	1	0	0	0	0
Sapelo	1	0	0	0	0	0	0
Sea Island	1	0	0	0	0	1	0
St. Simons	1	0	0	0	0	0	0
Blackbeard	0	2	1	0	0	1	0
Ossabaw	0	1	3	1	0	0	0
St. Catherines Island	0	0	1	0	0	0	0
TOTAL	7	4	11	1	0	2	3
South Carolina							
Edisto Beach State Park	3	0	0	0	0	0	0
Folly Beach	0	1	0	0	0	0	0
Hilton Head Island	0	1	3	0	0	0	1
Hunting Island	0	1	0	0	0	0	0
Huntington Island Beach State Park	0	0	1	0	0	0	1
Kiawah Island	0	0	0	1	0	0	0
Fripp Island	0	0	0	0	0	1	0
Pritchards Island	0	0	0	0	0	1	0
TOTAL	3	3	4	1	0	2	2
North Carolina							
Cape Hatteras NS	1	0	0	1	0	0	0
Carolina Beach	1	0	0	0	0	0	0
Northern Outer Banks	1	0	0	0	0	0	0
Bald Head Island	0	1	0	0	0	0	0
Holden Beach	0	1	0	0	0	0	0
Cape Lookout NS	0	0	0	4	0	0	0
TOTAL	3	2	0	5	0	0	0
TOTALS	13	9	15	7	0	4	5

Data from (Accessed 23 December 2015): http://www.seaturtle.org/nestdb/?view=1; http://www.seaturtle.org/nestdb/?view=2; http://www.seaturtle.org/nestdb/?view=3

METHODS

8.2.1 Data Collection

LMC staff biologists and technicians conducted nightly surveys for leatherbacks from early March through the end of June. The surveys were conducted from 21:00 to 5:00 on all-terrain vehicles along 12.2 km of beach. When a turtle was encountered on nightly surveys it was measured and checked for flipper tags, PIT tags, injuries, scars, epibiota, and other diagnostic markings. If the turtle was not tagged, researchers applied a flipper tag to the soft skin along the medial edge of each rear flipper and a PIT tag to the right shoulder (NMFS, 2008).

In conjunction with the nightly surveys, a morning survey was conducted to document and mark all leatherback nest sites along the same stretch of beach. These surveys continued until the end of October. During morning surveys, the location of each nesting and non-nesting emergence (false crawl) was collected through the use of a real-time corrected Differential Global Positioning System (DGPS) unit with sub-meter or sub-foot accuracy (Trimble Pathfinder ProXH®, Geo XH®, Geo XT® or Pathfinder Pro6T®).

For each crawl, the following information was collected and entered into a DGPS data logger, utilizing Trimble TerraSync software, and recorded on paper data forms:

- Survey zone
- Species of turtle
- Crawl type (nest or false crawl) above the high water line or below the high water line
- Estimated distance from the egg chamber or landward extent of the non-nesting emergence relative to the high tide line and the toe of the dune
- Number of abandoned body pits
- Number of abandoned egg chambers
- Obstructions (natural or man-made) encountered by the turtle and the response to that obstruction

The following additional parameters were recorded for marked nests:

- Unique nest code
- Designation as to whether the clutch was or was not located
- Distance from the egg chamber to the high tide line and the toe of the dune using a laser rangefinder
- Bearing and measured distance to an additional stake placed near the dune line using a laser rangefinder

Comments were recorded on paper data forms. Upon saving each feature into the data logger, precise coordinates (<1 m accurate) and date and time were logged for later retrieval and analysis. All DGPS data were later post-processed in GPS Pathfinder Office software (Trimble Navigation, Ltd., Sunneyvale, California), utilizing local base stations to obtain the highest possible accuracy.

Each morning, LMC survey crew would check the clutch location for signs of emergence or depredation, and excavated 72 hours post-emergence or at 80 days incubation. When nests are laid early in the season the date of excavation could be extended to 90+ days. This is due to longer incubation times for nests laid in colder weather as expected to occur earlier in the season. Nest contents were sorted into the following categories and recorded: live hatchlings, dead hatchlings, unhatched eggs, hatched shells, pipped live, pipped dead and unyolked eggs (spacer eggs). After the nest was excavated, the nest data was then entered into a database at LMC.

8.2.2 Conversion from GPS coordinates to distance

Conversion of GPS data to distance in meters was done utilizing the Universe Transverse Mercator Coordinate (UTM) system. This system divides the earth into 60 zones, and each zone serves as a reference point for the UTM coordinates with each zone. The system displays the earth as a two-dimensional flat surface. The coordinates are expressed as easting and northing points and are measured in meters. The northing points are measured in meters north in relation to the equator and the easting points are measured in meters east in relation to the central meridian. The northing points were calculated using the Beta Coordinate Conversion program from the National

Geodetic Survey, a division of the National Oceanic and Atmospheric Administration. This program converts GPS coordinates into northing and easting points based on the UTM coordinate system. The inputs for this program are latitude, longitude, ellipsoid height, and datum.

This system provided the basis for calculating the mean distance in meters the population of leatherbacks migrated each year subsequent to 2003. The mean latitude of nesting location per year was calculated from the DPGS data that was collected for the years 2004-2015. The data from 2003 was not included in this analysis because LMC only performed counts for Juno beach at this time. In 2004, LMC expanded the project to include Jupiter Carlin. Thus, the analysis of total nest locations began in 2004. The mean longitude for the 2004-2015 period was -80.05437994. The mean longitude per year was not utilized since the stretch of beach was approximately on the same longitude line throughout the 12.2 Km length and therefore assumed to be north to south. Datum NAD83, the default for the program, was selected instead of NAD27. The Federal government recognizes NAD83 as the legal horizontal datum for the United States. The ellipsoid height was kept at the default, 0. This datum uses the standard GRS-80 ellipsoid shell. While it is recognized that there are fluctuations in ellipsoid height throughout the coast of Florida, these fluctuations were negligible to the calculation of the northing points in the area encompassed by the study site. This was also justification for the use of UTM instead of XYZ, which does not account for ellipsoid height since the study area is at sea level. The difference between the northing points, in meters, was calculated for each year relative to 2004.

8.2.3 Sea Surface Temperature, Air Temperature and Precipitation

Sea surface temperature was obtained from NOAA's Extended Reconstructed Sea Surface Temperatures v4 dataset (http://www.esrl.noaa.gov/psd/data/gridded/data.noaa.ersst.v4.html#detail) [87, 88], NOAA_ERSST_V4 data provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, USA, from their website at http://www.esrl.noaa.gov/psd/ (accessed 12/11/2015). Resolution of this data is 2° latitude by 2° longitude. The two pixels closest to the nesting beach were utilized for analysis. The north pixel covers areas between -91 and

-79 degrees longitude and 27 and 29 degrees latitude. The south pixel covers areas between -91 and -79 degrees longitude and 25 and 27 degrees latitude. Sea surface temperatures are in degrees Celsius.

Air temperature and precipitation data for the City of North Palm Beach were also obtained from NOAA, Daily Summary Observations, Accessed 12/24/15; (https://gis.ncdc.noaa.gov/map/viewer/#app=cdo&cfg=obs&theme=ghcn). Temperature lows, highs, and averages were obtained for nest start dates for all nests observed from 2003 to 2015. Precipitation data was gathered for each nest start date from 2003 to 2015.

8.3 Results

8.3.1 Nesting Dates

There is no overall pattern of change or temporal variation on the dates of nesting in this population (Figure 15). The boxplots for 2004, 2008, 2010 and 2013 appeared to be skewed towards an earlier nesting date. None of the plots are skewed to nesting later in the season. Even though the plots are skewed toward earlier nesting, there appears to be a greater variation on the end of the nesting season. This would indicate that nesting females arrive at the nesting beaches at approximately similar dates, but would finalize nesting at different dates, which may depend on the conditions for each particular nesting female. This is consistent with the nesting patterns of Leatherbacks since they are known to nest multiple times in a season. In this population the estimated clutch frequency has been approximated to 4.4 ± 1.1 nests/year, and observed nesting period was measured at 10.2 ± 1.3 days between nests [66].

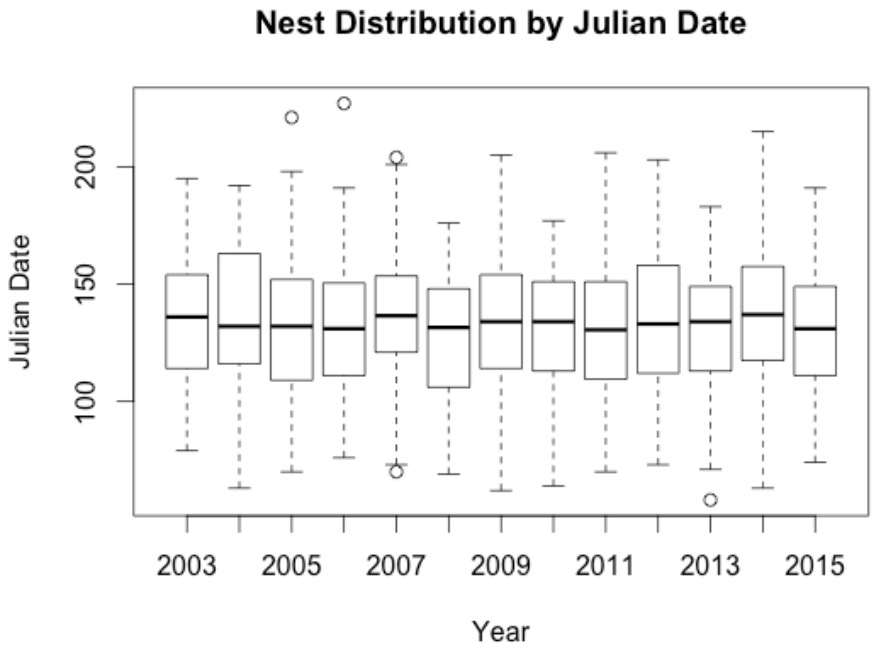


Figure 15 - Nest Distribution By Julian Date

The majority of the nests in this population occur in the months of April and May. This data set had 5 outliers:

2005 - Julian date 221 (9 August 2005), 2006 - Julian date 227 (15 August 2006), 2007 - Julian dates 70, 204 (11 March 2007, 23 July 2007, respectively), 2013 - Julian date 58 (27 February 2013).

Assuming a non-leap year, the season would start approximately on 11 March (Julian date 70) and continue until 14 July (Julian date 195) (Table 26). About 50% of the nests are laid between April 23rd (Julian date 113) and June 2nd (Julian date 153). \

Table 26 - Quartile Results for Nesting Dates

Year	Earliest Date	25 % Quartile	50 % Quartile	75 % Quartile	Latest Date
2003	79	114	136	154	195
2004	63	116	132	163	192
2005	70	109	132	152	198
2006	76	111	131	150.5	191
2007	73	121	136.5	153.5	201
2008	69	106	131.5	148	176
2009	62	114	134	154	205
2010	64	113	134	151	177
2011	70	109.5	130.5	151	206
2012	73	112	133	158	203
2013	71	113	134	149	183
2014	63	117.5	137	157.5	215
2015	74	111	131	149	191
Mean	70	113	133	153	195

The date of the first nest of the season is constant (Figure 16-A). The mean nest start date appears to be decreasing (negative correlation) with each year overall (Figure 16-B). However, the data was not statistically significant (R^2 = -0.0539, p = 0.55) and was not an indication that the nests were being laid earlier in the season.

A

B

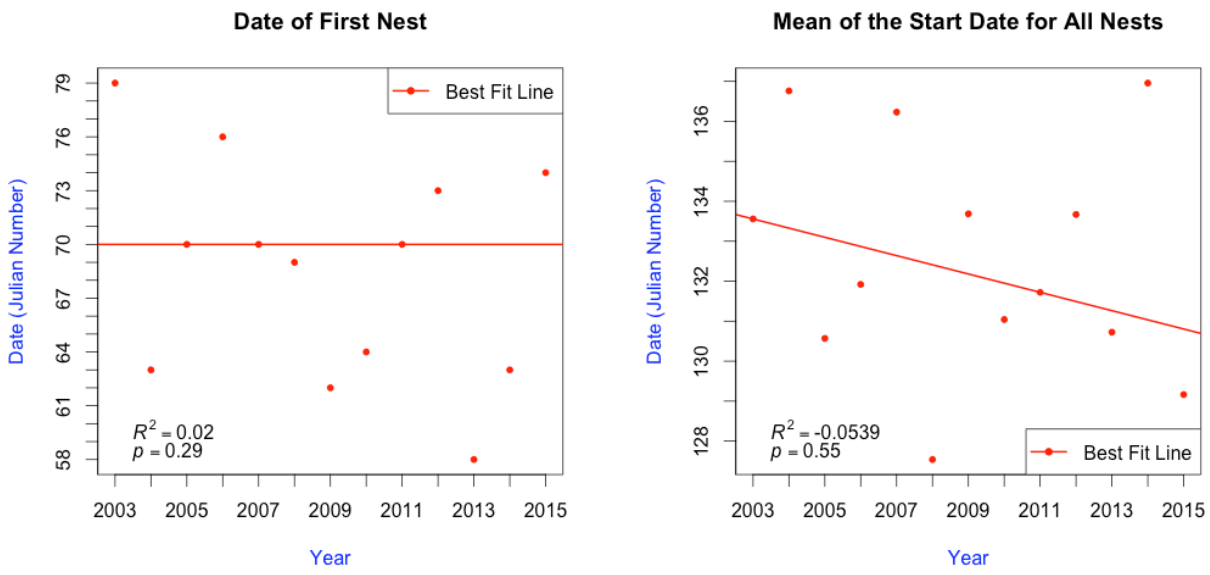


Figure 16 - Date Of First And Mean Of Nests

For the data analysis, the date of the first nest of each season was converted to Julian number and plotted as dates against the year. The data did not show specific changes in date of the first nest of the season overall. The data did show a negative trend for the mean of the nests.

A. - Year of nesting season vs. Julian date for the first nest of the season. There appears to be no relationship to the start date of the nesting season.

Date of first nest: y = -0.505x + 1084, (R^2 = 0.02, p = 0.29)

B. - Mean of the start date for all the nests laid during the season showing negative correlation indicating an overall decrease in temporal distribution of the population.

Mean of start date: y = -0.138x + 408.7, (R^2 = -0.0539, p = 0.55)

8.3.2 Latitude

Data from 2004 to 2015 was selected for consistency. Prior to 2004, LMC did not survey the north part of the beach at Jupiter/Carlin. Surveys of the northern part of the nesting area did not begin until 2004, therefore older data would bias the latitude towards the south end of the nesting site. The data set analyzed includes all the nests in the season. None of the nests that were predated, poached or lost to erosion were removed from the nesting data for location and date, since this data is valid for the nests.

It was assumed that the longitude at this location would vary minimally with latitude. The beach is situated on the east coast of the peninsula and there is only slight variation on the longitude at this location. Latitude at the nesting site was measured to 6 decimal places.

The mean of latitude was calculated as the mean of all the nests in the season for each season (R^2 = 0.549, p = 0.0035). This indicates that there is an increase in latitude with each nesting season. Most of the data points fall within the 95% interval for the best-fit line. There are only two points that fall outside of this window in 2009 and 2013. The minimum (southernmost) latitude observed was 26.83643 and the maximum (northernmost) was 26.94277 (Figure 17).

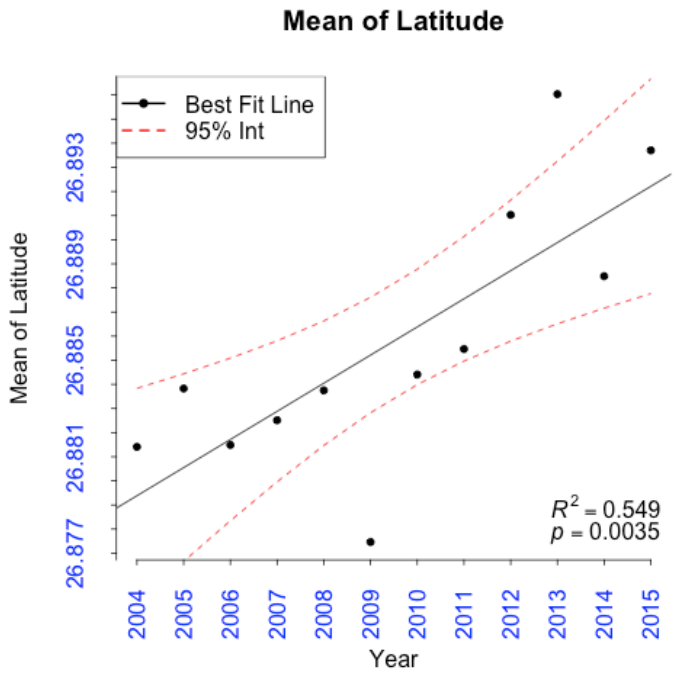


Figure 17 - Mean Of Latitude vs. Year

The mean of latitude was calculated as the mean of all the nests in the season for each season (Year). $R^2 = 0.549$, $p = 0.0035$. The black line is the best fit line and the red lines are the 95% interval for the best-fit line. Mean for 2009 and 2013 fall outside of the 95% interval. The minimum (southernmost) latitude observed was 26.83643 and the maximum (northernmost) was 26.94277.

8.3.3 Sea Surface Temperature (SST)

Modeling has shown that SST has a direct effect on the re-migration interval of green sea turtles in the Atlantic Ocean [89], therefore, as part of the analysis, nesting dates and latitudes were correlated with SST. First, the nesting dates were evaluated against the April and May SST since the majority of the nests occurred in this time period. Moreover, analysis of monthly average SST values showed an increase in April and May SST over the 2003-2015 nesting period (Fig. 7.8). There was no correlation between April and May SST with first nest dates nor the average nest start date.

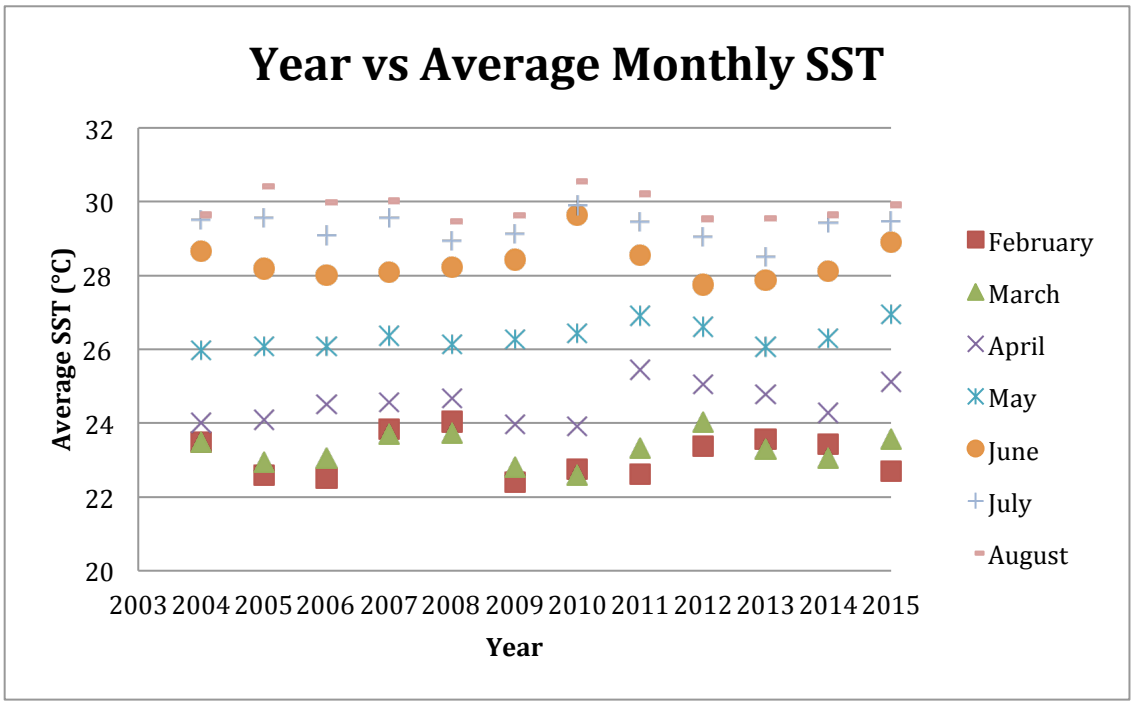


Figure 18 - Average SST for each year

Average SST for each month of the nesting season (February to August) from 2003 to 2015. Average SST is the average of the north and south pixels for North Palm Beach County obtained from the NOAA database.

When the latitude of each nest was evaluated, there was a positive correlation between the April and May SST and latitude. The correlation with each nest date was not apparent, particularly since there was a large variation on the nest location latitude in relation to the year of nesting. The data was then analyzed with respect to the mean nest latitude as the distance in meters from the 'base' point in 2004 (Figure 19). There was weak statistical evidence of a positive correlation in April and a strong statistically significant correlation in May (April = R^2 = 0.197, p = 0.084 and May = R^2 = 0.34, p = 0.027).

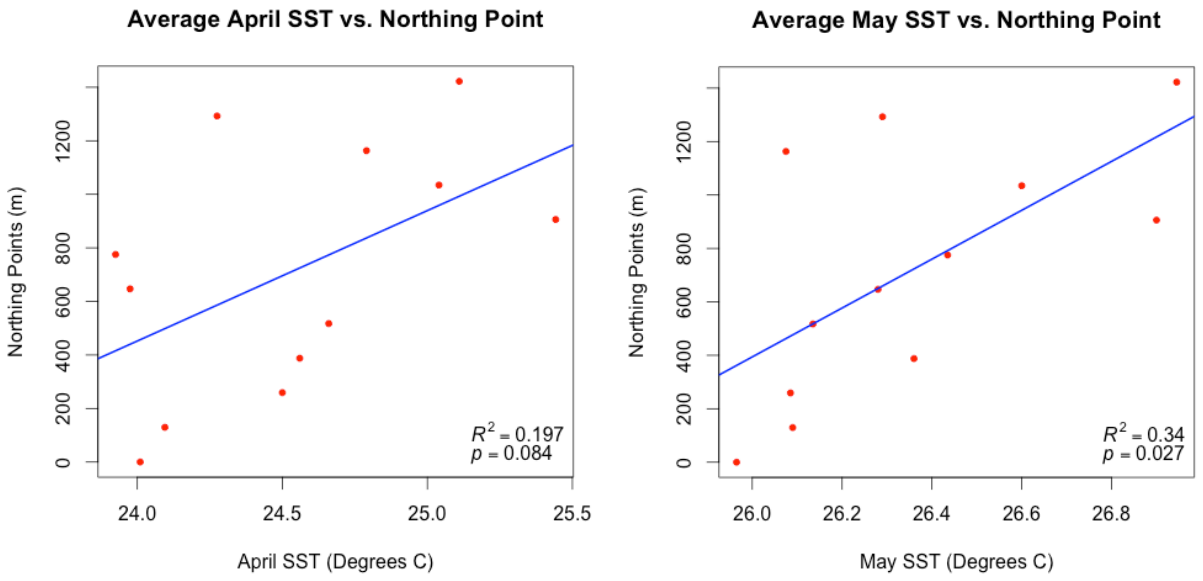


Figure 19 - Northing Points vs. Average SST

Average Yearly Northing Points for the 2004-2015 periods were plotted against Average April and May SST for the corresponding years. Average SST is the average of the north and south pixels for Palm Beach County obtained from the NOAA database.

8.4 Discussion

This study provides evidence that leatherback sea turtles' nest locations, as measured by the latitude, are increasing with each nesting season without an apparent change in date of nesting. This finding is consistent with the leatherback models proposed by Dudley et al [82, 90]. In these models the best way to ameliorate the effects of climate change for nesting mothers it to change nesting location and not the nesting date. Changes in nesting date do not appropriately account for the expected temperature changes associated with climate change.

Changes in nesting timing have been documented in other species [80, 81], however there is not documentation of changes in location associated with SST. Weishampel et

al showed a relationship between nesting time and SST in green and loggerhead sea turtles, the two other main species nesting in Florida [81]. In the case of leatherbacks a stronger effect on location, and not date of nesting, was expected from the models. For this population there was no indication that nests were being laid earlier in the season. The first nest of the season and the mean nest start date did not change from 2004 to 2015 and nesting dates were not associated with changes in SST (R^2 = -0.0539, p = 0.55). However, as expected from the model by Dudley and Porter, there is an increase in latitude for this local population. The mean of the nesting location latitude has moved north by 1.422 Km over the 2004 to 2015 seasons and is associated with an increase in SST. There appears to be an association between changes in the SST and the location of the nesting site (April = R^2 = 0.197, p = 0.084 and May = R^2 = 0.34, p = 0.027) (Figure 19). This would indicate that the turtles are moving north as the SST increases. However, there is not enough evidence to confirm that this is the main driving force. The relationship with SST does not directly indicate that this is the factor that is causing the movement of the nesting location. Further work on the location of nests and date of nesting as it relates to SST for leatherbacks is necessary.

There are other possible explanations for this spatial change, such as (1) better nest site location with respect to latitude, (2) sea turtles select areas of least erosion as determined by the slope of the beach, and (3) changes in the foraging grounds.

A secondary explanation is the movement towards a more amenable section of the beach further north. The study site is located on the southern section of the highest density in the main nesting area (Figure 6). It is possible that the best nesting area is located just north (higher latitude) of the study site and the sea turtle population is moving in this direction. This is supported by the fact that Martin County, the county north of the nesting site studied, historically had lower nesting counts than Palm Beach County and they currently have higher DC nest counts (Table 24). From 1979 through 2008 Palm Beach County accounted for 38.7%, with Martin County accounting for 32.1% of the total nesting sited in Florida [8]. From 2010 through 2014 Martin County accounted for 39.8% and Palm Beach County 31.2% of the total nesting counts.

Further work on the nesting beach conditions and nest latitude of the entire population over a greater time period would be necessary to confirm this hypothesis.

The third possibility is that sea turtles select areas of least erosion as determined by the slope of the beach. The hypothesis of nesting site selection where individual nesting females avoid erosion-prone areas was postulated by Spanier [51]. In this scenario, the nesting females would select areas of the beach that are more suitable for nesting. In our study site, there is little indication that beach nesting conditions are changing due to erosion. During the 2010 to 2015 period there were only 34 (2.89%) nests that were lost because of erosion (unpublished data). This is due in part to the high stability of the beaches in this part of Florida. In these nesting beaches, the beach line is relatively constant. There is consistent human controlled sand transfer to eroded beaches as part of the Palm Beach County shoreline enhancement & restoration program (Palm Beach County Department of Environmental Resources Management: Environmental Enhancement & Restoration Division 2014). Therefore, the slope of the beach should remain constant and not have an effect on the nesting location. However, continued analysis of the slope and conditions of the nesting beaches could ascertain if this is could have an effect on changes in latitude.

Information on the foraging grounds could also help in identifying the driving force for the apparent changes in location. Climatic changes in these areas could prompt the turtles to begin the migration at different times. Leatherbacks are known to nest multiple times in a season [61]. If the turtles were using SST cues to select a nest location they would select beaches further south earlier in the season and then move north as they continue to lay multiple nests. On the other hand, if the migration started at a later date the turtles would tend to arrive later in the season and nest further north. The necessary information on the foraging grounds for all the members of the population is not available so this can not be ascertained at this time. Further work on this area is necessary to determine if the nesting population is affected by climatic changes in the foraging grounds.

The lower latitude in 2009 could be attributed to the expansion of the nesting territory due to an increase in population. This latitude change occurred the year there was the

largest number of nests at the study beaches. The shift to lower latitude could indicate that as the population increases in size, the area where nests are deposited could also increase due to randomness of the nesting site location. Sea turtle populations have normal variation in nesting numbers [89]. The remigration interval for the leatherbacks at this nesting site has been calculated to 2.7 ± 1.0 yr. [66]. There were not events identified within 2 or 3 years prior to any other years that could account for the changes in latitude. Other possible anomalies that would account for the variation in 2009, including SST, were investigated without success.

It is important to state that this is a limited size and time study. There appears to be a genetic component to the nesting site location for leatherback sea turtles [61, 91]. The changes could be a part of this genetically controlled behavior that would take multiple generations to manifest itself. This would indicate that individual sea turtles could have the ability to change the location of the nesting site according to the nesting conditions they encounter. This analysis is meant as a study of part of the population and not of any one specific individual. Leatherbacks are known to nest in multiple locations along their nesting beach [61]. However, there were not enough subsequent encounters to correlate one individual's nest locations over time. Because of this, the nest location to a specific nesting female and the individual variation in nesting site was not able to evaluated. The area of this survey is limited and individual nesting females would likely nest outside of the survey area since leatherbacks are known to have large distances between nesting sites in a season [66].

8.5 Conclusions

This work presents the first concrete evidence of changes in spatial location for a sea turtle nesting local population and as related to changes in SST. Changes in nesting location are consistent with models predicting variations in nesting location as climate changes without changes in nesting dates.

8.6 Acknowledgments

Special thanks to Timothy Van Deelen for his invaluable input on the evaluation of data. His perspective has greatly enhanced the proper analysis of the data. Thanks to Paul D. Mathewson for his help with temperature data and maps. Thanks to all past and present LMC staff members and technicians who monitored the beaches from 2003 to 2015. Thanks to Tommy Cutt for his invaluable support. Finally, thanks to my mother, for her ever-undivided understanding and care, you are missed.

9 Chlorinated Pesticides In Sea Turtle Eggs

Relevant abbreviations used:

GC/ECD (Gas Chromatography/Electron Capture Detector)

POPs (Persistent organic pollutants)

9.1 Specific Aims

This exploratory work was proof of concept of use of the Accelerated Solvent Extractor for the analysis of chlorinated POPs.

- Develop a method for quantitating chlorinated pesticides in sea turtle egg and sea turtle tissue samples utilizing the Accelerated Solvent Extractor (ASE).
- Identify the chlorinated pesticides found in sea turtles' egg and sea turtle hatchling tissue samples.

9.2 Background

Chlorinated pesticides are a widespread class of xenobiotics, found as common pollutants in biological, soils and water samples [92-95]. Chlorinated pesticides are man made chemicals introduced into the environment as a direct result of their use as pesticides. Chlorinated pesticides have been demonstrated to have carcinogenic, mutagenic, and teratogenic properties and are known to bioaccumulate [96-99].

Leatherback and loggerhead sea turtle eggs and hatchlings were used as a monitor for the presence of these pollutants. Maternal transfer has been documented in sea turtles [23]. Maternal transport is the hypothesized mode of transmittance of POPs to the offspring [1]. The concentration of toxicants found in the eggs, and the deceased hatchlings, should been passed on from the mother if there are not external contaminants at the nesting site. It is expected that the concentration of chlorinated pesticides will determine the effects on the fitness of the hatchlings and reduce hatchling success.

9.3 Methods

The method was adapted from PAH method described in the Methods chapter for the PAHs ASE extraction procedure and adapted from the method used at the Wisconsin State Laboratory of Hygiene (WSLH). Originally the method called for the use of Soxhlet reflux extraction followed by a Florisil ™ column cleanup procedure with that is time consuming. This method was modified to use an ASE extraction to reduce extraction time.

9.3.1 Reagents

All reagents used were Pesticide, HPLC grade or better as listed in the PAH method with the exception of

- Florisil ™ - 100-200 mesh; Sigma Aldrich, St. Louis, Missouri, USA; CAS 1343-88.

9.3.2 Standards

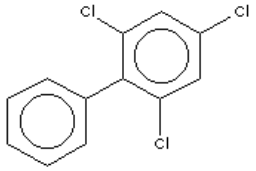
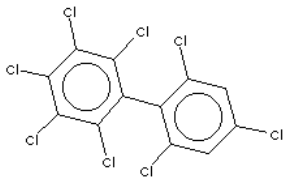
Commercially available stock standards were used. These were dissolved in toluene, diluted to volume in a 100 mL volumetric flask. Standards were stored in Teflon-sealed screw-cap bottles in a freezer and protect from light. Concentrations used were from 1 ng/mL to 100 ng/mL. CLP Organochlorine Pesticide Mix - Supelco, Bellefonte, Pennsylvania, USA, CA, Cat. No. 47426-U.

Table 27 - Chlorinated Pesticides Tested

	Compounds	CAS #
1	Aldrin	309-00-2
2	α-BHC	319-84-6
3	α-Chlordane	5103-71-9
4	β-BHC	319-85-7
5	δ-BHC	319-86-8
6	Dieldrin	60-57-1
7	α-Endosulfan	959-98-8
8	β-Endosulfan	33213-65-9
9	Endosulfan sulfate	1031-07-8
10	Endrin	72-20-8
11	Endrin aldehyde	7421-93-4
12	Endrin ketone	53494-70-5
13	γ-BHC	58-89-9
14	γ-Chlordane	5103-74-2
15	Heptachlor	76-44-8
16	Heptachlor Epoxide Isomer B	1024-57-3
17	Methoxychlor	72-43-5
18	4,4′-DDD	72-54-8
19	4,4′-DDE	72-55-9
20	4,4′-DDT	50-29-3

- Internal Standard - PCB mix in isooctane - An internal standard was used to verify extraction of each sample. This consisted of two known PCB, 30 and 206. Spike 25 uL for a final concentration of:
  - PCB 30 = 14.2 ng/mL
  - PCB 204 = 15.6 ng/mL

Table 28 - Internal Standard Used

	Compound	CAS #	Molecule
1	PCB 30	35693-92-6	
2	PCB 204	74472-52-9	

9.3.3 Equipment

As listed in the PAH method with the exception of:

- GC/ECD - Agilent Technologies 6890N Network GC System, 7683 Series Injectors, G23978A Electron Capture Detector, with GC ChemStation Rev. A.09.03 - 1417 software.
- GC column (Front Injector) - Agilent Technologies, Inc., DB-1, 60 m x 0.250 mm, 0.10 micron film, Narrowbore, Cat No. 122-1061.
- GC column (Back Injector) - Agilent Technologies, Inc., DB-17MS, 60 m x 0.250 mm, 0.25 micron film, Narrowbore, Cat No. 122-4762.

9.4 Sample Collection

As described in the PAH method of this document.

9.5 Sample Analysis

The method was an extension of the PAH method described in this document with the following alterations.

9.5.1 ASE Extraction

Standards were those of for pesticide as listed above. Add 25 µL of the internal standard (PCB mix) spiking solution onto each sample.

Add 0.1 mL of the standard matrix spiking solution onto sample chosen for spiking as a control.

The ASE parameter were kept constant with the PAH method, however the solvent was changed to a 70:30 dichloromethane:acetone solution.

Fat Percent Calculation and GPC extraction was performed as listed in the PAH method.

Table 29 - Collection fractions (Presence or Absence of compounds)

Component	Frac 1 21-25.5 min	Frac 2 25.5-30 min	Frac 3 30-34.5 min	Frac 4 34.5-39 min	Frac 5 39-43.5 min	Frac 6 43.5-48 min	Frac 7 48-52.5 min	Frac 8 >52.5 min
Aldrin				PRE	PRE	PRE		
α-BHC				PRE	PRE			
α-Chlordane (cis)			PRE	PRE	PRE			
β-BHC				PRE	PRE	PRE		
δ-BHC				PRE	PRE	PRE		
Dieldrin			PRE	PRE	PRE			
α-Endosulfan I			PRE	PRE	PRE			
β-Endosulfan II			PRE	PRE	PRE			
Endosulfan sulfate			PRE	PRE	PRE			
Endrin			PRE	PRE	PRE			
Endrin aldehyde *			PRE	PRE	PRE	PRE	PRE	PRE
Endrin ketone			PRE	PRE	PRE			
γ-BHC (Lindane)				PRE	PRE			
γ-Chlordane (trans)			PRE	PRE	PRE			
Heptachlor				PRE	PRE			
Heptachlor Epoxide Isomer B			PRE	PRE	PRE			
Methoxychlor			PRE	PRE				
trans-Nonachlor			PRE	PRE	PRE			
cis-Nonachlor				PRE	PRE	PRE		
4,4′-DDD				PRE	PRE			
4,4′-DDE			PRE	PRE	PRE			
4,4′-DDT			PRE	PRE	PRE			
ISTD PCB 30			PRE	PRE	PRE			
ISTD PCB 204				PRE	PRE			

(PRE = Present in fraction collected).

* Endrin Aldehyde continues to elute at minute concentrations from the GPC at the last 2 collections. The last two fractions insignificant amounts below the limit of quantitation.

9.5.2 GC/ECD Parameters

Different from the PAH procedure the analysis of samples was done by GC/ECD

Extracts were analyzed with the following instrument conditions:

Oven:

- Start at 50°C, hold 10 min
- Ramp at 10°C/min to 300°C
- Hold 15.5 min at 300°C
- Carrier Gas: Helium
- Injector: 280°C
- Transfer Line: 320°C
- Injection: Split/Splitless, Volume: 1 µL

Interferences extracted from the samples varied considerably from sample to sample. There were interferences with the early eluting compounds with each of the two columns used. Quantitation of α-BHC, β-BHC and δ-BHC was not possible using this extraction procedure.

Standards

9.5.3 Quality Control

- Before a batch of samples can be run, a method blank was analyzed to verify that each target compound's background concentration is below its LOD. If these criteria were not met samples were re-extracted and reanalyzed.
- During each batch of samples, a laboratory control was analyzed. The sample was spiked with all of the target analytes at 100 ng/mL.
- Recovery was used to determine if the extraction efficiency was acceptable. Concentration of samples was corrected to the recover of each of the compounds.
- Method Calibration - Internal Standard Procedure

1. Calibration standards: A minimum of five calibration standards at different concentrations, were used. Each calibration standard, plus solvent blank, contained

the appropriate amount of internal standard. The correlation coefficients (R^2) had to be ≥0.995.

2. Internal standard was added to all samples.
3. Quantitation was based on area.

1. The calibration points were constructed by calculating an amount ratio and a response ratio for each level of a particular peak in the calibration table.
2. The amount ratio is the amount of the compound divided by the amount of the internal standard at this level.
3. The response ratio was the abundance of the compound divided by the abundance of the internal standard at this level.
4. An equation for the curve through the calibration points was calculated using the linear type of curve fit (Equation 1 - Response Factor Calculation).
5. The results were used to plot a calibration curve of response vs. amount ratio.
6. ChemStation software calculated the above ratios. Each calibration table had at least five levels.

Table 30 - Chlorinated Pesticide Retention Times for each column

Component	RT	DB-1	RT	DB-17	Change
α-BHC	1	10.426	1	21.556	0
β-BHC	2	11.160	4	25.749	2
γ-BHC (Lindane)	3	11.523	3	24.201	0
ISTD PCB 30	4	11.862	2	22.232	-2
δ-BHC	5	12.009	6	27.688	1
Heptachlor	6	15.025	5	26.074	-1
Aldrin	7	16.654	7	28.099	0
Heptachlor Epoxide Isomer B	8	18.382	8	31.964	0
γ-Chlordane (trans)	9	19.510	9	33.220	0
α-Endosulfan (Endosulfan I)	10	20.149	12	34.283	2
α-Chlordane (cis)	11	20.391	11	34.036	0
trans-Nonachlor	12	20.883	10	33.290	-2
Dieldrin	13	21.465	14	36.263	1
4,4′-DDE	14	21.799	13	35.892	-1
Endrin	15	22.346	15	38.465	0
β-Endosulfan (Endosulfan II)	16	22.545	18	40.227	2
Endrin Aldehyde	17	23.388	20	42.248	3
4,4′-DDD	18	23.693	17	39.715	-1
cis-Nonachlor	19	23.855	16	38.649	-3
Endosulfan Sulfate	20	24.762	21	43.262	1
4,4′-DDT	21	25.881	19	41.630	-2
Endrin Ketone	22	26.789	24	47.159	2
Methoxychlor	23	29.063	23	46.621	0
ISTD PCB 204	24	29.502	22	44.208	-2

RT changes form the DB-1 column dimethyl polysiloxane, non-polar to the DB-17 (50%-phenyl)-methyl polysiloxane column of mid polarity column

Calibration curve

9.6 Results

The ASE method allows for the quantitation of 19 different Chlorinated Pesticides in egg and hatchling tissue samples. A calibration using all standards from 0.08 to 6.40 ug/mL was performed on the GC/ECD.

The linearity for each run was plotted using Microsoft Excel. Each dilution was compared to the others to determine the concentration in which the LOQ and LOD down to 1 ng/mL.

9.6.1 Sea Turtle Sample Results

We analyzed a total of 16 sea turtle egg and tissue samples for the presence of Chlorinated Pesticides. We analyzed 8 samples from each collection year, 2011 and 2012). One sample for 2012 failed to extract.

Table 31 - Loggerhead Chlorinated Pesticides

Compound	n = 15	Low ng/g	High ng/g
γ-BHC (Lindane)	2	5.9	22.6
Heptachlor	1	3.9	3.9
trans-Nonachlor	2	1.9	3.5
Dieldrin	1	2.5	2.5
4,4′-DDE	12	2.0	11.5

Chicken Egg	n = 4		
Heptachlor	2	3.5	3.9
Aldrin	1	0.9	0.9
4,4′-DDE	3	1.1	20.7

Table 32 - Leatherback Chlorinated Pesticides

γ-BHC (Lindane)									x	x			2
Aldrin									x				1
Heptachlor Epoxide					x	x		x		x			4
γ-Chlordane										x			1
α-Endosulfan		x		x	x	x		x		x		x	7
4,4′-DDE	x	x	x	x	x	x	x	x		x		x	10
β-Endosulfan		x		x	x	x		x		x		x	7
4,4′-DDD		x	x	x	x			x		x			6
Endosulfan sulfate		x		x				x		x			4
4,4′-DDT		x		x	x			x		x			5
Methoxychlor		x		x	x	x		x		x		x	7
Total	1	7	2	7	7	5	1	8	2	10	0	4	54

If there was peak within the RT window of acceptance of the assay by one column the peak was then evaluated by the second column. Not only would the RT had to match, but the concentration of the compound had to be within 20% of the second peak.

9.7 Discussion

This study provides method for analyzing chlorinated pesticides in egg and tissue samples. The first part of this study was to determine the limits of quantitation (LOQ), the limits of detection (LOD) and the upper limit of linearity (ULL). The second purpose is to quantitate the Chlorinated Pesticides present in sea turtle eggs and then determining the effects of varying concentrations of Chlorinated Pesticides on fitness and survival rate of hatchlings.

The findings are concordant with the findings in other matrices, specially the finding of DDTs in high number of samples. Keller et all reported the presence of PCBs, mirex, Dieldrin, trans-chlordane, oxychlordane, trans-nonachlor, cis-nonachlor, 4,4′-DDE and total DDTs in blood samples from loggerhead seaturtles [100]. In a second study fat

tissue and blood were also compared. Here Total PCBs, mirex, dieldrin, heptachlor epoxide, trans-chlordane, cis-chlordane, trans-nonachlor, cis-nonachlor, oxychlordane, 4,4', DDD, 4,4'-DDE, 2,4'-DDT and Total DDTs were detected in both blood and fat. In the case of a-HCH, b-HCH, g-HCH and HCB, these compounds were only detected in fat tissue [101].

10 Thesis Acknowledgments

10.1 Committee

- PI - Warren Porter, PhD
- Chris Bradfield, PhD
- Curtis Hedman, PhD
- Fariba Assadi-Porter, PhD
- Joel Pedersen, PhD
- Pat Gorski, PhD
- Timothy Van Deelen, PhD
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- Sarah Hirsch
- Tommy Cutt

10.6 Wisconsin State Laboratory of Hygiene

- Carol Buelow
- Dave Rogers
- Steve Geis
- Curtis Hedman

10.7 Awards/Funding/Other

- Nominated as an Honored Instructor for the Fall 2011 and 2013 Semesters (University Housing's Office of Academic Initiatives)

- Graduate Student Collaborative Vilas Research Travel Grant, March 2011
- Bunde Fund Award for support of research, May 2010
- Vilas Welcome Award, May 2009
- Advanced Opportunity Fellowship through the Science & Medicine Graduate Research Scholars (SciMed GRS), May 2009 (For two years of funding)
- To everyone in Biocore including the Staff and students, which had made me realize that teaching is a gift.

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12 Appendices

12.1 Appendix 12.1 - WSLH - PAHs - ESS ORG METHOD 1461

- Attached method for the extraction of PAHs - ESS ORG METHOD 1461, Revision 1, Effective Date: 5/26/2009 - present

12.2 Appendix 12.2 - WSLH - PAHs Standards

- Attached listing of controls used.

Appendix 12.1 - WSLH - PAHs - ESS ORG METHOD 1461

Polynuclear Aromatic Hydrocarbons in Fish Tissue by GC/MS-SW846 Method 8270D - Revision 4, February 2007, Effective 26, May 2009

Matrix: Tissue Analysis

PAHs in Fish Tissue by GC/MS

ESS ORG METHOD1461

Revision 1

Effective Date: 5/26/2009 - present

Replaces: ESS ORG Method 1460 revision 3.1

1. Scope and Application

1.1. Method 1461 is used to determine the concentration of certain poly-nuclear aromatic hydrocarbons (PAHs) in fish tissue.

1.2.

Parameter	**Report limit in ng/g**
Naphthalene	<15.0
Acenaphthylene	<15.0
Acenaphthene	<15.0
Fluorene	<15.0
Phenanthrene	<15.0
Anthracene	<15.0
Fluoranthene	<15.0
Pyrene	<15.0

Benz(a) anthracene	<15.0
Chrysene	<15.0
Benzo (b) fluoranthene	<15.0
Benzo (k) fluoranthene	<15.0
Benzo (a) pyrene	<15.0
Dibenz (a,h) anthracene	<20.0
Benzo (g,h,i) perylene	<15.0
Indeno (1, 2, 3-cd) pyrene	<20.0

2. Summary of Method:

2.1. The method provides Gas Chromatograph/Mass Spectrometer for the detection of ppb levels of certain PAHs. A measured mass of sample (~10 g) is Soxhlet extracted with methylene chloride. The resulting extract is dried, concentrated and a gel-permeation cleanup is performed. The extract is concentrated to 1.0 ml and analyzed by GC/MS.

2.2. List Regulatory Deviations: This section is not applicable to this method.

3. Safety and Waste Management:

3.1. List extreme hazards that are specific to the method.

3.2. General safety practices for all laboratory operations are outlined in the Chemical Hygiene Plan for Environmental Sciences

3.3. All laboratory waste, excess reagents and samples will be disposed of in a manner that is consistent with applicable rules and regulations.

Waste disposal guidelines are described in the University of Wisconsin Chemical Safety and Disposal Guide.

4. Sampling Handling and Preservation:

4.1. Tissue samples are coarsely ground and frozen in glass jars with aluminum foil lined caps.

4.2. See the Quality Assurance Manual for more information.

5. **Interferences:** Interferences coextracted from the samples will vary considerably from source to source. Although a general cleanup technique is provided with this method, individual samples may require additional cleanup to eliminate matrix artifacts.

6. Reagents and Standards:

6.1. Reagents

6.1.1. Methylene chloride, - pesticide grade

6.1.2. Sodium sulfate - ACS grade. Purify by heating at 450°C for four hours in a glass dish.

6.2. Standards

6.2.1. Prepare stock standard solutions by accurately weighing the pure material. Dissolve the material in GC/MS quality methylene chloride, dilute to volume in a 100-ml volumetric flask. Larger volumes can be used at the convenience of the analyst. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source. Label bottle with appropriate lab nomenclature for tracking purposes.

6.2.2. Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store in a freezer and protect from light. Stock standard solutions should be checked frequently for signs of degradation or

evaporation, especially just prior to preparing calibration standards from them.

6.2.3. Stock standard solutions must be replaced after one year, or sooner if comparison with check standards indicates a problem.

6.2.4. A second source standard, additionally needs to be prepared. Supelco offers a certified solution designated as “second source” with accompanying certification. Prepare in 100mL volumetric flask and transfer to 125 ml Teflon-sealed screw-cap amber bottle. This solution should be stored in a freezer until needed.

6.2.5. Surrogate standard. Certified deuterated solutions are purchased and diluted to 50mL. Document on standard prep sheet the volume diluted and final concentration. Transfer to a 50 ml screw top amber bottle and store in standard freezer. Label bottle with appropriate lab nomenclature for tracking purposes.

6.2.6. Internal standard solution. Certified deuterated solutions are purchased and diluted to a volume appropriate for analytical use. Transfer internal standard to amber screw top bottle and store in standard freezer until needed. Label bottle with appropriate lab nomenclature for tracking purposes.

7. Apparatus:

7.1. Industrial blender, 1000-ml and 100-ml blender cups.

7.2. Analytical balance

7.3. Micro-syringes

7.4. Büchi Rotovapor, model R-114. Accompanied with temperature controlled water bath, model B-490.

7.5. Nitrogen blow-down apparatus

7.6. Gel-Permeation Chromatography system, with 65 cm x 2.8 cm I.D. glass column. S-X3 select 200-400 mesh BioBeads. (O.I. Analytical Part # 091-203)

7.7. 10-ml syringe with a Luer-Lok fitting

7.8. Volumetric flasks, graduated cylinders – 10-, 25-, 100- or 500-ml

7.9. 250- & 500-ml boiling flasks

7.10. Teflon boiling chips.

7.11. Beakers, 100-, 250-, and 400-ml

7.12. 1.0mL or greater volumetric pipettes.

7.13. Spatulas

7.14. Rheostat controlled Soxhlet element banks.

7.15. Soxhlet condenser

7.16. Hot plate and water tray

7.17. Gas Chromatograph/Mass Spectrometer - analytical system complete with temperature programmable GC suitable for use with capillary columns and all required accessories including syringes, columns, gases, detector and a PC-based integrator.

7.18. Glass Wool

8. Quality Control

8.1. For general quality control procedures see the Quality Assurance Manual. For specific quality control acceptance limits that apply to laboratory control samples, surrogates, calibration check standards, matrix spikes, and duplicates for this analytical procedure please consult the laboratory's LIMS system. For details, see the standard operating procedure "ESS ORG QA0001 QAWRKSHT".

8.2. The quality assurance procedures followed for this method are a composite of the requirements found in EPA Method 8270D, EPA Method 8000B (Revision 2, December, 1996), the Wisconsin Laboratory Certification Code (Ch. NR 149, Register November 2009, No. 647), and the Quality Assurance Manual. The specific quality assurance procedures adopted are outlined below.

8.3. The minimum requirements consist of an initial demonstration of laboratory capability and an ongoing analysis of spiked samples to evaluate and document quality data. The laboratory should maintain records to document the quality of the data generated. Ongoing data quality checks are compared with established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

8.4. Before a batch of samples can be run, a method blank must be analyzed in order to verify that each target compound's background concentration is below its LOD. If this criterion is not met corrective action will be taken to locate and reduce the contamination. Samples will be re-extracted and reanalyzed.

8.5. During a batch of samples, a matrix-spike, matrix-spike duplicate, and laboratory control sample (LCS) will be analyzed. The sample will be spiked with all of the target analytes near the mid-point of the calibration range. A standard mix separate from that used to generate the initial calibration curves will be used for spiking. In general one member of the matrix-spike pair will be run at the beginning of the batch and one at the end. The percent recoveries of the spikes must fall within three standard deviations (the control limit) of the in-house generated data to be in control. If they do not fall within the limits, the samples will be rerun or the data will be appropriately flagged. The only exception to this applies if the sample chosen for spiking contains

the spiked compound in large amounts (i.e. a sample result equal to, or greater than, the spiking concentration). If any of these values fall within two to three standard deviations (the warning zone), then the analyst must evaluate whether the most recent data is drifting towards the control limits, and take whatever precautions to prevent this continued trend.

8.6. A matrix effect is indicated if the LCS recovery data are within the control limits, but the matrix-spike data exceed the control limits. Surrogate recoveries from these runs will also be used to help make this determination. If all recoveries for the matrix-spike compounds and the surrogate compounds in the LCS are in control, then the batch can be run, with all sample results being flagged due to matrix-spike criteria not being met. If LCS recoveries are not all met, then this indicates a laboratory performance problem, and resolution of the problem must take place before any samples can be analyzed. Until in-house data are generated for the LCS, limits of 70-130% will be used.

8.7. For each analytical batch a matrix spike/matrix spike duplicate pair, or a field duplicate should be analyzed. The decision on whether to prepare and analyze field duplicate samples or a field matrix spike/field matrix spike duplicate must be based on knowledge of the samples in the sample batch. If samples are expected to contain target analytes, then laboratories may use one field matrix spike and a field duplicate analysis of an unspiked field sample. If samples are not expected to contain target analytes, the laboratories should use a field matrix spike and field matrix spike duplicate pair. The precision acceptance criteria will be generated in house. Until in house data is generated a limit of 35% RPD will be used.

8.8. The experience of the analyst performing liquid chromatography is invaluable to the success of the methods. Each day that analysis is performed, the daily calibration sample should be evaluated to determine if the chromatographic system is operating properly. Questions that should be asked are: Do the peaks look normal? Is the response obtained comparable to the response from previous calibrations? Careful examination of the standard chromatogram can indicate whether the column is still good, the system is leaking, etc. If any changes are made to the system (e.g. column changed), recalibration of the system should take place.

8.9. A quality control (QC) check sample concentrate, containing each analyte of interest, is required annually or whenever new calibration standards are prepared. The QC check sample concentrate may be prepared from pure standard materials, or purchased as certified solutions. If prepared by the laboratory, the QC check sample concentrate should be made using stock standards prepared independently from those used for calibration.

8.10. If surrogate recovery is not within limits, the following is required.

8.10.1. Check to be sure there are no errors in calculations, surrogate solutions and internal standards. Also, check instrument performance.

8.10.2. Reanalyze the extract if any of the above checks reveal a problem or flag the data as exceeding the quality control limit.

9. Method Calibration - Internal Standard Procedure

9.1. The GC/MS is initially autotuned and must pass before proceeding.

9.2. GC/MS tuning standard: Decafluorotriphenylphosphine (DFTPP) assesses the systems capability of fracturing analytes over a wide mass range. Analysis cannot proceed unless the DFTPP passes.

Prepare a stock 50 ng/µL solution and dilute to 5.0 ng/µl, this is the working standard. Total mass injected should not exceed 50 ng. Injecting less, helps for sensitive MS systems. The DFTPP should be assessed by reasonably spanning both sides of the apex. The criteria for passing tune parameters are listed under table 11.2. Within the run, the analyst shall inject the DFTPP at 12 hour intervals. If the tune passes, the proceeding data is considered valid. Instrument maintenance and/or source cleaning would be needed if the DFTPP fails. All DFTPP reports should be included in the batch folder. When not in use, store stock and working standard in freezer.

9.3. Calibration standards: Prepare a minimum of five calibration standards at different concentrations, spanning the limit of detection to source saturation. Each calibration standard, plus solvent blank, will contain the appropriate amount of internal standard. When generating a calibration, each curve will be assessed against a second source standard, which is at or near the mid point calibration. The results from the second source standard shall be ±30% of the curve. The correlation coefficients (r) should be ≥0.995. All standards, blanks, and curves are printed and stored with batch folder. Copies of the curve are generated and included in subsequent batch folder.

9.4. All samples, spikes, duplicates and standards should have known and sufficient amount of IS to assess instrument drift and matrix affects. The internal standard (IS) solution contains naphthalene-d_8, acenaphthalene-d_{10}, phenanthrene-d_{10}, chrysene-$d_{12,}$ and perylene-d_{12}. Currently, 1.0 ml of sample extract is fortified with 10 µL at ~100 ng/µl. When assessing the IS response, all samples, spikes, duplicates and blanks shall not exceed the ranges of 50-200% of the calibration IS average. In order to achieve desired IS response it may

be necessary to adjust voltage or perform maintenance on GC/MS system. Note: Ideally, the initial IS response should fall between 75-125%. The late eluting perylene d-12's response typically increases over time, and will probably not pass later if >125% initially.

9.5. The individual IS and accompanying target analytes are listed below:

Deuterated Internal Standards	**Target compounds**
Naphthalene-d_8	Naphthalene, 2-methylnaphthalene, and 1-methylnaphthalene.
Acenaphthene-d_{10}	2,7-dimethylnaphthalene, acenaphthylene, acenaphthene, and fluorene.
Phenanthrene-d_{10}	Phenanthrene, anthracene, and Fluoranthene.
Chrysene-d_{12}	Pyrene, p-terphenyl-d_{14} (surrogate), benzo (a) anthracene, and chrysene.
Perylene-d_{12}	Benzo (b&k) fluoranthene, benzo (e&a) pyrene, Indeno (1,2,3-cd) pyrene, benzo (g,h,i) perylene, & dibenzo(a,h)anthracene.

9.6. Quantitation will be based on primary ions extracted from the total ion scan. The secondary ions are used to confirm target analytes. The

ions used are listed in Table: Primary and secondary ions used for quantification and confirmation for select PAHs, section 11.3.

9.7. Once a calibration has been generated, the analyst may perform a daily continuing calibration check standard (CCCS) to verify the instrument calibration. After the instrument passes the autotune and DFTPP, a mid-range check standard is injected and assessed for IS response, percent difference, peak shape and retention time. All PAHs should be within ±20%, exhibit proper peak shape and accurately identified. The IS response needs to be within 50-200% of the IS calibration average. The analyst may adjust the IS responses by adjusting the millivolts in the ionization chamber. The CCCS must be reinjected if voltage has changed and pass all criteria mentioned earlier. If the CCCS fails again, the instrument probably needs maintenance at inlet, column, and/or source. Additionally, it might be necessary to recalibrate at this point. Retention times are updated, if column maintenance has occurred.

9.8. The analyst will continue to run check standards after every ten samples. The PAHs should continue to be ±20% of the true value to verify the calibration curve. If some PAHs in the CCCS are outside the ±20% limit, the sample will be rerun or appropriately flagged. In the case where the instrument response has increased, it is still possible to confirm a “non-detect” if it is obviously not present.

9.9. When assessing the calibration curve and correlation coefficients, it may become apparent that low-level quantitation is suspect due to poorly responding analytes that fall nowhere near the origin. To remedy this, a “low level” calibration may be employed with more points around the detection limit and ranging only to the mid point. The analyst will have to generate different calibration standards and inject on the GC/MS. A “low level” calibration curve has to be

generated, with the accompanying correlation coefficients for each analyte. All PAH correlation coefficients (r) will be >0.995. Additionally, the analyst needs to look at each analyte curve and determine whether the curve properly quantitated near the origin and lowest level standard. An ongoing CCCS will be used to assess the low-level curve prior to batch analysis and needs to meet the criteria outlined in 9.7 and 9.8, only if a low level calibration curve already exists.

9.10. Select PAHs will be monitored for minimum response factors (RF) prior to analysis when assessing the CCCS. This is done to interpret whether or not the system is operating properly. Listed below are the PAHs with the targeted RFs.

9.11. Response Factors:

PAH	**Response Factor**
Naphthalene	0.700
2-Methylnaphthalene	0.400
Acenaphthylene	0.900
Acenaphthene	0.900
Fluorene	0.900
Phenanthracene	0.700
Anthracene	0.700
Fluoranthene	0.600
Pyrene	0.600

Benzo(a)anthracene	0.800
Chrysene	0.700
Benzo(b&k)fluoranthene	0.700
Benzo(a)pyrene	0.700
Indeno(1,2,3-cd)pyrene	0.500
Benzo(g,h,i)perylene	0.500
Dibenzo(a,h)anthracene	0.400

9.12. The additional of internal standard shall not be more than 1% difference by volume. For most applications in this method, that means the extracts will be concentrated to 1.0 ml and have 10 µL of IS added.

9.13. The calibration points are constructed by calculating an amount ratio and a response ratio for each level of a particular peak in the calibration table.

9.14. The amount ratio is the amount of the compound divided by the amount of the internal standard at this level.

9.15. The response ratio is the abundance of the compound divided by the abundance of the internal standard at this level.

9.16. An equation for the curve through the calibration points is calculated using the linear type of curve fit.

$$RFx = \frac{\text{Amount Ratio}}{\text{Response Ratio}}$$

The results can be used to plot a calibration curve of response vs. amount ratio.

9.17. Chemstation software calculates the above ratios and it is up to the analyst to properly enter the amounts of analytes and IS. Each calibration table may have at least six levels, so it is imperative that the analyst correctly enter pre calibration data to insure proper quantitation.

9.18. Each standard analyzed on the GC/MS should be identified for traceability purposes. This unique identifier will correspond with in-lab nomenclature.

10. Procedure

10.1. Sample Handling

10.1.1. Blend tissue with dry ice at high speed to produce a free flowing powder. Rinse the blender jar between samples with ethanol. Let the dry ice sublime overnight in a freezer. Mix 10.0 g of tissue with 60 g of anhydrous sodium sulfate stirring frequently for about 30 minutes. The tissue should appear dry and free flowing prior adding to Soxhlet tube.

10.1.2. Add 20-40 µL of the surrogate standard spiking solution onto each sample.

10.1.3. Add 1.0 ml of the matrix spiking solution onto sample chosen for spiking. (See EPA SW-846 Method 3500B for the appropriate choice of matrix spiking compounds and concentrations.)

10.1.4. Place the tissue/sodium sulfate mixture in a Soxhlet extractor with a glass wool plug.

10.1.5. Add a second glass wool plug on top of the tissue/sodium sulfate mixture. Pour 300 ml of methylene chloride into Soxhlet and let cycle to the attached 500-ml boiling flask containing solvent rinsed boiling chips. Attach the flask and Soxhlet extraction tube to Soxhlet bank and extract for 24 hours at 4-6 cycles per hour.

10.1.6. After extraction, allow the extract to cool and dry it by passing it through a drying column containing about 10 cm of anhydrous sodium sulfate. The eluate should be collected in a 500-ml boiling flask. After sample addition, rinse the drying column with 30 ml of methylene chloride.

10.1.7. Concentrate the extract using the Rotovapor, with the water bath temperature ~45°C to <10 ml and transfer to 10mL volumetric flask with at least two methylene chloride rinses of the 500mL boiling flask. Dilute to 10mL with methylene chloride.

10.1.8. Take a 2 ml aliquot and transfer it to an aluminum weighing dish tared to the nearest 0.1 milligram using an analytical balance. Place weighing dish in a hood and allow evaporate to dryness. Weigh the dish again to the nearest 0.1 milligram. Determine the fat content using the following equation:

% fat = (residue + dish weight - tare) x 100/sample weight.

10.2. Sample Cleanup

10.2.1. Automated GPC is used to separate the PAHs, from the bulk of the lipid. A 60-g bed of SX-3 Bio-Beads gel resin (Bio Rad) is used with a methylene chloride solvent system. The resin is packed in a 2.8 cm I.D. x 65 cm glass column fitted with two adjustable end plungers (Glenco Scientific). The column is placed

on an automated low-pressure GPC Autoprep 1001 chromatograph (ABC Labs), and solvent is pumped through the column at 5 ml/min.

10.2.2. Five milliliters (but not more than 1 gm. of lipid) of the sample extract is placed on the GPC column. The GPC eluate is split into two fractions. The first 100 - 140 ml is dumped (discarded) as this should contain only the extract lipids. The second fraction of 80 - 100 ml is collected in 250 ml boiling flasks. The exact volume eluted for each fraction is determined from time settings on the GPC control unit. The times are determined and periodically adjusted by "calibrating" the gel resin column with standards spiked into solvent.

10.2.3. The GPC extracts are rotovaped, @ 45°C to ≈ 3 mL . Transfer and rinse to a calibrated 5mL centrifuge tube and concentrate to 1 ml.

10.2.4. Add internal standard prior to injection.

11 Instrument Operating Parameters

11.1 Gas Chromatography/Mass Spectroscopy

11.1.1 Extracts are analyzed with an Agilent 6890N – gas chromatogram, with an Agilent 4973 mass selective detector. The analytical column is a HP-5MS, 30 m, 0.25 mm ID, and 0.25µm film.

11.1.2 The instrument conditions are:

11.1.2.1 Mass range: 50-550 amu

11.1.2.2 Oven:

11.1.2.2.1 Start at 50°C, hold 10 min.

11.1.2.2.2 Ramp at 10°C/min to 300°C.

11.1.2.2.3 Hold 15.5 min at 300°C

11.1.2.3 Carrier Gas: Helium

11.1.2.4 Injector: 280°C

11.1.2.5 Transfer Line: 320°C

11.1.2.6 Injection:

11.1.2.6.1 Split/Splitless, Grob

11.1.2.6.2 Volume: 2 µL

11.1.2.7 Scan Time: 2.91 scans/sec

11.2 See below for DFTTP tuning parameters. These parameters are taken from EPA Method 525 and though followed while performing this method, they are considered advisory.

Target Mass	**Rel. to Mass**	**Lower Limit%**	**Upper Limit%**
51	198	10	80
68	69	0.00	2
70	69	0.00	2
127	198	10	80
197	198	0	1

198	442	50	100
199	198	5	9
275	198	10	60
365	198	1	100
441	443	0.01	100
442	442	100	100
443	442	15	24

11.3 Primary and secondary ions used for quantification and confirmation for select PAHs

11.3.1

11. **Data Management:** Data is collected using HP Chemstation software. It is then transferred to the laboratory worksheet. All data is reviewed (by peers or section supervisors) and then manually entered onto the Laboratory's LIMS system.

12. **Definitions:** General definitions of other terms that may be used in this method are found in Section 19 of the SLH Quality Assurance Manual

13. **Method Performance:** Where applicable the laboratory's initial accuracy and precision data (MDLs and IDCs) were generated in compliance with the reference method and the Departments standard operating procedure "ESS ORG QA0012 LOD and LOQ Determinations". Data generated within the last two years will be located in the filing cabinet in the Department supervisor's cubicle. Any data older than two years is stored in the Department filing cabinet in the basement.

14. **References:**

14.1. "Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS)" EPA Method 8270D, (Revision 4, February 2007).

14.2. "Methods for Organic Chemical Analysis of Municipal and Industrial Wastewater" EPA/600/4-82-057.

14.3. "Soxhlet Extraction", EPA EPA Method 3540C (Revision 3, December, 1996).

14.4. "Determinative Chromatographic Separations", EPA Method 8000B (Revision 2, December, 1996).

14.5. "Laboratory Certification & Registration", Wisconsin Laboratory Certification Code (Ch. NR 149, Rev. 6, July, 1997), Wisconsin Department of Natural Resources.

14.6. "Quality Assurance Procedures and Policies", The ESS QA Manual.

14.7. "Constitution, Bylaws, and Standards", National Environmental Laboratory Accreditation Conference, (July 1999)

15. **Tables, figures, diagrams, charts, checklists, appendices:** None

16. Signatory Page:

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Appendix 12.2 - WSLH - PAHs Standards

Listing of standards used for analysis

Table of All Standards

	Standard Number	Standard Number	Standard Number	Standard Number	Standard Number	Standard Number	Standard Number	Standard Number	Standard Number	Standard Number
	1:2 of 0.16	OC-031214-12	OC-031314-1	OC-031314-2	OC-031314-3	OC-031314-4	OC-031314-5	OC-031314-6	OC-031314-7	OC-031314-8
Compound	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)	Conc (ug/mL)
Naphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
2-Methylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
1-Methylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
2,7-Dimethylnaphthalene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Acenaphthylene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Acenaphthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Fluorene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Phenanthrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Anthracene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Fluoranthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Retene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(a)anthracene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Chrysene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(b)fluoranthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(k)fluoranthene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(e)pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(a)pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Indeno(1,2,3-c,d)pyrene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Benzo(g,h,i)perylene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Dibenz(a,h)anthracene	0.08	0.16	0.40	0.60	0.80	1.0	1.2	2.0	4.0	6.4
Coronene	0.24*	0.48*	0.80*	1.2*	1.9*	2.7*	3.8*	5.4*	7.6*	10.9*
p-Terphenyl-d14	0.25	0.50	0.96	1.4	1.7	2.0	3.2	3.0	4.4	6.0
Internal std mix	0.925	0.925	0.925	0.925	0.925	0.925	0.925	0.925	0.925	0.925

Analyst: DTR

Standard Number
OC-031214-12

Name: 0.16µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/12/14	3/12/15

Methylene Chloride lot# DJ142

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	0.40	100.0	Methylene Chloride	Naphthalene	0.16
↑(PAH mix)↑			↓	2-Methylnaphthalene	0.16
				1-Methylnaphthalene	0.16
				2,7-Dimethylnaphthalene	0.16
				Acenaphthylene	0.16
				Acenaphthene	0.16
				Fluorene	0.16
				Phenanthrene	0.16
				Anthracene	0.16
				Fluoranthene	0.16
				Pyrene	0.16
				Retene	0.16
				Benzo(a)anthracene	0.16
				Chrysene	0.16
				Benzo(b)fluoranthene	0.16
				Benzo(k)fluoranthene	0.16
				Benzo(e)pyrene	0.16
				Benzo(a)pyrene	0.16
				Indeno(1,2,3-c,d)pyrene	0.16
				Benzo(g,h,i)perylene	0.16
				Dibenz(a,h)anthracene	0.16
↓(Surrogate mix)↓				Coronene	0.48*
OC-031214-8	0.25	100		p-Terphenyl-d14	0.50
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	0.71 @ 44.8ppm	100		Coronene	0.48*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-1

Name: 0.40µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	1.0	100.0	Methylene Chloride	Naphthalene	0.40
↑(PAH mix)↑			↓	2-Methylnaphthalene	0.40
				1-Methylnaphthalene	0.40
				2,7-Dimethylnaphthalene	0.40
				Acenaphthylene	0.40
				Acenaphthene	0.40
				Fluorene	0.40
				Phenanthrene	0.40
				Anthracene	0.40
				Fluoranthene	0.40
				Pyrene	0.40
				Retene	0.40
				Benzo(a)anthracene	0.40
				Chrysene	0.40
				Benzo(b)fluoranthene	0.40
				Benzo(k)fluoranthene	0.40
				Benzo(e)pyrene	0.40
				Benzo(a)pyrene	0.40
				Indeno(1,2,3-c,d)pyrene	0.40
				Benzo(g,h,i)perylene	0.40
				Dibenz(a,h)anthracene	0.40
↓(Surrogate mix)↓				Coronene	0.80*
OC-031214-8	0.48	100		p-Terphenyl-d14	0.96
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	0.90 @ 44.8ppm	100		Coronene	0.80*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-2

Name: 0.60µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	1.5	100.0	Methylene Chloride	Naphthalene	0.60
↑(PAH mix)↑			↓	2-Methylnaphthalene	0.60
				1-Methylnaphthalene	0.60
				2,7-Dimethylnaphthalene	0.60
				Acenaphthylene	0.60
				Acenaphthene	0.60
				Fluorene	0.60
				Phenanthrene	0.60
				Anthracene	0.60
				Fluoranthene	0.60
				Pyrene	0.60
				Retene	0.60
				Benzo(a)anthracene	0.60
				Chrysene	0.60
				Benzo(b)fluoranthene	0.60
				Benzo(k)fluoranthene	0.60
				Benzo(e)pyrene	0.60
				Benzo(a)pyrene	0.60
				Indeno(1,2,3-c,d)pyrene	0.60
				Benzo(g,h,i)perylene	0.60
				Dibenz(a,h)anthracene	0.60
↓(Surrogate mix)↓				Coronene	1.2*
OC-031214-8	0.70	100		p-Terphenyl-d14	1.4
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	1.34 @ 44.8ppm	100		Coronene	1.2*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-3

Name: 0.80µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	2.0	100.0	Methylene Chloride	Naphthalene	0.80
↑(PAH mix)↑			↓	2-Methylnaphthalene	0.80
				1-Methylnaphthalene	0.80
				2,7-Dimethylnaphthalene	0.80
				Acenaphthylene	0.80
				Acenaphthene	0.80
				Fluorene	0.80
				Phenanthrene	0.80
				Anthracene	0.80
				Fluoranthene	0.80
				Pyrene	0.80
				Retene	0.80
				Benzo(a)anthracene	0.80
				Chrysene	0.80
				Benzo(b)fluoranthene	0.80
				Benzo(k)fluoranthene	0.80
				Benzo(e)pyrene	0.80
				Benzo(a)pyrene	0.80
				Indeno(1,2,3-c,d)pyrene	0.80
				Benzo(g,h,i)perylene	0.80
				Dibenz(a,h)anthracene	0.80
↓(Surrogate mix)↓				Coronene	1.9*
OC-031214-8	0.85	100		p-Terphenyl-d14	1.7
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	2.46 @ 44.8ppm	100		Coronene	1.9*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-032614-3

Name: 1.0µg/mL **Second Source** PAH check standard solution.

Prep Date	Exp Date
3/26/14	3/26/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-032614-2	5.0	100.0	Methylene Chloride	Naphthalene	1.0
↑(PAH mix)↑			↓	2-Methylnaphthalene	1.0
				1-Methylnaphthalene	1.0
				2,7-Dimethylnaphthalene	1.0
				Acenaphthylene	1.0
				Acenaphthene	1.0
				Fluorene	1.0
				Phenanthrene	1.0
				Anthracene	1.0
				Fluoranthene	1.0
				Pyrene	1.0
				Retene	1.0
				Benzo(a)anthracene	1.0
				Chrysene	1.0
				Benzo(b)fluoranthene	1.0
				Benzo(k)fluoranthene	1.0
				Benzo(e)pyrene	1.0
				Benzo(a)pyrene	1.0
				Indeno(1,2,3-c,d)pyrene	1.0
				Benzo(g,h,i)perylene	1.0
				Dibenz(a,h)anthracene	1.0
↓(Surrogate mix)↓				Coronene	2.7*
OC-031214-8	1.0	100		p-Terphenyl-d14	2.0
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031814-3	0.44 @ 390ppm	100		Coronene	2.7*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-9

Name: 1.0µg/mL PAH spike solution.

Prep Date	Exp Date
3/13/14	3/13/15

Acetone lot# DK008

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	2.5	100.0	Acetone	22 component PAH mix	1.0
OC-031214-11	3.8mL X 44.8 µg/ml			Coronene	2.7*

*Coronene supplemented by 3.8mLs to achieve final concentration 2.7µg/mL

Analyst: DTR

Standard Number
OC-032714-1

Name: 1.0µg/mL PAH calibration standard solution **+ DFTTP**.(L5 & LoL4)

Prep Date	Exp Date
3/27/14	3/27/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	2.5	100.0	Methylene Chloride	Naphthalene	1.0
↑(PAH mix)↑			↓	2-Methylnaphthalene	1.0
				1-Methylnaphthalene	1.0
				2,7-Dimethylnaphthalene	1.0
				Acenaphthylene	1.0
				Acenaphthene	1.0
				Fluorene	1.0
				Phenanthrene	1.0
				Anthracene	1.0
				Fluoranthene	1.0
				Pyrene	1.0
				Retene	1.0
				Benzo(a)anthracene	1.0
				Chrysene	1.0
				Benzo(b)fluoranthene	1.0
				Benzo(k)fluoranthene	1.0
				Benzo(e)pyrene	1.0
				Benzo(a)pyrene	1.0
				Indeno(1,2,3-c,d)pyrene	1.0
				Benzo(g,h,i)perylene	1.0
				Dibenz(a,h)anthracene	1.0
↓(Surrogate mix)↓				Coronene	2.7*
OC-070214-1	1.00	100		p-Terphenyl-d14	2.00
OC-031214-9	1.0	100		Internal std mix	0.925
OC-090412-3	5.0	100		DFTPP TUNING STD.	12.5
OC-031214-11	3.8 @44.8ppm	100		Coronene	1.7

*Coronene fortified to elevate final concentration to 2.7µg/mL

Analyst: DTR

Standard Number
OC-031314-4

Name: 1.0µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	2.5	100.0	Methylene Chloride	Naphthalene	1.0
↑(PAH mix)↑			↓	2-Methylnaphthalene	1.0
				1-Methylnaphthalene	1.0
				2,7-Dimethylnaphthalene	1.0
				Acenaphthylene	1.0
				Acenaphthene	1.0
				Fluorene	1.0
				Phenanthrene	1.0
				Anthracene	1.0
				Fluoranthene	1.0
				Pyrene	1.0
				Retene	1.0
				Benzo(a)anthracene	1.0
				Chrysene	1.0
				Benzo(b)fluoranthene	1.0
				Benzo(k)fluoranthene	1.0
				Benzo(e)pyrene	1.0
				Benzo(a)pyrene	1.0
				Indeno(1,2,3-c,d)pyrene	1.0
				Benzo(g,h,i)perylene	1.0
				Dibenz(a,h)anthracene	1.0
↓(Surrogate mix)↓				Coronene	2.7*
OC-031214-8	1.0	100		p-Terphenyl-d14	2.0
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	3.8 @ 44.8ppm	100		Coronene	2.7*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-10

Name: EPA 8270 Base/Neutrals Surrogate Spike Mix @1.0µg/mL

Prep Date	Exp Date
3/13/14	3/13/15

Acetone lot#: DK008

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-8	1.0	200.0	Acetone	Nitrobenzene-d5	1.0
				P-Terphenyl-d14	1.0
				2-Fluorobiphenyl	1.0

"COA-OC-111413-2"

Analyst: DTR

Standard Number
OC-090214-11

Name: EPA 8270 Base/Neutrals Surrogate Spike Mix @1.0µg/mL

Prep Date	Exp Date
9/2/14	9/2/15

Acetone lot#: DK809

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-8	1.0	200.0	Acetone	Nitrobenzene-d5	1.0
				P-Terphenyl-d14	1.0
				2-Fluorobiphenyl	1.0

"COA-OC-111413-2"

Analyst: DTR

Standard Number
OC-031314-5

Name: 1.2µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	0.75	25.0	Methylene Chloride	Naphthalene	1.2
↑(PAH mix)↑			↓	2-Methylnaphthalene	1.2
				1-Methylnaphthalene	1.2
				2,7-Dimethylnaphthalene	1.2
				Acenaphthylene	1.2
				Acenaphthene	1.2
				Fluorene	1.2
				Phenanthrene	1.2
				Anthracene	1.2
				Fluoranthene	1.2
				Pyrene	1.2
				Retene	1.2
				Benzo(a)anthracene	1.2
				Chrysene	1.2
				Benzo(b)fluoranthene	1.2
				Benzo(k)fluoranthene	1.2
				Benzo(e)pyrene	1.2
				Benzo(a)pyrene	1.2
				Indeno(1,2,3-c,d)pyrene	1.2
				Benzo(g,h,i)perylene	1.2
				Dibenz(a,h)anthracene	1.2
↓(Surrogate mix)↓				Coronene	3.8*
OC-031214-8	0.40	100		p-Terphenyl-d14	3.2
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	1.45 @ 44.8ppm	100		Coronene	3.8*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-032614-4

Name: 2.0µg/mL **Second source** PAH calibration standard solution.

Prep Date	Exp Date
3/26/14	3/26/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-032614-2	10.0	100.0	Methylene Chloride	Naphthalene	2.0
↑(PAH mix)↑			↓	2-Methylnaphthalene	2.0
				1-Methylnaphthalene	2.0
				2,7-Dimethylnaphthalene	2.0
				Acenaphthylene	2.0
				Acenaphthene	2.0
				Fluorene	2.0
				Phenanthrene	2.0
				Anthracene	2.0
				Fluoranthene	2.0
				Pyrene	2.0
				Retene	2.0
				Benzo(a)anthracene	2.0
				Chrysene	2.0
				Benzo(b)fluoranthene	2.0
				Benzo(k)fluoranthene	2.0
				Benzo(e)pyrene	2.0
				Benzo(a)pyrene	2.0
				Indeno(1,2,3-c,d)pyrene	2.0
				Benzo(g,h,i)perylene	2.0
				Dibenz(a,h)anthracene	2.0
↓(Surrogate mix)↓				Coronene	5.4*
OC-031214-8	1.5	100		p-Terphenyl-d14	3.0
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031814-3	0.88 @ 390ppm	100		Coronene	5.4*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-6

Name: 2.0µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	5.0	100.0	Methylene Chloride	Naphthalene	2.0
↑(PAH mix)↑			↓	2-Methylnaphthalene	2.0
				1-Methylnaphthalene	2.0
				2,7-Dimethylnaphthalene	2.0
				Acenaphthylene	2.0
				Acenaphthene	2.0
				Fluorene	2.0
				Phenanthrene	2.0
				Anthracene	2.0
				Fluoranthene	2.0
				Pyrene	2.0
				Retene	2.0
				Benzo(a)anthracene	2.0
				Chrysene	2.0
				Benzo(b)fluoranthene	2.0
				Benzo(k)fluoranthene	2.0
				Benzo(e)pyrene	2.0
				Benzo(a)pyrene	2.0
				Indeno(1,2,3-c,d)pyrene	2.0
				Benzo(g,h,i)perylene	2.0
				Dibenz(a,h)anthracene	2.0
↓(Surrogate mix)↓				Coronene	5.4*
OC-031214-8	1.5	100		p-Terphenyl-d14	3.0
OC-031214-9	1.0	100		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	7.59 @ 44.8ppm	100		Coronene	5.4*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-7

Name: 4.0µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	2.5	25.0	Methylene Chloride	Naphthalene	4.0
↑(PAH mix)↑			↓	2-Methylnaphthalene	4.0
				1-Methylnaphthalene	4.0
				2,7-Dimethylnaphthalene	4.0
				Acenaphthylene	4.0
				Acenaphthene	4.0
				Fluorene	4.0
				Phenanthrene	4.0
				Anthracene	4.0
				Fluoranthene	4.0
				Pyrene	4.0
				Retene	4.0
				Benzo(a)anthracene	4.0
				Chrysene	4.0
				Benzo(b)fluoranthene	4.0
				Benzo(k)fluoranthene	4.0
				Benzo(e)pyrene	4.0
				Benzo(a)pyrene	4.0
				Indeno(1,2,3-c,d)pyrene	4.0
				Benzo(g,h,i)perylene	4.0
				Dibenz(a,h)anthracene	4.0
↓(Surrogate mix)↓				Coronene	7.6*
OC-031214-8	1.5	25		p-Terphenyl-d14	4.4
OC-031214-9	1.0	25		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	2.0 @ 44.8ppm	25		Coronene	7.6*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-031314-8

Name: 6.4µg/mL PAH calibration standard solution.

Prep Date	Exp Date
3/13/14	3/13/15

Methylene Chloride lot# DJ420

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031214-10	0.40	25.0	Methylene Chloride	Naphthalene	6.4
↑(PAH mix)↑			↓	2-Methylnaphthalene	6.4
				1-Methylnaphthalene	6.4
				2,7-Dimethylnaphthalene	6.4
				Acenaphthylene	6.4
				Acenaphthene	6.4
				Fluorene	6.4
				Phenanthrene	6.4
				Anthracene	6.4
				Fluoranthene	6.4
				Pyrene	6.4
				Retene	6.4
				Benzo(a)anthracene	6.4
				Chrysene	6.4
				Benzo(b)fluoranthene	6.4
				Benzo(k)fluoranthene	6.4
				Benzo(e)pyrene	6.4
				Benzo(a)pyrene	6.4
				Indeno(1,2,3-c,d)pyrene	6.4
				Benzo(g,h,i)perylene	6.4
				Dibenz(a,h)anthracene	6.4
↓(Surrogate mix)↓				Coronene	10.9*
OC-031214-8	0.75	25.0		p-Terphenyl-d14	6.0
OC-031214-9	0.25	25.0		Internal std mix	0.925
↑(8270D Istd.)↑					
OC-031214-11	2.5 @ 44.8ppm	25.0		Coronene	10.9*

* Coronene supplimented to help with increased LOD.

Analyst: DTR

Standard Number
OC-032614-2

Name: 20.0µg/mL PAH **Second Source** stock standard solution.

Prep Date	Exp Date
3/26/14	3/26/15

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-031814-1	1.0	100.0	Methylene Chloride	18 component PAH mix	≈20.0
OC-032614-1	2.05	100.0	Methylene Chloride	Benzo(e)pyrene	≈20.0
OC-031814-3	5.15	100.0	Methylene Chloride	Coronene	≈20.0
OC-032114-1	1.87	100.0	Methylene Chloride	2,7-Dimethylnaphthalene	≈20.0
OC-031814-4	1.58	100.0	Methylene Chloride	Retene	≈20.0

Methylene Chloride lot# DJ420

Analyst: DTR

Standard Number
OC-031214-10

Name: 40.0µg/mL PAH stock standard solution.

Prep Date	Exp Date
3/12/14	3/12/15

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-111413-1	2.0	100.0	Methylene Chloride	18 component PAH mix	≈40.0
OC-031214-2	5.0	100.0	Methylene Chloride	Benzo(e)pyrene	≈40.0
OC-031214-1	7.15	100.0	Methylene Chloride	Coronene	≈40.0
OC-031214-3	2.7	100.0	Methylene Chloride	2,7-Dimethylnaphthalene	≈40.0
OC-031214-4	5.27	100.0	Methylene Chloride	Retene	≈40.0

Methylene Chloride lot# DJ142

Analyst: DTR

Standard Number
OC-031214-9

Name: **92.5 ng/µL internal standard solution for method 8270D**

Prep Date	Exp Date
3/12/14	3/12/15

Methylene Chloride lot# DJ142

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ng/µL)
OC-111413-6	2.31	50	DCM	Internal std.	92.5

Analyst: DTR

Standard Number
OC-031214-8

Name: EPA 8270 Base/Neutrals Surrogate Spike Mix @200ppm

Prep Date	Exp Date
3/12/14	3/12/16

Stock Std No.	Aliquot (mL)	Volume (mL)	Solvent	Compound	Conc (ug/mL)
OC-111413-2	1.0	25.0	Methylene chloride	Nitrobenzene-d5	200
				P-Terphenyl-d14	200
				2-Fluorobiphenyl	200

Methylene Chloride lot# DI398

"COA-OC-060613-1"