

**Studies Using Aquatic Turtles (the Diamondback Terrapin and Snapping Turtle) to Assess the Potential Long-term Effects of Oiling of Nests During Early Embryonic Development**

**A Final Report Submitted to**

**The Coastal Response Research Center (CRRC)**

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## Abstract

Oil spills near coastal habitats in temperate and tropical regions have the potential to contaminate substrates used for nesting by aquatic turtles, presenting the question of the most protective strategies for spill response. Percolation of water accommodated fractions of oil through the overlying nest substrate is likely to expose eggs to oil derived compounds. The use of chemical dispersants, if employed in such habitats, results in a chemically enhanced water accommodated fraction (CEWAF) characterized by a different mix of compounds relative to non-dispersed water accommodated fraction (WAF), resulting in different exposure regimes for eggs depending upon the decision as to whether to employ dispersants. Thus the response strategies employed (to disperse or not) may determine the relative risks posed to developing turtle embryos in the spill area. We therefore designed a study to test the long term effects on embryonic and hatchling turtles following embryonic exposure to WAF and CEWAF under exposure conditions simulating those that may exist in natural nests.

Using snapping turtles as models, we constructed artificial nests in the laboratory in which eggs were exposed to two concentrations each (0.5 and 10 g weathered Arabian light crude oil per L water; “Low” and “High,” respectively) of WAF and CEWAF, as well as dispersant-only and control solutions. The dispersant Corexit 9500 was employed to create the CEWAF and dispersant-only solutions. Eggs were placed in replicate nests containing a substrate of sand and gravel at a depth of 3 cm. Solutions were percolated through a column containing 18 cm of the nest substrate prior to addition to the nests to mimic the composition of solutions that would contact eggs when buried in a nest. Nests also contained an absorptive substrate (Amberlite XAD<sup>TM</sup> resin) near the eggs for determination of PAH profiles. Six days after addition of solutions, two eggs and one XAD substrate were removed for chemical analyses. At the time of hatching, the remaining XAD substrates were removed to examine changes in PAH profiles over time. Solutions, eggs, and XAD were analyzed for 52 PAH congeners. At hatching, a subset of individuals was assessed for DNA strand breakage in liver tissue. Hatchlings were raised for 13 months during which time metabolic rate, behaviors, growth, and body composition were measured, following which animals were dissected to determine sex ratio and hepatosomatic index.

Prior to percolation through the substrate column, total PAH (“tPAH”) in WAF solutions were similar (High, 43; Low 67 ppm). Following percolation, tPAH was also similar in physically-dispersed fractions (High, 14; Low 24 ppm). Addition of dispersant (CEWAF solutions) increased tPAH prior to percolation in the High treatment (300 ppm) relative to Low (13 ppm), but percolation resulted in nearly equal concentrations in both treatments (High, 30; Low, 22 ppm) due to physical trapping of dispersed oil by the nest substrate. In both WAF and CEWAF treatments, percolation reduced low molecular weight (MW) compounds such that embryos were exposed to primarily mid- to high MW compounds. Total PAH in eggs differed 15-fold between the CEWAF High and WAF High treatments (560 and 36 ppb respectively), the former characterized by higher MW compounds than the latter. Alteration of PAH profiles during percolation to egg depth demonstrates that the composition of PAHs in water that arrives on a beach following a spill do not reflect the suite of compounds to which embryos are exposed. While we observed no biological

effects of exposure, our results suggest that experiments with subsurface organisms should be designed to account for compositional changes that occur as the solutions percolate through the substrate.

**Keywords:** snapping turtle, Arabian light crude oil, WAF, CEWAF, Corexit 9500

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## 1.0 Introduction

Oil spills near coastal habitats in temperate and tropical regions can contaminate areas used by aquatic turtles for nesting, especially during storm events or unusually high tides which may transport contaminants to the high beach nesting zone (Milton et al., 2003). Following an oil spill, wave and wind action entrains (disperses) or dissolves a portion of the floating slick into the water column (physical dispersion). This fraction is chemically distinct from the overlying slick, typically being enriched in low molecular weight (MW) polycyclic aromatic hydrocarbons (PAH) relative to the parent oil. Arrival of the oil-contaminated parcel of water on beaches comprised of porous substrates provides potential for the oil slick or underlying water-entrained fraction to percolate through the substrate, exposing subsurface organisms to potentially toxic compounds. Furthermore, an oil-slicked beach may allow for continued percolation of water-entrained fractions after rain or further storm/high tide events.

The use of chemical dispersants to treat oil slicks is intended to increase entrainment of droplets and dissolution of some compounds from the slick into the water column. This treated fraction containing a greater proportion of mid to high MW compounds and oil droplets differs compared to that produced when dispersants are not added. Dispersants are typically employed to treat oil slicks in marine open water systems in which constituents of the treated oil slick become highly diluted prior to entering shallow or coastal systems. However, consideration of use of dispersants in near shore habitats raises concerns regarding effects on shallow benthic habitats and beaches (NRC, 2005). The addition of a dispersant creates a sub-surface plume of the dispersant itself, as well as PAHs in the dissolved, colloidal, or particulate phase (fine oil droplets) which rapidly migrates to a greater depth than the plume of PAHs produced from natural dispersion. Thus following addition of dispersant to a slick, organisms inhabiting benthic habitats may be at risk of exposure at depths at which they would not otherwise be exposed. Furthermore, for subsurface, beach dwelling organisms, the action of dispersants to produce fine droplets of oil provides a possible route for delivery of the parent oil itself to greater depth than may occur with non-dispersed oil. As a result, percolation of dispersant-treated oil through beach substrates may expose buried organisms (such as turtle eggs) to a greater total concentration of oil-derived compounds than the naturally-dispersed oil plume or the residual slick, as well as exposing them to a suite of compounds different from those present in a naturally-dispersed fraction (including the dispersant itself). Relative to the parent oil or physically-dispersed fraction, chemically-dispersed oil may thus induce different types or magnitudes of effects on subsurface organisms than those induced by exposure to constituents of the naturally-dispersed plume or the residual slick.

Sea turtles typically nest in sandy areas of high beaches, and coastal oil spills may expose nests to PAHs or PAHs/dispersant-derived compounds that percolate through the substrate. Shipping accidents and associated oil spills frequently occur during times of storms and heavy surf (NOAA, 2003), allowing transport of oil to the high beach zone, where turtle nests are typically constructed. While the initial contamination event may be somewhat brief (e.g., the spill itself will disperse relatively rapidly naturally or through

enhancement by dispersants), residual oil-derived compounds entrained in the matrix of the nest substrates may chronically expose turtle eggs to potentially toxic compounds.

Saturation of turtle nests with oil is known to induce mortality in embryos. For example, following a spill that released 336,000 gallons of fuel oil in Tampa Bay, Florida in 1993, producing a slick covering 23 km of sandy beach, loggerhead sea turtle nests heavily saturated with the oil produced survival rates of ~ 5 %, compared to a normal rate of 50 to 90 % (Yender and Mearns, 2003). Whether effects of direct exposure to oil reflect toxic effects on the embryos or simply suffocation due to reduced gas exchange across the egg shells, however, remains unknown (e.g., Phillott and Parmenter, 2001; Yender and Mearns, 2003).

Despite observations from such natural experiments, controlled dose-response studies of the severity and possible residual effects of compounds present in oil and dispersant-treated oil fractions that percolate to the eggs have not been conducted. The lack of experimental data from studies in which exposures were controlled and conducted under conditions similar to those in natural sites precludes assessing how sensitive turtles may be to oil-derived contaminants, as well as how effects would be expressed physiologically. This information gap critically constrains spill responders and regulators in determining the proper remediation strategy (e.g., whether to use dispersants in near shore systems) to employ following oil spills to best protect turtle nests.

The threatened or endangered status of sea turtles worldwide precludes large scale toxicity studies on them for both ecological and ethical reasons. Use of surrogate species that are abundant and share traits with sea turtles provides an alternative to manipulating at-risk species while serving as models from which experimental results can be applied to other species. We conducted a study in which common snapping turtles (*Chelydra serpentina*) were used as surrogates for sea turtles to assess chronic effects of exposure of embryos in the nest to naturally- and chemically-dispersed oil. Experiments using diamondback terrapins (*Malaclemys terrapin*) were also proposed, but low survival in initial studies led us to subsequently focus only on snapping turtles. To standardize the oil-fractions, we prepared oil fractions based on the CROSERF protocols (e.g. Singer et al., 2000). Eggs were exposed in artificial nests to water-accommodated fractions (WAF) derived from weathered Arabian light crude oil and to the chemically-enhanced water accommodated fraction (CEWAF) produced by application of a commonly used dispersant (Corexit 9500<sup>TM</sup>) to the crude oil (Burns et al., 1999). We quantified the individual PAHs in the exposure regime, accumulation of PAHs by the embryos, and numerous physiological and behavioral traits in hatchlings over a period of 13 months following hatching.

## 2.0 Objectives

Our overall objective was to quantify the potential developmental constraints experienced by an estuarine and a fresh-/brackish water turtle species as a result of exposure to physically or chemically dispersed crude oil (WAF and CEWAF) *in ovo* due to contamination of nesting beaches. The overall hypothesis was that a single nest-

contamination event consisting of exposure to WAF or CEWAF occurring early in the embryonic period would influence embryonic development and molecular, cellular, bioenergetic, and behavioral traits of hatchlings throughout the first one year of the juvenile period.

Our objectives and hypotheses followed several lines of justification. First, permeability of turtle eggs to gases (and potentially some fluids) can provide routes of entry for contaminants such as volatile polycyclic aromatic hydrocarbons (PAHs) and non-volatile, fluid-borne contaminants via processes normally associated with respiratory gas exchange (i.e. soluble PAHs and dispersant components). Second, polycyclic aromatic hydrocarbons (PAHs) and other oil spill-related contaminants are known to illicit sub-cellular responses in vertebrates and invertebrates. Effects include induction of metabolic pathways (e.g. cytochrome P450; Kirchin and Winston, 1992; Ertl and Winston, 1998; Yawetz et al., 1998), DNA damage adducts and strand breakage (Kurelech et al., 1992; Sole et al., 1996; Mitchelmore et al., 1998; Taban et al., 2004), and anomalous endocrinological processes (Navas and Segner, 2000) such as production of vitellogenin; (an egg precursor compound typically found only in females) by males (Johnson et al., 1997; Livingstone et al., 2000). Thus exposure to contaminants during the embryonic period may bring about cumulative developmental irregularities in embryos and juveniles due to sub-cellular damage or induced repair mechanisms. Third, translation of cellular and sub-cellular irregularities into overall metabolic anomalies can have overall influences on bioenergetic processes regulating survival, energy storage, and growth functions of individuals during embryonic development and juvenile growth periods (Rowe et al., 1998, Rowe et al., 2001a, Rowe et al., 2001b, Steyermark, 2002). For example, overall narcotic effects of some PAHs (Newman and Unger, 2003) would be expected to modify rates of conversion of energy stores to somatic tissues, influencing rates of differentiation and growth. Finally, irregularities in developmental and physiological traits that ultimately influence bioenergetics and behaviors (e.g. abnormal neurological and morphological development) can translate into overall effects on individual fitness via impaired growth rates, predator avoidance, and foraging behaviors (e.g. Raimondo et al., 1998; Newman and Unger, 2003). Thus, via numerous routes originating in sub cellular modifications during embryonic development, contamination of turtle nests by WAF and CEWAF may have overall significance to fitness of individuals long after the initial exposure event.

### **3.0 Methods**

#### **3.1. Hypotheses and design:**

Our original proposal called for exposure of eggs of two species to crude oil, WAF + the residual surface oil slick, and CEWAF + the residual slick. Following recommendation from CRRCs Scientific Advisory Panel and proposal reviewers, we modified the protocol to examine only WAF and CEWAF fractions (not including residual slick) prepared as standard CROSERF exposures. The protocol that we initially employed was designed to identify effects of exposure to WAF (2 concentrations), CEWAF (2 concentrations,

dispersant = Corexit 9500), and water (control) on terrapins and snapping turtles. However, poor hatching success in all nests due to fungal infection of eggs required conducting a second experiment be conducted, adopting procedural changes and focusing only on snapping turtles. This report addresses only the second study due to the unreliable nature of the data from the first. We chose to use snapping turtles only due to limited funds available to support work with two species. Snapping turtles were selected as the best model for statistical and biological reasons. First, the large clutch size produced by snapping turtles provides for representation of eggs from each female in each replicate nest, avoiding confounding of results due to female-specific influences on development of the eggs. Terrapin egg clutches are typically much smaller (terrapins ~ 10 -16 eggs, snapping turtles ~ 20 – 50 eggs). Second, snapping turtles produce spherical eggs similar to sea turtles, presumably providing a more similar surface area to volume ratio than the oblong terrapin eggs. As exposure would require passage of compounds through the egg from the substrate, we opted for the species having the most similar shape and size to sea turtles.

We tested the following specific hypotheses:

**H1:** A single exposure of WAF or CEWAF occurring shortly after egg laying results in uptake of PAHs by eggs from the nest substrate.

**H2:** Exposure in the nest alters developmental patterns such that hatching success is reduced compared to control nests.

**H3:** Individuals that succeed in hatching from exposed nests will be smaller at hatching and display a higher frequency of morphological abnormalities compared to individuals from control nests.

**H4:** Exposure will induce DNA fragmentation (single strand breaks).

**H5:** Exposure to WAF and CEWAF will produce a greater frequency of females or intersex individuals than control nests, despite incubation at a male-producing temperature.

**H6:** Embryonic exposure to oil will have physiological and behavioral ramifications over the long-term post hatching leading to reduced growth rates and increased risks of predation-induced mortality.

**H7:** In the above responses, the relative severity of response will follow the pattern: CEWAF High > CEWAF Low > WAF High > WAF Low > Dispersant only > Water-only

Treatments consisted of two concentrations each of WAF and CEWAF, a filtered (0.2 µm) water-only control, and control water containing only Corexit 9500. An additive (J. Clark, pers. com.) was added as required in order that the dispersant would be effective in freshwater. Nominal concentrations of WAF and CEWAF were 0.5 (WAF and CEWAF "Low") and 10 g oil/L (WAF and CEWAF "High"). Mixing and ratios of oil to dispersant followed the CROSERF standardized test protocol (Singer et al., 2000). Briefly, weathered Arabian light crude oil and where appropriate a 1:20 (dispersant:oil) volume of Corexit 9500 (with additive), were added to glass (10L) aspirator bottles and the containers sealed. Mixing (approx. 25% vortex depth using stir bars) was carried out

for 24 hours in the dark at the exposure experiment temperature ( $25 \pm 1$  °C). After 24 hours the mixing was stopped and the oil/water/dispersant mixtures were allowed to separate for 6 hours. After 6 hours the exposure test solutions were carefully drained from the bottom of the vessels, leaving the 'slick' behind. These stock solutions were sub-sampled for PAH chemical analyses. The exposure solutions were then prepared by percolating the initial stock solutions through 18 cm of sand/gravel. (see below). Sub-samples of these exposure solutions following percolation were also analyzed for PAHs.

### 3.2 Biological and chemical exposure protocol:

We collected eggs in May, 2006 from a commercial turtle farm in Louisiana and transported them to our laboratory in MD. After a two week holding period, eggs were placed in shallow nests (3 cm) deep, a technique that has provided good hatching success in the past (Rowe, pers. obs.). Eggs from 12 clutches were distributed evenly among all nests such that eggs from each clutch were represented in all treatments. Half of the nests (2 per treatment) contained 2 PAH binding substrates (Amberlite XAD<sup>TM</sup> resin) in paper tea-bag material to assess PAH congener patterns in solutions when present in the vicinity of the buried eggs. Two additional eggs were placed in 2 nests per treatment (with the exception of WAF Low and CEWAF Low due to analytical cost constraints) for analysis of accumulation of PAHs by embryos over the first six days following addition of solutions, when they had presumably been exposed to the highest concentrations of PAHs. . Dosing solutions (1 L per nest) were first passed through an 18 cm long column containing nest substrate (3 parts rinsed medium sand to 1 part pea gravel) prior to addition of 1 L of the solution to each nest. This protocol was employed so that the solutions delivered to the eggs would reflect alterations to concentrations or composition that would occur as the solutions percolate to eggs in a nest. Samples from stock solutions prior to and following percolation through the sand column were collected from two replicate nests per treatment and analyzed as described below.

Following dosing of the nests, they were housed in a dark, 25 ° C constant-temperature room until hatching. The incubation temperature was chosen as it typically produces predominately male hatchlings (Yntema, 1976), thus providing for assessment of potential sex reversal following embryonic exposure to PAHs. Six days after dosing, the overlying substrate was removed and replaced with moist, autoclaved sphagnum moss, and one XAD substrate and the 2 additional eggs were removed for chemical analyses. The remaining XAD substrates were removed at the onset of hatching.

At 2 to 3 day intervals, the overlying moss was removed from each nest and eggs inspected for viability (normal coloration and hydration) and indications of imminent hatching (sloughing of outer shell layers). Non-viable eggs were noted and discarded. Moss was replaced at approximately 2 week intervals. As eggs pipped (shell began to split) they were removed to individual 0.5 L polyethylene containers containing moist sphagnum until hatching was complete.

As PAHs are known to illicit DNA strand breakage (Kurelech et al., 1992; Sole et al., 1996; Mitchelmore et al., 1998; Taban et al., 2004), we assessed DNA damage on a subset of hatchlings. One hatchling from each of 3 nests per treatment was sacrificed one

week after hatching for assessment of DNA damage in a portion of liver tissue. DNA damage was assessed by the comet assay (see Mitchelmore and Chipman, 1998a; Livingstone et al., 2000). Briefly, livers were washed with ice cold HEPES HBSS solution (pH 7.6). Tissues were minced with a Teflon-coated razor blade and passed through a 70um filter. 10ul of the filtrate containing cells was mixed with 100ul of 0.75% low-melting point agarose (in HEPES/HBSS buffer) and placed on a pre-coated slide of dried 1% NMA (in PBS) agarose. Triplicate slides from each turtle liver were prepared. The rest of the solution was used to assess cell viability using the trypan blue exclusion method. After solidification slides were coated with a further 100ul of 0.75% LMPA, allowed to solidify and then placed in ice-cold lysing solution for 1 hour. Following lysing, slides were rinsed and placed in an electrophoresis chamber containing unwinding buffer for 10 minutes. Slides were then electrophoresed for 10 minutes at 25V, 300mA. Slides were rinsed three times with neutralization buffer, stained with 20µg/µl Ethidium bromide and viewed under a fluorescent scope and quantified using KOMET 5.0 image analysis. Results are reported as tail % DNA, tail length and tail moment.

The number of hatchlings produced in each nest was counted to determine embryonic survival. Following hatching and resorption of residual yolk, animals were held individually in a 20 - 24 ° C laboratory in 1 L polyethylene containers filled to approximately 5 cm depth with well water for the duration of the study (13 months post-hatching). Survival was monitored daily and growth was measured monthly as carapace length (CL; straight line distance from the anterior- to posterior-most carapacial scutes) and wet weight (WW). As growth trajectories did not differ, only final sizes are presented and discussed here. Hatchlings were fed commercial turtle food (Fluker Laboratories, Baton Rouge, LA) *ad libitum* every 2 days. Water in the holding containers was changed weekly.

At the end of the experiment, juveniles were sacrificed by inhalation of an anesthetic (isoflurane) followed by decapitation. Animals were dissected for sex identification based upon gonadal morphology (Yntema, 1976), and the livers were removed for calculation of hepatosomatic indices (HSI; ratio of liver WW to carcass WW).

We quantified bioenergetic properties of hatchlings (metabolism, energy storage) due to the overall narcotic effects induced by some PAHs (Newman and Unger, 2003) which can potentially reduce metabolism, growth, and storage of energy as lipids. Metabolic rates of a subset of four to five hatchlings from each replicate nest were measured as O<sub>2</sub> consumption at rest in post-absorptive individuals by microrespirometry (MicroOxymax<sup>TM</sup>, Columbus Instruments, Columbus, OH). We chose individuals from clutches that were represented in all nests in order to reduce variability due to maternal effects on bioenergetics (Steyermark and Spotila 2001). Hatchlings were acclimatized to 20 ° C for 48 hrs prior measurement of metabolic rates, during which time they were not fed. Metabolic rates were measured on the same individuals in each of 3 trials (6, 10, and 11 months post hatching). During measurement of oxygen consumption, hatchlings were held individually in 500 ml jars containing a damp, unbleached paper towel in an unlit, 20 ° C incubator. Eight to 10 measurements were made at ~ 3 hr intervals for 24 to 30 hr, following which individuals were weighed and returned to the holding laboratory.

Energy storage (total body lipid content) was measured non-destructively on the same subset of hatchlings used for measurement of oxygen consumption using total body electrical conductivity (TOBEC Model SA-2, EM SCAN, Springfield, IL; see Walsberg, 1988; Kintner and Johnson, 1998) at 6, 9, and 11 months post hatching. Using carcasses from a prior study with the same species, we derived the following algorithm as a best fit relationship between electrical conductivity and body composition:

**Equation 1:** *% wet weight comprised of lipid* =  $-9.975 + 0.216 * E - 0.001 * E^2$ ,

where E = electrical conductivity parameter produced by the instrument ( $R^2 = 0.974$ ,  $P < 0.001$ ). These values were then corrected for body water content for comparison with other studies in which body composition are typically reported based upon percentage of lipid per unit dry body weight. Note that values that we calculated using this technique were comparable to those reported for hatchling and juvenile turtles for which lipids were quantified using destructive methods (Rowe et al., 1995; Nagle et al., 1998).

Behavioral assays: Potential irregularities in neurological development were measured using two behavioral assays on hatchlings. On two occasions (8 and 10 months post hatching) we assessed the response of juveniles to a visual stimulus using a technique adapted from Winkelman (1996), in which a simulated avian predator was used to cast a shadow on or near the test animal. Six individuals per treatment, consisting of 1 or 2 per replicate nest were randomly selected and each was placed individually in a 38 L aquarium filled to 4 cm water depth and lighted by a unidirectional light source overhead. A bird-shaped silhouette was passed through the light path in a standardized fashion from behind a blind to cast a shadow on the turtle. Three assays were run per individual on each occasion. Response behaviors were scored based upon relative activity level: no response, head twitch, full body twitch, or active swimming. Each activity was scored as either having occurred (assigned a score of 0) or not occurred (1) for each assay on each individual and the mean of the three scores per response calculated for each behavior. On one occasion we also assessed the righting response of individuals (the time required for the individual to return itself from a ventrally-upright to normal position; e.g. Steyermark and Spotila, 2001). We placed individuals on their back on a packed sand substrate and monitored times to righting. Measurements were made under indirect light, and observations were made from behind a blind.

### 3.3 Chemical analysis protocol:

Extracts were concentrated in hexane and eluted through a baked (550° C, 4 hr minimum) silica gel column consisting of approximately 3 g of silica gel packed into a 10 ml pipette and topped with sodium sulfate. Columns were pre-eluted with 20 ml 80:20 hexane:dichloromethane and eluted with 20 ml of the same mixture. Ultra high purity nitrogen was used to push samples through the column, which removed polar compounds prior to purified extracts being analyzed by GCMS. PAHs were identified and quantified using a capillary gas chromatograph (Hewlett Packard 5890) and a mass spectrometer

(Hewlett Packard 5970A) operated in selected ion monitoring mode with helium as the carrier gas. PAHs were identified by retention time relative to that of mixed standards (Supelco Separation Technologies, Bellefonte, PA). Identification was confirmed by the abundance of a secondary mass fragment relative to the molecular ion.

Surrogate PAH standards (perdeuterated PAHs  $d_8$ -naphthalene,  $d_{10}$ -fluorene,  $d_{10}$ -fluoranthene, and  $d_{12}$ -perylene) were added to each sample prior to extraction to quantify overall method performance. Internal standards ( $d_{10}$ -acenaphthalene,  $d_{10}$ -phenanthrene,  $d_{12}$ -benzo[a]anthracene,  $d_{12}$ -benzo[a]pyrene,  $d_{12}$ -benzo[g,h,i]perylene) were added to samples and calibration standards prior to analysis and the instrument was calibrated by comparing the response of each analyte to that of the respective internal standard. Surrogate recoveries ranged from 64 (naphthalene) to 109 % (fluorene) and did not vary systematically across treatments.

**Table 1. Specific congeners, molecular weights (MW) and Log Kow of PAHs quantified.**

<b>Congener</b>	<b>MW</b>	<b>Log Kow</b>	<b>Congener</b>	<b>MW</b>	<b>Log Kow</b>
1. Naphthalene	128.00	3.30	27. 1-Methylphenanthrene	192.00	5.14
2. 2-Methylnaphthalene	142.00	3.86	28. 9-Methylanthracene	192.00	5.07
3. Azulene	128.00	3.20	29. 9,10-Dimethylanthracene	206.00	5.25
4. 1-Methylnaphthalene	142.00	3.86	30. Fluoranthene	202.00	5.22
5. Biphenyl	154.00	3.97	31. Pyrene	202.00	5.18
6. 2,7-Dimethylnaphthalene	156.23	4.31	32. 3,6-Dimethylphenanthrene	206.00	5.44
7. 1,3-Dimethylnaphthalene	156.23	4.42	33. Benzo[a]fluorene	216.00	5.32
8. 1,6-Dimethylnaphthalene	156.23	4.44	34. Retene	234.34	6.35
9. 1,4-Dimethylnaphthalene	156.23	4.37	35. Benzo[b]fluorene	216.00	5.77
10. 1,5-Dimethylnaphthalene	155.23	4.26	36. Cyclopenta[c,d]pyrene	222.29	5.70
11. Acenaphthylene	152.00	3.90	37. Benz[a]anthracene	228.00	5.91
12. 1,2-Dimethylnaphthalene	156.23	4.31	38. Chrysene+Triphenylene	228.00	5.79
13. 1,8-Dimethylnaphthalene	156.23	4.26	39. Naphacene	228.00	5.84

*(continued)*

Table 1 (continued)

14. Acenaphthene	154.00	3.97	40. 4-Methylchrysene	242.31	6.07
15. 2,3,5-Trimethylnaphthalene	170.25	4.81	41. Benzo[b]fluoranthene	252.00	6.44
16. Fluorene	166.00	4.18	42. Benzo[k]fluoranthene	252.00	6.44
17. 1-Methylfluorene	180.00	4.97	43. Dimethylbenz[a]anthracene	256.00	5.80
18. Dibenzothiophene	184.25	4.38	44. Benzo[e]pyrene	252.00	5.98
19. Phenanthrene	178.00	4.57	45. Benzo[a]pyrene	252.00	6.44
20. Anthracene	178.00	4.54	46. Perylene	252.00	6.44
21. 2-Methyldibenzothiophene	192.00	4.71	47. 3-Methylchloanthrene	268.00	7.11
22. 4-Methyldibenzothiophene	198.28	4.71	48. I0eno[1,2,3-c,d]pyrene	276.00	7.04
23. 2-Methylphenanthrene	192.00	4.86	49. Dibenz[a,h+ac]anthracene	278.00	7.19
24. 2-Methylanthracene	192.00	5.00	50. Benzo[g,h,i]perylene	276.00	7.10
25. 4,5-Methylenephenanthrene	190.00	4.60	51. Anthanthrene	276.00	7.04
26. 1-Methylanthracene	192.00	4.89	52. Corenene	300.00	7.64

### 3.4 Statistical analyses:

Survival, growth, righting response, and DNA damage were analyzed by single factor analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. For responses that may vary allometrically with animal size (metabolic rate, body composition) we employed analysis of covariance (ANCOVA) using wet weight as the covariate. To assess predator avoidance, the average number of each response over the three replicate trials on each individual were calculated and treatments compared using multiway analysis of variance (MANOVA). Statistical significance was judged based upon a Type I error rate of  $\alpha = 0.05$ . Prior to statistical analyses, data were tested to verify that assumptions of the models were met and transformed if necessary. Data for PAH concentrations in solutions, eggs, and XAD substrates were not analyzed statistically because, due to costs associated with chemical analyses, only 2 samples per treatment were measured.

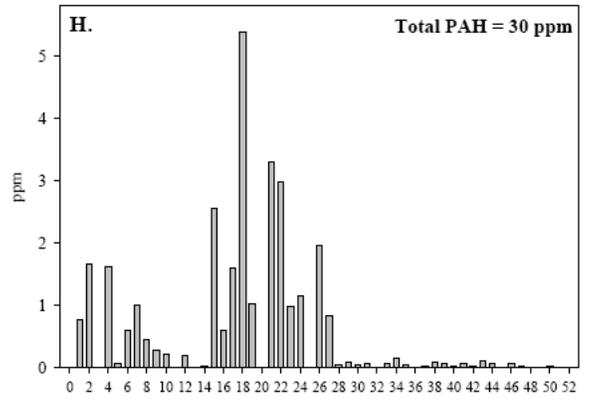
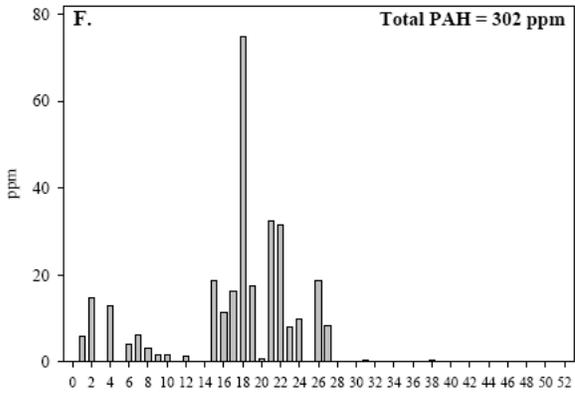
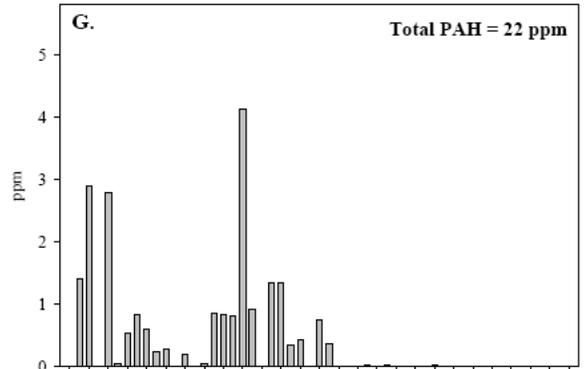
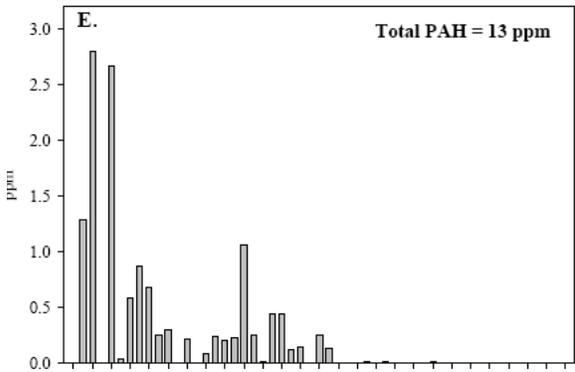
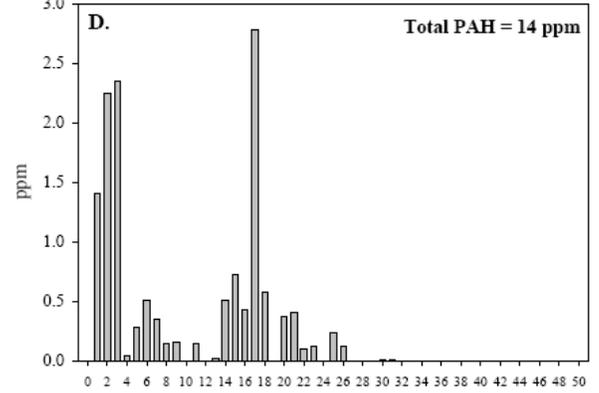
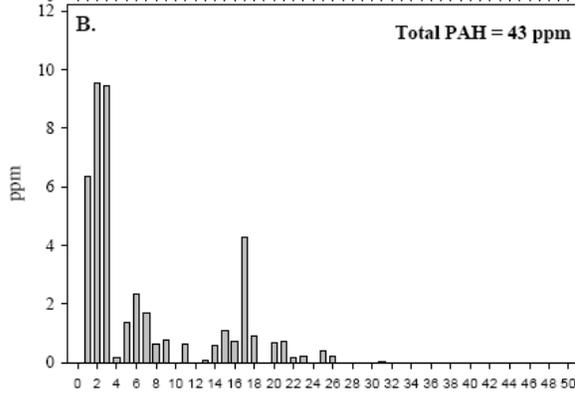
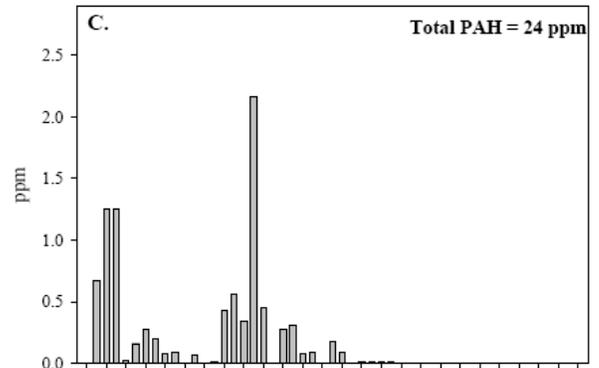
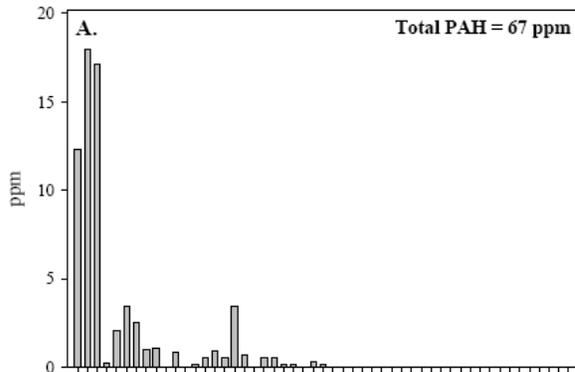
## 4.0 Results

Total PAH concentrations in stock solutions prior to and following passage through the sand column and the patterns of specific congener constituents are presented in Table 2 and Figure 1. Concentrations of tPAH in the Low and High WAF stocks were similar between doses prior to (67 and 43 ppm) and after percolation (24 and 14 ppm). In both of these Low and High WAF stocks, tPAHs were reduced (2.8 and 3.1-fold respectively) by percolation. This reduction in tPAHs following percolation of the WAF stocks was mainly due to a reduction in concentrations of low molecular weight (MW) PAHs. CEWAF Low and High stocks differed considerably prior to percolation between the two doses used (Low = 13 ppm; High = 302 ppm; not analyzed statistically) and both doses were enriched in higher MW compounds relative to WAF stocks prepared with the same amount of oil.

Percolation substantially reduced (over 10-fold) tPAH concentration in CEWAF High (prior to percolation = 302 ppm; following percolation = 30 ppm). Following percolation through the sand substrate, a greater proportion of PAHs ranging from approximately log  $K_{OW}$  of 4.5 to 5.5 (i.e. phenanthrene through pyrene; Table 1) were retained in the sand treated with CEWAF than with WAF (Fig. 1). Total PAH concentrations were similar between CEWAF and WAF after percolation through the sand (range 14-30 ppm for the four treatments; Fig. 1), although in CEWAF there was a greater proportional removal of the "mid  $K_{OW}$ " compounds remaining relative to WAF.

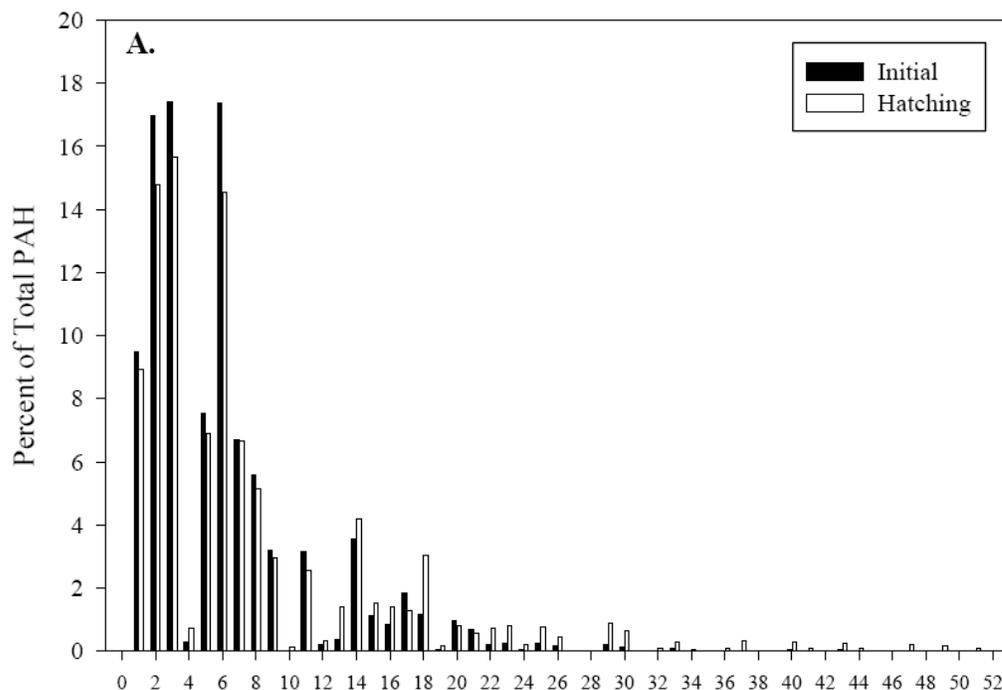
**Table 2. Concentrations of total PAHs in stocks prior to and following percolation through sand column and in contents of eggs removed from nests 6 d following addition of solutions to nests. Stock concentrations in ppm, egg concentrations in ppb. DNM = did not measure.**

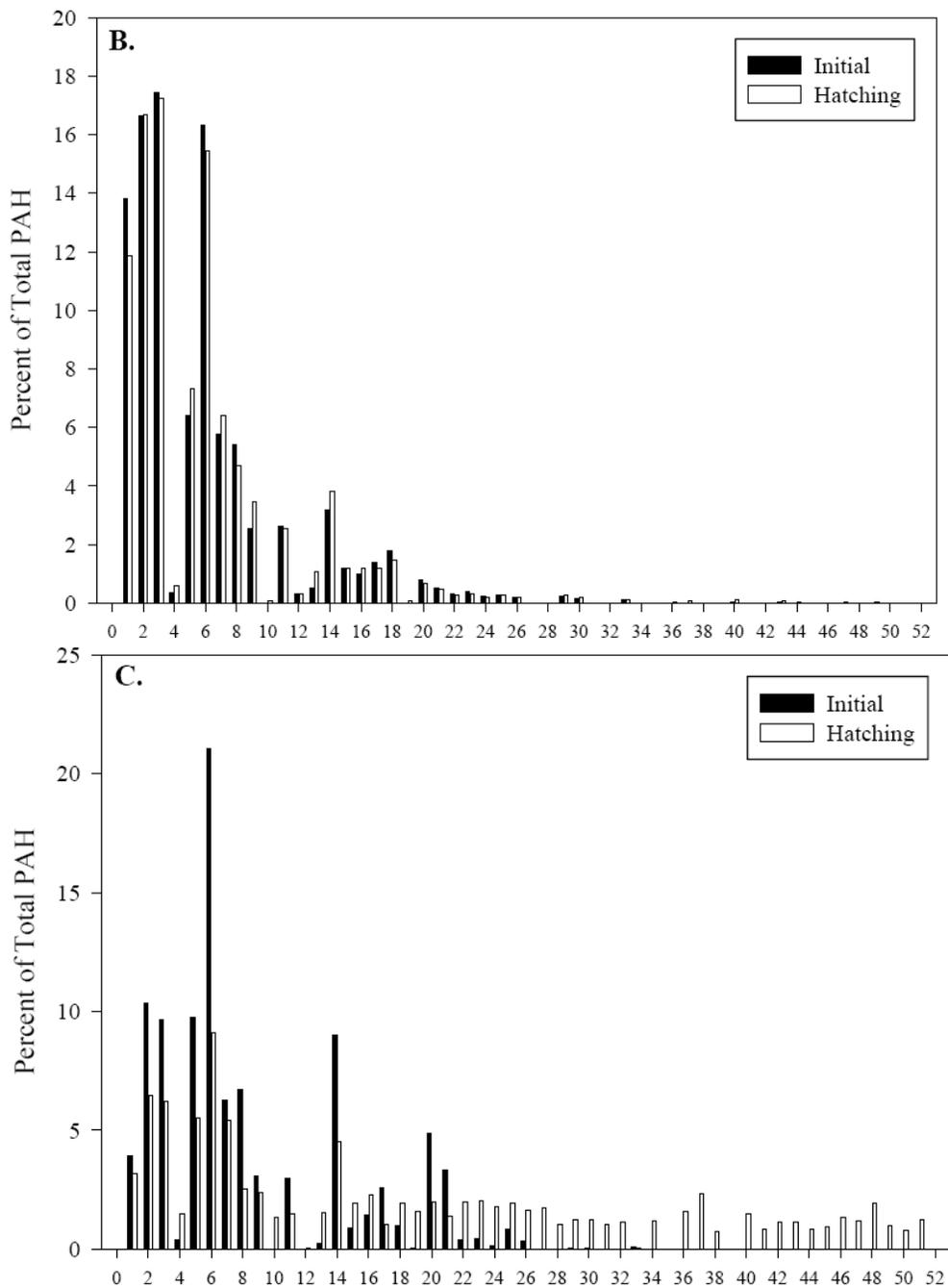
Treatment	Stock, prior to percolation (ppm)	Stock, following percolation (ppm)	Egg contents (ppb)
Control	0	0	4.8
Dispersant	1	DNM	5.9
WAF Low	67	24	DNM
WAF High	43	14	36.4
CEWAF Low	13	22	DNM
CEWAF High	302	30	563



**Figure 1. Congener patterns and total PAH (ppm) in WAF and CEWAF solutions, prior to and after percolation through the substrate. A. WAF low prior; B. WAF High prior; C. WAF low after; D. WAF High after; E. CEWAF Low prior; F. CEWAF High prior; G. CEWAF Low after; H. CEWAF High after. Note the differences in scales. Numbers on the horizontal axis correspond to designations in Table 1.**

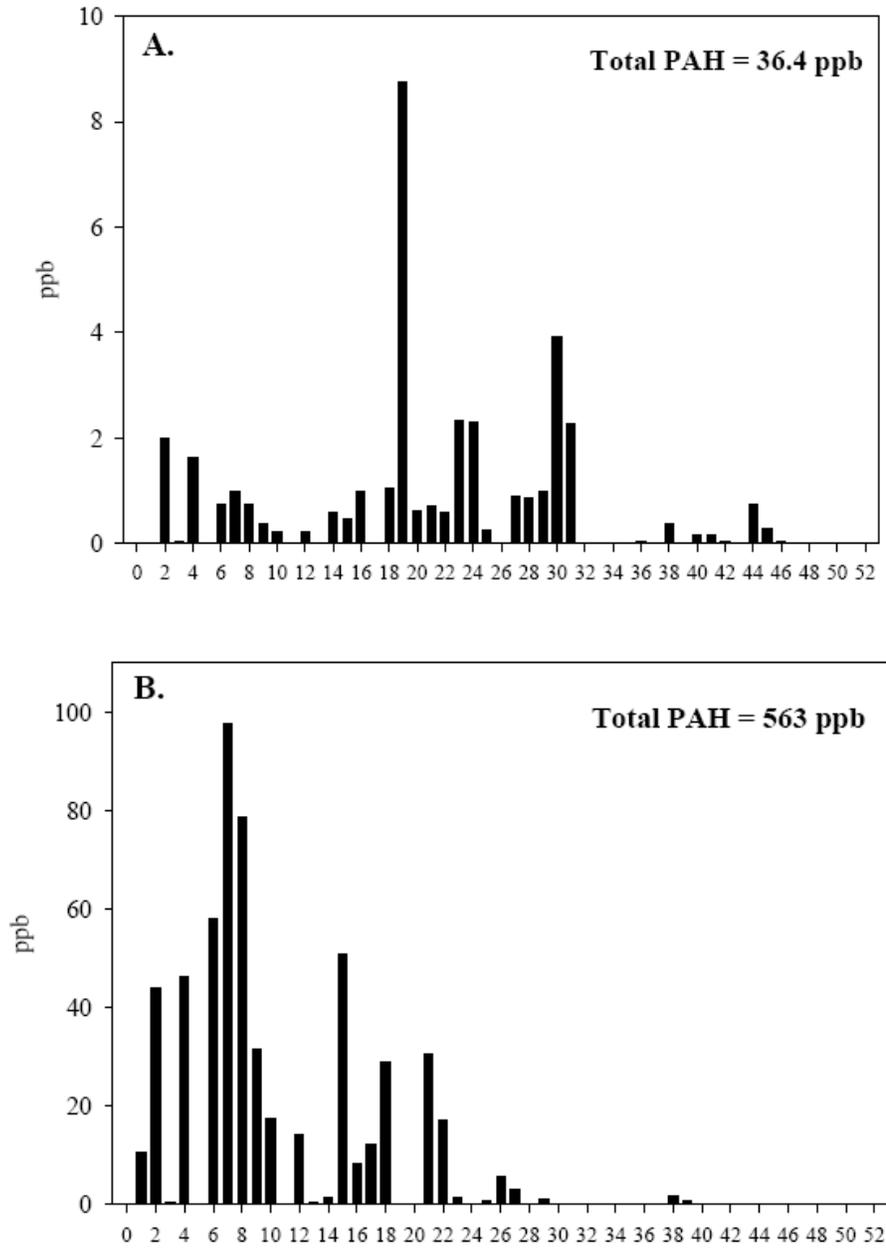
XAD substrates were used only to compare congener patterns rather than absolute levels of exposure among treatments due to potential differences in absorption of solutions among nests. Analysis of PAH in XAD substrates buried near the eggs illustrated that over the course of the study, the PAH profiles in CEWAF treatments shifted somewhat toward mid-molecular weight congeners, together with a depletion of low molecular weight compounds (e.g anthracene and below; Fig. 2). However, in both the CEWAF and WAF treatments, the PAH congener patterns at egg-depth were still strongly skewed toward low to mid-molecular weight compounds (Fig. 2). As several XAD bags disintegrated over time, we have no profiles of PAH congeners in XAD from CEWAF High at the beginning of the study or at the time of hatching, nor do we have profiles for dispersant-only treatment at the time of hatching.





**Figure 2. Percent of specific congeners contributing to total PAH in XAD resin substrates removed from nests six days following addition of solutions (“initial”) and at the time of hatching. A. WAF Low; B. WAF High; C. CEWAF Low. Limited recovery of substrates from CEWAF High initially and at time of hatching, as well as dispersant-only treatments at the time of hatching precluded inclusion. Numbers on the horizontal axis correspond to designations in Table 1.**

Concentrations of tPAH in eggs removed six days following dosing revealed that eggs treated with CEWAF High accumulated much greater concentrations than did those from the WAF High, Dispersant, or Controls (Table 2, Fig. 3). Concentrations of tPAH in eggs from WAF High were only ~ 6.5 % of those in CEWAF High.



**Figure 3. Congener patterns and total PAH (ppb) in eggs six days following addition of solutions to nests. A. WAF High; B. CEWAF High. Note the differences in scales. Numbers on the horizontal axis correspond to designations in Table 1.**

Hatching success was high in all treatments. CEWAF High exposure resulted in the lowest hatching success (60 %), yet it did not differ statistically from other treatments ( $P>0.05$ ; Table 3). The duration of the embryonic period was very consistent and did not differ among treatments, varying only by an average of one day among treatments (Table 3). Neither WW nor CL of hatchlings differed among treatments. Despite accumulation of PAHs in eggs (CEWAF High in particular), there was no evidence of elevated DNA damage in hatchlings from any treatment (Table 3). Sizes of juveniles at the end of the study did not differ among treatments (Table 3), nor was there evidence of behavioral anomalies in juveniles from any treatment.

There were no differences in metabolic rate among treatments during any trial. In the initial assay of body lipid content (7 months post hatch), lipid content of hatchlings from the WAF Low treatment was significantly lower than that for hatchlings having been embryonically exposed only to the dispersant, but there were no other differences among treatments (Table 3). We observed no significant differences in body lipid content among treatments in the assays conducted at 9 and 11 months post hatching, despite what appeared to be a considerable reduction in lipid content in CEWAF High relative to other treatments.

Mean survival of hatchlings through the end of the study ranged from 54 to 76 % among treatments, being lowest on average in CEWAF High relative to other treatments, although the differences were not statistically significant (Table 3). There were no differences in HSI or sex ratios of hatchlings among treatments (Table 3). We observed few gross abnormalities in any treatment.

**Table 3. Biological responses of embryonic and hatchling snapping turtles to experimental treatments. Values are means  $\pm$  1 S.E. Different superscripts represent those treatments that differed following multiple comparisons tests. CL = carapace length; WW = wet weight; HSI = hepatosomatic index.**

Treatment	Survival to hatching (%)	Time to hatch (d after dosing)	Final size (CL, mm)	Final size (WW, g)	Total body lipid content, 6 months post hatch (% dry weight)
<b>Control</b>	75.0 $\pm$ 7.6	61.0 $\pm$ 0.3	44.7 $\pm$ 1.7	31.3 $\pm$ 3.9	11.3 $\pm$ 3.5
<b>Dispersant</b>	68.8 $\pm$ 5.2	60.1 $\pm$ 1.1	42.6 $\pm$ 1.9	28.3 $\pm$ 4.0	25.7 $\pm$ 1.1 <sup>A</sup>
<b>WAF Low</b>	75.0 $\pm$ 5.9	60.9 $\pm$ 0.5	41.1 $\pm$ 0.9	23.6 $\pm$ 1.8	10.3 $\pm$ 5.4 <sup>B</sup>
<b>WAF High</b>	72.9 $\pm$ 7.1	59.9 $\pm$ 0.5	45.7 $\pm$ 2.7	33.2 $\pm$ 5.3	14.9 $\pm$ 3.0
<b>CEWAF Low</b>	81.3 $\pm$ 4.0	61.2 $\pm$ 0.9	47.4 $\pm$ 0.9	34.9 $\pm$ 2.5	14.2 $\pm$ 3.5
<b>CEWAF High</b>	60.4 $\pm$ 9.2	60.7 $\pm$ 1.0	42.9 $\pm$ 3.5	28.7 $\pm$ 6.3	17.8 $\pm$ 2.2
	F <sub>5,13</sub> = 1.10 P = 0.396	F <sub>5,13</sub> = 0.50 P = 0.772	F <sub>5,13</sub> = 1.14 P = 0.378	F <sub>5,13</sub> = 0.90 P = 0.503	F <sub>5,13</sub> = 3.82 P = 0.024

(continued)

Table 3 (continued)

<b>Treatment</b>	<b>Total body lipid content, 9 months post hatch (% dry weight)</b>	<b>Total body lipid content, 11 months post hatch (% dry weight)</b>	<b>Survival to end of study (%)</b>	<b>HSI</b>	<b>Sex ratio (% male)</b>	<b>DNA damage (% tail DNA)</b>
<b>Control</b>	24.0 ± 2.1	13.5 ± 2.8	75.2 ± 9.4	4.0 ± 0.3	76.5 ± 9.4	14.5 ± 2.4
<b>Dispersant</b>	18.5 ± 2.5	13.4 ± 1.9	65.9 ± 10.8	4.1 ± 0.3	87.5 ± 12.5	16.6 ± 3.1
<b>WAF Low</b>	22.7 ± 1.2	14.1 ± 1.4	76.3 ± 10.3	3.5 ± 0.4	79.3 ± 3.7	13.1 ± 4.2
<b>WAF High</b>	16.6 ± 1.1	11.1 ± 2.0	72.1 ± 5.6	4.2 ± 0.2	60.6 ± 8.2	13.3 ± 2.9
<b>CEWAF Low</b>	19.0 ± 1.9	12.2 ± 1.0	69.5 ± 6.6	4.6 ± 0.3	74.6 ± 12.7	16.9 ± 2.2
<b>CEWAF High</b>	17.9 ± 1.9	8.8 ± 1.1	54.5 ± 8.4	3.9 ± 0.4	100 ± 0	15.9 ± 0.5
	F <sub>5,13</sub> = 2.44 P = 0.074	F <sub>5,13</sub> = 1.19 P = 0.353	F <sub>5,13</sub> = 0.84 P = 0.541	F <sub>5,13</sub> = 1.37 P = 0.281	F <sub>5,13</sub> = 2.16 P = 0.105	F <sub>5,7</sub> = 0.36 P = 0.868

## 5.0 Discussion and Importance to the Oil Spill Response/Restoration

Chemical analyses revealed that relative to corresponding WAF treatments, CEWAF treatments delivered considerably higher concentrations of total PAH to nesting substrates (XAD as a proxy) and to egg contents. As would be expected, treatment with the dispersant (CEWAF) resulted in enrichment in somewhat higher molecular weight congeners in dosing solutions relative to non-dispersed, WAF treatments. Passage of CEWAF solutions through the sand column substantially reduced the total PAH concentrations delivered to the eggs (particularly in CEWAF High). Retention of PAHs from CEWAF in the sand likely reflects the presence of colloids of high MW compounds produced by the dispersant which likely adhered to particles in the substrate. On the other hand, total PAH concentrations in WAF solutions were less affected (i.e. 1.4 versus 10-fold in WAF and CEWAF High respectively) by percolation, having originally had much lower concentrations of PAH than the CEWAF High solutions and being characterized by lower compounds of lower MW and log Kow which would be more likely to be transported with water through the substrate.

Analyses of egg PAH concentrations revealed that those exposed to the highest CEWAF solutions accumulated much higher concentrations than those in WAF, although statistical analyses were not conducted (N=2 composite samples per treatment). Eggs from the WAF High treatment accumulated primarily mid-molecular weight PAHs (phenanthrene through pyrene), likely reflecting selective removal of low molecular weight congeners by percolation through the substrate. CEWAF High eggs accumulated low through mid-molecular weight congeners. While the CEWAF solution was also reduced in low molecular weight PAHs relative to higher molecular weight PAHs during percolation, there remained relatively high concentrations of the former being delivered to the substrate surrounding the eggs and thus being available for accumulation.

Despite what appeared to be trends suggesting that juveniles from CEWAF High treatments experienced lower lipid content late in the study and perhaps reduced survival, these effects were not statistically significant. The general lack of significant responses of embryos or hatchlings that we observed seems somewhat surprising, particularly because the eggs accumulated low to mid-molecular weight congeners (particularly when exposed to CEWAF) which have been shown to induce toxic responses such as genetic damage in other organisms (Incardona et al., 2005). However, PAHs vary substantially in their toxicity among congeners and taxa (e.g. Eisler, 1987). Birds for example (mallard ducks) exposed embryonically to PAHs were most substantially affected by the high molecular weight congener dimethylbenz(a)-anthracene (log Kow = 5.8), which induced embryonic toxicity at a concentration of only approximately 36 ppb ww (Hoffman and Gay, 1981). This compound was present at very low concentrations (below analytical detection limits) in the stock solutions and eggs in our study. As well, PAH profiles of eggs in our studies were dominated by congeners that have been shown to have little or no effects on the aryl hydrocarbon receptor in fish ("Ahr," an indicator of onset of toxicological response), which tends to respond to higher molecular weight compounds (Barron et al., 2004). Additionally, Matson et al., (2005) reported correlations between environmental concentrations of low molecular weight 3-ring

congeners (fluorene through anthracene) and DNA damage in adult turtles in Azerbaijan. These compounds were present in eggs in our study, yet we observed no effects on DNA strand integrity in hatchlings. The sites in which Matson et al. (2005) observed prevalent DNA damage had total low molecular weight 3-ring PAH concentrations of 165 ppb in sediment (tissue concentrations not reported), whereas the highest total concentration of these congeners in egg contents in our study was 50 ppb (in CEWAF High; Fig. 3 D).

It is interesting that PAH exposures did not induce toxicological effects in our studies, as turtles and other reptiles have been shown to be sensitive to some organic and inorganic contaminants under natural exposure regimes. Exposure of turtle eggs to PCBs, organic pesticides, and heavy metals via maternal transfer have been shown to affect sexual development, metabolic efficiency, hatching success, morphological development, and long term survival (Bishop et al., 1998, Nagle et al., 2001, de Solla et al., 2002, Rowe, 2008). With respect to PAH exposure specifically, a recent study of turtle eggs and hatchlings collected from a contaminated site suggested a relationship between total PAH in fat and hatchling deformities (Bell et al., 2006). Total PAH in fat was reported as 2.3 to 4.5 ppm, an order of magnitude higher than the highest total PAH concentration that we measured in whole egg samples (0.56 ppm in CEWAF High). As many as 50 % of embryos and hatchlings collected from the contaminated site displayed deformities, suggesting that exposure to such high concentrations of PAH during development may have been responsible. However numerous other organic and inorganic compounds were also present at the site (but not measured in turtles), thus confounding interpretation of direct relationship between PAH and deformities.

An important result of our study was in the finding that in none of the numerous endpoints that we measured, ranging from the molecular to physiological to behavioral traits, did embryonic exposure to WAF or CEWAF induce responses that would be expected to compromise health or fitness. Furthermore, our highest exposure concentrations were likely much higher than those that would be expected to be present on a beach following a spill (NRC, 2003, 2005). Although, repeated percolation from a oil impacted (particularly from a residual slick) beach could conceivably present increased toxicological risks given the increased time for continued exposure. Thus, under the specific conditions in which we conducted our experiments, and based upon the suite of endpoints that we quantified, it appears that the developing turtles were quite tolerant of exposure to compounds derived from WAF and CEWAF, as well as the dispersant itself. However as there have been no dose-response studies correlating embryonic effects in turtles with PAH exposure, we cannot interpret the accumulated concentrations of total PAH or specific congeners relative to known thresholds of effects.

Whether the snapping turtle was an adequate model for estimating effects on sea turtles cannot be explicitly determined. However, as the structure of the egg shell of snapping turtles is characterized by abundant, better defined pores relative to sea turtles (Packard et al., 1982), we predicted that accumulation by snapping turtle eggs may exceed that of sea turtle eggs, providing a conservative estimate of exposure of the latter. Thus, assuming that response correlates with accumulation, sea turtle eggs would be

expected to be less affected than our surrogate species (which was not discernibly affected) when exposed to the same solutions.

## **6.0 Technology Transfer**

The results of this study are intended to provide the oil spill response community with information for making decisions regarding the use of dispersants near turtle nesting beaches during the nesting and ensuing embryonic period.

## **7.0 Achievement and Dissemination**

- Results have been presented at two workshops sponsored by CRRC in 2006 (New Hampshire) and 2007 (Seattle, WA)
- Results were presented to the Caribbean Regional Response Team Meeting, 2007 (U.S. Virgin Islands)
- Overviews of the study approach and results were presented in 3 outreach seminars at Chesapeake Biological Laboratory (approximate attendance 25 – 40 people each seminar)
- Overviews of the study were presented to the summer secondary school teacher education program at Chesapeake Biological Laboratory in 2006 and 2007 (attendance 9 – 11 people)
- We have submitted a manuscript describing the results of this study for publication in a peer reviewed journal (*Science of the Total Environment*).

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