Evaluation of toxicity of *Deepwater Horizon* slick oil on spat of the oyster *Crassostrea virginica*

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Abstract :

The 2010 explosion of the Deepwater Horizon (DWH) oil rig generated the largest marine oil spill in US history with millions of barrels of crude oil released in the Gulf of Mexico (GoM). The eastern oyster, *Crassostrea virginica*, is an ecologically and economically important species in the northern GoM. Due to its biological characteristics (sessile, filter feeding), juvenile oysters may have been affected. This study investigated the effects of surface-collected DWH oil prepared as high-energy water-accommodated fraction (HEWAF) on the survival of 2-month-old oyster spat, and evaluated the potential impacts of HEWAF on particle clearance rate and spat tissue. Exposure of oysters to a range of oil/HEWAF (0-7-66-147-908-3450 $\mu\text{g tPAH50}$ (sum of 50 polycyclic aromatic hydrocarbons) L⁻¹) resulted in non-dose-dependent mortalities and reduced clearance rates of algal food (*Tisochrysis lutea*). A morphometric study of the digestive tubules (DGTs) indicated a dose-dependent response to oil exposure on lumen dilation, on epithelium thinning of the DGT, and a significant change in DGT synchrony (LOEC = 66 $\mu\text{g tPAH50 L}^{-1}$). This finding suggests that structural changes occurred in the digestive gland of exposed oysters most likely due to an oil-related stress. In addition, histological observations showed that tissues in contact with HEWAF (gills, palp, connective tissue, digestive gland) were adversely impacted at $\geq 7 \mu\text{g tPAH50 L}^{-1}$, and exhibited pathological symptoms typical of an inflammatory response (e.g., hemocyte diapedesis and infiltration, syncytia, epithelium sloughing).

Keywords : Oyster spat, *Deepwater Horizon* oil, Sublethal, Tissue, Inflammatory response, Histopathology

1. Introduction

On April 20th 2010, the explosion at the *Deepwater Horizon* (DWH) oil drilling rig located 50 miles southeast of the Louisiana Coast in Mississippi Canyon Block 252 (28°55'12''N, 88°23'14''W), led to the largest marine oil spill in United States history (Carriger & Barron, 2011; National Commission, 2010). Until the well was capped and the flow finally stopped, 87 days after the DWH oil rig sank, several million barrels of light Louisiana crude oil were released into the Gulf of Mexico (GoM) (U.S District Court, 2014; 2015). The escaping oil created a deep sea “cloud” or “plume”, and a surface slick covering up to 112,000 km² of the Gulf waters (Boesch, 2014; ERMA, 2015)]. The coastal extent of the spill was from Louisiana to Florida, with at least 2,113 km of shoreline visibly oiled (Nixon et al., 2015).

Petroleum hydrocarbons or crude oil constituents are one of the most common contaminants released into the marine environment: some of them are of particular concern due to their high chemical stability, low solubility, low degradation, and lipophilic nature. Although oil spills represent a small fraction of the total crude oil discharge into the sea, they have acute and long term impacts on marine ecosystems, including effects from physical damage (physical contamination and smothering) and toxicity from chemical compounds (NRC, 2003). Polycyclic aromatic hydrocarbons (PAHs) are considered to be the most acutely toxic components of crude oil (Neff, 1985; Neff & Stubblefield, 1995). Given their lipophilic nature, these contaminants can bind to particulate organic matter, sediment and microalgae, thus entering the food web (Graham et al., 2010; Lee et al., 1978). Sedentary, benthic filter-feeding organisms such as oysters have an enormous filtration capacity and are particularly effective in taking up hydrocarbons *via* filtration (Newell, 2004). Following ingestion of hydrocarbons, oysters may accumulate high concentrations within their tissues and may be susceptible to the negative effects of these contaminants. For this reason, bivalve mollusks, such as oysters and mussels, are employed on a world-wide scale as sentinel organisms to monitor

concentrations of contaminants in estuarine and coastal environments (Farrington et al., 1983; O'Connor & Lauenstein, 2006).

The Eastern oyster (*Crassostrea virginica*) is one of the most commercially important shellfish species in the GoM, representing about \$74 million in landings value in coastal regions of the northern Gulf for 2012 (NMFS, 2012). In addition to its economic value, it is also an ecologically vital species. Oyster reefs provide food, shelter and habitat for many fish and shellfish species, improve water quality, stabilize bottom areas, and influence water circulation patterns within estuaries (Coen et al., 2007; Newell, 2004; Wells, 1961). Although the location of the spill was in deep water, currents carried the oil to shallow coastal waters and estuaries, which are spawning and nursery grounds for many marine species, including oysters. In the North of the GoM, two peaks in settlement of *C. virginica* occur in early and late summer (Supan, 1983). Given the time of the DWH spill, newly settled spat and juveniles of *C. virginica* are thus likely to have been exposed to DWH crude oil and associated PAHs. The physiological and ecological effects of environmental stress are numerous. While the ultimate endpoint of stress is mortality, sub-lethal effects may interfere with normal metabolic processes of the animals, thus increasing expenditure of energy reserves at the expense of growth, survival and ultimately reproduction and recruitment (Capuzzo, 1996).

Given their role of sentinel in coastal environment, bivalve species have the capacity to survive in highly polluted waters, despite the fact that pollutant levels often overload the normal physiological mechanism of biotransformation or detoxification present in the cell (Gilewicz et al., 1984; Moore, 1985). Exposure to some petroleum hydrocarbons can cause alterations in the condition of certain molluscs, by reducing the feeding efficiency of the animals (Axiak & George, 1987; Barszcz et al., 1977; Galtsoff et al., 1935). As a result, variations in clearance rates of oil-exposed oysters may influence the degree of assimilation and exposure of specific tissues, and, subsequent damage. After long-term and sub-lethal exposure to petroleum hydrocarbons, vacuolization and atrophy of the digestive cells leading to a thinning of the digestive epithelium and subsequently to an increase in tubule lumen size have been reported for molluscs (Cajaraville et al., 1989; Lowe et al., 1981;

Widdows et al., 1982). In addition, a loss of the normal synchrony of the digestive cells of mussels exposed long-term to oil has been found, to a point where almost all the tubules exhibited a similar appearance (i.e. reconstituting stage) (Lowe et al., 1981). To avoid some of the subjectivity associated with descriptive histology, quantitative techniques, such as stereology and morphometric analysis, have been employed to measure alterations in cells following contaminant exposure (Cajaraville et al., 1989; Lowe et al., 1981). Accordingly, in the present study, morphometric analyses were employed to quantify alterations on the digestive tubules of oil-exposed *C. virginica*. Histopathological changes are powerful indicators of prior exposure to environmental stressors, especially xenobiotics, and histopathology is often seen as the easiest method for assessing both short- and long-term toxic effects in the field (Kim et al., 2006). The long-term effects of oil exposure in oysters collected from the field were studied after the *Amoco Cadiz* spill and included necrosis, inflammation, and lesions of the digestive tract epithelium, gonads, connective tissue, and gills (Berthou et al., 1987; Neff & Haensly, 1982). After an exposure to petroleum hydrocarbons, degeneration of epithelial cells in the foot and alimentary canal, activation of mucus secretory cells and inflammatory responses in the gills of the clam *Venus verrucosa* were observed (Axiak et al., 1988). These studies suggest that after exposure to oil, an inflammatory response will likely occur, mainly located in the gills and the digestive system of exposed bivalves. Studies have also shown evidence that contaminant-stimulated “reactive oxygen species” (ROS) production and resulting oxidative damage may be a mechanism of toxicity in aquatic organisms exposed to pollution (Di Giulio et al., 1989; Livingstone et al., 1989; 2001).

The objective of the present study was to determine the physiological (e.g. clearance rates), tissue (e.g. digestive tubules staging, tubule lumen sizes, inflammatory responses) and biochemical (e.g. lipid peroxidation) responses of *C. virginica* spat exposed to increasing concentrations of high-energy water accommodated fraction (HEWAF) of oil.

2. Material and methods

2.1 Preparation of exposure solutions

Surface oil, also referred to as “Slick A”, was collected on July 29th 2010, from the hold of barge number CTC02404, which was receiving surface slick oil from various skimmer vessels near the *Macondo* well, and was delivered under chain of custody (sample CTC02404-02). Slick A incurred significant natural weathering at sea before collection. HEWAF exposure solutions were prepared following a standardized procedure extensively detailed in Vignier et al. (2015). Briefly, two-liters of UV-sterilized and 0.1 µm-filtered seawater (FSW) were added to a stainless-steel blender pitcher (WaringTM CB15 commercial food blender) and 4 g of Slick A were added using a gastight syringe (1:500 dilutions of oil). After blending for 30 s at low speed, the solution was transferred to a 2-L aspirator bottle and left to settle for at least one hour to allow separation of the solution from residual floating oil. The stock solution (2 g oil L⁻¹) was obtained by carefully draining the bottom layer of the mixture from the aspirator bottle and used for PAH analysis and test dilutions with FSW. Four liters of stock solution were prepared every other day with fresh oil. The solution was not filtered, and thus contained whole particulate oil in addition to dissolved PAHs.

2.2 Water quality

Throughout the exposure period, temperature and dissolved oxygen (DO) were monitored daily by means of a Pro ODO optic probe (YSITM), while salinity and pH were measured daily using a refractometer (Pentair Aquatic Eco-systems Inc.TM) and a “Pinpoint” pH monitor (American Marine Inc.TM) respectively. Total ammonia was measured in each treatment, and analyzed using a SEAL Analytical Auto-Analyzer 3. All water quality parameters were measured daily, and from both discarded and fresh exposure solutions. Each exposure chamber was aerated to maintain oxygen levels above 5 mg L⁻¹ and light photoperiod was maintained at 12h: 12h using fluorescent lamps.

2.3 Experimental design

2.3.1 Test organisms

Two month-old single seed oyster spat (mean shell length $\approx$ 10-15 mm; mean individual dry weight $\approx$ 0.5 mg) from the Auburn University shellfish laboratory (Dauphin Island, AL) were used during spring 2013. After reception, oyster spat were gradually acclimated at the experimental hatchery in a flow-through tank for 2 weeks. Using a sump and a submersible pump, seawater was semi-recirculated and salinity was gently increased over several days, from its origin (i.e. 20 PSU $\pm$ 2) to the local conditions (ambient temperature of 23°C $\pm$ 1 and salinity of 30 PSU $\pm$ 2). Oyster were fed *ad libitum* with Instant Algae/Shellfish Diet[®] (Reed Mariculture Inc. TM) for optimal growth.

2.3.2 Experiment 1: HEWAF exposure and clearance rate measurements

Clearance rate (or filtration) measurements were carried out in 600 mL glass beakers for 24 h using the unicellular microalgae *Tisochrysis lutea* (CCMP 1324). Gentle central aeration by means of a glass pipette was provided at 100 mL min⁻¹ to each beaker to homogenize exposure solutions. Ten spat per beaker were exposed, in triplicate, to increasing concentrations of HEWAF ranging from 100 to 2000 mg oil L⁻¹. Total PAH content was not quantified in exposure solutions used in the 24-h filtration experiment. As a result, exposure treatments will be referred to as nominal concentrations of HEWAF and expressed in mg oil L⁻¹. At the start of the exposure, spat were fed *ad libitum* with 1 billion cells (equivalent to 100 mL) of *T-lutea* per beaker. After thorough mixing, residual algal cells were assessed through the exposure (T₀ and T₂₄) by taking a 1.5 mL aliquot from each replicate and then fixing the sample by addition of 100 μ L of Glutaraldehyde. After thorough agitation, three 10 μ L subsamples were taken from the fixed aliquots and counted under a microscope using a hemocytometer. Algal cells counts were then averaged for each sampling point and 24 h clearance rates were determined for each HEWAF condition. To compare different treatments, clearance rates

(CR) were normalized per gram of whole dry tissue weight of oyster, and determined using the following equation, adapted from Coughlan (1969):

$$CR = (V/(n \times t)) \times \ln (C_{ti}/C_{tf})/\varpi$$

where “CR” is the clearance rate in L h⁻¹ g⁻¹; “V” is the volume of each container in L (i.e. 0.5L); “n” is the number of spat; “t” is the time of experiment in h; “C_{ti}” and “C_{tf}” are the initial and final counts of *T. lutea* cells at t_i or t_f; “ϖ” is the mean individual dry weight of spat in g (ϖ=0.0005g).

We assumed that pumping rate of animal was constant, particle retention was 100 % efficient and the algal concentration was homogenous at all times thanks to aeration.

2.3.3 Experiment 2: Acute exposure to HEWAF for 10 days

2.3.3.1 Mortality measurement

Single oyster spat were haphazardly distributed at a density of 15 per beaker, in triplicate (5 replicates for the control). Gentle central aeration by means of a glass pipette was supplied to each beaker at 100 mL min⁻¹. Spat were exposed for 10 days to increasing concentrations of HEWAF (0 – 10 – 50 – 100 – 500 – 2000 mg oil L⁻¹ corresponding to 0 – 7 – 66 – 147 – 904 – 3450 µg tPAH50 L⁻¹). This range of doses tested and exposure duration were chosen according to partial mortality results observed during preliminary range finding tests. Exposure was performed in 600 mL glass beakers filled up with 500 mL of solution, under static-renewal conditions, with the exposure media renewed every other day (i.e. new fresh stock of HEWAF was prepared every other day). Spat from each beaker were fed daily with 2 mL of Instant Algae[®]. Every other day, spat were collected on a 2-mm stainless steel sieve, and observed using a dissecting microscope for examination of dead spat, measured by their failure to close their valves in seawater. Beakers were cleaned using deionized water and Kim-Wipes[®], and refilled with new exposure media.

2.3.3.2 Histopathological study

168 *a) Histopathological observation*

169 At the start of the experiment and following acclimation, histopathological condition of 26 individual
170 oyster spat was examined (Control Day 0; results presented in Table 2). At the end of the 10 day
171 exposure to HEWAF, 15 spat (excluding dead) from each concentration were randomly selected for
172 histological examination. The number of individuals sampled represented a third of the population.
173 Oyster spat were then fixed in a solution of Davidson's fixative for 48 h at 4°C. After rinsing
174 thoroughly with tap water, animals were placed in individual histological cassettes in a solution of 10
175 % EDTA (pH = 7.5) for decalcification (Howard et al., 2004). Solution of EDTA was changed every
176 other day during the week of decalcification process. After decalcification and thorough rinsing, spat
177 were transferred to cold 70% ethanol solution. Following dehydration through an ascending ethanol
178 series, tissues were dealcoholized with xylene using a tissue processor (Shandon Citadel 1000), and
179 embedded in paraffin wax. Sections (5 µm) were cut on a rotary microtome (Microm HM325),
180 stained with Harris' hematoxylin and eosin (Winstead, 1995), and examined with light microscopy.
181 A four-level semi-quantitative scale, from 0 to 3, was established to assess the intensity of each
182 histopathological condition observed in the gastro-intestinal system (i.e. hemocyte diapedesis,
183 hemocyte infiltration, mucus infiltration, and hyperplasia of mucus secretory cells). 0 = no
184 occurrence of the pathological condition was examined in the tissue area; 1= light (i.e. covering less
185 than 5% of the tissue); 2 = moderate (i.e. covering about 10% of the tissue); and 3 = severe (i.e.
186 covering about 25% of the tissue).

187 *b) Morphometric analysis of the digestive tubules*

188 Using an Olympus IX73 inverted microscope equipped with an Olympus DP73 camera and the
189 CellSens image analysis software, digestive tubules were measured at random from histological
190 sections of 6 surviving animals haphazardly selected in each treatment. Morphometric analysis
191 followed a protocol adapted and modified from Cajaraville et al. (1989) and Winstead (1995).
192 Histological cross sections of the digestive gland were divided into 4 fields of observation per

animal. Eight randomly selected tubules from each field (32 total tubules per spat) were measured. Two sets of measurements, internal (luminal) surface and total tubule surface, for each tubule were determined using the CellSens image analysis software, and tubule luminal ratio (luminal surface/total surface) was calculated.

c) Grading of digestive tubules

The phasic activity of the digestive gland was assessed by means of a subjective grading of the same tubules selected previously for morphometric measurements. Thus, at least 30 tubules per individual and a minimum of 180 per experimental group (6 oysters) were therefore categorized. Four tubule types were distinguished as described by Cajaraville (1989) (Fig. 1): holding/absorbing (combined as stage I), disintegrating (stage II), reconstituting (stage III), and a fourth tubule type, not related to the digestion process, and named “necrotic” (stage IV). The percentage occurrence of the 4 different tubule types with respect to the total number measured was then calculated.

Figure 1: Subjective grading of digestive tubules into the 4 different stages observed. I: Absorbing / Holding; II: Disintegrating; III: Reconstituting; IV: Necrotic (sloughing).

2.3.3.3 Biochemical analysis (lipid peroxidation assay)

Surviving oysters, which were not processed for histological analyses, were used for lipid peroxidation assay. Lipid peroxides derived from polyunsaturated fatty acids are unstable and decompose to other complex compounds of which malondialdehyde (MDA), a marker for oxidative

stress, is the most abundant by-product. MDA was measured by a spectrophotometric assay using a commercial BIOXYTECH[®] MDA-586 kit (Oxis Research). The method is based on the reaction of a chromogenic agent, N-methyl-2-phenylindole with MDA at 45°C. A minimum of 100 mg wet weight of oyster tissue was homogenized by sonication for 15 min in 990 µL of Phosphate Buffer Saline (PBS, pH = 7.4) and 10 µL of Butylated Hydrotoluene (BHT) to avoid intra-assay oxidation. The homogenate was then centrifuged at 10,000 g for 10 min, and the supernatant (200 µL) was added to a mixture of probucol in methanol, N-methyl-2-phenylindole, and hydrochloric acid as indicated in the MDA-586 kit. The reaction was conducted at 45°C for 60 min. After centrifugation at 10,000 g for 10 min, the clear supernatant was transferred into a microtitration plate and the absorbance was measured at 586 nm. A standard curve was established with a solution of tetramethoxypropane (TMP) hydrolyzed during the acid incubation step generating MDA. Results were expressed in µmol of MDA per g of wet mass of oyster tissue.

2.4 Analytical chemistry

During experiment 2, samples of every preparation of HEWAF (including FSW control, stock solution and dilutions) were collected at the start and before the first exposure solution renewal, and shipped (4°C) to ALS Environment (Kelso, WA, USA) for chemical analyses. After that and throughout the 10-day exposure, only stock solutions of oil prepared every 2 days with fresh oil and FSW control were sent for chemical analyses. Polycyclic aromatic hydrocarbons (PAHs), including alkyl homologues, were determined by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM) and a sum of 50 different PAHs were quantified (Table 1). The sum concentrations of these 50 PAH analytes are hereafter referred to as tPAH50. The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the targeted analytes (US EPA, 1986). Additional details regarding the methods used (e.g. standards used, QC criteria for surrogate recovery, internal standards, spiked blanks) can be found in the analytical QAPP provided by the analytical laboratory

and applied to all samples analyzed for the Deepwater Horizon Natural Resource Damage Assessment (DWH NRDA): <https://pub-dwhdatadiver.orr.noaa.gov/dwh-ar-documents/945/DWH-AR0101767.pdf>

2.5 Statistical analyses

All percentage data (mortality, tubule types) were arcsine-square root transformed to improve normality prior to statistical analyses. Effects of oil on mortality, tubule stages, tubule luminal ratio and lipid peroxidation were analyzed by one-way analysis of variance (ANOVA), after checking for normality (Shapiro-Wilk test) and homogeneity of variances between treatments (Levene's test). Whenever significant differences among groups were found ($p < 0.05$), a Tukey post-hoc test for multiple comparisons was performed. Non-parametric Kruskal-Wallis one-way ANOVA on ranks and Dunnett's T3 post-hoc test were completed whenever normality and homogeneity of variance requirements were not met after transformation of the data.

For histopathological results, a non-parametric Mann-Whitney/U-test ($p = 0.1$) was used to assess the effect of oil treatment on the prevalence of various pathological conditions compared to the control at day 10. Additionally, the total number of pathologies observed per individual as well as gastrointestinal pathologies, scored by intensity, were analyzed using the Mann-Whitney/U-Test ($p = 0.1$) and compared between treatments. All analyses were performed using SPSS® 22.0 statistical package (IBM Corp., NY).

3. Results and Discussion

3.1 Water quality and analytical chemistry of oil solutions

Temperature and salinity for all experiments were $25.4^{\circ}\text{C} \pm 1.0$ and $25.4 \text{ PSU} \pm 0.8$, respectively. DO and pH averaged $6.3 \text{ mg L}^{-1} \pm 0.4$ and 8.09 ± 0.2 respectively, and total ammonia concentration

remained below deleterious levels ($\text{NH}_3 = 0.0147 \text{ mg L}^{-1} \pm 0.005$). Two faulty aeration lines in 2 replicates within the control treatment of experiment 2 caused levels of DO to drop below 2 mg L^{-1} on day 9, resulting in a mass mortality; as a result, only 3 replicates for the control condition were included. Seawater used for control treatments contained background concentrations of PAHs ($\text{tPAH50} = 0.03 \text{ } \mu\text{g L}^{-1} \pm 0.01$) considered negligible for the present study. Nominal concentrations tested with corresponding sum concentration of 50 PAHs quantified by GC/MS-SIM are presented in Table 1, whereas PAH profile of the stock solutions of HEWAF throughout the 10-day exposure is shown in the Supplementary File.

Table 1: Range of nominal test dilutions in percentage of stock of HEWAF (2 g L^{-1}), nominal concentrations in mg oil L^{-1} , and corresponding measured tPAH50 (i.e. sum of 50 PAHs) concentrations, in $\mu\text{g L}^{-1}$, of the initial exposure solutions. Measured tPAH50 were only quantified in the stock solutions at renewal throughout the 10-day exposure, i.e. at Day 4, Day 6, and Day 8.

	% Stock	Nominal (mg oil L^{-1})	Day 0 ($\mu\text{g tPAH50 L}^{-1}$)	Day 4 ($\mu\text{g tPAH50 L}^{-1}$)	Day 6 ($\mu\text{g tPAH50 L}^{-1}$)	Day 8 ($\mu\text{g tPAH50 L}^{-1}$)
	100	2000	3450	3588	3710	3383
	25	500	904	-	-	-
HEWAF	5	100	147	-	-	-
Slick A	2.5	50	66	-	-	-
	0.5	10	7	-	-	-
	0	0	0.03	-	-	-

HEWAF = high-energy water accommodated fraction; tPAH50 = sum concentration of 50 polycyclic aromatic hydrocarbons analytes quantified by GC-MS/SIM by ALS (Kelso, WA).

Bi- and tri-aromatic compounds were the major contributor to the PAH content of the stock solutions of HEWAF used throughout the spat exposure. Furthermore, alkylated PAHs such as C1-C4 naphthalene, fluorene or phenanthrene were more abundant than parent PAHs, a result in accordance with our previous toxicity tests (Vignier et al., 2015; 2016) and other studies (Achten & Andersson, 2015; Neff & Anderson, 1981). These alkylated derivatives were shown to have increased toxicity to invertebrates as a result of increased lipophilicity (Hawthorne et al., 2006).

3.2 Effects of HEWAF on mortality

Oyster spat ($\approx 10\text{--}15$ mm) were exposed for 10 days to concentrations of HEWAF ranging from 7 to 3450 $\mu\text{g tPAH50 L}^{-1}$. Non-parametric statistical tests showed no significant differences in mortality between control and exposed treatments (Kruskal-Wallis: $p=0.280$) (Fig. 2). Overall, only partial mortalities were encompassed by the broad range of HEWAF concentrations tested, and no clear dose-dependent mortality response in exposed oyster spat was observed. Highest mortalities were observed at a relatively low concentration of oil ($66.7\% \pm 16.8$ at $66 \mu\text{g tPAH50 L}^{-1}$) and at the highest concentration ($57.8\% \pm 15.5$ at $3450 \mu\text{g tPAH50 L}^{-1}$) compared to control ($22.2\% \pm 8.9$) (Fig. 2).

Figure 2: Mortality of spat following 10 days of exposure to increasing concentrations of HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$ (initial oil loading rate of 2 g L^{-1}). Data are presented as mean percentages ($n=3$) $\pm$ standard errors (SE). Seawater control treatment corresponds to $0.03 \mu\text{g tPAH50 L}^{-1}$. TPAH50 = sum concentration of 50 polycyclic aromatic hydrocarbons analytes quantified.

This is in accordance with our preliminary spat exposures (data not shown) and our previous study exposing veliger stage of oyster larvae to HEWAF in which we found a non-dependent dose-

response (Vignier et al., 2016). This lack of a clear dose-response relationship is noteworthy. A recent study by Forth et al. (2017) determined how different WAF preparation methods and concentrations influenced the chemical composition and concentration of PAHs in the dissolved and particulate phases over time. In HEWAF solutions prepared with the same starting oil-to-water ratio (i.e. 2 g oil L⁻¹), the composition and concentration of the dissolved and particulate fractions varied substantially across a range of dilutions (same range used in the present toxicity testing) (Forth et al., 2017). The authors also found that in the stock solutions of HEWAF (containing oil droplets), the dissolved phase was a small fraction of the total PAH concentration whilst the dissolved phase became the dominant fraction when diluted to lower concentrations. Previous studies have demonstrated that most toxicity of oil to aquatic organisms can be imputed to the PAH derived from its dissolved fraction rather than its particulate form (Barron et al, 1999; 2003; Carls et al., 2008; Hansen et al., 2012). Consequently, the dynamic nature of HEWAF preparations (i.e. proportion of the dissolved and particulate phases) may explain the accrued lethal effects observed on oyster spat at low doses of oil tested in the present work.

In addition, spat in the seawater control suffered over 20 % mortality. This may have been caused by a stress due to change of salinity conditions between the hatchery where the spat came from (20 PSU) and the salinity conditions at the laboratory (26-30 PSU). Ultimately, if we consider solely the mortality data obtained in the current study, it must be concluded that oyster spat were relatively tolerant to DWH oil (LC50 value exceeded the range of concentrations tested, i.e. LC50 > 3450 µg tPAH50 L⁻¹). However, the sub-lethal impacts of mechanically dispersed DWH oil on filtration, histopathological features and lipid peroxidation of exposed spat were further investigated to determine any long-term effects on the population dynamics of oysters and on the whole estuarine ecosystem.

3.3 Effects of HEWAF on clearance rates

Clearance rates of spat fed *Tisochrysis lutea* were significantly impacted at all concentrations of HEWAF tested for 24h ($F_{4, 10} = 59.08$, $p < 0.001$; Fig. 3). Each treatment reduced oyster feeding activity by at least 5-fold compared to the control ($F_{4, 10} = 59.08$, $p < 0.05$). In response to an environmental stress such as oil pollution, filter-feeding oysters can reduce their clearance rates by closing their valves, as a defense mechanism (Akberali & Trueman, 1985). Concentrations of oil $\geq 100 \text{ mg L}^{-1}$ (equivalent to $147 \mu\text{g tPAH50 L}^{-1}$) caused a significant reduction in the clearance rate of oyster spat regardless of the concentration tested (Fig. 3).

Figure 3: Clearance rates of *Tisochrysis lutea* by oyster spat exposed for 24 h to nominal concentrations of HEWAF (in mg oil L^{-1}), expressed in $\text{L}^{-1} \text{h}^{-1} \text{g}^{-1}$ DW of oyster tissue. Data are presented as mean percentages ($n=3$) $\pm$ standard errors (SE). Different letters denote statistical differences compared to control (ANOVA; $p=0.05$). HEWAF = high-energy water accommodated fraction; DW = Dry weight.

This inhibition of clearance rates, similar across all treatments, suggests valve closure as a potential avoidance response. Other studies have also reported a decline in filtration rates of bivalves in response to hydrocarbons (Barszcz et al., 1977; Galtsoff et al., 1935; Widdows et al., 1982). Several reasons could explain this reduced feeding activity, the most obvious being a mechanical action of oil droplets by coating of the gills, thus disabling the normal efficiency of the ctenidia to sort and absorb food particles (Galtsoff et al., 1935). Exposure of the gill tissue of hard clams to toxic algae exerted

an irritation, causing a cessation of ciliary activity (Gainey & Shumway, 1991). In the same way, oil/PAHs could have induced a cessation of the ciliary motion, inhibiting feeding rates. In the current study, instances of erosion of gill filaments were observed in some individuals, indicative of an apparent irritation and toxicity by contact. Our results (i.e. high concentrations of 2 and 3 ring alkylated PAHs; see supplementary file) could also be explained by another study which suggested that bi- and tri-aromatic hydrocarbons could induce a narcotizing effect on the ciliary feeding mechanisms of *Mytilus edulis*, reducing clearance rates (Donkin and Widdows, 1990).

3.4 Effects of HEWAF on digestive morphometry

Measurements of the digestive tubule (DGT) lumen ratio in tissue sections of both control and experimental oysters indicated that exposure to increasing concentrations of slick oil prepared as HEWAF induced a significant increase of the luminal tubule ratio compared to control ($F_{5, 30} = 7.817$, $p < 0.001$; Fig. 4A). The number of DGT with dilated lumens (Type III, Fig. 1) increased in a dose-dependent manner, with concentrations of oil/HEWAF (e.g. 66, 904 or 3450 $\mu\text{g tPAH50 L}^{-1}$) having a significant effect ($F_{5, 30} = 7.817$, $p \leq 0.01$; Fig. 4A).

Figure 4: (A) Mean tubule luminal ratio ($\pm$ SD) from 6 oysters exposed for 10 days to increasing concentrations of HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$. Different letters denote a statistical difference (ANOVA: $p=0.05$). (B) Proportion (in percent) of the different digestive tubule (DGT) types observed in histological sections of spat ($n=6$), exposed for 10 days to HEWAF ($\mu\text{g tPAH50 L}^{-1}$). Scoring method

(2.3.3.2c) as follow: stage I: absorption; stage II: disintegration; stage III: reconstitution; stage IV: necrotic. Asterisks denote a statistical difference compared to control (ANOVA: $p=0.05$).

Furthermore, compared to control oysters, 10 days of exposure to oil induced significant changes in phasic activity between the different stages of tubules observed among treatments ($F_{5, 30} = 11.221$, $p<0.001$; Fig. 4B). Concentrations of HEWAF, i.e. 66, 904, and 3450 $\mu\text{g tPAH50 L}^{-1}$, significantly impacted the proportion of tubule types ($F_{5, 30} = 11.221$, $p\leq 0.05$; Fig. 4B) in a dose-dependant manner with a significant shift observed from stage I to III (Fig. 4B). Overall, histological sections of oil-exposed oysters showed that digestive diverticula were typical in appearance to that seen during starvation of mollusks (Morton, 1977). Symptoms such as severe atrophy of the DGT, with increased dilation of the lumina and a loss of epithelial cell height (stage III) were noted, but more markedly than during starvation observed by the above cited study. This result suggests that crude oil/PAHs might disrupt the normal food uptake by exposed oysters, hence inducing a nutritional stress. This is in agreement with other studies, which showed that environmental stress can cause or mimic starvation (Barszcz et al., 1977; Winstead, 1995; Wikfors & Smolowitz, 1995). Oil and PAH exposures were previously reported to lead to atrophy of the digestive diverticula and severe changes in the structure of the cells comprising the epithelium in the digestive gland of molluscs (Cajaraville et al., 1992; Lowe et al., 1981; Luna-Acosta et al., 2017). As described by Moore (1986) and Lowe (1981), PAHs could affect lysosomal activity, by altering their structure (size, number, membrane permeability) and function, leading to damage of digestive cells, thus impacting intracellular digestion.

3.5 Effects of HEWAF on tissue condition

A variety of pathological conditions were observed after 10 days of exposure to mechanically dispersed oil (HEWAF). Our observations focused on connective tissue (Fig. 5A), gills, labial palp (Fig. 5C), and mantle which were in direct contact with the oil solutions. In addition, organs of the digestive system, such as stomach (Fig. 6), intestine, digestive ducts and tubules (Fig. 7) were

specifically studied. Conditions such as; erosion of gill filaments, hemocyte infiltration in the water tubules of the gills or in the labial palp, high instances of mucus secretory cells in the digestive tubules and digestive ducts epithelia (i.e. mucus diapedesis), or parasitic infestation (*Tylocephalum*) were commonly observed in the oysters after 10 days, but distributed in all experimental treatments, exposed or not to oil. The histopathological condition of non-exposed spat at the start of the exposure (i.e. control Day 0; Table 2) suggests that a severe change in salinity from the original to the experimental condition during acclimation, as well as a potential poor health condition of the spat to begin with may have caused the observed high mortality in the non-exposed, control oysters after 10 days.

Nonetheless, oil-exposed spat differed from the control group in a number of aspects. In the stomach for instance, little food or digested material was found, particularly in those exposed to 66 µg tPAH50 L⁻¹ and higher. This finding is in line with our observations of oyster spat upon exposure, i.e. filtration reduction and occurrence of atrophy in the digestive diverticula.

One of the main histological features observed in exposed animals was the significant presence of syncytia in the connective tissue, in very high numbers compared to control (Fig. 5A & B) (U-test: $p < 0.05$, Table 2). In the gills, the presence of syncytia was consistently observed in all oil treatments except the 66 µg tPAH50 L⁻¹ dose, reaching up to 38.5% of the oysters at 7 µg tPAH50 L⁻¹ as compared to 5.3% in control (Table 2). Syncytia are defined as a multinucleate mass of protoplasm produced by the fusion of several cells (Dorland, 2011). Syncytia can be normal cells in animal biology (e.g. some type of fungi), but hemocytes are mononuclear cells and their merging together to form syncytia is a serious pathology, indicative of a severe inflammatory response. For instance, syncytia can form when cells are infected by certain types of viruses such as HIV, causing changes in immunology and surface antigens of the cells and ultimately apoptosis (Huerta et al., 2009). Formation of polykaryons cells (giant cells, syncytia) has been recorded in vertebrates in response to inflammation (Lewis & Lewis, 1926; Mariano & Spector, 1974) but more rarely reported in invertebrates (Anderson, 1987). The presence of syncytia may impair the immune response of

affected organisms, and the relationship between syncytia formation and oil-exposure requires further research.

In the labial palp epithelium, hemocytic diapedesis was observed more often in oil-exposed spat (Fig. 5D) than in non-exposed spat (Fig. 5C), particularly at the highest doses (100% of the oysters) (U-test: $p \leq 0.05$, Table 2). Combined with hemocyte infiltration in the water tubules, these features indicate a direct contact between soluble PAHs and/or oil droplets with the labial palp via filtration.

Figure 5: (A) healthy connective tissue with normal hemocytes and presence of phagocytosing hemocytes (dashed arrows), observed in non-exposed group after 10 days; (B) connective tissue observed in oil-exposed spat (at 10 mg oil L⁻¹ treatment equivalent to 7 μg tPAH50 L⁻¹) after 10 days showed high instance of syncytia (arrows); (C) Normal labial palp observed after 10 days in control oyster; (D) abnormal labial palp exhibiting major hemocyte infiltration (100 mg L⁻¹ treatment equivalent to 147 μg tPAH50 L⁻¹).

Acute exposure to oil prepared as HEWAF negatively affected the digestive system of oyster spat. In addition to DGT atrophy mentioned previously, histological examination of the digestive diverticula

and the gastro-intestinal tract revealed a major inflammatory response upon oil-exposure. After 10 days, the occurrence of severe pathological conditions such as ulcers (Fig. 6B) and epithelial sloughing (Fig. 6D) were found in a greater number of oysters at 66 and 147 $\mu\text{g tPAH50 L}^{-1}$ exposure concentrations compared to controls (U-test, $p \leq 0.1$, Table 2). Hemocyte proliferation in the connective tissue surrounding the digestive diverticula, as well as severe hemocyte diapedesis (Fig. 6C) and infiltration into the lumen of the alimentary tract (Fig. 6D) occurred significantly more in oil-exposed oysters than in non-exposed oysters (U-test, $p \leq 0.1$, Table 2).

A recent study from Luna-Acosta (2017) also found that the digestive gland of oysters exposed to mechanically dispersed oil showed hemocyte infiltrations. Significant migration of hemocytes by diapedesis from the vascular system into the stomach and the intestine is part of a defense mechanism in bivalves (Feng, 1966; Galimany et al., 2008). In the presence of oil, oyster hemocytes can migrate to engulf and encapsulate foreign material (Sami et al., 1992). Enzyme activities can be activated in hemocytes of *C. gigas* exposed to crude oil, and may play an important role in protection against xenobiotics (Luna-Acosta et al., 2011). Thus, as an attempt to eliminate oil, hemocytes may migrate from the connective tissue through the epithelia of the stomach/intestine by diapedesis, carrying the compounds to the alimentary canal (intestine) for elimination by excretion in the feces. This process was confirmed in our study by observation of actively phagocytosing hemocytes in the connective tissue (Fig. 5A & 6C) and apoptotic hemocytes in the lumen of the stomach/intestine (Fig. 6D). Such a detoxification pathway has been described in mussels challenged with environmental stressors or toxic algae; this process was associated with an increase of tissue lipid peroxidation due to an oxidative stress (Alves de Almeida et al., 2007; Galimany et al., 2008).

Figure 6: Section of the stomach of experimental oyster after 10 days of exposure to HEWAF. (A) Normal epithelium in non-exposed oyster; (B) example of ulcer and consequent hemorrhaging of hemolymph in the stomach lumen observed in oysters exposed to $66 \mu\text{g tPAH50 L}^{-1}$ treatment; (C) heavy hemocyte diapedesis (arrows) from the connective tissue into the lumen of the stomach ($7 \mu\text{g tPAH50 L}^{-1}$). Asterisks note the presence of phagocytosing hemocytes as well as syncytia; (D) severe hemocyte infiltration coupled with epithelium sloughing and diapedesis in the stomach ($147 \mu\text{g tPAH50 L}^{-1}$); dashed arrows show apoptotic hemocytes; arrows show oil accumulated in epithelia. ct = connective tissue; lu = lumen; ep = epithelium.

An excess of mucus in the alimentary canal and a high prevalence of hypertrophic mucus secretory cells in the epithelia (referred as mucus diapedesis) of the stomach/intestine were also consistently observed in exposed spat (U-test: $p \leq 0.05$, Table 2, Fig. 6D). Although mucus secretory cells are a normal cell type in epithelia of the digestive gland, their increased number and abnormal size in oil-exposed oyster may indicate hyperplasia, which could be a severe pathology directly imputable to contact with oil droplets/dissolved PAHs (Domingos et al., 2007).

Table 2: Histological parameters recorded in *Crassostrea virginica* tissues at day 0 and after 10 days of exposure to DWH oil (HEWAF), expressed in $\mu\text{g tPAH50 L}^{-1}$. Data are presented in percent of occurrence per treatment. Mann-Whitney/U-test compares treatments with control Day10: statistical difference at $p=0.1$.

Histological features	Measured HEWAF ($\mu\text{g tPAH50 L}^{-1}$)						
	Control Day 0	Control Day 10	7	66	147	908	3450
GILLS							
<i>Syncytia</i>	0	5.3	38.5**	0	15.8	18.2	9.1
<i>Hemocyte infiltration</i>	0	0	15.4#	12.5	5.3	9.1	9.1
<i>Erosion of filament</i>	8.6	0	0	12.5	0	0	9.1
PALP							
<i>Diapedesis</i>	5.2*	46.2	75	66.7	57.1	100*	100*
<i>Hemocyte infiltration</i>	0	7.7	50*	33.3	14.3	57.1*	28.6
CONNECTIVE TISSUE							
<i>Syncytia</i>	11.5	31.8	92.3***	75*	84.2***	92.3***	84.6**
STOMACH/INTESTINE							
<i>Epithelium sloughing</i>	0	5.3	11.1	33.3#	26.7#	15.4	0
<i>Ulcer</i>	0	0	0	33.3**	20*	7.7	0
<i>Hemocyte diapedesis</i>	40*	79	100	100	100#	100#	84.6
<i>Hyperplasia mucus cells</i>	25	21.1	78**	66.7*	60**	76.9**	69.2**
<i>Hemocyte infiltration</i>	15*	57.9	100*	83.3	93.3#	92.3*	76.9
<i>Mucus infiltration</i>	10	31.6	88.9**	50	46.7	53.9	61.5#
DIGESTIVE DIVERTICULA							
<i>Epithelium sloughing</i>	0	31.8	100***	37.5	89.5***	76.9**	66.7*
<i>Digestive cells in lumen</i>	7.7*	36.4	100***	50	94.7***	84.6**	66.7#
<i>Hemocyte diapedesis</i>	0	13.6	53.9**	50*	47.4*	61.5**	8.3
<i>Hyperplasia mucus cells</i>	7.7	9.1	30.8	12.5	15.8	30.8#	33.3#
<i>Oil in lumen®</i>	0	0	0	0	5.3	7.7	8.3

Data were categorized as 0 or 1, according to the absence (0) or presence (1) of the observed feature; Mann-Whitney/U-tests were performed: # $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, @ Three oysters had this feature, all in the highest doses tested ($> 147 \mu\text{g tPAH50 L}^{-1}$) see Fig. 7D.

Compared to non-exposed oysters (Fig. 7A), the digestive diverticula of oil-exposed oysters showed a predominance of dilated lumina of the DGT (Fig. 7B). In addition, migration of hemocytes by diapedesis into the lumen of the digestive ducts (DD), epithelium sloughing and presence of digestive cells in the lumina of the DD and DGT, were observed in most of the exposure groups (U-test, $p < 0.05$, Table 2) (Fig. 7C). More specifically, a prevalence (10 to 18%) of necrotic tubules was noted at the highest HEWAF concentrations tested (904 and $3450 \mu\text{g tPAH50 L}^{-1}$), with cellular debris and digestive cells, most often in apoptosis, sloughed into the lumen of tubules and ducts (Fig. 7C). It is evident from these observations that a degeneration of the digestive cells lining the DGT epithelia and subsequent sloughing into the lumen was related to exposure to hydrocarbons. Likewise, epithelium sloughing was described in oysters collected from the field following the *Amoco Cadiz* spill (Neff & Haensly, 1982) and with chronic experimental exposures (Barszcz et al., 1977). Instead of partially digested food material ready to be taken in by digestive cells for intracellular digestion, abnormal excretion product (i.e. mucus) and possibly oil were observed in the lumen of the DD and DGT from three spat exposed to the highest doses of HEWAF (Fig. 7D, Table 2). In light of these observations, it is possible that oysters directly ingested oil as droplets, carried it to the stomach via the labial palps, and transferred it to the lumen of DGT by the ciliated epithelia of the DD. As part of the DWH natural resource damage assessment, Forth et al. (2017) characterized the oil droplets size distribution in HEWAF dilutions used in our toxicity testing. Their results revealed that the initial mean diameters of the HEWAF droplets were $8 \mu\text{m}$ and decreased to $5 \mu\text{m}$ after 48h, a size range of particle well within the food spectrum available to filter-feeding oyster.

Figure 7: Histological preparations of *Crassostrea virginica* cross-sections (A) show predominantly normal (Type I) tubules (arrows); (B) arrows show severe metaplastic changes (Type III), with dilation of the lumina and loss of epithelial cell height; (C) section of digestive diverticulum (100 mg oil L⁻¹ treatment equivalent to 147 µg tPAH50 L⁻¹) showing necrotic stage of DGT (Type IV) with sloughing of digestive cells (dashed arrow) in the lumen of digestive tubules as well as hemocyte diapedesis (arrows) in epithelia of tubules and digestive ducts; (D) example of tubules and ducts with presence of oil in lumen observed in the 2000 mg L⁻¹ treatment (corresponding to 3450 µg tPAH50 L⁻¹). dgt = digestive tubules; dd = digestive ducts.

Figure 8A summarizes the mean number of pathological conditions observed per individual for each concentration of HEWAF tested. For most concentrations of HEWAF tested, an increase in the number of pathologies was observed in every organ studied in comparison to control oysters. Acute exposure to oil also affected the digestive system, where most histopathological conditions were reported, particularly in the gastro-intestinal system (Fig. 8A). A focus on the stomach and intestine revealed a significant increase in the intensity of pathologies compared to control (Fig. 8B). Surprisingly, concentration of 7 µg tPAH50 L⁻¹ seemed to induce more intense pathologies compared

to the highest dose of HEWAF tested (i.e. 3450 $\mu\text{g tPAH50 L}^{-1}$); for example, hemocyte diapedesis and hemocyte infiltration were significantly more intense at 7 $\mu\text{g tPAH50 L}^{-1}$ than at 3450 $\mu\text{g tPAH50 L}^{-1}$ (U-test, $p \leq 0.05$, Fig. 8B). Altogether, the gastro-intestinal system appeared to be the most impacted tissues with a significant increase of pathology intensity as compared to control. It may be that the chemical characteristics of low concentrations of HEWAF and particularly the proportion of dissolved to particulate (i.e. droplets associated) PAHs - which increases with decreasing total concentration through time (Forth et al., 2017) - could have contributed to these augmented sub-lethal effects. Filtration results, which indicated no difference of clearance between oil treatments, and the variable mortalities observed could support this hypothesis. Further research should be conducted on the respective cause of toxicity from dissolved and particulate oil/droplets.

Figure 8: (A) Organ distribution of total pathological conditions observed per oyster ($n \geq 9$) following 10 days of exposure to increasing concentrations of HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$. (B) Occurrence of various pathological conditions observed in the gastro-intestinal system (stomach/intestine) of oyster spat ($n \geq 9$) after 10 days of exposure to HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$. Occurrence of pathological conditions are categorized by intensity following a semi-quantitative scale (see details in 2.3.3.2.a); 0: absence, 1: light, 2: moderate, 3: severe. Different letters within each organ/pathological condition denote statistical difference at $p \leq 0.1$ (Mann-Whitney/U-test). Lettering differed between categories because separate analyses were performed for each organ/condition.

3.5 Effects of HEWAF on lipid peroxidation

For oysters exposed to increasing HEWAF concentrations, a trend of increased malondialdehyde (MDA, i.e. a marker for oxidative stress) level from 0 to $147 \mu\text{g tPAH50 L}^{-1}$ and a trend of decreased MDA level from $147 \mu\text{g tPAH50 L}^{-1}$ to $3450 \mu\text{g tPAH50 L}^{-1}$ (Fig. 9) were observed in the tissue. Furthermore, concentrations of the lipid peroxidation byproduct were relatively high in the tissue sampled from the $147 \mu\text{g tPAH50 L}^{-1}$ group ($13.6 \mu\text{mol g}^{-1}$). However, none of these results show any statistical significance ($F_{5, 15} = 0.946$, $p = 0.480$) due to the high individual variability. Chronic,

long-term exposure of bivalves to crude oil can induce an oxidative stress and impair membrane integrity, associated with lipid peroxidation (Di Giulio et al., 1989; Downs et al., 2002; Livingstone et al., 1989; 2001). Perhaps the duration of exposure tested in our study was too short to result in a significant oxidative stress measurable using the MDA biomarker.

Figure 9: Mean malondialdehyde (MDA) concentration ($\pm$ SE), expressed in $\mu\text{mol g}^{-1}$ of oyster tissue, in spat exposed for 10 days to increasing doses of oil prepared as HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$.

4. Conclusions

This study revealed lethal and sublethal effects of DWH oil on an ecologically important organism of the Gulf of Mexico, the oyster *Crassostrea virginica*. Relatively high concentrations of DWH oil (HEWAF) were necessary to elicit a lethal response ($> 3450 \mu\text{g tPAH50 L}^{-1}$ or $> 2 \text{ g oil L}^{-1}$) in oyster spat. If we consider the mortality data in conjunction with the histopathological changes observed on surviving spat however, exposure to relatively low doses of PAH ($< 66 \mu\text{g tPAH50 L}^{-1}$) caused severe alterations in the physiology and metabolism of these animals. The present study also demonstrated that an inflammatory response occurred, with various degrees of severity depending of HEWAF doses, mostly located around the alimentary canal and the connective tissue. Stress associated with shipping, acclimation and holding oysters in static-renewal conditions during

exposure could have contributed to the apparent poor condition of oysters in the control (both day 0 and day 10) and the unexpectedly elevated mortality in the control at the end of the exposure (> 20%). However, the differences between controls and experimental treatments showed significant difference suggesting that oil had an impact.

Oysters are the principal benthic filter-feeders in the northern region of the Gulf of Mexico and appear to be, from the present study, sensitive to crude oil. Although these laboratory results showed significant sub-lethal effects in oysters exposed to DWH oil, these results cannot be extrapolated directly to the environment or to the natural oyster population. The lowest effective dose of PAHs determined in the current study (i.e., 7 $\mu\text{g tPAH50 L}^{-1}$ for tissue damage) was greater than most tPAH50 concentrations measured in coastal-water samples during and after the DWH spill (e.g., 0.17 $\mu\text{g tPAH33 L}^{-1}$; Allan et al., 2012). It must be noted however that in the natural environment, oil can potentially interact with other abiotic factors (e.g. temperature, salinity, UV irradiance, xenobiotics) which may exacerbate the toxicity of its compounds to oysters (Pelletier et al., 1997; Powers et al., 2015). Further research is warranted to elucidate the effects of multiple stressors in combination with petroleum hydrocarbons on the physiology of oyster spat. Finally, information collected in the present study can be used in the selection of meaningful endpoints in addition to lethality for assessing oil spill effects.

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Supplementary file: Initial concentrations, expressed in $\mu\text{g L}^{-1}$, for the 50 PAH analytes (parent and alkyl homologs) measured in the fresh stock solutions of HEWAF (oil loading rate of 2 g L^{-1}) throughout the 10 day-exposure of oyster spat. Generalized subclasses are divided by dotted lines and noted on the figure

Figure 2

Figure 3

Figure 5

Figure 6

Figure 7

Figure 9

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Supplementary Material

Supplementary File PAH profile stocks.tif