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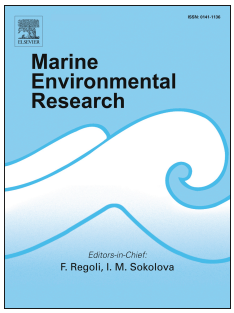

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Lethal and sub-lethal effects of *Deepwater Horizon* slick oil and dispersant on oyster (*Crassostrea virginica*) larvae

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Abstract

In April 2010, crude oil was spilled from the *Deepwater Horizon* (DWH) oil platform for 87 days, coincident with the spawning season and recruitment of the oyster, *Crassostrea virginica*, in the Gulf of Mexico. Impacts of acute exposures to surface-collected DWH oil (HEWAF), dispersed oil (CEWAF) and dispersant alone (Corexit 9500A®) on planktonic larval stages of *C. virginica* (veliger, umbo and pediveliger) were tested in the laboratory. Exposures to HEWAF, CEWAF and dispersant were toxic to larvae impairing growth, settlement success and ultimately survival. Larval growth and settlement were reduced at concentrations of tPAH50 ranging from 1.7 to 106 μg L^{-1} for HEWAF and 1.1 to 35 μg L^{-1} for CEWAF, concentrations well within the range of water sampled during the DWH oil spill. Sublethal effects induced by oil and dispersant could have significant ecological implications on oyster populations and on the whole estuarine ecosystem.

Keywords: *Deepwater Horizon* oil spill, *Crassostrea virginica*, Corexit 9500A®, larvae, growth, settlement, PAH

1. Introduction

The Eastern oyster, *Crassostrea virginica* is one of the most commercially important shellfish species propagating along the east coasts of the United States, from Maine to the Gulf of Mexico (GoM) (Galtsoff, 1964) and an ecologically vital species for the GoM region. Oyster reefs, which are the result of successive settlement of larvae onto existing reef structure, provide food, shelter and habitat for many fish and shellfish species, improve water quality, stabilize bottom areas, and influence water circulation patterns within estuaries (Wells, 1961; Newell, 2004; Volety et al., 2014). In addition to its ecological significance, it is also an economically important species, with total landings of *C. virginica* in Louisiana representing about $42 million in value for 2012 (National Marine Fisheries Service, 2012). In the northern part of the GoM, oyster spawning season typically occurs from mid-spring through late fall when water temperature is above 25˚C (Ingle, 1951; Stanley & Sellers, 1986), with two peaks in settlement in early and late summer (Supan, 1983).

The explosion of the *Deepwater Horizon* (DWH) oil drilling rig led to the largest marine oil spill in United States history, with millions of barrels of crude oil released into the GoM (U.S. District Court, 2015). In addition, several millions liters of the chemical dispersant Corexit 9500A® were used directly at the wellhead and at the surface to disperse the oil slicks (OSAT-1, 2010; U.S. Coast Guard, 2011). From April 20th until the final capping of the leak on July 15th, DWH crude oil spilled from the *Macondo* well (U.S. District Court, 2014), a period that coincided with the natural spawning and recruitment season of eastern oysters in the GoM. The developing pelagic larvae spend 2 to 3 weeks in the water column, generally floating near the surface, until they sink and settle on a suitable substrate (Bahr and Lanier, 1981). Among the biological components of marine ecosystems, planktonic organisms are particularly susceptible to oil pollution (Walsh, 1978; Graham et al., 2010; Almeda et al., 2013, 2014). Zooplankton such as oyster larvae cannot overcome the effects of currents, limiting their capacity to avoid crude oil patches and potentially forcing them to drift into highly polluted waters after oil spills.

Natural oil seepage, transportation, extraction, atmospheric deposition, surface run-offs and consumption are the main sources of crude oil into the sea (National Research Council, 2003). Oil spills can have strong acute and long-term impacts on marine ecosystems, including effects from physical damages (asphyxia, physical contamination or coating of oil) to toxicity from their chemical compounds that constitute crude oil (NRC, 2003). Crude oil is a complex mixture of both hydrocarbons, such as alkanes, cycloalkanes and aromatic

hydrocarbons, and non-hydrocarbon compounds. Polycyclic Aromatic Hydrocarbons (PAHs) are often considered to be the most acutely toxic components of crude oil (Neff, 1985; Barron et al., 1999). PAHs are also associated with potential carcinogenic, mutagenic and teratogenic effects in humans and aquatic animals (De Flora et al., 1990, 1991; Hylland, 2006).

Most bioassays have focused on acute embryo-toxicity, one of the most sensitive tests to marine pollutants (His et al., 1999). However, numerous studies have reported that larval growth assays were even more sensitive to organic contaminants than embryo toxicity assays (Hidu, 1965; His and Robert, 1985; Geffard et al., 2002; Mottier et al., 2013; Gosling, 2015), with growth inhibition occurring at much lower concentrations than those required to induce embryo abnormality. Studies on the toxicity of crude oil and/or dispersant on larval growth of oyster are very limited though, making comparison very difficult. Of particular relevance, a recent study by Laramore et al. (2014) exposed veliger and pediveliger larvae of *C. virginica* to CEWAF of artificially weathered DWH oil. Unfortunately, they only reported adverse effects of CEWAF on larval survival and larval growth was not assessed. In a previous study, fertilization success and particularly early larval growth of oysters were shown to be negatively affected by exposure to DWH oil/dispersant and to be sensitive toxicological endpoints (Vignier et al., 2015). At equivalent nominal concentrations, dispersed oil (CEWAF) and dispersant alone also showed similar toxicity responses of early life stages of oysters, indicating that most of the toxicity of CEWAF was associated with the Corexit itself (Vignier et al., 2015). It is also expected that sensitive processes such as metamorphosis and settlement of bivalves would likely be affected by acute exposure of the larvae to pollutant, even for a short period of time and/or at relatively low levels of contaminants (Crisp & Austin, 1960). Despite the fact that the bivalve metamorphosis assay has been shown to be a rapid, sensitive, reliable and easy method (Phelps and Warner, 1990), only a few studies have used it as a biological response to contaminants (Beiras and His, 1994; His et al., 1997; Mottier et al., 2013).

The aims of the present study were i) to examine the lethal and sublethal effects of surface-collected DWH oil, dispersed oil and dispersant on different stages of the rapidly developing *C. virginica* larvae, and ii) to evaluate the validity of larval development and metamorphosis of oyster as toxicity endpoints for ecotoxicology assessment of DWH oil spill and dispersant assessment. Three separate experiments were carried out in the laboratory to test the effects of increasing concentrations of HEWAF, CEWAF and dispersant using static acute exposure

on i) 24 h-old veliger, ii) late umbo larvae (10 day-old), and iii) pediveliger larvae (14 day-old).

2. Material and Methods

2.1. Water Accommodated Fractions

Crude oil was obtained under chain of custody during the *Deepwater Horizon* response efforts. The DWH slick oil ("Slick A") was collected near the source on July 29, 2010, from the hold of barge number CTC02404, which received surface slick oil from various skimmer vessels near the *Macondo* well (sample CTC02404-02). The dispersant Corexit 9500A® (Nalco Environmental Solutions LLC, Sugar Land, TX, USA) was provided by the DWH Trustees. For all exposure solutions, contaminants were added to UV-sterilized and 0.1 µm-filtered seawater (FSW), maintained at a salinity of 20–25 PSU.

2.1.1. HEWAF

The oil-only exposure solutions or High Energy Water Accommodated Fractions (HEWAFs) were prepared at 25˚C and under fluorescent lights. The High Energy method was used to artificially recreate the action of waves, currents and stormy conditions, hence dispersing oil mechanically. Two-liters of FSW were added to a stainless steel blender pitcher (Waring™ CB15 commercial food blender) and 4 g of "Slick A" (1:500 dilutions of oil) were added using a gastight syringe. 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 (Incardona et al., 2013; Vignier et al., 2015). 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. The solution was not filtered, and thus contained whole particulate oil in addition to dissolved PAHs.

2.1.2. CEWAF

The oil/dispersant mixtures or Chemically Enhanced Water Accommodated Fractions (CEWAFs) were also prepared at 25°C, under artificial light, according to the CROSERF method (Aurand and Coelho, 2005). Stocks of CEWAF were prepared by adding slick oil (4

g) to dispersant (400 mg) at a ratio of 10:1 (wt:wt) using a gastight syringe and an aspirator bottle, previously filled with 2 L of FSW. The mixture was stirred for 18 h at a vortex adjusted to 25% of solution height. To allow separation of the solution from residual floating oil, the oil and dispersant mixture was left to stand for 3 h. The lower portion of the solution was then drained for PAH analysis and utilization in test dilutions.

2.1.3. Corexit

Dispersant exposure solutions were prepared as described for CEWAF above, except that no oil was added. The dispersant stock was collected by draining the aspirator bottle and, the different exposure concentrations were obtained by diluting the stock solution with FSW.

2.2. Experimental oysters and algae

Adult specimens of *Crassostrea virginica* (average weight of 75 g ± 20) were collected in June 2013 from natural populations in Estero Bay, Florida (Lat. 26˚19'50''N, Long. 81˚50'15''W). Adult oysters were maintained in the hatchery at 23˚C ± 1, in a flow-through system supplied with coarsely filtered (30 µm sand filter) seawater, at ambient salinity (20-30 PSU), under natural light conditions, and fed a mixture of laboratory-cultured fresh microalgae (*Tetraselmis chui*, *Chaetoceros sp*. and *Tisochrysis lutea)* at a daily ration of 3% of oyster dry body weight for conditioning (Utting and Millican, 1997).

Microalgae cultures were grown in f/2 culture medium (Guillard, 1975) prepared with FSW, and held in 10-L carboys at 22-23 ˚C and 30-32 PSU salinity on a 12:12 light:dark cycle with cool-white fluorescent lights and appropriate aeration.

Pediveliger larvae (≈ 14 day-old) used for the settlement assays were sent from the Auburn University Shellfish Laboratory (Dauphin Island, AL) in a chilled (≈ 5ºC) Styrofoam container and shipped overnight. Once received, the pediveliger larvae were placed in a sterile beaker filled with 2 L of FSW at 25ºC and allowed to acclimate for 30 min.

2.3. Spawning and larval culture

Mature oysters were induced to spawn by thermal stimulation. Spawning females were isolated in ≈ 500 mL of FSW for collection of oocytes; whereas spawning males were placed in ≈ 200 ml of FSW, to obtain a dense sperm solution. Gametes were examined under a microscope for motility (sperm), shape and absence of atresia (oocytes) for selection of the

best products. After filtration through a 55 µm mesh to remove debris, sperm from several males ($n \geq 3$) were pooled in a 1-L sterile beaker. Oocytes from several females ($n \geq 3$), after successive sieving through 150 µm and 55 µm mesh to remove tissue and debris were rinsed on 20 µm sieve and transferred into a sterile beaker filled with 2 L of FSW. Oocytes were fertilized with 2.5 % of sperm solution (v:v), and gently mixed. Five subsamples of 50 µL of newly fertilized embryos were stained with Lugol and counted using a Sedgwick-Rafter® cell and a dissecting microscope. After microscopic observation of the first cell cleavage, fertilization success was determined and embryos were thereafter transferred to hatching tank filled up with 50 L of FSW, at a density of 40 embryos mL^{-1}.

About 24 h after fertilization at 28°C, embryos developed to swimming straight-hinge larvae or veliger and were retained on a 35 µm sieve. Veliger larvae (1 day old) were then re-suspended in 2 L of FSW, counted as previously described, and used for the first acute exposure.

The left-over veligers were placed in a tank at a density of 10 mL^{-1}, and were cultured in the hatchery to the late umbo (10 days) stage. Filtered seawater, maintained at 28°C, was changed every other day, and larvae were fed with live microalgae according to Helm and Bourne (2004).

2.4. Acute exposure of early veliger, late umbo, and pediveliger larvae

2.4.1 Acute exposure of early veliger larvae (day 1)

One day old veliger (mean initial length = 70.8 µm ± 1.6; n=25) were distributed at a density of 15 larvae mL^{-1} (approximately 3000 individuals per beaker) into 400 mL beakers filled with 200 mL of the different exposure concentrations of HEWAF, CEWAF or dispersant (Table 1). Control and treatment groups, in quadruplicate, were maintained for 96 h at 25.5 °C ± 1 and 25 PSU ± 1, with no renewal of the exposure solutions. Gentle aeration (≈ 1 bubble s^{-1}) was provided for each beaker in order to maintain D.O levels above 4 mg L^{-1}. Fresh cultured microalgae (*T. lutea* and *C. muelleri*) were added to each beaker at day 0 and day 2 (1×10^5 cells mL^{-1}). A 10-mL subsample was collected on the first day from the stock (T0), and after 48 h from each exposure beaker and preserved by addition of 300 µL of 10% buffered formalin for measurements of shell length and mortality. After 96 h of exposure, larvae from each beaker were concentrated by filtering larvae through a 35 µm mesh, and preserved with 0.9 mL of 10% buffered formalin for later examinations of survival and shell measurements, to obtain a final volume of 30 mL. Final survival was assessed by taking 5

subsamples of 300 µL (n=5) from the concentrate (30 mL) of each of the 4 replicates after homogenization, and examined under a microscope to evaluate live and dead larvae (translucent shell or opened valves). At each sampling time (0, 48 h and 96 h), shell lengths of 25 randomly selected live larvae from each replicate were measured (total of 100 per treatment group) using an inverted microscope (Olympus IX73) equipped with a camera Olympus DP73, and the CellSens Software.

2.4.2 Acute exposure of late umbo larvae (day 10)

Ten day-old umbo larvae were retained on a 90-µm sieve, rinsed, re-suspended in FSW in a sterile 2-L beaker and counted as previously described. Acute exposure of late umbo (mean initial length = 139.4 µm ± 3.5; n=200) were performed using the same protocol previously described, i.e. same nominal exposure concentrations with 4 replicates per condition, excepted that larvae were loaded at a density of ≈ 2000 in 300 mL of FSW, and 50 larvae were randomly selected from each replicate to measure shell lengths at 0, 48 and 96 h, and final survival (considering the initial number of stocked larvae and the final number of survivors). Total PAH content was not quantified in exposure solutions of umbo larvae: hence we used nominal tPAH50 estimates based on concentrations measured during the veliger exposure tests.

2.4.3 Acute exposure of pediveliger larvae (day 14)

After reception from the Dauphin Island hatchery (Auburn, AL) and acclimation in FSW at 25ºC, pediveliger or "eyed" larvae were collected on a 200-µm sieve, rinsed, re-suspended in FSW in a 2-L beaker, and counted as previously described. Selected pediveligers were distributed at ≈ 1000 individuals into 600-mL beakers filled with 450 mL of the different exposure concentrations of HEWAF and CEWAF. Exposure consisted of 5 to 6 nominal concentrations and a FSW control, with 4 replicates per concentration (Table 1). Our previous work showed that, at equivalent nominal doses, Corexit only and CEWAF exposures impaired fertilization success and early larval development in a similar manner (Vignier et al., 2015). As a result, we only tested HEWAF and CEWAF exposures on pediveliger.

Pediveligers were exposed, for 72 h, in a static system at 23 ˚C ± 2 and 23 PSU ± 1 with no renewal of contaminant. Two settlement plates consisting of HardieBacker® cement board tiles (120mm x 58mm), previously soaked/conditioned in seawater for a minimum of 2

weeks, were set-up vertically in the water column of each container. Gentle aeration (≈ 1 bubble s^{-1}) was supplied to each beaker for 30 min every 2 h using a timer-controlled air pump, in order to maintain dissolved oxygen (D.O) levels > 4 mg L^{-1}. Fresh cultured microalgae (*T. lutea* and *C. muelleri*) were added to each exposure beaker at days 0 and 2 (1 x 10^5 cells mL^{-1}).

After 72 h of exposure, developmental success of pediveliger was determined by their progression to settlement as well as mortality. Settlement plates and container walls were examined under a dissecting microscope, and newly settled oysters counted. For later estimation of survival, remaining larvae were collected on a 150-µm sieve, rinsed with FSW and re-suspended in a 50-mL centrifuge tube. Samples were then preserved with 10% buffered formalin (0.9 mL) and adjusted with FSW to a final volume of 30 mL. Final survival was assessed by taking 3 subsamples of 1 mL from the concentrate (30 mL) of each replicate after homogenization, and observed under a microscope to discriminate between live and dead larvae. Live larvae were distinguished by clarity of internal organs (Fig. 1A). Dead larvae were often grey and opaque, with opened valves, and no evidence of internal organization (Fig. 1B). Some dead larvae showed retraction or partial decomposition of tissue, and some with invasion of bacteria and protozoa (ciliates). Newly settled larvae were identified by their larger size (> 400 µm) and their attachment to the substrate, and by the transition from rounded pediveliger to a flat shape with the new dissoconch (Fig. 1C). Settlement success was calculated by considering total settled larvae on tiles versus the total number of pediveligers unsettled, and a median effective concentration (EC50) inhibiting settlement was determined for HEWAF and CEWAF (Table 3).

Figure 1: (A) Live *Crassostrea virginica* pediveliger larva, (B) dead larva, and (C) newly settled spat on tile.

2.4.4 Water quality and analytical chemistry

Temperature, dissolved oxygen, salinity, and pH were measured daily using Pro ODO optic probe (YSI), a refractometer, and a "Pinpoint" pH monitor (American Marine Inc.)

respectively. Total ammonia was measured at the start and the end of each exposure experiment, using a Seal Analytical Auto Analyzer 3 and the G-171-96 method.

Chemical analyses of hydrocarbon constituents of the different HEWAF, CEWAF, dispersant concentrations and the FSW control were performed by ALS Environment (Kelso, WA, USA). The 250-mL unfiltered water samples were collected for the veliger and the pediveliger tests (no chemistry samples were taken for the umbo test), and stored in amber-bottles at 4˚C until shipment to the analytical laboratory by expedited courier. Samples were then extracted and processed for GC-MS. 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 (tPAH50) was quantified. 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. Additional information regarding the PAH analytes and the tPAH50 sum can be found in Forth et al. (2015). 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

Nominal concentrations used during exposure to HEWAF, CEWAF and dispersant, as well as corresponding tPAH50 contents, are listed in Table 1. Chemical analyses of tPAH50 concentrations were not performed for any of the umbo larval exposures with oil. Instead, we used tPAH50 concentrations measured during the veliger larvae tests using the same exposure preparation methods to estimate concentrations during the umbo exposures. We refer to these throughout as “nominal tPAH50”.

2.5. Statistical analyses

Analyses of variance (ANOVA) were performed on shell lengths and settlement success data to obtain lowest observed effective concentrations (LOECs). Before ANOVA analysis, all percentage data were arcsine-square root transformed to improve normality. Normality (Shapiro-Wilk test) and homogeneity of variances (Levene’s test) were verified using the SPSS® 22.0 statistical package. When significant effects of treatment were found (ANOVA: $p \leq 0.05$), post-hoc tests were performed. We used Tukey post-hoc tests unless data did not

meet homogeneity of variances requirements. In those cases, we used Dunnett's T3 post-hoc tests. In addition to ANOVAs, dose-response curves were fitted using log-logistic models with the *drc* package in R version 3.1.1 (2014) (Ritz and Streibig, 2005; Ritz, 2010). For binomial response variables (mortality, settlement), we fitted a three-parameter log-logistic model, while for growth, we fitted a 4-parameter log-logistic model. We estimated median lethal concentrations (LC50) and effective concentrations (ECx) from these fitted models. Final survival was calculated using the number of live larvae observed at the end of the exposure, divided by the total number initially stocked. All results are reported with 95% confidence intervals (CIs).

3. Results

3.1. Water quality and PAH analysis

Overall, temperature and salinity were 25.6 ˚C ± 1.2 and 24.9 PSU ± 1.4 respectively. Dissolved oxygen (D.O.) and pH averaged 6.8 mg L^{-1} ± 0.3 and 8.1 ± 0.2 respectively. For each tested concentration of oil and/or dispersant, total ammonia concentration remained below levels causing deleterious effects to tested organisms (NH_3 = 0.212 mg L^{-1} ± 0.312). Filtered seawater (FSW) used for the control treatments showed levels of PAHs at background concentrations (mean tPAH50 = 0.45µg L^{-1} ± 0.12), considered negligible for the present study. Sum of 50 PAHs (tPAH50) measured for each concentration of HEWAF and CEWAF preparations are shown in Table 1 with corresponding nominal concentrations; whereas, PAH profiles of the stock solution of CEWAF and HEWAF are presented in supplementary files.

Table 1: Range of nominal concentrations (mg L^{-1}) of test solutions used for exposures of 1 day-old veliger larvae, 10 day-old umbo larvae, and 14 day-old pediveliger larvae, and corresponding PAH concentrations (in µg L^{-1} = sum of 50 PAHs or tPAH50). PAH = polycyclic aromatic hydrocarbons; HEWAF = high-energy water accommodated fraction; CEWAF = chemically enhanced water accommodated fraction.

Larval stage exposed:	**Veliger**	**Umbo***	**Pediveliger**
Oil Preparation	**Nominal (mg L^{-1}) => tPAH50 (µg L^{-1})**		
HEWAF	0 => 0.5	0 => 0.5	0 => 0.1
	62.5 => 95.3	62.5 => 95.3	31.25 => 47.8
	125 => 202.0	125 => 202.0	62.5 => 112.9
	250 => 389.9	250 => 389.9	125 => 191.0
	500 => 761.7	500 => 761.7	250 => 399.1
	1000 => 1605.4	1000 => 1605.5	500 => 719.0
	2000 => 2985.2	2000 => 2985.2	
CEWAF	0 => 0.4	0 => 0.4	0 => 0.8
	62.5 => 14.0	62.5 => 14.0	31.25 => 10.1
	125 => 25.3	125 => 25.3	62.5 => 19.1
	250 => 44.9	250 => 44.9	125 => 43.6
	500 => 91.2	500 => 91.2	250 => 80.9
	1000 => 178.5	1000 => 178.5	500 => 177.3

*: Nominal tPAH50.

3.2. Lethal effects on veliger and umbo larvae

Control veliger and umbo larvae exposed only to filtered seawater had a mean percent mortality of 28 % (± 10) and 21 % (± 23) after 96 h respectively. All exposure solutions

tested induced significant mortalities regardless of the life stage initially exposed, i.e. veliger or umbo (Fig.2). At the highest concentrations tested, dead larvae were noted with extruded and vacuolated tissues as well as translucent shells and opened valves (Fig. 1B). With the exception of the HEWAF exposure of veliger larvae which did not induce a clear dose-related response in terms of mortalities (Fig. 2A), dose-dependent mortalities were generally observed for both stages after 96 h of exposure to HEWAF, CEWAF and Corexit (Fig. 2B-F).

Median lethal concentrations after 96 h ($LC50_{96h}$), expressed as PAH concentrations (tPAH50) or nominal Corexit concentrations, are shown in Table 3, which summarizes all of the experiments carried out in the present work. Exposing veliger larvae to CEWAF, $LC50_{96h}$ reached 41.8 µg tPAH50 L^{-1} (corresponding to 22.5 mg Corexit L^{-1}) (Table 3). By comparison, exposure of veliger larvae to oil only (HEWAF) and dispersant only (Corexit) generated $LC50_{96h}$ reaching 715 µg tPAH50 L^{-1} and 22.9 mg Corexit L^{-1} respectively (Table 3). Higher $LC50_{96h}$ value for HEWAF as compared to CEWAF (715 *vs* 41.8 µg tPAH50 L^{-1}) suggests that CEWAF was potentially more toxic to veliger than HEWAF. Similar trends were observed with the umbo larvae assay, with $LC50_{96h}$ values for HEWAF exposures higher than for CEWAF exposure (2790 *vs* 72 µg nominal tPAH50 L^{-1}).

When comparing $LC50_{96h}$ values expressed in nominal Corexit between CEWAF and dispersant only exposures of veliger larvae, similar results were found (22.5 *vs* 22.9 mg Corexit L^{-1}). In contrast, $LC50_{96h}$ values, expressed in nominal Corexit, reported during umbo exposure to CEWAF were significantly lower from the one reported during dispersant only exposure (39.6 *vs* 58 mg L^{-1}) (Table 3). These results suggest that, at equivalent nominal concentrations, umbo larvae were more sensitive to CEWAF-associated dispersant than to dispersant alone; whereas, veliger larvae were as sensitive to CEWAF-associated dispersant than dispersant alone. Lastly, at equivalent nominal concentrations of Corexit tested, $LC50_{96h}$ values obtained during veliger exposures to CEWAF and Corexit only were significantly lower than values obtained during the umbo exposures (Table 3). This indicates a higher sensitivity of veliger larvae to Corexit compared to umbo.

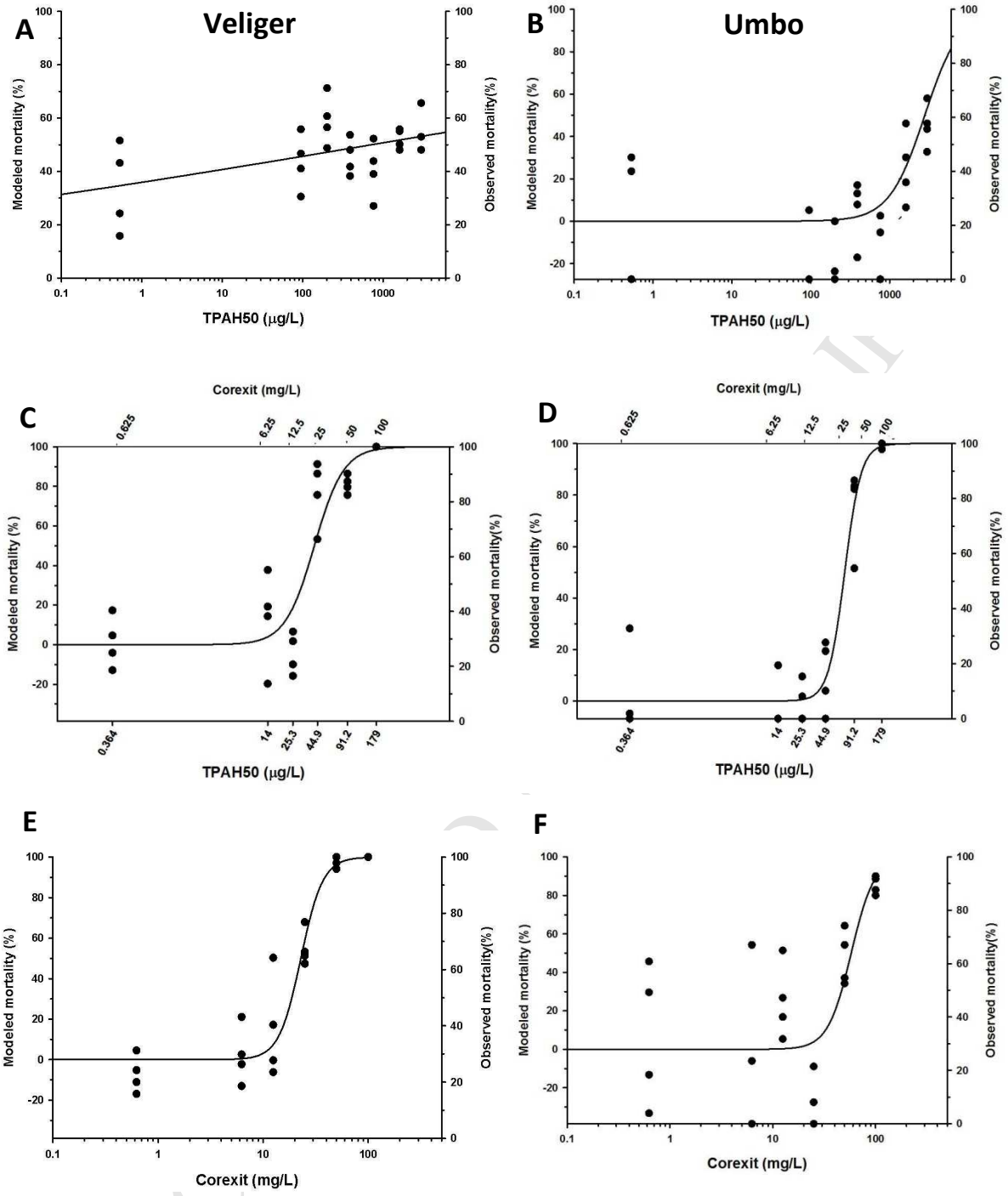


Figure 2: Dose response curves for HEWAF (A, B), CEWAF (C, D) and Corexit alone (E, F) exposures of 1-day old veliger larvae (A, C, E) and 10-day old umbo larvae (B, D, F). Observed mortalities (in %) were reported after 96-h of exposure, for 4 replicates per treatment, and calculated from initial stocking numbers and final number of survivors. Model for CEWAF mortalities (C, D) was fitted to measured TPAH50 (sum of 50 PAHs) exposure concentrations (µg L^{-1}), and the corresponding nominal concentration of dispersant (mg L^{-1}) is shown. Modeled mortalities for HEWAF (A, B) and Corexit only (E, F) were fitted to TPAH50 exposure concentrations (µg L^{-1}) and nominal Corexit (mg L^{-1}) respectively. TPAH50 values for umbo tests are nominal

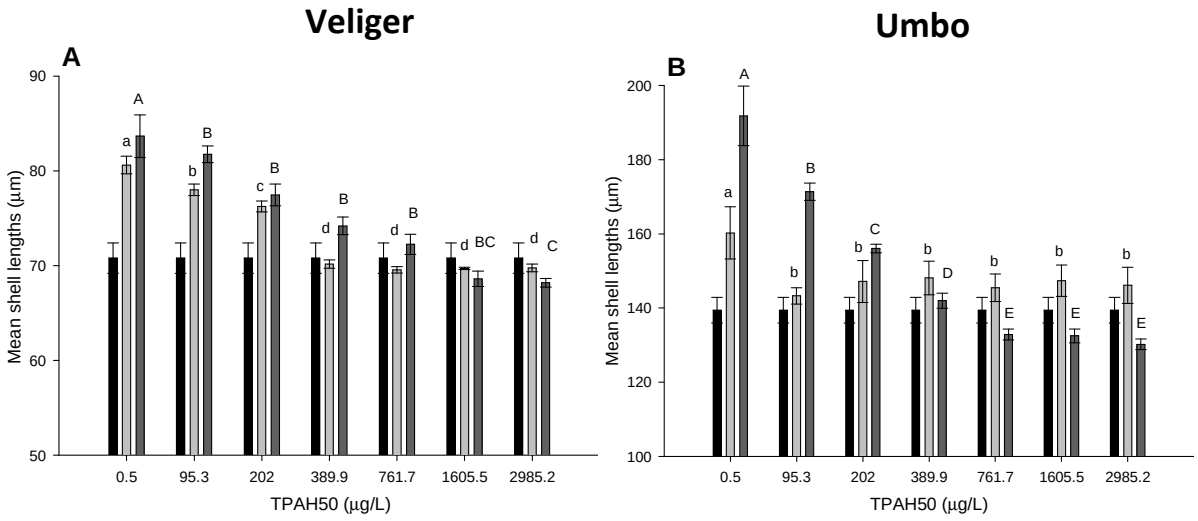
3.3. Sub-lethal effects on growth of veliger and umbo larvae

In the control groups, the mean shell length (n≥100 per condition) of veliger larvae increased approximately 13 µm in 96 h, while it increased by 52 µm in 96 h for the umbo larvae (Fig. 3). Conversely, the mean shell length of exposed larvae consistently declined with increasing HEWAF, CEWAF, and dispersant concentrations (Fig. 3).

The lowest concentration of HEWAF inhibiting shell length (LOEC) of veliger was 95.3 µg tPAH50 L^{-1}, while shell growth of veliger larvae exposed to HEWAF was completely inhibited at 389.9 µg tPAH50 L^{-1} (Fig. 3A). Furthermore, the LOEC of HEWAF inhibiting shell lengths for umbo larvae was 95.3 µg nominal tPAH50 L^{-1}, whereas concentrations of HEWAF of 389.9 µg nominal tPAH50 L^{-1} completely inhibited the growth of exposed larvae (Fig. 3B).

For CEWAF exposure, shell lengths of exposed veliger and umbo larvae were significantly and negatively affected at LOEC of 14 µg tPAH50 L^{-1} (corresponding to 6.3 mg Corexit L^{-1}) ($F_{4, 23} = 187.6$, $p = 0.002$) and of 25.3 µg nominal tPAH50 L^{-1} (equivalent to 12.5 mg Corexit L^{-1}) ($F_{5, 26} = 15.8$, $p < 0.001$) respectively (Fig. 3C & 3D). In addition, growth of veliger was completely inhibited at 14 µg tPAH50 L^{-1} whereas growth of umbo larvae was inhibited at 44.9 µg nominal tPAH50 L^{-1} of CEWAF.

At equivalent nominal concentrations, exposure to dispersant alone showed similar responses as CEWAF exposure, with shell increment of veliger larvae and umbo larvae completely inhibited at 6.3 mg L^{-1} (Fig. 3E) and at 25 mg L^{-1} (Fig. 3F) respectively.



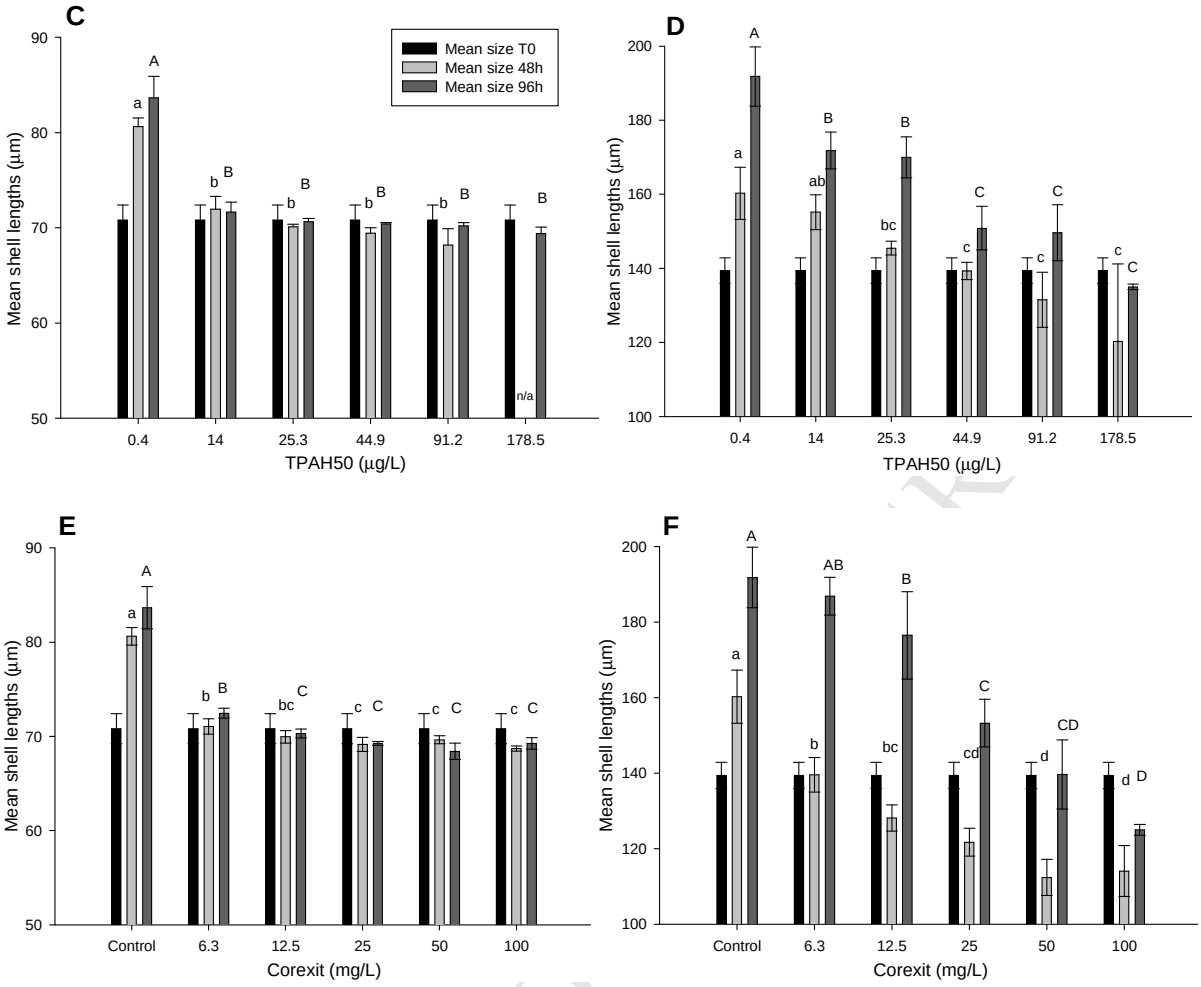


Figure 3: Mean shell lengths (µm ± SD; n=4 replicates) of veliger larvae (1 day-old) and umbo larvae (10 day-old) after 96 h of exposure to HEWAF (A & B), CEWAF (C & D) and dispersant Corexit (E & F), expressed as measured TPAH50 concentrations (µg L^{-1}) for oil solutions, or nominal Corexit (mg L^{-1}) for dispersant solutions.
TPAH50 values for umbo tests are nominal. n/a: no live larvae were observed, i.e. 100% mortality.
Different letters denote statistical difference at α=0.05 (ANOVA). Tukey post-hoc tests were performed for exposure B, E, and F; Dunnett's post-hoc tests were performed for exposure A, C, and D.

Similarly to the lethal responses, concentrations of CEWAF (expressed as tPAH50) inhibiting 20% of veliger and umbo larvae growth were substantially lower (7 to 10 times) than $EC20_{96h}$ values reported for HEWAF exposure (1.1 *vs* 106 µg tPAH50 L^{-1} and 8.6 *vs* 61 µg nominal tPAH50 L^{-1} respectively; Table 3).

The $EC20_{96h}$ values (3.5 mg L^{-1}) during veliger exposure to Corexit alone showed a significantly lower effective concentration (about 3 times) compared to umbo larvae (10.7 mg L^{-1}) (Table 3). Similarly, $EC20_{96h}$ values observed with CEWAF exposure, expressed as nominal Corexit concentration, were lower for the veliger exposure (25 mg. L^{-1}) than the umbo exposure (37 mg. L^{-1}) (Table 3). Furthermore, although not statistically different, EC20 values (expressed as nominal Corexit) for larvae exposed to dispersant alone were lower than EC20 values obtained for larvae exposed to CEWAF (Table 3).

3.4. Lethal and sub-lethal effects on pediveliger larvae

Our previous work (Vignier et al., 2015) as well as present results from the veliger/umbo assays showed that, at equivalent nominal doses, Corexit alone and CEWAF exposures impaired larval development and survival in a similar manner (Fig. 2 and 3). Therefore, only HEWAF and CEWAF exposures were tested on pediveliger. Mean settlement of pediveliger larvae exposed to HEWAF (A) and CEWAF (B) are shown in Figure 4. Control group exhibited a mean settlement of 37.6 % (± 7.3) after 72 h. Exposing pediveligers acutely for 72 h to increasing concentrations of HEWAF, significant settlement inhibition occurred compared to non-exposed larvae ($F_{5, 22} = 21.6$, $p<0.001$; Fig. 4A). The lowest concentration of HEWAF having an effect (LOEC) on settlement was 48 µg tPAH50 L^{-1} ($F_{5, 22} = 21.6$, $p<0.001$; Fig. 4A). Effective concentration of HEWAF that inhibited 50 % of settlement success (EC50) could not be precisely determined due to a lack of intermediate responses, but was estimated to be 1.7 µg tPAH50 L^{-1} (Table 3).

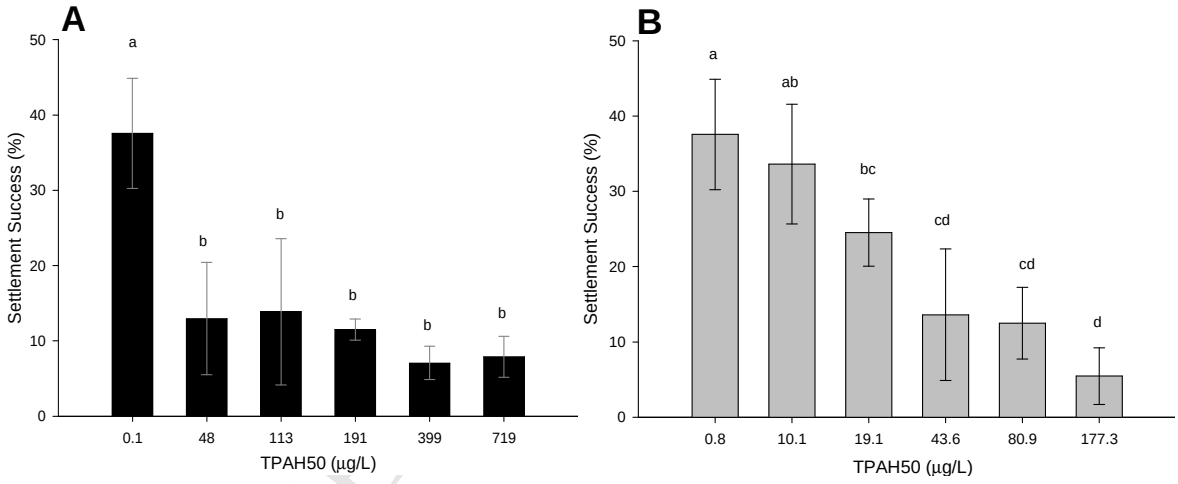


Figure 4: Mean percent settlement success (± SD) of pediveliger larvae after 72 h of acute exposure to HEWAF (A), and CEWAF (B), expressed as measured TPAH50 concentrations (µg L^{-1}). Different letters denote significant differences between treatments (ANOVA, Tukey: $p<0.05$).

Exposure of pediveliger larvae for 72 h to increasing doses of CEWAF induced settlement inhibition in a dose-dependent manner ($F_{5, 22} = 18.6$, $p<0.001$; Fig. 4B). The LOEC reducing significantly settlement success was 19.1 µg tPAH50 L^{-1} ($F_{5, 22} = 18.6$, $p=0.036$; Fig. 4B). In addition, the dose of CEWAF inhibiting 50 % of settlement success ($EC50_{72h}$) was 35 µg tPAH50 L^{-1} (corresponding to 10.6 mg Corexit L^{-1}) (Table 3).

Exposure of pediveliger larvae to HEWAF impacted more severely settlement success ($EC50_{72h}$ = 1.7 µg tPAH50 L^{-1}) compared to CEWAF exposure ($EC50_{72h}$ = 35 µg tPAH50 L^{-1}) (Table 3). However, it must be noted that due to a lack of intermediate responses in the HEWAF exposure, we could not calculate reliable confidence intervals for the estimate of EC50.

Finally, LC50 values after 72 h of exposure of pediveligers to HEWAF and CEWAF reached 1530 µg tPAH50 L^{-1} and 88 µg tPAH50 L^{-1} (corresponding to 26 mg Corexit L^{-1}) respectively (Table 3).

Table 3: Effective (ECx) and median lethal concentrations (LC50) of veliger (day 1 to 5), umbo (day 10 to 14) and pediveliger larvae (day 15 to 18) exposed to HEWAF, CEWAF and the Corexit dispersant (1:10 oil ratio) (± 95% CI). ECx and LCx values are expressed as measured tPAH50 in µg L^{-1} for oil alone (HEWAF), as nominal Corexit (in mg L^{-1}) and measured tPAH50 (in µg L^{-1}) for dispersed oil (CEWAF), and as nominal concentration of Corexit (in mg L^{-1}) for dispersant alone.

Exposure solution	Veliger		Umbo		Pediveliger	
	$EC20_{96h}$ (growth)	$LC50_{96h}$	$EC20_{96h}$ (growth)	$LC50_{96h}$	$EC50_{72h}$ (settlement)	$LC50_{72h}$
HEWAF (µg tPAH50L^{-1})	**106** (75-137)	**715** (NC)	**61**[a] (41-80)	**2814**[a] (2738-2875)	**1.7** (NC)	**1530** (1370-1760)
CEWAF (mg Corexit L^{-1})	**25** (0.02-46)	**22.5** (22.1-22.8)	**37** (13-68)	**39.6** (39-40)	**10.6** (9.3-11.8)	**26** (NC)
CEWAF (µg tPAH50L^{-1})	**1.1** (NC)	**41.8** (41.2-42.4)	**8.6**[a] (3.5-14.5)	**72**[a] (71-73)	**35** (31-39)	**88** (85-91)
Corexit (mg L^{-1})	**3.5** (0.7-4.9)	**22.9** (22.5-23.3)	**10.7** (7.6-14)	**58** (57-59)	---	---

[a]: nominal tPAH50.
NC: could not be calculated due to a lack of intermediate responses

4. Discussion

Effects on larval survival

For both veliger and umbo, mortality figures following 96 h of exposure to chemically enhanced WAF (CEWAF) or Corexit alone were dose-dependent. Overall, CEWAF and Corexit showed similar toxic responses at equivalent nominal doses of dispersant tested, particularly in the veliger exposure: this potentially suggests that most of the toxic effect of

the dispersed oil may come from its Corexit fraction. At the highest concentrations of CEWAF and dispersant tested, high instances of larvae with translucent, opened shells and partial decomposition of tissue were consistently observed, indicating a detrimental impact of these substances on oyster larvae. Corexit 9500A® contains hazardous substances including petroleum distillates (solvent), propylene glycol (stabilizer), dioctyl sodium sulfosuccinate or DOSS (surfactant), sorbitan and other ingredients (Nalco Energy Services, 2012). Corexit 9500A® was the main dispersant type used to disperse the *Deepwater Horizon* oil spill in the Gulf of Mexico (National Commission, 2010) with millions of liters released in the Gulf of Mexico (OSAT-1, 2010; U.S. Coast Guard, 2011). Acute toxicity of dispersant alone is usually attributed to its surface active effects on bio-membranes, which include disruption of respiratory cells resulting from electrolytic and/or osmotic imbalances (Singer et al., 1991; 1996). Likewise, our previous work on *C. virginica* revealed severe damages on exposed oocytes and embryos and subsequent larvae (e.g. membrane disruption, extrusion of larval tissue), likely attributable to the dispersant fraction. In addition, gamete and embryo exposed to CEWAF and Corexit generally exhibited similar responses in terms of lethal and sublethal doses (Vignier et al., 2015).

In the case of umbo exposure, CEWAF was shown to induce higher mortalities than dispersant alone (+ 150%) at equivalent nominal doses of Corexit tested (Table 3), suggesting that umbo larvae were more sensitive to CEWAF-associated dispersant than to dispersant alone. Similarly, in a study exposing marine mesozooplankton to Corexit 9500A® alone and chemically dispersed DWH oil (CEWAF), Almeda et al. (2013) observed increasing mortalities after 48 h, from 48% to 72%, which they associated to the additive effects of oil and dispersant. Adams et al. (2014) found that exposure of fish embryos to CEWAF induced higher mortality than exposure to Corexit alone based on just the Corexit concentrations in these exposure solutions. The authors hypothesized that the higher apparent toxicity of the dispersant in the CEWAF was due to the toxicity of the oil in the CEWAF and not the dispersant. In fact, the authors conducted a test using a CEWAF produced with dispersant and Nujol, a non-toxic mineral oil, and found no toxicity on fish. Adams et al. (2014) also found no difference in toxicity between CEWAF and HEWAF exposures and concluded that the toxicity of the CEWAF was due entirely to oil and that there was no synergistic or additive toxicity in the CEWAF due to the presence of dispersant. This result is consistent with comparisons of the toxicity of HEWAF *vs* CEWAF for various fish species that were tested as part of the DWH NRDA (Morris et al., 2015) in which there was no apparent difference in toxicity. However, similar to the results we report for this study, an increased toxicity in

CEWAFs compared to HEWAFs was found for various invertebrate species (e.g., Table 4.3-3 in Chapter 4 of DWH NRDA 2016; Morris et al., 2015), suggesting an additive or synergistic effect of the dispersant on oil toxicity in CEWAF exposures of invertebrates. Similarly, our results from this study suggest that the increased lethality observed in CEWAF-exposed oyster umbo larvae compared to HEWAF-exposed larvae are also associated with the additive or synergistic effects of oil-derived PAHs and dispersant in solution.

Overall, we observed mortalities in all exposed larvae regardless of the initial stage exposed. For instance, we found $LC50_{96h}$ values for CEWAF exposure of veliger and pediveliger ranging from 41.8 to 88 µg tPAH50 L^{-1} respectively. In comparison, Laramore et al. (2014) obtained lower LC50 values for veliger (18 µg tPAH L^{-1}) and pediveliger (16 µg tPAH L^{-1}) exposure to CEWAF. Slight variation in oil preparation or quality of organisms tested could explain these differences using the same species. More specifically, umbo larvae as compared to veliger, showed an increased tolerance (2 to 3 times) to Corexit exposure, alone or in association with oil (CEWAF). In terms of measured tPAH50, our results also showed that pediveliger for instance were more tolerant to CEWAF than veliger larvae ($LC50_{96h}$ = 88 µg tPAH50 L^{-1} *vs* 41.8 µg tPAH50 L^{-1}, respectively). These results are in agreement with the previous study of Vignier et al. (2015), which showed a stage-dependent sensitivity: larvae derived from exposed embryos were 2 to 3 times more tolerant to dispersed oil and dispersant than larvae derived from exposed gametes (e.g. CEWAF: $LC50_{96h}$ = 17.7 µg tPAH50 L^{-1} *vs* 8.5 µg tPAH50 L^{-1}, respectively). Laramore et al. (2014) also showed an enhanced tolerance to oil of more developed (eyed) larval stages of *C. virginica* compared with early larval stages (D-stage). This differential tolerance based on size is likely related to the higher surface area to volume ratio of small organisms which may increase the uptake of dissolved PAHs and/or toxic compounds of dispersant *via* passive diffusion. Similarly, results from other studies on coral larvae (Goodbody-Gringley et al., 2013) and copepods (Jiang et al., 2012) suggested that body size was inversely correlated with oil/PAH toxicity and that difference of sensitivity was related to variations in respiration rates. This size relationship could explain the difference in toxicity observed between the larval stages tested.

Sub lethal effects on growth

In the present work, a consistent decline of shell lengths was observed in larvae exposed to HEWAF, CEWAF and Corexit solutions. This is in agreement with Renzoni (1975) who

reported that *Mulinia lateralis* larvae previously exposed to crude oil were significantly smaller than non-exposed ones. In addition, a consistent decrease of the amount of food present in the gut of larvae exposed to increasing concentrations of CEWAF, HEWAF and dispersant was observed, suggesting a relationship between feeding alteration and growth inhibition. This observation is in agreement with Strathmann (1987) and Hart & Strathmann (1995) who postulated that smaller larvae typically encounter and filter less food, and are therefore more prone to starvation than larger ones. Hence, oil, dispersed oil and dispersant may reduce the fitness of affected larvae by reducing feeding efficiency, and alter larval growth. Rapid valve closure and withdrawal into the shell in response to toxics is a well-known defense mechanism in oyster larvae (Wisely and Blick, 1967). These authors also found that larvae exposed to the highest dose exhibited shells that were sometimes snapped together before the velum had completely retracted, leaving it protruding. In the same way, protruded velum in larvae exposed to high concentrations of oil and dispersant were often observed in our study, indicating a sudden retraction of the larvae in their shell. Narcotic effects manifested as sluggish behavior and/or a cessation of swimming is another sublethal effect resulting from oil exposure, and commonly observed in marine plankton species (Berdugo et al., 1977; Saiz et al., 2009; Almeda et al., 2013). Although narcosis is reversible in most aquatic organisms after recovery in unpolluted waters (Berdugo et al., 1977), a prolonged exposure to narcosis may reduce feeding, growth, and consequently lead to death. Almeda et al. (2013) demonstrated that narcotic effects in copepods may be associated to both volatile components of hydrocarbons (BTEX) and low molecular weight (LMW) PAHs such as naphtalene and acenaphtene. Analysis of exposure media showed that naphtalene was one of the most abundant PAH quantified in the present study (see supplementary file).

In the current study, CEWAF and HEWAF exposure affected larval growth of oyster at values (LOEC and EC20) for tPAH50 well within the range (0 to 100 µg tPAH50 L^{-1}) of reported concentrations of PAHs in water samples collected during the DWH oil spill (Diercks et al., 2010; Allan et al., 2012). In the present work, growth of CEWAF-exposed veliger larvae were reduced at similar levels of tPAH50 ($EC20_{96h}$ = 1.1 µg tPAH50 L^{-1}) than those affecting embryogenesis ($EC20_{24h}$= 9.7 µg tPAH50 L^{-1}) in the embryotoxicity test of Vignier et al. (2015), suggesting that larval growth is a valid endpoint as sensitive as embryogenesis. This is in accordance with other ecotoxicological studies exposing oyster larvae to toxicants (Hidu, 1965; Brereton et al., 1973; Watling, 1982; His and Robert, 1985; Beiras and His, 1994). These results are of particular significance as a marked reduction in

larval growth may lengthen the larval period and increase the risks of predation, disease or dispersion (Davis and Hidu, 1969; Calabrese et al, 1973; Beiras and His, 1994).

For Corexit alone and CEWAF exposures, similar toxic responses on shell length were observed (Fig. 3), suggesting again that most of the toxicity of the CEWAF could be attributed to the chemical properties of the dispersant itself. In terms of growth inhibition, however, our results indicated that exposure to Corexit alone had greater adverse effects on larval growth (lower $EC20_{96h}$) compared to CEWAF-associated Corexit, at equivalent concentration of dispersant (1:10). These results are in agreement with data from Hemmer et al. (2011) which indicated that Corexit 9500 concentrations in CEWAF of Louisiana Sweet Crude were much higher than concentrations of Corexit alone causing lethal acute toxicity to mysid shrimps or inland silversides. Adams et al. (2014) determined that the toxicity of dispersant was vastly mitigated in CEWAFs generated using non-toxic mineral oil, which suggests that the bioavailability of some or all of the toxic components of the dispersant was reduced by the mineral oil. However, potential changes in the composition of active dispersant compounds after mixing with oil has not been studied and reported extensively in the literature. Reviews of existing data by the National Research Council (2005) on the efficacy and effects of dispersants suggests that different components of the dispersant, such as the surfactants, will become bound to oil particles while other components, such as solvents, will likely remain in solution. This stands to reason as the main purpose of the solvents in the dispersant is to help dissolve the surfactants and other additives and not necessarily interact directly with the oil (NRC, 2015). Therefore, it is possible that the toxic constituents in the dispersant that drove the reduced larval growth in our tests were somehow bound to oil or otherwise removed from the exposure solution during the CEWAF mixing and settling process, which would have resulted in a different, somehow less toxic dispersant exposure composition than in the dispersant only exposures. Alternatively, there could be a physiological explanation for the apparent decrease in the dispersant's sublethal toxicity in the CEWAF, whereby the combined exposure to oil and dispersant results in a different mode of action than when oyster larvae are exposed to dispersant alone. For Corexit alone exposures, $EC20_{96h}$ values, expressed as nominal concentrations of Corexit, were lower for veliger compared to umbo larvae (Table 3) indicating that veligers were more sensitive to dispersant than umbo larvae.

For oil alone exposure (HEWAF), veliger or umbo larvae were both impacted in a dose-dependent way, shell lengths being significantly reduced compared to control. Interestingly, a lower $EC20_{96h}$ was found for the umbo exposure compared to the veliger exposure with the

HEWAF (61 *vs* 106 µg tPAH50 L^{-1}). However, this is not a significant difference (confidence limits overlap) and the umbo $EC20_{96h}$ value was calculated based on nominal concentrations. In contrast to the CEWAF and Corexit exposures, the HEWAF exposure did appear to generally have a more severe effect on growth of umbo larvae than veliger larvae. This could be explained by the fact that umbo larvae have a higher filtration capacity compared to veliger larvae. As a result, a mechanical action of the particulate oil on gills and velum may impair the normal physiology of the larvae and explain the difference observed between veliger and umbo larvae exposed to HEWAF. Many studies investigating oil toxicity on aquatic organisms highlighted the fact that most toxic effect of crude oil was related to the dissolved fraction of PAH (Barron et al., 1999, 2003; Ramachandran et al., 2004; Carls et al., 2008; Nordtug et al., 2011). However, it has been shown recently by several authors (Lee et al., 2012; Almeda et al., 2013, 2014) that crude oil toxicity could also be associated with its particulate fraction. It has been well documented that some filter-feeding plankton species could ingest these oil droplets, which are in the same range as their food spectrum (Lee et al., 1978, 2012; Hansen et al, 2012; Almeda et al., 2014). Given the fact that oil droplets were observed within our exposed organisms, direct ingestion of particulate oil by oyster larvae is a potential route of exposure.

Effects on settlement success

We found that following 72 h of exposure to HEWAF or CEWAF, settlement success of pediveliger was impaired in a dose-dependent manner. The present study reported sublethal effects of dispersed oil (HEWAF and CEWAF) causing a 50% decrease in settlement success at concentrations ranging from 1.7 to 35 µg tPAH50 L^{-1}. During oil spills, total PAHs concentration may frequently range from 1 to 150 µg L^{-1} (Neff and Stubblefield, 1995; Law et al., 1997). Reported concentrations of total PAHs in water samples collected during the DWH oil spill ranged from 146 mg tPAH L^{-1} near the wellhead to below detection limit in distant waters (Diercks et al., 2010; Boehm et al., 2011; Wade et al., 2011), including reported value of 1.7 µg tPAH33 L^{-1} in coastal waters (Allan et al., 2012). The finding of the present study implies that relatively low concentrations of PAHs (e.g. 1.7 µg tPAH50L^{-1}), at levels realistically found in the environment at the time of the DWH oil spill, could have detrimental consequences on metamorphosis/settlement of *C. virginica* larvae. Our study also demonstrated that HEWAF solutions reduced larval settlement at concentrations much lower

than the doses of HEWAF inhibiting larval growth (1.7 *vs* 106 µg tPAH50L^{-1}), suggesting that larval settlement inhibition is a very sensitive endpoint. It would thus be interesting to include it in toxicological assessment of crude oil and dispersant. Several studies investigating the negative effects of heavy metals (Watling, 1983; Beiras & His, 1994), pesticides (Mottier et al., 2013), or oil-contaminated sediments (Phelps & Warner, 1990, His et al., 1997) on the settlement of oyster larvae have shown that metamorphosis failure is a valid bio-indicator of general toxicity for exposure of *C. gigas* to contaminants. However, to our knowledge, this is the first time settlement success was studied as an endpoint using *C. virginica* pediveliger exposed acutely to oil and dispersant, particularly without the use of the chemical inducer epinephrine.

As opposed to mortality response (LC50), settlement success was relatively more sensitive to HEWAF than CEWAF solutions, with $EC50_{72h}$ for HEWAF much lower than $EC50_{72h}$ reported for CEWAF (1.7 µg tPAH50 L^{-1} vs 35 µg tPAH50 L^{-1}). Chemical characteristics of HEWAF and the contribution of dissolved PAH found in higher proportion in low doses of HEWAF preparations (Forth et al., 2015), or the cumulative effects of dissolved and droplet-associated PAHs, may explain the accrued impact of HEWAF on larval settlement, at equivalent nominal concentrations. In addition to the toxic effect of PAHs on pediveliger, we could also suspect that coating of settlement substrate by crude oil or oil-associated droplets might have been deleterious to the settlement of competent larvae. Similarly, Smith & Hackney (1989) found that setting of larvae on oil-treated shells was delayed and spat recruitment on oiled-shells was significantly lower than control shells. Banks & Brown (2002) showed that clay tiles previously exposed to hydrocarbons in the laboratory depressed settlement success of *C. virginica* larvae. Further research is required to elucidate the mechanisms by which oil/PAHs and/or dispersant affect the processes of larval metamorphosis and settlement.

Observed effects of oil/dispersant on larval development and metamorphosis led to wonder whether larvae could recover from a temporary acute exposure. Following a 24-h exposure of *C. virginica* larvae to sublethal concentration of CEWAF (12 µg tPAH L^{-1}), Laramore et al. (2014) monitored the subsequent larval development for 3 weeks in clean seawater, and did not find significant reduction of larval growth. However, they found that survival was negatively affected 3 weeks post-exposure to 12 µg tPAH L^{-1} of CEWAF. In this light, more research should be done on the impacts of oil exposure to larvae and the subsequent capacity

to recover in non-exposed seawater. Mild effects on the physiology of oyster larvae (e.g. filtration) may only be deleterious for a few days, and larvae could recover rapidly as shown by Ben Kheder et al. (2010b) with *C. gigas*. Other processes such as larval settlement might be delayed substantially due to delays in growth, and potentially never occurs because of a temporary oil/dispersant exposure. Such contaminant-induced delay combined or not with other adverse effects may increase the larval predation risk and thus affect population dynamic and structure.

5. Conclusion

Results of the present work demonstrated that oil alone (HEWAF), dispersed oil (CEWAF) and dispersant alone (Corexit 9500A) were highly toxic to *C. virginica* larvae, regardless of the stage of development. DWH oil alone, dispersant alone or the combination of both significantly inhibited larval growth and settlement success, and reduced survival. Oyster larvae were sensitive to toxic effects from oil/PAHs and particularly the Corexit, alone or in combination with oil (CEWAF). Moreover, HEWAF exposure of pediveliger larvae (and to a lesser extent umbo larvae) highlighted the necessity of considering both particulate oil and the dissolved oil fraction and the associated toxicity mechanisms in ecotoxicological study. Overall, larval growth and settlement success are sensitive physiological endpoints since these bioassays allowed detection of toxic effects at environmentally relevant concentrations (i.e. < 10 µg tPAH50 L^{-1}). Accordingly they are useful indicators for a realistic assessment of the impact of a major oil spill like the DWH event.

It has to be denoted that in the natural environment, toxicity of crude oil depends not only on the concentration and the duration of exposure, but also on environmental conditions. For instance, temperature, UV radiation or salinity may increase substantially the toxicity of crude oil to marine organisms (Jewell, 1994; Pelletier et al., 1997; Lyons et al., 2002; Ramachandran et al., 2006; Almeda et al., 2013; Alloy et al., 2015). As a result, estimated lethal and sublethal concentrations of tPAH50 from the current study are most likely conservative. Alone or in combination with other environmental stressors, deleterious effects of DWH oil and dispersant on growth and metamorphosis/settlement could have important implications on the recruitment for the following year, and cause long-term negative impacts on the population dynamics of oysters in the Gulf of Mexico.

Acknowledgment

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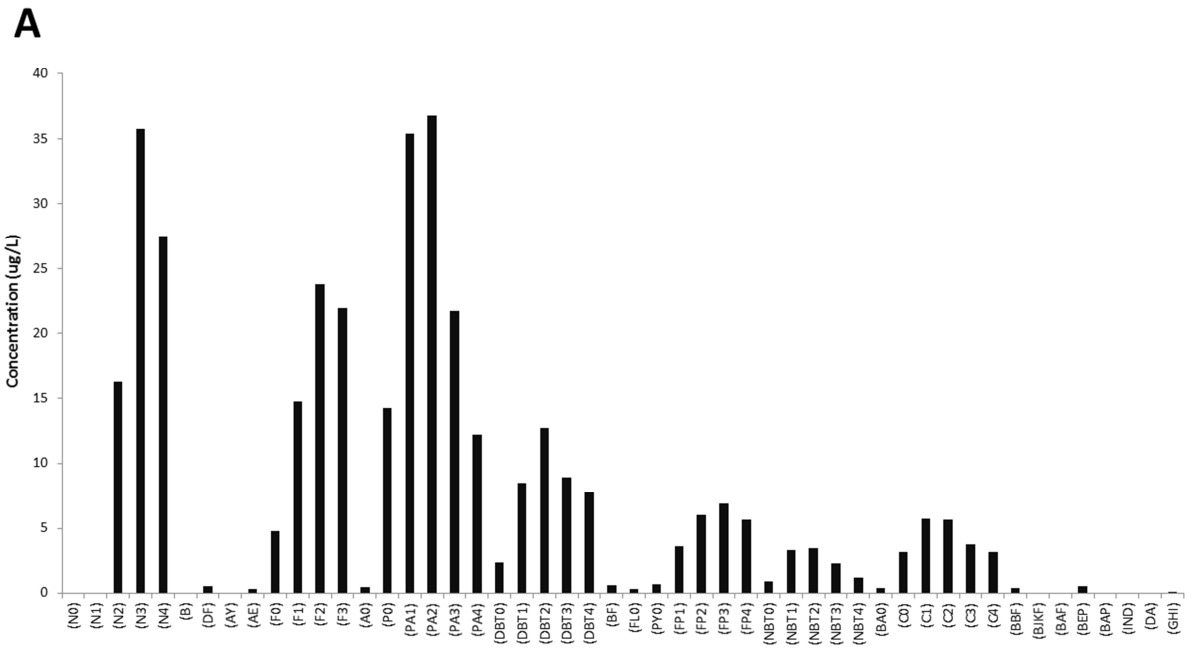

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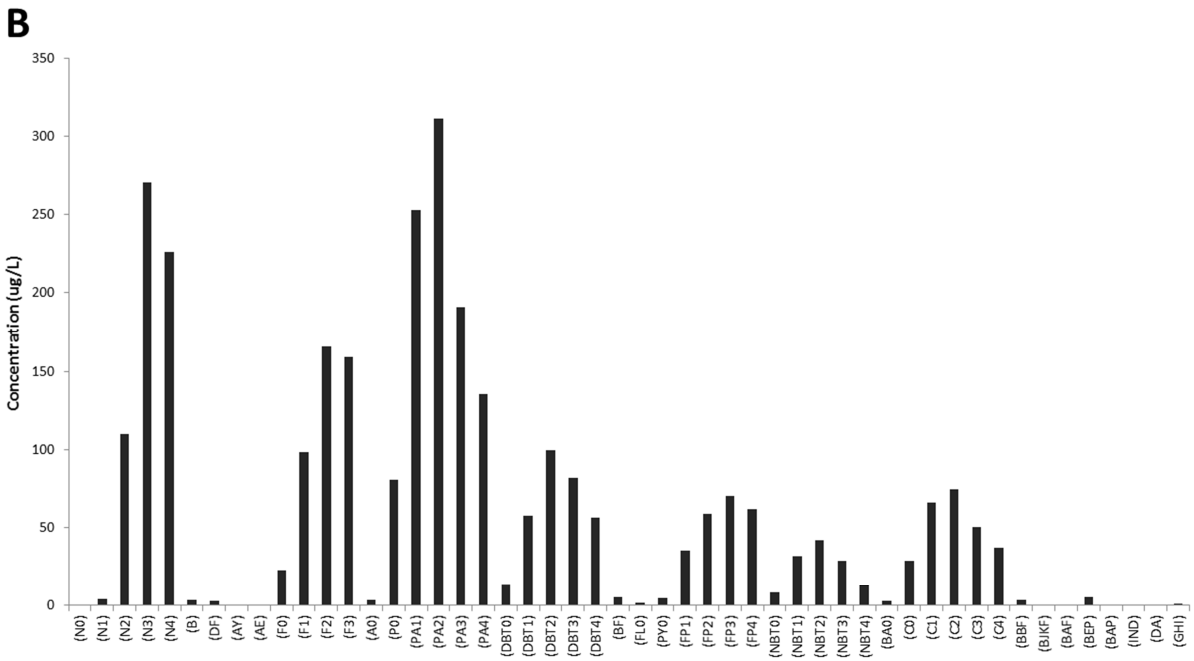

2

3 **Supplementary file:** PAHs content in stock solutions of (A) CEWAF, and (B) HEWAF, expressed
4 in µg L^{-1} quantified by GC/MS-SIM. Stock solutions correspond to nominal oil load of 2 g oil L^{-1}. N0-
5 4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene;
6 PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0:
7 Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4: Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-
8 4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF:
9 Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA:
10 Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0),
11 while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc.
12 *Target method detection limit range: 1 - 5 ng/L.* Additional information regarding the PAH analytes
13 and the tPAH50 sum can be found in Forth et al. (2015).

Highlights

- *Deepwater Horizon* oil spill coincided with oyster spawning and recruitment season.
- Different *C. virginica* larval stages were exposed to oil and/or Corexit for 72-96h.
- HEWAF, CEWAF and Corexit reduced larval growth, settlement success and survival.
- Toxic effects were observed at environmentally relevant concentrations of tPAH50.
- Sublethal doses of PAH may impact oyster populations and the whole ecosystem