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Photo-enhanced toxicity of two weathered Macondo crude oils to early life stages of the eastern oyster (*Crassostrea virginica*)

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) have been reported to absorb ultraviolet (UV) light, resulting in enhanced toxicity. Early developmental stages of bivalves may be particularly susceptible to photo-enhanced toxicity during oil spills. In the current study, toxicity tests were conducted with sperm and three larval ages of the eastern oyster (*Crassostrea virginica*) to evaluate the photo-enhanced toxicity of low-energy water-accommodated fractions (WAFs) of two weathered Macondo crude oils collected from the Deepwater Horizon incident. Larvae exposed to oil WAFs under UV-filtered light demonstrated consistently higher survival and normal development than larvae exposed to WAFs under UV light. The phototoxicity of weathered Macondo oil increased as a function of increasing UV light intensity and dose. Early developing oyster larvae were the most sensitive to photo-enhanced toxicity, whereas later shelled prodissoconch larvae were insensitive. Comparisons between two weathered crude oils demonstrated that toxicity was dependent on phototoxic PAH concentration and UV light intensity.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) occur naturally as well as anthropogenically and can be acutely toxic to aquatic organisms through a narcosis mechanism (Bradbury et al., 1989). Polycyclic aromatic hydrocarbons have demonstrated the ability to absorb ultraviolet (UV) light, thereby enhancing their toxicity to aquatic organisms (Newsted and Giesey, 1987; Mekenyan et al., 1994). Absorption of UV light can lead to photo-enhanced toxicity of PAHs, a phenomenon dependent on the PAH compound, aqueous concentration, and UV light dose (Newsted and Giesey, 1987; Finch and Stubblefield, 2015). Polycyclic aromatic hydrocarbons with 3–5 rings are of greatest environmental concern for phototoxicity as a result of their amphipathic properties, thereby maximizing solubility in water, while retaining lipophilic properties that promote uptake into tissues of aquatic organisms. The generation of reactive oxygen species induced from photo-excitation is recognized as the primary mode of toxic action of photoactive PAHs (Choi and Oris, 2000), resulting in damage to cell membranes and biomolecules (Landrum et al., 1987; Sinha and Hader, 2002).

Polycyclic aromatic hydrocarbons are commonly detected in the environment and often occur in complex mixtures, such as in crude oils. Environmental weathering of oil results in loss of substantial portions of lighter-ringed PAHs, while heavier PAHs and other hydrocarbons are typically retained (Riley et al., 1980; Wang et al., 1998). Weathering is attributed to various chemical, physical and biological processes, including photolysis, microbial degradation, dissolution, wave action, evaporation, and wind action (Sivadier and Mikolaj, 1973; Larson et al., 1977; Jordan and Payne, 1980) that can substantially alter the composition and toxicity of oil to aquatic organisms. However, weathered crude oil has been reported to contain phototoxic compounds that result in phototoxicity (Cleveland et al., 2000; Barron et al., 2003). Other modifying factors that determine photo-enhanced toxicity due to effects on UV light intensity include water quality characteristics (turbidity, dissolved organic carbons, chlorophyll), photoperiod, sun angle, cloud cover, ozone, aerosol thickness, shaded habitat, and organism life history traits (Finch and Stubblefield, 2015; Baker et al., 1980; Smith and Baker, 1981; Ireland et al., 1996; Gensemer et al., 1998; Nikkila et al., 1999). Such variation in atmospheric and hydrospheric conditions can have strong influences on photo-enhanced toxicity, making it a site-specific phenomenon.

Oil in aquatic environments is of particular concern to broadcast spawners such as bivalves, pelagic fish, copepods, echinoderms, and corals, which often release free-floating gametes on or near the water's surface increasing the likelihood of exposure to oil constituents and UV light. Bivalve larvae are particularly sensitive to organic contaminants, especially in the presence of UV light (Bellas et al., 2008). However, little data is available on the photo-enhanced toxicity of PAHs to aquatic

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invertebrates and even fewer studies have examined effects on bivalves. Spehar et al. (1999) found that fluoranthene in the presence of UV light resulted in a >40-fold increase in toxicity to developing embryos and larvae of the coot clam (*Mulinia lateralis*) compared to fluoranthene in the absence of UV light. Furthermore, Pelletier et al. (1997) reported a 50,000-fold increase in toxicity of pyrene to embryos of coot clams (*Mulinia lateralis*) in the presence of UV light compared to fluorescent lighting. Pyrene was also reported to impair 100% of embryonic development of the Pacific oyster (*Crassostrea gigas*) at 25 nM under UV light, whereas 500 nM caused 50% inhibition in embryonic development under fluorescent light (Lyons et al., 2002). Boese et al. (1997) examined photo-enhanced toxicity of seven benthic crustaceans that occupied different habitats and found that UV exposure enhanced fluoranthene toxicity in five of the seven species tested (*Rhepoxynius abronius*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, *Grandidierella japonica*, *and Corophium insidiosum*). Susceptibility to photo-enhanced toxicity was found to be dependent on life history traits of crustaceans, with organisms commonly exposed to UV light being less sensitive than those less likely to be exposed to sunlight in their natural habitat.

The Deepwater Horizon (DWH) oil spill resulted in the release of an estimated 3.19 million barrels of crude oil into the Gulf of Mexico (United States District Court for the Eastern District of Louisiana, 2015), some of which was present as floating weathered crude oil that may have affected exposed gametes of broadcast spawners. Macondo crude oil released from the DWH wellhead traveled 1500 m from the seafloor and had to be transported considerable distances before reaching shorelines, resulting in extensive oil weathering. Therefore gametes of broadcast spawners located near-shore were more likely exposed to weathered Macondo crude oil rather than source oil.

The Gulf of Mexico is a highly productive area that supports an abundant and diverse number of species (Moretzsohn et al., 2010), including the eastern oyster (*Crassostrea virginica*). The eastern oyster is of interest due to its economic and environmental importance. Annual average harvests of the eastern oyster in the Gulf of Mexico from 2007 to 2009 were 22 million pounds, with an economic value of $67 million (NOAA, 2011). The eastern oyster also serves as a keystone species for benthic communities through the provision of shelter and biodeposition of nitrogen and phosphorus (Newell, 2004). The toxicity of oil components to larvae of the eastern oyster has been well studied (Stefansson et al., 2016; Langdon et al., 2016), but little is known about their susceptibility in the presence of UV light. The objectives of the current study were to: 1) determine the relative sensitivities of early larval developmental stages of eastern oysters to combinations of UV light and water-soluble fractions of weathered crude oils collected from the DWH oil spill, and 2) to evaluate the influence of UV light intensity on the photo-enhanced toxicity of two weathered DWH oils for early oyster larvae.

2. Methods

2.1. Test materials

Test oils used in the current study were collected from the Gulf of Mexico during the DWH oil spill (BP Gulf Science Data, 2014). Weathered CTC oil was recovered from the CTC02404 barge on July 29, 2010 by skimming the surface of Gulf of Mexico waters. Weathered Juniper oil was recovered from the Gulf of Mexico by skimming the surface approximately 25 miles northwest of the MC-252 wellhead by the Coastguard cutter "Juniper" on July 19, 2010. Test oils were stored in a refrigerator at 4 °C in the dark prior to use.

2.2. Experimental design

Eastern oyster broodstock were obtained from a commercial supplier in Washington State (Taylor Shellfish, Shelton, WA, USA). Broodstock were conditioned in the laboratory for 3 to 8 weeks, depending on reproductive condition. Oysters were held in flowing 10 μm filtered seawater from Yaquina Bay (Newport, OR, USA) at 20 °C. They were fed a mixed algal diet of *Isochrysis galbana* and *Chaetoceros* sp. during the conditioning period. Spawning was induced by thermal shock at a maximum temperature of 30 °C, resulting in a fertilization rate of >90%.

Acute static tests (48-h) were conducted with eastern oyster larvae using USEPA (1995) and ASTM E724-98 (ASTM, 2004) methods. The 48-h acute static tests in this study deviated from standard bivalve methods in that test vials were horizontally oriented on their side rather than positioned upright, in order to expose larvae to the UV light source without interference from the vial's cap. Additional sets of upright control vials held in the dark were included in each test, in which vials were kept upright in the dark to ensure that gamete quality was sufficient to meet acceptable larval survival criteria for bioassays. Test chambers consisted of 20 mL glass scintillation vials with high UV transmission (Kimble Chase, KG-33 borosilicate glass) and Teflon-lined screw caps. Treatments were conducted with four replicates, each with 10–15 larvae/mL of test solution (20 mL total; 200–300 larvae per replicate). Test treatments consisted of a dilution series of water-accommodated fractions (WAFs; 100%, 50%, 25%, 12.5%, 6.25%) of crude oils, a seawater and gamete control in upright vials in the dark, horizontal vials in the dark or under a UV-filter, and a fluoranthene (Sigma Aldrich, >98% purity, Saint Louis, MO, USA) positive control (30 μg/L). The fluoranthene positive control was prepared in acetone (0.01%), and therefore, a solvent control was also included in toxicity testing. Fluoranthene is a known phototoxic PAH that was expected to elicit phototoxic effects on larval eastern oyster survival and development.

Low energy WAFs of test oils were generated according to methods described in Chemical Response to Oils Spills: Ecological Effects Research Forum (Singer et al., 2000, 2001a,b) and Critical Evaluation of CROSERF Test Methods for Oil Dispersant Toxicity Testing under Subarctic Conditions (Barron and Ka'aihue, 2003). Seawater used in testing was obtained from Yaquina Bay (Newport, OR, USA) and filtered through a 0.2 μm filter prior to use. Water-accommodated fractions were prepared at oil to water ratios of 1 g of oil per liter of seawater (±20%) in a 4-L glass liter aspirator bottle with a bottom dispensing port (VWR; Radnor, PA, USA). Aspirator bottles were placed on stir plates and slowly stirred without a vortex at 60 rpm. Oil-in-water suspensions were stirred for 20 h followed by a 4-hour settling period. After 24 h, WAFs were collected for analytical characterization and toxicity testing. Water quality of test solutions were recorded at 0 h (test initiation) and 48 h (test termination) by measuring dissolved oxygen (DO), temperature, and pH were measured using a Hach meter (Model No. HQ40D, Loveland, CO, USA) and salinity was measured using an Oakton SALT 6+ salinity meter (Vernon Hills, IL, USA).

Test solutions (WAFs) for photo-enhanced toxicity tests were placed under either artificial UV light or UV-filtering plexiglass (Acrylite OP-3 UV Filtering Museum Quality Plexiglass, EVONIK Industries, Parsippany, NJ, USA). The spectral transmittance of OP-3 plexiglass is presented in the Supplemental Data, Fig. S1. Ultraviolet light was produced from a solar simulator (Atlas SolarConstant 1200 Single Control, Germany) that was equipped with metal halide lamps, each with a max power of 1200 W. The spectral distribution of each lamp was from 280 to 3000 nm, emulating natural UV sunlight conditions (Supplemental Data, Fig. S2). Irradiance was measured for UV-A, UV-B, and RFF (visible light) at the beginning and end of each exposure period at the water's surface using a Macam portable radiometer Model UV203-3 (Macam Photometries, Livingston, Scotland; annually calibrated at the National Physical Laboratory, Teddington, Middlesex, UK). Light transmission for the glass test vials used in the current study is presented in Fig. S3 of the Supplemental Data. UV-A and UV-B transmission rates through test vials ranged from 70 to 90% and 35–65%, respectively, depending on wavelength and were similar to those reported with borosilicate glass jars reported in Finch and Stubblefield (2015). Comparisons among light intensities were made using measured UV-A ($\lambda = 320$–400 nm) values. UV-A is present at higher intensities in sunlight and

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can penetrate to deeper depths in seawater compared with UV-B, which represents only 8% of total available UV radiation (Lean, 1998).

At the end of the 48-h exposure period, larvae in vials were counted to determine the total number of abnormal and normal D-shaped larvae. Larvae were considered abnormal if they had incomplete shell development, severely misshapen shells, or if the velum protruded from between the shell valves. Survival and normal shell development were determined at the conclusion of the study and used to calculate the median lethal concentration (LC50) and effective concentration that resulted in 10% abnormal larvae in the test population (EC10).

2.3. Larval stage sensitivity

Phototoxicity tests to compare sensitivities of different larval developmental stages were conducted with CTC oil WAFs (100, 50, 25, 12.5, 6.25% WAF dilutions), seawater alone control, and a seawater plus UV light control. Studies were conducted with sperm and three larval developmental stages designated as early (2–4 h post-fertilization (pf)), mid (8–10 h pf), and late (44–46 h pf) larvae. The two-hour variation in initial larval ages was due to differences among experiments in the time required to start tests after egg fertilization. For each larval stage, WAF exposure began 2 to 4 h after egg fertilization. Developing larvae were exposed to UV light for 6 h at nominal UV-A intensities of 3, 6, 12, and 18 W/m^2 or to UV-filtered light. Ultraviolet light exposures began at test initiation for the early life stage (2–4 h pf), after 6-h of development in WAF media for the mid larval stage (8–10 h pf), and after 42-h of development in WAF media for the late larval stage (44–46 h pf).

Eastern oyster sperm were exposed to CTC oil WAFs in combination with UV light prior to fertilization to determine their susceptibility to photo-enhanced toxicity. Sperm were exposed for 1 h at nominal intensities of 3, 6, 12, and 18 W/m^2 UV-A or to UV-filtered light. Subsequently, UV-exposed sperm was used to fertilize eggs that were then placed in the dark for the remainder of the 48 h assay (1:47 h light:dark cycle). Sperm were also exposed to a CTC oil WAF in the dark to compare the effects of WAF in the absence of UV light exposure. Experimental UV-A, UV-B, and visible light intensities and total UV energy doses for both sperm and larval tests are presented in Supplemental Data, Table S1.

2.4. Photo-enhanced toxicity of two weathered Macondo crude oils

Eastern oyster larvae were used to compare the photo-enhanced toxicity of WAFs of two weathered Macondo oils (CTC and Juniper) at dilutions of 100, 50, 25, and 12.5% or seawater alone, with exposure to nominal UV-A light intensities of 3, 6, 12, and 18 W/m^2 UV-A or to UV-filtered light for 6 h. Exposure to WAFs began at test initiation (2–4 h pf) and UV light exposure began when oysters reached the mid larval age (8–10 h pf), resulting in a 6 h PAH loading period. The mid larval age was chosen because this life stage was both sensitive to photo-enhanced toxicity and was a more convenient larval stage for use in bioassays than earlier stages.

2.5. Analytical chemistry methods

Chemical analyses were performed on all parent WAF solutions (100% solution) evaluated in toxicity tests. Samples were analyzed for parent and alkylated polycyclic aromatic hydrocarbons (PAHs), saturated hydrocarbon compounds (SHCs), and volatile organic compounds – paraffins, isoparaffins, aromatics, naphthenes, olefins (VOC-PIANO; see Table 2 of Stefansson et al., 2016 for list of analytes). A total of 46 PAHs were used to calculate total PAHs (tPAHs). To estimate the concentrations of phototoxic PAHs in WAFs, the sum of parent and alkylated (C1–C4) pyrenes (including benzo[*a*]pyrene and benzo[*e*]pyrene), anthracenes (including benz[*a*]anthracene and dibenz[*ah*]anthracene), chrysenes, and fluoranthenes (including benzo[*a*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*b*]fluoranthene) were recorded. Alkylated anthracenes and phenanthrenes were measured in combination; however, phenanthrene has zero to very little phototoxic potential (depending on alkylation; Finch et al., submitted for publication) and comprised a large fraction of Macondo crude oils. Consequently, phototoxic PAH concentrations were presented with and without phenanthrene/anthracene measurements (Table 1).

Samples of the initial 100% WAFs solutions were collected during each test for analytical characterization. Samples were also collected at the end of each test (48 h) from extra test vials filled with 100% WAF solutions that were placed under the different UV light treatments to assess the effects of UV light on WAF composition during the assays. WAF samples were shipped overnight to Battelle (Norwell, MA, USA) for trace volatile and semi-volatile hydrocarbon analyses and processed via liquid-liquid extraction with methylene chloride. Saturated hydrocarbons were analyzed by gas chromatography – flame ionization detector (GC-FID). Parent and alkylated PAHs were analyzed by gas chromatography/mass spectrometry using selected ion monitoring (GC/MS-SIM) and modifications of EPA SW-846 Method 8270. Quantification methods were modifications of protocols described in NOAA's Technical Memorandum #130, Sampling and Analytical Methods of the National Status and Trends Program Mussel Watch Project: 1993–1996 Update (NOAA, 1998). Samples for VOC-PIANO, were analyzed by Battelle by purge-and-trap GC/MS (EPA SW-846 Method 8260). Fluoranthene was analyzed using methods presented in Ramirez et al. (2014).

2.6. Statistical analysis

Larval survival and development data were used to calculate LC50 and EC10 values, respectively. EC10 values were defined as the number of normal larvae at test termination (48 h) divided by the initial number of embryos added at test initiation. Toxicity values are presented on a percent WAF basis to enable comparisons of the two weathered crude oils and on a tPAH basis to represent exposure concentrations. Toxicity values (LC50s and EC10s) were converted to a tPAH basis by

Table 1
Mean total PAH (tPAH), saturated hydrocarbons (SHC), benzene, toluene, ethylbenzene, and xylene (BTEX), and phototoxic PAHs measured in 100% WAFs. Values are the means of samples collected at test initiation (0 h) and test termination (48 h) unless otherwise noted.

Life stage	Nominal UV-A intensity (W/m^2)	Oil type	tPAH (μg/L)	SHC (μg/L)	BTEX (μg/L)	Phototoxic PAHs[a–c] (μg/L)
Life stage sensitivity and uv intensity studies						
Sperm	No UV	CTC	14.9[c]	2.64[c]	0[c]	0.52 (2.57[d])
Sperm	3	CTC	14.9[c]	2.64[c]	0[c]	0.52 (2.57[d])
Sperm	6	CTC	14.9[c]	2.64[c]	0[c]	0.52 (2.57[d])
Sperm	12	CTC	14.9[c]	2.64[c]	0[c]	0.52 (2.57[d])
All larvae	UV filtered	CTC	12.4	0.97	0.40	0.52 (2.57[d])
All larvae	3	CTC	12.4	0.93	0.40	0.52 (2.57[d])
All larvae	6	CTC	12.2	1.04	0.40	0.52 (2.57[d])
All larvae	12	CTC	12.1	0.98	0.40	0.52 (2.57[d])
Photo-enhanced toxicity comparisons among weathered crude oils						
Larvae	UV filtered	CTC	13.2	1.99	0.04	0.62 (2.60[d])
Larvae	3	CTC	13.3	1.73	0.04	0.62 (2.60[d])
Larvae	6	CTC	13.1	1.86	0.04	0.62 (2.60[d])
Larvae	12	CTC	13.0	1.75	0.04	0.62 (2.60[d])
Larvae	UV filtered	Juniper	3.15	0.51	0	0.28 (1.25[d])
Larvae	3	Juniper	3.07	0.88	0.05	0.28 (1.25[d])
Larvae	6	Juniper	3.02	0.54	0.05	0.28 (1.25[d])
Larvae	12	Juniper	2.88	0.51	0.05	0.28 (1.25[d])

[a] Concentrations of phototoxic PAHs at test initiation.
[b] Sum of all parent and alkylated anthracenes (parent only), pyrenes, chrysenes, and fluoranthenes.
[c] Chemical analysis occurred only at test initiation.
[d] Phototoxic PAH concentration including alkylated phenanthrenes/anthracenes (see methods).

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multiplying percent WAFs by tPAH concentrations in the 100% WAF (Tables 2-3). UV light alone was deleterious to sperm and oyster larvae and, therefore, LC50s and EC10s of WAFs were corrected for mortality due to UV light alone, using Abbott's formula (Emmens, 1948). LC50 and EC10 values were derived by probit regression analysis with R statistical software using the MASS package ($\alpha = 0.05$; R Core Development Team, 2010). Significant differences between LC50 values were determined using the LC50 ratio test (Sprague and Fogels, 1977). The LC50 ratio test compares the ratio of LC50s to 1; a method with significantly more power than the confidence interval overlap procedure (Wheeler et al., 2006). Fluoranthene and solvent controls were compared using two-sample *t*-tests.

To accurately compare toxicity values of two weathered Macondo crude oils with different chemical compositions, phototoxicity estimates were based on the relative proportions and potencies of individual phototoxic PAHs in WAFs as well as UV-A dose ($\mu W \cdot h/cm^2$). Using methods described by Sellin Jeffries et al. (2013), comparisons between LC50 values of CTC and Juniper oils were carried out using dose metrics that integrate UV-A intensity and predicted total phototoxic PAH equivalents ($tpPAH_{eq}$) in tissues. Measured concentrations of phototoxic PAHs in water were converted to tissue concentrations using bioconcentration factors (BCFs) estimated using the equation presented in Donkin et al. (1991) for blue mussels (*Mytilus edulis; logBCF* $= -2.220 + 0.965\ \log K_{ow}$), as it has been shown to be the best approximation for BCFs in short-term PAH exposures (Okay and Karacik, 2008). To account for differences in potencies among individual phototoxic PAHs, relative photodynamic activities (RPA) were calculated from data presented in Newsted and Giesey (1987). Potencies of phototoxic PAHs were determined by calculating the RPA of each PAH relative to anthracene as described in Sellin Jeffries et al. (2013). The RPAs were then multiplied by the predicted phototoxic PAH concentrations in tissues. Total PAH equivalents were calculated by taking the sum of phototoxic PAHs in tissues. Integrated dose was subsequently calculated by multiplying UV-A dose and total PAH equivalents. Alkylated phototoxic PAHs were considered to have the same RPA values as parent phototoxic PAHs excluding the combined anthracene/phenanthrene measurement. A mean RPA value for pyrene and fluoranthene were used, as they were measured in tandem during analytical measurements. A comprehensive list of phototoxic PAHs, RPAs, and BCFs used in integrated dose calculations is included in the Supplemental Data, Table S2.

Table 3
Comparison of median lethal concentrations (LC50), and effective concentrations resulting in 10% abnormal larvae (EC10s) of CTC and Juniper weathered crude oils to eastern oyster larvae (8–10 h post-fertilization) exposed to different UV light intensities for 6 h.

Oil type	UV-A intensity[a] (W/m^2)	LC50[a,b] (% WAF; 95% CI)	EC10[a,b] (% WAF; 95% CI)
CTC	0.243 (UV filter)	>100 [A]	31.2 (10.3–52.1) [A]
Juniper	0.243 (UV filter)	>100 [A]	>100 [B]
CTC	3.27	>100 [A]	64.0 (17.9–107.1) [A]
Juniper	3.27	>100 [A]	60.8 (0.0–121.9) [A]
CTC	6.59	50.5 (40.6–60.3) [A]	13.5 (7.85–19.1) [A]
Juniper	6.59	61.4 (49.7–73.1) [A]	7.26 (1.42–13.1) [A]
CTC	11.2	42.1 (34.1–50.0) [A]	9.86 (4.99–14.7) [A]
Juniper	11.2	48.9 (40.5–57.3) [A]	13.7 (7.19–20.1) [A]

[a] Comparisons were made at each UV intensity via the LC50 ratio test [Sprague and Fogels, 1977].
[b] Values sharing the same capital letters in brackets are not significantly different ($p > 0.05$).

3. Results

Oyster larvae in seawater controls placed upright in the dark resulted in ≥70% survival (actual survival was 87%) and ≥90% normal development, which met criteria of EPA's standard methods for bivalve larval assays and indicated that gamete and seawater quality were satisfactory (Supplemental Data, Fig. S4). Survival of oyster larvae in control vials oriented on their sides was 66%, which is slightly below EPA's criteria (Supplemental Data, Fig. S4); however, horizontal orientation of vials was necessary in order to allow exposure to UV light without interference from the vials' opaque cap. The slight negative effect of side orientation may have been due to the reduced seawater depth available for vertical larval swimming, resulting in larvae more frequently encountering the upper walls of the vials.

In the phototoxicity bioassays, UV light alone was deleterious to a proportion of early oyster larvae and therefore all data were corrected for UV light mortality (Supplemental Data, Fig. S4). The UV-A intensity of 18 W/m^2 alone (in the absence of WAFs) resulted in only approximately 10% survival of early and mid larvae; therefore data were not analyzed due to inadequate numbers of surviving larvae for accurate estimations of toxicity values. Survival of early larvae at the next highest intensity (12 W/m^2 UV-A) in seawater was as low as 43%; however, each replicate contained 200–300 oyster larvae at test initiation,

Table 2
Comparison among median lethal concentrations (LC50s) and effective concentrations resulting in 10% abnormal larvae (EC10s) of CTC oil WAFs tested with different developmental stages (sperm and early, mid, and late larvae) of the eastern oyster under artificial UV light for 6 h or under UV-filtering plexiglass. Sperm controls were exposed to CTC oil WAF in the dark instead of using a UV-filter. Toxicity values are presented in terms of percentages of the 100% WAF solution and total PAH (tPAH) concentrations.

Age at UV exposure (hours)	UV Exposure duration (hours)	Life stage	Measured UV-A intensity (W/m^2)	LC50[a,b] (% WAF; 95% CI)	LC50 (μg/L tPAH; 95% CI)	EC10[a,b] (% WAF; 95% CI)	EC10 (μg/L tPAH; 95% CI)
0	1	Sperm	0 (No UV)	>100 [A]	>14.9 [A]	>100 [A]	>14.9 [A]
0	1	Sperm	3.38	>100 [A]	>14.9 [A]	>100 [A]	>14.9 [A]
0	1	Sperm	6.26	>100 [A]	>14.9 [A]	26.2 (9.74–42.7) [B]	3.90 (1.45–6.36) [B]
0	1	Sperm	12.0	>100 [A]	>14.9 [A]	24.7 (14.4–35.1) [B]	3.68 (2.15–5.23) [B]
2–4 (early)	6	Larvae	0.344 (UV filter)	>100 [A]	>12.4 [A]	>100 [A]	>12.4 [A]
2–4	6	Larvae	3.08	>100 [A]	>12.4 [A]	>100 [A]	>12.4 [A]
2–4	6	Larvae	6.74	97.6 (71.8–123.4) [A]	11.9 (8.76–15.1) [A]	21.4 (13.7–29.0) [B]	2.61 (1.67–3.54) [B]
2–4	6	Larvae	11.4	41.6 (34.1–49.0) [B]	5.03 (4.13–5.30) [B]	10.7 (5.22–16.1) [C]	1.29 (0.63–1.95) [C]
8–10 (mid)	6	Larvae	0.352 (UV filter)	>100 [A]	>12.4 [A]	>100 [A]	>12.4 [A]
8–10	6	Larvae	3.21	>100 [A]	>12.4 [A]	46.8 (29.8–63.7) [B]	5.80 (3.70–7.90) [B]
8–10	6	Larvae	6.72	>100 [A]	>12.2 [A]	27.7 (18.1–37.3) [C]	3.38 (2.21–4.55) [C]
8–10	6	Larvae	11.2	59.2 (47.1–71.2) [B]	7.16 (5.70–8.62) [B]	13.2 (9.50–16.9) [D]	1.60 (1.15–2.04) [D]
44–46 (late)	6	Larvae	0.301 (UV filter)	>100 [A]	>12.4 [A]	>100 [A]	>12.4 [A]
44–46	6	Larvae	3.16	>100 [A]	>12.4 [A]	>100 [A]	>12.4 [A]
44–46	6	Larvae	6.26	>100 [A]	>12.2 [A]	>100 [A]	>12.2 [A]
44–46	6	Larvae	10.6	>100 [A]	>12.1 [A]	>100 [A]	>12.1 [A]

[a] Pairwise comparisons were made within each age group using the LC50 ratio test [Sprague and Fogels, 1977].
[b] Values sharing the same capital letters in brackets are not significantly different ($p > 0.05$).

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resulting in 86 to 129 surviving larvae, which was sufficient for accurate toxicity value estimates. Water quality test values for pH (0 h: 8.18–8.42; 48 h: 8.15–8.42), DO (0 h: 7.18–7.76 mg/L; 48 h: 7.02–7.64 mg/L), temperature (0 h: 24.3–26.0 °C; 48 h: 24.0–26.0 °C), and salinity (0 h: 27.9–32.9 g/L; 48 h: 27.6–31.6 g/L) were within optimal ranges for larval survival and development at both initiation and termination of bioassays.

Chemical analyses of WAF solutions in the present study are presented in Table 1. All Macondo crude oils had high phenanthrene concentrations and, therefore, phototoxic PAH concentrations are presented with and without phenanthrene/anthracene measurement. Calculated tPAHs ranged from 11.8 to 14.9, and from 2.87 to 3.15 μg/L for CTC and Juniper oil WAFs, respectively. Phototoxic PAH concentrations were 0.52–0.63 μg/L for CTC oil WAFs. Juniper oil WAFs (0.28 μg/L) contained phototoxic PAHs concentrations that were approximately 2-fold less than CTC oil WAFs. Concentrations of tPAHs in CTC and Juniper oil WAFs under different UV light intensities decreased over the 48-h test period, with an average loss of 17.3% in vials under UV-filtering plexiglass and 23.5% in vials exposed to UV light.

3.1. Larval stage sensitivity

During larval stage studies, no significant differences in survival or development were observed between fluoranthene and solvent controls for larvae or sperm studies in the dark, suggesting fluoranthene alone was not toxic. In larval stage studies, 12 W/m^2 UV-A and fluoranthene (29.2 μg/L) resulted in significant reductions in the proportion of normal early, mid and late-aged larvae compared to larvae exposed to solvent alone, while a significant reduction in survival was only observed with the mid-aged larval group. Fluoranthene had no effect on UV-exposed sperm's ability to fertilize larvae.

Data indicated that a combined exposure to CTC oil WAF and UV light resulted in substantial effects on eastern oyster larval survival and development compared to exposure to WAF under a UV-filter (Table 2; Fig. 1). Significant differences between LC50s were observed among all larval ages (2–4, 8–10, and 44–46 h pf) exposed CTC WAFs at 11 W/m^2 UV-A (Fig. 1). Sensitivities of larval eastern oysters to CTC oil WAFs in combination with UV light decreased with increasing age: early (2–4 h pf) > mid (8–10 h) > late (44–46 h; Table 2; Fig. 1). The proportion of normal larvae was significantly less for early and mid life stage larvae when compared with late stage larvae at 6.6 and 11 W/m^2 UV-A (Fig. 1). Phototoxic effects were observed for survival of eastern oyster larvae at UV intensities at or above 6.6 W/m^2 UV-A but effects on normal development were observed at all tested UV intensities for at least one larval stage (Fig. 1).

Sperm exposed to combinations of CTC oil WAF and UV light prior to egg fertilization resulted in no significant differences among larval LC50 values at all UV intensities, suggesting that egg fertilization was not affected by these exposure conditions. However, the proportion of normal larvae resulting from sperm exposed to 0 and 3.38 W/m^2 UV-A were significantly greater than those from sperm exposed to 6.26 and 12.0 W/m^2 UV-A (Table 2).

3.2. Phototoxicity of two weathered Macondo crude oils

During comparative studies with the weathered oils, survival and the proportion of normal larvae exposed to fluoranthene (20.7 μg/L) were significantly greater for larvae in the dark compared to larvae exposed to 6 W/m^2 UV-A. Similarly, phototoxic effects of oyster larvae exposed to CTC and Juniper oils were only observed at UV intensities above 6.5 W/m^2 UV-A. Generally, LC50s and EC10s of larvae decreased with increasing UV light intensities for WAFs of both CTC and Juniper oils (Table 3). Water accommodated fractions of CTC oil were slightly more toxic than those of Juniper oil under both UV-filtered light and UV light (Table 3), which is in agreement with the total phototoxic PAH concentrations measured in each WAF (Table 1). Oyster larvae exposed to WAFs of CTC and Juniper oil had slight, but non-significant differences in LC50 values at UV-A intensities of 0.243, 3.27, 6.59, and 11.2 W/m^2. The proportion of normal larvae was significantly reduced in CTC oil WAFs compared to Juniper oil WAFs under UV filtered light (Table 3); however, no significant differences in development of oyster larvae were observed between CTC and Juniper oil WAFs at any of the tested UV-A exposures (Table 3).

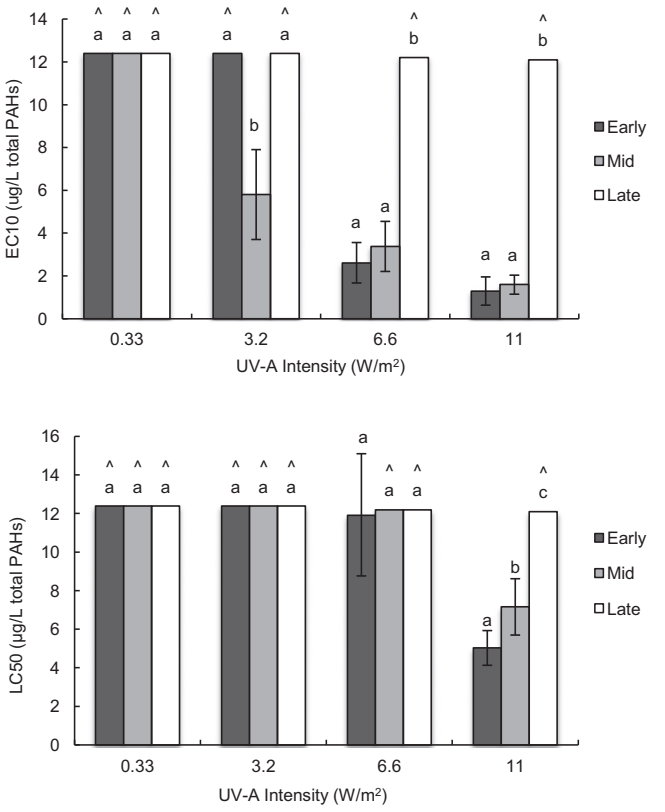

Fig. 1. Comparisons among median lethal concentrations (LC50 ± 95% confidence intervals) and the effective concentration resulting in 10% abnormal larvae (EC10 ± 95% confidence intervals) of early (2–4 h post fertilization [pf]), mid (8–10 h pf), and late (44–46 h pf) larval stages of eastern oysters exposed to a CTC oil WAF at different UV-A intensities (mean values; 6 h UV exposure). Statistical comparisons were made among life stages for each UV intensity. Values sharing the same letters among life stages at each UV intensity were not significantly different ($p > 0.05$; LC50 ratio test). Toxicity values beyond test concentrations were denoted with ^.

To compare the phototoxicity of the two weathered oils, the potency and concentration of phototoxic PAHs (tpPAH$_{eq}$) and UV-A intensities were combined, resulting in a linear relationship between LC50 values and integrated photoactive PAH doses ($I_{UV\text{-}A}$ x tpPAHeq; Fig. 2). Using this approach, we found that the phototoxicity of the CTC oil WAF at 6.59 W/m^2 UV-A was similar to that of Juniper oil WAF at 11.2 W/m^2 UV-A (Fig. 2), demonstrating that at equal UV-A exposures, CTC oil would be expected to be more phototoxic based on higher phototoxic PAH concentrations. Phototoxicity did not have an effect on survival of larval oysters below 6.59 W/m^2 UV-A for either oil.

4. Discussion

Bivalve mollusks have been reported to be one of the most sensitive taxa to photo-enhanced toxicity (Spehar et al., 1999; Weinstein, 2001). The current study has identified sensitive developmental stages of eastern oyster larvae and demonstrated that larval sensitivity of eastern oyster larvae to photo-enhanced toxicity decreases with increasing larval age (Table 2; Fig. 1). Early and mid-stage developing eastern oyster larvae (2–4 and 8–10 h pf, respectively) were the most sensitive life stages to combinations of UV light and WAFs of CTC oil (Table 2; Fig.

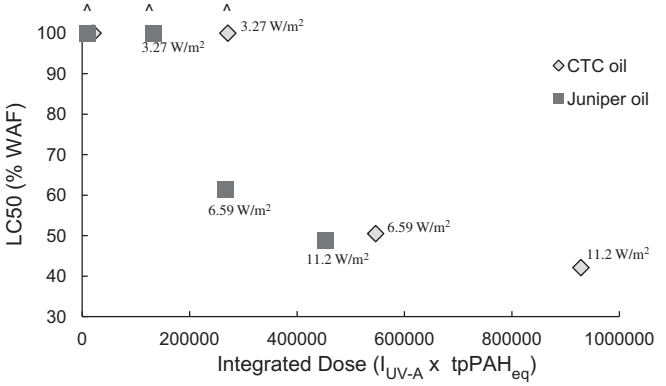

Fig. 2. Relationship between the integrated dose (see Sellin Jeffries et al., 2013) of UV-A light intensity (I_{UV-A}; μW·h/cm^2) and total phototoxic PAH equivalents (tpPAHeq), versus median lethal concentrations (LC50) for CTC and Juniper weathered Macondo crude oils. Values next to LC50 data points represent UV-A intensities. Toxicity values beyond test concentrations were denoted with ^.

1) and demonstrated decreased survival and higher proportions of abnormal larvae at low to moderate UV intensities. However, when eastern oyster larvae reached the shelled veliger stage (44–46 h old), they became resistant to photo-enhanced toxicity (Table 2; Fig. 1). Calcium carbonate (in the form of aragonite) present in larval shells has the capability to block UV light penetration (Mitra, 1996) and, thereby, limit penetration of UV light into tissues and the photo-excitation of absorbed PAHs. As organisms age, they typically develop protective mechanisms against UV light that limit the photo-enhanced toxicity of PAHs. Pelletier et al. (1997) reported juvenile coot clams to be less sensitive than embryos, suggesting protective mechanisms at later life stages. Reductions in phototoxicity in aquatic organisms have been reported to result from increases in pigmentation in fish and shrimp with increasing age (Finch and Stubblefield, 2015). Boese et al. (1997) found that organisms rarely exposed to UV light were more sensitive to photo-enhanced toxicity than those typically exposed to UV in their natural habitat. These studies suggest that changes during development may have multiple purposes, one of which could be reducing the damaging effects of UV light. Our data suggest that there is a brief window of sensitivity in which early non-shelled, developing oyster larvae are susceptible to photo-enhanced toxicity; however, the development of a shell in late larval stages may provide UV protection. While studies demonstrated stark differences in toxicity between life stages, the horizontal orientation of the test vials may have increased the sensitivity of the bioassays. Compared with vials placed upright, horizontal vial orientation slightly reduced larvae survival over the 48-h test period. Slight effects to the physiology of the larvae as a result of vial orientation may have increased organism susceptibility to combinations of oil and UV light. However, effects of horizontal orientation were minor and were necessary to maximize UV light penetration into test chambers during phototoxicity studies. The effects of vial orientation were consistent across all life stages of oyster larvae examined, and therefore effected each life stage equally, resulting in accurate comparisons among each larvae age group.

Eastern oyster sperm were also sensitive to photo-enhanced toxicity under experimental conditions of these studies. The proportion of normal larvae was significantly reduced when sperm were exposed CTC WAFs and UV intensities >6.26 W/m^2 UV-A, compared seawater controls in the dark (Table 2). However, UV exposure did not reduce the proportion of fertilized eggs that developed into larvae, suggesting that fertilization success was unaffected. Both Li et al. (2000) and Kijima (1992) reported chromosomal damage (genetic inactivation) and morphological changes of Pacific oyster sperm as a result of UV radiation that resulted in reduced fertilization and development of early stage larvae. Similarly, abnormal development of oyster larvae in the present study could be attributed to chromosomal damage due to phototoxic effects. The lack of effects on the proportion of eggs that developed into larvae (normal and abnormal) after fertilization by UV-exposed sperm may be attributed to the short duration of UV exposure (1 h) or limited UV exposure due to the dense sperm suspension used in the assays. Short UV exposure periods for sperm, however, are likely representative of some field conditions where gametes are typically released from males and females in close proximity to each other, resulting in short periods of gamete dispersal before egg fertilization (Galtsoff, 1964; Thompson et al., 1996).

The photo-enhanced toxicity of WAFs to eastern oyster larvae was dependent on UV light intensity (Table 2). Results are in agreement with other studies demonstrating that photo-enhanced toxicity is dependent, in part, on UV light intensity (Finch and Stubblefield, 2015; Lyons et al., 2002; Ankley et al., 1995). Differences in toxicity between low- and high-intensity UV light may be attributed to the degree of UV light penetration into dermal layers, with higher intensity UV light penetrating deeper into tissue layers or alternatively, the differences in overall UV doses (Supplemental Data, Table S1; Finch and Stubblefield, 2015). Separating the effects of UV intensity versus overall dose was not the focus of this study; however, Finch and Stubblefield (2015) reported significant differences in phototoxicity for mysid shrimp (*Americamysis bahia*), inland silverside (*Menidia beryllina*), and sheepshead minnow (*Cyprinodon variegatus*) between UV-A intensities of 8 and 24 W/m^2, even when each intensity was provided at equivalent UV doses (different durations), suggesting that UV light intensity alone can contribute to toxicity. Therefore, both UV dose and intensity are likely contributing factors associated with the observed increase in phototoxicity with increasing UV intensity.

CTC and Juniper crude oils are both weathered oils, however, CTC had an approximately four-fold higher concentration of tPAHs than found in WAFs of Juniper crude oil (Table 1), suggesting Juniper oil is more weathered of the two oils. Given the low concentration of BTEX components in weathered crude oils, the proportion of phototoxic PAHs was likely the primary driver in the observed toxicity under UV light. Water accommodated fractions of CTC oil had approximately 2-fold greater concentrations of phototoxic PAHs than WAFs of Juniper oil (Table 1), thereby explaining the slightly but consistently greater phototoxic potential of CTC oil WAFs. Despite the higher concentrations of photoactive PAHs in WAFs of CTC oil, no significant differences in larval survival and development were measured between WAFs of CTC and Juniper oils at the tested UV light intensities (Table 3). When accounting for the relative proportion of individual phototoxic PAHs in both oils, CTC crude oil had a greater phototoxic potential, evident by similar LC50 values to Juniper oil but at a much lower UV-A intensity (Fig. 2).

Results indicated that weathered (CTC and Juniper) crude oil WAFs have potential to cause photo-enhanced toxicity to eastern oyster larvae at environmentally relevant UV intensities. Ultraviolet light measurements recorded for natural sunlight at the OSU Aquatic Toxicology Laboratory (Albany, OR, USA) reached a maximum of 28 W/m^2 UV-A during peak summer months in 2014 (unpublished). UV-A intensities in the Gulf of Mexico (Barataria Bay, LA, USA; unpublished) were observed as high as 28 W/m^2 at the seawater surface in late September 2014 (Barataria Bay, LA, USA; unpublished). Therefore, UV intensities (0 to 11 W/m^2 UV-A) and durations (6 h) used in the current study are likely representative of summer UV intensities and doses for oyster gametes and larvae near the seawater surface in the Gulf of Mexico.

PAH concentrations tested in the current study were greater than the majority of PAH concentrations reported in samples taken from Gulf of Mexico nearshore waters during and after the DWH spill, which are defined as waters within 3 nautical miles (nmi) from the coast, except for Florida that defines nearshore to be within 9 nmi of its coastline (OSAT, 2010). Total PAH concentrations measured in seawater samples from the Gulf of Mexico from June 2010 to July 2012 exceeded the lowest LC50 values for CTC oil WAFs (2.94 μg/L tPAHs)

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in only 3.0% (578 out of 19,149) of all samples and 0.12% of near-shore samples, while exceeding the lowest EC10 for CTC oil WAFs (0.467 μg/L tPAHs) in only 6.9% (1315 out of 19,149) of all samples and 0.6% of near-shore samples (BP Gulf Science Data, 2014). Similarly, tPAH concentrations measured in field-collected water samples from the Gulf of Mexico were greater than those of the lowest LC50 for WAFs of Juniper oil (0.913 μg/L tPAHs) in 4.9% (935 out of 19,149) of all samples and 0.3% of near-shore samples and exceeded the lowest EC10 of Juniper oil WAFs (0.280 μg/L tPAHs) in 9.6% (1835 out of 19,149) of all samples and 0.6% of near-shore samples (BP Gulf Science Data, 2014). Oil reaching nearshore oyster beds may have been more weathered than either CTC or Juniper oils with lower PAH levels. However, samples of such oils were not available in this study. It is not known if water samples collected by research teams during and after the DWH oil spill were representative of PAH concentrations in oyster habitat.

In summary, evaluation of phototoxic effects of WAFs of weathered crude oils on oyster larvae demonstrated a dependence on the stage of larval development and UV light intensity. Early stages of eastern oyster larvae, up to 10 h post-fertilization, were sensitive to simultaneous exposure to UV and phototoxic PAHs at environmentally relevant UV intensities, but later stages (44–48 h post-fertilization) were much less sensitive, possibly because of inhibition of UV light penetration by the larval shell. UV-exposed sperm also showed sensitivity to photo-enhanced toxicity at environmentally relevant UV intensities, as evident in the higher proportion of abnormal larvae derived from eggs fertilized with exposed sperm. When evaluating phototoxic effects of oil to oyster larvae in the environment, life history traits as well as climatic and water conditions should be considered.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.10.008.

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