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Multi-stressor Effects of Ultraviolet Light, Temperature, and Salinity on Louisiana Sweet Crude Oil Toxicity in Larval Estuarine Organisms

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Abstract

When oil is spilled into the environment its toxicity is affected by abiotic conditions. The cumulative and interactive stressors of chemical contaminants and environmental factors are especially relevant in estuaries where tidal fluctuations cause wide variability in salinity, temperature, and ultraviolet (UV) light penetration, which is an important modifying factor for polycyclic aromatic hydrocarbon (PAH) toxicity. Characterizing the interactions of multiple stressors on oil toxicity will improve prediction of environmental impacts under various spill scenarios. This study examined changes in crude oil toxicity with temperature, salinity, and UV light. Oil exposures included high-energy, water-accommodated fractions (HEWAFs) and thin oil sheens. Larval (24–48 h post hatch) estuarine species representing different trophic levels and habitats were evaluated. Mean 96 h LC_{50} values for oil prepared as a HEWAF and tested under standard conditions (20 ppt, 25 °C, No-UV) were 62.5 µg/L $tPAH_{50}$ (mud snails), 198.5 µg/L (grass shrimp), and 774.5 µg/L (sheepshead minnows). Thin oil sheen 96 h LC_{50} values were 5.3 µg/L $tPAH_{50}$ (mud snails), 14.7 µg/L (grass shrimp), and 22.0 µg/L (sheepshead minnows) under standard conditions. UV light significantly increased the toxicity of oil in all species tested. Oil toxicity also was greater under elevated temperature and lower salinity. Multi-stressor (oil combined with either increased temperature, decreased salinity, or both) LC_{50} values were reduced to 3 µg/L $tPAH_{50}$ for HEWAFs and < 1.0 µg/L $tPAH_{50}$ for thin oil sheens. Environmental conditions at the time of an oil spill will significantly influence oil toxicity and organismal response and should be taken into consideration in toxicity testing and oil spill damage assessments.

Marine organisms may be exposed to oil through many routes, including dissolved fractions in the water column, droplets in the water column, thin sheens on the surface, or dietary exposure. Sheens are thin layers of oil (0.3–5 µm in thickness) on the water surface (Garcia-Pineda et al. 2020). Once oil enters the marine environment, abiotic factors, such as wave energy, ultraviolet (UV) light, temperature, and salinity, contribute to the fate and transformation of oil chemical components. Polycyclic aromatic hydrocarbons (PAHs) are the primary toxic component of naturally weathered crude oil (Heintz et al. 1999). The toxicity of PAHs in marine organisms has been shown to increase 10- to 100-fold in the presence of ultraviolet (UV) light (Alloy et al. 2017; Diamond et al. 2003; Sweet et al. 2017; Roberts et al. 2017). Photoenhanced toxicity can occur through photosensitization, when an organism is exposed to UV light after biouptake of PAHs. PAH molecules within the organism's tissues absorb UV light and promote electrons to a higher energy state. When the electrons return to ground-state, the energy is released and transferred, causing a cellular response (oxidative damage) without any change to the PAH molecule itself (Barron and Ka'aihue 2001; Finch and Stubblefield 2016). Alternatively, photomodification occurs when UV light oxidizes PAH molecules in the water column, forming more potent molecules. The photo-modified products can then be incorporated into the surrounding biota (Barron and Ka'aihue 2001). When UV light interacts with PAHs, the PAH molecules are excited and are able to donate

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electrons to other molecules, such as oxygen, which can then become more reactive. Reactive oxygen species interact with macromolecules, such as DNA, proteins, and fatty acids, leading to oxidative stress and subsequent cellular and tissue damage in the organism (Sweet et al. 2017). Effects of photo-enhanced oil toxicity in marine organisms have included mortality, decreased fecundity (Alloy et al. 2017), defects in morphology that result in heart failure (Pasparakis et al. 2019; Alloy et al. 2017), and feeding inhibition (Hatch and Burton 1999).

Estuaries serve as nurseries for the early developmental stages of commercially and recreationally important fish and shellfish species. The early life stages of marine organisms have been found to be especially vulnerable to developmental toxicity from PAHs (Pasparakis et al. 2019). This report provides data for early life stages of estuarine organisms, including grass shrimp, *Palaemonetes pugio*, sheepshead minnow, *Cyprinodon variegatus*, and mud snail, *Tritia obsoleta*. These species represent different trophic levels, habitats, and feeding strategies, and they are common along the East and Gulf coasts of the United States.

Grass shrimp are a key contributor to the pelagic macrofaunal biomass of tidal creeks, with densities as high as 40 shrimp/m^3 (Leight et al. 2005). Depending on food availability, grass shrimp act as detritivores, primary consumers, and secondary consumers (Anderson 1985) and also are important prey species for commercially and recreationally important marine organisms, such as spotted seatrout (*Cynoscion nebulosus*), red drum (*Sciaenops ocellatus*), mummichogs (*Fundulus heteroclitus*), crab species, and other shrimp species (Anderson 1985). The ecological importance of grass shrimp and their sensitivity to a variety of contaminants make them an ideal species for toxicity testing (Key et al. 2006; DeLorenzo et al. 2016).

The sheepshead minnow is a common estuarine fish species that resides in estuarine waters ranging from the Atlantic coast of Cape Cod to Mexico (Magnuson et al. 2018). Spawning events occur from February to October in creeks or small bodies of brackish waters. Sheepshead minnows are tolerant of a wide range of temperature (Bennett and Beitinger 1997) and salinity (Nordlie 1985). They grow to an adult length of approximately 4.6 cm and feed on detritus, algae, or microcrustaceans (Page and Burr 2011). *C. variegatus* is a standard test organism for the Environmental Protection Agency (2002) and the American Society of Testing and Materials (2004).

The Eastern mud snail is a common estuarine species found along marsh flats (Kelaher et al. 2003). The larval life stage is swimming and filter-feeding and resides in the water column, whereas adult mud snails live and feed on the sediment surface. Both the larval and adult life stages have served as a model gastropod species for a variety of studies (Collier 2002), including fuel oil toxicology (Miller and Pechenik 1983). Eastern mud snails have nonselective feeding habits, depending more on opportunity than nutritional need (Curtis and Hurd 1981). The Eastern mud snail plays an important ecological role in regulating estuarine intertidal soft-sediment community structure (Kelaher et al. 2003) as their foraging habits accelerate nutrient cycling (Connor et al. 1982) and modulate annelid densities (Kelaher et al. 2003).

Estuaries can experience rapid and dynamic changes in their water quality conditions, causing physiological stress for estuarine organisms. For example, continuous monitoring data (2007–2008) from Leadenwah Creek, SC, showed temperature ranged from 2.79 to 37.88 °C and salinity ranged from 0 to 36.83 ppt (Supplemental Figure 1). Adaptation for organisms living in such a wide range of environmental factors may come at an energetic cost, such as reduced growth (da Silva Rocha et al. 2005), and increased rates of metabolism (Lannig et al. 2010) and respiration (Fernandes and Rantin 1994). Chemical toxicity might exacerbate temperature and salinity stress and cause an increase in mortality for estuarine organisms, and both temperature and salinity may alter contaminant uptake and metabolism (DeLorenzo et al. 2009). Salinity has been shown to affect biotransformation rates and toxicity for several classes of chemicals (DeLorenzo 2015). An increase in chemical toxicity when combined with salinity stress has been attributed to decreased physiological functions, such as contaminant metabolism and detoxification processes (DeLorenzo 2015).

Several studies have examined the effect of salinity on oil toxicity in species, such as oysters (Zanette et al. 2011), amphipods (Tedengren et al. 1988), and mussels (Tedengren and Kautsky 1987). Additionally, increasing temperature has been shown to increase benzo[a]pyrene (BAP) toxicity (decrease in detoxification enzyme activity and an increase in BAP accumulation) in the mussel, *Mytilus galloprovincialis* (Kamel et al. 2012). Higher temperatures also were observed to increase toxicity of water-accommodated No. 2 fuel oil on development in *Fundulus heteroclitus* (National Research Council 2005).

Early life stages are particularly vulnerable to oil pollution due to increased capacity for contaminant uptake because of size (greater surface area to volume ratios), higher metabolic rates, rapid development, and less developed detoxification mechanisms (Key et al. 2006). In addition, many larvae lack pigmentation and often congregate at the surface, increasing their sensitivity to UV light exposure (Alloy et al. 2017; Barron and Ka'aihue 2001; Finch and Stubblefield 2016; Roberts et al. 2017). While effects thresholds for Louisiana Sweet Crude oil have been established for a range of marine and estuarine species, fewer studies have examined the toxicity of thin oil sheens, and even less experimentation has included multiple stressors.

The objective of this research was to determine the role abiotic stressors, such as UV light, temperature, and salinity play on survival of larval estuarine organisms in co-exposures with oil. The study examined effects of dissolved oil (high-energy, water-accommodated fractions (HEWAF)) and thin oil sheens, under different light, temperature, and salinity conditions.

Materials and Methods

Collection of Test Animals

Palaemonetes pugio

Adult ovigerous grass shrimp (2–3 cm in length) were collected from Leadenwah Creek (N 32°38′51.00″; W 80° 13′ 18.05″) a tidal tributary of the North Edisto River, SC, USA. The shrimp were acclimated 7–14 d in 76-L tanks with 20 ppt saltwater and were fed Tetramin® fish flakes. *P. pugio* larvae were obtained by placing gravid adult shrimp in brooding chambers within 10-L aquaria. The brooding chambers were designed to allow the embryos to hatch and the larvae to escape through the mesh. The larvae were fed newly hatched brine shrimp (*Artemia salina*) before testing and tested at 24–48 h old.

Tritia obsoleta

Adult mud snails also were collected from the Leadenwah Creek location during low tide. Mud snails (15–18 mm in length) were acclimated in the laboratory in 20-L aquariums at a density of approximately 250 snails per aquarium and were fed Tetramin® fish flakes daily ad libitum. Adult snails deposited egg capsules on the glass sides of the aquarium. Egg capsules were scraped from the side of the tank using a razor blade and transferred to a glass finger bowl containing filtered (0.22 µm) 20 ppt seawater, covered with aluminum foil and kept aerated in an environmental chamber until larvae hatched. Mud snail larvae were fed cultured algae (*Isochrysis galbana*) before testing and were tested at 24–48 h old.

Cyprinodon variegatus

Adult sheepshead minnows were collected from a tidal pond located on the Hollings Marine Laboratory property (N 32°74′82.24″; W 79°90′12.35″) using minnow traps. Adult fish were acclimated to laboratory conditions and then placed in spawning chambers within 76-L aquariums. Fish were fed Tetramin® fish flakes twice daily. Egg collection trays were used to retrieve eggs produced. Eggs were then transferred to glass finger bowls and allowed to hatch. Larval fish were fed newly hatched brine shrimp (*Artemia salina*) before testing and were tested at 24–48 h old.

Oil Treatment Preparations

HEWAF Methods

Seawater for all bioassays was acquired from Charleston Harbor estuary (N 32°45′11.52″; W 79° 53′ 58.31″), filtered (5 µm), UV-sterilized, activated carbon filtered, and diluted as needed to produce the appropriate test salinity. High-energy, water-accommodated fractions (HEWAFs) were prepared (DWH methods according to Forth et al. 2017a) at an initial concentration of 1 g/L by mixing 3.75 L of seawater with fresh LSC oil (3.75 g by mass) in a commercial blender, on low power, for 30 s. The mixture was transferred to a glass aspirator bottle with bottom outlet. The mixture was allowed to settle for 1 h in the dark, after which the bottom outlet was opened, and the HEWAF was dispensed into a collection container, without disturbing the upper slick layer. The 100% HEWAF was then diluted with seawater to achieve the nominal exposure concentrations (12.5%, 4.17%, 1.39%, 0.46%, and 0.15%).

Sheen Methods

Static, 96-h exposures were conducted with thin oil sheens. Seawater (200 mL) was added to glass crystallizing dishes and then the animals were added. Fresh LSC oil was then pipetted onto the surface of the water to achieve an oil sheen. A range of nominal sheen thicknesses (0.25, 0.5, 1.0, 2.0, and 4.0 µm) was tested. The volume (V) of oil needed to achieve the desired sheen thickness (h) was determined using the radius (r) of the container, ($V = \pi r^2 h$): 1.42 µL, 2.84 µL, 5.67 µL, 11.34 µL, and 22.68 µL, respectively. A minimum of three replicates were used for each oil treatment and control.

Experimental Conditions

Light

Each HEWAF and oil sheen treatment and control were tested under UV versus no-UV conditions. All experiments were conducted in environmental chambers (Percival Scientific IntellusUltra C8 incubators) with a photoperiod of 16-h light/8-h dark. Within the 16-h light photoperiod, the duration of the UV light exposure was 8 h. UV-A and UV-B intensity were measured using an ILT2400 light meter (International Light Technologies, Inc., Peabody, MA) placed at the level of the exposure chambers in each incubator. "No-UV" conditions were tested under fluorescent bulbs (3.8×10^{-6} W/cm^2 UV-A and 1.2×10^{-6} W/

cm^2 UV-B). "UV" conditions were established using T5 AgroMax UV-A PLUS bulbs (2.4×10^{-3} W/cm^2 UV-A, 1.1×10^{-6} W/cm^2 UV-B). Environmental intensity of UV light can be quite variable (Alloy et al. 2017; Roberts et al. 2017; Sweet et al. 2017). The bulbs used in this study emitted UV intensity similar to that recorded outside the Charleston laboratory in full sun, during July 2019, at 1.8×10^{-3} W/cm^2 UV-A and 7.1×10^{-5} W/cm^2 UV-B.

Temperature

Two temperatures were selected for testing: the standard bioassay temperature of 25 °C, and 32 °C, which represents the upper range of temperature found in southeastern United States estuarine habitats (DeLorenzo et al. 2009). Temperature was controlled using the incubators and varied within ± 2 °C.

Salinity

Three salinities were selected for testing: 10, 20, and 30 ppt. The 20-ppt salinity corresponds with the standard toxicity test conditions for these estuarine species, the 10-ppt salinity is within the lower range of salinity typical of southeastern United States estuarine habitat (and approximately the lower tolerance level for *T. obsoleta* (Scheltema 1965)), and the 30-ppt salinity is within the upper bound of typical estuarine conditions (DeLorenzo et al. 2009). Full-strength seawater was diluted with deionized water to achieve the exposure salinities.

Acclimation

Newly hatched larvae were maintained in 20-ppt seawater in a 25 °C incubator under fluorescent lighting (standard conditions) and then acclimated to the appropriate multi-stressor conditions. Salinity changes did not exceed 5 ppt per hour and temperature was adjusted approximately two degrees per hour. For the full-factorial design, larvae were split into four bowls with filtered 20 ppt seawater. Half of the larvae were kept in the 25 °C incubator with no UV for 1 h. The other half of the larvae were acclimated in a 32 °C incubator with no UV for 1 h. The 20-ppt seawater in both 25 °C incubator and 32 °C incubator was then diluted to 15 ppt, and the larvae were acclimated for 2 h. The 15-ppt seawater in both incubators was further diluted to 10 ppt, and the larvae were acclimated for 2 h.

Testing

All exposures were static and the animals were not fed during testing. The mortality of the larvae in each dish was assessed at 24 h, 48 h, 72 h, and 96 h. At 96 h, water-quality (temperature, dissolved oxygen, salinity, and pH) was measured.

The study began by establishing LC_{50} values for LSC oil as a HEWAF and as a sheen for each test species under standard test conditions of 25 °C and 20-ppt salinity, with either UV or No-UV conditions. This first level of testing consisted of two-factor (oil and light) experiments with five oil concentrations plus a control (Level 1 testing, Fig. 1a). After establishing the initial toxicity values, salinity or temperature was added as an experimental factor. During these three-factor experiments, three concentrations of HEWAF or oil sheen were tested plus a control under both light conditions, plus three salinity levels (10, 20, or 30 ppt) or two temperature levels (25 °C or 32 °C) (Level 2 testing, Fig. 1a). Ultimately, a full four-factor design was used to test the potential interactive effects of all variables (oil, UV light, temperature, and salinity). The treatments included three oil concentrations (0.25%, 1%, 4% HEWAF or 0.25-µm, 1.0-µm, 4.0-µm sheen), two temperatures (25 °C or 32 °C), two salinities (10 ppt or 20 ppt), and two light conditions (UV or No-UV) (Level 3 testing, Fig. 1a). Three replicates each with 10 larvae per replicate were used for each oil treatment and control, for a total of 32 treatments and 960 larvae per test (Fig. 1b). Animal care and use protocols for experiments with sheepshead minnow were consistent with those required for the use of vertebrates in research by the Institutional Animal Care and Use Committee (IACUC) in the United States.

Chemical Analysis

Water samples for chemical analysis were collected immediately after settling from each 100% HEWAF solution and from a subset of the HEWAF dilutions. Water samples also were collected from thin oil sheens. Before the addition of water and oil, a standpipe (Teflon straw) was established in the crystallizing dish. After 24 h, the water beneath the sheen was collected from the standpipe using a siphon, without disturbing the overlying oil layer. Chemistry samples collected from thin oil sheens were prepared without test organisms. A total of 50 PAHs ($tPAH_{50}$) were analyzed, including both parent and alkylated PAHs (Supplemental Table 1) using liquid/liquid extraction and gas chromatography mass spectrometry (GC/MS) (detailed methods in Supplemental Methods 1). The mean $tPAH_{50}$ value quantified for each of the HEWAF treatments and under each oil sheen was calculated.

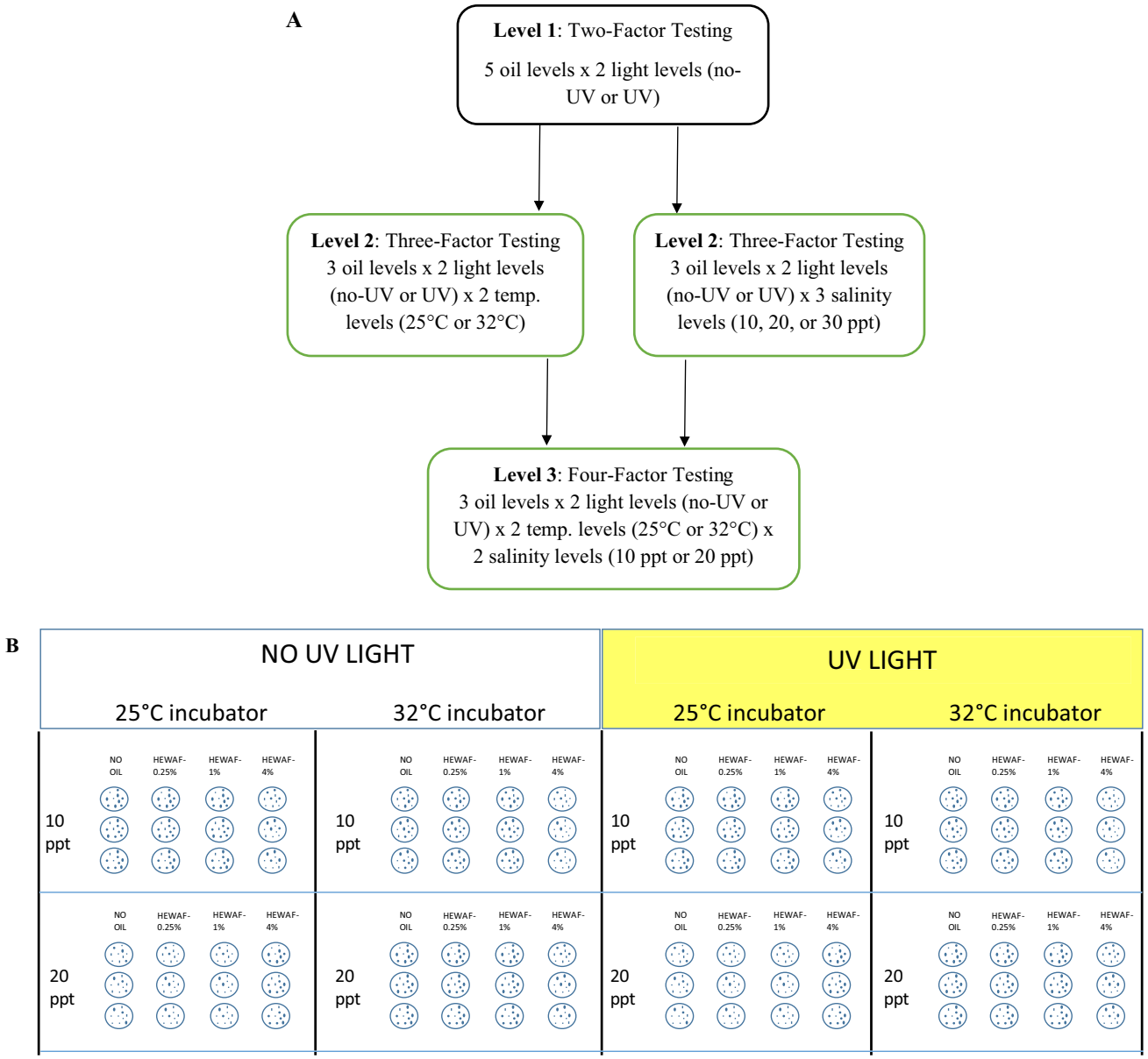

Fig. 1 Flow-chart of all testing (**a**) and experimental design graphic for full factorial HEWAF test (**b**) with oil, UV, temperature and salinity as factors. The full factorial Sheen testing was designed in a similar manner, with sheen thicknesses of 0.25, 1.0, and 4.0 µm

Statistical Methods

Lethal concentration (LC) toxicity thresholds (96 h LC_{10} and LC_{50} values) with 95% confidence intervals (CI) were determined using Statistical Analysis Software (SAS) Probit Analysis (PROC PROBIT, SAS V.9.4, Cary, NC). The LC_{50} ratio test (Wheeler et al. 2006) was used to test for significant differences between LC_{50} values. The mean toxicity values across multiple experiments for each test species and condition were calculated. Dunnett's test for multiple comparisons and Kruskal–Wallis one-way analysis were used to determine which treatments were significantly different from the control. Multifactorial *x*–*y* plots to display treatment interactions were generated using R statistical software (R Core Team 2020).

Results

Chemistry

Mean measured concentrations of $tPAH_{50}$ were determined for both HEWAF (Table 1) and thin oil sheen exposures (Table 2). The range of concentrations in the HEWAF

Table 1 Mean measured average $tPAH_{50}$ (µg/L) concentrations from the HEWAF exposures, standard deviation, and number of samples from different tests used to calculate the mean

Nominal % HEWAF	Mean measured $tPAH_{50}$ (µg/L)	Standard deviation	*n*
100.00 (1 g/L LSC oil)	7949.28	1443.91	16
12.50	1030.27	228.56	5
4.17	316.95	28.93	3
1.39	84.69	20.50	3
0.46	32.47	2.88	3
0.15	12.85	3.08	3

Table 2 Mean measured average $tPAH_{50}$ (µg/L) concentrations in the water column from the oil sheen exposures, standard deviation, and number of samples from different tests used to calculate the mean

Nominal oil sheen thicknesses (µm)	Mean measured $tPAH_{50}$ (µg/L)	Standard deviation	*n*
4.0	14.47	7.31	12
2.0	7.45	3.46	8
1.0	5.26	4.21	18
0.5	3.00	2.00	17
0.25	2.75	1.12	6

dilutions (0.15–12.5%) collected immediately after preparation (t = 0 h) was 12.85 to 1030.27 µg/L $tPAH_{50.}$ Water chemistry from samples collected beneath the sheen exposures of 0.25-µm to 4-µm thickness 1 day after preparation (t = 24 h) yielded a range of concentrations from 2.75 to 14.47 µg/L $tPAH_{50.}$ Mean measured concentrations of $tPAH_{50}$ were used to calculate the LC_{50} values for both HEWAF and thin oil sheen exposures.

Species Sensitivity

Larval mud snails were the most sensitive species tested in both LSC oil preparations with no UV. The mean 96-h LC_{50} value for LSC oil prepared as a HEWAF and tested under standard conditions (20 ppt, 25 °C, No-UV) was 62.5 µg/L $tPAH_{50}$, compared with 198.5 µg/L for grass shrimp and 774.5 µg/L for sheepshead minnows (Table 3). The HEWAF LC_{50} values for each species were all significantly different from one another based on LC_{50} ratio testing ($p < 0.0001$). The LC_{10} value for the most sensitive species tested, larval mud snails, was 15.5 µg/L $tPAH_{50}$ (Supplemental Table 2).

The order of species sensitivity was the same for thin oil sheens; larval mud snails more sensitive than grass shrimp, followed by sheepshead minnows, with 96-h LC_{50} values under standard test conditions of 5.3 µg/L $tPAH_{50}$ (snails), 14.7 µg/L (shrimp), and 22.0 µg/L (fish) (Table 4). The thin oil sheen LC_{50} value for mud snails was significantly lower than grass shrimp (LC_{50} ratio test, $p = 0.0039$) and

Table 3 Mean 96-h LC_{50} values (95% confidence intervals) for larval (24–48 h old) estuarine organisms exposed to LSC HEWAF under each test condition. The standard test condition is 20 ppt salinity, 25 °C temperature, and no UV exposure. The data represent a summary of 60 individual 96-h experiments. Values calculated using measured $tPAH_{50}$ values (µg/L), SAS Probit Analysis. ND = not determined (outside range of Probit analysis)

	Mean	20 ppt 25 °C No-UV	20 ppt 25 °C UV	10 ppt 25 °C No-UV	10 ppt 25 °C UV	30 ppt 25 °C No-UV	30 ppt 25 °C UV	20 ppt 32 °C No-UV	20 ppt 32 °C UV	10 ppt 32 °C No-UV	10 ppt 32 °C UV
Shrimp	LC_{50}	198.5 (180.5–214.5)	52.8 (33.3–82.0)	101.0 (81.0–135.5)	37.0 (32.5–41.5)	310.0 (248.0–296.0)	69.0 (66.0–73.0)	139.5 (118.5–158.0)	25.5 (17.5–36.0)	20.0 (8.0–30.0)	ND
Fish	LC_{50}	774.5 (457.0–2620.0)	60.5 (29.3–102.3	ND	100.0 (28.0–224.5)	ND	119.0 (ND)	55.5 (29.5–88.0)	16.0 (3.5–29.0)	198.0 (84.0–1032.0)	3.0 (ND-31.0)
Snail	LC_{50}	62.5 (47.3–76.5)	24.8 (19.8–29.8)	18.0 (10.5–25.0)	6.0 (ND-14.0)	23.0 (23.0–23.0)	13.0 (ND)	15.5 (12.5–20.0)	15.0 (13.0–21.0)	9.0 (6.0-ND)	ND

Table 4 Mean 96 h LC_{50} values (95% confidence intervals) for larval (24–48 h old) estuarine organisms exposed to LSC thin oil sheens under each test condition. The standard test condition is 20-ppt salinity, 25 °C temperature, and no UV exposure. The data represent a summary of 56 individual 96 h experiments. Values calculated using measured $tPAH_{50}$ values (µg/L), SAS Probit Analysis. ND = not determined (outside range of Probit analysis)

	Mean	20 ppt 25 °C No-UV	20 ppt 25 °C UV	10 ppt 25 °C No-UV	10 ppt 25 °C UV	30 ppt 25 °C No-UV	30 ppt 25 °C UV	20 ppt 32 °C No-UV	20 ppt 32 °C UV	10 ppt 32 °C No-UV	10 ppt 32 °C UV
Shrimp	LC_{50}	14.7 (11.3–24.7)	3.5 (3.5–4.5)	8.0 (7.0–9.0)	2.0 (ND)	11.0 (9.0–13.0)	2.0 (ND)	10.0 (8.0–12.0)	4.0 (ND)	ND	ND
Fish	LC_{50}	22.0 (6.5-ND)	3.7 (1.7–4.0)	17.5 (7.5–15.0)	3.0 (2.5–3.0)	16.0 (12.0–103.0)	2.0 (0.1–4.0)	5.0 (2.0–6.0)	ND	14.0 (12.0–19.0)	1.0 (ND-3.0)
Snail	LC_{50}	5.3 (4.3–6.3)	2.0 (1.0–2.7)	2.5 (1.5–3.0)	< 1 (ND)	ND	1.3 (ND-3.2)	3.0 (1.0–4.0)	ND	ND	ND

sheepshead minnows (LC_{50} ratio testing, $p = 0.003$), whereas the grass shrimp and sheepshead minnow values were not significantly different from one another (LC_{50} ratio test, $p = 0.3513$). The LC_{10} value for larval mud snails, again the most sensitive species tested, was 1.5 µg/L $tPAH_{50}$, and this concentration is estimated at a sheen thickness of < 0.25 µm (Supplemental Table 3).

Effect of UV Light

The addition of UV light increased the toxicity of oil for all species tested, with mean 96-h HEWAF LC_{50} values of 24.8 µg/L $tPAH_{50}$ for mud snails, 52.8 µg/L for grass shrimp, and 60.5 µg/L for sheepshead minnows (Table 3). The addition of UV light reduced the LC_{50} values by 60% for snails, 73% for shrimp, and 92% for fish. UV HEWAF exposure LC_{50} values for all three species were significantly lower than No-UV exposures (LC_{50} ratio test, $p < 0.0001$ for fish and snails, and $p = 0.0071$ for shrimp). Thin oil sheen testing revealed similar increases in toxicity with the addition of UV light. Mean 96-h LC_{50} values were 2.0 µg/L $tPAH_{50}$ (snails), 3.5 µg/L $tPAH_{50}$ (shrimp), and 3.7 µg/L $tPAH_{50}$ (fish) (Table 4), representing 62%, 76%, and 83% reductions in the median lethal concentrations determined under standard testing conditions. UV sheen exposure LC_{50} values for all three species were significantly lower than No-UV exposures (LC_{50} ratio test, $p < 0.0001$).

Effect of Salinity

Salinity effects varied by species. Compared with standard test conditions (20 ppt, 25 °C, No-UV), lower salinity (10 ppt, 25 °C, No-UV), increased the toxicity of oil to mud snails and grass shrimp, with mean 96 h HEWAF LC_{50} values of 18.0 µg/L $tPAH_{50}$ for mud snails and 101.0 µg/L $tPAH_{50}$ for grass shrimp (Table 3). Decreasing salinity significantly reduced the grass shrimp LC_{50} by 49% and the snail LC_{50} by 71% (LC_{50} ratio test, $p < 0.0001$). However, there was no significant effect of decreasing salinity on the toxicity of HEWAFs to larval sheepshead minnows (LC_{50} ratio test, $p = 0.663$). Increasing salinity significantly reduced HEWAF toxicity to the grass shrimp (LC_{50} ratio test, $p < 0.0001$). There was no significant effect of increasing salinity (30 ppt, 25 °C, No-UV) compared with standard test conditions (20 ppt, 25 °C, No-UV), on the toxicity of HEWAFs to fish or snails (LC_{50} ratio test, $p = 0.627$ and 0.9848, respectively).

Decreasing salinity also significantly increased oil toxicity to the grass shrimp in thin oil sheen exposures (No-UV 96-h LC_{50} approximately 45% lower at 10-ppt compared with 20-ppt salinity; LC_{50} ratio test, $p < 0.0001$; Table 4). In addition, grass shrimp were more sensitive to thin oil sheens under increased salinity conditions (No-UV 96 h LC_{50} approximately 25% lower at 30 ppt compared with 20 ppt; LC_{50} ratio test, $p = 0.0262$). Larval mud snails also were more sensitive to thin oil sheens at both lower salinity (No-UV 52% decrease in LC_{50} value at 10-ppt salinity than 20-ppt salinity, LC_{50} ratio test, $p < 0.0001$) and higher salinity (No-UV 75% decrease in LC_{50} value at 30-ppt salinity than 20-ppt salinity, LC_{50} ratio test, $p < 0.0001$). Sheepshead minnow response to thin oil sheens was not significantly affected by decreasing salinity to 10 ppt (LC_{50} ratio test, $p = 0.9160$), nor increasing salinity to 30 ppt (LC_{50} ratio test, $p = 0.5266$).

Effect of Temperature

Compared with standard test conditions (20 ppt, 25 °C, No-UV), higher temperature (20 ppt, 32 °C, No-UV), significantly increased the toxicity of oil to all species tested, with mean 96-h LC_{50} values of 15.5 µg/L $tPAH_{50}$ for mud snails, 55.5 $tPAH_{50}$ for fish, and 139.5 µg/L $tPAH_{50}$ for grass shrimp (Table 3). Increasing temperature decreased the HEWAF LC_{50} values by 30% in shrimp, 75% in snails, and by 93% in fish (LC_{50} ratio test, $p = 0.0269$ for shrimp, $p < 0.0001$ for snails, and $p < 0.0001$ for fish).

Increasing temperature also significantly increased the toxicity of thin oil sheens to all species tested (Table 4).

The 96-h LC_{50} values under thin oil sheens with UV light exposure were 10.0 µg/L $tPAH_{50}$ (shrimp), 5.0 µg/L $tPAH_{50}$ (fish), and 3.0 µg/L $tPAH_{50}$ (snail). Thin oil sheen LC50 values decreased by 32% (shrimp), 43% (snails), and 77% (fish) with increased temperature (LC_{50} ratio test, $p = 0.0007$ for shrimp, $p < 0.0001$ for snails, and $p = 0.0025$ for fish, respectively).

UV, Temperature, and Salinity Interactions

To reduce the number of treatments, the 30-ppt salinity was not included in the full factorial test because it had a less of an effect on toxicity than the 10-ppt salinity treatment. When reduced salinity and UV light were combined as a treatment for mud snails exposed to HEWAF, the combined stressor percent mortality was generally greater than for either stressor alone (Supplemental Table 4). When increased temperature and UV light were combined as a treatment for mud snails exposed to HEWAF, the combined stressor percent mortality also was generally greater than for either stressor alone.

Larval grass shrimp mortality in the HEWAF exposure was most affected by the addition of UV light, as seen by the steep increase in the dose response (Supplemental Table 5). When reduced salinity and UV light were combined as a treatment for larval sheepshead minnows exposed to HEWAF, the combined stressor percent mortality was generally not different than for either stressor alone (Supplemental Table 6). However, the combined effect of UV light and increased temperature did increase mortality (20–73%) compared with either stressor alone (Supplemental Table 6).

Similar multistressor responses were observed under thin oil sheens. Larval mud snails exposed to thin oil sheens had the greatest mortality when oil exposures were combined with increased temperature, decreased salinity, and UV light (Supplemental Table 7). A similar trend of oil toxicity influenced primarily by UV light was observed for grass shrimp in the thin oil sheen exposures. When an additional abiotic stressor of either increased temperature or decreased salinity was added to the UV sheen exposures, the interaction resulted in > 80% grass shrimp mortality (Supplemental Table 8). As with the HEWAF exposures, sheepshead minnows responded strongly to an increase in temperature combined with UV light under thin oil sheens (Supplemental Table 9). Decreased salinity alone did not lead to enhanced sheen toxicity over standard test conditions, and adding decreased salinity to increased temperature and UV light as a third abiotic factor did not increase toxicity over the effect of increased temperature and UV light (Supplemental Table 9).

The lowest concentration tested for both HEWAF and thin oil sheens in the full factorial tests was 0.25% and 0.25 µm, respectively. The percent mortality under each testing condition was graphed to visualize the effect of abiotic factors at one oil level. A 0.25% HEWAF exposure resulted in larval mud snail mortality ranging from 30 to 100%, depending on test conditions (Fig. 2). The combination treatments of UV light with either lower salinity or higher temperature at the same oil concentration resulted in nearly complete mortality to the mud snails (Fig. 2). A similar effect was seen for the mud snails and a thin oil sheen exposure of 0.25 µm, where strong influence of UV light was seen on oil toxicity under all temperatures and salinities but with greatest mortality observed under the combined UV light, temperature, and salinity treatment (Fig. 3). The HEWAF mortality graphed at 0.25% demonstrates that larval grass shrimp were more tolerant of decreased salinity and increased temperature than the larval snails, but that the combined stressors of 10-ppt salinity, 32 °C, and UV light again resulted in the highest HEWAF mortality (Fig. 2). For the multifactor sheen experiment, there was very little influence of temperature or salinity on grass shrimp mortality at 0.25 µm (mortality < 20%); however, the addition of UV light resulted in 80–100% mortality (Fig. 3). The 0.25% HEWAF graph demonstrates that larval sheepshead minnows are more sensitive to increased temperature and UV light than to decreased salinity, with the greatest mortality observed in the 20 ppt, 32 °C, and UV light treatment (Fig. 2). The temperature and UV light interactions also resulted in the greatest sheepshead minnow mortality under the 0.25-µm sheen (Fig. 3). In most cases, the combination of reduced salinity, increased temperature, and UV light yielded the highest larval mortality.

There was no significant difference between the eight seawater controls, No-UV, and UV at both temperatures and both salinities for any of the species tested in the HEWAF experiments (grass shrimp ANOVA, $p = 0.2499$; mud snail ANOVA, $p = 0.1171$; sheepshead minnow ANOVA, $p = 0.6615$) (Supplemental Tables 4–6). Similarly, there was no significant difference between the eight seawater controls, No-UV, and UV at both temperatures and both salinities for any of the species tested in the sheen experiments (grass shrimp, all 100% survival; mud snail ANOVA, $p = 0.0569$; and sheepshead minnow, all 100% survival) (Supplemental Tables 7–9).

Discussion

Sheens Versus HEWAFs

This study examined two very different types of oil exposure, with thin oil sheen exposures generating lower 96-h LC_{50} values than HEWAF exposures. Thin oil sheens are a particularly important oil exposure mechanism to study due to the lack of toxicity data for sheens. Thin sheens often are present in coastal oil spills, and evaluation of their potential toxicity to estuarine organisms, especially larval forms that

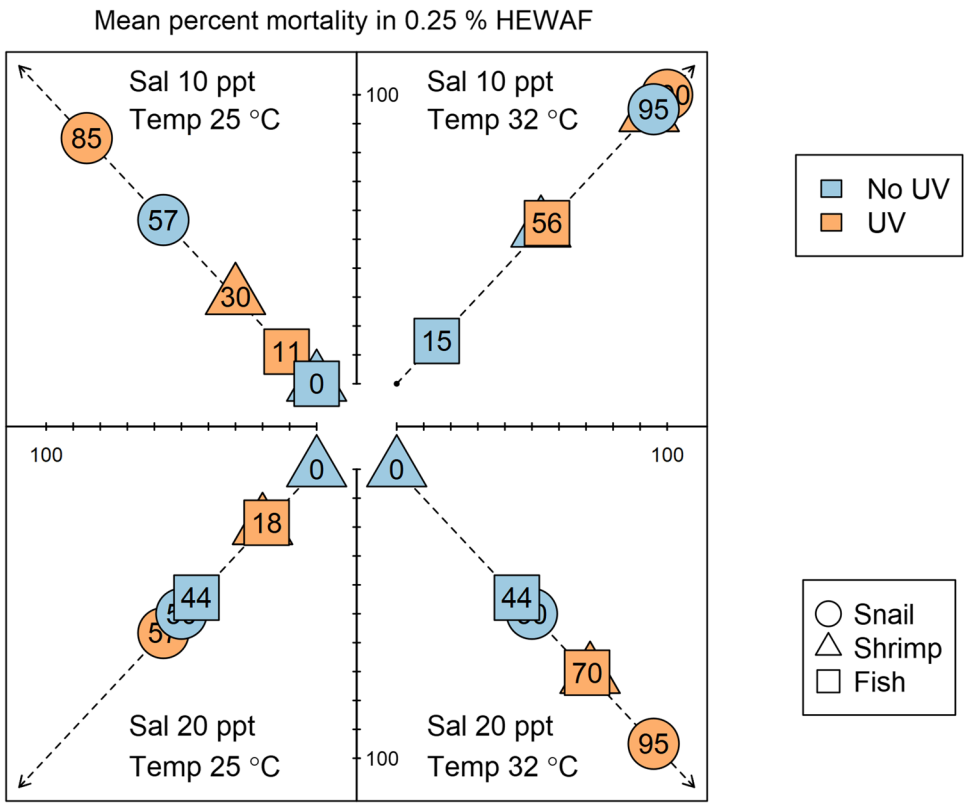

Fig. 2 Mean percent mortality for larval snails (circle), shrimp (triangle), and fish (square) exposed to a nominal 0.25% LSC oil HEWAF for 96 h under various light [no UV (blue) and UV (orange)], temperature, and salinity conditions

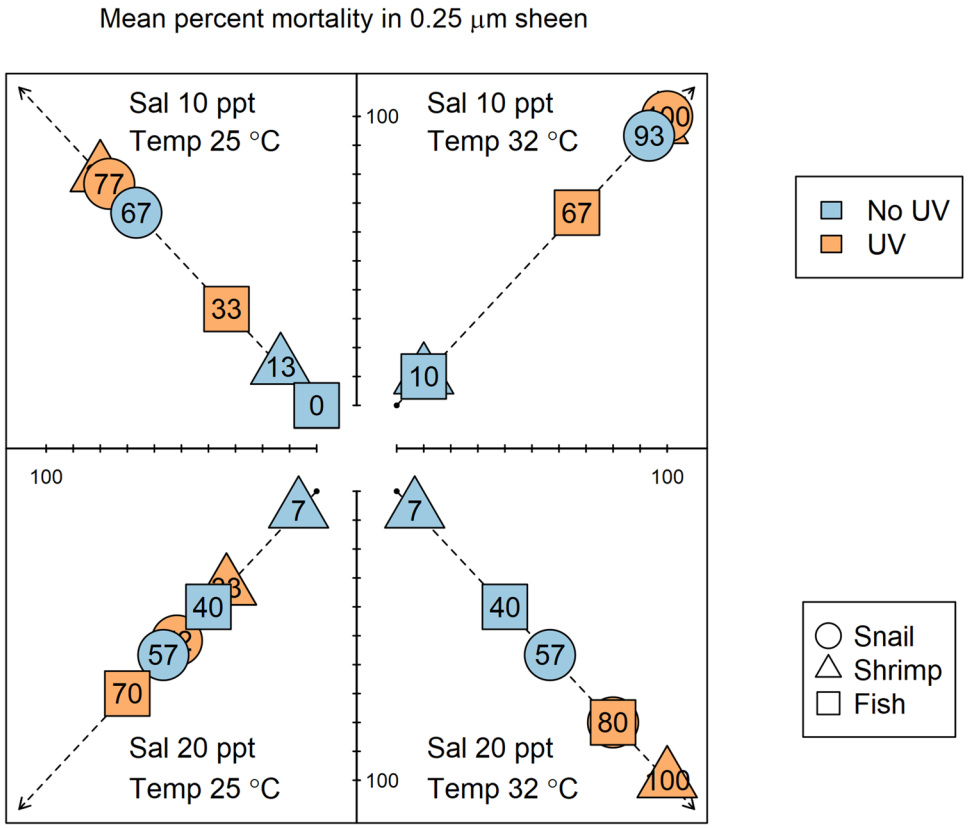

Fig. 3 Mean percent mortality for larval snails (circle), shrimp (triangle), and fish (square) exposed to a nominal 0.25 µm LSC oil sheen for 96 h under various light [no UV (blue) and UV (orange)], temperature, and salinity conditions

occur near the water surface, will benefit oil spill impact assessments. Observed differences in LC_{50} thresholds for HEWAF and sheens may be due to differences in the actual exposures larvae experienced or experimental design. Direct comparisons of toxicity across oil exposures cannot be made, because we do not have sufficient information regarding the physics of oil in the different oil preparations, nor the changes in PAH concentration over time given the difference in sampling times (sheens were measured 24 h after preparation, whereas HEWAFs were measured after 1 h) (Liu et al. 2012). The HEWAF preparations potentially provided a more homogeneous chemical exposure than the sheens, although there is known to be heterogeneity due to droplets versus dissolved oil in HEWAFs and that the ratio of droplets to dissolved phase decreases as HEWAFs are diluted (Forth et al. 2017a, b). Given the complex nature of oil and water mixtures, there also could be differences in the amount of hydrocarbons delivered to the test solutions by the different test preparations, potentially driven by the starting ratio of oil to water, and also differences in the composition of oil between HEWAF and sheen exposure (e.g., changes in the volatile component), based on preparation method and time of sampling (O'Shaughnessy et al. 2018). In addition, it is difficult to compare thin oil sheens created in the laboratory with those present in the environment due to the small, shallow container effect, and volatilization from fresh oil. In open water, the ratio of sheen thickness to volume beneath the sheen would be much greater. Not much is known about the relationship between sheen thickness and tPAH50 concentrations beneath thin oil sheens during oil spills.

The $tPAH_{50}$ threshold concentrations (96-h LC_{50} values) determined for larval grass shrimp, mud snails, and sheepshead minnow under standard testing conditions in this study (62.5–774.5 µg/L for HEWAF and 5.3–22 µg/L for sheens) are within the range of surface PAH concentrations (0–84.8 µg/L) reported in the DWH spill area (Diercks et al. 2010). The $tPAH_{50}$ values in the present study are based on chemistry samples taken immediately after HEWAF preparation and 24 h after sheen preparation, and because the exposure solutions were not renewed during the 96 h test, the threshold values calculated in this study may underestimate toxicity because of reduction in PAH concentration in the exposure solutions over time, especially with fresh oil.

Abiotic Factors

In the environment, estuarine organisms experience salinities ranging from 0–36 ppt, temperatures ranging from 2 to 37 °C (DeLorenzo et al. 2009), and various levels of UV light, all depending on season, precipitation, and tidal cycle. The three estuarine species selected for testing in this study are all tolerant of a wide range of environmental conditions (Anderson 1985; DeLorenzo et al. 2009; Haney 1999; Scheltema 1965). In this study, there was no significant effect of the abiotic factors tested (UV light, salinity, or temperature) on larval survival in the control treatments. While the control survival for all exposures was within acceptable range for standard toxicity testing, we should note that sublethal effects from the abiotic stressors were not evaluated. In fact, physiological stress from elevated temperature and decreased salinity is assumed to play a role in the multistressor interaction between environmental conditions and chemical exposure.

Oil and Abiotic Factor Interactions

The cumulative and interactive stressors of chemical contaminants and environmental factors are especially relevant in estuaries where tidal fluctuations cause wide variability in salinity and temperature. In addition, many larval organisms lack pigmentation and spend their early life stages in the upper mixing layer of coastal waters, thus increasing their UV light exposure (Alloy et al. 2017; Barron and Ka'aihue 2001; Finch and Stubblefield 2016; Roberts et al. 2017). Photo-enhanced toxicity is an important consideration in oil spill response, because the spatial and temporal extent of negative effects to aquatic organisms may be underestimated if based on standard laboratory bioassays with fluorescent lighting. In this study, UV light significantly increased the toxicity of LSC oil in all species tested. In general, LSC oil toxicity also was significantly greater under elevated temperature conditions and lower salinity conditions.

The toxicity of other chemical contaminants has also been shown to be influenced by salinity, although not always in the same direction. For example, toxicity of the heavy metal cadmium to *C. variegatus* was shown to increase significantly with decreasing salinity (96-h LC_{50} value of 495.5 µg/L at 25 ppt, compared with 312.14 µg/L at 15 ppt and 180.3 µg/L at 5 ppt) (Hall et al. 1995). *C. variegatus* also was more sensitive to the insecticides 4-nitrophenol and 2,4-dinitro-phenol at lower salinities, however increasing salinity increased toxicity of two organophosphate insecticides: terbufos and trichlorfon (Brecken-Folse et al. 1994). Hall et al. (1994) demonstrated decreased chemical sensitivity for larval sheepshead minnows at low salinities (96-h LC_{50} values for the herbicide atrazine were 16.2 mg/L, 2.3 mg/L, and 2.0 mg/L at salinities of 5 ppt, 15 ppt, and 25 ppt, respectively). Larval mud snails were more sensitive to oil dispersants at lower salinities, where toxicity increased 62% with lower salinity (DeLorenzo et al. 2017). Similarly, oil dispersant toxicity to larval grass shrimp increased 51% with lower salinity (DeLorenzo et al. 2016). The effects of salinity on chemical contaminant toxicity, therefore, vary with the type of contaminant and species tested (DeLorenzo 2015).

Temperature also has been demonstrated to alter toxicity of chemical contaminants. The oil dispersant Corexit was shown to be more toxic to grass shrimp at higher temperatures (Fisher and Foss 1993). Brecken-Folse et al. (1994) found increasing temperature increased the toxicity of two organophosphate insecticides, terbufos, and trichlorfon, in both *P. pugio* and *C. varietagus*. The toxicity of the pesticides chlorothalonil and Scourge® (active ingredients resmethrin and piperonyl butoxide) also were found to be more toxic to both larval and adult grass shrimp at higher temperatures (25 °C vs. 35 °C) (DeLorenzo et al. 2009). In response to thermal stress, cell membrane structure and fluidity are known to change, allowing the membrane to become more permeable which may result in increased uptake of chemicals (Holmstrup et al. 2014). Metabolism also is affected by temperature and may impact the organism's ability to bio-transform and detoxify foreign compounds (DeLorenzo 2015).

Consistent with previous studies examining early life stages of marine fish species (Alloy et al. 2017; Sweet et al. 2017), UV light significantly increased the toxicity of LSC oil in all the larval estuarine species tested. UV light penetration can vary widely in estuarine waters, with depth, tides, and seasonal productivity as key factors. Average incident UV 380 in the Gulf of Mexico during the DWH oil spill was estimated at 1550 mW/s/cm^2 (Bridges et al 2018), which is of a similar intensity as the UV exposure in this study. Few studies have examined the interactions of multiple abiotic factors on contaminant toxicity. In this study, we characterized the interaction of oil with UV light, temperature, and salinity. While there were species-specific differences in response, generally, LSC oil toxicity also was exacerbated under elevated temperature conditions and under lower salinity conditions. It is likely that homeostatic energy needed to survive low salinity and high temperature, combined with photo-induced oil toxicity provided additional stress and increased larval mortality rates. Multi-stressor interactions significantly reduced toxicity thresholds for the larval species tested, eliciting effects at PAH concentrations far lower than would be predicted under standard test conditions. LC_{50} values dropped to 20, 3, and 6 µg/L $tPAH_{50}$ for shrimp, fish, and snails, respectively, under HEWAF plus abiotic stressor conditions, and to 1–2 µg/L $tPAH_{50}$ under thin oil sheens.

Chemical risk assessments are typically performed using toxicity data from standardized tests conducted at uniform water quality parameters; yet chemical spills and accidental releases occur under a wide range of environmental conditions. The research findings discussed herein demonstrate that changes in temperature, salinity, and UV light interact to alter the toxicity of oil pollution in larval estuarine organisms, making predictions about the impacts of oil in estuaries especially challenging. Multi-stressor testing is a valuable step for refining the accuracy of chemical risk assessments and understanding how real-world conditions can impact toxicity. The effects thresholds presented in this report for oil in early life stages of estuarine organisms characterize how toxicity changes with environmental conditions and provides data that can be used to inform oil spill response and assessment.

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