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The effects of weathering and chemical dispersion on Deepwater Horizon crude oil toxicity to mahi-mahi (*Coryphaena hippurus*) early life stages

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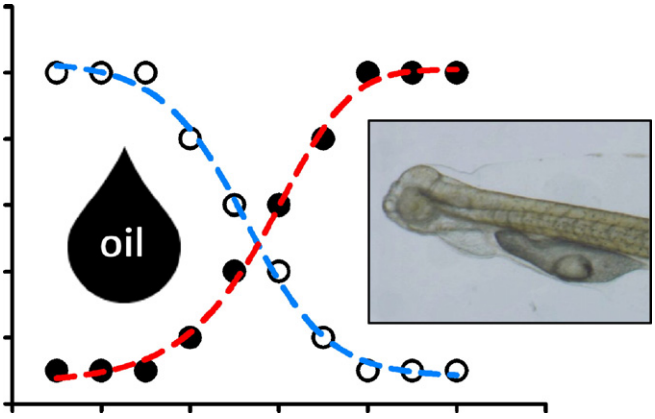
HIGHLIGHTS

- Weathering of crude oil increases the toxicity of water accommodated fractions.
- Dispersant does not change lethal or sub-lethal oil toxicity in larval fish.
- Pelagic larvae are sensitive to weathered oil at low μg/l ΣPAH concentrations.
- There is a close relationship between survival and cardiotoxicity between oil types.

GRAPHICAL ABSTRACT



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ABSTRACT

To better understand the impact of the *Deepwater Horizon* (*DWH*) incident on commercially and ecologically important pelagic fish species, a mahi-mahi spawning program was developed to assess the effect of embryonic exposure to *DWH* crude oil with particular emphasis on the effects of weathering and dispersant on the magnitude of toxicity. Acute lethality (96 h LC50) ranged from 45.8 (28.4–63.1) $\mu g\ l^{-1}$ ΣPAH for wellhead (source) oil to 8.8 (7.4–10.3) $\mu g\ l^{-1}$ ΣPAH for samples collected from the surface slick, reinforcing previous work that weathered oil is more toxic on a ΣPAH basis. Differences in toxicity appear related to the amount of dissolved 3 ringed PAHs. The dispersant Corexit 9500 did not influence acute lethality of oil preparations. Embryonic oil exposure resulted in cardiotoxicity after 48 h, as evident from pericardial edema and reduced atrial contractility. Whereas pericardial edema appeared to correlate well with acute lethality at 96 h, atrial contractility did not. However, sub-lethal cardiotoxicity may impact long-term performance and survival. Dispersant did not affect the occurrence of peri-

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Dispersant
Corexit 9500
Pericardial edema
Atrial contractility

cardial edema; however, there was an apparent reduction in atrial contractility at 48 h of exposure. Pericardial edema at 48 h and lethality at 96 h were equally sensitive endpoints in mahi-mahi.

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1. Introduction

During the spring and summer of 2010 the *Deepwater Horizon* (*DWH*) incident released millions of barrels of crude oil into the northern Gulf of Mexico (GOM), making it the largest marine oil spill in United States history (Crone & Tolstoy, 2010; Ryerson et al., 2012; Camilli et al., 2012; McNutt et al., 2012). This resulted in extensive oiling of the pelagic zone and fouling of shoreline habitats. The timing of the *DWH* spill also coincided with the spawning seasons for many commercially important fish species (Rooker et al., 2012; Rooker et al., 2013; Block et al., 2005; Brown-Peterson et al., 2001; Gibbs & Collette, 1959) and likely resulted in exposure to both breeding and early life stage fish. The embryos of marine fish are particularly susceptible to developmental toxicity from crude oil-derived polycyclic aromatic hydrocarbons (PAHs), which cause a suite of sub-lethal morphological and cardiovascular defects (Incardona et al., 2004; Incardona et al., 2009; Incardona et al., 2011a; Incardona et al., 2012; Dubansky et al., 2013; Zhang et al., 2012; Huang et al., 2012). In aquatic environments crude oil releases a complex mixture (Carls & Meador, 2009) of PAHs into the dissolved phase, many of which have their own unique toxicity characteristics (Incardona et al., 2004; Incardona et al., 2006; Incardona et al., 2011b). The composition of individual PAHs in the water column results from a combination of solubility and natural weathering processes. Weathering typically removes low molecular weight hydrocarbons from oil water mixtures through evaporation, subsequently producing oil slicks with proportionally higher molecular weight and low solubility PAHs (Carls & Meador, 2009). However, the *DWH* spill was distinct from past surface spills in that the crude oil was released from the seafloor, facilitating greater dissolution of low molecular weight hydrocarbons into the water column during ascent (Reddy et al., 2012). This was further complicated by the unprecedented 1.8 million gallons of chemical dispersants that were applied to the surface and sub-surface in an effort to solubilize crude oil and minimize slick formation (Lehr et al., 2010). Dispersants such as the heavily used Corexit 9500 influence oil solubility and PAH bioavailability. Importantly, these dispersants have also been shown to be toxic to fish, such as the Atlantic herring and inland silverside (Hemmer et al., 2011; Adams et al., 2014a).

Historically, oil exposure was thought to exhibit toxic effects primarily through narcotic mechanisms, which led to the target lipid model of toxicity that related oil constituent toxicity to the respective octanol/water partitioning coefficient (Di Toro et al., 2000). The solubility of individual constituents was subsequently integrated with the model to estimate the toxic potential of complex oil water mixtures (Di Toro et al., 2007). Under this scenario low molecular weight PAHs and volatile BTEXs were estimated to be the more harmful components in complex mixtures regardless of the organism (Di Toro et al., 2007). However, over the last 15 years a range of complementary studies have established that cardiotoxicity in early life stage fish following crude oil exposure is specifically associated with water soluble PAHs (Incardona et al., 2009; Marty et al., 1997; Carls et al., 1999; Incardona et al., 2005; Adams et al., 2014b). The primary injury phenotype was extensively characterized in boreal species following the 1989 Exxon Valdez oil spill in Alaska (Peterson et al., 2003), and is marked by fluid accumulation around the heart and other secondary malformations (Incardona et al., 2011a). Interestingly, studies indicated that 3 ring PAHs were the primary cause of embryonic heart failure by disrupting cardiac function (Incardona et al., 2004; Incardona et al., 2009; Incardona et al., 2005), and furthermore that the relative proportion of 3 ring PAHs is predictive of embryonic cardiotoxicity to a complex oil mixture (Incardona et al., 2004). This has raised the important question of whether natural weathering reduces toxicity by eliminating the higher solubility toxic compounds, or increases toxicity by concentrating those components with greater individual toxicity.

Despite the rich literature on PAH toxicity to larval and embryonic fish, relatively little is known about species native to the pelagic zone of the GOM. We recently demonstrated that four representative pelagic species – bluefin tuna, yellowfin tuna, amberjack and mahi-mahi – show characteristic embryonic cardiotoxicity when exposed to high energy water accommodated fractions (WAFs) of naturally or artificially weathered MC252 crude oil at low μg l^{-1} total ($\sum$) PAH concentrations (Incardona et al., 2014; Mager et al., 2014). However, a systematic assessment of acute lethality and cardiotoxicity that incorporates the state of weathering and degree of chemical dispersion has yet to be performed on GOM pelagic species. This information is crucial when attempting to assess the damage to fish and fisheries resulting from the *DWH* oil spill. To this end, the current study established a mahi-mahi (*Coryphaena hippurus*) spawning brood stock culture specifically to obtain newly fertilized embryos for toxicity testing with a variety of DWH-relevant PAH mixtures. The main objective was to assess the differential toxicity of source, artificially weathered and naturally weathered oil, as well as the influence of chemical dispersion on the toxicity of these field-collected samples. A secondary objective was to directly compare standardized lethal concentration 50 (LC50) tests to sub-lethal measures of cardiotoxicity to the different oil types with the intent of defining the proposed link between the two endpoints.

2. Experimental methods

2.1. Animals

Mahi-mahi broodstock (*C. hippurus*) were captured off the coast of Miami, FL using hook and line angling. The fish were subsequently transferred to the University of Miami Experimental Hatchery (UMEH), where they were acclimated in 80 m^3 fiberglass maturation tanks equipped with re-circulated and temperature controlled water (IACUC protocol # 12–064). All embryos used in the experiments described herein were collected within 2–10 h following a volitional (non-induced) spawn using standard UMEH methods (Stieglitz et al., 2012). A prophylactic formalin treatment (37% formaldehyde solution at 100 μl/l for 1 h) was administered to the embryos, followed by 30 min of flushing with a minimum of 300% water volume in the treatment vessel using filtered, UV-sterilized seawater. A small sample of eggs was collected from each spawn to microscopically assess fertilization rate and embryo quality. Spawns demonstrating low fertilization rate (<85%) or frequent developmental abnormalities (>5%) were not used.

2.2. Preparation of water accommodated fractions

Three distinct sources of MC252 crude oil were used to generate WAFs. A field sample was collected on July 29, 2010 from the hold of barge number CTC02404, which was receiving slick oil from various skimmer vessels. This oil type had a DOSS concentration below detection limit (<1 μg g^{-1}). This sample is referred to as slick A throughout. An artificially weathered source sample was generated by slow heating at 70 °C until the total volume was reduced by 20%. The third sample was unweathered source oil that was obtained from the riser pipe. Corexit 9500 was used as dispersant. In all cases, samples were delivered to the University of Miami under chain of custody. High energy

water accommodated fractions (HEWAFs) were prepared as described previously (Incardona et al., 2014; Incardona et al., 2013) at a loading rate of 1 g of oil per liter of seawater. Chemically enhanced water accommodated fractions (CEWAFs) were prepared in 2 l or 5 l aspirator bottles at a loading rate of 1 g of oil per liter of filtered (1 μm), UV-sterilized seawater. Corexit 9500 was added at a rate of 100 mg per g of oil and mixed for 18–24 h at an approximately 10% vortex using a stir plate and Teflon coated magnetic stir bar. After mixing the CEWAF was allowed to settle for 3–6 h, after which the lower 90% was collected and used for exposures. All WAFs were prepared the day of use. Chemical composition of the respective WAF types is shown in supplemental Figs. S1–3.

Due to logistical constraints, only unfiltered samples were measured for toxicity assessments. The dissolved fraction of each PAH analyte was subsequently estimated for each WAF preparation type. Because *Deepwater Horizon* (DWH) oil is a complex mixture with many unknown components, we used empirical data for these estimates. Our participation in the DWH NRDA provided access to chemical analyses of many samples of WAF preparations using the same oil types and methods as the exposure treatments. These samples included paired filtered and unfiltered samples taken from dilutions of each of the six WAF preparations. To produce filtered samples, WAF dilutions were passed through two stacked 0.3-μm glass fiber filters under low suction (<5 cm–10 cm Hg); this filtered out the oil droplets, leaving only the dissolved analytes in the filtered sample. Regressions of the filtered concentration versus unfiltered concentration were performed for each of 50 PAH analytes within each WAF (see Fig. S4). For each regression, we fitted an equation of the form:

$$[\text{dissolved}] = a\left(1 - e^{-b[\text{unfiltered}]}\right)$$

where "a" is the modeled maximum found in the solution, and "b" is proportional to the slope of the relationship approaching the maximum. These models were fitted using the *nls* function in the *stats* package in (R Core Team, 2015). When this equation fit poorly for insoluble analytes (e.g., $K_{ow} > 5.3$) (Redman et al., 2012) or analytes with low concentrations in the oils used to produce the WAFs, we assumed that those analytes did not dissolve, and estimated the dissolved concentration as zero. For a few highly soluble analytes (naphthalene, C1-naphthalene, and biphenyl), measurements of filtered concentrations were often greater than the corresponding unfiltered concentrations. For these analytes, we estimated the dissolved concentrations as 100% of the unfiltered concentrations. The regression results and solubility assessments described above were applied to measured unfiltered concentrations to estimate the dissolved fraction of each analyte (see Supplemental Table S1).

2.3. Toxicity testing

All tests were performed in a temperature controlled environmental chamber (26 °C) with a 16:8 light/dark cycle. Treatments were generated by spiking 5 l of filtered and sterile seawater with varying levels of WAF using a glass syringe. The treatment was mixed on a stir plate for 5 min and then aliquoted into 4 1 l replicates. A single replicate consisted of 20 embryos in 1 l of test solution held in a 1 l glass beaker, with four replicates per treatment. Survival tests consisted of five test concentrations and a control, while sub-lethal tests consisted of four concentrations and a control. This difference was due to the length of time needed to process sublethal endpoints relative to survival. Tests were monitored daily for survival. Mortality was assessed visually and by lack of response to prodding. All dead animals were removed from the test daily using glass transfer pipettes. Survival tests were deemed unreliable if control survival at hatch was less than 70%, or if the subsequent survival of hatched larvae was less than 80%. The defined hatch point for the purposes of test reliability was 48 h as mahi-mahi typically hatch at 35–40 hpf. No water changes were performed.

Survival tests were concluded after 96 h while cardiotoxicity endpoints were assessed after 48 h. For all imaging, 2–3 larvae were captured at a time and transferred to a petri dish, where they were individually mounted atop 2% methylcellulose in seawater. This ensured rapid imaging of specimens, avoiding potential temperature elevation on the microscope stage. Two stereoscope stations allowed sequential processing of 2 replicates at a time; larvae were imaged continuously until all replicates were completed. Crude oil exposure replicates were processed in random order with unexposed control fish evenly spaced throughout. Images (640 × 480) were collected using FireI-400 industrial digital video cameras (Unibrain, San Ramon, CA) mounted on Nikon SMZ800 stereomicroscopes, using MacBook laptops and BTV Carbon Pro software. Images were calibrated using a stage micrometer.

2.4. Image analysis

For scoring the presence or absence of edema, still frames and videos were assessed for the shape of the yolk mass. Larvae were scored as normal if the anterior portion of the yolk sac was smooth and rounded with a bullet-shaped tip and if there were are no obvious indentations on the yolk sac due to pressure from fluid buildup in the pericardial area. Edema was scored positive if the anterior portion of the yolk sac was concave or pushed to a sharp point, and/or if indentations indicated by dark, angular lines were seen pushing on the yolk sac due to pressure from fluid buildup in the pericardial area. Atrial diameter was measured in digital video frames stopped at diastole (maximal relaxation) and systole (maximal contraction), and used to measure contractility calculated as fractional shortening using the formula (diastolic diameter − systolic diameter) / diastolic diameter × 100. All scoring of sublethal endpoints was performed blind, whereby the oil dosage information was withheld from the scoring individual.

2.5. Water chemistry analysis

Samples for total sum PAH (ΣPAH) analysis were collected from each WAF preparation immediately after generation, as well as from each treatment concentration immediately after dilution. Each sample was collected into a 250 ml amber glass bottle with no head space and immediately stored at 4 °C. The ΣPAH measurements comprised a panel of 50 different PAH analytes. This is more than the 33–40 PAHs measured in past studies, with the consequence of shifting the ΣPAH metric upwards. For CEWAF samples, four 10 ml samples were collected for dioctylsulfosuccinate sodium salt (DOSS) analysis and stored at 4 °C. For source oil WAFs, three additional 20 ml samples were collected in glass vials with no head space for volatile analysis, including benzene, toluene, ethylbenzene and xylenes (BTEX). Preliminary analyses showed artificially weathered source and slick A oil WAF preparations contained negligible BTEX concentrations. All samples were then shipped overnight on ice to ALS Environmental (Kelso, WA) for analysis by gas chromatography and mass spectrometry – selective ion monitoring (GC/MS-SIM; based on EPA method 8270D).

To complement GC/MS-SIM quantitation of PAHs, a previously established fluorescence method was also used to calculate ΣPAH for each treatment (Greer et al., 2012). Briefly, a percent WAF standard curve was generated for each prepared WAF in a 50% ethanol solution. A 10 ml sample from each treatment concentration was diluted in 10 ml of ethanol. All standards and samples were sonicated for 3 min and centrifuged at 10,000 rpm for 10 min to precipitate salts. The supernatant was then transferred to a quartz cuvette and measured for fluorescence using a LS-45 spectrophotometer (PerkinElmer). The exact wavelengths were determined for each individual WAF using an excitation-emission scan ranging from 200 to 600 nm. Two distinct peaks were generally observed at excitation wavelengths of

approximately 224 and 258 nm. Instrument software was used to calculate the peak area (relative fluorescence units; rfu), which was used to calculate a standard curve ($R^2 \geq 0.99$) of log[rfu] versus log[% WAF]. The commercially measured PAH concentrations in the stock WAF were then used to convert all fluorescence values to ΣPAH. An independent clean seawater sample from the UMEH facility was used to account for background fluorescence. This analysis provided ΣPAH values for one concentration of the 96 h LC50 source HEWAF test, as well as four concentrations for the slick A CEWAF test.

The following water quality parameters were also monitored for all tests: temperature, pH, dissolved oxygen (DO), salinity and total ammonia. Temperature and DO were measured daily in all replicates using a ProODO handheld optical DO probe and meter (YSI, Inc., Yellow Springs, OH) and pH was measured daily using a PHM201 meter (Radiometer, Copenhagen, Denmark) fitted with a combination glass electrode. Both pH and DO probes were calibrated daily. Salinity was measured daily using a refractometer and total ammonia was measured in final water samples using the colorimetric assay (Verdouw et al., 1978).

2.6. Statistics

Exposure-response curves were fit using the U. S. Environmental Protection Agency's TRAP software package. All data were first fit to a tolerance type Gaussian model with 3 parameters using log-transformed data. In some instances this method yielded a poor fit with no convergence; therefore, a non-linear regression method was used (see Table S2 and S3). Comparison of LC50 and cardiotoxicity effective concentration 50 (EC50) values was based on overlapping 95% confidence intervals, with non-overlapping intervals signifying a significant difference between estimates. Hypothesis testing of cardiac contractility was performed using a one-way analysis of variance and Dunnett post-hoc test ($P \leq 0.05$) using the SigmaPlot 12.5 software package. When data did not conform to test assumptions an analysis of variance on ranks with a Dunn's post-hoc test was used. The effect of a single concentration of dispersant on cardiac endpoints was tested using a student's t-test. Note that hypothesis testing was used in lieu of EC50 because the upper bounds of the endpoint (maximal response) were not apparent in the data set. All raw data from toxicity tests are presented in supplemental Tables S4–6.

3. Results

3.1. Chemical composition of WAF preparations

Of the three oil types, slick A was the most enriched in 3 ringed PAHs, with tricyclics accounting for approximately 60% of the total ΣPAH in both HEWAF and CEWAFs (Figs. S1–3). Source and artificially weathered source HEWAFs had similar profiles that were approximately 50% 2-ring PAHs. The weathered source CEWAF profile was similar to the HEWAF; however, the source CEWAF contained 80% 2-ring PAHs. The estimated dissolved fraction of all WAF preparations was more enriched in 2-ringed PAHs, and accounted for 84–94% of ΣPAH in weathered and source WAFs, but only 27–33% in slick A WAFs. The measured BTEX were generally similar in total concentration between source HEWAF and CEWAF (Fig. S4), but when calculated relative to the total ΣPAH the CEWAF contained approximately 17 BTEX per unit PAH while the HEWAF contained only 0.7 BTEX per unit PAH.

3.2. Lethal concentration toxicity tests

96 h LC50 estimates for the six WAF preparations are shown in Table 1. There were marked differences in toxicity between oil samples related to weathering state. When expressed on the basis of either total or dissolved ΣPAH, source oil was significantly less toxic than the slick A oil in both HEWAF and CEWAF preparations. Toxicity of the artificially weathered source oil was not significantly different from either source or slick A when expressed as dissolved ΣPAH; however, when expressed as total ΣPAH weathered source HEWAF was also significantly more toxic than source oil. When expressed based on the dissolved 3 ring PAH composition, LC50s were nearly indistinguishable between all three oil samples and WAF types. The only notable exception was a marginally, but significantly, higher estimate for slick A HEWAF and CEWAF relative to the source oil CEWAF. No differences between CEWAFs and HEWAFs were apparent within an oil type when expressed as dissolved ΣPAH or 3 ring PAH; however, the CEWAF was more toxic than the HEWAF for source oil based on total ΣPAH.

To assess the influence of chemical dispersion on crude oil toxicity, survival data were analyzed based on the measured DOSS concentrations in the respective CEWAFs. The toxicity of all three oil types was significantly more severe than the Corexit 9500 alone treatment (Fig. 1). Corexit 9500 alone resulted in a DOSS LC50 of 3.9 mg l^{-1}, but ranged between 22 and 918 μg l^{-1} in the presence of oil.

3.3. Sub-lethal cardiotoxicity tests

The occurrence of pericardial edema and reduced cardiac contractility were used as endpoints to assess sub-lethal toxicity after 48 h of exposure to the various WAF types. No differences in the EC50 for the occurrence of pericardial edema were apparent between oils or preparation methods when expressed as total or dissolved ΣPAH (Table 2); although, a suitable pericardial edema EC50 could not be calculated for slick A HEWAF. When expressed as dissolved 3 ring PAHs, the slick A CEWAF had a significantly higher EC50 than other CEWAFs, but this value was within a similar range to the HEWAF preparation. The range of test concentrations was also not sufficient to define a maximum response in cardiac contractility, and therefore LOEC values were used in place of EC50s to define toxicity. This precluded quantitative comparisons of toxicity between oil types and WAF preparation methods. For HEWAF assays the LOEC values for reduction of contractility (Table 3) followed a qualitatively similar trend to that described for survival and pericardial edema; however, HEWAF LOEC values are 4 to 7 times higher than the respective CEWAF LOECs (Table 3). Overall, the different

Table 1
Acute lethality estimates (96 h LC50s) for mahi-mahi exposed to different *Deepwater Horizon* oil types using both high energy and chemically dispersed preparations. Water-accommodated fractions (WAFs) are expressed as total ΣPAH, estimated dissolved ΣPAH and estimated dissolved 3 ring polycyclic aromatic hydrocarbon (PAH) concentrations. Bracketed values represent 95% confidence intervals.

WAF preparation		96 h LC50		
		Total (μg l^{-1})	Dissolved (μg l^{-1})	3 ring (μg l^{-1})
High energy	Source	45.8 (28.4–63.1)	21.3 (5.4–37.3)	1.3 (0.3–2.3)
	Weathered	12.3 (9.5–16.1)	5.4 (2.1–13.6)	0.8 (0.3–2.1)
	Slick A	8.8 (7.4–10.3)	3.5 (3.1–4.1)	2.1 (1.8–2.4)
Chemically enhanced	Source	25.3 (23.2–27.7)	20.6 (18.9–22.5)	1.3 (1.2–1.4)
	Weathered	8.7 (0–59.8)	3.7 (0–19.9)	0.4 (0–2.4)
	Slick A	9.5 (8.5–10.5)	3.4 (3.2–3.7)	2.1 (2–2.3)
Corexit 9500 only	DOSS (mg l^{-1})	3.9 (3.7–4.1)	–	–

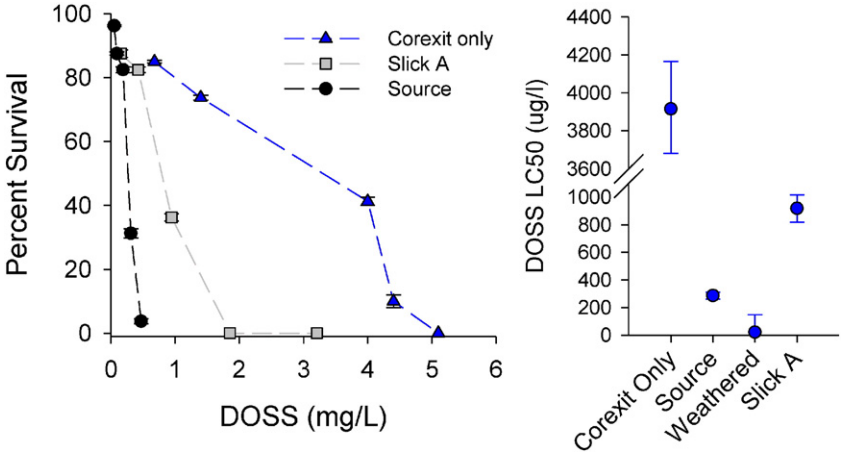

Fig. 1. The effects of the dispersant Corexit 9500 on PAH toxicity to larval mahi-mahi. Left panel: Percent survival plotted against dispersant derived dioctyl sodium sulfosuccinate (DOSS) in various exposure preparations (mean ± SEM). Note that the artificially weathered oil type is not included due to a steep dose response. Right panel: the calculated DOSS 96 h LC50 values (±95% confidence interval) for chemically enhanced WAF exposures.

WAFs showed a close relationship between toxicity manifested as either mortality or pericardial edema (Fig. 2). Importantly, effective concentrations for mortality (96 h) were in a similar range as the concentrations producing edema (measured at 48 h); the latter occurred below the LC50 in only one test (Source CEWAF).

4. Discussion

In the wake of the *DWH* oil spill many challenges arose for injury assessment related to fish and fisheries in the GOM. These included a lack of relevant toxicity information, as little was known about the effects of PAH exposure on pelagic larvae native to the GOM. A second challenge was incorporating natural weathering and chemical dispersion processes into toxicity estimates for potentially affected species. To our knowledge this is the first study to describe acute mortality for embryonic PAH exposure in a species native to the GOM pelagic zone. We also provide additional information to a now expanding body of literature (Incardona et al., 2014; Mager et al., 2014) describing the cardiotoxicity of PAHs on pelagic embryos from the GOM. Finally, by using a variety of oil types and WAF preparation methods we demonstrate the effects of natural weathering processes and chemical dispersion on PAH toxicity as determined through both acute lethality and sub-lethal cardiotoxicity.

Acute lethality for mahi-mahi, as defined by LC50, varied from 8.8 to 45.8 μg l^{-1} total ∑PAH depending on the state of weathering. The naturally weathered slick A WAFs were more acutely lethal than non-weathered source oil; a trend unaffected by chemical dispersion from Corexit 9500. These results reinforce the importance of accounting for the chemical composition of oil as part of the injury assessment process for the *DWH* spill. For example, available field data from the *DWH* oil spill report ΣPAH concentrations ranging from <0.01–77 μg l^{-1} (Bejarano et al., 2013) and as high as 85 μg l^{-1} (Diercks et al., 2010). While the upper range of the reported concentrations is above the mahi-mahi LC50 regardless of oil type, if these reported concentrations are chemically similar to the slick A oil then the upper range exceeds the LC50 by almost an order of magnitude. These data support previous conclusions drawn for early life stage PAH toxicity, including studies that have shown oil becomes more toxic to embryos and larvae of Pacific herring (Carls et al., 1999) and pink salmon (Heintz et al., 1999) as it weathers. However, these earlier findings have recently been challenged (Neff et al., 2013; Page et al., 2012). As described earlier, the high solubility and purported narcosis mechanism of toxicity should mean a higher toxic potential of low molecular weight PAHs and BTEX (Di Toro et al., 2007), which are proportionately and progressively lost during weathering. Nonetheless, the current results clearly demonstrate that weathered oil WAFs are more toxic than non-weathered source oil preparations on a ΣPAH basis. It is well established that sub-lethal cardiotoxicity to complex oil mixtures is attributable to high molecular weight PAHs (Incardona et al., 2004; Incardona et al., 2006; Incardona et al., 2011b), most notably the 3 ring compounds (Incardona et al., 2004). As expected, HEWAF preparations of the slick A oil were enriched in high molecular weight PAHs relative to the source oil and artificially weathered source oils; a trend that was magnified in the dissolved fraction (Fig. S4). In fact, expressing acute lethality in terms of dissolved 3 ring PAH concentration largely eliminated the difference between oil types. This may suggest that different toxicity expressed in terms of total ΣPAH between oil types was the result of the relative abundance of 3 ring PAHs; however, the complex nature of oil mixtures

Table 2
Incidence of pericardial edema (48 h EC50) for mahi-mahi exposed to different *Deepwater Horizon* oil types using both high energy and chemically dispersed preparations. Water-accommodated fractions (WAFs) are expressed as total ΣPAH, estimated dissolved ΣPAH and estimated dissolved 3 ring PAH concentrations. Bracketed values represent the 95% confidence intervals.

WAF preparation		48 h EC50		
		Total (μg l^{-1})	Dissolved (μg l^{-1})	3 ring (μg l^{-1})
High energy	Source	7.3 (1.4–36.7)	6.0 (1.7–21.9)	0.4 (0.1–1.3)
	Weathered	5.7 (1.4–24.0)	2.6 (0.7–9.7)	0.4 (0.1–1.5)
	Slick A	>5.1	>2.6	>1.5
Chemically enhanced	Source	11.5 (7.4–17.9)	9.9 (6.4–15.4)	0.6 (0.4–0.9)
	Weathered	11.3 (7.6–16.8)	5.2 (3.5–7.7)	0.6 (0.4–0.9)
	Slick A	13.0 (8.1–20.7)	3.6 (1.6–7.9)	2.7 (1.2–5.9)
Corexit 9500 only	DOSS	NA (1400)		

Table 3
Reduction in atrial contractility (48 h LOEC) for mahi-mahi exposed to different *Deepwater Horizon* oil types using both high energy and chemically dispersed preparations. Water-accommodated fractions (WAFs) are expressed as total ΣPAH, estimated dissolved ΣPAH and estimated dissolved 3 ring PAH concentrations. Bracketed values represent the NOEC.

WAF preparation		PAH 48 h LOEC — contractility		
		Total (μg l^{-1})	Dissolved (μg l^{-1})	3 ring (μg l^{-1})
High energy	Source	21.6 (10.3)	15.2 (8)	0.88 (0.47)
	Weathered	15.9 (7.9)	6.9 (3.6)	1.05 (0.54)
	Slick A	4.8 (1.2)	2.6 (0.6)	1.49 (0.37)
Chemically enhanced	Source	2.4 (1.2)	2.1 (1.1)	0.13 (0.07)
	Weathered	5.4 (2.7)	1.3 (2.4)	0.28 (0.15)
	Slick A	3.5 (0.3)	0.4 (0.04)	0.29 (0.03)
Corexit 9500 only	DOSS	NA (1400)	–	–

makes this conclusion somewhat difficult. At the very least, acute lethality is similar to cardiotoxicity whereby high molecular weight PAH concentration (≥3 ring) determines toxicity to complex PAH mixtures (Incardona et al., 2004).

ΣPAH measurements represent the sum of two distinct PAH fractions: the dissolved fraction and the micro-droplet fraction (Redman et al., 2012). A dissolution model was used to apply dissolved PAH correction factors to the total ΣPAH measures of toxicity and thereby assess toxicity with respect to the dissolved fractions. Both CEWAF and HEWAF dissolved toxicity patterns were consistent with those for total ΣPAH. More importantly, the significant difference in total ΣPAH acute lethality observed between HEWAF and CEWAF source oil preparations was no longer present. The impact of Corexit 9500 on acute lethality was further addressed by expressing toxicity based on measured DOSS concentrations — a principle ingredient of Corexit 9500. Acute lethality based on DOSS concentration was between 4 and 177 times more severe in the presence of oil than in the presence Corexit 9500 alone. In combination, these data show that the effects of Corexit 9500 on acute lethality are limited to the chemical solubility of PAHs as no additive effects at the biological level were observed when considering the dissolved ΣPAH LC50s. This is generally consistent with survival endpoints using a variety of oil types and fish species, with either dispersant and oil showing no difference or in some cases reduced toxicity relative to oil alone (Hemmer et al., 2011; Fuller et al., 2004). Furthermore, the effect of dispersant and oil was no different than oil alone in seabass, *Dicentrarchus labrax*, exposed to a series of environmental challenge tests (Claireaux et al., 2013). However, dispersant and oil did result in an increased CYP1a response relative to oil alone using three different oil types in rainbow trout, *Oncorhynchus mykiss* (Ramachandran et al., 2004). In the context of developing fish embryos, the toxicity of Corexit 9500 would be expected to arise from its surfactant action on biological membranes, and thus occur at concentrations above which surfactants form membrane-active micelles, or its critical micellar concentration (CMC). The CMC of DOSS in seawater is 1.25 mg l^{-1} (Steffy et al., 2011), and consistent with toxicity based on micellar membrane action, the DOSS LC50 for mahi-mahi larvae following embryonic exposure was above this at 3.9 mg l^{-1} (Table 1).

As anticipated from previous studies on mahi-mahi (Mager et al., 2014) and other species (Incardona et al., 2004; Incardona et al., 2009; Incardona et al., 2011a; Incardona et al., 2012), mahi-mahi showed sub-lethal cardiotoxicity after 48 h of exposure to WAF preparations. This was most consistently evident as reduced atrial contractility and pericardial edema, which were both observed in all tests; no secondary malformations (e.g., jaw or body axis defects) were observed. It has generally been assumed that larval cardiotoxicity results in reduced survival and the data provided here support that viewpoint. A qualitative assessment of LC50 and pericardial edema EC50 across oil types supports the premise that acute lethality occurs from complications stemming from circulatory failure. Interestingly, cardiotoxicity after 48 h of exposure was not found to be a more sensitive endpoint than acute lethality following 96 h of exposure. It seems likely that fast developing species – a trait common for many pelagic GOM species – may suffer the effects of cardiac impairment earlier than slower developing species. The clear link between cardiotoxicity and acute lethality in the current study also allows for the extrapolation of acute survival thresholds of other commercially important GOM species with similar early life histories, ontogenetic timing, and defined pericardial edema sensitivity (Incardona et al., 2014). In particular, bluefin and yellowfin tuna have 48 h EC50s of 0.9 and 2.5 μg l^{-1}, respectively, for pericardial edema when exposed to artificially or naturally weathered oil HEWAF (Incardona et al., 2014). The current study would therefore predict 96 h LC50 values in a similar range, which in the case of bluefin tuna is nearly 100-times lower than the upper range of reported field concentrations.

The relationship between acute lethality and atrial contractility is less robust than that for pericardial edema; however, the difference in endpoint metric makes comparisons more difficult. Significant effects on atrial contractility did not arise until concentrations exceeded the pericardial edema EC50 in two of three HEWAF preparations. In fact,

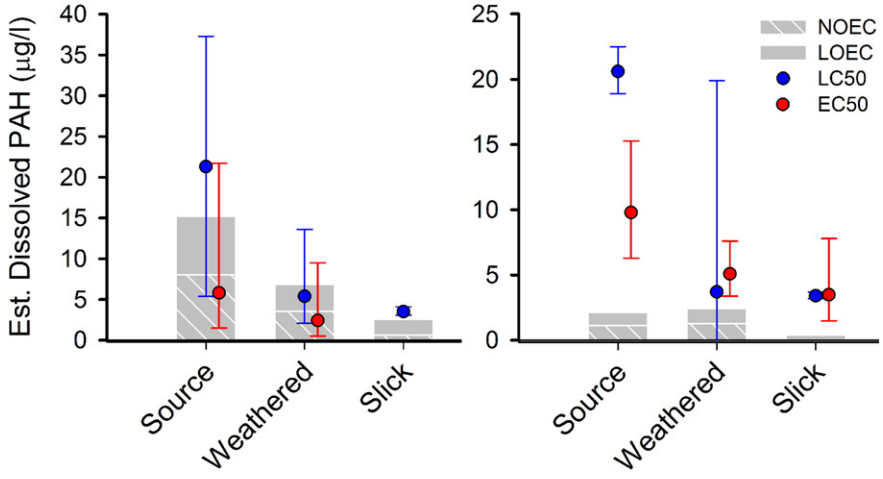

Fig. 2. Comparison of the effects of dissolved ΣPAH exposure on acute lethality, the occurrence of pericardial edema and reduced atrial contractility. Left panel: no dispersant high energy WAF. Right panel: chemically enhanced WAF. NOEC = no observed effect concentration for the reduced atrial contractility, LOEC = lowest observed effect concentration for reduced atrial contractility. Error bars denote the 95% confidence interval for LC50 estimates. A suitable pericardial edema EC50 estimate could not be generated for the slick A HEWAF.

the 48 h LOEC for the weathered HEWAF exceeded the 96 h LC50. Interestingly, the atrial contractility LOECs for all CEWAF preparations were lower than the respective HEWAF NOECs. This difference persisted even when accounting for the dissolved only fraction and 3 ring composition of the respective WAF types, which suggests that some interactive effect is present for this particular endpoint. The different responses between pericardial edema and atrial contractility also suggest different performance consequences. The occurrence of pericardial edema correlates with acute lethality, reflecting more severe cardiac dysfunction (Brette et al., 2013) with clear population level significance. In contrast, inhibited atrial contraction will reduce performance through reduced cardiac output and may alter heart development due to a transient phase of reduced contractility, potentially leading to reduced cardiorespiratory function or even delayed mortality. However, at present it is unclear how persistent such cardiac effects are in surviving fish. Both mahi-mahi and zebrafish show reduced swim performance in recovery and grow-out studies following embryonic PAH exposure (Mager et al., 2014; Hicken et al., 2011); however, no differences were observed in aerobic scope in mahi-mahi (Mager et al., 2014) suggesting that the effects on aerobic swim performance were not due to reduced cardiorespiratory capacity. In contrast, zebrafish showed persistent effects on cardiac morphology a year after embryonic exposure, which likely contributed to reduced swim performance in this species; however, aerobic scope was not evaluated (Hicken et al., 2011). Clearly future work is necessary to further elucidate the functional significance and fitness consequences of sub-lethal cardiotoxicity in larval fish species, especially with respect to the persistence of compromised cardiorespiratory phenotypes.

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Appendix A. Supplementary data

Supplementary material.

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