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Developmental abnormalities and differential expression of genes induced in oil and dispersant exposed *Menidia beryllina* embryos

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ABSTRACT

Exposure of fish embryos to relatively low concentrations of oil has been implicated in sub-lethal toxicity. The objective of this study was to determine the effects of the exposure of *Menidia beryllina* embryos at 30–48 h post-fertilization to the water accommodated fractions of oil (WAF, 200 ppm, v/v), dispersants (20 ppm, v/v, Corexit 9500 or 9527), and mixtures of oil and each of the dispersants to produce chemically enhanced water accommodated fractions (CEWAFs) over a 72-hour period. The polyaromatic hydrocarbon (PAH) and benzene, toluene, ethylene and xylene (BTEX) constituents of the 5X concentrated exposure solutions (control, WAF, dispersants and CEWAFs) were determined and those of the 1× exposures were derived using a dilution factor. PAH, BTEX and low molecular weight PAH constituents greater than 1 ppb were observed in WAF and the dispersants, but at much higher levels in CEWAFs. The WAF and CEWAFs post-weathering were diluted at 1:5 (200 ml WAF/CEWAF: 800 ml 25 ppt saltwater) for embryo exposures. Mortality, heartbeat, embryo normalcy, abnormality types and severities were recorded. The qPCR assay was used to quantify abundances of transcripts of target genes for sexual differentiation and sex determination (*StAR, dmrt-1, amh, cyp19b*, *vtg* and *chg-L*,), growth regulation (*ghr*) and stress response (*cyp1a* and *Hsp90*); and *gapdh* served as the housekeeping gene. Temperature was 21 ± 1.5 °C throughout the experimental period, while mortality was low and not significantly different ($p = 0.68$) among treatments. Heartbeat was significantly different (0.0034) with the lowest heartbeats recorded in Corexit 9500 (67.5 beats/min) and 9527 (67.1 beats/min) exposed embryos compared with controls (82.7 beats/min). Significantly more treated embryos were in a state of deterioration, with significantly more embryos presenting arrested tissue differentiation compared with controls ($p = 0.021$). Exposure to WAF, dispersants and CEWAF induced aberrant expression of all the genes, with *star, dmrt-1, ghr* and *hsp90* being significantly down-regulated in CEWAF and *cyp19b* in Corexit 9527. The *cyp1a* and *cyp19b* were significantly up-regulated in CEWAFs and WAF, respectively. The molecular endpoints were most sensitive, especially the expression of *star, cyp19b, cyp1a, hsp90* and could therefore be used as early indicators of long term effects of Corexit 9500 and 9527 usage in oil spill management on *M. beryllina*, a valid sentinel for oil pollution events.

1. Introduction

Oil dispersing agents have been employed in spill response for decades. Although the exact constituents of Corexit 9500 and 9527 in common use today is proprietary; they have been reported to consist of non-ionic and/or anionic surfactants in a solvent base designed to enhance oil miscibility under varying temperature and salinity conditions (Major et al., 2012; Place et al., 2010; Singer et al., 1996). When an oil pollution event and the subsequent cleanup occur, polycyclic aromatic hydrocarbons (PAHs) and other components of oil and the dispersant used in the cleanup process may persist in the marine environment for a long time thereby creating pathways for lingering biological exposure and associated adverse effects. The toxicity, carcinogenic, mutagenic, and teratogenic properties of PAHs are well documented (Boström et al., 2002; Stegeman et al., 1991). Studies have reported extreme impacts of crude oil and PAHs on the heart of exposed fish embryos including what was described as cardiogenic fluid accumulation syndrome (Brette et al., 2014; Carls et al., 1999; Heintz et al., 1999), and craniofacial and body axis defects (Incardona et al., 2005). George-Ares and Clark (2000) reported that oil spill dispersants, Corexit® 9500 and Corexit® 9527 induced low to moderate toxicity in most

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aquatic species in laboratory tests; while Berninger et al. (2011) studied the effect of oil and oil/9500 mixtures on fish and shrimp species and concluded that the toxicity of oil increases when mixed with dispersant 9500. A similar study by Khan and Payne (2005) looked at the toxicity of 9527 to four different fish species and revealed results similar to Berninger et al., (2011) with an exception of Cunner fish (*Tautogolabrus adspersus*), where mortality was higher in fish exposed to oil alone. Thus they concluded that toxicity of oil and/or dispersants might be species specific. Adams et al., (2014) also provided evidence in support of the contention that dispersants increase the concentration of oil in test solutions without affecting the toxicity of the dispersed oil.

Although aquatic pollutants do impact an extensive range of fish species, more studies use standard laboratory models such as medaka, fathead minnow and zebrafish to determine the impact of pollution (Ankley et al., 2008; Segner, 2009; Scholz and Mayer, 2008). Most environmental risk assumptions about sensitivity and long-term effects of pollutants on fish are based on these few species, which actually may not be natural inhabitants of the aquatic environment under consideration. Hence, *Menidia beryllina*, an estuarine Atherinid fish commonly known as the inland silverside has been proposed as a more widely distributed fish model, especially for North America because they are found in estuarine and brackish habitats throughout the coast (Brander et al., 2013, 2012; Middaugh and Hemmer, 1992). Atherinids are also listed by USEPA (2002) as model fish species for the Whole Effluent Toxicity Testing Program, while being reported to be more sensitive to toxicants when compared with other species (Clark et al., 1985). Moreover, estuaries are utilized by many species of fish for at least part of their lives and are therefore subject to a wide array of pervasive environmental contaminants. Gene expression profiles from the liver (Whitehead et al., 2012) and gill (Dubansky et al., 2013) tissues of the non-Artherinid Gulf killifish, *Fundulus grandis* have been used to identify exposure of fish to the toxic components of oil. These have been shown, especially in early life stages, to reflect the types of responses that are expected to precede long-term population-level effects. These include compensatory responses in genes associated with regulation of transcription, cell cycle progression, RNA processing, DNA damage, and apoptosis (Pilcher et al., 2014).

Oil pollution from exploration and production processes, natural seeps, atmospheric contribution, freight accidents, industrial discharge, and urban run-off is a significant hazard for the marine environment (Wilson and LeBlanc, 2000). Dispersants are generally used as a frontline means of mitigating the impact of oil spills; nevertheless, the ecological implication of their use is still ambiguous. Dispersants increase the rates of natural hydrocarbon degradation by breaking up oil slicks, which increases the surface area for access to the oil by hydrocarbon-degrading bacteria. While this accelerates biodegradation, dispersion may also increase the amounts of PAH present in the water column resulting in a large increase (5–50 times) in the amount of aromatics and PAHs in the water column (Fingas and Banta, 2008). Exposure of fish embryos to relatively low concentrations of oil has been implicated in sub-lethal toxicity. However, the effect of oil and dispersants; singly and in combination should be more thoroughly evaluated to better understand and anticipate the ecological impacts. The present study therefore looked into the effect of oil and commonly used dispersants (Corexit 9500 and 9527), singly and in combination on the *M. beryllina* embryos. We also determined the PAHs benzene, toluene, ethylbenzene and xylenes (BTEX) and low molecular weight PAH constituents of the water-accommodated fraction of oil (WAF), dispersants and chemically enhanced water-accommodated fractions of dispersants and oil (CEWAFs).

We hypothesized that exposure of *Menidia* embryos to WAF, CEWAFs, and each dispersant used alone would (1) disrupt tissue differentiation and hence hatching; (2) result in abnormal development of hatched embryos; and (3) disrupt the expression of transcripts of genes associated with sexual differentiation, growth and the stress response.

2. Materials and methods

2.1. Preparation of exposure solutions

Crude oil from the Deep Water Horizon used in this study was collected from the leaking well riser by BP (using Entrix sampler) and provided to us by AECOM Environment Toxicology Laboratory. The Corexit 9500 and 9527 were also obtained as a gift. Solutions of BP's Deep Water Horizon crude oil collected from the leaking well riser, Corexit 9500, Corexit 9527, oil/9500, and oil/9527 mixed in artificial seawater (25 ppt Instant Ocean) were weathered as described by Hemmer et al. (2011). Each solution was prepared in 2.0 L pyrex glass containers and mixed for 7 days by vortexing with a stir bar, and allowed to degas under a fume hood (25 °C). Mixing intensity was maintained so that a vortex extended 2–3 inches under the water surface. Oil (1000 ppm, v/v, 1 ml/L) and dispersants (100 ppm, v/v, 0.1 ml/L) were weathered singly and in combination at a ratio of 10:1 (oil: dispersant, v/v) by adding oil to the mixing seawater, followed by the dispersants according to the manufacturer's recommended application rate. After 7 days, the solutions were allowed to settle overnight, and the WAF and CEWAF were separated from the oil using separatory funnels. The WAF and CEWAF solutions were further diluted 1:5 into 1 L pyrex glass holding containers with artificial seawater (25 ppt) for embryo exposures. The holding containers were sealed with a lid and opened temporarily to refresh each treatment daily. The final exposure solutions for WAF were the dissolved fraction from 200 ppm oil, 20 ppm for each of the dispersants alone, and the dissolved fraction from a mixture of 200 ppm oil/20 ppm dispersant for the two CEWAFs. This diluted solution was used for fish exposures.

One liter subsamples from each undiluted preparation (control water, WAF (oil), dispersants (Corexit 9500 and 9527) and CEWAF (oil/9500, oil/9527)) were collected and shipped to Columbia Analytical Services (Kelso, WA) for analyses of PAH, BTEX and low molecular weight PAHs. As a preservative, 1 mL hydrochloric acid was added to each sample and the samples were stored at 4 °C and shipped on ice. The PAH and BTEX constituents of the 1× exposure waters were derived based on a dilution factor of 5. Values of PAH or BTEX greater than 1 ppb in at least one of control, WAF, dispersants or CEWAFs are presented in the results. Analyses were conducted using gas chromatography/mass spectrometry (GC/MS) and GC MS/MS according to EPA 8270D standard operating method (USEPA, 2007). Oil fingerprinting was achieved using an HPLC/MS/MS following the EPA 3580A method (USEPA, 1992) and volatile organic constituents were determined using the EPA 5030B method with GC/PID (USEPA, 1996).

2.2. Embryo exposures

M. beryllina (inland silversides) embryos at 30–48 h post-fertilization were exposed in groups of 35–40 embryos per container in 100 ml of either control water or exposure medium that had been diluted 1:5 from the original weathered solutions (WAF, Corexit 9500 or 9527, and CEWAFs (oil/9500, oil/9527)). Exposures were performed in quadruplicate in uncovered glass containers for 72 h. The exposure solutions were renewed with a 50% change on a daily basis. All exposure vessels and measurement devices were made of glass throughout this experiment. Embryos were obtained from Aquatic Biosystems (Fort Collins, CO).