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DISPERSANT AND SALINITY EFFECTS ON WEATHERING AND ACUTE TOXICITY OF SOUTH LOUISIANA CRUDE OIL

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Abstract: Chemical dispersants are an important technology in the remediation of oil spills in the aquatic environment, facilitating degradation of crude oil and salinity is an important factor in dispersant effectiveness. The aim of the present study was to explore the role of salinity on the degradation chemistry of crude oil polycyclic aromatic hydrocarbons (PAHs) and acute toxicity of the water-accommodated fraction (WAF) of the dispersant COREXIT 9500A and chemically dispersed crude oil on a common estuarine fish. Laboratory microcosms were designed at salinities of 4 parts per thousand (ppt), 12 ppt, or 18 ppt and spiked with crude oil, COREXIT 9500A, or a combined exposure to crude oil and COREXIT and allowed to biodegrade for 1 wk, 4 wk, and 16 wk. The WAF was harvested for analytical PAH analysis and acute toxicity testing in juvenile *Fundulus grandis*. Compared with undispersed oil, COREXIT exponentially increased the PAH concentrations in the WAF for up to 16 wk; hopane-normalized concentrations indicated that biodegradation was slowed for the first 4 wk. Dispersed crude oil and COREXIT were acutely toxic following 1 wk of biodegradation with no correlation between PAH concentrations and crude oil WAF mortality. Both dispersant and dispersant oil mixtures remained toxic for at least 4 wk at the lowest salinity tested, suggesting increased sensitivity or reduced biodegradation of toxic components in low-saline environments. At the lowest salinity, oil dispersed with COREXIT was more toxic than either the COREXIT alone or oil alone, even after 16 wk of biodegradation. *Environ Toxicol Chem* 2013;32:2611–2620. © 2013 SETAC

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INTRODUCTION

Chemical dispersants are an important technology in the remediation of oil spills in the aquatic environment and facilitate the chemical, physical, and biological degradation of crude oil. The purposes of surfactants are to reduce oil–water interfacial surface tension and to facilitate the formation of small ($<100\,\mu$m) oil-surfactant micelles [1]. These micelles are then dispersed into the water column through mixing energy or simple diffusion. The direct effect of this dispersion is to reduce the risk of oil slick formation and subsequent fouling of productive coastal ecosystems, reducing exposure for surface-dwelling organisms and increasing the oil–water surface area to potentially increase microbial degradation [2].

Movement of dispersant, crude oil, and their degradation products into the water column imposes risks to aquatic organisms. Early dispersants originated from solvent-based degreasing agents and were characterized as highly toxic [3]. Dispersants today have proven to be more effective while reducing toxicity as measured by median lethal concentration (LC50; 190–500 mg/L) [2]. Aquatic organisms are unlikely, however, to be exposed to dispersants alone, and oil/dispersant mixtures increase toxicity by increasing the uptake and exposure of oil constituents [4–6].

Crude oil is a mixture of tens of thousands of hydrocarbon compounds [7,8]. Aromatics are a major component of crude oil, and the toxicity, mutagenicity, and carcinogenicity of these compounds—specifically, of polycyclic aromatic hydrocarbons (PAHs)—have been well documented [9,10]. Many highly soluble and toxic constituents (low molecular weight alkanes and monocyclic hydrocarbons) are also highly volatile, resulting in an oil plume that is enriched in PAHs, including parent compounds and alkylated homologues [2]. The remaining polar hydrocarbons (including the resins and asphaltenes) comprise the majority of the remaining fraction, but there is limited evidence for their bioavailability [11] or toxicity [12]. Various hydrocarbon measurements (including several PAH analyses) are conducted in routine monitoring programs and in assessing the impact of oil spills, but researchers have not yet identified a chemical measurement that accurately predicts toxicity under a wide range of conditions [13].

Chemically dispersed oil is a complex mixture of dispersant, petroleum hydrocarbons, oil/dispersant droplets, and nondispersed oil. Numerous environmental factors can greatly affect both the solubility of crude oil constituents and the performance of the dispersant. Salinity, for example, has a direct relationship on hydrocarbon solubility and dispersant efficacy. A study of 12 aromatic hydrocarbons showed a decrease in PAH solubility moving from freshwater to seawater [14]. Thus, oil spilled in estuarine environments would likely have greater concentrations of hydrocarbons in the water column than would oil spilled in the open ocean. Low salinities, however, also increase the solubility of the dispersant into the water phase, which lessens dispersant efficacy by reducing the interaction of the dispersant with the oil [15]. Although decreased dispersant solubility may inhibit the dispersion of hydrocarbons into the water column, it may also reduce biodegradation and sequestration rates [16,17].

The National Research Council has recommended testing the effects of dispersant on crude oil biodegradation using laboratory microcosms rather than field-scale investigations [2]. Additionally, many recent studies have used microcosms for biodegradation to simulate actual oil spill conditions without regulatory restrictions, high costs, or loss of control over environmental

variables [18–20]. The gulf killifish (*Fundulus grandis*) is one of the most abundant aquatic species in the productive northern Gulf of Mexico (USA) coastal estuaries in both number and biomass and represents an important secondary productive species [21]. Gulf killifish have been used as a model species to assess the biological consequences of the Deepwater Horizon oil spill for resident estuarine species [22]. Furthermore, *Fundulus* have been shown to tolerate and thrive at a wide range of salinities observed in Louisiana coastal estuaries and thus make them an excellent model to study the toxicity of weathered crude oil over a range of salinity [23].

Approximately 6.9 million liters of the chemical dispersant COREXIT 9500A was injected into the wellhead or applied to surface waters following the Deepwater Horizon oil spill. Although these dispersants were applied to saline waters, weather and currents potentially carried dispersant and dispersed crude oil to coastal habitats with reduced salinities. Thus, the objectives of the present study were 1) to explore the role of salinity and weathering age on partitioning a major class of crude oil components (PAHs) and biodegradation of chemically dispersed and nondispersed crude oil in laboratory microcosms; 2) to examine the acute toxicity of a chemical dispersant (COREXIT 95000A), nondispersed crude oil, and chemically dispersed crude oil in the water-accommodated fraction (WAF) on juvenile *F. grandis* at multiple salinities and weathering stages and model the role of these variables on mortality; and 3) to determine if a common environmental sampling analysis (WAF PAH concentrations) accurately reflects acute toxicity of dispersed and nondispersed crude oil.

MATERIALS AND METHODS

Sediment collection and microcosm preparation

Coastal sediments were collected from Rockefeller Wildlife Refuge in southwestern Louisiana, USA (29° 36′ 51″ N; 92° 34′ 50″ W), on 18 May 2011. This location was selected because it is considered a reference site that did not experience oiling following the Deepwater Horizon oil spill (http://resources.geoplatform.gov/news/mapping-response-bp-oil-spill-gulf-mexico). Sediment was homogenized and transferred in approximately 15-L aliquots to 30 microcosms—that is, 150-L glass aquaria, prepared in a greenhouse laboratory at the Louisiana State University Agricultural Center's Aquaculture Research Station (Baton Rouge, LA, USA). Sediment was then overlaid with approximately 80 L artificial seawater at nominal 4–parts per thousand (ppt), 12-ppt, and 18-ppt salinities prepared using Crystal Sea Marinemix (Marine Enterprises International) as measured with a salinity meter (YSI Model 85; YSI Incorporated) and matured for 12 wk to 16 wk. Mature microcosms were designed to simulate Louisiana coastal estuaries consisting of oxic water overlying sediment with an oxic surface layer and an anoxic subsurface. Microcosms were randomly distributed across 2 metal racks with a top and bottom shelf and were shaded to ensure equal light exposure among all aquaria as measured by a spherical quantum sensor (LI-193; LI-COR Environmental). To prevent fouling of the probe, salinity was not measured in microcosms following the addition of COREXIT and/or crude oil. Appropriate salinities were maintained by adding deionized water weekly to replace evaporation as denoted by exterior markings on the microcosm aquaria.

Treatment of microcosms with oil and dispersants

All microcosm weathering experiments were conducted from October 2011 through February 2012. Microcosms were spiked with varying combinations of South Louisiana crude oil and COREXIT 9500A chemical dispersant. The crude oil was supplied by BP America Production Company and was described as sweet petroleum crude oil (MC 252; sample id: SO-20110211-MPKF-002). Crude oil fingerprinting analysis via gas chromatography–mass spectrometry (GC-MS) demonstrated that this oil was consistent with unweathered oil released during the Deepwater Horizon oil spill in April 2010 (E. Overton, Louisiana State University, Baton Rouge, LA, USA, personal communication). The COREXIT 9500 was supplied by Nalco. Three microcosms, 1 at each salinity, received no treatments and served as controls for background PAH concentrations. The other 27 microcosms received 1 of 3 treatments: water mixed with crude oil ($n = 9$; 3 at each salinity), COREXIT 9500A ($n = 9$; 3 at each salinity), or a combined exposure to crude oil and COREXIT 9500A ($n = 9$; 3 at each salinity). Oil and dispersant were subjected to an initial "preweathering" prior to addition to the microcosms to simulate the loss of volatile organics seen immediately following an oil spill in the environment and to resemble more closely the likely exposure of estuarine systems after an offshore spill event [17]. This preweathering was performed in 4000-mL beakers containing approximately 2600 mL of artificial seawater at salinities of 4 ppt, 12 ppt, or 18 ppt and the appropriate treatment (control, COREXIT 9500A, and/or oil). Oil was initially mixed at a ratio of approximately 1:4 oil:water by volume, and the COREXIT 9500A was used at a dispersant:oil ratio of 1:10 as recommended by the manufacturer. Each beaker was continuously stirred at a uniform speed (220 rpm) overnight (approximately 18 h) under a fume hood. Stirring speed was maintained such that a vortex was created; however, the vortex did not contact the stir bar. After preweathering, mixtures were weighed to measure evaporative loss, and the oil fraction was separated from the water fraction using a separatory funnel. Fractions were weighed to allow later addition to microcosms on on the basis of weight rather than volume. Each fraction was stored refrigerated in amber glass bottles until both oil and aqueous fraction were applied to the microcosms. Separation and storage were required to allow the addition of similarly composed treatments to replicate microcosms, because the exposures were started at different times.

Oil, dispersant, and oil and dispersant treatments were added to microcosms based on weight equivalent to an initial volume of 220 mL of oil or 22 mL of COREXIT 9500A per approximately 80 L of microcosm water. This volume corresponded to roughly 40% surface area coverage of the oil-alone microcosms, which is slightly lower than previous work [13,17] but is more representative of the peak coverage produced by the Deepwater Horizon oil spill (http://sero.nmfs.noaa.gov/ClosureSizeandPercentCoverage.htm). Oil was allowed to weather for 1 wk, 4 wk, and 16 wk at 4-ppt, 12-ppt, and 18-ppt salinities. Water remained at ambient temperature and was monitored using LogTag recorders (LogTag Recorders Ltd) recording every 1 h for the duration of each weathering period. Mean kinetic temperature was calculated (LogTag Analyzer, Ver 2.2) as a measure of the effect of temperature fluctuations during a specified period. Mean kinetic temperature is commonly used in pharmaceutical and food industries as a measure of potential degradation or spoiling during shipment and storage. It differs from the arithmetic mean in that mean kinetic temperature disproportionately weighs higher temperatures in calculations. This is a more informative measure of temperature because crude oil partitioning and biodegradation has been shown to be influenced by higher temperatures [24]. Following weathering

periods, the WAF was collected into buckets lined with modified polytetrafluoroethylene (PTFE) bags to prevent permeation and stored frozen and protected from light until used for acute toxicity studies and PAH analysis.

Chemical analysis

Water samples were collected from all 18 oil-exposed and oil and Corexit–exposed microcosms, as well as from a representative control and COREXIT-only microcosm. Seventy-six PAHs and associated homologues were extracted and analyzed from these water samples by Columbia Analytical Services according to laboratory standard operating procedures. Samples were extracted from 1 L of microcosm water according to US Environmental Protection Agency (USEPA) method 3510C. The samples were serially extracted with methylene chloride using a separatory funnel, dried, concentrated, and exchanged into the appropriate solvent and diluted. Extracted samples were then analyzed by USEPA method 8270D, by GC-MS in the selective ion-monitoring mode (GC-MS-SIM). Reporting limits were typically $<0.03\,\mu g/L$, with the exception of the chemically dispersed 18-ppt salinity 1 wk weathered samples, which was $0.11\,\mu g/L$ (Supplemental Data, Table S1).

Results of the chemical analyses were used to create target PAHs and expanded target PAHs. A target PAH was calculated similar to the calculations of Overton et al. [25] by combining target PAHs from the 24 PAHs listed in the National Marine Fisheries Service methods and in the USEPA methods. A target PAH in the present study differed from Overton et al. [25] in that 1) 2,3,5-trimethyl naphthalene replaced 1,6,7-trimethyl naphthalene, and 2) benzo(*a*)anthracene was included in the present study. Expanded target PAHs included all of the constituents in the target PAH but also all 76 analytes available in the complete analysis. Specifically, in the present study, we included the alkylated forms of naphthalene, fluorene, fluoranthenes/pyrenes, and chrysenes in our expanded target PAHs. Polycyclic aromatic hydrocarbon concentrations also were normalized to C30 hopane. Hopanes are a 5-ring PAH (C28 through C33) that are highly resistant to biodegradation, and hopane normalization eliminates PAH variability caused by abiotic factors (e.g., adsorption, solubility) to indirectly measure biodegradation [26,27]. Very low hopane concentrations, approaching the lower reporting limit $(<0.02\,\mu g/L)$, exponentially skewed the normalization of remaining PAHs to unrealistic values. Samples with hopane concentrations $<0.05\,\mu g/L$ were excluded from hopane normalization and not included in the presented data. Polycyclic aromatic hydrocarbons (and subsequent hopane concentrations) were generally much lower in the nondispersed microcosm treatments; therefore, the 4-ppt salinity at 1 wk, 12 ppt at 4 wk, the 18 ppt at 4 wk and 16 wk were excluded from hopane normalization.

Acute toxicity bioassays

Broodstock gulf killifish were obtained from a cultured population held in indoor recirculation systems maintained at $24.0 \pm 0.5\,^\circ C$ and a salinity of approximately 12 ppt at the Louisiana State University Agricultural Center's Aquaculture Research Station. Juveniles were acclimated for salinity treatment groups by adding premixed saltwater or municipal freshwater to increase or decrease the salinity by 2.5 ppt/d to 3 ppt/d until target salinities were reached. Fish were maintained at the target salinity for a minimum of 2 d prior to initiation of acute toxicity bioassays. Salinity treatments were mixed using Crystal Sea Marinemix (Marine Enterprises International) and verified using a YSI Model 85 meter (YSI Incorporated).

Juveniles were approximately 4 mo to 6 mo of age at the initiation of exposures. Feed was withheld 48 h prior to treatments.

Static-renewal 96-h acute toxicity bioassays were conducted on juvenile *F. grandis* (0.17 ± 0.002 g; mean $\pm$ standard error of the mean [SEM]) using the WAF from each treated microcosm at 5 serial dilutions (100% v/v, 50% v/v, 25% v/v, 12.5% v/v, and 6.25% v/v) along with water collected from a control nontreated microcosm and acclimation water used in serial dilutions. If 100% mortality was observed within 30 min of the initiation of exposure in the 100% v/v treatment groups, an additional 75% v/v dilution was included. Exposures were conducted in triplicate 600-mL glass exposure chambers with 6 fish in each replicate in a water bath at $25 \pm 1\,^\circ C$ with constant aeration. All water was changed daily and mortalities removed twice daily. Water samples were collected and analyzed for water quality at the termination of each exposure. Water-quality measurements included pH, temperature, total ammonia nitrogen, salinity, and alkalinity. Protocol was approved by the Louisiana State University Institution Animal Care and Use Committee prior to initiation of exposures.

Statistical analyses

Correlation analysis was performed between mortality of *F. grandis* in undiluted microcosm water (100% v/v) and 96 chemical constituents or classes of constituents, including the aforementioned list of target PAHs and expanded target PAHs. Significance was determined at an alpha of 0.10 rather than 0.05 as this analysis was less concerned with the risk of incorrectly concluding that a chemical measurement was correlated with toxicity and more centered on the risk of incorrectly concluding that a chemical measurement was unrelated to toxicity. For the same reason, adjustments in the overall error rate to account for making 96 independent statistical tests were not performed even though this would cause identification of 9.6 spurious correlations on average.

For acute toxicity tests, nominal dilution concentrations in v/v percentages were used to calculate the median lethal dose (LD50) and 95% confidence intervals (CIs) following 96-h exposure using PROC PROBIT (SAS, Ver 9.3) with a normal distribution. Generalized linear mixed models were used to identify treatment conditions that most influenced 48-h mortality (PROC GLIMMIX; SAS, Ver 9.3). For the generalized linear mixed models, error distribution was binomial, the link function was logit, and estimation was by maximum likelihood. Model variables included salinity (both linear and quadratic), time, treatment, and dilution. Ninety-one candidate models were selected to include only those variables and interactions with biological relevance. Akaike's information criteria corrected for small sample sizes was used to rank and select the most parsimonious model [28]. Akaike weight, as calculated from the difference of a particular model from the best fit model, represents likelihood of the model and is interpreted as the probability that a particular model is the best approximation in the candidate set [28]. Goodness of fit was calculated by Pearsons χ^2/degrees of freedom (*df*) and r^2_{GIC} (as defined by $1 - (e^{(GICbest - GICnull)/sample\ size})$, which represents the improvement of the model as compared to the null model [29].

RESULTS

PAH analysis and microcosm biodegradation

Microcosm temperatures were generally consistent throughout weathering dates (Table 1). As weathering was conducted in

an outdoor greenhouse, daily fluctuations in ambient weather conditions influenced microcosm temperatures. This influence was more noticeable in the shorter weathering periods because outlying high or low temperatures would have a greater impact on the average of the entire weathering period. As such, the lowest and highest mean kinetic temperatures were noted in the 18-ppt 1-wk exposure (16.4 °C) and 12-ppt 1-wk exposure (21.1 °C).

Visual examination of the chemical measurements suggested treatment differences among salinities, weathering ages, and presence of the chemical dispersant (Figure 1). Control and COREXIT 9500A PAH levels were all at or near the reporting limit, with only the alkylated naphthalenes and decalins $>0.1\,\mu g/L$, and all $< 0.6\,\mu g/L$. The difference in target PAHs and expanded target PAHs in the dispersed versus nondispersed oil treatments was greater than 10-fold (Figure 1A and B). The distribution of the PAHs into the water column at the different salinities and weathering stages in the dispersed and non-dispersed oil were unique. In the nondispersed oil at 4 ppt, PAH concentrations were low at 1 wk and increased at 4 wk before returning to concentrations comparable to controls by 16 wk. At 12 ppt, PAH concentrations were highest at 1 wk and returned to low concentrations by week 4 and remained low through week 16. The 18-ppt salinity treatment had minimal dissolved PAHs at any time point.

The partitioning of PAHs into water following the addition of dispersant results in a pattern different than that observed in the nondispersed oil. The 4-ppt dispersed oil treatments contained less water accommodated PAH than higher salinities. However, the weathering pattern was similar to the nondispersed in that PAH concentrations peaked at 4 wk before returning to reference levels by 16 wk. The COREXIT–dispersed oil treatments at 12 ppt had the highest and most persistent PAH concentrations of any salinity. Polycyclic aromatic hydrocarbons concentrations in dispersed microcosms following 16 wk of weathering at 12 ppt were 70 times higher than either the 4-ppt or 18-ppt salinities at the same weathering age. In contrast to the 4-ppt and 12-ppt treatments, dispersed 18-ppt microcosm PAH concentrations peaked at 1 wk and also contained the highest concentrations of any single treatment. Concentrations at this salinity subsequently decreased rapidly, falling by more than 85% by 4 wk and returning to reference concentrations by 16 wk.

Normalized PAH concentrations in the nondispersed oil were highly variable across time and salinity (Figure 2A). At a salinity of 4 ppt, there was an increase in hopane normalized PAH concentrations, whereas 12 ppt demonstrated little change following 16 wk of weathering. At 18 ppt, only the 1-wk
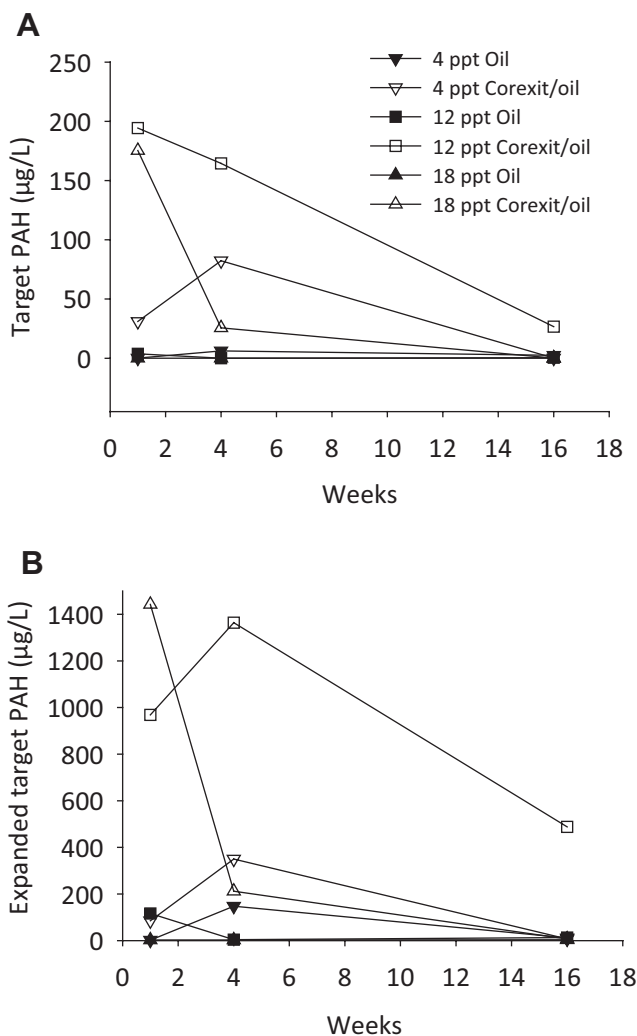

Figure 1. Changes over time in (**A**) target polycyclic aromatic hydrocarbons (PAHs) and (**B**) an expanded list of target PAHs from microcosms containing water with 1 of 3 salinities (parts per thousand; ppt) receiving either south Louisiana crude oil or south Louisiana crude oil dispersed with COREXIT 9500.

weathering age contained hopanes levels sufficient for normalization, and thus no trends could be observed. Normalized PAHs in dispersed oil show a generalized decrease in relative PAHs at all 3 salinities as the oil ages (Figure 2B). The greatest decreases occurred at 4-ppt salinity between 4 wk and 16 wk, and in the 12-ppt treatments between the 1-wk and 4-wk weathering stages. Both water-only and dispersant-only controls were near or below the reporting limit and thus excluded from hopane normalization.

Relative ratios of normalized small (2–3-ring) and large (4–6-ring) PAHs were calculated to examine the biodegradation stage of the WAF (Table 2). In the nondispersed oil microcosms, the ratio of 2-ring to 3-ring PAHs to the large 4-ring to 6-ring compounds was highly variable, with no apparent pattern of change, which may suggest very little biodegradation occurring in the nondispersed oil microcosms. In contrast, there was a slight decrease noted in 2-ring to 3-ring PAHs relative to larger 4-ring to 6-ring PAHs in the dispersed oil microcosms. This was more apparent in the 4-ppt and 12-ppt salinities. At 18-ppt dispersed oil microcosms, however, the ratio at 1 wk was similar to that seen at 16 wk at salinities of both 4 ppt and 12 ppt. This suggests that the stage of weathering of chemically dispersed oil

Table 1. Mesocosm weathering dates with mean temperature (standard error of the mean [SEM]), maximum and minimum temperature (max/min), and mean kinetic temperature (MKT) during the weathering period

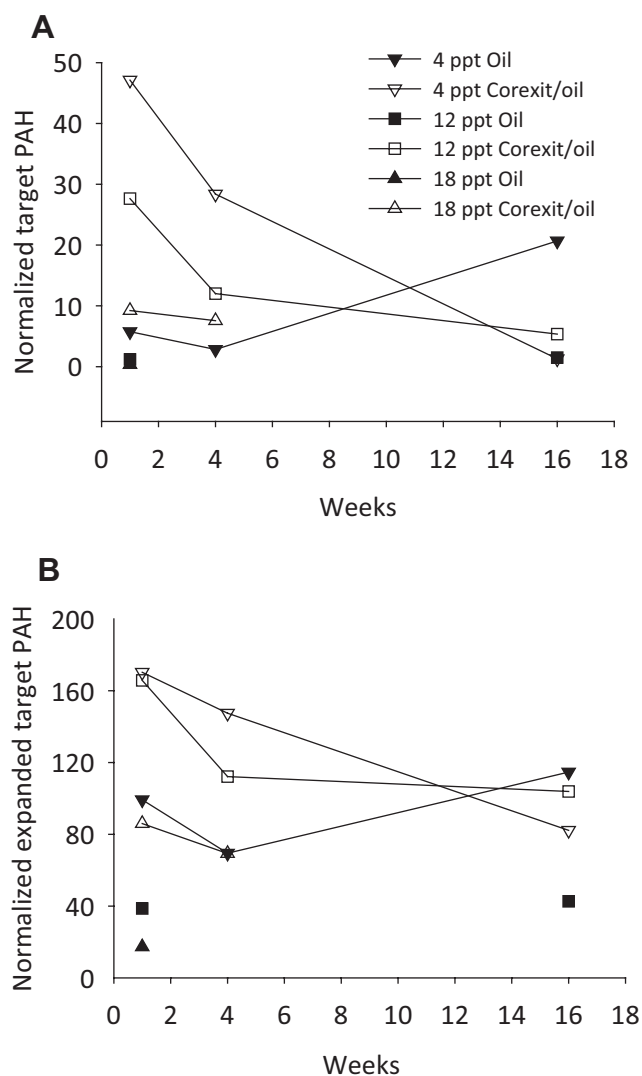| Treatment	Weathering dates	Mean temperature (SEM; °C)	Max/Min (°C)	MKT (°C)
4 ppt				
1 wk	11/7/11–11/14/11	19.0 (0.29)	26.8/11.0	19.8
4 wk	10/31/11–11/28/11	20.1 (0.15)	28.3/10.6	20.8
16 wk	10/31/11–02/13/12	17.3 (0.08)	28.3/5.6	18.4
12 ppt				
1 wk	10/24/11–10/31/11	20.3 (0.30)	27.5/12.8	21.1
4 wk	10/10/11–11/07/11	20.2 (0.15)	27.6/9.7	21.0
16 wk	10/06/11–01/26/12	18.1 (0.09)	28.3/5.6	19.3
18 ppt				
1 wk	11/28/11–12/05/11	15.4 (0.34)	23.0/7.0	16.4
4 wk	11/14/11–12/12/11	17.1 (0.22)	28.3/5.6	18.7
16 wk	11/14/11–02/27/12	17.3 (0.08)	28.3/5.6	18.3

A

B

Figure 2. Hopane normalized polycyclic aromatic hydrocarbon (PAH) concentrations for (**A**) target PAHs and (**B**) extended target PAHs as they weather at each salinity for estimation of PAH biodegradation. Hopane normalization values were excluded for samples with hopane concentrations $< 0.05 \mu g/L$. Biodegradation in nondispersed oil was negligible at all measurable salinity (parts per thousand; ppt) and weathering periods.

following 1 wk at 18 ppt was similar to that observed in the lower salinities at 16 wk.

Acute toxicity

Dispersed oil was more toxic than nondispersed oil at all salinities after 1 wk of biodegradation (Figure 3A, D, and G) and

Table 2. Percentage of small (2–3 ring) and large (4–6 ring) hopane normalized expanded target polycyclic aromatic hydrocarbon concentrations for nondispersed and dispersed oil for each nominal salinity (parts per thousand; ppt) and weathering period

	4 ppt			12 ppt			18 ppt		
	1 wk	4 wk	16 wk	1 wk	4 wk	16 wk	1 wk	4 wk	16 wk
Nondispersed									
2–3 ring	98	83	92	65	–	74	69	–	–
4–6 ring	2	17	8	35	–	26	31	–	–
Dispersed									
2–3 ring	93	91	90	92	89	87	86	85	–
4–6 ring	7	9	10	8	11	13	14	15	–

after 4 wk of biodegradation at the lowest salinity (Figure 3B). At no time was the dispersed oil less toxic than the nondispersed oil (Figure 4A–I). Both COREXIT and COREXIT and oil mixtures induced significant mortalities dependent on salinity and weathering age. After 1 wk of biodegradation, COREXIT alone induced complete mortality at full-strength microcosm water at all salinities and toxicity persisted only at the lowest salinity. At a salinity of 4 ppt, dispersant alone resulted in an LD50 of 30% v/v, and the COREXIT and oil mixture was slightly less toxic at an LD50 of 42% v/v (Table 3). At 4 wk, however, toxicity was reversed between the COREXIT and the COREXIT and oil mixture (Figure 3B). The COREXIT alone was not sufficient to induce at least 50% mortality and thus resulted in an LD50 > 100% v/v (114%), whereas the COREXIT and oil mixture resulted in an LD50 of 72% v/v. All treatments were similar to control by 16 wk (Figure 3C).

At a salinity of 12 ppt, the difference in acute toxicity between dispersant alone and dispersant with oil was reversed (Figure 3D) at 1 wk of weathering. Dispersant and oil treatments resulted in an LD50 of 37% v/v, whereas the dispersant alone was slightly less toxic at 51% v/v (Table 3). Both treatments were comparable to controls by 4 wk (Figure 3E) and remained similar to controls through 16 wk (Figure 3F).

At 18 ppt following 1 wk of microcosm weathering, acute mortality was similar between dispersant alone and dispersant and oil mixtures (Figure 3G). The LD50 of the COREXIT was 28% v/v, and the LD50 of the COREXIT and oil mixture was 36% v/v at this weathering age, and both were comparable to the control by 4 wk (Table 3). Mortalities increased again in the COREXIT-only treatments following 16 wk of weathering (Figure 3I), resulting in an LD50 of 56%. A significant mortality event occurred following 72 h in this treatment, and mortality was comparable to controls at the 48-h time point (data not shown). This treatment is the only 16-wk weathering age that resulted in mortalities dissimilar to control microcosms.

Controls consisted of 100% v/v acclimation water used for serial dilutions and 100% v/v control microcosm water for all salinity and weathering age combination. Mortality in all controls was less than 15%, except in the 4-ppt treatment at 1 wk (28%) and the 18-ppt treatment at 16 wk (22%) (Figure 3). Water-quality measurements were within recommended ranges, with the exception of the 18-ppt microcosm water at 4-wk, in which un-ionized ammonia was 8-fold higher than USEPA recommended concentrations [30]. However, no significant mortality was noted in these treatments.

Twelve chemical measurements were significantly correlated with survival of *F. grandis* in undiluted microcosm water (Table 4). Survival was significantly correlated only with these individual constituents and not with the remaining 64 chemicals measured, as well as all PAH classes measured, such as target PAHs, expanded PAHs, 2-ring PAHs, 3-ring PAHs, and so on. Scatterplots between survival and the 12 statistically significant chemicals, with one exception, exhibited little actual dependence of survival on the chemical measured (data not shown). The exception was the relationship between survival and benzo(*a*)pyrene, which was detected in only 3 samples. Scatterplots between survival and classes of chemicals likewise showed little dependence of survival on chemical classes.

Acute mortality modeling

Analysis of 48-h mortality using generalized linear mixed models identified 3 top models accounting for more than 97% of Akaike weight or likelihood, and the 2 most parsimonious regression models resulting in more than 87% of Akaike weight.
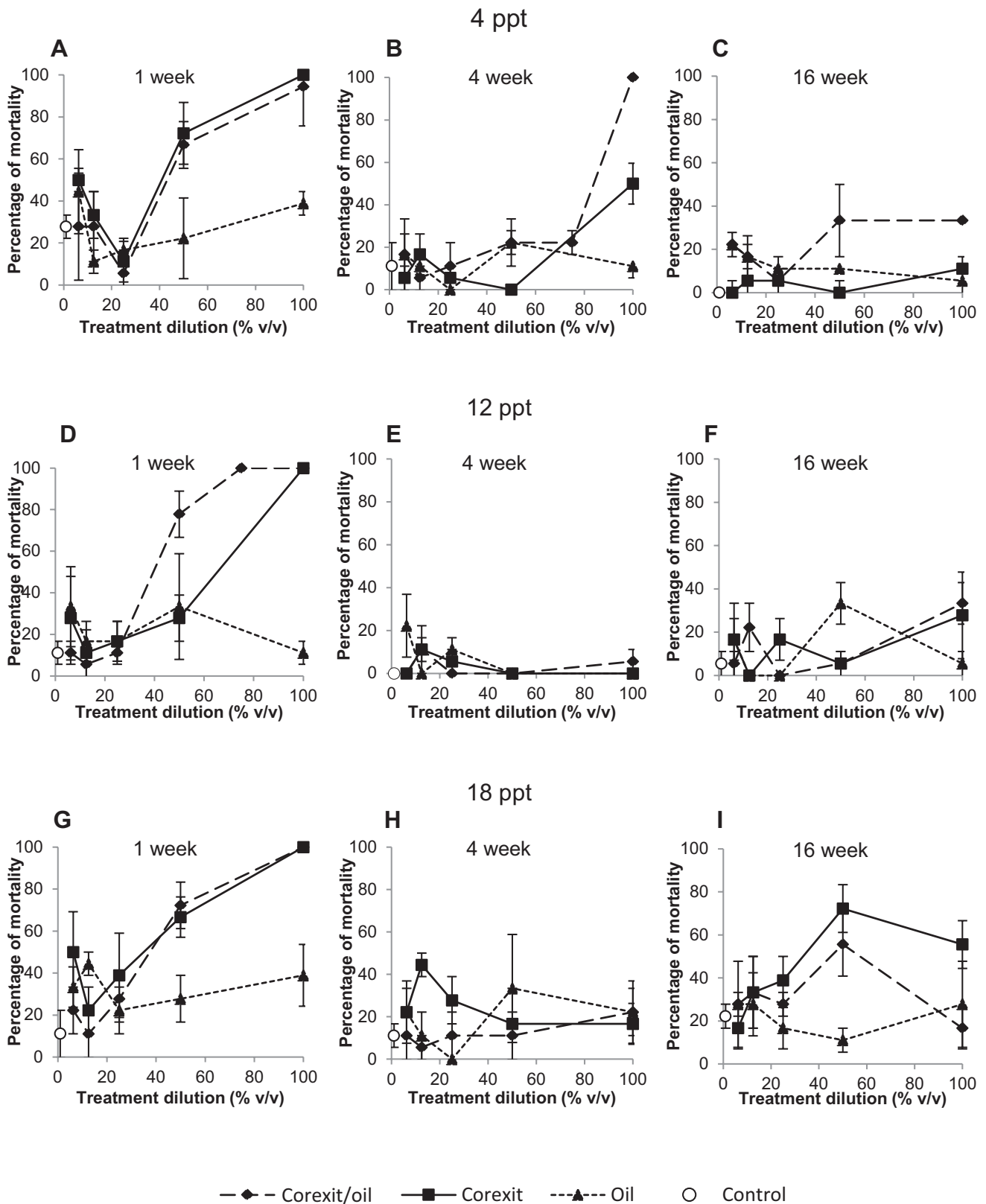
4 ppt

12 ppt

18 ppt

Figure 3. Mortality curves for salinities of 4 parts per thousand (ppt) (**A–C**), 12 ppt (**D–F**), and 18 ppt (**G–I**), with each curve plotting survival percentage against dilution percentage (% v/v) of COREXIT 9500A, oil, and a mixture of COREXIT 9500A and oil for each weathering period (1 wk **A**, **D**, **G**; 4 wk **B**, **E**, **H**; 16 wk **C**, **F**, **I**). Error bars represent standard error of the mean.

All 3 models include a term representing an interaction between weathering age, treatment, and dilution factor and a permutation of an interaction including terms indicating a pairwise interaction between salinity, treatment, and weathering age (Table 5). The most parsimonious model (accounting for 46% likelihood)

indicated that salinity, when considered alone, has a parabolic effect on mortality, representing that at the low and high salinities, there is greater probability of mortality across all treatments, ages, and dilutions. When interactions between age and treatment were taken into consideration, the effects of
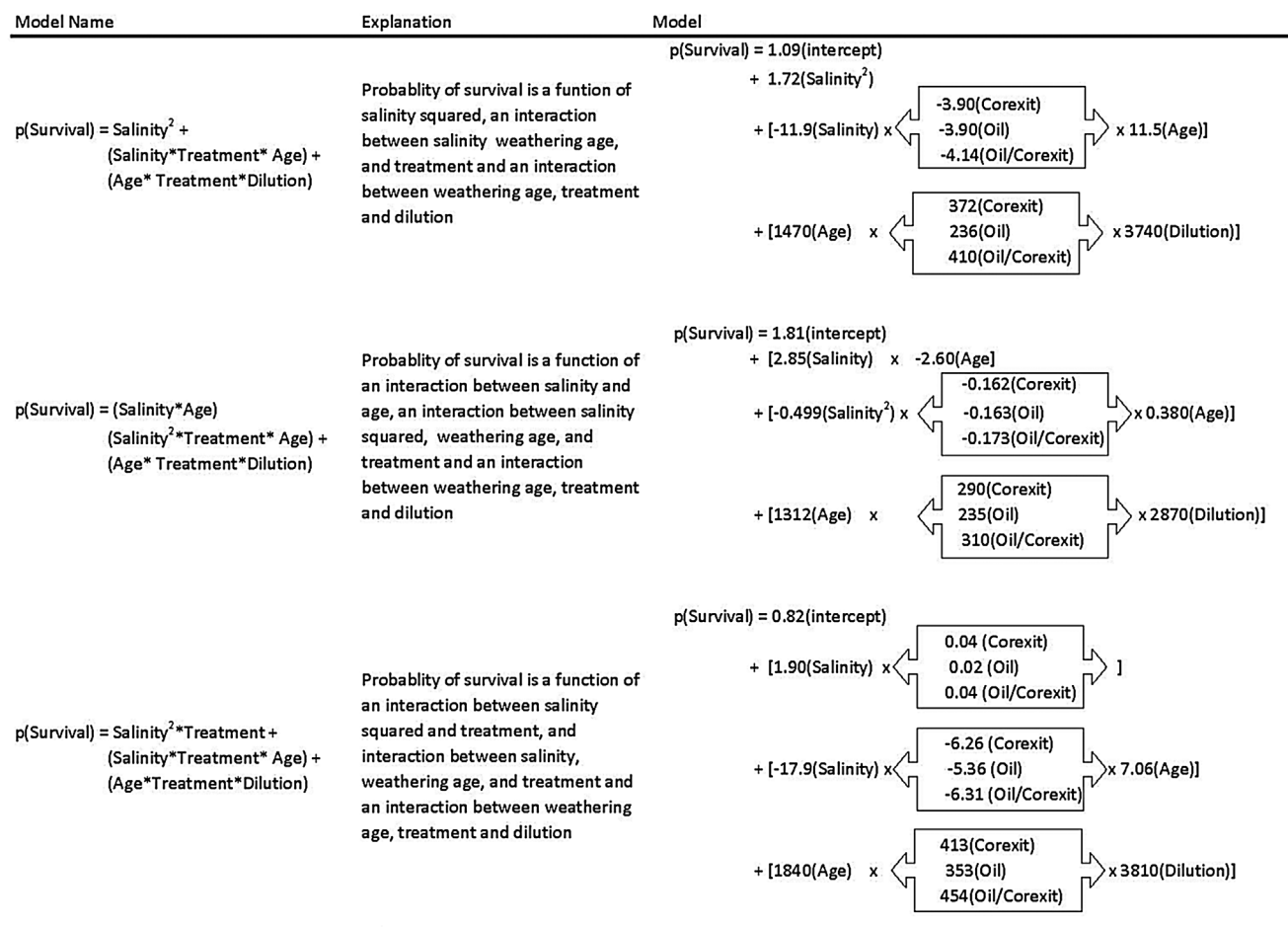
Model Name	Explanation	Model
p(Survival) = Salinity^2 + (Salinity*Treatment* Age) + (Age* Treatment*Dilution)	Probablity of survival is a funtion of salinity squared, an interaction between salinity weathering age, and treatment and an interaction between weathering age, treatment and dilution	p(Survival) = 1.09(intercept) + 1.72(Salinity^2) + [-11.9(Salinity) x { -3.90(Corexit), -3.90(Oil), -4.14(Oil/Corexit) } x 11.5(Age)] + [1470(Age) x { 372(Corexit), 236(Oil), 410(Oil/Corexit) } x 3740(Dilution)]
p(Survival) = (Salinity*Age) (Salinity^2*Treatment* Age) + (Age* Treatment*Dilution)	Probablity of survival is a function of an interaction between salinity and age, an interaction between salinity squared, weathering age, and treatment and an interaction between weathering age, treatment and dilution	p(Survival) = 1.81(intercept) + [2.85(Salinity) x -2.60(Age] + [-0.499(Salinity^2) x { -0.162(Corexit), -0.163(Oil), -0.173(Oil/Corexit) } x 0.380(Age)] + [1312(Age) x { 290(Corexit), 235(Oil), 310(Oil/Corexit) } x 2870(Dilution)]
p(Survival) = Salinity^2*Treatment + (Salinity*Treatment* Age) + (Age*Treatment*Dilution)	Probablity of survival is a function of an interaction between salinity squared and treatment, and interaction between salinity, weathering age, and treatment and an interaction between weathering age, treatment and dilution	p(Survival) = 0.82(intercept) + [1.90(Salinity) x { 0.04 (Corexit), 0.02 (Oil), 0.04 (Oil/Corexit) }] + [-17.9(Salinity) x { -6.26 (Corexit), -5.36 (Oil), -6.31 (Oil/Corexit) } x 7.06(Age)] + [1840(Age) x { 413(Corexit), 353(Oil), 454(Oil/Corexit) } x 3810(Dilution)]

Figure 4. Model explanation and parameter estimates for the 3 most parsimonious models describing morality as a function of salinity, treatment, age, and dilution.

changes in salinity were linear. This likely represents the mortalities that were only observed at a salinity of 4 ppt following 4 wk of weathering.

In contrast, the second most parsimonious model (accounting for 41% of Akaike weight) indicated that salinity alone did not affect mortality but had a parabolic affect when weathering age and treatment are considered. This is likely represented by the slightly reduced toxicity of the dispersant only at a salinity of 12 ppt (LD50 = 51% v/v) versus 4 ppt (LD50 = 30% v/v) and 18 ppt (LD50 = 28% v/v) following 1 wk of weathering.

The interaction of weathering age, treatment, and concentration was logical. Concentration affected the probability of mortality only when age and treatment are considered. For example, high concentrations were toxic only in less-weathered dispersant and oil/dispersant treatments. At 16-wk weathered oil, there was no variation in mortality regardless of treatment or concentration.

Parameter estimates calculated to describe the magnitude of each term demonstrated that the interaction between weathering age, treatment and dilution has the greatest influence on the probability of survival with both weathering age and dilution factor estimates >1400 (Figure 4). The effect each individual treatment in this interaction was also calculated with the influence of oil/COREXIT > COREXIT alone > oil alone, indicating that the dispersed oil is more acutely toxic to juvenile *F. grandis* depending on age and dilution. Although the parameter estimates of salinity, treatment, and weathering age interactions are considerably lower, the rank of treatment influences were similar (oil/COREXIT > COREXIT alone > oil) for both of the most likely models. The parabolic response of salinity alone had the least influence on the model with a parameter estimate of 1.72. In the second most likely model, salinity^2, treatment, and weathering age interactions contribute the least to the overall model.

Table 3. Median lethal dilution (LD50) with 95% confidence intervals of oil alone, COREXIT 9500A alone, and a COREXIT 9500A and oil mixture in 96-h acute toxicity studies in juvenile *Fundulus grandis*

	4 ppt			12 ppt			18 ppt		
	1 wk	4 wk	16 wk	1 wk	4 wk	16 wk	1 wk	4 wk	16 wk
Oil LD50 (% v/v)	–	–	–	–	–	–	–	–	–
COREXIT LD50 (% v/v)	30% (18, 44)	114% (84, 213)	–	51% (40, 68)	–	–	28% (15, 41)	–	56% (28, 134)
COREXIT/oil LD50 (% v/v)	42% (31, 56)	72% (60, 90)	–	37% (31, 46)	–	–	36% (28, 48)	–	–

ppt = parts per thousand.

Table 4. Twelve hydrocarbons correlated with mortality of *Fundulus grandis* after 96 h of exposure to undiluted mesocosm water

Hydrocarbon	r^2	Probability of a greater r	Sample size
2-Methylanthracene	0.41228	0.0133	14
Biphenyl	0.36150	0.0138	16
C1–Fluorenes	0.31516	0.0294	15
Anthracene	0.33643	0.0297	14
C2–Naphthalenes	0.22787	0.0451	18
C2–Fluorenes	0.22824	0.0524	17
C1–Dibenzothiophenes	0.18191	0.0776	18
Benzo(a)pyrene	0.98331	0.0825	3
C1–Phenanthrenes/Anthracenes	0.17491	0.0841	18
1-Methylphenanthrene	0.19764	0.0845	16
4-Methyldibenzothiophene	0.19084	0.0907	16
2,6-Dimethylnaphthalene	0.17448	0.0999	15

Table 5. Three best models (of 91 tested) ranked from most to least parsimonious by Akaike's information criterion corrected for a small sample size (AIC_c)

Model	AIC_c	ΔAIC_c	w_i	χ^2/df [a]	r^2_{GIC} [a]
s^2, s a t, a t d	973.31	0.00	0.45931	1.33	0.767
s a, s^2 a t, a t d	973.54	0.23	0.40942	1.24	0.767
s^2 t, s a t, a t d	976.31	3.00	0.10249	1.30	0.765

[a] Both χ^2/DF and r^2_{GIC} are representations of goodness of fit.
ΔAIC_c = the difference in AIC_c between each model and the most parsimonious model; w_i = the probability that the model is the most parsimonious model; χ^2/DF = Pearson's chi-squared divided by the degrees of freedom; r^2_{GIC} = the improvement over the null model; s = salinity; a = weathering age; t = treatment; d = dilution.

The goodness-of-fit of all 3 models as represented by Pearsons's χ^2 divided by the *df* is 1.33 (1.00 suggests perfect fit), and the improvement over the null-model as represented by the r^2_{GIC} is all 0.76, which indicates that all 3 models are good representations of the probability of survival.

DISCUSSION

Crude oil partitioning and biodegradation

The addition of chemical dispersants can drastically alter crude oil chemistry and partitioning with the goal to reduce oil/water surface tension and move oil from the water surface to the water column. In the present study, the addition of a chemical dispersant resulted in a >10-fold overall increase in PAH levels in the WAF. However, the effectiveness of the dispersant and movement of PAHs into the water fraction may have been affected by salinity and weathering age. The highest target and expanded target PAH levels in the dispersed oil treatments were in the 18-ppt 1-wk microcosms, and the lowest were in the 4-ppt treatments. This is consistent with published reports that show that chemical dispersants are most effective at higher salinities [31]. In contrast to the chemically dispersed oil, there was a generalized decrease in PAH concentrations in non-dispersed treatments as waters became more saline (Figure 1A). In the present study, microcosms at the lowest salinities had the highest target and expanded target PAH concentrations, while microcosms at the highest salinities were comparable to control tanks at all weathering ages. Dissolution dictates partitioning of PAHs between both the oil/water and sediment/water fractions and resulting water fraction and can be influenced by a number of factors, including temperature and salinity [32,33]. Studies have demonstrated a 3-fold to 4-fold increase in PAH solubility as

temperatures increased from 5 °C to 30 °C [34], whereas solubility decreased 68 4.4% in saltwater as compared to freshwater [14,35]. Our studies demonstrated that in the absence of dispersant and a significant difference in weathering temperatures, lower salinities correlated with increased solubility of crude oil.

One of the justifications for using chemical dispersants is to increase removal of oil, but our measurements of target PAHs and expanded target PAHs indicated that the dispersant increased the concentration of hydrocarbons in the water column at all salinities for at least 4 wk and at intermediate salinities for at least 16 wk. Concentrations of PAHs are affected by evaporation and binding to particles, such as sediment and plankton, and biodegradation. Biodegradation has been shown to be the major mechanism for PAH removal in sediment and water [36] and is affected by temperature [37], oxygen content [38], nutrient load [39], and salinity [40]. As such, analysis of biodegradation rate is highly dependent on adsorption/desorption from the sediments, dissolution into the water, and vaporization [36], making the analysis of biodegradation difficult. In the present study, hopane normalization was unable to accurately normalize PAH concentrations in samples that approached the hopane limit of detection and thus overestimated the remaining dissolved PAHs. In all remaining measurable nondispersed oil treatments, hopane normalized expanded target PAH concentrations following 16 wk of weathering is as similar to that following 1 wk. In contrast, dispersed oil demonstrated much higher concentrations and a generalized decrease in normalized expanded target PAH levels across all salinities; but even after 16 wk of weathering, decreases were not enough to reach the low levels observed in nondispersed oil treatments except in the highest salinity treatment.

The rate of biodegradation of aromatic hydrocarbons is directly related to the number of rings, with high-molecular-weight 4-ring to 6-ring compounds biodegrading at a much lower rate than smaller 2-ring to 3-ring compounds [41]. As mixtures of PAHs are biodegraded, there is an increase in the relative proportion of the larger PAHs to the total PAHs due their resistance in biodegradation. In the present study, little change in the hopane normalized PAH ring size ratio of nondispersed treatments suggests that PAH degradation was potentially negligible regardless of salinity or weathering stage. Salinity does appear to have an effect on the degradation rate in the dispersed oil treatments. The ratio of small to large PAHs at 18 ppt following 1 wk of weathering is consistent with the ratios observed in the 4-ppt and 12-ppt treatment following 16 wk of weathering suggesting a decrease in biodegradation rates as salinity decreases. Together, these results demonstrate the effect low-saline environments can have on dispersed oil chemistry, sequestration, and biodegradation as it moves into coastal estuaries.

Acute mortality and modeling

The WAF of nondispersed crude oil did not induce acute toxicity at any salinity or weathering age. This is consistent with numerous studies showing minimal relative acute toxicity in nondispersed oil versus dispersed oil in many taxa and conditions [2]. Because many toxic crude oil components are highly insoluble or rapidly volatized, without dispersion (physical or chemical) into the water column, acutely toxic concentrations for nekton often cannot be achieved by simple diffusion alone.

Dispersant alone, however, was shown to be acutely toxic within the range of the manufacturer's recommended application

ratio at all salinities, and this toxicity persisted at least 4 wk at our lowest salinity. Nominal concentrations of COREXIT 9500A used in the present study were approximately 225 mg/L. A review of acute toxicity of COREXIT 9500A in fish found LC50 concentrations ranging from as low as 25.2 mg/L to greater than 400 mg/L [42] with *F. heteroclitus* displaying a 96-h LC50 of approximately 140 mg/L [42]. Although LC50 concentrations were not calculated in the present study, sufficient COREXIT remains after 1 wk of biodegradation to induce mortality at all salinities and at 4 wk in the lowest salinity.

Salinity influences acute toxicity of a variety of compounds in aquatic organisms [43] and also appears to play a role in the COREXIT acute toxicity observed in the present study. Models from the present study suggest a potential parabolic relationship between toxicity and salinity. The primary components of Corexit 9500A as released by the manufacturer include sorbitan, butanedioic acid, propanol, hydro-treated petroleum distillates, and the anionic surfactant dioctyl sodium sulfosuccinate (DOSS). Aquatic toxicity studies of DOSS and dispersant mixtures containing DOSS have resulted in gill epithelial ruptures, hyperplasia, and lesions on gill lamellae and on filaments, altering respiratory and osmoregulatory functions [44]. Recent work with Corexit 9500, crude oil, and chemically dispersed crude oil on Na^+, Cl^-, and K^+ ion flux in gills indicated a potential role of dispersant and dispersed oil in osmoregulation [45]. Taken with the results from the present study, this indicates a role of COREXIT 9500A and specifically DOSS on osmoregulation and possibly potentiating toxicity under hypo- and hyperosmotic conditions.

Toxicity persisted beyond 1 wk only at the lowest salinity (4 ppt); it persisted for 4 wk for the dispersant and the dispersed oil. Toxicity in both dispersant and dispersed oil was comparable to controls at the intermediate and high salinities at this weathering stage. Although this increased toxicity at 4 ppt may suggest an increased sensitivity in hypo-osmotic conditions, it may also be indicative of reduced degradation of components of COREXIT and toxic crude oil components in low-saline environments. Our observations agree with previous observations that dispersed oil remained more toxic than either oil or COREXIT 9500 even after 6 mo of biodegradation at low salinity [46]. Additional research is necessary into the degradation of components of COREXIT and crude oil at low salinities to determine the mechanism of persistent toxicity.

Polyaromatic hydrocarbons are a highly toxic fraction in spilled crude oil [47,48], and PAH concentrations were analyzed extensively during the 2010 Deepwater Horizon oil spill research and response. However, current results suggest that dissolved PAH concentrations may not accurately represent potential crude oil toxicity. In juvenile *F. grandis* crude oil, acute toxicity was not correlated to any of the individual petroleum chemicals, PAH classes, or target PAHs and expanded target PAHs measured as potential indicators of toxicity. Crude oil WAF toxicity is assumed to be more closely related to total petroleum hydrocarbons (TPHs), which include the heterocyclic aromatics, monocyclic aromatics, and alkyl benzenes [49]; but our observation that toxicity measurements and chemical measurements were only weakly related agreed with the observations of previous researchers who have found no or only weak relationships between TPHs, total PAHs, and/or individual hydrocarbons when such assumptions are tested [13,50–52]. We hypothesize that chemical measurements and toxicity measurements are poorly related in the present study and in previous work [12,50–52] because there are tens of thousands of compounds in crude oil [7,8], but GC-MS currently

quantifies less than 150 of those compounds [25]. Together, these results justify the continued use of toxicity testing in oil-spill response until the chemicals causing toxicity can be identified.

Examination of the fate of DOSS following the Deepwater Horizon oil spill showed that COREXIT applied to the underwater plume persisted at measurable levels at a distance of 300 km from the application site and up to 64 d following the cessation of application [53]. Although it should be noted that, as a result of Gulf of Mexico currents, no DOSS was measured in near-shore samples at concentrations greater than 20 μg/L (study limit of detection) [54], the 3-mile shoreline application limit of COREXIT makes it important to know what the potential impacts of dispersants and dispersed oil in these variable saline environments. Thus, the aim of the present study was to explore the role of salinity on the degradation chemistry and acute toxicity of the dispersant COREXIT 9500A and chemically dispersed crude oil. The present results suggest that low-saline environments reduce the dispersant efficacy and thus also reduce the PAH biodegradation rate. There did not appear to be a correlation between PAH concentrations and crude oil WAF acute toxicity; treatments with the highest PAH concentrations did not consistently demonstrate the greatest acute toxicity. In contrast, COREXIT alone was toxic within manufacturer recommended ratios and appeared to be greatest outside of iso-osmotic conditions. Salinity did not appear to affect toxicity of lightly weathered chemically dispersed oil, but may affect toxicity persistence as both COREXIT and COREXIT/oil mixtures were still toxic following 4 wk of weathering in low saline environments only. Potential mechanisms for this persistence include reduced degradation (COREXIT) or greater toxin bioavailability.

SUPPLEMENTAL DATA

Table S1. (1.1 MB PDF).

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