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EFFECT OF DISPERSANT ON THE COMPOSITION OF THE WATER-ACCOMMODATED FRACTION OF CRUDE OIL AND ITS TOXICITY TO LARVAL MARINE FISH

CATHERINE M. COUILLARD,*† KENNETH LEE,‡ BENOÎT LÉGARÉ,† and THOMAS L. KING‡
†Environmental Sciences Branch, Department of Fisheries and Oceans, Maurice Lamontagne Institute, P.O. Box 1000, Mont-Joli, Quebec G5H 3Z4, Canada
‡Centre for Offshore Oil and Gas Environmental Research, Bedford Institute of Oceanography, Fisheries and Oceans Canada, P.O. Box 1006, Dartmouth, Nova Scotia B2Y 4A2, Canada

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Abstract—Newly hatched mummichog (*Fundulus heteroclitus*) were exposed in a 96-h static renewal assay to water-accommodated fractions of dispersed crude oil (DWAF) or crude oil (WAF) to evaluate if the dispersant-induced changes in aqueous concentrations of polycyclic aromatic hydrocarbons (PAH) affected larval survival, body length, or ethoxyresorufin-*O*-deethylase (EROD) activity. Weathered Mesa light crude oil (0.05–1 g/L) and filtered seawater with or without the addition of Corexit 9500® were used to prepare DWAF and WAF. At 0.2 g/L, the addition of dispersant caused a two- and fivefold increase in the concentrations of total PAH (ΣPAH) and high-molecular-weight PAH (HMWPAH) with three or more benzene rings. Highest mortality rates (89%) were observed in larvae exposed to DWAF (0.5 g/L; ΣPAH, 479 ng/ml). A reduction in body length was correlated with increased levels of ΣPAH ($r^2 = 0.65$, $p = 0.02$) and not with HMWPAH. The EROD activity increased linearly with HMWPAH ($r^2 = 0.99$, $p = 0.001$) and not with ΣPAH. Thus, chemical dispersion increased both the ΣPAH concentrations and the proportion of HMWPAH in WAF. Dispersed HMWPAH were bioavailable, as indicated by a significantly increased EROD activity in exposed mummichog larvae, and this may represent a significant hazard for larval fish.

Keywords—*Fundulus* sp. Dispersant Petroleum oil Polycyclic aromatic hydrocarbons Ethoxyresorufin-*O*-deethylase

INTRODUCTION

Petroleum oils are complex mixtures of hundreds of different compounds, more than 98% of which usually are hydrocarbons, and their toxicity varies as a function of their composition [1]. Polycyclic aromatic hydrocarbons (PAH) are viewed as a major determinant of petroleum oil toxicity [2]. Early life stages of fish are very sensitive to the toxic effects of PAH. Detrimental effects on Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*) have been observed at total PAH (ΣPAH) concentrations of 0.4 and 1 ng/ml, respectively, in exposure studies with water-accommodated fractions of weathered Alaska North Slope crude oil [2,3]. Biological responses include edema, hemorrhages, spinal deformities, growth retardation, and increased activity of cytochrome P450 enzymes.

The individual components within a crude oil exhibit different levels of toxicity. Low-molecular-weight hydrocarbons (LMWH) with one or two benzene rings are relatively water-soluble (octanol/water partition coefficient [log K_{ow})], <4) and quickly reach high concentrations in the water column a few hours after an oil spill. However, they are not considered to be highly persistent in the environment, because they are very susceptible to physical processes, such as evaporation [4]. Nevertheless, they remain an environmental concern, because they are capable of penetrating cell membranes to cause toxicity through a nonspecific mode of action, such as narcosis [5]. High-molecular-weight PAH (HMWPAH) with three or more benzene rings are less water soluble, more persistent, and 10- to 1,000-fold more toxic than LMWH [4]. Some HMWPAH, such as benzo[*a*]pyrene and retene, induce the activity of cytochrome P4501A (CYP1A). This enzyme may metabolize PAH to more toxic forms and induce a variety of toxic effects, including oxidative stress and genotoxicity. The detrimental effects observed in fish embryos exposed to crude oils are similar to those observed in several species of fish embryos exposed to a variety of CYP1A-inducing compounds [6–8].

Chemical oil dispersants are applied to spills at sea to reduce the risk of environmental impacts to near-shore and coastal areas and surface inhabitants, such as birds or marine mammals. Their use is based on the premise that oil is diluted to nontoxic levels and that dispersed oil enhances the rate of oil degradation by physical and chemical processes. However, before dilution by natural processes to less than the toxicity threshold limits, the application of chemical dispersants could increase the risk of toxicity to pelagic organisms, including early life stages of fish, because they elicit a rapid increase in water-column PAH concentrations [9]. Concentrations of LMWH and HMWPAH are higher in the water column following application of chemical dispersants to surface slicks. Several studies have shown that dispersant additions influenced the introduction of PAH into the water column by increasing the relative concentrations of HMWPAH [10,11].

Evidence suggests that the dispersed oil water-accommodated fraction (DWAF) is significantly more toxic than the oil water-accommodated fraction (WAF) or dispersant alone [10,12–15]. Increased toxicity of DWAF may be attributable mostly to higher concentrations of LMWH that would represent short-term risk for acute toxicity but quickly disappear from the water column. Our knowledge regarding the bioavailability of chemically dispersed HMWPAH is limited. An assessment of their potential impact on aquatic toxicity must

* To whom correspondence may be addressed (couillardc@dfo-mpo.gc.ca).

Table 1. Standardized method for preparation of water-accommodated fractions of dispersed crude oil (DWAF) or crude oil (WAF)[a]

	WAF	DWAF
Water preparation	Filtered natural seawater, salinity adjusted to 30‰	Filtered natural seawater, salinity adjusted to 30‰
Petroleum oil	Weathered Mesa light crude oil, 0.2–1.0 g oil/L water	Weathered Mesa light crude oil, 0.05–0.2 g oil/L water
Vessel	2-L aspirator glass bottle with 1.8 L of water	2-L aspirator glass bottle with 1.8 L of water
Dispersant	No dispersant	Corexit 9500, 1:30 (ratio of dispersant to oil)
Vortex	Low mixing speed, no vortex	Vortex 20–25% of water depth
Mixing duration	24 h	18 h
Settling duration	No settling	6 h

[a] Singer et al. [18].

be determined, because they represent a greater risk for long-term chronic toxicity. Concern exists that chemical dispersants may increase the bioavailability of the HMWPAH and, thus, the risk for chronic toxicity to aquatic biota, including early life stages of fish.

To evaluate if the dispersant-induced change in composition of aqueous PAH represents a significant hazard for fish larvae, several WAF and DWAF of varying compositions were prepared, and their toxic effects on mummichog (*Fundulus heteroclitus*) larvae were examined. Mummichog, which is one of the most productive and abundant fish in tidal marshes on the east coast of North America from Texas to the Gulf of St. Lawrence, was selected as the test species, because it is easily raised in the laboratory and is sensitive to petroleum oil toxicity [16,17]. Using a reference crude oil, our objectives were to compare the concentration and composition of PAH in WAF and DWAF, to compare their toxicity to mummichog larvae, and to investigate the relationship between PAH composition and toxicity.

MATERIALS AND METHODS

Preparation of WAF and DWAF

Weathered Mesa light crude oil and filtered seawater with or without the addition of the dispersant Corexit 9500® (Nalco/Exxon Energy Chemicals, Houston, TX, USA), were used to prepare DWAF and WAF. To simulate weathered oil to which dispersant would be added during an emergency response event, the crude oil was preweathered by sparging with air at room temperature for 24 to 48 h until 20% of the oil volume had been lost. Dilution water was filtered (pore size, 0.2 μm) natural seawater collected from the Maurice Lamontagne Institute marine laboratory system, which is located on the south shore of the St. Lawrence Estuary (PQ, Canada). The salinity was adjusted to 30‰ with Instant Ocean® (Aquarium Systems, Mentor, OH, USA).

Both DWAF and WAF for toxicity testing were prepared with a standard, widely accepted method developed by the Chemical Response to Oil Spills Ecological Effects Research Forum [18]. Moderate mixing energy is used to produce DWAF and low mixing energy to produce WAF to limit entrainment of particulate oil in the water column and prevent emulsification. Application of this standardized procedure enhanced reproducibility in the preparation of WAF and is applicable to a wide variety of oils. In contrast, Barron and Ka'aihue [19] recommend comparative studies using an identical defined energy level for producing DWAF and WAF that was considered to be representative of environmental conditions during an actual oil-spill event. However, under this scenario, the chemical composition of WAF produced under similar conditions may be variable because of the entrainment of bulk oil in the water.

The DWAF and WAF (Table 1) were prepared with 1.8 L of water in 2-L glass aspirator bottles covered with aluminum foil [18]. To prepare a typical stock solution of DWAF, oil and dispersant were sequentially injected into a vortex (20–25% of water depth) formed in the aspirator bottle with a magnetic stirring bar. Dispersant was added at a ratio of dispersant to oil of 1:30 (v/v) as recommended by the manufacturer for field application [20]. An 18-h mixing period was followed by a 6-h settling period to allow recoalescence and surfacing of bulk oil particles. To prepare WAF, the speed of the magnetic stirrer was adjusted at the maximal speed possible without the formation of a vortex (195–200 rpm). A 24-h mixing period was used with no settling period before sample recovery, because bulk oil did not become entrained in the WAF fraction prepared under these experimental conditions. The aqueous phase was then poured into a glass separatory funnel for recovery of the WAF and DWAF. A variety of WAF and DWAF were prepared at various oil loadings to generate solutions with a range of ΣPAH concentrations, including WAF and DWAF with similar ΣPAH concentrations and different compositions. Controls with or without dispersant (at the concentration used for the highest oil loading) were prepared exactly like the DWAF or WAF but without the addition of petroleum oil.

Extraction and analyses of PAH in the WAF

Water samples were transferred (600–780 ml) into a 2-L separatory funnel for extraction using a modified version of U.S. Environmental Protection Agency Method 3510 C (http://www.epa.gov/epaoswer/hazwaste/test/main.htm). Each water sample was spiked with 1,000 μl of a surrogate standard mixture containing d_{10}-phenanthrene, d_{10}-pyrene, d_{12}-benzo[*a*]pyrene, d_{12}-benzo[*b*]fluoranthene, and d_{14}-dibenz[*a*,*h*]anthracene as well as the predeuterated alkanes d_{26}-dodecane, d_{36}-heptadecane, d_{50}-*n*-tetracosane, d_{66}-*n*-dotricontane, and 5α-cholestane (Cambridge Isotope Laboratories, Andover, MA, USA) and extracted three times with 50 ml of dichloromethane (Caledon, Georgetown, ON, Canada). The extracts were combined and concentrated to 1.0 ml using a Turbo-Vapp II concentrator (Zymark, Hopkinton, MA, USA) for storage at 4°C in amber glass vials until further analysis.

The extracts were purified according to the method of Telli-

Karakoc et al. [21] with modifications using a solid-phase extraction column (VWR-Canlab, Mont-Royal, PQ, Canada, Catalog BJ9400) packed with silica gel (activated 200°C for 17 h and deactivated 5% w/v with high-performance liquid chromatography [HPLC]-grade water; Whatman Laboratory Division, Clifton, NJ, USA; 60A 70–230 mesh American Society for Testing and Materials for HPLC, catalog no. 4791-010). The column was washed with hexane (Caledon), the sample applied as a 1-ml extract, and PAH eluted with 10 ml of hexane:dichloromethane (4:1 v/v).

Extracts of water were analyzed using high-resolution gas chromatography (GC; Agilent 6890, Wilmington, DE, USA) coupled to a mass-selective detector (Agilent 5973N) in the selective ion-monitoring mode. The following GC (MDN-5S column; length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; Supelco, Bellefonte, PA, USA) conditions were used: Cool on-column injection with oven-track mode (track 3°C higher than the oven-temperature program), 80°C hold for 2 min, ramp at 4°C/min to 280°C, and hold for 10 min.

Quantification criteria for PAH included retention time matching—that is, within ±0.010 min of the retention time of the standard—and comparing the relative abundance of the qualifying ions (±10%), the molecular ion, and one or more qualifier ions in the mass spectrum of the compound with the commercial standard. A calibration curve using six PAH (Ultra Scientific, Kingstown, RI, USA; Absolute Standards, Hemden, CT, USA) working standards (concentration range, 50–2,000 pg/ml) was updated for each batch of samples analyzed. Analytes detected in extracts were interpreted and quantified using the Auto-Quant software (Agilent). The same method was used to measure PAH in one sample of the weathered Mesa light crude oil.

Operational blanks were prepared with each batch of 10 samples. The method before GC–mass spectrometry requires internal standardization. Typical recoveries of added deuterated PAH surrogates before extraction averaged as follows (mean ± SD): d_{10}-phenanthrene, 91% ± 7%, d_{10}-pyrene, 108% ± 8%; d_{12}-benzo[*a*]pyrene, 114% ± 11%; d_{12}-benzo[*b*] fluoranthene, 114% ± 11%; and d_{14}-dibenz[*a*,*h*]anthracene, 137% ± 11%. Recoveries of added predeuterated alkanes before extraction averaged as follows: d_{26}-dodecane, 76% ± 14%; d_{36}-heptadecane, 102% ± 9%; d_{50}-*n*-tetracosane, 138% ± 12%; d_{66}-*n*-dotricontane, 101% ± 13%; and 5α-cholestane, 80% ± 32%.

Toxicity test

Mummichog collected from a reference site in Horton's Creek (NB, Canada) were maintained at the Maurice Lamontagne Institute in a 420-L fiberglass tank with flowing water (salinity, 26‰). To induce gonadal maturation, the water temperature was raised progressively from 10 to 19°C, and the photoperiod was adjusted to 16:8-h light:dark [22]. Fish were fed ad libitum twice daily with pelleted cichlid food (Nutrafin®; Rolf C. Hagen, Montreal, PQ, Canada) supplemented with dried shrimp and *Tubifex* worms. Spawning in the laboratory was triggered by introducing spawning substrates (artificial plants) into the tank. Eggs were collected and incubated at 22.6 ± 0.15°C until hatching was triggered by water immersion (day 11).

Two experiments were conducted to expose fish larvae to a variety of WAF and DWAF with overlapping ΣPAH concentrations. Larvae were exposed to six treatments in triplicate. In experiment 1, larvae were exposed to three WAF prepared with 0.2, 0.5, or 1 g oil/L without dispersant to, one DWAF prepared with 0.2 g oil/L with the addition of dispersant, and to two controls, one with water alone and the other with water and dispersant at a concentration equal to that used for the highest oil loading. In experiment 2, larvae were exposed to four DWAF prepared with 0.05, 0.1, 0.2, or 0.5 g oil/L with dispersant added, and to two controls, one with water alone and the other with water and dispersant. Water-accommodated fractions of crude oil and dispersed crude oil were prepared fresh daily to renew the exposure solution. A sample of each WAF, DWAF, or control was taken each day immediately after preparation for chemical analysis. In addition, one pooled sample for each treatment was taken from the test chambers at the end of each 24-h exposure period to monitor the temporal changes of the PAH concentrations.

Newly hatched mummichog larvae were assigned randomly into groups of 18 larvae and distributed into 500-ml glass bottles with 360 ml of WAF, DWAF, water with dispersant, or water alone, in a static renewal assay for 96 h at 20°C under fluorescent light with a photoperiod adjusted to 14:10-h light:dark. Solutions were not aerated during the test [18]. Water temperature (experiment 1, 20.2 ± 0.3°C; experiment 2, 21.3 ± 1.5°C), dissolved oxygen (experiment 1, 83% ± 4%; experiment 2, 83% ± 12%), and salinity (experiment 1, 30.0‰ ± 0.1‰; experiment 2, 30.2‰ ± 0.1‰) for the test chambers were measured at the beginning and the end of each 24-h exposure period in one replicate per treatment. No significant deviations were found in water temperature, oxygen, or salinity between experiments or among treatments.

All bottles were checked daily for mortality, and dead larvae were noted and removed. Cumulative mortality was assessed in each bottle. At the end of the 96-h exposure period, body length was measured for all live larvae using a microscope fitted with an ocular micrometer. All live larvae in each bottle were then frozen in liquid nitrogen and stored at −80°C for analysis of EROD activity encoded by CYP1A. Ethoxyresorufin-*O*-deethylase activity was measured in the S9 fraction prepared from homogenates of a pool of 10 whole mummichog larvae. It was measured at 22°C by a microplate spectrofluorometric assay using a Cytofluor II® plate reader (Perseptive Biosystems, Framingham, MA, USA; excitation wavelength, 530 nm; emission wavelength, 585 nm) [23]. Reaction mixtures contained 50 μl of S9 in *N*-2-hydroxyehtylpiperazine-*N*′-2-ethanesulfonic acid buffer (0.1 M, pH 7.8), 10 μl of nicotinamide adenine dinucleotide phosphate (20 mg/ml), and 50 μl of 7-ethoxyresorufin (0.024 mg/ml in dimethyl sulfoxide). Activity was quantified by measuring the fluorescence of resorufin at 60-s intervals over a total scan time of 13 min. Fluorescence readings were compared to a resorufin standard curve. All samples were assayed in triplicate. Total protein content of the S9 fraction was measured with Bradford reagent using bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA, USA). Ethoxyresorufin-*O*-deethylase activity was calculated as pmol resorufin/min/mg protein.

Statistical analyses

For EROD activity and percentage mortality, the sample size per treatment was small ($n = 3$); thus, it was not possible to test normality and homogeneity of variance. Therefore, these variables were compared among treatments within experiments with a Kruskal–Wallis test followed by, when significant, analysis of variance (ANOVA) on ranked data with Tukey's studentized range test. Normality and homogeneous variance

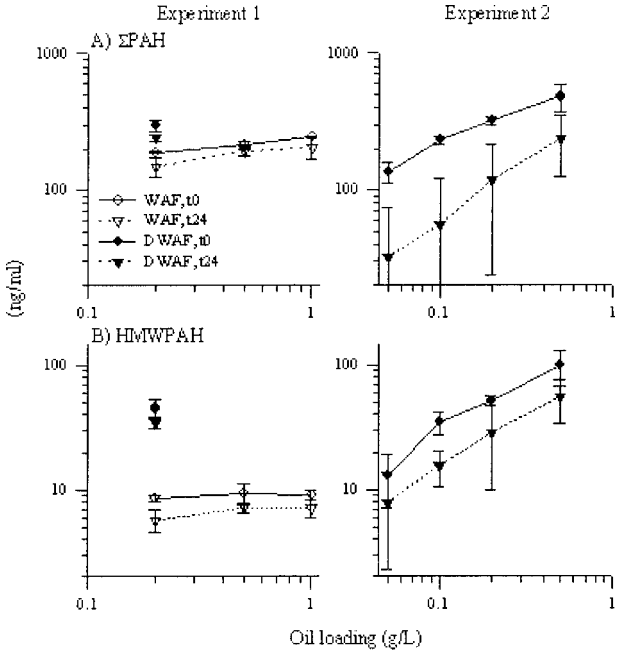

Fig. 1. Temporal variations of the aqueous concentrations of (**A**) total polycyclic aromatic hydrocarbons and (**B**) high-molecular-weight PAH (HMWPAH) in water-accommodated fractions of oil (WAF) and dispersed oil (DWAF) from the beginning (t_0) to the end (t_{24}) of the 24-h exposure periods (n = 4) during 96-h static bioassays with renewal. Values are expressed as the mean ± standard deviation.

were observed for body length (n = 18), and body length was compared among treatments within experiments using a nested ANOVA with Tukey's studentized range test. In experiment 2, body length was slightly higher (p = 0.04) in one of the three jars exposed to DWAF prepared with 0.1 g/L compared to that in the two other jars. The exclusion of this experimental unit did not affect the results of the ANOVA.

The relationships between the biological responses and the initial aqueous concentrations of ΣPAH or HMWPAH were investigated by linear regression. For these analyses, combining the data from both experiments, body length was converted to the percentage decrease relative to controls, and the ratio of EROD activity in exposed fish compared to controls was calculated (X-fold induction). For EROD activity, DWAF prepared with an oil loading of 0.2 g/L or greater were excluded from this analysis, because these concentrations were acutely toxic and caused a nonmonotonous exposure response for EROD activity.

RESULTS

PAH concentrations

The addition of dispersant caused an increase in the concentration of ΣPAH. For example, at an oil loading of 0.2 g/L, the addition of dispersant caused a 1.6-fold increase in ΣPAH concentrations (Fig. 1A). Concentrations of ΣPAH ranged from 190 to 243 ng/ml in WAF prepared with an oil loading from 0.2 to 1 g/L, whereas concentrations of ΣPAH ranged from 136 to 479 ng/ml in DWAF prepared with an oil loading from 0.05 to 0.5 g/L (Fig. 1A). Concentrations of ΣPAH in controls were at background levels (0–0.1 ng/ml).

The addition of dispersant markedly increased the aqueous concentrations of HWPAH. Water-accommodated fraction of crude oil prepared with 1 g oil/L and DWAF prepared with 0.1 g oil/L had similar ΣPAH concentrations but different compositions, with fourfold more HMWPAH in the DWAF (Fig. 1). At 0.2 g/L, the addition of dispersant caused a 5.3-fold increase in HMWPAH, whereas they caused only a 1.6-fold increase in ΣPAH. The dispersant-facilitated amplification of PAH concentrations in water can be expressed as the ratio of the concentration of individual PAH in WAF relative to those in DWAF with a similar oil loading [11]. A positive relationship (r^2 = 0.73) was observed between this ratio and the log K_{ow} (Fig. 2).

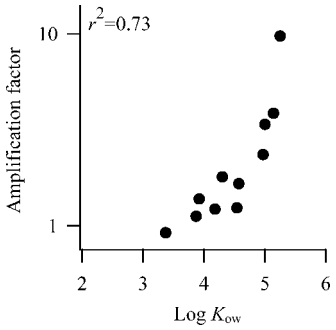

Fig. 2. Relationship between the amplification factor and the octanol/water partition coefficient (log K_{ow}) of individual polycyclic aromatic hydrocarbons (PAH). The amplification factor is the ratio of the concentration of an individual PAH in dispersed oil to its concentration in oil water-accommodated fractions, with both prepared at an oil loading of 0.2 g/L (experiment 1).

Dispersant increased the aqueous concentrations of alkyl homologues, particularly of naphthalenes (N1 through N4), dibenzothiophenes (DBT1 through DBT4), and phenanthrenes (P1 through P4), which have higher partition coefficients compared to the parent PAH (Fig. 3). The PAH profile in DWAF is intermediate between the PAH profile in WAF and the PAH profile in crude oil. Crude oil contains higher proportions of chrysene and pyrene compared to DWAF, and these compounds are not detected in WAF. Percentages of naphthalene are sixfold higher in WAF, and threefold higher in DWAF, compared to crude oil. Percentages of chrysene are sixfold

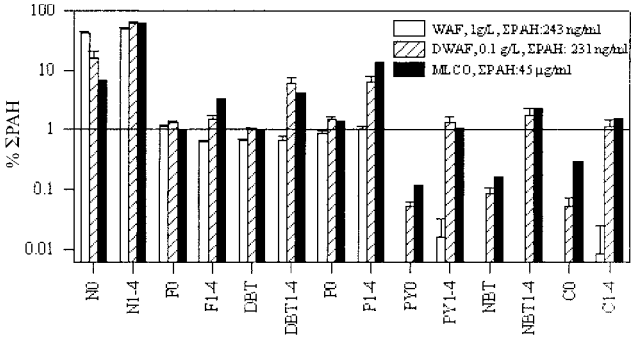

Fig. 3. Comparison of the composition of dispersed oil water-accommodated fraction (DWAF) and oil water-accommodated fraction (WAF) with similar concentration of total polycyclic aromatic hydrocarbons (ΣPAH) and of the weathered Mesa light crude oil (MLCO) used to prepare WAF and DWAF. Percentages of specific resolved components are shown. N0 = naphthalene; N1–N4 = C1–C4 alkylated naphthalenes, respectively; F0 = fluorene; F1–4 = C1–C4 alkylated fluorenes, respectively; DBT = dibenzothiophene; DBT1–DBT4 = C1–C4 alkylated dibenzothiophenes, respectively; P0 = phenanthrene; P1–P4 = C1–C4 alkylated phenanthrenes, respectively; PY0 = pyrene; PY1–4 = C1–C4 alkylated pyrenes, respectively; NBT = naphthobenzothiophene; NBT1–4 = C1–C4 alkylated naphthobenzothiophenes, respectively; C0 = chrysene; C1–C4 = C1–C4 alkylated chrysenes.

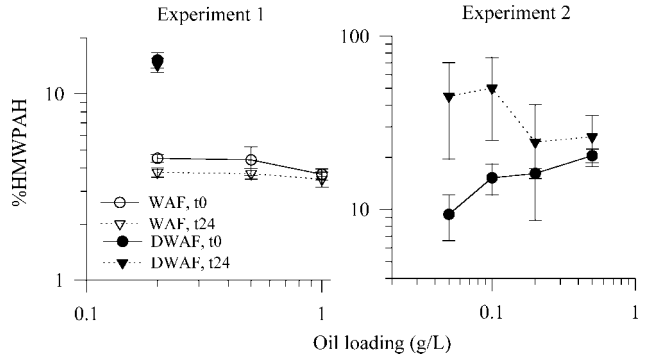

Fig. 4. Temporal variations of the percentage of high-molecular-weight hydrocarbons (HMWPAH) in water-accommodated fractions of oil (WAF) or dispersed oil (DWAF) from the beginning (t_0) to the end (t_{24}) of the 24-h exposure periods ($n = 4$) during 96 h static bioassays with renewal. Values are expressed as mean ± standard deviation.

lower in DWAF than in crude oil, and chrysene was not detected in WAF.

The addition of dispersant increased the proportion of HMWPAH within the ΣPAH. In DWAF, as oil loading increased, the initial percentage of HMWPAH increased from 9 to 20%, whereas the initial percentage of naphthalene decreased from 86 to 70% (Fig. 4). In contrast, the composition of WAF varied little (3.4–4.0% HMWPAH) with oil loading.

Temporal variation of PAH concentrations

The variation in the initial concentration of ΣPAH within treatments and among different days was relatively low, indicating that the method of preparation was reliable. From day to day, initial concentrations of ΣPAH tended to be more variable for DWAF (coefficient of variation [CV], 6–23%) than for WAF (CV, 2–10%) (Fig. 1A).

Concentrations of ΣPAH and HMWPAH in the WAF or DWAF decreased during the 24-h period between solution renewals (Fig. 1). Concentrations of ΣPAH decreased more markedly in DWAF during experiment 2 than in DWAF or WAF during experiment 1. In DWAF prepared at an oil loading of 0.2 g/L, a reduction in ΣPAH with time of 20% and 60%, respectively, was found in the two experiments, despite the fact that the initial ΣPAH concentrations were similar (Fig. 1A).

In experiment 2, with a preferential loss of LMWH, an increase was found in the relative proportion of HMWPAH in DWAF with time, particularly at the lowest oil loading, where more than 40% of the ΣPAH are HMWPAH after 24 h (Fig. 4). In experiments 1 and 2, higher concentrations of HMWPAH were observed in DWAF compared to WAF throughout the exposure period (Fig. 1B).

Mortality

The highest mortality rate (89%) was observed in fish larvae exposed to the highest initial concentrations of ΣPAH (479 ± 109 ng/ml) in 0.5 g oil/L DWAF (Fig. 5). No mortality was observed in control larvae exposed to seawater alone or to seawater and dispersant.

Mortality rates were not linearly related to ΣPAH concentrations (regression analysis, $p > 0.05$). In experiment 1, a 19% mortality rate in larvae exposed to DWAF prepared with 0.2 g oil/L (ΣPAH, 295 ± 28 ng/ml; 15% HMWPAH) was 10-fold higher than that for the same treatment (ΣPAH, 321 ± 20 ng/ml; 16% HMWPAH) in experiment 2 (Fig. 5).

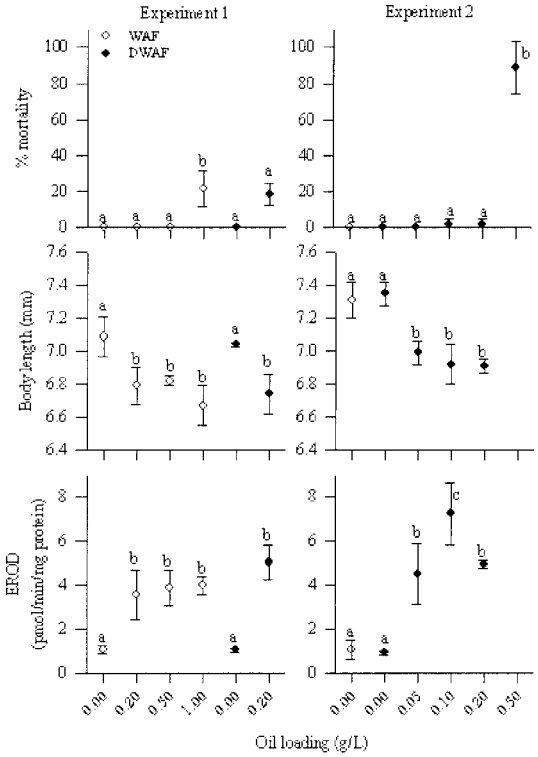

Fig. 5. Mortality, body length, and ethoxyresorufin-*O*-deethylase (EROD) activity in mummichog larvae exposed to water-accommodated fractions of oil (WAF) or dispersed oil (DWAF). Mortality and EROD activity are expressed as the mean ± standard deviation and body length as the mean ± standard error. Different letters (a and b) indicate significant differences among treatments within experiments ($p \leq 0.05$).

Mortality rates plotted against HMWPAH concentrations showed weak correlations (regression analysis, $p > 0.05$). In experiment 1, WAF prepared with 1 g oil/L (initial ΣPAH, 243 ± 6 ng/ml; HMWPAH, 9 ± 0.8 ng/ml) caused 22% mortality, whereas in experiment 2, DWAF prepared with 0.1 g oil/L having similar ΣPAH (231 ± 16 ng/ml) but higher HMWPAH (35 ± 7 ng/ml) caused only 2% mortality. These differences were similar to those observed between experiments for DWAF prepared with 0.2 g/L.

Body length

Both WAF and DWAF caused a reduction in body length. The highest reduction in body length was observed in the few larvae surviving exposure to DWAF prepared with 0.5 g oil/L (7.9%). Body length was reduced by 4.9 to 7.9% in larvae exposed to DWAF prepared with oil loadings of 0.05 to 0.5 g/L, compared to reduction of 3.8 to 6.0% in larvae exposed to WAF prepared with oil loadings of 0.5 to 1 g/L (Fig. 5). Body length did not differ between control larvae exposed to seawater alone and those exposed to seawater and dispersant.

The percentage reduction in body length increased with increasing ΣPAH ($r^2 = 0.56$, $p = 0.03$) (Fig. 6). Larvae exposed to DWAF prepared with 0.2 g oil/L and having similar initial ΣPAH and HMWPAH concentrations suffered similar reductions in body length of 4.8% ± 1.7% and 6.0% ± 1.4% for experiments 1 and 2, respectively.

The percentage reduction in body length increased with the concentration of HMWPAH in DWAF but not in WAF. In experiment 1, the WAF prepared with 1 g oil/L caused the same reduction in body length (6.0% ± 2.6%) as that in the

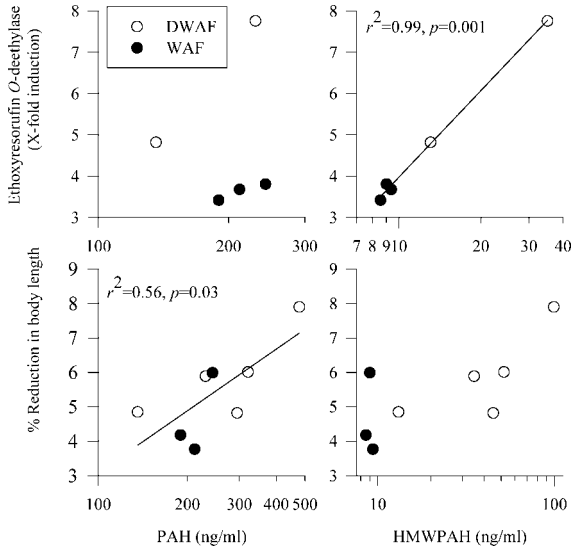

Fig. 6. Relationships between percentage reduction of body length or the mean ethoxyresorufin-*O*-deethylase (EROD) activity and initial aqueous concentrations of total polycyclic aromatic hydrocarbons (ΣPAH) or high-molecular-weight hydrocarbons (HMWPAH) in water-accommodated fractions of oil (WAF) and dispersed oil (DWAF).

DWAF prepared in experiment 2 using 0.1 g oil/L (5.9% ± 2.6%). Both had similar ΣPAH concentrations, but the DWAF had higher concentrations of HMWPAH.

The no-observed-effect concentration (NOEC) was less than the lowest concentrations tested for both WAF (0.2 g/L; ΣPAH, 190 ng/ml; HMWPAH, 9 ng/ml) and DWAF (0.05 g/L; ΣPAH, 135 ng/ml; HMWPAH, 13 ng/ml).

EROD activity

In larvae exposed to DWAF, a nonmonotonic concentration–response relationship was found for EROD activity. The highest EROD activity (eightfold relative to control) was observed in larvae exposed to DWAF prepared with 0.1 g oil/L. Lower EROD activity (fivefold relative to control) was observed in larvae exposed to DWAF prepared with the higher oil loading of 0.2 g/L, which caused mortality (Fig. 5). Ethoxyresorufin-*O*-deethylase activity could not be assessed in larvae exposed to DWAF prepared with 0.5 g oil/L, because insufficient numbers of live larvae survived. In larvae exposed to WAF, EROD activity was significantly higher compared to control and did not increase significantly as oil loading increased (Fig. 5). Ethoxyresorufin-*O*-deethylase activity did not differ between control larvae exposed to seawater alone and those exposed to seawater and dispersant.

Ethoxyresorufin-*O*-deethylase activity showed positive correlation with the log of HMWPAH ($r^2 = 0.99$, $p = 0.001$, $n = 5$) and not with ΣPAH concentrations ($p = 0.7$) (Fig. 6). The positive linear relationship between EROD activity and HMWPAH was still significant even if the highest value of HMWAPH was removed from the analysis ($r^2 = 0.97$, $p = 0.02$, $n = 4$). The WAF prepared with 1 g oil/L caused a 3.8-fold increase in EROD activity, whereas the DWAF prepared with 0.1 g oil/L and having similar ΣPAH concentration but higher HMWPAH caused an eightfold increase in EROD activity.

The NOEC was less than the concentrations tested for both WAF and DWAF.

DISCUSSION

Effect of dispersant on PAH concentration and composition

The addition of dispersant caused a 1.6-fold increase in ΣPAH concentrations in DWAF compared to WAF at an oil loading of 0.2 g/L. In contrast, Cohen et al. [24] found a fivefold increase in ΣPAH concentrations in DWAF compared to WAF using Corexit 9527 at a 1:30 ratio of dispersant to oil but with a different test oil and at a much higher oil loading (100 g/L). Dispersant efficacy may vary as a function of the composition of the dispersed oil, of the type of dispersant, and of the energy used to mix the solution [19]. Greater dilution of the dispersant in water also may decrease its efficacy [25]. In the present study, at an oil loading of 0.2 g/L, only 13 μl of dispersant were added to 1.8 L of water (6.5 μl/L), whereas Cohen et al. [24] added more than 50 ml of dispersant to 15 L of water (3.3 ml/L).

The application of dispersant altered the suite of PAH introduced into the water column and increased the relative concentrations of HMWPAH. The addition of dispersant induces the formation of small oil droplets with a high surface to volume ratio, increasing the dissolution rate of PAH [10,25]. Chemical dispersion particularly enhanced the aqueous concentrations of the less water-soluble PAH, the HMWPAH. The dispersant-facilitated amplification of PAH concentrations in water increased as the log K_{ow} increased. A similar relationship was observed for DWAF and WAF prepared with the same mixing energy in a large mesocosm, which is a more environmentally realistic system [11]. Our data also demonstrate that the addition of dispersant in particular increased the concentrations of alkyl homologues, which have a higher log K_{ow} compared to the parent PAH [26]. Our method of analysis does not differentiate between PAH contained within oil droplets and PAH dissolved in water. The PAH profile of DWAF is intermediate between the PAH profile in WAF and the PAH profile in crude oil, indicating that at least part of the PAH measured in DWAF are dissolved in water.

In DWAF, as the oil loading increased, the initial percentage of HMWPAH increased. Compositional changes in the aqueous phase with variable oil loading has been reported previously and was the basis for the recommendation of using serial loading rather than serial dilution for toxicity testing of WAF of petroleum [18].

Temporal variation of PAH concentration and composition

Concentrations of PAH in the WAF or DWAF decreased during the 24-h period between solution renewals more markedly in DWAF in experiment 2 than in WAF or DWAF in experiment 1. Possible causes for the highest decline in ΣPAH concentrations in experiment 2 are higher rates of adsorption on the experimental jars, droplet coalescence and resurfacing, volatilization, biodegradation, and/or uptake by fish larvae. At the end of the exposure period, control fish larvae were larger in experiment 2 (7.31 ± 0.12 mm) than in experiment 1 (7.09 ± 0.11 mm), and larger fish may have taken up a larger PAH load [27]. Body weight (mg) increases linearly with body length (mm; body weight = 1.35(body length) − 7.39; $r^2 = 0.63$, $p \leq 0.05$, $n = 40$) in mummichog larvae (unpublished data). Predicted total biomass per jar would be 44 mg in experiment 2, compared to 39 mg in experiment 1. Further studies are needed to evaluate the effect of larval size on PAH uptake in our experimental conditions.

In experiments 1 and 2, the amplification of HMWPAH concentrations in DWAF prevailed over the temporal decline in the concentrations of PAH, so higher concentrations of HMWPAH were observed in DWAF compared to WAF throughout the exposure period (Fig. 1B). In their mesocosm experiment, Yamada et al. [11] also observed that amplification of HMPAH in DWAF prevailed over the facilitated biodegradation. Thus, following the application of dispersant, as the concentrations of ΣPAH decrease, the proportion of HMWPAH may increase, and the toxicity of the mixture of PAH in the water column may vary with time.

Mortality

The highest mortality rate was observed in fish larvae exposed to the highest initial concentrations of ΣPAH (479 ng/ml) in DWAF at an oil loading of 0.5 g oil/L. At high ΣPAH concentrations, all petroleum hydrocarbons, but most importantly the LMWH, may contribute to lethality through a narcotic effect characterized by the accumulation of liposoluble compounds in the cell membranes [28]. The higher HMWPAH aqueous concentrations in DWAF compared to those in WAF did not seem to play a major role in the acute mortality response. The lethal effects of HMWPAH may be underestimated in a 96-h test, because the lethal body burden for insoluble chemicals may not be reached until significantly later than 96 h. The median lethal concentrations of mixture containing HMWPAH should be assessed using 10- or 28-d bioassays [29]. In herring larvae exposed to WAF, the 4-d median lethal concentration was only half that of the 8-d exposure [25].

For similar treatments, mortality rates were higher in experiment 1 than in experiment 2. The smaller-size larvae in experiment 1 may respond more rapidly than the larger ones in experiment 2, because the lethal body burden is reached more rapidly in smaller-sized organisms [2,27]. The more important loss of LMWH in experiment 2 during the 24-h exposure period also may contribute to the observed lower mortality rate.

Body length

Mummichog larvae exposed to DWAF had lower body length compared to larvae exposed to WAF prepared with similar oil loading. The percentage of reduction in body length increased with increasing ΣPAH concentrations, and it increased with the concentration of HMWPAH in DWAF but not in WAF. Further studies are needed to evaluate the ecological impacts of the observed reduction in the body length of the larvae. Minor reduction in body length may reduce survival rates of fish larvae by different mechanisms, including altered swimming speed and increased predation rates [30,31]. The NOEC for body length reduction was less than the lowest concentration tested (ΣPAH, 135 ng/ml; HMWPAH, 13 ng/ml).

Again, a longer-term bioassay (≥7 d) would be needed to better assess the relationship between PAH concentrations and larval growth [32]. In the present study, larvae relied on their yolk sac for feeding, but it also would be important to evaluate the effect of exposure to WAF or DWAF on their capacity to capture live prey. The toxic effects of oil on fish growth may be expressed long after the end of exposure. Delayed effects of oiled gravel on growth of pink salmon were demonstrated four to six months after exposure [4].

EROD activity

Higher EROD activity was observed in larvae exposed to DWAF prepared with 0.1 g oil/L compared to DWAF prepared with a higher oil loading of 0.2 g/L. This nonmonotonous concentration response likely resulted from the PAH toxicity at the highest test concentration. Inhibition of CYP1A activity in fish exposed to high concentrations of PAH has been reported previously and may be related to a variety of mechanisms, including hepatotoxicity, oxidative inactivation, and exposure to inhibitory PAH [33–35]. These findings indicate that EROD activity may underestimate exposure to petroleum oil PAH when fish larvae are exposed to relatively high concentrations of ΣPAH (ΣPAH in the present study, 300 ng/ml).

Ethoxyresorufin-*O*-deethylase activity increased linearly with the concentration of HMWPAH. Thus, the dispersed CYP1A-inducing HMWPAH were bioavailable, and chemical dispersant significantly increased exposure to these potentially toxic PAH. The identification of the petroleum oil hydrocarbons inducing CYP1A is still underway. Induction of CYP1A has been demonstrated in early life stages of several fish species exposed to alkyl phenanthrenes, and are a major component of HMWPAH in DWAF, and cause blue sac disease [8,36]. Cytochrome P4501A activity is involved in PAH metabolism and excretion, and the CYP1A-generated metabolites of PAH or oxidant radicals are thought to play a role in chronic toxicity to larval fish. Increased exposure of fish larvae to CYP1A-inducing substances may enhance the risk for CYP1A-mediated toxicity in early life stages, such as blue sac disease, genotoxicity, and delayed effect on growth and reproduction [7,36,37]. It is recommended that dispersant-use guidelines take into account the possibility of an added risk of toxic effects during early life stages of fish because of enhanced exposure to HMWPAH.

The NOEC was less than the lowest concentrations tested for both WAF (0.2 g/L; ΣPAH, 190 ng/ml; HMWPAH, 9 ng/ml) and DWAF (0.05 g/L; ΣPAH, 135 ng/ml; HMWPAH, 13 ng/ml). Pacific herring eggs exposed for 16 d postfertilization to a ΣPAH of 0.4 ng/ml had sublethal effects (yolk sac edema and premature hatching) suggestive of exposure to CYP1A-inducing HMWPAH [3]. The water was contaminated by passage through gravel mixed with highly weathered Alaska North Slope crude oil, and more than 80% of the initial ΣPAH was accounted for by HMWPAH, mostly alkyl PAH. The lowest observed effective concentration was higher (ΣPAH, 9.1 ng/ml) for water contaminated with gravel mixed with less weathered oil and HMWPAH representing less than 20% of the initial ΣPAH, indicating the importance of HMWPAH in the toxicity.

In terms of reducing environmental impacts, the success of chemical dispersant is based on the premise that oil on the surface transported into the water column is rapidly diluted by turbulent diffusion to a concentration less than the toxicity threshold limits. Indeed, this operational objective has been verified in controlled field trials and response operations. For example, McAuliffe et al. [38] noted that total oil concentrations (Prudhoe Bay crude) over a period from 15 to 220 min after aerial application of dispersants declined from a maximum concentration range of 30 to 50 ng/ml (depth, 1 m) to a range of 0.5 to 2.3 ng/ml (depth, 1–9 m). During cleanup operations following the Sea Empress spill off the coast of Wales in February 1996, oil dispersant additions resulted in water-column concentrations of approximately 10 ng/ml to a

depth of 4 m, with a rapid decline in oil concentration over a period of days to weeks. By the end of March, concentrations had diluted to less than 0.1 ng/ml over most of the affected area, with the exception of a few hot spots that had values of up to 0.5 ng/ml [39]. Despite these reports, however, for large spills within near-shore regions or smaller spills in confined areas, turbulent transport may not be adequate to disperse oil rapidly, and concentrations similar to those tested in the present study may be encountered in the environment [40]. Furthermore, as illustrated by the results of the present study, the transport of bioavailable components from crude oils to the water column may be influenced by the addition of chemical dispersants.

The present results comparing the chemistry and toxicity of DWAF and WAF suggest that the application of oil dispersants increases the hazard for marine larval fish because of an increase not only in the concentration of ΣPAH but also in the relative proportion of HMWPAH. Chemically dispersed petroleum HMWPAH were bioavailable and were taken up by fish larvae, as indicated by higher EROD activity, which is a biomarker of exposure to CYP1A-inducing HMWPAH. A primary issue with approval of dispersant use has been the lack of scientific information to quantify fully their impact on biota in realistic environmental conditions. To date, most studies have been focused on the concentration of dispersed oil rather than alterations in the bioavailability of specific constituents of biological concern. This information, as well as that for chronic low-level exposures, is needed in models of risk assessment to evaluate the net environmental benefits from the use of chemical oil dispersant.

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