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Environmental Toxicology and Chemistry, Vol. 33, No. 1, pp. 107–114, 2014
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OIL AND OIL DISPERSANT DO NOT CAUSE SYNERGISTIC TOXICITY TO FISH EMBRYOS

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(Submitted 3 July 2013; Returned for Revision 11 August 2013; Accepted 12 September 2013)

Abstract: Atlantic herring (*Clupea harengus*) embryos were exposed to water accommodated fractions (WAFs; oil dissolved in water) and chemically enhanced water accommodated fractions (CEWAFs; oil dispersed in water with Corexit 9500A) of Medium South American (MESA) crude oil. The CEWAF was approximately 100-fold more toxic than WAF based on nominal loadings of test solutions (% v/v). In contrast, the ratio of WAF and CEWAF toxicity expressed as measured oil concentrations approximated 1.0, indicating that the higher toxicity of CEWAFs was caused by an increase in exposure to hydrocarbons with chemical dispersion. In a second experiment, the chronic toxicity of Corexit 9500A and chemically dispersed heavy fuel oil 7102 (HFO 7102) to rainbow trout (*Oncorhynchus mykiss*) embryos was compared to chemically dispersed Nujol, a nontoxic mineral oil. Dispersant alone was toxic, but caused different signs of toxicity than HFO 7102. Nujol at a dispersant-to-oil ratio of 1:20 was nontoxic, suggesting that dispersant was sequestered by oil and not present at toxic concentrations. In contrast, the same nominal loadings of dispersed HFO 7102 caused concentration-dependent increases in toxicity. Both experiments suggest that chemically dispersed oil was more toxic to fish embryos than solutions created by mechanical mixing due to the increased exposure of fish to petroleum hydrocarbons and not to changes in hydrocarbon toxicity. The Nujol control discriminated between the toxicity of oil and chemical dispersant and would be a practical addition to programs of dispersant testing. *Environ Toxicol Chem* 2014;33:107–114. © 2013 SETAC

Keywords: Oil spill Chemical dispersant Chronic toxicity Mixture Exposure

INTRODUCTION

Chemical dispersants are often used to remove spilled oil from the water's surface. The active ingredients of dispersants are amphoteric (i.e., molecules with both hydrophobic and hydrophilic regions). Consequently, the active ingredients of chemical dispersants applied to oil align at the oil–water interface and decrease interfacial tension. In combination with mechanical energy, dispersants can break oil slicks into smaller oil–surfactant micelles or droplets, which readily disperse into the water column. Such micro-droplets are relatively stable in water due to the interactions between molecules of water and the hydrophilic region of the surfactant [1,2]. Chemical and mechanical dispersion in combination produce more and smaller droplets than mechanical dispersion alone. Dispersion of oil–surfactant micelles increases the concentration of petroleum hydrocarbons in the water column but also increases the rate of oil decomposition through dilution and biodegradation [3].

Advancements in formulating dispersants have decreased their toxicity [4] but have shifted the concern from the toxicity of the dispersant itself to the toxicity of the dispersed oil mixtures. Although many studies have compared the toxicity of dispersants, oils, and dispersed oil mixtures to aquatic species under varying exposure conditions [4–9], debate continues about why chemically dispersed oil seems more toxic than mechanically dispersed oil. Questions remain about whether the enhanced toxicity of dispersed oil mixtures is caused by the dispersant, the oil, or a synergistic interaction between the pair. If there are synergistic interactions, the toxicity of oil and dispersant mixed would be greater than the sum of the toxicity of the oil and dispersant tested separately. Potentially, solutions of dispersed oil include dissolved hydrocarbons, dissolved dispersant, and oil–surfactant micelles, which confound the interpretation of cause and effect [5]. Many laboratory studies, however, fail to report the concentration and composition of oil in test solutions. For example, Rico-Martínez et al. [6] measured the acute toxicity of Corexit 9500A (a commonly used dispersant, hereafter referred to as Corexit) and Corexit-dispersed Macondo crude oil to rotifers. They concluded that there was synergism between the 2 substances because chemically enhanced water accommodated fractions (CEWAFs) were 47 to 52 times more toxic than water accommodated fractions (WAFs), based on nominal loadings. Because the hydrocarbon concentrations of test solutions were not measured—a recommended requirement for dispersed oil studies [7]—comparisons of toxicity among treatments may have been incomplete [8]. Although Rico-Martínez et al. [6] acknowledged that previous studies have found increased hydrocarbon concentrations in solutions of CEWAFs, they did not consider how the lack of measured concentrations could alter their interpretation of comparisons between WAF and CEWAF treatments.

Previous studies in which the concentrations of oil in test solutions were measured demonstrate that dispersants increased the concentration of hydrocarbons in test solutions and shifted the composition of hydrocarbons in solution to higher molecular weight compounds [9,10,11]. The concentration of dissolved hydrocarbons in dispersed oil solutions depends on the rate of partitioning of hydrocarbons from oil droplets to water, and the rate of partitioning depends on the size of droplets (i.e., the surface area available for partitioning) and the concentration and solubility of each hydrocarbon in oil and water. Therefore, observed differences in the toxicity of oil test solutions to aquatic biota with respect to loading may relate directly to the availability of, or exposure to, hydrocarbons.

If the difference in toxicity between undispersed and chemically dispersed oil is due primarily to an increase in

All Supplemental Data may be found in the online version of this article.
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Published online 23 September 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.2397

EX5002-000001-TRB

hydrocarbon exposure, comparisons of toxicity between WAFs and CEWAFs should be based on measured concentrations of hydrocarbons, particularly polycyclic aromatic hydrocarbons (PAHs). The increase in the concentration and bioavailability of PAHs in dispersed oil solutions is of concern because PAHs are thought to be the most chronically toxic components of oil [12]. Nevertheless, there is still a need to test the assumption that the hydrocarbons partitioning from oil droplets are responsible for toxicity and that any freely dissolved dispersant, or dispersant mixed with oil, is not contributing to the observed toxicity. The present study combined 2 distinctly different experiments to distinguish the toxicity of oil from the toxicity of dispersant or dispersant–oil interactions.

To illustrate the effect of chemical dispersion on both the exposure and toxicity of oil, we measured the chronic toxicity of WAFs and CEWAFs of Medium South American (MESA) crude oil to embryos of Atlantic herring (*Clupea harengus*). Chronic toxicity was expressed in terms of nominal loadings (amount of WAFs or CEWAFs added to test solutions; % v/v) and in terms of measured concentrations of oil in test solutions. Based on loading, the effect of dispersants on exposure should be evident when the ratio of WAF/CEWAF toxicities calculated from nominal loadings significantly exceeds 1.0. If the dispersant is highly efficient at increasing exposure, a much smaller amount of CEWAFs will be needed to cause toxicity relative to WAFs. In contrast, when WAF and CEWAF toxicities are expressed as measured concentrations of oil in test solutions, the WAF/CEWAF toxicity ratio should approximate 1.0, assuming that only petroleum hydrocarbons in dispersed oil mixtures are toxic. If chemical dispersants are toxic by themselves at the concentrations typical of CEWAFs, or if the dispersants interact synergistically with hydrocarbon toxicity, the ratios based on measured hydrocarbon concentrations would deviate significantly from 1.0; that is, the hydrocarbons in CEWAFs would be more toxic than the hydrocarbons in WAFs.

In a second test of the chronic toxicity of heavy fuel oil 7102 (HFO 7102) to rainbow trout (*Oncorhynchus mykiss*) embryos, we included new control treatments to assess the interactive toxicity of dispersants and oil. In addition to chemically dispersed HFO 7102 prepared with high-energy mechanical mixing (HE-CEWAF), controls included dispersant alone, mechanically dispersed Nujol, a nontoxic mineral oil used in mammalian cell culture (Nujol alone), and dispersant mixed with Nujol (Nujol HE-CEWAF). If the Nujol HE-CEWAF is toxic but not Nujol alone, the dispersant is likely bioavailable at toxic concentrations. If the Nujol alone and Nujol HE-CEWAF are nontoxic, the dispersant would be at subtoxic concentrations in test solutions, likely because it is sequestered in dispersed oil. If the dispersed HFO 7102 is more toxic than chemically dispersed Nujol, the difference in toxicity would represent the toxicity of petroleum hydrocarbons released from droplets of HFO 7102.

These 2 separate lines of evidence lead to the same conclusion: that chemical dispersants do not change the toxicity of oil in laboratory toxicity tests of chemically dispersed oil. We present for the first time some practical experimental controls that use Nujol, a nontoxic oil, to assess whether dispersants are bioavailable at toxic concentrations in dispersed oil solutions, which can be adapted to the specific conditions of any study.

METHODS

All experiments were conducted under Queen's University's Animal Care Protocol (Hodson-2003-23-Or, Hodson-2007-032-R2, and Hodson-2011-038-Or) following the 2011 Guidelines of the Canadian Council on Animal Care. Water quality (dissolved oxygen, ammonia, temperature, and pH) was monitored throughout the exposures and was within an acceptable range for handling and care of Atlantic herring and rainbow trout.

Oil stock

Weathered MESA crude oil was provided by the Centre for Offshore Oil and Gas and Energy Research (Bedford Institute of Oceanography). The oil was weathered artificially by sparging with air at 18 psi for 130 h, resulting in a weight loss of 13.8% [13]. The Emergencies Science and Technology Division, Environment Canada provided HFO 7102. Nujol mineral oil, Corexit, and 7-isopropyl-1-methylphenanthrene (retene) were purchased from Sigma-Aldrich, Ondeo Nalco Energy Services, and MP Biomedicals, respectively.

Toxicity of undispersed and dispersed MESA to Atlantic herring embryos

Test species. Sexually mature Atlantic herring were acquired by Fisheries and Oceans Canada (Gulf Region) from a fisherman in Clam's Harbour, Nova Scotia, on 21 October 2004. Within 15 h of capture, the sperm and eggs of 5 males and 5 females were pooled separately in glass jars wrapped with cloth and shipped on ice to Queen's University. The sticky eggs were coated sparsely on glass microscope slides and placed in a suspension of sperm in salt water for 60 min at 10 °C to achieve a fertilization rate greater than 60%. Slides of fertilized eggs were rinsed with salt water and placed immediately into 250-mL jars of test solutions at 10 °C.

Preparation of test solutions

Test solutions were prepared from Kingston, Ontario municipal water, dechlorinated with charcoal filtration and 1 mg/L sodium bisulphite and adjusted to 15‰ salinity with Kent Sea Salt (Kent Marine) at least 24 h before use. Solutions of WAFs and CEWAFs of weathered MESA oil were prepared following Singer et al. [11], using a 1:9 ratio of water-to-oil stirred for 18 h with 25% vortex, followed by a 1:10 dispersant (Corexit)-to-oil ratio (DOR). After 1 h of settling in a separatory funnel, the bottom aqueous layer (the WAF or CEWAF stock) was decanted.

Dilutions of WAFs (ranging from 0.032% to 32.0% v/v) and CEWAFs (ranging from 0.032% to 3.20% v/v) were prepared in test volumes of 200 mL, with 6 replicates of all treatments, including negative (water) and positive (320 μg/L of retene) controls. Retene was used as a positive control because it is known to cause signs of blue sac disease (BSD) and mortality in fish embryos, similar to those caused by exposure to oil [14]. Test solutions were renewed every 48 h until herring hatched, when they were scored for pathology.

Herring bioassays. Unfertilized eggs were removed from glass slides 2 d post-fertilization (dpf) and were not included in the bioassays. Measurements included mortality, hatching success, time to hatch, the frequency and intensity of pericardial edema, and heart rate. The prevalence of pericardial edema was enumerated before hatch (10 dpf), whereas percent live hatch and percent normal were enumerated at hatch (13–19 dpf). The intensity of pericardial edema was observed using a dissecting microscope and scored by the volume of fluid accumulation in the pericardial cavity; a score of 0 indicated no edema and a score of 3 indicated severe edema. Heart rate was observed visually through the transparent chorion and was measured as beats per minute for 10 randomly selected embryos from each treatment group at 9 dpf. The experiment terminated when controls completed hatching at 19 dpf. Thereafter, all embryos were

anesthetized with an overdose of tricaine methane sulfonate (MS-222; 100 mg/L).

Toxicity of dispersant and chemically dispersed HFO and mineral oil to rainbow trout embryos

Test species. Rainbow trout were purchased from Rainbow Springs Hatchery at the eyed stage, approximately 12 d prior to hatching. Eyed eggs are characterized by melanin pigment visible in the developing optic cup [15]. Developing eggs were held at 10 °C in stainless steel bowls containing dechlorinated municipal water replenished every 24 h until hatch, when the exposures began.

Preparation of test solutions. Solutions of dispersed HFO 7102 and Nujol were prepared by a high energy mechanical and chemical mixing method, creating the HE-CEWAF solutions (as described in Adams et al., unpublished manuscript). The HE-CEWAF was prepared in glass scintillation vials with Teflon-lined septum caps with a 1:9 oil-to-water (filtered water; 18.2MΩ-cm, PURELAB Ultra water system, Siemens Water Technologies) ratio and a 1:20 DOR. In addition to the 1:20 DOR, Nujol HE-CEWAF was prepared with higher DOR ratios of 1:10, 1:5, and 1:2.5. Dispersion consisted of 5 min of vigorous mixing (100% vortex) with a hand vortex and 5 min of sonication, after which the mixture was left to settle for 90 min. During the settling period, the vial was placed upside down to enable the bottom aqueous phase to be collected by puncturing up through the septum cap and to minimize disturbance of the bulk oil phase at the surface. The lower aqueous phase was decanted with an airtight glass syringe and referred to as the HE-CEWAF stock. Dilutions of the HE-CEWAF stock in dechlorinated municipal water were prepared daily to renew the test solutions.

This high-energy mechanical and chemical dispersion method facilitated the formation of more and smaller droplets of oil to maximize surface area- to-volume ratios for partitioning of hydrocarbons in solution. This method generated solutions of HFO 7102 and Nujol that represented the worst-case scenario of environmental exposure. Oil droplets remained suspended in the water column, maximizing the concentrations of dissolved PAH in solution. Test solutions of Corexit alone were prepared by dilution of Corexit in water. Corexit was added to the surface of 1.0 L of water and mixed with an additional 1.0 L of water. When fish were added to the test solutions, no oil sheen was visible on the surface, which suggested complete mixing.

Retene was the positive control (100 μg/L), and the negative controls were methanol (100 μg/L; the solvent carrier for retene), Nujol (dilution of Nujol in water; 0.8 mL/L, the maximum oil loading of 1.6 mL in the HE-CEWAF stock diluted in 2.0 L of exposure water), and water. All controls were static daily renewals run in triplicate ($N = 25$ per treatment), except for a single Nujol control. The average responses of control embryos are included in all graphs.

Trout bioassays. Rainbow trout embryos were exposed from hatch to swim-up (24 d) to dilutions of Corexit, and to dilutions of HE-CEWAF stocks prepared from combinations of Corexit and HFO 7102 (DOR = 1:20) or Nujol (DOR = 1:20, 1:10, 1:5, and 1:2.5). The dilutions of Corexit ranged from 0.0001% to 0.01% v/v, whereas dilutions of HFO 7102 HE-CEWAF and Nujol HE-CEWAF ranged from 0.01% to 0.32% v/v. Test solutions were renewed daily with newly prepared dispersed oil solutions.

The estimated concentrations of Corexit in test solutions were calculated from the dilutions of Corexit in HE-CEWAF stocks, with the assumption that no dispersant was lost in preparing the HE-CEWAF mixtures. The estimated amount of dispersant in Corexit alone, HFO 7102, and Nujol HE-CEWAFs prepared with a DOR of 1:20 ranged from 0.48 mg/L to 95 mg/L (Supplemental Data, Table S1).

During the chronic exposure, treatments were checked for dead embryos. The number was recorded, and the embryos were observed for signs of morphological abnormalities. The exposure was terminated at swim-up, approximately 24 d post hatch, when embryos swim to the surface of the water, signifying their readiness to feed. The embryos were anaesthetized with an overdose of MS-222, and fish were measured individually for total length (mm) and examined under a dissecting microscope for signs of BSD.

Measurement of oil in test solutions. The concentrations of oil in test solutions for the herring and trout bioassays were measured with a QMI Fluorescence Spectrometer (Photon Technologies International, Felix software Ver 1.4, PTI). Water samples (1.5 mL) were mixed with equal parts anhydrous ethanol in glass scintillation vials and stored in the dark at 4 °C.

Samples were vortexed and sonicated prior to fluorescence measurement in a quartz cuvette. Emission scan wavelengths were optimized for MESA with excitation at 240 nm and an emission range of 248 nm to 327 nm and for HFO 7102 with excitation at 290 nm and emission range of 300 nm to 450 nm. This range of wavelengths targeted the measurement of the 2- to 4-ringed PAHs [16–18] using linear standard curves relating to known concentrations of MESA (diluted in 50:50 salt water: ethanol) or HFO 7102 (diluted in 50:50 freshwater:ethanol) to fluorescence area. The oil concentration for unknown samples was calculated from the measured peak area after the removal of the background signal (50:50 salt water or freshwater:ethanol blank). To account for the dilution of water samples in equal parts ethanol during storage, measured concentrations were multiplied by 2.

Statistical analysis. For the herring bioassays, treatment effects were analyzed by one-way analysis of variance (ANOVA; percent normal; heart rate) followed by a Student–Newman–Keul post hoc test for multiple comparisons, where significance was determined ($p < 0.05$). As percent live hatch did not meet the assumption of equal variance, a nonparametric Kruskal Wallis one-way ANOVA on ranks was completed, followed by Dunn's post hoc test for multiple comparisons, where significance was determined ($p < 0.05$). Dunnett's test for multiple comparisons was used to compare heart rates between each treated group and the water control group. Median lethal concentrations (LC50s) and median effective concentrations (EC50s) were estimated with the trimmed Spearman–Karber method [19]. All results were reported with 95% confidence intervals (CIs).

Statistical analyses for the trout bioassays were conducted using Microsoft Excel 2010 and GraphPad Prism Ver 5. Although treatments were not replicated, the regression design allowed the estimation of LC50s and EC50s using LC50 DOS 2.0 (Probit analysis) and GraphPad Prism Ver 5 (nonlinear regressions).

RESULTS

Toxicity of MESA WAFs and CEWAFs to Atlantic herring embryos

Measured oil concentrations in CEWAF solutions were approximately 100-fold higher than in WAF solutions with the same nominal loadings (Figure 1). The nominal loading (% v/v) that caused 50% hatch of embryos was approximately 100-fold lower for CEWAFs than for WAFs (Figure 2A). When toxicity

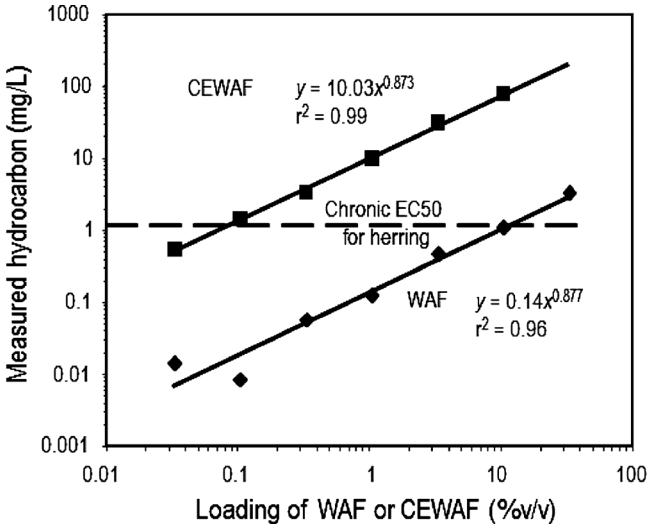

Figure 1. The measured concentration of oil in toxicity test solutions created by different loadings of the water accommodated fraction (WAF) and the chemically enhanced water accommodated fraction (CEWAF) of Medium South American crude oil to test tanks. Analytical methods are described in the Supplemental Data. The slopes (exponents) of the regressions were not significantly different, but the intercept of the regression for the CEWAF was approximately100-fold higher than the intercept for the WAF. The 19-d median effective concentration (EC50) was taken from Figure 2.

was expressed against measured oil concentrations, the WAF and CEWAF regressions overlapped (Figure 2B).

The prevalence and intensity of pericardial edema increased with increasing concentrations of WAFs until 3.20% v/v, after which they decreased to control values (Supplemental Data, Figure S1). Similarly, the lowest concentration of CEWAFs (0.032% v/v) caused the greatest effects on pericardial edema (frequency 99% and intensity 1.8). The retene control had a frequency of pericardial edema of 100% and an average intensity of 2.34. The WAF concentrations greater than or equal to 1.0% v/v and all CEWAF concentrations caused a significant decrease in heart rate from the control values (Dunnett's, $p < 0.05$; Supplemental Data, Figure S2).

At the end of the experiment (19 dpf), none of the embryos exposed to CEWAFs or to retene, the positive control, appeared normal, nor did any of the embryos exposed to nominal WAF concentrations greater than 0.32% v/v (Supplemental Data, Figure S3A). In contrast, 78% of control embryos appeared normal. When the percent normal was plotted against measured concentrations of oil in solution, the data for WAFs and CEWAFs were consistent, forming one continuous distribution with a 19-d EC50 of approximately 0.15 mg/L of oil as estimated by fluorescence (Supplemental Data, Figure S3B). This result is virtually the same as that for the percentage of embryos that hatched (Figure 2B; 19 d EC50 1.02 mg/L).

Premature hatching was observed in herring exposed to high concentrations of WAFs and CEWAFs (Supplemental Data, Figure S4). When treatments were separated based on similarities in hatching patterns, embryos exposed to clean water or to 0.032% to 0.32% v/v WAFs hatched on average 16 ± 2 dpf. On average, those exposed to 10.0% v/v WAFs or to 0.032% to 0.10% v/v CEWAFs hatched 2 d earlier (14 ± 2 dpf). For those exposed to 0.32% v/v CEWAFs, more than 70% had already hatched by the first day of scoring (13 dpf), at least 3 d premature relative to controls (average peak hatch of water controls 15 dpf).

Toxicity of dispersant and chemically dispersed HFO and mineral oil to rainbow trout

A concentration-dependent increase in the cumulative percent mortality was observed with higher concentrations of dispersant alone in the Corexit treatment (Figure 3A). In contrast, there was no mortality observed in the Nujol alone, methanol, water, or any 1:20 DOR Nujol HE-CEWAF treatments (Figure 3A). The average cumulative percent mortality was 42% for the retene control. Exposure to HFO 7102 HE-CEWAF treatment increased the cumulative percent mortality for rainbow trout embryos to 100% mortality at 0.32% v/v, with equivalent nominal loadings and DOR as the 1:20 DOR Nujol HE-CEWAF treatment (Figure 3A). Over the same range of nominal loadings, the Nujol HE-CEWAF treatment prepared with higher DORs caused mortality of trout embryos. At the highest loading of 0.32% v/v, mortality ranged from 19% to 23% at a DOR of 1:10 and 1:5, respectively, and to 100% at a DOR of 1:2.5 (Figure 3A).

By nominal loading (% v/v), Corexit alone was more than 16-fold more toxic than HFO 7102 HE-CEWAF (24-d LC50; Figure 3A). The 24-d LC50 for Corexit alone was 0.00311% v/v (95% CI 0.00309–0.00313) compared with 0.052% v/v for HFO 7102 HE-CEWAF (95% CI 0.049–0.054). When treatments were compared based on the estimated concentrations of dispersant in test solutions, the order of toxicity was reversed; HE-CEWAF of HFO 7102 (24-d LC50 = 2.5 mg/L; 95% CI 2.2–2.8) was approximately 12-fold more toxic than Corexit alone (29.5 mg/L).

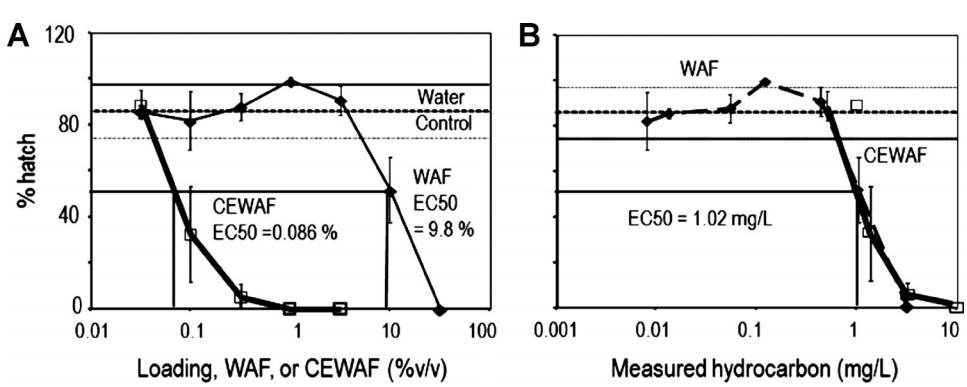

Figure 2. The effect of exposure to the water accommodated fraction (WAF) and the chemically enhanced water accommodated fraction (CEWAF) of Medium South American crude oil on hatching success of Atlantic herring embryos. The estimated concentrations causing a 50% reduction in hatch (EC50s) after 19-d exposure are expressed as a function of nominal loadings (% v/v; **A**) and of measured hydrocarbon concentrations of the solutions of dispersed (CEWAF) and undispersed (WAF) oil (mg/L; **B**). The horizontal dashed lines represent the average percent hatch and 95% confidence interval of the water control treatment.

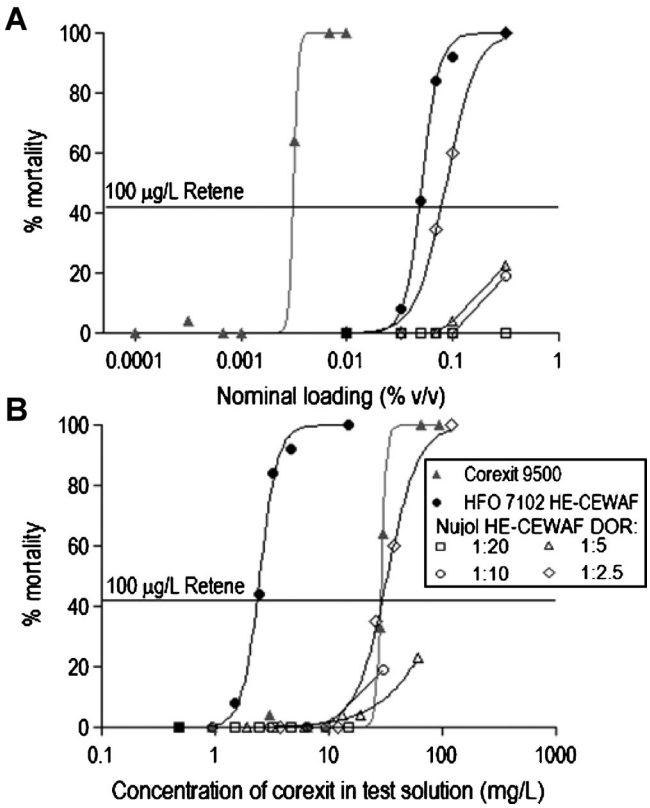

Figure 3. Cumulative percent mortality of rainbow trout exposed to 24-d static daily renewals of Corexit, Nujol high-energy chemically enhanced water accommodated fraction (HE-CEWAF, prepared with a dispersant-to-oil ratio [DOR] of 1:20) and heavy fuel oil 7102 (HFO 7102) HE-CEWAF (DOR = 1:20). Rainbow trout were also exposed chronically to Nujol HE-CEWAF prepared with varying DORs (1:10, 1:5, and 1:2.5). Toxicity was reported as the cumulative percent morality against nominal HE-CEWAF loading (% v/v; **A**), and by the estimated concentration of Corexit in test solutions (mg/L; **B**). Water controls and Nujol alone (water accumulated fraction; 0.8 mL/L) caused no mortality of rainbow trout embryos.

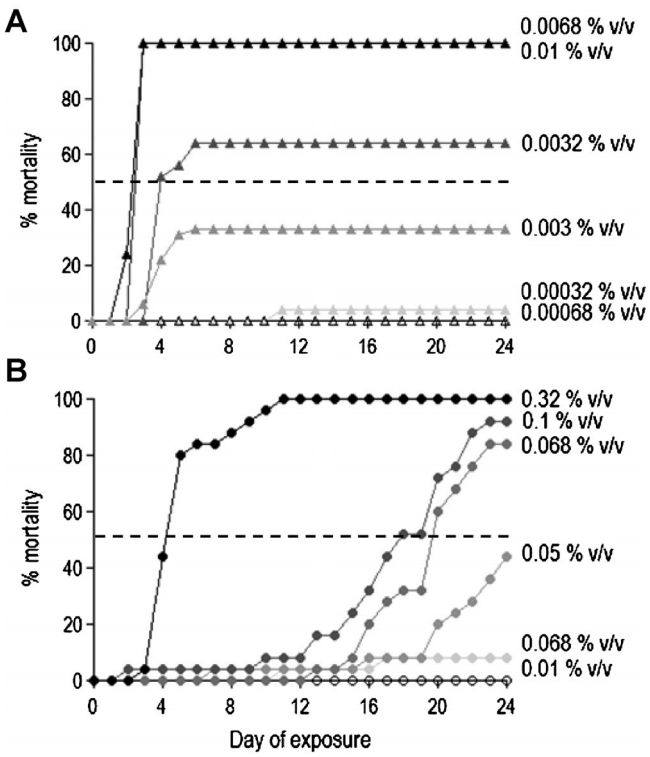

Figure 4. Cumulative percent mortality of embryos exposed to static daily renewal (24 d) of Corexit (**A**) and heavy fuel oil #7102 (HFO 7102) high-energy chemically enhanced water accommodated fraction (HE-CEWAF) dilutions (**B**) over the days of embryo exposure. The horizontal dashed line represents 50% mortality.

The toxicity of dispersed Nujol (DOR 1:2.5; 24-d LC50 = 32.8; 95% CI 27.3–40.0) was not significantly different from that of Corexit alone, as indicated by the overlapping 95% confidence interval (Figure 3B). There was insufficient mortality to calculate LC50s at DORs of 1:10 and 1:5.

The time to mortality and the signs of toxicity observed in the embryos also differed between dispersant and dispersed oil treatments. Most mortality in the Corexit treatments occurred within the first 4 d of the exposure, while the mortality in the HFO 7102 treatments was higher in the latter half of the 24-d exposure period following the expression of severe signs of BSD (Figure 4). The more gradual increase in cumulative mortality caused by HFO 7102 HE-CEWAF was exposure-dependent, so that mortality was 100% by day 11 in the 0.32% v/v loading. Over the same nominal loadings, all mortality in the Nujol HE-CEWAF treatments occurred prior to day 6, consistent with the time to mortality observed in the Corexit treatment (Figure 4, Supplemental Data, Figure S5).

In the HFO 7102 HE-CEWAF treatments, embryos developed BSD, which was first observed on day 8 of the exposure period and was followed by mortality (Figure 4). Embryos that died in the Corexit treatments did not have the same signs of toxicity but had severe disruption of the gills, opaque yolk sacs, loss of epidermal pigmentation, and spinal curvature (Supplemental Data, Figure S6). The signs of sublethal toxicity in the dispersant treatments were also not observed prior to embryo mortality.

DISCUSSION

The Atlantic herring bioassay demonstrated that the toxicity of chemically dispersed MESA crude oil was markedly greater than that of undispersed oil when expressed as a dilution of stock solutions. However, no difference was found between WAF and CEWAF toxicity when expressed as the measured concentrations of oil in water. The ratio of WAF to CEWAF EC50s approaching 1.0 suggests no interaction between the toxicities of the dispersant and of petroleum hydrocarbons. These results support the contention that dispersants increase the concentration of oil in test solutions without affecting the toxicity of the dispersed oil.

The observed abnormalities in Atlantic herring embryos exposed to crude oil suggest a delayed development and may explain the apparent decrease in the frequency and intensity of pericardial edema at high levels of exposure. For WAF, the first significant decrease in heart rate occurred at 1.0% v/v, the same concentration that caused a marked increase in pericardial edema. Although the frequency and intensity of pericardial edema decreased to control values at concentrations above 3.2% v/v, there was no corresponding increase in heart rate, which continued to decrease with increasing exposure. Therefore, the delay in development and decreased heart rate caused by oil toxicity likely reduced blood pressure and the accumulation of fluid in the pericardium and hence the severity of edema. Embryos that have delayed development may be less viable in the natural environment and subject to high rates of predation.

This conclusion is consistent with induction of CYP1A enzyme activity in groups of juvenile rainbow trout exposed for 48 h to WAFs and CEWAFs prepared with Corexit from 3 different crude oils. Induction of CYP1A enzymes in fish indicates the degree of exposure to PAHs [20], which comprise up to 6% by weight of crude and fuel oils [21]. When the 48-h EC50s for CYP1A induction for dispersed and undispersed crude oil were expressed based on the loading of WAFs or CEWAFs to test solutions, the ratio of WAF/CEWAF EC50s for Terra Nova, MESA, and Scotian light were approximately 1100, 100, and 6, respectively [22]. When EC50s were expressed as total PAHs measured by gas chromatography–mass spectroscopy, the EC50s were similar between CEWAF and WAF treatments, and the ratio of WAF to CEWAF EC50s was approximately 1.0 (Figure 5). Similarly, Wu et al. [23] reported that ratios of WAF to CEWAF LC50s and EC50s (22-d exposure of rainbow trout embryos; percent normal, BSD severity index, and ethoxyresorufin-*O*-deethylase activity) of Alaska North Slope crude oil, Federated crude, MESA, and Scotian light crude oils were approximately 1.0 when expressed as measured hydrocarbon concentrations in test solutions.

The effects of dispersants on exposure of fish to petroleum hydrocarbons and the absence of dispersant effects on oil toxicity have been replicated in studies of chronic toxicity to rainbow trout embryos of WAF and CEWAF of diesel fuel. The chronic toxicity curves of percent mortality and BSD score for WAFs and CEWAFs overlapped when toxicity was expressed as measured concentrations of diesel [24]. Greer et al. [25] also reported an overlap in measured concentrations of hydrocarbons in WAF and CEWAF solutions causing mortality, reduced percent hatch, reduced percent normal, and increased BSD severity in Atlantic herring embryos exposed to dispersed Alaska North Slope and Arabian Light crude oil. As a result, the authors could combine the WAF and CEWAF toxicity data for each oil into single, nonlinear exposure-response regressions.

The contrast in perceived risk between toxicity expressed as measured concentrations and toxicity expressed as nominal oil loading can also be seen in acute lethality data reported by the US Environmental Protection Agency for mysids (*Americamysis bahia*) and inland silversides (*Menidia beryllina*) (Figure 6). Loading LC50s were calculated for each of the WAF and CEWAF tests reported by Hemmer et al. [26] by dividing the measured total petroleum hydrocarbon (TPH) concentration in stock solutions by the calculated LC50s. When the WAF/CEWAF ratios were calculated with nominal loadings, the ratios ranged from 3.0 to 137 for mysids and from 1.0 to 429 for silversides, and the geometric means of the ratios were 34 and 29, respectively. These values overlap the ranges reported in the present study for herring and trout. When the ratio was calculated from the estimated TPH concentration of the LC50s (estimated from dilutions of the stock concentration), the ratios ranged from 0.1 to 3.6 for mysids and from 0.2 to 3.7 for silversides, and the geometric means of ratios were 0.94 and 0.95, respectively (Figure 6). Overall, the similarity in the ratio of measured concentrations of the WAFs and CEWAFs with different dispersants indicates that the toxicity of the dispersant in dispersed oil is not of concern, but the toxicity of the components of oil is.

Slight variations around a WAF-to-CEWAF ratio of 1.0 are expected due to the multiphase nature of CEWAF solutions and because the EC50 estimates represent complex mixtures of hydrocarbons that differ among oils and among the same oil treatments with different experimental conditions [5]. Ratios below 1.0 may be related to the array of PAHs in the dispersed oil solution; dispersion shifts the composition of PAHs in solution

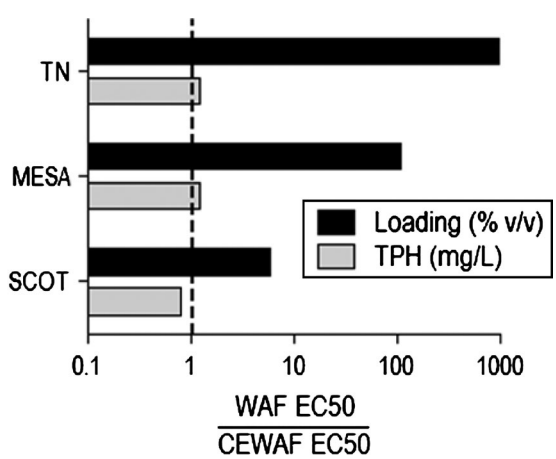

Figure 5. The ratios of 48-h median effective concentrations (EC50s) for concentrations of water accommodated fraction (WAF) and chemically enhanced water accommodated fraction (CEWAF) from 3 crude oils, Terra Nova (TN), Medium South American crude (MESA), and Scotian Light (SCOT), that induced ethoxyresorufin-*O*-deethylase activity in livers of juvenile rainbow trout (calculated from [22]). The EC50s were calculated from the loading of WAF and CEWAF stock solutions to test tanks and from the measured concentrations of polycyclic aromatic hydrocarbons (PAH) in the final test solutions [22]. CEWAF solutions were prepared using Corexit.

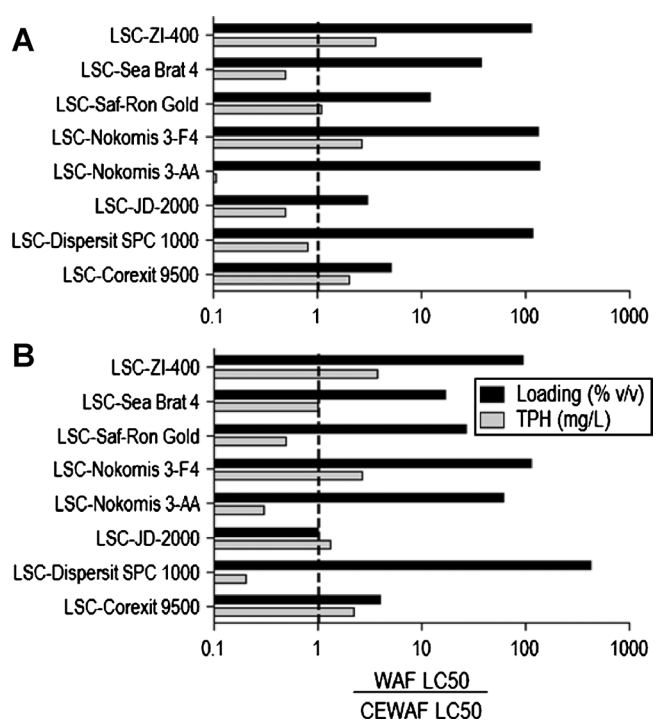

Figure 6. Ratio of median lethal concentrations (LC50) for mysids (**A**; *Americamysis bahia*) and inland silversides (**B**; *Menidia beryllina*) exposed for 48 and 96 h, respectively, to water accommodated fractions (WAF) of Louisiana Sweet crude oil (LSC) and to chemically enhanced water accommodated fractions (CEWAF) prepared with 8 dispersants. Data reported were calculated from Hemmer et al. [26]. Comparison of the LC50s was based on nominal loadings (% v/v) and calculated total petroleum hydrocarbon concentrations (TPH; mg/L) from dilution of the measured concentration of the CEWAF stock. When WAF/CEWAF LC50 ratios were calculated with nominal loadings, the geometric mean for mysids was 34 (range = 3.0–137), and for inland silversides, the geometric mean was 29 (range = 1.0–429). For WAF/CEWAF ratios calculated with TPH, the geometric means were 0.94 (range = 0.1–3.6) and 0.95 (range = 0.1–3.7) for mysids and silversides, respectively.

toward the higher–molecular weight PAHs [9] by changing the rate of dissolution of PAHs. The measured concentrations of CEWAFs can also be higher than for WAFs based on the inclusion of oil droplets in test solutions. In the Hemmer et al. study [26], an additional variation in the values around 1.0 may reflect differences among products in dispersant effectiveness.

The rainbow trout bioassay examining the toxicity of Nujol (mineral oil), Corexit, and HFO 7102 provided further evidence of the lack of dispersant toxicity in dispersed oil mixtures. The toxicity of Corexit was clearly evident in tests of dispersant alone, but the concentrations of dispersant in the HFO 7102 HE-CEWAF and Nujol HE-CEWAF (DOR 1:20) test solutions were insufficient to cause mortality to trout embryos at the loadings tested. Based on the estimated concentrations of Corexit in test solutions, HFO 7102 HE-CEWAF was more than 10-fold more toxic than Corexit alone. The toxicity of HFO 7102 (DOR of 1:20) over the same loadings as dispersed Nujol (DOR of 1:20) indicated that dispersed oil toxicity was due primarily to the constituents of oil that partition from dispersed oil. Nujol dispersions prepared with higher DORs (1:10, 1:5, and 1:2.5) caused toxicity at the highest nominal loadings of 0.32% v/v, suggesting a potential for dispersant toxicity in exposures to dispersed oil due to free dispersant in solution. Corexit is a multicomponent solution, and we have assumed that the amphoteric active ingredient in the dispersant mixture is the compound that is toxic when free or unbound.

When treatment effects were compared with the estimated concentration of dispersant in test solutions, the exposure–response curves for dispersed Nujol and Corexit alone overlapped. This suggests that dispersions prepared with higher DORs may have insufficient oil to sequester Corexit, resulting in higher concentrations of unbound dispersant in solution. The concentration of dispersant in test solutions was also estimated for several studies using the reported DOR, oil-to-water ratios, nominal loadings or measured concentrations, and toxicity data (Supplemental Data, Table S2). The concentrations of dispersed oil required to cause 50% mortality were less than the concentration of dispersant alone to cause the same effect. In addition, the estimated concentrations of Corexit at the dispersed oil LC50s were lower than concentrations of dispersant alone that caused toxicity, except results from Hemmer et al. [26]. Their data indicated that Corexit concentrations in dispersed Louisiana Sweet Crude were 3 to 5 times higher than concentrations of Corexit alone that cause acute lethality, consistent with a low bioavailability of Corexit when mixed with oil. For all other reported LC50s, mortality due to Corexit was unlikely; at higher nominal loadings, however, the concentrations of unbound dispersant may have been sufficient to cause exposure–dependent increases in toxicity. Unfortunately, the concentrations of Corexit in test solutions were not measured in the present study; therefore, the hypothesis that Corexit is nontoxic when sequestered by oil could not be tested directly. Analyses of dispersant in test solutions are recommended for future dispersed oil toxicity tests.

The present experiments demonstrated by a weight-of-evidence approach that dispersant and oil toxicity could be discriminated by examining differences in time to mortality, time to onset of toxicity, and signs of sublethal toxicity in embryos among treatments. The dispersed oil was chronically toxic, with the majority of embryo mortality occurring during the second half of the exposure duration. The dispersant alone was acutely toxic, with the majority of embryo mortality occurring within the first 4 d of the exposure. The difference in the Nujol HE-CEWAF and HFO 7102 HE-CEWAF toxicity, in combination with pathology in embryos exposed to HFO 7102 HE-CEWAF, was consistent with oil toxicity, not dispersant toxicity, and provided sufficient evidence to conclude that Corexit was not toxic in dispersed oil mixtures at the concentrations tested.

It is recommended that similar nontoxic oil controls be included in future tests of dispersed oil toxicity because the conditions that are specific to each study, such as the characteristics of the oil, DORs, energy input when preparing CEWAF, and toxicity test conditions, determine the interaction between the oil and dispersant in the mixture. For example, dispersed oil solutions prepared with low energy mixing of viscous oils could produce solutions with higher concentrations of dispersant dissolved in water compared to less viscous oils. Indeed, dispersants are less effective on heavier oils resulting in less oil–water interface available for dispersant interaction [27,28]. Nujol controls also allow for the discrimination between oil and dispersant effects at higher nominal loadings, where the concentration of Corexit in test solutions may be sufficient to cause mortality.

Characterization of the test solutions is also essential for defining exposures and comparing oils, particularly where measured concentrations are expected to increase with chemical dispersion. Analysis of PAHs is recommended, because it is believed that the toxicity of oil is related to the concentration of 3- to 4-ringed alkyl PAHs ([29]; J. Adams et al., unpublished manuscript). One assumption in the present study (and many others that have compared dispersed oils) is that only the dissolved hydrocarbons partitioning from oil droplets into solution are contributing to toxicity. It is clear that the dissolved phase is responsible for toxicity in fish [30], but particulate oil could also contribute to toxicity through direct contact and uptake in fish tissues [31,32]. Ramachandran et al. [33] observed crude oil droplets on the gills of rainbow trout exposed to undispersed and dispersed solutions of MESA crude oil. Droplets on the gills could allow direct partitioning of hydrocarbons from oil droplets into fish [33].

Images of particulate oil in the HFO 7102 HE-CEWAF stock and in test solutions have been captured by fluorescence microscopy (J. Adams et al., unpublished manuscript); examining the distribution of measured hydrocarbon concentrations in bulk oil, HE-CEWAF stock, and test solutions by gas chromatography–mass spectroscopy confirmed the presence of particulate oil in HE-CEWAF test solutions. Singer et al. [11] also found that Prudhoe Bay crude oil was entrained in the water as particulate oil with higher mixing energies. Entrained oil in water samples analyzed by gas chromatography–flame ionization detector had the same n-alkane signature as bulk oil. The impact of oil droplets in the MESA treatments is likely less than that of the HFO 7102 treatments because of the lower energy mixing used to prepare test solutions. More detailed assessments of CEWAF stocks and test solutions are needed to define the role of oil droplets in toxicity.

The present study emphasizes the importance of measuring the concentration of hydrocarbons in test solutions when comparing the toxicity of oils [7,8,11]. Contrary to Rico-Martínez et al. [6], neither experiment in the present study was consistent with synergistic toxicity of oil and dispersant in dispersed oil mixtures. Rather, the dispersant in the mixture increased the exposure of embryos to hydrocarbons, without changing or contributing to their toxicity.

The data presented by Hemmer et al. [26] demonstrated that dispersant toxicity was highly variable, but the dispersants tested alone were less toxic than undispersed or dispersed oil. These

observations, however, were isolated from the broader perspective of an ecological risk assessment. Oil dispersion can increase the exposure of aquatic species to hydrocarbons by up to 1100-fold, likely in proportion to the solubilization of specific toxic constituents such as alkyl PAHs [23]. Hence, depending on the oil tested and the efficacy of the chemical dispersants, chemical dispersion will increase the risk of oil toxicity to fish by up to 1100-fold compared to oil left as a slick on the surface. In practice, there is an added risk of toxicity due to dispersant applied from the air that does not land on oil slicks and remains free in solution. In future field and lab studies, it will be important to measure concentrations of dispersants in water to verify that dispersants are not aggravating effects on biota by acting independently of oil toxicity.

In summary, the toxicity of petroleum hydrocarbons did not change with chemical dispersion. Two lines of evidence are consistent with independent actions of dispersant and hydrocarbons in dispersed oil. There were no synergistic interactions between petroleum hydrocarbons and chemical dispersant, as the dispersant did not contribute to the increased toxicity of CEWAFs compared to WAFs. We conclude that chemical dispersion of oil increases the bioavailability of petroleum hydrocarbons by increasing the surface area-to-volume ratio of oil droplets and the rate of partitioning of hydrocarbons from droplets into aqueous solution.

SUPPLEMENTAL DATA

Tables S1–S2.
Figures S1–S6. (494 KB DOCX).

Acknowledgment—The present research was supported by contracts from the Center for Offshore Oil and Gas Energy Research, Department of Fisheries and Oceans Canada (Dartmouth, NS, Canada) and the Emergencies Science and Technology Division, Environment Canada (Ottawa, ON, Canada). The authors declare they have no potential sources of conflict of interest that would influence their objectivity.

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