

OIL DISPERSION INCREASES THE APPARENT BIOAVAILABILITY AND TOXICITY OF DIESEL TO RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)ALLISON SCHEIN,[†] JASON A. SCOTT,[†] LIZZY MOS,[‡] and PETER V. HODSON^{*†}[†]School of Environmental Studies, Queen's University, Kingston, Ontario K7L 3N6, Canada[‡]Hemmera, 4th Floor, 19 Bastion Square, Victoria, British Columbia V8W 1J1, Canada

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Abstract—Diesel is a complex mixture containing polycyclic aromatic hydrocarbons, which persist after a spill, pass readily from water into tissues, and are toxic to early life stages of fish. The bioavailability and chronic toxicity of hydrocarbons dissolved into water from floating diesel (water-accommodated fraction) and chemically dispersed diesel (chemically enhanced water-accommodated fraction) were measured by the extent of ethoxyresorufin-*O*-deethylase (EROD) induction in juvenile rainbow trout (*Oncorhynchus mykiss*) and by the severity of blue sac disease in embryos. The water-accommodated fraction of floating diesel was virtually nontoxic to embryos at nominal concentrations up to 1,000 mg/L, causing only small weight changes. Liver EROD induction in juvenile trout was only observed at the highest nominal water-accommodated fraction concentration (10,000 mg/L). Chemical dispersion increased the bioavailability and toxicity of diesel to trout by 100-fold. Diesel chemically enhanced water-accommodated fraction induced EROD activity, caused blue sac disease, and impaired development and growth of embryonic trout at nominal concentrations as low as 10 mg/L; 88% mortality occurred at 100 mg/L. However, when total hydrocarbon concentrations were measured, differences between dispersed and undispersed diesel disappeared, with a median lethal concentration of 8 mg/L of total hydrocarbons and sublethal median effective concentrations ranging from 1.3 to 6.1 mg/L. Dispersion of diesel by high-energy mechanical mixing was recently reported to cause acute lethality to juvenile trout between 40 and 200 mg/L. Therefore, dispersion of oil by any means increases the bioavailability and apparent toxicity of diesel to fish embryos without changing the toxicity of its components. Nevertheless, in an actual spill, dispersion of diesel increases the effects of oil on fish populations.

Keywords—Oil spill Polycyclic aromatic hydrocarbons Fish Ethoxyresorufin-*O*-deethylase Blue sac disease

INTRODUCTION

Oil spills constitute significant risks to aquatic habitat, in particular acute and chronic toxicity to aquatic organisms such as fish [1,2]. These risks are not restricted to large-scale oil spills along coastlines and into marine ecosystems, which receive much public attention. They also include frequent small spills of refined hydrocarbon products in freshwater originating from their transport by road or rail [3]. Polycyclic aromatic hydrocarbons (PAHs), which constitute a small percentage of crude and refined oils, are of greatest concern during spill events, as they are more toxic than aliphatic hydrocarbons and persist in anoxic sediments [4,5].

The PAHs in oil can continue to become bioavailable after a spill following dispersion of oil by high-energy turbulence or by the application of chemical dispersants. Dispersion reduces the impact of oil on surface-dwelling animals and enhances biodegradation but also creates a larger reservoir of oil in the water column, increasing the concentration of dissolved PAHs and subsequent risks of toxicity to fish [6,7]. In the early life stages of fish, oil toxicity is characterized as blue sac disease (BSD), an array of signs that includes pericardial and yolk sac edema, craniofacial and spinal deformities, hemorrhaging, and induction of cytochrome P450 (CYP1A) enzymes [1,2,4].

In the present study, spills of diesel with and without dispersion were simulated in laboratory exposures to assess the

effect of dispersion on the bioavailability and toxicity of oil-derived hydrocarbons to rainbow trout (*Oncorhynchus mykiss*) and whether the toxicity of diesel to free-swimming embryonic trout is similar to its toxicity to free-swimming fry. Oil dispersants, detergents that have surfactant properties that reduce the surface tension between oil and water [6], were used to mimic the effects of mechanical dispersion in a reproducible way.

MATERIALS AND METHODS

All experiments included semistatic tests (24-h daily renewal) of water-accommodated fraction ([WAF]; components of oil dissolved in water through mechanical mixing) and chemically enhanced WAF ([CEWAF]; components introduced by combining chemical dispersion with mechanical mixing), using ultralow sulfur Diesel No. 2 (Chemical Abstracts Service 68476-34-6) and Corexit 9500[®] (Nalco Energy Services, Sugar Land, TX, USA) as a dispersant. The chemical components of this diesel sample were previously described [8] and included 69% short-chain aliphatic hydrocarbons, 22% longer-chain components, minor contributions of volatile hydrocarbons (5%), and benzene, toluene, ethylbenzene, and xylene ([BTX]; 0.3%). The total amount of 14 unsubstituted PAHs constituted 0.2% by weight, but alkylated PAHs, which typically comprise 85 to 90% of total PAHs in crude oil [5], may have contributed most to the 3.5% of the weight of oil that was unaccounted for [8].

Test species

Rainbow trout fingerlings and eyed eggs were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada)

* To whom correspondence may be addressed (peter.hodson@queensu.ca).

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Table 1. Water quality of test solutions^a

Water-quality parameter	Value	
	CYP1A induction assay ^b	Chronic toxicity assay ^c
Temperature	15.7 ± 0.2°C	9.2 ± 1.2°C
pH	7.55 ± 0.04	8.21 ± 0.05
Conductivity	252 ± 3 mS	265 ± 51 mS
Dissolved oxygen	59 ± 6.6% saturation (no relationship to oil treatment)	82 ± 17% saturation
Unionized ammonia ^d	NA	11.3 ± 3.6 µg/L

^a CYP1A = cytochrome P450; NA = data not available.

^b Water-quality parameters were measured at the end of the 24-h exposure.

^c Water temperature was measured daily; pH, dissolved oxygen, and conductivity were measured every 3 d.

^d Unionized ammonia was measured on the last day of the experiment, 23 h after dosing.

and acclimated before experiments; juvenile trout were not fed 48 h before testing. The water for all acclimation and testing was Kingston, Ontario, Canada, municipal water (hardness 135 mg/L as CaCO₃), dechlorinated by charcoal filtration and the addition of 1 mg/L of sodium bisulfite [9]. All other water-quality parameters met the requirements for optimum fish health (Table 1).

Test solutions

Solutions of WAF and CEWAF were prepared by a standardized method described by Ramachandran et al. [6], which allows for comparisons among studies. For WAF, oil and distilled water (1:9) were stirred gently for 18 h and left to stand for 1 h. The clear bottom layer was removed for dilution in test solutions, ensuring that fish were only exposed to hydrocarbons dissolved in water during mixing. Solutions of CEWAF were prepared by adding dispersant in a dispersant-to-oil ratio of 1:9 after the 18 h of mixing. The turbid suspension of oil droplets was stirred for 1 h, left to stand for 1 h, and separated from traces of residual oil before dilution in test solutions. All tests included negative controls of water and 10 µ/L of dispersant (the concentration typical of the highest CEWAF concentration) and positive controls of β-naphthoflavone (10 µg/L; Sigma Chemical, Oakville, ON, Canada), a known CYP1A inducer [10], or retene (100 µg/L; ICN Biomedical, Aurora, ON, Canada), an alkyl PAH causing BSD [11].

Characterizing test solutions

Ideally, test solutions should be characterized by analyzing those components of a complex mixture that cause the observed effects. However, the array of PAHs in crude and refined oil is very large, given the number of two- to six-ringed PAH congeners that may contribute to toxicity and the permutations and combinations of alkyl side-chain size and position. For example, more than 500 C1 to C4 phenanthrenes are possible (R. Stephen Brown, Department of Chemistry, Queen's University, Kingston, ON, Canada, personal communication). The problem is exacerbated by the very low concentrations of PAHs associated with biological effects. For example, CYP1A enzymes of trout were induced by WAF and CEWAF solutions of medium South American crude at nominal oil concentrations of 0.1 mg/L of total hydrocarbon or greater [6], equivalent to 1 µg/L of total PAHs or greater, assuming 1% PAHs by weight. For nominal oil concentrations of 56 mg/L or greater, approximately 20 individual PAHs were detectable by gas chromatography-mass spectrometry (GC-MS), while at concentrations of 18 mg/L or less, only four PAHs were measurable at concentrations above the limits of detection [6].

Given the high numbers of analyses needed to characterize toxicity test exposures, the high cost of GC-MS, and the inability to detect more than a few PAHs at relevant exposure concentrations, we chose fluorescence spectroscopy as an alternative analytical method because of its greater sensitivity and lower cost. This method is indirect in that the concentrations of total oil hydrocarbons, or of a subset such as PAHs, are assumed to correlate with the total fluorescence associated with aromatic components. The method provides a measure of total hydrocarbon exposure that demonstrates whether target concentrations were achieved, whether concentrations changed over time, and whether concentrations differed among treatments.

Test water (1.5 ml) was diluted with methanol (1.5 ml) for analysis of hydrocarbon concentrations using a QMI® Fluorescence Spectrometer (Photon Technologies International, Monmouth Junction, NJ, USA). For CYP1A experiments, water samples were collected at 0 and 24 h. For chronic toxicity tests, water was sampled from each treatment once and daily from the highest WAF and CEWAF treatments (30–300 min after dosing). Water samples were also taken from all replicates of the highest WAF and CEWAF concentrations at 0, 1, 2, 4, 8, 19, and 24 h from dosing to describe the decay of concentrations between each renewal of test solutions. Emission spectra were measured between 245 and 343 nm at an excitation wavelength of 240 nm, and the integrated peak area of samples was compared to standard curves generated from known concentrations of diesel diluted in methanol, with $r^2 \geq 0.90$. Responses at these wavelengths reflect primarily the concentrations of naphthalenes, fluorenes, and phenanthrenes (R. Stephen Brown, personal communication).

CYP1A induction potential of diesel

For fingerling trout, CYP1A induction was indicated by the increased activity of liver ethoxyresorufin-*O*-deethylase (EROD). Groups of five 1- to 5-g trout were exposed to WAF (0.1–10% v/v) or CEWAF (0.01–1.0% v/v) for 24 h. Each treatment consisted of 10 L of nonaerated water in covered, randomly distributed black plastic buckets lined with food-grade polyethylene bags at 15.7 ± 0.2°C in a 16:8-h (light:dark) photoperiod. After 24 h, trout were killed by tricaine methane sulfonate (MS-222 or ethyl 3-aminobenzoate methanesulfonic acid) anesthesia (Sigma Chemical) and spinal section. Wet weights were recorded, and livers were removed, weighed, and homogenized in HEPES (5.206 g/L of 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; 11.184 g/L of KCl; pH 7.5) grinding buffer. Homogenates were centrifuged at 9,000 g, and supernatants (S9 fractions) [12] were flash-frozen in liquid nitrogen and stored at –80°C. The EROD

activity of S9 fractions was measured at room temperature using a microplate kinetic enzyme assay [10] and was normalized to water controls to show induction.

For embryos, CYP1A protein was measured by Western blots [9] using mouse antifish CYP1A peptide monoclonal antibody C10-7 (Cayman Chemical, Ann Arbor, MI, USA), and horseradish peroxidase-goat antimouse IgG (H+L) conjugate (Zymed Laboratories, South San Francisco, CA, USA). Band densities were assessed with ImageJ[®] software (U.S. National Institutes of Health, Bethesda, MD) and divided by the sample weight to obtain a density relative to weight.

Chronic toxicity of diesel

Triplicate groups of 25 free-swimming trout embryos (eleutheroembryos; [12]) were exposed from hatch to swim-up (24 d) to WAF (0.01–1.0% v/v) or CEWAF (0.001–0.1% v/v) in 2.0 L of aerated test solutions in stainless steel bowls, randomly distributed in a constant temperature room ($9.2 \pm 1.2^\circ\text{C}$). The photoperiod was 0:24 h (light:dark), except during daily renewal of test solutions. Mortality was recorded daily, and dead embryos were removed. When controls reached swim-up, all fish were anesthetized in MS-222 and scored for signs of BSD, based on severity (0–3) or presence or absence (0–1) of yolk sac edema (0–3), pericardial edema (0–3), yolk or body hemorrhaging (0–1), ocular hemorrhaging (0–1), cranial facial deformities (0–1), spinal deformities (0–1), and fin rot (0–1). Based on the presence of BSD, it was assumed that mortality beyond exposure day 9 resulted from BSD, and these fish were given the highest possible score of 11.5. Fish that died before day 9 showed few signs of BSD, and mortalities were unrelated to oil exposure; they were assumed to have died from other causes and were not scored. All BSD scores were divided by 11.5 to create a severity index of 0 to 1.0.

After scoring, embryos were weighed in groups of five before and after removal of the yolk sac and were frozen in liquid nitrogen for analysis of CYP1A protein. The average weights of embryos without yolk sacs and of residual yolk were calculated as an index of growth ($n = 9$ –12 groups per treatment). The ratio of yolk sac-to-fish weight was calculated as an index of development, assuming that higher ratios reflected a reduced conversion of stored energy to biomass due to yolk sac edema, slowing of development, or both.

Data analysis

The EROD induction data were log-transformed to correct for nonhomogeneity of variance, and treatment effects were assessed by analysis of variance (SigmaStat[®], ver 3.0, Systat Software, San Jose, CA, USA); the 95% confidence limits about geometric means were calculated from the pooled error variance. For toxicity tests, two-way analysis of variances tested WAF or CEWAF concentrations and replicates as independent variables, with the BSD severity index as the dependent variable, followed by multiple treatment comparisons using a Holm-Sidak post hoc test.

Median effective concentrations (EC50s) were estimated by nonlinear regressions (GraphPad Prism[®], GraphPad Software, San Diego, CA, USA). For the BSD severity index, the data series was truncated and the EC50 was estimated by assuming that the next-highest test concentration (0.32% v/v) would be lethal and cause a maximum response. In all other cases where the data did not fit the computer requirements, EC50s were estimated graphically. Triplicate median lethal concentrations (LC50s) were calculated using probit analysis

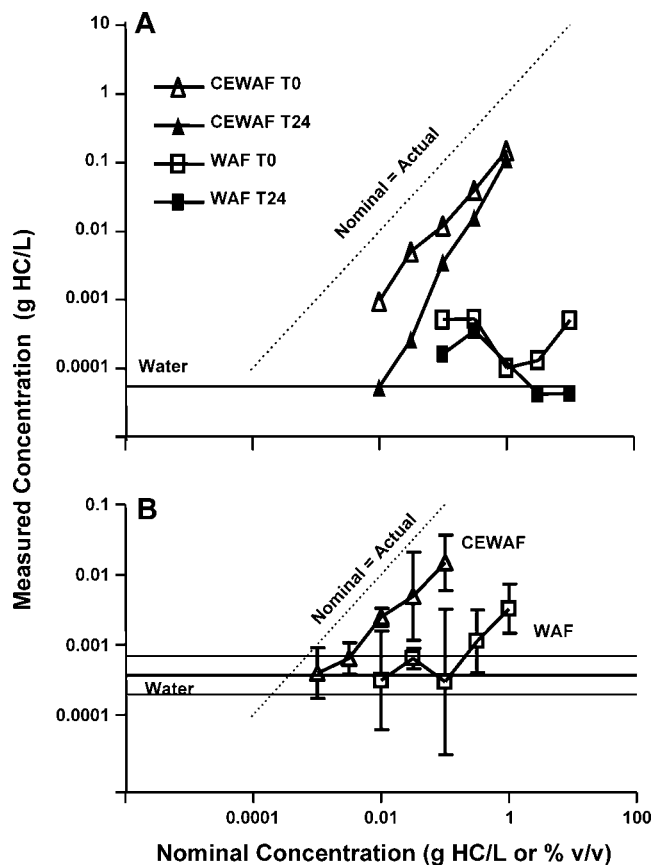


Fig. 1. Actual versus nominal concentrations of diesel hydrocarbons (HC) in the water of ethoxyresorufin-*O*-deethylase (EROD) induction assays (A; $n = 1$) and chronic toxicity tests (B; $n = 2$ –3) for water-accommodated fraction (WAF) and chemically enhanced WAF (CEWAF). For the EROD assays, results are shown for the start (time 0) and end (time 24 h) of the test. The dotted black lines show where measured concentrations would equal nominal. The horizontal solid line is the water control at time 0, and error bars for chronic toxicity data represent ± 1 standard deviation. A solution of 1% (v/v) WAF or CEWAF, which is made with a 1:10 ratio of oil to water, is equivalent to adding 1 g of oil per liter of water, assuming that all oil dissolves in water. The difference between nominal and measured represents the oil that was not incorporated into WAF or CEWAF.

(LC50 BAS 2.0, 1986, [13]) and were reported as the mean and standard deviation. Endpoints were estimated from nominal dilutions of WAF and CEWAF (% v/v converted to mg/L of total hydrocarbons) and from measured concentrations of total hydrocarbons, assuming that our one survey of all test concentrations represented the entire exposure period.

RESULTS

Hydrocarbon concentrations in test solutions

Chemical dispersion of oil (CEWAF) increased the amount of hydrocarbons in solution relative to WAF by 10- to 1,000-fold at 0 and 24 h after dosing (CYP1A induction experiment). Measured concentrations averaged 82% of nominal (expected concentration if all diesel dissolves in water) for CEWAF (range, 75–86%; $n = 5$) and 42% for WAF (range, 33–54%; $n = 5$). Over 24 h, hydrocarbon concentrations in WAF and CEWAF decreased from initial measurements by 0- to 10-fold, depending on the concentration (Fig. 1A). During the chronic test, measured concentrations of CEWAF averaged 83% of nominal (range, 80–87%; $n = 5$), while WAF averaged 58% of nominal (range, 49–62%; $n = 5$; Fig. 1B). At the highest

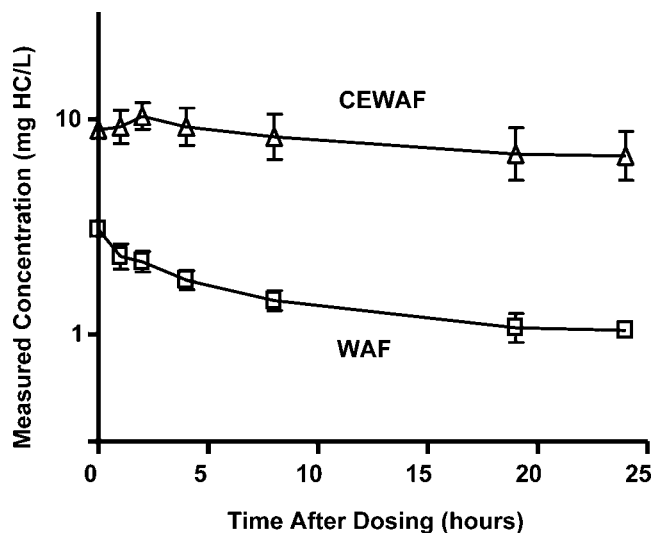


Fig. 2. The decline in concentration of hydrocarbons (HC) from diesel in 1% (v/v) water-accommodated fraction (WAF; $n = 3$) and 0.1% (v/v) chemically enhanced WAF ($n = 2$) between initial dosing and solution renewal after 24 h. Error bars represent ± 1 standard deviation.

concentrations of WAF or CEWAF, measured concentrations of total hydrocarbons were relatively constant between daily renewals (Fig. 2), declining by approximately 20% in CEWAF and approximately 33% in WAF solutions.

CYP1A induction potential of diesel

For CYP1A induction, CEWAF was 1,000 times more potent than WAF and equivalent to 10 $\mu\text{g/L}$ of β -naphthoflavone. The EC₅₀ was less than the lowest concentration tested (0.01% v/v, or 10 mg/L of total hydrocarbons). The WAF exposures resulted in an estimated EC₅₀ of 6,170 mg/L, with only the highest concentration (10,000 mg/L) causing EROD induction (Fig. 3).

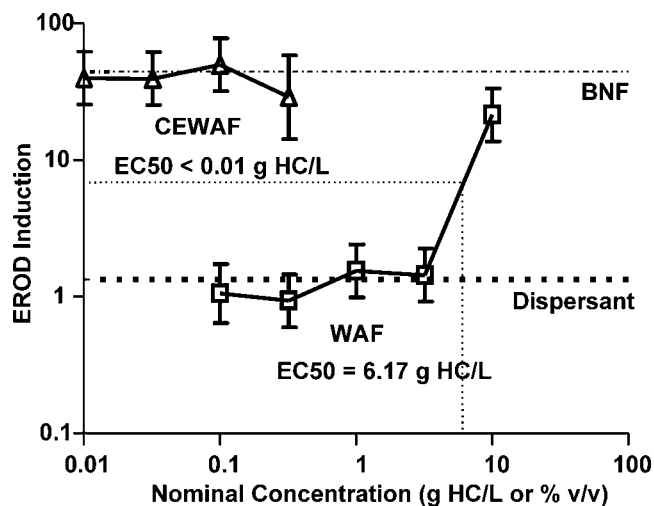


Fig. 3. Induction of ethoxyresorufin-*O*-deethylase activity in trout exposed to water-accommodated fraction (WAF) and chemically enhanced WAF (CEWAF) of diesel. The response of fish to negative control (dispersant, 10 $\mu\text{L/L}$) and positive control (β -naphthoflavone [BNF], 10 $\mu\text{g/L}$) treatments is also shown. Error bars are 95% confidence intervals. The median effective concentration (EC₅₀) was estimated graphically as the concentration of WAF associated with the geometric mean induction of BNF and water control fish. $n = 5$ per treatment except WAF 0.1 ($n = 4$) and CEWAF 0.32 ($n = 2$).

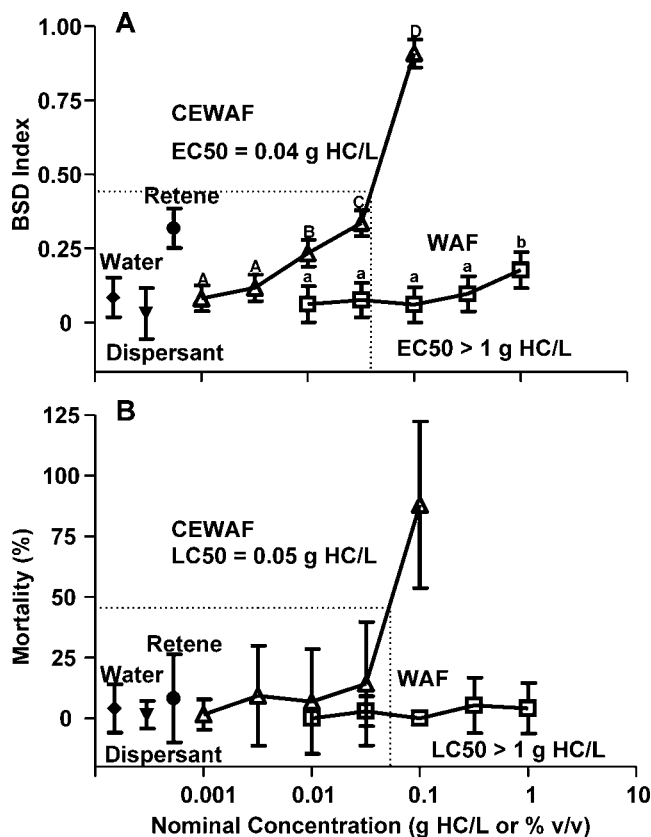


Fig. 4. The effect of exposure of free-swimming rainbow trout embryos to the water-accommodated fraction (WAF) and chemically enhanced WAF (CEWAF) of diesel on the severity of blue sac disease (BSD; A) and mortality (B). The responses of fish to negative control (water or 10 $\mu\text{L/L}$ of dispersant) and positive control (retene, 100 $\mu\text{g/L}$) treatments are also shown. Error bars are 95% confidence intervals based on three replicates ($n = 61$ –74 per treatment). Means sharing the same letter were not significantly different from one another ($p > 0.05$), as determined by a two-way analysis of variance and a Holm-Sidak post hoc test. The BSD severity index was statistically greater than controls at the highest WAF concentration ($F = 3.4$, degrees of freedom [df] = 5, $p = 0.006$) and at CEWAF concentrations of at least 0.01 g of hydrocarbon (HC) per liter ($F = 112.4$, $df = 5$, $p < 0.001$). The median effective concentrations (EC₅₀) and median lethal concentrations (LC₅₀) for CEWAF are the averages of three replicates.

Western blots demonstrated CYP1A protein synthesis in embryos from the two highest CEWAF treatments (10 and 32 mg/L) and from all WAF treatments (results not shown). No CYP1A protein was found in control embryos, and the standards worked on both gels. Relative densities increased eightfold with increasing WAF concentration, except for 320 mg/L, which was barely detectable. Similarly, staining density increased with CEWAF concentration, but the WAF and CEWAF results were derived from different gels and were not directly comparable.

Chronic toxicity of diesel

Blue sac disease and mortality. The lowest-observable-effect concentration of CEWAF for signs of BSD was 100-fold lower than that of WAF (Fig. 4A). Before day 9, there was 0 to 24% mortality, but it was unrelated to treatments or to BSD and likely due to handling stress at day 0. After day 9, mortality ranged from 0 to 8% in controls and increased to 96% with increasing exposure to CEWAF (Fig. 4B). The EC₅₀ and LC₅₀ for CEWAF averaged 40 and 50 mg/L, respectively, approx-

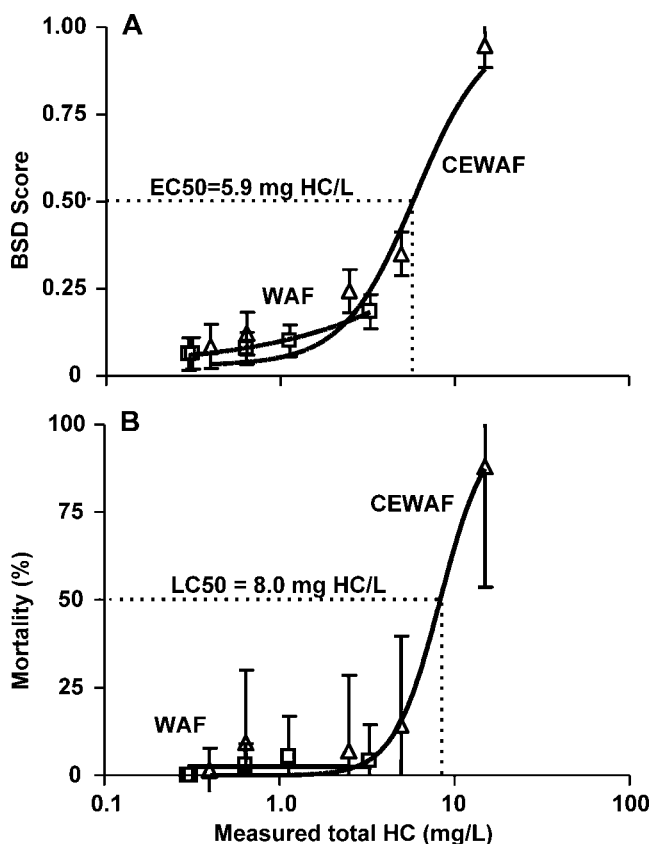


Fig. 5. The toxicity of water-accommodated fraction (WAF) and chemically enhanced WAF (CEWAF) of diesel on free-swimming rainbow trout embryos when expressed as measured concentrations of total hydrocarbons (HC) (mg/L). Toxicity is measured as blue sac disease ([BSD]; A) and mortality (B). Error bars are 95% confidence intervals based on three replicates ($n = 61\text{--}74$ per treatment). The median effective concentrations (EC50) and median lethal concentrations (LC50) are shown only for the CEWAF exposures because the measured concentrations of WAF solutions did not cause more than a 50% response.

imately 100-fold lower than those for WAF, which exceeded 1% (v/v) or 1,000 mg/L. Corexit 9500 alone was nontoxic relative to water controls.

When effects on embryos were compared to measured con-

centrations of total hydrocarbons, the differences between WAF and CEWAF treatments disappeared (Fig. 5). Concentrations of hydrocarbons associated with effects overlapped between the two experiments, with the primary difference being that exposure-response relationships for WAF were truncated because less hydrocarbon was entrained in test solutions compared to CEWAF tests. Where a sufficient response to WAF was found, estimated endpoints differed little among WAF, CEWAF, and the combined data (Table 2).

Effects on growth and development

Both CEWAF and WAF reduced the weight of embryos at swim-up, with lowest-observable-effect concentrations of 3.2 and 320 mg/L, respectively (Fig. 6A). For CEWAF, less growth was reflected in higher weights of residual yolk. The effects of oil on development were more apparent from the ratio of the residual yolk weight to the weight of the fish without the yolk sac. Based on nominal concentrations, CEWAF was 100 times more toxic than WAF (Fig. 6B), with graphically interpolated EC50s of 5.6 mg/L for CEWAF and 560 mg/L for WAF. When expressed as measured hydrocarbons, EC50s for yolk-to-fish ratios ranged from 1.3 to 3.3 mg/L of total hydrocarbons (Table 2) and were the most sensitive of the responses measured.

DISCUSSION

Diesel spills are often considered an acute toxicity risk to aquatic organisms, with most volatile components evaporating or breaking down rapidly in the environment. However, the present study showed that the constituents of diesel remaining after 18 h of stirring (subjected to processes of weathering and solubilization) were chronically toxic to rainbow trout, in particular when chemically dispersed. Exposure to the remaining water soluble components of diesel caused EROD induction in juvenile trout (the present study; [8]) and all hallmark signs of BSD in embryonic trout. These effects were accompanied by a significant reduction in growth of embryos, a cessation of development, or both, as indicated by a smaller size and reduced absorption of yolk. Such responses are consistent with the toxic effects observed in an array of fish species exposed to various crude oils [1,2,7,14,15] and suggest that diesel contains similar toxic components.

The toxicity of crude oil has been associated with fractions

Table 2. Summary of endpoints expressed as nominal and measured concentrations of hydrocarbons^a

Endpoint ^b	Exposure solution ^c	Hydrocarbon concentration (mg/L)				
		Estimate	Nominal		Measured	
			95% Confidence limit	Estimate	95% Confidence limit	
LC50	WAF	>1,000	ND	>30	ND	
	CEWAF	49	35–74	8.0	6.0–11.6	
	Combined	ND	ND	ND	ND	
EC50–BSD score	WAF	>1,000	ND	>30	ND	
	CEWAF	38	17–82	5.9	3.6–9.9	
	Combined	ND	ND	6.1	4.8–7.9	
Ratio of yolk to fish weight	WAF	1,765	135–23,140	3.3	1.9–5.6	
	CEWAF	5.7	1.2–2.7	1.3	0.7–2.5	
	Combined	ND	ND	1.5	1.0–2.4	

^a LC50 = median lethal concentration; EC50 = median effective concentration; BSD = blue sac disease; WAF = water-accommodated fraction; CEWAF = chemically enhanced water-accommodated fraction; ND = no data.

^b Where responses did not exceed 50% of the maximum possible response, the endpoint is listed as “greater than the highest concentration tested.”

^c Combined refers to endpoints calculated from responses and measured hydrocarbon concentrations from tests of WAFs and CEWAFs.

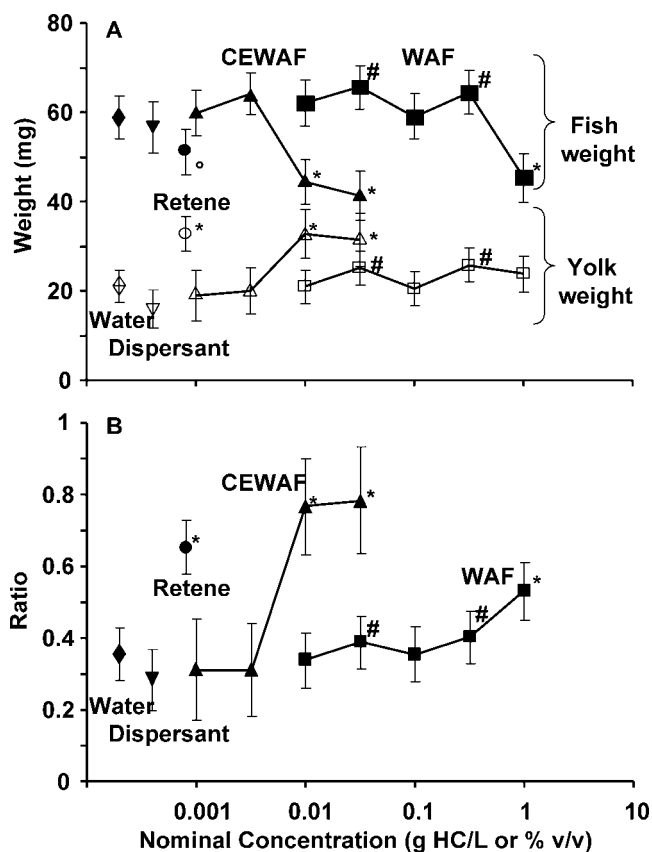


Fig. 6. The weights of trout embryos and of their yolk sacs (A) and the ratio of yolk to fish weights (B) at swim-up after exposure to the water-accommodated fraction (WAF) and chemically enhanced WAF (CEWAF) of diesel. Weights and ratios are also shown for negative control (water or 10 μ L/L of dispersant) and positive control (retene, 100 μ g/L) treatments. Error bars are 95% confidence intervals based on three replicates ($n = 9-12$). Means that were significantly different from both water and solvent control values are shown by an asterisk (*), while those different from water only are indicated by a degree sign ($^{\circ}$) and from dispersant only by a pound sign (#). (Fish weights: CEWAF $F = 12.4$, degrees of freedom [df] = 6, $p < 0.001$; WAF $F = 7.32$, $df = 7$, $p < 0.001$. Yolk weights: CEWAF $F = 7.03$, $df = 6$, $p < 0.001$; WAF $F = 6.28$, $df = 7$, $p < 0.001$. Yolk-fish ratios: CEWAF $F = 11.8$, $df = 6$, $p < 0.001$; WAF $F = 9.79$, $df = 7$, $p < 0.001$.)

rich in alkyl phenanthrenes, fluorenes, naphthobenzothio-phenes, chrysenes, and pyrenes [16]. Alkyl phenanthrenes such as retene are known to cause concentration-dependent increases in BSD in single-compound exposures to the embryo-larval stages of fish [10,11,17]. In addition, such aromatic hydrocarbons are generally more persistent and more water soluble than aliphatics [18] and hence are more likely to be bioavailable to fish. Therefore, PAHs or their alkylated homologs, present in both crude and diesel oil, are likely the toxic agents causing the observed induction of CYP1A protein synthesis and enzyme activity and the signs of BSD. Lower molecular weight compounds, such as BTEX, have a narcotic mode of action and could contribute to acute lethality. However, mortality occurred only late during embryonic exposures, so this seems unlikely. This conclusion is consistent with the weathering (loss of volatiles) that would occur during the 18 h of slow stirring required to produce WAF. At the measured concentrations of total hydrocarbons associated with lethality (Fig. 5), the maximum concentration of BTEX (0.03% of whole oil) would only be 60 μ g/L.

Concentrations of dispersed diesel (CEWAF) were relatively stable over 24 h, with fluorescence of test solutions declining to 67 to 77% of initial values. However, concentrations of undispersed diesel (WAF) decreased more, to 35 to 50% of nominal, likely because there were no droplets to act as sources of additional hydrocarbon. The decreases in hydrocarbon concentrations observed in both test solutions are likely due to evaporation, partitioning to tank walls, and uptake by fish.

The bioavailability and toxicity of the components of diesel appeared to increase when diesel was dispersed. Relative to WAF, CEWAF entrained more hydrocarbons in water, as indicated by fluorescence spectrometry. A corresponding increase in exposure of fish to PAHs was supported by an increased concentration of CYP1A protein and severity of BSD in embryonic trout. Exposure to CEWAF also affected the size and development of embryos and the severity of BSD at nominal concentrations as low as 0.01% v/v (10 mg/L). In contrast, WAF appeared virtually nontoxic, even at nominal concentrations up to 1.0% v/v, equivalent to 1,000 mg/L of whole oil added to water.

The 1,000-fold increase in bioavailability of diesel and exposure to PAHs in fish following chemical dispersion (as measured by EROD induction) is consistent with previous studies using dispersed crude oil. Chemical dispersion of medium South American crude caused a 1,100-fold increase in EROD activity of trout [6] and a 56-fold increase in toxicity to freshwater medaka embryos (*Oryzias latipes*) [7]. In saline water, dispersion of medium South American crude increased toxicity to estuarine mummichog embryos (*Fundulus heteroclitus*) by only fourfold [15], partly because of the reduction in solubility and dispersibility of oil components in saltwater compared to freshwater [15] and possible species differences in toxicity [7].

However, the increase in the bioavailability and toxicity of oil following chemical dispersion may not reflect any change in the toxicity of its components. The similarity of endpoints based on measured hydrocarbons concentrations (Fig. 5 and Table 2) suggest that the primary difference between WAF and CEWAF toxicity is that WAF entrains much less hydrocarbon in solution than CEWAF. Ramachandran et al. [6] found similar results for EROD activity in trout exposed to WAF and CEWAF of medium South American crude oil. At concentrations of WAF and CEWAF where PAHs could be measured by GC-MS, the measured EC50 concentrations of total PAHs associated with increased CYP1A activity in trout differed little between WAF and CEWAF exposures, even though the effective dilutions differed by more than 1,000-fold [6]. In other words, the bioavailability of CYP1A-inducing PAHs had not changed with oil dispersion. Therefore, while diesel is chronically toxic to fish, it is the transfer of toxic constituents from oil to water that limits exposure, uptake, and toxic effects. In a spill situation, the expression of diesel toxicity depends on the extent of its dispersion, which can be determined by the energy of mechanical mixing under the conditions of the spill or the application of chemical dispersants.

An alternative explanation for the enhanced toxicity of diesel CEWAF might be the toxicity of the chemical dispersant, but our results do not support this idea. The responses of dispersant control fish were similar to those of water control fish, and the expression of endpoints on the basis of measured total hydrocarbons demonstrated no marked difference between WAF and CEWAF exposures that might be attributed

to dispersant toxicity. While dispersant is toxic to fish embryos, Ramachandran [7] reported that a mineral oil-dispersant mixture was three times less toxic to medaka embryos than dispersant alone. Because the dispersant is amphiphilic (both hydrophobic and hydrophilic), it coats oil droplets and is less bioavailable and toxic to fish.

While these results reinforce the necessity of measuring actual exposure concentrations in toxicity tests and oil spills to recognize the stochastic nature of oil exposures, the application of GC-MS techniques can be costly for large numbers of treatments and lengthy exposure periods. Past experience has also indicated that measurements of most PAHs using GC-MS were below the detection limit at concentrations of WAF and CEWAF that increased CYP1A protein concentrations and enzyme activities [6,19]. Fluorescence spectrometry, as used in the present experiment, was a sensitive alternative for describing exposure conditions based on total hydrocarbons, although it lacks specificity for PAHs. As a second alternative, measurement of EROD activity in exposed fish is a rapid, sensitive, and inexpensive dose metric that demonstrates the recent history of PAH exposure [20]. The response is sensitive and specific to many PAHs that typify crude and refined oils [5], with the exception of its responsiveness to some polychlorinated aromatics (e.g., polychlorinated biphenyls), which generally do not co-occur with PAHs in most oils. Hence, it is a practical substitute for expensive chemical analyses of complex mixtures, particularly those where multiple PAHs at different concentrations may cause induction. Similarly, Western blots of CYP1A protein, while nonquantitative, indicate PAH exposure for embryonic trout. As a sensitive indicator of exposure to PAHs, CYP1A induction typically precedes other signs of toxicity to embryonic fish [21].

Parent PAHs, or their metabolites [9], appear to accumulate to a threshold tissue concentration before becoming toxic, but the threshold may change as a fish grows and develops. In this study, a higher nominal concentration of WAF was needed to induce EROD activity in juveniles than to increase CYP1A protein concentrations in embryos. In addition, within-species differences in diesel toxicity were seen between embryos and fry. For fry, the nominal WAF 14-d LC50 was approximately 90 mg/L (estimated from [8]). In the present study, WAF was not lethal to embryos at concentrations of 1,000 mg/L (1.0% v/v) over 24 d. The difference may be related to the way in which WAF solutions were prepared. For the fry study, WAF was prepared by 30 min of vigorous mixing [8] that would mechanically disperse oil and mimic CEWAF. As well, the brief period of mixing may not have purged highly volatile compounds such as BTEX and low molecular weight aliphatics (5.3% by weight of diesel), which could cause acute toxicity. In the present study, WAF was prepared by 18 h of slow stirring and 1 h of no stirring, following a protocol standardized to permit comparisons of toxicity among oils [6,22,23]. Slow, prolonged stirring would minimize the dispersion of small oil droplets and facilitate the loss of volatile compounds so that embryonic fish would react predominantly to residual components, such as PAHs. Therefore, the important feature in the expression of diesel toxicity is the extent to which the oil is dispersed in water, whether by mechanical or by chemical means.

The developmental stages tested may also be an important factor. In this study, newly hatched free-swimming embryos (sac fry) would depend on energy derived from egg yolk and would be exposed to components of diesel taken up across the

skin, the primary respiratory surface. Fry, which feed actively on small particles, may be exposed to diesel components by ingestion and by absorption across gill membranes. Hence, the molecular signature of diesel exposure in fry [8] may differ from that of embryos because the array of compounds taken up may differ. This is an important research need.

Our results are also consistent with genomic responses of fry observed by Mos et al. [8], which suggested oxygen deprivation, endocrine disruption, and altered immune function. Some of these responses, particularly oxygen deprivation, may reflect the cardiovascular toxicity typical of PAH effects on larval fish [24,25].

CONCLUSION

The present study shows that diesel toxicity, like that of crude oil, could impair the health and recruitment of fish populations, resulting in long-term reductions in economic returns to fisheries. As this study and that by Mos et al. [8] have shown, diesel toxicity can vary widely, depending on the extent of chemical or mechanical dispersion and the life stage exposed. While dispersants are not commonly used in freshwater, diesel may be spilled into fast-flowing, turbulent rivers, where mechanical dispersion may entrain oil droplets in the water column and sediments and cause similar effects. Therefore, it is important to assess the risks of diesel spills to fish and fisheries in terms of the spill location and timing relative to fish spawning and development. In marine systems, critical decisions about applying dispersants must be based on our current knowledge of diesel to reduce short-term and long-term impacts to aquatic organisms.

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