FINAL PROJECT REPORT

JOINT PROJECT AGREEMENT F5244-080736

Toxicity Effects of Dispersed Alaska North Slope Oil on Fish

Submitted to:

Prince William Sound Regional Citizens' Advisory Council
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Submitted by:

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March 1, 2013

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EXECUTIVE SUMMARY

This report describes the experiments conducted by research groups located at the St. Andrews Biological Station, Gulf Fisheries Centre, Bedford Institute of Oceanography; and Queen's University.

Environmental conditions, such as water temperature and salinity, may affect the toxicity of chemically and mechanically dispersed crude oil when accidental spills occur. Impacts of oil spills on local fish populations will also vary depending on the relative sensitivities of resident species and stocks. The objectives of the herring studies conducted by the Gulf Fisheries Centre were to: 1) determine the influence of rearing temperatures (7, 10 and 15ºC) and salinities (7.5, 15 and 30‰) on the toxicity of Arabian Light (ALC) to Atlantic herring (*Clupea harengus*) embryos; 2) determine if different species (Atlantic vs. Pacific herring [*Clupea pallasii]*) and spawning stocks (spring vs. fall spawning Atlantic, British Columbia vs. Alaska Pacific) of herring embryos respond similarly to chemically and mechanically dispersed crude oils; and 3) compare the toxicity of three crude oils (ALC; Alaska North Slope, ANS; Mediterranean South American, MESA). Toxicity was assessed from survival-to-hatch, length-at-hatch and the prevalence and severity of developmental abnormalities, incorporated into the blue sac disease severity index (BSD SI), in herring exposed throughout the embryo stage. All end-points assessed indicated greater toxicity of ALC when Atlantic fall embryos were reared at 7°C compared to 10 or 15°C. This increased toxicity at a low rearing temperature did not appear to result from dispersant effectiveness or PAH solubility as expected but to cold stress, increased exposure duration or reduced metabolic processes. Lower salinities (7.5 and 15‰) seemed to increase ALC toxicity by increasing PAH concentrations in these rearing salinities, likely resulting from higher PAH solubility. Atlantic herring were found to be more sensitive than Pacific herring. Within species, sensitivity differences were detected between stocks of Pacific herring, with herring from Alaska being more sensitive than herring from British Columbia. However, elevated BSD SI in control embryos from the Atlantic fall stock limited our assessment of the sensitivity of the Atlantic herring spawning stocks. Of the three oils tested, MESA was more toxic than ANS or ALC to Pacific herring embryos. Results of the present study suggest chronic toxicity of hydrocarbon exposure for herring embryos at concentrations two orders of magnitude below previously reported thresholds of 0.4 ug/L. Oil spill mitigation measures need to be conservative in their assessment of potentially harmful concentrations of hydrocarbons since the present study demonstrates high sensitivity, and a substantial range of sensitivity, to hydrocarbons among species and stocks of herring. Mitigation measures also need to consider the influence of environmental factors, especially temperature, on hydrocarbon toxicity.

As an intermediate model between a full-scale spill at sea and a laboratory test, a wave tank was used to simulate dispersion of spilled oil and to determine if the resultant concentrations were sufficient to cause toxicity in Atlantic herring embryos. Wave tank exposures were up to four times more toxic than laboratory-prepared test solutions, but in general, wave tank exposures followed the same toxicity trends as laboratory exposures. Additionally, toxicity increased with exposure time and concentration. Overall, laboratory tests can be used to reasonably estimate the potential for toxicity from a spill at sea. Although additional experimentation would be required

in order to make a definitive statement, our testing also shows that differences toxicological response of Atlantic and Pacific herring during the early life stages we examined is not likely to be significant. This finding supports the cautious use of data collected for the two species interchangeably.

Research conducted by the Gulf Fisheries Centre and Queens University comparing the sensitivity of Atlantic and Pacific herring stocks to spilled oil was somewhat contradictory. While experiments conducted by Queens University find that the difference in sensitivity between the two herring species is not likely to be significant, similar research by the Gulf Fisheries Centre shows that Atlantic herring are potentially more sensitive to spilled oil than their Pacific counterparts. For this reason, we suggest that additional research is required in order to definitively answer the question of where or not toxicological results for Atlantic and Pacific herring can be used interchangeably in risk assessments involving spilled oil.

Results from studies conducted by the Biological Effects group at the St Andrews Biological Station show that water accommodated fractions (WAF) of oil mixed either in baffled flasks or in the BIO wave tank is toxic to Atlantic salmon (*Salmo salar*) or various life stages of Atlantic cod (*Gadus morhua*) only at high concentrations. Atlantic salmon survived all exposures to WAF. Egg fertilisation, hatching success, larval and juvenile survival were affected only at concentrations unlikely to be seen in the real world. Similarly, sub-lethal indicators of impact are observed only at environmentally irrelevant concentrations.

Chemically enhanced water accommodated fractions (CEWAF) of oil, mixed in baffled flasks or in the BIO wave tank affect the endpoints mentioned above at much lower concentrations (higher dilution factors). Dilutions representing as low as 0.5% of CEWAF have been shown to reduce fertilisation of cod eggs and reduce survival of larvae compared to unexposed eggs and larvae. Once fertilised, hatching success is unaffected. CEWAF is also lethal to Atlantic salmon and Atlantic cod juveniles. However, the concentration required to kill these fish is quite high (*ca* 3 to 8% v/v). Our work has shown that exposure to CEWAF may induce responses at the biochemical level (EROD activity) in juvenile cod. We have also shown that water temperature affects the timing and magnitude of this response.

Risk, however, can only be assessed in the context of wind, current, quantity of oil, use and effectiveness of chemical oil dispersants, wave energy, proximity to shore and presence of aquatic species. While we conclude that WAF is not of concern to marine fish, non-dispersed oil clearly has greater impact on shores, seabirds and marine mammals. Chemical dispersion of oil makes oil-based compounds more available to marine species. It is therefore, more hazardous and may present a greater risk to marine fish. Our work is designed to identify hazards and provide data for professional risk assessors so they might have a better idea of effects thresholds upon which to base their predictions.

A paper presented at the 2011 International Oil Spill Conference (IOSC) also summarizes many of the findings of this project and can be found in Appendix A of this report.

INTRODUCTION

Oil spills can have devastating effects on the receiving aquatic environment. Chemical dispersants may be applied to reduce impacts of spilled oil on fauna and coastal habitats such as mangroves, marshes and beaches because once oil reaches these habitats, the complexity of clean-up efforts increases, hence, oil can remain in these areas for many years following a spill (Exxon Valdez Oil Spill Trustee Council 2009). Chemical dispersion removes the oil slick from the water surface by creating small oil droplets which dissolve throughout the water column (Fiocco and Lewis, 1999). However, once dispersed these oil droplets can be absorbed by aquatic biota, such as fish, increasing exposure to the toxic components of the oil such as polycyclic aromatic hydrocarbons (PAHs).

Environmental factors such as water temperature and salinity may influence the toxicity of dispersed oil by altering the effectiveness of the dispersant and the solubility of PAHs. Dispersant effectiveness should increase in warmer water temperatures because lower water temperatures can increase the viscosity of both the oil and the dispersant (Chandrasekar et al., 2005). In addition, the greater viscosity of the oil would necessitate greater mixing energy to disperse the oil (Chandrasekar et al., 2005). The solubility of PAHs should also increase at warmer temperatures (Whitehouse 1984). However, under natural conditions, low water temperatures could reduce biological degradation of the oil; hence oil concentrations within the water column could be lower but remain present for a longer period of time. Low water temperatures also reduce growth rates of cold blooded animals such as fish, which prolong exposure during critical developmental periods such as the embryonic and larval stages. Furthermore, low water temperatures can reduce the activity of detoxifying enzymes (Lyons et al., 2011). Therefore, conflicting factors make predictions of the influence of temperature on oil toxicity complex and for this reason, further studies are essential.

Chemical dispersant effectiveness is also influenced by salinity. Highly saline waters increase chemical dispersant effectiveness because the solubility of the dispersant is reduced which results in greater surfactant availability at the oil-water interface, promoting droplet formation (Mackay et al. 1984; Chandrasekar et al. 2006). However, an increase in salinity reduces the solubility of PAHs, nonetheless, the influence of temperature on PAH solubility is far greater than the influence of salinity (Whitehouse, 1984; Ramachandran et al., 2006). The dispersant Corexit 9500 was developed to have an optimal effectiveness in 25-34‰ environments (personal communication, T. King, Bedford Institute of Oceanography, Dartmouth, NS, Canada), though one study (Ramachandran et al., 2006) showed lower liberation of EROD active PAHs at high salinities (30 ppt). Therefore, the question of salinity effects on the toxicity of dispersed oil deserves further research.

Past research on the toxicity of chemically-dispersed crude oil to fish embryos has focused on freshwater and estuarine fish species and on Medium South American (MESA) crude oil, with chemical dispersion by a standard lab method. Few data exist for commercial marine species or other oil types, and none for oil dispersed under field conditions. Hence, there is a need to confirm the toxicity of other oil types to representative commercial fish species, and compare the properties of dispersed oil solutions generated in the laboratory and those which more closely

approximate field conditions. Previous work on the relationship between time and EC50s for embryo toxicity suggests that the risk of toxicity following the use of oil dispersants could be as great for brief exposures, before the dispersed oil is diluted, as for the chronic exposures typical of past studies. In addition, in the case of herring, due of the difficulties in working with non-aquaculture species, data on Atlantic herring are not abundant. In contrast, there are many studies of oil toxicity to Pacific herring which could be incorporated into risk assessments of oil dispersion if the relative sensitivities of the two species are known.

This report covers work carried out between 2008 and 2011, and describes laboratory and wave tank experiments conducted on the toxicity of dispersed Alaska North Slope (ANS) and Mediterranean South American (MESA) medium grade crude oils, and Arabian Light Crude (ALC); physical characteristics and chemical composition of these oils can be found in Appendix B. The research conducted includes experiments on commercially significant species including Atlantic and Pacific herring, as well as Atlantic cod and salmon. The report describes the experiments undertaken by scientists at Department of Fisheries and Oceans Canada located at the St. Andrews Biological Station (cod and salmon), Gulf Fisheries Centre (herring), and the Bedford Institute of Oceanography (wave tank facility). Additional herring toxicity studies conducted by Queen's University have also been included in this report.

LABORATORY HERRING STUDIES

MATERIALS AND METHODS

Test Species

Local fishermen collected Pacific herring from Neck Point, British Columbia (BC) on March 1, 2010 and Sitka Sound, Alaska (AK) on April 6, 2010 arriving at the Gulf Fisheries Centre (Moncton, New Brunswick, Canada) on March 3, 2010 and April 8, 2010 respectively. Atlantic herring were collected from Petit-Cap, New Brunswick on April 30, 2010 for the salinity bioassay and from Pictou, Nova Scotia on September 9, 2010 and September 23, 2010 for the temperature experiment. Excised gonads or whole fish were stored in plastic bags and packed on ice packs or crushed ice in a cooler for transport to the Gulf Fisheries Centre the same day the fish were caught. Herring embryos used in the species comparison were taken from the Pacific British Columbia stock and the Atlantic spring spawning stock.

Fertilization

Eggs for all experiments were fertilized on the day they arrived at the laboratory to maximize the fertilization rate. Eggs were extracted by ventral pressure or from gonads by making an incision and subsequently squeezing to remove the eggs. The eggs were pooled (from 3 to 16 females), mixed and dispersed homogeneously on glass microscope slides, to which they stick, using a dissecting needle (approximately 80-100 eggs/slide). Milt from the testes was extracted by ventral pressure or from gonads by removing a small section of the gonad and mixing in water. Milt was pooled (from 3 to 10 males) and mixed with 30‰ artificial seawater (Kent Sea Salt, Kent Marine, Acworth, GA, USA in reverse osmosis treated municipal water) in a 38 x 27 x 5 cm Pyrex dish. The egg-covered slides were transferred to the milt solution for approximately 10 minutes for fertilization to occur. The embryos used in the salinity experiment were rinsed and placed in the respective salinities (7.5, 15 and 30‰) by lowering the initial salinity of 30‰ by adding fresh water gradually over a one-hour period. The embryos used in the temperature experiment were rinsed and placed in the respective temperatures (7, 10 and 15°C) by reducing the initial temperature of 15°C by gradual cooling over a one-hour period. Fertilization success was assessed one hour post-fertilization (PF) by observing a definite cellular blastomere of blastula forming at the pole of the egg. The slides were then transferred immediately to the respective test solutions of chemically-enhanced water accommodated fraction (CEWAF) or water accommodated fraction (WAF). Since eggs and milt were combined into one common source, replication was the slide.

Test Solutions

The test oils were Alaska North Slope (ANS) and Mediterranean South American (MESA), medium grade crude oils and Arabian Light (ALC), a light grade crude oil, in the Pacific herring experiments while only ALC was tested in the Atlantic herring spring (salinity) and fall

(temperature) experiments. The crude oils and the dispersant, Corexit 9500, were provided by Department of Fisheries and Oceans, Centre for Offshore Oil, Gas and Energy Research (COOGER). ANS was weathered 10% by volume using nitrogen sparging in 2009, MESA was weathered by sparging with air for 130 hours to simulate the loss of 13.8% volatile components in 1998 and ALC was weathered 7% by volume by sparging with air in 2009. To generate CEWAF, a 1:9 mixture of oil and water (Ramachandran et al., 2006) was mixed for 18 hours in 500-mL baffled flasks with screw caps and a stopcock at the bottom of the flask (Venosa et al., 2002), at an estimated speed of 1200 rpm. Corexit was then added at a ratio of 1:10 dispersant-oil (Hemmer et al., 2010) and further mixed for one hour prior to one hour of settling. After settling, the bottom water fraction (CEWAF) was drained and diluted to the respective concentrations for testing. The same protocol was used to generate WAF except the dispersant was not added. Fresh CEWAF and WAF mixtures were prepared daily.

Test Conditions

Unfertilized eggs were removed and live embryos were reduced haphazardly to 20 per slide within ~72 hours of fertilization. The concentrations tested for the Pacific herring experiments and Atlantic herring spring experiment were 0.0001, 0.001 and 0.01% v/v for CEWAF and 0.01, 0.1 and 1.0% v/v for WAF (comparative oil toxicity and salinity experiment). The concentrations tested in the Atlantic herring fall (temperature) experiment were 0.0001, 0.001, 0.01, 0.1, 1.0 and 10% v/v CEWAF only. Concentrations were chosen based on previous research in this field (Ramachandran et al. 2004, Boudreau et al. 2009 and McIntosh et al. 2010). Controls for the bioassays included a water control (negative control), 320µg/L retene positive control (Billiard et al. 1999, Sweezey 2005; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and a dispersant treatment (0.1% Corexit 9500 for the Atlantic herring temperature experiment). Exposures were static from one hour PF until hatch with daily renewal of test solutions (non-aerated and static; 200 mL in 250 mL Mason jars) and maintained at 10°C (± 1°C) in 30‰ (± 1‰) artificial seawater. The salinity experiment was maintained at 10°C (± 1°C) in 7.5, 15 or 30‰ (± 1‰) artificial seawater while the temperature experiment was maintained at 7, 10 and 15°C (± 1°C) in 30‰ (± 1‰) artificial seawater. All experiments were performed with three replicates per concentration. Experiments were terminated when 100% of viable embryos hatched. At hatch, larvae were evaluated for Blue Sac Disease (BSD, see below). Once larvae were evaluated they were euthanized using ethyl 3-aminobenzoate methanesulfonate salt (MS-222, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at a concentration of 200 mg/L (Ramachandran et al., 2006).

Toxicological Response

Toxicity was based on survival-to-hatch, length-at-hatch and the prevalence and severity of BSD observed in the hatched larvae. Heart rates and time-to-hatch were quantified but showed no interpretable responses to oil treatment so are not presented (Appendix C, Tables C1 to C4).

At hatch, swimming ability was noted and each larva was photographed to later determine length-at-hatch and the presence and severity of morphological abnormalities. Swimming ability (SA) was determined by observing the motility of each individual larva in the test container (250 mL mason jar) on a scale of 0-2 (0 represents no movement, 1 represents twitches, 2 represents complete swimming ability). Presence and severity were recorded for spinal curvature (SC), pericardial edema (PE; accumulation of fluid in the pericardial sac), and yolk sac edema (YSE; accumulation of fluid in the vitelline vasculature). Severities were determined using the graduated severity index method (Carls et al. 1999) in which 1 represents slight defect, 2 moderate defect, and 3 severe defect (Boudreau et al. 2009; McIntosh et al. 2010). Presence or absence was recorded for jaw malformation (JM; inability to close lower jaw), and skin lesion (SL; rough, darkened appearance of the epithelial tissue). Abnormalities were integrated with mortality into a modified version of the McIntosh et al. (2010) BSD severity index (BSD SI):

$$\text{BSD SI} = \frac{\Sigma SC + \Sigma PE + \Sigma YSE + \Sigma JM + \Sigma SL - \Sigma SA + (13.5?D)}{n*13.5}$$

where D represents the total number of dead embryos for the treatment, n represents the total number of embryos exposed per treatment replicate (20 embryos) and the value 13.5 represents the maximum BSD score a larva can receive. In other words, the BSD SI is a normalized value (between 0 and 1) produced by the sum of the BSD scores (including mortality) and averaged within each treatment replicate.

Observations and measurements were made with a computer-based image analysis system (Matrox Inspector, version 3.0, Matrox Imaging, Dorval, QC, Canada) linked to a microscope (Leitz, Wild Photomakroskop M400, Leica Microsystems, Willowdale, ON, Canada) (16x – 90x) through a video camera (Hitachi, HV-D25, Fisher Scientific, Nepean, ON, Canada).

Characterization of Hydrocarbon Concentrations

Gas Chromatography/Mass Spectroscopy (GC-MS)

Gas chromatography-mass spectroscopy (GC-MS) was used to quantify the concentration of the following parent and alkyl-homologue PAHs: naphthalene, acenaphene, acenaphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzothiophene, benzo[a]anthracene, chrysene and perylene. Individual PAH concentrations were quantified in WAF (1.0% v/v) and CEWAF (0.01% v/v for the Pacific herring and Atlantic herring salinity bioassays and 0.1, 1.0, 10.0% v/v for the temperature bioassay) treatments at T=0 h for all temperatures and salinities and at T=24 h for 30‰ and 10ºC. Water samples (500 mL) were placed in hexane-acetone rinsed amber glass bottles, preserved with 500 uL 6N HCl, capped, sealed with Teflon tape and refrigerated until analyzed (extracted within 3 months and analyzed within 4 months of collection). Depending on the volume of the sample extracted, the detection limit ranged from 0.065-0.189 ug/L. GC-MS (Environmental Protection Agency 1996; King and Lee 2004) analysis was performed by COOGER in Dartmouth, NS.

Synchronous Scan Fluorescence

To quantify the total petroleum hydrocarbon (TPH) concentration (fluorescing compounds, mainly PAHs; Lyons et al., 2011), water samples were taken immediately following water changes (T = 0 h) and also before water changes (T = 24 h). Generally, water samples from the highest concentration of WAF (1.0% v/v for Pacific herring and Atlantic herring salinity experiments) and CEWAF (0.01% v/v for all experiments) were collected. Although 0.01% v/v CEWAF was not the highest concentration tested in the Atlantic herring temperature experiment (highest concentration was 10% v/v CEWAF for the temperature experiment only) this concentration was chosen for quantification to ensure consistency between experiments. Additionally, WAF (1.0% v/v for Atlantic herring salinity experiment) and CEWAF (0.01% v/v for both Atlantic herring salinity and temperature experiments) water samples were also collected at T = 2, 4, and 8 hours to monitor how the test solution changed over time. Water samples (50mL) were placed in hexane-acetone rinsed glass test tubes, preserved with 50 uL 6N HCl, capped, sealed with Teflon tape and refrigerated until analyzed (within 6 months of collection). Water samples were analyzed by synchronous scan fluorescence (excitation from 230 to 523 nm, λ57 nm) using a Varian Cary Eclipse Fluorescence Spectrofluorometer with Varian BIO Package version 1.1 software (Varian Inc., Paolo Alto, USA; Lyons et al. 2011). The limit of quantification was 4 ug/L.

Statistical Analysis

Replication was the jar, with three jars per treatment group. Survival-to-hatch was analyzed by median lethal concentration (LC50) in the temperature bioassay since the number of exposure concentrations was increased from three to six. LC50 could not be calculated for the Pacific or salinity bioassays due to a lack of data points. To calculate LC50, top constraints were set at the average control values for each temperature (7ºC = 96.67%; 10ºC = 88.33%; 15ºC = 95.00%) and lower constraints were set at zero. To improve the fit of the top plateau of the curve, control values were included as part of the dose-response curve. A four parameter nonlinear regression was employed to calculate LC50, along with an F-test and non-overlapping confidence limits to establish significant differences among the LC50 estimates (Environment Canada, 2005). The LC50 was calculated based on nominal concentrations not measured concentrations of test solutions.

The concentration that affected development in 75% of embryos (EC75% abnormal) and the BSD SI were calculated for the temperature bioassay only. Because the incidence of morphological abnormalities was above 50% in most treatment groups, an EC75 was calculated instead of an EC50. To calculate EC75% abnormal or EC75BSD SI, top constraints were set at 100 or 1 and lower constraints were set at the average control values for each temperature (EC75% abnormal: 7ºC = 12.07%; 10ºC = 30.19%; 15ºC = 35.08% and EC75BSD SI: 7ºC = 0.07; 10ºC = 0.16; 15ºC = 0.11). To improve the fit of the bottom plateau of the curve, control values were included as part of the dose-response curve. An ECanything nonlinear regression was employed to calculate the EC75, along with an F-test and non-overlapping confidence limits

to establish significant differences among these values (Environment Canada, 2005). EC75 estimates were also calculated based on nominal concentrations.

Because the number of concentrations in the salinity bioassay was not sufficient to calculate an LC50 or EC75, survival-to-hatch and BSD SI were analyzed by a 2-factor analysis of variance (ANOVA; salinity and concentration as factors). For all bioassays, length-at-hatch was analyzed by a 2-factor nested ANCOVA to remove the variability associated with replicates (nested within concentration) to test the influence of treatment only and because length-at-hatch could be positively correlated with incubation period, time-to-hatch was included as a covariate. Interactions were explored by ANOVAs analyzing each factor separately. Data were tested for normality (probability plot) and homoscedasticity (Levene/Bartlett test) and log (log X+0.01) or arcsine square-root transformed (survival and BSD SI) when needed. ANOVAs were followed by Tukey multiple comparison tests.

For both Pacific herring bioassays there was only one set of negative controls (water) and positive controls (retene) for the three crude oils, each tested at three concentrations. Therefore, the two controls and each of the nine oil-concentration combinations, considered individual treatments, were compared by 1-factor ANOVA for survival and BSD SI and by 1-factor nested ANCOVA for length-at-hatch. These end-points were also analysed for spawning stocks and species comparisons by a 1-factor ANOVA models assessing the effect of concentration.

Analyses were performed with Systat version 11.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.04 (San Diego, CA, USA). The level of significance was $p<0.05$. Means, LC50s and EC75s are accompanied by their 95% confidence interval, where possible.

RESULTS

Temperature experiment

Characterization of Hydrocarbon Concentration

Summed PAH concentrations measured by GC-MS were proportional to serial dilutions starting at 16µg/L (0.1% v/v; Table 1). Although stock CEWAF concentrations were 2.2X higher in 15°C than at 7°C (Table 1), this effect of temperature was less apparent in exposure concentrations which contained 1.2-1.5X more summed PAHs at 15°C than at 7°C, depending on the dose (Table 1). This pattern was also observed in water samples analyzed by synchronous scan fluorescence (Table 1). During the 24-hour period between test solution changes, summed PAH concentrations at 10°C dropped 16-28% from 0.1-10% v/v CEWAF (Table 1). Similarly, TPH concentration dropped 24-55%, depending on nominal concentration and exposure temperature (Table 1). At higher concentrations (1% v/v) there was a lower percent drop in TPH concentrations than at lower concentrations (0.01% v/v; 24-37% compared to 36-55%; Table 1). At warmer temperatures (15°C) there was a higher percent drop (37-55%) in TPH concentrations than in colder test conditions (24-37%; Table 1).

Table 1. Water samples analyzed by gas chromatography-mass spectroscopy (GC-MS) to determine summed PAH (methylated and non-methylated) concentrations and synchronous scan fluorescence to estimate total petroleum hydrocarbon (TPH) concentrations to which Atlantic herring embryos were exposed in Arabian Light WAF and CEWAF at 30‰ and 7, 10 and 15°C. The detection limit (DL) for GC-MS was ~0.065-0.189µg/L and the quantification limit for fluorescence was 4µg/L. Blank cells represent water sample data that are not available.

				Summed PAH (µg/L)		
Crude Oil Stock				12,792,270		
	Conc. (% v/v		N	7°C	10°C	15°C
CEWAF	Stock		1	17,850	16,850	39,960
	0.1	T=0 h	1	16	16	24
	1.0		1	180	190	220
	10.0		1	1,910	2,550	2,300
	0.1	T=24 h	1		12	
	1.0		1		160	
	10.0		1		1,840	
	0.1	24 h % Δ			25%	
	1.0				16%	
	10.0				28%	
Water		T=0 h	1		<DL	
Retene		T=0 h	1		61	
Corexit		T=0 h	1		2.3	
				TPH (µg/L)		
	Conc. (% v/v)		N	7°C	10°C	15°C
CEWAF	0.01	T=0 h	3	133	119	185
		T=2 h	1	86	106	149
		T=4 h	1	88	98	115
		T=24 h	3-7	84	76	83
		24 h % Δ		37%	36%	55%
	1.0	T=0 h	3	12,305	11,188	18,979
		T=24 h	3-7	8,269	8,556	11,977
		24 h % Δ		33%	24%	37%
Controls	Water	T=24 h	3-7	6.5	6.3	6.1

Survival-to-Hatch, Length-at-Hatch and Developmental Abnormalities

LC50 dose response curves exhibited the steepest slope at 7ºC, producing an LC50 of 0.01% (0.0089-0.0176; 95% confidence interval) at 7ºC compared to 0.41% (0.1779-0.6372) at 10ºC and 0.34% (0.1072-0.5730) at 15ºC (Figure 1; F-test, $F_{2, 57} = 25.97$, $p<0.0001$). Non-overlapping LC50 confidence intervals indicate significantly greater toxicity at 7ºC than at 10ºC and 15ºC (Environment Canada 2005). All embryos were killed by 10% v/v CEWAF and only a low percentage survived 1% CEWAF and only at the two warmer temperatures (Table 2). Similarly, all embryos exposed to the Corexit treatment experienced 0% survival (Table 2). Embryos exposed to the retene positive control responded similarly to the higher CEWAF concentrations (Figure 1, Table 2).

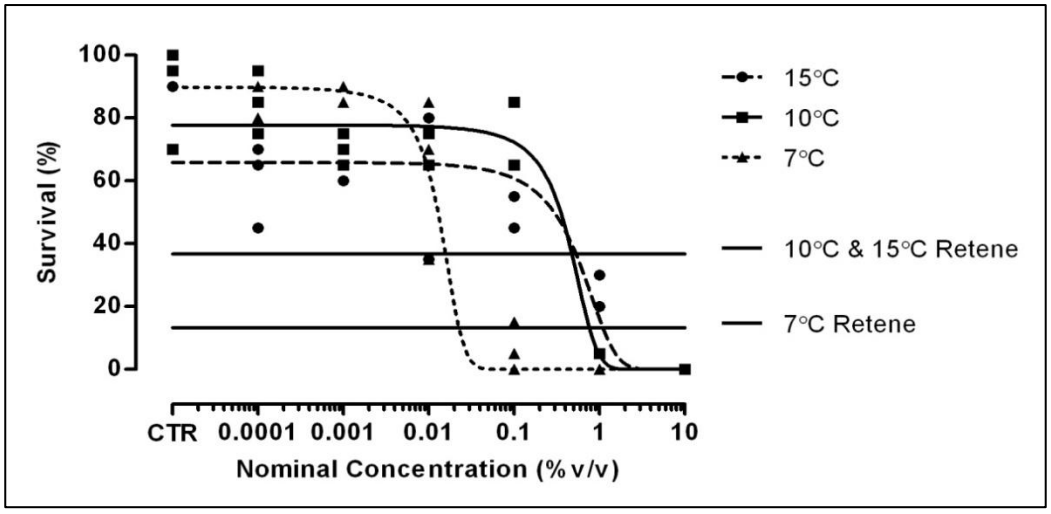


Figure 1. Percent survival of Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7, 10 and 15°C. Dose response curves for LC50 were calculated by 4-parameter nonlinear regression. N=3 jars/concentration.

Table 2. Atlantic herring embryos exposed to Arabian Light CEWAF at 7, 10 and 15°C. Reported are the mean percentages that survived-to-hatch, developed abnormally, and afflicted by individual abnormalities as well as the average swimming ability (0-2) including 95% confidence intervals. Upper 95% confidence interval of backtransformed values for survival was set at a maximum value of 100%.

Temperature		Conc. (% v/v)	Survival	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
7°C	CEWAF	0.0001	89 (65-100)	54±33	0±0	9±7	36±30	15±10	19±42	1.91±0.10
		0.001	84 (62-97)	49±25	0±0	4±9	37±35	10±10	19±43	1.78±0.17
		0.01	64 (6-100)	96±17	0±0	62±20	85±37	46±73	33±10	1.14±0.18
		0.1	4 (8-42)	100±0	0±0	0±0	100±0	0±0	100±0	0.50±0.92
		1.0	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch
		10.0	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch
	Controls	Water	98 (79-100)	12±31	0±0	0±0	10±23	5±1	9±27	1.88±0.11
		Retene	13 (7-21)	100±0	0±0	0±0	100±0	0±0	100±0	0.00±0.00
		Corexit	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch
10°C	CEWAF	0.0001	86 (53-100)	50±23	6±2	18±12	29±54	11±25	34±18	1.84±0.12
		0.001	70 (57-82)	76±24	0±0	10±29	41±51	12±24	49±79	1.71±0.19
		0.01	68 (53-82)	54±33	5±10	17±13	44±26	29±6	22±22	1.61±0.19
		0.1	72 (39-95)	97±11	3±11	39±20	74±27	52±9	59±39	1.28±0.18
		1.0	5 (5-5)	100±0	0±0	0±0	100±0	0±0	100±0	1.00±0.00
		10.0	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch
	Controls	Water	93 (30-100)	31±20	0±0	11±32	19±51	6±12	18±10	1.93±0.09
		Retene	36 (5-76)	100±0	4±18	8±36	100±0	0±0	100±0	0.86±0.21
		Corexit	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch
15°C	CEWAF	0.0001	60 (28-88)	61±18	0±0	33±13	19±26	21±25	37±32	1.86±0.17
		0.001	60 (60-60)	72±32	0±0	28±43	33±0	25±21	36±48	1.83±0.15
		0.01	61 (9-99)	71±25	0±0	26±7	32±12	19±25	37±15	1.92±0.10
		0.1	48 (34-63)	100±0	20±21	45±24	65±22	65±22	52±15	1.38±0.28
		1.0	17 (0-59)	100±0	0±0	0±0	100±0	0±0	100±0	0.36±0.34
		10.0	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch
	Controls	Water	97 (69-100)	35±19	2±7	3±8	25±21	10±12	12±29	1.83±0.11
		Retene	37 (19-56)	100±0	8±19	0±0	96±16	0±0	93±31	0.55±0.23
		Corexit	0 (0-0)	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch	No Hatch

Larval length-at-hatch was also affected by temperature but the effect differed with CEWAF concentration [Figure 2; Table C5 (Table 5 in Appendix C)]. For the control group and low CEWAF concentrations (0.0001 and 0.001% v/v), length-at-hatch decreased with increasing rearing temperature and no significant effect of CEWAF treatment was observed (Figure 2; Table C5). The opposite relationship was seen in embryos exposed to ≥0.01% v/v: an increase in length-at-hatch with increasing temperature. Starting at this concentration (0.01% at 7ºC) there was also a significant reduction in length-at-hatch with increasing CEWAF concentration (Figure 2; Table C5). Embryos exposed to retene followed a similar pattern as embryos exposed to higher concentrations of CEWAF (Figure 2; Table C5).

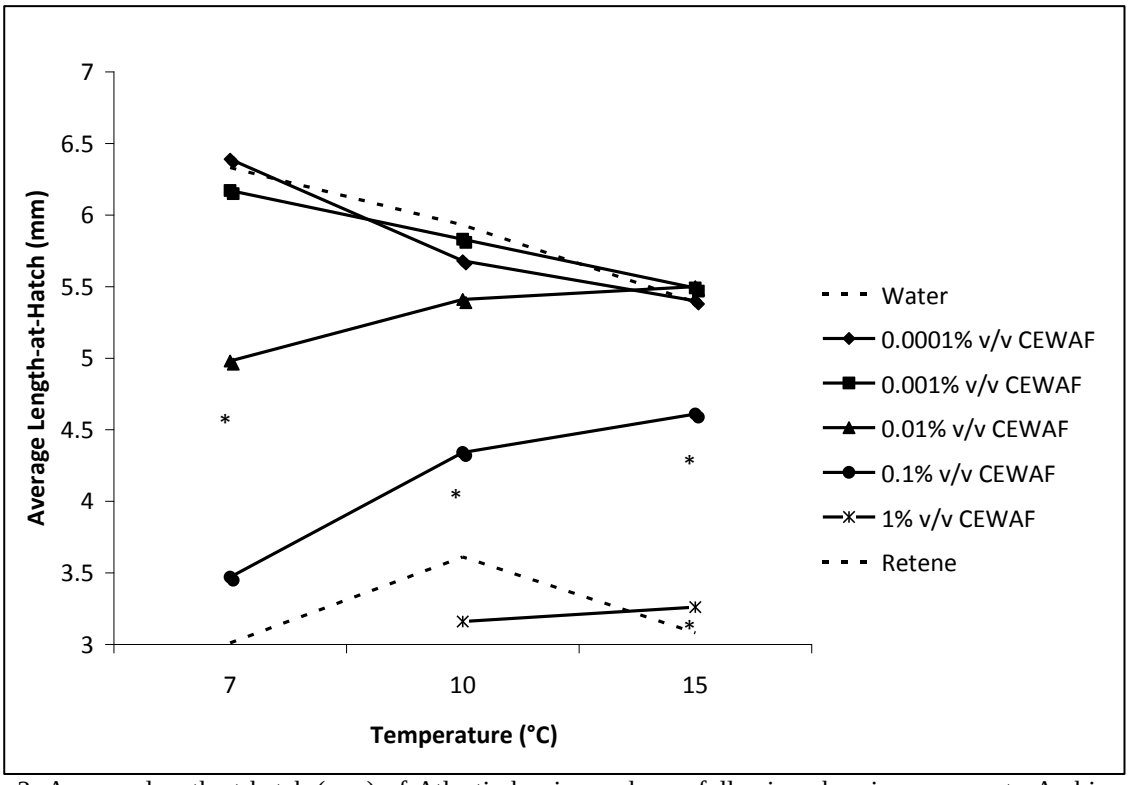
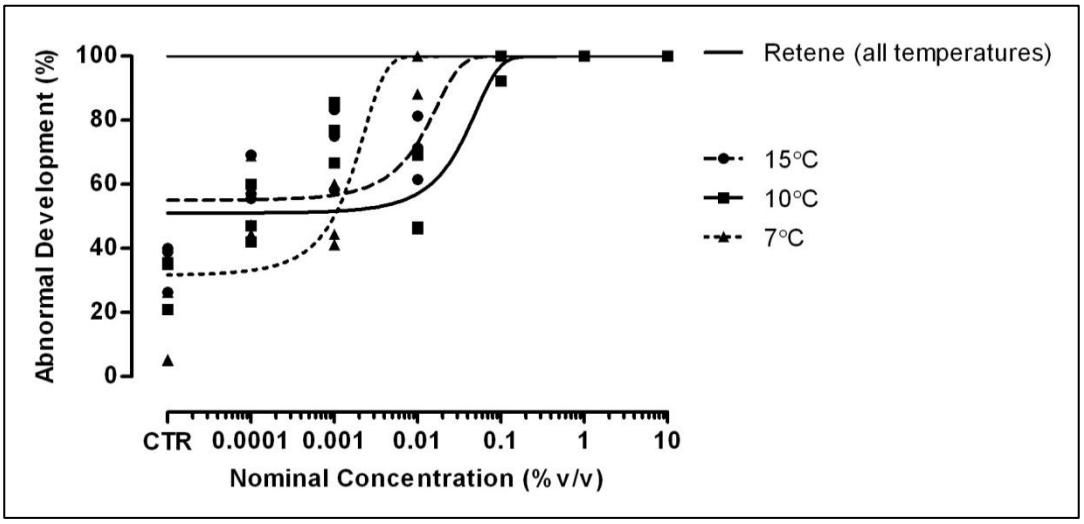


Figure 2. Average length-at-hatch (mm) of Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7, 10 and 15°C. Individual temperatures were analyzed separately (nested ANCOVA) following significant interaction of factors in 2-Factor nested ANCOVA. Asterisks (*) indicate treatments that experienced significantly reduced length-at-hatch compared to the negative control (Tukey test, $p<0.05$). Embryos exposed to Corexit all died. N=3 jars/concentration. For clarity, the checked line representing retene is the lower of the two checked lines.

Effect of temperature on oil toxicity was assessed by EC75 % abnormal which showed greater toxicity at 7ºC (0.002%, 0.0002-0.0046) than 10ºC (0.050%, -0.0169-0.1169) and also 15ºC (0.017%, 0.0026-0.0309). Although a significant effect of temperature was detected (Figure 3A; F-test, $F_{2,57} = 10.33$, $p=0.0001$), which temperatures differed could not be determined because of overlapping confidence intervals. Embryos that survived to hatch following exposure to the positive control (retene) experienced 100% abnormal development (Table 2).

A



B

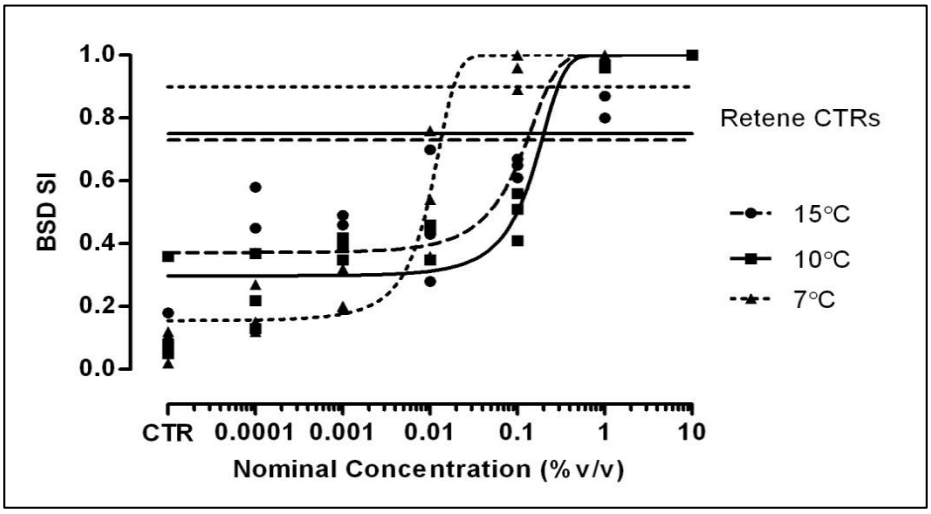


Figure 3. Percent abnormal development (**A**) and average blue sac disease severity index (BSD SI; **B**) values for Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7, 10 and 15°C. Dose response curves for EC75 were calculated by an ECanything nonlinear regression. N=3 jars/concentration.

The BSD SI showed clear dose responses at all three temperatures reaching the maximum score of 1 (all embryos dead) at 1.0% v/v at 7ºC and 10.0% v/v at 10ºC and 15ºC (Figure 3B). The EC75BSD SI estimate was lower at 7°C (0.014%, 0.0110-0.0175) than 10°C (0.207%, 0.0841-0.3301) and also 15°C (0.150%, 0.0472-0.2535; F-test, $F_{2,57}$ = 21.61, p<0.0001). Non-overlapping LC50 confidence intervals indicate significantly greater toxicity at 7ºC than both 10ºC and 15ºC (Environment Canada 2005). Embryos exposed to the positive retene control responded similarly to the higher CEWAF concentrations (Figure 3B).

For all temperatures, the observable toxicity changed as CEWAF concentrations increased (Figure 4). The percentage of embryos that developed pericardial edema, yolk sac edema, spinal curvature, jaw malformations and skin lesion increased as concentration increased until the highest concentration in which embryos still survived. At this concentration the percentage of

pericardial edema, yolk sac edema and jaw malformation dropped to 0% while 100% of embryos developed spinal curvatures and skin lesions (Table 2).

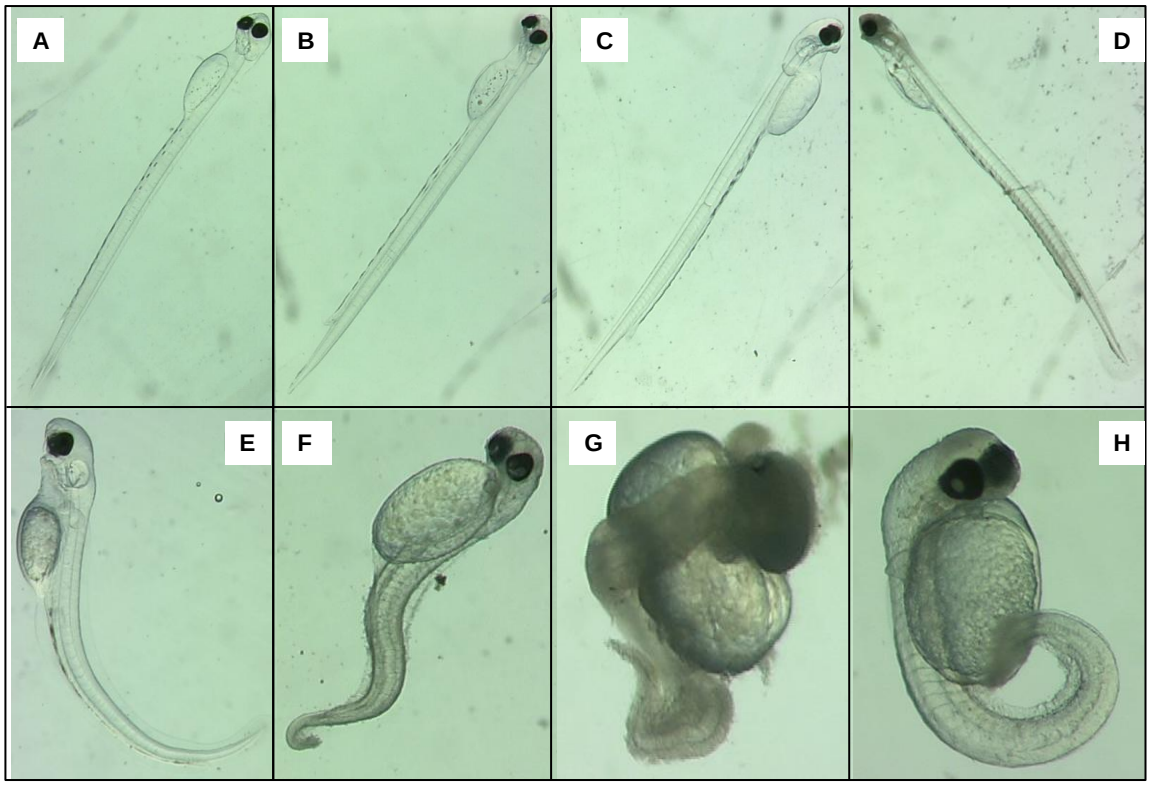


Figure 4. Typical sub-lethal effect trends observed in Atlantic herring larva from the negative control (**A**; normal larva), 0.0001% v/v (**B**; normal larva), 0.001% v/v (**C**; slight pericardial edema, jaw), 0.01% v/v (**D**; yolk sac edema, slight spinal curvature, skin lesion on head), 0.1% v/v (**E**; yolk sac edema, pericardial edema, jaw, spinal curvature), 1% v/v (**F**; spinal curvature, skin lesion over whole body), 10% v/v (**G**; dead larva) and the retene positive control (**H**; spinal curvature, yolk sac edema, skin lesion over whole body). The observed toxicity trends changed as concentrations increased. Edemas and jaw deformities became more prevalent and severe as concentration increased until the highest concentration in which embryos still hatched, at which point 0% of live larvae possessed either edema or jaw deformities (**F**); while spinal curvature and skin lesion became more prevalent and severe as concentration increased until 100% of live larvae possess both spinal curvature and skin lesion (**F**).

Salinity Bioassay

Characterization of Hydrocarbon Concentration

Herring embryos in the salinity bioassay were exposed only to the lower range of CEWAF concentrations tested in the temperature bioassay (i.e., 0.0001–0.01% v/v and not 0.1–10% v/v). However, this experiment also included WAF exposures of 0.01–1% v/v. As observed in the temperature bioassay, concentrations of summed PAHs at these high dilutions were generally undetectable by GC-MS (Table 3). Measured concentrations of summed PAHs in stock solutions were approximately 2.0X and 1.8X higher in 7.5‰ relative to 30‰ CEWAF and WAF, respectively (Table 3). TPH concentrations measured by synchronous scan fluorescence were

measurably elevated in the highest dose tested of both CEWAF and WAF but showed little effect of salinity and declined approximately 50% over the 24 hours between test solution replacements (Table 3).

Table 3. Water samples analyzed by gas chromatography-mass spectroscopy (GC-MS) to determine summed PAH (methylated and non-methylated) concentrations and synchronous scan fluorescence to estimate total petroleum hydrocarbon (TPH) concentrations to which Atlantic herring embryos were exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 10°C. The detection limit (DL) for GC-MS was ~0.065-0.189µg/L and the quantification limit for fluorescence was 4µg/L. Blank cells represent water sample data that are not available.

				Summed PAH (µg/L)		
Crude Oil Stock				12,792,270		
	Conc. (% v/v)		N	7.5‰	15‰	30‰
CEWAF	Stock		1	33,530	26,760	16,850
	0.01	T=0 h	1	<DL	<DL	0.34
WAF	Stock		1	3,020	3,960	1,650
	1.0	T=0 h	1	4.5	0.31	0.4
Controls	Water	T=0 h	1			<DL
	Retene	T=0 h	1			61
	Corexit	T=0 h	1			2.3
				TPH (µg/L)		
	Conc. (% v/v)		N	7.5‰	15‰	30‰
CEWAF	0.01	T=0 h	1	109	139	103
		T=2 h	1	80	90	95
		T=4 h	1	64	73	
		T=8 h	1	61	58	79
		T=24 h	7	54	44	95
		24 h % Δ		51%	68%	8%
WAF	1.0	T=0 h	1	85	134	108
		T=2 h	1	78	114	96
		T=4 h	1	74	109	83
		T=8 h	1	58	82	78
		T=24 h	7	46	57	43
		24 h % Δ		46%	58%	60%
Controls	Water	T=24 h	7	<DL	<DL	6.4

Survival-to-Hatch, Length-at-Hatch and Developmental Abnormalities

Survival-to-hatch, which ranged from 99% to 100%, was not significantly reduced by exposure to WAF or CEWAF test solutions at any salinity (Tables 4, C6). However, embryos exposed to the positive control retene suffered significantly reduced survival-to-hatch in all salinities and greatest reduction in saltiest water (30‰; Tables 4, C6).

Table 4. Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰. Reported are the mean percentages that survived-to-hatch, developed abnormally, and afflicted by individual abnormalities as well as the average swimming ability (0-2) including 95% confidence intervals. Salinities were analyzed separately (1-Factor ANOVA) following significant interaction of factors in 2-Factor ANOVA. Asterisks (*) indicate a significant reduction in survival-to-hatch from the negative control (Tukey test, $p<0.05$; N=3 jars/concentration). Upper 95% confidence interval of backtransformed values for survival was set at a maximum value of 100%.

Salinity		Conc. (% v/v)	Survival	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
CEWAF	7.5‰	0.0001	100 (100-100)	30±33	0±0	12±14	17±19	3±15	3±15	1.92±0.07
		0.001	100 (100-100)	47±26	0±0	15±43	25±12	12±19	12±19	1.93±0.07
		0.01	99 (71-100)	95±12	10±25	81±13	91±26	61±45	18±22	1.22±0.13
	15‰	0.0001	100 (100-100)	40±12	2±7	22±7	15±12	13±15	8±8	1.90±0.08
		0.001	100 (100-100)	42±26	0±0	22±28	13±19	18±8	2±7	1.98±0.03
		0.01	100 (100-100)	90±25	7±14	85±37	55±22	60±43	8±19	1.52±0.17
	30‰	0.0001	100 (100-100)	18±19	0±0	3±15	5±12	12±7	5±12	1.97±0.07
		0.001	100 (100-100)	37±31	0±0	8±36	12±19	8±19	20±12	1.90±0.10
		0.01	100 (100-100)	63±32	7±19	30±12	17±28	20±12	42±51	1.83±0.12
WAF	7.5‰	0.01	100 (100-100)	38±32	3±15	17±31	12±26	20±25	10±0	1.97±0.05
		0.1	99 (85-100)	44±24	0±0	22±13	10±12	20±15	8±19	1.90±0.11
		1.0	99 (85-100)	69±15	0±0	61±20	34±21	39±32	10±1	1.71±0.13
	15‰	0.01	99 (85-100)	36±25	0±0	12±31	12±31	19±13	3±15	1.93±0.07
		0.1	100 (100-100)	32±28	3±8	12±14	5±12	23±19	3±8	1.98±0.03
		1.0	100 (100-100)	82±14	12±19	63±19	45±22	60±22	30±22	1.67±0.12
	30‰	0.01	100 (100-100)	37±26	0±0	2±7	10±12	2±7	33±32	1.97±0.07
		0.1	100 (100-100)	27±19	0±0	2±7	5±12	2±7	22±14	2.00±0.00
		1.0	100 (100-100)	67±31	2±7	25±22	32±47	18±29	45±50	1.78±0.14
Controls	7.5‰	Water	100 (100-100)	20±22	0±0	8±19	10±12	3±15	0±0	2.00±0.00
		Retene	94* (85-99)	100±0	23±26	93±7	98±8	20±41	50±30	0.84±0.11
	15‰	Water	100 (100-100)	22±7	0±0	5±12	3±15	15±0	2±7	2.00±0.00
		Retene	86* (53-100)	100±0	4±9	75±52	100±0	0±0	51±8	0.88±0.09
	30‰	Water	100 (100-100)	20±12	0±0	2±7	7±7	3±8	7±14	2.00±0.00
		Retene	50* (26-74)	100±0	3±15	35±33	100±0	0±0	97±12	0.43±0.19

Embryos exposed to the highest concentration of WAF and CEWAF were significantly shorter at hatch than control embryos for all salinities tested (Figure 5; Table C5). At the highest concentration of WAF, embryos were significantly shorter at hatch when reared in 7.5 and 15‰ than 30‰ but there was no significant difference among salinities at the highest concentration of CEWAF (Table C5). Embryos exposed to retene showed the opposite effect, with significantly shorter larvae hatching in 30‰ compared to 15 and 7.5‰ (Figure 5; Table C5). The LOEC for CEWAF that significantly reduced length-at-hatch in the salinity bioassay (0.01 % v/v) is consistent with the lowest LOEC observed in the temperature bioassay (7ºC; Figures 2, 5; Table C5).

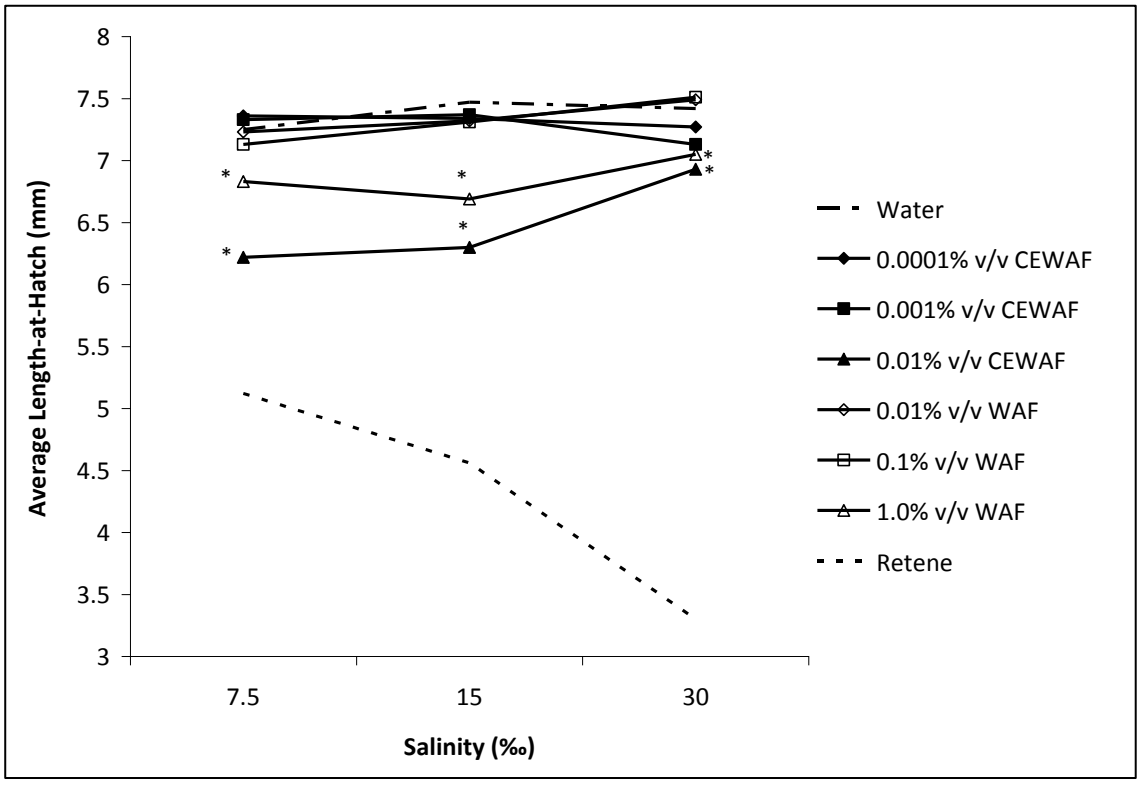


Figure 5. Average length-at-hatch (mm) of Atlantic herring embryos following chronic exposure to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰. Individual salinities were analyzed separately (nested ANCOVA) following significant interaction of factors in 2-Factor nested ANCOVA. Asterisks (*) indicate treatments that experienced significantly reduced length-at-hatch compared to the negative control (Tukey test, $p<0.05$). N=3 jars/concentration.

BSD SI was significantly increased at the lowest exposure concentration of CEWAF (0.0001% v/v) in embryos reared in 15‰ while embryos reared in the 7.5 and 30‰ salinities only suffered from an increase BSD SI at the highest concentration tested (0.01% v/v, Figure 6). For WAF, the embryos reared in 7.5‰ showed a significantly elevated BSD SI at a lower concentration (0.1% v/v) than 15 and 30‰ (1% v/v; Figure 6; Table C7). Within concentration comparisons also demonstrated greater toxicity at lower salinities with significantly higher BSD-SI observed in the lowest salinity for the highest concentration of CEWAF and in the lower salinities in the two

highest concentrations of WAF (Table C7). Embryos exposed to retene at all salinities also responded with increased BSD symptoms compared to the negative control and all WAF and CEWAF concentrations (Table C7). However, as for length-at-hatch, the effect of salinity on morphological abnormalities was the reverse of the effect noted for oil treatments: highest BSD-SI at the highest salinity tested.

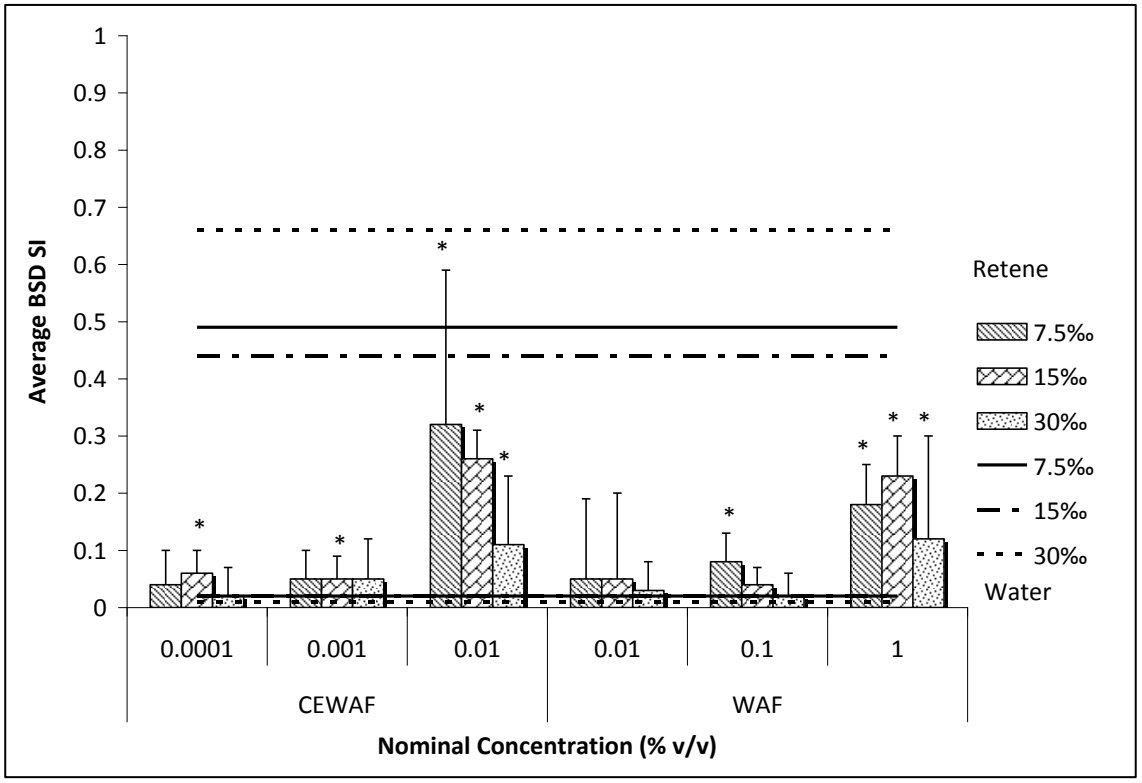


Figure 6. Average blue sac disease severity index (BSD SI) values for Atlantic herring embryos following chronic exposure to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰. Individual salinities were analyzed separately (ANOVA) following significant interaction of factors in 2-Factor ANOVA. Asterisks (*) indicate treatments that experienced significantly higher BSD symptoms compared to the negative control (Tukey test, $p<0.05$). N=3 jars/concentration.

Pacific Herring Experiments

Characterization of Hydrocarbon Concentration

Analysis by GC-MS indicated that the lowest detectable concentration of summed PAH was observed in MESA WAF and CEWAF at 0.38 µg/L (1.0% v/v) and 3.1 µg/L (0.01% v/v), respectively (Table 5). Summed PAH concentrations were higher in stocks of CEWAF than WAF: approximately 40, 15 and 10X higher in ANS, MESA and ALC respectively (Table 5). The concentration of PAHs was also dependent on the crude oil with MESA CEWAF stocks containing 2-3.2X more summed PAHs than either ANS or ALC CEWAF stocks (Table 5). TPH concentrations measured by synchronous scan fluorescence also demonstrated that MESA CEWAF test solutions (0.01% v/v) contained 2-4X more hydrocarbons than ANS or ALC

CEWAF test solutions (Table 6). During the 24 hours between solution changes, in the highest concentration of CEWAF and WAF, the TPH concentrations dropped 31-41% and 57-86%, respectively, depending on the crude oil (Table 6).

Table 5. Water samples analyzed by gas chromatography-mass spectroscopy (GC-MS) to determine summed PAH (methylated and non-methylated) concentrations to which Atlantic and Pacific herring embryos were exposed at 30‰ and 10°C. The detection limit (DL) was approximately 0.065-0.189µg/L. Blank cells represent water sample data that are not available. N=1 sample/concentration.

			Summed PAH (µg/L)			
			ANS	MESA	ALC	Controls
Crude Oil Stock			10,571,360	10,058,890	12,792,270	
CEWAF		Stock	27,080	53,680	16,850	
	T=0 hr.	0.01% v/v	<DL	3.1	<DL	
	T=24 hr.	0.01% v/v	<DL	<DL	NA	
WAF		Stock	720	3,570	1,650	
	T=0 hr.	1.0% v/v	2.3	0.39	<DL	
	T=24 hr.	1.0% v/v	0.94	0.38	<DL	
Water	T=0 hr.					<DL
Retene	T=0 hr.					61

Table 6. Water samples analyzed by synchronous scan fluorescence to estimate total petroleum hydrocarbon (TPH) concentrations to which Atlantic and Pacific herring embryos were exposed at 30‰ and 10°C. The quantification limit was 4µg/L. Blank cells represent water sample data that are not available. N=1-3 samples/concentration for samples taken at T=0, 2, 4 and 8h while N=3-7 samples/concentration for samples taken at T=24 h.

			TPH (µg/L)							
			Atlantic		Pacific					
	Conc. (% v/v)		Spring	Fall	BC			AK		
Oil			ALC	ALC	ANS	MESA	ALC	ANS	MESA	ALC
CEWAF	0.01	T=0 h	103	119	158	267	75			
		T=2 h	95	106						
		T=4 h		98						
		T=8 h	79	76						
		T=24 h	95		94	182	52	115	208	54
		24 hr. Δ	8%		41%	32%	31%			
WAF	1.0	T=0 h	108		109	264	146			
		T=2 h	96							
		T=4 h	83							
		T=8 h	78							
		T=24 h	43		47	36	25	43	25	34
		24 hr. Δ	60%		57%	86%	83%			
Controls	Water	T=0 h			7.0	7.0	7.0			
		T=24 h	6.4	6.3	8.4	8.4	8.4	8.4	8.4	8.4

Toxicity of Three Crude Oils to Geographical Spawning Stocks of Pacific Herring

In the Pacific herring experiments, embryos responded as expected to the positive control, retene. Exposure to retene resulted in lower survival-to-hatch, reduced length-at-hatch and a higher BSD SI than negative controls and in most cases, also than all WAF and CEWAF treatment concentrations. Retene produced similar abnormalities to those produced by the WAF and CEWAF of oils tested in the present study.

Survival-to-hatch indicated greater toxicity of MESA than ANS or ALC CEWAF, but not WAF, for AK, but not BC (Table 7), herring embryos. AK embryo survival was reduced by CEWAF doses starting at 0.001% v/v for ANS and ALC and 0.0001% v/v for MESA (Tables 8 and C8). Length-at-hatch of surviving AK, but not BC, embryos was significantly reduced by 0.01% v/v MESA and ANS, but not ALC, CEWAF (Tables 7, 8, C9).

Exposure of BC embryos to both WAF and CEWAF of all oils did not significantly increase the BSD SI compared to the negative control, except in the highest concentration of MESA CEWAF (Figure 7; Table C10). However, embryos from AK were significantly affected with LOECs being 0.0001% v/v for MESA CEWAF, 0.001% v/v for both ANS and ALC CEWAF and 0.01% v/v for MESA and ANS WAF and 0.1% v/v for ALC WAF (Figure 7; Table C10).

Table 7. Pacific herring embryos (BC stock) exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) WAF and CEWAF. Reported are the mean percentages that survived to hatch, developed abnormally, and were afflicted by individual abnormalities as well as the average larval length-at-hatch (mm) and swimming ability (0-2) including 95% confidence intervals. CEWAF and WAF were analyzed separately and arrows indicate a significant difference from the negative control [1-Factor ANOVA (oil-concentration combination); Tukey test $p<0.05$; N=3 jars/concentration]. Upper 95% confidence interval of backtransformed values for survival was set at a maximum value of 100%.

		Conc. (% v/v)	Survival	Length	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
CEWAF	ANS	0.0001	98 (58-100)	7.97±0.19 ↑	50±37	0±0	2±8	34±42	14±10	25±44	1.88 ±0.09
		0.001	99 (71-100)	7.85±0.17 ↑	57±7	0±0	4±7	26±31	19±13	33±18	1.91±0.07
		0.01	87 (5-100)	7.26±0.35	84±13	42±48	31±22	60±2	51±41	18±33	1.67±0.16
	MESA	0.0001	85 (49-100)	7.71±0.32 ↑	70±46	10±21	4±9	54±45	32±55	49±24	1.84±0.12
		0.001	98 (47-100)	7.70±0.16 ↑	63±6	2±9	4±8	39±45	34±5	31±16	1.82±0.10
		0.01	82 (52-99)	7.21±0.20	93±18	37±5	50±46	57±14	81±21	31±10	1.78±0.13
	ALC	0.0001	85 (71-95)	7.70±0.24 ↑	49±25	4±8	10±15	44±34	18±2	35±36	1.71±0.17
		0.001	92 (74-100)	7.55±0.30	42±1	2±7	5±13	24±12	26±24	26±10	1.78±0.15
		0.01	89 (69-99)	7.41±0.31	72±15	10±22	8±17	40±30	51±21	38±30	1.83±0.13
WAF	ANS	0.01	95 (95-95)	7.61±0.24	58±57	0±0	2±7	35±53	23±7	35±33	1.86±0.11
		0.1	92 (74-100)	7.56±0.25	52±40	0±0	2±8	26±24	31±25	20±14	1.87±0.10
		1.0	85 (49-100)	7.45±0.27	72±38	22±26	18±10	34±13	45±47	29±22	1.88±0.11
	MESA	0.01	90 (74-99)	7.81±0.28	63±62	0±0	6±1	45±67	26±60	43±24	1.87±0.09
		0.1	79 (47-98)	7.83±0.42	64±13	4±17	15±22	32±7	47±1	32±13	1.83±0.14
		1.0	94 (85-99)	7.91±0.32 ↑	82±20	18±16	29±16	37±11	61±12	34±48	1.80±0.11
	ALC	0.01	86 (53-100)	7.72±0.24	59±33	2±8	8±6	42±39	26±4	36±23	1.80±0.13
		0.1	93 (43-100)	7.58±0.26	61±17	7±15	7±8	25±44	32±30	23±25	1.85±0.11
		1.0	94 (52-100)	7.51±0.25	78±10	7±10	9±10	49±11	49±18	52±20	1.71±0.14
Controls	Water		95 (48-100)	7.26±0.29	30±17	6±1	5±13	22±20	7±6	14±25	1.84 ±0.11
	Retene		23 (4-51) ↓	5.49±0.39	100±0	22±96	69±67	100±0	11±48	26±63	1.07±0.42

Table 8. Pacific herring embryos (AK stock) exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) WAF and CEWAF. Reported are the mean percentages that survived-to-hatch, developed abnormally, and afflicted by individual abnormalities as well as the average larval length-at-hatch (mm) and swimming ability (0-2) including 95% confidence intervals. CEWAF and WAF were analyzed separately and arrows indicate a significant difference from the negative control [one-way ANOVA (oil-concentration combination); Tukey test $p<0.05$; N=3 jars/concentration]. Upper 95% confidence interval of backtransformed values for survival was set at a maximum value of 100%.

		Conc. (% v/v)	Survival	Length	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
CEWAF	ANS	0.0001	100 (100-100)	7.36±0.17	63±19	8±19	13±15	23±32	47±51	18±36	1.90 ±0.08
		0.001	88 (81-94) ↓	7.31±0.26	78±27	13±23	25±31	32±44	49±26	34±41	1.85±0.11
		0.01	84 (66-96) ↓	6.46±0.25 ↓	98±9	24±11	76±25	56±26	84±34	32±46	1.56±0.14
	MESA	0.0001	85 (85-85) ↓	7.53±0.14	78±8	6±15	12±25	18±38	51±30	39±23	1.94±0.07
		0.001	94 (38-100)	7.29±0.21	89±4	11±26	32±24	42±38	60±30	23±38	1.91±0.08
		0.01	79 (62-91) ↓	6.60±0.27 ↓	100±0	21±18	81±13	70±8	90±15	38±26	1.68±0.14
	ALC	0.0001	95 (95-95)	7.41±0.19	77±15	5±13	21±26	30±27	49±42	39±59	1.93±0.07
		0.001	90 (90-90) ↓	7.38±0.25	87±8	11±37	30±48	30±16	67±13	43±39	1.83±0.17
		0.01	92 (83-98) ↓	7.35±0.16	100±0	31±7	73±37	36±18	82±8	22±33	1.84±0.10
WAF	ANS	0.01	89 (65-100)	7.39±0.15	79±25	26±12	33±37	33±36	49±20	22±21	1.91±0.08
		0.1	93 (43-100)	7.18±0.23	91±8	23±19	46±8	34±34	71±10	19±31	1.91±0.08
		1.0	93 (43-100)	7.06±0.21	98±8	62±31	65±29	50±27	93±8	33±12	1.70±0.13
	MESA	0.01	90 (74-99)	7.34±0.22	83±15	9±21	28±29	39±36	59±26	48±25	1.89±0.10
		0.1	94 (85-99)	7.35±0.21	95±0	7±7	30±37	37±25	68±13	52±27	1.84±0.10
		1.0	88 (55-100)	7.10±0.19	100±0	8±11	57±37	45±40	84±12	46±21	1.81±0.11
	ALC	0.01	95 (48-100)	7.21±0.21	62±30	6±15	18±11	24±8	42±27	34±7	1.93±0.07
		0.1	84 (62-97)	7.28±0.21	85±19	8±8	35±22	38±18	58±25	36±19	1.84±0.11
		1.0	92 (74-100)	7.24±0.16	98±8	15±32	57±17	40±15	80±10	35±54	1.86±0.10
Controls	Water		100 (100-100)	7.43±0.11	38±19	2 ± 7	17±18	7±7	27±8	7±28	1.98 ±0.03
	Retene		22 (15-29) ↓	4.66±0.46 ↓	100±0	23±63	20±86	92±36	0±0	62±31	0.69±0.29

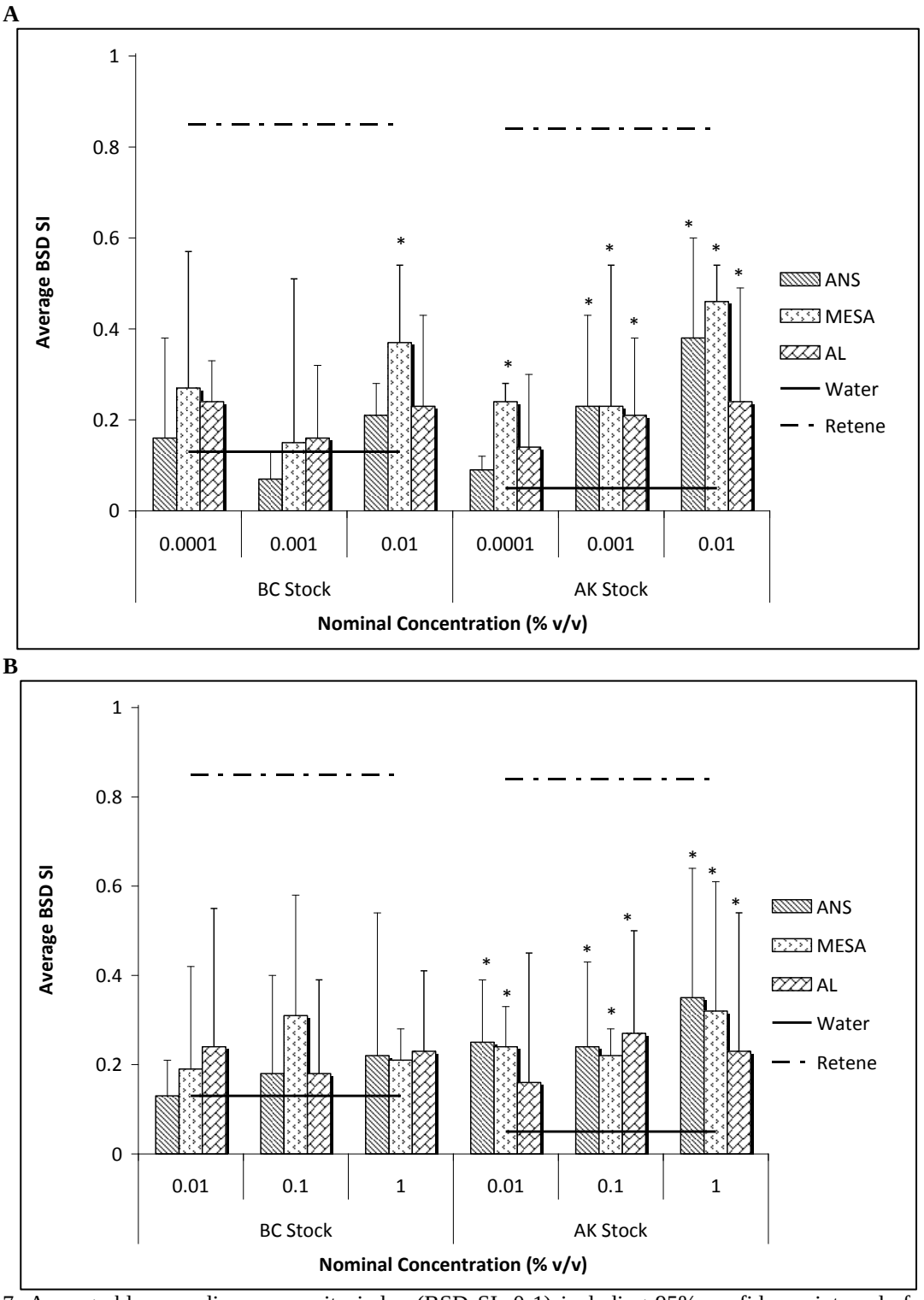


Figure 7. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Pacific herring embryos exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) CEWAF (**A**) and WAF (**B**). Stocks were analyzed separately and asterisks (*) indicate treatments that were significantly different from the negative control [1-Factor ANOVA (oil-concentration combination); Tukey test, $p<0.05$]. N=3 jars/concentration.

Toxicity of Chemically Dispersed Arabian Light Crude to Seasonal Spawning Stocks of Atlantic Herring

Survival-to-hatch was not significantly reduced by exposure to ALC CEWAF for either stock of Atlantic herring (Tables 9, C11). Length-at-hatch was significantly reduced compared to controls at the highest concentration tested in the spring stock (0.01% v/v; Table 9, C12). Both the frequency (Table 9) and severity (BSD-SI; Figure 8) of abnormalities were higher in the fall than the spring stock, including negative controls, but significant elevation of abnormality by Al CEWAF was seen only in the spring stock at the highest dose tested (0.01% v/v; Figure 8; Table C12).

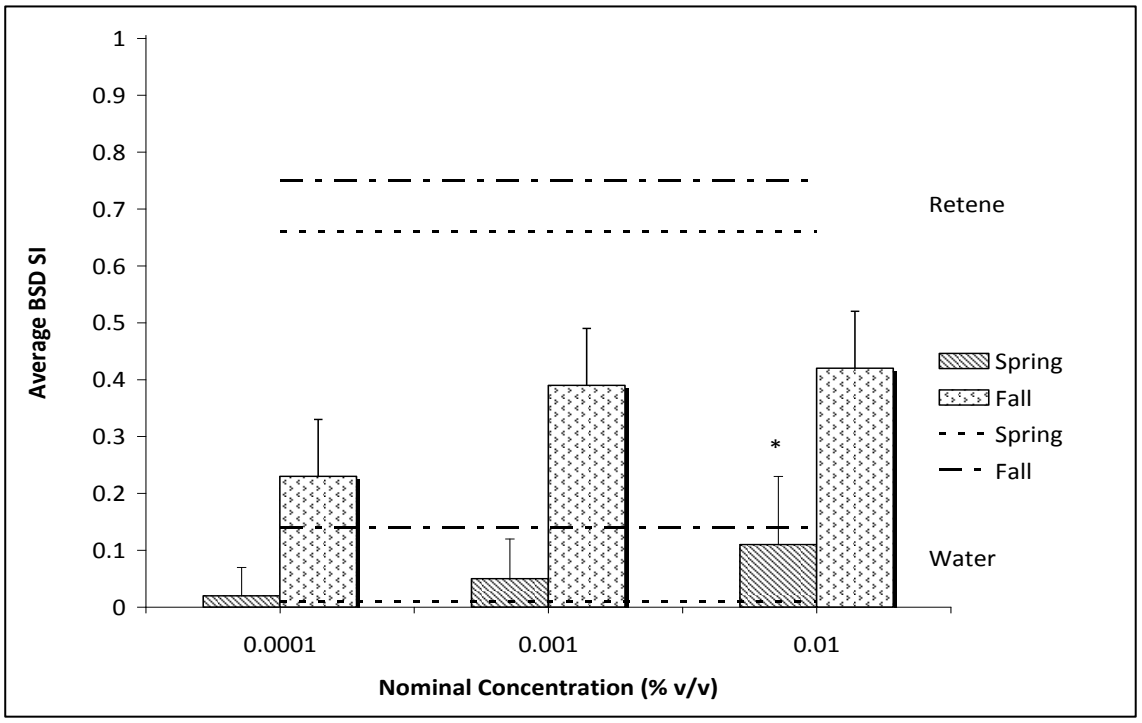


Figure 8. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Atlantic herring embryos exposed to Arabian Light CEWAF. Stocks were analyzed separately and asterisks (*) indicate treatments that were significantly different from the negative control (ANOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

Table 9. Atlantic herring embryos from spring and fall spawning stocks exposed to Arabian Light WAF and CEWAF. Reported are the mean percentages that survived-to-hatch, developed abnormally, and afflicted by individual abnormalities as well as the average larval length-at-hatch (mm) and swimming ability (0-2) including 95% confidence intervals. Stocks were analyzed separately and arrows indicate a significant difference from the negative control (one-way ANOVA; Tukey test, $p<0.05$; N=3 jars/concentration). Upper 95% confidence interval of backtransformed values for survival was set at a maximum value of 100%.

		Conc. (% v/v)	Survival	Length	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
Spring	CEWAF	0.0001	100 (100-100)	7.27±0.16	18±19	0±0	3±15	5±12	12±7	5±12	1.97±0.07
		0.001	100 (100-100)	7.13±0.19	37±31	0±0	8±36	12±19	8±19	20±12	1.90±0.10
		0.01	100 (100-100)	6.93±0.23 ↓	63±32	7±19	30±12	17±28	20±12	42±51	1.83±0.12
	WAF	0.01	100 (100-100)	7.49±0.15	37±26	0±0	2±7	10±12	2±7	33±32	1.97±0.07
		0.1	100 (100-100)	7.51±0.10	27±19	0±0	2±7	5±12	2±7	22±14	2.00±0.00
		1.0	100 (100-100)	7.05±0.23 ↓	67±31	2±7	25±22	32±47	18±29	45±50	1.78±0.14
	Controls	Water	100 (100-100)	7.42±0.12	20±12	0±0	2±7	7±7	3±8	7±14	2.00±0.00
		Retene	50 (26-74) ↓	3.30±0.16 ↓	100±0	3±15	35±33	100±0	0±0	97±12	0.43±0.19
Fall	CEWAF	0.0001	86 (53-100)	5.68±0.22	50±23	6±2	18±12	29±54	11±25	34±18	1.84±0.12
		0.001	70 (57-82)	5.83±0.28	76±24	0±0	10±29	41±51	12±24	49±79	1.71±0.19
		0.01	68 (53-82)	5.41±0.37	54±33	5±10	17±13	44±26	29±6	22±22	1.61±0.19
	Controls	Water	93 (30-100)	5.93±0.21	31±20	0±0	11±32	19±51	6±12	18±10	1.93±0.09
		Retene	36 (5-76) ↓	3.61±0.17 ↓	100±0	4±18	8±36	100±0	0±0	100±0	0.86±0.21

Sensitivity of Pacific vs Atlantic herring to Arabian Light

Herring species responses to Arabian Light oil were compared using the BC Pacific stock and the Atlantic spring stock to minimize latitudinal and seasonal differences. Embryonic survival-to-hatch was not significantly reduced by ALC WAF or CEWAF exposure for either species (85-94% survival in Pacific herring and 100% survival in Atlantic herring; Table 7, 9, C8, C11). Of the surviving embryos, length-at-hatch was significantly reduced at a lower concentration for Atlantic herring compared to Pacific herring (CEWAF: 0.01% v/v compared to >0.01% v/v and WAF: 1.0% v/v compared to >1.0% v/v; Tables 7, 9, C9, C12). A greater percentage of Pacific herring embyos developed abnormally compared to Atlantic herring embryos (42-78% compared to 18-67%; Tables 7, 9). However, exposure to ALC CEWAF and WAF elicited significantly elevated BSD in Atlantic herring embryos at a lower concentration compared to Pacific herring (0.01 and 1.0% v/v compared to >0.01 and >1.0% v/v; Figure 9; Tables C10, C12).

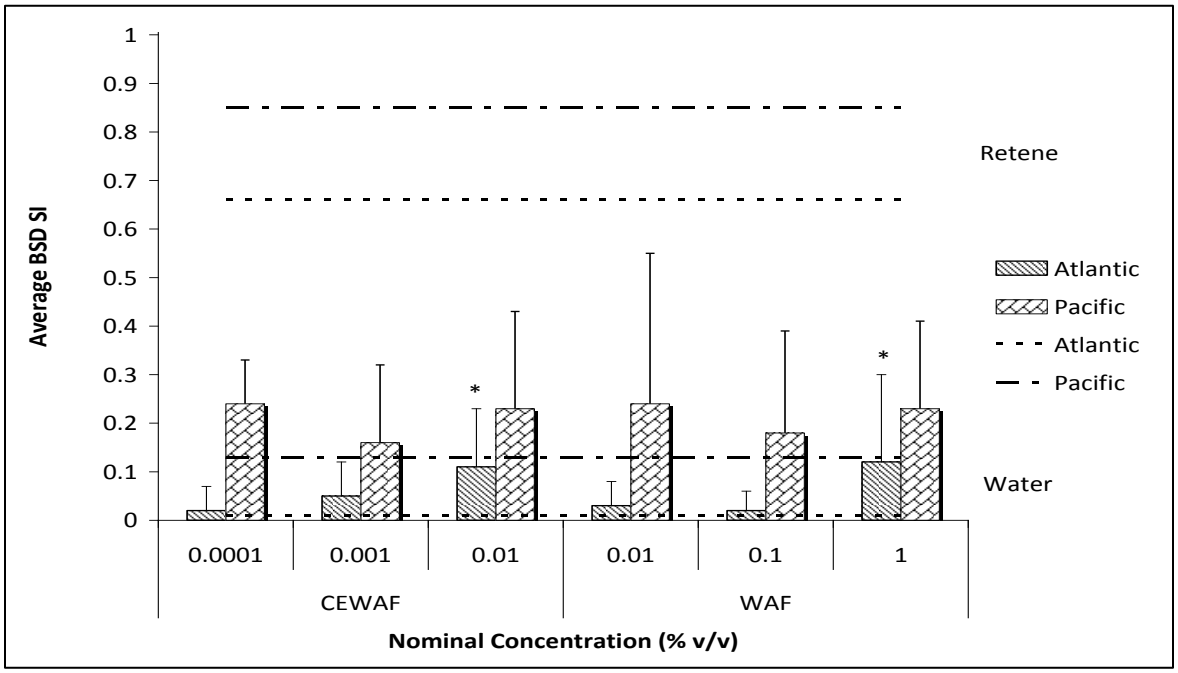


Figure 9. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Pacific (BC stock) and Atlantic herring (spring stock) embryos exposed to Arabian Light WAF and CEWAF. Species were analyzed separately and asterisks (*) indicate treatments that were significantly different from the negative control (ANOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

DISCUSSION

Temperature Experiment

There was a clear trend for all endpoints (survival (LC50), length-at-hatch, and abnormalities (EC75 % abnormal and EC75 BSD SI,)) suggesting greater toxicity of ALC CEWAF in the coldest rearing temperature (7ºC) compared to 10ºC and 15ºC.

The increased toxicity observed in the colder rearing conditions might be a result of the natural physiology or exposure duration of the herring stock exposed to ALC CEWAF. Fall spawning Atlantic herring spawn in relatively warm water, ~15°C in the Gulf of St. Lawrence and 10°C off south-western Nova Scotia, compared to spring spawners which spawn in colder waters of ~5ºC (Steward and Arnold 1994). Since the herring exposed to ALC CEWAF in the temperature experiment were taken from a fall spawning stock, rearing at low temperature might, in itself, have constituted a stress. We consider this likely because our initial experimental design, which called for rearing at temperatures as low as 5ºC, repeatedly failed; fertilization of eggs was only successful when the temperature was increased to 7ºC. Alderdice and Velsen (1971) found a similar temperature tolerance limit of 4-5ºC in Pacific herring. Alternatively, or in addition to cold stress, the increased toxicity observed in the colder rearing conditions might have resulted from a slower developmental rate and considerably longer exposure duration. Embryos hatched in 15-19 days (depending on CEWAF treatment) at 7ºC compared to 6-7 days at 15ºC (Table C2).

Colder temperature would also be expected to reduce metabolic processes including excretion of contaminants. For example, Lyons et al. (2011) observed a temperature effect linked to metabolic reactions. Juvenile Atlantic cod (Gadus morhua) ethoxyresorufin-O-deethylase (EROD) activity following exposure to CEWAF of Mediterranean South American (MESA) crude oil was 9X and 12X lower at 2ºC than at 7ºC and 10ºC, respectively. PAH concentrations were also lower at 2ºC relative to 7ºC and 10ºC but the authors concluded that the lower EROD induction was likely related to reduced metabolic rate of cod as opposed to the lower concentration of PAHs in the exposure water (Lyons et al. 2011).

The observed pattern of increased toxicity at lower temperature is contrary to what might be predicted from higher PAH concentrations in warmer water as a result of dispersant effectiveness and PAH solubility. Theoretically, chemical dispersants are more effective in warmer environments because the viscosity of the oil is reduced (Daling 1988; Moles et al. 2001; Chandrasekar et al. 2005) and PAHs are more soluble (Whitehouse 1984; May and Wasik 1978), which ultimately increases the concentration of PAHs in the water. In the present study, dispersant effectiveness was quite similar for all temperatures (1.3-3.1%) so the increased PAH solubility in warmer water likely caused the increase in summed PAH concentrations (2.2X) in the 15ºC CEWAF stock relative to the 7°C CEWAF stock. The fact that greater toxicity was observed at low temperature indicates a greater role of either prolonged exposure or thermal stress on herring embryos than PAH concentration in the water.

Herring spawning occurs over a wide range of temperature (0 to 15ºC) depending on the stock (Blaxter, 1985). With this in mind the present study was initially designed to include a test temperature of 5ºC in which repeated attempts to fertilize eggs at 5ºC failed so the lowest test temperature was raised to 7ºC. Therefore, 7ºC may be close to the lower limit of temperature tolerance for the stock studied, and rearing near this limit may render these embryos more susceptible to oil exposure. Studies have suggested that spawning is triggered mainly by temperature (Blaxter, 1985; Haegele and Schweigert, 1985), which would suggest that spawning may be infrequent at sub-optimal temperatures. However, a study by Winters and Wheeler (1996) assessed the reproductive period of Newfoundland Atlantic herring from 1970 to 1992 and determined that the reproductive period of herring was much more plastic than previously described by other studies. These authors found that the reproductive period of Newfoundland herring could be adjusted, by as much as 4-5 weeks, in order to be synchronised with favourable environmental conditions to maximise recruitment success. Furthermore, reproduction is not initiated by favourable sea temperatures in spring, as previously believed, but is triggered well in advance by January sea temperatures (spring spawners). This early trigger of gonad maturation and spawning may not be able to avoid fluctuating sea temperature at the time of spawning resulting in eggs maturing at a range of temperature including those tested in the present study. Hence, even if increased toxicity of oil at low temperature in our experiment resulted from sub-optimal rearing conditions, this may well be a situation that occurs naturally.

Embryos exposed to the Corexit treatment in the temperature bioassay experienced 100% mortality regardless of exposure temperature. These experiments were done in 30‰ water which is near optimal for dispersant stability and effectiveness (35‰ for Corexit 9500; Fingas 2004). Singer et al. (1996) reported that red abalone (Haliotis rufescens) embryos exposed to Corexit 9500 stopped developing during early embryogenesis, consistent with findings that cellular toxicity of surfactants occurs at the membrane, which produces permanent membrane damage such as loss of barrier function (Partearroyo et al. 1990). In the present study, mortality in the Corexit treatment also occurred during early embryogenesis. Hence, mortality may also have been caused by similar mechanism as proposed by Singer et al. (1996) and these mechanisms may have been increased at higher salinities because of the increased stability of the surfactant droplet which may better penetrate the membrane of the embryo.

The Corexit treatment included in the temperature bioassay, 0.1% or 1000 ppm Corexit, corresponded to the amount present in the highest concentration tested of ALC CEWAF (10% v/v). This Corexit concentration exceeds the 96h LC50 of larval and juvenile fish species which ranges from 25 – 143 ppm (George-Ares and Clark, 2000; Hemmer et al., 2010). The ALC CEWAF LC50s calculated from the temperature bioassay varied from 0.01 – 0.41% v/v. At these CEWAF concentrations, the amount of Corexit could vary from 1 to 41 ppm which is in the range of Corexit LC50 values observed by other studies (George-Ares and Clark, 2000; Hemmer et al., 2010). However, given that much of the Corexit present in the CEWAF solution should bind to the hydrocarbon molecules, concentrations of Corexit which may remain in the CEWAF are likely lower than LC50 values observed by other studies. Therefore, the toxicity observed in our CEWAF treatments should be related to the PAHs present in the CEWAF. To validate this assumption, future studies should measure the amount of Corexit remaining in CEWAF solutions to confirm the source of its toxicity.

Salinity Experiment

There was some suggestion in the present study that lower salinities may increase oil toxicity. Embryos exposed to 1% v/v ALC WAF were smaller at hatch when incubated in 7.5‰ and 15‰ than 30‰ and embryos showed higher rates of abnormality (BSD-SI) when exposed to higher concentrations of both WAF and CEWAF at lower salinities.

Increased toxicity associated with less saline test solutions in the present study likely resulted from higher hydrocarbon concentration in exposure solutions since PAH solubility is higher in fresh water (May and Wasik 1978; Whitehouse 1984; Ramachandran et al. 2006). In the present study, summed PAHs were 2.0X (CEWAF) and 1.8X (WAF) higher in 7.5‰ than in 30‰ stock solutions. Ramachandran et al. (2006) also reported an increase in EROD activity in juvenile mummichog (Fundulus heteroclitus) and rainbow trout (Oncorhynchus mykiss) as salinity decreased, suggesting that lower salinity increased hydrocarbon exposure.

While these observations suggest greater oil toxicity at low salinity, to produce more conclusive results we suggest that a larger experiment incorporating a wide enough range of CEWAF and WAF concentrations to support dose-response curves be conducted. We suggest further experimentation because in the present study the effects on length-at-hatch and morphological abnormalities that were seen with WAF were not seen consistently with CEWAF, as would be expected because TPH concentrations were comparable in the highest doses of WAF and CEWAF tested (Table 3). Secondly, salinity impacts on length-at-hatch and morphological abnormality responses observed were the reverse of what was observed in the positive, retene controls. Therefore, these results should be regarded as preliminary.

Toxicity of Three Crude Oils to Geographical Spawning Stocks of Pacific Herring

Pacific herring embryos from Alaska were more sensitive to oil exposure than embryos from British Columbia as observed through survival-to-hatch, length-at-hatch and morphological abnormalities (BSD SI). Paulson and Smith (1977) reported that adult Pacific herring fecundity increased with latitude from California to British Columbia to Alaska. Because herring fecundity varies inversely with egg size (Kelly and Stevenson 1985), herring from Alaska likely produce smaller, lower quality eggs that may be more sensitive. Conversely, Pacific herring from Puget Sound, Washington and Prince William Sound, Alaska exposed throughout embryonic development responded similarly to Prudhoe Bay crude oil exposure (Kocan et al. 1996). Therefore, there is evidence both for and against the suggestion that Pacific herring are stock sensitive to oil exposure which may be further evidence that egg quality can vary both between and within spawning populations (Brooks et al. 1997).

Pacific herring embryos exposed to MESA showed increased toxicity compared to the other oils tested in the present study. Couillard (2002) exposed mummichog embryos to both weathered MESA and ANS and also determined that MESA was more toxic to embryos than ANS. MESA

is a medium crude oil that contains more PAHs than the other oils (2X-3.2X more in the present study), which could explain the increased toxicity.

Toxicity of Chemically Dispersed Arabian Light Crude to Seasonal Spawning Stocks of Atlantic Herring

In the present study, survival-to-hatch was inconclusive when comparing the sensitivity to oil of spring and fall spawned embryos. Length-at-hatch was only significantly reduced at the highest concentration tested in spring-spawned embryos. Developmental abnormalities combined in the BSD SI, was also significantly elevated compared to control embryos at the highest concentration tested in spring embryos. However, in the fall spawning stock, a large variation in the BSD SI values of the control group may have masked significant increases in BSD SI from oil exposure. A previous study illustrated that fall spawning stocks of Atlantic herring were more sensitive to MESA exposure than spring spawning stocks (McIntosh et al. 2010).

Fall spawning stocks were also expected to be more sensitive because Haegele and Schweigert (1985) reported that spring spawning Atlantic herring typically produce larger but fewer eggs than fall spawning stocks. This suggests that the adult spring-spawning females might invest more energy in producing high quality eggs that may be more robust and have a greater tolerance to chemical insult, such as oil exposure.

Furthermore, in the northwest Atlantic, herring spawn from northern Labrador to Virginia with spring spawners dominating in the northern part of the Atlantic while fall spawners dominate in the south (Haegele and Schweigert, 1985). Therefore, fall spawners may be less adapted to environmental conditions of the northern Atlantic. During the course of the present study, bioassays with fall spawners often had to be repeated due to very low fertilization rates (<10%) and a high incidence of naturally occurring abnormal/underdeveloped embryos. However, this was never observed during spring bioassays for which high fertilization rates (>90%) were always observed with lower rates of naturally occurring abnormal embryos. Hence, better egg quality in spring spawners was observed during the course of our study. Other researchers have also observed this pattern (D.P. Swain, research scientist, Gulf Fisheries Centre, Moncton, NB, pers. comm.). The low gamete quality of the fall stock likely concealed significant impacts of oil exposure, for this reason, and because of the low fertilization rates of these gametes, we do not recommend the use of eggs from Atlantic herring fall spawners in toxicology studies.

Toxicity of Arabian Light Crude to Herring Species

Atlantic herring were more sensitive to ALC exposure than Pacific herring as observed through length-at-hatch and morphological abnormalities. Similarly, Greer (2011) observed that Atlantic herring were four times more sensitive to CEWAF then Pacific herring in their normal development and the incidence of BSD. While Greer concluded that this difference in sensitivities did not merit species-specific advice, because the results of the present study

indicate greater sensitivity of Atlantic herring, the range of sensitivity of these species should be considered in risk assessments of dispersed oil.

Differences in sensitivity to crude oil, in the present study, may be the result of biological (or ecological) differences between these species, such as natural rearing conditions. For example, the laboratory rearing temperature of 10ºC may have been more appropriate for Pacific herring because spawning in British Columbia occurs at temperatures ranging between 6.5ºC-9.8ºC (Haegele and Schweigert, 1985) compared to spring spawning Atlantic herring which typically spawn at lower temperatures of 5ºC (Steward and Arnold 1994). However, these differences are unlikely to be related to egg quality, as it took two days for the Pacific herring gametes to travel from the West Coast (BC or AK) to our laboratory on the East Coast (NB, Canada), while gametes of Atlantic herring were fertilized on the same day the fish were captured.

Characterization of Hydrocarbon Concentration

Even though PAH concentrations were high enough to affect herring embryos, results of GC-MS and fluorescence indicate that hydrocarbon concentrations in the test solutions were much lower than expected. Comparison of summed PAH measured by GC-MS in crude oil stocks and those in the CEWAF stocks showed a dispersant effectiveness ranging from 1.32 – 5.34%. Dispersant effectiveness is defined as the amount of oil that is dispersed into the water column compared to the amount of oil that remains on the surface (Chandrasekar et al. 2005). In the present study we calculated our dispersant effectiveness as = (((summed PAH in CEWAF stock / (summed PAH in crude oil stock * 10%))*100); the summed PAH in the crude oil stock is multiplied by 10% because this was the amount of stock used to produce the CEWAF. Because of the low dispersant effectiveness, our test concentrations were often below detection limits for GC-MS. Fluorescence was able to detect fluorescing compounds in our test solutions although test concentrations were only analysed by fluorescence for the highest concentrations of WAF and CEWAF. Chandrasekar et al. (2005) tested different factors which can affect dispersant effectiveness. One of these factors, mixing speed, increased dispersant effectiveness from 21 to 70% when mixing speed increased from 150 to 250 rpm. Mixing speed in the present study was approximately 1200 rpm, in order to produce a vortex 20-25% of the water depth following Singer et al. (2000) recommendations. The dispersant to oil ratio chosen in the present study was also high (1:10 dispersant:oil; Hemmer et al., 2010) which should increase dispersion (Fiocco and Lewis, 1999). Dispersant effectiveness also varied depending on the physical (viscosity, density, surface tension, pour point) and chemical composition of the oil and weathering of the oil (Chandrasekar et al., 2005). However, the most probable rationalization for the low concentrations in our exposure solutions would be the coalescence of oil droplets during the 1h settling period. The small surface area of the 500 mL baffled flask and the large amount of oil, 20 mL, compared to 100 uL in a 150 mL flask in other studies (Chandrasekar et al., 2005), would facilitate the coalescing of oil droplets, therefore removing them from our CEWAF stock solutions (personal communication, T King, Bedford Institute of Oceanography, Dartmouth, NS, Canada).

Environmental Implications

One of the beneficial outcomes of the low concentrations tested in our study was the ability to observe that measured concentration as low as 0.38 ug/L (1% v/v MESA WAF) produced 100% abnormally developed larvae and a 100-fold reduction in this concentration (0.01% v/v MESA WAF) which was below detection levels of GC-MS (0.065-0.189 ug/L), caused 83% of embryos to develop abnormally (Tables 5, 8). These results suggest chronic toxicity to hydrocarbons for herring embryos at concentrations lower than reported previously. Carls et al. (1999, 2002) demonstrated that exposing herring embryos to 0.4-0.7 ug/L PAH would result in edema and reduced growth, while the present study illustrates significantly reduced survival and normal development at concentrations potentially two orders of magnitude lower. Exposure concentrations measured by GC-MS were lower than those reported by other studies (McIntosh et al. 2010).

Chronic embryonic exposures can be criticized for overestimating the toxicity of oil spills because once dispersed, toxic oil concentrations are not likely to last for the entire embryonic period, i.e. 13-14 days at 10ºC in herring, and may be present for only a few hours (McIntosh et al., 2010). However, directly following the Exxon Valdez spill, PAH concentrations were measured at up to 6.24 ug/L and concentrations of up to 1.59 ug/L persisted for at least five weeks near heavily oiled beaches (Short and Harris, 1996). During the Deepwater Horizon oil spill, which lasted for approximately three months, PAH concentrations measured from 24 May 2010 to 6 June 2010 ranged from 0.01 to 59 ug/L, while TPH concentrations were measured from 2 to 422 ug/L (Wade et al., 2011). More specifically, Diercks et al. (2010) reported that total PAH concentrations were 189 ug/L at 1320 metres and 29 ug/L at 1160 metres near the wellhead site. Therefore, chronic exposures to low level PAHs in the present study may reflect environmental conditions following major oil spills. These chronic exposures to low PAH levels would be considered realistic for early life stages with limited or no motility and, as shown in the present study, are able to reduce embryonic growth and alter normal development in herring.

CONCLUSION

The results of the present study show that rearing temperature was the more influential of the two environmental factors tested. However, the increased toxicity at a lower temperature didn't appear to be the result of dispersant effectiveness or PAH solubility as expected but to natural adaptation to warmer temperature by the herring stock. In addition to cold stress, the increased toxicity may have resulted from a slower developmental rate, hence considerably longer exposure duration, and to reduced metabolic processes. Our results suggest that lower salinities may increase oil toxicity. However, the lack of consistency between WAF and CEWAF results and also with the positive control retene suggest the need for further experiments. Results of the present study corroborate those of Greer (2011) which showed that Atlantic herring were more sensitive to oil toxicity than Pacific herring. Stocks within each species of herring also showed differences in sensitivity to oil exposure, with the Pacific herring from Alaska being more sensitive than those from British Columbia. However, elevated BSD SI in the control group of the fall spawning stock of Atlantic herring limited our assessment of the sensitivity of the

Atlantic herring spawning stocks. MESA appeared to be the most toxic of the three oils tested to Pacific herring embryos. Results of the present study suggest chronic toxicity of hydrocarbon exposure for herring embryos at concentrations lower than reported previously. Therefore, mitigation measures in the event of an oil spill need to be conservative in their assessment of potentially harmful concentrations of hydrocarbons since the present study demonstrates high sensitivity, and a substantial range of sensitivity, to hydrocarbons among species and stocks of herring. Mitigation measures also need to consider the influence of environmental factors, especially temperature, on hydrocarbon toxicity.

Additional details of this work can be found in Sarah Johnson's M.Sc. thesis which accompanies this report (Appendix D).

WAVE TANK FACILITY

WAVE TANK

Figure 10 shows a photograph and schematic diagram of the wave tank used in this research. The tank measures 32 m long, 0.6 m wide, and 2 m high, with an average water depth of 1.5 m. Different waves types are generated by a computer-controlled flap-type wave maker situated at one end of the tank. The wave maker is linked to an adjustable cam that controls stroke length to alter wave-height characteristics, and wave frequency is controlled by the rotation speed of the cam. The wave tank can produce both regular non-breaking waves and breaking waves, with their mixing energy levels quantified in terms of the energy dissipation rate per unit mass of water (ε). An autocorrelation function (Boufadel et al., 2008; Wickley-Olsen et al., 2008; and Venosa et al.,2008) applied to time-series velocity measurements obtained by an Acoustic Doppler Velocimeter (SonTec/YSI, Inc. San Diego, CA) is used to evaluate energy dissipation rate.

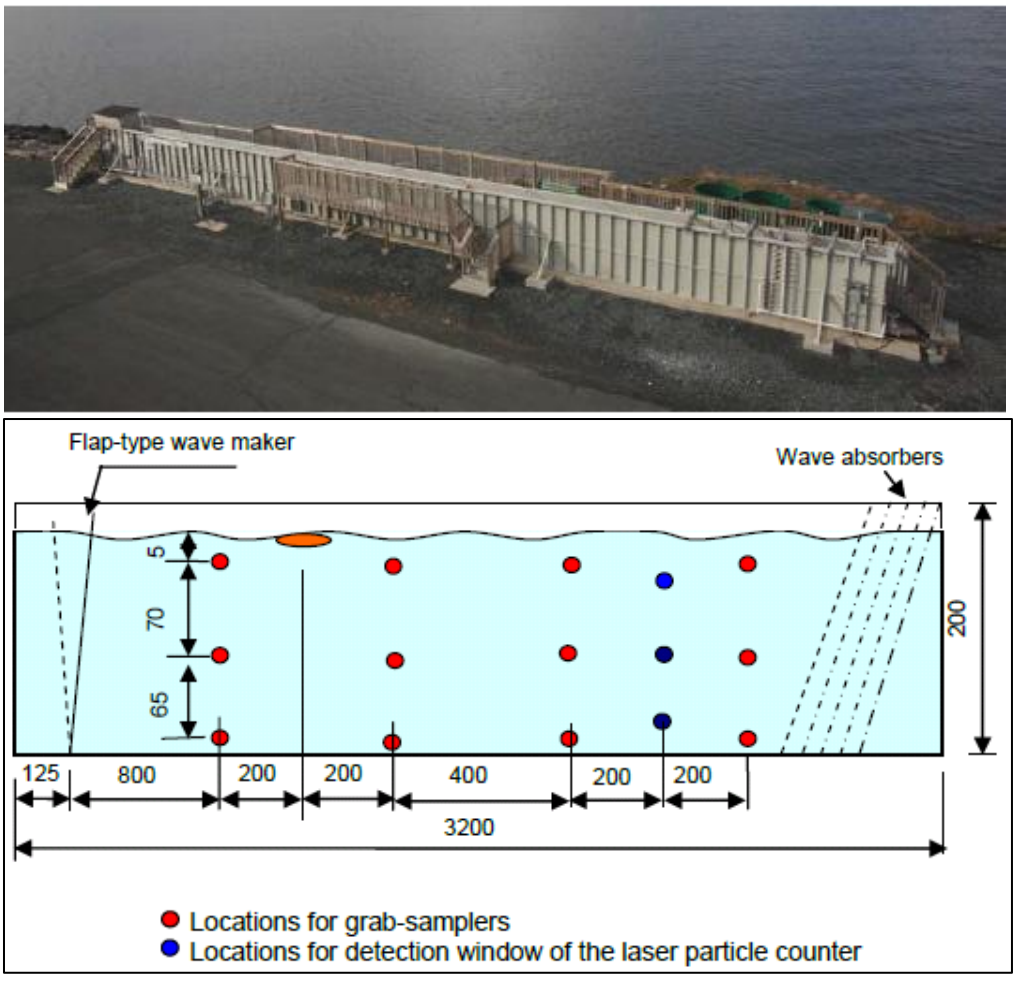


Figure 10. Photograph (upper panel) and schematic representation (lower panel, all dimensions in cm, not to scale) of the wave tank facility at the Bedford Institute of Oceanography

Prior to each dispersion experiment, wave hydrodynamic conditions were characterized based on past research by Li et al. (2008). Regular non-breaking waves were generated with the flap stroke set at 12 cm to produce a constant frequency wave of 0.80 Hz, a wave length of 2.44 m and a wave height of approximately 23 cm. Low energy spilling breakers were created with an 8.4 cm flap stroke by generating alternating trains of high-frequency waves (0.85 Hz, wave length 2.16 m, wave height 18 cm, and duration of 20 s) and low-frequency waves (0.48 Hz, wave length 6.77 m, wave height 6 cm, and duration of 5 s). Plunging breaking waves were produced with a 12 cm flap stroke and alternating trains of high-frequency waves (0.85 Hz, wave length 2.16 m, wave height 26 cm, and duration of 20 s) and low-frequency waves (0.5 Hz, wave length 6.24 m, wave height 9 cm, and duration of 5 s).

GENERATION OF WAVE TANK DISPERSED WAF AND CEWAF

In order to produce wave tank generated WAF and CEWAF, fresh seawater is pumped from the Bedford Basin through a double layer sock-filter (Atlantic Purification Ltd, Dartmouth, NS, Canada) with a pore size of 25 and 5 μm for the coarse and fine filters, respectively. Background temperature, salinity, and particle size distribution are recorded before each experiment. To start an experiment, 300 ml of crude oil is gently poured onto the water surface within a 40 cm (inner diameter) ring (constructed of NSF-51 reinforced clear PVC tube) located 10 m from the wave-maker. Immediately after oil addition, 12 ml of dispersant (or water for the control) are sprayed on to the top of the oil through a pressurized nozzle (60 psi; 0.635 mm i.d.) resulting in a dispersant-to-oil ratio (DOR) of 1:25. The ring is then lifted immediately prior to the arrival of the first wave, and waves are generated continually for the duration of the mixing phase. Samples are collected using four sets of 100 ml syringes connected to a stainless steel manifold. Following collection, samples are extracted in dichloromethane (DCM) according to Cole et al., 2007 (liquid-liquid partitioning) and analyzed for total petroleum hydrocarbons (TPH) with a Genesys 20 ultraviolet spectrophotometer (Thermo Fisher Scientific; Calgary Canada).

Data collected during previous wave tank studies shows the mixing properties of the wave tank during WAF and CEWAF generation (Li et al., 2009). During these studies both MESA and ANS oils were used and water samples were collected over a 120 min period. Samples were collected at four horizontal locations (8, 12, 16, and 20 m downstream from the wave maker), three depths (5, 75, and 140 cm from the average water surface), and various time points (e.g., 5, 30, 60, 120 min after initial mixing) during the run; the results of this sampling can be found in Table 10. Wave tank oil concentration contours, using MESA oil, during one of these runs can be seen in Figures 11 and 12. The ANS oil distributions in the wave tank (data not shown) were similar to those of MESA oil. Under plunging breaking waves, spreading of the oil plume was pronounced compared to the non-breaking and spilling breaking waves (data not shown). In the no-dispersant control condition, spreading at the surface was higher, as shown by the reduced net drifting of oil at the surface (Figure 11). In the presence of chemical dispersants, the oil plume appeared virtually homogenized in the wave tank at all depths due to the combined effect of more vigorous turbulent diffusion created by the plunging breaking waves and the presence of chemical dispersants (Figure 12).

Table 10. Average water column dispersed oil concentrations (mg/l) as a function of time. Data reported as the average ± one standard deviation of independent triplicate runs.

Oil	Wave	Dispersant	5 min	30 min	60 min	120 min
MESA	Regular	Water	0.31 ± 0.02	0.44 ± 0.26	0.86 ± 0.65	1.05 ± 0.73
		Corexit	0.39 ± 0.28	3.39 ± 2.74	3.89 ± 1.85	4.05 ± 1.40
		SPC	0.69 ± 0.79	1.36 ± 1.09	2.00 ± 1.54	2.09 ± 1.51
	Spilling	Water	0.31 ± 0.09	0.39 ± 0.14	0.42 ± 0.22	0.71 ± 0.22
		Corexit	1.46 ± 1.57	5.04 ± 1.05	5.19 ± 1.46	4.79 ± 1.46
		SPC	2.40 ± 2.39	2.84 ± 2.71	3.61 ± 2.96	3.64 ± 2.83
	Plunging	Water	0.77 ± 0.27	1.55 ± 0.58	1.66 ± 0.43	2.07 ± 0.46
		Corexit	4.31 ± 0.95	7.09 ± 0.85	6.50 ± 0.29	5.43 ± 0.15
		SPC	3.39 ± 1.85	4.65 ± 1.62	4.31 ± 1.29	4.40 ± 0.70
ANS	Regular	Water	0.67 ± 0.77	0.81 ± 0.57	1.19 ± 0.57	1.37 ± 0.69
		Corexit	2.26 ± 2.17	3.32 ± 2.61	3.68 ± 1.03	3.93 ± 0.75
		SPC	1.49 ± 1.06	2.12 ± 1.60	2.21 ± 1.71	2.59 ± 1.93
	Spilling	Water	0.25 ± 0.08	0.25 ± 0.09	0.47 ± 0.47	0.55 ± 0.33
		Corexit	4.77 ± 1.00	8.58 ± 0.65	8.21 ± 1.05	6.55 ± 0.34
		SPC	1.97 ± 2.14	3.14 ± 3.64	2.92 ± 2.86	2.97 ± 2.21
	Plunging	Water	0.28 ± 0.15	1.01 ± 0.61	1.39 ± 0.65	1.61 ± 0.36
		Corexit	4.09 ± 3.65	8.37 ± 1.61	7.73 ± 0.75	6.91 ± 0.50
		SPC	2.91 ± 1.04	5.89 ± 3.06	5.69 ± 1.92	5.75 ± 1.06

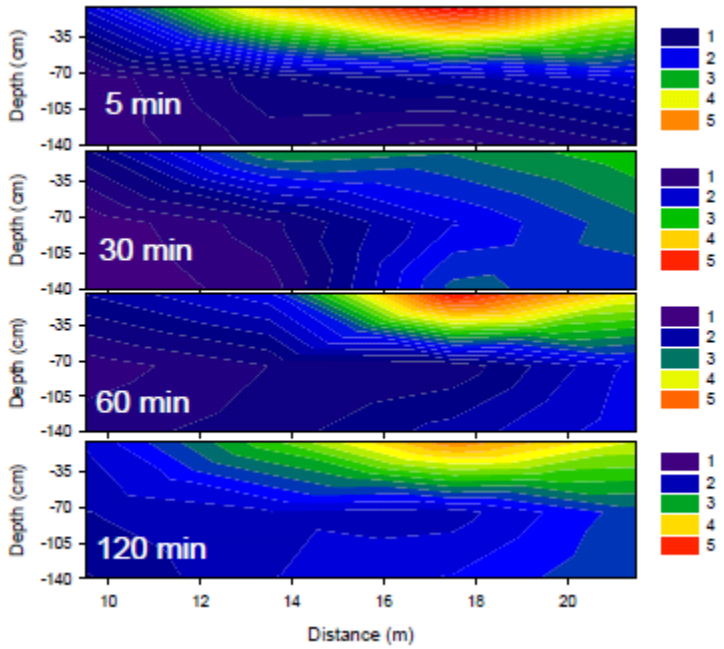


Figure 11. Dispersed MESA oil concentration ($mg \cdot L^{-1}$) as a function of time and space under plunging breaking waves with no dispersant (WAF).

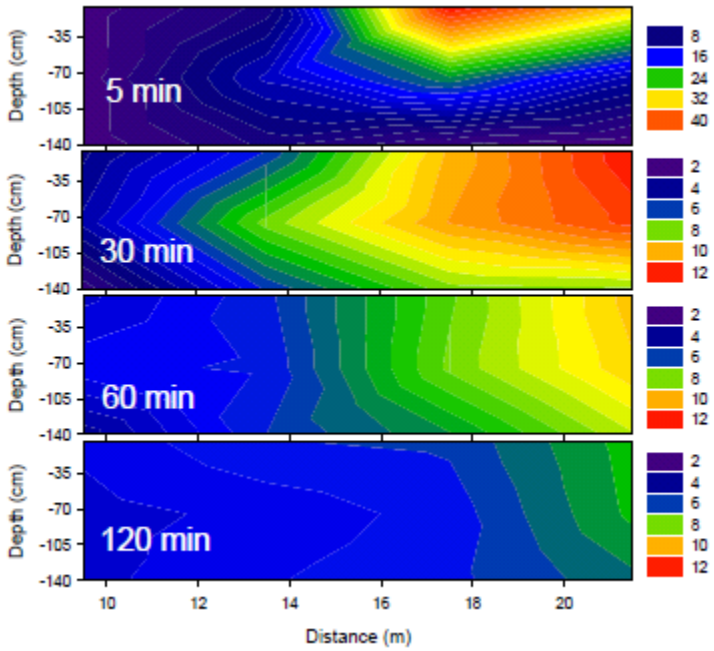


Figure 12. Dispersed MESA oil concentration ($mg \cdot L^{-1}$) as a function of time and space under plunging breaking waves with Corexit 9500 (CEWAF).

The effect of breaking waves on the oil distribution (in particular the penetration depth of dispersed oil) is related to a number of contributing factors. As waves break, it is estimated that 30 to 50% of the dissipated wave energy entrains oil droplets into the water column (Lamarre and Melville 1991; Tkalich and Chan 2002), and effectively determines the first-order oil entrainment rate (Tkalich and Chan 2002). Breaking waves develop a mixing layer in the upper water column, and the penetration of oil results in a uniform mixing of the droplets, with the mixing layer proportional to the height of breaking waves (Delvigne and Sweeney 1988; Tkalich and Chan 2002). Moreover, breaking waves generate micro-scale turbulence with the smallest eddies having the greatest velocity gradients, leading to deformation, elongation, and eventual breakup of larger droplets, forming a large number of small droplets that have lower buoyancy and more rapid diffusion efficiency (Delvigne et al. 1987; Li and Garrett 1998).

WAVE TANK HERRING STUDIES

MATERIALS AND METHODS

The toxicity of chemically dispersed crude oil to herring embryos

In order to assess whether solutions of chemically dispersed oil produced in the lab are suitable for predicting dispersed oil toxicity in the field, we ran wave tank experiments at the Canadian Offshore Oil and gas energy research labs at the Bedford Institute of Oceanography (COOGER). A fixed volume of Alaska North Slope Crude (ANSC, viscosity of 17.5cP), or Arabian Light Crude (ALC; viscosity of 15.5cP) were dispersed in the wave tank with Corexit 9500A and sufficient agitation to create breaking waves; preliminary lab tests were made with Medium South American crude (MESA; viscosity of 26.1cP). Solutions were sampled from 12 sites at 5 to 60 min post dispersion and hydrocarbon concentrations were measured by fluorescence against a standard curve of crude oil, and by GC/FID. The test solutions were diluted 50:50 with freshwater to achieve 15 ppt salinity, the optimum for development of Atlantic herring embryos (McIntosh, 2009). The toxicity of dispersed oil was compared to equivalent solutions of chemically-enhanced water accommodated fractions (CEWAF) prepared in the lab following a standardized CROSERF method (Singer et al, 2000). The toxicity test exposed herring embryos to wave-tank CEWAF samples for 24 h immediately after fertilization, followed by 14 d in fresh water. At the end of the exposure, the prevalence of signs of blue sac disease (BSD) a sign of oil toxicity, were compared among solutions.

An exposure time-response experiment exposed herring embryos to lab-prepared and wave tank-prepared CEWAF for 2.4, 8, 24, and 336 h (14 d) to determine whether brief exposures were toxic. The toxicity of lab-prepared CEWAF was also compared between Atlantic herring and Pacific herring obtained from Fisheries and Oceans, Nanaimo, BC, to determine if data from one species of herring could be used to assess the risks to another.

RESULTS AND DISCUSSION

The toxicity of chemically dispersed crude oil to herring embryos

Fluorescence measures of oil dispersed in water provided a precise measure of the relative amounts of petroleum hydrocarbons in test solutions. There was a linear relationship among measured concentrations of fluorescing petroleum hydrocarbons and nominal loadings of oil (% v/v) for ANSC and ALC for both water-accommodated fraction (WAF) and CEWAF (Figure 13). All lines were parallel ($P = 0.90$) and shared a slope of 0.89. Chemical dispersion of crude oil (CEWAF) caused a 100-fold increase in concentrations of fluorescing hydrocarbons in water compared to un-dispersed oil (WAF; $P < 0.0001$), and ALC had more soluble fluorescent hydrocarbons than ANSC in both CEWAF ($P < 0.0001$) and WAF ($P = 0.0053$). Hatched embryos exposed to crude oil exhibited exposure-dependent signs of toxicity, including yolk sac edema, pericardial edema, spinal curvature (Figure 14). While the differences in LC50 and $EC50_{\%Hatch}$ values between ANSC and ALC were non-significant, ANSC was ~ two times more

toxic than ALC based on the $EC50_{\%Normal}$ and $EC50_{SI}$ (P = 0.04 and 0.003, respectively; Figure 15). Oil toxicity to early life stages of fish is likely caused by PAHs (Hodson et al. 2007), so that toxicity should increase with the concentrations of soluble PAHs (Di Toro et al. 2007). Assuming that fluorescence reflects the concentrations of PAHs, the toxicity of the two oils should have been the same for the same intensity of fluorescence. The higher toxicity of ANSC may be explained by the nature of the PAH in each oil. ANSC has two times more total phenanthrene concentrations, the alkyl PAH thought to be the most toxic to early life stages of fish (Barron et al. 2004; Hodson et al. 2007). For ALC, the higher fluorescence coupled with lower toxicity may reflect the predominance of low molecular weight PAH (i.e., naphthalenes, thiophenes) and C-1 to C-2 alkylPAH over C3-C4. The lower toxicity of ALC will also reflect the greater proportion of hydrocarbons that would evaporate from test solutions over a 24-h period.

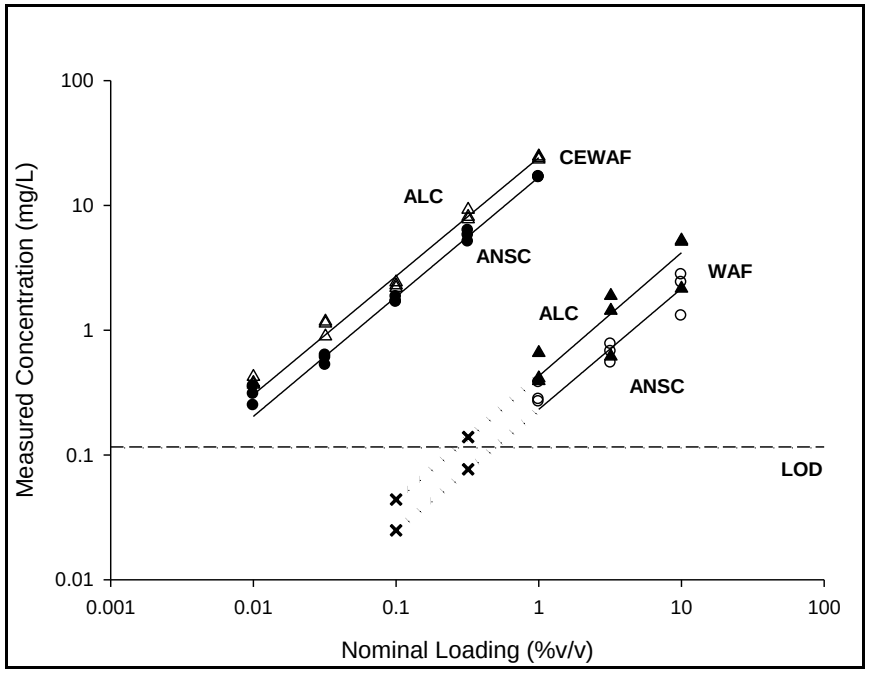


Figure 13. The concentration of petroleum hydrocarbons in test solutions at different loadings of the water accommodated fraction (WAF) and the chemically-enhanced water accommodated fraction (CEWAF). Open triangles show the Arabian Light Crude (ALC) CEWAF, closed triangles represent the ALC WAF, closed circles show the Alaska North Slope Crude (ANSC) CEWAF, and open circles represent the ANSC WAF. The x's show the extrapolated concentration from the line of best fit below the limit of detection. Figure taken from Greer, 2011.

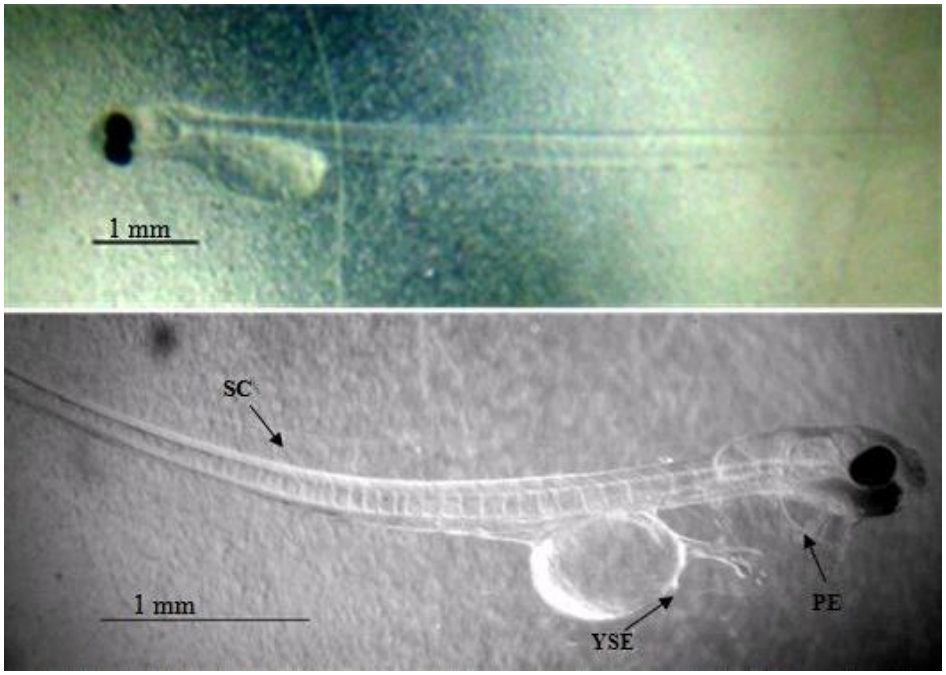


Figure 14. Hatched Atlantic herring embryos. The top panel represents a hatched embryo showing no signs of toxicity ("normal"). The lower panel depicts a hatched embryo that was exposed to 2.7 m/L of Arabian light crude (ALC) chemically-enhanced water accommodated fraction (CEWAF). The lower embryo has yolk sac edema (YSE), pericardial edema (PE), and a spinal curvature (SC). Figure taken from Greer, 2011.

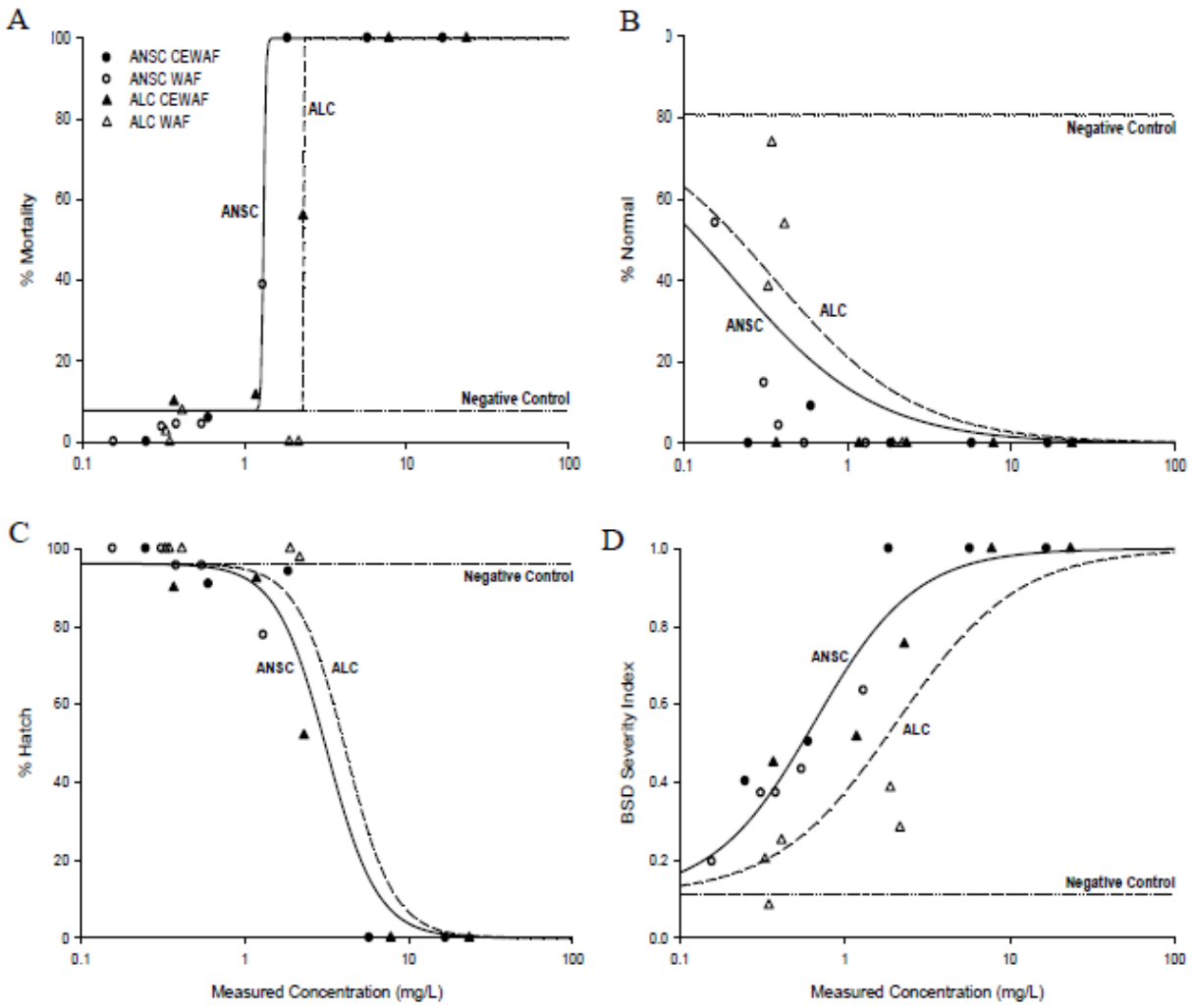


Figure 15. Toxicity of the water accommodated fraction (WAF) and chemically-enhanced water accommodated fraction (CEWAF) of Alaska North Slope Crude (ANSC) and Arabian Light Crude (ALC) oils to Atlantic herring embryos expressed as (a) % Mortality, (b) % Normal, (c) % Hatch, and (d) BSD Severity Index in relation to the measured concentration of fluorescing compounds (mg/L). Figure taken from Greer, 2011.

The toxicity of ANSC and ALC CEWAF solutions increased progressively with exposure time (Figure 16), based on % Normal ($P < 0.0001$). When EC50s for both oils were compared to exposure time, there was a linear decrease (increase in toxicity) with increasing time (log EC50 (mg/L) = 1.491-0.8847 log time; $r = 0.93$). The predicted EC50 at 2.4 h, the shortest exposure time, was about 14 mg/L, slightly greater than observed (10.7 mg/L), and typical of many oil spills. Thus, even brief exposures of newly-fertilized herring eggs to waterborne petroleum hydrocarbons will have significant impacts on recruitment.

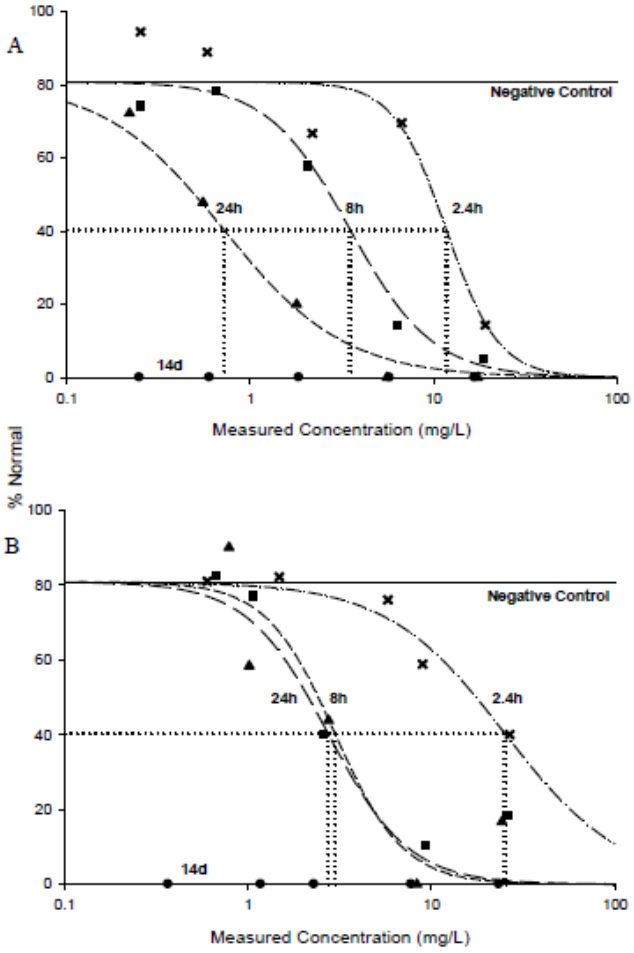


Figure 16. The effect of exposure time and petroleum hydrocarbon concentration on percentage of normal embryos at hatch exposed to Alaska North Slope Crude (ANSC; A) and Arabian Light Crude (ALC; B). Percent normal indicates percentage of embryos alive and showing no signs of toxicity at hatch. Embryos were exposed immediately following fertilization for 2.4 h, 8 h, 24 h or 14 d (static daily renewal). Figure taken from Greer, 2011.

For wave tank experiments, samples of dispersed ALC were non-toxic to herring embryos in 24 h exposures (Figure 17), and below the concentrations predicted to be toxic in 24 h exposures to lab-prepared CEWAF. For ANSC, CEWAF samples drawn from the tank 15 minutes post-dispersion were significantly more toxic than controls (P=0.025), and somewhat more toxic than lab-prepared CEWAF (reference curve in Figure 17A). The greater toxicity of the 15 minute samples compared to the reference curve and the 30-minute samples may be a function of the rate of partitioning and subsequent dilution of the acutely lethal components of oil (e.g., BTEX) from the suspended droplets created by chemical dispersion. The results for solutions of ALC (low toxicity) and ANSC (greater toxicity) from the wave tank are consistent with results from lab-prepared CEWAF. Thus, lab-derived solutions provide a reasonable basis for assessing the risks of spilled and dispersed oil.

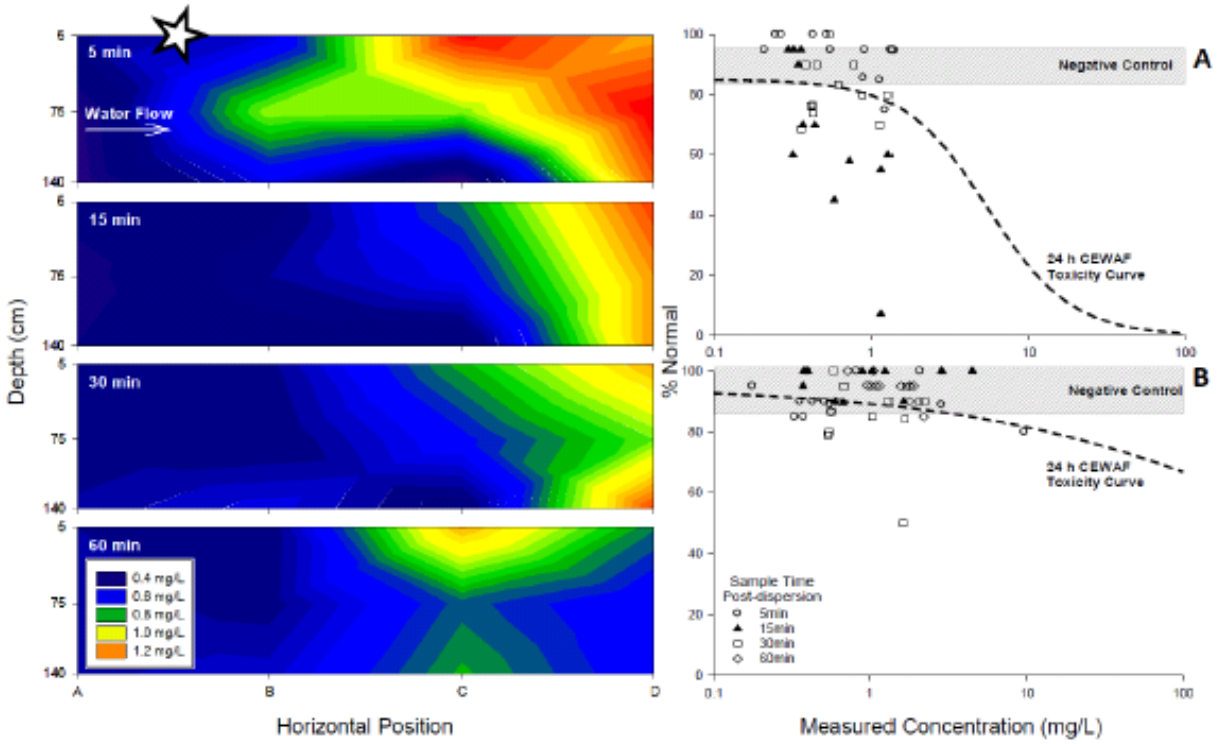


Figure 17. Wave tank data showing the movement of fluorescing hydrocarbons in Alaska North Slope Crude oil (ANSC) through the tank over time (left panel), and the change in percent normal with increasing concentration of the chemically-enhanced water accommodated fraction (CEWAF) of ANSC (A) and Arabian Light Crude oil (ALC; B). Left panel: Times represent sampling time after addition of Corexit 9500A to ANSC. The white star represents the oil spill site. Right panel: The effects on herring of a 24 h exposure to laboratory-prepared CEWAF has been superimposed onto the graphs for comparison (dotted lines). The measured concentrations represent samples drawn from bioassay tanks. Figure taken from Greer, 2011.

Comparisons between Atlantic and Pacific herring exposed under the same conditions to chemically-dispersed ANSC oil demonstrated no difference in 14-d LC50s or EC50s for percent hatch (data not shown). However, the prevalence of sub-lethal signs of toxicity varied between the oils. There was a 4-fold greater sensitivity of Atlantic herring ($P < 0.05$) when toxicity was expressed as the EC50 for the percentage of fish that were normal after 14 days (Figure 18) or the severity of blue sac disease (data not shown). The difference between the two species may be an artifact of variability among females and egg quality, an important issue when working with Atlantic herring taken from commercial fisheries. Thus, the toxicological significance of this difference is likely small, which supports the idea of using data interchangeably between the two species for risk assessments. In future, capturing live adults and holding them in seawater until they ripen would increase the rate of successful fertilization (J. Rice, NOAA, Juneau AK, personal communication). When Pacific herring were exposed to CEWAF of MESA crude oil, MESA appeared 2-fold more toxic than ANSC, but the difference was not statistically significant. We have also found no difference between the toxicities to trout embryos of chemically-dispersed ANSC and MESA oils, consistent with their similar concentrations of alkyl PAH (unpublished data).

Additional detail on these experiments can be found in the paper (Appendix E) and M.Sc. thesis (Appendix F) written by Colleen Greer.

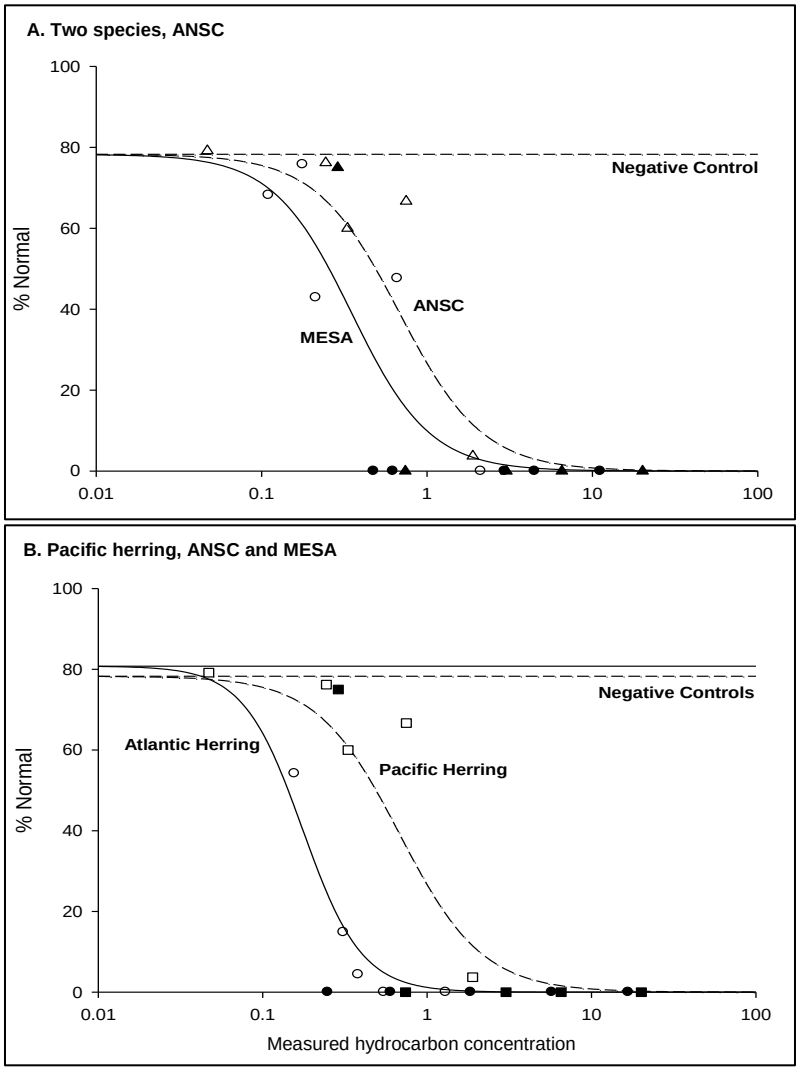


Figure 18. Toxicity of the water accommodated fraction (WAF) and chemically-enhanced water accommodated fraction (CEWAF) of crude oil to Atlantic and Pacific herring embryos, expressed as the percentage that were normal at the end of the experiment. Panel A. Pacific herring exposed to both ANSC and Medium South American Crude (MESA) oils. Panel B. Both species exposed to Alaska North Slope Crude (ANSC) oil. The concentration of hydrocarbons compounds were measured by fluorescence against a standard curve of the oil samples.

SALMON AND COD STUDIES

Water temperature influences - cod sub-lethal dose response tests

Juvenille Atlantic cod (*Gadus morhua*) were exposed to either water accommodated fraction (WAF) or chemically enhanced water accommodated fraction (CEWAF) of Mediterranean South American (MESA) crude oil and Corexit 9500 dispersant at three different water temperatures (2, 7 and 10°C). Two concentrations (0.2 and 1% v/v) of WAF and CEWAF were tested at these temperatures, exposures were conducted in duplicate.

Results showed that EROD induction was dependent on exposure concentration and water temperature. Induction was higher in fish exposed to CEWAF compared to WAF. EROD activity was higher after 24 and 48 h exposure at 7 and 10°C compared to 2°C suggesting that the lower temperature resulted in lowered metabolism in the fish thus lowering the rate of EROD induction. This may be the case since at 72 h post start of exposure (2°C), the EROD activity was still increasing whereas for the higher temperatures (7 and 10°C) it had started to decline.

This work has been published and is presented in Appendix G.

Sub-lethal dose response studies using Atlantic cod and salmon

To justify the comparability of the dispersed oil in the baffled flasks and in the wave tank (similar to those observed in the field), a series of experiments were conducted to compare the dispersed droplet size distribution in the laboratory baffled flasks with the oil droplets that have been measured in the BIO wave tank facility. The in-situ dispersed oil droplet size distribution and the total volume dispersed oil concentration were recorded with the laser in-situ scattering and transmissiometry (LISST-100X). The characterization results indicated that the droplet size distributions of the chemically dispersed crude oil in the baffled flasks at 200 rpm for more than 30 min were consistent with droplets generated in the wave tank under plunging breaking wave conditions (data not shown). This means the generation of dispersed oil solutions could be done in the laboratory facilities for a large number of experiments.

A study was conducted to determine the sub-lethal effects in Atlantic cod and salmon (*Salmo salar*) held at 12 ± 2°C following a 4h exposure to various combinations of WAF and CEWAF. Exposures to MESA oil and Corexit 9500 were done using oil dispersed in the wave tank, while the remainder of the exposures used laboratory generated WAF and CEWAF. Results showed that salmon exposed to WAFs (4.7 to 75% v/v) and CEWAFs (0.5 to 64% v/v) showed significant levels of EROD induction compared to the controls. Significant levels of induction occurred at >18.75% v/v for WAF and 2% v/v for CEWAF. Maximum levels were achieved at 24h post start of exposure then declined, with levels still above background by 72h post start of exposure. Cod were then exposed to the same combinations of WAF and CEWAF. No induction was seen in the WAF exposed fish but maximum EROD induction was seen at 48h post start of exposure then declining by 72h with levels still higher than controls in the CEWAF exposed fish.

The difference in maximum EROD time points suggests that cod can metabolise and excrete PAHs faster than Atlantic salmon at the temperature range investigated.

A paper detailing this research is in progress, a draft copy of which is presented in Appendix H.

Cod and salmon 24h lethality tests

To determine toxicity of dispersants and dispersed oil, lethality tests were conducted at the St. Andrews Biological Station (SABS) using cod and salmon. Tests were conducted using Corexit and SPC-1000 dispersants alone, WAF of ANS and MESA oils and CEWAF of oil and dispersants. Dispersed oil (WAF) was prepared by mixing oil and water in baffled flasks and for CEWAF, dispersant was added to the mix. Standard procedures were followed for the mixing.

SPC1000 dispersant was determined to be 10 times more toxic to cod than Corexit 9500 (9.26 ppm and 94.78 ppm respectively). We also ran a range-finding lethality test with 50% WAF (MESA oil and seawater). One fish out of five died in this test. When exposed to various combinations of CEWAF, the LC_{50} was similar for ANS/Corexit, ANS/SPC-1000 and Mesa/Corexit (in the range 6.12 to 7.86% v/v) whereas Mesa/SPC-1000 was determined to be approximately twice as toxic at 3.01% v/v.

Results are detailed in Table 12.

Cod early life stage effect tests

Fertilisation and hatching success

Cod eggs were fertilized in a range of concentrations of WAF, CEWAF and oil dispersant. Survival, fertilization success, cell division and hatching success were all monitored. Newly hatched cod larvae (up to 3 days post-hatch) were exposed to a range of concentrations of WAF, CEWAF or dispersant. WAF was prepared using one part oil to nine parts water with dispersant added as one part to 25 parts oil for CEWAF. Solutions of dispersant alone were prepared by adding dispersant to sea water (no crude oils used) in the same ratios as for CEWAF.

The following procedures were carried out in a cold room environment at *ca* 6°C. Alaskan North Slope (ANS) or Mesa crudes were used to prepare WAF. CEWAF was prepared using the same oils along with Corexit 9500 and SPC-1000 dispersants. Eggs and milt were mixed in the presence of either WAF, CEWAF or dispersant at two concentration ranges, 0.5-25% and 0.01-0.5%, followed by "hardening" for 24 h in WAF or CEWAF. The eggs were then rinsed, transferred to clean seawater, and fertilisation success assessed. Viable fertilised eggs were transferred to multi-well plates containing untreated seawater and monitored to assess hatching rates. Hatching success and rates were compared against control eggs.

Results indicated that fertilisation in the presence of WAF had no effect on fertilisation success or hatching success except for Mesa at 25% v/v. Fertilisation success of eggs exposed to each CEWAF was similar over the concentrations used except at 0.5% v/v when there was lower fertilisation overall. The same was true for eggs fertilised in dispersant alone. This may suggest that the toxicity of the chemically dispersed oils is related to the dispersant rather then the oil itself since the fertilisation success of the CEWAFs and dispersants alone follow similar profiles. This remains speculation as we have not tested this experimentally. Hatching success of viable fertilised eggs was not affected except in SPC-1000 dispersant, ANS/SPC-1000 and Mesa/SPC-1000 at 0.5% v/v.

Exposure dilutions and determined total polycyclic aromatic hydrocarbons (PAHs) in the respective dilutions are presented in Table 11.

Larval lethality

Eggs and milt from single parent Atlantic cod were mixed and placed in 50 L incubator pots. Larvae hatched approximately 17 days post fertilisation at water temperatures of *ca* 6°C. Larvae, less than 3 days old, were used for the 24 h lethality studies. ANS and Mesa crude oils and Corexit and SPC-1000 dispersants were used to prepare the test solutions.

Larvae were exposed to ANS and Mesa WAFs in the range 1.23 to 100% v/v with no LC_{50} calculable for MESA WAF (even at 100% v/v), whereas ANS WAF had a LC_{50} of 42.6% v/v. For ANS/Corexit (0.37 to 30% v/v) and ANS/SPC-1000 (0.04 to 3.33% v/v) CEWAFs, calculated LC_{50} were 0.89% v/v and 0.67% v/v respectively. For Mesa/Corexit (0.123 to 10% v/v) and Mesa/SPC-1000 (0.04 to 3.33% v/v) CEWAFs, calculated LC_{50} were 1.92% v/v and 0.62% v/v respectively. For dispersants alone, larvae were exposed to Corexit and SPC-1000 in the range 2.2 to 180% v/v resulting in LC_{50} at 68.5% v/v and 29.6% v/v respectively. Lethality of the prepared solutions showed that SPC-1000 dispersant is more toxic to newly hatched larvae compared to Corexit. Mesa WAF was not lethal to larvae, even at 100% exposure concentration whereas ANS WAF had an LC_{50} at 42.6% v/v. The CEWAF combinations of ANS, Mesa oils and Corexit, SPC-1000 dispersants showed similar LC_{50} with Mesa/Corexit being slightly less toxic than the other 3 combinations.

Lethality data presented as exposure dilutions (% v/v) and determined total polycyclic aromatic hydrocarbons (PAHs) in those dilutions are presented in Table 12.

Table 11. Fertilisation and hatching success of cod eggs

Test material	Fertilisation exposure concentrations	Fertilisation success	Hatching success
Corexit dispersant	0.436, 2.182, 4.364, 21.818 ppm*	114.7, 93.0, 90.7, 45.0%	77.8, 76.4, 82.4, 76.0%
SPC-1000 dispersant	*equivalent concentrations to those in CEWAF	76.7, 76.1, 50.6, 32.3%	88.2, 87.5, 74.7, 59.1%
WAF of weathered Mesa oil	0.701, 1.423, 7.812, 14.265 µg/mL[#] (0.5, 2.5, 12.5, 25% v/v)	75.1, 67.9, 71.9, 43.5%	55.6, 62.5, 65.3, 66.0%
WAF of weathered ANS oil	0.397, 0.696, 3.684, 7.619 µg/mL[#] (0.5, 2.5, 12.5, 25% v/v)	99.5, 82.0, 90.5, 83.3%	79.2, 81.3, 82.0, 81.2%
CEWAF of weathered ANS/Corexit 9500	0.589, 1.945, 3.428, 15.708 µg/mL[#] (0.01, 0.05, 0.1, 0.5% v/v)	64.6, 63.7, 57.0, 34.7%	83.0, 85.5, 81.9, 79.6%
CEWAF of weathered ANS/SPC-1000	0.304, 1.519, 4.219, 14.876 µg/mL[#] (0.01, 0.05, 0.1, 0.5% v/v)	73.2, 68.0, 70.2, 43.4%	82.7, 87.3, 78.5, 57.2%
CEWAF of weathered MESA oil/ Corexit 9500	0.492, 2.073, 3.328, 20.015 µg/mL[#] (0.01, 0.05, 0.1, 0.5% v/v)	158.7, 130.2, 131.7, 94.4%	78.5, 81.6, 83.3, 78.1%
CEWAF of weathered MESA oil/ SPC-1000	0.451, 0.633, 0.987, 3.956 µg/mL[#] (0.01, 0.05, 0.1, 0.5% v/v)	83.0, 85.2, 72.3, 55.2%	82.6, 75.0, 71.5, 48.8%

[#]: Determined total PAH concentration by GC-FID
(% v/v): Nominal exposure dilution

Table 12. Lethality of oil and oil dispersants to larval cod, juvenile cod and salmon smolts

Test material	LC_{50} (Exposure temperature range)		
	Larval cod (Temp. = *ca* 6°C)	Juvenile cod (Temp. = 10 to 13.6°C)	Salmon smolt (Temp. = 8.8 to 11.4°C)
Corexit dispersant	68.5ppm	94.78 ppm	61.56 ppm
SPC-1000 dispersant	29.6 ppm	9.26 ppm	Not conducted
WAF of weathered Mesa oil	Not calculable, no mortalities at 63.321 µg/mL[#] (100% v/v)	One mortality at 50% v/v	Not conducted
WAF of weathered ANS oil	24.27 µg/mL[#] (42.6% v/v)	Not conducted	No mortalities at 50% v/v
CEWAF of weathered ANS oil/Corexit 9500	39.3 µg/mL[#] (0.89% v/v)	6.41% v/v	Not conducted
CEWAF of weathered ANS oil/SPC-1000	59.06 µg/mL[#] (0.67% v/v)	7.86% v/v	Not conducted
CEWAF of weathered MESA oil/ Corexit 9500	50.06 µg/mL[#] (1.92% v/v)	6.12% v/v	6.05% v/v
CEWAF of weathered MESA oil/ SPC-1000	40.19 µg/mL[#] (0.62% v/v)	3.01% v/v	Not conducted

[#]: Determined total PAH concentration by GC-FID
% v/v: Calculated LC_{50} exposure dilution

RESULTS AND DISCUSSION

A number of chemical and environmental factors, temperature, salinity and altered composition of weathered oil, for example make assessing risk on the toxicity effects of dispersed Atlantic North Slope oil on fish complicated. The waters of Prince William Sound can range in temperature from 3°C in winter to 10°C in summer with varying salinity depending on freshwater runoff. The near shore habitats in the subarctic waters of the Gulf of Alaska may serve as natal and rearing grounds for many commercially important marine fishes, life stages that are very susceptible to long term damage from oil (Moles et al, 2001).

Early life stages vs. juvenile cod

Current risk assessments are performed mainly on adult organisms. If experiments indicate that early life stages are predominately the most sensitive life forms, their use rather than adults as test species for LC_{50} values will be a more ecologically relevant approach to establishing a conservative measure of risk in the assessment procedures. Comparison of LC_{50} values among adults and their early life stages could serve as a starting point for such an evaluation (Olsen et al, 2011). Our 24 hour LC_{50} results indicate that larval Atlantic cod (LC_{50} = 0.89% v/v CEWAF) are 7 times more sensitive to CEWAF prepared with weathered ANS oil and Corexit dispersant than juvenile Atlantic cod (LC = 6.41% v/v CEWAF) and 12 times more sensitive to CEWAF prepared with weathered ANS oil and SPC1000 dispersant (larval Atlantic cod LC_{50} = 0.67% v/v CEWAF, juvenile Atlantic cod LC_{50} = 7.86% v/v CEWAF).

The results of our lethality tests show that larval stages of Atlantic cod are more sensitive to chemically dispersed oil than juvenile Atlantic cod. The risk of dispersant use on an oil spill to eggs and larval fish in near shore areas would have to be compared to the long term damage to the near shore from oil left to disperse naturally. Fertilization and hatching success of Atlantic cod eggs was only affected at the highest concentrations of SPC1000 dispersant tested alone, 0.5% v/v CEWAF prepared from weathered ANS oil/ SPC1000 dispersant and 0.5% v/v CEWAF prepared from weathered ANS oil/ Corexit dispersant). There were no significant hatching success effects from WAF of ANS oil when compared to controls.

Chemical dispersants are usually applied at a dispersant to oil ratio of 1:20-25 during an oil spill so it is highly unlikely that fish would be exposed to concentrations of dispersant that would cause acute toxicity in the open ocean.

Using dilution factors developed from a particle tracking model for the dispersion of produced water discharged from an oil production platform, rapid dilution by at least 240X occurs within 50-100m, 1000X by 4-5 km and up to 9000X at 20 km from the discharge site (Pérez-Casanova et al, 2010). Larval cod exposed to CEWAF prepared from ANS/Corexit had an LC_{50} of 0.89% v/v which is equal to a 112X dilution of the stock CEWAF. There may be risk to larval cod directly at the sight of a dispersed oil spill but that risk would diminish rapidly within a short distance. Total PAH measured for the 0.89% v/v dilution of CEWAF was 39.3 µg/ml and for the WAF of ANS LC_{50} of 42.6% v/v the measured concentration was 24.27 µg/ml. PAHs derived

from *Exxon Valdez* crude oil contaminated seawater to depths of at least 5 meters at concentrations ranging to 6.24±0.63 µg/l 1 to 2 weeks after the grounding. Seawater PAH concentrations were measured at 1.59 µg/l by 5 weeks post grounding (Short et al, 1996). A model of a hypothetical dispersed oil spill in Prince William Sound showed maximum measurement of PAHs of 85 ppb which is equivalent to 85 µg/l (Cox, 2004).

Temperature

The sub-lethal effect of hepatic EROD induction on juvenile cod was seen at three temperatures for fish exposed for 72 hours. The induction was delayed at 2°C. Our data indicate that cod exposed to CEWAF made from weathered MESA oil/ Corexit dispersant, at moderate sea water temperatures (7 and 10°C) will react (biochemically) more quickly than at colder temperatures. Water temperature should be included as a variable in risk assessment of the use of dispersants on oil spills in subarctic waters. The exposure concentrations used are equivalent to100X and 500X dilution of the stock WAF and CEWAF. Again the rapid dilution of the WAF and CEWAF would minimize risk.

Lethal vs. sub-lethal effects

Performing lethal or sub-lethal tests at low water temperatures reduce chemical solubility and this in combination with the low metabolism of fish at subarctic temperatures may cause delays in chemical uptake. The LC_{50} may not be an appropriate endpoint for risk assessment at colder temperatures. Sub-lethal effects could be incorporated into risk assessment and used in the estimation of ecosystem recovery time. Biomarkers of effects could be included in risk assessment (Olsen et al, 2011).

A range of different species with different ecology, feeding and habitat must be tested and evaluated for their applicability in risk assessment procedures (Olsen et al, 2011). We studied the sub-lethal effect of hepatic EROD induction on juvenile cod and Atlantic salmon smolts after a 4 hour exposure to WAF and CEWAF using 2 weathered oils – MESA and ANS and 2 dispersants – Corexit and SPC1000. Species specific differences were seen in EROD induction profiles of the two fish species.

An LC_{50} could not be calculated for WAF prepared from weathered MESA oil and weathered ANS oil at 50% v/v when exposing juvenile Atlantic cod. WAF prepared with weathered MESA oil was not lethal to larval cod even at the highest concentration tested. The LC_{50} of 42.6% WAF prepared with weathered ANS oil is not an environmentally relevant concentration for larval cod. It seems that not enough of the weathered oil components entered the aqueous phase to be toxic. The results suggest that the larval and juvenile cod were not sensitive to the oil that remained in the immiscible layer.

In conclusion, the present work with Atlantic cod and Atlantic salmon has shown that dispersed or non-dispersed oil is unlikely to pose an acute risk to these species of fish in the subarctic

environment beyond the immediate spill site. Rapid dilution would occur so that exposure to harmful concentrations of PAHs would be localized and for a short period of time.

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APPENDIX A

Toxicity Effects of Chemically-Dispersed Crude Oil on Fish

This appendix can be found as a supplemental PDF file accompanying this report.

APPENDIX B

Physical characteristics and chemical compositions of oils used in the study

This appendix can be found as a supplemental PDF file accompanying this report.

APPENDIX C

Statistical Analysis for Laboratory Herring Study

This appendix can be found as a supplemental PDF file accompanying this report.

APPENDIX D

Sarah Johnson M.Sc. Thesis

This appendix can be found in a supplemental PDF file accompanying this report.

APPENDIX E

Toxicity of Crude Oil Chemically Dispersed in a Wave Tank to Embryos of Atlantic Herring (*Culpea harengus*)

This appendix can be found as a supplemental PDF file accompanying this report.

APPENDIX F

Colleen Greer M.Sc. Thesis

This appendix can be found as a supplemental PDF file accompanying this report.

APPENDIX G

The influence of water temperature on induced liver EROD activity in Atlantic cod (*Gadus morhua*) exposed to crude oil and oil dispersants

This appendix can be found as a supplemental PDF file accompanying this report.

APPENDIX H

Comparison of the Effects to Atlantic Cod (*Gadus morhua*) and Atlantic Salmon (*Salmo salar*) When Exposed to WAF and CEWAF of MESA and ANS Crude Oil and Corexit 9500 and SPC-1000 Dispersants

This appendix can be found as a supplemental PDF file accompanying this report.