Toxicity Effects of Chemically-Dispersed Crude Oil on Fish

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ABSTRACT

Studies were conducted to: (1) evaluate the toxicity of polycyclic aromatic hydrocarbons (PAHs), the water accommodated fraction (WAF) and chemically enhanced water accommodated fraction (CEWAF) from Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) crude oils on life stages of commercially valuable herring and cod; (2) use biomarkers as indicators of exposure and effects; (3) assess environmental, physical, chemical, biological and temporal factors affecting toxicity; and (4) compare data using identical protocols for different oils and dispersants (Corexit 9500 and SPC-1000). Chronic LC50s for Pacific herring embryos were 3 (ANS) and 1.5 (MESA) mg-petroleum-hydrocarbons/L. ANS was 2.5-3 times more toxic than AL to Atlantic herring embryos. The apparent greater toxicity of a medium compared to a light crude might have been due to higher concentration and increased degree of alkyl PAHs with three or more rings, but the difference was not statistically significant. Increased toxicity with higher concentrations of hydrocarbons in CEWAF produced in a wave tank (measured by fluorescence - data not presented) was also reflected in EC50s from short, episodic laboratory tests. Higher salinities reduced sub-lethal toxicity of AL CEWAF (EC50 = 0.0072% v/v at 30 ppt vs. 0.0024% v/v at 15 ppt and 0.0015% v/v at 7.5 ppt) whereas colder water increased its lethal toxicity (LC50 = 0.0130% v/v at 7˚C vs. 0.0917% v/v at 10˚C and 0.0768% v/v at 15˚C). The similar toxicity results for Atlantic and Pacific herring embryos suggested that data from one species could be used to generate an accurate risk assessment for the other. Short term exposure to CEWAF and to high WAF concentrations significantly increased ethoxyresorufin *O*-deethylase (EROD) activity in juvenile Atlantic cod. Maximum EROD induction occurred at 24 h post-exposure (5-8 pmol· mg^{-1}·min^{-1} MESA WAF and 11-13 pmol· mg^{-1}·min^{-1} MESA CEWAF) and remained elevated over the next 48 h compared to controls. The highest level of EROD activity (27 pmol· mg^{-1}·min^{-1}) with ANS and SPC-1000 corresponded to a 1:12 dilution (8% v/v CEWAF). In MESA-exposed fish (2-64% v/v WAF or CEWAF) an induction maximum, possibly due to liver damage, occurred at 14-18 pmol· mg^{-1}·min^{-1}.

INTRODUCTION

Chemical dispersants are applied to crude oil spills at sea to reduce the impact of oil on biota inhabiting the littoral zone, on marine birds (Khan and Payne, 2005; Kanicky et al., 2001; Perez et al., 2010; Iverson and Esler, 2010), marine mammals, eggs and larvae of many fish and invertebrates, and obligate dwellers in the neuston layers (Zaitsev, 1970). To alleviate marine and coastal impacts, consideration is given to enhancing the dispersion of oil slicks at sea into small droplets (< 70µm) in the water column, to facilitate oxygen transport at the air-water interface, reduce contact with organisms that use the air-water interface (although pelagic organisms initially receive higher exposure potential), and to encourage the biodegradation of the oil by micro-organisms (Kanicky et al., 2001; Blondina et al., 1999). This process may be enhanced by applying chemical dispersants, which are surfactants in carrier solvents, that reduce interfacial tension at the oil-water interface (Kanicky et al., 2001). Dispersant use is predicated on the concept of dilution of oil to reduce its concentration below toxicity threshold limits and the enhancement of natural microbial degradation, as small oil droplets offer greater surface area for access to nutrients and oil degrading microbes (Bobra et al., 1989).

Dispersant effectiveness is influenced by sea energy, temperature, salinity and the nature of the crude oil (Ramachandran et al., 2004; Chandrasekar et al., 2005; NRC, 2005; Sorial et al., 2004). When oil and water are mixed, a certain percentage enters the water column as oil droplets. This oil-water mixture is referred to as the water accommodated fraction of oil, or WAF. When chemical dispersants are applied along with mixing energy in the form of waves and currents, the volume of oil in the water increases markedly, and the mixture is referred to as the chemically enhanced water accommodated fraction, or CEWAF. Due to toxicity concerns, hydrocarbon concentrations in WAF and CEWAF are frequently measured on the basis of polycyclic aromatic hydrocarbon (PAH) content.

Research on the impacts of the Exxon Valdez Oil Spill (EVOS) to support Natural Resources Damage Assessment demonstrated that embryos of Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*), both commercially important fish species, were very sensitive to exposure to oil. Signs of toxicity included cardiac pathology, pericardial and yolk sac edema, craniofacial deformities, spinal curvatures, fin erosions, and induction of cytochrome P-450 enzymes (Carls and Rice, 2007). The consequences of toxicity were impaired recruitment, as fewer embryos survived to the feeding stage, and latent effects in surviving salmon smolts that significantly reduced the number of adults returning to spawn. Effects were associated most strongly with concentrations of PAH. In light of these findings, it has been proposed that the environmental risk of accidental oil spills to fish may be assessed in terms of exposure to PAHs and changes in exposure to PAHs after the use of dispersants (Ramachandran et al., 2004). PAH exposure can be estimated by a standardized laboratory assay of cytochrome P-450 (CYP1A) induction (Hodson et al., 1996). The ethoxyresorufin-*O*-deethylase (EROD) assay monitors the induction of the xenobiotic-metabolizing enzyme, CYP1A, and is a widely used biomarker for exposure of wildlife to substances that bind the aryl hydrocarbon (Ah) receptor. EROD is induced in fish in the presence of xenobiotics including PAHs (Hodson et al., 1991). It should be noted that EROD induction in itself is not a toxic response, but an indication that the organism is responding to a xenobiotic compound.

Since the EVOS, there have been a variety of laboratory studies that confirmed the high toxicity of an array of crude and refined oils to different species of fresh and saltwater fish (e.g. Schein et al., 2008; McIntosh et al., 2010). Effects driven investigations of crude oil chemical fractions has found toxic effects on embryos to be associated with the concentrations of alkyl

PAH (Hodson et al., 2007) which comprise more than 90% of total PAH in petroleum. Individual alkyl PAH are highly toxic to embryonic stages, and are generally more toxic than non-alkylated PAH (Incardona et al., 2004, 2005, 2006; Farwell et al., 2006; Kiparissis et al., 2003; Rhodes et al., 2005; Scott et al., 2010; Turcotte et al., 2011). When oil is chemically dispersed, exposure of embryos to petroleum-derived PAH is significantly increased, although there is little change in the toxicity of the PAH in solution (Schein et al., 2008; Ramachandran et al., 2004; Carls et al., 2008).

While lab experiments have broadened our understanding of the range of fish species affected, the types of oil that cause toxicity, and the impacts of chemical dispersion, the capacity to apply these data to risk assessments during an actual oil spill is limited. Factors that impede practical application include: a lack of inter-species extrapolations because multiple species have not been tested with the same oil under the same conditions; a lack of inter-oil extrapolations because oils are often tested one at a time and under different conditions; and a focus on chronic exposure scenarios that typify stranded oil in spawning shoals, with few studies on brief or episodic exposures.

To facilitate risk assessment, we examined the toxicity of three commonly tested oils, ANS, MESA, and AL, to embryos and juveniles of Atlantic herring (*Clupea harengus)* and Atlantic cod (*Gadus morhua*), commercially important fish. The studies included both chronic exposures to undispersed and dispersed oil, and episodic exposures to dispersed oil generated by standard lab protocols and by chemical dispersion of oil in an outdoor wave-tank. To tie these studies to the large body of research arising from the EVOS, parallel chronic toxicity studies with ANS oil compared the response of Pacific herring embryos to that of Atlantic herring. The influence of environmental factors such as water temperature and salinity on the toxicity of dispersed oil was also tested. Short term exposures of juvenile Atlantic cod (*Gadus morhua*) were employed to determine the magnitude of hepatic CYP1A (EROD) induction from WAF and CEWAF, and the duration of elevation. The observations presented here represent work in progress.

METHODS

Chronic toxicity of oil to Atlantic and Pacific herring

"Ripe and running", stage IV Atlantic and Pacific herring were acquired from roe herring fishermen in Nova Scotia and fisheries officers in British Columbia. Embryos were prepared by fertilizing herring eggs in the laboratory. Directly after fertilization, embryos were exposed for 12-16 days — depending on the development rate of each batch of eggs — to laboratory-prepared water accommodated fraction (WAF) or chemically-enhanced water accommodated fraction (CEWAF) prepared using the stir bar method adapted from Singer et al. (2000) and reported previously (McIntosh et al., 2010). Oils tested were MESA, ANS, and AL. CEWAF was prepared using Corexit 9500A dispersant. Stock solutions of WAF and CEWAF were spiked into saltwater (15 ppt) to create gradients of oil loading (% v/v) for static daily renewal bioassays.

The consistency of test solutions was assessed by fluorescence analysis of aliquots of the water samples, compared to standard curves of the test oils. Fluorescence is primarily an indicator of single ring aromatics and PAH, those compounds with a conjugated bonding arrangement that are most closely associated with chronic toxicity of oil solutions.

Wave tank CEWAF for herring exposures

To assess the toxicity of chemically dispersed crude oil under conditions closer to that encountered in the open-ocean by Atlantic herring embryos and juvenile Atlantic cod, a wave tank was used as a practical intermediate model between a lab test and a full-scale spill in the environment. A wave tank facility at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia, was used to generate chemically-dispersed ANS and AL crude oils under breaking wave conditions (Li et al., 2008; Li et al., 2009). For all experiments, 300 mL of crude oil was added, followed by spraying of 12 mL Corexit 9500A chemical dispersant to achieve a dispersant-to-oil ratio of 1:25, a standard operational parameter for dispersant application in the field. Breaking waves were then generated. Water samples were collected simultaneously at 12 subsurface points in the wave tank at 5, 15, 30, and 60 minutes post-dispersion. The sampling points were located at four horizontal distances, one upstream and three downstream, of the initial point of release of oil and dispersant, and three depths, to cover the range of the dispersed oil plume in the water column. Embryos were exposed to each sample for 24 h (one set of embryos in each wave tank sample) and then transferred to clean water until hatch, when they were scored for pathology. During the oil exposure, water samples were drawn at time 0 and 24 h for analysis of total oil by fluorescence. This technique detects compounds in oil that have a conjugate bonding arrangement (i.e. PAHs), so it gives an estimate of the relative concentration of oil, not the absolute concentration, since fluorescing components may not have the same solubility as non-fluorescing.

Influence of Environmental factors on WAF and CEWAF toxicity

To test the influence of varying environmental conditions on the toxicity of WAF and CEWAF, herring embryos were exposed to AL at salinities of 7.5, 15 and 30 ppt and a temperature of 10ºC, and at 30 ppt and temperatures of 7, 10 and 15ºC. Artificially fertilised Atlantic herring embryos adhered to glass microscope slides were exposed in 200 mL of test solution (250 mL mason jars) to 0.01-1% WAF or 0.0001-0.01% CEWAF (salinity assay), or 0.0001-10% CEWAF (temperature assay) from fertilisation until hatch (6-20 d) in static daily renewal bioassays as described above for Atlantic herring chronic toxicity testing. These tests were independent of the wave tank experiments. Percent survival and normal development (i.e. absence of blue-sac disease characteristics) were assessed at hatch and EC50s calculated based on normal development.

Juvenile Atlantic cod

Atlantic cod (*Gadus morhua*) used in this study were hatched and raised to juveniles by the cod genome project and Fisheries and Oceans Canada staff at the St. Andrews Biological Station, New Brunswick. WAF and CEWAF were collected from either the wave tank at the Bedford Institute of Oceanography or prepared using a baffle flask method (Venosa et al., 2002). The production of WAF and CEWAF in the wave tank was done in batch mode following the same procedures as described for the herring exposure study. After oil (and dispersant for CEWAF) was exposed for 30 min to breaking waves, the wave tank effluent valves were opened and the effluent pump was engaged to drain the tank at a flow rate of 3.8 L/s for at least 5 min to thoroughly purge the lines. After purging, the freshly generated WAF or CEWAF was diverted into a series of glass aquaria to prepare WAF or CEWAF exposure water at various concentrations. Exposure dilutions (6.25% to 100% v/v) were prepared using seawater.

For laboratory prepared WAF, 2.5 mL of crude oil (ANS or MESA) were added to sand-filtered seawater (2500 mL) in a 4 L spouted baffled Erlenmeyer flask. The sample was placed on an orbital shaker and mixed at 250 rpm for 18 h. After 18 h, the sample was allowed to settle for 10 min then the oil droplet size checked using a LISST particle size analyser. Exposure dilutions (4.7% to 75% v/v) were prepared from this stock using seawater.

For CEWAF, 2 mL of crude oil (ANS or MESA) were added to 2 L seawater followed by dispersant (either Corexit or SPC-1000, 80 µL) in a 4 L spouted baffled Erlenmeyer flask. The sample was shaken at 250 rpm for 30 min followed by a 10 min settling period. The oil droplet size was determined by diluting the CEWAF stock (1.5 mL) with sea water (98.5 mL) and analysing the solution using a LISST particle size analyser. Exposure dilutions (0.5% to 100% v/v) were prepared from the stock using filtered seawater.

Juvenile cod were exposed to each combination of WAF and CEWAF for 4 h then transferred to clean seawater for up to 68 h. A known CYP1A inducer, ß-naphthoflavone (BNF), at a concentration of 10 $\mu g \cdot L^{-1}$ was used as a positive control. Fish lengths and weights, and tissue samples were collected from replicate fish (n = 5) at set time points of T = 4, 24, 48 and 72 h after the start of exposure. Over the course of the exposures, mean fish body weights ranged from 20.8 g ± 1.0 to 37.7 ± 1.7 g (Table 1). Blood samples were collected and stored on ice until processed for plasma. Gill, muscle and liver samples were quick frozen in liquid nitrogen and stored at -80°C until analysed.

Table 1 Weight of experimental fish during each exposure.

Treatment	Mean Fish Weight (g) ± SEM
ANS WAF	27.3 ± 0.8
	30.7 ± 1.5
MESA WAF	26.1 ± 1.4
	28.6 ± 0.9
ANS/ Corexit CEWAF	24.7 ± 1.2
	34.7 ± 1.6
ANS/ SPC1000 CEWAF	27.9 ± 1.6
	30.5 ± 1.4
MESA/ Corexit CEWAF	23.2 ± 1.1
	33.3 ± 1.6
MESA/ SPC1000 CEWAF	20.8 ± 1.0
	37.7 ± 1.7

Water temperatures for the exposures ranged from 9.3 to 13.5°C. Exposures to each WAF and CEWAF combination were conducted in duplicate. Exposure water samples were collected at T = 0 h and 4 h for determination of total PAH content using synchronous scanning fluorescence spectroscopy. Duplicate samples were taken from the highest concentration from each exposure for detailed PAH determination at the Bedford Institute of Oceanography by GC-MS or GC-FID. In addition, levels of total PAHs in each of the collected samples were also determined by fluorescence spectrophotometry. Calibration lines were prepared with MESA or ANS crude oils. These standards were fortified into hexane to give the equivalent PAH concentrations in water (2

to 50 $\mu g \cdot mL^{-1}$ for CEWAF, 4 to 160 $ng \cdot mL^{-1}$ for WAF). Water samples, 1, 2 or 5 mL for CEWAF and 50 mL for WAF were extracted using hexane (5 mL and 4 mL, respectively) and levels of PAHs quantified against the prepared calibration lines providing a concentration in crude oil equivalents. Sample analysis was conducted using a Varian Cary Eclipse fluorescence spectrophotometer coupled with Varian BIO Package version 1.1 software (Varian Inc., Paolo Alto, USA). Synchronous scan fluorescence mode was employed (excitation start/finish = 230/523 nm, $\Delta\lambda = 57$ nm, Ramachandran et al., 2004). Background subtraction was performed for each sample by subtracting the hexane spectra from each sample spectra prior to integration of peaks and quantification.

Collected liver samples were analysed for EROD induction (Hodson et al., 1996). Liver samples were homogenised in a buffer of 20 mM HEPES sodium salt and 150 mM potassium chloride at pH 7.5. Samples were centrifuged at 9000 g (20 min, 2°C) and the S9 fraction collected and stored at -80°C until analysed. EROD activity for each sample was determined by a micro-plate method employing spectrofluorometry where the conversion of ethoxyresorufin to resorufin by the S9 fractions was monitored over a set time. The protein content of each sample was determined by UV absorbance at 600 nm. Overall EROD induction was expressed as pmol $resorufin \cdot min^{-1} \cdot mg^{-1}protein$.

Statistical analyses were performed using SPSS software. Analyses of variance (ANOVA) were performed for EROD activity values that had been log transformed to achieve normal distribution (Hodson et al., 1996). The post-hoc Tukey's HSD was used to make all pair-wise comparisons and to identify significant differences in EROD activity between treated fish and seawater controls.

RESULTS

Chronic toxicity of oil to Atlantic herring

Preliminary results from an ongoing study (Greer, 2011) suggest that chemical dispersion of oil (ANS and AL) increased the concentration of measured fluorescent hydrocarbons within test solutions by 10 to 100-fold. When either Pacific or Atlantic herring embryos were exposed chronically from fertilization to hatch, there was little difference between the toxicity to Pacific herring of the two medium crude oils, ANS and MESA (chronic LC50 = 1.94 and 1.75 mg/L of fluorescent petroleum hydrocarbons, respectively, Table 2), but ANS was about 2 times more toxic to Atlantic herring than AL. The same relationship was evident in tests of chronic toxicity to trout embryos of the WAF and CEWAF of MESA, ANS, Federated crude, and Scotian Light crude oils (Hodson, unpublished data).

Table 2 Toxicity of Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) crude oil chemically-enhanced water accommodated fraction (CEWAF) to Atlantic and Pacific herring embryos. LC50s (mg/L) are based on measured concentrations of fluorescing petroleum hydrocarbons calibrated as total oil.

	Atlantic Herring		Pacific Herring	
CEWAF	14 day LC50	Standard Error	14 day LC50	Standard Error
ANS	2.03	1.12	1.94	1.61
MESA	not tested	not tested	1.75	<0.01
AL	4.33	1.58	not tested	not tested

Chemical data clearly indicated that the medium grade oils, ANS and MESA, had a greater percentage of aromatics compared to the lighter grade AL (Table 3).

Table 3 Chemical (crude oil characterization) and physical (% weathered, density, viscosity) characteristics of Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL); Alk = alkanes, Aro = aromatics, Res = resins, Asph = asphaltenes.

Oil	Chemical				Physical		
	Alk	Aro	Res	Asph	Weathered	Density (g/mL)	Viscosity (20°C, cStk)
ANS	32.0%	39.3%	24.4%	4.3%	10%	0.8607	17.5
MESA	34.2%	36.5%	20.8%	8.5%	15%	0.8802	26.1
AL	32.7%	18.9%	46.9%	1.5%	7%	0.8691	15.5

Pacific Herring comparison

CEWAF was much more toxic than WAF for MESA and ANS. It was clear that chemical dispersion of oil did not change the oil's toxicity, but caused a substantial 100-fold increase in the loading of toxic constituents to the water (data not shown), which would increase the exposure of sub-surface species to oil constituents. For this reason, LC50s for Atlantic and Pacific herring were calculated from nonlinear regressions of the mortality of embryos on the measured concentrations of oil in each WAF and corresponding CEWAF solution. From the relationships (not presented here) it was found that the responses of the two species of herring embryos to MESA and ANS were statistically indistinguishable, with LC50s of 1.75 and 1.94 mg/L respectively. Thus, data for the two species would be interchangeable for risk assessments.

Exposure time versus toxicity

During short episodic exposures (typical of spills in pelagic systems) the toxicity of dispersed ANS and AL CEWAF increased progressively with exposure time from 2.4 h to 14 d (Table 4). The 2.4 h EC50 for the measured concentration of fluorescing petroleum hydrocarbons reducing the number of herring embryos that were normal at hatch was about 11 mg/L. The EC50 decreased to 3 mg/L for an 8 h exposure, and to 0.49 mg/L after a 24 h exposure, but decreased thereafter to <0.25 mg/L for exposures up to 14 d. A similar result was found for AL crude but the overall toxicity was generally lower (higher EC50s).

Table 4 Exposure duration and embryo sublethal toxicity of Alaska North Slope (ANS) and Arabian Light (AL) chemically-enhanced water accommodated fraction (CEWAF) to Atlantic herring. EC50s (mg/L) and standard errors (SE) represent the concentrations causing a reduction in the percentage of normal embryos at hatch. Values are based on measured concentrations of fluorescing petroleum hydrocarbons calibrated to total oil.

CEWAF	2.4 h EC50	SE	8 h EC50	SE	24 h EC50	SE	14 day EC50	SE
ANS	11.08	<0.01	3.07	0.88	0.49	0.72	<0.25*	no data
AL	18.00	29.21	2.21	3.97	1.94	5.84	<0.37*	no data

*below the lowest concentration tested

Wave Tank CEWAF and herring

The observed concentrations of dispersed ANS in the wave tank were mostly <3 to 5 mg/L, which bracket the concentrations shown to be lethal to Atlantic herring embryos with lab-prepared solutions after 14 d of exposure (Table 4). In contrast, for sublethal responses, the highest concentrations generated within the wave tank under the experimental conditions (> 0.3 mg/L) were associated with a reduced number of normal herring at hatch (Greer, 2011). For AL, CEWAF generated by wave tank experiments caused no lethal or sublethal effects.

Influence of Environmental factors on WAF and CEWAF toxicity to Atlantic herring

Preliminary results also showed high survival at all concentrations of both WAF (range: 98.33 to 100%) and CEWAF (96.67 to 100%) of AL at all three salinities (7.5, 15 and 30 ppt). The range of concentrations tested were 0.0001 to 0.01% CEWAF and 0.01 to 1 % WAF. EC50s for normal development for WAF, calculated from nominal concentrations, were similar for both 7.5 (EC50 = 0.40%) and 15 ppt (EC50 = 0.36%) and only slightly higher at 30 ppt (EC50 = 0.64%; Table 5). There seemed to be a greater difference in EC50s of CEWAF, as the 30 ppt EC50 was 3-4.5 times higher than the 7.5 and 15 ppt EC50s (EC50 at 30 ppt = 0.0072% vs. EC50 at 7.5 ppt = 0.0015% and at 15 ppt = 0.0024%; Table 5). Ramachandran et al. (2006) who showed that increased salinity decreases the solubility of PAHs.

To determine the toxicity of AL CEWAF at different temperatures, the range of tested concentrations were 0.0001 to 10% v/v. The lower water temperature seemed to increase the toxicity of dispersed oil from LC50s of 0.0917% at 10˚C and 0.0768% at 15˚C to 0.0130% at 7˚C. The EC50s calculated at the different temperatures were lower than the lowest concentration tested of 0.0001% for the 7 and 15˚C treatment groups and at 0.0008% for 10˚C. The lower rearing temperature substantially increased the embryonic period. For the control groups, the embryonic period was an average of 18.9 (± 0.3; 95% CI) days to hatch at 7ºC compared to 13.3 (± 0.2) days to hatch at 10ºC and only 7.3 (± 0.2) days to hatch at 15ºC. The increased duration of the embryonic period consequently increased the exposure period which may be responsible for the lower EC50 at this temperature.

Table 5 Effect of salinity on sublethal toxicity (EC50) of water accommodated fractions (WAF) and chemically-enhanced water accommodated fraction (CEWAF) of Arabian Light in chronic exposures of Atlantic herring embryos from fertilization until hatch. EC50s with 95% confidence intervals (CI) are based on nominal concentrations and represent the concentrations causing a reduction in the percentage of embryos that were normal at hatch. Chemical analyses are underway to determine measured concentrations of PAHs in all tested WAF and CEWAF solutions.

	EC50 (% v/v) for % Normal at Hatch					
Salinity	7.5 ppt	95% CI	15 ppt	95% CI	30 ppt	95% CI
WAF	0.40	0.21-0.77	0.36	0.27-0.48	0.64	0.37-1.12
CEWAF	0.00153	0.00102-0.00229	0.0024	0.0014-0.0041	0.0072	0.0033-0.0157

Juvenile Atlantic cod

Table 6 shows the concentration of hydrocarbons in water samples collected at the beginning of the 4 h exposures of juvenile cod to the different types of WAF and CEWAF. Generally, the WAF and CEWAF of MESA oil had higher concentrations of all analytes compared to ANS oil regardless of the dispersant used.

Table 6 Representative results ($\mu g \cdot L^{-1}$) of GC-MS analysis of water collected at onset of cod exposures. Total petroleum hydrocarbons (TPH) measured by GC-FID; all others by GC-MS.

Compounds	**ANS 75% WAF**	**MESA 75% WAF**	**ANS + SPC 1000 64% CEWAF**	**ANS + Corexit 64% CEWAF**	**MESA + SPC 1000 64% CEWAF**	**MESA + Corexit 64% CEWAF**
Σ Alkanes	15.7	38.8	235.8	256.9	319.3	390.5
Σ Methylated PAHs	0.4	1.6	39.9	47.7	45.5	53.3
Σ PAHs	0.6	1.4	4.7	5.6	4.7	5.4
TPH	380	400	4820	4120	5360	5810

Corresponding fluorescence analyses confirmed the presence of elevated concentrations of fluorescing compounds in CEWAF. However, in contrast to the TPH analysis, the CEWAF of ANS had more fluorescing compounds in the mixture than CEWAF of MESA.

All fish survived and appeared to be unaffected by exposure to WAF and CEWAF under our experimental conditions. Figures 1 and 2 show the induction of EROD in livers collected from cod exposed for 4 hours to wave tank effluent WAF or CEWAF of MESA dispersed with Corexit 9500. The pattern of response was consistent across all exposures (data not shown). EROD was not significantly induced in cod livers immediately after the 4 h exposure to WAF or CEWAF. BNF, the positive control, caused significant EROD induction after only 4 h (Figure 2). In the exposures shown in Figure 1Figure 2, the level of EROD activity in cod livers was significantly elevated compared to livers from unexposed fish 20 h after transfer to clean

seawater. Only the highest dilution (lowest concentration, 6.25%) of WAF failed to significantly induce hepatic EROD activity relative to controls (Figure 1). Maximum EROD induction occurred at the 24 h sample time. Over the next 48 hours the level of EROD activity remained elevated compared to pre-treatment levels or controls sampled at the same time but were declining relative to the 24-h sample (Figure 1 and Figure 2).

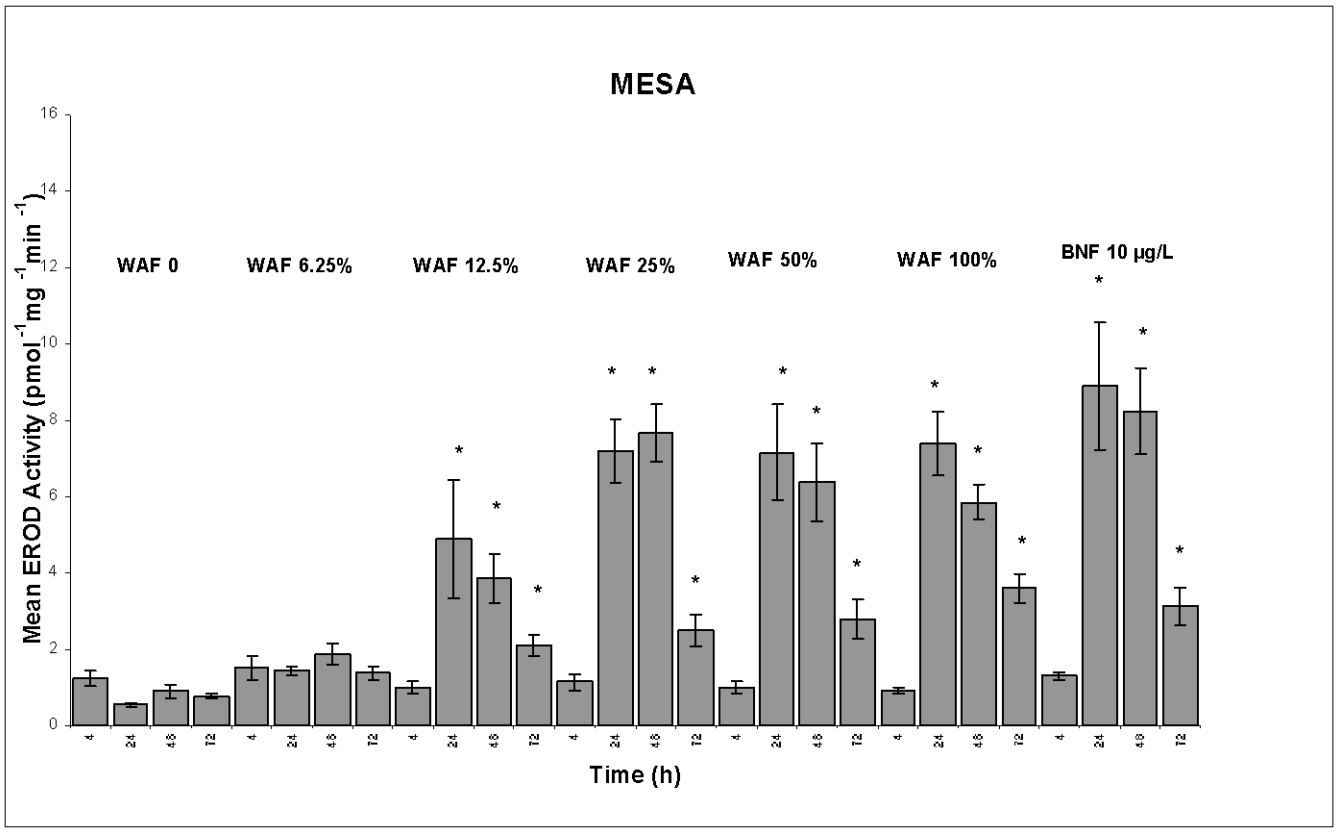

Figure 1 EROD induction in juvenile cod exposed for 4 h to MESA oil WAF (as percentage of the mixed WAF in seawater) and ß-naphthoflavone (BNF); asterisk (*) represents significant difference ($P<0.05$) from seawater control; bars represent ± standard error

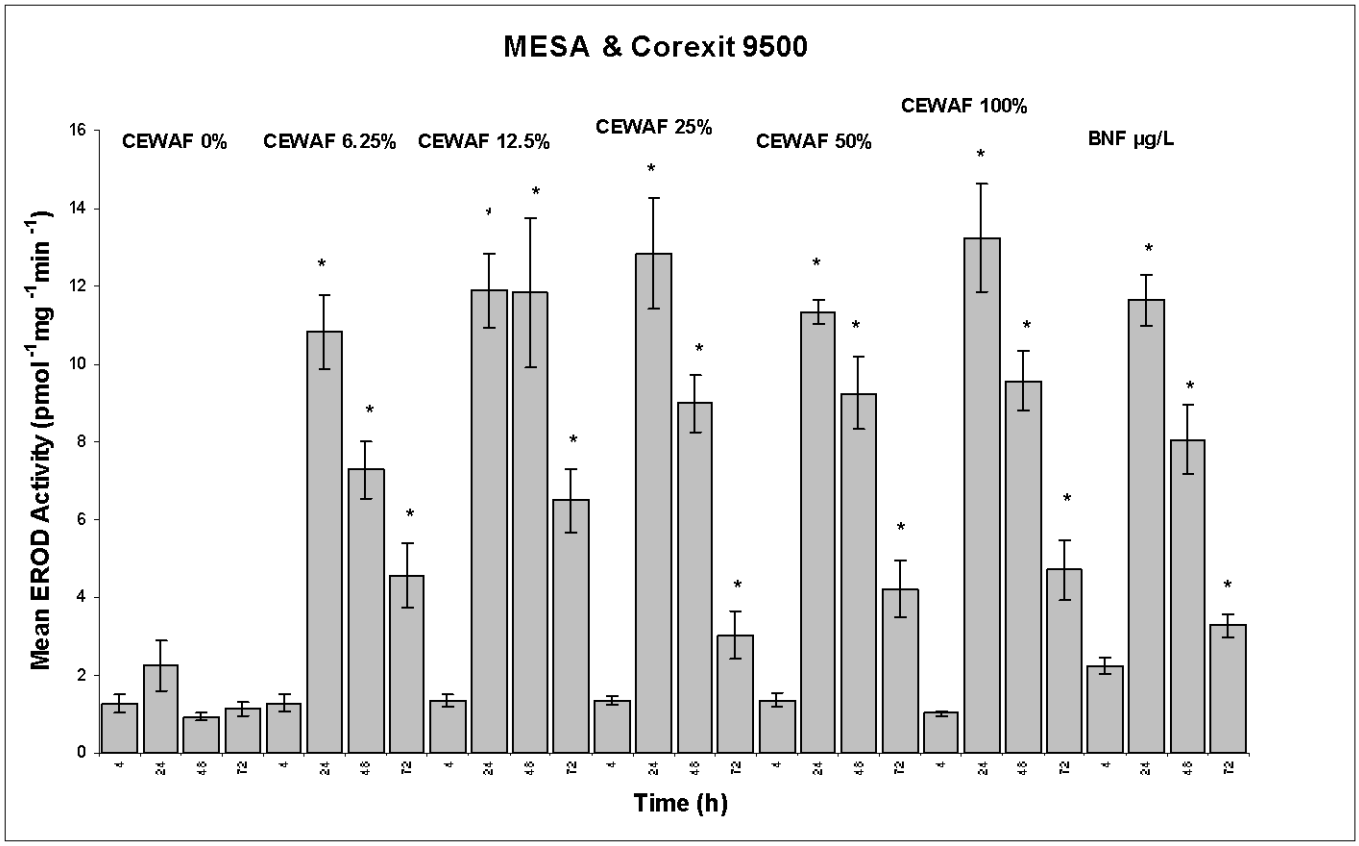

Figure 2 EROD induction in juvenile cod after 4 h exposure to ß-naphthoflavone (BNF) and to CEWAF of MESA oil dispersed with Corexit 9500; concentrations are expressed as percentage of the mixed CEWAF in seawater; asterisk (*) represents significant difference (P<0.05) from seawater control; bars represent ± standard error

Figure 3 shows a summary of EROD induction at various dilutions of WAF and CEWAF, prepared with ANS oil and SPC-1000 at the 24 h sample time. At the highest dilution of CEWAF (0.5%) there was no significant induction of EROD. At all other CEWAF concentrations EROD activity was significantly elevated (P<0.05%). WAF significantly affected EROD activity only at the highest concentrations.

The elevation of EROD activity at 24 hours was greater in CEWAF-exposed fish than in WAF exposed fish (Figure 3). It also appeared that, despite the difference in concentrations of PAHs in ANS-treated water compared to MESA-treated water, that the EROD response was at least as large in ANS-exposed fish. There was significant elevation of EROD activity in all CEWAF exposures at concentrations greater than 2% (Figure 3). The level of EROD induction showed no dose-response at the higher concentrations of WAF or CEWAF (Figure 3). In fact, the highest level of EROD activity seen with ANS and SPC-1000 corresponds with a dilution factor of 1:12 (8% CEWAF by volume). EROD induction at lower dilutions was significantly lower. In MESA-exposed fish an induction maximum appeared to have been reached at ~20 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein.

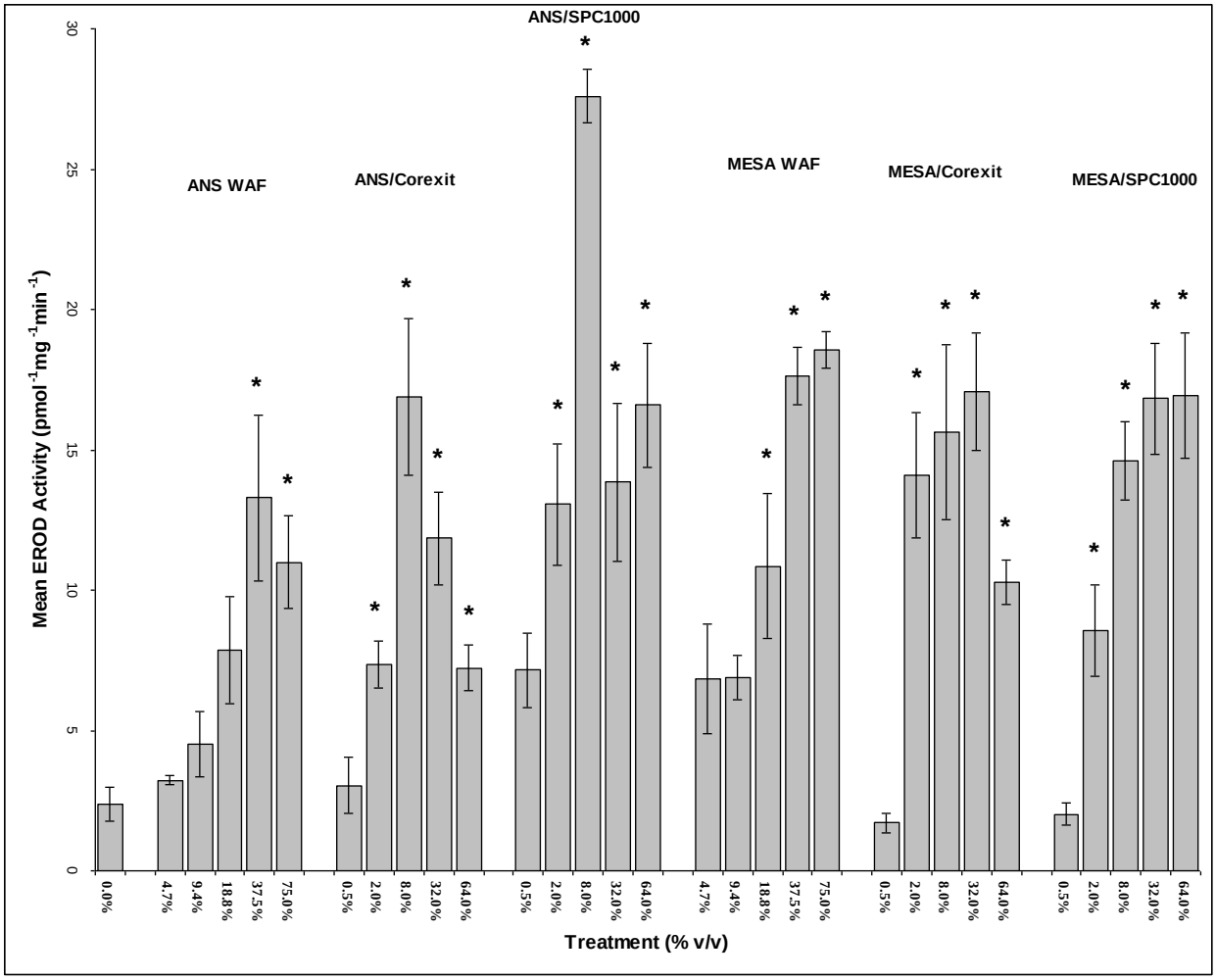

Figure 3 Mean EROD induction at T = 24 h post-exposure, in livers of cod exposed for 4 hours to WAF or CEWAF of ANS or MESA dispersed with either Corexit 9500 or SPC-1000; asterisk (*) represents significant difference ($P<0.05$) from seawater control; bars represent ± standard error

DISCUSSION

The experiments with herring embryos demonstrated that while the CEWAFs of the medium test oils ANS and MESA were more toxic than that of the lighter AL oil there was little difference in sensitivity between herring species, and toxicity increased with longer exposure times. The wave tank experiments demonstrated that the highest concentrations of petroleum hydrocarbons in wave tank samples as measured by fluorescence were in the same range as the measured concentrations that were lethal to herring embryos in tests of lab-prepared oil solutions. Hence, toxicity measured for wave tank samples were in general agreement with toxicity measured for lab-prepared solutions. Preliminary results also indicated that environmental conditions may affect the sub-lethal and lethal toxicity of dispersed oils.

The greater toxicity to herring of medium crudes relative to lighter oils is likely due to their higher concentrations of 3-5 ringed PAH (Hodson et al., 2007), and perhaps to a higher degree of alkyl substitution. A more detailed analysis of the composition of the oils and their proportion of

different PAHs would be required to draw specific conclusions about cause and effect. The nature of effects of crude oil on the development and survival of herring embryos were consistent across oils, and there was similarity in sensitivity to oil between Atlantic and Pacific herring, suggesting that data from one species could be used to generate an accurate risk assessment for the other. For herring, this means that the data base will expand considerably if the two species are considered as one.

The decrease in CEWAF EC50s (increased toxicity) with increasing exposure times is typical of most exposure-response relationships. Although toxicity decreased for brief exposures, the EC50s for exposure times ranging from 2.4 to 24 h remained within the range of concentrations observed in actual oil spills. Thus, the impacts of real oil spills on fish embryos and recruitment will vary directly with the rate of dilution of the oil droplets. To generate accurate risk assessments when applying chemical dispersants to a spill, it would be necessary to determine how long and at what concentration hydrocarbons will remain within the area. A recent study by McIntosh et al. (2010) provided such an analysis for Atlantic herring embryos, creating three-dimensional response surfaces relating effects to exposure concentration and exposure duration. McIntosh et al. (2010) added a third dimension by testing embryos at different stages of development. Sensitivity was greatest immediately after egg fertilization and immediately after hatch. Oviparous fish eggs have a structure called the micropyle on the surface of the egg envelope through which the sperm must enter to attach to the oocyte plasma membrane. The heightened sensitivity may have been due to high rates of exposure to petroleum hydrocarbons associated with their rapid uptake before micropyle closure following fertilization, and then again after egg envelope chorion removal during hatching.

The trend of increasing responses of fish embryos to increasing concentrations of hydrocarbons in CEWAF sampled from the wave tank corresponded to measured effect concentrations in laboratory toxicity tests of short, episodic exposures to CEWAF. All responses were sublethal (i.e. a reduced percentage of newly hatched herrings that appeared normal), indicating that the concentrations generated in the wave tank under breaking wave conditions were at the threshold of toxicity. If this holds true in subsequent experiments, it supports the use of lab data in risk assessments that compare measured concentrations of hydrocarbons in environmental samples to established toxicity thresholds for realistic exposure times. While these toxicity results may be considered conservative, because water samples recovered from the wave tank were diluted by 50% with fresh water to attain a salinity of 15 ppt, the optimum for Atlantic herring development, it is important to emphasize that the experiments were conducted using the wave tank as a closed system (no flow-through). The introduction of a dynamic flow-through using the wave tank would generate CEWAF that would more closely stimulate natural conditions. In addition, Corexit 9500 was designed for application in the open ocean in saline waters of >25 ppt (Blondina et al., 1999) and thus its effectiveness may be affected by the level of salinity (Blondina et al., 1999; Chandrasekar et al., 2006; Ramachandran et al., 2006). Interestingly, the sub-lethal toxicity of AL CEWAF decreased at a 30 ppt salinity compared to lower salinities of 7.5 and 15 ppt, perhaps indicating decreased PAH solubility rather than reduced effectiveness of Corexit 9500.

CEWAF induced higher EROD activity in juvenile Atlantic cod compared to mechanically dispersed oil (WAF). Chemical dispersion of oil affects the particle size of oil droplets (Li et al., 2008; Li et al., 2009) and the toxicity of oil is influenced by particle size (Bobra et al., 1989), because PAHs are partitioned into the water phase, thereby becoming more bioavailable for

uptake by fish. Since the wave tank samples were diluted to 15 ppt salinity prior to exposure, a bias was introduced into the toxicity measurements.

EROD activity reported in this work is similar to the levels reported by other authors. Goksøyr et al. (1994) reported a dose-dependent response of hepatic EROD activity in the range of 50-150 pmol·min^{-1}·mg^{-1}protein for juvenile Atlantic cod caged in a polluted fjord. Aas et al. (2000) exposed juvenile cod for 30-days to mechanically dispersed crude oil. They showed a dose-dependent response of EROD activity with an initial increase during the first three days. They reported hepatic EROD activity in control fish around 2 pmol·min^{-1}·mg^{-1}protein and around 6 pmol·min^{-1}·mg^{-1}protein in the 1 ppm nominal concentration of mechanically dispersed crude oil exposure group after three days. Sturve et al. (2006) exposed juvenile Atlantic cod to 0.5 ppm North Sea oil for fifteen days and saw a significant increase in EROD activity. Hepatic EROD activity in control fish was 20 pmol·min^{-1}·mg^{-1}protein and fish exposed to 0.5 ppm oil had a mean EROD level of 50 pmol·min^{-1}·mg^{-1}protein. Aas et al. (2000) and Sturve et al. (2006) also reported elevated EROD activity in Atlantic cod chronically exposed to North Sea oil. Our fish were exposed directly to WAF and CEWAF but for only 4 hours and showed EROD ranged from ~1 pmol·min^{-1}·mg^{-1} for control fish and up to ~20 pmol·min^{-1}·mg^{-1}protein for CEWAF-treated fish.

In some cases EROD activity in cod exposed to WAF and CEWAF did not increase in a dose-dependant manner. At high concentrations of CEWAF the EROD activity was actually lower than that observed with lower concentrations (Figure 3). This result could be due to liver damage (Holdway et al., 1994). Occasional differences in mean hepatic EROD activity were seen between replicate treatments. In particular, the replicates of the MESA/SPC1000 CEWAF treatment had the greatest size difference in fish yet there were only significant differences between EROD response for the control fish at T = 48 h, the 2% CEWAF treated fish at T = 72 h and the 64% CEWAF treated fish at T = 72 h. Our data lead us to conclude that the difference in size of the experimental fish between replicates has no bearing on the EROD induction between replicate exposures or interpretation of the data.

It is clear from our results that short term exposure to CEWAF, and short term exposure to high concentrations of WAF, can both significantly increase EROD activity in juvenile Atlantic cod. The elevation is persistent over several days. The biological significance of elevated EROD remains unclear as elevated EROD is not considered a toxic response but is a clear indicator of biochemical change (Whyte et al., 2000). The risk associated with WAF exposure appears low. However, exposure to low levels of CEWAF for short periods of time can induce EROD activity. Despite the fact that pre-exposure levels were not reached over 72 h, the trend is for enzyme activity to fall with time (Figure 1 and Figure 2). Risk is a function of concentration and exposure. In situations where chemical dispersants are used for short periods of time the risk to individuals and populations is probably small. However, risks associated with long term application of chemical dispersants or with chronically elevated EROD activity in livers of cod remain unknown.

These data enhance the ability to monitor the environmental impacts of crude oil spills. They provide further insight into the potential application of oil dispersants through evaluation of the factors controlling the toxicological responses of early life stages of fish to dispersed and non-dispersed oil. The long-term persistence of CEWAF and the dispersants themselves in the environment, a subject under debate, needs to be carefully considered in risk assessment.

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Table B1. Physical characteristics and chemical compositions of oils used in the study.

Oil	Chemical Composition				Physical Characteristics		
					Weathered	ρ	η
	Saturates	Aromatics	Resins	Asphaltenes	By Volume	20 °C	20 °C
	%	%	%	%	%	g/cm^3	cP
ALC	32.7	18.9	46.9	1.5	7	0.869	15.5
ANS	32.0	39.3	24.4	4.3	10	0.874	17.5
MESA	34.2	36.5	20.8	8.5	15	0.881	26.1

The chemical composition of the oils was determined using thin-layer chromatography coupled with flame ionization detection (Maki & Sasaki, 1997). The physical and chemical measurements were determined in the BIO COOGER chemistry labs. It is also important to note that all oils were weathered prior to use. Therefore, there were no acute toxicity effects from volatiles such as BTEX.

Table C1. Average heart rate (beats/min) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 7, 10 and 15°C (CEWAF only). 2-Factor nested ANOVAs were performed using salinity or temperature and concentration as the two factors with the interaction term reported. Uppercase letters indicate significant differences between salinities or temperatures (individual concentrations analyzed; Tukey test, $p<0.05$). Lowercase letters indicate significant differences between concentrations (individual salinities and temperatures analyzed; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration. Heart rates for embryos were measured on day 7 post-fertilisation for the salinity bioassay and on days 5, 7 and 12 post-fertilisation when reared at 15, 10 and 7°C, respectively (Boudreau et al., 2009).

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{8, 425} = 14.83$, $p<0.001$				
Water	41.80±1.71 A ac	48.60±2.52 B a	40.40±1.85 A a	$F_{2, 87} = 17.28$, $p<0.001$
0.0001	44.20±1.71 A a	45.80±2.67 AB ab	48.20±2.08 B b	$F_{2, 87} = 3.46$, $p=0.036$
0.001	36.20±2.16 A bd	42.40±2.63 B b	52.00±3.13 C b	$F_{2, 87} = 36.77$, $p<0.001$
0.01	39.6±2.47 A bc	43.80±1.97 B b	38.60±2.01 A a	$F_{2, 87} = 6.63$, $p=0.002$
Retene	33.80±1.71 A d	42.00±2.20 B b	42.00±3.17 B a	$F_{2, 87} = 15.01$, $p<0.001$
ANOVA	$F_{4, 135} = 21.23$, $p<0.001$	$F_{4, 135} = 7.31$, $p<0.001$	$F_{4, 135} = 24.19$, $p<0.001$	
WAF Interaction: $F_{8, 425} = 6.59$, $p<0.001$				
Water	41.80±1.71 A a	48.60±2.52 B a	40.40±1.85 A a	$F_{2, 87} = 17.28$, $p<0.001$
0.01	48.00±2.20 A b	52.80±2.23 B b	49.40±2.10 AB b	$F_{2, 87} = 5.33$, $p=0.007$
0.1	35.80±1.25 A cd	49.53±1.90 B ab	44.60±2.61 C ab	$F_{2, 87} = 54.38$, $p<0.001$
1.0	39.40±1.63 A ad	45.00±2.10 B ac	40.80±2.38 A a	$F_{2, 87} = 8.12$, $p=0.001$
Retene	33.80±1.71 A c	42.00±2.20 B c	42.00±3.17 B a	$F_{2, 87} = 15.01$, $p<0.001$
ANOVA	$F_{4, 135} = 46.77$, $p<0.001$	$F_{4, 135} = 16.67$, $p<0.001$	$F_{4, 135} = 11.26$, $p<0.001$	
Conc. (% v/v)	7°C	10°C	15°C	ANOVA
CEWAF Interaction: $F_{10, 238} = 15.77$, $p<0.001$				
Water	52.11 (49.08-55.33) A a	57.14 (52.47-62.22) AB a	63.23 (57.01-70.14) B a	$F_{2, 42} = 5.98$, $p=0.005$
0.0001	35.31 (32.80-38.01) A bd	54.57 (50.11-59.56) B ab	86.09 (79.79-92.67) C b	$F_{2, 42} = 149.27$, $p<0.001$
0.001	38.27 (36.30-40.35) A b	49.65 (46.44-52.96) B ab	77.97 (70.95-85.69) C b	$F_{2, 42} = 110.53$, $p<0.001$
0.01	27.79 (25.11-30.82) A c	51.63 (48.07-55.33) B ab	89.94 (84.71-95.49) C b	$F_{2, 42} = 250.91$, $p<0.001$
0.1	29.23 (26.60-32.13) A d	38.27 (34.50-42.45) B c	62.80 (56.48-69.81) C a	$F_{2, 37} = 60.85$, $p<0.001$
1.0	NA[a]	46.12 (42.26-50.46) b	48.07 (41.68-55.33) c	NS
10.0	NA	NA	NA	NA
Retene	23.06 (19.94-26.60) A e	36.05 (31.98-40.63) B c	54.19 (49.31-59.42) C ac	$F_{2, 42} = 57.18$, $p<0.001$
Corexit	NA	NA	NA	NA
ANOVA	$F_{5, 68} = 43.54$, $p<0.001$	$F_{6, 84} = 24.05$, $p<0.001$	$F_{6, 84} = 36.39$, $p<0.001$	

[a]NA = not available because of high rates of embryonic mortality in these groups.

Table C2. Average time-to-hatch (dpf) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 7, 10 and 15°C (CEWAF only). 2-Factor nested ANOVAs were performed using salinity or temperature and concentration as the two factors with the interaction term reported. Uppercase letters indicate significant differences between salinities or temperatures (individual concentrations analyzed; Tukey test, $p<0.05$). Lowercase letters indicate significant differences between concentrations (individual salinities and temperatures analyzed; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{8, 830} = 21.92$, $p<0.001$				
Water	15.10±0.10 A a	15.02±0.09 A a	14.70±0.12 B a	$F_{2, 177} = 16.32$, $p<0.001$
0.0001	15.05±0.07 A a	14.77±0.11 B ab	15.03±0.05 A b	$F_{2, 177} = 15.33$, $p<0.001$
0.001	15.05±0.06 A a	14.53±0.14 B b	14.63±0.14 B a	$F_{2, 177} = 22.33$, $p<0.001$
0.01	15.00±0.16 A a	14.77±0.14 B ab	14.28±0.13 C c	$F_{2, 175} = 27.21$, $p<0.001$
Retene	15.45±0.19 A b	14.90±0.22 B a	13.77±0.36 C d	$F_{2, 134} = 43.13$, $p<0.001$
ANOVA	$F_{4, 279} = 8.33$, $p<0.001$	$F_{4, 276} = 7.78$, $p<0.001$	$F_{4, 255} = 47.84$, $p<0.001$	
WAF Interaction: $F_{8, 829} = 17.709$, $p<0.001$				
Water	15.10±0.10 A a	15.02±0.09 A	14.70±0.12 B a	$F_{2, 177} = 16.32$, $p<0.001$
0.01	14.98±0.13 a	14.86±0.11	14.80±0.10 a	NS
0.1	14.88±0.12 a	14.97±0.05	14.82±0.11 a	NS
1.0	14.88±0.16 A a	14.87±0.10 A	14.63±0.14 B a	$F_{2, 176} = 4.33$, $p=0.015$
Retene	15.45±0.19 A b	14.90±0.22 B	13.77±0.36 C b	$F_{2, 134} = 43.13$, $p<0.001$
ANOVA	$F_{4, 279} = 11.042$, $p<0.001$	NS	$F_{4, 255} = 26.47$, $p<0.001$	
Conc. (% v/v)	7°C	10°C	15°C	ANOVA
CEWAF Interaction: $F_{10, 649} = 8.77$, $p<0.001$				
Water	19.31 (19.00-19.58) A a	13.60 (13.39-13.83) B a	7.44 (7.27-7.61) C a	$F_{2, 165} = 2{,}798.59$, $p<0.001$
0.0001	19.53 (19.22-19.90) A a	12.38 (12.10-12.64) B b	7.61 (7.42-7.81) C a	$F_{2, 137} = 1{,}806.40$, $p<0.001$
0.001	19.49 (19.09-19.90) A a	13.42 (13.08-13.76) B a	7.56 (7.39-7.73) C a	$F_{2, 125} = 1{,}771.18$, $p<0.001$
0.01	18.15 (17.61-18.70) A b	12.84 (12.38-13.86) B ab	7.51 (7.34-7.68) C a	$F_{2, 112} = 792.08$, $p<0.001$
0.1	15.34 (14.05-16.70) A c	12.24 (11.79-12.73) B b	7.23 (7.00-7.47) C a	$F_{2, 73} = 233.75$, $p<0.001$
1.0	No Hatch	9.88 (8.48-11.47) A c	6.00 (5.54-6.51) B b	$F_{1, 12} = 46.82$, $p<0.001$
10.0	No Hatch	No Hatch	No Hatch	No Hatch
Retene	17.73 (16.97-18.53) A b	11.03 (10.63-11.42) B c	6.61 (6.36-6.88) C c	$F_{2, 49} = 483.03$, $p<0.001$
Corexit	No Hatch	No Hatch	No Hatch	No Hatch
ANOVA	$F_{5, 194} = 19.16$, $p<0.001$	$F_{6, 234} = 24.99$, $p<0.001$	$F_{6, 206} = 13.63$, $p<0.001$	

Table C3. Average heart rate (beats/min) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) crude oil WAF and CEWAF. Arrows indicate significant differences from the negative control (1-Factor nested ANOVA among oil-concentration combinations and control groups; Tukey test, $p<0.05$). Stocks were analyzed separately and N=3 jars/concentration. Heart rates were measured on day 8 post-fertilisation (Boudreau et al., 2009).

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	ALC	Controls
CEWAF 1-Factor ANOVA: $F_{10, 297} = 60.21$, $p<0.001$; WAF 1-Factor ANOVA: $F_{10, 297} = 83.56$, $p<0.001$					
Water					47.4±2.84
CEWAF	0.0001	53.2±3.31	43.4±2.68	59.2±3.66 ↑	
	0.001	48.2±2.73	40.6±1.63 ↓	48.2±1.81	
	0.01	30.8±2.02 ↓	35.0±2.36 ↓	39.8±2.08 ↓	
WAF	0.01	61.8±3.01 ↑	52.3±2.37	63.0±2.87 ↑	
	0.1	51.8±2.73	42.8±2.33	44.0±1.79	
	1.0	39.8±2.79 ↓	30.6±1.89 ↓	37.4±2.01 ↓	
Retene					31.0±2.77 ↓
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	ALC	Controls
CEWAF 1-Factor ANOVA: $F_{10, 297} = 52.62$, $p<0.001$; WAF 1-Factor ANOVA: $F_{10, 297} = 46.00$, $p<0.001$					
Water					44.0±3.02
CEWAF	0.0001	43.6±2.82	47.4±2.45	46.2±2.36	
	0.001	48.4±2.56	48.6±2.45	41.0±2.29	
	0.01	26.4±2.25 ↓	34.8±2.79 ↓	33.4±2.10 ↓	
WAF	0.01	51.2±2.81 ↑	53.6±2.88 ↑	39.6±2.00	
	0.1	37.4±2.68 ↓	50.2±2.32 ↑	45.0±2.34	
	1.0	35.6±1.85 ↓	45.6±2.09	42.8±2.18	
Retene					28.6±1.52 ↓

Table C4. Average time-to-hatch (dpf) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) WAF and CEWAF. Arrows indicate significant differences from the negative control (1-Factor nested ANOVA among oil-concentration combinations and control groups; Tukey test, $p<0.05$). Stocks were analyzed separately and N=3 jars/concentration.

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	ALC	Controls
CEWAF 1-Factor ANOVA: $F_{10, 513} = 19.44$, $p<0.001$; WAF 1-Factor ANOVA: $F_{10, 515} = 21.64$, $p<0.001$					
Water					15.78±0.22
CEWAF	0.0001	15.61±0.18	15.22±0.22 ↓	15.39±0.17	
	0.001	15.40±0.18	15.36±0.18	15.40±0.15	
	0.01	15.04±0.26 ↓	14.88±0.25 ↓	15.15±0.24 ↓	
WAF	0.01	15.30±0.17 ↓	15.46±0.15	15.00±0.26 ↓	
	0.1	15.38±0.21	15.51±0.18	15.07±0.15 ↓	
	1.0	15.22±0.20 ↓	15.14±0.16 ↓	15.31±0.15 ↓	
Retene					12.86±1.03 ↓
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	ALC	Controls
CEWAF 1-Factor ANOVA: $F_{10, 521} = 11.79$, $p<0.001$; WAF 1-Factor ANOVA: $F_{10, 523} = 9.43$, $p<0.001$					
Water					15.17±0.20
CEWAF	0.0001	15.20±0.19	15.08±0.22	15.25±0.21	
	0.001	14.68±0.23	14.94±0.25	14.96±0.21	
	0.01	14.32±0.33 ↓	14.40±0.23 ↓	14.53±0.27 ↓	
WAF	0.01	15.21±0.26	15.07±0.24	15.40±0.29	
	0.1	14.76±0.24	15.20±0.23	14.94±0.25	
	1.0	14.54±0.25 ↓	14.62±0.23 ↓	14.89±0.24	
Retene					13.39±0.58↓

Table C5. Average length-at-hatch (mm) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 7, 10 and 15°C (CEWAF only). 2-Factor nested ANCOVAs were performed using salinity or temperature and concentration as the two factors with the interaction term reported. Uppercase letters indicate significant differences between salinities or temperatures (individual concentrations analyzed; Tukey test, $p<0.05$). Lowercase letters indicate significant differences between concentrations (individual salinities and temperatures analyzed; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{8, 829} = 10.82$, $p<0.001$				
Water	7.25±0.12 A a	7.47±0.09 B a	7.42±0.12 AB a	$F_{2, 176} = 4.78$, $p=0.010$
0.0001	7.36±0.10 a	7.34±0.15 a	7.27±0.16 ab	NS
0.001	7.33±0.11 AB a	7.37±0.15 A a	7.13±0.19 B ab	$F_{2, 176} = 3.63$, $p=0.028$
0.01	6.22±0.80 b	6.30±0.25 b	6.93±0.23 b	NS
Retene	5.12±0.14 A c	4.56±0.10 B c	3.30±0.16 C c	$F_{2, 133} = 159.40$, $p<0.001$
ANOVA	$F_{4, 278} = 24.02$, $p<0.001$	$F_{4, 275} = 221.57$, $p<0.001$	$F_{4, 254} = 149.48$, $p<0.001$	
WAF Interaction: $F_{8, 828} = 20.924$, $p<0.001$				
Water	7.25±0.12 A a	7.47±0.09 B a	7.42±0.12 AB a	$F_{2, 176} = 4.78$, $p=0.010$
0.01	7.23±0.16 A a	7.32±0.12 AB a	7.49±0.15 B a	$F_{2, 175} = 3.39$, $p=0.036$
0.1	7.13±0.20 A ab	7.31±0.10 AB a	7.51±0.10 B a	$F_{2, 175} = 6.75$, $p=0.001$
1.0	6.83±0.25 A b	6.69±0.19 A b	7.05±0.23 B b	$F_{2, 175} = 6.69$, $p=0.002$
Retene	5.12±0.14 A c	4.56±0.10 B c	3.30±0.16 C c	$F_{2, 133} = 159.40$, $p<0.001$
ANOVA	$F_{4, 278} = 103.40$, $p<0.001$	$F_{4, 274} = 330.28$ $p<0.001$	$F_{4, 254} = 226.72$, $p<0.001$	
Conc. (% v/v)	7°C	10°C	15°C	ANOVA
CEWAF Interaction: $F_{10, 648} = 4.33$, $p<0.001$				
Water	6.33±0.27 A a	5.93±0.21 B ab	5.39±0.23 C a	$F_{2, 164} = 22.36$, $p<0.001$
0.0001	6.39±0.22 A a	5.68±0.22 B a	5.40±0.28 C a	$F_{2, 136} = 19.52$, $p<0.001$
0.001	6.17±0.33 A a	5.83±0.28 B ab	5.49±0.20 C a	$F_{2, 124} = 22.77$, $p<0.001$
0.01	4.98±0.42 A b	5.41±0.37 B b	5.50±0.22 C a	$F_{2, 111} = 52.99$, $p<0.001$
0.1	3.47±0.75 A a	4.34±0.35 B c	4.61±0.36 C b	$F_{2, 72} = 7.03$, $p=0.002$
1.0	No Hatch	3.16±2.18 a	3.26±0.39 c	NS
10.0	No Hatch	No Hatch	No Hatch	No Hatch
Retene	3.01±0.34 A c	3.61±0.17 A c	3.08±0.33 B c	$F_{2, 48} = 10.18$, $p<0.001$
Corexit	No Hatch	No Hatch	No Hatch	No Hatch
ANOVA	$F_{5, 193} = 15.12$, $p<0.001$	$F_{6, 233} = 14.01$, $p<0.001$	$F_{6, 205} = 23.73$, $p<0.001$	

Table C6. Results for survival-to-hatch in Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰. Two-Factor ANOVAs were performed using salinity and concentration as the two factors with the interaction term reported. Salinities were then analyzed separately and results indicate which treatment concentrations significantly reduced survival compared to the negative control (Tukey test, $p<0.05$). Non-significant results are labeled as NS. N=3 jars/concentration.

		Conc. (% v/v)	Survival
CEWAF	Interaction $F_{8, 30} = 11.008$, $p < 0.0001$		
	One-way ANOVA: $F_{4,10} = 5.110$, $p = 0.017$		
	7.5‰	0.0001	NS
		0.001	NS
		0.01	NS
	One-way ANOVA: $F_{4,10} = 19.578$, $p < 0.0001$		
	15‰	0.0001	NS
		0.001	NS
		0.01	NS
	One-way ANOVA: $F_{4,10} = 182.568$, $p < 0.0001$		
	30‰	0.0001	NS
		0.001	NS
		0.01	NS
WAF	Interaction $F_{8, 30} = 9.542$, $p < 0.0001$		
	One-way ANOVA: $F_{4,10} = 4.498$, $p = 0.025$		
	7.5‰	0.01	NS
		0.1	NS
		1.0	NS
	One-way ANOVA: $F_{4,10} = 10.478$, $p = 0.001$		
	15‰	0.01	NS
		0.1	NS
		1.0	NS
	One-way ANOVA: $F_{4,10} = 182.568$, $p < 0.0001$		
	30‰	0.01	NS
		0.1	NS
		1.0	NS
Retene	7.5‰		$p < 0.05$
	15‰		$p < 0.05$
	30‰		$p < 0.05$

Table C7. Average blue sac disease severity index (0-1) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰. 2-Factor ANOVAs were performed using salinity and concentration as the two factors with the interaction term reported. Arrows indicate significant differences from the negative control (individual salinities analyzed; Tukey test, $p<0.05$). Uppercase letters indicate significant differences between salinities (individual concentrations analyzed; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{8,30} = 9.82$, $p<0.001$				
Water	0.02 (0.01-0.04)	0.02 (0.01-0.04)	0.01 (0.00-0.03)	NS
0.0001	0.04 (0.00-0.10)	0.06 (0.02-0.10) ↑	0.02 (0.00-0.07)	NS
0.001	0.05 (0.02-0.10)	0.05 (0.02-0.09) ↑	0.05 (0.01-0.12)	NS
0.01	0.32 (0.10-0.59) A ↑	0.26 (0.21-0.31) AB ↑	0.11 (0.04-0.23) B ↑	$F_{2,6} = 8.25$, $p=0.019$
Retene	0.49 (0.46-0.53) A ↑	0.44 (0.36-0.53) A ↑	0.66 (0.47-0.84) B ↑	$F_{2,6} = 16.86$, $p=0.003$
ANOVA	$F_{4,10} = 71.25$, $p<0.001$	$F_{4,10} = 194.28$, $p<0.001$	$F_{4,10} = 112.66$, $p<0.001$	
WAF Interaction: $F_{8,30} = 6.61$, $p<0.001$				
Water	0.02 (0.01-0.04)	0.02 (0.01-0.04)	0.01 (0.00-0.03)	NS
0.01	0.05 (0.00-0.19)	0.05 (0.00-0.20)	0.03 (0.01-0.08)	NS
0.1	0.08 (0.04-0.13) A ↑	0.04 (0.02-0.07) B	0.02 (0.00-0.06) B	$F_{2,6} = 14.31$, $p=0.005$
1.0	0.18 (0.12-0.25) AB ↑	0.23 (0.17-0.30) A ↑	0.12 (0.02-0.30) B ↑	$F_{2,6} = 5.86$, $p=0.039$
Retene	0.49 (0.46-0.53) A ↑	0.44 (0.36-0.53) A ↑	0.66 (0.47-0.84) B ↑	$F_{2,6} = 16.86$, $p=0.003$
ANOVA	$F_{4,10} = 87.25$, $p<0.001$	$F_{4,10} = 76.90$, $p<0.001$	$F_{4,10} = 102.21$, $p<0.001$	

Table C8. Results of survival-to-hatch of Pacific herring embryos exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) WAF and CEWAF. Results (NS or $p<0.05$) indicate significant differences from the negative control (1-Factor ANOVA among oil-concentration combinations and control groups; Tukey test, $p<0.05$). Stocks were analyzed separately. N=3 jars/concentration.

Survival				
	Crude Oil	Conc. (% v/v)	BC Stock Bioassay	AK Stock Bioassay
CEWAF	1-Factor ANOVA		$F_{10, 22} = 5.109$, $p=0.001$	$F_{10,22} = 31.884$, $p<0.0001$
	ANS	0.0001	NS	NS
		0.001	NS	$p < 0.05$
		0.01	NS	$p < 0.05$
	MESA	0.0001	NS	$p < 0.05$
		0.001	NS	NS
		0.01	NS	$p < 0.05$
	ALC	0.0001	NS	NS
		0.001	NS	$p < 0.05$
		0.01	NS	$p < 0.05$
WAF	1-Factor ANOVA		$F_{10, 22} = 7.722$, $p<0.0001$	$F_{10,22} = 9.983$, $p < 0.0001$
	ANS	0.01	NS	NS
		0.1	NS	NS
		1.0	NS	NS
	MESA	0.01	NS	NS
		0.1	NS	NS
		1.0	NS	NS
	ALC	0.01	NS	NS
		0.1	NS	NS
		1.0	NS	NS
Controls	Retene		$p < 0.05$	$p < 0.05$

Table C9. Average length-at-hatch (mm) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) WAF and CEWAF. Arrows indicate significant differences from the negative control (1-Factor nested ANCOVA among oil-concentration combinations and control groups; Tukey test, $p<0.05$). N=3 jars/concentration.

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	ALC	Controls
CEWAF 1-Factor ANOVA: $F_{10, 512} = 5.15$, $p<0.001$; WAF 1-Factor ANOVA: $F_{10, 514} = 5.52$, $p<0.001$					
Water					7.26±0.29
CEWAF	0.0001	7.97±0.19 ↑	7.71±0.32 ↑	7.70±0.24 ↑	
	0.001	7.85±0.17 ↑	7.70±0.16 ↑	7.55±0.30	
	0.01	7.26±0.35	7.21±0.20	7.41±0.31	
WAF	0.01	7.61±0.24	7.81±0.28	7.72±0.24	
	0.1	7.56±0.25	7.83±0.42	7.58±0.26	
	1.0	7.45±0.27	7.91±0.32 ↑	7.51±0.25	
Retene					5.49±0.39
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	ALC	Controls
CEWAF 1-Factor ANOVA: $F_{10, 520} = 18.33$, $p<0.001$; WAF 1-Factor ANOVA: $F_{10, 522} = 14.20$, $p<0.001$					
Water					7.43±0.11
CEWAF	0.0001	7.36±0.17	7.53±0.14	7.41±0.19	
	0.001	7.31±0.26	7.29±0.21	7.38±0.25	
	0.01	6.46±0.25 ↓	6.60±0.27 ↓	7.35±0.16	
WAF	0.01	7.39±0.15	7.34±0.22	7.21±0.21	
	0.1	7.18±0.23	7.35±0.21	7.28±0.21	
	1.0	7.06±0.21	7.10±0.19	7.24±0.16	
Retene					4.66±0.46 ↓

Table C10. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Mediterranean South American (MESA) and Arabian Light (ALC) WAF and CEWAF. Arrows indicate significant differences from the negative control (1-Factor ANOVA among oil-concentration combinations and control groups; Tukey test, $p<0.05$). Stocks were analyzed separately. Non-significant results are labelled as NS. N=3 jars/concentration.

British Columbia Stock Bioassay				
Conc. (% v/v)	ANS	MESA	ALC	Controls
1-Factor ANOVA: $F_{10, 22} = 17.56$, $p<0.001$				
Water				0.13 (0-0.44)
0.0001	0.16 (0.03-0.38)	0.27 (0.05-0.57)	0.24 (0.16-0.33)	
0.001	0.07 (0.03-0.13)	0.15 (0-0.51)	0.16 (0.05-0.32)	
0.01	0.21 (0.15-0.28)	0.37 (0.22-0.54) ↑	0.23 (0.08-0.43)	
Retene				0.85 (0.60-0.98) ↑
1-Factor ANOVA: $F_{10, 22} = 13.68$, $p<0.001$				
Water				0.13 (0-0.44)
0.01	0.13 (0.07-0.21)	0.19 (0.04-0.42)	0.24 (0.04-0.55)	
0.1	0.18 (0.04-0.40)	0.31 (0.10-0.58)	0.18 (0.04-0.39)	
1.0	0.22 (0.02-0.54)	0.21 (0.15-0.28)	0.23 (0.09-0.41)	
Retene				0.85 (0.60-0.98) ↑
Alaska Stock Bioassay				
Conc. (% v/v)	ANS	MESA	ALC	
1-Factor ANOVA: $F_{10, 22} = 35.09$, $p<0.001$				
Water				0.05 (0.01-0.09)
0.0001	0.09 (0.07-0.12)	0.24 (0.20-0.28) ↑	0.14 (0.03-0.30)	
0.001	0.23 (0.08-0.43) ↑	0.23 (0.03-0.54) ↑	0.21 (0.09-0.38) ↑	
0.01	0.38 (0.18-0.60) ↑	0.46 (0.39-0.54) ↑	0.24 (0.06-0.49) ↑	
Retene				0.84 (0.79-0.89) ↑
1-Factor ANOVA: $F_{10, 22} = 19.58$, $p<0.001$				
Water				0.05 (0.01-0.09)
0.01	0.25 (0.13-0.39) ↑	0.24 (0.16-0.33) ↑	0.16 (0.01-0.45)	
0.1	0.24 (0.09-0.43) ↑	0.22 (0.17-0.28) ↑	0.27 (0.09-0.50) ↑	
1.0	0.35 (0.11-0.64) ↑	0.32 (0.09-0.61) ↑	0.23 (0.03-0.54) ↑	
Retene				0.84 (0.79-0.89) ↑

Factors Affecting Toxicity of Dispersed and Undispersed Crude Oils: Herring Species, Spawning Stocks and Environmental Conditions

by

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ABSTRACT

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 this study 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 (AL) crude oil 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 (Alaska North Slope, ANS; Medium South American, MESA; AL). 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 AL 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 AL 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 AL 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 μg/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.

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List of Abbreviations

AL	Arabian Light crude oil
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
ANS	Alaska North Slope crude oil
AhR	Aryl Hydrocarbon Receptor
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
BSD	Blue Sac Disease
CEWAF	Chemically Enhanced Water Accommodated Fraction
CI	Confidence Interval
CYP1A	Cytochrome P450 1A
EC75	75% Effective Concentration
EROD	Ethoxyresorufin-O-deethylase
GC-MS	Gas Chromatography-Mass Spectrometry
LC50	Median Lethal Concentration
LOEC	Lowest Observed Effect Concentration
MESA	Medium South American crude oil
PAH	Polycyclic Aromatic Hydrocarbon
PBC	Prudhoe Bay Crude oil
ROS	Reactive Oxygen Species
SARA	Saturates, Aromatics, Resins, Asphaltenes
TPH	Total Petroleum Hydrocarbon
WAF	Water Accommodated Fraction

Chapter 1: General Introduction

This thesis investigates factors that may affect the toxicity associated with oil spills to herring (*Clupea* sp.) embryos. Oil toxicity can be influenced by environmental conditions such as salinity in coastal and estuarine environments and varying temperature. Risk assessment for oil spills should also consider the differing sensitivity of different species and spawning stocks. Therefore, the first objective of this thesis was to investigate the effect of temperature and salinity on hydrocarbon toxicity to Atlantic herring embryos (*Clupea harengus*). The second objective was to determine if different species (Atlantic and Pacific, *Clupea pallasii*) and spawning stocks of herring differ in sensitivity to chemically dispersed and undispersed crude oils. The third objective was to compare the toxicity of dispersed and undispersed fractions of three different crude oils (Alaska North Slope, ANS; Medium South American, MESA; and Arabian Light, AL) to Pacific herring embryos.

The thesis is presented as two data chapters, each intended to be submitted for publication in the primary literature. Chapter 2 reports results for objective 1. Authors on this paper are Sarah Johnson, Monica Boudreau, Ken Lee and Simon Courtenay and the intended journal is Environmental Toxicology and Chemistry. Chapter 3 reports results for objectives 2 and 3. Authors on this paper are Sarah Johnson, Monica Boudreau, Peter Hodson, Ken Lee and Simon Courtenay and the intended journal is Aquatic Toxicology. Results of the two data chapters are integrated in a final discussion (chapter 4) in which next steps for this line of research are suggested.

1.1 Risk of Oil Spills

Globally, oil transport via seaborne routes has been on the rise since the 1980s while the number of oil spills and the volume released has declined from 1970-2009 (ITOPF 2010a). Most of these oil spills are small (<7,950 litres; ITOPF 2010a) and occur during loading and discharging of oil at ports or terminals. However, occasional accidents at sea typically result in large quantities (>7,950 litres) of oil being spilled, with groundings and collisions accounting for 64% of the total oil lost due to accidents from 1970-2009 (ITOPF 2010a). The average number of these large oil spills during the 2000s was just an eighth of that during the 1970s (from 25.3 to 3.3 spills on average per year; ITOPF 2010a). Similarly, the average volume of oil spilled annually has also been on the decline since the 1970s to 2009 (from ~3 billion litres to 219 million litres; ITOPF 2010a). Although the number of oil spills has been on the decline, the size of tankers is getting larger. This suggests that modern spills, though infrequent, are likely to be large.

Canada produces, imports and exports crude oil, thereby increasing the risk of an oil spill in Canadian coastal waters. In 2011, Canada produced ~6.2 million litres of crude oil per day and exported ~4 million litres per day (~98% of this oil is exported to the United States; National Energy Board retrieved from http://www.neb.gc.ca/clf-nsi/rnrgynfmtn/sttstc/crdlndptrlmprdct/crdlndptrlmprdct-eng.html on August 14, 2012). However, in 2011 Canada imported ~0.086 million litres of heavy crude oil per day and ~1.2 million litres of light crude oil per day into Ontario, Quebec and the Atlantic provinces (National Energy Board retrieved from http://www.neb.gc.ca/clf-nsi/rnrgynfmtn/sttstc/crdlndptrlmprdct/crdlndptrlmprdct-eng.html on August 14, 2012).

Not only is heavy crude oil being transported throughout Atlantic waters but oil wells are being drilled offshore of the Atlantic Provinces. Offshore drilling is occurring off Newfoundland and Labrador at Hibernia, Terra Nova, White Rose and North Amethyst oil fields (Canada-Newfoundland and Labrador Offshore Petroleum Board retrieved from http://www.cnlopb.nl.ca/ on February 22, 2012). Gas platforms are also present off Nova Scotia near Sable Island (Sable Offshore Energy Project; retrieved from http://www.gov.ns.ca/energy/oil-gas/offshore/current-activity/sable-offshore.asp on December 7, 2012). Further exploration is currently underway at Orphan Basin oil field off Newfoundland and at Old Harry oil field in the Gulf of St. Lawrence's Laurentian Channel.

In the past, oil spills have occurred globally and released various crude oils into a variety of environments. For instance, light, medium and heavy crude oil was spilled from the Odyssey tanker (spilled ~136.4 million litres off the coast of Nova Scotia in 1988), the Exxon Valdez tanker (spilled ~42 million litres in Prince William Sound, AK in 1989) and the ABT Summer tanker (spilled ~269 million litres off the coast of Angola in 1991), respectively (ITOPF case history retrieved from http://www.itopf.com/information-services/data-and-statistics/case-histories/index.aspx on July 10, 2011). Oil has also been released in fresh water (pipeline rupture spilling 985,000 litres light crude oil into Pine River, BC in 2000; BC Ministry of Environment retrieved from www.env.gov.bc.ca/eemp/incidents/earlier/pembina_00.htm on July 23, 2012), in brackish water (Mystra tanker in Delaware Bay; National Research Council 1997) and in saltwater of varying temperatures. For instance, the Exxon Valdez spill occurred in 3.5-5.5°C (~42 million litres into Prince William Sound, AK in 1989; Gay

and Vaughn 2001; ITOPF 2010a), the Metula spill in 8-10°C (~50 million litres off Chile in 1974; ITOPF case history retrieved from http://www.itopf.com/information-services/data-and-statistics/case-histories/index.aspx on July 10, 2011), the Prestige spill in 13°C (~65 million litres off northern Spain in 2002; ITOPF case history retrieved from http://www.itopf.com/information-services/data-and-statistics/case-histories/index.aspx on July 10, 2011; Peteiro *et al.* 2006). Recently, the Deepwater Horizon oil platform explosion (~840 million litres over ~90 days into the Gulf of Mexico in 2010 at ~1500m; Diercks *et al.* 2010; McNutt *et al.* 2011) occurred in 28°C at the surface (retrieved from http://www.aoml.noaa.gov/phod/dhos/xbt_ctd.php on February 22, 2012) and 4.5°C at the wellhead (retrieved from www.earthgauge.net/wp-content/EG_Gulf_oil_spill_Microbes.pdf on February 22, 2012). Therefore, it is clear that different crude oils are spilled into waters with varying salinity and temperature. Since the toxicity associated with these oil spills may depend to a great extent on the particular crude oil spilled and the receiving environment, toxicity testing of crude oils at various salinity and temperature conditions require further experimentation.

1.2 Contamination Risk

Following an oil spill, the initial impact of floating crude oil can have severe effects on wildlife living at the water surface, such as birds and marine mammals. For instance, an oil slick could mimic floating food and attract fish to the surface, which could attract predatory birds to dive into the slick in an effort to catch their prey (Australian Maritime Safety Authority retrieved from http://www.amsa.gov.au/marine_environment_protection/educational_resources_and_information/teachers/the_effects_of_oil_on_wildlife.asp on October 25, 2011). Birds may

also be attracted to an oil slick if the floating oil appears to be calmer water from an aerial view (Timoney and Ronconi 2010). Crude oil contamination of birds and marine mammals could lead to hypothermia (oil disrupts the insulation and waterproofing of feathers and fur), weight loss (due to increased metabolic rate while trying to maintain body temperature), starvation (due to abandonment or lack of feeding ability), or drowning (due to the oil weight; Timoney and Ronconi 2010; Australian Maritime Safety Authority retrieved from http://www.amsa.gov.au/marine_environment_protection/educational_resources_and_information/teachers/the_effects_of_oil_on_wildlife.asp on October 25, 2011). Following the Exxon Valdez oil spill, approximately 36,000 birds and 1,000 sea otters died from March to September 1989 (Maki 1991). More recently, one year after the Deepwater Horizon oil spill (April 2011), mortality was tallied at 6,147 birds and 157 mammals with an additional 2,086 birds and 2 mammals oiled but still alive (Deepwater Horizon Response Consolidated Fish & Wildlife Collection Report retrieved from dailydeadbirds.com on October 25, 2010). These numbers are likely to be underestimates since oiled birds have been observed to sink out of sight (Timoney and Ronconi 2010).

Coastline ecosystems are also at risk of contamination following an oil spill since ocean currents and wind can drive oil slicks ashore and physically contaminate plants and trees (such as sea grasses and mangroves) as well as the shores and microscopic inhabitants (Amoco Cadiz, Exxon Valdez, Sea Empress, Metula; ITOPF case history retrieved from http://www.itopf.com/information-services/data-and-statistics/case-histories/index.aspx on July 10, 2011). Coastal ecosystems are ecologically important since they provide feeding, breeding, and nursery grounds for plants, animals, and

insects crucial to the food chain (Burke *et al.* 2001). Shores are difficult to restore once oil contamination has occurred. For example, large amounts of sediment can be combined with the oil on high wave energy shores to form tar mats, while oil may remain in muddy sediments (typical of sheltered shores) for an extended period of time (ITOPF 2002).

Oil spills may also affect marine life including pelagic and benthic organisms, which rely on estuaries and coastal waters as spawning and nursery grounds (Maki 1991; Locke and Courtenay 1995; Carls *et al.* 2002). This is especially important since oil droplets can bind to suspended particulates in the water column, causing the oil to sink to the bottom (ITOPF 2002) where benthic spawners deposit their eggs, resulting in oil exposure during sensitive early-life stages. McIntosh *et al.* (2010) determined that the most sensitive stages of Atlantic herring early-life development were gametes, embryos one to three days old and newly hatched larvae. In the field, the impact of spilled oil was observed in Pacific herring embryos present during the Exxon Valdez oil spill. Spawning activity was average in the year of the spill (1989) and the next year (Maki 1991) but mortality of embryos and larvae resulted in such a severe reduction to the 1993 spawning population (~75-86%) that the herring fishery collapsed (Carls *et al.* 2002; Exxon Valdez Oil Spill Trustee Council 2010).

1.3 Hydrocarbon Toxicity

Crude oil contains saturates, aromatics, resins and asphaltenes (SARA; Environmental Protection Agency, EPA 2003; Wang *et al.* 2006). Saturates are non-aromatic hydrocarbons in crude oil, including both normal and branched alkanes and cycloalkanes (EPA 2003), which contain no double bonds (Wang *et al.* 2006). The n-

alkanes (paraffins) range from C_5 to C_{40}, while large n-alkanes ($>C_{18}$) are known as waxes (Wang and Fingas 2006). In general, n-alkanes are often the predominant compounds found in crude oil (Wang and Fingas 2006). Aromatics are cyclic, planar hydrocarbons that include BTEX (benzene, toluene, ethylbenzene and xylene), PAH (polycyclic aromatic hydrocarbons) and some heterocyclic aromatics (EPA 2003; Wang *et al.* 2006). Resins and asphaltenes are large, polar hydrocarbons that are non-volatile (EPA 2003). ANS, a medium crude oil, and AL, a light crude oil, contain 72.1% and 73.3% saturates, 16.0% and 16.9% aromatics, 7.4% and 6.9% resins, 4.4% and 3.8% asphaltenes and 2.9% waxes for both oils, respectively (EPA 2003). PAHs typically comprise 0.5-4% of crude oil (Wang *et al.* 2006), more specifically ANS crude contained 1.1-1.5% total PAH and AL crude contained 0.8-0.9% total PAH (1.4-1.7X more total PAH in ANS than AL; EPA 2003).

The composition of crude oil following a spill, and ultimately the toxicity, is influenced by the degree of weathering through physical, chemical and biological processes that change the properties of the crude oil (Wang *et al.* 2006). Lightly weathered oils typically have lost volatile components (C_3-benzenes and BTEX) due to evaporation (EPA 2003; Wang *et al.* 2006). As oil continues to weather, saturates are degraded by microbes and removed from the oil more quickly than aromatics (Wang and Fingas 2003; Wang *et al.* 2006). Although saturates are not as water-soluble as aromatics and are readily broken down through microbial activity, these lower molecular weight (LMW) compounds can be acutely toxic at high concentrations (Khan 2007). However, since saturates do not persist as long as aromatics, they are not a concern in terms of chronic effects in the early life stages of fish (Khan 2007). Resins and asphaltenes are also not a direct concern in causing chronic toxicity since they have very low water solubility and

are likely too large to be taken up by embryos (Khan 2007). Therefore, the components in weathered oil that contribute to chronic toxicity are primarily aromatics and more specifically PAHs.

PAHs are considered to be one of the most toxic classes of hydrocarbons found in crude oils (Carls *et al.* 1999; Heintz *et al.* 1999; Baussant *et al.* 2001; Cohen *et al.* 2001; Billiard *et al.* 2002; Barron *et al.* 2004; Billiard *et al.* 2008; Carls *et al.* 2008) and are believed to be responsible for morphological abnormalities resulting from oil exposure (Carls *et al.* 1999; Barron *et al.* 2004; Incardona *et al.* 2004; Billiard *et al.* 2006; Billiard *et al.* 2008; Boudreau *et al.* 2009; McIntosh *et al.* 2010). This suite of morphological abnormalities is known as blue sac disease (BSD) which includes: spinal curvature, pericardial edema (fluid collecting in the pericardial sac), yolk sac edema (fluid collecting in the vitelline vasculature), jaw malformations, haemorrhaging, fin rot, cardiovascular dysfunction, decreased length and increased mortality (Brinkworth *et al.* 2003; Winchester 2003; Billiard *et al.* 2008; Scott and Hodson 2008; Boudreau *et al.* 2009).

Even though PAHs are considered to be one of the most toxic classes of hydrocarbons found in crude oils, exposures to certain PAHs results in morphological abnormalities while others do not. Khan (2007) exposed juvenile rainbow trout and medaka *(Oryzias latipes)* to fractions of ANS and Scotian light crude oil. Fractions containing saturates, monoaromatics (BTEX), naphthalenes and dibenzothiophenes did not induce CYP1A (see below) or produce BSD in juvenile rainbow trout and medaka, while exposure to a fraction containing phenanthrenes, napthobenothiophenes and fluorenes and another fraction containing pyrenes and chrysenes did induce CYP1A and BSD. More specifically, Incardona *et al.* (2004) exposed zebrafish (*Danio rerio*)

embryos to individual PAHs and found that pyrene, fluorene, dibenzothiophene, and phenanthrene (PAH containing 2-4 aromatic rings) were biologically active, while naphthalene, anthracene, and chrysene (also contains 2-4 aromatic rings) did not cause morphological abnormalities. Likewise, Laughlin and Neff (1979) found that mud crab (*Rhithropanopeus harrisii)* larvae exposed to phenanthrene experienced reduced survival while exposure to naphthalene did not affect survival. Therefore, PAHs responsible for embryo toxicity are likely phenanthrenes, dibenzothiophenes, fluorenes and pyrenes while naphthalenes, chrysenes and anthracenes do not appear to be embryo toxic.

1.4 Mechanism of Toxicity

BSD was first described as being common in hatcheries as a result of poor water quality (containing products of fish metabolism such as ammonia and urea; Wolf 1957). Exposure of fish embryos to dioxins (Walker *et al.* 1991) and PAHs (Brinkworth *et al.* 2003) has also resulted in BSD. The induction of CYP1A enzymes, and their potential role in toxicity (see below), is what distinguishes chemically induced BSD (through PAH or dioxin exposure).

The etiology of PAH induced lesions is through cardiac dysfunction (Walker *et al.* 1991; Billiard *et al.* 1999; Couillard 2002; Incardona *et al.* 2004; Billiard *et al.* 2006; Incardona *et al*. 2009), which may cause subsequent abnormalities in different systems (Couillard 2002; Incardona *et al.* 2004). Cardiac dysfunction can be measured through heart rate, which is reduced following exposure to PAHs (Incardona *et al.* 2004). Reduced circulation could promote the formation of both pericardial and yolk sac edemas because the heart is too weak to move the fluid throughout the body (Incardona

et al. 2004). It is also plausible that PAH produce oxidative stress resulting in peroxidation of lipid membranes that would lead to edema and haemorrhaging (Cantrell *et al.* 1998). Since the blood in herring embryos and larvae is transparent, it is difficult to determine if an organism has haemorrhaged by gross observation. However, Guiney *et al.* (1997) exposed lake trout to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and observed that the proteins in the yolk sac edema fluid were similar to those found in plasma. This suggests that edemetous fluid is blood leaking from the endothelial vasculature of the yolk sac. Reduced circulation could also be causing jaw malformations either directly (Billiard *et al.* 1999; Incardona *et al.* 2004) or indirectly by producing a large edema that prevents the jaw from closing. Spinal curvatures can also be the result of a lack of blood flow through the embryo and may ultimately reduce swimming ability (Billiard *et al.* 1999; Incardona *et al.* 2004). Larvae that cannot swim in a directed manner are unlikely to be able to actively feed and escape predators. Reduced blood flow through the vitelline vasculature could also reduce the amount of yolk nutrients absorbed and could therefore reduce the growth and overall development of the embryo (Walker *et al.* 1991; Billiard *et al*. 1999).

Exposure to hydrocarbons has been shown to cause both premature and delayed hatching in herring (Linden 1978; Kocan *et al.* 1996). According to Linden (1978), herring time-to-hatch was delayed following exposure to light fuel oil, while embryos hatched prematurely following exposure to Venezuelan crude oil. Delayed hatching could be due to the high concentration of aromatic hydrocarbons, which could cause the metabolic rate to be reduced during incubation (Linden 1978). Exposure to low concentrations of benzene accelerated the developmental rate (Struhsaker *et al.* 1974) and could lead to premature hatching (Linden 1978).

Abnormalities on the epithelial surface of herring larvae following hydrocarbon exposure are associated with BSD but are likely caused by bacterial infection. Fin rot is a skin abnormality associated with BSD (Scott and Hodson 2008). A similar lesion was observed in herring larvae, classified as peduncle disease, following exposure to hydrocarbons (Boudreau *et al.* 2009). *Flavobacterium psychrophilum* (historically from the bacterium genus *Cytophaga*) is the etiological agent responsible for causing cold-water peduncle disease and has the ability to lyse fish erythrocytes and bacterial cells (Lorenzen *et al.* 1997; Nematollahi *et al.* 2003). Even if the organism is not invaded by this pathogen the body may appear ragged and rough (Post 1987). *Flavobacterium psychrophilum* survives in fresh and brackish waters with a tolerance limit of approximately 8‰ to 10‰ (Pacha 1968; Lorenzen *et al.* 1997; Madetoja *et al.* 2001; Namatollahi *et al.* 2003) and temperatures between 3°C and 20°C (Pacha 1968; Post 1987; Madetoja *et al.* 2001; Nematollahi *et al.* 2003). Similar lesions may be observed in saltwater fish due to *Tenacibaculum maritimum* infection (a saltwater pathogen belonging to the same family as *Flavobacterium psychrophilum;* Namatollahi *et al.* 2003).

Although the specific mechanism causing the BSD symptoms is not well understood, it is widely acknowledged that the toxicity observed is linked to the aryl hydrocarbon receptor (AhR) binding and cytochrome P450 1A (CYP1A) induction (Billiard *et al*. 1999; Billiard *et al.* 2002; Barron *et al.* 2004; Bauder *et al.* 2005; Incardona *et al.* 2005). PAHs bind to the AhR, a ligand-activated transcription factor, which then causes the CYP1A gene to be expressed and the CYP1A enzyme to be produced (Billiard *et al.* 2002; Bauder *et al*. 2005; Incardona *et al.* 2005; Incardona *et al.*

2006; Scott and Hodson 2008). These CYP1A enzymes have been shown to both increase and decrease the toxicity of PAHs (Billiard *et al.* 2008). PAHs metabolized by CYP1A enzymes into reactive intermediates, that are more toxic than the parent PAH, can cause toxicity to be higher (Billiard *et al.* 2008). Reactive intermediates may cause oxidative stress (Schlezingler *et al.* 1999; Scott and Hodson 2008; Billiard *et al.* 2008) that can overwhelm antioxidant defenses and damage proteins, lipids and DNA (Bauder *et al*. 2005). Lipid peroxidation can damage endothelial membranes of the vascular system and cause edema and haemorrhaging (Bauder *et al*. 2005; Incardona *et al.* 2005; Incardona *et al.* 2006). CYP1A inhibition can also increase toxicity of PAHs since lowering the rate of metabolism extends the half-life of the PAHs, allowing the PAH to persist longer (Billiard *et al.* 2008). Generally, blocking CYP1A enzyme activity results in the protection against PAH exposure, likely due to the inability to metabolize PAHs into reactive intermediates (Billiard *et al.* 2008). However, functional CYP1A enzymes may also confer protection to embryos exposed to PAH by metabolizing the PAHs to reactive intermediates with relatively low toxicity (Billiard *et al.* 2008). Therefore, the pathway of AhR binding and subsequent CYP1A induction in predicting toxicity is complex.

1.5 Oil Spill Countermeasures

1.5.1 Mechanical cleanup

In an effort to reduce contamination risks to wildlife and sensitive areas following an oil spill, oil slicks may be contained using booms and removed from the surface by skimming, pumping, or *in-situ* burning (Fingas 2004; ITOPF 2010b). However, problems may be encountered that reduce the effectiveness of removal

techniques. For instance, water temperatures are usually lower than vessel temperatures; consequently once spilled the viscosity of the crude oil increases making skimming and pumping more difficult (ITOPF 2002). *In-situ* burning requires that the volatile components have not evaporated and sufficient oil thickness be maintained within fireproof booms (ITOPF 2002; Fingas 2004; ITOPF 2010b). Therefore, timely deployment of appropriate equipment is essential for *in-situ* burning to be successful.

In the event that oil goes ashore, the result may be a thick emulsion, which could require labour intensive removal by shovel (ITOPF 2002). Less viscous forms could be removed by vacuum, washing at high and low temperatures and pressures, or applying absorbent materials (Fingas 2004; ITOPF 2010b). However, washing at high pressure and temperature could result in further damage to the microbial community that would otherwise aid in degradation (ITOPF Shoreline Clean Up retrieved from http://www.itopf.com/spill%2Dresponse/clean%2Dup%2Dand%2Dresponse/shoreline%2Dclean%2Dup/ on October 27, 2011).

1.5.2 Chemical Dispersion

Chemical dispersants are a mixture of surfactants and solvents used to accelerate the dispersion of an oil slick into the water column (Fiocco and Lewis 1999). Following their application, the solvent distributes the surfactants (molecules containing both lipophilic and hydrophilic ends) throughout the oil. The surfactant positions at the oil-water interface with the lipophilic end in contact with the oil and the hydrophilic end in contact with the water. This reduces surface tension and promotes the formation of oil droplets that disperse throughout the water column as a result of turbulent mixing (Fingas 2004; ITOPF 2005). Surfactant molecules remain on the oil droplet surface to

ensure that the oil does not re-coalesce and return to the surface of the water (ITOPF 2005). The trade-off between reducing physical impacts of a surface oil slick to birds, mammals and coasts is increased chemical impacts on biota throughout the water column. For instance, chemical dispersants were applied following the Deepwater Horizon oil spill and resulted in plumes of dispersed oil lingering at depths between 500 and 1300m (Diercks *et al*. 2010).

Oil droplets are not directly toxic to fish embryos since the dispersed oil droplet is too large to cross the chorion; the PAHs must dissolve in the water to be biologically available (Carls *et al.* 2008). Carls *et al.* (2008) observed that oil droplets could drift past zebrafish eggs without adhering to the chorion, indicating that the egg is hydrophilic. The chorions of zebrafish, pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring are smooth and do not contain filaments that could trap oil particles on the surface of the egg (Carls *et al.* 2008). The chorion pore size of various fish species varies between 0.13-0.53 µm (Markle and Frost 1985; Carls *et al.* 2008). Therefore, oil droplets within this range would be able to pass through the chorion but larger oil droplets would be blocked. Chemically dispersed oil droplets are smaller than those generated by physical dispersion (Bobra *et al.* 1989; Li *et al*. 2009; Li *et al.* 2011) and are typically 1-70 µm in diameter if effective dispersion was achieved, but droplets are most stable if less than 45 µm in diameter (ITOPF 2005). Li *et al.* (2011) reported that chemical dispersion of MESA and ANS crude oils produced oil droplets <10 µm in diameter. Since oil droplets are larger than the pore diameter, the passage of oil droplets through the chorion into the egg would be blocked (Carls *et al.* 2008). The oil droplet size also influences PAH solubility. As the droplet size is reduced, the surface area is

increased for a particular volume of oil, which increases the rate at which PAH partition to water to reach their solubility limits (Ramachandran *et al.* 2004). In summary, the oil droplet is acting as a reservoir for PAHs and the PAHs dissolved from that reservoir are responsible for causing toxicity.

1.7 Dispersant Effectiveness and PAH Solubility

Hydrocarbon concentration in the water column following chemical dispersion of an oil spill is influenced by the dispersant effectiveness and solubility of PAHs. 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). Many factors influence the effectiveness of chemical dispersants such as the crude oil composition, degree of weathering, sea energy, water temperature and salinity (Mackay *et al.* 1984; Daling 1988; Moles *et al.* 2001; Moles *et al.* 2002; EPA 2003; Fingas 2004; Chandrasekar *et al.* 2005; Nordvik 2005; Chandrasekar *et al.* 2006).

Crude oil composition is a key factor in determining chemical dispersant effectiveness. Crude oils containing higher proportions of smaller aliphatic hydrocarbons and PAHs will experience higher dispersant effectiveness, while oils high in large aliphatic hydrocarbons (>C20) will likely result in lower dispersant effectiveness (Fingas 2008). According to the EPA (2003), chemical dispersion of ANS using Corexit 9500 resulted in 15-47% dispersion while dispersion of AL resulted in 8-19% dispersion, depending on the degree of weathering. Increased dispersant effectiveness on ANS could be due to higher PAH concentrations relative to AL.

Chemical dispersants are most effective if applied immediately onto an oil spill, since the dispersibility of crude oil is reduced as it weathers (Moles *et al.* 2001; EPA

2003; Nordvik 2005). Weathering increases the viscosity of the oil and decreases the effectiveness of the chemical dispersant (Chandrasekar *et al.* 2005). Dispersant effectiveness of Corexit 9527 on ANS crude was reduced as the oil weathered and the oil was no longer dispersible after 120 hours of weathering (Nordvik 2005). Moles *et al.* (2002) measured dispersant effectiveness of Corexit 9500 on fresh and weathered ANS crude to be approximately 25% and <10%, respectively. Chandrasekar *et al.* (2005) also reported that increased dispersant effectiveness was observed on less weathered Prudhoe Bay crude (PBC) oil.

Improved mixing of the oil and dispersant will lead to increased dispersant effectiveness, which is directly influenced by sea energy. High wave energy will produce more dispersed oil droplets and keep more of the droplets suspended in the water column (Fingas 2004). In the laboratory, as mixing energy increased from 150 to 250 rpm (using an orbital mixer) the dispersant effectiveness on fresh South Louisiana crude and Prudhoe Bay Crude oil (PBC) increased from 55% to 90% and 21% to 70%, respectively (Chandrasekar *et al.* 2005).

Water temperature is another factor that influences the effectiveness of a chemical dispersant by altering oil viscosity. In colder water the oil and dispersant become more viscous (EPA 2003; Chandrasekar *et al.* 2005) requiring more energy to mix the dispersant into the oil. Laboratory tests have illustrated that increasing the temperature from 3ºC to 35ºC increased the dispersant effectiveness on various crude oils (Daling 1988; Moles *et al.* 2001; Chandrasekar *et al.* 2005, 2006). Dispersant effectiveness also increased on PBC oil as temperature increased from 5ºC to 22ºC. However, when the temperature was further increased from 22ºC to 35ºC, the dispersant effectiveness was reduced (Chandrasekar *et al.* 2005, 2006). It is possible that if the

water temperature becomes too high for a particular oil that further weathering could occur, thereby, increasing the oil viscosity and reducing dispersant effectiveness (Chandrasekar *et al.* 2005).

Salinity influences the solubility of the chemical dispersant and ultimately determines dispersant effectiveness. Increasing salinity causes the surfactant to be less soluble in water, which causes the surfactant to remain at the oil-water interface, promoting droplet formation (Mackay *et al.* 1984; Chandrasekar *et al.* 2006). Researchers (Blondina *et al.* 1999; Moles *et al.* 2001 Chandrasekar *et al.* 2006) have found that dispersant effectiveness increased as salinity increased from 10‰ to 34‰.

The solubility of PAHs is also influenced by a combination of the water temperature and salinity. Whitehouse (1984) studied these effects simultaneously and found that salinity and temperature both influenced PAH solubility, but these factors did not interact. In general, the solubility of PAHs is higher in less saline and also warmer water (May and Wasik 1978; Whitehouse 1984; Ramachandran *et al.* 2006). Whitehouse (1984) determined that PAHs (3 to 5 aromatic rings: phenanthrene, anthracene, 2-methylanthracene, 2-ethylanthracene, benzo (a) pyrene) required a small increase in temperature and a large decrease in salinity in order to substantially increase the solubility of these PAHs. Ramachandran *et al.* (2006) also reported that as salinity increased there was a greater decrease in parent and alkylated PAHs with two (napthalenes and dibenzothiophrenes) and three aromatic rings (phenanthrenes) while PAHs with four or greater (pyrenes and chrysenes) aromatic rings were only slightly reduced.

1.8 PAH Concentrations and Duration Following Oil Spills

Experimental oil spills have been conducted to determine the dynamics of hydrocarbon concentrations in the water column. An experimental spill of 15,000 litres was conducted in cold (-0.8°C to -1.8°C), saline (19‰ to 31‰) water off Baffin Island, Northwest Territories in August 1981. Measured concentrations of hydrocarbons under the dispersed oil spill were greater than 50,000 µg/L for 12 hours and were detectable for more than four days at a depth of 10 metres, while the undispersed spill only generated hydrocarbon concentrations of 1,000 µg/L at a depth of 1 metre at low tide and was undetectable at high tide (Buckley *et al.* 1987; Humphrey *et al*. 1987). Off the coast of New Jersey, experimental spills were conducted by releasing 1,670 litres into relatively warm water (13°C). The average total hydrocarbon concentration measured 20-60 minutes after dispersion at depths of 1, 3, 6 and 9 metres was 700, 700, 300 and 200 µg/L for La Rosa crude oil and 3100, 2400, 500 and 400 µg/L for Murban crude oil (McAuliffe *et al.* 1980). Another experimental spill conducted in warmer (28.5°C), saline (32‰) water, off the Caribbean coast of Panama, released 715 litres of dispersed oil and 953 litres of undispersed oil. Measured concentrations of hydrocarbons at 3-5 metres under the spill reached 222,000 µg/L and 90 µg/L immediately following dispersed and undispersed oil release, respectively. After three days the total measured hydrocarbons were 8.9 µg/L and 10.2 µg/L under the dispersed and undispersed oil spills (Ballou *et al.* 1987).

Hydrocarbon concentrations have also been quantified following accidental spills. Directly following the Exxon Valdez spill (42 million litres oil into saltwater at 3.5°C to 5.5°C), total PAH concentrations in the water column (1-5 metres deep) were

higher adjacent to beaches that were heavily oiled (1.26-6.24 µg/L) than adjacent to unoiled beaches (0.91-2.23 µg/L; Short and Harris 1996). After five weeks, the total PAH concentration was still 1.59 µg/L in the water column (1 metre depth) adjacent to a heavily oiled beach (Short and Harris 1996). More recently, three weeks after the Deepwater Horizon oil platform explosion, (795 million litres into saltwater at 28°C at the surface and 4.5°C at the wellhead) total PAH concentrations in the water column were quite high: 84.8 µg/L at the surface, 29.4 µg/L at 1160 metres and 189 µg/L at 1320 metres (Diercks *et al.* 2010). Since oil induced lesions have been observed at these concentrations (Carls *et al.* 1999; Carls *et al.* 2002) a spill could have the potential to impair early life stages of fish in the receiving environment.

1.9 Herring: Test Organism

Herring are fish species of ecological and economic importance. They connect the base of the food web (plankton) to higher order marine organisms, since many fish, marine mammals and waterfowl rely on this pelagic species as their food source (Stewart and Arnold 1994). The Clupeoidae suborder, which includes Atlantic and Pacific herring, is economically important since it contributes to one-third of the global fish catch (Stewart and Arnold 1994) and supports markets for fresh, frozen and smoked flesh as well as herring roe, oil and fishmeal (Stewart and Arnold 1994; Hay *et al.* 2001; Burke and Phyne 2008). The landed value of Atlantic herring from Eastern Canada was $27.9 million in 1992 (Stewart and Arnold 1994) and increased to $40.4 million by 2005 (Burke and Phyne 2008). The value has been consistently between $30 million and $43 million over the past seven years (DFO Commercial Fisheries Statistics retrieved from

http://www.dfo-mpo.gc.ca/stats/commercial/land-debarq/sea-maritimes/s2011av-eng.htm on October 31, 2012).

The distribution of herring is circumpolar. Atlantic herring are found on both sides of the North Atlantic between Iceland and the Strait of Gibraltar in the east and between northern Labrador and Greenland to North Carolina in the west (Stewart and Arnold 1994). Pacific herring, which is a closely related species (Grant and Utter 1984; Grant 1986; Shaw *et al.* 1999), are found on both sides of the Pacific from southern California to Alaska and the Bering Sea in the east, and south to Japan in the west (McHugh 1954; Haegele and Schweigert 1985).

Herring spawn in the intertidal and upper subtidal zones with each stock having its characteristic spawning time and place (Iles and Sinclair 1982; Winters and Wheeler 1996; Bekkevold *et al*. 2005). Atlantic herring have a spring and fall spawning stock that spawn from April to June and August to November, respectively (Haegele and Schweigert 1985). Spring spawning herring typically spawn in the north while fall spawning is predominately in the south (Haegele and Schweigert 1985). Pacific herring only have a spring spawning stock that may begin spawning in November in California and spawn as late as July in Northern Alaska (Haegele and Schweigert 1985). Atlantic herring typically spawn offshore at depths of 5 to 150m (Blaxter 1985), depending on the temporal stock (Steward and Arnold 1994). Atlantic herring spring spawning stocks typically spawn on coarse substrate in water less than 5 metres deep in the Gulf of St. Lawrence and up to 10 metres deep in eastern Newfoundland (Steward and Arnold 1994). Fall spawning stocks of Atlantic herring typically spawn in deeper waters of 10-25 metres in the Gulf of St. Lawrence and up to 40 metres off southwestern Nova Scotia (Steward and Arnold 1994). Pacific herring spawn in shallow intertidal and subtidal

regions at depths <20 metres, but usually within 10 metres (Haegele and Schweigert 1985). At these shallow depths, spilled oil could expose embryos to toxic concentrations of PAHs.

Herring typically spawn in high-energy environments. Therefore, it is imperative that the eggs be deposited on stable material (marine vegetation, algae, sea grass and rocky substrate) to prevent translocation (Blaxter 1985; Haegele and Schweigert 1985). Demersal eggs adhere to substrates due to mucopolysaccharides that are present on the zona radiate externa (part of the egg envelope; Riehl and Appelbaum 1991). These high-energy environments benefit the developing embryos by providing oxygen, removing metabolic wastes, preventing siltation and sustaining high levels of primary production (Haegele and Schweigert 1985). However, in the event of an oil spill, water turbulence can cause crude oil to naturally disperse into the water, thereby increasing the risk of exposure (ITOPF 2002).

Herring are considered to be euryhaline and stenothermal. Studies have demonstrated their ability to tolerate salinities between 2‰ and 37.5‰, with optimal survival at salinities of 12‰ to 17‰ (Ford 1930; McMynn and Hoar 1953; Alderdice and Velsen 1971; Blaxter 1985; Haegele and Schweigert 1985). Pacific herring also spawn in environments where salinity varies greatly (8‰ to 28‰; Queen Cove, Vancouver Island, BC; McMynn and Hoar 1953). Herring embryos can tolerate temperatures between 0.5°C to 20°C (Ford 1930; McMynn and Hoar 1953; Alderdice and Velsen 1971; Blaxter 1985; Haegele and Schweigert 1985; Reid *et al.* 1999). Temperature tolerance during spawning of herring is limited, with spawning typically occurring at 6°C to 15°C in the Gulf of Maine, more specifically at 12°C to 15°C on Georges Bank, 6°C to 13°C on Nantucket Shoals, and 8°C to 12°C near Grand Manan

Island (Reid *et al.* 1999). However, the temperatures at which herring spawn depend to a great extent on the spawning season. Spring spawning Atlantic herring typically spawn in relatively cool water (approximately 5°C) while fall spawning herring spawn in warmer water (approximately 15°C in Gulf of St. Lawrence and 10°C off southwest Nova Scotia; Steward and Arnold 1994).

Herring are extremely sensitive to oil exposure. Rice *et al.* (1979) tested the sensitivity of 39 Alaskan species of marine fish and invertebrates to crude oil and found that adult Pacific herring had the second lowest 96-h LC50. Of the nine marine fish species, Pacific herring was also the most sensitive with 96-h LC50s ranging from 1,220 to >11,720 µg/L (Rice *et al.* 1979). Atlantic herring embryos have also been shown to be more sensitive to Orimulsion-400 (70% bitumen and 30% water) exposure than another Atlantic coastal fish, the mummichog (*Fundulus heteroclitus;* Boudreau *et al.* 2009).

The Exxon Valdez oil spill led to significant research into the toxicological effects of dispersed oil on Pacific herring embryos, including the development of the sticky-egg bioassay. To enable field toxicity testing following the spill, herring eggs were adhered to glass microscope slides within an exposure container and positioned within the water column (Kocan *et al.* 1996). This and other studies demonstrated that exposure to crude oil resulted in morphologic, genetic and histopathologic damage in Pacific herring embryos following the Exxon Valdez spill (Brown *et al.* 1996; Hose *et al.* 1996; Kocan *et al.* 1996; Norcross *et al.* 1996; McGurk and Brown 1996), with as little as 0.4-0.7µg/L PAH causing edema and reducing growth (Carls *et al.* 1999; Carls *et al.* 2002). A modified sticky-egg bioassay has also been validated with Atlantic herring, which facilitates transfer into fresh test solutions and observations under the

microscope to assess toxicity (Boudreau *et al.* 2009; McIntosh *et al.* 2010; Greer *et al.* 2012).

1.10 Objectives

Since the majority of oil spills occur in coastal waters, where herring species spawn, it is important to establish the toxic of dispersed and undispersed crude oil to early life stages of Atlantic herring and Pacific herring, depending on environmental conditions and the particular crude oil released. The primary objectives of the present study were to:

1) Determine if temperature and salinity influence hydrocarbon toxicity to Atlantic herring;
2) Compare the toxicity of three chemically dispersed and undispersed crude oils (Alaska North Slope, ANS; Medium South American, MESA; and Arabian Light, AL) to Pacific herring; and
3) Determine whether different species (Atlantic and Pacific) and spawning stocks of herring respond similarly to chemically dispersed and undispersed AL crude oil.

The data collected in the present study will provide more detailed information to guide decisions on use of chemical dispersants in coastal waters. Specifically, this research will tell responders whether they need to take into consideration environmental conditions (temperature and salinity) and presence of herring (different stocks of Pacific and Atlantic herring) in assessing potential toxicity of dispersed oil.

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Chapter 2: Effect of salinity and temperature on the toxicity of dispersed oil to Atlantic herring (*Clupea harengus)* embryos exposed to Arabian Light crude oil

2.1 Abstract

In the event of an oil spill managers considering use of a chemical dispersant need to know and/or consider how dispersed oil may affect the early-life stages of coastal fish species under different environmental conditions. In this study, the toxicity of graded concentrations of chemically dispersed (0.0001-10% v/v CEWAF; Corexit 9500) and undispersed water accommodated fractions (0.01-1% v/v WAF) of Arabian Light (AL) crude oil were tested throughout the embryonic development of Atlantic herring (*Clupea harengus*) at 7.5, 15 and 30‰ and at 7, 10, and 15°C. Toxicity was based on survival-to-hatch, length-at-hatch, and the Blue Sac Disease Severity Index (BSD SI; incorporating mortality, swimming ability, prevalence and severity of spinal curvature, pericardial edema, yolk sac edema, and prevalence of jaw malformation and skin lesion). Greater toxicity of dispersed oil was observed at the lowest temperature (7°C) tested, at least for fall-spawned Atlantic herring, which normally spawn at higher temperatures. Greater toxicity at low temperature appeared to result from one or more of: cold stress, prolonged exposure period or decreased metabolism of toxicants rather than from increased dispersant effectiveness or PAH solubility. Effects of salinity were less clear but reduced length-at-hatch and increased morphological abnormalities suggested greater toxicity at lower salinity. Additional tests with higher doses of CEWAF and WAF will be required to confirm this effect.

2.2 Introduction

Oil spills can have devastating impacts on aquatic environments. To protect fauna and reduce impacts of spilled oil on coastal habitats, chemical dispersants can be applied to the surface of an oil slick to remove and dilute the oil throughout the water column (ITOPF 2010b). The potential benefits to coastal habitats is countered by an increased exposure to toxic components of oil, such as polycyclic aromatic hydrocarbons (PAHs), for pelagic and benthic fish species which may use the receiving environment as nursery grounds (Cohen *et al.* 2001; Ramachandran *et al.* 2004; Clarke 2007; Greer 2011). Nursery grounds are especially sensitive to oil exposure as indicated by the declines in Pacific herring (*Clupea pallasii*) populations following the 1989 Exxon Valdez spill in Prince William Sound, Alaska (Carls *et al.* 2002; Exxon Valdez Oil Spill Trustee Council 2010).

In the past, spills have occurred in fresh water (pipeline rupture spilling 985,000 litres light crude oil into Pine River, BC in 2000; BC Ministry of Environment retrieved from www.env.gov.bc.ca/eemp/incidents/earlier/pembina_00.htm on July 23, 2012), brackish water (Mystra tanker in Delaware Bay; National Research Council 1997) and salt water of varying temperatures ranging from 3.5-5.5°C (Exxon Valdez grounding; Gay and Vaughn 2001; ITOPF 2010a) to 28°C at the surface (Deepwater Horizon blowout; retrieved from http://www.aoml.noaa.gov/phod/dhos/xbt_ctd.php on February 22, 2012) and 4.5°C deep under water at the wellhead (retrieved from www.earthgauge.net/wp-content/EG_Gulf_oil_spill_Microbes.pdf on February 22, 2012).

Water temperature and salinity may influence the toxicity of an oil spill, particularly if a chemical dispersant is applied. The concentration of PAHs in the water column following dispersant application depends on the effectiveness of the chemical dispersant and the solubility of PAHs, both of which are affected by temperature and salinity. Higher water temperatures typically increase PAH solubility (May and Wasik 1978; Whitehouse 1984) and dispersant effectiveness (Daling 1988; Moles *et al.* 2001) due to reduced viscosity of the oil and dispersant, which then require less mixing energy for dispersion to occur (Chandrasekar *et al.* 2005).

Although colder waters may dissolve less oil, they may also reduce biological degradation of the oil (Atlas 1975) exposing biota for longer periods of time. Low water temperatures also reduce growth rates of cold-blooded animals such as fish (Alderdice and Velsen 1971), which may 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.

Increased salinity also causes chemical dispersants to be more effective (Moles *et al.* 2001). Because the solubility of dispersants decreases with increased salinity, this promotes the movement of surfactant molecules to the oil-water interface to bind and mix with the oil, thereby producing oil droplets that will subsequently disperse throughout the water column (Mackay *et al.* 1984; Chandrasekar *et al.* 2006). Even though increased salinity increases dispersant effectiveness, higher salinity reduces the solubility of PAHs due to 'salting-out' (May and Wasik 1978; Whitehouse 1984). Ramachandran *et al.* (2006) observed this phenomenon by demonstrating that at high

salinities (30‰) the concentration of potentially toxic PAHs was lower. Whitehouse (1984) also observed reduced solubility of PAHs following large changes in salinity although the influence of temperature on PAH solubility was far greater. Therefore, the effects of salinity on the toxicity of dispersed oil require further research.

Herring (*Clupea* sp.) were chosen as the test organism in the present study since they spend some of their most sensitive life stages (embryos and larvae) in coastal waters where oil may be spilled. This is important because herring have been found to be extremely sensitive to oil. Rice *et al.* (1979) exposed 39 Alaskan marine species to crude oil and found that adult Pacific herring had the second lowest 96-h LC50 and the lowest 96-h LC50 of the nine marine fish species (1,220 µg/L compared to >11,720 µg/L). The Exxon Valdez oil spill highlighted this sensitivity of herring embryos and lead to significant research into the toxicological effects of dispersed oil to early-life stages of this species (Brown *et al.* 1996; Hose *et al.* 1996; Kocan *et al.* 1996; McGurk and Brown 1996; Norcross *et al.* 1996). Herring also have the ability to survive in various salinity and temperature environments, as they are considered to be euryhaline and stenothermal (Ford 1930; McMynn and Hoar 1953; Alderdice and Velsen 1971; Blaxter 1985; Haegele and Schweigert 1985; Reid *et al.* 1999).

Since the majority of oil spills occur in coastal waters, where salinity and temperature fluctuations can be significant, the influence of these environmental factors on the toxicity of dispersed oil must be established and considered during oil spill response. Therefore, the primary objective of the present study was to determine whether temperature and salinity influence the toxicity of dispersed oil to Atlantic herring (*Clupea harengus*) embryos.

2.3 Materials and Methods

2.3.1 Test Species

Local fishermen collected Atlantic herring from the Northumberland Strait. 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 bioassay. Whole fish were placed in plastic bags, wrapped in paper and transported in coolers with crushed ice to the laboratory at the Department of Fisheries and Oceans, Gulf Fisheries Centre, Moncton, NB, Canada.

2.3.2 Fertilization

Eggs were fertilized on the day of collection to ensure high fertilization rate. In the laboratory, eggs were extracted by ventral pressure and pooled to minimize interfemale variability (15 females from NB and 3 and 7 females from NS; Kocan *et al.* 1996). The pooled eggs were 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 and pooled (6 males from NB and 5 and 7 males from NS; Kocan *et al.* 1996). The milt was mixed with 30‰ water (Kent Sea Salt, Kent Marine, Acworth, GA, USA in reverse osmosis treated municipal water) in a 38 x 27 x 5 cm glass Pyrex pan. The egg-covered slides were transferred to the milt solution for approximately 10 minutes for fertilization to occur. The embryos used in the salinity bioassay were rinsed with clean water and placed in the respective salinities (7.5, 15 and 30‰) by lowering the salinity from 30‰ by adding fresh water gradually over a one-hour period. The embryos used in the temperature bioassay were rinsed with clean

water and placed in the respective temperatures (7, 10 and 15°C) by reducing the temperature from 15°C by gradually cooling over a one-hour period. Embryos fertilized on September 9, 2010 were exposed to 10°C and 15°C while embryos fertilized on September 23, 2010 were exposed to 7°C. Fertilization success was assessed one hour post-fertilization (PF; Kocan *et al.* 1996) by observing a definite cellular blastomere forming at the pole of the egg, in the blastula developmental stage (C. Bourque, Department of Fisheries and Oceans, Moncton, NB, Canada, personal communication). The slides were then transferred immediately to the respective chemically enhanced water accommodated fraction (CEWAF) and water accommodated fraction (WAF) test solutions. Since eggs and milt were combined into one common source, replication was the jar (each containing a single slide).

2.3.3 Test Solutions

Arabian light (AL) crude oil and the dispersant, Corexit 9500, were provided by Department of Fisheries and Oceans, Centre for Offshore Oil, Gas and Energy Research (COOGER, Dartmouth, NS, Canada). AL was weathered 7% by volume by sparging with air in 2009.

To generate CEWAF, a 1:9 mixture of oil and water was mixed with a stir bar 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 9500 was 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. Dispersant effectiveness was defined as the concentration of summed PAH in the CEWAF stock divided by the

concentration of summed PAH in the crude oil, which is based on the total volume of oil added to the flask (Sorial *et al.* 2004). The same protocol was used to generate WAF except that dispersant was not added. Fresh CEWAF and WAF mixtures were prepared daily.

Nominal concentrations tested for the salinity bioassay were 0.0001, 0.001 and 0.01% v/v CEWAF and 0.01, 0.1 and 1.0% v/v WAF. Atlantic herring embryos exposed to various temperatures were only exposed to CEWAF at nominal concentrations of 0.0001, 0.001, 0.01, 0.1, 1.0 and 10% v/v. Concentrations were chosen based on previous studies (Ramachandran *et al.* 2004; McIntosh *et al.* 2010). Controls for the bioassays included a negative control (clean water), retene positive control (320 µg/L; Billiard *et al.* 1999; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and a dispersant treatment (0.1% Corexit 9500) for the temperature bioassay.

2.3.4 Test Conditions

Unfertilized eggs were removed and live embryos were reduced randomly to 20 per slide within ~72 hours of fertilization. Exposures were performed in glass Mason jars (non-aerated and static) containing 200 mL of test solution with each treatment concentration performed in triplicate. Embryos were exposed to test solutions from one hour PF until hatch with daily renewal of test solutions. Test conditions were maintained at 10°C in 7.5, 15 or 30‰ water or at 30‰ in 7, 10 or 15°C water. Temperature and salinity were recorded daily throughout the bioassay and never deviated more than ± 1°C and ± 1‰. Experiments were terminated when 100% of viable embryos hatched. At hatch, larvae were evaluated for BSD symptoms (see below) and then 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).

2.3.5 Characterization of Hydrocarbon Concentrations

2.3.5.1 Gas Chromatography-Mass Spectroscopy

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 salinity bioassay 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 10°C. Water samples (500 mL) were placed in hexane-acetone rinsed amber glass bottles, preserved with 500 µL 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 summed PAH detection limit ranged from 0.065-0.189 µg/L. GC-MS analysis (EPA 1996; King and Lee 2004) was performed by COOGER in Dartmouth, NS.

2.3.5.2 Synchronous Scan Fluorescence

To quantify the total concentration of petroleum hydrocarbons (fluorescing compounds, mainly PAHs; Lyons *et al.* 2011), WAF (1.0% v/v) and CEWAF (0.01% v/v) from the salinity bioassay were collected at T=0, 2, 4, 8 and 24 h. CEWAF water samples from the temperature bioassay were collected at T=0, 2, 4 and 24 h (0.01% v/v)

and at T=0 and 24 h (1.0% v/v). In the salinity bioassay, water samples from the highest concentrations of WAF and CEWAF were chosen for quantification since lower concentrations were below detection limits. The 0.01% v/v CEWAF concentration was also quantified for the temperature bioassay to ensure consistency between experiments. Water samples (50mL) were placed in hexane-acetone rinsed glass test tubes, preserved with 50 µL 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; Singer *et al.* 1996, Ramachandran *et al.* 2006 as modified by Lyons *et al.* 2011). The TPH limit of quantification was 4 µg/L.

2.3.6 Toxicological Response

Toxicity was based on survival-to-hatch, length-at-hatch and the prevalence and severity of BSD observed in the hatched larvae. BSD is characterized by the presence of spinal curvatures, pericardial edema, yolk sac edema and jaw malformations and by the reduction in heart rate, reduced time-to-hatch and length-at-hatch and overall reduced survival (Billiard *et al.* 1999; Brinkworth *et al.* 2003; Carls *et al.* 2008; Boudreau *et al.* 2009; McIntosh *et al.* 2010). Heart rate and time-to-hatch were quantified but did not show interpretable responses to oil treatment and therefore are not presented (Appendix A).

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 (200 mL test solution in a Mason jar) and scored on a scale of 0-2 (0 represents no movement, 1 represents twitches, 2 represents complete swimming ability). Presence and severity was 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) where 1 represented slight defect, 2 represented moderate defect, and 3 represented 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).

2.3.7 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 six compared to three for the salinity bioassay. 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 (maximum response). 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 concentration that affected development and the BSD SI in 75% of embryos ($EC75_{\%\ abnormal}$ and $EC75_{BSD\ SI}$, respectively) were calculated for the temperature bioassay. Because the prevalence of morphological abnormalities was above 50% in most treatment groups, an EC75 was calculated instead of an EC50. To calculate $EC75_{\%\ abnormal}$ or $EC75_{BSD\ SI}$, top constraints were set at 100 or 1 (maximum response) 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 $EC75_{BSD\ SI}$: 7ºC = 0.07; 10ºC = 0.16; 15ºC = 0.11). 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).

The LC50 and EC75 estimates were calculated based on nominal concentrations not measured concentrations of test solutions since only the three highest concentrations were analyzed (0.1-10% v/v) by GC-MS.

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 both 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. ANOVAs were followed by Tukey multiple comparison tests. Prior to analyses, data were tested for normality (probability plot) and homoscedasticity (Levene/Bartlett test) and were arcsine square-root transformed (survival-to-hatch and BSD SI for salinity bioassay).

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.

2.4 Results

2.4.1 Temperature Experiment

2.4.1.1 Characterization of Hydrocarbon Concentration

Summed PAH concentrations measured by gas chromatography-mass spectroscopy (GC-MS) were proportional to serial dilutions starting at 16µg/L (0.1% v/v; Table 2-1). Although stock CEWAF concentrations were 2.2X higher in 15°C than at 7°C (Table 2-1), 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 2-1). This pattern was also observed in water samples analyzed by synchronous scan fluorescence (Table 2-1). 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 2-1). Similarly, TPH concentration dropped 24-55%, depending on nominal concentration and exposure temperature (Table 2-1). 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 2-1). At warmer temperatures (15°C) there was a higher percent drop (37-55%) in TPH concentrations than in colder test conditions (24-37%; Table 2-1). Water sampling techniques and length of sample storage time may have caused some inconsistencies in both summed PAH and TPH measured values.

2.4.1.2 Positive Control Response

For all three endpoints, embryos exposed to retene (positive control) responded to the toxicant in a similar manner as the higher concentrations of CEWAF (Figures 2-1, 2-2, 2-3; Tables 2-2, 2-3). Retene also produced similar morphological abnormalities to those produced by CEWAF in the present study.

2.4.1.3 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% (0.0078-0.0206; 95% confidence interval) at 7ºC compared to 0.39% (0.2236-0.5621) at 10ºC and 0.24% (0.0826-0.3953) at 15ºC (Figure 2-1; F-test, $F_{2, 48} = 40.41$, $p<0.0001$). 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 2-2). Similarly, all embryos exposed to the Corexit treatment died (Table 2-2).

Larval length-at-hatch was also affected by temperature but the effect differed with CEWAF concentration (Figure 2-2; Table 2-3). For the negative (water) 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 2-2; Table 2-3). 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% v/v) there was also a significant reduction in length-at-hatch with increasing CEWAF concentration (Figure 2-2; Table 2-3).

Effect of temperature on oil toxicity was assessed by $EC75_{\%\ abnormal}$, which showed greater toxicity at 7ºC (0.005%, 0.0025-0.0074) than 10ºC (0.052%, 0.0026-0.1010) and also 15ºC (0.028%, -0.0049-0.0613). Although a significant effect of temperature was detected (Figure 2-3A; F-test, $F_{2, 48} = 18.63$, $p<0.0001$), which temperatures differed could not be determined because of overlapping confidence intervals.

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 7°C and 10.0% v/v at 10°C and 15°C (Figure 2-3B). The $EC75_{BSD\ SI}$ estimate was lower at 7°C (0.015%, 0.0111-0.0194) than 10°C (0.278%, 0.0918-0.4646) and also 15°C (0.575%, 0.2344-0.9150; F-

test, $F_{2, 48} = 32.97$, $p<0.0001$). Non-overlapping LC50 confidence intervals indicate significantly greater toxicity at 7ºC than both 10ºC and 15ºC (Environment Canada 2005).

For all temperatures, the observable toxicity changed as CEWAF concentrations increased (Figure 2-4). 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 2-2).

2.4.2 Salinity Bioassay

2.4.2.1 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 2-4). 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 2-4). 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 2-4).

2.4.2.2 Positive Control Response

For all endpoints, embryos exposed to retene (positive control) responded to the toxicant but responded more severely than the highest test concentrations of both WAF and CEWAF (Figures 2-5, 2-6, 2-7, 2-8; Tables 2-3, 2-5, 2-6). Retene also produced similar morphological abnormalities to those produced by WAF and CEWAF in the present study.

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

Survival-to-hatch, which ranged from 99% to 100% (Table 2-5), was not significantly reduced by exposure to WAF or CEWAF test solutions at any salinity.

Embryos exposed to the highest concentration of WAF and CEWAF were significantly shorter at hatch than control embryos for all salinities tested (Figures 2-5, 2-6A; Table 2-3). Embryos exposed to WAF were significantly shorter in test solutions of 7.5 and 15‰ compared to 30‰ (Figure 2-6B; Table 2-3). The LOEC (lowest observed effect concentration) 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 2-2, 2-5; Table 2-3).

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 2-7; Table 2-6). At the highest CEWAF concentration, BSD SI was influenced by salinity and was significantly increased at 7.5‰ compared to 30‰ (Table 2-6). Embryos exposed to WAF experienced significantly increased BSD SI at all concentrations compared to the negative control (Figure 2-8A; Table 2-6). There was

also a salinity effect with exposure to 7.5 and 15‰ resulting in significantly elevated BSD SI compared to 30‰ (1% v/v; Figure 2-8B; Table 2-6).

In general, where a salinity effect was observed, toxicity was increased in lower salinities (Figures 2-6B, 2-8B; Tables 2-3, 2-6).

2.5 Discussion

2.5.1 Temperature Bioassay

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 AL 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 AL 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 AL CEWAF in the temperature experiment were taken from a fall spawning stock, rearing at low temperature might 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 4ºC to 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 to 19 days (depending on CEWAF treatment) at 7ºC compared to 6 to 7 days at 15ºC (Table A2-2).

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 Medium 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 (May and Wasik 1978; Whitehouse 1984), which ultimately increases the concentration of PAHs in the water. In the present study, dispersant effectiveness was quite similar for all temperatures (1.3% to 3.1%) so the increased PAH solubility in warmer water likely caused the increase in summed PAH concentrations (2.2X) in the 15°C CEWAF stock relative to the 7°C CEWAF stock. However, PAHs were measured in both the solution and also in the droplets. Since the concentration in droplets was also included in the analyses, the values may be overestimating the amount of dissolved PAHs at these

different salinities. 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 15°C) depending on the stock (Blaxter 1985). With this in mind, the present study was initially designed to include a test temperature of 5ºC. However, 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. Alderdice and Velsen (1971) found a similar temperature tolerance limit of 4ºC to 5ºC in British Columbia herring with optimal survival occurring at 8.7ºC. Studies have suggested that spawning is triggered mainly by temperature (Blaxter 1985; Haegele and Schweigert 1985), which would suggest that spawning might 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 maximize 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). Since gonad maturation is triggered earlier the fish may not be able to avoid spawning in fluctuating sea temperatures. This would result in eggs potentially developing in temperatures 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 conducted 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). 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 AL CEWAF (10% v/v). This Corexit concentration exceeds the 96h LC50 of larval and juvenile fish species, which ranges from 25 to 143 ppm (George-Ares and Clark 2000; Hemmer *et al.* 2010). The AL CEWAF LC50s calculated from the temperature bioassay varied from 0.01 to 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. However, it is conceivable that the toxicity observed in our CEWAF treatments could also result from Corexit or some combination of the PAHs and Corexit. To validate the source of toxicity of CEWAF mixtures, future studies should measure the amount of Corexit remaining in CEWAF solutions.

2.5.2 Salinity Bioassay

There was some indication in the present study that lower salinities may increase oil toxicity. Embryos exposed to 1% v/v AL WAF were smaller at hatch when incubated in 7.5‰ and 15‰ than 30‰ (Table 2-3). Embryos also showed higher rates of abnormality (BSD SI) when exposed to higher concentrations of both WAF and CEWAF at lower salinities (Table 2-7).

Increased toxicity associated with less saline test solutions likely result 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), as observed 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 observed 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 2-4). 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.

2.5.3 Environmental Implications

In the present study, Atlantic herring exposed to 0.34, 16, 190 and 2,550 µg/L summed PAH (nominal concentrations of 0.01, 0.1, 1 and 10% v/v AL at 10ºC and 30‰, T=0 h) experienced 100, 72, 5 and 0% survival-to-hatch and only 37, 2 and 0% developed normally, respectively (Tables 2-1, 2-2, 2-4, 2-5). At the lowest detectable concentration (0.31 µg/L for 1.0% v/v AL WAF at 15‰), all the embryos survived but only 18% developed normally. Even treatments containing PAH concentrations too low to be quantified (below the detection limit of 0.065-0.189 µg/L) still reduced normal development (5% and 10% of embryos developed normally in 0.01% v/v at 7.5‰ and 15‰, respectively).

Even though the concentrations of PAHs in the present study were much lower than anticipated, they were within the range of concentrations reported at depths of 1 to 5 metres following the Exxon Valdez spill and at greater depths throughout the Deepwater Horizon spill. Directly following the Exxon Valdez spill total PAH concentrations ranged from 0.91 to 2.23 µg/L and from 1.26 to 6.24 µg/L on adjacent

heavily oiled beaches. Concentrations persisted for at least five weeks at 1m depths ranging from 0.92 to 1.59 µg/L (Short and Harris 1996). During the Deepwater Horizon oil spill, which lasted for approximately three months, total PAH concentrations measured from May 24, 2010 to June 6, 2010 ranged from 0.01 to 59 µg/L, while TPH concentrations were measured from 2 to 422 µg/L (Wade *et al*. 2011). More specifically, Diercks *et al.* (2010) reported that total PAH concentrations were 189 µg/L at 1320 metres and 29 µg/L at 1160 metres near the wellhead site.

Potential oil exposure to early life stages of herring could depend on the spawning habitat depth. Atlantic herring off eastern Nova Scotia would typically spawn at depths of 15 to 60 metres. In contrast, Pacific herring off the coast of British Columbia and Western United States may deposit eggs at depths of 20 metres but most spawn is deposited within 10 metres of the mean tide level at spawning (Haegele and Schweigert 1985). At these spawning depths, herring embryos would be at risk of exposure to PAHs at toxic concentrations if a major oil spill were to occur over spawning shoals.

2.5.4 Limitations and Future Research

The results of this study suggest temperature influences the toxicity of oil to herring embryos; with embryos being more sensitive to colder test condition. Lower saline waters may also increase the toxicity of oil but further experiments are required to validate these results.

One of the major limitations in the present study is not being able to quantify the inter-assay variance within a given stock of herring. Embryos exposed to 7°C were stripped from different adults than embryos exposed to 10°C and 15°C. The adults were

taken from the same stock of Atlantic herring but there is some suggestion that the quality of eggs varied within this stock since 88% of embryos reared in clean water developed normally at 7°C while only 65 to 70% of embryos developed normally at 10°C and 15°C. However, the quality of gametes is not likely responsible for the increased toxicity observed at 7°C since the control embryos survived better than the embryos exposed to warmer conditions. Nevertheless, the variance in egg quality within this stock could influence the results of bioassays done with this species and may lead to over- or underestimating sensitivity. Future experiments are needed to quantify this variance by replicating whole experiments, as was done by Couillard (2002).

Additional research is also needed to determine inter-stock variability to establish if the observed salinity and temperature effects on the toxicity of crude oil would also be observed for other stocks of herring or whether it is characteristic only of spring or fall-spawned Atlantic herring, respectively. Further experimentation to test the hypothesis that cold stress could be explaining the higher toxicity in fall-spawned herring would be a next step. To test this hypothesis, the temperature bioassay would need to be repeated with spring-spawned herring. If temperature adaptation affects response to dispersed oil, spring-spawned herring should show the opposite response to fall herring: greater toxicity at high temperature. Preliminary results from ongoing research support this hypothesis. Atlantic herring from a spring spawning stock exposed to Venture produced water (containing hydrocarbons and metals) experienced significantly higher toxicity at 15°C relative to 7 and 10°C (M. Boudreau, Gulf Fisheries Centre, Moncton, NB, Canada, personal communication). Identifying the physiological pathway of enhanced toxicity at lower temperatures could shed light on whether the

higher toxicity associated with colder environments is a result of natural adaptation, exposure duration, or simply the variance within the stock of herring.

In the temperature bioassay, the survival, abnormality and BSD SI data (LC50 and EC75) were analyzed by a four-parameter non-linear regression and ECanything non-linear regressions, however, the distribution of these data were not ideal for these analyses. Hence, the precision of these values (LC50 and EC75) could be improved in future experiments by 1) including more data points to permit the use of non-standard hill slopes; 2) including concentrations with similar responses to the control group which would improve the fit of the curve near the control (constraint) value; 3) including a wider range of concentrations with responses between 20-80% and reducing the amount of groups with maximal responses.

The effect of salinity was limited but implied a potential increase in toxicity at lower salinity test conditions. However, the salinity experiment was conducted with only a limited range of test concentrations (0.0001 to 0.01% v/v) compared to herring exposed at different temperatures (0.0001 to 10.0% v/v). Therefore, additional salinity experiments are needed with wider range of CEWAF concentrations to estimate traditional toxicity endpoints, such as LC50s and EC50s.

Exposure concentrations in the present study were much lower than expected. Water sampling techniques and length of sample storage time may have caused some inconsistencies in both summed PAH and TPH measured values in exposure solutions. Dispersant effectiveness could also influence the concentration of hydrocarbons in the test solutions. The most probable explanation for the low dispersant effectiveness observed in the present study is the re-coalescing of oil droplets during the settling phase of CEWAF production. Laboratory tests to determine dispersant effectiveness combine

different volumes of oil and water than laboratory protocols to generate CEWAF and also mix and settle for different durations. The baffled flask test (BFT) and swirling flask test (SFT) are used to determine dispersant effectiveness (Moles *et al.* 2002; Venosa *et al.* 2002; Sorial *et al.* 2004; Chandrasekar *et al.* 2005). The SFT mixes 100 µL of dispersant with 2.5 mL of oil (dispersant to oil ratio of 1:25) and then mixes 100 µL of this mixture into 120 mL water for 20 minutes at 150 rpm on an orbital shaker prior to 10 minutes of settling (Moles *et al.* 2002). The BFT, which is a new method adopted by the US EPA, mixes 120mL water with 100 µL oil and 4 µL dispersant (dispersant to oil ratio of 1:25) for 10 minutes at 200 rpm on an orbital shaker prior to 10 minutes of settling (Venosa *et al.* 2002; Sorial *et al.* 2004; Chandrasekar *et al.* 2005). In order for a dispersant to be included in the national contingency plan product schedule, the dispersant must be at least 45% (50±5%) effective in dispersing Prudhoe Bay and South Louisiana crude oils in the laboratory test (Venosa *et al.* 2002). The laboratory protocol followed in the present study was modeled after Singer *et al*. (2000). In the present study, 180 mL water was mixed with 20 mL oil for 18 hours on a stir plate with a vortex achieving 20-25% depth. The oil-water mixture (1:9) was further mixed for one hour with 2 mL dispersant (1:20) and then settled for one hour. Since the volume of oil is larger (20 mL versus 100 µL) and is allowed to settle for a longer period (1 hour versus 10 minutes) in the present study, there would be a greater chance of oil droplets colliding and forming larger droplets that could re-coalesce and be removed from the stock test solution (T. King, Bedford Institute of Oceanography, Dartmouth, NS, Canada, personal communication).

2.5.5 Conclusions

The results of the present study show that rearing temperature was the more influential of the two environmental factors tested. The increased toxicity of oil exposure in colder conditions may reflect an adaptation to warmer environments by the herring stock exposed (i.e., exposure close to natural temperature tolerance limits) or longer exposure duration. The increased toxicity is less likely to be affected by factors that influence dispersed oil toxicity such as dispersant effectiveness or PAH solubility. Possible increased toxicity in less saline conditions appeared to be a reflection of increased PAH solubility and hence hydrocarbon exposure. Following an oil spill, concentrations may be reduced very quickly in the water column but even these low concentrations are sufficient to produce severe effects in early-life stages of Atlantic herring.

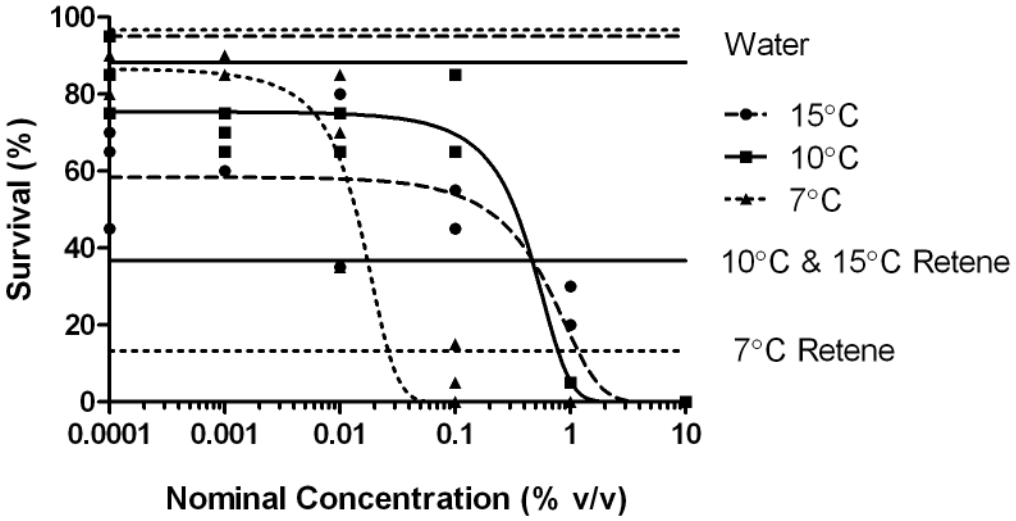

Figure 2-1. Percent survival of Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7, 10 and 15°C. Dose response curves for LC50 were calculated by 4-parameter nonlinear regression. N=3 jars/concentration.

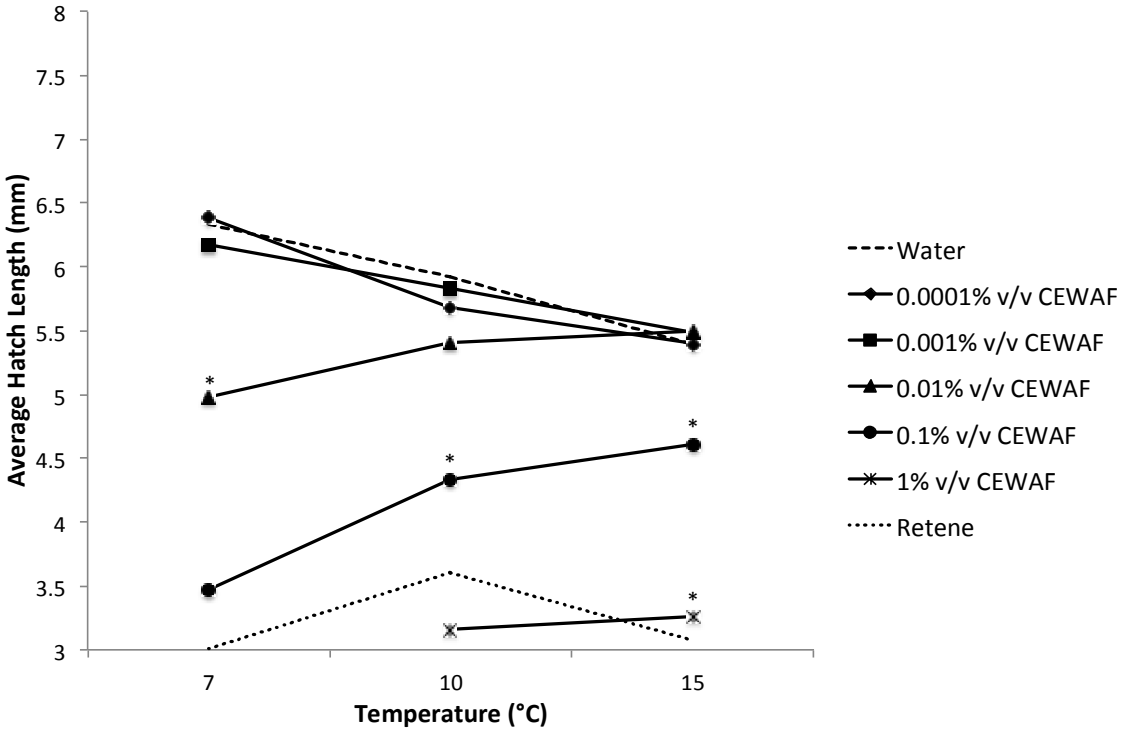

Figure 2-2. Average length-at-hatch (mm) of Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7, 10 and 15°C. Individual temperatures were analyzed separately and asterisks (*) indicate treatments that experienced significantly reduced length-at-hatch compared to the negative control (ANCOVA; Tukey test, $p<0.05$). Embryos exposed to Corexit all died. N=3 jars/concentration.

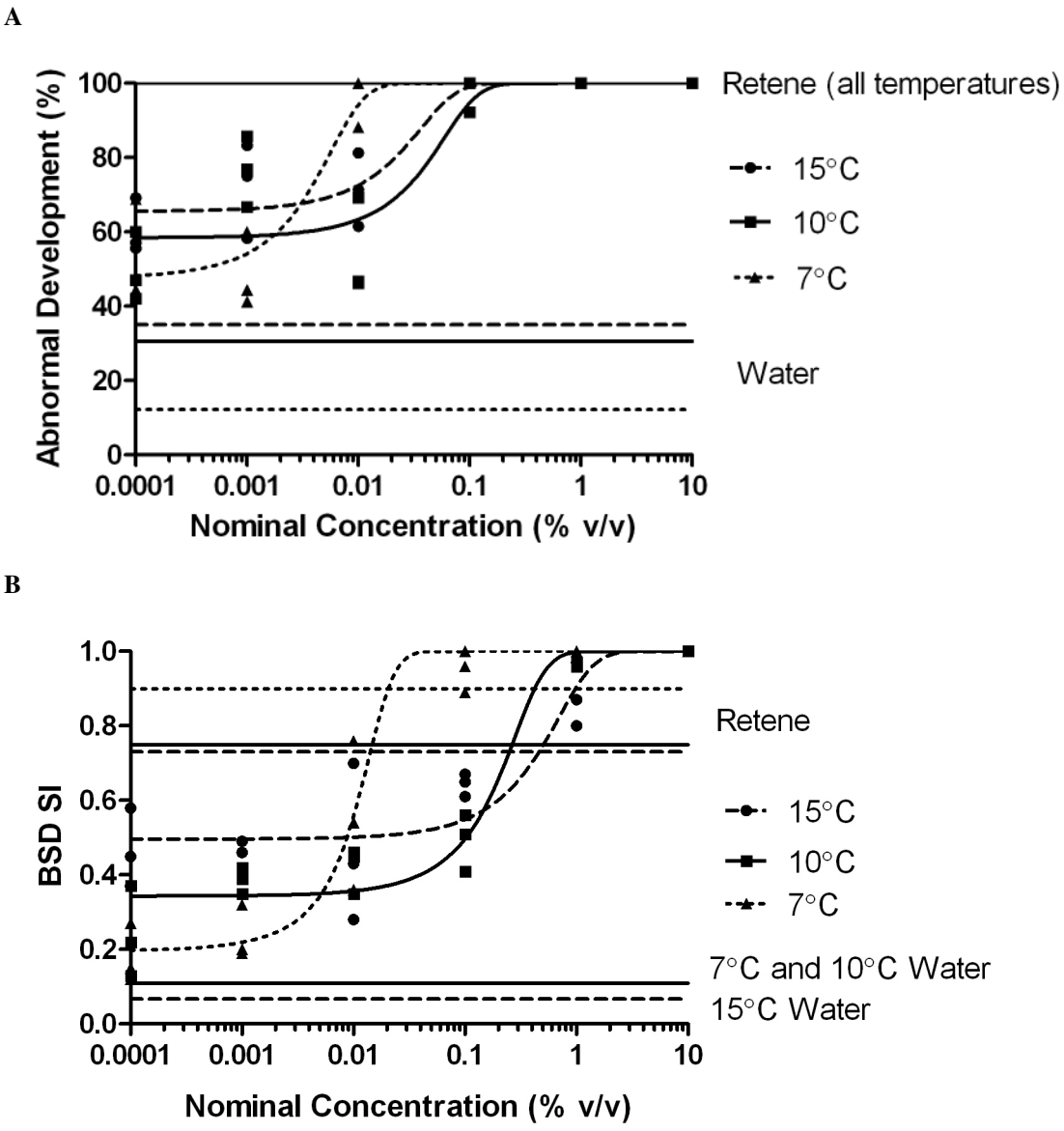

Figure 2-3. Percent abnormal development (**A**) and average blue sac disease severity index (BSD SI; **B**) values for Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7, 10 and 15°C. Dose response curves for EC75 were calculated by an ECanything nonlinear regression. N=3 jars/concentration.

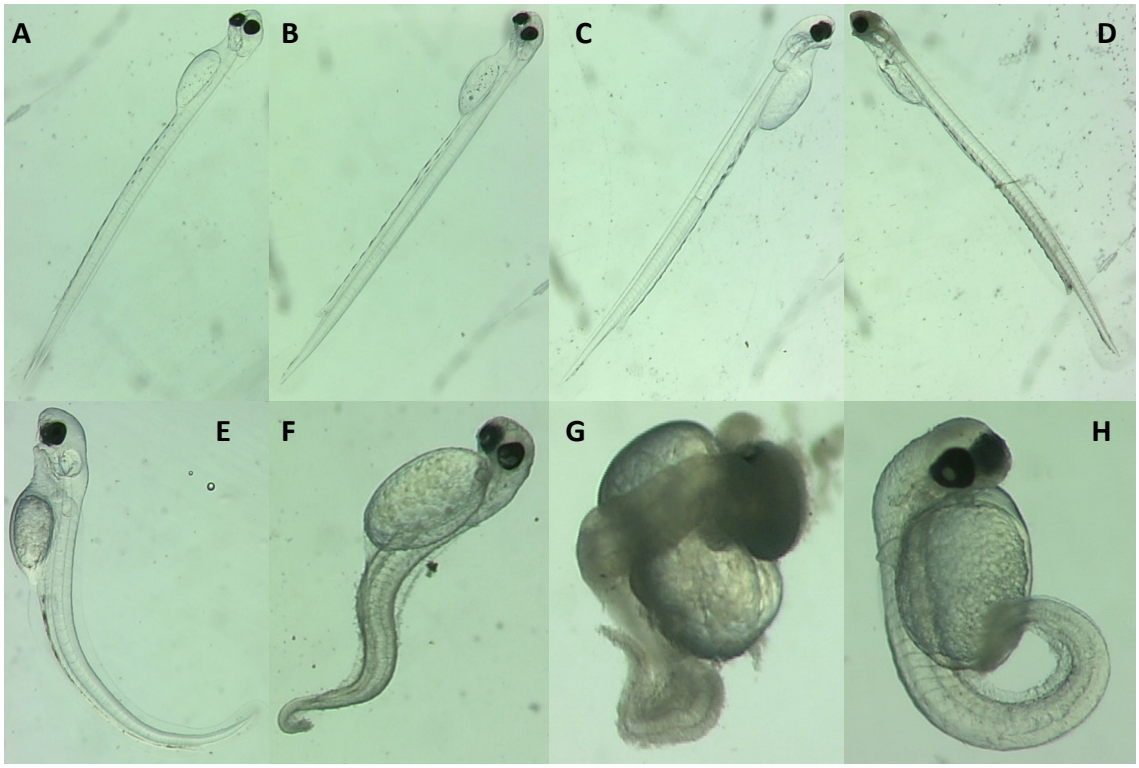

Figure 2-4. Typical sublethal effect trends observed in Atlantic herring larva from the negative control (**A**; normal larva), 0.0001% v/v (**B**; normal larva), 0.001% v/v (**C**; slight pericardial edema, jaw), 0.01% v/v (**D**; yolk sac edema, slight spinal curvature, skin lesion on head), 0.1% v/v (**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**).

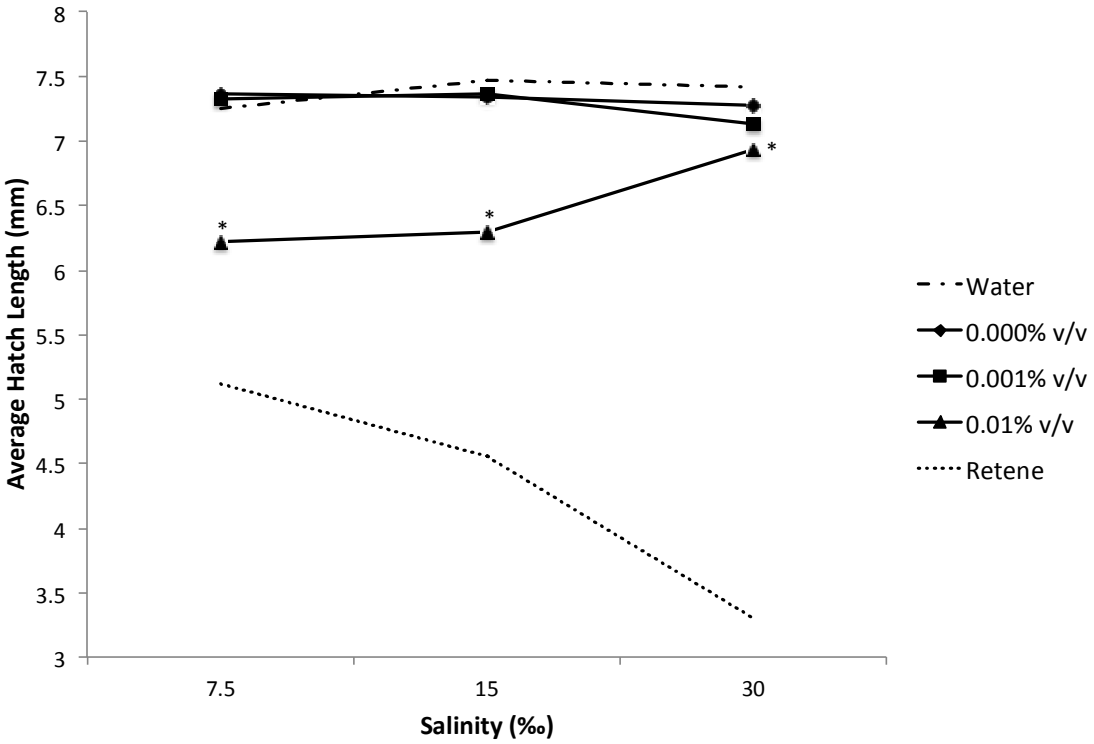

Figure 2-5. Average length-at-hatch (mm) of Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7.5, 15 and 30‰. Individual salinities were analyzed separately and asterisks (*) represent concentrations that are significantly shorter than the negative control (ANCOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

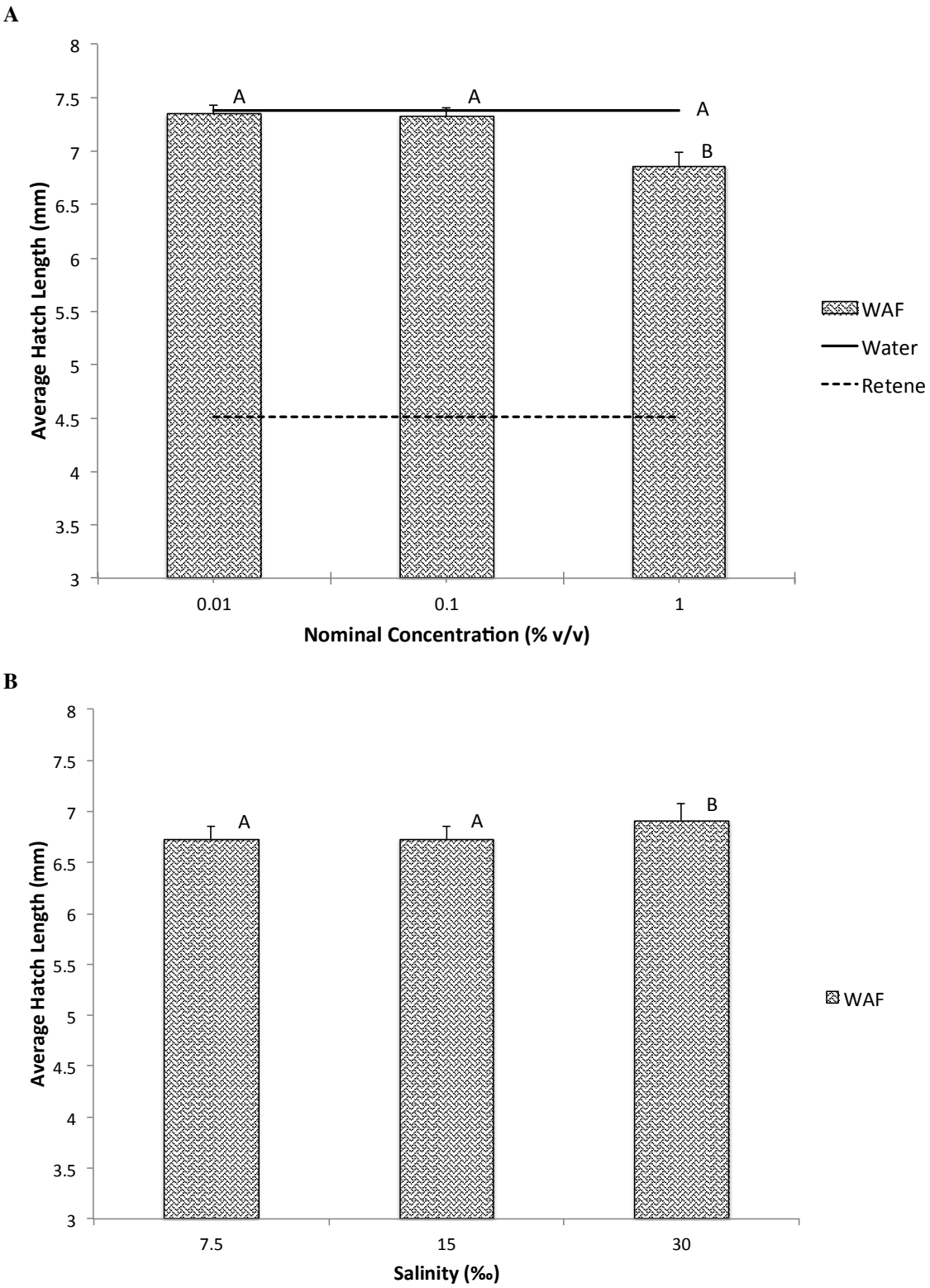

Figure 2-6. Average length-at-hatch (mm) of Atlantic herring embryos following chronic exposure to Arabian Light WAF at 7.5, 15 and 30‰. Non-significant interaction was encountered and factors (concentration (**A**) and salinity (**B**)) were analyzed separately (ANCOVA; Tukey test, $p<0.05$). Concentrations and salinities grouped by different letters are significantly different from each other. N=3 jars/concentration.

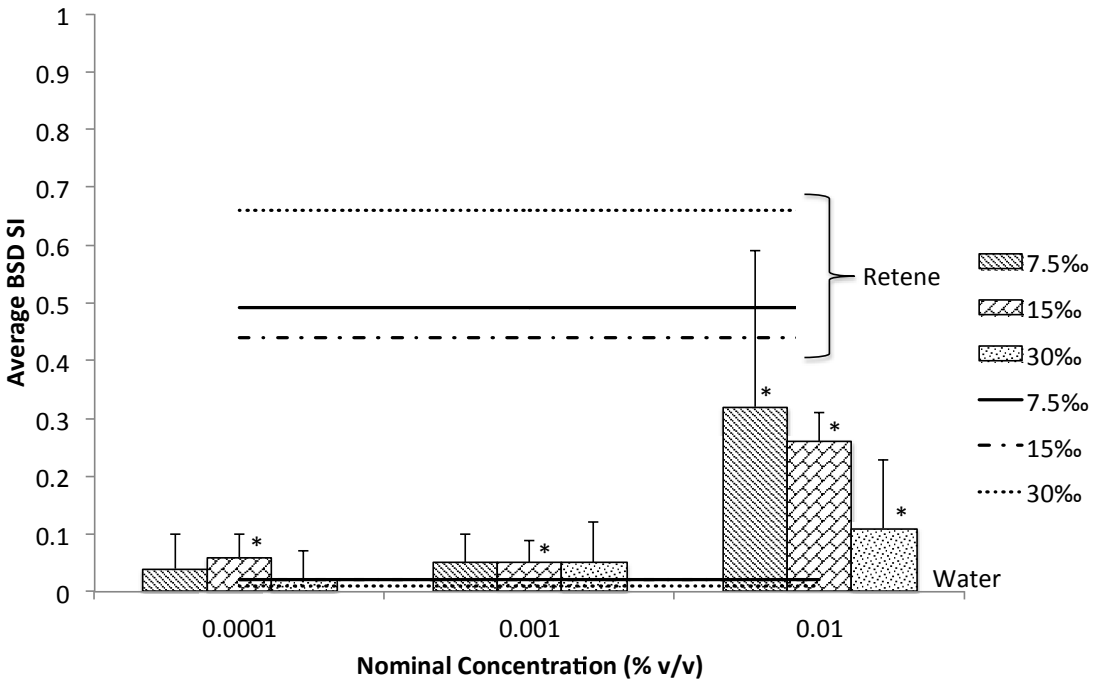

Figure 2-7. Average blue sac disease severity index (BSD SI) values for Atlantic herring embryos following chronic exposure to Arabian Light CEWAF at 7.5, 15 and 30‰. Individual salinities were analyzed separately and asterisks (*) indicate concentrations with significantly higher BSD SI compared to the negative control (ANOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

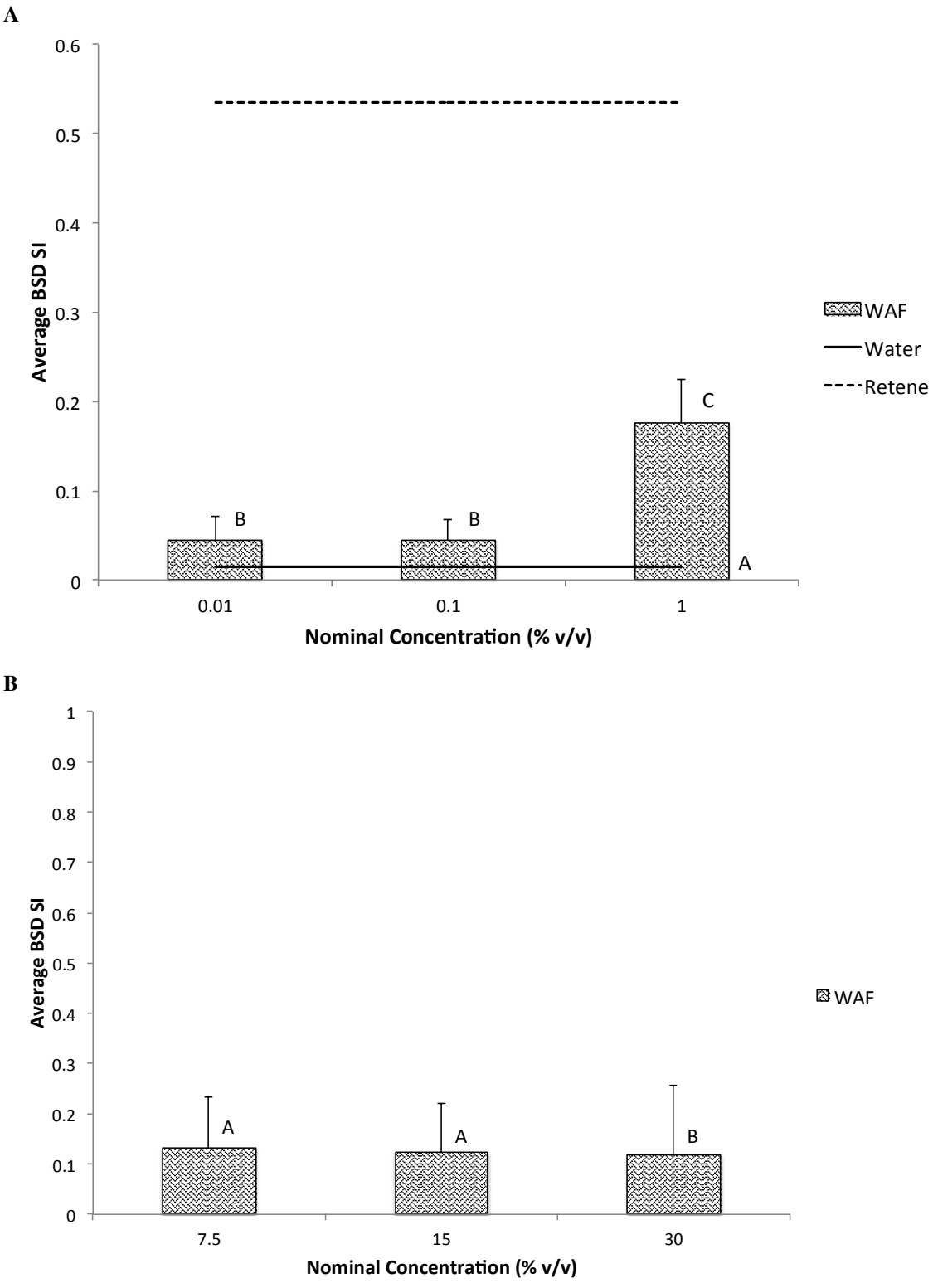

Figure 2-8. Average blue sac disease severity index (BSD SI) values for Atlantic herring embryos following chronic exposure to Arabian Light WAF at 7.5, 15 and 30‰. Non-significant interaction was encountered and factors (concentration (**A**) and salinity (**B**)) were analyzed separately (ANOVA; Tukey test, $p<0.05$). Concentrations and salinities grouped by different letters are significantly different from each other. N=3 jars/concentration.

Table 2-1. 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 ~0.065-0.189 µg/L and the quantification limit for fluorescence was 4 µg/L. Blank cells represent water sample data that are not available. Dispersant effectiveness was calculated ((amount of PAH in CEWAF stock/[amount of PAH in crude oil stock*10%])*100) and is defined as the amount of oil products that are dispersed into the water column compared to the amount of oil products that remain on the water surface in the slick. Therefore, only 1.3-3.1% of the different crude oils went into the water column (CEWAF).

				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%	
	Dispersant Effectiveness			1.3%	1.3%	3.1%
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

Table 2-2. 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 back transformed 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

Table 2-3. Average length-at-hatch (mm) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 7, 10 and 15°C. 2-Factor nested ANCOVAs were performed using salinity or temperature and concentration as the two factors with the interaction term reported. Non-significant interaction was analyzed by separate 1-factor ANOVAs with lowercase letters representing significant differences among treatments (separate 1-factor ANOVAs for concentration and salinity; Tukey test, $p<0.05$). Significant interactions were explored using 1-factor ANOVAs with uppercase letters representing significant differences among salinities or temperatures (1-factor ANOVA analyzing individual concentrations; Tukey test, $p<0.05$). Asterisks (*) represent treatments that were significantly shorter than the negative control (1-factor ANOVA analyzing individual salinities and temperatures; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{6, 697} = 2.95$, p=0.008				
Water	7.25±0.12 A	7.47±0.09 B	7.42±0.12 AB	$F_{2, 176} = 4.78$, p=0.010
0.0001	7.36±0.10	7.34±0.15	7.27±0.16	NS
0.001	7.33±0.11 AB	7.37±0.15 A	7.13±0.19 B	$F_{2, 176} = 3.63$, p=0.028
0.01	6.22±0.80 *	6.30±0.25 *	6.93±0.23 *	NS
Retene	5.12±0.14	4.56±0.10	3.30±0.16	
ANOVA	$F_{3, 225} = 7.22$, p<0.001	$F_{3, 227} = 43.80$, p<0.001	$F_{3, 227} = 2.85$, p=0.038	
Conc. (% v/v)		Salinity		
WAF Interaction: NS				
Concentration: $F_{3, 696} = 25.49$, $p < 0.001$		Salinity: $F_{2, 696} = 15.92$, $p < 0.001$		
Water	7.38±0.06 a			
0.01	7.35±0.08 a	7.5‰	6.73±0.12 a	
0.1	7.32±0.08 a	15‰	6.73±0.13 a	
1.0	6.86±0.13 b	30‰	6.91±0.17 b	
Retene	4.51±0.14			
Conc. (% v/v)	7°C	10°C	15°C	ANOVA
CEWAF Interaction: $F_{8, 601} = 3.18$, p=0.002				
Water	6.33±0.27 A	5.93±0.21 B	5.39±0.23 C	$F_{2, 164} = 22.36$, p<0.001
0.0001	6.39±0.22 A	5.68±0.22 B	5.40±0.28 C	$F_{2, 136} = 19.52$, p<0.001
0.001	6.17±0.33 A	5.83±0.28 B	5.49±0.20 C	$F_{2, 124} = 22.77$, p<0.001
0.01	4.98±0.42 A *	5.41±0.37 B	5.50±0.22 C	$F_{2, 111} = 52.99$, p<0.001
0.1	3.47±0.75 A	4.34±0.35 B *	4.61±0.36 C *	$F_{2, 72} = 7.03$, p=0.002
1.0	No Hatch	3.16±2.18	3.26±0.39 *	NS
10.0	No Hatch	No Hatch	No Hatch	No Hatch
Retene	3.01±0.34	3.61±0.17	3.08±0.33	
Corexit	No Hatch	No Hatch	No Hatch	No Hatch
ANOVA	$F_{4, 188} = 4.79$, p=0.001	$F_{5, 214} = 13.50$, p<0.001	$F_{5, 186} = 8.97$, p<0.001	

Table 2-4. 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 ~0.065-0.189 µg/L and the quantification limit for fluorescence was 4 µg/L. Blank cells represent water sample data that are not available. Dispersant effectiveness was calculated ((amount of PAH in CEWAF stock/[amount of PAH in crude oil stock*10%])*100) and is defined as the amount of oil products that are dispersed into the water column compared to the amount of oil products that remain on the water surface in the slick. Therefore, only 1.3-2.6% of the different crude oils went into the water column (CEWAF).

				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
	Dispersant Effectiveness			2.6%	2.0%	1.3%
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

Table 2-5. 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. N=3 jars/concentration. Upper 95% confidence interval of back transformed 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

Table 2-6. Average blue sac disease severity index (0-1) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰. 2-Factor ANOVAs were performed using salinity and concentration as the two factors with the interaction term reported. Non-significant interaction was analyzed by separate 1-factor ANOVAs with lowercase letters representing significant differences among treatments (separate 1-factor ANOVAs for concentration and salinity; Tukey test, $p<0.05$). Significant interactions were explored using 1-factor ANOVAs with uppercase letters representing significant differences among salinities (1-factor ANOVA analyzing individual concentrations; Tukey test, $p<0.05$). Asterisks (*) represent treatments that were significantly shorter than the negative control (1-factor ANOVA analyzing individual salinities; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{6,24} = 5.07$, $p=0.002$				
Water	0.02 (0.01-0.04)	0.02 (0.01-0.04)	0.01 (0.00-0.03)	NS
0.0001	0.04 (0.00-0.10)	0.06 (0.02-0.10) *	0.02 (0.00-0.07)	NS
0.001	0.05 (0.02-0.10)	0.05 (0.02-0.09) *	0.05 (0.01-0.12)	NS
0.01	0.32 (0.10-0.59) A *	0.26 (0.21-0.31) AB *	0.11 (0.04-0.23) B *	$F_{2,6} = 8.25$, $p=0.019$
Retene	0.49 (0.46-0.53)	0.44 (0.36-0.53)	0.66 (0.47-0.84)	
ANOVA	$F_{3,8} = 31.59$, $p<0.001$	$F_{3,8} = 102.13$, $p<0.001$	$F_{3,8} = 12.95$, $p=0.002$	
Conc. (% v/v)		Salinity		
WAF Interaction: NS				
Concentration: $F_{3,24} = 56.37$, $p<0.001$		Salinity: $F_{2,24} = 6.51$, $p=0.006$		
Water	0.015 (0.011-0.019) a			
0.01	0.045 (0.024-0.071) b	7.5‰	0.132 (0.057-0.233) a	
0.1	0.044 (0.026-0.068) b	15‰	0.123 (0.051-0.221) a	
1.0	0.176 (0.132-0.225) c	30‰	0.118 (0.029-0.257) b	
Retene	0.535 (0.450-0.619)			

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Chapter 3: Sensitivity of early-life stages of herring (*Clupea* sp.) species and stocks to the toxicity of three dispersed and undispersed oils

3.1 Abstract

The toxicity of chemically-enhanced water accommodated fraction (CEWAF) and water accommodated fraction (WAF) of Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) crude oils were tested during the embryonic development of two Pacific herring (*Clupea pallasi*) stocks. The toxicity of AL was also tested during the embryonic development of two Atlantic herring (*Clupea harengus*) stocks in 30‰ water at 10°C. Toxicity was determined based on survival-to-hatch, length-at-hatch, and the Blue Sac Disease Severity Index (BSD SI; incorporates mortality, swimming ability, prevalence and severity of spinal curvature, pericardial edema and yolk sac edema, and prevalence of jaw malformation and skin lesion). Atlantic herring were more sensitive than Pacific herring suggesting a need for species-specific advice for risk assessment. Pacific herring from Alaska were more sensitive than a stock from British Columbia. However, elevated BSD SI in the control group of the fall stock of Atlantic herring limited our assessment of the sensitivity of the Atlantic herring spawning stocks. MESA was the most toxic crude oil for Pacific herring embryos. This study suggests that oil spill managers considering dispersant use can minimize the toxicity to coastal fish by considering information on the particular crude oil spilled and the species or stocks that could be exposed if an oil spill occurred.

3.2 Introduction

During the last decade, ~219 million litres of oil has been spilled annually in aquatic habitats (ITOPF 2010a). Once spilled, oil can harm wildlife living at the water surface, as well as coastal ecosystems. To reduce these impacts, chemical dispersants can be applied to the oil slick to remove the oil from the surface, dilute it throughout the water column and prevent it going ashore (ITOPF 2010b). Dispersants were not widely used offshore following the 1989 Exxon Valdez oil spill (42 million litres released) in Prince William Sound, Alaska and as of 2003 an estimated 84,000 litres of oil still remained in intertidal sediments and was decreasing at a rate of only 0-4% per year (Exxon Valdez Oil Spill Trustee Council 2009). In comparison, the 2010 Deepwater Horizon spill in the Gulf of Mexico resulted in the largest ever application of chemical dispersants to protect coastal marshes. While approximately 1,100 kilometres of shores (Ramseur 2010) were contaminated by this massive oil spill (840 million litres; McNutt *et al.* 2011), coastal habitats would likely have suffered greater damages if dispersants had not been used (EPA 2011).

Increased exposure of pelagic fish to hydrocarbons in the water column resulting from the use of dispersants presents a challenge to the protection of wildlife and coastal habitats (Cohen *et al.* 2001; Ramachandran *et al.* 2004; Clarke 2007; Greer 2011). Especially vulnerable are nursery grounds because early-life stages, embryos and larvae, are considered to be among the most sensitive life stages of fish (McIntosh *et al.* 2010). Exposures during sensitive early-life stages could result in large population declines as observed following the Exxon Valdez spill, when 75-86% of the exposed Pacific herring population did not return to spawn in 1993 (Carls *et al.* 2002; Exxon Valdez Oil Spill

Trustee Council 2010). However, causes other than oil exposure have been offered to explain the drastic 1993 decline in spawning herring including over-harvesting, predation, very cold water temperature, increased disease and over-population which could have led to poor conditions in Prince William Sound (Pearson *et al.* 1999). This is a prime example of the fundamental weakness of observational studies in the field, illustrating the need for controlled laboratory experiments.

Oil toxicity has been attributed to polycyclic aromatic hydrocarbon (PAH) exposure (Carls *et al.* 1999; Heintz *et al.* 1999; Baussant *et al.* 2001; Cohen *et al.* 2001; Billiard *et al.* 2002; Barron *et al.* 2004; Billiard *et al.* 2008; Carls *et al.* 2008). Exposure to oil or PAHs during early-life stages can result in morphological abnormalities, collectively termed as blue sac disease (BSD). This syndrome is characterized by spinal curvature, pericardial edema (fluid collecting in the pericardial sac), yolk sac edema (fluid collecting in the vitelline vasculature), craniofacial malformations, haemorrhaging, fin rot, cardiovascular dysfunction, decreased length, reduced heart rate and increased mortality (Billiard *et al.* 1999, 2008; Brinkworth *et al.* 2003; Carls *et al.* 2008; Boudreau *et al.* 2009; McIntosh *et al.* 2010; Scott and Hodson 2008; Winchester 2003). It is widely acknowledged that these symptoms are mitigated through the cytochrome P450 1A (CYP1A) pathway.

The concentration of PAHs in the water column following chemical oil dispersion depends on the dispersant effectiveness which is influenced by the type of crude oil spilled and the degree of weathering (Moles *et al.* 2001; Environmental Protection Agency, EPA 2003; Fingas 2004; Chandrasekar *et al.* 2005; Nordvik 2005). Weathering refers to the evaporation of volatile components (C_3-benzenes and BTEX:

benzene, toluene, ethylbenzene and xylenes; EPA 2003), which increases the viscosity of the oil. As the oil becomes more viscous the effectiveness of the dispersant is reduced (Moles *et al.* 2001; EPA 2003; Nordvik 2005). Lower dispersion rates are also observed in lighter crude oils. For example, Corexit 9500 dispersed 8 to 19% of Arabian Light (AL) crude oil compared to 15 to 47% dispersion of Alaska North Slope (ANS) crude, a medium crude oil (EPA 2003). According to the EPA (2003), ANS crude oil contained 1.3 to 1.7X more PAH than AL crude oil (based on 3 to 5 ring PAHs: phenanthrenes, fluorenes, chrysenes, pyrene, benzo-a-pyrene, anthracene and benzo-a-anthracene). Therefore, highest waterborne PAH concentrations, and associated toxicity, would be expected from spills of non-weathered, chemically dispersed, heavier crude oils.

The sensitivity to oil or PAH exposure can vary greatly among fish species. Herring (*Clupea* sp.) are very sensitive to PAHs compared to other marine species. Rice *et al.* (1979) exposed 39 Alaskan marine species (including nine fish species) to crude oil and found that adult Pacific herring was the second most sensitive species and the most sensitive of the nine fish species tested, with 96-h LC50s ranging from 1,220 µg/L (Pacific herring) to >11,720 µg/L. Further research by Carls *et al.* (1999, 2002) has illustrated that exposure to as little as 0.4-0.7 µg/L total PAH caused edema and reduced growth in Pacific herring embryos. Following the Exxon Valdez oil spill, Pacific herring embryos demonstrated sensitivity to oil exposure, which resulted in morphologic, genetic and histopathologic damage (Brown *et al.* 1996; Hose *et al.* 1996; Kocan *et al.* 1996; McGurk and Brown 1996; Norcross *et al.* 1996). Atlantic herring embryos have also been shown to be more sensitive to Orimulsion-400 (70% bitumen

and 30% water) exposure than another common Atlantic coastal fish species, the mummichog (*Fundulus heteroclitus;* Boudreau *et al.* 2009).

Populations of both Atlantic and Pacific herring spawn in distinct geographic regions, producing spatially distinct stocks (Iles and Sinclair 1982; Haegele and Schweigert 1985; Winters and Wheeler 1996; Bekkevold *et al*. 2005). Of these spatially distinct stocks of Atlantic herring, some stocks spawn in the spring, others in the fall, and a few in between during the summer months (Haegele and Schweigert 1985). Assessing stock and species sensitivities equips responders with a range of sensitivities for groups of fish highly susceptible to oil such as herring and also tests the transferability of oil toxicity data between species.

Since the majority of oil spills occur in coastal waters, where herring species spawn, it is important to establish the toxicity of dispersed and undispersed oil to early life stages of Atlantic and Pacific herring, as a function of oil type released. Therefore, the primary objective of the present study was to determine the toxicity of three mechanically and chemically dispersed crude oils and to establish relative sensitivities of herring stocks and species to oil exposure.

3.3 Materials and Methods

3.3.1 Test Species

Local fishermen collected Pacific herring from Neck Point, British Columbia (BC) and Sitka Sound, Alaska (AK) on March 1, 2010 and April 6, 2010, respectively. Gonads were excised, stored in plastic bags and packed on ice packs in a cooler for transport to the Gulf Fisheries Centre laboratory (Department of Fisheries and Oceans, Moncton, NB, Canada), arriving March 3, 2010 from British Columbia and April 8,

2010 from Alaska. Local fishermen also collected Atlantic herring from the Northumberland Strait. Herring were collected from Petit-Cap, New Brunswick on April 30, 2010 from a spring-spawning stock and from Pictou, Nova Scotia on September 9, 2010 from a fall-spawning stock. Whole fish were placed in plastic bags, wrapped in paper and transported in coolers with crushed ice to the Gulf Fisheries Centre laboratory 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.

3.3.2 Fertilization

Eggs for all bioassays were fertilized on the day they arrived at the laboratory to maximize the fertilization rate. Eggs were extracted from mature adults by ventral pressure or from gonads by making an incision and subsequently squeezing to remove the eggs. The eggs were pooled to minimize interfemale variablity (9 females from BC; 16 females from AK; 15 females from Atlantic spring stock; 3 females from Atlantic fall stock; Kocan *et al.* 1996) and dispersed homogeneously on glass microscope slides using a dissecting needle (approximately 80-100 eggs/slide). Milt from the testes was extracted from mature adults by ventral pressure or from gonads by removing a small section of the gonad and mixing in water. Milt was pooled (3 males from BC; 10 males from AK; 6 males from Atlantic spring stock; 5 males from Atlantic fall stock; Kocan *et al.* 1996) and mixed with 30‰ water (Kent Sea Salt, Kent Marine, Acworth, GA, USA in reverse osmosis treated municipal water) in a 38 x 27 x 5 cm glass Pyrex pan. The egg-covered slides were transferred to the milt solution for approximately 10 minutes for fertilization to occur, then rinsed with and placed in clean 30‰ water. Fertilization

success was assessed one hour post-fertilization (PF; Kocan *et al.* 1996) by observing a definite cellular blastomere, in the blastula developmental stage, forming at the pole of the egg (C. Bourque, Department of Fisheries and Oceans, Moncton, NB, Canada, personal communication). The slides were transferred immediately to the respective test solutions. Since eggs and milt were combined into one common source, replication was the jar (each containing a single slide).

3.3.3 Test Solutions

Test solutions were made using medium (Alaska North Slope, ANS and Medium South American, MESA) and light (Arabian Light, AL) crude oils. Groups of Pacific herring embryos were exposed to one of the three crude oils while Atlantic herring were exposed only to AL crude. The crude oils and dispersant, Corexit 9500, were provided by Department of Fisheries and Oceans, Centre for Offshore Oil, Gas and Energy Research (COOGER, Dartmouth, NS, Canada). ANS was weathered 10% by volume using nitrogen sparging in 2009, MESA was weathered 13.8% by sparging with air for 130 hours in 1998, and AL was weathered 7% by volume by sparging with air in 2009.

Embryos were exposed to water accommodated fraction (WAF) and chemically enhanced water accommodated fraction (CEWAF) of these oils. To generate CEWAF, a 1:9 mixture of oil and water was mixed with a stir bar 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 added at a ratio of 1:10 dispersant:oil (Hemmer *et al.* 2010) and mixed further 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. Dispersant effectiveness was defined as the concentration of

summed PAH in the CEWAF stock divided by the concentration of summed PAH in the crude oil, which is based on the total volume of oil added to the flask (Sorial *et al.* 2004). The same protocol was used to generate WAF except the dispersant was not added. Fresh CEWAF and WAF mixtures were prepared daily.

Nominal concentrations tested for the Pacific herring bioassays and the Atlantic herring spring stock bioassay were 0.0001, 0.001 and 0.01% v/v CEWAF and 0.01, 0.1 and 1.0% v/v WAF. Atlantic herring from the fall stock bioassay were exposed to only CEWAF at nominal concentrations of 0.0001, 0.001 and 0.01% v/v. Concentrations were chosen based on previous studies (Ramachandran *et al.* 2004; McIntosh *et al.* 2010). Controls for the bioassays included a negative control (clean water) and a retene positive control (320 µg/L; Billiard *et al.* 1999; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada).

3.3.4 Test Conditions

Unfertilized eggs were removed and live embryos were reduced randomly to 20 per slide within ~72 hours of fertilization. Exposures were performed in glass Mason jars (non-aerated and static) containing 200 mL of test solution with each treatment concentration performed in triplicate. Embryos were exposed to test solutions from one hour PF until hatch with daily renewal of test solutions. Test conditions were maintained at 10°C and 30‰. Temperature and salinity were recorded daily throughout the bioassay and never deviated more than ± 1°C and ± 1‰. Experiments were terminated when 100% of viable embryos hatched. At hatch, larvae were evaluated for BSD symptoms (see below) and 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).

3.3.5 Characterization of Hydrocarbon Concentrations

3.3.5.1 Gas Chromatography-Mass Spectroscopy

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) at T=0 h and T=24 h for all three crude oils. Water samples (500 mL) were placed in hexane-acetone rinsed amber glass bottles, preserved with 500 µL 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 summed PAH detection limit ranged from 0.065-0.189 µg/L. GC-MS (Environmental Protection Agency 1996; King and Lee 2004) analysis was performed by COOGER in Dartmouth, NS.

3.3.5.2 Synchronous Scan Fluorescence

To quantify the total petroleum hydrocarbon (TPH) concentration (fluorescing compounds, mainly PAHs; Lyons *et al.* 2011), WAF (1.0% v/v for Pacific herring and Atlantic herring spring experiments) and CEWAF (0.01% v/v for all experiments) water samples were collected at T=0 h and T=24 h. Additionally, WAF (1.0% v/v for Atlantic herring spring experiment) and CEWAF (0.01% v/v for both Atlantic herring spring and

fall experiments) water samples were also collected at T=2, 4, and 8 h to monitor how the test solution changed over time. Water samples (50mL) were placed in hexane-acetone rinsed glass test tubes, preserved with 50 µL 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; Singer *et al.* 1996, Ramachandran *et al.* 2006 as modified by Lyons *et al.* 2011). The TPH limit of quantification was 4 µg/L.

3.3.6 Toxicological Response

Toxicity was based on survival-to-hatch, length-at-hatch and the prevalence and severity of BSD observed in the hatched larvae. BSD is characterized by the presence of spinal curvatures, pericardial edema, yolk sac edema and jaw malformations and by the reduction in heart rate, time-to-hatch and length-at-hatch and overall reduced survival (Billiard *et al.* 1999; Brinkworth *et al.* 2003; Carls *et al.* 2008; Boudreau *et al.* 2009; McIntosh *et al.* 2010). Heart rate and time-to-hatch were quantified but did not show interpretable responses to oil treatment and are therefore not presented (Appendix B).

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 (200 mL test solution in a Mason jar) and scored 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).

3.3.7 Statistical Analysis

Replication was the jar, with three jars per treatment group. 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 negative control and each of the nine oil-concentration combinations, considered individual treatments, were compared by 1-factor ANOVA for survival-to-hatch and BSD SI. Length-at-hatch analyses included 1-factor nested ANCOVA models to remove the variability associated with replicates (nested within concentration) to test the influence of treatment only. Since length-at-hatch could be positively correlated with incubation period, time-to-hatch was included as a covariate. These end-points were also analyzed for spawning stocks and species comparisons by a 1-factor ANOVA (survival and BSD SI) or 1-factor nested ANCOVA (length-at-hatch) assessing the effect of concentration. ANOVAs were followed by Tukey multiple comparison tests. Prior to analyses, data were tested for normality (probability plot) and homoscedasticity (Levene/Bartlett test) and arcsine square-root transformed (survival and BSD SI).

Analyses were performed with Systat version 11.0 (SPSS, Chicago, IL, USA). The level of significance was $p<0.05$. Means are accompanied by their 95% confidence interval, where possible.

3.4 Results

3.4.1 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 3-1). Summed PAH concentrations were higher in stocks of CEWAF than WAF: approximately 40, 15 and 10X higher in ANS, MESA and AL CEWAF compared to WAF, respectively (Table 3-1). 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 AL CEWAF stocks (Table 3-1). 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 AL CEWAF test solutions (Table 3-2). 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 3-2).

3.4.2 Positive Control Response

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

3.4.3 Toxicity of Three Crude Oils to Geographically Separate Spawning Stocks of Pacific Herring

Survival-to-hatch was not affected in BC embryos following exposure to WAF or CEWAF, nor was survival-to-hatch affected in AK embryos exposed to WAF (Tables 3-3, 3-4, 3-5; ANOVA, $p<0.05$). AK embryo survival was reduced by CEWAF doses

starting at 0.001% v/v for ANS and AL and 0.0001% v/v for MESA (Tables 3-4, 3-5). MESA is considered to be the most toxic oil to AK embryos exposed to CEWAF since survival-to-hatch was significantly reduced at a lower concentration than the other test oils.

Length-at-hatch of surviving AK, but not BC, embryos was significantly reduced by 0.01% v/v MESA and ANS, but not AL, CEWAF (Table 3-6).

Common morphological abnormalities observed in this study are presented in Figure 3-1. 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 3-2; Table 3-7). However, embryos from AK were significantly affected with the LOEC (lowest observe effect concentration) being 0.0001% v/v for MESA CEWAF, 0.001% v/v for both ANS and AL CEWAF and 0.01% v/v for ANS and MESA WAF and 0.1% v/v for AL WAF (Figure 3-2; Table 3-7).

3.4.4 Toxicity of Chemically Dispersed Arabian Light Crude to Atlantic Herring that Spawn in Different Seasons

Survival-to-hatch was not significantly reduced by exposure to AL CEWAF for either stock of Atlantic herring (Table 3-8). Length-at-hatch was significantly reduced compared to controls at the highest concentration (0.01% v/v) tested in the spring stock, while length-at-hatch was not reduced in embryos from the fall spawning stock (Table 3-9). Both the frequency (Table 3-8) and severity (BSD SI; Table 3-9; Figure 3-3) of abnormalities were higher in the fall than the spring stock, including negative controls.

However, a significant elevation of abnormality by AL CEWAF was seen only in the spring stock at the highest dose tested (0.01% v/v; Figure 3-3; Table 3-9).

3.4.5 Sensitivity of Herring Species to Arabian Light Crude

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 AL WAF or CEWAF exposure for either species (85-94% survival in Pacific herring and 100% survival in Atlantic herring; Tables 3-3, 3-5, 3-8). 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 3-6, 3-9). Similarly, exposure to AL 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 3-4; Tables 3-7, 3-9).

3.5 Discussion

3.5.1 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. A possible explanation for this observation could be related to the dispersant effectiveness of Corexit 9500 on the crude oils. 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). Comparison of summed PAHs measured by GC-MS in crude oil stocks and those in the CEWAF stocks showed dispersant effectiveness to be 5.3, 2.6 and 1.3% for MESA, ANS and AL, respectively. This means that only 1.3-5.3% of the different crude oils went into the water column (CEWAF) and ultimately into the test solutions.

Factors that can influence dispersant effectiveness include mixing speed and the degree of weathering (Chandrasekar *et al.* 2005). Increasing mixing speed causes the dispersant to be more effective at dispersing the crude oil. To illustrate, dispersant effectiveness on Prudhoe Bay crude increased from 21% to 70% as mixing speed increased from 150 to 250 rpm (Chandrasekar *et al*. 2005). 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. However, the size and shape of the mixing container and stir bar can influence the mixing rpm speed. In the present study, one-inch stir bars were used in 500 mL baffled flasks. Therefore, sufficient mixing speed was likely achieved to produce successful dispersion and thus does not explain the low dispersant effectiveness observed in the present study, although the weathering of the crude oil may help explain this dilemma. The viscosity of crude oil increases as it weathers, which causes the chemical dispersant to be less effective (Chandrasekar *et al.* 2005). Moles *et al.* (2002) reported that Corexit 9500 effectiveness decreased from 25% for fresh ANS crude to <10% for 20% weathered ANS crude at 10°C and 32‰. Since the oil used in the present study was weathered (7-13.8%) and dispersed under similar conditions it is possible that weathering contributed to the low dispersant effectiveness.

The most probable explanation for the low dispersant effectiveness and ultimately low exposure concentrations observed in the present study would be the re-coalescing of oil droplets during the one-hour settling phase. As outlined in section 2.5.4, since the volume of oil mixed with water was relatively large (20 mL oil compared to 100 µL used in effectiveness tests; Singer *et al.* 2000; Moles *et al.* 2002; Venosa *et al.* 2002; Sorial *et al.* 2004; Chandrasekar *et al.* 2005) and settled for a longer period (1 hour versus 10 minutes; Singer *et al.* 2000; Moles *et al.* 2002; Venosa *et al.* 2002; Sorial *et al.* 2004; Chandrasekar *et al.* 2005), there would be a greater chance of oil droplets colliding and forming larger droplets that could re-coalesce and effectively be removed from the stock test solution (T. King, Bedford Institute of Oceanography, Dartmouth, NS, Canada, personal communication).

In summary, the unexpectedly low hydrocarbon concentrations in the test solutions may be caused by the very low dispersant effectiveness, likely due to re-coalescing oil being removed from the stock CEWAF solution. Further explanation for low measured hydrocarbon concentrations could simply be related to chemical analysis. The quality of sample preservation, storage time and extraction analysis could also be issues leading to reduced hydrocarbon detection.

3.5.1 Toxicity of Three Crude Oils to Geographically Separate Spawning Stocks of Pacific Herring

Pacific herring embryos from Sitka Sound, Alaska appeared to be more sensitive to oil exposure than embryos from Neck Point, British Columbia as observed through survival-to-hatch, length-at-hatch and developmental abnormalities through the BSD SI (Table 3-10). The difference in stock sensitivities within Pacific herring could be due to

egg quality, which may be influenced by the age of spawners and the geographical location of the stock. Herring spawning for the first time typically produce eggs of lower quality (Brooks *et al.* 1997). Since Pacific herring eggs used in these experiments were stripped from wild stocks, the particular age class of the adults is unknown. Therefore, the age of adults may have been different between the stocks of Pacific herring and caused the quality of eggs to also be different. Pacific herring fecundity (number of eggs) increases with latitude from California to British Columbia to Alaska (Paulson and Smith 1977). Since 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).

MESA was the most toxic oil tested in the present study for Pacific herring embryos since MESA was either the single most toxic or among the most toxic of the three oils tested. 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 to 3.2X more in the present study), which could explain the increased toxicity. Wu *et al.* (2012) tested the toxicity of four weathered crude oils to rainbow trout embryos. The lightest crude (Scotian light) was found to be the least toxic, while the other three crude oils

(ANS, MESA and Federated crude) varied little in toxicity. The authors hypothesized that the similar toxicity observed in the heavier oils was likely due to only small differences in the concentrations of three- and four-ringed alkyl PAHs. Scotian light crude was the least toxic, likely because there was very little three- and four-ringed alkyl PAHs. The EPA (2003) also reported that medium crude oils contain more PAHs than light crude oil; weathered ANS contains 2X to 4X more PAHs than AL (based on phenanthrenes, fluorenes, chrysenes, pyrene, benzo-a-pyrene, anthracene and benzo-a-anthracene).

3.5.2 Toxicity of Chemically Dispersed Arabian Light Crude to Atlantic Herring that Spawn in Different Seasons

In the present study, survival-to-hatch was not reduced in either spring or fall spawned embryos (Table 3-10). Length-at-hatch was only significantly reduced at the highest concentration in spring-spawned embryos. Conversely, developmental abnormalities combined in the BSD SI, was significantly elevated compared to control embryos at the highest concentration tested in spring embryos. In the fall spawning stock, a large variation in the BSD SI values of the control group may have masked significant increases in the 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 low 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, Department of Fisheries and Oceans, Moncton, NB, Canada, personal communication). 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.

3.5.3 Toxicity of Arabian Light Crude to Herring Species

In the present study, length-at-hatch was significantly reduced and developmental abnormalities observed through the BSD SI were significantly increased compared to the control group at a lower concentration in spring-spawned Atlantic herring than Pacific herring from BC (Table 3-10). Atlantic herring are concluded to be more sensitive to oil exposure than Pacific herring. Similarly, Greer (2011) observed that Atlantic herring embryos were 4X more sensitive to ANS CEWAF exposure than

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 corroborate the greater sensitivity of Atlantic herring, the range of sensitivity of these species should be considered in risk assessments of dispersed oil. It is interesting to note that similar results were found between the present study and Greer (2011) even though Atlantic herring were taken from different stocks (spring spawners from Petit-Cap, NB compared to fall spawners from Escuminac, NB) and exposed to different test salinities (30‰ compared to 15‰).

Difference in sensitivity to crude oil, in the present study, may be a result of biological (or ecological) differences between the Atlantic and Pacific 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). In contrast, spring spawning Atlantic herring, which were used in this bioassay, typically spawn at lower temperatures of 5ºC (Steward and Arnold 1994). However, these differences are unlikely 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.

3.5.4 Environmental Implications

One of the beneficial outcomes associated with the low concentrations tested in our study was the ability to observe that measured concentrations as low as 0.38 µg/L summed PAH (1.0% v/v MESA WAF) produced 100% abnormally developed larvae.

Even a 100-fold dilution of this concentration (0.01% v/v MESA WAF), which was below detection levels (~0.065-0.189 µg/L) by GC-MS, produced developmental abnormalities in 83% of embryos (Tables 3-1, 3-4). These results suggest chronic toxicity to hydrocarbons for herring embryos at concentrations lower than reported previously (Carls *et al*. 1999, 2002). Exposure concentrations measured by GC-MS were lower than those reported by other studies (McIntosh 2009). Since water samples experienced delayed analysis time, it is possible that the concentration of PAHs reported may not reflect the actual exposures. However, TPH concentrations measured by fluorescence were comparable to those reported by Greer (2011).

Chronic embryonic exposures can be criticized for overestimating the toxicity of oil spills because, once dispersed, toxic oil concentrations are not likely to persist 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, this may not always be true. Following the Exxon Valdez spill, total PAH concentrations in the open seawater (1-5 metres deep) ranged from 0.91-2.23 µg/L and from 1.26-6.24 µg/L adjacent to heavily oiled beaches. Concentrations ranging from 0.92-1.59 µg/L persisted for at least five weeks at 1m depths (Short and Harris 1996). Therefore, chronic exposures to low level PAHs in the present study may reflect environmental conditions following some 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.

The potential for exposure of freshly spawned herring embryos will depend on the depths of their spawning habitats. Atlantic herring typically spawn offshore at

depths of 5 to 150 metres (Blaxter 1985), depending on the temporal stock (Steward and Arnold 1994). Atlantic herring from spring spawning stocks typically spawn in water less than 5 metres deep in the Gulf of St. Lawrence and up to 10 metres deep in eastern Newfoundland (Steward and Arnold 1994). However, fall spawning stocks typically spawn in deeper waters of 10 to 25 metres in the Gulf of St. Lawrence and up to 40 metres off southwestern Nova Scotia (Steward and Arnold 1994). Pacific herring spawn in shallow intertidal and subtidal regions at depths <20 metres, but usually within 10 metres (Haegele and Schweigert 1985). These spawning depths can be reached by toxic concentrations of dispersed oil (McAuliffe *et al.* 1980; Ballou *et al.* 1987; Buckley *et al.* 1987; Humphrey *et al*. 1987; Short and Harris 1996; Diercks *et al.* 2010). Therefore, a spill could have the potential to impair early life stages of many herring populations.

3.5.5 Limitations and Future Research

The observed sensitivity between different stocks and species may be influenced by the time from collection to fertilization. Eggs were fertilized as quickly as possible, but the time elapsed varied between experiments. McIntosh (2009) found that gamete viability decreased with time until approximately four days post-collection, at which point they were no longer viable.

Damage to the gametes during transport could also account for variation between experiments. Although fish and gametes were both wrapped in paper for protection it is possible that the gametes became damaged from low temperatures. However, gametes can sustain low temperatures (0.5°C) and still remain viable (Haegele and Schweigert 1985).

The efficiency of mixing WAF and CEWAF daily could also produce varied exposure concentrations. Since test solutions of WAF and CEWAF are not homogenous mixtures (Fingas 2008) the concentration of hydrocarbons may vary between batches. Therefore, responses between stocks and species may have been influenced by differences in chemical composition both within and between experiments.

Further research is needed to validate the results in the present study. Each experiment should be repeated to determine inter-assay variance within each stock. Couillard (2002) tested this variance by replicating each experiment three times using adults from a wild population of mummichog and found experiments to be quite consistent. However, the experiments were completed using the same sub-population of mummichog instead of repeating the experiment with different samples from the same population. The number of test concentrations in future experiments should also be increased from three to six to cover a greater range in order to estimate toxicity endpoints, such as the LC50 (median lethal concentration) and the EC50 (median effective concentration) for sublethal responses.

3.5.6 Conclusion

There is evidence in the present study to support a need for species-specific advice for risk assessment since Atlantic herring were found to be more sensitive than Pacific herring. Pacific herring from Alaska were more sensitive than stocks from British Columbia. However, elevated BSD SI in the control group of the fall stock of Atlantic herring limited our assessment of the sensitivity of the Atlantic herring spawning stocks. MESA test solutions 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.

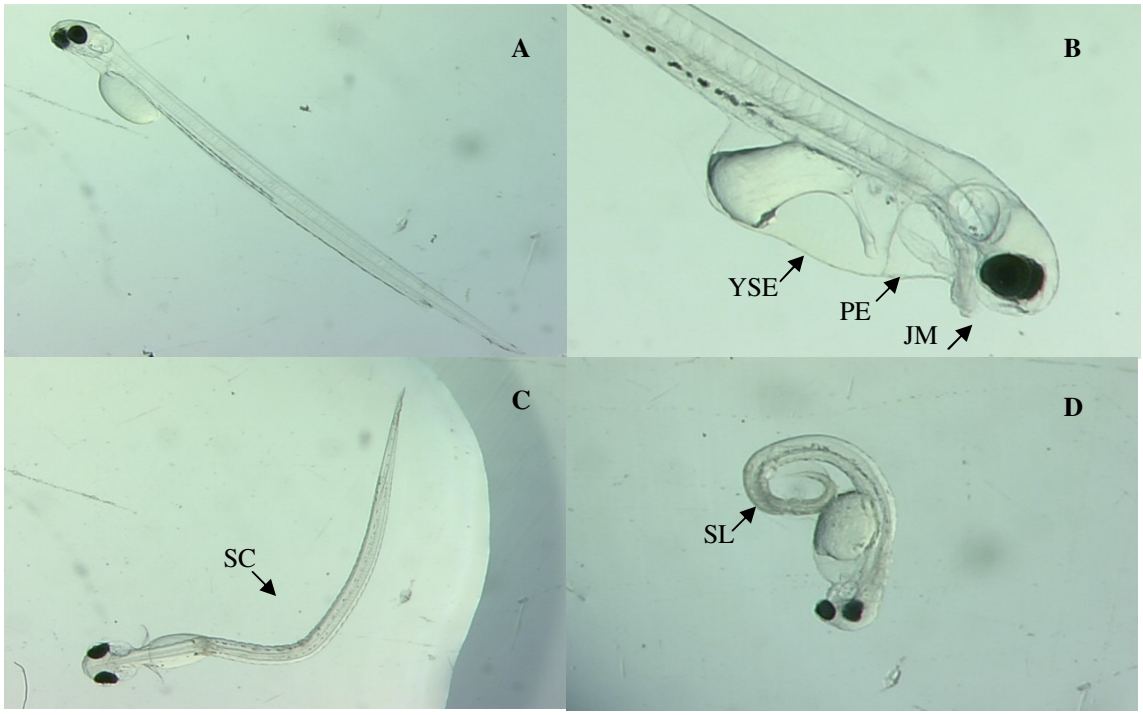

Figure 3-1. Most common abnormalities observed in herring (*Clupea sp.*) embryos exposed to crude oil WAF and CEWAF from fertilization until hatch. Shown are a normal larva approximately 7mm in length (**A**), pericardial edema (PE), yolk sac edema (YSE) and jaw malformation (JM; **B**), spinal curvature (SC; **C**) and skin lesion (SL; **D**).

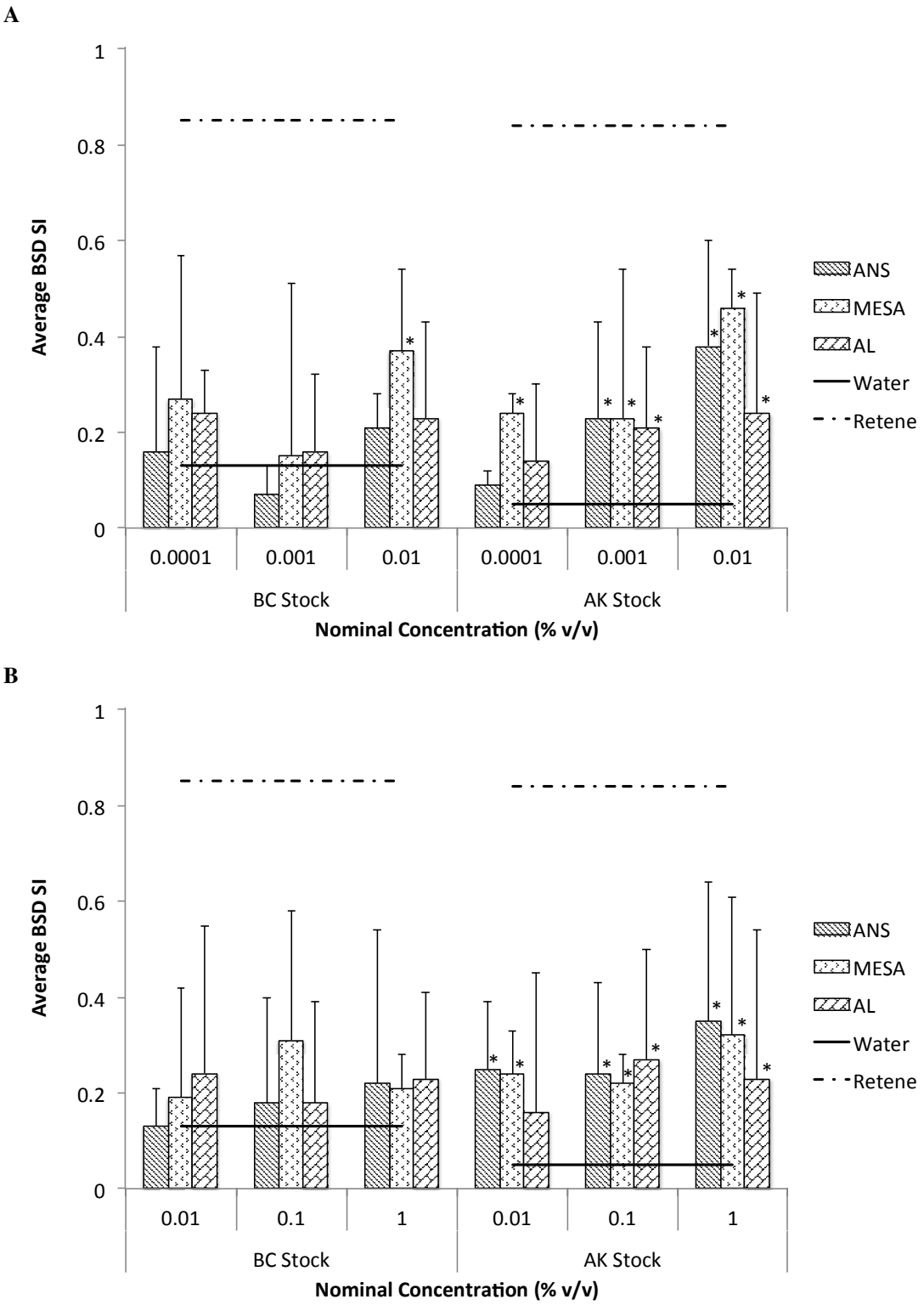

Figure 3-2. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Pacific herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) CEWAF (**A**) and WAF (**B**). Stocks were analyzed separately and asterisks (*) indicate treatments that were significantly different from the negative control (ANOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

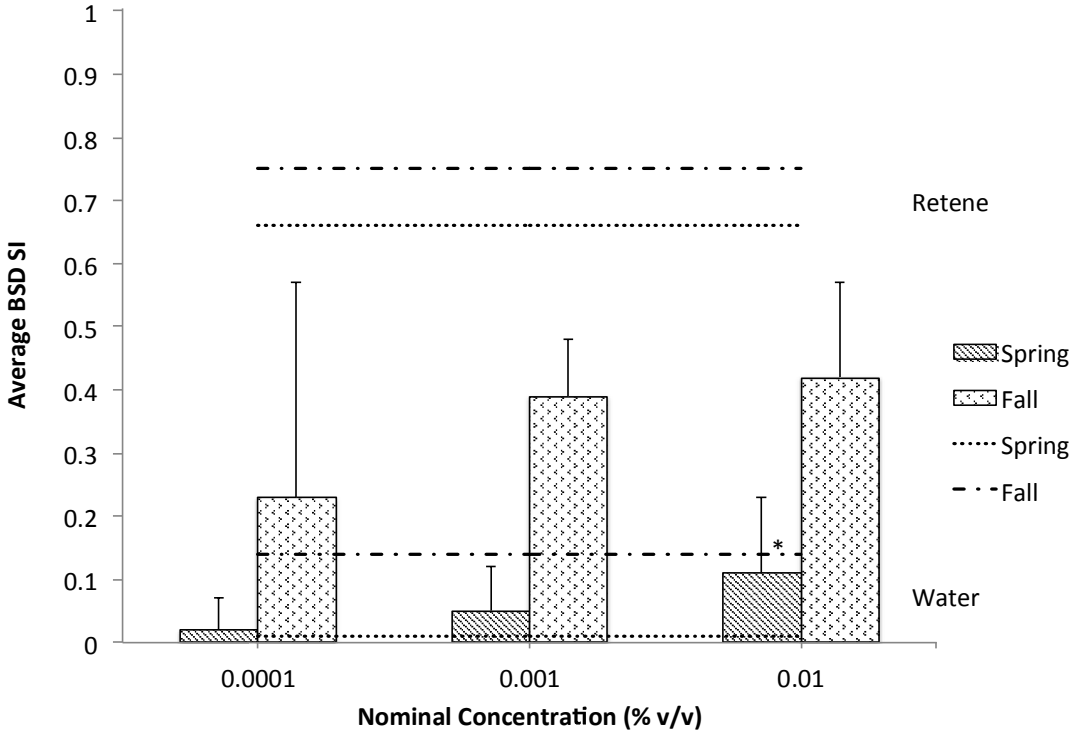

Figure 3-3. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Atlantic herring embryos exposed to Arabian Light CEWAF. Stocks were analyzed separately and asterisks (*) indicate treatments that were significantly different from the negative control (ANOVA; Tukey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

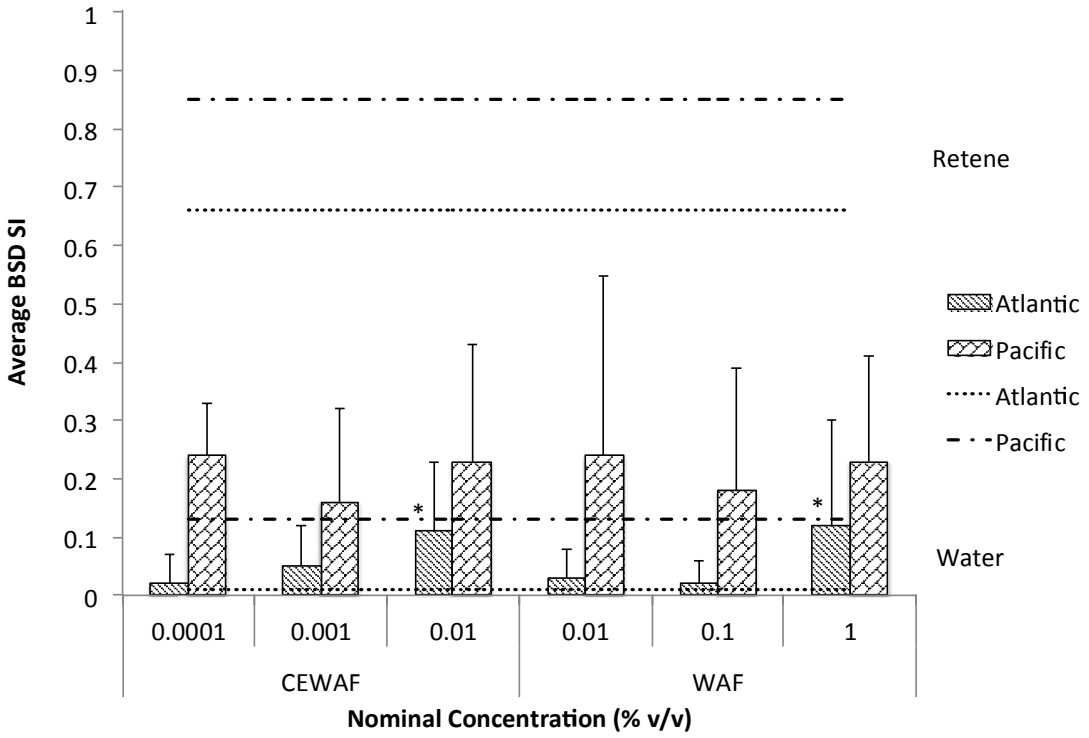

Figure 3-4. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Pacific (BC stock) and Atlantic herring (spring stock) embryos exposed to Arabian Light WAF and CEWAF. Species were analyzed separately and asterisks (*) indicate treatments that were significantly different from the negative control (ANOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

Table 3-1. 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. Dispersant effectiveness was calculated ((amount of PAH in CEWAF stock/[amount of PAH in crude oil stock*10%])*100) and is defined as the amount of oil products that are dispersed into the water column compared to the amount of oil products that remain on the water surface in the slick. Therefore, only 1.3-5.3% of the different crude oils went into the water column (CEWAF).

			Summed PAH (µg/L)			
			ANS	MESA	AL	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	
	Dispersant Effectiveness		2.6%	5.3%	1.3%	
WAF		Stock	7206	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 3-2. 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			AL	AL	ANS	MESA	AL	ANS	MESA	AL
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

Table 3-3. Pacific herring embryos (BC stock) exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) 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 swimming ability (0-2) including 95% confidence intervals. Upper 95% confidence interval of back transformed values for survival was set at a maximum value of 100%. N=3 jars/concentration.

		Conc. (% v/v)	Survival	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
CEWAF	ANS	0.0001	98 (58-100)	50±37	0±0	2±8	34±42	14±10	25±44	1.88 ±0.09
		0.001	99 (71-100)	57±7	0±0	4±7	26±31	19±13	33±18	1.91±0.07
		0.01	87 (5-100)	84±13	42±48	31±22	60±2	51±41	18±33	1.67±0.16
	MESA	0.0001	85 (49-100)	70±46	10±21	4±9	54±45	32±55	49±24	1.84±0.12
		0.001	98 (47-100)	63±6	2±9	4±8	39±45	34±5	31±16	1.82±0.10
		0.01	82 (52-99)	93±18	37±5	50±46	57±14	81±21	31±10	1.78±0.13
	AL	0.0001	85 (71-95)	49±25	4±8	10±15	44±34	18±2	35±36	1.71±0.17
		0.001	92 (74-100)	42±1	2±7	5±13	24±12	26±24	26±10	1.78±0.15
		0.01	89 (69-99)	72±15	10±22	8±17	40±30	51±21	38±30	1.83±0.13
WAF	ANS	0.01	95 (95-95)	58±57	0±0	2±7	35±53	23±7	35±33	1.86±0.11
		0.1	92 (74-100)	52±40	0±0	2±8	26±24	31±25	20±14	1.87±0.10
		1.0	85 (49-100)	72±38	22±26	18±10	34±13	45±47	29±22	1.88±0.11
	MESA	0.01	90 (74-99)	63±62	0±0	6±1	45±67	26±60	43±24	1.87±0.09
		0.1	79 (47-98)	64±13	4±17	15±22	32±7	47±1	32±13	1.83±0.14
		1.0	94 (85-99)	82±20	18±16	29±16	37±11	61±12	34±48	1.80±0.11
	AL	0.01	86 (53-100)	59±33	2±8	8±6	42±39	26±4	36±23	1.80±0.13
		0.1	93 (43-100)	61±17	7±15	7±8	25±44	32±30	23±25	1.85±0.11
		1.0	94 (52-100)	78±10	7±10	9±10	49±11	49±18	52±20	1.71±0.14
Controls	Water		95 (48-100)	30±17	6±1	5±13	22±20	7±6	14±25	1.84 ±0.11
	Retene		23 (4-51)	100±0	22±96	69±67	100±0	11±48	26±63	1.07±0.42

Table 3-4. Pacific herring embryos (AK stock) exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) WAF and CEWAF. 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 back transformed values for survival was set at a maximum value of 100%. N=3 jars/concentration.

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

Table 3-5. Percent survival-to-hatch of Pacific herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) WAF and CEWAF. 1-Factor ANOVAs were performed using treatment (oil-concentration combinations) as the factor. Asterisks (*) represent treatments with significantly reduced survival compared to the negative control (Tukey test, $p<0.05$). Stocks were analyzed separately. Non-significant results are labeled as NS. N=3 jars/concentration.

Survival-to-Hatch				
	Crude Oil	Conc. (% v/v)	BC Stock Bioassay	AK Stock Bioassay
CEWAF	1-Factor ANOVA		NS	$F_{9, 20} = 9.29$, $p<0.0001$
	ANS	Water	95 (48-100)	100 (100-100)
		0.0001	98 (58-100)	100 (100-100)
		0.001	99 (71-100)	88 (81-94) *
		0.01	87 (5-100)	84 (66-96) *
	MESA	0.0001	85 (49-100)	85 (85-85) *
		0.001	98 (47-100)	94 (38-100)
		0.01	82 (52-99)	79 (62-91) *
	AL	0.0001	85 (71-95)	95 (95-95)
		0.001	92 (74-100)	90 (90-90) *
		0.01	89 (69-99)	92 (83-98) *
		Retene	23 (4-51)	22 (15-29)
WAF	1-Factor ANOVA		NS	NS
	ANS	Water	95 (48-100)	100 (100-100)
		0.01	95 (95-95)	89 (65-100)
		0.1	92 (74-100)	93 (43-100)
		1.0	85 (49-100)	93 (43-100)
	MESA	0.01	90 (74-99)	90 (74-99)
		0.1	79 (47-98)	94 (85-99)
		1.0	94 (85-99)	88 (55-100)
	AL	0.01	86 (53-100)	95 (48-100)
		0.1	93 (43-100)	84 (62-97)
		1.0	94 (52-100)	92 (74-100)
		Retene	23 (4-51)	22 (15-29)

Table 3-6. Average length-at-hatch (mm) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) WAF and CEWAF. 1-Factor ANOVAs were performed using treatment (oil-concentration combinations) as the factor. Asterisks (*) represent treatments that are significantly shorter compared to the negative control (Tukey test, $p<0.05$). Stocks were analyzed separately. Non-significant results are labeled as NS. N=3 jars/concentration.

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
CEWAF 1-Factor ANOVA: $F_{9, 501} = 3.97$, $p<0.001$; WAF 1-Factor ANOVA: $F_{9, 503} = 2.14$, $p=0.025$					
Water					7.26±0.29
CEWAF	0.0001	7.97±0.19	7.71±0.32	7.70±0.24	
	0.001	7.85±0.17	7.70±0.16	7.55±0.30	
	0.01	7.26±0.35	7.21±0.20	7.41±0.31	
WAF	0.01	7.61±0.24	7.81±0.28	7.72±0.24	
	0.1	7.56±0.25	7.83±0.42	7.58±0.26	
	1.0	7.45±0.27	7.91±0.32	7.51±0.25	
Retene					5.49±0.39
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
CEWAF 1-Factor ANOVA: $F_{9, 510} = 4.28$, $p<0.001$; WAF 1-Factor ANOVA: NS					
Water					7.43±0.11
CEWAF	0.0001	7.36±0.17	7.53±0.14	7.41±0.19	
	0.001	7.31±0.26	7.29±0.21	7.38±0.25	
	0.01	6.46±0.25 *	6.60±0.27 *	7.35±0.16	
WAF	0.01	7.39±0.15	7.34±0.22	7.21±0.21	
	0.1	7.18±0.23	7.35±0.21	7.28±0.21	
	1.0	7.06±0.21	7.10±0.19	7.24±0.16	
Retene					4.66±0.46

Table 3-7. Average blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) WAF and CEWAF. 1-Factor ANOVAs were performed using treatment (oil-concentration combinations) as the factor. Asterisks (*) represent treatments with significantly increased BSD SI compared to the negative control (Tukey test, $p<0.05$). Stocks were analyzed separately. Non-significant results are labeled as NS. N=3 jars/concentration.

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
1-Factor ANOVA: $F_{9, 20} = 3.72$, p=0.007					
Water					0.13 (0-0.44)
CEWAF	0.0001	0.16 (0.03-0.38)	0.27 (0.05-0.57)	0.24 (0.16-0.33)	
	0.001	0.07 (0.03-0.13)	0.15 (0-0.51)	0.16 (0.05-0.32)	
	0.01	0.21 (0.15-0.28)	0.37 (0.22-0.54) *	0.23 (0.08-0.43)	
Retene					0.85 (0.60-0.98)
1-Factor ANOVA: NS					
Water					0.13 (0-0.44)
WAF	0.01	0.13 (0.07-0.21)	0.19 (0.04-0.42)	0.24 (0.04-0.55)	
	0.1	0.18 (0.04-0.40)	0.31 (0.10-0.58)	0.18 (0.04-0.39)	
	1.0	0.22 (0.02-0.54)	0.21 (0.15-0.28)	0.23 (0.09-0.41)	
Retene					0.85 (0.60-0.98)
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	
1-Factor ANOVA: $F_{9, 20} = 12.20$, p<0.001					
Water					0.05 (0.01-0.09)
CEWAF	0.0001	0.09 (0.07-0.12)	0.24 (0.20-0.28) *	0.14 (0.03-0.30)	
	0.001	0.23 (0.08-0.43) *	0.23 (0.03-0.54) *	0.21 (0.09-0.38) *	
	0.01	0.38 (0.18-0.60) *	0.46 (0.39-0.54) *	0.24 (0.06-0.49) *	
Retene					0.84 (0.79-0.89)
1-Factor ANOVA: $F_{9, 20} = 4.48$, p=0.003					
Water					0.05 (0.01-0.09)
WAF	0.01	0.25 (0.13-0.39) *	0.24 (0.16-0.33) *	0.16 (0.01-0.45)	
	0.1	0.24 (0.09-0.43) *	0.22 (0.17-0.28) *	0.27 (0.09-0.50) *	
	1.0	0.35 (0.11-0.64) *	0.32 (0.09-0.61) *	0.23 (0.03-0.54) *	
Retene					0.84 (0.79-0.89)

Table 3-8. 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 swimming ability (0-2) including 95% confidence intervals. Upper 95% confidence interval of back transformed values for survival was set at a maximum value of 100%. N=3 jars/concentration.

		Conc. (% v/v)	Survival	Abnormal	Pericardial Edema	Yolk Sac Edema	Spinal Curvature	Jaw	Skin Lesion	Swim Ability
Spring	CEWAF	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	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	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
Fall	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
	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

Table 3-9. Average length-at-hatch (mm) and blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Atlantic herring embryos exposed to Arabian Light WAF and CEWAF. 1-Factor ANOVAs were performed using concentration as the factor. Asterisks (*) represent treatments with significantly decreased length and significantly increased BSD SI compared to the negative control (Tukey test, $p<0.05$). Stocks were analyzed separately. N=3 jars/concentration.

		Conc. (% v/v)	Length-at-Hatch (mm)	BSD SI
Spring	CEWAF	Water	7.42±0.12	0.01 (0.00-0.03)
		0.0001	7.27±0.16	0.02 (0.00-0.07)
		0.001	7.13±0.19	0.05 (0.01-0.12)
		0.01	6.93±0.23 *	0.11 (0.04-0.23) *
		Retene	3.30±0.16	0.66 (0.47-0.84)
		ANOVA	$F_{3, 227} = 2.85$, $p=0.038$	$F_{3, 8} = 12.95$, $p=0.002$
	WAF	Water	7.42±0.12	0.01 (0.00-0.03)
		0.01	7.49±0.15	0.03 (0.01-0.08)
		0.1	7.51±0.10	0.02 (0.00-0.06)
		1.0	7.05±0.23 *	0.12 (0.02-0.30) *
		Retene	3.30±0.16	0.66 (0.47-0.84)
		ANOVA	$F_{3, 227} = 6.54$, $p<0.001$	$F_{3, 8} = 12.28$, $p=0.002$
Fall	CEWAF	Water	5.93±0.21	0.14 (0.03-0.66)
		0.0001	5.68±0.22	0.23 (0.02-0.57)
		0.001	5.83±0.28	0.39 (0.30-0.48)
		0.01	5.41±0.37	0.42 (0.27-0.57)
		Retene	3.61±0.17	0.75 (0.46-0.95)
		ANOVA	$F_{5, 214} = 13.50$, $p<0.001$	NS

Table 3-10. Summary of endpoints to assess oil mediated toxicity in Pacific and Atlantic herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) WAF and CEWAF. End-points considered were reduced percent embryo survival-to-hatch, reduced length-at-hatch and increased developmental abnormalities (BSD SI) compared by testing the lowest observed effect concentration (LOEC; ANOVA, $p<0.05$).

		Survival LOEC	Length LOEC	Abnormalities LOEC
British Columbia Stock Bioassay				
CEWAF	ANS	>0.01	>0.01	>0.01
	MESA	>0.01	>0.01	0.01
	AL	>0.01	>0.01	>0.01
WAF	ANS	>1.0	>1.0	>1.0
	MESA	>1.0	>1.0	>1.0
	AL	>1.0	>1.0	>1.0
Alaska Stock Bioassay				
CEWAF	ANS	0.001	0.01	0.001
	MESA	<0.0001	0.01	<0.0001
	AL	0.001	>0.01	0.001
WAF	ANS	>1.0	>1.0	<0.01
	MESA	>1.0	>1.0	<0.01
	AL	>1.0	>1.0	0.1
Atlantic Spring Spawning Stock Bioassay				
CEWAF	AL	>0.01	0.01	0.01
WAF	AL	>1.0	1.0	1.0
Atlantic Fall Spawning Stock Bioassay				
CEWAF	AL	>0.01	>0.01	>0.01

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Chapter 4: General Discussion

4.1 Overview

Crude oil can be introduced into North American waters following accidents at sea or during loading and unloading in coastal bays and estuaries. Current knowledge illustrates that use of chemical dispersants should be considered if oil is spilled near environmentally sensitive ecosystems where the oil would need to be removed from the surface in order to protect the ecosystem (i.e. coastlines, salt marshes, coral reefs, or mangroves; ITOPF 2010; ITOPF 2011). However, chemical dispersion may be detrimental if the spill occurred over herring (*Clupea* sp.) spawning shoals during the reproductive cycle. In the present study, knowledge gaps were addressed regarding the chronic toxicity of chemically dispersed oil on herring embryos by testing factors that influence toxicological responses such as dispersant application, oil concentration, type of crude oil, temperature, salinity and the relative sensitivity of different herring species and stocks. Toxicity tests were conducted using two stocks from both Atlantic herring (*Clupea harengus*) and Pacific herring (*Clupea pallasii*). These embryos were exposed to graded concentrations of WAF and CEWAF of three different crude oils (Alaska North Slope, ANS; Medium South American, MESA; Arabian Light, AL) or three different environmental salinities (7.5, 15, 30‰) or temperatures (7, 10, 15°C).

Chapter 2 results illustrate that Atlantic herring embryos exposed to crude oil contamination in colder and also possibly less saline water exhibited increased sensitivity to oil exposure. Results of chapter 3 illustrate that the sensitivity to hydrocarbon exposure among species and stocks of herring differed substantially. Elevated BSD SI in the control group of the fall stock of Atlantic herring limited our

assessment of the sensitivity of the Atlantic herring spawning stocks. However, Pacific herring embryos from Alaska were more sensitive to crude oil exposure than embryos from British Columbia, with MESA being more toxic than ANS or AL. Atlantic herring were more sensitive to AL exposure than Pacific herring.

4.2 Synthesis

Considering results from chapters 2 and 3, a few conclusions become apparent:

Chapter 3 demonstrated that MESA crude oil was the most toxic of three oils tested to Pacific herring embryos. Since Atlantic herring were found to be more sensitive than Pacific herring, the impact observed of environmental conditions on oil toxicity (Chapter 2) might have been even more pronounced if MESA crude had been used rather than AL crude. Since MESA contained 2X to 3.2X more PAHs than ANS and AL, and lower salinity increased the solubility of PAHs (approximately 2X higher in 7.5‰ than 30‰), a combination of these two factors could have demonstrated a more significant effect of environmental factors, especially salinity, for which effects on oil toxicity were unclear. Therefore, further testing could determine if the influence of environmental factors is more pronounced for oils with greater toxicity.

Chapter 3 also suggested that different sensitivities between Atlantic herring spawning stocks could not be addressed due to the elevated BSD SI in the fall stock control embryos. This implies that the influence of environmental salinity and temperature (chapter 2 results) could be similar for both spawning stocks. However, the similarity in the response for early-life development and embryonic survival does not necessarily imply that other biomarkers and end-points such as genetic damage (Carls *et al.* 1999) or biochemical responses (mixed function oxygenase (MFO); Thomas *et al.*

1997) would also respond similarly. Genetic differences among individuals or populations of the same fish species exist and may affect the toxicity of PAHs (Logan 2007). Further studies should assess responses of other end-points in these two stocks and in older fish.

Further results of chapter 3 suggest that Atlantic herring are more sensitive to AL exposure than Pacific herring. It may also be that temperature and salinity effects on toxicity would be less important for Pacific than Atlantic herring. However, the assessment that Atlantic herring were more sensitive than Pacific herring is very dependent on which stocks of each species were compared. In the present study, to limit geographic (latitudinal) and temporal variability, the British Columbia stock (Pacific herring) was compared to the spring stock of Atlantic herring. However, if the Alaska stock of Pacific herring had been compared to the spring spawning stock of Atlantic herring, the Atlantic species would probably have been found to be more robust. Similarly, if the British Columbia Pacific stock had been compared to the fall Atlantic stock, the Pacific species may have been found to be more robust. Greer (2011) observed that Atlantic herring fall spawned embryos were 4X more sensitive to ANS CEWAF exposure than Pacific herring from BC in their normal development and the incidence of BSD.

4.3 Environmental Implications

Following an oil spill, both salinity and temperature may influence potential toxicity. Oil toxicity can be greater in less saline environments due to both chemical and ecological influences. The solubility of PAH is higher in less saline water, which could result in higher PAH concentrations in the water column (May and Wasik 1978;

Whitehouse 1984; Ramachandran *et al.* 2006). This was evidenced in the present study by summed PAH concentrations being 2.0 and 1.8X higher in 7.5‰ relative to 30‰ CEWAF and WAF stock solutions, respectively. Ecologically, the risk of hydrocarbon contamination and subsequent toxicity in less saline water also has the potential to be greater since these lower salinity environments (estuaries and coastal zones) are physically closer in proximity to very dynamic and sensitive ecosystems (coastlines, vegetation and spawning grounds). Contamination of estuaries could have devastating impacts since more than 75% of commercially important fish species in North America rely on estuaries at some point during their life history (Environmental Protection Agency, EPA 2005). Therefore, an oil spill in these regions would likely have greater impacts than a spill offshore.

Water temperature plays an important role in predicting oil toxicity following a spill. Toxicity could potentially be higher in both warmer and colder conditions due to chemical and biological influences. Chemically, PAH solubility is higher in warmer environments (May and Wasik 1978; Whitehouse 1984), which could lead to higher PAH concentrations in the water column. This was observed in the present study since summed PAH concentrations were 2.2X higher in the CEWAF stock at 15°C relative to 7°C. This effect of temperature was less apparent in exposure concentrations, which contained 1.2X to 1.5X more summed PAHs at 15°C than at 7°C, depending on the dose. However, embryonic development is temperature dependent, being slower in colder water. Even though the PAH concentration is lower in colder water, the embryos are exposed for a longer time due to their slower development (i.e., more than twice as long at 7°C than 15°C in the present experiment). For instance, if an oil spill occurred

over habitats occupied by sensitive species and resulted in toxic hydrocarbon levels reaching the spawning beds continuously over a period of a few weeks, then the potential toxicity of PAH exposure could be enhanced in colder environments. However, if the same spill only resulted in toxic hydrocarbon levels reaching spawning beds for only a couple of days, then the potential toxicity of PAH exposure would likely be enhanced in warmer environments due to the higher PAH concentration. Further research is warranted into impacts of chronic versus short-term exposure to dispersed and undispersed oil. McIntosh *et al.* (2010) showed that some periods of development are more sensitive to oil toxicity than others so chronic effects are unlikely to be a simple cumulative function.

The environmental temperature may also influence toxicity by affecting the spawning season and therefore the density of embryos and larvae available to be exposed to spilled oil. In warm conditions fish stocks typically spawn over a longer period of the year, while in colder environments the spawning season has a relatively small temporal and spatial distribution (Hjermann *et al.* 2007). This trend is observed with Atlantic herring spawning off the Canadian eastern coast in both the spring and fall. The spring stock typically spawns in relatively cold water (~5ºC; Steward and Arnold 1994), which may be responsible for the short spawning season observed in the spring. However, fall spawning stocks generally spawn over a longer time period, which may be in response to spawning in warmer water (10-15ºC; Steward and Arnold 1994). As a result, embryos and larvae (most sensitive life-stages) spawned in cold conditions would likely have a smaller spatial and temporal distribution than those spawned in warmer environments (Hjermann *et al.* 2007). Therefore, oil spilled over

spawning shoals in colder water (where embryos and larvae are more concentrated) has the potential to affect a larger proportion of the population than the embryos and larvae that are more spatially and temporally dispersed in warmer conditions.

Oil spills can have devastating impacts at both the population and ecosystem levels. Hjermann *et al.* (2007) suggested that the collapse of a fish stock would likely affect population levels of their prey, predators and alternative prey of its predators. For instance, if an oil spill resulted in large declines in herring, their prey (plankton; Checkley 1982) populations could increase due to lack of predation but their predator populations (demersal fish, marine mammals, large pelagic fish and seabirds; Overholtz and Link 2007) could decrease due to a lack of food. However, oil contamination also has the potential to negatively affect both the prey and predator populations directly, so it is difficult to predict the impact that an oil spill could have on an ecosystem. To illustrate, following the Exxon Valdez oil spill, marine mammals suffered high mortality (20-40% killer whales and ~50% sea otters) resulting from contact with crude oil (Peterson *et al.* 2003). Since sea otters forage on sea urchins, the reduced population of sea otters allowed sea urchins to grow larger. Larger sea urchins could over-graze the macroalgae and lead to a loss of structural habitat. Although there is no evidence directly linking sea urchin grazing to macroalgae destruction, rockweed (*Fucus gardneri*) was initially lost following the spill. The destruction of the protective algal canopy caused grazing gastropod populations to be reduced, while opportunistic barnacles took advantage of free space on the rocks and thrived for a period (Peterson *et al.* 2003).

4.4 Management Implications

Results of the present study illustrate that response techniques following a spill need to be conservative in their assessment of potentially harmful concentrations since summed PAH concentrations as low as 0.34 µg/L (detected) and at or below 0.065-0.189 µg/L (detection limit) caused a significant reduction in normal development of herring embryos. Embryos that develop abnormally are thought to not recruit into the spawning population since they possess abnormalities (lack of swimming or feeding ability) that make them more susceptible to predation and starvation (Silverstone and Hammell 2002). These findings are important since, as of 2009, the state of Alaska's most stringent control on water contamination allows up to 10 µg/L total PAH for both fresh and marine water used for aquaculture, growth and propagation of fish, shellfish and other aquatic life and wildlife (Department of Environmental Conservation 2009). To protect the organisms that rely on coastal waters, it is important to have standards that reflect the sensitivity of organisms occupying the water.

4.5 Future Research

Knowledge gaps still exist in the field of oil toxicity with respect to environmental salinity and temperature influences addressed in this study. Below, questions concerning the influence of environmental adaptation, exposure duration and the interactive effect of salinity and temperature on oil toxicity are stated and further experiments are proposed to address these confounding factors.

Are organisms adapted to a particular environment more tolerant to oil exposure in those conditions? This could be addressed by exposing spring spawning Atlantic herring (which spawn in colder water) to different temperatures to determine if this

stock is more tolerant to oil exposure at low temperatures, in the same way that fall spawning stocks (spawn in warmer water) were more tolerant to oil exposure in warmer water. Further experimentation could also include exposing numerous organisms with different salinity and temperature requirements to identify if organisms, in general, are more tolerant to oil exposure within their respective natural range.

Is oil toxicity for herring embryos best quantified as a metabolic or developmental rate? If so, then herring exposed to a range of temperature/duration regimes equalling the same number of degree-days should show comparable toxicity.

Does the influence of temperature interact with salinity in predicting the toxicity associated with hydrocarbon exposure? The present study explored the effect of different temperatures at 30‰ and different salinities at 10ºC but did not investigate interactions of these two environmental factors. Whitehouse (1984) investigated the effect of salinity and temperature on the solubility of individual PAHs, and the results did not suggest that the solubility of PAHs is influenced by an interaction between these factors. However, in the present study, the toxicity and PAH concentration did not always correlate. For instance, herring embryos exposed at 7ºC were more sensitive to oil exposure than at warmer conditions, even though the PAH concentration was lowest at this cold temperature. Therefore, one cannot assume that the toxicity of salinity and temperature effects would not interact just because Whitehouse (1984) found the solubility to not interact. Hence, further research is needed to determine if there is an interactive effect between salinity and temperature on the toxicity of crude oil for herring embryos.

4.6 Summary

There is evidence in the present study to support a need for species-specific advice for risk assessment since Atlantic herring were found to be more sensitive than Pacific herring. However, stocks of Atlantic herring responded similarly to oil exposure while stocks of Pacific herring from Alaska were more sensitive than stocks from British Columbia. Rearing temperature was the more influential of the two environmental factors tested. Atlantic herring embryos exposed to colder test conditions (7°C) were more sensitive to AL crude oil. There was some suggestion, from the present study, of greater oil toxicity in lower salinity water but this will need to be corroborated in a more extensive study.

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Appendix A

Embryonic Heart Rate and Time-to-Hatch Analysis for Chapter 2

Methods

Embryonic heart rates in the salinity bioassay were measured on day 7 PF, while embryonic heart rates in the temperature bioassay were measured on day 5, 7 and 12 PF when reared at 15, 10 and 7°C, respectively. Embryos were placed in a temperature-controlled petri dish and heart rate was measured by counting the number of beats under a dissecting microscope for 10 seconds.

Heart rate and time-to-hatch was analyzed by a 2-factor (temperature or salinity and concentration) analysis of variance (ANOVA) model followed by Tukey multiple comparison tests. Interactions were explored by ANOVAs analyzing each factor separately. Data were tested for normality (probability plot) and homoscedasticity (Levene/Bartlett test) and only required transformation (log X+0.01) for both endpoints in the temperature bioassay. Analyses were performed with Systat version 11.0 (SPSS, Chicago, IL, USA). The level of significance was $p<0.05$. Means are accompanied by their 95% confidence interval.

Results

Embryonic heart rate and time-to-hatch were influenced by temperature and exposure concentration. As expected, embryos exposed to CEWAF at 7°C experienced significantly lower heart rates and significantly longer time-to-hatch compared to embryos exposed at 15°C (Tables A2-1, A2-2). For both heart rate and time-to-hatch, a dose-related reduction compared to the control group was observed at a lower concentration at 7°C compared to 10 and 15°C (Table A2-1 and A2-2).

Analysis of embryonic heart rate and time-to-hatch of embryos exposed to various salinities produced significantly different results from the negative control at treatment concentrations sporadically (Tables A2-1, A2-2). In general, exposure to oil or PAH reduced both embryonic heart rate and time-to-hatch (Boudreau *et al*. 2009). However, test concentrations in the salinity bioassay may have been too low (Tables 2-1, 2-2) to produce very clear and uniform trends distinguishing among salinities. Therefore, these endpoints were not included in the assessments.

Table A2-1. Average heart rate (beats/min) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 7, 10 and 15°C. 2-Factor nested ANOVAs were performed using salinity or temperature and concentration as the two factors with the interaction term reported. Significant interactions were explored using 1-factor ANOVAs with uppercase letters representing significant differences among salinities or temperatures (1-factor ANOVA analyzing individual concentrations; Tukey test, $p<0.05$). Arrows represent treatments that were significantly different than the negative control (1-factor ANOVA analyzing individual salinities and temperatures; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{6, 340} = 19.34$, $p<0.001$				
Water	41.80±1.71 A	48.60±2.52 B	40.40±1.85 A	$F_{2, 87} = 17.28$, $p<0.001$
0.0001	44.20±1.71 A	45.80±2.67 AB	48.20±2.08 B ↑	$F_{2, 87} = 3.46$, $p=0.036$
0.001	36.20±2.16 A ↓	42.40±2.63 B ↓	52.00±3.13 C ↑	$F_{2, 87} = 36.77$, $p<0.001$
0.01	39.6±2.47 A	43.80±1.97 B ↓	38.60±2.01 A	$F_{2, 87} = 6.63$, $p=0.002$
Retene	33.80±1.71 A	42.00±2.20 B	42.00±3.17 B	$F_{2, 87} = 15.01$, $p<0.001$
ANOVA	$F_{3, 108} = 13.70$, $p<0.001$	$F_{3, 108} = 6.97$, $p<0.001$	$F_{3, 108} = 35.10$, $p<0.001$	
WAF Interaction: $F_{6, 340} = 6.76$, $p<0.001$				
Water	41.80±1.71 A	48.60±2.52 B	40.40±1.85 A	$F_{2, 87} = 17.28$, $p<0.001$
0.01	48.00±2.20 A ↑	52.80±2.23 B ↑	49.40±2.10 AB ↑	$F_{2, 87} = 5.33$, $p=0.007$
0.1	35.80±1.25 A ↓	49.53±1.90 B	44.60±2.61 C ↑	$F_{2, 87} = 54.38$, $p<0.001$
1.0	39.40±1.63 A	45.00±2.10 B	40.80±2.38 A	$F_{2, 87} = 8.12$, $p=0.001$
Retene	33.80±1.71 A	42.00±2.20 B	42.00±3.17 B	$F_{2, 87} = 15.01$, $p<0.001$
ANOVA	$F_{3, 108} = 41.36$, $p<0.001$	$F_{3, 108} = 9.52$, $p<0.001$	$F_{3, 108} = 16.92$, $p<0.001$	
Conc. (% v/v)	7°C	10°C	15°C	ANOVA
CEWAF Interaction: $F_{8, 195} = 23.20$, $p<0.001$				
Water	52.11 (49.08-55.33) A	57.14 (52.47-62.22) AB	63.23 (57.01-70.14) B	$F_{2, 42} = 5.98$, $p=0.005$
0.0001	35.31 (32.80-38.01) A ↓	54.57 (50.11-59.56) B	86.09 (79.79-92.67) C ↑	$F_{2, 42} = 149.27$, $p<0.001$
0.001	38.27 (36.30-40.35) A ↓	49.65 (46.44-52.96) B	77.97 (70.95-85.69) C ↑	$F_{2, 42} = 110.53$, $p<0.001$
0.01	27.79 (25.11-30.82) A ↓	51.63 (48.07-55.33) B	89.94 (84.71-95.49) C ↑	$F_{2, 42} = 250.91$, $p<0.001$
0.1	29.23 (26.60-32.13) A ↓	38.27 (34.50-42.45) B ↓	62.80 (56.48-69.81) C	$F_{2, 37} = 60.85$, $p<0.001$
1.0	NA[a]	46.12 (42.26-50.46) ↓	48.07 (41.68-55.33) ↓	NS
10.0	No Hatch	No Hatch	No Hatch	
Retene	23.06 (19.94-26.60) A	36.05 (31.98-40.63) B	54.19 (49.31-59.42) C	$F_{2, 42} = 57.18$, $p<0.001$
Corexit	No Hatch	No Hatch	No Hatch	
ANOVA	$F_{4, 56} = 50.44$, $p<0.001$	$F_{5, 72} = 17.39$, $p<0.001$	$F_{5, 72} = 38.16$, $p<0.001$	

[a]NA = not available because of high rates of embryonic mortality in these groups.

Table A2-2. Average time-to-hatch (dpf) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF at 7.5, 15 and 30‰ and 7, 10 and 15°C. 2-Factor nested ANOVAs were performed using salinity or temperature and concentration as the two factors with the interaction term reported. Non-significant interaction was analyzed by separate 1-factor ANOVAs with lowercase letters representing significant differences among treatments (separate 1-factor ANOVAs for concentration and salinity; Tukey test, $p<0.05$). Significant interactions were explored using 1-factor ANOVAs with uppercase letters representing significant differences between salinities or temperatures (1-factor ANOVA analyzing individual concentrations; Tukey test, $p<0.05$). Arrows represent significant differences from the negative control (1-factor ANOVA analyzing individual salinities and temperatures; Tukey test, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

Conc. (% v/v)	7.5‰	15‰	30‰	ANOVA
CEWAF Interaction: $F_{6, 698} = 14.13$, $p<0.001$				
Water	15.10±0.10 A	15.02±0.09 A	14.70±0.12 B	$F_{2, 177} = 16.32$, $p<0.001$
0.0001	15.05±0.07 A	14.77±0.11 B ↓	15.03±0.05 A ↑	$F_{2, 177} = 15.33$, $p<0.001$
0.001	15.05±0.06 A	14.53±0.14 B ↓	14.63±0.14 B	$F_{2, 177} = 22.33$, $p<0.001$
0.01	15.00±0.16 A	14.77±0.14 B ↓	14.28±0.13 C ↓	$F_{2, 175} = 27.21$, $p<0.001$
Retene	15.45±0.19 A	14.90±0.22 B	13.77±0.36 C	$F_{2, 134} = 43.13$, $p<0.001$
ANOVA	NS	$F_{3, 228} = 13.65$, $p<0.001$	$F_{3, 228} = 44.82$, $p<0.001$	
Treatment		Salinity		
WAF Interaction: NS				
Treatment: $F_{3, 697} = 3.44$, $p<0.017$		Salinity: $F_{2, 697} = 18.42$, $p<0.001$		
Water	14.94±0.06 a			
0.01	14.88±0.07 ab	7.5‰	15.05±0.07 a	
0.1	14.89±0.06 ab	15‰	14.92±0.05 a	
1.0	14.79±0.08 b	30‰	14.63±0.07 b	
Retene	14.88±0.17			
Conc. (% v/v)	7°C	10°C	15°C	ANOVA
CEWAF Interaction: $F_{8, 602} = 9.95$, $p<0.001$				
Water	19.31 (19.00-19.58) A	13.60 (13.39-13.83) B	7.44 (7.27-7.61) C	$F_{2, 165} = 2{,}798.59$, $p<0.001$
0.0001	19.53 (19.22-19.90) A	12.38 (12.10-12.64) B ↓	7.61 (7.42-7.81) C	$F_{2, 137} = 1{,}806.40$, $p<0.001$
0.001	19.49 (19.09-19.90) A	13.42 (13.08-13.76) B	7.56 (7.39-7.73) C	$F_{2, 125} = 1{,}771.18$, $p<0.001$
0.01	18.15 (17.61-18.70) A ↓	12.84 (12.38-13.86) B ↓	7.51 (7.34-7.68) C	$F_{2, 112} = 792.08$, $p<0.001$
0.1	15.34 (14.05-16.70) A ↓	12.24 (11.79-12.73) B ↓	7.23 (7.00-7.47) C	$F_{2, 73} = 233.75$, $p<0.001$
1.0	No Hatch	9.88 (8.48-11.47) A ↓	6.00 (5.54-6.51) B ↓	$F_{1, 12} = 46.82$, $p<0.001$
10.0	No Hatch	No Hatch	No Hatch	
Retene	17.73 (16.97-18.53) A	11.03 (10.63-11.42) B	6.61 (6.36-6.88) C	$F_{2, 49} = 483.03$, $p<0.001$
Corexit	No Hatch	No Hatch	No Hatch	
ANOVA	$F_{4, 189} = 21.74$, $p<0.001$	$F_{5, 215} = 19.84$, $p<0.001$	$F_{5, 187} = 9.35$, $p<0.001$	

Appendix B

Embryonic Heart Rate and Time-to-Hatch Analysis for Chapter 3

Methods

Pacific herring embryonic heart rates were measured on day 8 PF, while Atlantic herring embryonic heart rates were measured on day 7 PF. Embryos were placed in a temperature-controlled petri dish and heart rate was measured by counting the number of beats under a dissecting microscope for 10 seconds.

Heart rate and time-to-hatch were analyzed by analysis of variance (ANOVA) models followed by Tukey multiple comparisons test. Data were tested for normality (probability plot) and homoscedasticity (Levene/Bartlett test) prior to analysis and only required transformation (log X+0.01) for both endpoints in the Atlantic fall-spawning stock. 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, each of the nine oil-concentration combinations was considered an individual treatment and compared to the negative control by 1-factor ANOVA. This illustrated which treatment concentrations caused a significantly different response compared to negative control. For both Atlantic herring bioassays, these end-points were also analysed for spawning stocks and species comparisons by a 1-factor ANOVA assessing the effect of concentration. Nested ANOVA models removed the variability associated with replicates. Analyses were performed with Systat version 11.0 (SPSS, Chicago, IL, USA). The level of significance was $p<0.05$. Means are accompanied by their 95% confidence interval.

Results

In the present study, analysis of embryonic heart rate and time-to-hatch produced significantly different results from the negative control at treatment concentrations sporadically (Tables B3-1, B3-2, B3-3). Although clear trends were not observed on the toxicity of different crude oils, a concentration effect was generally observed in heart rate and time-to-hatch of Pacific herring embryos. Past studies have reported that exposure to oil or PAHs generally reduce both embryonic heart rate and time-to-hatch (Boudreau *et al*. 2009). Test concentrations in the present study may have been too low (Tables 3-1, 3-2) to produce interpretable results distinguishing among oils. Therefore, these endpoints were not included in the assessments.

Table B3-1. Average heart rate (beats/min) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) crude oil WAF and CEWAF. Arrows indicate significant differences from the negative control (1-factor nested ANOVA among oil-concentration combinations; Tukey test, $p<0.05$). Stocks were analyzed separately. N=3 jars/concentration.

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
CEWAF 1-Factor ANOVA: $F_{9, 270} = 53.68$, $p<0.001$; WAF 1-Factor ANOVA: $F_{9, 270} = 74.97$, $p<0.001$					
Water					47.4±2.84
CEWAF	0.0001	53.2±3.31 ↑	43.4±2.68	59.2±3.66 ↑	
	0.001	48.2±2.73	40.6±1.63 ↓	48.2±1.81	
	0.01	30.8±2.02 ↓	35.0±2.36 ↓	39.8±2.08 ↓	
WAF	0.01	61.8±3.01 ↑	52.3±2.37	63.0±2.87 ↑	
	0.1	51.8±2.73	42.8±2.33	44.0±1.79	
	1.0	39.8±2.79 ↓	30.6±1.89 ↓	37.4±2.01 ↓	
Retene					31.0±2.77
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
CEWAF 1-Factor ANOVA: $F_{9, 270} = 42.46$, $p<0.001$; WAF 1-Factor ANOVA: $F_{9, 270} = 27.94$, $p<0.001$					
Water					44.0±3.02
CEWAF	0.0001	43.6±2.82	47.4±2.45	46.2±2.36	
	0.001	48.4±2.56	48.6±2.45	41.0±2.29	
	0.01	26.4±2.25 ↓	34.8±2.79 ↓	33.4±2.10 ↓	
WAF	0.01	51.2±2.81 ↑	53.6±2.88 ↑	39.6±2.00	
	0.1	37.4±2.68 ↓	50.2±2.32 ↑	45.0±2.34	
	1.0	35.6±1.85 ↓	45.6±2.09	42.8±2.18	
Retene					28.6±1.52

Table B3-2. Average time-to-hatch (dpf) including 95% confidence intervals of Pacific herring embryos exposed to Alaska North Slope (ANS), Medium South American (MESA) and Arabian Light (AL) WAF and CEWAF. Arrows indicate significant differences from the negative control (1-factor nested ANOVA among oil-concentration combinations; Tukey test, $p<0.05$). Stocks were analyzed separately. N=3 jars/concentration.

British Columbia Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
CEWAF 1-Factor ANOVA: $F_{9,502} = 6.75$, $p<0.001$; WAF 1-Factor ANOVA: $F_{9,504} = 6.80$, $p<0.001$					
Water					15.78±0.22
CEWAF	0.0001	15.61±0.18	15.22±0.22 ↓	15.39±0.17	
	0.001	15.40±0.18	15.36±0.18	15.40±0.15	
	0.01	15.04±0.26 ↓	14.88±0.25 ↓	15.15±0.24 ↓	
WAF	0.01	15.30±0.17 ↓	15.46±0.15	15.00±0.26 ↓	
	0.1	15.38±0.21 ↓	15.51±0.18	15.07±0.15 ↓	
	1.0	15.22±0.20 ↓	15.14±0.16 ↓	15.31±0.15 ↓	
Retene					12.86±1.03
Alaska Stock Bioassay					
Treatment	Conc. (% v/v)	ANS	MESA	AL	Controls
CEWAF 1-Factor ANOVA: $F_{9,511} = 8.84$, $p<0.001$; WAF 1-Factor ANOVA: $F_{9,513} = 5.76$, $p<0.001$					
Water					15.17±0.20
CEWAF	0.0001	15.20±0.19	15.08±0.22	15.25±0.21	
	0.001	14.68±0.23	14.94±0.25	14.96±0.21	
	0.01	14.32±0.33 ↓	14.40±0.23 ↓	14.53±0.27 ↓	
WAF	0.01	15.21±0.26	15.07±0.24	15.40±0.29	
	0.1	14.76±0.24	15.20±0.23	14.94±0.25	
	1.0	14.54±0.25 ↓	14.62±0.23 ↓	14.89±0.24	
Retene					13.39±0.58

Table B3-3. Average heart rate (beats/min) and time-to-hatch (dpf) including 95% confidence intervals of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF. Stocks were analyzed separately and arrows indicate significant differences from the negative control (1-factor ANOVA; Tukey test, $p<0.05$). N=3 jars/concentration.

		Conc. (% v/v)	Heart Rate	Time-to-Hatch
Spring	CEWAF	Water	40.40±1.85	14.70±0.12
		0.0001	48.20±2.08 ↑	15.03±0.05 ↑
		0.001	52.00±3.13 ↑	14.63±0.14
		0.01	38.60±2.01	14.28±0.13 ↓
		Retene	42.00±3.17	13.77±0.36
		ANOVA	$F_{3, 108} = 35.10$, $p<0.001$	$F_{3, 228} = 44.82$, $p<0.001$
	WAF	Water	40.40±1.85	14.70±0.12
		0.01	49.40±2.10 ↑	14.80±0.10
		0.1	44.60±2.61 ↑	14.82±0.11
		1.0	40.80±2.38	14.63±0.14
		Retene	42.00±3.17	13.77±0.36
		ANOVA	$F_{3, 108} = 16.92$, $p<0.001$	NS
Fall	CEWAF	Water	57.14 (52.47-62.22)	13.60 (13.39-13.83)
		0.0001	54.57 (50.11-59.56)	12.38 (12.10-12.64) ↓
		0.001	49.65 (46.44-52.96)	13.42 (13.08-13.76)
		0.01	51.63 (48.07-55.33)	12.84 (12.38-13.86) ↓
		Retene	36.05 (31.98-40.63)	11.03 (10.63-11.42)
		ANOVA	$F_{5, 72} = 17.39$, $p<0.001$	$F_{5, 215} = 19.84$, $p<0.001$

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Publications:

Lee K, King T, Robinson B, Li Z, Burridge L, Lyons M, Wong D, MacKeigan K, Courtenay S, Johnson S, Boudreau M, Hodson P, Greer C, Venosa A. 2011. Toxicity of Chemically-Dispersed Crude Oil on Fish. International Oil Spill Conference report no. 2011-163.

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TOXICITY OF CRUDE OIL CHEMICALLY DISPERSED IN A WAVE TANK TO EMBRYOS OF ATLANTIC HERRING (*CLUPEA HARENGUS*)

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Abstract—Tests of crude oil toxicity to fish are often chronic, exposing embryos from fertilization to hatch to oil solutions prepared using standard mixing procedures. However, during oil spills, fish are not often exposed for long periods and the dynamic nature of the ocean is not easily replicated in the lab. Our objective was to determine if brief exposures of Atlantic herring (*Clupea harengus*) embryos to dispersed oil prepared by standard mixing procedures was as toxic as oil dispersed in a more realistic model system. Embryos were first exposed to chemically dispersed Alaska North Slope crude and Arabian light crude oil for 2.4 h to 14 d from fertilization to determine if exposure time affected toxicity. Toxicity increased with exposure time, but 2.4-h exposures at realistic concentrations of oil induced blue-sac disease and reduced the percentage of normal embryos at hatch; there was little difference in toxicity between the two oils. Secondly, oil was chemically dispersed in a wave tank to determine if the resultant oil solutions were as toxic to herring embryos as laboratory-derived dispersed oil using a single exposure period of 24 h. Samples taken 15 min postdispersion were more toxic than laboratory-prepared solutions, but samples taken at 5, 30, and 60 min postdispersion were less toxic. Overall, the laboratory- and wave tank-derived solutions of dispersed oil provided similar estimates of toxicity despite differences in the methods for preparing test solutions, suggesting that laboratory and wave tank data are a reliable basis for ecological risk assessments of spilled oil. Environ. Toxicol. Chem. 2012;31:1324–1333. © 2012 SETAC

Keywords—Fish Embryo Crude oil Toxicity Wave tank

INTRODUCTION

The *Exxon Valdez* oil spill occurred in Prince William Sound, Alaska, USA, on March 24, 1989, coinciding with the return of Pacific herring (*Clupea pallasi*) to spawning grounds in the area [1]. By 1994, the spawning area of Pacific herring had decreased from a range of 106 to 273 km of shoreline to only 12 km, marking a massive population crash and leading to the closure of the roe herring fishery [2]. Although other opinions exist [3], it is assumed that the oil was the leading cause of the crash, corroborating lab-scale tests showing the toxicity of crude oil to Pacific herring [4,5].

As oil sits on water, soluble compounds will partition from the oil, forming the water accommodated fraction of oil in water (WAF) [6]. Chemical dispersants, which were widely used during the Deepwater Horizon oil spill on both surface and subsurface oil [7], decrease the interfacial tension between oil and water, allowing the oil to break into smaller droplets that could be more easily dispersed. Smaller droplets have an increased surface area relative to a slick of oil at the surface, which allows a larger quantity of petroleum compounds to partition into the water, composing the chemically enhanced water accommodated fraction (CEWAF) [6]. Compared to naturally dispersed crude oil, chemical dispersion of oil markedly increases the concentration of oil in water in contact with the droplets [8,9]. Dispersants are applied under the premise that oil will be diluted quickly to subtoxic concentrations, will be more bioavailable to hydrocarbon-degrading microorganisms, and will reduce the risk of oil damage to coastal areas [10]. However, before this can occur, there is a surge in the concentration of oil below the water surface that could induce toxicity in early life stages of fish [11,12].

Polycyclic aromatic hydrocarbons (PAHs) comprise the most chronically toxic fraction of crude oil to fish embryos [13]. By making droplets smaller and increasing the partitioning of oil into water, chemical dispersion of crude oil could increase the exposure of fish embryos to PAH. Exposure of early life stages of fish to PAH can result in blue-sac disease (BSD) [14], signs of which include yolk-sac edema, pericardial edema, spinal curvature, fin rot, and craniofacial deformations [12]. Severe deformities would hinder an embryo's ability to forage for food, escape predators, and swim unimpeded, reducing overall recruitment [1,15].

Oil tanker routes in eastern Canada often pass through fish spawning grounds, particularly those of the Atlantic herring around New Brunswick, Nova Scotia, and Newfoundland, Canada. Atlantic herring may be a useful model for other marine species because they are an accessible source of gametes for four to six months of the year. In particular, the results of research on Atlantic herring could be extended to other *Clupea* species in the North Atlantic and even to Pacific herring (C. Greer, 2011, Master's thesis, Queen's University, Kingston, Ontario, Canada). Atlantic herring spawn in shallow intertidal and subtidal (0–20 m) areas. Although chemical dispersants have not yet been used in these areas, they represent depths where dispersed oil is likely to reach [15].

Most studies of the toxicity of crude oil to fish embryos have used chronic exposure conditions [3,5,9]. Chronic toxicity tests involving continuous exposures of embryos to oil from

All Supplemental Data may be found in the online version of this article.
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fertilization to hatch provide a reproducible and controlled environment and a "worst-case exposure scenario." However, maintaining the same concentration of PAH from fertilization to hatch may not be realistic. In dispersant-effectiveness tests, hydrocarbon concentrations can remain high in the water phase for a few hours to a few days following a spill, but perhaps not to the extent of a chronic toxicity test [16]. Additionally, fish sensitivity is not constant throughout development; fish are most sensitive to oil exposure as gametes and as embryos immediately following fertilization or hatch [12]. The blastula and gastrula stages (within 24–48 h of fertilization) are critical for development, and exposure during this time can decrease hatching success by 40%, whereas exposures 72 h after fertilization do not [1]. By pinpointing the most sensitive stages of development, experiments can be designed to better estimate the potential for toxicity under reasonable oil-exposure scenarios.

According to a National Research Council report [10], oil dispersion in laboratory tests does not naturally model the scale or complexity of conditions at sea, but opportunities to test dispersion during a real spill in the field are rare. As such, a practical intermediate model is needed to validate laboratory studies of CEWAF prepared using the standard protocol of the Chemical Response to Oil Spills: Ecological Effects Research Forum that was reported by Singer et al. [6]. To simulate chemical and natural dispersion of oil spills at sea, a wave tank at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada, was used to generate plunging breaking waves similar to white caps on a windy day (when the crest of the wave appears white as it breaks). Breaking waves cause velocity shear, increasing the mixing energy among the oil, dispersant, and water, thereby enhancing both the chemical and physical dispersion of the oil [17]. With the use of chemical dispersants, oil-surfactant droplets in the wave tank have a volumetric mean diameter smaller than 50 μm [16]. Compared to laboratory studies, preparation of CEWAF in a wave tank takes into account variable mixing energies and current effects, simulating the natural environment [16].

The main objective of the present study was to determine if oil chemically dispersed within a wave tank could induce similar toxicity to Atlantic herring embryos as laboratory-prepared test solutions. Two oils were tested, Alaska North Slope crude (ANSC), as a reference oil that has been well studied since the *Exxon Valdez* oil spill in 1989, and Arabian light crude (ALC), which is shipped in large volumes to refineries in Atlantic Canada. First, WAF and CEWAF of each oil were analyzed by fluorescence spectrometry to determine if chemical dispersion increased the concentration of oil in the water phase. Second, newly fertilized embryos were exposed to lab-prepared WAF and CEWAF to provide a baseline measure of toxicity and to CEWAF prepared in a wave tank to compare toxicity between the two preparation methods and the two oils. Finally, brief episodic exposures were used to determine the relationships among exposure time, concentration, and toxicity.

METHODS

All experiments were part of an approved Queen's University Animal Care Protocol (Hodson 2007 032) following the guidelines of the Canadian Council on Animal Care (www.ccac.ca).

Wave tank

The wave tank was located at the Bedford Institute of Oceanography and operated by the Centre for Offshore Oil, Gas, and Energy Research, a national Centre of Expertise of the Department of Fisheries and Oceans Canada. The wave tank was made from carbon steel, was 32 m long, 0.6 m wide, and 2 m high, and was located beside the Bedford Basin. It was filled to an average depth of 1.5 m (~29,000 L) with filtered (5 μm) saltwater from the Bedford Basin for each experiment. Using a frequency sweep technique [18], recurrent breaking waves were generated by a computer-controlled flap-type wave maker situated at one end of the tank. A high-frequency, slow-moving wave was superimposed by a low-frequency, fast-moving wave, causing wave heights to increase until the wave broke ($f = 0.85$ Hz for 20 s, followed by $f = 0.50$ Hz for 5 s). The tank provided a useful model of oil dispersion at sea because it simulated deepwater waves (depth ≥0.28 times wavelength), low oil-to-water ratios (300 ml of oil to 29,000 L of water), and low dispersant-to-oil ratios, set at 1:25 to follow past practice for actual spills [16].

The wave tank was equipped with 12 water sampling ports at four horizontal locations (2 m upstream and 2, 6, and 10 m downstream) and three depths (5, 75, and 140 cm from the water surface) from the spot where oil was added (Fig. 1). At time 0, 300 ml of oil was poured onto the surface of the water inside an oil containment ring, and 12 ml of dispersant (Corexit 9500A) was sprayed onto the surface of the oil slick, at a target ratio of 1:25. Water was sampled from each of the 12 sample points at 5, 15, 30, and 60 min during dispersion.

Test solutions

All oils and the dispersant were supplied by the Centre for Offshore Oil, Gas, and Energy Research (Dartmouth, Nova Scotia, Canada). Oils were weathered by evaporation and sparging with air to simulate loss of volatiles at sea: 10% loss by weight of ANSC (final viscosity, 17.5 cP) and 7% loss by weight of ALC (final viscosity, 15.5 cP). The oils were characterized by methods previously described (Table 1) [19,21]. Corexit 9500A (Nalco Energy Services), which has a recommended dispersant-to-oil ratio of 1:50 to 1:10 (U.S. Environmental Protection Agency [U.S. EPA] Technical Product Bulletin D-4), was used to make CEWAF. In contrast to the wave tank, CEWAF prepared in the lab was dispersed at 1:10 to follow the standard lab protocol for CEWAF and WAF described in the Chemical Response to Oil Spills: Ecological Effects Research Forum method that was adapted by Singer et al. [6] and reported previously [12,20]. The intent of this method was standardization among labs, not realism. Briefly, oil was added to water at a ratio of 1:9 and stirred for 18 h. For CEWAF, dispersant was applied to the oil surface after 18 h of stirring and stirring continued for 1.0 h. After stirring, the WAF and CEWAF solutions were allowed to settle for 1.0 h and the solutions below the residual oil slick were decanted and diluted for test solutions.

Positive controls included retene (320 μg/L using methanol as the carrier solvent) and laboratory-prepared CEWAF. Retene (7-isopropyl-1-methyl phenanthrene) is an alkyl-phenanthrene known to cause BSD in early life stages of fish [14]. Negative controls included lab water, wave tank water drawn before oil application, and a dispersant control. The water used in the wave tank was from the Bedford Basin (30 g/L salinity), and samples were diluted with dechlorinated freshwater to 15 g/L before toxicity testing and chemical analyses as this is the optimal salinity for Atlantic herring fertilization and hatching (S. McIntosh, 2009, Master's thesis, Queen's University, Kingston, Ontario, Canada).

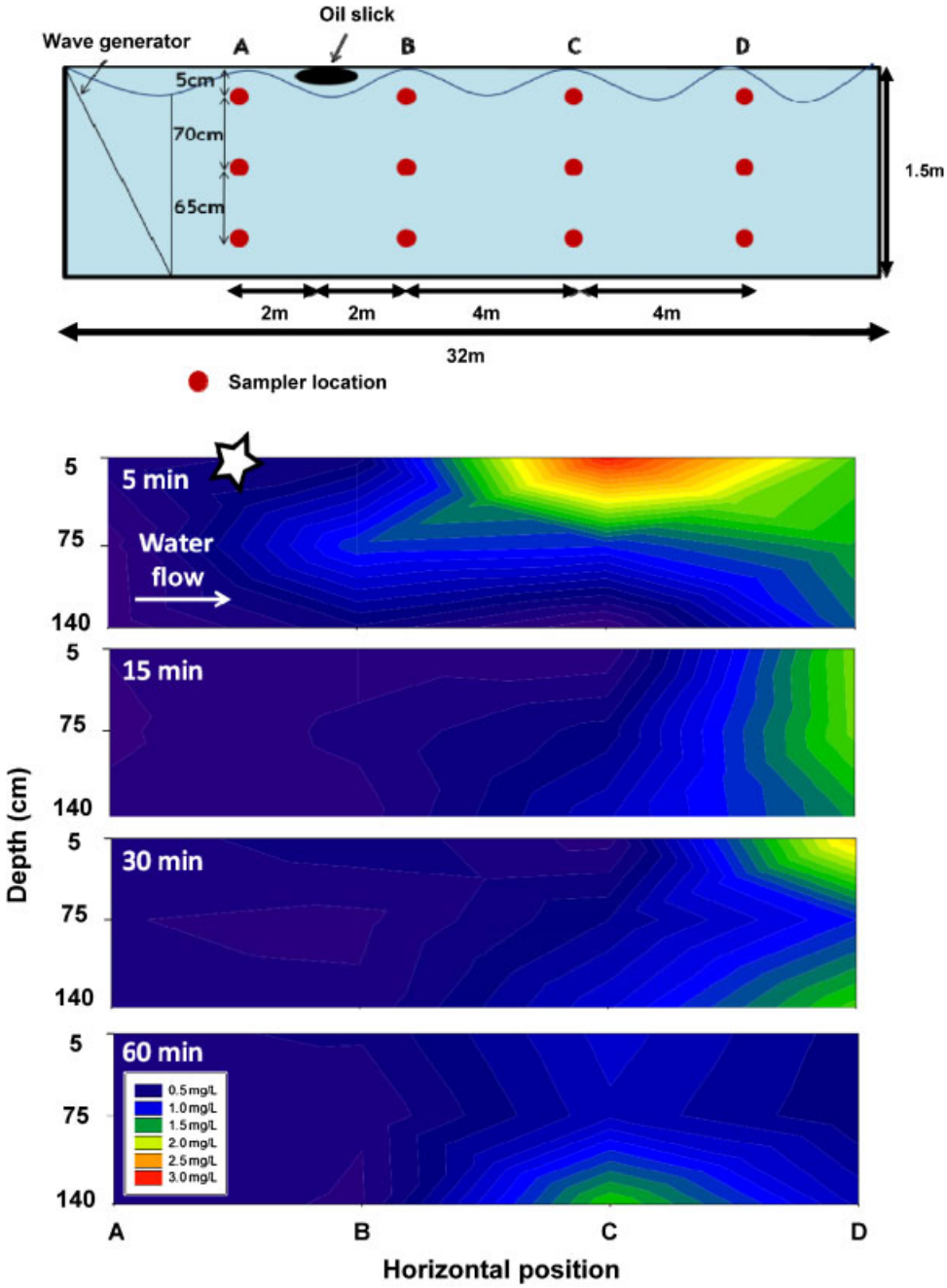

Fig. 1. Schematic of the wave tank with sampling locations (upper panel). The movement of fluorescing hydrocarbons through the wave tank over 5 to 60 min following addition of Corexit 9500 dispersant to Alaska North Slope Crude oil (lower panel). White star represents the location where oil was initially added.

Test species

"Ripe and running" stage VI herring were acquired from the roe herring fishery in Eastern Passage, Nova Scotia, Canada [22,23]. Ripe herring have transparent eggs, and the eggs and sperm are released from the fish with little external pressure [23]. Gonads were removed from the fish at the wharf using a scalpel and transported in resealable plastic bags in a cooler to the Bedford Institute of Oceanography. Eggs and sperm were used within 24 h of capture, to ensure freshness.

Egg fertilization

Freshly fertilized eggs were used for these experiments because they are the most sensitive life stage for herring [12]. Each experiment, from egg fertilization to scoring hatched embryos, was maintained at 10°C (± 1°C) with a 2:22 h dim light:dark photoperiod. Approximately 50 to 100 eggs were spread onto glass slides and separated into groups of two or three using a dissection needle. Once the eggs were attached to glass slides, the slides were placed into a milt solution that was prepared by mashing one-fifth of a testis, from a pool of all testes collected (approximately six males), in approximately 500 ml of saltwater (15 g/L salinity). Slides were left in the milt solution for approximately 15 min, after which they were rinsed with clean saltwater to remove excess milt and placed into test solutions. Individual experiments used eggs from a single female that had been fertilized by sperm from multiple males.

Table 1. Physical and chemical characterization of Alaska North slope crude (ANSC) and Arabian light crude (ALC) oils[a]

Oils	ANSC	ALC
Type	Medium	Light
%Weathered by weight	10	7
Density (g/ml)	0.8607	0.8691
Viscosity (20°C, cStk)	17.5	15.5
%Alkanes	32.0	32.7
%Resins	24.4	46.9
%Asphaltenes	4.3	1.5
%Aromatics	39.3	18.9
Σ Naphthalenes (μg/g)	4,665	3,221
Σ Fluorenes (μg/g)	461	540
Σ Phenanthrenes (μg/g)	1,687	1,086
Σ Pyrenes (μg/g)	284	202
Σ Chrysenes (μg/g)	562	188
Σ Dibenzothiophenes (μg/g)	1727	3788
Σ Naphthobenzothiophenes (μg/g)	851	3683
Σ Alkyl-PAH (μg/g)	90.07	96.33
Σ Nonalkyl PAH (μg/g)	126.7	8.3
Total PAH (μg/g)	10,363	12,716
Total PAH (% of oil)	1.03	1.27

[a] Oils were weathered by evaporation and sparging with air to simulate loss at sea. Density and viscosity were measured after weathering (Lee et al. [19]). The aromatics include the polycyclic aromatic hydrocarbons (PAHs), which were characterized by gas-chromatographymass spectrometry as described previously [20] and presented in detail in Supplementary Data Table S1.

Experimental setup

To assess the effect of exposure time on oil toxicity, slides of herring eggs were exposed to dilutions of WAF for 14 d or CEWAF for 2.4 h, 8 h, 24 h, or 14 d. After exposure, embryos were transferred to clean Mason jars containing fresh saltwater (15 g/L). After 24 h, water and test solutions for 14-d exposures of WAF and CEWAF were renewed daily until hatch.

To assess the toxicity of oil dispersed in the wave tank, exposure solutions were drawn from the 12 sampling ports at 5, 15, 30, and 60 min after oil and dispersant application. Solutions were diluted 1:1 with dechlorinated freshwater to reach a salinity of 15 g/L. Slides of herring eggs were exposed in the lab to the diluted wave tank samples for 24 h, after which the embryos were transferred to clean saltwater and raised to hatch. Embryos remained attached to the glass slides until hatch. Seven days postfertilization, unfertilized eggs were removed from the slides and fertilized embryos were randomly culled to approximately 20 per slide. Every other day from this point to hatch, dead embryos were removed and survivors were monitored to determine the day of hatch.

Although each slide contained 20 eggs, the slides were the unit of experimentation (i.e., $n = 1$). The measurement of oil concentrations in each of a wide array of test concentrations enabled the calculation of regressions and of median lethal concentrations (LC50s) and median effective concentrations (EC50s) without replication. The size of these experiments (50–70 treatments) and initial difficulties in obtaining fertilized eggs made replication impractical.

Because herring were captured by a commercial fishery, fish were usually not at the peak of fertility, and many experiments failed because eggs were not fertilized. High variability was observed among and within females in terms of egg quality and in terms of response to oil exposure. It is not uncommon that 50% of naturally spawned herring in the wild exhibit morphological deformities [5,24], a background response rate that would reduce reproducibility among experiments. As a result, we only included slides in the 14-d experiment that had a minimum of five fertilized eggs. All slides in the wave tank and exposure-time experiments had at least 18 eggs. Experiments with abnormally high rates of malformed embryos in the controls (<75% normal) were discarded. Future experiments would be more successful if herring were captured live and held in fresh seawater until ready to spawn (S.D. Rice, National Oceanographic and Atmospheric Administration, Juneau, AK, USA, personal communication).

Scoring signs of toxicity

Within 24 h of hatch, embryos were scored for signs of BSD. These included pericardial edema (score 0–3), yolk sac edema (0–3), spinal curvature (0–3), fin rot (0–1), craniofacial deformities (0–1), and mortality. A graduated scale was used for scoring pathologies: 3 indicated the most severe response and 0 indicated no response. Fin rot and craniofacial deformities were scored as either present (1) or absent (0) because it was assumed they would have less of a lasting impact on the fish, and the responses were subtle and could not be scored on a graduated scale. Edemas and spinal curvature were the most sensitive responses to oil exposure and could clearly be scored on a graduated scale. Mortality of embryos is often a result of edema, and edema and spinal curvatures can prevent an embryo from swimming effectively, escaping predators, or foraging for food [25,26]. We assumed that fish with lower BSD scores were more likely to survive and contribute to the next generation, despite the deformities.

Embryos that died from day 7 to hatch received the highest possible BSD score (11.5) to indicate the most severe response beyond sublethal toxicity. The BSD Severity Index was the average BSD score for all embryos within a treatment, normalized to the maximum score, and included both lethal (mortality) and sublethal (BSD) signs of toxicity (modified from Villalobos et al. [27]). Alternatively, embryos were considered normal if they were alive at hatch, did not show any signs of BSD, and swam normally. These fish would be most likely to grow, reproduce, and contribute to the next generation.

Water chemistry

Water samples were drawn from exposure solutions to quantify the concentration of hydrocarbons by fluorescence (detailed operating procedure in Supplemental Data, SOP S1; Fig. S1). Fluorescence measures compounds in oil with conjugated double bonds that fluoresce when bombarded with ultraviolet light [28]. Water samples (3 ml) were drawn from exposure solutions at the start (time, 0 h) and at the end of the exposure period, then averaged to determine concentration. They were added to 3 ml of absolute ethanol in 7-ml glass scintillation vials with foil-lined caps and stored at 4°C. Samples were sonicated for 3 min before chemical analysis to reduce the loss of hydrocarbons to the container from storage. On mixing with ethanol, salt from the water samples precipitated from the solution. To reduce interference by salt particles, samples were centrifuged at 9,100 *g* for 10 min to separate the salt from the sample but not to remove hydrophobic compounds. An aliquot of the supernatant was placed in a quartz cuvette and analyzed for total fluorescence using the RF-5301PC scanning spectrofluorometer (Shimadzu) with Panorama fluorescence 1.1 software (LabCognition). The excitation (278 nm) and emission (300–450 nm) wavelengths used for the experiments were the same for both ALC and ANSC. The fluorometric response was measured by integrating the area

under the curve from 300 to 450 nm. A comparison of fluorescence to a standard curve prepared from undispersed whole oil dissolved in 50:50 ethanol:salt water (15 ppt) provided an estimate of the compounds in the test solution that fluoresce (Supplemental Data, Fig. S2). The limit of detection, or method detection limit, is the concentration of chemical that can be determined above the level of the blank and reported with 99% confidence (U.S. EPA method 40 CFR 136, Appendix B, revision 1.11; www.epa.gov/region9/qa/pdfs/40cfr136_03.pdf). The limit of detection is $t \times S$, where t is the Student's t value at the 99% confidence level for $n - 1$ degrees of freedom and S is the standard deviation of 10 replicates of a sample. The limit of detection was 0.028 mg/L.

Water samples (100 ml) were also drawn from a subset of exposures to quantify the concentration of total petroleum hydrocarbons. Samples were preserved with 10 ml of dichloromethane and sent to the Centre for Offshore Oil, Gas, and Energy Research, Bedford Institute of Oceanography, to be analyzed by gas chromatography-flame ionization detection (GC-FID, [20]). The relationship between total petroleum hydrocarbon concentrations measured by GC-FID and by fluorescence was used to estimate total petroleum hydrocarbon concentrations in other exposures characterized by fluorescence (Supplemental Data, Fig. S2).

Statistics

SigmaPlot 11.0 (Systat Software) and GraphPad Prism 5.00 (GraphPad Software) were used to derive nonlinear regressions from a four-parameter logistic equation [29] and to estimate LC50s and EC50s (details are in the Supplemental Data, Table S2). For logistic equations, the responses of negative control fish and the maximum possible responses of oil-exposed fish were used to establish the upper and lower limits for regressions. Statistical comparisons were made by comparing the confidence intervals (CIs) for overlap and by using the extra sum-of-squares F-test function in GraphPad Prism, where parameters were shared to determine if data were more strongly correlated when combined or as separate functions.

RESULTS

Water chemistry

Chemical dispersion of crude oil (CEWAF) caused an approximately 100-fold increase in concentrations of fluorescent petroleum hydrocarbons in the saltwater phase compared to physically dispersed oil (WAF; F test, $p < 0.001$). Above the detection limit of 0.028 mg/L, there were linear relationships between measured concentrations of fluorescent petroleum hydrocarbons and nominal loadings of oil (% v/v) for ANSC and ALC oils of both WAF and CEWAF (Fig. 2). All lines were parallel (F test, $p = 0.39$) and shared a slope of 1.06. At $x = 1$, the y values were significantly different between ANSC and ALC CEWAF (F test, $p < 0.001$), but the y values were not significantly different between the WAFs (F test, $p = 0.98$).

To test the efficiency of the fluorescence method, GC-FID was also used to analyze the concentration of total petroleum hydrocarbons in some exposure solutions. When compared to the concentrations obtained through spectrofluorometry, a linear relationship of log-transformed data was observed (Supplemental Data, Fig. S2), demonstrating that as total petroleum hydrocarbons increased, so did the fluorescent signal. Although a decrease in linearity was observed at low concentrations, the two methods were in agreement at total petroleum hydrocarbon concentrations >0.028 mg/L. While the fluorescence method targets PAH, it may underestimate other components of oil that do not dissolve in water to the same extent. In both cases, the preservation of samples with either ethanol (fluorescence) or dichloromethane (GC-FID) would solubilize oil droplets so that the assays measured total oil in test solutions.

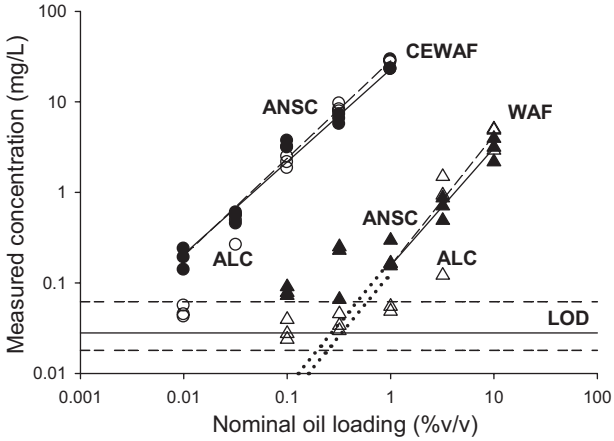

Fig. 2. The concentration of petroleum hydrocarbons in test solutions at different loadings of water accommodated fraction (WAF) or chemically enhanced water accommodated fraction (CEWAF). Open circles and triangles represent Arabian light crude (ALC) CEWAF ($y = 10^{(1.08*\log(x) + 1.45)}$, $r^2 = 1.0$) and WAF ($y = 10^{(1.44*\log(x) - 0.81)}$, $r^2 = 0.89$), respectively. Closed circles and triangles represent Alaska North Slope crude (ANSC) CEWAF ($y = 10^{(1.02*\log(x) + 1.36)}$, $r^2 = 0.99$) and WAF ($y = 10^{(1.29*\log(x) - 0.80)}$, $r^2 = 0.89$), respectively. Dotted lines show the extrapolated concentration of the WAF samples below the limit of detection (solid horizontal line, 0.028 mg/L). Data points close to the limit of detection deviate from linearity and were not used to calculate linear regressions. Upper and lower 95% confidence limits of the limit of detection (LOD) are shown as dashed lines.

Comparison of oils

Atlantic herring embryos responded to dilutions of laboratory-prepared WAF and CEWAF of ANSC and ALC in an exposure-dependent manner (Fig. 3). Based on measured oil concentrations by fluorescence and the combined data for WAF and CEWAF, ANSC appeared to be no more toxic than ALC, as evidenced by LC50s (Table 2). Assuming that the proportion of total PAH in exposure solutions was the same as that in whole oil (Table 1), the 14-d LC50s for ANSC and ALC were equivalent to 20 and 26 μg/L total PAH, respectively. Similarly, for percentage of hatch and the BSD Severity Index, EC50s for the two oils did not differ markedly. The estimated total PAH concentrations associated with 14-d EC50s for the BSD Severity Index were 0.4 and 0.3 μg/L for ANSC and ALC, respectively. For percentage of normal, the EC50s based on fluorescence were higher for ALC than for ANSC for three of four sampling times, suggesting that ALC was somewhat less toxic than ANSC. All the differences, however, were relatively small and statistically insignificant (overlapping 95% CI), suggesting no real difference in toxicity between the two oils.

Exposure time

The toxicities of CEWAF prepared from ANSC and ALC increased linearly with exposure times from 2.4 to 24 h (Fig. 4, Table 2), and there was no significant difference between the slopes (F test, $p = 0.70$) or intercepts (F test, $p = 0.33$) of the lines of best fit of the $EC50_{\%Normal}$ values versus exposure time (Fig. 5). For ANSC CEWAF there was a significant difference among the $EC50_{\%Normal}$ values for all exposure times (Fig. 5; F test, $p < 0.001$), but for ALC there was no significant

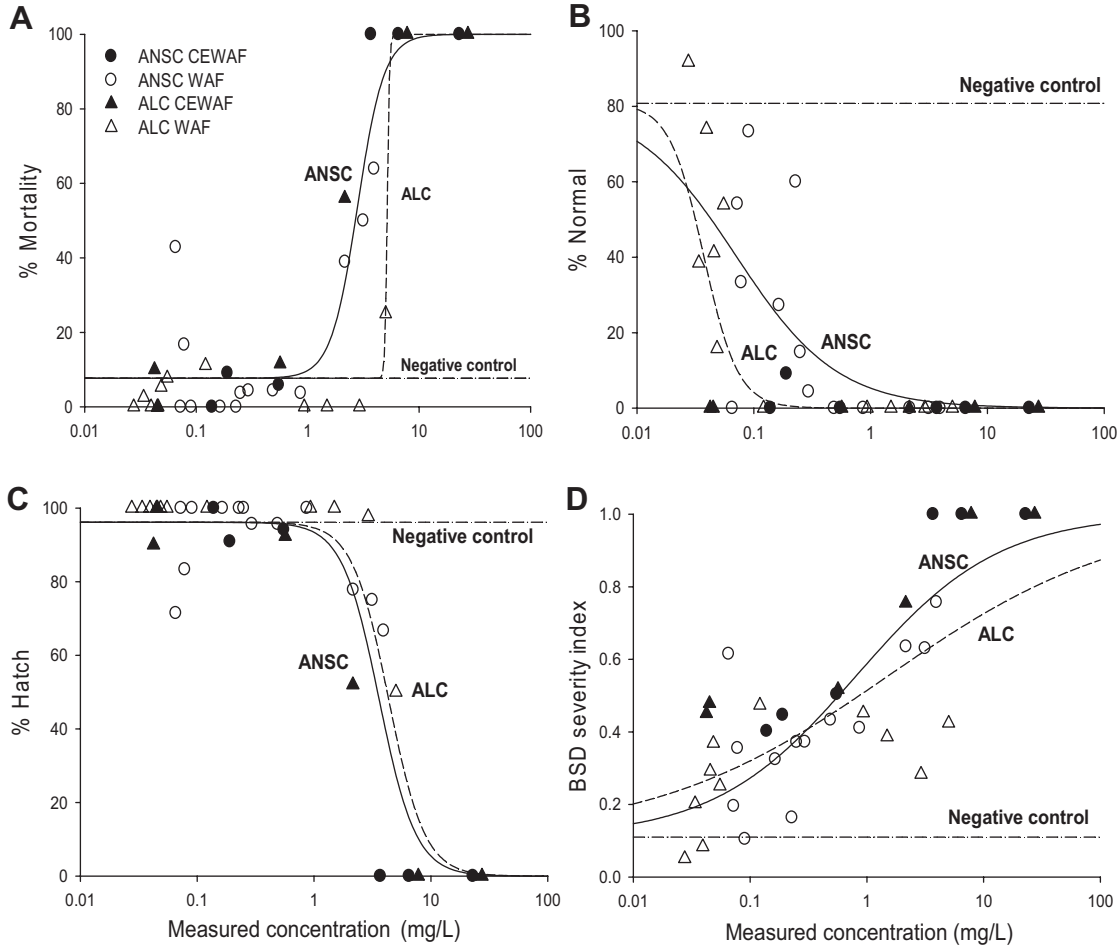

Fig. 3. Toxicity of the water accommodated fractions (WAF) and chemically enhanced water accommodated fractions (CEWAF) of Alaska North Slope crude (ANSC) and Arabian light crude (ALC) oils prepared in the lab to Atlantic herring embryos expressed as (**A**) %mortality, (**B**) %normal, (**C**) %hatch, and (**D**) blue-sac disease (BSD) Severity Index in relation to the measured concentration of fluorescing compounds. Regression lines and median lethal concentration (LC50) and median effective concentration (EC50) values were calculated using WAF (open symbols) and CEWAF (closed symbols). Upper and lower constraints for nonlinear regressions were control and maximal (**A** = 100%, **B** and **C** = 0%, **D** = 1.0) responses. Dashed lines represent the response to ALC exposure, and solid lines represent the response to ANSC exposure. $n = 20$ embryos per treatment.

difference between 8 and 24 h for CEWAF exposures (F test, $p = 0.86$). For mortality, LC50s were higher than the highest concentrations tested, except for the 14-d exposures, which produced an LC50 of 1.9 mg/L (95% CI too wide to estimate) for ANSC and 2.1 mg/L (95% CI 1.9–2.3 mg/L) for ALC (Supplemental Data, Fig. S3). The $EC50_{\%Hatch}$ values were also higher than the highest concentrations tested, except for the 14-d exposures, which produced an $EC50_{\%Hatch}$ of 0.86 mg/L (95% CI 1.5–5.9 mg/L) for ANSC and 2.2 mg/L (95% CI 1.9–2.7 mg/L) for ALC (Supplemental Data, Fig. S3).

Wave tank

Hydrocarbon concentrations produced by dispersing ANSC and ALC oils in the wave tank were too low to affect %hatch, the BSD Severity Index, or mortality (Supplemental Data, Fig. S4). For percentage of normal, the 24-h EC50 for ANSC CEWAF calculated by combining all wave tank samples was 16 mg/L (95% CI 0.32–761 mg/L), while the $EC50_{\%Normal}$ for ALC CEWAF was higher than the highest concentration tested (>10.4 mg/L) (Fig. 6). The toxicity of ANSC CEWAF was especially evident in samples drawn from the tank at 15 min, with an $EC50_{\%Normal}$ of 1.2 mg/L (95% CI 0.61–2.5 mg/L). In contrast, the toxicity of laboratory-prepared ANSC CEWAF was sixfold lower ($EC50_{\%Normal} = 6.6$ mg/L, 95% CI 4.9–8.8 mg/L; F test, $p = 0.026$) when tests were run with embryos from the same female as the wave tank experiment. However, exposures drawn at other sampling times did not show a significant deviation from the toxicity of laboratory-prepared CEWAF. While ANSC CEWAF from the wave tank appeared at times more toxic than laboratory-prepared solutions, concentrations of ALC CEWAF from the tank were lower than the laboratory-prepared $EC50_{\%Normal}$ and did not cause toxicity.

Quality assurance/quality control

Hatched embryos exposed to crude oil exhibited exposure concentration- and time-dependent signs of toxicity, including yolk-sac edema, pericardial edema, spinal curvature, fin rot, and craniofacial deformities. The mean responses of controls were as follows: %mortality = 3.2 ± 3.7%; %normal = 86.3 ± 6.1%; %hatch = 98.2 ± 2.0%; and BSD Severity Index = 0.058 ± 0.049.

DISCUSSION

The present study is the first to compare the embryotoxicity of dispersed oil solutions generated in an outdoor wave tank with those generated by standard laboratory protocols. As such, it provides perspective on the applicability of lab-derived toxicity data to ecological risk assessment of chemical dispersion of oil.

Table 2. Effect of exposure time on the toxicity of the chemically enhanced water accommodated fraction (CEWAF) of Alaska North Slope crude (ANSC) and Arabian light crude (ALC) oils[a]

		Atlantic herring (LC50s and EC50s in mg/L)							
Oil	Exposure time	%Mortality	CI	%Normal	CI	%Hatch	CI	BSD Severity Index	CI
ANSC									
FL	14 d	1.95	Very wide	<0.14	—	0.86	Very wide	0.44	0.16–1.24
TPH		7.12	Very wide	<1.41	—	4.31	Very wide	2.85	1.51–5.39
FL	24 h	>24.15	—	0.74	0.35–1.57	>24.15	—	63.70	Very wide
TPH		>33.11	—	3.94	2.49–6.23	>33.11	—	60.48	22.31–163.9
FL	8 h	>26.28	—	4.24	3.26–5.53	>26.28	—	40.09	8.27–194.3
TPH		>35.48	—	11.48	9.76–13.51	>35.48	—	45.53	17.29–119.9
FL	2.4 h	>26.93	—	14.66	6.02–33.70	>26.93	—	39.77	16.02–98.66
TPH		>35.65	—	24.56	14.22–42.41	>35.65	—	45.30	25.94–79.11
ALC									
FL	14 d	2.07	1.88–2.28	<0.04	—	2.25	1.89–2.70	0.25	0.02–2.63
FL	24 h	>28.51	—	2.44	0.36–16.56	>28.51	—	84.38	Very wide
FL	8 h	>30.96	—	2.79	1.02–7.62	>30.96	—	73.70	Very wide
	2.4 h	>31.79	—	29.73	16.02–55.18	>31.79	—	419.7	Very wide

[a] Median lethal concentrations (LC50s) represent lethal toxicity, specifically an increase in the mortality of embryos. Median effective concentrations (EC50s) represent sublethal toxicity, specifically the concentrations causing a reduction in the percent normal and percentage of hatch and an increase in the blue-sac disease (BSD) Severity Index of Atlantic herring embryos. Values are based on measured concentrations of fluorescing (FL) petroleum hydrocarbons of CEWAF or estimations of total petroleum hydrocarbon (TPH) concentrations (relationship shown in Supplemental Data, Fig. S3) and shown with 95% confidence intervals (CIs). " > " and " < " indicate that the LC50 or EC50 was greater than the highest concentration tested or less than the lowest concentration tested.

Chemical dispersion of both ALC and ANSC oils increased the concentration of waterborne hydrocarbons by approximately 100-fold, and the concentration of fluorescent hydrocarbons in the water phases of dispersed ALC and ANSC were similar. Little difference in toxicity was seen between ANSC and ALC when embryos were exposed from fertilization until hatch, consistent with the very similar concentrations of PAH in the two oils. The toxicity of oil to early life stages of fish has been associated with concentrations of alkyl PAH [13], implying that a greater quantity of waterborne PAH (or aromatics), particularly the alkyl phenanthrenes, will result in a higher degree of toxicity [20,30]. Both oils contained just over 1.0% PAH by weight, and the concentrations of most classes of alkyl PAH were similar between the two. The exceptions were the sulfur heterocycles, alkyl dibenzothiophenes, and naphthobenzothiophenes, which were about two to four times more concentrated in ALC than in ANSC (Table 1). Because the toxicity of the two oils was not correlated with the relative concentrations of thiophenes, the sulfur heterocycles may not contribute much to oil chronic toxicity. The similarity in fluorescence between the two oils may also relate to the relative amounts of all fluorescent compounds in each oil, as many organic molecules contain conjugated double bonds and will fluoresce (e.g., high molecular weight asphaltenes) [28]. Additionally, compounds vary in their ability to fluoresce according to their chemical composition, and ring substitutions can increase fluorescence; aniline can be 50 times more fluorescent than benzene [31].

In actual oil spills, exposures of embryos may only last a few hours to days if oil is dispersed rapidly by currents and waves [32,33]. As a result, estimates of oil toxicity may be unrealistic if embryos are exposed chronically from fertilization to hatch [12]. The effect of exposure time was examined using laboratory-prepared CEWAF to simulate pulse exposure scenarios, in which embryos were exposed to oil in water over increasing exposure times. In accordance with McIntosh et al. [12], increasing exposure time to CEWAF of ANSC decreased the percentage of normal embryos at hatch. Even the shortest exposure times (2.4 h) caused toxicity, and the longer the embryos were exposed, the higher the degree of embryo malformation and the greater the risk of failure to recruit to future generations. This relationship however, was not as consistent for ALC CEWAF because there was no significant difference in toxicity between 8- and 24-h exposures. This could indicate that the toxic agents in the 24-h exposure had been depleted from solution by 8 h or that uptake had reached equilibrium with excretion and metabolism. Signs of toxicity (%normal, %hatch, %mortality, BSD Severity Index) were more pronounced in embryos exposed chronically (14 d) because soluble hydrocarbons were replenished by the daily static renewal protocol.

Although lethality depends on both exposure time and concentration [12,32], toxicity resulting from exposure times of 24 h or less was primarily determined by sublethal end points, because LC50s could not be estimated within the concentration range tested; %normal and, to a lesser extent, BSD Severity Index were the most sensitive end points used for determining toxicity. In contrast, all end points could be used to estimate toxicity to Atlantic herring embryos exposed for 14 d. However, short-term episodic exposures are likely to be more typical during an oil spill.

Oil is a complex mixture, and the kinetics of weathering and uptake are difficult to mimic in the laboratory. While CEWAF prepared in a wave tank provided more realistic spill and dispersion conditions, solutions of ALC dispersed in the wave tank did not cause toxicity, likely because the concentrations of hydrocarbons were less than those observed to cause toxicity in the lab. In contrast, solutions of ANSC dispersed in the wave tank and drawn from the tank at 15 min postdispersion were approximately six times more toxic than laboratory-prepared CEWAF. Solutions drawn from the tank at 5 and 30 min postdispersion showed little toxicity, most likely because the concentrations were below those found to be toxic in laboratory-prepared CEWAF. The significant increase in toxicity of wave tank solutions of ANSC sampled at 15 min may be due to interactions among droplet dispersion, oil–water partitioning, and exposure to fish, that is, peak concentrations of PAH dissolved in water may have been produced at 15 min. However, because sample preservation solubilized oil droplets, measured

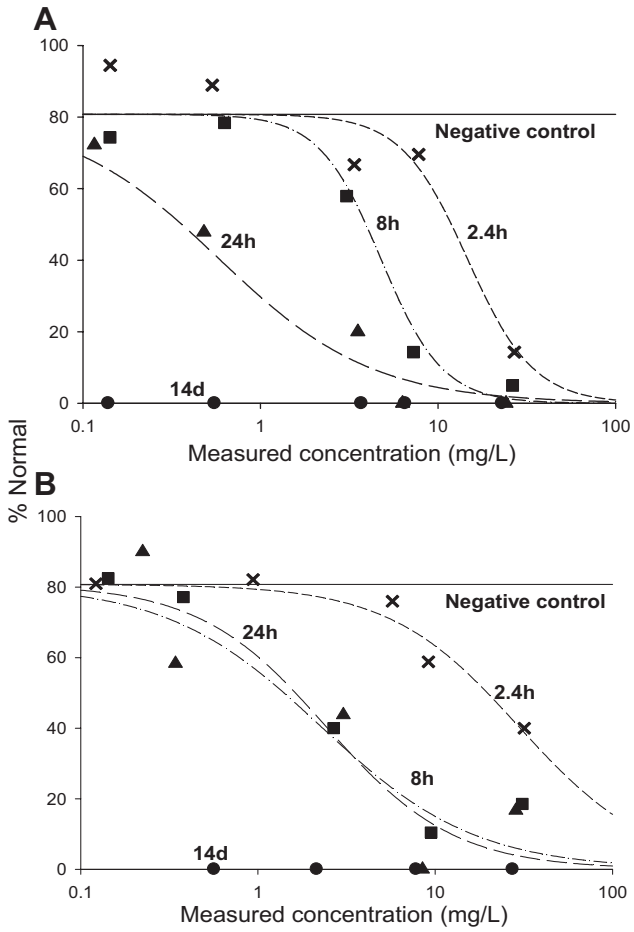

Fig. 4. Effect of exposure time and petroleum hydrocarbon concentration on the percentage of normal embryos at hatch for herring exposed to lab-prepared Alaska North Slope crude (ANSC; **A**) and Arabian light crude (ALC; **B**). %Normal indicates percentage of embryos alive and showing no signs of toxicity at hatch. Embryos were exposed immediately following fertilization for 2.4 (x's), 8 (squares), or 24 h (triangles) or 14 d (circles) (static daily renewal). Upper and lower constraints for nonlinear regressions were control responses and zero (maximum possible response). $n = 20$ embryos per treatment.

concentrations of oil in water do not allow discrimination between dissolved and droplet oil.

While toxicity was greatest at 15 min (Fig. 6), hydrocarbon concentrations within the wave tank were highest toward the end of the tank and at the sampling times immediately following dispersion (5 min). Previous testing using the wave tank has shown that average droplet size decreased quickly from 300 to 50 μm within 10 min of adding oil and dispersant [16]. Smaller droplets would increase partitioning and make hydrocarbons more bioavailable to fish embryos, which could explain why the wave tank exposures were more toxic at 15 min than at 5 min. Although the research done by Li et al. [16] was performed in a wave tank using a flow-through mode, they also observed quick dilution of oil within the wave tank and rapid movement of oil to the end of the tank. In a batch mode operation, the distribution of dispersed oil in the wave tank would be nonuniform, with the highest concentration measured near the surface at the end of the wave tank because of horizontal transport due to Stokes drift and vertical transport from the buoyancy of oil droplets; this distribution was not due to edge effects. This suggests that even though a spill may not occur directly over fish spawning grounds, chemical dispersion, currents, and wave action could quickly move the hydrocarbons to those areas.

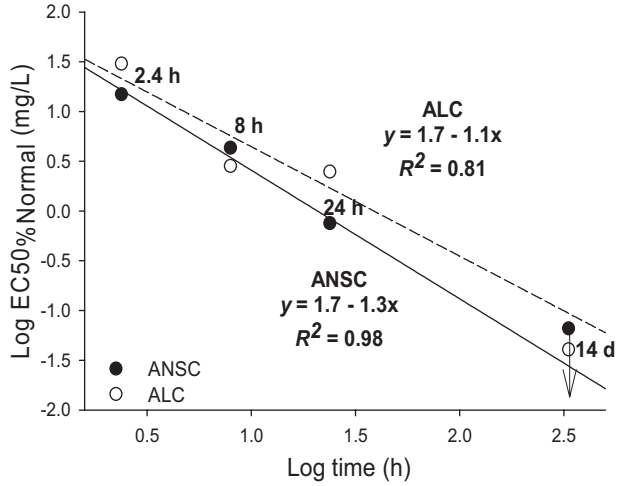

Fig. 5. Effect of exposure time on median effective concentrations ($EC50_{\%Normal}$) of Alaska North Slope crude oil (ANSC) chemically enhanced water accommodated fraction (CEWAF) and Arabian light crude oil (ALC) CEWAF affecting %normal. The lowest concentration tested is shown for the 14-d data point as there was still 100% response at the lowest concentrations; arrow indicates that the actual EC50 would be lower than this concentration. Therefore, linear regressions were calculated using only three data points (2.4, 8, and 24 h).

Wave tank-prepared and laboratory-prepared CEWAF may exhibit differences in the types of hydrocarbons within the water column, the degree of weathering and dilution, and the chemical characteristics and physical composition of the oil. Further chemical analysis and a change in experimental conditions would be required to assess these possibilities. Increasing the volume of oil added to the wave tank for dispersion may increase the concentration of dissolved oil generated by the tank to better pinpoint LC50s and EC50s but may stray from environmentally realistic wave-tank conditions. Further, a difference in toxicity could result from the different dispersant-to-oil ratios used in the wave tank and laboratory tests; the volume of dispersant in laboratory tests could be adjusted to parallel wave tank experiments. If samples were tested undiluted at 30 ppt salinity, the concentration of oil in the water samples would be double those of samples diluted to 15 ppt as was done in the present experiments. Preliminary tests at 30 ppt showed a higher range of toxicity, presumably because samples were undiluted; but there was no difference in survival or rates of pathology among controls between salinities of 15 and 30 ppt.

The wave tank system provides a model for oil spills in the field; but the ocean is a dynamic system, and each oil spill would occur under different conditions and regimes. The wave tank experiments were designed to simulate specific high-energy conditions in the ocean on a windy day and to generate a droplet size distribution similar to that expected at sea. Physical and chemical measurements have verified that the tank is a success in this regard [16]. The demonstration that solutions of oil from the wave tank had effects on herring embryos consistent with those observed with other fish species in field and laboratory studies of oil toxicity [1,34] indicates that the wave tank can be applied successfully to studies of the biological impacts of spilled oil.

CONCLUSION

Atlantic herring embryos were sensitive to exposure to chemically dispersed crude oil, with 0.1- to 14-d LC50s above 1 mg/L total hydrocarbons. The EC50s for chronic toxicity

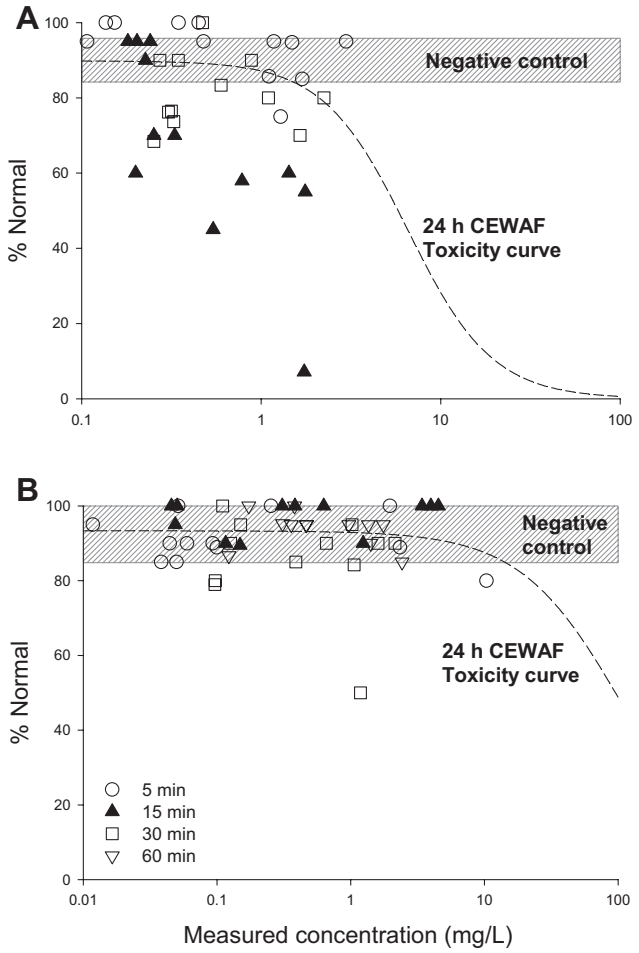

Fig. 6. Change in %normal of herring embryos exposed to water samples drawn from the tank after chemical dispersion of Alaska North Slope crude oil (ANSC; **A**) and Arabian light crude oil (ALC; **B**). Effects on Atlantic herring of 24-h exposure of the corresponding laboratory-prepared CEWAF exposures have been superimposed for comparison (dashed lines). Measured concentrations were derived from samples of toxicity test solutions. $n = 20$ embryos for each exposure.

typical of PAH exposure fell between 0.04 and 84 mg/L, depending on exposure time and end point. Exposure times as short as 2.4 h (0.1 d) caused toxicity at concentrations similar to those observed near oil spills. The estimated total PAH concentrations corresponding to these EC50s ranged from 0.4 to 840 μg/L, and ANSC was no more toxic to Atlantic herring embryos than ALC, reflecting similar concentrations of alkyl PAH.

The wave tank experiments demonstrated that chemical dispersants increased the amount of petroleum hydrocarbons in the water column to toxic concentrations. They also demonstrated that toxicity did not persist, supporting the use of dispersants to rapidly dilute concentrated oil. The risk of toxicity under these circumstances will depend on the amount of oil dispersed, the wave action and mixing energy acting on the dispersed oil, and the proximity of fish embryos to the spill site. While there were differences in toxicity between wave tank and laboratory solutions, they were neither large nor consistent in direction. Thus, data from both laboratory and wave tank experiments could be used to support ecological risk assessments of oil spills. Additional trials at higher concentrations of oil in the wave tank are required to fully understand the chemistry of spilled oil in the wave tank, and these experiments could be extended to achieve a greater degree of realism by conducting toxicity tests entirely in the wave tank. By suspending slides of eggs within the tank during oil dispersion under continuous-flow conditions, the effects of brief pulses of exposure could be measured to support computer modeling of potential spill effects.

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TOXICITY OF CHEMICALLY DISPERSED CRUDE OIL TO HERRING EMBRYOS

by

Colleen Diane Greer

A thesis submitted to the Department of Biology

In conformity with the requirements for

the degree of Master of Science

Queen's University

Kingston, Ontario, Canada

(May, 2011)

Abstract

The use of chemicals to disperse oil spills raises concerns for organisms living below the surface of the water. While decreasing the surface area of the slick, chemical dispersants increase the amount of oil in the water column, the surface-to-volume ratio of droplets, the partitioning to water of the toxic constituents of oil, and the bioavailability of oil to pelagic and benthic organisms. Chemical dispersion can increase the exposure to polycyclic aromatic hydrocarbons (PAH) by 100-fold. As a model for a full-scale spill at sea, a wave tank was used to simulate chemical and natural dispersion of spilled oil to determine if the concentrations of chemically dispersed oil were sufficient to cause toxicity to embryos of Atlantic herring (*Clupea harengus)*. While the hydrocarbon concentrations of dispersed oil from the wave tank were not large, the exposure response relationship was consistent with that of laboratory-prepared dispersed oil. Additionally, the toxicities of chemically dispersed oil prepared in the lab to Pacific (*Clupea pallasi*) and Atlantic herring were compared to ensure that the wealth of literature available on Pacific herring could be used for assessing the risk of oil exposure to Atlantic herring. Exposures to low concentrations of dispersed oil for short periods (2.4 to 24 h) consistently increased the incidence of blue sac disease, and decreased the percentage of normal embryos at hatch, indicating that even brief exposures to oil could be detrimental to the survival and recruitment of herring.

Co-Authorship

Chapters 2 and 3 were co-authored by Dr. Peter Hodson. Dr. Hodson contributed to experimental design, data analysis and interpretation, and editing. Chapter 2 was also co-authored by Dr. Zhengkai Li, Thomas King, and Dr. Kenneth Lee, who contributed to experimental design and operation of the wave tank.

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It's funny how things turn out. In April of 2008, I was leaving Kingston with a BScH of Biology in my back pocket and determined never to return to Kingston. A year later and apparently wiser, I came back, and what a whirlwind it has been! Returning has certainly been the best decision I have ever made.

First and foremost, I would like to thank my supervisor, Peter Hodson. Thank you for the wonderful opportunity to study in your lab. Your guidance and patience has taught me more than I could have imagined; both about science and about myself.

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Colleen

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List of Abbreviations

A. herring	Atlantic herring
ANSC	Alaska North Slope Crude oil
ALC	Arabian Light Crude oil
BIO	Bedford Institute of Oceanography
BSD	Blue sac disease
CEWAF	Chemically-enhanced water accommodated fraction
CI	95% Confidence intervals
CYP1A	Cytochrome P450A
EC50	Median effective concentration
$EC50_{\%Hatch}$	Median effective concentration of percent hatch
$EC50_{\%Normal}$	Median effective concentration of percent normal
$EC50_{SI}$	Median effective concentration of the BSD Severity Index
GC-FID	Gas Chromatography-Flame Ionized Detection
GC-MS	Gas Chromatography-Mass Spectrometry
LC50	Median lethal concentration
MESA	Medium South American Crude oil
P. herring	Pacific herring
PAH	Polycyclic aromatic hydrocarbons
tPAH	Total polycyclic aromatic hydrocarbon concentration
WAF	Water accommodated fraction

Chapter 1

Introduction

The *Exxon Valdez* Oil Spill, the 2nd largest spill in US Maritime history, occurred on March 24, 1989 (Paine *et al.* 1996). Approximately 41 million litres of Alaska North Slope crude oil were spilled in Prince William Sound, Alaska, coating 1750 km of shoreline (Figure 1-1; Bragg *et al.* 1994). Immediately following the spill, conditions were ideal for recovery, but emergency response was not prepared (Ginsburg 1993). An argument about whether or not to use chemical dispersants, lead primarily by ExxonMobil with the desire to disperse all oil spilled, further delayed cleanup (Ginsburg 1993). Now dispersants are stockpiled, but approval for use is only granted if mechanical recovery (booming, skimming) is deemed inefficient (PWSRCAC 1999; PWSRCAC 2009).

As oil exploration, development, and transport expand into more remote and inaccessible regions of the world, the potential for large-scale spills and ecological damage increases. The more remote the area, the harder it would be to get the necessary equipment into the vicinity for spill cleanup and the larger the expense (Etkin 1999). This will encourage spill response to rely heavily on chemical dispersants to mitigate the effects of the oil as efficiently and economically as possible. Unfortunately, there is still considerable controversy surrounding the use of chemical dispersants. Dispersants are much less toxic since the tragic events of the *Torrey Canyon* spill (1967), where the use

of dispersants caused widespread mortality of benthic organisms, but have also become more efficient, allowing a greater quantity of oil to enter the water column. Although chemical dispersants spare surface-dwelling organisms and shorelines from oiling, they induce a surge of oil below the surface, increasing the potential for toxicity to organisms inhabiting these areas. There is a deficiency in the knowledge available to oil spill responders to enable them to make educated decisions about where and when to use chemical dispersants.

This chapter will provide the knowledge necessary to understand the concept of oil toxicity to early life stages of fish, and introduce the main thesis, where I will assess the toxicity of chemically dispersed and un-dispersed crude oil to Atlantic and Pacific herring embryos.

1.1 Crude Oil

Crude oil is a fossil fuel naturally generated from geological and geochemical processes. The threat oil poses to the environment involves not only the volume of oil spilled and the location of the spill, but also the chemical composition of the oil and the proportion of its toxic components. Most crude oils consist of about 97% hydrocarbons or related compounds (saturates, aromatics, resins, and asphaltenes) and the remaining 3% is composed of nitrogen, sulphur, and oxygen (NRC 2003). While oil sits on the surface of the water and is being naturally dispersed by waves and currents, compounds will partition into the water to a degree proportional to their solubility in water. This produces a water accommodated fraction of oil in water (WAF). Natural dispersion also causes the

oil to weather, whereby the physical and chemical characteristics of the oil are altered; the oil becomes thicker, and lighter, more volatile compounds are released.

BTEX (benzene, toluene, ethylbenzene, and xylene) are monoaromatic compounds that are highly volatile, acutely toxic, and cause narcosis, but the high volatility of BTEX decreases the potential for harm as the oil weathers (Barron *et al.* 2004). Asphaltenes and resins are polar compounds bonded with sulphur, nitrogen, or oxygen and have a significant effect on the behaviour of oil (NRC 2003). The higher the composition of asphaltenes and other high molecular weight compounds, the more viscous the oil is, which can pose many negative physical effects on organisms (e.g. smothering, skin contact effects), as well as increase the persistence of oil in the environment and along shorelines (NRC 2003). Polycyclic aromatic hydrocarbons (PAH) compose the most toxic component of oil, causing chronic toxicity, and include known carcinogens (Ramachandran *et al.* 2004).

With expansion of the oil and gas industry there is an increasing number of tankers transporting oil throughout the world. In response, increased regulations on how oil can be transported have led to a significant decrease in the number of large tanker-related spills over the years (1970-2009), but small-scale spills remain inconsistently documented (ITOPF 2010). Extraction, transportation, and consumption can all release oil in great quantities to the environment as a result of human activities, and natural seeps continuously release low levels of hydrocarbons (NRC 2003). It is estimated that more

than nine million barrels of petroleum are released into the environment worldwide annually (NRC 2003).

1.2 Chemical Dispersants

Oil spill cleanup depends on a number of factors, with the primary concern being the amount of visible oil. Whether or not the oil will reach the shoreline is of utmost importance, since the general consensus is that this is where it would cause the most long-term damage and be the most difficult to clean up (Etkin 1999). Before cleanup is initiated, a decision must be made as to which method will minimize the negative impacts of the oil. For chemical dispersants, there is a trade-off between the shoreline and surface dwelling organisms, and organisms that live within the water column.

Chemical dispersants are composed of surfactants and solvents. The surfactants contain both a lipophilic and a hydrophilic end, which effectively decrease the interfacial tension between the oil and water. This allows the oil to break into small oil-surfactant micelles that can more easily be diluted by wave action and currents than the original oil (Fiocco and Lewis 1999). The surfactants stabilize the oil droplets to prevent resurfacing and recoalescence with the oil (Fiocco and Lewis 1999). In this way, oil droplets can spread throughout the water column, quickly diluting the concentration of total oil and becoming more bioavailable to hydrocarbon-degrading bacteria.

During the events of the *Torrey Canyon* spill, the solvents used in dispersants were aromatic based (e.g. benzene), rendering the dispersant highly toxic. Modern dispersants use solvent systems that allow a higher proportion of surfactant per volume of

dispersant, decreasing the required application rate for successful dispersion (Fiocco and Lewis 1999). A commonly used dispersant today and the only dispersant stockpiled for use in Canada is Corexit 9500A (contains: dioctyl sodium sulphosuccinate (active ingredient); sorbitans; butanedioic acid, 2-sulfo-, 1,4-bis(2-ethylhexyl) ester, sodium salt (1:1); propanol; petroleum distillates). The US Environmental Protection Agency has recommended a dispersant to oil ratio of 1:10 to 1:50 for Corexit 9500A which is reported to be up to 45.3% effective at dispersing Prudhoe Bay Crude oil (USEPA Technical Product Bulletin #D-4). The effectiveness of Corexit 9500A was measured using the swirling flask dispersant effectiveness test and calculated from the oil remaining after dispersant application.

The ability of oil to be dispersed is a function of its physical and chemical properties. Heavy oils are highly viscous, making it difficult for the dispersant to penetrate and break the oil into smaller droplets, and light oils are very buoyant, requiring high doses of dispersant to break the oil into small enough droplets to overcome buoyancy (Venosa *et al.* 2005). Water-based dispersants are less effective and have a lower affinity to oil, but might be successful at dispersing lighter oils (Fiocco and Lewis 1999).

Before a dispersant is applied, oil will form a slick on the surface of the water. Few compounds in oil will readily mix into the water, as crude oil is generally immiscible in water. As chemical dispersants are applied and small droplets of oil in water are formed, the surface area of oil in water greatly increases. This allows more compounds

to reach their limits of solubility in the water and increases the rate of partitioning, forming the chemically-enhanced water accommodated fraction (CEWAF; Figure 1-3).

Compared to undispersed crude oil, chemical dispersion of oil markedly increases the water concentration of polycyclic aromatic hydrocarbons (PAH), particularly high molecular weight PAH, below the water surface (Couillard *et al.* 2005; Cohen *et al.* 2001). Application of dispersants occurs under the premise that oil will not reach the shoreline and will be quickly diluted into the water column to concentrations below toxic levels. However, if the concentration of PAH is high enough, even for a short period of time, it could induce toxicity in early life stages of fish and other organisms (Wolfe *et al.* 1994).

1.3 Polycyclic Aromatic Hydrocarbons (PAH) and Bioavailability

Polycyclic aromatic hydrocarbons consist of two or more fused benzene rings and occur naturally in crude oil (Wang and Fingas 2003). Three to five-ring alkyl-PAH comprise the most toxic fraction of oil (Khan 2007; Hodson *et al.* 2007) and are known to cause dioxin-like signs of toxicity in early life stages of fish (Billiard *et al.* 1999). Heavier oils contain a higher percentage of aromatics than their light counterparts (Reviewed by Incardona *et al.* 2008; Appendix A). For example, the Bunker C fuel oil spilled during the *Cosco Busan* spill (2007) has three times more PAH per unit mass than the medium crude spilled by the *Exxon Valdez* (Alaska North Slope Crude; Reviewed by Incardona *et al.* 2008).

The bioavailability of oil is a function of the compounds present in the oil and the portion of chemicals that are in a form that can be absorbed by the organism and can reach the sites of toxic action (Rand *et al.* 1995). Additionally, biotic and abiotic transformations, such as metabolism, hydrolysis, oxidation, and photolysis, have the potential to increase the availability and toxicity of chemicals (Rand *et al.* 1995). The composition of PAH within the oil determines toxicity, as some PAH are more toxic than others and if found in high quantities would contribute greatly to toxicity (Carls *et al.* 1999). In fact, the larger the quantity of high molecular weight PAH (≥ 3 rings) the greater the toxicity to Pacific herring embryos (Carls *et al.* 1999).

Carls et al (2008) tested the bioavailability of oil to zebrafish embryos and found that the dissolved PAH fraction is most toxic. Only 16% of mechanically-dispersed Alaska North Slope Crude (ANSC) oil dissolved in water and was bioavailable to zebrafish, and total PAH (tPAH) concentrations were up to 17 times less in the dissolved PAH exposure compared to the exposure containing oil droplets (Carls *et al.* 2008). This shows that bioavailability of oil to fish embryos is consistent with hydrocarbon compounds that are soluble in water. In contrast, phytane, a highly insoluble tracer compound for oil, was present in the droplet exposure, but not the dissolved oil exposure (Carls *et al.* 2008).

Retene (7-isopropyl-1-methyl phenanthrene) is C-4 alkyl phenanthrene and chronic exposure of early life stages of fish results in signs of Blue Sac Disease (BSD) and the induction of cytochrome P450A (CYP1A; Billiard *et al.* 1999). Exposure to

whole crude oil, of which retene may be a component, can also induce CYP1A. As such, retene can be used as a model for oil exposure and a positive control for BSD (Ramachandran *et al*. 2004).

Retene induces the production of the CYP1A protein by binding to the aryl hydrocarbon receptor (AhR) protein in the liver (Billiard *et al.* 2002). CYP1A catalyzes oxygenation reactions, which might generate reactive oxygen species and other reactive byproducts, and could result in oxidative stress and BSD (Halliwell and Gutteridge 1999). Others have found that chronic exposure of embyronic rainbow trout to retene increases toxicity (signified as BSD and mortality) in a concentration-dependent manner and that the metabolites may be the toxic component (Billiard *et al.* 1999; Hodson *et al.* 2007). In addition, there is evidence for a wide range of toxic effects among structurally similar PAH (Turcotte *et al.* 2011). This could account for various toxicities among oils, which are complex mixtures that contain a wide variety of PAH and other compounds.

Ramachandran *et al.* (2004) found that induction of CYP1A was greatest when rainbow trout were exposed to chemically dispersed oil versus non-dispersed oil and greatest for dispersed oils containing a higher percentage by weight of PAH, suggesting that CEWAF contains larger concentrations of PAH with higher toxicity than WAF. By increasing the concentration of dissolved PAH, chemical dispersion increases the bioavailability of oil to early life stages of fish.

Heintz *et al.* (1999) have demonstrated that PAH such as phenanthrene and chrysene are persistent in the environment, extending the potential period for toxicity.

Phenanthrenes and chrysenes are the most toxic, relatively abundant PAH in Alaska North Slope Crude oil (ANSC), the oil spilled during the *Exxon Valdez* spill. Stranded oil that has been relatively protected from weathering can persist and leach for years, while continuously exposing organisms in the vicinity (Short and Harris 1996).

Blue sac disease is a chronic, non-contagious syndrome exhibited in early life stages of fish that have been exposed to persistent contaminants such as PAH (Wolf 1957; Brinkworth *et al.* 2003). It is believed that BSD in early life stages of fish is a result of oxidative damage and membrane destabilization (Carls *et al.* 1999). Signs include, but are not limited to, pericardial edema, yolk sac edema, spinal curvature, craniofacial malformations, fin rot, and CYP1A induction. Edema is the most sensitive sign of toxicity in pink salmon embryos (Marty *et al.* 1997), zebrafish embryos (Incardona *et al.* 2009), and herring embryos (Carls *et al.* 1999) exposed to PAH, and often leads to mortality. Edema can reduce cardiac output, impede circulation, and negatively affect swimming ability (Carls *et al.* 1999). Spinal curvature is also an important sign of toxicity because it can occur in varying degrees and inhibits swimming ability, reducing the capacity to forage for food and escape predators. Addtionally, BSD could increase the susceptibility of fish to disease and parasitism, reduce movement and feeding ability, and eventually lead to mortality (Heintz *et al.* 1999). Severe deformities would hinder an embryo's ability to forage for food, escape predators, swim unimpeded, and ultimately be reproductively viable (Kocan *et al.* 1996; Smith and Cameron 1979).

1.4 Early Life Stages of Fish

Chemically dispersed crude oil is 50 to 1000 times more toxic than un-dispersed oil, and older, more toxic dispersants (e.g. Finasol SC) can further increase toxicity (Linden 1975; McIntosh *et al.* 2010; Schein *et al.* 2009). Additionally, newly fertilized and newly hatched fish embryos are the most sensitive to oil contamination (McIntosh *et al.* 2010). Damages to fish eggs, embryos, and larvae are more threatening to the future of the fish population and fishing industry than injury to adult fish because the young represent future recruitment to the population.

Pink salmon (*Oncorhynchus gorbuscha*) were the most economically important fish species in Prince William Sound during the *Exxon Valdez* oil spill and all life stages, including spawning adults, were exposed to oil in the area (Marty *et al.* 1997). Pink salmon spawn in the intertidal region of Prince William Sound, which represented the most heavily oiled area in the region (Marty *et al.* 1997). Weidmer *et al.* (1996) found elevated CYP1A activity, a known sign of oil exposure in fish, in pink salmon embryos, which indicated exposure for up to 2 years following the spill. Additionally, Bue *et al.* (1996) found high levels of egg mortality in oiled streams around Prince William Sound from 1989 to 1993, and developmental impairment in salmon fry was reported at initial concentrations of 4.4 µg/L tPAH (Marty *et al.* 1997). *Exxon Valdez* oil at concentrations representative of the environment can be lethal to pink salmon embryos at concentrations as low as 1 µg/L tPAH (Heintz *et al.* 1999).

1.5 Herring

Herring are especially sensitive to oil exposure (Carls *et al.* 1999). Just as Pacific herring (P. herring) were starting to spawn in Prince William Sound, Alaska, the *Tanker Vessel* (*T/V) Exxon Valdez* collided with Bligh Reef, spilling its load of Alaska North Slope Crude oil (ANSC; Brown *et al.* 1996). Prior to the *Exxon Valdez* oil spill, P. herring (*Clupea pallasi*) were observed to spawn along 106-273 km of the Prince William Sound shoreline and the population peaked at over 110,000 metric tons in 1989 (Figure 1-2; Funk 1994; Brown and Carls 1998). In 1994, the spawning area decreased to 12 km, marking a massive population crash and leading to the closure of the roe herring fishery (Funk 1994). A Natural Resource Damage Assessment estimated that the spill directly caused a 52% reduction in herring productivity (Brown *et al.* 1996). By 1997 the population had only recovered to 34,100 metric tons, but a limited commercial fishery in Prince William Sound re-opened (Brown and Carls 1998). Although there is evidence of a link between the abrupt population decline and over-population (Pearson *et al.* 1999), oil is believed to be the leading cause, corroborated by lab-scale tests showing the toxicity of crude oil to P. herring embryos (Paine *et al.* 1996; Carls *et al.* 1999). There is a positive correlation between oil-exposed areas in Prince William Sound directly following the *Exxon Valdez* oil spill and physical deformities in herring embryos, compared with un-oiled areas (Hose *et al.* 1996).

Herring (*Clupea sp.*) are more at risk of oil exposure than some other species because the eggs are sessile when laid, sticking to stationary surfaces like kelp, and

leaving the embryos vulnerable to an oil spill (Smith and Cameron 1979). Herring are a significant species both economically for the fishing industry and ecologically, as a vital component of the local food web. Pacific herring and many populations of Atlantic herring (A. herring; *Clupea harengus*) spawn in shallow intertidal and subtidal (0-20 m) areas, and Baltic herring (*Clupea harengus membras*) frequent the upper 10 m of the water column (Haegele and Schweigert 1985; Smith and Cameron 1979; Ackefors 1974). Pacific herring normally spawn in sheltered estuarine areas where oil is likely to strand and persist as a result of reduced wave action (Haegele and Schweigert 1985). Additionally, CEWAF is more likely to reach shallow depths where herring generally spawn.

Atlantic herring consist of both Spring and Fall-spawning stocks, while P. herring prefer Winter-Spring spawning (Haegele and Schweigert 1985). Personal observations of A. herring have indicated that Spring spawners are less fecund than Fall spawners and that Fall spawning occurs over a larger area and longer time period, as not all fish on the spawning grounds are "ripe and running" at the same time. It has been suggested that Fall spawning herring are more fecund because of a high mortality rate of newly hatched embryos and larvae during the first winter (Liamin 1959). During the Spring spawn, lower fecundity could be a result of poorer food availability over the winter and lower predation risk (Cushing 1967; Kerr and Dickie 1985). Additionally, A. herring spawn in waters ranging from 0.5 – 19.0 ºC and from "virtually freshwater" to 35 ppt salinity

(summarized in Haegele and Schweigert 1985). Higher water temperatures generally decrease the time to hatch, resulting in smaller hatchlings (Alderdice and Velson 1971).

Herring are most sensitive to oil exposure immediately following fertilization, specifically during the blastula and gastrula stages of embryonic development, as determined by pulse exposures (McIntosh *et al.* 2010; Kocan *et al.* 1996). Embryos exposed within the first 24 - 48 h of development showed a 40% decrease in hatching success, while embryos exposed beginning 72 h post-fertilization did not (Kocan *et al.* 1996). This stage-specific sensitivity may relate to damage to the anterior neural plate during gastrulation, when embryos are particularly susceptible to teratogenesis, and exposure to chemicals that affect DNA synthesis or integrity (Rogers and Kavlock 2007). Carls *et al.* (1999) found that low concentrations of ANSC caused significant lethal and sublethal effects on Pacific herring embryos, including genetic damage, malformations, reduced swimming ability, earlier onset of hatch, and reduced growth. Exposures to water containing 0.4 µg/L tPAH of highly weathered oil and consisting mainly of high molecular weight PAH induced sublethal effects in the embryos, while 0.7 µg/L tPAH induced genotoxicity (Carls *et al.* 1999). Edema was primarily responsible for mortality in the embryos (Carls *et al.* 1999), indicating that it has the greatest effect on survival in oil-exposed fish. Both edema and spinal curvature inhibited swimming and feeding ability, which also indirectly leads to death (Carls *et al.* 1999).

Immediately following the *Exxon Valdez* Oil spill, concentrations of tPAH were up to 6.24 µg/L in open water and concentrations of at least 1.59 µg/L tPAH persisted for

five weeks (Short and Harris 1996). Additionally, dissolved PAH concentrations were found to a depth of 25 m in Prince William Sound and ranged from 0.9 to 6.2 µg/L near heavily oiled beaches immediately following the spill (Short and Harris 1996). However, Carls *et al.* (1999) observed toxicity in Pacific herring embryos exposed to concentrations as low as 0.4 µg/L tPAH, indicating that conditions to observe embryotoxicity to Pacific herring were suitable in Prince William Sound. The Alaskan guidelines for acceptable levels of total aromatic hydrocarbons in water were set at 10 µg/L in 2009, significantly higher than those observed to cause toxicity to herring (State of Alaska Quality Standard Regulations 18 AAC 70, September 19, 2009).

Pacific herring were also affected in San Francisco Bay following the *Cosco Busan* oil spill in 2007. The spill corresponded with herring spawning events and oil coated many of the spawning grounds in the Bay (Incardona *et al.* 2008). In an attempt to quantify the percentage of embryos that would be lost from oil exposure, embryos were monitored for deformities upon hatch (Incardona *et al.* 2008). Laboratory and field testing confirmed toxic effects from oil exposure, as indicated by signs of BSD (Incardona *et al.* 2008).

Much research has been published on the toxicity of crude oil to Pacific herring, but there is a lack of data available for Atlantic herring. Coincidently, Atlantic herring spawning grounds (Stephenson *et al.* 2009) overlap with tanker routes monitored by the International Tanker Owners Pollution Federation (ITOPF) where oil is transported (ITOPF 2011) through the Eastern seaboard of North America (Figure 1-4). There are

many individual stocks of Atlantic herring throughout the northeastern and northwestern Atlantic, each with different spawning times and locations (Iles and Sinclair 1982; Cushing 1969). For the purposes of this thesis, we will focus on herring within the northwestern Atlantic, specifically those spawning around Nova Scotia and New Brunswick.

McIntosh *et al.* (2010) also studied Atlantic herring spawning near Nova Scotia. They found gamete fertilization and newly fertilized embryos to be the most sensitive life stages to oil exposure, with 1 h EC50s approximately equal to 21 mg/L and 100 mg/L tPAH of MESA CEWAF, respectively; the 24 h EC50 of newly fertilized embryos was 8.5 mg/L tPAH. Often out of convenience, toxicity testing begins 24 h post-fertilization, omitting the most sensitive stages of development and underestimating toxicity. With this in mind, McIntosh *et al.* (2010) emphasized the need for more research on the sensitive life stages of fish under conditions and for durations that represent possible real-world scenarios.

1.6 Modeling Oil Spills

Researchers can model oil spills to determine dispersant efficacy and toxicity. These methods can range from simple bench-top mixing systems to large scale *in situ* spill experiments. Laboratory preparation methods allow for consistency and reproducibility among experiments, as well as a controlled environment, but may not be realistic because preparation in the lab does not take into account variable mixing energy, currents, or wind. The laboratory method utilized for the following experiments is based

on the Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) method and reported elsewhere (Singer *et al.* 2000; Appendix B).

Preparation of CEWAF in a wave tank allows for a practical intermediate model between laboratory-prepared CEWAF and a full-scale *in situ* field experiment, which is very expensive and uncommon. In contrast to laboratory studies, preparation of CEWAF in a wave tank integrates mixing energy and current effects, in an attempt to simulate the natural environment (Li *et al.* 2009). A wave tank at the Bedford Institute of Oceanography (BIO), Dartmouth, NS has been used to simulate chemically dispersed oil spills at sea (Li *et al.* 2008; Li *et al.* 2009). Within the wave tank, breaking waves are formed in a reproducible manner, occurring at the same location in the tank with each wave (Venosa *et al.* 2005). Breaking waves cause velocity shear, increasing the mixing energy among the oil, dispersant and water, thereby enhancing both chemical and mechanical dispersion of the oil (Li *et al.* 2006) and forming oil-surfactant droplets (Li *et al.* 2009).

Most testing and literature show the toxicity of crude oil to fish embryos under chronic exposure conditions (Couillard *et al.* 2005; Hose *et al.* 1996; Paine *et al.* 1996). Aside from the recent Deepwater Horizon Oil Spill, oil is rarely released from a spill source and replenished on a continuous basis in the environment, maintaining the same concentration of PAH from fertilization to hatch. Dispersant efficacy tests have shown that hydrocarbon concentrations remain high for a few hours to a few days following a spill, but not to the extent of a chronic toxicity test (Li *et al.* 2009). It is beneficial to test

a range of exposure times to estimate sublethal and lethal thresholds, and to provide a more complete view of toxicity in relation to realistic exposure times (Sprague *et al.* 1969). Standard US Environmental Protection Agency operating procedures for dispersant toxicity testing are 24, 48 and 96 h acute toxicity experiments (Weber *et al.* 1993), which are not generally maintained beyond the exposure time and not suitable for observing the chronic effects associated with acute exposure. To better analyze the effect of more realistic exposure times on fish, embryos were exposed to both lab-prepared and wave tank-prepared dispersed oil solutions for exposures ranging from 2.4 to 24 h post-fertilization. By using an acute sublethal exposure and scoring for toxicity post-hatch, we can observe the chronic toxicity observed at hatch resulting from a brief exposure coinciding with fertilization.

1.7 Purpose

The primary goal of this research was to determine if environmentally relevant exposure scenarios can be replicated in a laboratory setting. Previous research suggests that Atlantic herring embryos are most sensitive to oil exposure immediately following fertilization (McIntosh *et al.* 2010), and environmentally relevant exposure concentrations and durations have not been thoroughly studied. Most literature examines the toxicity of oil exposure to Pacific herring embryos, yet oil exploration, exploitation and transportation also occur around Atlantic herring spawning grounds. Most importantly, if a spill were to occur near spawning grounds, chemical dispersion of oil may increase the potential for toxicity. As such, my null hypotheses were: chemical

dispersion of oil would not increase the concentration of petroleum hydrocarbons in the water; the toxicity of CEWAF prepared in a wave tank would not be more toxic than CEWAF prepared in the lab; CEWAFs prepared from different oils would not differ in toxicity; exposure duration would not affect toxicity; and Atlantic and Pacific herring would not respond differently to oil exposure.

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1.9 Figures

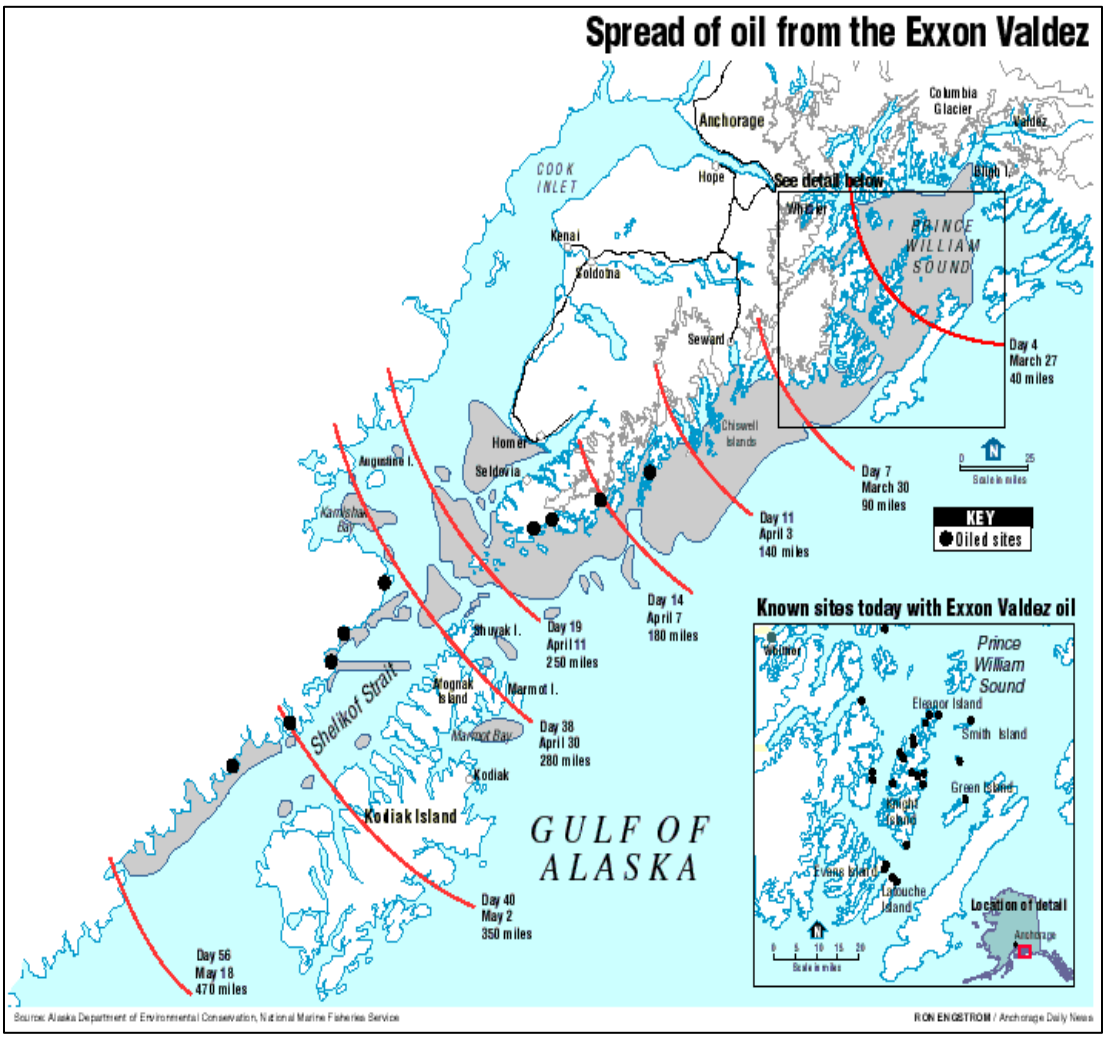

Figure 1-1: Extent oil spread from the *Exxon Valdez* crash site (source: Alaska Department of Environmental Conservation, National Marine Fisheries Service). The top right corner is Prince William Sound. The inlaid panel shows the extent of oil still present today.

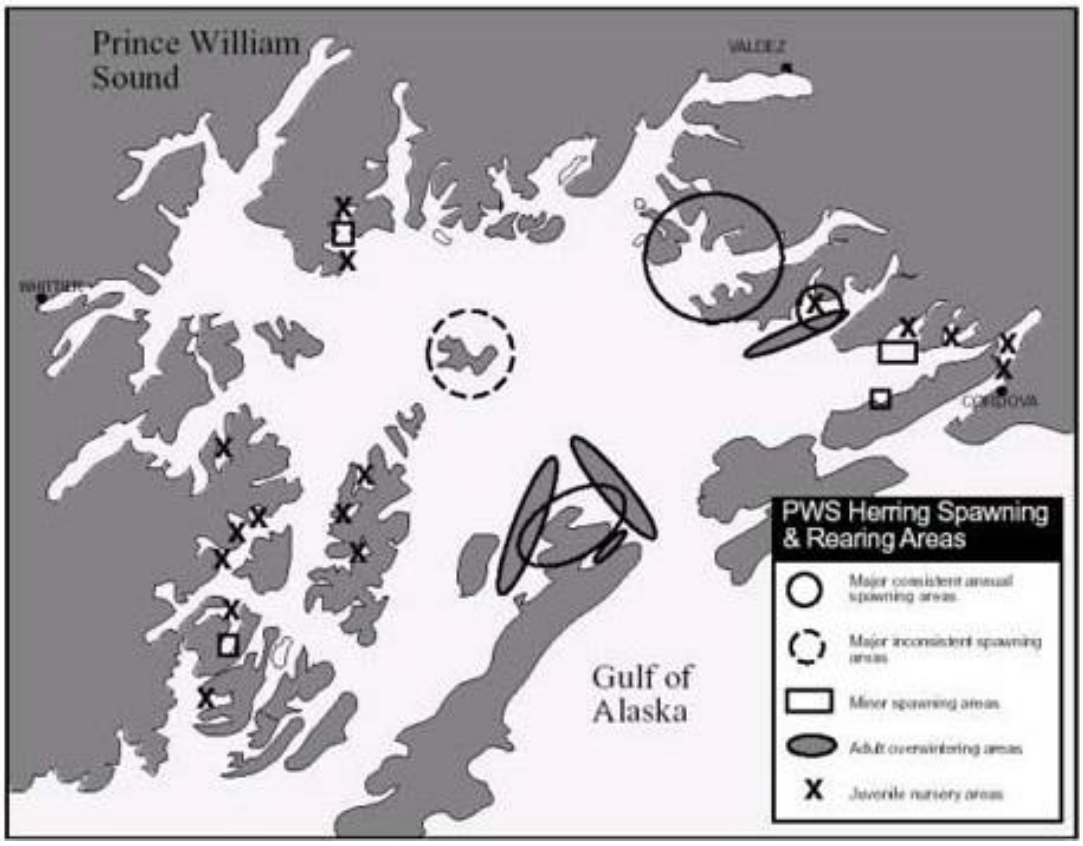

Figure 1-2: Pacific herring spawning sites overlap with oiled areas resulting from the *Exxon Valdez* Oil Spill. The figure shows the major spawning sites for Pacific herring within Prince William Sound (Brown and Carls 1998). The circled areas indicate the major spawning sites and the x's indicate the juvenile nursery areas. Refer to previous figure to identify areas still oiled today.

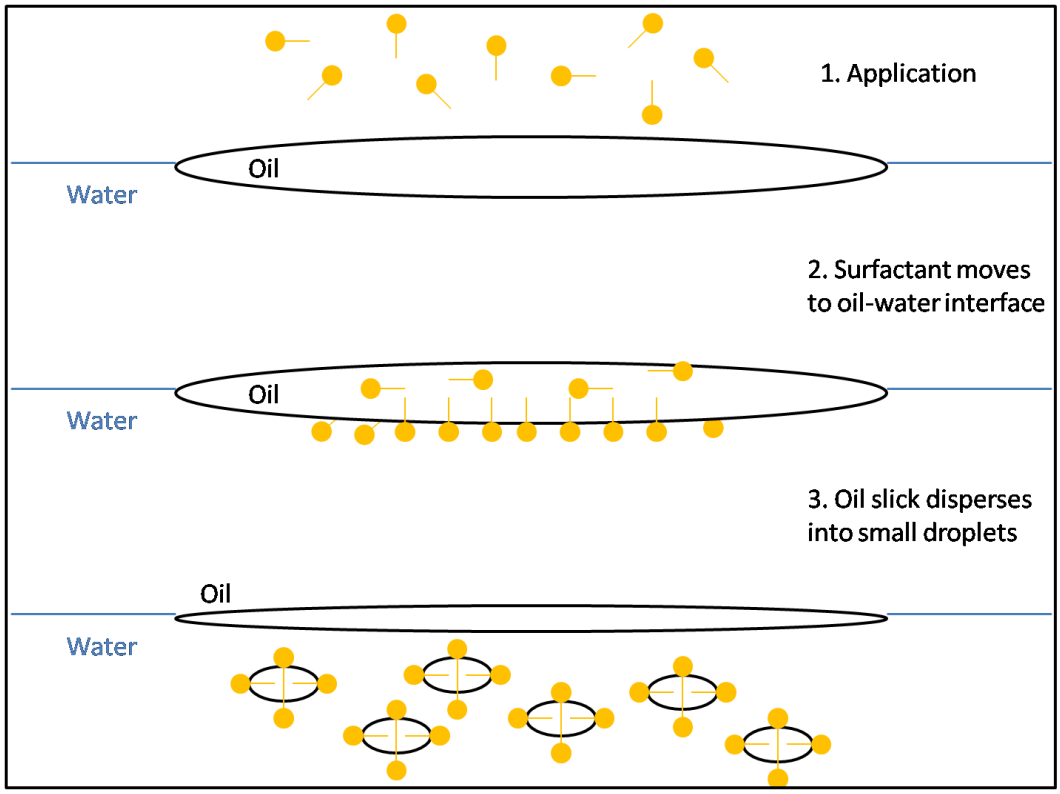

Figure 1-3: Application of a chemical dispersant onto an oil slick. Before dispersants are applied, petroleum compounds partition into the water by natural dispersion forming the water accommodated fraction (WAF). Chemical dispersants promote the formation of smaller droplets of oil, increasing the surface area of oil exposed to water and increasing the rate of partitioning of petroleum compounds into the water. This forms the chemically-enhanced water accommodated fraction (CEWAF).

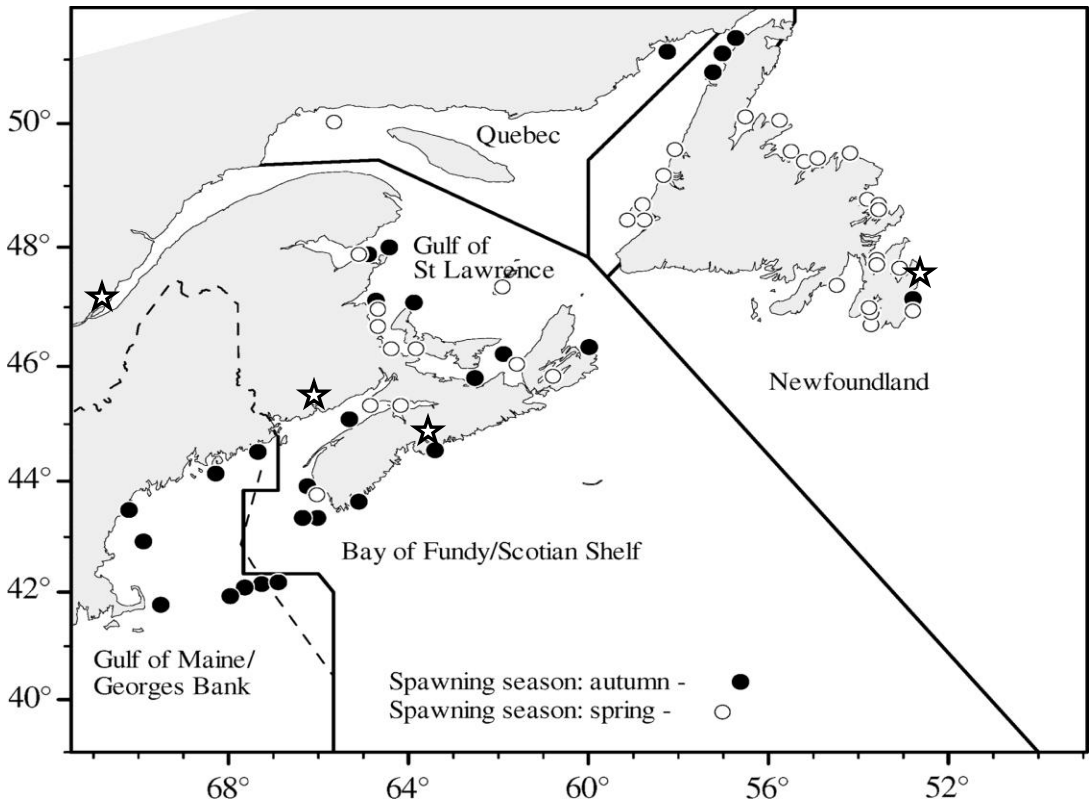

Figure 1-4: Atlantic herring spawning beds overlap with tanker routes transporting crude oil in Eastern Canada. The map shows the Fall (closed symbols) and Spring (open symbols) spawning stocks of Atlantic herring (borrowed with permission from Stephenson *et al.* 2009). The stars represent the major ports in Eastern Canada with large oil refining operations. Transportation routes to and from the ports would likely be close to herring spawning grounds.

Chapter 2

Toxicity of crude oil dispersed in a wave tank to Atlantic herring (*Clupea harengus*) embryos

2.1 Abstract

The use of chemical dispersants to clean up oil spills raises many concerns for organisms living below the surface of the water. Chemical dispersants break oil slicks into smaller droplets suspended in the water column, thereby increasing the rate of dilution and biodegradation. However, by decreasing the volume of surface oil, chemical dispersion increases the partitioning of hydrocarbons into water and the bioavailability of polycyclic aromatic hydrocarbons (PAH) to pelagic and benthic organisms by 100-fold. Laboratory tests of chronic toxicity are often scrutinized because they do not adequately reflect the conditions of actual oil spills. 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 (*Clupea harengus*) 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.

2.2 Introduction

The *T/V Exxon Valdez* collided with Bligh Reef in Prince William Sound, Alaska on March 24, 1989, coinciding with the return of Pacific herring to the spawning grounds in Prince William Sound (Kocan *et al.* 1996). Over 40 million litres of Alaska North Slope crude oil were spilled, coating 1750 km of shoreline (Bragg *et al.* 1994). Emergency response was not prepared for the spill, which delayed cleanup and allowed the oil to spread, causing widespread damage.

Prior to the 1989, annual spawning of Pacific herring (*Clupea pallasi*) took place over 106-273 km of shoreline. In 1994, the spawning area decreased up to 96%, to only 12 km (Funk 1994). Although other opinions exist (Pearson *et al.* 1999), it is assumed that the oil was the leading cause of the population crash. Following the spill, Paine *et al.* (1996) and Hose *et al.* (1996) observed physical deformation of Pacific herring embryos in Prince William Sound, which they have linked to oil exposure. The grounding of the *Exxon Valdez* and subsequent ecological damage lead to more stringent measures for tankers travelling through the Sound and for preparations in anticipation of another disaster, including stockpiling of chemical dispersants.

As oil sits on water, soluble compounds will partition into the water, forming the water accommodated fraction of oil in water (WAF). Chemical dispersants decrease the interfacial tension between oil and water, allowing the oil to break into smaller droplets of oil-surfactant micelles that can more easily be dispersed by wave action and currents. Smaller droplets have an increased surface area, allowing a larger quantity of petroleum compounds to partition into the water, composing the chemically-enhanced water

accommodated fraction (CEWAF). As such, chemical dispersion of oil markedly increases the concentration of polycyclic aromatic hydrocarbons (PAH) below the water surface compared to un-dispersed crude oil, and increases the water solubility of high molecular weight PAH (Couillard *et al.* 2005; Cohen *et al.* 2001). Application of dispersants occurs under the premise that oil will be quickly diluted into the water column to concentrations below toxic levels, and will be more bioavailable to hydrocarbon-degrading microorganisms. However, before this can occur, there is a surge in concentration of oil below the water surface, and if the concentration of petroleum hydrocarbons is high enough, even for a short period of time, it could induce toxicity in early life stages of fish (Wolfe *et al.* 1994; McIntosh *et al.* 2010).

PAH comprise the most toxic fraction of crude oil (Hodson *et al.* 2007). By making droplets smaller and increasing the partitioning of oil into water, chemical dispersion of crude oil could increase the exposure of fish embryos to PAH. Exposure of early life stages of fish to PAH can result in dioxin-like toxicity, inducing blue sac disease (Billiard *et al.* 1999). Pathological signs of blue sac disease in hatched herring embryos include yolk sac edema, pericardial edema, spinal curvature, fin rot, and craniofacial deformations (Figure 2-1; McIntosh *et al.* 2010). Severe deformities would hinder an embryo's ability to forage for food, escape predators, swim unimpeded, and ultimately be reproductively viable (Kocan *et al.* 1996; Smith and Cameron 1979).

Clupea sp. is at further risk because eggs stick to the substrate, preventing the embryos from avoiding an oil spill (Smith and Cameron 1979). Atlantic herring (*Clupea*

harengus) spawn in shallow intertidal and subtidal (0-20 m) areas, putting the embryos at further risk because CEWAF is more likely to reach these depths (Smith and Cameron 1979). Additionally, oil tanker routes in Eastern Canada pass through Atlantic herring spawning grounds. McIntosh *et al.* (2010) found that Atlantic herring are most sensitive to PAH directly following fertilization and hatch, increasing the potential for a high risk to spawning grounds should a spill occur there during spawning season.

Aside from the work done by McIntosh *et al.* (2010), most studies of the toxicity of crude oil to fish embryos in the literature have used chronic exposure conditions (Couillard *et al.* 2005; Hose *et al.* 1996; Paine *et al.* 1996). However, maintaining the same concentration of PAH from fertilization to hatch may not be realistic. Chronic toxicity testing (fertilization to hatch) allows for a reproducible and controlled environment for exposure, while giving the "worst-case scenario" for oil exposure. However, dispersant efficacy tests have shown that hydrocarbon concentrations can remain high for at most only a few days following a spill (Li *et al.* 2009). Additionally, fish sensitivity is not constant throughout development. Fish are most sensitive to oil exposure while gametes, immediately following fertilization, and immediately following hatch (McIntosh *et al.* 2010). Research has shown that the blastula and gastrula stages (within 24 – 48 h of fertilization) are critical for development, and exposure during this time can decrease hatching success by 40%, while exposures 72 h after fertilization do not (Kocan *et al.* 1996). By pinpointing the most sensitive stages of development,

exposures can be designed to better estimate the potential for toxicity under reasonable petroleum hydrocarbon exposure scenarios.

Laboratory tests may not be realistic, but opportunities to test the conditions of a real spill in the field are rare. As such, a practical intermediate model is needed to validate studies of CEWAF prepared in the lab using a standard protocol. To simulate chemical and natural dispersion of oil spills at sea, a wave tank at the Bedford Institute of Oceanography (BIO), Dartmouth, NS, was used (Appendix C). Plunging breaking waves (f = 0.85 Hz for 20 s followed by f = 0.50 Hz for 5 s), which are similar to white caps visible on a windy day, were used to simulate the open ocean. With the use of chemical dispersants, oil-surfactant droplets in the wave tank have a volumetric mean diameter smaller than 50 µm (Li *et al.* 2009). Compared to laboratory studies, preparation of CEWAF in a wave tank takes into account variable mixing energies and current effects, simulating the natural environment (Li *et al.* 2009).

The objective of this study was to determine if crude oil chemically dispersed within a wave tank can induce similar toxicity to Atlantic herring embryos as caused by laboratory-prepared test solutions. Newly fertilized embryos were exposed to lab-prepared WAF and CEWAF to provide a baseline for toxicity and to CEWAF prepared in a wave tank to compare toxicity between the two preparation methods. Finally, brief episodic exposures were used to determine the relationships among exposure time, concentration and toxicity.

2.3 Methods

2.3.1 Wave Tank

To assess the toxicity of chemically dispersed crude oil to Atlantic herring embryos, a wave tank was used as a practical intermediate model between a lab-prepared bench top method and a full-scale spill in the environment. The wave tank was located at the Bedford Institute of Oceanography (BIO), Dartmouth, NS, and operated by the Centre for Offshore Oil, Gas and Energy Research, a division of the Department of Fisheries and Oceans Canada. The wave tank was made from carbon steel, was 32 m long, 0.6 m wide, and 2 m high, and located beside the Bedford Basin. It was filled to an average depth of 1.5 m with filtered saltwater from the Bedford Basin for each experiment. Waves were generated by a computer-controlled flap-type wave maker situated at one end of the tank. Using a frequency sweep technique (Funke and Mansard 1979), recurrent breaking waves were generated for these experiments. A low-frequency, fast-moving wave was super-imposed on a high-frequency, slow-moving wave, causing wave heights to increase continuously until they broke.

The wave tank was equipped with water samplers at four horizontal locations (2 m upstream, and 2, 6 and 10 m downstream) and three vertical locations (5, 75 and 140 cm from the water surface) from the spill site (Appendix C). Thus, twelve water samples, or exposure solutions, were collected at each sampling time. At time 0, 300 mL of oil was poured onto the surface of the water at the spill site and 12 mL of dispersant (Corexit

9500A) was sprayed onto the surface of the oil. Water was sampled from the twelve sample points at 5, 15, 30, and 60 min post-dispersion.

2.3.2 Test Species

"Ripe and running", stage VI herring were acquired from the roe herring fishery in Eastern Passage, Nova Scotia (Dinnel *et al.* 2002; Hay 1985). Ripe herring have transparent eggs, and the eggs and sperm flow without external pressure (Hay 1985). Gonads were removed from the fish at the wharf and transported in re-sealable plastic bags in a cooler to BIO. Eggs and sperm were used within 24 h of capture to ensure freshness.

2.3.3 Experimental Setup

The experiments, from egg fertilization to scoring hatched embryos, were maintained at 10 C (±1C) with a 2:22 h dim light:dark photoperiod. Approximately 50-100 eggs were spread onto glass slides and separated into groups of two or three using a dissection needle. Individual experiments used eggs from a single female, and subsequent experiments were replicated with eggs from a different female. Once the eggs were attached to the glass slides, the slides were placed into a milt solution that was prepared by mashing 1/5 of a testis, from a pool of all testes collected (6 males), in approximately 500 mL of saltwater (15 g/L salinity). Slides were left in the milt solution for about 15 min after which they were rinsed with clean saltwater to remove excess milt and placed into test solutions. Freshly fertilized eggs were used for the experiments because they are the most sensitive life stage for herring (McIntosh *et al*. 2010).

2.3.4 Test Solutions

Crude oils tested included Alaska North Slope (ANSC, viscosity of 17.5cP) and Arabian Light (ALC, viscosity of 15.5cP). Oils were weathered by evaporation and sparging with air to simulate loss of volatiles at sea; 10% loss of ANSC, by weight and 7% loss of ALC, by weight (Appendix A; King 2011; Lee *et al.* 2011). Oil viscosities were measured after weathering. Corexit 9500A (Nalco Energy Services, L.P.; active ingredient is dioctyl sodium sulphosuccinate) was used to make CEWAF because it is the only dispersant stockpiled for use in Canada. All oils and the dispersant were supplied by the Centre for Offshore Oil, Gas and Energy Research, Dartmouth, NS. The recommended application is a dispersant:oil ratio (DOR) of 1:50 to 1:10 (US EPA Technical Product Bulletin #D-4). For our purposes, oil in the wave tank was dispersed using a DOR of 1:25 to follow the specifications of the tank, and CEWAF prepared in the lab was dispersed at a DOR of 1:10 to maximize effect.

Exposure solutions were either drawn from the wave tank or prepared in the laboratory. Wave tank solutions were drawn from the twelve sampling points at 5, 15, 30, and 60 min post-dispersion. Positive controls included retene (320 µg/L using methanol as the carrier solvent) and laboratory-prepared CEWAF. Retene (7-isopropyl-1-methyl phenanthrene) is an alkyl-phenanthrene and known to cause BSD in early life stages of fish (Billiard *et al.* 1999). Negative controls included lab water, wave tank water drawn before oil application, and a dispersant control. The optimum salinity for Atlantic herring fertilization and hatching is 15 g/L (McIntosh 2009). The water used in the wave tank was from the Bedford Basin (30 g/L salinity) and samples were diluted

with dechlorinated freshwater to 15 g/L. The CROSERF methods used to prepare CEWAF and dispersant controls are outlined in Appendix B, adapted from Singer *et al.* (2000), and reported previously (McIntosh *et al.* 2010).

2.3.5 Design

Slides of herring eggs were exposed to WAF (14 d exposure) and CEWAF for 2.4 h, 8 h, 24 h, or 14 d. After exposure, embryos were transferred to clean Mason jars containing fresh saltwater (15 g/L). Water and test solutions for 14 d exposures were renewed daily until hatch. Embryos remained attached to the glass slides until hatch. Seven days post-fertilization, unfertilized eggs were removed from the slides and fertilized embryos were randomly culled to about 20 per slide. Every other day from this point to hatch, dead embryos were removed and survivors were monitored to determine the day of hatch.

Within 24 h of hatch, embryos were scored for signs of blue sac disease (BSD). These included pericardial edema (Score = 0-3), yolk sac edema (0-3), spinal curvature (0-3), fin rot (0-1), craniofacial deformities (0-1), and mortality. Scoring was done using a graduated scale; three indicated the most severe response and zero indicated no response. Fin rot and craniofacial deformities were scored as either present (1) or absent (0) because it was assumed they would have less of a lasting impact on the fish, and the response was subtle and could not be scored on a graduated scale. We assumed that fish with lower BSD scores were more likely to survive and contribute to the next generation, despite the deformities. Abnormal fin and jaw development are thought to be results of

early hatch induced by PAH exposure and could be reversible (Carls *et al.* 1999; Humphrey *et al.* 1995). Jaw growth and development continue throughout the larval stage, increasing the probability that a fish with this type of deformity could still develop a normal jaw if the water quality improved (Alderdice and Velsen 1971). On the other hand, edema was the most sensitive response to oil exposure and primarily responsible for mortality, and residual edema may reduce the ability to feed (Carls *et al.* 1999; Marty *et al.* 1997).

Embryos that were dead upon hatch received the highest possible BSD score (11.5) to indicate the most severe response beyond sublethal toxicity. The BSD Severity Index was calculated from the average BSD scores for all embryos within a treatment and normalized to the maximum score. The Index included both lethal (mortality) and sub-lethal (BSD) signs of toxicity (modified from Villalobos *et al.* 2000). Alternatively, embryos were considered 'normal' if they were alive at hatch, did not show any signs of BSD, and swam normally. These fish would be most likely to grow, reproduce, and contribute to the next generation.

2.3.6 Water Chemistry

Water samples were drawn from exposure solutions to quantify the concentration of hydrocarbons by fluorescence (Detailed SOP in Appendix D). Water samples (3 mL) were drawn from exposure solutions at the start (time 0 h) and end of the exposure period and added to 3 mL of absolute ethanol in 7 mL glass scintillation vials with foil-lined caps and stored at 4C. Samples were sonicated for 3 min before chemical analysis. Upon

mixing with ethanol, salt from the water samples precipitated from solution. To reduce interference by salt particles, samples were centrifuged at 9055 x g for 10 min to separate the salt from the sample. An aliquot of the sample was placed in a quartz cuvette and analyzed for total fluorescence using a RF-5301PC scanning spectrofluorometer (Shimadzu Corporation, Kyoto, Japan) with Panorama fluorescence 1.1 software (LabCognition, Dortmund, Germany). The excitation (278 nm) and emission (300-450 nm) wave lengths used for the experiments were the same for both ALC and ANSC. A comparison of fluorescence to a standard curve prepared from each test oil dissolved in 50:50 ethanol:water provided an estimate of total petroleum hydrocarbons. The limit of detection (LOD), or instrument detection limit, is the concentration of chemical required to produce a signal three standard deviations above the noise level of the instrument, and the concentration above which we can accurately determine the concentration of chemical. The LOD was calculated as three times the standard deviation of the mean fluorescence of a standard oil concentration run ten times on the fluorometer.

Water samples (100 mL) were also drawn from a subset of exposures to quantify the concentration of total petroleum hydrocarbons for comparison to concentrations estimated by fluorescence. Samples were preserved with 10 mL of dichloromethane and sent to the Centre for Offshore Oil, Gas and Energy Research, Bedford Institute of Oceanography, to be analyzed by Gas Chromatography-Flame Ionized Detection (GC-FID).

2.3.7 Statistics

SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA) and GraphPad Prism 5.00 (GraphPad Software, San Diego, CA) were used to prepare nonlinear regressions and estimate LC50s (median lethal concentration) and EC50s (median effective concentration). To calculate LC50s, lower constraints for percent mortality were set at the negative control (14 d and exposure time = 7.69%; Wave tank = 0.00%; Supplementary ANSC wave tank = 5.00%) for the corresponding experiments and upper constraints were set at 100% to represent the maximum possible response. EC50s were calculated for percent normal, percent hatch and the BSD Severity Index. The upper constraints for percent normal (14 d and exposure time = 80.77%; ANSC wave tank = 89.82%; ALC wave tank = 93.33%; Supplementary ANSC wave tank = 81.23%) and hatch (14 d and exposure time = 96.15%; ANSC and ALC wave tank = 100%; Supplementary ANSC wave tank = 96.67%) were set at the corresponding negative controls and the lower constraints were zero. The BSD Severity Index is a normalized value based on the sum of the BSD scores for each fish (including mortalities) and the maximum possible score, and averaged within treatments. Lower constraints were set at the response level of negative controls (14 d and exposure time = 0.11; ANSC wave tank = 0.02; ALC wave tank = 0.01; Supplementary ANSC wave tank = 0.09) and the upper constraints at the maximum possible score of 1.0, where all embryos would be dead. Statistical comparisons were made by comparing the confidence intervals for overlap and by using the Extra Sum-of-Squares F-Test function in GraphPad Prism, where parameters are shared to determine if correlations are better with shared data or as separate functions.

2.4 Results

Hatched embryos exposed to crude oil exhibited exposure-dependent signs of toxicity, including yolk sac edema, pericardial edema, spinal curvature, fin rot, and craniofacial deformities (Figure 2-1). Mean survival of controls was: % Mortality = 3.2 ± 3.7%; % Normal = 86.3 ± 6.1%; % Hatch = 98.2 ± 2.0%; BSD Severity Index = 0.058 ± 0.049.

There was a linear relationship among measured concentrations of fluorescing petroleum hydrocarbons and nominal loadings of oil (%v/v) for ANSC and ALC oils of both WAF and CEWAF (Figure 2-2). All lines were parallel (F-test, $P = 0.90$) and shared a slope of 0.89. Chemical dispersion of crude oil (CEWAF) caused a roughly 100-fold increase in concentration of fluorescing petroleum hydrocarbons in the water compared to un-dispersed oil (WAF; F-test, $P < 0.001$). ALC had more soluble fluorescent hydrocarbons than ANSC in both CEWAF (F-test, $P < 0.001$) and WAF (F-test, $P = 0.005$).

Atlantic herring embryos responded to dilutions of WAF and CEWAF of ANSC and ALC in a dose-response manner. ANSC was more toxic than ALC, as evidenced by lower EC/LC50s (median effective/median lethal concentrations) of percent mortality (LC50), percent normal ($EC50_{\%Normal}$), percent hatch ($EC50_{\%Hatch}$), and the BSD Severity Index ($EC50_{SI}$; Figure 2-3; Table 2-1). There were no significant differences in LC50 and $EC50_{\%Hatch}$ values (wide 95% confidence intervals (CI)) for ANSC and ALC. However, the $EC50_{\%Normal}$ and $EC50_{SI}$ values indicated that ANSC was approximately two times more toxic than ALC (F-test, $P = 0.040$ and 0.003, respectively).

The toxicity of CEWAF prepared from ANSC increased with exposure time (Figure 2-4; Table 2-1). There was a significant difference among the $EC50_{\%Normal}$ values for Atlantic herring embryos for all exposure times (F-test, $P < 0.001$). LC50s were larger than the highest concentrations tested, except for the 14 d exposure which produced an LC50 of 1.3 mg/L (95% CI too wide to estimate). $EC50_{\%Hatch}$ values were also larger than the highest concentrations tested, except for the 14 d exposure which produced an $EC50_{\%Hatch}$ of 3.0 mg/L (95% CI = 1.5- 5.9 mg/L). $EC50_{SI}$ values were not significantly different between 2.4 and 8 h (F-test, $P = 0.99$), between 8 and 24 h (F-test, $P = 0.57$), or between 2.4 and 24 h (F-test, $P = 0.48$), but there was a significant difference between 24 h and 14 d (F-test, $P = <0.001$).

The toxicity of CEWAF prepared from ALC increased with exposure time (Figure 2-4; Table 2-1). There were significant differences between the $EC50_{\%Normal}$ values for 2.4 h and 8 h (F-test, $P = 0.002$) and between 2.4 h and 24 h exposure times (F-test, $P = 0.003$), but not between 8 h and 24 h (F-test, $P = 0.94$). The 14 d $EC50_{\%Normal}$ was significantly lower than all other exposure times tested (F-test, $P = 0.008$). LC50s were larger than the highest concentrations tested, except for the 14 d exposure which resulted in an LC50 of 2.3 mg/L (95% CI too wide to estimate). $EC50_{\%Hatch}$ values were also larger than the highest concentrations tested, except for the 14 d exposure which produced an $EC50_{\%Hatch}$ of 2.6 mg/L (95% CI too wide to estimate). $EC50_{SI}$ values were not significantly different between 2.4 and 8 h (F-test, $P = 0.39$), between 8 and 24 h (F-

test, P = 0.98), or between 2.4 and 24 h (F-test, P =0.55), but there was a significant difference between 8 h and 14 d (F-test, P = 0.001).

Hydrocarbon concentrations produced by dispersing ANSC and ALC oils in the wave tank were too low for $EC50_{\%Hatch}$, $EC50_{SI}$ and LC50 values to be calculated (Figure 2-5). The wave tank 24 h $EC50_{\%Normal}$ for ANSC was 27 mg/L (95% CI = 0.0004 – $1.7x10^6$ mg/L), while the $EC50_{\%Normal}$ for ALC was larger than the highest concentration tested (> 9.6 mg/L). The increasing trend of toxicity at the highest concentrations of ANSC was especially evident in the samples drawn from the tank 15 minutes post-dispersion, where the $EC50_{\%Normal}$ was 1.0 mg/L (95% CI = 0.62 – 1.7 mg/L). Some ANSC samples from the wave tank appeared to be more toxic than the laboratory-prepared controls, while ALC concentrations were lower than the laboratory-prepared $EC50_{\%Normal}$ and did not produce a trend in toxicity. The $EC50_{\%Normal}$ for ANSC prepared in the laboratory with the same females as the ANSC wave tank-prepared experiment was 4.9 mg/L (95% CI = 3.5 - 6.7 mg/L). Exposure solutions sampled from the wave tank at 15 minutes post-dispersion were significantly more toxic than those produced in the lab (F-test, P = 0.025).

An additional wave tank experiment using ANSC is shown in the supplementary material (Appendix C). The overall $EC50_{\%Normal}$ estimated by combining all data points (33 mg/L; 95% CI = 10 – $1.2x10^6$ mg/L) and the $EC50_{\%Normal}$ of the laboratory-prepared CEWAF (4.3 mg/L; 95% CI = 0.63 – 30 mg/L) were not significantly different (95% CIs overlap). The $EC50_{\%Normal}$ values for exposures drawn 5 (3.4 mg/L) and 60 (3.4 mg/L)

minutes post-dispersion were less than the $EC50_{\%Normal}$ for laboratory-prepared CEWAF, but the 95% CIs were too wide to estimate and therefore we could not determine if they were significantly different. The $EC50_{\%Normal}$ values for 15 and 30 minutes post-dispersion could not be estimated because no clear trend was evident.

2.5 Discussion

Hatched embryos exposed to oil exhibited signs of toxicity known to correlate with exposure to PAH (Hodson *et al.* 2007). Chemical dispersion in the lab increased the concentration of fluorescing petroleum hydrocarbons in the water by 100-fold and ALC released significantly more fluorescing hydrocarbons into the water than ANSC. However, ANSC was more toxic than ALC to Atlantic herring embryos, as evidenced by significantly lower EC50s of percent normal and BSD Severity Index. This difference was likely a result of chemical composition. Additionally, toxicity of the two oils increased linearly with exposure time. Toxicity resulting from exposures of 24 h or less was primarily sublethal, which was indicated largely by a reduction in percent normal and an increase in BSD without mortality. There was no toxicity from ALC dispersed in the wave tank and concentrations were below the EC50 for laboratory-prepared solutions. However, some exposures of ANSC dispersed in a wave tank were more toxic than laboratory-prepared CEWAF. Overall, laboratory prepared exposure solutions were considered a good surrogate for solutions prepared under conditions expected at sea.

Chemical dispersion of oil increased the concentration of water-borne hydrocarbons by 100-fold and a higher concentration of hydrocarbons partitioned into the

water from ALC than from ANSC in both CEWAF and WAF. Conversely, ANSC was nearly twice as toxic as ALC when embryos were exposed from fertilization until hatch. Oil toxicity to early life stages of fish is generally a result of PAH (Hodson *et al.* 2007), implying that the greater quantity of soluble PAH (or aromatics) will result in a higher degree of toxicity (Di Toro *et al.* 2007). The toxicity of ANSC was in agreement with the chemical characteristics of the oils, which indicate that ANSC has two times more aromatics than ALC (Appendix A; King 2011). Additionally, ANSC has two times more phenanthrenes, which are known to be highly toxic to early life stages of fish (Barron *et al.* 2004). The higher fluorescence coupled with lower toxicity may relate to the relative composition of fluorescent compounds present in the tested oils, as many organic molecules which are aromatic or contain conjugated double bonds will fluoresce (Williams and Bridges 1964). Additionally, compounds can vary in their ability to fluoresce; aniline can be 50 times more fluorescent than benzene (Bridges and Williams 1962). This introduces limitations to the methods chosen for water chemistry because compounds present in oil other than PAH fluoresce, including high molecular weight asphaltenes that have both aromatic and aliphatic components. ALC contains three times more thiophenes than ANSC (Appendix A), which may account for the discrepancy between fluorescence and toxicity. Although thiophenes have a toxic potential, they are not considered PAH (Williams and Bridges 1964) and may contribute more to the fluorescent properties of oil in water than to toxicity.

To estimate the highest potential toxicity, as indicated by McIntosh *et al.* (2010), embryos were exposed immediately following fertilization for the desired exposure time. In a similar study, Kocan *et al.* (1996) found that the blastula and gastrula stages of embryonic development (within 48 h of fertilization) were the most sensitive to oil exposure, documented by a decrease in the percent normal at hatch. They found that exposures occurring 72 h after fertilization did not have the same lethal effects as those occurring within 24 and 48 h following fertilization, which decreased hatching success by 40% (Kocan *et al.* 1996).

Toxicity estimations are often unrealistic because embryos are exposed chronically (fertilization to hatch), while in actual fact, most oil spills would expose embryos in an acute contact scenario of a few hours to days. Exposure time was examined using laboratory-prepared CEWAF to reflect a pulse exposure scenario, where embryos were exposed to a constant concentration of oil in water over a pre-determined exposure time. In accordance with McIntosh *et al.* (2010), increasing exposure time decreased the percentage of normal embryos. The exposure-toxicity relationships indicate that even very short exposure times (2.4 h) can result in toxicity, and the longer the embryo is exposed, the higher the risk for toxicity and for failure to recruit to future generations. This relationship however, was not as clear for ALC CEWAF. There was no significant difference in toxicity between 8 h and 24 h exposures. This could indicate that the solubility of PAH in water has reached a maximum by 8 h, meaning the toxic agents had been depleted from solution, or that uptake and excretion/metabolism have

reached equilibrium within the embryos, so that no increase in toxicity would be observed. It is also possible that the threshold in toxicity is a result of experimental error or random variability among embryos. Signs of toxicity (percent normal, percent hatch, percent mortality, BSD Severity Index) were more pronounced in embryos exposed chronically (14 d), which could be the effect of daily static renewal of solutions, where soluble hydrocarbons were replenished daily.

Toxicity resulting from exposure times of 24 h or less was primarily determined by sublethal endpoints, as LC50s could not be estimated. It was found that percent normal and to a lesser extent, the BSD Severity Index were the most sensitive endpoints used for determining toxicity (Appendix F). This is consistent with McIntosh *et al.* (2010) and Rice *et al.* (1987) who found lethality to be both exposure time and concentration dependent. On the other hand, all endpoints could be used to estimate toxicity to Atlantic herring embryos exposed for 14 d.

Oil is a complex mixture, and the kinetics of weathering and uptake are difficult to mimic in the laboratory. As such, CEWAF was also prepared in a wave tank to allow for more realistic spill and dispersion conditions. Exposure solutions of ALC dispersed in the wave tank did not show toxicity and the concentrations of hydrocarbons were less than those observed to cause toxicity in the lab. In contrast, exposure solutions of ANSC dispersed in the wave tank and drawn from the tank at 15 minutes post-dispersion were over four times more toxic than laboratory-prepared CEWAF. Exposures drawn at 5, 30, and 60 minutes post-dispersion from the tank did not show the same negative trend and

the concentrations were too low to determine if toxicity mimicked laboratory-prepared CEWAF. A second wave tank experiment using ANSC is shown in the supplementary material (Appendix C). No increase in toxicity was evident for exposure solutions drawn at 15 and 30 minutes post-dispersion, but the 5 and 60 minute samples were slightly more toxic than the laboratory-prepared CEWAF. The significant increase in toxicity to embryos exposed to water sampled at 15 minutes for the first wave tank experiment suggests that oil droplets in the wave tank had been weathered enough to release toxic concentrations of hydrocarbons. After 15 minutes, the toxic constituents may have been degraded or diluted beyond their toxic potential at concentrations found within the water column. It is also possible that there is a difference between wave tank-prepared and laboratory-prepared CEWAF in terms of the types of hydrocarbons within the water column, the degree of weathering and dilution, and the chemical characteristics and physical composition of the oil. Further chemical analysis would be required to support these possibilities.

The wave tank system is merely a model for oil spills in the field. The ocean is a dynamic system and each oil spill would occur under different conditions and regimes. The wave tank experiments were designed to simulate specific high energy conditions in the ocean on a windy day. It is more of a stream channel that has boundaries and a shallow depth, which prevent the oil from spreading horizontally and vertically. As a result, hydrocarbon concentrations and potential toxicity cannot be extrapolated directly to conditions expected at sea.

Within the wave tank, hydrocarbon concentrations were highest towards the end of the tank and at the sampling times directly following dispersion (5 minutes), and toxicity was greatest at 15 minutes (Figure 2-5). This suggests that even though a spill may not occur directly over a fish spawning bed, chemical dispersion, currents, and wave action could quickly move the hydrocarbons to that area. Increasing the volume of oil added to the wave tank for dispersion may increase the concentration of dissolved oil generated by the tank to better pinpoint LC50s and EC50s. The volume of dispersant could also be increased to a maximum DOR of 1:10. Additionally, maintaining the salinity in toxicity tests at 30 ppt would double the concentration of oil in the water samples (as opposed to diluting the sample to 15 ppt as was done in the present experiments) and increase exposure. Preliminary experiments performed at 30 ppt showed a higher range of toxicity, presumably because samples were undiluted, and there was no difference in toxicity among CEWAF controls between salinities (Appendix C-4). Unfortunately, these earlier experiments could not be linked to hydrocarbon concentrations. It is possible however, that oil toxicity would increase with salinity (Johnson 2011; Fingas *et al.* 1991).

There are many methods available for measuring the concentration of oil in water, depending on the target compounds. Most methods can be very time consuming and costly (eg. Gas Chromatography-Mass Spectrometry, Gas Chromatography-Flame Ionized Detection), extending the length of a project and/or decreasing the number of samples possible for testing. To overcome these hurdles, fluorescence was used to

analyze the quantity of hydrocarbons within the exposure solution by comparing total fluorescence to a standard curve prepared from whole oil. This method was employed for monitoring the bioassay because a large number of samples required processing, the analysis and equipment were relatively simple, and samples were processed on-site at a low cost. The largest proportion of oil that fluoresces is the PAH component, which is also known to be the most toxic and the cause of dioxin-like toxicity that we observed in fish embryos (Hodson *et al.* 2007). To quantify hydrocarbon concentration, a standard curve was prepared for comparison to unknowns and the wavelengths targeted were specific to phenanthrenes (three-ringed PAH), which are known to cause toxicity and have a higher toxic potential than other PAH (Di Toro *et al.* 2007). Other experiments that compare toxicity to total petroleum hydrocarbon concentrations would produce a higher concentration simply because these methods also measure alkanes, which do not fluoresce. By targeting the most toxic fraction of the oil, as is done in fluorescence, the measured concentration is more accurately estimated by the PAH component causing toxicity and not erroneously including other compounds (Di Toro *et al.* 2007).

Gas Chromatography-Flame Ionized Detection was also used to analyze the concentration of total petroleum hydrocarbons within some of the exposure solutions. When compared to the concentrations obtained through spectrofluorometry, a linear relationship was observed (Figure 3-1 in Chapter 3), indicating that the methods provided are comparable to measures of relative concentration among exposures. If need be, total petroleum hydrocarbon concentrations can be extrapolated from spectrofluorescence

results. Further GC-MS analysis on the toxicity of oil fractions would enable more precise EC50 and LC50 values based solely on PAH, the most toxic component of oil.

Obtaining high quality eggs was an issue in these experiments. Many experiments failed because eggs were not fertilized, likely because females were not sufficiently mature prior to harvesting eggs. There was high variability among and within females in terms of egg quality and there was variability in terms of response to oil exposure (personal observations). Fertilization success was observed to be much greater during the fall spawning season compared to the spring spawning season, which corroborates observations made by McIntosh *et al.* (2010). It is not uncommon that 50% of naturally spawned herring in the wild exhibit morphological deformities (Purcell *et al.* 1990; Hose *et al.* 1996), a background response rate that would reduce reproducibility among experiments and place the relative importance of this response in perspective. As a result, we found it necessary to only include replicates that had a minimum of five fertilized eggs per slide and to view toxicity data with respect to the negative control for that experiment. Replicates that had abnormally high rates of malformed embryos (< 75% normal) in the controls were discarded. Hose *et al.* (1996) also found high variability in oil sensitivity of Pacific herring among spawning sites, but nevertheless, hydrocarbon concentrations present directly following the *Exxon Valdez* spill were high enough to elicit severe physical deformities and genetic damage to embryos. Although possibly unrealistic for time management, many experiments are needed to compile a

sufficient database that risk assessors can use to define the average threshold of toxicity to Atlantic herring.

2.6 Conclusion

Atlantic herring embryos were sensitive to dispersed crude oil exposure and exhibited signs of toxicity known to correlate with PAH exposure. Chemical dispersants increased the concentration of petroleum hydrocarbons in the water column by 100-fold, increasing the availability of oil to early life stages of fish. ANSC appeared to be more toxic to Atlantic herring embryos than ALC, and exposure times to CEWAF of either oil for periods as short as 2.4 h caused toxicity. Exposure solutions of ANSC dispersed by the wave tank were up to four times more toxic than laboratory-prepared CEWAF, but embryos exposed to ALC dispersed in the wave tank did not exhibit signs of toxicity and concentrations were lower than those found in the laboratory-prepared CEWAF exposures. The results suggest that preparation of CEWAF in the lab is a suitable alternative to large-scale experiments for determining the toxicity of dispersed oil to herring embryos.

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2.9 Figures

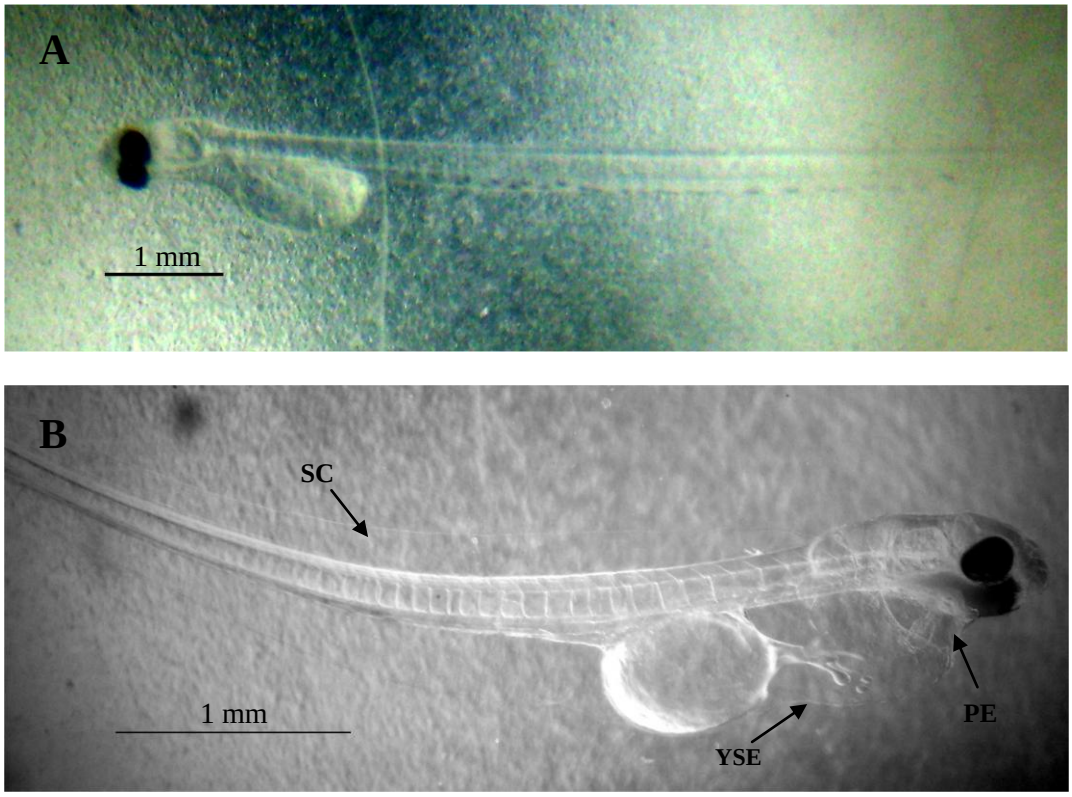

Figure 2-1: Hatched Atlantic herring embryos. The top panel (A) represents a hatched embryo showing no signs of toxicity ("normal"). The lower panel (B) depicts a hatched embryo that was exposed to 2.7 m/L of Arabian light crude (ALC) chemically-enhanced water accommodated fraction (CEWAF). The lower embryo has yolk sac edema (YSE), pericardial edema (PE), and a spinal curvature (SC). Contrast and brightness have been adjusted for clarity. Panel B shows a larger magnification than Panel A to enhance visibility of edemas. Additional photos can be found in Appendix E.

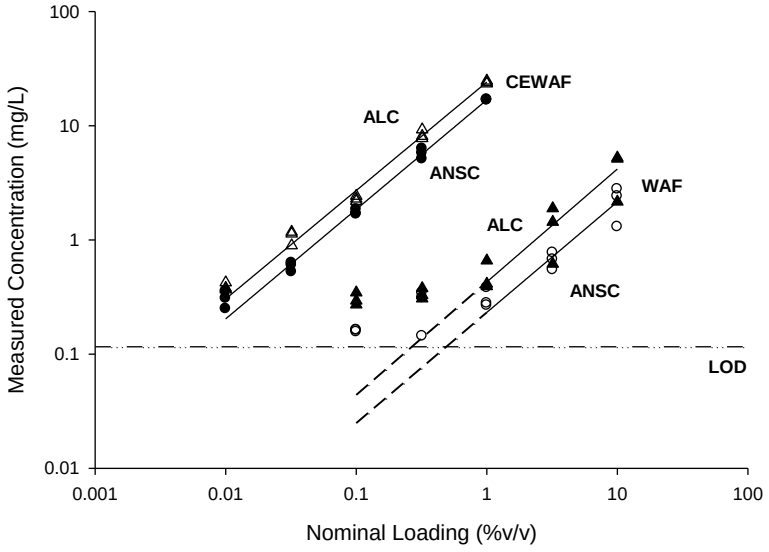

Figure 2-2: The concentration of petroleum hydrocarbons in test solutions at different loadings of the water accommodated fraction (WAF) and the chemically-enhanced water accommodated fraction (CEWAF). Open triangles show the Arabian Light Crude (ALC) CEWAF ($R^2 = 0.99$), closed triangles represent the ALC WAF ($R^2 = 0.76$), closed circles show the Alaska North Slope Crude (ANSC) CEWAF ($R^2 = 0.99$), and open circles represent the ANSC WAF ($R^2 = 0.82$). The dashed lines show the extrapolated concentration of the WAF samples below the limit of detection (LOD; calculation in section 2.3.6). Data points at or below the LOD are shown for comparison.

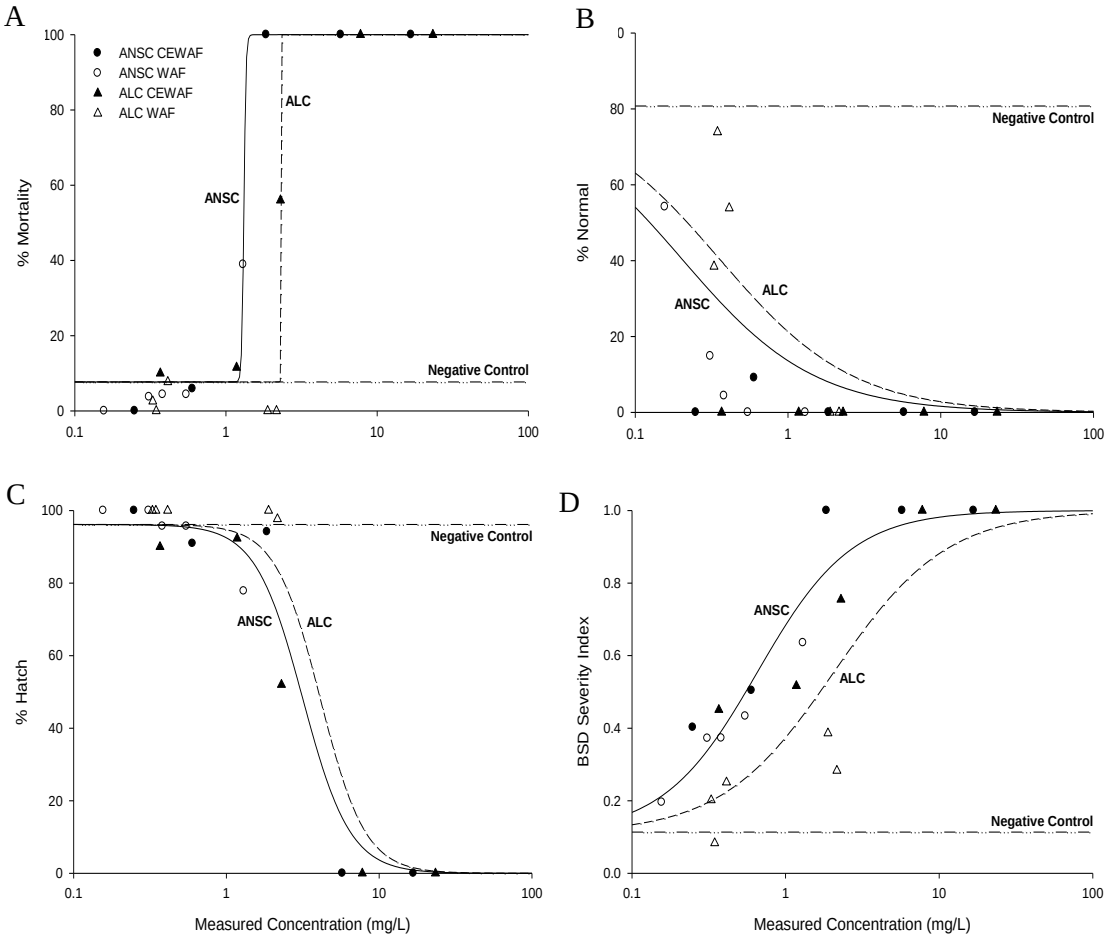

Figure 2-3: Toxicity of the water accommodated fractions (WAF) and chemically-enhanced water accommodated fractions (CEWAF) of Alaska North Slope Crude (ANSC) and Arabian Light Crude (ALC) oils prepared in the lab to Atlantic herring embryos expressed as (a) % Mortality, (b) % Normal, (c) % Hatch, and (d) BSD Severity Index in relation to the measured concentration of fluorescing compounds (mg/L). Regression lines and LC50/EC50 values were calculated using WAF (open symbols) and CEWAF (closed symbols). Regression equations can be found in Appendix G. Dash lines represent response from ALC exposure, and solid lines represent response from ANSC exposure. N = 20 embryos per treatment.

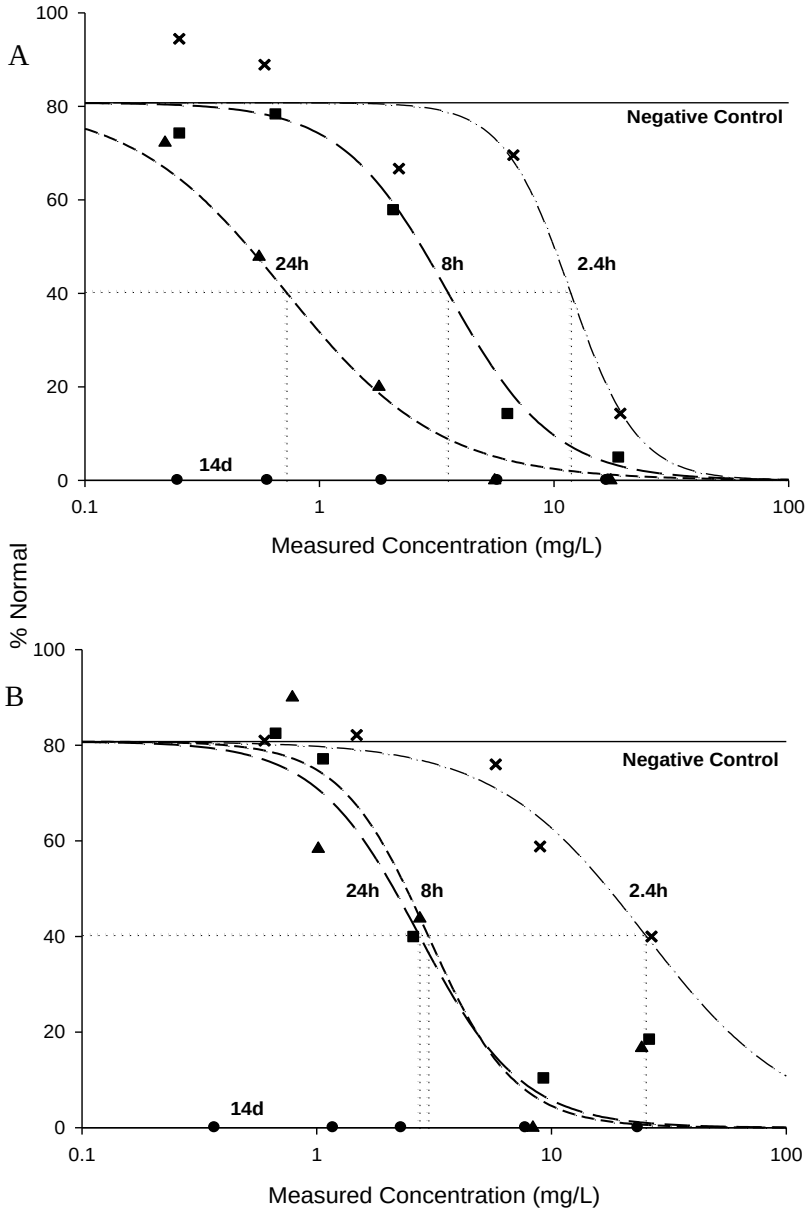

Figure 2-4: The effect of exposure time and petroleum hydrocarbon concentration on the percentage of normal embryos at hatch exposed to lab-prepared Alaska North Slope Crude (ANSC; A) and Arabian Light Crude (ALC; B). Percent normal indicates percentage of embryos alive and showing no signs of toxicity at hatch. Embryos were exposed immediately following fertilization for 2.4 h, 8 h, 24 h or 14 d (static daily renewal). Percent mortality, percent hatch, and BSD Severity Index data can be found in Appendix F. Regression equations can be found in Appendix G. Dotted lines show estimation of median effective concentrations. N = 20 embryos per treatment.

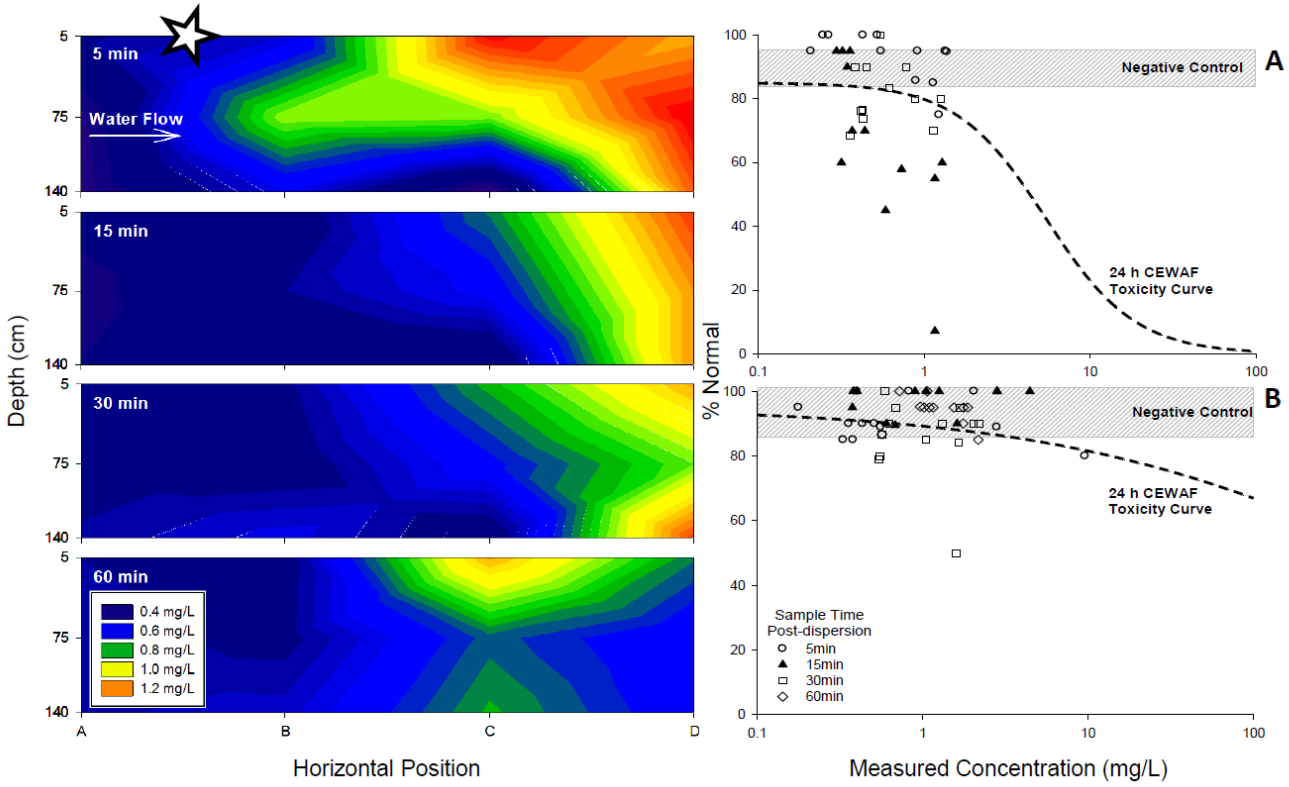

Figure 2-5: Wave tank data showing the movement of fluorescing hydrocarbons in Alaska North Slope Crude oil (ANSC) through the tank over time (left panel), and the change in percent normal with increasing concentration of the chemically-enhanced water accommodated fraction (CEWAF) of ANSC (A) and Arabian Light Crude oil (ALC; B) exposures drawn from the tank. 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 Atlantic herring of a 24 h exposure of the corresponding laboratory-prepared CEWAF exposures have been superimposed for comparison (dash lines). The measured concentrations were derived from samples of toxicity test solutions. N = 20 embryos for each exposure.

Table 2-1: The effect of exposure time on the toxicity of the chemically-enhanced water accommodated fraction (CEWAF) of Alaska North Slope Crude (ANSC) and Arabian Light Crude (ALC) oils. Median lethal concentrations (LC50s) represent lethal toxicity, specifically an increase in the mortality of embryos. Median effective concentrations (EC50s) represent sublethal toxicity, specifically the concentrations causing a reduction in the percent normal and percent hatch, and an increase in the BSD Severity Index of Atlantic herring embryos. Values are based on measured concentrations of fluorescing petroleum hydrocarbons of CEWAF in mg/L and shown with 95% confidence intervals (CI). ">" or "<" indicate that the LC/EC50 was greater than the highest concentration tested or less than the lowest concentration tested. "NA" indicates confidence limits were too wide to estimate.

		Atlantic Herring (LC50s and EC50s in mg/L)							
Oil	**Exposure Time**	**% Mortality**	**CI**	**% Normal**	**CI**	**% Hatch**	**CI**	**BSD Severity Index**	**CI**
ANSC	**14 d**	1.32	NA	<0.25	NA	2.99	1.51-5.93	0.66	0.48-0.89
	24 h	>17.46	-	0.76	0.58-0.99	>17.46	-	27.13	11.89-61.89
	8 h	>18.77	-	3.16	2.36-4.23	>18.77	-	27.33	7.22-103.50
	2.4 h	>19.17	-	11.74	5.32-25.91	>19.17	-	41.37	7.48-228.80
ALC	**14 d**	2.29	NA	<0.37	NA	2.29	NA	2.07	1.01-4.24
	24 h	>24.23	-	2.75	1.01-7.47	>24.23	-	55.26	2.58-1186
	8 h	>26.06	-	2.86	1.26-6.52	>26.06	-	53.68	13.47-213.90
	2.4 h	>26.68	-	25.06	14.66-42.84	>26.68	-	215.90	0.015-3.3x10^6

Chapter 3

Oil Toxicity: A Comparison Between Pacific and Atlantic Herring

3.1 Abstract

Significant declines in Pacific herring (*Clupea pallasi*) populations have followed two major oil spill events in the United States, the *Exxon Valdez* oil spill and the *Cosco Busan* oil spill. As a result of oil-related declines in Pacific herring, many studies have documented the risk to Pacific herring of oil exposure. Tanker transportation routes throughout the Northwestern Atlantic Ocean coincide with Atlantic herring (*Clupea harengus*) spawning grounds, but little research has been done to study the risk of oil exposure to Atlantic herring. The main objective of this research was to determine if Atlantic herring and Pacific herring embryos respond similarly to oil exposure, so that available knowledge on Pacific herring can be included in risk assessments for Atlantic herring. Under the same test conditions, embryos of Atlantic herring were four times more sensitive to chemically dispersed crude oil than Pacific herring embryos, as determined by the percentage of normal embryos at hatch and the incidence of BSD. Additionally, medium crude oils were two times more toxic to Atlantic herring than light crude oils. The differences in sensitivity were sufficiently small that data on the toxicity of chemically dispersed oil may be interchangeable between the two species.

3.2 Introduction

As Pacific herring (P. herring; *Clupea pallasi*) were returning to the spawning shoals in Prince William Sound, Alaska in March of 1989, the *T/V Exxon Valdez* collided with Bligh Reef, spilling Alaska North Slope Crude oil (ANSC; Kocan *et al*. 1996). Prior to the spill, P. herring spawned along 106-273 km of shoreline in Prince William Sound. In 1994, the spawning area decreased to 12 km, forcing the roe herring fishery to close (Funk 1994). Although there is evidence to support a theory of over-population (Pearson *et al.* 1999), most believe exposure to oil in sensitive breeding grounds was the leading cause of the population crash. This has been supported by lab-scale tests showing the toxicity of crude oil to P. herring embryos in Prince William Sound (Paine *et al.* 1996) and correlations have been made between areas of oil contamination in the Sound and the prevalence of deformed herring embryos (Hose *et al.* 1996).

Clupea sp. are very important economically and ecologically, as integral components of commercial fisheries and local food webs. Herring stocks have declined in many areas from natural and anthropogenic factors. The Cherry Point stock, for example, was once the largest Pacific herring stock in Washington State (mid-1970s), but has since declined precipitously to less than ten percent of the original spawning biomass (Dinnel *et al.* 2002). To promote recovery of the population, anthropogenic influences need to be eliminated, including sources of pollution that are a leading cause of fish stock declines. Herring, for example, are known to be especially sensitive to oil exposure (Carls *et al.* 1999).

Should an oil spill occur, chemical dispersants can be sprayed onto the oil slick to decrease the negative effects of the spill more quickly. Dispersants reduce the interfacial tension between the oil and water, allowing the oil to break into small droplets, which can then be 'dispersed' by mechanical and natural means. While decreasing the surface area of the slick, dispersants increase the amount of oil in the water column, the surface-to-volume ratio of droplets, the partitioning to water of the toxic constituents of oil, and the bioavailability of oil to pelagic and benthic organisms. The water accommodated fraction (WAF) is composed of the portion of petroleum hydrocarbons, including polycyclic aromatic hydrocarbons (PAH), that partition into the water from an oil slick that has been naturally dispersed by wave action and currents. The chemically-enhanced water accommodated fraction (CEWAF) has a higher concentration of these constituents as a result of the application of chemical dispersant, which decreases the size of oil droplets and increases the partitioning of oil into water. Considering it is the dissolved PAH fraction that is the most acutely toxic constituent of oil, an increase in solubility would effectively increase the exposure and effects on fish (Heintz *et al.* 1999).

Exposure of fish embryos to PAH can induce a chronic, non-contagious syndrome known as blue sac disease (BSD) and may be a result of oxidative damage and membrane destabilization (Cantrell *et al.* 1996). Signs of BSD could include pericardial edema, yolk sac edema, spinal curvature, fin rot, or craniofacial malformations. Edemas are the most sensitive signs of toxicity in herring embryos and can reduce cardiac output, impede circulation, and negatively affect swimming ability, often resulting in death

(Carls *et al.* 1999). Additionally, spinal curvatures can vary in severity, inhibiting swimming ability, which would reduce the capacity to forage for food and to escape predators.

Highly weathered oil can induce lethal and sublethal effects in P. herring embryos at concentrations as low as 0.7 µg/L total PAH (tPAH; lowest observed effective concentration; Carls *et al.* 1999). This is lower than concentrations observed following the *Exxon Valdez* Oil Spill, where tPAH in open seawater was measured at 6.24 µg/L directly following the spill, and persisted at 1.59 µg/L for at least five weeks thereafter (Short and Harris 1996). Additionally, dissolved PAH were found to a depth of 25 m in Prince William Sound and ranged from 0.9 to 6.2 µg/L near heavily oiled beaches immediately following the spill (Short and Harris 1996).

Because of the *Exxon Valdez* and *Cosco Busan* spills, most literature has reported studies of P. herring and few researchers have attempted to make correlations with Atlantic herring (A. herring; *Clupea harengus*). On the eastern seaboard of North America, A. herring spawn in areas frequented by tanker traffic transporting oil products and in areas where oil exploration has become prevalent. This increases the probability that a major spill event will occur in A. herring habitat. The abundant literature on P. herring could strengthen assessments of the risk to A. herring of oil toxicity if the response of the two species to oil is similar. Since a direct comparison between the species has not been done, the purpose of this research was to compare the toxicity of chemically dispersed crude oil to A. herring and P. herring to determine if risk

assessment decisions can be made on A. herring based on available literature regarding oil toxicity to P. herring.

3.3 Methods

3.3.1 Test Species

"Ripe and running", stage VI herring were acquired from roe herring fisheries based in Escuminac, NB (A. herring) and in Nanaimo, BC (P. herring). Fish selection and transportation procedures followed those outlined in Section 2.3.2, except that P. herring were shipped to Queen's University for experiments.

3.3.2 Experimental Setup

Experimental setup was identical to section 2.3.3. Eggs were spread onto glass slides and fertilized in milt solution, then transferred to glass Mason jars for exposure. Embryos were raised at 10C (±1C) from fertilization to hatch.

3.3.3 Test Solutions

Test solutions were prepared using the CROSERF laboratory-preparation method (Singer *et al.* 2000) outlined in section 2.3.4 and a dispersant to oil ratio of 1:10 was used for preparing oil dispersed with Corexit 9500A. Crude oils tested included Alaska North Slope (ANSC; viscosity of 17.5cP), Medium South American (MESA; viscosity of 26.1cP), and Arabian Light (ALC; viscosity of 15.5cP). Oils were weathered to achieve a 15% loss of MESA, by weight, a 10% loss of ANSC, by weight, and a 7% loss of ALC,

by weight (King 2011). Quality control exposures included a negative water control and a position control made from retene, a C-4 alkyl-phenanthrene.

Water for exposure solutions was maintained at a salinity of 15 g/L and prepared using Kent Sea Salt (Kent Marine Inc., Georgia, USA) and dechlorinated freshwater. Freshwater used for the P. herring experiments was drawn from Lake Ontario and treated by the city of Kingston, ON, while freshwater for the A. herring experiments was drawn from Dartmouth, NS municipal water.

3.3.4 Design

The basic experimental design followed that outlined in section 2.3.5. Slides of herring eggs were exposed to WAF and CEWAF from fertilization to hatch with daily static renewal in glass Mason jars. Jars were cleaned and solutions were renewed on a daily basis. Embryos remained attached to the glass slides until hatch (approximately 14 days for A. herring and 21 days for P. herring). At 5 days post-fertilization (dpf), unfertilized eggs were removed from the slides and the number of fertilized eggs was counted. Dead embryos were removed every other day and recorded until hatch. Pathologies associated with BSD were measured at hatch and included pericardial edema, yolk sac edema, spinal curvature, fin rot, and craniofacial deformation.

3.3.5 Water Chemistry

Water samples were drawn from exposure solutions to quantify the concentration of hydrocarbons by fluorescence and by Gas Chromatography-Mass Spectrometry as outlined in section 2.3.6. Two different centrifuges were used for the experiments to

remove salt from samples. Samples generated from the P. herring experiment were centrifuged at 10,621 x g, while those from the A. herring experiments were centrifuged at 9,055 x g. Water samples generated from the P. herring experiment were analyzed for total fluorescence using a RF-5301PC scanning spectrofluorometer (Shimadzu Corp., Kyoto, Japan; Panorama Fluorescence 1.1 Software, LabCognition, Dortmond, Germany) , while those from the A. herring experiments were analyzed using a Quanta-Master Fluorescence Spectrometer and Felix Software (Photon Technology International Ltd., London, ON, Canada). The excitation wavelengths were 295 nm for MESA, 278/293 nm for ANSC, and 278 nm for ALC, and the emission range was 300- 450 nm for all oils.

3.3.6 Statistics

Nonlinear regressions and median lethal (LC50) and effective (EC50) concentrations were estimated using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA) and GraphPad Prism 5.00 (GraphPad Software, San Diego, CA) statistical packages. To calculate LC50s, lower constraints for percent mortality were set at the negative control (A. herring = 7.69%; P. herring = 8.69%) for the corresponding experiments and upper constraints were set at 100% to represent the maximum possible response. EC50s were calculated for percent normal, percent hatch and BSD Severity Index. The upper constraints for percent normal (A. herring = 80.77%; P. herring = 78.26%) and hatch (A. herring = 96.15%; P. herring = 95.65%) were set at the corresponding negative controls and the lower constraints were zero. The BSD Severity Index is a normalized value based on the sum of the BSD scores for each fish (including mortalities) and averaged

within treatments. Lower constraints were set at the response level of the negative controls (A. herring and P. herring = 0.11) and the upper constraints at the maximum possible score of 1.0, where all embryos would be dead. Statistical comparisons were performed as outlined in section 3.3.6.

3.4 Results

The concentration of petroleum hydrocarbons measured by fluorescence for exposures of MESA and ANSC, WAF and CEWAF, increased linearly with the volume of oil added (Figure 3-1). There was approximately a 100-fold increase in the concentration of fluorescing petroleum hydrocarbons in the water as a result of chemical dispersion (F-test, $P < 0.001$). The slopes of MESA and ANSC CEWAF were statistically parallel (F-test, $P = 0.25$), and there was no difference in soluble fluorescent hydrocarbon concentrations in CEWAFs of the two oils (F-test, $P = 0.09$). Although, the slopes of MESA and ANSC WAF were not parallel (F-test, $P = 0.02$), the y-values at log fluorescence of 1.0 were not significantly different (F-test, $P = 0.45$).

The hydrocarbon concentrations deduced from GC-FID and spectrofluorescence formed a linear relationship with log-transformed data (Figure 3-1). The relationships for MESA and ANSC formed linear functions with equations $y = 1.09x – 0.61$ and $y = 1.29x – 0.84$, respectively. The lines were parallel (F-test, $P = 0.36$) and when data were combined graphically, they shared a slope of 1.17. The lines are steeper than a 1:1 line and would intersect the 1:1 line at about 100 mg/L. Additionally, the y-values at log fluorescence of 1.0 were not significantly different (F-test, $P = 0.22$). Total petroleum

hydrocarbon (TPH) concentrations were estimated from the linear log-log relationship presented in Figure 3-1 (Table 3-1). Because of the deviation from the 1:1 line, fluorescence in relation to TPH would be increasingly inaccurate with decreasing concentration, meaning the greatest uncertainty would be at the threshold of toxicity.

Hatched embryos of both species showed signs of BSD, including spinal curvature, pericardial edema, yolk sac edema, fin rot, and craniofacial malformation. Severe spinal curvatures often accompanied edema and resulted in an inability to swim in a directed fashion. The frequency and severity of BSD observed in hatched embryos increased with increasing concentration and exposure time. The mean response of negative controls was: % Mortality = 8.2 ± 0.99%; % Normal = 80 ± 1.7%; % Hatch = 96 ± 0.35%; BSD Severity Index = 0.11 ± 0.

Atlantic herring embryos appear to be about 4 times more sensitive to ANSC exposure than P. herring embryos for most parameters examined (Figure 3-2). For mortality, the confidence limits for the A. herring LC50 for ANSC were too wide to estimate, so a test of significance between the LC50s of the two species was not possible. Alternatively, there was a significant difference between the EC50s of percent normal ($EC50_{\%Normal}$; F-test, $P < 0.001$) and the BSD Severity Index ($EC50_{SI}$; F-test, $P < 0.001$) for the two species, but the EC50s for percent hatch ($EC50_{\%Hatch}$) were not significantly different (F-test, $P = 0.57$).

MESA exposure resulted in exposure-related toxicity to P. herring embryos (Figure 3-3). MESA appeared to be two times more toxic than ANSC to P. herring

embryos; however the confidence intervals of all parameters overlapped and the relationships were not significantly different.

3.5 Discussion

Hatched embryos exhibited signs of BSD, including yolk sac edema, pericardial edema, spinal curvature, fin rot, and craniofacial malformations, which are known to correlate with exposure to PAH (Hodson *et al.* 2007). A 100-fold increase in concentration of fluorescing petroleum hydrocarbons resulted from chemical dispersion of the crude oils tested, and the concentrations did not differ between MESA and ANSC oils at equal nominal loadings. Exposures of ANSC appeared four times more toxic to A. herring than P. herring, and P. herring responded similarly to ANSC and MESA exposures. Alternatively, ANSC appeared two times more toxic to A. herring embryos than ALC.

A comparison of hydrocarbon concentrations in exposure solutions deduced from GC-FID and fluorescence demonstrated a linear relationship (Figure 3-1). Fluorescence was chosen to monitor the bioassays because a large number of samples required processing, the analysis and equipment are relatively simple, and samples could be processed on-site at a lower cost. To quantify hydrocarbon concentrations, a standard curve was prepared from whole oil for comparison to unknowns, and the wavelengths targeted were specific to phenanthrenes (three-ringed PAH), which are abundant in oil, toxic to early life stages of fish (Turcotte *et al.* 2011), and identified as one of the most likely groups of PAH responsible for the toxicity of oil (Heintz *et al.* 1999; Hodson *et al.*

2007). The results indicate that while fluorescence measurements can be used to predict total hydrocarbon concentrations that would be attained by the much more costly and time consuming GC-FID method, there will be a bias towards over-stating toxicity at low concentrations. The underestimate in concentration is likely due to the relative solubilities of the components of oil because we are assuming that the compounds targeted by fluorescence will vary in proportion to the other components of oil (high molecular weight PAH and resins, waxes, and asphaltenes). In actual fact, the compounds measured by fluorescence using a standard curve of whole oil may not be representative of the soluble compounds present in CEWAF, which could explain why the comparison between fluorescence and GC-FID does not follow the 1:1 line (Figure 3-1b). When preparing a standard curve of whole oil, all compounds in oil are forced into solution using hexanes, but CEWAF is only composed of the compounds that are soluble in water.

There was no significant difference between the GC-FID – fluorescence relationships for MESA and ANSC oils, implying that the quantity of fluorescing petroleum hydrocarbons (e.g. phenanthrenes) within the solutions were present in similar proportions (Appendix A). Additionally, there was no significant difference between the two oils with regards to toxicity to P. herring embryos. One can conclude that the potencies of compounds causing BSD in P. herring embryos were similar in ANSC and MESA oils.

ANSC appeared four times more toxic to A. herring than to P. herring. It is plausible that the difference between the two species is an artifact of variability among females and egg quality (refer to Chapter 2). Should this be the case, the similarity between the two species is favourable for risk assessment, as there have been abundant data compiled on the risks of oil exposure to P. herring. If A. herring are in fact more susceptible to oil exposure, it would be best to focus on data from A. herring to ensure that limits on PAH exposure are set low enough to protect both species. Sweezey (2005) found the 14 day LC50 for A. herring exposed to MESA to be 1.02 mg/L (WAF and CEWAF) of fluorescing hydrocarbons and the 14 day EC50 (% Normal) to be 0.15 mg/L (based solely on WAF). In comparison with our data (Table 3-1), MESA appears to be about two times more toxic to A. herring embryos than P. herring. Lacking confidence limits, we cannot determine if these results are significantly different. However, the results fall just slightly outside of our confidence limits (Table 3-1), indicating that the two species would not be statistically different.

In addition, the comparison of toxicity to A. herring embryos demonstrated that ANSC is two times more toxic than ALC (refer to Chapter 2). Assuming the aromatic portion of oil is primarily responsible for toxicity, the chemical characteristics of the oils reflect the difference in toxicity, since ANSC has two times more aromatics per gram of oil than ALC (Appendix A). Although MESA appears to be two times more toxic than ANSC to P. herring, the relationship is not significant and the two oils have about the same percentage of aromatics, indicating that they have similar toxicity.

Fish are most sensitive to oil exposure during periods of embryonic development, particularly immediately following fertilization and hatch (Linden 1975; McIntosh *et al.* 2010). More specifically, herring embryos appear to be most sensitive during the blastula and gastrula stages of development, which occur within 48 h of fertilization (Linden 1975). As a result, we exposed embryos from fertilization to estimate the maximum potential toxicity.

Unfortunately, fertilization success and egg quality were not always ideal, resulting in numerous failed experiments from poor or negligible fertilization of eggs. Additionally, egg quality and exposure response among and within females were highly variable. Experiments can be affected by the quality of gonads, including age, condition, collection methods, transportation and storage time, and ripeness (Dinnel *et al.* 2002). In fact, fertilization success among females can range from 0-100% and is significantly different among sampling sites (Dinnel *et al.* 2002) and year-class (Kocan *et al.* 1996). Additionally, the percentage of normal embryos in a negative control treatment can range from 0-100% (Dinnel *et al.* 2002; Purcell *et al.* 1990). With this in mind, treatment replicates were discarded if there were fewer than five fertilized eggs and toxicity was normalized to the negative control. If percent normal of negative controls for a replicate was less than 75%, the entire replicate was discarded. Despite high variability among spawning sites, hydrocarbon concentrations present immediately following the *Exxon Valdez* spill were high enough to cause toxicity (Hose *et al.* 1996). Because of the nature of the experiments and the difficulty with egg fertilization, data pertaining to Atlantic

herring are scarce and valuable. An alternative to obtaining fish from roe herring fisheries would be to catch live adults and ripen them in the lab, which can produce consistent fertilization success (J. Rice, NOAA, Juneau, AK, personal communication).

Much of the toxicity to herring embryos during the *Exxon Valdez* Oil Spill was likely a result of photooxidation (Carls *et al.* 1999). It has been well documented that UV light increases the toxicity of PAH to herring embryos and other fish (Barron *et al.* 2004; Carls *et al.* 1999). In this experiment, the interaction between UV light and toxicity was avoided by raising the embryos in a dark environment with less than two hours per day exposure to very dim light during embryo examination and maintenance. Nevertheless, a comparison of UV-toxicity is an important research need.

Future research must include additional replicates to compile a sufficient database for better estimates of toxicity. Additional experiments will also increase the statistical power with which to make concrete conclusions about toxicity with regards to herring species and oils. It is recommended that additional oils be tested. One should also examine the differences in toxicity among exposure times (See Chapter 2). Since A. herring may be more sensitive to oil exposure, further experiments should be done to better understand the relationships among the *Clupea* species and to determine the variability among females and egg quality.

3.6 Conclusion

Chemical dispersion of oil increases the concentration of petroleum hydrocarbons in the water by 100-fold, which increases the risk to herring embryos should a spill be

dispersed over a spawning bed during spawning season. ANSC appears to be four times more toxic to A. herring embryos than to P. herring embryos, while P. herring respond similarly to ANSC and MESA, as indicated by the aromatic compositions of the oils and the lack of statistical differences among LC50s and EC50s. Alternatively, ANSC has two times more aromatics than ALC and appears to be two times more toxic to A. herring embryos. Further testing is required to determine if Atlantic herring are indeed more sensitive to oil exposure, but the sensitivity to oil exposures of the two species appear to be sufficiently similar that the data available can be used for risk assessment for both species.

3.7 Acknowledgements

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3.9 Figures

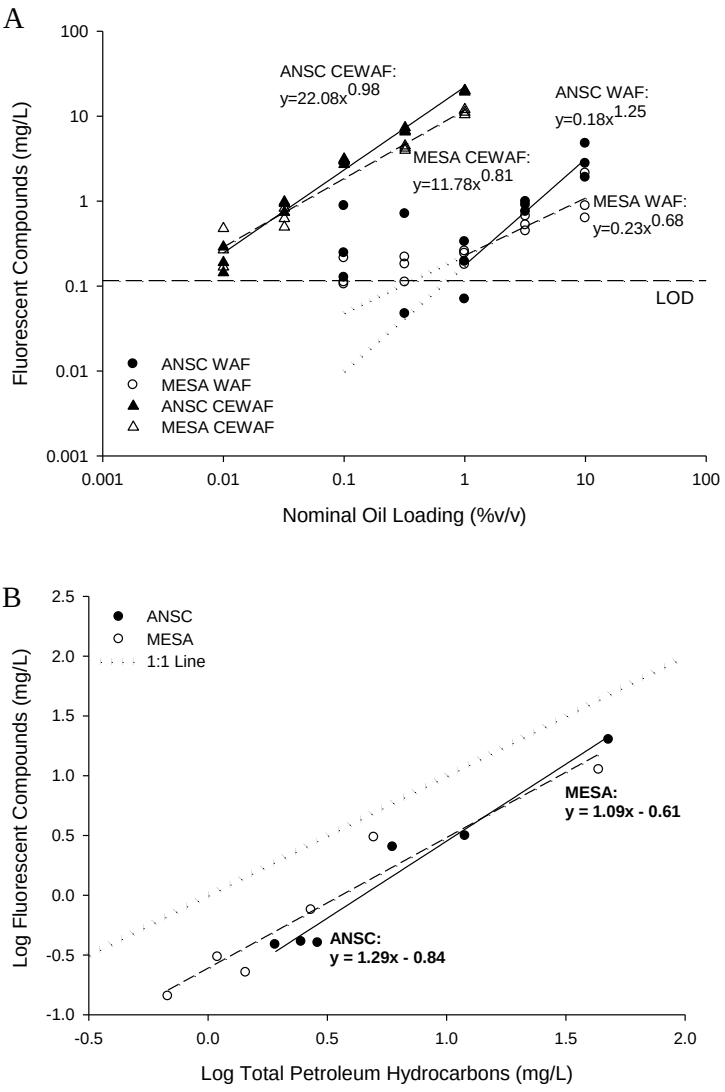

Figure 3-1: The concentration of petroleum hydrocarbons in test solutions at different loadings of the water accommodated fraction (WAF) and the chemically-enhanced water accommodated fraction (CEWAF) of Alaska North Slope Crude (ANSC; WAF $R^2 = 0.87$; CEWAF $R^2 = 0.98$) and Medium South American Crude (MESA: WAF $R^2 = 0.79$; CEWAF $R^2 = 0.95$) oils (A). The dotted line shows the extrapolated concentration from the line of best fit below the limit of detection (LOD). The data points at Nominal Oil Loadings of 0.32 and 0.1%, which are at or below the LOD have been shown for comparison. The lower panel (B) shows the relationship between fluorescent hydrocarbon compounds measured by spectrofluorescence and total petroleum hydrocarbons measured by GC-FID for CEWAF of ANSC ($R^2 = 0.96$) and MESA ($R^2 = 0.93$). The dotted line is a 1:1 line added for comparison.

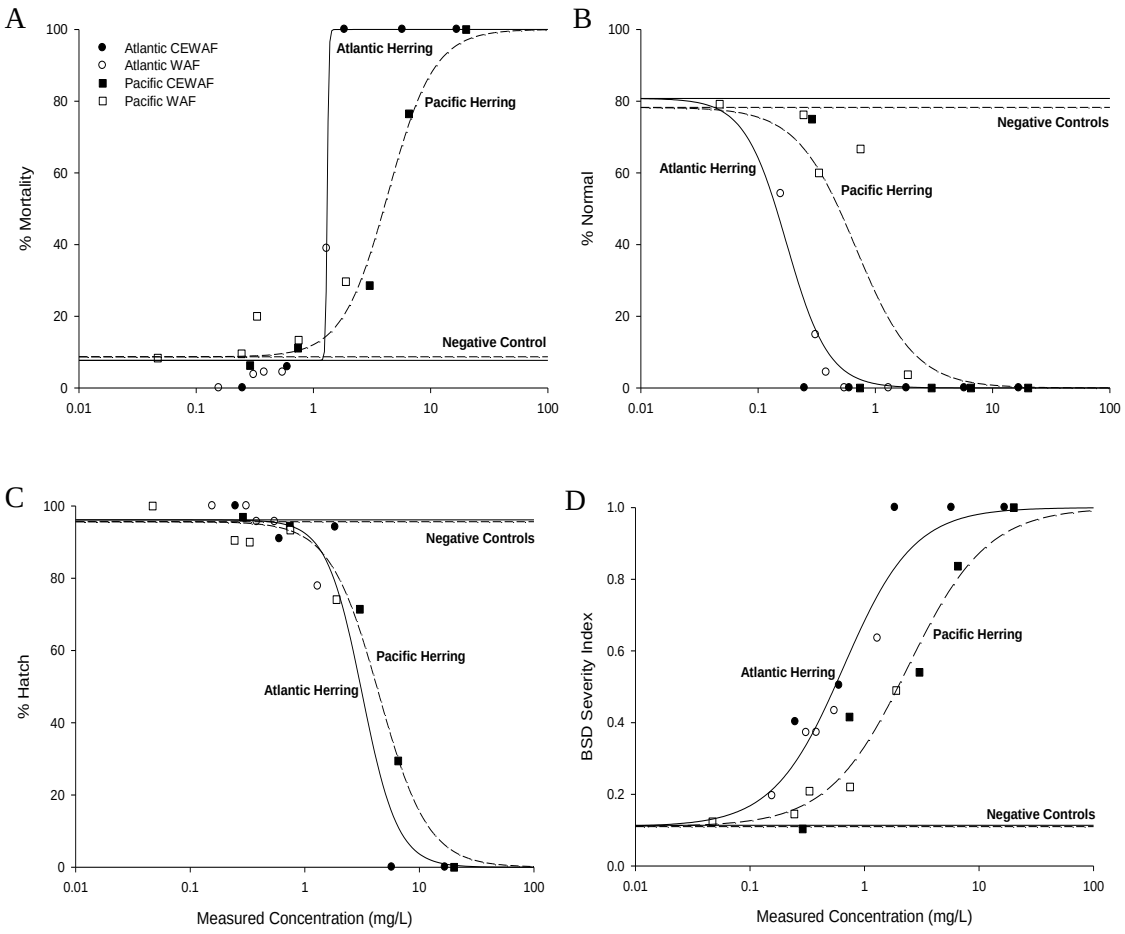

Figure 3-2: Toxicity of the water accommodated fraction (WAF) and chemically-enhanced water accommodated fraction (CEWAF) of Alaska North Slope Crude (ANSC) to Atlantic and Pacific herring embryos, expressed as (A) % Mortality, (B) % Normal, (C) % Hatch, and (D) BSD Severity Index in relation to the measured concentration of fluorescing compounds (mg/L). Regression analysis was done by grouping the WAF and CEWAF data and equations can be found in Appendix G. The Atlantic herring data were imported from Figure 2-3. N = 20 embryos for each exposure.

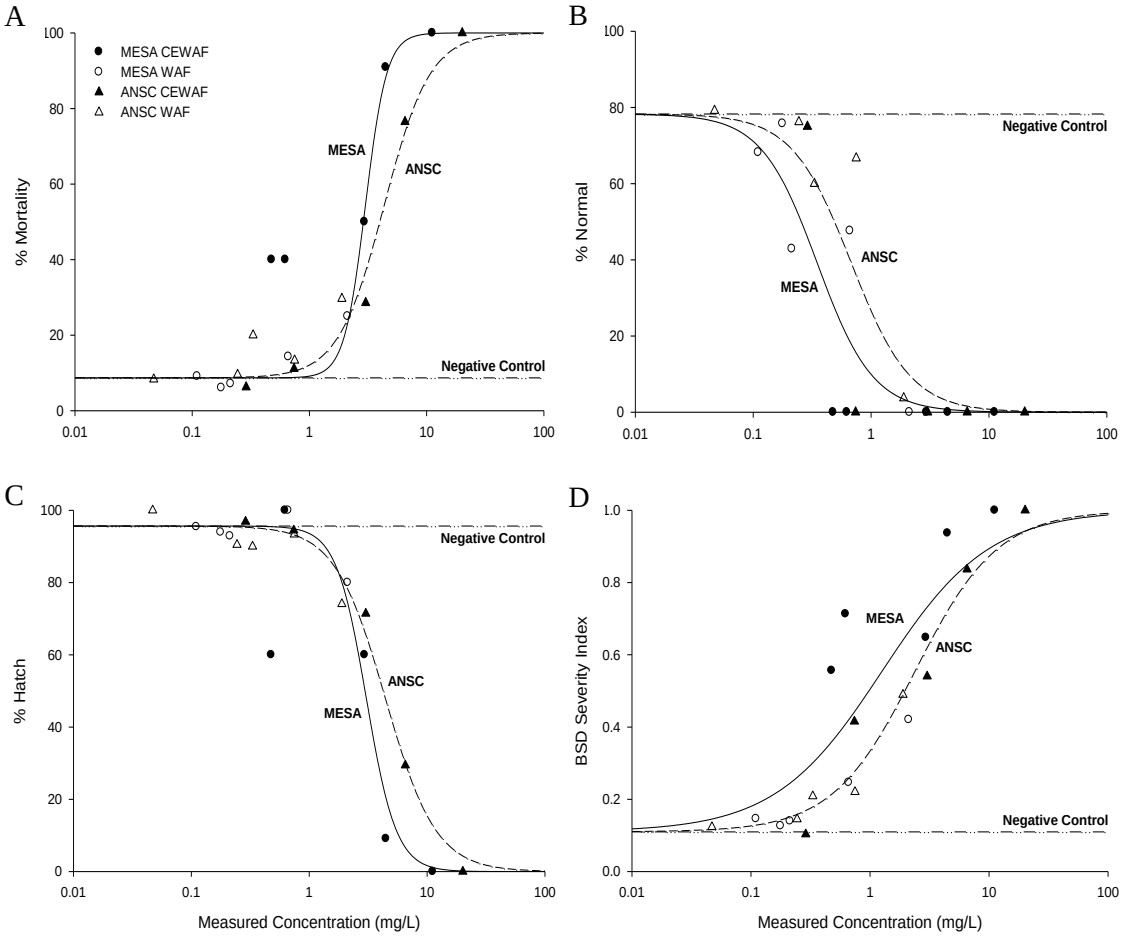

Figure 3-3: Toxicity of the water accommodated fraction (WAF) and chemically-enhanced water accommodated fraction (CEWAF) of Alaska North Slope Crude (ANSC) and Medium South American Crude (MESA) oils to Pacific herring embryos, expressed as (A) % Mortality, (B) % Normal, (C) % Hatch, and (D) BSD Severity Index in relation to the measured concentration of fluorescing compounds (mg/L). Regression analysis was done by grouping the WAF and CEWAF data and equations can be found in Appendix G. The ANSC data were imported from Figure 3-2. N = 20 embryos for each exposure.

Table 3-1: The toxicity of Medium South American Crude (MESA), Alaska North Slope Crude (ANSC), and Arabian Light Crude (ALC) oils to Atlantic and Pacific herring embryos, expressed as median lethal concentrations (LC50s; % Mortality) and median effective concentrations (EC50s; % Normal, % Hatch, BSD Severity Index). Values are based on measured concentrations of fluorescing petroleum hydrocarbons (FL) and total petroleum hydrocarbons (TPH) estimated from Figure 1, and shown with 95% confidence intervals (CI).

		Atlantic Herring (LC50s and EC50s in mg/L)					
		MESA		ANSC		ALC	
		FL	TPH	FL	TPH	FL	TPH
% Mortality	LC50	-	-	1.32	5.50	2.29	-
	CI	-	-	Wide	Wide	Wide	-
% Normal	EC50	-	-	0.17	1.14	0.37	-
	CI	-	-	0.15-0.19	1.03-1.24	0.24-0.57	-
% Hatch	EC50	-	-	2.99	10.5	2.29	-
	CI	-	-	1.51-5.93	5.89-17.8	Wide	-
BSD	EC50	-	-	0.66	3.24	2.07	-
	CI	-	-	0.48-0.89	2.51-4.08	1.01-4.24	-
		Pacific Herring (LC50s and EC50s in mg/L)					
		MESA		ANSC		ALC	
		FL	TPH	FL	TPH	FL	TPH
% Mortality	LC50	2.53	8.51	4.32	13.8	-	-
	CI	1.32-4.82	4.68-15.5	3.49-5.42	11.7-16.6	-	-
% Normal	EC50	0.29	1.16	0.66	3.24	-	-
	CI	0.17-0.50	0.71-1.86	0.40-1.08	2.19-4.79	-	-
% Hatch	EC50	3.15	10.5	4.45	14.5	-	-
	CI	2.55-3.89	8.91-12.6	3.75-5.28	11.0-16.2	-	-
BSD	EC50	1.27	4.47	2.40	8.91	-	-
	CI	0.52-3.10	2.00-10.3	1.74-3.31	6.92-11.2	-	-

Chapter 4

General Discussion and Summary

4.1 Overview

Significant declines in Pacific herring (*Clupea pallasi*) populations have followed two major oil spill events in the United States, the *Exxon Valdez* oil spill and the *Cosco Busan* oil spill. As a result, many studies have been performed and documented in the literature to assess the risk to Pacific herring of oil exposure. In contrast, tanker transportation routes throughout the Northwestern Atlantic Ocean coincide with Atlantic herring (*Clupea harengus*) spawning grounds, but little research has been done to study the risk of oil exposure to Atlantic herring.

Polycyclic aromatic hydrocarbons (PAH), as major components of oil, are toxic to early life stages of fish and can cause blue sac disease (BSD; Billiard *et al.* 1999; Khan 2007). Chemical dispersion of crude oil increases the concentration of hydrocarbons, including PAH in the water column by 100-fold, which increases their bioavailability to early life stages of fish. Previous research on Atlantic herring embryos indicates that chemically dispersed crude oil is indeed more toxic than un-dispersed oil (McIntosh *et al.* 2010; Sweezey 2005). McIntosh *et al.* (2010) determined that fertilization was severely hindered when gametes were exposed to oil, and that herring were most sensitive to oil immediately following fertilization and hatch. Additionally, effects can be seen after exposures to chemically dispersed crude oil of only one hour (McIntosh *et al.* 2010), and Sweezey (2005) observed BSD in Atlantic herring embryos at concentrations as low as

0.12 mg/L of fluorescing compounds (14 d exposure; measured by fluorescence). As a result of these studies, it was determined that there is a research need for toxicity data obtained from experiments using more realistic exposure scenarios and times, and a comparison made between Atlantic and Pacific herring.

The main objective of this thesis was to determine if laboratory experiments are suitable alternatives for assessing risk of oil exposure to fish compared to an *in situ* field experiment at sea. This was done by dispersing oil in a model ecosystem (wave tank) and comparing the observed toxicity to parallel laboratory experiments. Additionally, dispersant effectiveness testing has shown that petroleum hydrocarbons are maintained in the water column after a real spill for a few hours to a few days, but not to the extent of a chronic laboratory toxicity test from fertilization to hatch (Li *et al.* 2009). As a result, we tested the effects of exposure times of 2.4, 8, 24 h, and 14 d post-fertilization. This thesis also measured the chronic toxicity of chemically dispersed Medium South American (MESA), Alaska North Slope (ANSC) and Arabian Light (ALC) crude oils to Pacific and Atlantic herring embryos to provide a greater knowledge base for risk assessment in the event of an oil spill off Canada's east coast.

The toxicities of two crude oils (ANSC and ALC) chemically dispersed in a wave tank to Atlantic herring embryos were compared to the toxicity of the chemically-enhanced water accommodated fraction (CEWAF) generated by the standard CROSERF lab method (Singer *et al.* 2000). The concentrations of petroleum hydrocarbons produced from ALC dispersed in the wave tank were lower than those produced from the lab

method and as a result, toxicity was not observed. On the other hand, toxicity was observed from ANSC dispersed in the wave tank. In fact, solutions sampled from the wave tank 15 minutes post-dispersion caused a greater toxicity than lab-prepared CEWAF, indicating that the oil droplets were releasing sufficient hydrocarbons to be toxic to the herring embryos. Other exposures from the wave tank appeared to follow the toxicity generated by the lab method. Thus, lab estimates of dispersed oil toxicity should not differ significantly from estimates of the toxicity of dispersed oil sampled from a model ecosystem (wave tank) or the receiving water of an oil spill.

The results of the exposure time experiments indicate that toxicity is dependent upon both concentration and exposure time, which corresponds to results found by McIntosh *et al.* (2010) and Rice *et al.* (1987). Toxicity of ANSC CEWAF increased linearly with exposure time, while toxicity of ALC CEWAF appeared to reach a maximum after 8 h. This suggests that by 8 h, the bioavailable hydrocarbons in the ALC CEWAF exposures had been taken up by the embryos, but toxicity to ANSC CEWAF continued beyond 8 h. Most importantly, toxicity can be observed at exposure times that are more likely to be observed following a real oil spill.

Chronic toxicity testing showed that Atlantic herring embryos were two times more sensitive to exposures of ANSC than ALC, and there was no difference in toxicity to Pacific herring embryos between ANSC and MESA exposures. The observed difference in toxicity corresponded with the chemical composition of the oils, specifically the concentration of PAH, i.e., ANSC and MESA oils have two times more aromatics than ALC. In addition, ANSC and MESA have two times more alkyl-phenanthrenes than

ALC, which are a component of PAH and known to be highly toxic to early life stages of fish (Barron *et al.* 2004; Turcotte *et al.* 2011). These data highlight a connection between the aromatic composition of oil and its toxicity.

To compare Atlantic and Pacific herring, embryos of both species were exposed to ANSC. Atlantic herring appeared to be four times more sensitive to oil exposure than Pacific herring embryos, as determined by the percentage of normal embryos at hatch and the incidence of BSD. The sensitivity 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. However, negative control data were virtually identical between the two experiments. The results indicate that chemical dispersion of oil increases the risk to herring embryos and exposure limits for risk assessment need to be set conservatively to protect the two species because variability in egg quality increases uncertainty in estimates of toxicity.

4.2 Significance of Findings and Future Work

This research was implemented in response to a need for more detailed toxicological information on the effects of chemical dispersants on the toxicity of spilled oil. The wave tank studies also have direct relevance to research on wave tank conditions that affect the extent of oil dispersion. Externally, the results are linked to current research by the US EPA on methods for generating, testing, and characterizing dispersed oil. The next step would be to extend these tests in the wave tank and then in the field with increasing degrees of realism of exposure conditions. For example, herring embryos

can be tested directly in the wave tank by suspending glass slides coated with eggs within the tank, or suspended in cages within the trajectory of an oil spill in the field. To expand our knowledge, different oils and species could also be tested.

A direct comparison of Atlantic and Pacific herring has not been made before, and the difference in sensitivity is sufficiently small and variable that the two species could be considered to have essentially the same sensitivity to oil. This significantly increases the capacity to predict chemical effects for models of oil spread and ecological impacts, and for ecological risk assessments where data are often sparse and not suited for specific questions. 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).

Herring are a multi-million dollar (USD) resource along the Eastern and Western coasts of North America (Brown and Carls 1998), and are more at risk of oil exposure than some other species because the eggs are sessile when laid and unable to avoid a spill (Smith and Cameron 1979). Additionally, spawning often occurs in shallow areas where oil is more likely to reach (Haegele and Schwiegert 1985; Smith and Cameron 1979). Spawning seasons vary with latitude and species (Brown and Carls 1998), with Atlantic herring spawning primarily in the Spring and Fall, and Pacific herring in the Winter and Spring (Haegele and Schweigert 1985). Incubation time is dependent on temperature, but Atlantic herring hatch approximately 14 d post-fertilization and Pacific herring approximately 21 d, which would place herring spawning beds at risk for at least three weeks. Additionally, the window of sensitivity is highly dependent on spill location, as

herring spawn could be present from February to October, and oil exposure could be very detrimental to both the economy and the future of the species.

Most importantly, toxicity can be observed at concentrations and exposure times relevant to real spills (Short and Harris 1996; Carls *et al.* 1999) and medium crude oils have a higher potential for toxicity when compared to light oils. As found by Carls *et al.* (1999), toxicity to Pacific herring embryos is evident at tPAH concentrations up to 15 times lower than those observed following the *Exxon Valdez* oil spill (Short and Harris 1996) and we have determined that Atlantic herring respond similarly to oil exposure. Following the *Prestige oil spill*, Gonzalez *et al.* (2006) observed tPAH concentrations up to 5.80 µg/L in the water column, which too is above that indicated by Carls *et al.* (1999) to be toxic to Pacific herring embryos. This suggests that PAH concentrations following real oil spills would be high enough to cause toxicity to Atlantic herring embryos in the event of a large spill on the East Coast of Canada.

4.3 Summary

1. Chemical dispersion of oil increased the concentration of hydrocarbons in the water column 100-fold, and ALC had more soluble fluorescent hydrocarbons than ANSC in both CEWAF and WAF.
2. There was no difference in toxicity to Pacific herring embryos of two chemically-dispersed medium crude oils (MESA and ANSC) and ANSC was two times more toxic to Atlantic herring embryos than ALC, a light crude oil.

3. These results provide a linkage between toxicity and chemical composition of oil, as the medium oils contain equal quantities of aromatic compounds, including phenanthrenes, and the light oil contains two times fewer aromatics.
4. Toxicity to Atlantic herring embryos increased with exposure time to chemically-dispersed ANSC.
5. Toxicity of chemically-dispersed ALC to Atlantic herring embryos reached a maximum at an exposure duration of 8 h, but toxicity increased when exposure solutions were renewed.
6. In exposure time experiments, toxicity was endpoint dependent and % Normal was the most sensitive response to oil exposure.
7. Lab estimates of dispersed oil toxicity should not differ from estimates of the toxicity of dispersed oil sampled from a model ecosystem (wave tank).
8. Atlantic herring appeared to be four times more sensitive to ANSC than Pacific herring embryos.

4.4 References

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Appendix A

Physical and Chemical Characteristics of Oils

Table A-1: Physical and chemical characterization of Medium South American Crude (MESA), Alaska North Slope Crude (ANSC), and Arabian Light Crude (ALC) oils. Oils were weathered by evaporation and sparging with air to simulate loss at sea. Density and viscosity were measured after weathering. The aromatics include the polycyclic aromatic hydrocarbons (PAHs). Adapted from King, 2011[1].

Oils	Type	% Weathered by weight	Density (g/mL)	Viscosity (20ºC, cStk)	% Alkanes	% Aromatics	% Resins	% Asphal-tenes
MESA	Medium	15	0.8802	26.1	34.2	36.5	20.8	8.5
ANSC	Medium	10	0.8607	17.5	32.0	39.3	24.4	4.3
ALC	Light	7	0.8691	15.5	32.7	18.9	46.9	1.5

[1] King, T. 2011. Do surface films negatively bias the effect of oil spill chemical dispersants in a wave tank test facility? MSc. thesis, Saint Mary's University, Halifax, NS.

Table A-2: Chemical characterization by GC-MS of Medium South American Crude (MESA), Alaska North Slope Crude (ANSC), and Arabian Light Crude (ALC) oils. Values are percentages of the polycyclic aromatic hydrocarbon (PAH) composition, including thiophenes. Total PAH (tPAH) is a sum of the non-alkylated PAH, alkylated PAH, and thiophenes. Concentrations below the method detection limits were treated as 0.

	Composition of PAH Fraction (%)		
Compound Group	**MESA**	**ANSC**	**ALC**
Naphthalenes	54.07	44.52	25.14
Fluorenes	3.85	4.37	4.21
Anthracene	0.00	0.00	0.06
Phenanthrenes	16.42	15.99	8.48
Fluoranthene	0.00	0.06	0
Pyrenes	3.22	4.46	2.33
Chrysenes	3.30	5.32	1.46
5–ring PAH	0.62	0.84	0
Dibenzothiophenes	13.47	16.37	29.57
Naphthobenzothiophene	5.06	8.07	28.74
Σ Non-alkylated PAH	**8.38**	**9.76**	**1.89**
Σ Alkylated PAH	**74.41**	**67.79**	**41.35**
Σ Thiophenes	**18.53**	**24.44**	**58.31**

Table A-3: Raw data of chemical characterization by GC-MS of Medium South American Crude (MESA), Alaska North Slope Crude (ANSC) and Arabian Light Crude (ALC) oils.

Compound	MESA (µg/g)	ANSC (µg/g)	ALC (µg/g)
n-decane	2028.3	2005.5	4251.8
undecane	2818.0	2339.7	4729.5
dodecane	3420.7	2657.1	4681.7
tridecane	3891.9	3022.5	4485.1
tetradecane	4474.1	3425.0	4516.2
pentadecane	4422.6	3443.7	4217.1
hexadecane	3967.1	3098.7	3351.0
heptadecane	4048.6	3237.4	3421.4
octadecane	3377.2	2759.4	3131.8
nonadecane	3101.3	2620.0	2919.7
eicosane	2738.9	2324.6	2356.5
heneicosane	2610.0	2299.9	2240.7
docosane	2475.0	2220.1	1903.5
tricosane	1589.2	2222.0	1685.1
tetracosane	2136.7	1972.0	1604.8
pentacosane	2044.1	1861.6	1339.6
hexacosane	1989.7	1771.3	1190.5
heptacosane	1857.5	1464.9	945.7
octacosane	1634.7	1245.7	771.8
n-nonacosane	1726.2	1134.1	620.9
tricontane	1195.6	795.1	439.1
n-heneicontane	1151.0	661.1	327.0
dotriacontane	882.2	536.8	203.1
tritriacontane	918.3	549.6	146.3
tetratriacontane	901.9	641.6	104.9
n-pentatriacontane	907.6	708.4	71.6
17beta(H), 21 alpha (H)-	7.4	7.3	7.0

hopane			
naphthalene	477.5	367.6	134.4
methylnaphthalene	1539.2	1015.5	462.3
dimethylnaphthalene	2152.7	1392.9	938.5
trimethylnaphthalene	1648.4	1166.8	1086.5
tetramethylnaphthalene	804.2	722.2	599.7
acenphene	35.8	15.6	<0.025
acenaphthalene	20.6	15.9	<0.025
fluorene	143.0	117.0	26.0
methylfluorene	243.9	234.2	85.1
dimethylfluorene	57.7	55.8	160.7
trimethylfluorene	31.3	53.9	267.7
dibenzothiophene	163.0	210.5	197.1
methyldibenzothiophene	369.7	377.4	673.9
dimethyldibenzothiophene	513.7	490.4	1213.7
trimethyldibenzothiophene	386.8	400.3	1079.0
tetramethyldibenzothiophene	230.0	248.1	624.6
phenanthrene	268.1	304.9	60.4
anthracene	0.0	0.0	8.3
methylphenanthrene	514.1	158.5	217.3
dimethylphenanthrene	556.8	558.5	323.4
trimethylphenanthrene	444.1	395.0	279.7
tetramethylphenanthrene	244.3	270.3	205.7
fluoranthene	0.0	6.7	<0.025
pyrene	13.8	12.0	<0.025
methylpyrene	50.6	57.1	29.4
trimethylpyrene	116.5	174.3	97.1
tetramethylpyrene	113.4	131.3	105.5
dimethylpyrene	103.0	95.4	67.1
naphthobenzothiophene	28.0	41.1	31.6

methylnaphthobenzothiophene	117.5	257.9	174.0
dimethylNBenzothiophene	473.0	532.5	2910.3
tetramethylNbenzothiophene	6.7	9.1	248.5
trimethylNbenzothiophene	0.0	10.7	318.1
benz[a]anthracene	47.8	52.8	<0.025
chrysene	0.0	102.0	12.6
methylchrysene	80.7	85.5	24.9
dimethylchrysene	119.8	182.4	31.5
trimethylchrysene	126.3	116.8	62.1
tetramethylchrysene	80.4	75.0	56.5
benzo[b]fluoranthene	7.3	9.7	<0.025
benzo[k]fluoranthene	0.0	0.0	<0.025
benzo[e]pyrene	10.6	13.1	<0.025
benzo[a]pyrene	3.2	3.5	<0.025
perylene	0.0	0.0	<0.025
indeno[1,2,3-cd]pyrene	0.0	0.0	<0.025
dibenz[a,h]anthracene	4.0	4.2	<0.025
benzo[ghi]perylene	3.6	5.2	<0.025

Appendix B

Standard Operating Procedure – Laboratory Preparation of WAF and CEWAF

Prepared by: Colleen Greer and Peter Hodson©

1. This SOP follows the methods outlined by Singer *et al.* 2000
    a. Singer, M.M., Aurand, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L., and Tjeerdema, R.S. 2000. Standardization of the Preparation and Quantitation of Water-Accommodated Fractions of Petroleum for Toxicity Testing. Mar. Pollut. Bull 40(11): 1007-1016.

2. Set-up
    a. Add 90mL of exposure water to a 250 mL beaker with 1 cm stir bar
        i. The same procedure can be done with a side-arm flask with vertical sides, similar in shape (and surface area) to the beaker. The side-arm should be positioned at the bottom of the flask, and the tubing should NOT be silicone or rubber. A Teflon stopcock and glass tubing are ideal.
    b. Let the test oil reach room temperature then add 10mL of oil to water.
    c. The water-oil mixture should be 1 part oil: 9 parts water.
3. Stirring
    a. Place beaker onto a stir plate.
    b. Cover with tin foil or a glass plate.

c. Start stirring and adjust the speed so that the vortex formed in the oil is 1/3 the height of the water.

d. Stir for 18h at this speed.

4. WAF (Water Accommodated Fraction):

    a. After 18h, stop stirring and let the 2 phase system sit for 1h so that oil droplets (if any) coalesce at the surface.

    b. After 1h, carefully decant the WAF, i.e., the bottom phase of the mixture, being careful to avoid any droplets of oil. The WAF should be virtually clear and colorless without suspended droplets or floating oil. There will be a significant amount of oil left floating on the surface of the 2-phase system, about the same volume as was added.

        i. WAF can be decanted with a glass syringe with polyethylene tubing attached to the tip. Draw a small amount of air into the syringe, insert the tubing through the oil, expel the air, and draw the CEWAF solution into the syringe.

        ii. Where a side-arm flask is used, the stopcock is opened carefully and the WAF drained into a glass beaker.

    c. Dilute the WAF solution at the desired concentration (nominal oil loading %v/v) into test solutions as soon as possible after recovering the WAF stock solution,

5. CEWAF (Chemically Enhanced WAF):

a. After 18h of stirring, add 1mL of dispersant (1 part dispersant: 10 parts Oil; or alter for the required dispersant to oil ratio) (Another common ratio is 1:20).
b. Continue stirring for 1h
c. After 1h, stop stirring and let sit for 1h
d. After 1h, carefully decant off the bottom phase of the mixture as described for WAF. CEWAF is usually turbid and yellow to brown in colour with few visible droplets of oil (most are too tiny to see) and no floating oil. After dispersion, there will be less oil left on the surface of the stirred oil-water 2-phase system than there was with WAF.

6. Clean-up
    a. Consumables (pipette tips, syringes, tubing) should be disposed of in the appropriate oily waste receptacle.
    b. Dispose of leftover oil, WAF, and CEWAF into the appropriate liquid waste receptacle.
    c. Rinse glassware with hexane to remove remaining oil stuck to the glass and dispose of the waste hexane in the solvent waste container
    d. Clean glassware with Sparkleen (or equivalent) and rinse thoroughly with distilled water.

Appendix C

Wave Tank

Figure C-1: Wave tank located at the Bedford Institute of Oceanography, Dartmouth, NS

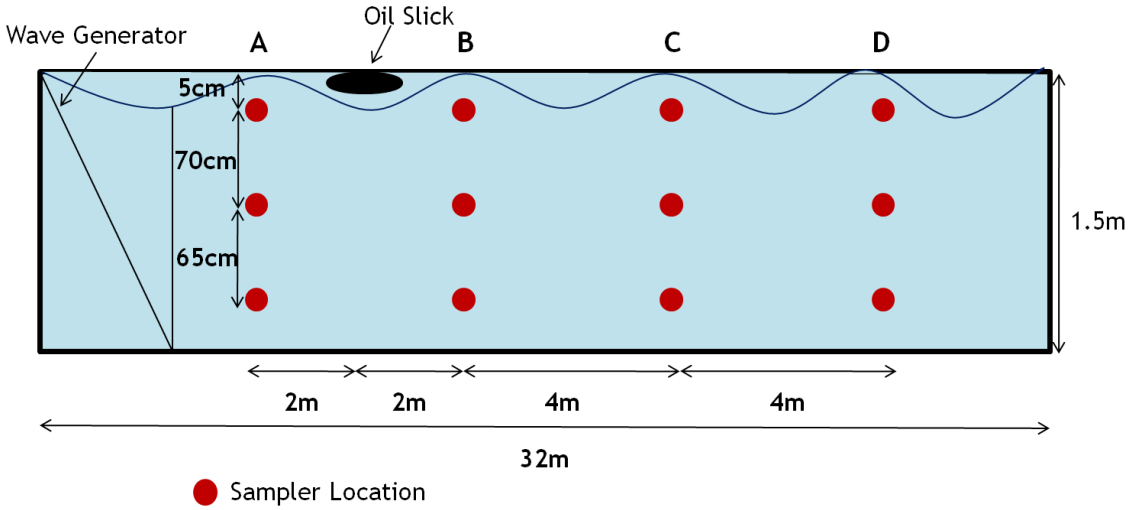

Figure C-2: Wave tank schematic showing sampling locations and depths. Water flow is from left to right.

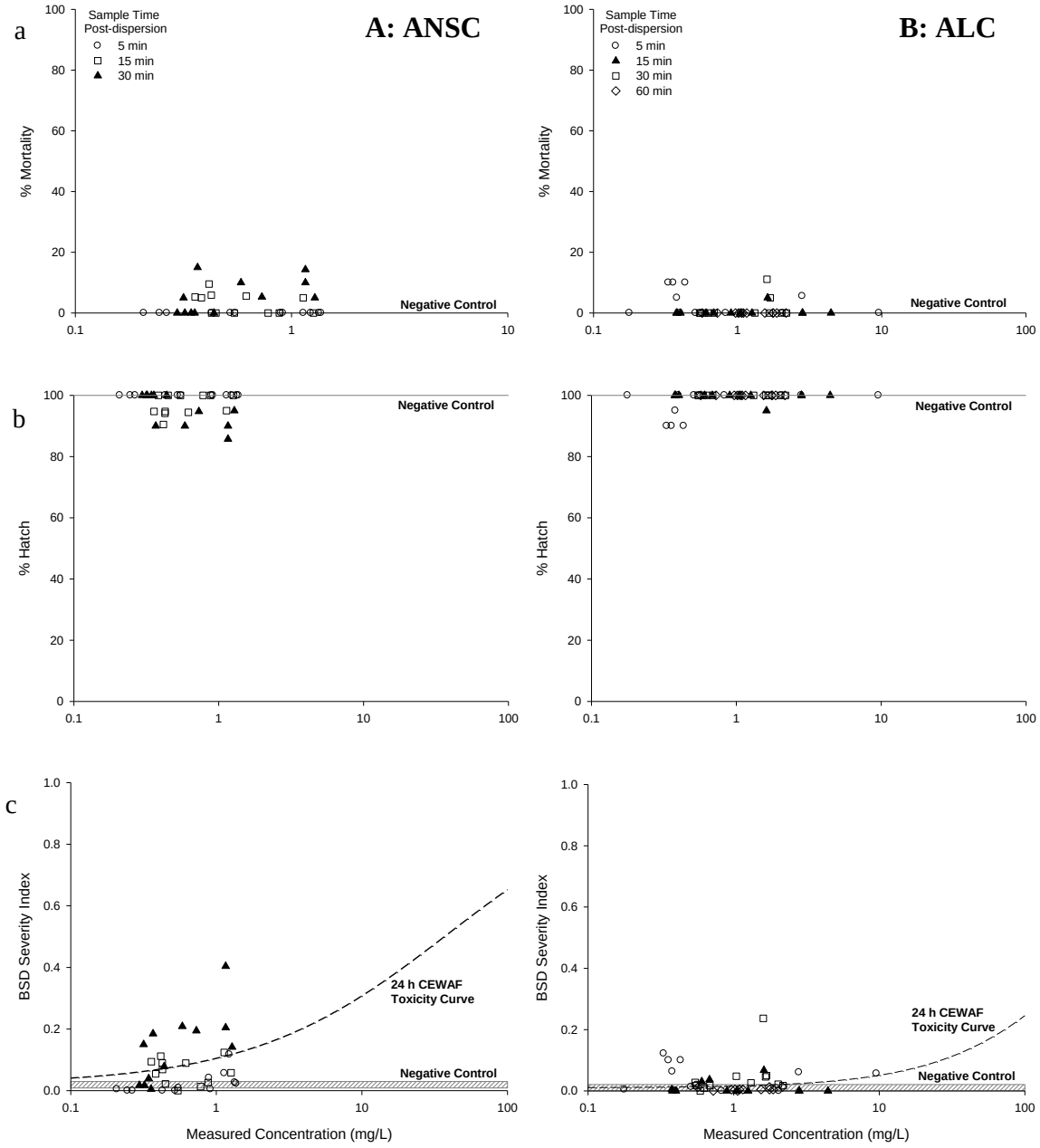

Figure C-3: Wave tank data corresponding to Chapter 2. Graphs show change in % Mortality (a), % Hatch (b), and the BSD Severity Index (c) with increasing concentration of the chemically-enhanced water accommodated fraction of Alaska North Slope Crude (ANSC; Panel A) and Arabian Light Crude (ALC; Panel B) oils. The effects on Atlantic herring of a 24 h exposure of laboratory-prepared CEWAF (dash lines) have been superimposed onto the graphs for comparison when the response was greater than control. N = 20 for each treatment.

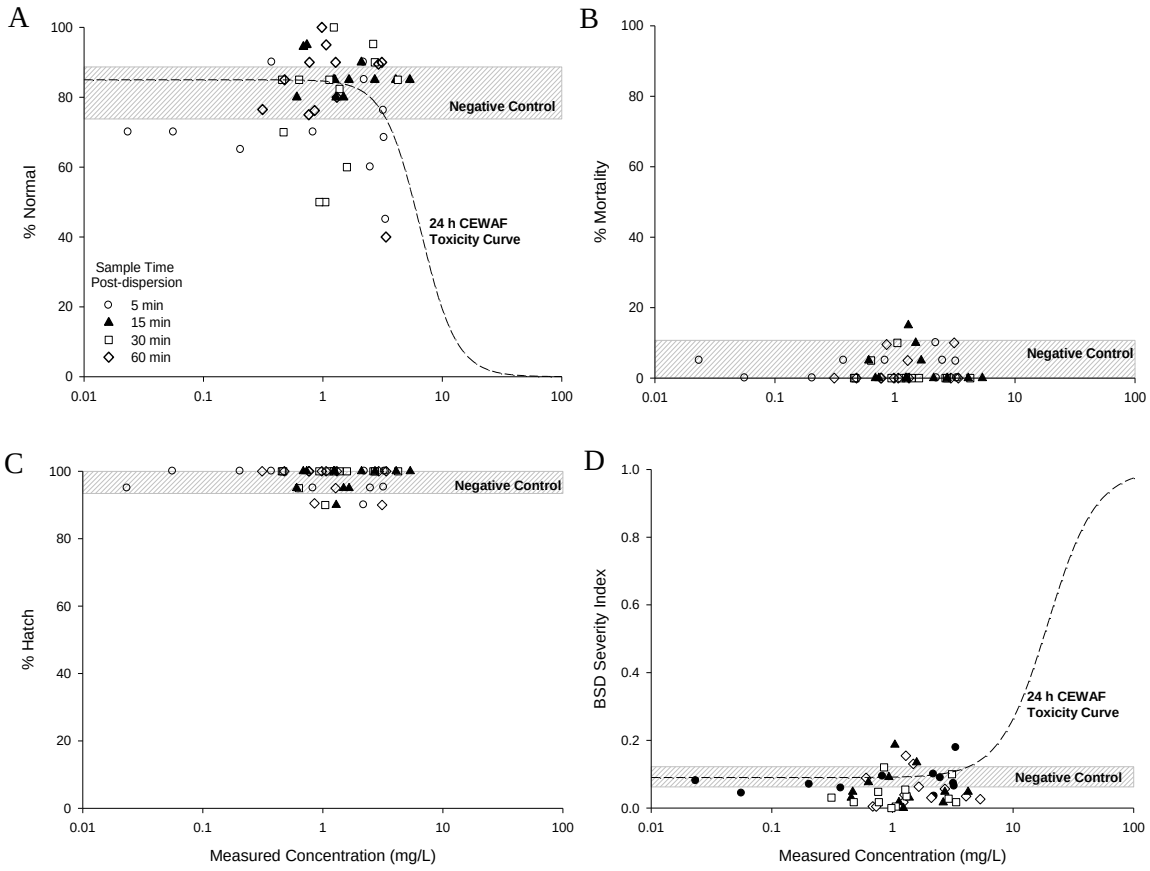

Figure C-4: Supplementary wave tank data corresponding to Chapter 2. Graphs show change in % Normal (A), % Mortality (B), % Hatch (C), and the BSD Severity Index (D) with increasing concentration of the chemically-enhanced water accommodated fraction (CEWAF) of Alaska North Slope Crude (ANSC) oil. The effects on Atlantic herring of a 24 h exposure of laboratory-prepared CEWAF (dash lines) have been superimposed onto the graphs for comparison when the response was greater than control. N = 20 for each treatment.

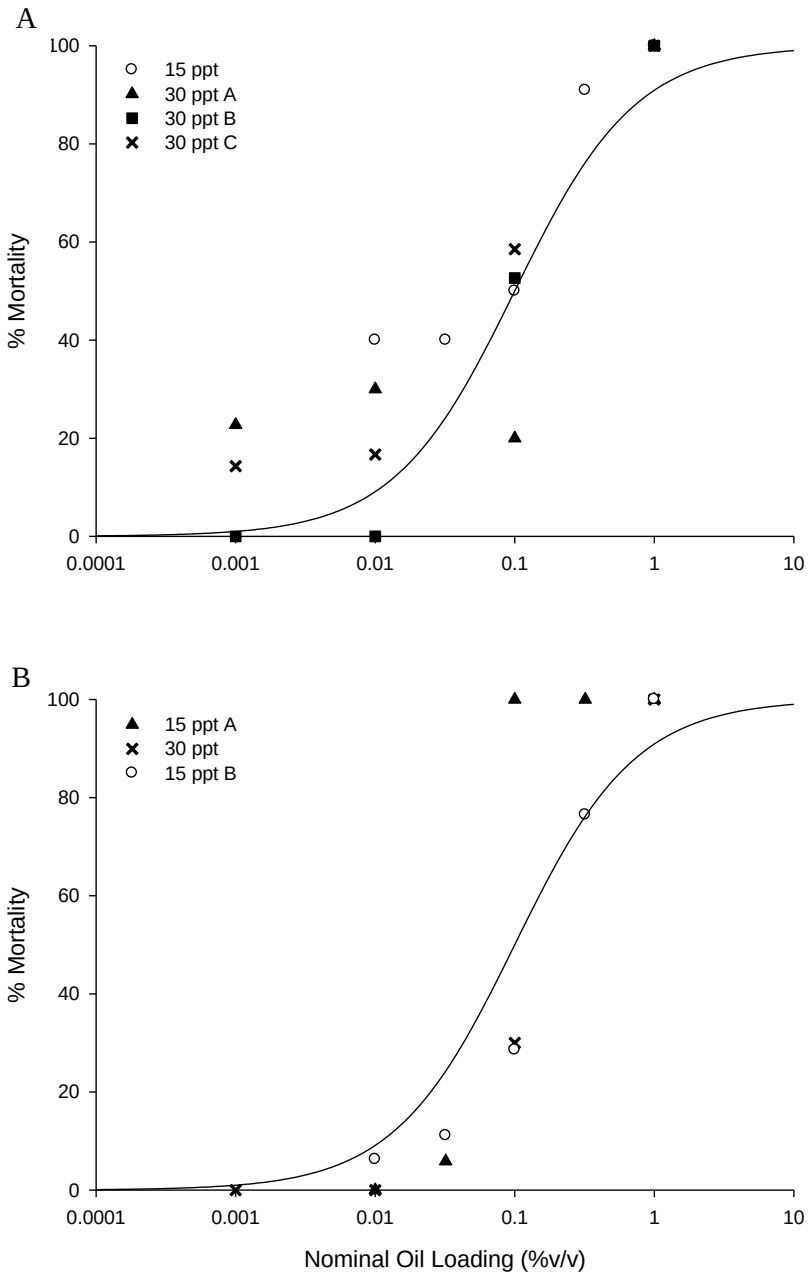

Figure C-5: Preliminary laboratory experiments to assess the acute toxicity of CEWAF to herring embryos under different salinities (15 and 30 ppt) of Medium South American Crude (MESA; A) and Alaska North Slope Crude (ANSC; B) chemically-enhanced water accommodated fractions (CEWAF). Due to water sample contamination, measured concentrations were unavailable. Non-linear regressions include all replicates. There did not appear to be a difference in oil toxicity between salinities.

Appendix D

Standard Operating Procedure – Analysis of Saline Water Samples Containing Oil by Fluorescence

Prepared by: Colleen Greer and Peter Hodson©

1. Draw water samples from exposure solutions and add to an equal volume of absolute ethanol (un-denatured) in a scintillation vial. Vials with teflon-lined caps are best for preserving sample volume, but foil-lined caps are acceptable if samples will be stored for less than one month. Vial volume should be selected according to sample volume to minimize headspace. A minimum of 3mL of water sample is required for analysis. Make sure caps are tightened well on vials. Store vials in fridge until ready to analyze.
2. Prepare and run a standard curve for the oil to be analyzed to determine the excitation and emission wavelengths, and the concentration of PAH in the water samples. Standards should be prepared using a serial dilution as outlined below. Water used to prepare standards should be from the same source as the water used for the exposure solutions. Sonicate for 3 minutes between each step.
    a. 1:9 oil-hexanes (stock)
        i. 100µL of oil by weight
        ii. ~900µL hexanes
    b. 100µL of stock + 9.9mL absolute ethanol (1000ppm)
    c. 1mL of 1000ppm solution + 4.5mL ethanol + 4.5mL water (100ppm)

d. 1mL of 100ppm solution + 4.5mL ethanol + 4.5mL water (10ppm)

e. 1mL of 10ppm solution + 4.5mL ethanol + 4.5mL water (1ppm)

f. 0.5mL of 10ppm solution + 4.75mL ethanol + 4.75mL water (500ppb)

g. 1mL of 1ppm solution + 4.5mL ethanol + 4.5mL water (100ppb)

h. 0.5mL of 1ppm solution + 4.75mL ethanol + 4.75mL water (50ppb)

i. 1mL of 100ppb solution + 4.5mL ethanol + 4.5mL water (10ppb)

j. 1mL of 10ppb solution + 4.5mL ethanol + 4.5mL water (1ppb)

3. Sonicate standard to be run for 3 minutes.
4. If standards are prepared in salt water, after sonication step transfer sample to microcentrifuge tubes. If using 1.5mL microcentrifuge tubes, two tubes will be require for each sample. Spin samples at 10,000 rpm for 10 minutes. A salt pellet will be visible at the bottom of the tube. Remove the sample from the tube while avoiding the pellet and transfer the sample to a quartz cuvette.
5. Place cuvette into fluorometer and analyze. Start with the 1ppm sample (or the highest concentration standard to be analyzed) and optimize the emission spectrum using an excitation scan to determine the wavelength resulting in the highest peak.
6. Run the rest of the standards following the optimal specifications determined in step 5.
7. Calculate the area under the curve (either using the integrate area function specific to the equipment or by summing the peak heights over the desired bandwidth).

8. Using the areas and the known concentrations, plot the values to determine a standard curve for the oil. If the resulting regression has an R^2 value above 0.95, the standards are deemed correct. Prepare additional standards if necessary. Using the regression equation, concentrations of unknown samples can be determined using the area under the fluorescence curve.
9. Sonicate samples for 3 minutes.
10. If samples are in salt water, centrifuge (as outlined for standards) for 10 minutes at 10,000 rpm.
11. Analyze samples by fluorescence.
12. Calculate concentration using prepared standard curve. To determine the actual concentration of the samples, the value calculated from the standard curve will have to be multiplied by two to take into account the dilution factor from the addition of ethanol to preserve the samples.
13. Blanks should be run each day to account for any fluctuations in the bulb. The water-ethanol blank will act as a baseline or calibration for the standard curve and should be subtracted from the samples. The water-ethanol blank should be prepared using 1 part ethanol to 1 part water (drawn from the same source as the water samples). Separate water and ethanol blanks can also be run to determine if the equipment is functioning properly.
14. Between each sample, the cuvette should be cleaned. First rinse in distilled or de-ionized water then rinse twice using absolute ethanol.

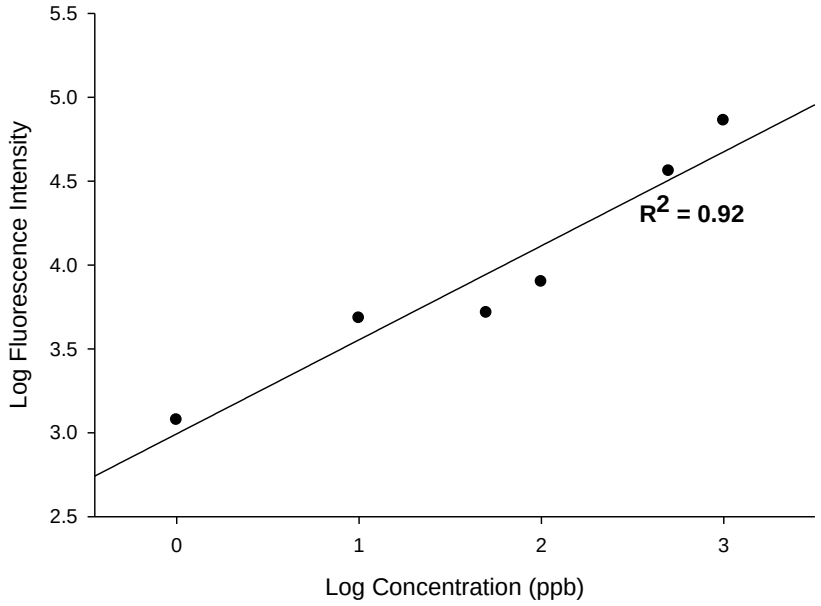

Figure D-1: Calibration curve of fluorescence methods using whole oil. Curve can be used to calculate concentration of exposure solutions from fluorescence intensity.

Appendix E

Photos of Hatched Atlantic Herring Embryos

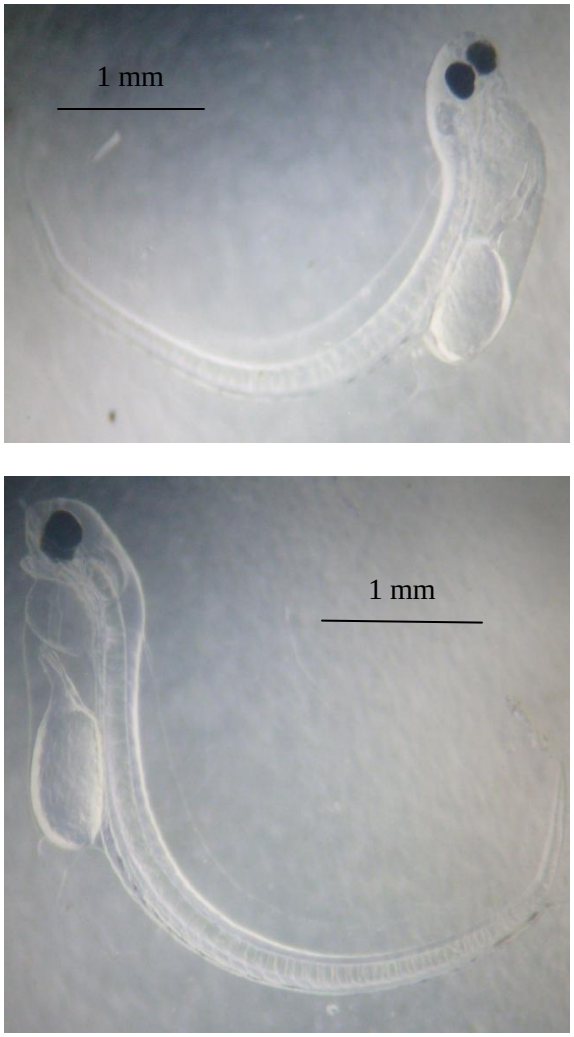

Figure E-1: Hatched Atlantic herring embryos exhibiting signs of blue sac disease from exposure to chemically dispersed crude oil.

Appendix F

Exposure Time

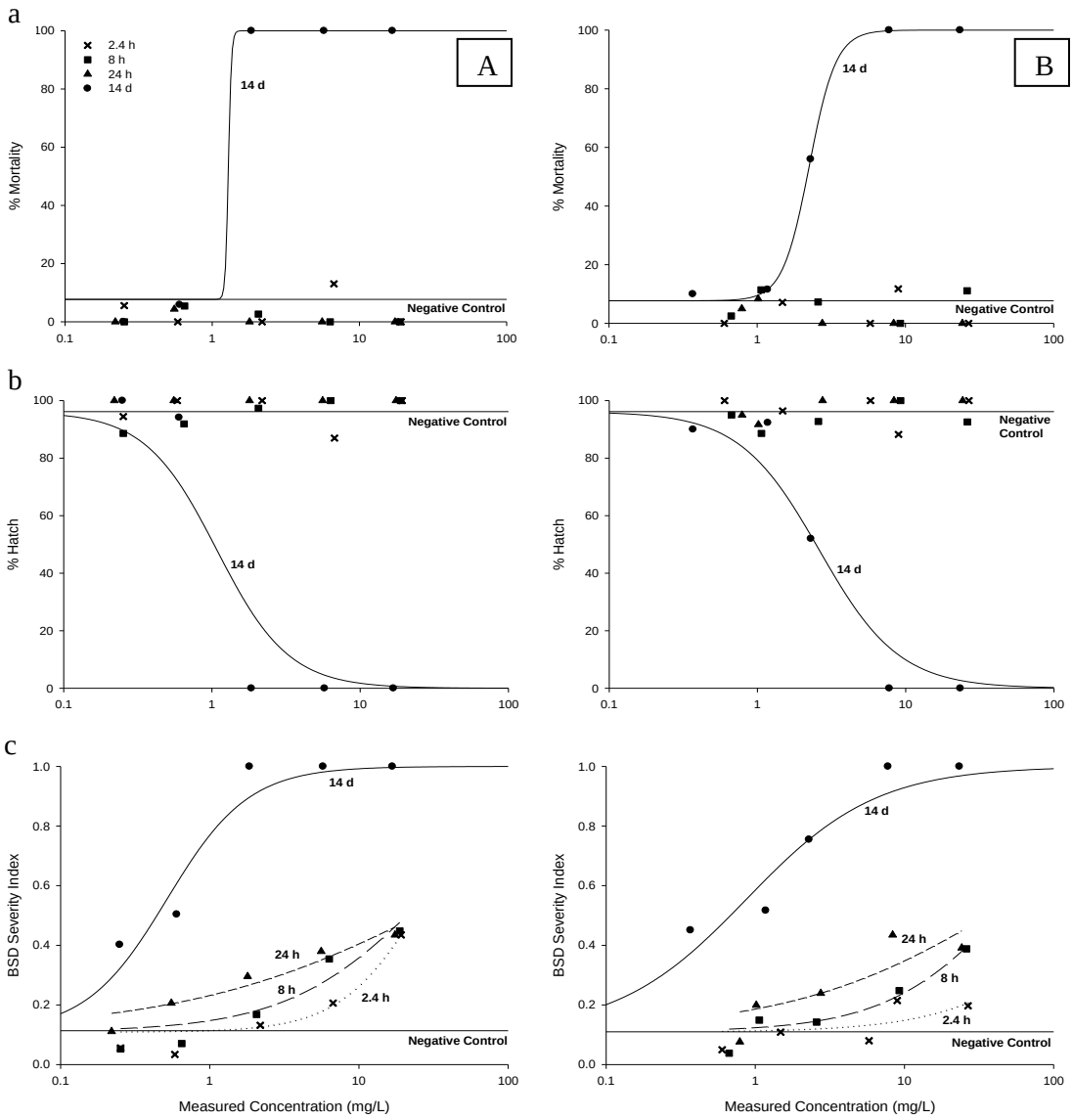

Figure F-1: Exposure time – response experiments from Chapter 2. The percent mortality (a), percent hatch (b), and BSD Severity Index (c) for Atlantic herring embryos exposed to Alaska North Slope Crude (ANSC, Panel A) and Arabian Light Crude (ALC, Panel B). The x's represent 2.4 h exposure, the squares represent 8 h exposure, the triangles represent 24 h exposure, and the circles represent a 14 day exposure from fertilization to hatch with daily static renewal of exposure solutions. Dose-response curves were included when applicable. N = 20.

Appendix G

Equations of Non-linear Regressions

Table G-1: Equations of Non-linear regressions from figures in Chapters 2 and 3. The oils tested were Arabian Light Crude (ALC), Alaska North Slope Crude (ANSC) and Medium South American Crude (MESA). The minimum and maximum are the constraints set on the regressions with respect to the negative controls and the maximum/minimum response as outlined in the Statistics section of their respective chapters. The median lethal concentrations (LC50) are specific for the % Mortality relationships and the median effective concentrations (EC50) are specific for the % Normal, % Hatch, and the BSD Severity Index relationships. Concentrations in mg/L are based on measured concentrations of fluorescing petroleum hydrocarbons and shown with 95% confidence intervals (CI). The Hillslope values were calculated by SigmaPlot 11.0 and shown with 95% confidence intervals (CI).

Figure	Figure Panel	Oil	Species	Endpoint	Minimum	Maximum	LC50/EC50 (mg/L)	CI (mg/L)	Hillslope	CI
2-3, 3-4	Upper Left	ALC	Atlantic	% Mortality	7.69	100	2.29	Very wide	282	Very wide
2-3, 3-4	Upper Left	ANSC	Atlantic	% Mortality	7.69	100	1.32	Very wide	48.2	Very wide
2-3, 3-4	Upper Right	ALC	Atlantic	% Normal	0	80.77	0.37	0.24-0.57	-2.53	(-9.5)-4.5

2-3, 3-4	Upper Right	ANSC	Atlantic	% Normal	0	80.77	0.17	0.15-0.19	-7.35	(-13.9)-(-0.77)
2-3, 3-4	Lower Left	ALC	Atlantic	% Hatch	0	96.15	2.29	Very wide	-226	Very wide
2-3, 3-4	Lower Left	ANSC	Atlantic	% Hatch	0	96.15	2.99	1.51-5.93	-5.92	(-13.8)-1.96
2-3, 3-4	Lower Right	ALC	Atlantic	BSD Severity Index	0.11	1.0	2.07	1.01-4.24	1.19	0.11-2.26
2-3, 3-4	Lower Right	ANSC	Atlantic	BSD Severity Index	0.11	1.0	0.66	0.48-0.89	1.42	0.77-2.06
2-4	Upper 2.4 h	ANSC	Atlantic	% Normal	0	80.77	11.74	5.32-25.9	-3.04	(-7.63)-1.56

2-4	Upper 8 h	ANSC	Atlantic	% Normal	0	80.77	3.16	2.36-4.23	-2.08	(-3.10)-(-1.06)
2-4	Upper 24 h	ANSC	Atlantic	% Normal	0	80.77	0.76	0.58-0.99	-1.54	(-2.08)-(-0.99)
2-4	Lower 2.4 h	ALC	Atlantic	% Normal	0	80.77	25.1	14.7-42.8	-1.35	(-2.32)-(-0.38)
2-4	Lower 8 h	ALC	Atlantic	% Normal	0	80.77	2.86	1.26-6.52	-2.11	(-5.43)-1.21
2-4	Lower 24 h	ALC	Atlantic	% Normal	0	80.77	2.75	1.01-7.47	-2.17	(-6.37)-2.04
2-5	Upper Right	ANSC	Atlantic	% Normal Toxicity Curve	0	89.82	4.85	3.50-6.71	-1.43	(-2.01)-(-0.84)

2-5	Lower Right	ALC	Atlantic	% Normal Toxicity Curve	0	93.33	>22.63	Very wide	-0.57	(-2.64)-1.51
3-2	Upper Left	ANSC	Atlantic	% Mortality	7.69	100	1.32	Very wide	48.2	Very wide
3-2	Upper Left	ANSC	Pacific	% Mortality	8.69	100	4.35	3.49-5.42	2.21	1.27-3.16
3-2	Upper Right	ANSC	Atlantic	% Normal	0	80.77	0.17	0.15-0.19	-7.35	(-13.9)-(-0.77)
3-2	Upper Right	ANSC	Pacific	% Normal	0	78.26	0.66	0.40-1.08	-2.61	(-5.91)-0.69
3-2	Lower Left	ANSC	Atlantic	% Hatch	0	96.15	2.99	1.51-5.93	-5.92	(-13.8)-1.96

3-2	Lower Left	ANSC	Pacific	% Hatch	0	95.65	4.45	3.75-5.28	-2.03	(-2.66)-(-1.40)
3-2	Lower Right	ANSC	Atlantic	BSD Severity Index	0.11	1.0	0.66	0.48-0.89	1.42	0.77-2.06
3-2	Lower Right	ANSC	Pacific	BSD Severity Index	0.11	1.0	2.40	1.74-3.31	1.25	0.80-1.70
3-3	Upper Left	MESA	Pacific	% Mortality	8.69	100	2.53	1.32-4.82	1.40	0.18-2.62
3-3	Upper Left	ANSC	Pacific	% Mortality	8.69	100	4.35	3.49-5.42	2.21	1.27-3.16
3-3	Upper Right	MESA	Pacific	% Normal	0	78.26	0.29	0.17-0.50	-2.30	(-4.58)-(-0.01)

3-3	Upper Right	ANSC	Pacific	% Normal	0	78.26	0.66	0.40-1.08	-2.61	(-5.91)-0.69
3-3	Lower Left	MESA	Pacific	% Hatch	0	95.65	3.15	2.55-3.89	-5.34	(-10.9)-0.22
3-3	Lower Left	ANSC	Pacific	% Hatch	0	95.65	4.45	3.75-5.28	-2.03	(-2.66)-(-1.40)
3-3	Lower Right	MESA	Pacific	BSD Severity Index	0.11	1.0	1.27	0.52-3.10	0.97	0.16-1.78
3-3	Lower Right	ANSC	Pacific	BSD Severity Index	0.11	1.0	2.40	1.74-3.31	1.25	0.80-1.70

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

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ABSTRACT

Juvenile Atlantic cod were exposed to either the water-accommodated fraction (WAF) or the chemically enhanced water-accommodated fraction (CEWAF) of Mediterranean South American (MESA), a medium grade crude oil at three different temperatures. Two concentrations of each mixture were tested, 0.2% and 1.0% (v/v) at 2, 7 and 10 °C. Corexit 9500 was the chemical dispersant tested. The liver enzyme ethoxyresorufin-*O*-deethylase (EROD) was measured during a 72-h exposure. The WAF of oil had significant ($P<0.05$) effect on enzyme activity compared to controls at only one sampling time: 48 h at 10 °C. Exposure of CEWAF of oil resulted in significantly ($P<0.05$) elevated EROD activity compared to controls. The level of EROD induction was temperature related with higher induction being observed in cod exposed to CEWAF at higher temperatures. Total polycyclic aromatic hydrocarbon (PAH) concentrations in exposure water were significantly higher in chemically dispersed mixtures. While PAH concentrations were lower in the 2 °C water compared to 7 or 10 °C (8.7 vs 11.9 μg mL^{-1}), the level of EROD induction was approximately 9 and 12 times lower at 2 °C compared to 7 or 10 °C, respectively, suggesting the metabolic rate of the cod plays a role in the enzyme response. These data suggest the risk of negative impacts associated with exposure to chemically dispersed oil may be affected by water temperature and that risk assessment of potential effects of WAF or CEWAF should consider the effects of water temperature on the physiology of the fish as well as the effectiveness of dispersants.

1. Introduction

Oil spills associated with marine transport and offshore production facilities often occur in the open sea. Birds, mammals and inter-tidal animals can be affected if the oil is allowed to move toward shorelines. Clean-up costs for shorelines are much higher than the cost of clean-up at sea. Chemical dispersants are used to disperse crude oils after spills and discharges in order to prevent coating of inshore areas inhabited by a variety of marine organisms (Khan and Payne, 2005). Dispersant effectiveness is influenced by sea energy, temperature, salinity and the nature of the crude oil (Ramachandran et al., 2004; Chandrasekar et al., 2005; NRC, 2005; Sorial et al., 2004). Dispersing oil markedly increases hydrocarbon concentrations in test solutions based on measured concentrations of polycyclic aromatic hydrocarbons (PAHs).

The risk to fish from oil and oil-dispersant treatments may be assessed in terms of exposure to PAHs and changes in exposure of PAHs after the use of dispersants (Ramachandran et al., 2006). PAH exposure can be estimated by a standardized laboratory assay of cytochrome P-450 (CYP1A) induction (Hodson et al., 1996). Mixed-function oxygenase (MFO) enzymes are a family of membrane-bound enzymes which increase the water solubility of aromatic and lipophilic compounds. The terminal oxidase enzyme of the MFO system is the iron-containing hemoprotein CYP1A. The CYP1A enzyme catalyzes the hydroxylation of PAH to a more soluble and excretable form in the bile. Assays of liver CYP1A activity provide a good biomarker of PAH exposure in fish (McCarty et al., 2002). Ethoxyresorufin-*O*-deethylase (EROD) is part of the family of cytochrome enzymes and is induced in the presence of xenobiotic compounds including PAHs (Hodson et al., 1991).

Fish within their natural environment are able to survive and remain active over a wide thermal range. A number of studies have reported compensatory changes of metabolic and physiological functions in fish responding to thermal fluctuations (Hazel and Prosser, 1974). It appears that environmental temperature also influences the level of various xenobiotic metabolizing enzyme activities in fish (Andersson and Koivusaari, 1985). Nahrgang et al. (2010) have reported that polar cod (*Boreogadus saida)* show differences in normal EROD activity relating to season. Abrahamson et al. (2008) exposed immature Atlantic cod (*Gadus morhua)* to crude oil in water for 24 hours at 2 and

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8 °C and measured EROD activity in gill filaments. The cod were acclimated for one week from 8 °C (rearing temperature) to 2 °C. EROD was significantly induced in a concentration-dependent manner at both temperatures. The same nominal concentrations of crude oil caused significantly higher induction at 2 °C compared to 8 °C. PAHs and other readily metabolized AhR (aryl hydrocarbon receptors) agonists may undergo first-pass metabolism in the gills and other tissues following absorption from water. Previous experiments have shown that rainbow trout (*Oncorhynchus mykiss)* injected with beta-naphthoflavone (BNF) may have elevated hepatic P450-dependent activity in decreasing temperatures but also that at low temperatures, the activity may be reduced or absent (review by Andersson and Förlin, 1992). Induction of P450 by BNF occurred in juvenile rainbow trout (*O. mykiss)* acclimated to both cold (5 °C) and warm (10 °C) water. EROD induction was about two times higher and the inductive response came faster in the warm acclimated fish than in the cold acclimated fish (Andersson and Koivusaari, 1985). Differences in water temperature and nutritional status of North Sea dab (*Limanda limanda*) between sampling locations obscured the effects of contamination with PCBs and PAHs on CYP1A levels (Sleiderink et al., 1995). Strong induction of CYP1A activity was seen at both 1 and 10 °C in Arctic char (*Salvelinus alpinus*) given an oral dose of benzo[α]pyrene. At 1 °C, the response was delayed and lasted longer than at 10 °C (Jørgensen and Wolkers, 1999). Polar cod (*B. saida*) kept at low temperatures (3–6 °C) and fed over 52 days with an artificial diet containing varying degrees of crude oil contamination and polar cod injected with crude oil and kept for 21 days at 0 °C showed induction of hepatic CYP1A (George et al., 1995).

Temperature is an environmental influence on Atlantic cod metabolism. Studies on metabolic rate of Atlantic cod have shown that both standard metabolic rate (SMR) and active metabolic rate (AMR) increase with temperature (Schurmann and Steffensen, 1997; Claireaux et al., 2000).

The objective of this study was to determine the effect of water temperature on hepatic CYP1A (EROD) activity in juvenile Atlantic cod exposed to the water accommodated fraction (WAF) and chemically enhanced (or dispersed) WAF (CEWAF) of crude oil.

2. Materials and methods

2.1. Fish stocks

Atlantic cod were hatched, raised and held at the St. Andrews Biological Station, St. Andrews, New Brunswick, Canada. Fish were held in sand-filtered seawater at ambient temperature ranging from 2 to 10 °C and simulated natural photoperiod for St. Andrews, New Brunswick, Canada (Lat=45°, 4.9 min North, Long=67°, 5.0 min West)

Oxygen and temperature were recorded daily. The fish were hand-fed once daily with a dry pellet mixture of Gemma starter feed and Europa, 2.0 mm feed from Skretting (Skretting, Inc., St. Andrews, NB). Feed was withheld 24 h prior to each exposure experiment. Protocols for all experiments were approved by DFO's regional animal care committee.

2.2. Experimental design

Experiments, lasting 72 h, were conducted to assess the effects of WAF and CEWAF at three environmentally relevant water temperatures: 2, 7 and 10 °C. The experiments were conducted twice at each temperature. The six experiments were conducted over the period December 2007 to June 2008 in ambient seawater. Experimental fish ranged in weight from 14 to 243 g. Mean weights are presented in Table 1. Both exposures at 2 °C were conducted in February, 2008. Exposures at 7 °C were conducted in December, 2007 and May 2008. Exposures at 10 °C were conducted in November 2007 and June, 2008.

2.3. Preparation of WAF and CEWAF

WAF and CEWAF were prepared using weathered MESA crude oil supplied by the Bedford Institute of Oceanography, Dartmouth, Nova Scotia. Weathering was achieved by bubbling compressed air through the oil until 13.8% of the volatiles in the oil had been driven off.

Table 1
Weight of experimental fish during each exposure.

Date	Exposure temperature (°C)	Mean fish weight (g) ± SEM
February 12–15	2	86.8 ± 3.4
February 26–29	2	85.5 ± 3.3
December 5–7	7	52.9 ± 2.3
May 6–9	7	107.3 ± 3.9
November 6–9	10	32.7 ± 1.2
June 17–20	10	147.8 ± 5.1

MESA oil and Corexit 9500 dispersant were used along with sand-filtered seawater to prepare the WAF and CEWAF following procedures described in Ramachandran et al. (2004). Briefly, a 10% v/v WAF mixture was prepared by using a 1:9 mixture of oil and water. CEWAF was prepared using the same 1:9 mixture of oil and water after which dispersant was added at a ratio of 1:25 (dispersant:oil). The WAF and CEWAF were prepared at ambient room temperature to give similar levels of PAH at the beginning of each exposure experiment.

2.4. Exposures

At each temperature, fish were exposed to a seawater control, WAF (0.2% and 1.0% (v/v)) or, CEWAF (0.2% and 1.0% (v/v)). The WAF and CEWAF dilutions were prepared in seawater using the WAF and CEWAF stock. BNF (10 μg L^{-1}), a known CYP1A inducer, was used as a positive control. Six 100 or 200 L glass aquaria (depending on fish size) were placed in a flow-through water bath to maintain temperature and fifteen juvenile cod were added to each aquarium. The aquaria were covered with plexiglass lids. Stocking densities were maintained at ~10 g L^{-1}. The water was aerated and test solutions were renewed every 24 h by removing half of the exposure water and replacing with seawater (for the control) and freshly prepared solutions of WAF, CEWAF or BNF.

2.5. Sampling

Temperature and dissolved oxygen were recorded daily in each aquarium. Water samples (500 mL) were taken during the daily renewal for total PAH analysis into amber glass jars (I-Chem Certified® 200 Series) containing 0.5 mL of 6 N HCl to act as a preservative. Samples were then stored at ca. 4 °C pending chemical analysis.

Five fish per treatment were sacrificed at 24, 48 and 72 h. Liver tissue was taken for the CYP1A (EROD) assay and gill, muscle and blood were stored for other biochemical analyses. Tissues were flash frozen in liquid nitrogen and stored at ca. −80 °C. Length, weight, liver weight, blood volume and sex were recorded for each fish.

2.6. Ethoxyresorufin-O-deethylase assay

EROD activity in cod livers was measured according to Hodson et al. (1996). The S9 fraction was isolated from homogenized liver samples and enzyme activity was measured using a kinetic fluorometric microplate method with excitation and emission wavelengths of 530 and 590 nm, respectively (Hodson et al., 1996). Protein levels in the same samples were quantified by UV absorbance at 600 nm using a BIORAD commercial reagent. BioTek FLx800 and Powerwave XS plate readers were used to measure fluorescence over time and absorbance. BioTek Gen 5 software was used to process the data. EROD activity was reported as picomoles resorufin per milligram protein per minute.

2.7. Spectrofluorometry

Levels of total PAHs in each of the collected water sample were determined by fluorescence spectrophotometry. Calibration lines using WAF and CEWAF prepared with MESA were created by weighing appropriate amounts of oil and preparing a series of dilutions using dichloromethane (DCM): hexane (1:1, v/v). These standards were then fortified into hexane to give the equivalent PAH concentrations in water (2–50 μg mL^{-1} for CEWAF, 4–160 ng mL^{-1} for WAF). Water samples, 1, 2 or 5 mL for CEWAF and 50 mL for WAF were extracted using hexane (5 and 4 mL, respectively) and levels of PAHs quantified against the prepared calibration lines providing a concentration in crude oil equivalents. The hexane layers were transferred to disposable methacrylate cuvettes (Fisher Scientific, clear sided, 4.5 mL, 10 mm pathlength) for analysis using a Varian Cary Eclipse fluorescence spectrophotometer coupled with Varian BIO Package version 1.1 software (Varian Inc., Paolo Alto, USA). Synchronous scan fluorescence mode was employed (excitation start/finish=230/523 nm, $\Delta\lambda$=57 nm, Ramachandran et al., 2006). Background subtraction was performed for each sample by subtracting the hexane spectra from each sample spectra prior to integration of peaks and quantification.

2.8. Statistical analysis

Statistical analyses were performed using SPSS software. Analyses of variance (ANOVA) were performed for EROD activity values that had been log transformed to achieve normal distribution (Hodson et al., 1996). The post-hoc Tukey's HSD was used to make all pair-wise comparisons and to identify significant differences in EROD activity between treated fish and seawater controls. The post-hoc least significant difference (LSD) pair-wise multiple comparisons test was used to identify significant differences in EROD activity between sampling time points and between temperatures for the CEWAF exposures ($P < 0.05$).

3. Results

3.1. EROD activity

Induction of EROD activity in Atlantic cod was dependent on concentration and water temperature. Exposure to CEWAF or BNF resulted in significant EROD induction at all temperatures. At 2 °C there was no significant difference between seawater controls and WAF-exposed fish at any of the sampling times (Fig. 1). EROD activity was significantly ($P < 0.05$) higher at 2 °C for CEWAF and BNF (positive control) treatments but significant elevation of EROD activity was observed only after 48 h in livers collected from cod exposed to 0.2% CEWAF at 2 °C and after 72 h in livers collected from cod exposed to 0.2% and 1.0% CEWAF at 2 °C. BNF-treated cod, exposed at 2 °C, showed elevated EROD activity at 48 and 72 h (Fig. 1). At 7 °C there was no significant difference between controls and WAF-exposed fish at any of the sampling times but EROD activity was significantly higher for both CEWAF treatments and BNF treatment at all three time points (Fig. 2). The highest induction of EROD activity was at 10 °C. It occurred in the BNF-treated cod and the 0.2 and 1.0% CEWAF-treated cod at all three time points (Fig. 3). WAF exposure had no significant effect on EROD activity compared to controls except in livers of fish exposed at 10 °C to 1.0% WAF for 48 h. After 24 h exposure at 10 °C to 1.0% WAF there was higher EROD activity compared to controls but the

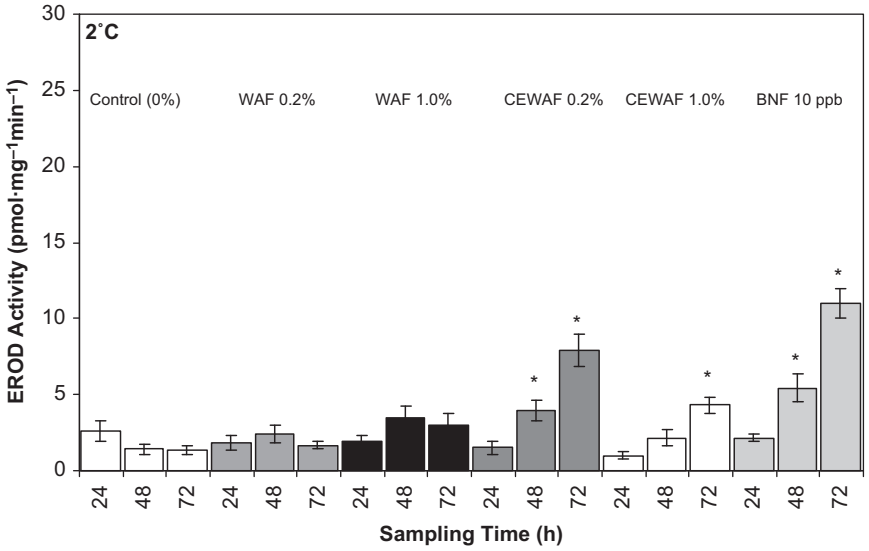

Fig. 1. EROD induction in Atlantic cod exposed to water accommodated fractions (WAF) or chemically enhanced water accommodated fractions (CEWAF) from MESA crude oil, MESA oil and Corexit 9500 and ß-naphthaflavone (BNF) at 2 °C. Bars represent ±1 Standard Error (SE). Asterisk (*) represent significant difference ($P < 0.05$) from seawater control.

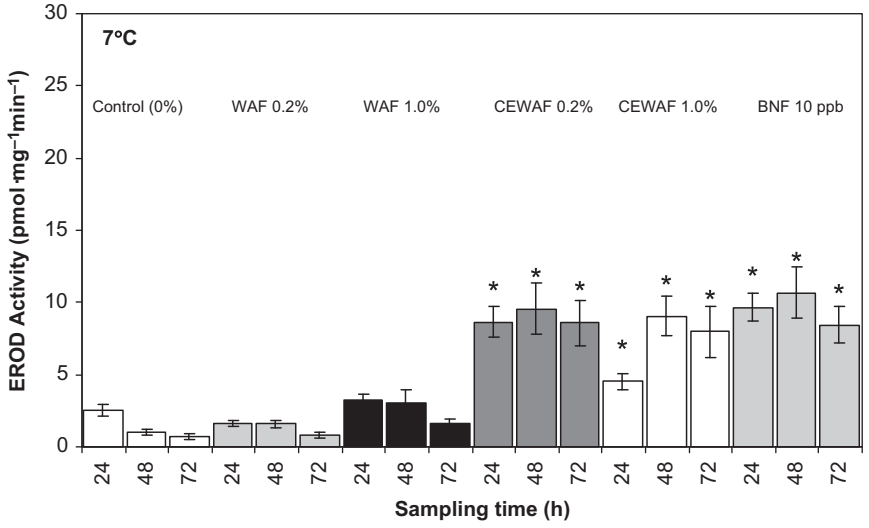

Fig. 2. EROD induction in Atlantic cod exposed to water accommodated fractions (WAF) or chemically enhanced water accommodated fractions (CEWAF) from MESA crude oil, MESA oil and Corexit 9500 and β-naphthaflavone (BNF) at 7 °C. Bars represent ±1 Standard Error (SE). Asterisk (*) represent significant difference ($P < 0.05$) from seawater control.

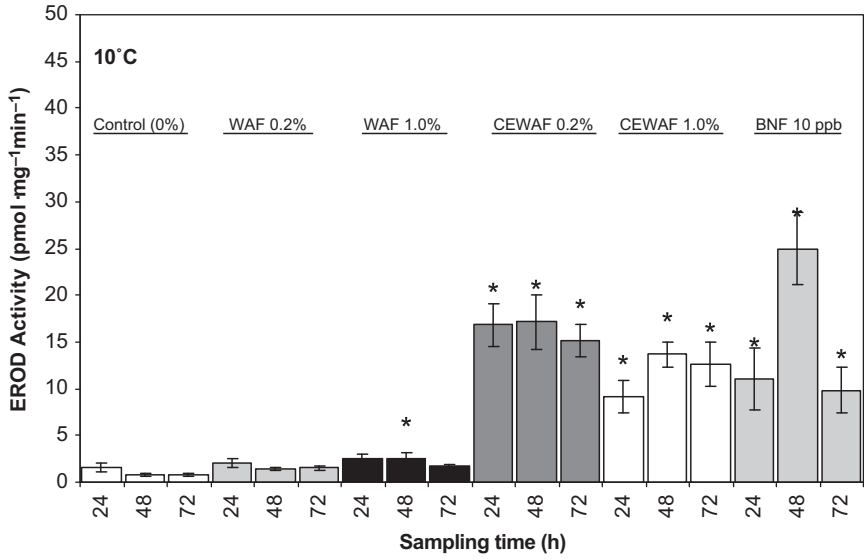

Fig. 3. EROD induction in Atlantic cod exposed to water accommodated fractions (WAF) or chemically enhanced water accommodated fractions (CEWAF) from MESA crude oil, MESA oil and Corexit 9500 and β-naphthaflavone (BNF) at 10 °C. Bars represent ±1 Standard Error (SE). Asterisk (*) represent significant difference ($P < 0.05$) from seawater control.

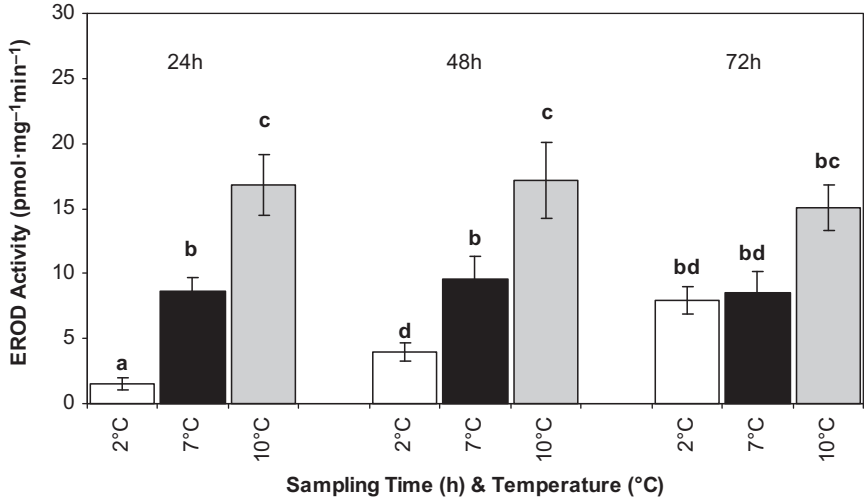

Fig. 4. Mean EROD activity in Atlantic cod exposed to 0.2% (v/v) chemically enhanced water accommodated fractions (CEWAF) from MESA crude oil and Corexit 9500. Bars represent ±1 Standard Error (SE). Significant differences between the groups within the figure are denoted by different letters ($P < 0.05$).

difference was not significant. The lack of significant difference can be attributed to a higher mean EROD activity for controls after 24 h exposure. After 72 h exposure there was no significant difference between WAF-exposed and control fish (Fig. 3).

Hepatic EROD activity in the 0.2% CEWAF exposed Atlantic cod was temperature dependent. After 24 and 48 h exposure there was a significant difference in EROD activity between all three temperatures. After 72 h there was significant difference in EROD activity between 2 and 10 °C and between 7 and 10 °C but no difference between 2 and 7 °C (Fig. 4). Hepatic EROD activity in the 1.0% CEWAF exposed cod showed temperature dependence to a lesser extent. There was no significant difference in EROD activity between 7 and 10 °C at any of the three sampling time points but there was a significant difference between 2 and 7 °C and 2 and 10 °C after 24 h. After 48 h there was significant difference only between 2 and 10 °C (Fig. 5).

EROD activity was significantly lower in cod exposed to 1.0% CEWAF compared to 0.2% CEWAF after 48 and 72 h exposure at 2 °C and after 24 h exposure at 7 and 10 °C. After 48 and 72 h EROD activity was still lower in cod exposed to the 1.0% CEWAF compared to 0.2% CEWAF but the differences were not significant (Fig. 6).

3.2. PAH concentrations in treatments

The total PAH concentrations in experimental tanks are shown in Table 2. Total PAHs in treatment water were markedly increased after mixing with chemical dispersants. PAHs were present in all water samples indicating the fish were exposed to PAHs during the full 72 h experimental periods. Analysis of water samples show that CEWAF samples (0.2% and 1%) contain higher levels of PAHs compared to the WAF samples at the same dilutions (Table 2). Over the 72 h exposure period, measured PAH levels in CEWAF decreased for all treatments except the 0.2% treatment at 10 °C. Water sampling technique and length of storage time of water samples may have caused some inconsistencies in measured PAH values.

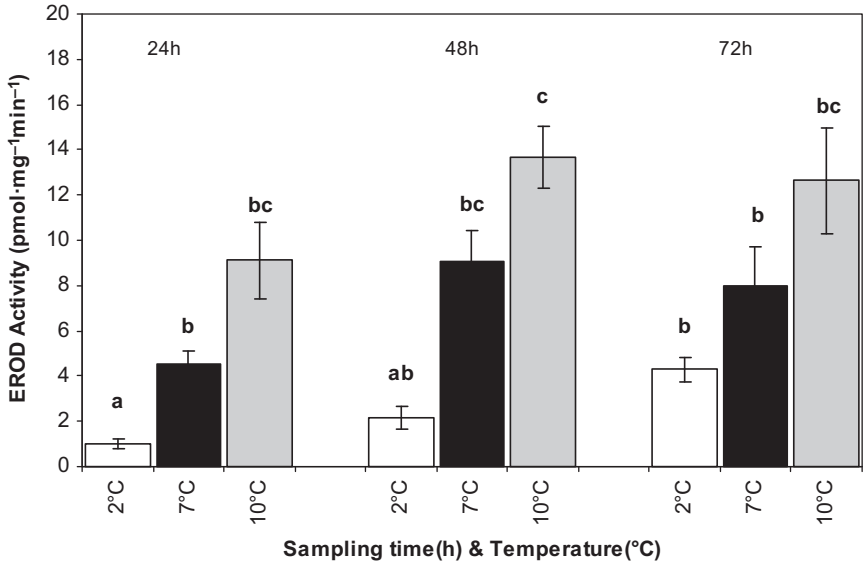

Fig. 5. Mean EROD activity in Atlantic cod exposed to 1.0% (v/v) chemically enhanced water accommodated fractions (CEWAF) from MESA crude oil and Corexit 9500. Bars represent ±1 Standard Error (SE). Significant differences between the groups within the figure are denoted by different letters ($P < 0.05$).

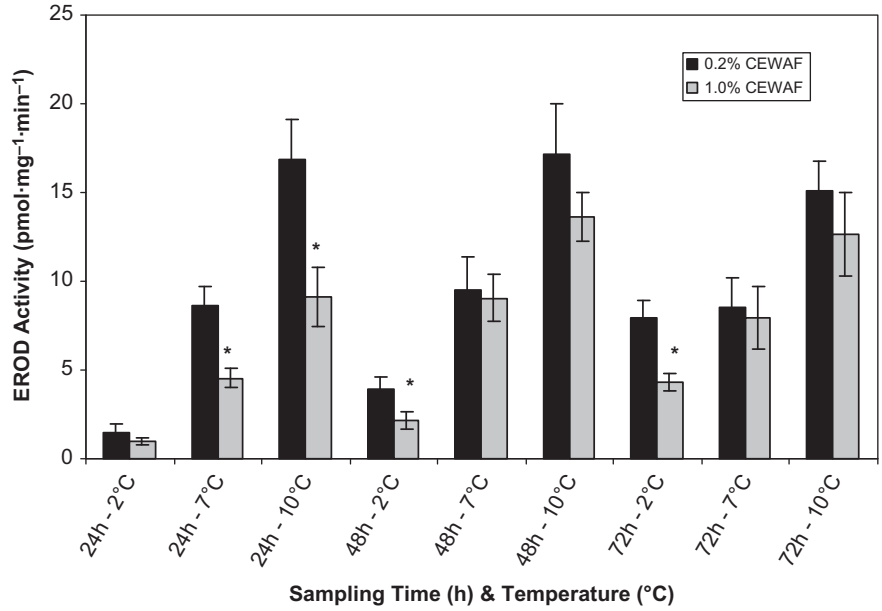

Fig. 6. Mean EROD activity in Atlantic cod exposed to 0.2% and 1.0% (v/v) chemically enhanced water accommodated fractions (CEWAF) from MESA crude oil and Corexit 9500. Significant differences between 0.2% CEWAF and 1% CEWAF within the figure are denoted by an asterisk ($P < 0.05$).

4. Discussion

In the present study EROD activities for juvenile cod were in the same range reported by other investigators. EROD levels in our control juvenile cod ranged from 1.1 to 2.9 pmol min^{-1} mg^{-1}protein. WAF-exposed cod had EROD levels from 1.1 to 5.3 pmol min^{-1} mg^{-1} protein and the CEWAF exposed cod had levels from 1.0 to 17.3 pmol min^{-1} mg^{-1}protein. Most previously reported experimental work on CYP1A and EROD related to oil contamination in fish has been performed with intraperitoneal, oral or sediment exposure. Ramachandran et al. (2004) exposed juvenile rainbow trout for 48 h to MESA oil and chemically dispersed MESA oil. They reported EROD activity in the range of 1–100 pmol mg^{-1} min^{-1}. EROD activity decreased at the highest concentration of

Table 2
Total PAH concentrations crude oil equivalents detected in WAF and CEWAF at three different seawater temperatures.

Temperature (°C)	Treatment (%)	CEWAF $T=0$ h PAH[a] (μg mL^{-1})	CEWAF $T=72$ h PAH[a] (μg mL^{-1})	WAF $T=0$ h PAH[a] (μg mL^{-1})	WAF $T=72$ h PAH[a] (μg mL^{-1})
2	1	8.68	4.78	0.069	0.036
2	0.2	2.18	1.20	0.015	0.011
7	1	11.94	4.95	0.018	0.015
7	0.2	1.47	1.18	0.006	0.009
10	1	11.59	5.75	0.014	0.017
10	0.2	1.80	4.14	0.005	0.013

[a] Expressed as crude oil equivalent.

CEWAF possibly due to liver damage (Holdway et al., 1994). Goksøyr et al. (1994) reported a dose-dependent response of hepatic EROD activity in the range of 50–150 pmol min^{-1} mg^{-1}protein for juvenile Atlantic cod caged in a polluted fjord. Beyer et al. (1996) measured hepatic EROD activity in the range of 13–34 pmol min^{-1} mg^{-1} protein in mature Atlantic cod caged in a polluted fjord. Aas and Klungsøyr (1998) measured hepatic EROD activity in cod from areas with oil production in the North Sea. They reported no difference in EROD levels in fish caught in the vicinity of oil production fields compared to those caught at a reference site. However a 30-day chronic exposure study of juvenile Atlantic cod to mechanically dispersed crude oil showed a dose-dependent response. EROD activity showed an initial increase during the first three days and remained elevated during the remainder of the exposure period (Aas et al., 2000). They reported hepatic EROD activity in control fish around 2 pmol min^{-1} mg^{-1}protein and around 6 pmol min^{-1} mg^{-1}protein in the 1 ppm exposure group after three days. Sturve et al. (2006) exposed juvenile Atlantic cod to 0.5 ppm North Sea oil for fifteen days and saw a significant increase in EROD activity. Hepatic EROD activity in control fish was 20 pmol min^{-1} mg^{-1}protein and fish exposed to 0.5 ppm oil had a mean EROD level of 50 pmol min^{-1} mg^{-1} protein. Holth et al. (2009) chronically exposed cod to different treatments of PAHs and alkylated phenols combined in a cocktail. Hepatic EROD activity ranged from 3 to 30 pmol min^{-1} mg^{-1}protein. Khan and Payne (2005) looked at changes in gill architecture in Atlantic cod exposed to oil and oil dispersants but they did not measure EROD activity. Chemically dispersed oil caused the highest prevalence of gill lesions in cod compared to the water-accommodated fraction or dispersant alone.

Abrahamson et al. (2008) reported that basal EROD activities in gills were higher in cod kept at 2 °C compared to those kept at 8 °C. In contrast, we saw no significant difference in basal EROD activity in cod at 2, 7 and 10 °C and saw no significant difference in liver EROD activity between cod exposed to weathered oil and controls except at 10 °C after 48 hours. Hepatic EROD activity may not be comparable to gill EROD activity as some metabolizing of PAHs may take place before the PAHs arrive in the liver. Another possible reason for differing results between the two studies is that our oil exposures were performed at ambient temperatures of 2, 7 and 10 °C as opposed to acclimating fish for a one week period from 8 to 2 °C.

Regardless of temperature, chemically dispersed oil in CEWAF induced higher EROD activity compared to mechanically dispersed oil in WAF. Chemical dispersion of oil has been shown to affect particle size of oil (Li et al., 2008, 2009). This allows PAHs to be more bioavailable for uptake by the fish.

The present study was designed to assess quantitative changes in enzyme (EROD) activity and our results support a conclusion that water temperature does affect the response to PAHs.

The hepatic EROD activity in our CEWAF-exposed Atlantic cod was temperature dependent. Greater induction occurred at 10 °C than at 2 or 7 °C.

Experiments were conducted at ambient water temperature and in the case of cod exposed at 7 and 10 °C the fish were significantly different in size (Table 1). There was no significant difference in hepatic EROD activity in control, BNF-treated fish at any temperature or sampling time. Nor were there differences between replicates at 2 or 10 °C. Statistically significant differences in activity between replicate treatments were only observed at 7 °C for the 1.0% WAF-treated cod after 48 h and the 1.0% CEWAF-treated cod after 72 h (data not shown). All other comparisons between replicates at 7 °C showed no differences. In particular, the replicates at 10 °C had the greatest size difference yet there was no difference in EROD response between the replicates. These data lead us to conclude that the difference in size of the experimental fish between replicates has no bearing on the results or interpretation of the data.

There was no significant difference in hepatic EROD activity of the WAF-exposed cod between temperatures except for livers collected from cod exposed to WAF for 48 h at 10 °C. Hepatic EROD activity in our CEWAF-exposed Atlantic cod was concentration dependent. This was comparable to results with rainbow trout and decreased EROD activity at higher exposure concentrations of CEWAF (Ramachandran et al., 2004). Correia et al. (2007) saw an increase of EROD activity in juvenile gilthead seabream at low exposure concentrations to the PAH phenanthrene and an inhibition of EROD at a high concentration. The same response may be taking place in our study where 1.0% CEWAF often does not induce EROD to the extent 0.2% CEWAF does.

Results from chemical analysis of the exposure water show that PAH concentrations generally decreased in the CEWAF exposure water after 72 h. Since it is unlikely that all the PAHs would be driven off by aeration, we suggest that they are being taken up by the cod. Our data would therefore indicate that the delay in EROD induction noted at 2 °C is attributable to the metabolism of the cod as opposed to the concentration of PAHs in the exposure water. At low temperature, uptake of PAHs could be slower because of a slower metabolic rate.

Moles et al. (2001) found that the effectiveness of dispersants on oil and seawater dispersion was greatest at 10 °C compared to 3 or 22 °C. Their results showed a drop in dispersant effectiveness at subarctic temperatures, probably due to the effect of low temperature on the oil's viscosity. Fingas et al. (1991) found a two-fold rise in dispersant effectiveness with each three-fold rise in temperature.

Low temperature will affect both dispersant and oil by increasing their viscosity making the oil harder to disperse (Chandrasekar et al., 2005). Srinivasan et al. (2007) showed low temperatures lead to lower dispersant effectiveness. There is reduced solubility of the oil compounds at low temperature reducing the bioavailability of PAHs. Our analysis of CEWAF exposure water at 2 °C showed PAH levels in the same range as CEWAF exposure water at 7 and 10 °C. Although PAH levels were in the same range at the three exposure temperatures, oil particle size may have been a factor at 2 °C where we saw lower and delayed induction of EROD. The oil may not have been as readily available to the fish. We did not measure oil particle size by Laser *in situ* Scattering and Transmissiometry (LISST) in our experiment.

The delay of EROD induction at low temperature in our study corresponds with results obtained by Jørgensen and Wolkers (1999) in a study with benzo-*a*-pyrene and Arctic char. These authors noted a delayed induction of an EROD response and a longer duration in fish exposed at 1 °C compared to those exposed at 10 °C. It has also been shown in other studies with petroleum hydrocarbons that tissues other than liver and gills are inducible (e.g. Payne and Fancey, 1982; Payne et al., 1984). Since we did not look at EROD in other tissues our conclusions regarding effects of temperature and consequent risk can only apply to EROD induction in hepatic tissue.

5. Conclusion

The hepatic EROD activity of the juvenile Atlantic cod responded to the presence of chemically dispersed oil at three relevant seawater temperatures. The induction response was delayed at the lowest exposure temperature of 2 °C. It remains unclear if this is solely due to the metabolic rate of the fish, although we believe this is a major factor in the response. Another possibility is the much colder temperature may have transiently decreased hydrocarbon bioavailability across the gut/intestinal wall and decreased entry of ingested hydrocarbons into the liver. Therefore during a short lag period, risk may be reduced. Water temperature will also affect solubility of some compounds as well as the effectiveness of the chemical dispersants. Further experimentation is required to determine how long hepatic EROD activity remains elevated after similar or shorter exposures. Should low temperature result in a significant delay, or even elimination, of the induction of hepatic

EROD activity in cod, risk assessment of the effects of oil or of use of dispersants will need to include temperature as a variable. Our data indicate that cod exposed to WAF or CEWAF at moderate seawater temperatures (7 or 10 °C) will react (biochemically) more quickly than those exposed to lower temperatures.

Acknowledgments

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Table C11. One-Factor ANOVA results of survival-to-hatch of Atlantic herring embryos exposed to Arabian Light WAF and CEWAF. Stocks were analyzed separately and results indicate which concentrations significantly reduced survival compared to the negative control (Tukey, $p<0.05$). Non-significant results are labelled as NS. N=3 jars/concentration.

		Conc. (% v/v)	Survival-to-Hatch
Spring	CEWAF	ANOVA	$F_{4,10} = 182.568$, $p<0.0001$
		0.0001	NS
		0.001	NS
		0.01	NS
	WAF	ANOVA	$F_{4,10} = 182.568$, $p<0.0001$
		0.01	NS
		0.1	NS
		1.0	NS
	Controls	Retene	$p < 0.05$
Fall	CEWAF	ANOVA	$F_{4,10} = 6.661$, $p=0.007$
		0.0001	NS
		0.001	NS
		0.01	NS
	Controls	Retene	$p < 0.05$

Table C12. Average length-at-hatch (mm) and blue sac disease severity index (BSD SI; 0-1) including 95% confidence intervals for Atlantic herring embryos exposed to Arabian Light WAF and CEWAF. Stocks were analyzed separately and arrows indicate significant differences from the negative control (1-Factor nested ANCOVA for length-at-hatch and 1-Factor ANOVA for BSD SI; Tukey test, $p<0.05$). N=3 jars/concentration.

		Conc. (% v/v)	Length-at-Hatch (mm)	BSD SI
Spring	CEWAF	Water	7.42±0.12	0.01 (0.00-0.03)
		0.0001	7.27±0.16	0.02 (0.00-0.07)
		0.001	7.13±0.19	0.05 (0.01-0.12)
		0.01	6.93±0.23↓	0.11 (0.04-0.23) ↑
		Retene	3.30±0.16↓	0.66 (0.47-0.84) ↑
		ANOVA	$F_{4, 254} = 149.48$, $p<0.001$	$F_{4, 10} = 112.66$, $p<0.001$
	WAF	Water	7.42±0.12	0.01 (0.00-0.03)
		0.01	7.49±0.15	0.03 (0.01-0.08)
		0.1	7.51±0.10	0.02 (0.00-0.06)
		1.0	7.05±0.23↓	0.12 (0.02-0.30) ↑
		Retene	3.30±0.16↓	0.66 (0.47-0.84) ↑
		ANOVA	$F_{4, 254} = 226.72$, $p<0.001$	$F_{4, 10} = 102.21$, $p<0.001$
Fall	CEWAF	Water	5.93±0.21	0.14 (0.03-0.66)
		0.0001	5.68±0.22	0.23 (0.02-0.57)
		0.001	5.83±0.28	0.39 (0.30-0.48)
		0.01	5.41±0.37	0.42 (0.27-0.57)
		Retene	3.61±0.17 ↓	0.75 (0.46-0.95) ↑
		ANOVA	$F_{4, 193} = 12.99$, $p<0.001$	$F_{4, 10} = 10.36$, $p=0.001$

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.

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Abstract

EROD induction in juvenille Atlantic cod (*Gadus morhua*) and Atlantic Salmon (*Salmo salar*) when exposed for a 4 hour period to various combinations of water accomodated fraction (WAF, 4.7 to 64% v/v) or chemically enhanced water accomodated fraction (CEWAF, 0.5 to 64% v/v) was investigated. Two sources of crude oil, Mediterranean South American (MESA) and Alaskan North Slope (ANS) were used along with 2 dispersants, Corexit 9500 and SPC-1000. Exposures were performed at water temperatures of 12 ± 2°C in dupliacte for each WAF and CEWAF combination used. Liver samples were collected periodically for determination of EROD induction. Water samples were collected from each exposure to determine the levels of total petroleum hydrocarbons and PAHs present. Oil droplet size was also checked prior to preparation of the treatment dilutions.

All exposures resulted in EROD induction in cod. Maximum induction was seen seen at 24 h post start of exposure then declined to levels near or slightly above that of the the

controls by 72 h. For salmon exposures, no significant EROD induction was seen with WAF. Exposure to CEWAF resulted in EROD induction with maximum levels observed at 48 h post start of exposure then declining, but still elevated relative to controls by 72 h.

Introduction

Extensive offshore exploration, production and transport of oil means that the probability of oil spills occurring is very high (Anderson and LaBelle, 1994). When such accidents occur, oil spill remediation can take 2 main forms. The oil can be left untreated to break up naturally with the aid of wind and wave action along with biological activity. Secondly, dispersants can be sprayed on the oil slick.. Dispersants, due to their hyrdophilic and hydrophobic properties, break the oil into smaller droplets (typically <100µm) (Canevari, 1978). Typically, the toxicity of the untreated oil is less than that of the chemically treated oil since it only contains low levels of dissolved hydrocarbons (*e.g.* PAHs). The treated oil on the other hand is more toxic due to the dissolved components and oil plus dispersant interactions resulting in smaller droplet sizes of the dispersed oil which increases its surface area and thus its bioavailbility to marine fish (Singer *et al*, 1998). Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*) may be vulnerable to any oil and oil dispersant exposure due to their natural habitat being within areas of oil exploration, production and shipping lanes in the North Atlantic sea. When exposed to contaminants such as PAHs, organisms take up then remove these xenobotic compounds *via* biliary excretion. Detoxification occurrs by oxidative metabolism of these compounds by the family of cytochrome P-450 enzymes into a more hydrophilic form to enable excretion from the body (Stegman and Kloepper-Sams, 1987). A widely accepted biomarker to monitor for PAH exposure to organisms is ethoxyresorufon-*O*-deethylase (EROD),

The aim of this study was to investigate EROD induction in 2 economically important species of marine fish after exposure to various combinations of 2 crude oils and 2 dispersants. Concentrations of treated oil were chosen to reflect environmentally relevant concentrations present in the sea after an oil spill. A 4 h exposure period was used, which reflects a realistic period in which fish may be exposed, followed by a 68 h depuration period in clean sea water.

Materials and Methods

Crude Oil and Dispersants

Pre-weathered, 90% of original by weight, ANS and MESA crude oils plus Corexit 9500 and SPC-1000 dispersants were supplied by the Bedford Institute of Oceanography (BIO), Dartmouth, NS. The crude oils were further weathered by *ca* 5% of the supplied volume prior to use. The weathering process mimics natural environmental weathering which drives off low molecular weight compounds from the crude oil.

Fish Stocks

Juvenille Atlantic cod used for this study were spawned and raised from broodstock held at the St. Andrews Biological Station. Fish were raised and held in ambient temperature sand filtered sea water and fed daily with a diet of commercial dry pellets designed for cod (Skretting, Bayside, NB, E5B 3T5).

Atlantic salmon, Saint John River strain, were purchased from the Mactaquac Biodiversity Center, Mactaquac, NB as parr. Fish were held in dechlorinated domestic fresh water until the ambient sea water temperature reached *ca* 7°C when they were then transferred to seawater by increasing the proportion of seawater delivered to the holding tank until the fish received 100% SW (T=5 days) Successful smoltification was

assessed by collecting blood samples from a number of fish and performing an osmolality test using a Vapro® vapor pressure osometer Model 5520 (Wescor Inc., Utah, USA) on the prepared plasma. The fish were fed once daily on commercial salmon pellet diet (Skretting, Bayside, NB, E5B 3T5). Feed was withheld from fish 24 h prior to each exposure occassion. The body weight ranges of the fish used throughout this study are presented in Table 1.

Preparation of WAF and CEWAF solutions for exposure

Stock solutions of WAF were prepared by a shaking flask method(REFERENCE). ANS or MESA crude oil, 2.5 mL, was added to 2.5 L of sand filtered sea water in a 4 L spouted Erlemeyer flask then mixed for 18 h at 250 rpm on an orbital mixer. After 18h, mixing was stopped and the stock solution allowed to settle for 0.5 h prior to oil droplet size determination using a LISST particle size analyser. The prepared WAF stock solutions were then diluted up to a total volume of 75 L with filtered sea water to give an exposure range of range from 0 to 75% v/v.

Stock solutions of CEWAF were also prepared by the shaking flask method. ANS or MESA crude oil, 2.5 mL, was added to 2.5 L of sand filtered sea water in a 4 L spouted Erlemeyer flask followed by 100 µL of either Corexit 9500 or SPC-1000 dispersant. The solutions were mixed on an orbital shaker at 250 rpm for 30 min then allowed to settle for 10 min prior to oil droplet size determination using a LISST particle size analyser. The prepared CEWAF stock solutions were then diluted up to a total volume of 75 L with filtered sea water to give an exposure range from 0 to 64% v/v.

Oil Droplet Size Distribution

The oil droplet size of selected WAF and CEWAF stock solutions was determined by the use of a Type C LISST-100X analyser (Sequoia Scientific Inc., Bellvue, WA, USA). This instrument measures particles in the range 2.5 to 500 µm and was used in the benchtop mode with the full-path test chamber. WAF and CEWAF stock solutions were diluted 50x (2mL stock + 98 mL sea water) and 67x (1.5 mL stock + 98.5 mL sea water) respectively with sea water prior to droplet size determination

Experimental Design

Static exposures were performed in 100L glass aquaria held in a flow through water bath to maintain water temperature during exposures. during each experiment, cod or salmon (5 fish per concentration level) were exposed to either WAF or CEWAF as detailed in Table 2. During some experiments an additional group of fish was exposed to *beta*-Naphthoflavone (βNF, 10 µg/L), a known CYP1A inducer to act as positive controls.

To minimise the effects of coalescence and adsorption of WAF or CEWAF onto the walls of the aquaria, the exposure water for each experiment was recirculated within the aquaria. This was achieved by positioning a glass funnel just below the water's surface and attaching a Supreme Mag Drive 3 pump (Danner, USA) to it. Each aquaria was also aerated to oxygenate the water during the exposure period, this also served to continuously mix the exposure water.

Table 1. Body weight ranges of cod and salmon used throughout this study.

Toxicant	Exposure No.	Body Weight Range (g)	
		Cod	Salmon
ANS WAF	1	8.5 – 78.4	19.0 – 158.2
	2	9.3 – 62.0	40.4 – 248.2

MESA WAF	1	6.1 – 90.6	19.1 – 131.5
	2	10.9 – 74.4	41.9 – 243.5
ANS + Corexit CEWAF	1	5.4 – 65.4	17.8 – 110.9
	2	12.5 – 93.3	15.6 – 179.4
ANS + SPC-1000 CEWAF	1	5.6 – 98.0	11.4 – 100.6
	2	9.1 – 82.9	23.0 – 568.0
MESA + Corexit CEWAF	1	6.7 – 84.3	33.7 – 214.8
	2	10.8 – 98.1	32.8 – 119.8
MESA + SPC-1000 CEWAF	1	5.4 – 60.7	20.5 – 129.7
	2	12.2 – 148.1	25.4 – 159.1

Table 2. Combinations of WAF and CEWAF used for cod and salmon exposures

		Exposure Concentrations (% v/v)
WAF	ANS MESA	0, 4.7, 9.4, 18.75, 37.5, 75
CEWAF	ANS + Corexit 9500 ANS + SPC-1000 MESA + Corexit 9500 MESA + SPC-1000	0, 0.5, 2, 8, 32, 64

Samples of blood gill, liver, gall bladder and muscle were collected from replicate fish (n=5) at pre-treatment and 4, 24, 48 and 72 h post start of exposure. Blood samples were stored on ice, centrifuged and the plasma decanted into microcentrifuge tubes and flash frozen in liquid nitrogen. All other tisses were flash frozen in liquid nitrogen at time of collection then stored at -80°C until analysed. Sub-samples of liver (right lobe) and gall bladder were pooled (n=5) and stored at -20°C then transported to BIO for (what kind of) analysis. In addition,the length, weight and sex of sampled fish was recorded. Water temperature and dissolved oxygen levels were also recorded during the exposure and depuration periods.

Ethoxyresorufin-O- deethylase Assay

EROD activity, reported as pmol/min/mg protein, was determined according to Hodson *et al*, 1996, using BioTek Powerwave XS UV and FLx800 fluorescence readers employing Gen 5 software. Aliquots of sampled liver were homogenised with buffer, the S9 fractions isolated then analysed using a kinetic fluorometric microplate method using 530 nm excitation and 590 nm emmission wavelengths to determine the conversion of ethoxyresorufin to resorufin. Protein concentration of the S9 fractions was determined by UV spectrophotometry at a wavelength of 600 nm.

Analysis of Exposure Water Samples

Water samples (400 mL) were collected from each exposure before addition of fish (T=0 h) and at the end of the 4 h exposure period. Samples were acidified with 6 N hydrochloric acid (0.5 mL) then stored at +4°C prior to extraction and determination of total PAH by fluorescence spectrophotometry (Lyons *et al*, 2011). In brief, analysis was performed by preparing calibration standards of ANS or MESA crude oils in dichloromethane:hexane (1:1, v/v). These standards were then fortified into hexane to give the equivalent PAH concentrations in water (2 to 50 $\mu g \cdot mL^{-1}$ for CEWAF, 4 to 160 $ng \cdot mL^{-1}$ for WAF). Water samples, 1, 2 or 5 mL for CEWAF and 50 mL for WAF were extracted using hexane (5 mL and 4 mL, respectively) and levels of PAHs quantified against the prepared calibration lines providing a concentration in crude oil equivalents. Sample analysis was conducted using a Varian Cary Eclipse fluorescence spectrophotometer running Varian Bio Package version 1.1 software (Varian Inc., Paolo Alto, USA). Synchronous scan fluorescence mode was employed (excitation start/finish = 230/523 nm, $\Delta\lambda$ = 57 nm, Ramachandran *et al*, 2006). Background subtraction was performed by subtracting hexane blanks from each sample spectra prior to integration of

peaks. The concentration of total PAHs in the WAF and CEWAF samples were interpolated against the respective calibration lines and presented as crude oil equivalents.

A duplicate set of water samples from the control and top dilution concentration from each exposure were taken at T=0 and 4h, acidified with 6 N hydrochloric acid and stored at +4°C before tranfer to BIO. Levels of total petroleum hydrocarbons (TPH) in these samples were determined by GC-FID, (Cole *et al*, 2007). Total PAH in certain samples was determined by GC-MS.

Statistical Analysis

Statistical analysis of EROD induction was performed using SPSS version 17.0 software (IBM, USA). Generated data were log transformed then analysed using a one way ANOVA test followed by the *post hoc* Tukey pairwise multiple comparisons test. For each of the combinations of WAF and CEWAF for cod and salmon, exposures 1 and 2 were compared against each other. This was to check for any significant differences between treatment concentrations over the 2 exposures. If no significant differences were noted, the data for both exposures were averaged to give one overall EROD induction profile for that toxicant used. For each species of fish, comparisons of were made against each WAF and CEWAF exposure to see if there were any significant differences related to the treatments.

Results

EROD Activity in Atlantic Cod

EROD activity in cod exposed to ANS and MESA WAF, 4.7 to 75% (v/v), showed some significant differences ($p < 0.05$) within the treatment groups when exposure 1 and 2

were compared against each other. This was most likey due to a difference in the ambient sea water temperature that the 2 exposures were conducted at (Lyons *et al*, 2011). Therefore additional exposures with these treatments were perfromed at 12 ± 2°C to be consistent with the rest of the exposure temperatures.

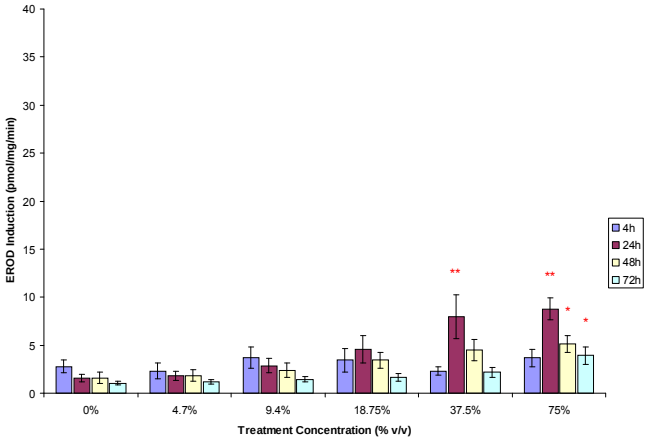
For ANS WAF, results show that significant differences ($p < 0.005$) compared to controls are evident at 37.5% and 75% (v/v) 24h post start of exposure. Induction declined after 24 h in these 2 groups but remained above those of the corresponding control. Significant differences ($p < 0.05$) were seen at 48 h and 72 h in the 75% (v/v) treatment group. Exposure concetrations below 37.5% did not show any significant difference from the corresponding control at any of the sampling time-points. For MESA WAF exposure, EROD induction was slightly elevated at the 9.4%, 18.75%, 37.5% and 75% level compared to ANS WAF with significant differences ($p < 0.05$ and $p < 0.005$) observed at 9.4% (72h) and 18.75% to 75% (24h to 72h). Maximum induction was again seen at 24 h post start of exposure with a gradual decline resulting in levels above those of the control by 72 h. Highest EROD induction was seen at 75% v/v for both MESA and ANS WAF exposures. Overall, exposure to MESA WAF gave higher EROD induction compared to that of ANS WAF.

When exposed to ANS+Corexit 9500 CEWAF, significant differences ($p < 0.005$) from controls were noted at 2, 8, 32 and 64% (v/v) from 24 h up to 72 h post start of exposure. Highest levels of EROD induction were seen at 24 h at these treatment concentrations, the exception being the 64% treatment which had the highest EROD induction at 48h. EROD induction decreased after 24 h but activity remained above background by 72 h. Exposure to ANS+SPC-1000 CEWAF gave a similar EROD induction profile to that of ANS+Corexit 9500 CEWAF. Maximum induction for each concentration was seen at 24

h post start of treatment with levels declining but remaining above background by 72 h. Significant differences ($p < 0.005$) from background were observed for 8%, 32% and 64% concentrations at 24 h, 48 h and 72 h. When exposed to MESA+Corexit 9500 CEWAF, EROD induction followed a similar trend to that of ANS CEWAF with maximum induction seen at 24 h post start of exposure with a gradual decline thereafter. Significant differences ($p < 0.05$ and $p < 0.005$) were seen at concentrations ranging from 2% to 64% at 24 h to 72 h post start of exposure. This trend was reflected in the MESA+SPC-1000 CEWAF exposure, however maximum induction was seen at 48 h rather than 24 h, followed by a decline to 72 h. There were significant differences ($p < 0.05$ and $p < 0.005$) at 24 h to 72 h time-points, except at 2% (72 h) and 8% (72 h). Overall, all combinations of CEWAF gave similar EROD induction to each other with the maximum EROD induction typically at the 8% v/v concentration, 24 h post start of exposure.

Cod and salmon exposed to β-Napthaflavone showed induction of EROD when compared against the respective control groups, these data are not presented.
A summary of maximum EROD induction is presented in Table 3.

a)



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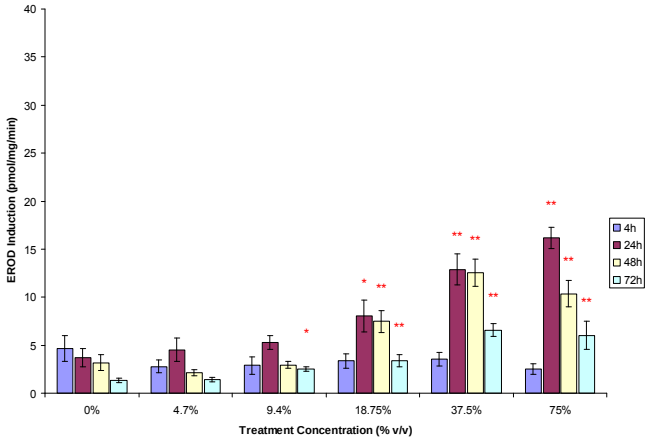

Figure 1. Mean EROD induction ± 1 SE for cod exposed to a) ANS WAF and b) MESA WAF for 4 hours (*=p<0.05; **=p<0.005)

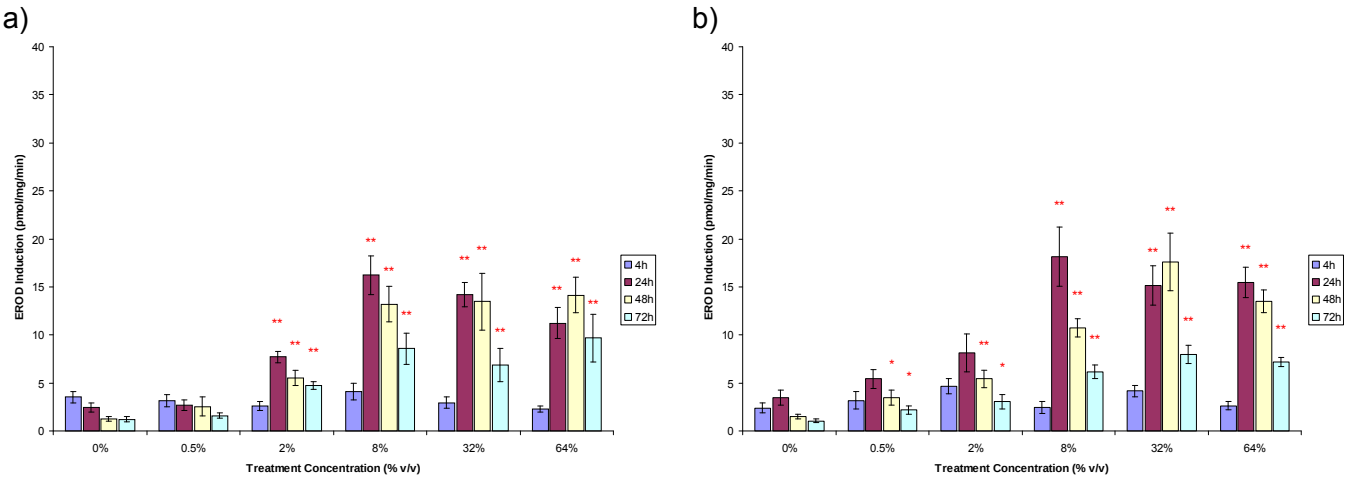

Figure 2. Mean EROD induction ± 1 SE for cod exposed to a) ANS+Corexit 9500 and b) ANS+SPC-1000 CEWAF for 4 hours (*=p<0.05; **=p<0.005)

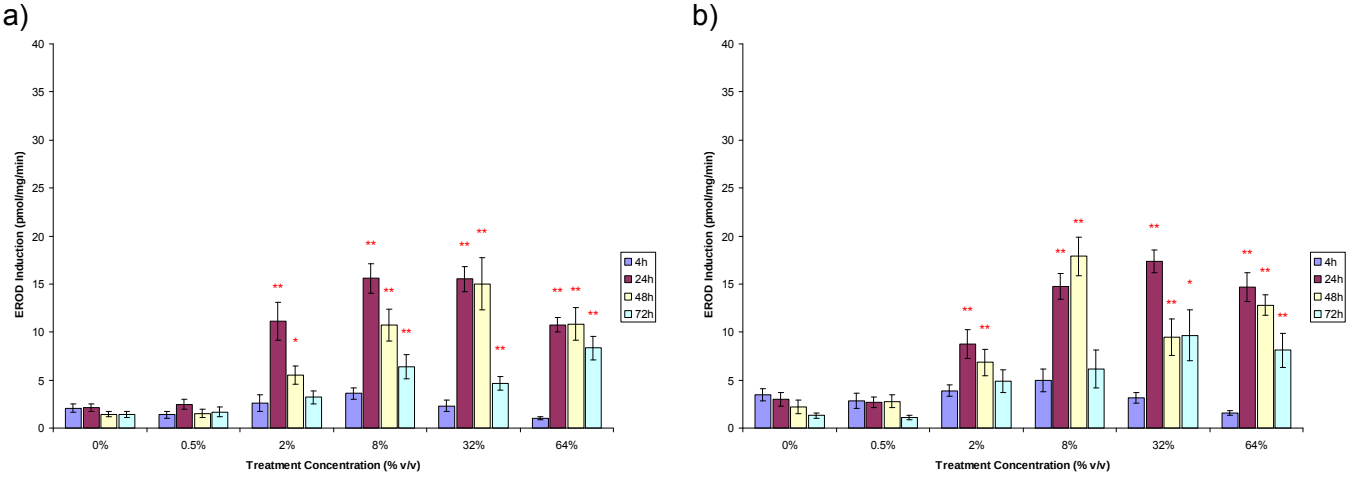

Figure 3. Mean EROD induction ± 1 SE for cod exposed to MESA+Corexit 9500 and b) MESA+SPC-1000 CEWAF for 4 hours (*=p<0.05; **=p<0.005)

EROD Induction in Atlantic Salmon

No major significant differences within treatment groups were noted between exposure 1 and 2.

Salmon exposed to ANS crude oil WAF for 4 hours showed significant hepatic EROD induction only in the 18.75% and 75% treatment groups at 24 h post start of expsoure. All other treatments did not show any induction when compared to the control group. Exposure to MESA WAF did not show any EROD induction in any treatment group that was significantly different from the countrol group.

After exposure to ANS+Corexit 9500 and ANS+SPC-1000 CEWAF, EROD induction was similar for both treatments. Up to 2% v/v, there was no evidence of EROD induction, however from 8% to 64%, induction was evident with significant differences ($p < 0.005$) at 24 h to 72 h post start of treatment. The highest EROD induction was seen after 48 h for both these treatments at the 64% v/v concentration. Exposure to ANS+SPC-1000 CEWAF gave a slightly higher overall induction compared to ANS+Corexit 9500 CEWAF.

Exposure to MESA+Corexit 9500 and MESA+SPC-1000 CEWAF produced higher EROD induction compared to the respective ANS CEWAFs although the induction profiles were similar. Maximum induction was again seen at the 64% v/v concentration, 48 h post start of exposure.

A summary of maximum EROD induction is presented in Table 3.

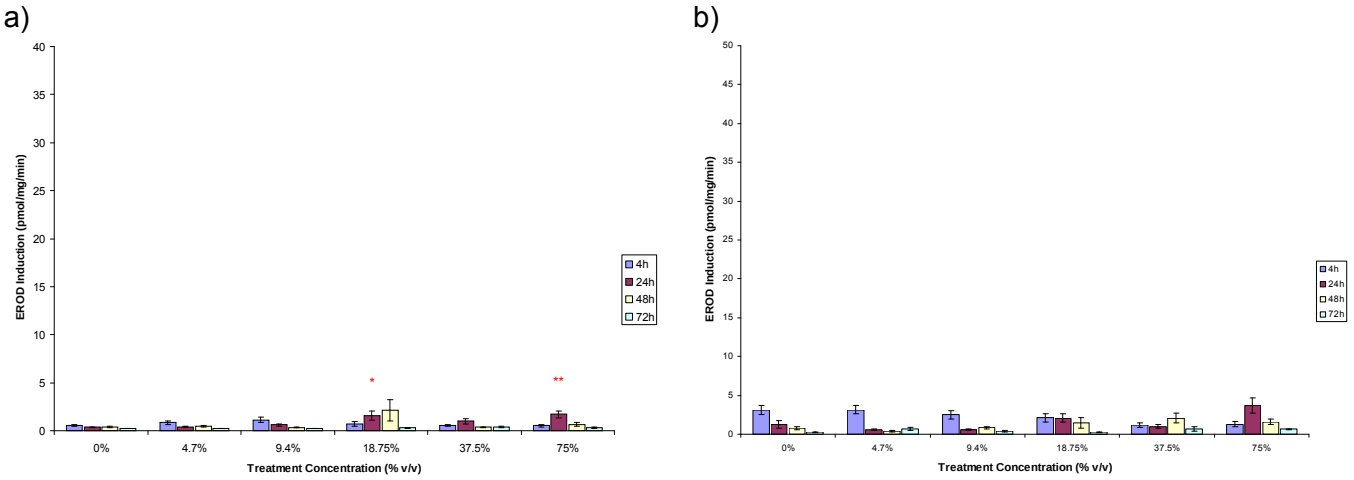

Figure 4. Mean EROD induction ± 1 SE for salmon exposed to a) ANS and b) MESA WAF for 4 hours (*=p<0.05; **=p<0.005)

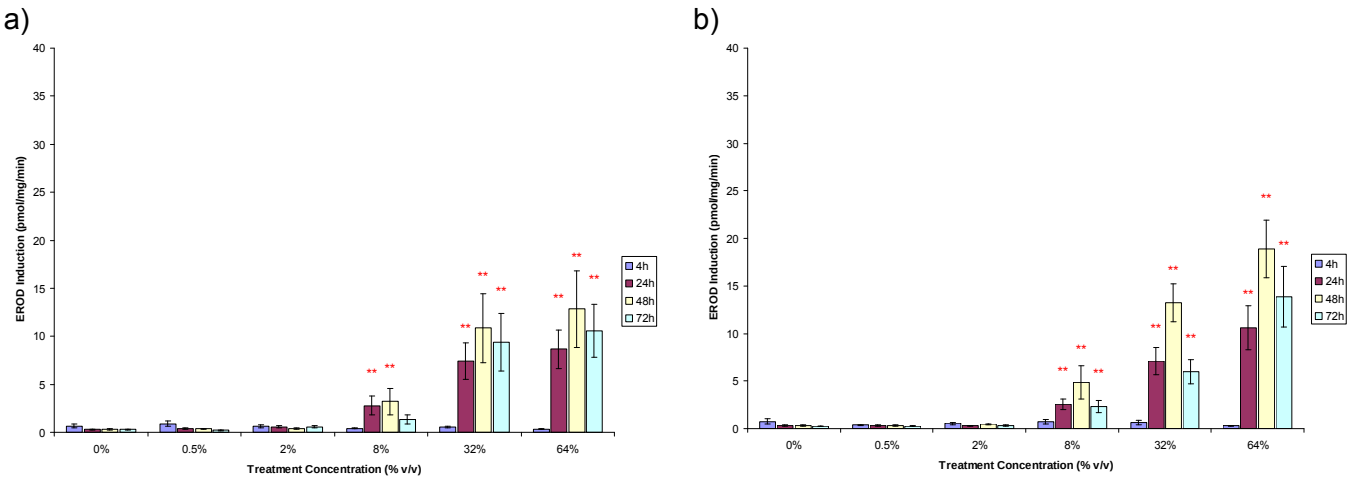

Figure 5 Mean EROD induction ± 1 SE for salmon exposed to a) ANS+Corexit 9500 and b) ANS+SPC-1000 CEWAF for 4 hours (*=p<0.05; **=p<0.005)

a) b)

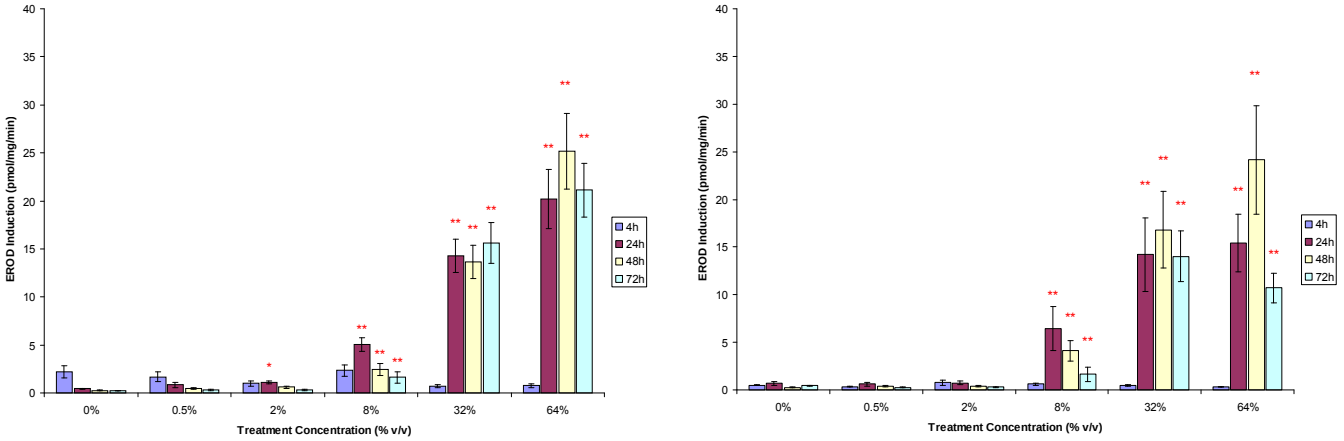

Figure 6. Mean EROD induction ± 1 SE for salmon exposed to a) MESA+Corexit 9500 and b) MESA+SPC-1000 CEWAF for 4 hours (*=p<0.05; **=p<0.005)

Table 3. Summary of maximum EROD induction (pmol/min/mg protein) for Atlantic cod and salmon exposed to various combinations of WAF and CEWAF.

	Cod			Salmon		
Toxicant	Concentration (% v/v)	Sampling Time-point (h)	EROD ± 1SE (pmol/min/mg protein)	Concentration (% v/v)	Sampling Time-point (h)	EROD ± 1SE (pmol/min/mg protein)
ANS WAF	75	24	8.79 ± 1.13	18.75	48	2.15 ± 1.11
MESA WAF	75	24	16.14 ± 1.11	75	24	3.71 ± 0.94
ANS + Corexit 9500 CEWAF	8	24	16.21 ± 2.01	64	48	12.88 ± 3.99
ANS + SPC-1000 CEWAF	8	24	18.12 ± 3.08	64	48	18.89 ± 3.04
MESA + Corexit 9500 CEWAF	8	24	15.58 ± 1.56	64	48	25.18 ± 3.95
MESA + SPC-1000 CEWAF	8	48	17.88 ± 2.01	64	48	21.14 ± 5.68

Comparison of mechanically and chemically dispersed oils.

From the obtained EROD data, comparisons were made against each of the different prepared WAFs and CEWAFs when exposed to each fish species to determine if there were any significant differences between treatments.

Table 4. Comparison of treatments for WAF and CEWAF exposures in Atlantic cod and salmon.

1) ANS WAF versus MESA WAF
2) ANS + Corexit 9500 versus MESA + Corexit
3) ANS + SPC-1000 Versus MESA + SPC-1000
4) ANS + Corexit 9500 versus ANS + SPC-1000
5) MESA + Corexit 9500 versus MESA + SPC-1000

When cod were exposed to WAF of ANS or MESA, the only significant difference in EROD induction was seen at 37.5% v/v at 48 and 72 h post start of exposure. No other significant differences were seen which suggests that the toxicity of the water fractions from both oils to cod is similar. With Atlantic salmon, the only significant differences were seen at 4.7% v/v (4 h) and 37.5% v/v (48 h). However, the 4.7% v/v concentration had a high result for the control at this time-point, therefore this significance is not considered valid.

When comparisons (of what ?) were made between the CEWAF combinations shown in Table 4 in either Atlantic cod or salmon, no significant differences were seen.

Water Analysis

Results from the analysis of collected water samples by fluorescence spectroscopy showed the prescence of PAHs in all exposure dilutions. This confirmed that the fish were exposed to levels of PAHs for the full 4 h experimental period. Some results however did not follow the expected dilution profile, these inconsistencies may be attributed to factors such as storage stability of the samples and non-homogenous sampling for analysis.

Levels of total PAH determined by GC-MS were much lower than that determined by fluorescence since this is a more selective technique. As would be expected, levels of PAHs were higher in CEWAF compared to the WAF samples. PAH concentrations were similar in each of the CEWAF samples indicating that the mixing method was consistent for each occasion.

Fish	Toxicant and Concentration (% v/v)		Fluorescence (Total PAH, µg/mL)		GC-FID (Total TPH, µg/mL)		GC-MS (Total PAH, ng/mL)	
			0 h	4 h	0 h	4 h	0 h	4 h
Cod	ANS WAF	0	0.005	0.013	-	-	-	-
		75	0.105	0.135	0.182	0.168	0	-
	MESA WAF	0	0.002	0.003	-	-	-	-
		75	0.181	0.136	0.482	0.327	0.711	-
	ANS+Corexit 9500 CEWAF	0	0.059	0.127	-	-	-	-
		64	27.453	17.75	5.724	1.701	3.622	-
	ANS+SPC-1000 CEWAF	0	ND	0.011	-	-	-	-
		64	60.609	13.647	5.293	1.355	2.791	-
	MESA+Corexit 9500 CEWAF	0	0.032	0.007	-	-	-	-
		64	36.515	7.383	4.252	1.535	2.863	-
	MESA+SPC-1000 CEWAF	0	0.008	0.012	-	-	-	-
		64	22.295	42.621	4.295	2.992	3.081	-
Salmon	ANS WAF	0	0.011	0.018	-	-	-	-
		75	0.147	0.168	0.825	0.407	-	-
	MESA WAF	0	0.004	0.008	-	-	-	-
		75	0.381	0.415	0.690	0.358	-	-
	ANS+Corexit 9500 CEWAF	0	1.171	0.676	-	-	-	-
		64	451.844	312.254	6.218	1.894	-	-
	ANS+SPC-1000 CEWAF	0	ND	0.004	-	-	-	-
		64	48.698	6.013	8.256	-	-	-
	MESA+Corexit 9500 CEWAF	0	0.379	0.166	-	-	-	-
		64	18.970	18.242	4.592	1.833	-	-
	MESA+SPC-1000 CEWAF	0	ND	0.009	-	-	-	-
		64	61.283	19.887	3.736	1.233	-	-

ND = Not detected
- = No sample analysed

Table 4. Total PAHs and TPH in exposure waters samples.

Oil droplet size determination

The nominal volume concentration for the WAF and CEWAFs was calculated to be 20 ppm and 15 ppm respectively based on the volumes of oil and sea water used to prepare the solutions and the sample size used for LISST analysis. For the majority of the prepared WAF and CEWAF stocks, the determined concentration volumes were close to nominal. For WAF, the mean determined concentration volumes ranged from *ca* 1 to 7 µL/L with an average volume mean diameter (VMD) of *ca* 44 to 229 µm. For CEWAF, the mean volume concentration ranged from *ca* 13 to 28 µL/L with an average VMD of *ca* 13 to 179 µm. These values for the most part stayed stable for the duration of the analysis for both WAF and CEWAF indicating that the oil droplets were quite stable and did not re-coalescence and there was little or no resurfacing of dispersed oil.

Li *et al* (2009) produced physically and chemically dispersed oils within a wave tank environment using the same crude oils and dispersants as used in this study. They found that the physically dispersed oils had VMD > 100 µm and chemically dispersed oil typically were < 150 µm depending on the type of wave energy used. These results reflected the oil droplet sizes obtained by the shaking flask method used in this study.

Discussion

The majority of studies reporting exposure of oil and oil dispersants to cod and salmon have concentrated on chronic exposure of these species to low concentrations of WAF and CEWAF.

In this study we found that when cod were exposed to WAF and CEWAF, the EROD induction profiles were different from those seen for salmon. Exposure to ANS WAF produced lower EROD induction in cod than that of MESA WAF, suggesting that MESA oil is the more toxic of the 2 oils. EROD induction increased up to the maximum exposure concentration (75% v/v) whereas EROD induction for CEWAF exposed cod peaked at 8% v/v then declined at the higher concentrations. Ramachandran *et al* (2004) saw this phenomenon at exposure concentrations >0.1% v/v when rainbow trout were exposed to the CEWAF of 3 different crude oils for 48 h. This decline was attributed to liver damage caused by the CEWAF (Gangnon and Holdway, 2000). Nava and Englehardt (1982) also noted this decreasing trend in EROD induction when the American eel, *Anguillia rostrata* were dosed with increasing levels of PAHs. However, the decline observed in the aforementioned studies may not be fully explained since they related to fresh water species and cod is a marine species. When salmon were exposed to the WAF in this study, no significant EROD induction was observed suggesting that the dissolved PAHs were not toxic to the fish. When exposed to CEWAF, EROD induction occurred and contrary to cod, activity increased to the highest exposure concentration of 75% v/v. A decline similar to Ramachandran's observations may have been expected with the salmon CEWAF exposures since both exposed groups of fish are salmonids, however this was not found to be the case. The fact that the salmon in this study were smolts and held in salt water may be a factor.

Steadmen *et al*, Nava *et al*, Payne *et al* postulate that there may be an upper limit beyond which increases in PAH concentration may result in no or deceased MFO activity

which may be species, age, sex *etc* dependant. This theory may explain the trends in EROD induction seen between the cod and salmon used in this study.

When comparisons were made against of the various combinations of CEWAFs used, there were no significant differences seen in the EROD induction of either cod or salmon. Wu *et al* (2012) found that for WAF, MESA was less toxic than ANS and for CEWAF, using Corexit 9500 dispersant, ANS was less toxic than for MESA when using Rainbow trout embryos. This contradicts the findings in this study as MESA WAF was found to be more toxic than ANS WAF in cod due to the higher EROD induction with no differences noted in salmon. For ANS plus Corexit 9500 and MESA plus Corexit 9500 CEWAFs, no differences were noted in either cod or salmon, therefore the toxicity of these dispersed oils are considered similar for both fish. Again, differences in toxicity could be explained by the fact that Wu *et al* used whole body Rainbow trout embryos and we used the livers from marine fish to determined ERDO activity.

Analysis of exposure water for TPH showed that the fish were exposed to similar levels of hydrocarbons, therefore the differences in response cannot be attributed to the respective exposure concentrations of the WAF and CEWAF. Levels of PAHs and TPHs did decrease from the start to the end of exposure. This may be attributed to adsorption of these compounds onto the glass of the aquaria and recirculation pump, volatisation of low molecular weight compounds caused by aeration of the exposure water and uptake by the fish, the latter was confirmed by the presence of EROD induction in the exposed fish.

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