



Impact of crude oil and the dispersant Corexit™ EC9500A on capelin (*Mallotus villosus*) embryo development



José Beirão^{a,b,*}, Lucie Baillon^c, Margaret A. Litt^a, Valérie S. Langlois^c, Craig F. Purchase^a

^a Fish Evolutionary Ecology Research Group, Biology Department, Memorial University of Newfoundland, St. John's, NL, A1B 3X9, Canada

^b Faculty of Biosciences and Aquaculture, Nord University, NO - 8049, Bodø, Norway

^c Institut national de la recherche scientifique (INRS), Centre Eau Terre Environnement, Quebec City, QC, G1K 9A9, Canada

ARTICLE INFO

Keywords:

Ecotoxicology
Petroleum hydrocarbons
Polycyclic aromatic hydrocarbons
Oil spill

ABSTRACT

Marine food webs are particularly vulnerable to oil spills if keystone species are impacted. To quantify lethal and sublethal toxicity in a key Holarctic forage fish, capelin embryos were exposed to Hibernia crude oil water accommodated fraction (WAF) produced at an oil-to-water ratio of 1:9 (v:v) and chemically-enhanced WAF (CEWAF) produced with the dispersant Corexit™ EC9500A at a dispersant-to-oil ratio of 1:10 (CEWAF H) or 1:50 (CEWAF L). Corexit alone yielded similar embryotoxicity to CEWAF. 10% CEWAF H, with total polycyclic aromatic hydrocarbons of 99.2 µg/L, decreased embryo survival following 10 h of exposure, while continual exposure to 1% CEWAF L decreased hatching and heart rates. Concentrations down to 0.1% CEWAF L increased in a dose-dependent manner the transcript level of cytochrome P4501a1 (*cyp1a1*) in hatched larvae. These data indicate that embryo-larval survival of capelin is likely at risk if an oil spill coincides in space and time with spawning.

1. Introduction

Marine oil spills are frequently treated with dispersants to disband floating oil and speed-up breakdown (Prince, 2015). For example, during the 2010 BP Deepwater Horizon oil spill, over 1.8 million gallons of dispersant were used (Rufe et al., 2011). Dispersants contain both solvents and surfactants that facilitate oil breakdown into tiny droplets that are more rapidly diluted and become more available for biodegradation (Major et al., 2012; Word et al., 2015). Because of their mode of action, dispersants can alone cause the disruption of biological membranes, and thus, are potentially dangerous for aquatic life (Word et al., 2015). In addition, the mixture of oil and dispersant releases greater concentrations of toxic oil components (e.g., polycyclic aromatic hydrocarbons - PAHs) into the water causing higher oil toxicity than untreated oil (e.g., Couillard et al., 2005; Berninger et al., 2011; Adams et al., 2014a). Consequently, there is great concern surrounding the long-term impacts of oil spills and the use of dispersants on marine ecosystems. Such impacts could be greater in relatively simple marine polar/temperate vs tropical food webs, particularly if keystone species are affected.

Capelin (*Mallotus villosus*) is the most important fish species in the Northwest Atlantic food web (and of significance in the Arctic, Pacific, and Northeast Atlantic), being the major forage for top predators, such

as cod, seabirds, and marine mammals (e.g., Davoren, 2013; Mullaney and Rose, 2014). Successful capelin reproduction and recruitment have direct implications on the functioning of the marine ecosystem in the Northwest Atlantic (see Carscadden and Vilhjálmsson, 2002 and references therein). However, their reproductive behaviour puts them at higher risk of oil spills than other fish. During their short reproductive season (a few weeks), capelin form dense schools in nearshore areas and then spawn either in demersal sites or on beaches (Penton and Davoren, 2013) making them particularly susceptible to the effects of oil spills that could concentrate nearshore. However, how petroleum products affect this keystone species is not well understood, despite current and substantial oil extraction and transport activities occurring near critical spawning habitats (e.g., Eastern Newfoundland, Canada).

Biological impacts of contaminants on aquatic life are often tied to reproduction (Couillard et al., 2005; Adams et al., 2014a). Much attention has been placed on sensitivity of embryos and larvae, particularly in species with external fertilization and embryo development (e.g., González-Doncel et al., 2008; Frantzen et al., 2012; Adams et al., 2014a; Martin et al., 2014; Incardona and Scholz, 2016). Different studies have reported that embryo exposure to the water accommodated fraction (WAF) of crude oil can lead to both lethal and sublethal effects, in a dose dependent manner, particularly during early cleavage stages and during heart development (Cherr et al., 2017).

* Corresponding author. Faculty of Biosciences and Aquaculture, Nord University, NO - 8049, Bodø, Norway.

E-mail address: jose.beirao-dos-santos@nord.no (J. Beirão).

<https://doi.org/10.1016/j.marenvres.2019.04.004>

Received 24 October 2018; Received in revised form 2 April 2019; Accepted 8 April 2019

Available online 10 April 2019

0141-1136/© 2019 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Sublethal effects such as larvae malformations, including edemas and skeletal and heart malformations (Adeyemo et al., 2015; Incardona and Scholz, 2016; Cherr et al., 2017), compromise later larval survival and could affect population recruitment. The measure of gene expression is increasingly used in ecotoxicology to detect precocious effects, like the induction of oxidative stress-related genes by toxic compounds (Madison et al., 2015). For example, PAHs are known to alter the expression of genes that control phase I and II detoxification and biotransformation of xenobiotics (Feng et al., 2013; Barjhoux et al., 2014; Vehniäinen et al., 2016).

How oil interactions with dispersant lethally and sub-lethally influence capelin embryo development is unknown. In the absence of dispersant, capelin embryo exposure to dissolved crude oil compounds, including hydrocarbons, affects hatching time and larvae morphology, with sublethal effects occurring at 10% of the lethal dose (Paine et al., 1991, 1992; Frantzen et al., 2012). Khan and Payne (2005) found that adult capelin are susceptible to both dispersant and chemically-dispersed crude oil, and Beirão et al. (2018) observed that dispersants can affect their sperm fertilization ability. The main objective of the present study was to quantify how crude oil treated with dispersant affects capelin embryo development in ways that would impair larval recruitment when embryos are exposed during all the embryonic development or only after late organogenesis. Early stage embryos are expected to be more sensitive to contaminants (McIntosh et al., 2010). Based on work in other species, we hypothesized that negative impacts of oil exposure are increased by the use of dispersant, and that acute lethal and sublethal responses are both important to adequately capture the overall embryotoxicity. To test this hypothesis, capelin embryos were exposed to different nominal concentrations of WAF from Hibernia crude oil and chemically-enhanced WAF (CEWAF) produced with the dispersant Corexit™ EC9500A. Different lethal (survival at different embryonic development stages and hatching) and sublethal effects (heart beat rate, larvae morphology, and expression of genes involved in xenobiotic biotransformation of phase I and II) were studied.

2. Material and methods

2.1. WAF and CEWAF production

Crude oil ($\rho = 0.759 \text{ g/ml}$) was obtained from the Hibernia offshore production platform located in the Northwest Atlantic, 315 km off the coast of Newfoundland, Canada. For exposure solutions, WAF and CEWAF were prepared according to the modified methods described in Beirão et al. (2018) and Martin et al. (2014) that largely follow Singer et al. (2000)'s recommendations using the dispersant Corexit™ EC9500A. Briefly, WAF was prepared by adding crude oil at an oil-to-water ratio (OWR) of 1:9 (v:v) using a salinity of 25 psu prepared with Instant Ocean® sea salt and distilled water, corresponding to 84 g of oil per L, and mixed for 18 h. We used 25 psu water as higher salinities are known to severely affect hatching performance (Purchase, 2018). For CEWAF preparation, we followed the same steps, but after the 18 h mixing, Corexit™ EC9500A was added at a dispersant-to-oil ratio (DOR) of 1:10 (CEWAF H) or 1:50 (CEWAF L), and mixed for an additional hour. These are the highest (H) and lowest (L) Corexit concentrations recommended by the producer (Nalco Environmental Solutions LLC 2012). The WAF, CEWAF H and CEWAF L solutions were then added to one of the 12 different glass jars containing 25 psu water to obtain 0.01, 0.1, 1, and 10% concentrations. Two solutions with dispersant alone were prepared by adding Corexit directly to water in the same proportion used in the CEWAF solution preparation (10 mL/L for Dispersant H and 2 mL/L for Dispersant L). The solutions were mixed 1:99 with 25 psu water to obtain the correspondent concentration of dispersant to the one used in the 1% CEWAF H and CEWAF L. A preliminary trial with dispersant concentration corresponding to 10% CEWAF H and CEWAF L resulted in 30–40% embryo survival at 8/

16 cell and close to 0% at the end of organogenesis. All solutions were freshly prepared each day and kept at 5 °C.

Chemical analyses of the treatments followed the procedures described by Beirão et al. (2018). Briefly, the samples were analyzed by gas chromatography mass spectrometry (GC-MS) using a C7–C40 saturated alkanes standard (Sigma Aldrich) in a combined scan/selected ion monitoring mode. Calibration curves were prepared with standards of 0.1, 2.0, 5.0 and 20.0 mg/L. The lowest calibration standard of 0.1 mg/L corresponds to 1.0 to 0.7 mg/L in the sample (given the concentration factor of 100–150), and anything lower than that was not quantified, but reported as ‘trace’. TPH were measured by GC – flame ionization detection. Parent PAHs were quantified using CRM48905 mix (Sigma Aldrich), that includes the 16 EPA PAHs. Calibration curves were prepared with standards of 0.01, 0.1, 2.0, 5.0, and 20.0 mg/L. For the samples without dispersant (seawater and WAF), the same rationale as above was followed: the 0.01 mg/L standard corresponds to 0.1 to 0.07 mg/L in the sample, and anything lower than that was not quantified. For the samples with dispersant (CEWAF) the baseline was noisier, which prevented proper integration of small peaks. The cut-off here was set at 0.5–1.0 mg/L. Of note, for the CEWAF H and for the dispersant alone solutions (Dispersant H and Dispersant L), there was visible breakthrough during the silica gel separation step and for the fraction containing the PAHs, there was co-elution of the internal standard with an unknown compound, so that no concentrations can be reported. Thus, we decided to only analyze the 10% treatment of both CEWAF H and CEWAF L. We assumed that the total petroleum hydrocarbon (TPH) and PAH concentrations of the tested treatments are proportional to the WAF stock solution dilutions and 10% CEWAF H and CEWAF L. For comparison with previous works looking at the crude oil toxicity in fish embryos, we present our data in relation to the TPAH instead of the nominal concentrations.

2.2. Capelin sampling and in vitro fertilization

Groups of beach spawning capelin were captured with a cast net repeatedly in July and August 2016 from different locations on the Avalon Peninsula, Newfoundland, Canada. Fish were transported 10–90 min in aerated coolers and then kept in flow through sea-water tanks between 7 and 10 °C until the next day. Gametes were collected within 24 h of capture. The experiment was repeated 10 times, with different pools of parent fish (randomized block design). Each block consisted of pooled eggs from 3 to 10 females, and pooled semen from 4 to 11 males. The fertilization procedure followed the technique described by Purchase (2018). All procedures followed Canadian guidelines on the use of research animals (Memorial University protocol 16-19-CP).

2.3. Embryo exposure

For each block, 15 treatments were tested (WAF 0.01, 0.1, 1, and 10%, CEWAF H 0.01, 0.1, 1, and 10%, CEWAF L 0.01, 0.1, 1, and 10%, dispersant H, dispersant L, and a negative control: 25 psu water). Three 50-mL glass beakers, each with 60 eggs, were used for each treatment, except the negative control for which only two beakers were used. From these three beakers, two were duplicate, in which the eggs were exposed to the treatments from 0 days (0 d) after fertilization until hatching. The third beaker of eggs had plain 25 psu water until the 6th day (6 d) after fertilization (corresponding to late organogenesis), after which, one of the 14 exposure treatments (all but the negative control) was added. This treatment is referred to as 6 d hereafter in contrast with 0 d for the embryos exposed since the day of fertilization. Thus, there were 44 incubating beakers, each with 60 fertilized eggs (exactly 2,640 embryos) for each of the ten blocks (see schematic Supplementary Fig. 1., N = 26,400 individually counted embryos). All beakers were kept in the dark at 10 °C following Purchase (2018) using plant growth chambers to control temperature. A semi-static exposure regime was

Table 1

Response variables measured in each block and the total number of embryos or larvae per block used per treatment to measure it. The exposure column indicates if the response variable was measured in embryos exposed to the treatments from 0 days after fertilization until hatching (0 d) or from the 6th day after fertilization (corresponding to late organogenesis) until hatching (6 d).

Response variable	Exposure	Block 1	Block 2 and 3	Block 4 - 10
Survival 8/16 cell	0 d	100–150	100–150	100–150
Survival end of organogenesis	0 d	20	20	20
Heart beating rate	0 d	–	–	5
Hatching	0 d and 6 d	100 (0 d) and 50 (6 d)	100 (0 d) and 50 (6 d)	100 (0 d) and 50 (6 d)
Larvae morphology	0 d and 6 d	10	10	10
Gene expression	0 d and 6 d	–	7	7

used. For this, following previous successful protocols (Purchase, 2018) approximately 50% of water (treatment) in each beaker was decanted off, and then replaced by new test solution every other day. To measure initial survival in each treatment, an additional group of 100–150 eggs were placed in 15 Petri dishes and exposed for 10 h at 10 °C (see Table 1), upon which they were scored as live (cell division, 8/16 cell stage) or dead (no cell division) as performed by Purchase (2018).

2.4. Embryo performance

At the 7th day of exposure, corresponding to the end of the organogenesis, embryo survival and the heart beating rate of survivors (Table 1) were evaluated under a stereomicroscope. The embryo survival rate was evaluated in 20 eggs per treatment randomly picked, 10 from each duplicate beaker, whereas the heart beating rate (beats per min) was evaluated in 5 embryos of these 20 eggs. If less than 5 embryos out of the 20, were alive at the end of organogenesis, heart beating rate was not evaluated. This only occurred in the treatments with higher nominal concentrations. The heart rate was measured as described by Adams et al. (2014a). In order to avoid temperature changes that could affect the heart rate, the embryos were moved one at the time from the incubation beaker into the stereomicroscope plate. The number of heart beats, visible through the transparent chorion, were then counted for 30 s. Only results from 7 blocks (blocks 4 to 10) were used to measure heart beating rate, because potential temperature problems were not considered in the first 3 blocks (Table 1). In order to keep the same density of embryos per beaker throughout the incubation period in all treatments, 10 randomly selected embryos were also removed from the 6 d group beakers on the same day the heart beating rate was evaluated in the sibling beakers.

At 10 °C, capelin eggs were expected to take 16–18 days to hatch (Purchase, 2018), so we began monitoring for hatch at day 11. The first 10 larvae to hatch in each treatment, if 10 hatched, were killed with an overdose of MS-222 (10 g/L) and preserved in 2.2% buffered formalin for larvae morphology measurements. In 9 of the 10 blocks, the subsequent 7 larvae to hatch were killed and placed in RNAlater buffer (Fisher Scientific) for gene expression analysis (Table 1). For a given beaker, once hatching started, if there were no new hatched larvae for three consecutive days (Purchase, 2018), it was assumed that no more would hatch and the beaker was discarded. The hatch success was calculated by dividing the total number of hatched larvae by 50 (number of eggs).

2.5. Larvae morphology

Fixed larvae were photographed with a measurement standard using a stereomicroscope camera and a 40× magnification lens. Using ImageJ (version 1.47f), the following measurements were taken:

standard length, from tip of the snout until the end of the notochord; preanal length, from the tip of the snout until the anal opening; head length, from the tip of snout to the end of the operculum; eye diameter; yolk area without the yolk sac; oil globule diameter; and myotome height, taken immediately posterior to the anal opening. Measures were taken to the nearest μm.

2.6. RNA extraction and qPCR analysis

Total RNA was extracted using RNeasy micro kits (QIAGEN, Ottawa, ON, CA) following the manufacturer's method. Larvae were pooled before RNA extraction in pools of 7 to obtain sufficient mRNA for qPCR analysis. A total of 9 pools per treatment were studied, corresponding to each block. Isolated RNA was re-suspended in 20 μL RNase free water and concentrations were measured using a NanoDrop-2000 spectrophotometer (Thermo-Fisher, Ottawa, ON, Canada). Total RNA (1 μg) was retro-transcribed in cDNA with a QuantiTect Reverse Transcription Kit (QIAGEN, Ottawa, ON, CA) according to the manufacturer's protocol and thermocycled for 15 min at 42 °C and inactivated at 95 °C for 3 min. Reverse-transcription and RT-qPCR methods were done following MIQE guidelines for qPCR (Bustin et al., 2009), that were validated by Madison et al. (2015, 2017). The cDNA products were diluted 80-fold prior to RT-qPCR amplification based on optimization runs for the genes of interest (cytochrome P4501A (*cyp1a*), aryl-hydrocarbon receptor (*ahr*), and glutathione-S-transferase (*gst-t*)). The primers were designed, cloned, sequenced, and blasted in NCBI database in order to verify that the qPCR product was the targeted sequence. These genes were selected based on literature showing that PAHs are known to induce the phase I and II of xenobiotic metabolism through AhR activation, and CYP1A induction was also described as a good biomarker in fish exposed to crude oil (Madison et al., 2015, 2017; Alsaadi et al., 2018). Each of the 9 samples were run in duplicate, including samples without reverse-transcriptase, no-template controls, and positive water controls. All genes of interest (Table 2) were assessed using relative change to the reference genes: *ribosomal protein subunit L8* (*rpl8*) and *elongation factor 1 α* (*ef1a*). The efficiency of all genes ranged between 90 and 110% and $R^2 > 0.99$. Data were normalized following both reference genes and the negative control group (25 psu water) in order to obtain the fold change compared to control.

2.7. Data analyses

Statistical tests were conducted using R 3.4.3 (R Core Team, 2017). In all cases, results were considered significantly different for $p < 0.05$. The underlying assumptions of our parametric tests were checked using the model's residuals with the Levene's test for homogeneity of variances and with histograms for the distribution of normality. Early

Table 2

Genes of interest and their associated biological function, custom designed primer sequences, and amplicon length (bp).

Biological function	Gene of interest	Primers (5'-3')	Amplicon length (bp)
Reference gene	<i>ef1a</i>	F: CCCAGGGTGAAAGCCAGGAG R: CCTGGACACAGGGACTTCATCC	284
Reference gene	<i>rpl8</i>	F: CGCCACCGTTATTTCCACACA R: GCAACAACACCAACAACGGC	89
Xenobiotic metabolism Phase I	<i>ahr</i>	F: GGTACCAGTTCATCCAGCAGC R: GCCACTGTTCTTGGTCAGCAACC	228
Xenobiotic metabolism Phase I	<i>cyp1a</i>	F: GACAAGGACAACATCCGTGACC R: GCCCAGGACAAAGCAGTGC	150
Xenobiotic metabolism Phase II	<i>gst-t</i>	F: TGGCTCAAAGGTGTTCTGGT R: CCCCAATGATGAAGGCTCTGT	150

Table 3

Concentrations in µg/L of petroleum hydrocarbons in the negative control (25 psµ water), water accommodated fraction (WAF) stock solution, and 10% chemically enhanced WAFs (CEWAF H and CEWAF L) used for embryo exposures at different concentrations (0.01%, 0.1%, 1%, and 10%). TPH: Total petroleum hydrocarbons.

n-alkane	25 psµ water (µg/L)	WAF (µg/L)	10% CEWAF H (µg/L)	10% CEWAF L (µg/L)
C11			31.8	
C12			129	1.8
C13			251	25.7
C14			348	52.9
C15		2.4	449	79.2
C16		13.8	413	85.9
C17		23.3	430	93.5
C18		23.4	358	80.6
C19		24.1	326	78.7
C20		24.7	318	78.1
C21		22.8	283	68.8
C22	0.7	21.9	255	62.2
C23	2.0	21.5	247	59.8
C24	2.9	21.0	223	53.5
C25	4.5	23.2	228	54.1
C26	3.8	20.0	199	46.5
C27	4.2	19.5	181	42.0
C28	4.4	16.3	145	34.5
C29	4.8	16.4	131	30.1
C30	4.8	14.5	10.0	24.6
C31	5.1	12.9	76.7	19.3
C32	5.2	11.3	55.0	15.1
C33		9.8	34.9	11.1
C34			26.7	10.2
Sum	43	352	5127	1108
Pristane	-	13.6	266	57
Phytane	-	17.8	290	62
TPH	43	1.85×10^3	33.6×10^3	6.00×10^3

The sum of the C11-C34 n-alkanes is highlighted in bold.

survival rate, survival at 7 days and heart rate were analyzed with a repeated measures ANOVA, using the 'lme' function of the nlme package, where group of eggs (block) was considered repeated (random effect) in each of the 15 treatments (fixed effects) (model 1).

DV ~ Treatment + Error (block/treatment) (model 1)

The hatching success results were analyzed with a similar 2-way ANOVA model, where both the 15 treatments and the start day of exposition to the different treatments, 0 d or 6 d, were considered fixed effects (model 2). There was a significant interaction between treatment and start day of exposition and the ANOVA model was simplified by creating one model for 0 d and another for 6 d (model 1).

DV ~ Treatment × Day + Error (block/treatment) (model 2)

The results for the larvae measurements and the gene expression were both analyzed with MANOVA models using the 'manova' function of the R stats package. The larvae measurements were closely related, thus to avoid the violation of multicollinearity in the MANOVA analysis, the seven measurements were first reduced by principal component analysis to two parameters (PC1 and PC2) using the 'princomp' function of the R stats package and calculated from the covariance matrix which explained 60% of the variance. The PC1, that explained 39% of the variance, mainly related with size parameters (total length, head length and pre-anal length), whereas the PC2, that explained 21% of the variance, was related with the yolk reserves (yolk area and oil globule diameter). A 2-way MANOVA model was used to test the effect of different treatments according to the start day of exposition on the larvae morphology, where PC1 and PC2 were considered the dependent variables (DV1 and DV2) (model 3). There was a significant interaction between treatment and start day of exposition and the model was simplified by creating one-way MANOVA models for 0 d and another for 6 d. Post-hoc ANOVAs were conducted for each PC individually

(model 1).

DV1, DV2, DV3 ~ Treatment × Day + Error (block) (model 3)

The gene expression was also analyzed using the model 3 MANOVA and each gene was considered a dependent variable (DV). The model was first analyzed saturated and then re-analyzed with the non-significant interaction removed. There were no significant differences between the two start days of exposition (0 d or 6 d), thus for the post-hoc analysis the results from embryos exposed from day 0 and from day 6 were pooled together. Three post-hoc 1-way ANOVA models (model 1) were conducted for each gene separately.

In all instances, when the ANOVA models detected significant differences, post-hoc analyses were conducted for multiple comparisons with Tukey's Honestly Significant Difference.

3. Results

3.1. Chemical effect of dispersant

Dispersant increased by several orders of magnitude the concentrations of individual petroleum hydrocarbon (Table 3) and PAHs (Table 4) in CEWAF compared to WAF for the same nominal concentration. The WAF stock solution had 1.85×10^3 µg/L of TPH and 12.0 µg/L of total PAHs (TPAH). Comparing both CEWAF solutions, the TPH concentration was about 5 times higher in the CEWAF H compared with the CEWAF L (Table 3), whereas the TPAH was about 4 times higher in the CEWAF H compared with the CEWAF L (Table 4). The 10% CEWAF H treatment had 33.6×10^3 µg/L TPH and 99.2 µg/L TPAH, whereas the 10% CEWAF L treatment had 6.0×10^3 µg/L TPH and 23.6 µg/L TPAH.

Table 4

List of polycyclic aromatic hydrocarbon (PAH) concentrations in µg/L in the negative control (25 psµ water), water accommodated fraction (WAF) stock solution and 10% chemically enhanced WAFs (CEWAF H and CEWAF L) used for embryo exposures at different concentrations (0.01%, 0.1%, 1%, and 10%).

PAHs ^a	25 psµ water (µg/L)	WAF (µg/L)	10% CEWAF H (µg/L)	10% CEWAF L (µg/L)
Naphthalene		T ^b	T	T
C-1 Naphthalenes		0.52	0.67	0.47
C-2 Naphthalenes		0.54	1.91	0.63
C-3 Naphthalenes		0.53	3.37	0.87
C-4 Naphthalenes		0.48	1.76	0.65
Acenaphthylene				
Acenaphthene				
Fluorene		2.37	13.4	0.99
C-1 Fluorenes		0.68	3.34	0.92
C-2 Fluorenes		0.67	3.33	1.01
C-3 Fluorenes				
Phenanthrene ^c	0.50	4.64	36.7	6.49
C-1 Phenanthrenes		0.87	8.30	1.94
C-2 Phenanthrenes		0.70	6.58	1.73
C-3 Phenanthrenes			2.76	1.02
Anthracene				
Fluoranthene				
Pyrene			2.02	1.10
Benz(a)anthracene				
Chrysene			8.66	2.44
C-1 Chrysenes			1.46	0.85
Benzo(b)fluoranthene ^d			2.20	1.41
Benzo(k)fluoranthene				
Benzo(a)pyrene				
Indeno(1,2,3-cd)anthracene				
Dibenzo(a)anthracene ^d			1.46	
Benzo(ghi)perylene ^d			1.33	1.12
Sum of all PAHs	0.5	12.0	99.2	23.6

^a Detection limits are estimated to be 0.1 µg/L for the 25 psµ water and WAF and 0.5–1 µg/L for the CEWAFs.

^b Trace amounts present, but not quantifiable.

^c Phenanthrene was present in dihydrophenanthrene, which was one of the surrogate standards added to all the samples. This adds a background level of approximately 0.5 µg/L to all the samples.

^d For these compounds, peaks were present at the expected retention time in the SIM trace, but their intensity in the scan trace was too low to allow for verification of identity by their mass spectrum.

3.2. Early survival and performance

After 10 h of exposure to the different treatments, when the embryos were between the 8- and 16-cell stage, there was already a significant ($F_{14, 124} = 10.8, p < 0.0001$) impact on survival in the highest CEWAF concentration (10%) corresponding to $33.6 \times 10^3 \mu\text{g/L}$ TPH and

$99.2 \mu\text{g/L}$ TPAH. Embryos kept in 25 psµ water had $91.3 \pm 1.5\%$ survival at this stage; whereas, only $68.8 \pm 7.1\%$ of the embryos exposed in the highest CEWAF H survived (Fig. 1, A). These values were measured in embryos kept separately in Petri dishes and could slightly differ from the results for embryos kept in the beakers for the whole embryonic development period (see 2.3. Embryo exposure). At the

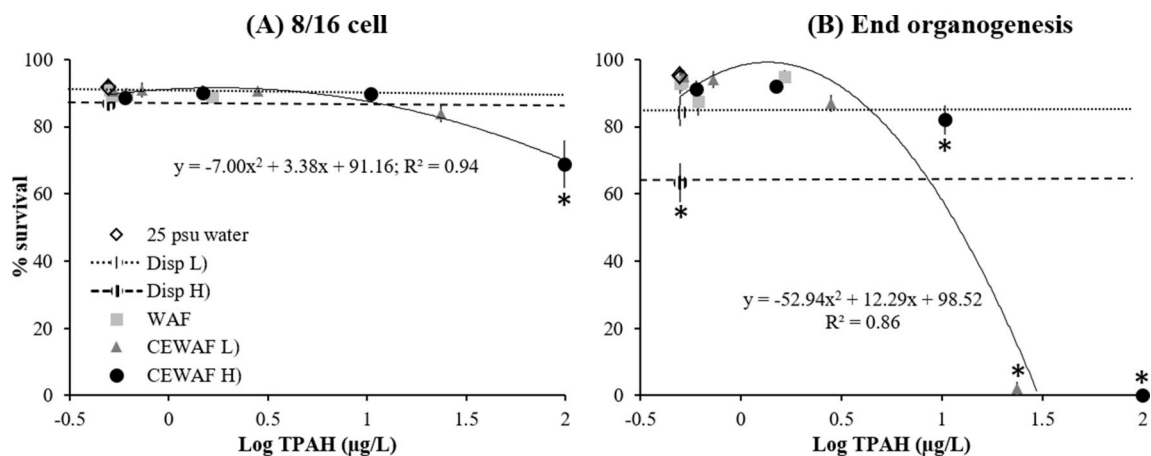


Fig. 1. Capelin embryo survival at (A) 8/16 cell stage and at (B) 7 days (end of organogenesis) in 25 psµ water (negative control), WAF, CEWAF H and L (0.01%, 0.1%, 1% and 10%), and dispersant Corexit™ EC9500A at the same concentration as CEWAF H 1% (Disp H) and CEWAF L 1% (Disp L). The data in the independent axis are plotted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Disp H and Disp L are represented by a horizontal dashed and a dotted line respectively. The symbols for these treatments and the correspondent error bars are placed in line with the 25 psµ water TPAH concentration, even though the PAH concentration could not be analyzed. Regressions for the effect of TPAH on the % survival are represented by a solid line (data from WAF and CEWAFs treatments merged). Significant differences between the treatments and the negative control (25 psµ water) are signed with a * ($p < 0.05$). Error bars represent SEM among different blocks ($n = 10$).

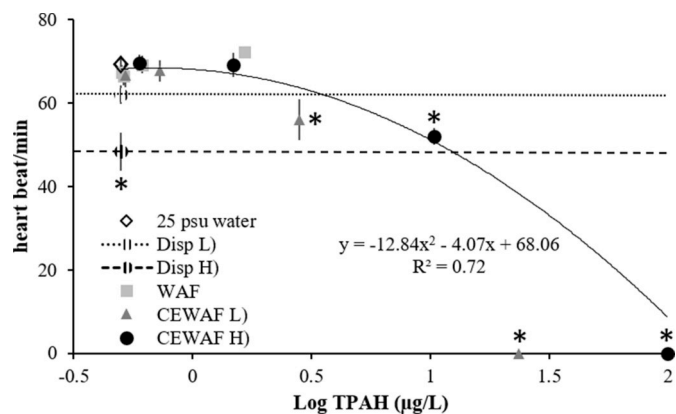


Fig. 2. Capelin embryo heart beat rate at the end of organogenesis in 25 psu water (negative control), WAF, CEWAF H and L (0.01%, 0.1%, 1% and 10%), and dispersant Corexit™ EC9500A at the same concentration as CEWAF H 1% (Disp H) and CEWAF L 1% (Disp L). The data in the independent axis are plotted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Disp H and Disp L are represented by a horizontal dashed and a dotted line respectively. The symbols for these treatments and the correspondent error bars are placed in line with the 25 psu water TPAH concentration, even though the PAH concentration could not be analyzed. A regression for the effect of TPAH on embryo heart rate is represented by a solid line (data from WAF and CEWAFs treatments merged). Significant differences between the treatments and the negative control (25 psu water) are signed with a * ($p < 0.05$). Error bars represent SEM among different blocks ($n = 7$).

seventh day of exposure, when the embryos reached the end of organogenesis, the negative effects of the CEWAF treatments were more evident ($F_{14, 125} = 144.9, p < 0.0001$). At this development stage, the survival rate in the 10% CEWAF H (corresponding to $33.6 \times 10^3 \mu\text{g/L}$ TPH and $99.2 \mu\text{g/L}$ TPAH) and 10% CEWAF L (corresponding to $6.0 \times 10^3 \mu\text{g/L}$ TPH and $23.6 \mu\text{g/L}$ TPAH) had decreased to 0 or close to 0% (Fig. 1, B). While the embryos kept in 1% CEWAF H ($\sim 34.0 \times 10^2 \mu\text{g/L}$ TPH and $\sim 10.4 \mu\text{g/L}$ TPAH) also had a significant decrease in survival with $82.0 \pm 4.5\%$ compared with $95.0 \pm 2.2\%$ for embryos kept in 25 psu water. Embryos exposed to the Dispersant H, also showed a lower survival at this stage ($63.3 \pm 5.8\%$).

The heart beat rate was also lower at the end of the organogenesis in the embryos kept in the higher CEWAF concentrations ($F_{14, 84} = 144.8,$

$p < 0.0001$). In the 10% CEWAF H and 10% CEWAF L, not enough embryos survived until this stage to measure the heart beat rate. Compared with the embryos kept in the 25 psu water with 69.3 ± 1.2 heart beats per min, there was a lower heart beat rate in live embryos kept in 1% CEWAF H ($\sim 34.0 \times 10^2 \mu\text{g/L}$ TPH and $\sim 10.4 \mu\text{g/L}$ TPAH), 1% CEWAF L ($\sim 6.4 \times 10^2 \mu\text{g/L}$ TPH and $\sim 2.8 \mu\text{g/L}$ TPAH), and Dispersant H, with respectively $52.0 \pm 1.9, 56.0 \pm 4.8,$ and 48.3 ± 4.5 heart beats per min (Fig. 2).

3.3. Hatching success

The 2-way ANOVA procedure on hatching success detected a significant interaction between treatment and the start day of exposition; thus, the main effects were not interpreted and separate ANOVAs for 0 d and 6 d were conducted. For the embryos exposed to the different treatments from day 0 (Fig. 3, A), there was a significant decrease in hatching success of embryos exposed to the treatments with higher dispersant concentration ($F_{14, 124} = 355.3, p < 0.0001$) compared with embryos kept in the 25 psu water ($85.9 \pm 5.3\%$). No hatching was observed for the highest CEWAF H ($33.6 \times 10^3 \mu\text{g/L}$ TPH and $99.2 \mu\text{g/L}$ TPAH) and CEWAF L ($6.0 \times 10^3 \mu\text{g/L}$ TPH and $23.6 \mu\text{g/L}$ TPAH); whereas, for the 1% CEWAF H ($\sim 34.0 \times 10^2 \mu\text{g/L}$ TPH and $\sim 10.4 \mu\text{g/L}$ TPAH) and the Dispersant H only a few larvae hatched in some blocks ($0.2 \pm 0.6\%$ and $0.7 \pm 1.3\%$ hatched in each of these treatments, respectively). Also the 1% CEWAF L ($\sim 6.4 \times 10^2 \mu\text{g/L}$ TPH and $\sim 2.8 \mu\text{g/L}$ TPAH) and the Dispersant L had a lower hatching ($59.4 \pm 10.1\%$ and $59.6 \pm 17.5\%$, respectively) compared with embryos kept in the 25 psu water. For embryos exposed only after the 6th day (late organogenesis) (Fig. 3, B), there was also a significant decrease in hatching success for several treatments ($F_{14, 126} = 89.4, p < 0.0001$). For the highest CEWAF H and CEWAF L there were no hatched larvae, whereas only a few larvae hatched in the Dispersant H ($2.4 \pm 1.6\%$). Moreover, the 1% CEWAF H and the Dispersant L also had a lower hatching ($50.6 \pm 7.1\%$ and $68.8 \pm 8.5\%$, respectively) compared with the 25 psu water ($85.4 \pm 1.6\%$). In this occasion, the embryos exposed to 1% CEWAF L showed similar hatching success ($73.8 \pm 3.8\%$) to the embryos in 25 psu water.

3.4. Larvae morphology

For the comparisons of the hatched larvae morphology, there was a

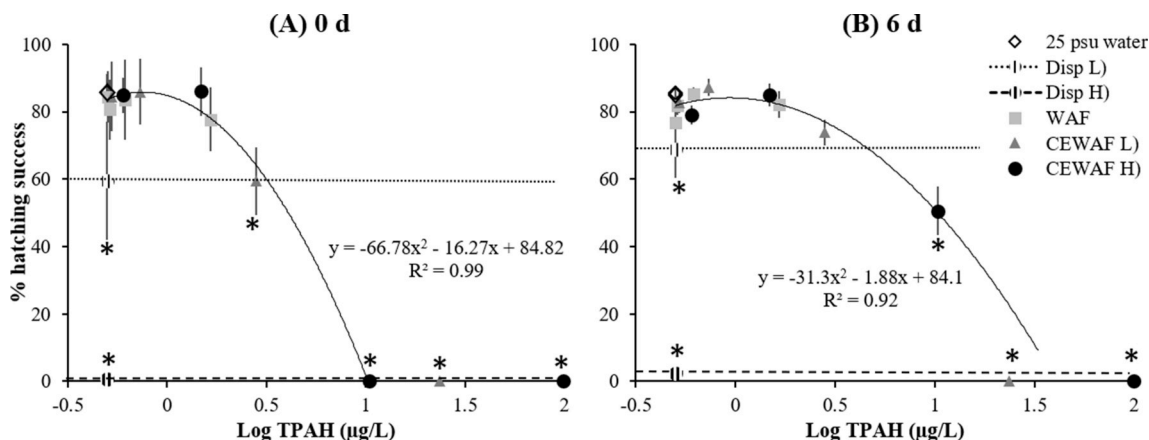


Fig. 3. Capelin embryo hatching success in 25 psu water (negative control), WAF, CEWAF H and L (0.01%, 0.1%, 1% and 10%) and dispersant Corexit™ EC9500A at the same concentration as CEWAF H 1% (Disp H) and CEWAF L 1% (Disp L). The left panel (A) represents embryos kept in the exposure treatments from day zero after fertilization until hatching (0d), whereas the right panel (B) represents embryos exposed to the different treatments from the 6th day after fertilization until hatching (6d). The data in the independent axis are plotted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Disp H and Disp L are represented by a horizontal dashed and a dotted line respectively. The symbols for these treatments and the correspondent error bars are placed in line with the 25 psu water TPAH concentration, even though the PAH concentration could not be analyzed. Regressions for the effect of TPAH on embryo hatching are represented by a solid line (data from WAF and CEWAFs treatments merged). Significant differences between the treatments and the negative control (25 psu water) are signed with a * ($p < 0.05$). Error bars represent SEM among different blocks ($n = 10$).

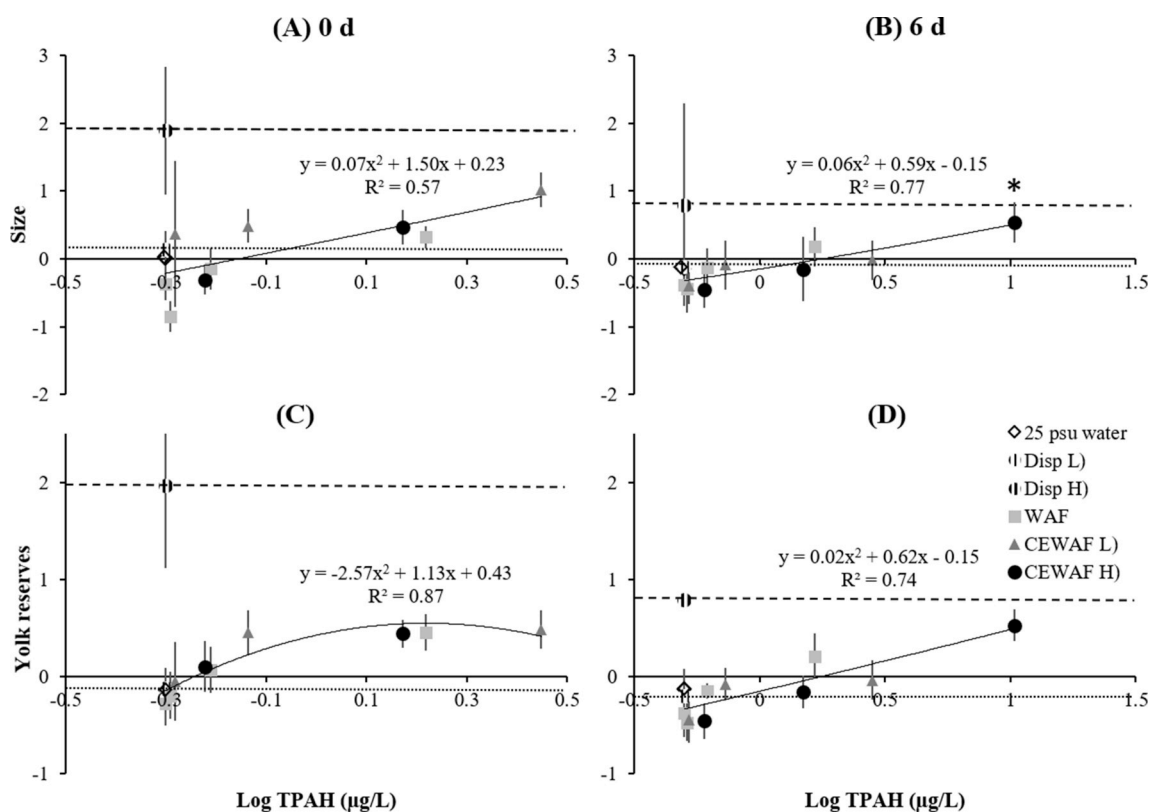


Fig. 4. Capelin larvae morphology for embryos kept in 25 psu water (negative control), and exposed to WAF, CEWAF H and L (0.01%, 0.1%, 1%, and 10%) and dispersant Corexit™ EC9500A at the same concentration as CEWAF H 1% (Disp H) and CEWAF L 1% (Disp L). The top panels (A and B) show larvae size (PC1), whereas the lower panels (C and D) show larvae yolk reserves (PC2). The left panels (A and C) represent larvae from embryos kept in the exposure treatments from day zero after fertilization until hatching (0 d), whereas the right panels (B and D) represent larvae from embryos exposed to the different treatments from the 6th day after fertilization until hatching (6 d). No data is displayed for some treatments because not enough larvae hatched in them. The data in the independent axis are plotted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Disp H and Disp L are represented by a horizontal dashed and a dotted line respectively. The symbols for these treatments and the correspondent error bars are placed in line with the 25 psu water TPAH concentration, even though the PAH concentration could not be analyzed. Regressions for the effect of TPAH on larvae morphology are represented by a solid line (data from WAF and CEWAFs treatments merged). Significant differences between the treatments and the negative control (25 psu water) are signed with a * ($p < 0.05$). Error bars represent SEM among different blocks ($n = 10$).

significant interaction in the MANOVA model between treatment and start day of exposition, thus, the main effects were not interpreted and separate MANOVAs for 0 d and 6 d were conducted. The post-hoc MANOVA model for embryos exposed from the first day (0 d) detected a significant effect of the treatments on larvae morphology (Pillai's = 0.1708, $F_{12, 1037}$, $p < 0.0001$). Similarly, the MANOVA model for embryos exposed to the different treatments only after late organogenesis (6 d) indicated that larvae morphology at hatching was affected by the different treatments (Pillai's = 0.2017, $F_{12, 1097}$, $p < 0.0001$). The post-hoc ANOVAs revealed that both larvae size (PC1) and yolk reserves (PC2) were affected by the different treatments ($F_{12, 86} > 2.2$, $p < 0.0196$ for 0 d; and $F_{12, 93} > 2.6$, $p < 0.0045$ for 6 d) (Fig. 4). Nonetheless, compared with larvae hatched from embryos kept in 25 psu water, the only difference was for larvae size (PC1) of 6 d larvae exposed to 1% CEWAF H ($\sim 34.0 \times 10^2 \mu\text{g/L}$ TPH and $\sim 10.4 \mu\text{g/L}$ TPAH). The morphology of the larvae exposed to the Dispersant H treatment was apparently different from the remaining treatments, but unfortunately, this data refers to a very low number of larvae (for this treatment only five larvae hatched for 0 d and four for 6 d in the 10 blocks), which decreases the power to detect significant differences with other treatments. Spine malformations were frequently observed in the higher concentration CEWAF treatments and in the dispersant alone treatments.

3.5. Gene expression

The MANOVA model for the gene expression detected significant differences between the treatments (Pillai's = 0.9130, $F_{13, 120}$, $p < 0.0001$), but not between the start day of exposition (Pillai's = 0.0072, $F_{1, 115}$, $p = 0.836$). The post-hoc ANOVAs indicate significant differences among treatments for both the *cyp1a* ($F_{13, 84} = 10.25$, $p < 0.0001$) and the *gst-t* expression ($F_{13, 85} = 3.08$, $p = 0.0009$) but not for *ahr* ($F_{13, 83} = 1.20$, $p = 0.29$) (Fig. 5). There was an increase in *cyp1a* mRNA level for the larvae from the embryos exposed to both 10% WAF and the 0.1% CEWAFs or higher, compared with larvae from embryos kept in 25 psu water by up 2-fold. There was no difference between larvae from embryos exposed to Dispersant L treatment and kept in 25 psu water. Only the larvae from embryos exposed to 0.1% CEWAF L ($\sim 1.0 \times 10^2 \mu\text{g/L}$ TPH and $\sim 0.7 \mu\text{g/L}$ TPAH) showed a significant different *gst-t* mRNA level compared with larvae kept in 25 psu water.

4. Discussion

The impacts of Hibernia crude oil on capelin embryo development were more pronounced in the presence of dispersant, but larval hatching alone failed to capture the toxicological sensitivity. Lifetime fitness of capelin larvae is likely compromised by sublethal impacts of oil exposure, including reduction in heart rate, abnormal morphology, and induction of the expression of some genes. The present study shows

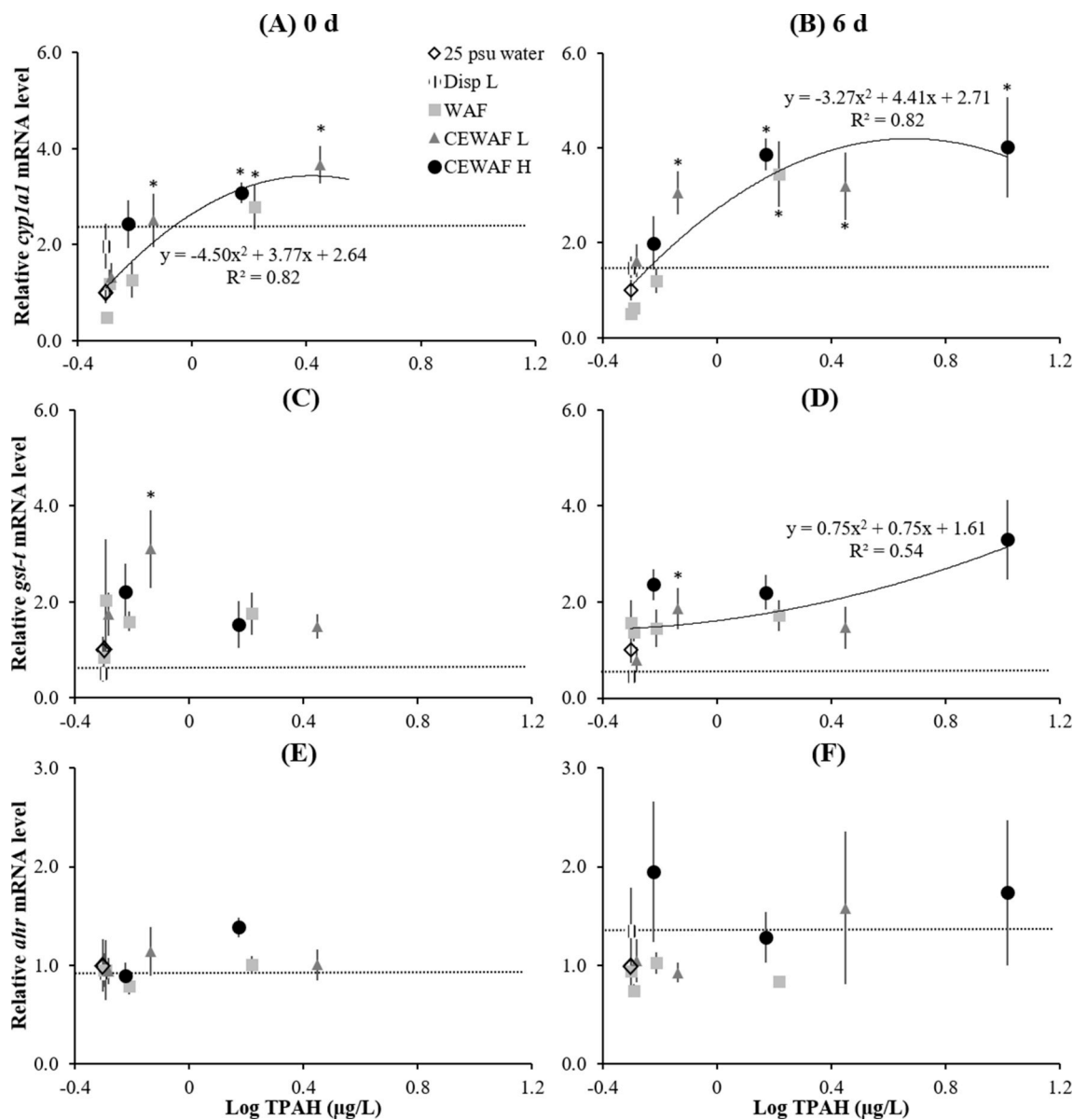


Fig. 5. Expression of genes involved in xenobiotic biotransformation of phase I and II: cytochrome P4501a (*cyp1a*) (A and B); glutathione-S-transferase (*gst-t*) (C and D) and; aryl hydrocarbon receptor (*ahr*) (E and F). Data are for capelin larvae hatched from embryos kept in 25 psu water (negative control), and exposed to WAF, CEWAF H and L (0.01%, 0.1%, 1% and 10%) and dispersant Corexit™ EC9500A at the same concentration as CEWAF L 1% (Disp L). The left panels (A, C and E) represent larvae from embryos kept in the exposure treatments from the day zero after fertilization until hatching (0 d), whereas the right panels (B, D and F) represent larvae from embryos exposed to the different treatments from the 6th day after fertilization until hatching (6 d). No data is displayed for some treatments because not enough larvae hatched in them. The data in the independent axis are plotted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Disp L is represented by a dotted line placed horizontally. The symbols for this treatment and the correspondent error bars are placed in line with the 25 psu water TPAH concentration, even though the PAH concentration could not be analyzed. Regressions for the effect of TPAH in the gene expression are represented by a solid line (data from WAF and CEWAFs treatments merged). Significant differences between the treatments and the negative control (25 psu water) are signed with a * ($p < 0.05$). For gene expression, there were no significant differences between the two start days of exposition (0d or 6d), thus the results were pooled together and the significant differences are the same for both 0d and 6d. Error bars represent SEM among different blocks ($n = 9$).

that in the event of an oil spill close in time and space to capelin spawning, the use of dispersants, such as Corexit™ EC9500A at high DOR may increase the concentration of crude oil toxicants, and dispersant compounds, to levels that could significantly reduce embryonal survival and later recruitment in affected areas.

As expected, the use of dispersant led to an increase (around 100-fold) in the amount of dissolved crude oil toxicants, namely petroleum hydrocarbons and PAHs in CEWAF treatments. Chemical dispersion increases fish embryotoxicity of oils (Wu et al., 2012; Adams et al., 2014a). It is known that chemical dispersion increases the exposure of embryos to PAHs in water (Adams et al., 2014a), and there is little

difference in toxicity between WAF and CEWAF when effects are compared with similar concentrations of hydrocarbons. According to Adams et al. (2014b), 3- and 4-ringed alkyl PAHs are the main compounds responsible for fish embryotoxicity. Nonetheless, other poorly characterized crude oil compounds, normally referred as unresolved complex mixtures, are also responsible for the dissolved crude oil toxicity (Melbye et al., 2009; Petersen et al., 2017). Most embryotoxicity that we observed in capelin embryos would have probably resulted from dissolved oil toxicants as observed by Carls et al. (2008) in zebrafish (*Danio rerio*).

Corexit producers recommend a dosage of dispersant-to-oil ratio

between 1:50 and 1:10 (Nalco Environmental Solutions LLC 2012). The CEWAF preparation methodology used was a modification of Martin et al. (2014), which is detailed in Beirão et al. (2018). During the CEWAF preparation, we added the dispersant in the center of the vortex on top of the oil; however, the short mixing time of 1 h could be insufficient for a complete oil chemical dispersion, especially when the highest DOR was tested (1:10), and consequently, there could be Corexit components free in the solution. To allow for full chemical dispersion of oil, some authors recommend adding the dispersant immediately after the oil vortex is established and stir the whole mixture for 18 h (e.g., DFO, 2017). Due to the procedures used, the presence of oil micro-droplets in the CEWAF treatments cannot be discarded. As observed in haddock (*Melanogrammus aeglefinus*) by Sørhus et al. (2015), these micro-droplets can adhere to the embryos and result in direct oil toxicants absorption by fish eggs. Furthermore, in the absence of oil, dispersant can act as a detergent on the external lipid membranes of the embryos, causing the destruction of the external embryo membranes and affecting oxygen transfer and osmotic regulation (Wu et al., 2012). Our data for Corexit™ EC9500A demonstrated that dispersant alone is toxic for capelin embryos and yielded similar levels of embryotoxicity to CEWAF treatments at the correspondent concentrations of Corexit. Indeed, apart from the *cyp1a1* expression, none of the other embryo-larvae performance measures were significantly different between the negative control (25 psu water) and the treatments without dispersant (WAF). This could indicate that part of the observed embryotoxicity is neither the result of additive or synergistic toxicity between crude oil toxicants and dispersant, but merely of the dispersants alone. Observations made by other authors in different fish species (e.g., Wu et al., 2012; Adams et al., 2014a), support the idea that the application of dispersants can lead to an additional source of toxicity to marine life whether dispersants reach the oil, or fail to reach it and remain free in the water column.

Developing embryos are susceptible to dissolved crude oil toxicants (McIntosh et al., 2010). In our study, oil alone had no effect on the earliest stages of development at the tested concentrations, but with the addition of dispersant, the highest tested CEWAF H concentration (10%), corresponding to $33.6 \times 10^3 \mu\text{g/L}$ TPH and $99.2 \mu\text{g/L}$ TPAH, caused a significant decrease in embryo survival after 10 h of exposure. In our previous study (Beirão et al., 2018), we observed that sperm fertilizing ability was affected by even lower concentrations of dissolved hydrocarbons ($\sim 16.1 \times 10^3 \mu\text{g/L}$ TPH and $\sim 47.9 \mu\text{g/L}$ TPAH). In the present study, embryos were only exposed to the contaminants after fertilization (oocytes and sperm mixing); thus, either the fertilized egg's activation pathway or mechanisms controlling early cell divisions were affected.

Hatching success was not affected by the tested WAF treatments, even at 10% ($\sim 2.2 \times 10^2 \mu\text{g/L}$ TPH and $\sim 1.7 \mu\text{g/L}$ TPAH). After adding the dispersant, 1% CEWAF L ($\sim 6.4 \times 10^2 \mu\text{g/L}$ TPH and $\sim 2.8 \mu\text{g/L}$ TPAH) or higher loadings caused a reduction in capelin hatching when embryos were exposed during the complete period of embryonic development. Similarly, when Carls et al. (1999) exposed herring eggs for 16 days (in our study capelin eggs were exposed between 15 and 25 days) to $0.7\text{--}7.6 \mu\text{g/L}$ TPAH, they observed an increase in embryo mortality, larvae malformations, reduced swimming, and genetic damages. On the other hand, Frantzen et al. (2012) also tested capelin embryotoxicity, but they only observed significant reductions in embryo survival and hatching at the concentration of $40 \mu\text{g/L}$ TPAHs of Kobbe crude oil from the Goliat field from Barents Sea. In their study, they exposed embryos from the end of gastrulation, tested water soluble fraction without dispersants and used a different exposition scenario, with decreasing TPAH with time in contrast with our study that kept the concentrations stable. Paine et al. (1992) exposed capelin embryos to $4.8 \times 10^3 \mu\text{g/L}$ TPH and did not obtain any hatchings. In our case, 10% CEWAF L ($60.0 \times 10^2 \mu\text{g/L}$ TPH and $23.6 \mu\text{g/L}$ TPAH) cause a complete fail to hatch, while for 1% CEWAF H ($\sim 34.0 \times 10^2 \mu\text{g/L}$ TPH and $10.4 \mu\text{g/L}$ TPAH), a few larvae hatched. Whereas, in Frantzen et al.

(2012), at $81.2 \mu\text{g/L}$ TPAH around 10% of capelin larvae hatched. Once again, this difference should be related to the different exposure scenarios that include the initial embryonic developmental stages in our study, semi-static vs flow-through settings, oil types and the use of dispersants. In our study, Corexit alone at a concentration of 2 mL/L also caused a decrease in the hatching rate. If embryos were only exposed after the organogenesis (6 d treatments), the lowest tested concentration that affected hatching rate was 1% CEWAF H. Similar to our results, Paine et al. (1992) also observed lower embryotoxicity when embryos were exposed at a later development stage. McIntosh et al. (2010) testing MESA crude oil in Atlantic herring described that the greatest sensitivity of embryos occurred during the early embryonic stages after fertilization. Under our exposition scenario, lethal effects are found at concentrations as low as $\sim 2.8 \mu\text{g/L}$ TPAH if the embryos are exposed throughout the embryonic development.

The WAF treatments did not affect the embryos' heart beating rate, probably because of the low concentration of dissolved crude oil toxicants, as reflected by the TPAH measured values in these treatments. On the other hand, the decrease in the heart rate (bradycardia) observed in the higher concentration CEWAF treatments, confirms the idea that the embryo heart malformations due to exposure to crude oil toxicants also occur early in capelin embryo development. Heart malformations at the embryonic stage are frequently associated with lower hatching rates or larval survival (e.g., Carls et al., 1999; Esbaugh et al., 2016; Incardona and Scholz, 2016). Indeed, as reviewed by Incardona and Scholz (2016), several morphological defects caused by exposure to PAHs are a result of cardiac dysfunction. Except for the embryos exposed to the 1% CEWAF L treatment ($\sim 2.8 \mu\text{g/L}$ TPAH), the remaining treatments that presented bradycardia at the end of the organogenesis, failed to hatch. However, we cannot attribute the hatching failure solely to the heart malformations at this early stage, since embryos were continuously exposed to the different treatments until the hatching stage, and thus, other malformations could have contributed. Indeed, exposure after this stage, in the 6 d treatments for example, also caused failure to hatch and alterations in the expression of some genes. The sublethal embryonic cardiotoxicity is usually attributed to high molecular weight PAHs (Incardona and Scholz, 2016). As explained by different authors (Carls et al., 1999; Adams et al., 2014a; Incardona and Scholz, 2016), bradycardia is normally associated with pericardial edema and tube heart malformation, which cause lower blood pressure and accumulation of fluid in the pericardium leading to delayed development. Additionally, our treatment with higher proportion of Corexit (10 mL/L ; Dispersant H) also caused bradycardia, similar to the observations by Adeyemo et al. (2015) in *Menidia beryllina* embryos. These authors attributed this effect to the dispersant interaction with cellular membranes. The higher tested concentrations of crude oil toxicants in our study clearly caused bradycardia in embryos at the end of organogenesis and this is likely to be one of the causes for hatching failure.

The appearances of larvae malformations after embryo exposure to petroleum hydrocarbons is a condition frequently reported in different fish species (Adeyemo et al., 2015; Nahrgang et al., 2016; Bosker et al., 2017), including for capelin (Paine et al., 1992), and normally associated with a secondary effect of heart malformations (Incardona and Scholz, 2016). In our study, we failed to detect overall malformations in the hatched larvae in both WAF and CEWAF treatments as well as any effect in time to hatch (data not shown). Likewise, Frantzen et al. (2012) failed to detect developmental deformities after capelin embryos were exposed to different concentrations of water soluble fraction of crude oil.

The expression of the molecular indicator of phase I detoxification, *cyp1a*, was altered by Hibernia crude oil exposure. 10% WAF concentration ($\sim 2.2 \times 10^2 \mu\text{g/L}$ TPH and $\sim 1.65 \mu\text{g/L}$ TPAH) increased *cyp1a* expression levels by up to 2-fold. Whereas for the CEWAF solutions, there was an upregulation in *cyp1a* for all the treatments with a concentration equal or higher than ($\sim 1.0 \times 10^2 \mu\text{g/L}$ TPH and

~0.73 µg/L TPAH) compared with larvae from embryos kept in 25 psu water. The *cyp1a* upregulation is linked to the activation of the xenobiotic response via induction of the phase I of detoxification and has been tested as a bio-indicator of PAH exposure in other fish embryos (e.g., Madison et al., 2015; Madison et al., 2017; Alsaadi et al., 2018; Bosker et al., 2017). In our study, there was an enhanced *cyp1a* expression proportional to the PAH bioavailability in both WAF and CEWAF solutions, but not to embryotoxicity, which seemed more related to dispersants exposure. Groups of embryos exposed to Corexit alone at a concentration of 2 mL/L (Dispersant L) during all the embryonic development and only after late organogenesis had decreased hatching rate, but the *cyp1a* expression did not change. Similar observations were made by Madison et al. (2015, 2017) in medaka (*Oryzias latipes*) that despite significant morphological modifications caused by dispersant control, there was no effect on *cyp1a* expression. Interestingly, no difference was observed between fish exposed from 1 or 6-days after fertilization. This suggests an early establishment of xenobiotic metabolism after fertilization, which has also been demonstrated in fish embryos exposed to diluted bitumen where *cyp1a* induction was considered as a good biomarker of PAH exposure (Nahrgang et al., 2010). The other two genes measured, that were also involved in xenobiotics metabolism, were not affected by the exposure to Hibernia crude oil (i.e., *ahr*) or did not exhibit a concentration-response profile (i.e., *gst-t*).

5. Conclusion

Oil drilling and transportation in the Newfoundland region creates risk of an oil spill, which could be particularly problematic if overlapping in space and time with capelin reproduction. How such an oil spill is treated is predicted to affect the impact. Chemical dispersion may benefit some species (e.g., seabirds (Prince, 2015)), but increases toxicity to fishes. We show that the higher tested concentration of chemically dispersed oil 10% CEWAF H (corresponding to 99.2 µg/L TPAH) was lethal to capelin embryos within only 10 h of exposure, and completely impaired their survival if exposed for longer periods. Corexit caused similar levels of embryotoxicity as the CEWAF treatments at the correspondent dispersant concentration. On the other hand, CEWAF L sublethal effects, which can subsequently affect larval survival, were observed at nominal loadings 100 times lower (~0.73 µg/L TPAH). Both of these concentrations are well within the range of what can be observed in the event of an oil spill, as an example, during the 2010 BP Deepwater Horizon oil spill, Diercks et al. (2010) reported concentrations of TPAH as high as 189 µg/L in subsurface waters.

Capelin eggs are not pelagic, but rather adhere to the substrate, either on beaches or offshore. In the event of an oil spill close to their spawning grounds, they will be continuously exposed to the components released in the water. The present study establishes embryotoxicity for capelin to dissolved crude oil toxicants, including TPAHs. In the event of an oil spill during capelin spawning season, this could be compounded by bioaccumulative effects on adults that are passed to the embryo. We analyzed the oil dispersant Corexit™ EC9500A because of its widespread use and for the several concerns regarding its effect on marine life that have been raised in the past decade (e.g., Berninger et al., 2011; Word et al., 2015). In the event of an oil spill that reaches coastal areas, where most Newfoundland capelin spawn, shoreline washing agents would normally be preferred to oil dispersants as an oil spill response. Shoreline washing agents are in general less toxic for marine organisms than dispersants (e.g., Hansen et al., 2014).

Capelin are a keystone species in the Northwest Atlantic food web, spawning for a short period in dense aggregations both offshore and on beaches, which are areas known to be vulnerable to the accumulation of crude oil and dispersants in the event of a spill. This study indicates that capelin embryo survival and development are likely to be compromised in areas impacted by an oil spill. Although more work would be needed to determine the impacts of spill-related embryo-larval toxicity on adult

capelin recruitment, given the importance of this species to the food-web in the Northwest Atlantic, actions should be taken to minimize risks of oil spills during times and places where capelin spawn.

Acknowledgments

Advice from Barry Madison (Queen's University) and Penny Morrill (MUN) helped in development of the WAF and CEWAF preparation methods. We are also thankful to Geert Van Biesen from the stable isotope facility at Memorial University for the chemical analyses of the solutions. Comments from two anonymous reviewers improved an earlier version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2019.04.004>.

Funding

This work was supported by grants to CFP and VSL from the National Contaminants Advisory Group of the Department of Fisheries and Oceans Canada. In addition, funding was provided to CFP from the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, and the Research and Development Corporation of Newfoundland and Labrador. VSL holds a Canada Research Chair (CRC 950-230442).

References

- Adams, J., Swezey, M., Hodson, P.V., 2014a. Oil and oil dispersant do not cause synergistic toxicity to fish Embryos. *Environ. Toxicol. Chem.* 33, 107–114. <https://doi.org/10.1002/etc.2397>.
- Adams, J., Bornstein, J.M., Munno, K., Hollebone, B., King, T., Brown, R.S., Hodson, P.V., 2014b. Identification of compounds in heavy fuel oil that are chronically toxic to rainbow trout embryos by effects-driven chemical fractionation. *Environ. Toxicol. Chem.* SETAC 33, 825–835. <https://doi.org/10.1002/etc.2497>.
- Adeyemo, O.K., Kroll, K.J., Denslow, N.D., 2015. Developmental abnormalities and differential expression of genes induced in oil and dispersant exposed *Menidia beryllina* embryos. *Aquat. Toxicol.* 168, 60–71. <https://doi.org/10.1016/j.aquatox.2015.09.012>.
- Alsaadi, F., Madison, B.N., Brown, R.S., Hodson, P.V., Langlois, V.S., 2018. Morphological and molecular effects of two diluted bitumens on developing fathead minnow (*Pimephales promelas*). *Aquat. Toxicol.* 204, 107–116. <https://doi.org/10.1016/j.aquatox.2018.09.003>.
- Barjhoux, I., Cachot, J., Gonzalez, P., Budzinski, H., Le Menach, K., Landi, L., Morin, B., Baudrimont, M., 2014. Transcriptional responses and embryotoxic effects induced by pyrene and methylpyrene in Japanese medaka (*Oryzias latipes*) early life stages exposed to spiked sediments. *Environ. Sci. Pollut. Res.* 21, 13850–13866. <https://doi.org/10.1007/s11356-014-2895-7>.
- Beirão, J., Litt, M.A., Purchase, C.F., 2018. Chemically-dispersed crude oil and dispersant affects sperm fertilizing ability, but not sperm swimming behaviour in capelin (*Mallotus villosus*). *Environ. Pollut.* 241, 521–528. <https://doi.org/10.1016/j.envpol.2018.05.080>.
- Berninger, J.P., Williams, E.S., Brooks, B.W., 2011. An initial probabilistic hazard assessment of oil dispersants approved by the United States National Contingency Plan. *Environ. Toxicol. Chem.* 30, 1704–1708. <https://doi.org/10.1002/etc.532>.
- Bosker, T., van Balen, L., Walsh, B., Sepúlveda, M.S., DeGuise, S., Perkins, C., Griffitt, R.J., 2017. The combined effect of Macondo oil and corexit on sheepshead minnow (*Cyprinodon variegatus*) during early development. *J. Toxicol. Environ. Health* 80, 477–484. <https://doi.org/10.1080/15287394.2017.1340208>.
- Bustin, S.A., Benes, V., Garson, J.A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55 (4), 611–622. <https://doi.org/10.1373/clinchem.2008.112797>.
- Carls, M.G., Holland, L., Larsen, M., Collier, T.K., Scholz, N.L., Incardona, J.P., 2008. Fish embryos are damaged by dissolved PAHs, not oil particles. *Aquat. Toxicol.* 88, 121–127. <https://doi.org/10.1016/j.aquatox.2008.03.014>.
- Carls, M.G., Rice, S.D., Hose, J.E., 1999. Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasii*). *Environ. Toxicol. Chem.* 18, 481–493. [https://doi.org/10.1897/1551-5028\(1999\)018%3c0481:SOFTFW%3e2.3.CO;2](https://doi.org/10.1897/1551-5028(1999)018%3c0481:SOFTFW%3e2.3.CO;2).
- Carscadden, J.E., Vilhjálmsson, H., 2002. Capelin - what are they good for? Introduction. *ICES J. Mar. Sci.* 59, 863–869. <https://doi.org/10.1006/jmsc.2002.1283>.

- Cherr, G.N., Fairbairn, E., Whitehead, A., 2017. Impacts of petroleum-derived pollutants on fish development. *Annu. Rev. Anim. Biosci.* 5, 185–203. <https://doi.org/10.1146/annurev-animal-022516-022928>.
- Couillard, C.M., Lee, K., Legare, B., King, T.L., 2005. Effect of dispersant on the composition of the water-accommodated fraction of crude oil and its toxicity to larval marine fish. *Environ. Toxicol. Chem.* 24, 1496–1504. <https://doi.org/10.1897/04-267R.1>.
- Davoren, G.K., 2013. Distribution of marine predator hotspots explained by persistent areas of prey. *Mar. Biol.* 160, 3043–3058. <https://doi.org/10.1007/s00227-013-2294-5>.
- DFO, 2017. A Framework for the development of standard methods to evaluate the toxicity of petroleum hydrocarbons on aquatic organisms. DFO Can. Sci. Adv. Sec. Sci. Adv. Rep. 24. 2017/053. http://publications.gc.ca/collections/collection_2018/mpo-dfo/fs70-6/Fs70-6-2017-053-eng.pdf.
- Diercks, A.-R., Highsmith, R.C., Asper, V.L., Joung, D., Zhou, Z., Guo, L., Shiller, A.M., Joye, S.B., Teske, A.P., Guinasso, N., Wade, T.L., Lohrenz, S.E., 2010. Characterization of subsurface polycyclic aromatic hydrocarbons at the Deepwater Horizon site. *Geophys. Res. Lett.* 37, L20602. <https://doi.org/10.1029/2010GL045046>.
- Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., Hoening, R., Brown, T.L., French, B.L., Linbo, T.L., Lay, C., Forth, H., Scholz, N.L., Incardona, J.P., Morris, J.M., Benetti, D.D., Grosell, M., 2016. The effects of weathering and chemical dispersion on Deepwater Horizon crude oil toxicity to mahi-mahi (*Coryphaena hippurus*) early life stages. *Sci. Total Environ.* 543, 644–651. Part A. <https://doi.org/10.1016/j.scitotenv.2015.11.068>.
- Feng, S., Cao, Z., Wang, X., 2013. Role of aryl hydrocarbon receptor in cancer. *Biochim. Biophys. Acta BBA - Rev. Cancer* 1836, 197–210. <https://doi.org/10.1016/j.bbcan.2013.05.001>.
- Frantzen, M., Falk-Petersen, I.-B., Nahrang, J., Smith, T.J., Olsen, G.H., Hangstad, T.A., Camus, L., 2012. Toxicity of crude oil and pyrene to the embryos of beach spawning capelin (*Mallotus villosus*). *Aquat. Toxicol.* 108, 42–52. <https://doi.org/10.1016/j.aquatox.2011.09.022>.
- González-Doncel, M., González, L., Fernández-Torija, C., Navas, J.M., Tarazona, J.V., 2008. Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: studies with Prestige oil and medaka (*Oryzias latipes*). *Aquat. Toxicol.* 87, 280–288. <https://doi.org/10.1016/j.aquatox.2008.02.013>.
- Hansen, B.H., Altin, D., Bonaunet, K., Overjordet, I.B., 2014. Acute toxicity of eight oil spill response chemicals to temperate, boreal, and Arctic species. *J. Toxicol. Environ. Health* 77, 495–505. <https://doi.org/10.1080/15287394.2014.886544>.
- Incardona, J.P., Scholz, N.L., 2016. The influence of heart developmental anatomy on cardiotoxicity-based adverse outcome pathways in fish. *Aquat. Toxicol.* 177, 515–525. <https://doi.org/10.1016/j.aquatox.2016.06.016>.
- Khan, R.A., Payne, J.F., 2005. Influence of a crude oil dispersant, corexit 9527, and dispersed oil on capelin (*Mallotus villosus*), Atlantic cod (*Gadus morhua*), longhorn sculpin (*Myoxocephalus octodecemspinosus*), and cunner (*Tautoglabrus adspersus*). *Bull. Environ. Contam. Toxicol.* 75, 50–56. <https://doi.org/10.1007/s00128-005-0717-9>.
- Madison, B.N., Hodson, P.V., Langlois, V.S., 2017. Cold Lake Blend diluted bitumen toxicity to the early development of Japanese medaka. *Environ. Pollut.* 225, 579–586. <https://doi.org/10.1016/j.envpol.2017.03.025>.
- Madison, B.N., Hodson, P.V., Langlois, V.S., 2015. Diluted bitumen causes deformities and molecular responses indicative of oxidative stress in Japanese medaka embryos. *Aquat. Toxicol.* 165, 222–230. <https://doi.org/10.1016/j.aquatox.2015.06.006>.
- Major, D., Zhang, Q., Wang, G., Wang, H., 2012. Oil-dispersant mixtures: understanding chemical composition and its relation to human toxicity. *Toxicol. Environ. Chem.* 94, 1832–1845. <https://doi.org/10.1080/02772248.2012.730202>.
- Martin, J.D., Adams, J., Hollebone, B., King, T., Brown, R.S., Hodson, P.V., 2014. Chronic toxicity of heavy fuel oils to fish embryos using multiple exposure scenarios. *Environ. Toxicol. Chem.* 33, 677–687. <https://doi.org/10.1002/etc.2486>.
- McIntosh, S., King, T., Wu, D., Hodson, P.V., 2010. Toxicity of dispersed weathered crude oil to early life stages of Atlantic herring (*Clupea harengus*). *Environ. Toxicol. Chem.* 29, 1160–1167. <https://doi.org/10.1002/etc.134>.
- Melbye, A.G., Brakstad, O.G., Hokstad, J.N., Gregersen, I.K., Hansen, B.H., Booth, A.M., Rowland, S.J., Tollefsen, K.E., 2009. Chemical and toxicological characterization of an unresolved complex mixture-rich biodegraded crude oil. *Environ. Toxicol. Chem.* 28, 1815–1824. <https://doi.org/10.1897/08-545.1>.
- Mullowney, D.R.J., Rose, G.A., 2014. Is recovery of northern cod limited by poor feeding? The capelin hypothesis revisited. *ICES J. Mar. Sci. J. Cons.* fst188. <https://doi.org/10.1093/icesjms/fst188>.
- Nahrang, J., Camus, L., Gonzalez, P., Jönsson, M., Christiansen, J.S., Hop, H., 2010. Biomarker responses in polar cod (*Boreogadus saida*) exposed to dietary crude oil. *Aquat. Toxicol.* 96, 77–83. <https://doi.org/10.1016/j.aquatox.2009.09.018>.
- Nahrang, J., Dubourg, P., Frantzen, M., Storch, D., Dahlke, F., Meador, J.P., 2016. Early life stages of an arctic keystone species (*Boreogadus saida*) show high sensitivity to a water-soluble fraction of crude oil. *Environ. Pollut.* 218, 605–614. <https://doi.org/10.1016/j.envpol.2016.07.044>.
- Nalco environmental solutions LLC Corexit TM EC9500A. <https://www.nalcoenvironmentalsolutionsllc.com/corexit/>.
- Paine, M., Leggett, W., Mcurer, J., Frank, K., 1992. Effects of Hibernia crude-oil on capelin (*Mallotus villosus*) embryos and larvae. *Mar. Environ. Res.* 33, 159–187. [https://doi.org/10.1016/0141-1136\(92\)90147-E](https://doi.org/10.1016/0141-1136(92)90147-E).
- Paine, M.D., Leggett, W.C., Mcurer, J.K., Frank, K.T., 1991. Effects of incubation in oiled sediment on emergence of capelin (*Mallotus villosus*) larvae. *Can. J. Fish. Aquat. Sci.* 48, 2228–2239. <https://doi.org/10.1139/f91-262>.
- Penton, P.M., Davoren, G.K., 2013. A common garden experiment on capelin (*Mallotus villosus*) early life history stages to examine use of beach and deep-water spawning habitats. *J. Exp. Mar. Biol. Ecol.* 439, 54–60. <https://doi.org/10.1016/j.jembe.2012.10.009>.
- Petersen, K., Hultman, M.T., Rowland, S.J., Tollefsen, K.E., 2017. Toxicity of organic compounds from unresolved complex mixtures (UCMs) to primary fish hepatocytes. *Aquat. Toxicol.* 190, 150–161. <https://doi.org/10.1016/j.aquatox.2017.06.007>.
- Prince, R.C., 2015. Oil spill dispersants: boon or bane? *Environ. Sci. Technol.* 49, 6376–6384. <https://doi.org/10.1021/acs.est.5b00961>.
- Purchase, C.F., 2018. Low tolerance of salt water in a marine fish: new and historical evidence for surprising local adaptation in the well-studied commercially exploited capelin. *Can. J. Fish. Aquat. Sci.* 75, 673–681. <https://doi.org/10.1139/cjfas-2017-0058>.
- R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- Rufe, R., Moore, C., Behler, D., Cunningham, J., Dietrick, L., Joves, A., Moore, D., Parker, B., Pollock, G., Shaneyfelt, R., Tarpley, J., 2011. BP Deepwater Horizon Oil Spill: Incident Specific Preparedness Review (ISPR): Final Report, January 2011.
- Sørhus, E., Edvardsen, R.B., Karlsen, O., Nordtug, T., van der Meeren, T., Thorsen, A., Harman, C., Jentoft, S., Meier, S., 2015. Unexpected interaction with dispersed crude oil droplets drives severe toxicity in Atlantic haddock embryos. *PLoS One* 10, e0124376. <https://doi.org/10.1371/journal.pone.0124376>.
- Vehniäinen, E.-R., Bremer, K., Scott, J.A., Junntila, S., Laiho, A., Gyenesei, A., Hodson, P.V., Oikari, A.O.J., 2016. Retene causes multifunctional transcriptomic changes in the heart of rainbow trout (*Oncorhynchus mykiss*) embryos. *Environ. Toxicol. Pharmacol.* 41, 95–102. <https://doi.org/10.1016/j.etap.2015.11.015>.
- Word, J.Q., Clark, J.R., Word, L.S., 2015. Comparison of the acute toxicity of Corexit 9500 and household cleaning products. *Hum. Ecol. Risk Assess.* 21, 707–725. <https://doi.org/10.1080/10807039.2014.920227>.
- Wu, D., Wang, Z., Hollebone, B., McIntosh, S., King, T., Hodson, P.V., 2012. Comparative toxicity of four chemically dispersed and undispersed crude oils to rainbow trout embryos. *Environ. Toxicol. Chem.* SETAC 31, 754–765. <https://doi.org/10.1002/etc.1739>.