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Early life stages of an arctic keystone species (*Boreogadus saida*) show high sensitivity to a water-soluble fraction of crude oil☆

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ABSTRACT

Increasing anthropogenic activities in the Arctic represent an enhanced threat for oil pollution in a marine environment that is already at risk from climate warming. In particular, this applies to species with free-living pelagic larvae that aggregate in surface waters and under the sea ice where hydrocarbons are likely to remain for extended periods of time due to low temperatures. We exposed the positively buoyant eggs of polar cod (*Boreogadus saida*), an arctic keystone species, to realistic concentrations of a crude oil water-soluble fraction (WSF), mimicking exposure of eggs aggregating under the ice to oil WSF leaking from brine channels following encapsulation in ice. Total hydrocarbon and polycyclic aromatic hydrocarbon levels were in the ng/L range, with most exposure concentrations below the limits of detection throughout the experiment for all treatments. The proportion of viable, free-swimming larvae decreased significantly with dose and showed increases in the incidence and severity of spine curvature, yolk sac alterations and a reduction in spine length. These effects are expected to compromise the motility, feeding capacity, and predator avoidance during critical early life stages for this important species. Our results imply that the viability and fitness of polar cod early life stages is significantly reduced when exposed to extremely low and environmentally realistic levels of aqueous hydrocarbons, which may have important implications for arctic food web dynamics and ecosystem functioning.

1. Introduction

Past experience has shown that releases of crude oil can have important long-term ecosystem effects at the regional level (Peterson et al., 2003), leading to significant economic consequences through the loss of ecosystem services (Cohen, 1995; Garza-Gil et al., 2006; Ocean Studies Board, 2013). The impact on specific ecosystems and their post-spill recovery potential is largely determined by their species composition, function, and life-cycle strategies but also by environmental characteristics (temperature, oxygen level, and salinity), additional anthropogenic factors (e.g. climate warming and overfishing) and their potential interactive effects (Soto et al., 2014; Thorne and Thomas, 2008; Whitehead, 2013).

The sensitivity of arctic marine ecosystems is becoming an increasing concern in an era of rapid and unprecedented climate change (Doney et al., 2012; Wassmann, 2011) and of concomitant increase in anthropogenic activities. Rapidly increasing temperatures measured across the entire Arctic in the past decade and the loss of perennial sea ice (Wang and Overland, 2009) poses a direct threat to many ice-associated arctic species (Michel et al., 2012). The poleward expansion of boreal species has the potential to alter the structure of high arctic ecosystems through changes in species interactions and replacement (Berge et al., 2015a; Fossheim et al., 2015; Kortsch et al., 2012). Last, but not least, the receding ice cover allows for an increase in anthropogenic activities including the opening of new shipping routes along the Arctic shelves (Smith and Stephenson, 2013) and new resource exploration and development of oil and gas extraction (Harsem et al., 2011). Recently, Norway modified the definition of the Arctic marginal ice zone in preparation for a new round of oil and gas licensing, enabling the

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oil and gas industry to operate further north in the Barents Sea. As the accessibility to the Arctic increases, the threat of accidental oil spills increases greatly. This will further be exacerbated by changes in sea ice structure and behavior in combination with expected increases in extreme weather events resulting from climate change (Harsem et al., 2011).

In ice-covered environments, oil recovery is considered exceptionally challenging because the remoteness of the Arctic renders detection and access to the impacted area difficult, especially during the polar night. Spilled oil can easily become encapsulated into the ice and released in the following melt seasons when it may be widely distributed over larger areas (Dickins et al., 2008; Fingas and Hollebone, 2003); this will potentially affect ecosystems during periods of high productivity (Leu et al., 2015) and at biological hotspots (Kuletz et al., 2015). In addition, our increasing understanding of ecosystem processes during the polar night shows a system with high activity levels and biological interactions across most trophic levels (Berge et al., 2015b,c) that may be more vulnerable than previously assumed. Weathering processes in the Arctic, and in particular those of crude oil encapsulated in sea ice, are significantly prolonged, thereby also increasing the time of exposure to marine organisms (Brandvik and Faksness, 2009). Water-soluble hydrocarbons within brine channels in the sea ice can also reach concentrations that are toxic to ice-associated organisms and they can be released over several months, potentially contaminating food webs (Faksness and Brandvik, 2008). The water-soluble fraction (WSF) of crude oil can exert significant developmental effects on fish embryos at levels in the μg/L range (Carls et al., 1999; Frantzen et al., 2012; Hicken et al., 2011; Incardona et al., 2012, 2014, 2015), which can lead to a reduced fitness and survival to adulthood (Heintz et al., 2000). These effects (reduced size at hatch, spinal malformations, pericardium and yolk sac edemas) are often associated with polycyclic aromatic hydrocarbons (PAHs) (Carls et al., 2008; Turcotte et al., 2011). Sensitivity of embryos and larvae to oil spills is important because they are considered to be the most vulnerable life stages of fish and represent a direct link to population consequences and resilience (Albers, 2002). Understanding the impact of environmentally relevant concentrations on these life stages and determining thresholds for effects are central objectives in the assessment of global ecosystem sensitivity and vitality.

Polar cod (*Boreogadus saida*) is the most abundant pan-arctic fish species, and it sustains the majority of other species that comprise higher trophic levels (Hop and Gjøsæter, 2013; Mueter et al., 2016). Populations of this small gadid are at risk of experiencing significant changes in ecosystem interactions (Renaud et al., 2012) and alterations in their life cycle strategies (Nahrgang et al., 2014) in regions of enhanced warming. Additional impacts from anthropogenic pollution, and in particular accidental oil spills, may accelerate the decline of this key species and thereby its central role in the Arctic food web. Although a series of studies have been performed in the last decades on the sensitivity of oil to adult polar cod (e.g. Andersen et al., 2015; Nahrgang et al., 2010a,b), to the best of our knowledge, investigations on its early life stages have not been performed yet. Therefore, our study focused on investigating the effects of a Barents Sea crude oil WSF on the embryonic development of this ractic keystone species. The present study provides novel insights into the sensitivity of the early life stages of polar cod to concentrations of crude oil WSF that are similar to what may be released from brine channels following an oil spill in ice-covered waters. Such spills will likely expose the epipelagic eggs of polar cod to extremely low levels of toxic compounds during embryonic development. We hypothesized that polar cod early life stages would show dose-dependent effects on mortality, hatching success, occurrence of cardiac dysfunction, malformations and length, at environmentally realistic concentrations of crude oil WSF. Our unique dataset provides evidence that this arctic keystone species is put at high risk from these petroleum compounds associated with increasing anthropogenic activities in the Arctic.

2. Material and methods

2.1. Ethical statement

All work was performed according to and within the regulations enforced by the Norwegian Animal welfare authorities and no specific permissions were required. The R/V Helmer Hanssen is owned by the UiT The Arctic University of Norway, which has all the necessary authorization from the Norwegian Fisheries Directorate to use a bottom trawl to collect fish for scientific purposes. The lead author has all necessary training and certificates (FELASA C) to perform the work. The organisms are neither protected nor endangered in the coastal waters of the Svalbard Archipelago.

2.2. Polar cod sampling

Polar cod were caught in Kongsfjorden (Svalbard; 78°95′02″N, 11°99′84″E) during a research cruise on the R/V Helmer Hanssen in January 2014. Trawling was conducted at a depth of about 150 m, at a speed of about 2 knots and for 5 min at a time using a Campelen Super 1800 bottom trawl with a fish-lift to avoid injuring the collected fish. During the cruise, fish were kept on deck in 500 L tanks with continuous seawater inflow. Injured and dead individuals were removed daily. Upon return to Tromsø (27 January 2014), the fish were transferred to the biological research station of the UiT The Arctic University of Norway in Kårvika and maintained in a 2000 L holding tank under continuous seawater inflow (filtered seawater, 20 μm, 3.1–3.3 °C, pH of 8, salinity of 33–34 psu) and constant darkness, mimicking the ambient January Svalbard light (polar night). Fish were preventively treated daily against microbial infections using Halamid® and fed *ad libitum* with natural prey *Calanus* spp. (purchased from Calanus AS).

2.3. Experimental design

The exposure system was designed to deliver a WSF of crude oil to polycarbonate incubators, using oiled-rock columns and a flow-through system similar to the procedure described by Carls et al. (1999). The oiled-rock columns (see Fig. S1 for technical details) contained 11 kg of gravel (size range 7–11 mm) each that had been coated with crude oil (Kobbe crude from the Barents Sea shelf) at four concentrations; control (no oil), low (0.5 g oil per kg gravel), medium (3 g oil per kg gravel) and high (6 g oil per kg gravel) concentrations, thoroughly mixed by manual shaking and dried at ambient air temperature (−10 °C) for 72 h. Filtered seawater (20 μm, 3.1–3.3 °C) ran through each column upwards into 3 replicate incubators (6 L each) for a total of 12 incubators receiving exposure water at a final flow of 0.3 L/minute per incubator. The exposure water ran through the columns for 10 days prior to the transfer of fertilized eggs to the incubators in order to decrease the concentrations of the WSF to levels in the lower μg/L range, i.e. expected to leak from brine channels following an oil spill in icy waters (Faksness and Brandvik, 2008).

Incubators were divided into a lower and upper half using fine mesh netting (pore size 1 mm; Fig. S1). Incubators were open, allowing for unrestricted gas exchange. Seawater quality parameters as well as photoperiod were the same as for the holding tanks (see section above). The daily routine consisted of controlling the temperature and dissolved oxygen levels and readjusting the water flow. No oil slick and oil odor was noticed during the exposure

period.

2.4. In vitro fertilization, exposure and sampling

On 9 February 2014, a total of 6 polar cod females were stripped of their eggs and following an immediate visual inspection of egg quality indicators (clearness, shape and size), a total of 3 egg batches (identified as batches A, B and C) were formed from pooling eggs of 2 different females per batch to account for qualitative differences between the eggs/larvae from different egg donors (Table S1). Each of the 3 egg batches was distributed to 1 of the 3 incubators per treatment and fertilization was conducted by placing 1000 eggs on a petri dish floating on the incubator water (3 °C). Fertilization began by adding 10 μL of the milt pool (8 males pooled in equal amounts) onto the eggs and 10 ml of incubation water from the control treatment. After 10 min, the excess milt was rinsed using incubation water from the control treatment and egg batches were placed within their respective incubators.

Water samples (2L per column, no replicates) were taken directly from the outflow of the oiled-rock columns and stored at −20 °C until further analyses. Samples for chemical analyses were taken 3 days after column flow initiation for 3 treatments (control, low, and high) and weekly for every treatment from the start of exposure.

Mortality counts were conducted on a daily basis throughout the whole incubation period (37 dpf). Living and healthy polar cod eggs are buoyant and transparent, and they float close to the water surface while dead or dying eggs turn white and sink to the bottom. Floating eggs that had turned white were also defined as dead and removed from the incubators daily.

Hatching started at 27 dpf in all incubators and lasted until 37 dpf, date chosen to stop the experiment. Sampling of embryos was done (1, 8, 15, 22 and 29 dpf) but data was not included in the present study. Larvae were sampled randomly at the water surface using a pipette with an enlarged opening at 31, 33, 36 and 37 dpf, for further morphological analyses. Group photographs (all time points except 36 dpf) of approximately 30–45 larvae per incubator and single-larva photographs (36 dpf) were taken for malformation analyses, using a LEICA M205 C microscope with a Leica MC170 HD camera.

Assessment of the cardiac activity (n = 15 per incubator) and arrhythmias (n = 3 per incubator) was conducted on larvae at 36 dpf that had been mildly sedated before recording using a Tricaine methanesulfonate (Finquel MS-222) solution (50 mg/L in control water). Single larvae were transferred onto a watch glass placed into an ice bath, water temperature was controlled at every measurement and maintained at 3 °C. Video recordings (1 min; 30 frames/second) of the cardiac activity and a photograph of the whole larvae (lateral view) were taken using the Leica M205 C microscope and Leica MC170 HD camera, for the malformation analysis at 36 dpf.

The remaining living larvae found both in the water column and on the bottom at 37 dpf were counted and sampled for image analysis. Total mortality and hatching success per batch and treatment were corrected for sampled individuals.

2.5. Chemical analyses of water samples

Water samples were analyzed for THC (C11–C40) by ALS Laboratory group Norway AS, following standard procedures (ISO 9377-2) and using a gas chromatography-flame ionization detector (GC-FID) on isooctane extracts using an Agilent 6890N GC with a capillary column, fitted with an Agilent 7638B Series auto sampler. Analysis of 26 PAHs (16 Environmental Protection Agency PAHs and 10 alkylated naphthalenes, phenanthrenes and dibenzothiophenes, see Table S1) in the collected water samples was performed by Unilab Analyse AS, Tromsø. Water samples for chemical analysis were added 1 ml of an internal standard mixture of five deuterated PAHs; naphthalene-d_8, biphenyl-d_{10}, phenanthrene-d_{10}, pyrene-d_{10} and benzo(a)pyrene-d_{12}. The water solution was then extracted with 3 × 60 ml dichloromethane (DCM), dried for at least 1 h with Na_2SO_4 (s), and concentrated to 1 ml. The extract was purified on an SPE column by elution with pentane and DCM. The eluted solution was concentrated to 0.5 ml, evaporated to dryness under N_2-gas, and finally added 100 μl isooctane.

Analysis for 26 PAHs was performed by GC–Mass Spectrometry (GC–MS) operated in selected ion monitoring (SIM) mode. The system comprised of an Agilent 7890N GC with a capillary column, and an Agilent 5975C quadrupole Mass Selective Detector with Helium as carrier gas. One μl of sample was injected into a 300 °C split/splitless injector. The oven temperature was heated to 50 °C for 2 min, and then heated through various steps up to a final temperature of 315 °C. Data and chromatograms were monitored and recorded using MSD ChemStation (version E.02.00.493) software. The MSD ion source temperature was 230 °C. Single PAH concentrations were calculated by quantification of the added deuterated standards, and a pre-determined calibration curve of 5 PAH-standards at different concentrations. Each sample extract was analyzed once on the GC-MS, as well as calibration solutions of known PAH-concentrations (including deuterated standards). Blank samples were processed and analyzed in the same way as real samples, and calculation of PAHs in real samples were corrected for the blank values determined. LOD were determined from the average value and standard deviation of a series of blank samples. See Table S2 for compounds analyzed.

2.6. Malformations and morphometrics

Spinal malformations and yolk sac alterations were determined in larvae, using group (31, 33 and 37 dpf), and single (36 dpf) larvae photographs that were randomly sampled at the water surface of the incubators. The evaluation of spinal malformations was based on a categorical severity index (0, none observed; 1, mild bend; 2, major bend), according to Carls et al. (1999). Yolk sac alterations were categorically scored for presence and severity (0, none observed; 1, area affected < 25%; 2, area affected >25%). The malformations were scored blind by two people independently and compared. In case of a disagreement, the larvae were reassessed. Larval spine length (mm) and yolk mass area (mm^2) were measured with the ImageJ software (version 1.47), using the same photographs as above, except for 33 dpf specimens that were excluded because of an erroneous scale bar. The spine length was measured from the back of the head to the end of the notochord using only individuals that did not exhibit signs of spine curvature. Spine length of individuals with severe spine curvature increased the standard deviation of the measured group, masking the effect observed on normal larvae. For the yolk mass area (mm^2), the outmost delineation of the sac was used. No other developmental abnormalities (e.g. haemorrhage, jaw deformities, pericardial edema etc.) were observed during this experiment.

2.7. Cardiac activity and arrhythmia

Larval heart rates at 36 dpf were counted from our video recordings and normalized to 60 s. Arrhythmia was assessed in form of interbeat variability, similar to that for heart rate, and assessments were conducted on 20-s segments of the same video recordings (Incardona et al., 2009). Briefly, the video frame numbers at the onset of each cardiac contraction were recorded using PotPlayer (version 1.5.45955). The number of frames between

contractions and its standard deviation were calculated and the latter used as an indicator of arrhythmia.

2.8. Statistical analyses

The Chi-square test of independence was used to determine significant differences between batches for mortality and hatching success, as well as significant differences among treatments and time for the occurrence of malformations and severity (both spine and yolk). When significant, the Chi-square test was followed by a pairwise comparison with Bonferroni corrections of the *p*-values. Differences in cardiac activity and arrhythmia were tested using a one-way ANOVA followed by a Tukey's post hoc test when requirements for normality and homogeneity of variance were met. When the variance failed to be homogenous (arrhythmia in Batch C), the Robust Test of Equality of Means (Welch ANOVA) was used. The program SSD Master was used to generate distribution plots and the 95th percentile confidence intervals for each length dataset. The best model was selected based on mean square error, distribution of residuals, and how well the data fit the model. All datasets were best fit to a Normal distribution model, except for the high dose of Batch B (Fisher-Tippett model). The Kolmogorov-Smirnoff test for distributions was performed pairwise between the control and the three treatment groups. For the length distributions, the test was repeated with bootstrap sampling using the Mersenne-Twister random number generator. The minimum significance level for all analyses was set at $\alpha = 0.05$. SYSTAT 13 (Systat, 2009) was used to calculate regression equations.

3. Results

Significant and dose-dependent sub-lethal effects during polar cod embryo development were detected despite low concentrations of petroleum hydrocarbons in the exposure water across all oil treatments and throughout the exposure. Assuming that each egg batch is equally represented in a population, only 24 ± 1% of the high dose treatment eggs would be viable and survive without any signs of spine malformation, compared to 67 ± 1% for control eggs. In addition, the length of WSF exposed larvae was reduced by between 1.6% ± 0.6% in the low dose treatment and 7 ± 0.7% in the high dose treatment compared to the control specimens (Table 1). These effects showed a significant and linear dose-dependent response for all batches combined (Table 1), and this significant dose-dependent relationship was also present within each batch (see results below).

3.1. Hydrocarbon concentrations of the crude oil water-soluble fraction

After three days of seawater flow from the oiled-rock columns (initial flush), the total hydrocarbon content (THC) of the high treatment column-outflow was 256 μg/L and differed only by its C11-C16 fraction (181 μg/L) compared to the control treatment (12 μg/L; Table S3). In addition, the high treatment water contained a concentration of sum of 26PAHs (Σ26PAHs) below 5 μg/L.

One week later, at the start of embryo exposure (day 0, also corresponding to the day of egg fertilization), THC levels in the high treatment had decreased to levels below the limit of detection (LOD <10 μg/L for each analyzed fraction). The Σ26PAHs in the high treatment group had decreased to 2.18 μg/L at day 0 and further declined to 0.059 μg/L at day 14 (Table 2). Only 2- and 3-ring alkylated PAHs (C1 to C3-naphthalenes, C0 to C3-dibenzothiophene and C0 and C1-anthr/phenanthrene) were detected (Table S2). For all other treatments, THC and PAH levels were continuously below the LOD during the entire exposure period (Table 2).

3.2. Mortality and hatching success

Embryo mortality resulting from the crude oil WSF treatment did not occur during the course of the experiment. During the hatching phase, a significant dose-dependent increase in the proportion of sinking and heavily malformed embryos was observed, even in the low and medium treatment groups (37 dpf, Fig. 1). These embryos and larvae were unable to hatch or swim, and therefore considered to be moribund. This suggests that these specimens would suffer from a delayed and dose-dependent mortality if the experiment had been pursued over time.

Despite some variability in egg quality across batches, the observed dose-dependent responses were consistent. Total hatching success among control batches ranged from 23% (Batch B) to 70% (Batch A) and did not significantly change in the exposed groups. High variability in egg quality with high mortality rates is common in wild-caught marine fish (Brooks et al., 1997; Buckley et al., 1991; Craik and Harvey, 1984; Springate et al., 1984). For instance, the survival rates from egg fertilization to yolk sac larvae of Atlantic herring and Atlantic mackerel were in total 0.5% and 6%, respectively (Dahlberg, 1979). Also, Atlantic cod showed highly variable hatching success (1–82%) across batches (Ouellet et al., 2001). In our study, variability in hatching success across batches was also thought to be a function of maternal factors, because milt pool and other exposure conditions were the same for all batches.

3.3. Larval malformations

The “swimming” larvae that were distributed in the upper water column of the incubators (Fig. 1) exhibited different degrees of sub-lethal effects. The occurrence of malformations, in the form of spine curvature and yolk sac alterations, were significantly different and increased in a dose-dependent fashion in larvae 31–37 dpf from all batches (Fig. 2). These malformations also significantly increased in severity with treatment in all three batches (Fig. S2). The batches responded to the treatments with different sensitivities, as seen by different regression slopes for both spine curvature and yolk sac

Table 1
Summary of the main developmental effects of larva surviving hatching. Estimation of the mean (±SEM) percentage of swimming hatchers, hatchers without spine curvature and the percentage length reduction of the latter compared to the control group.

Treatment	Oil loading (g/kg gravel)	% Swimming hatchers	% Hatchers without spine curvature	% Length reduction compared to control
Control	0	91 ± 1	67 ± 1	0.0
Low	0.5	83 ± 1	59 ± 1	1.6 ± 0.6
Medium	3	77 ± 1	39 ± 1	4.5 ± 0.6
High	6	65 ± 2	24 ± 1	7.0 ± 0.7

Numbers are based on the means from batches A, B and C (n = 3); the percentage swimming hatchers is based on data presented in Fig. 1; percentage of hatchers without spine curvature is based on data presented in Fig. 2A. The dose response for the percentage of individuals that hatched without spine curvature followed a significant (ANOVA, $p = 0.014$) linear relationship (% swim = 63.8–6.98 * Dose, $r^2 = 0.96$).

Table 2
Total hydrocarbon content (THC) and sum of 26PAHs (Σ26PAHs) in incubation water during the course of the experiment. Initial crude oil concentration on the gravel (g crude oil per kg gravel) and water concentrations of THC (μg/L, sum fractions C11-C40) and Σ26PAHs (μg/L) in the control, low, medium and high treatments of the prepared water-soluble fraction (WSF) at weekly intervals, i.e. 3 days following initial column flush (7 days pre-exposure) and during the experiment (0, 7, 14, 21 and 28 days post fertilization).

Treatment	Crude oil g/kg gravel	Time (days of exposure)	THC (μg/L)	Σ26PAH (μg/L)
Control	0	−7	92.5	<LOD
		0–28	< LOD	<LOD
Low	0.5	−7	n/a	1.09
		0–28	< LOD	<LOD
Medium	3	−7	n/a	n/a
		0–28	< LOD	<LOD
High	6	−7	256	4.90
		0	< LOD	2.18
		7	< LOD	0.64
		14	< LOD	0.06
		28	< LOD	<LOD

Oiled-rock columns were flushed for 10 days before exposure began.
LOD = limits of detection; n/a = not available. LOD was 10 μg/L for each THC group analyzed (C11-C12, C13-C16, C17-C35, C36-C40).
Detailed concentrations of THC fractions and PAH concentrations are presented in Tables S2 and S3.
Note that the days of exposure also correspond to the days post-fertilization (dpf) of the embryos.

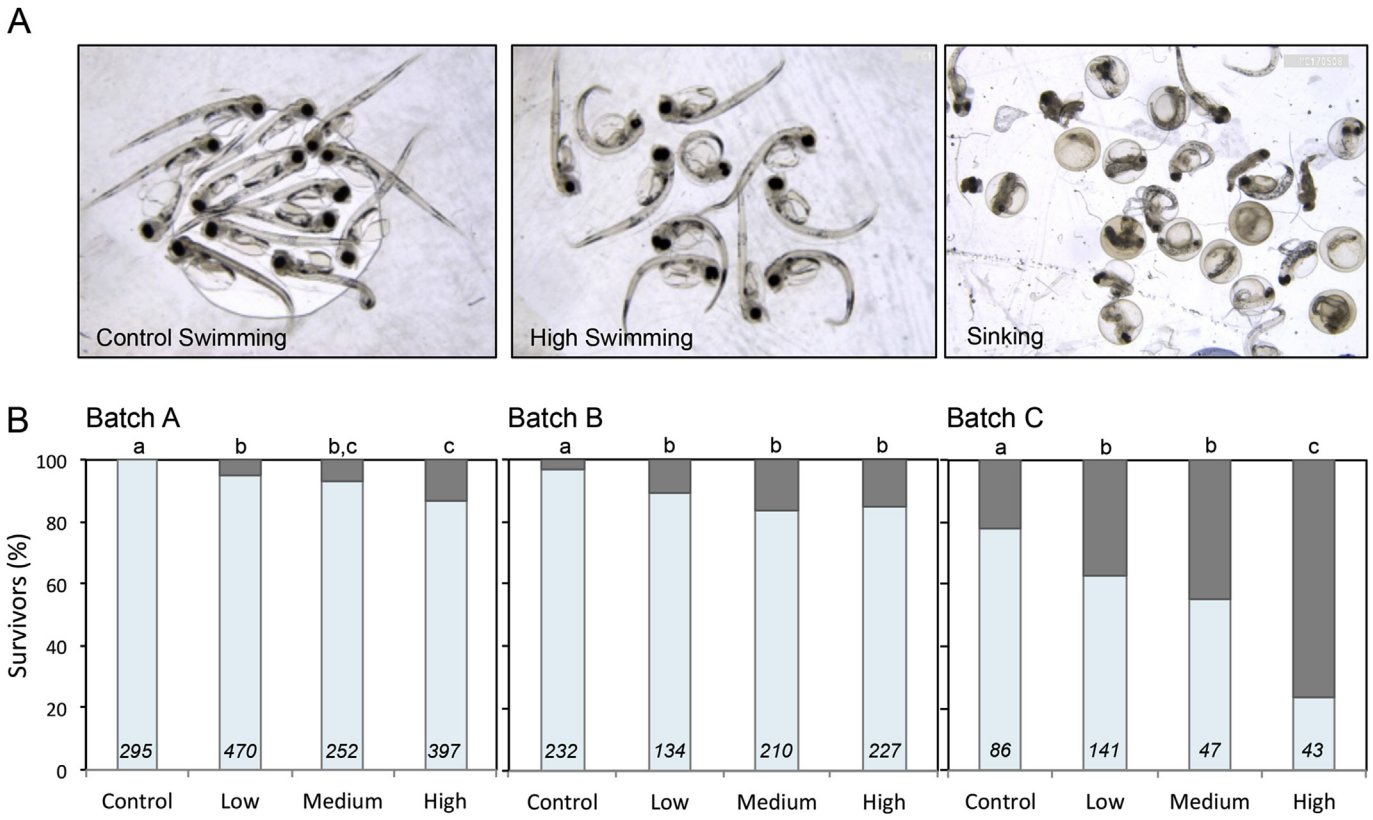

Fig. 1. Survivors (larvae) at 37 dpf. (A) Representative photographs of individuals from batch A at the end of the exposure (37 dpf) from the water column (swimming larvae) of the control treatment (left photo), of the high treatment (middle photo) and from the bottom of the incubators (sinking larvae and embryos) of the high treatment (right photo); and (B) percentage of individuals at 37 dpf in batches A, B and C collected from the water column (blue = larvae) and at the bottom (dark grey = larvae and embryos) of each incubators. Letters on the bars indicate significant (Chi-square, all p-values <0.001) differences among treatments. Numbers inside the bars indicate the total number of individuals collected.

alterations (Fig. 2). Batch C, which had the steepest dose-response slope (Fig. 2), also experienced the lowest hatching success (Fig. 1). By contrast, batch A was the most robust batch with both the weakest dose-response slope in malformations and the highest hatching success.

We classified yolk sac abnormalities as “alterations”, rather than an “edema”, because the lack of comparative studies on this species renders it impossible to unequivocally determine whether the observed alterations were a result of fluid retention in extracellular spaces (edema) or a reduction in yolk mass (Fig. S3). The abnormal visual aspect of the yolk sac and yolk mass observed in polar cod was different from edema commonly observed in other species after exposure to hydrocarbons, where the anterior margin of the yolk membrane is bound by an area of clear fluid (Carls et al., 1999). In polar cod, the visual appearance of this detachment did not resemble an accumulation of fluid but rather a reduction of yolk mass, leaving the yolk sac partly empty (Fig. S3). In support of this, the size of the yolk mass in our study decreased significantly over time in all treatments, including the controls (Fig. S4), reflecting a time-dependent resorption of the yolk (Aronovich et al., 1975). The

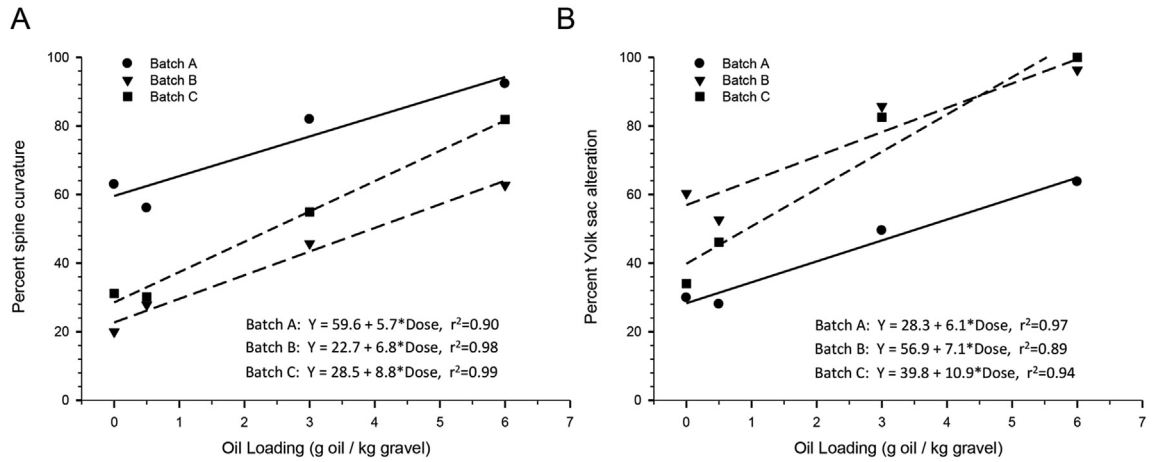

Fig. 2. Dose-dependent increase of malformations of larvae between 31 and 37 dpf. Relationship between frequency of occurrence (%) of (A) spinal curvature and (B) yolk sac alteration for larvae and oil loading in the columns. Data is presented for larvae from batches A, B and C collected from the water column (swimming individuals, Fig. 1A) at 31, 33 and 36 and 37 dpf. For detailed information on malformation scoring, see Figs. S2 and S3. Linear regressions and their r^2 are shown for each batch. *P*-values for the regression coefficients (intercept, slope) are presented in Table S4. Circles, triangles and squares are raw data.

mismatch between the clear dose-dependent increase in yolk sac alterations performed by visual scoring (Fig. 2 and S3) and the apparent lack of dose-dependent reduction in yolk mass area (Fig. S4) is likely related to a systematic overestimation of the yolk mass area in specimens with visually strong yolk sac alterations. This is due to the conservative yolk mass area measurement performed (see Materials and Methods section). In addition, there is a mathematical relationship between volume and area that systematically underestimates a change in volume when represented as an area.

3.4. Larval morphometrics

The spine length of larvae was measured at 31, 36 and 37 dpf and only on individuals that did not show malformations of the spine to avoid bias and allow comparisons among treatments. Each dataset for larval length was best represented by a normal distribution, except the high dose, which was best fitted with a Fisher-Tippet model (Fig. 3). The reduction in length was significant for specimens in the medium and high treatments in batch A, and all treatments in batch B compared to the control (Table S5). Bootstrap resampling used to explore the accuracy of our small sample estimates also indicated significant differences for the medium- and high-dose treatments compared to controls for batch C (Table S5). The mean reduction in spine length was also highly correlated to the oil loading of the columns (Fig. S5). On average, the reduction in length of normal larvae from the high treatment exhibited a decline of 8.8%, 10.2% and 2.0% for batches A, B and C, respectively, compared to the control specimens.

3.5. Cardiac activity and arrhythmia

Larvae that were randomly collected from the water column in batches A and C at 36 dpf exhibited a significant decreases in cardiac activity in the oil treatments compared to controls, but this occurred without a clear dose-dependent pattern (Fig. 4A). Although not significant, the interbeat variability showed a similar but increasing trend with dose (Fig. 4B).

4. Discussion

The present study is the first to report significant sub-lethal developmental effects for polar cod at environmentally relevant levels of exposure to the dissolved fraction of crude oil. The observed effects include dose-dependent spine curvature, yolk sac alterations, and reduced length. Our study shows a decrease of up

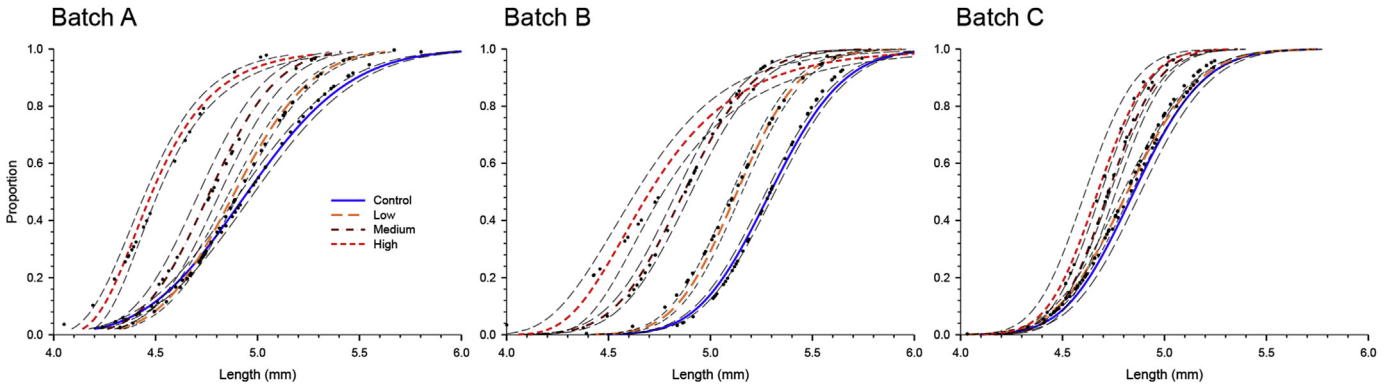

Fig. 3. Length of larvae between 31 and 37 dpf. Cumulative frequency distribution of spine length (mm) for larvae that did not show any spine curvature at 31, 36 and 37 dpf combined. Batch A: control n = 39, low n = 39, medium n = 18 and high n = 20; batch B: control n = 44, low n = 33, medium n = 26 and high n = 15; and batch C: control n = 27, low n = 42, medium n = 19 and high n = 12. Regression analyses are shown for each dose as colored dashed lines with a solid line for the control and their 95% confidence interval (thin black dashed line). Circles are raw data. The Kolmogorov-Smirnoff test results are presented in Table S5.

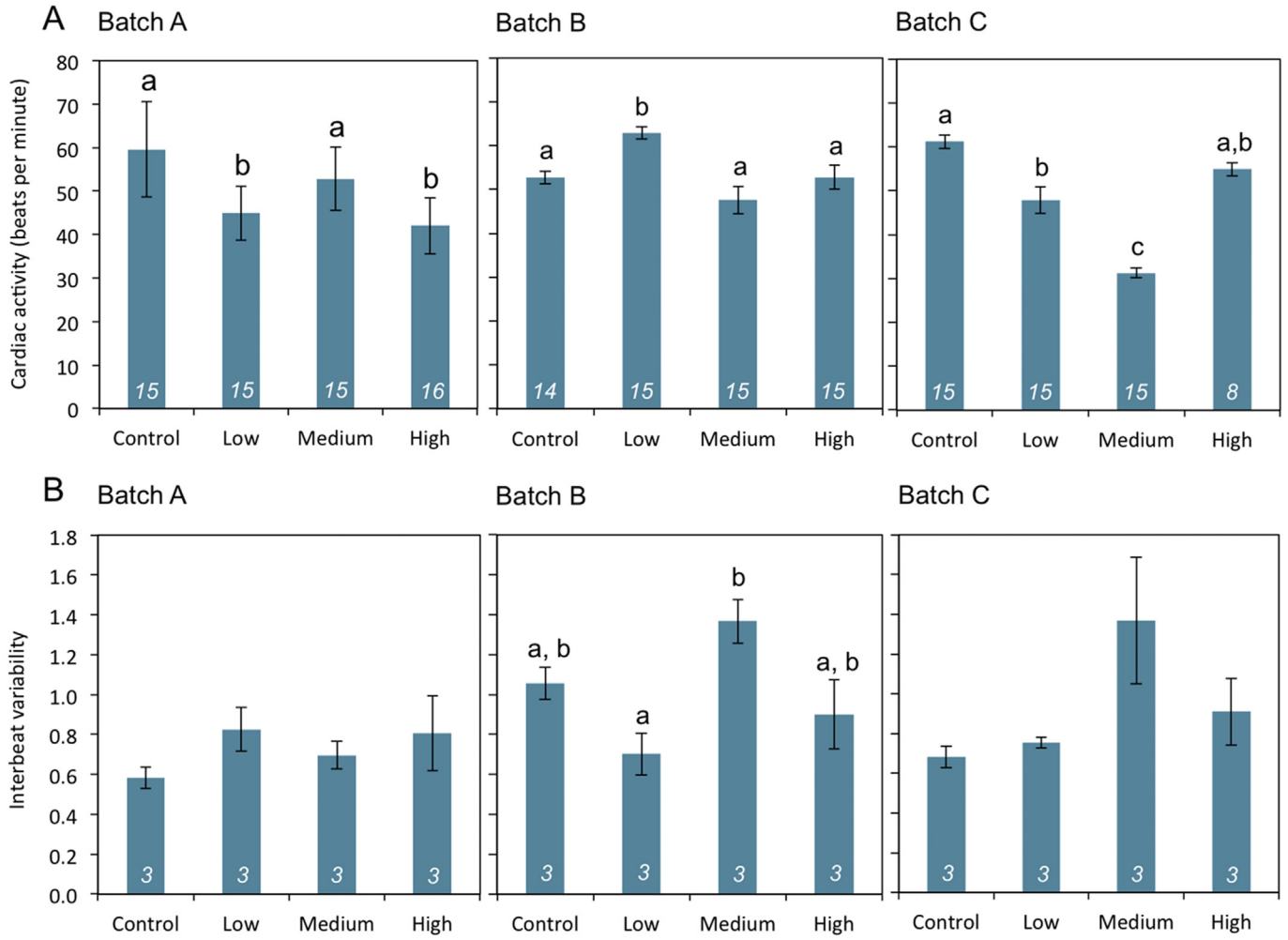

Fig. 4. Cardiac dysfunction of larvae at 36 dpf. (A) Cardiac activity (mean ± SEM, beats per min) and (B) arrhythmia (mean ± SEM, interbeat variability) of larvae (swimming individuals, Fig. 1A) from batches A, B and C at 36 dpf. Letters on bars indicate significant differences (one-way ANOVA, $p < 0.05$) among treatments. Numbers inside the bars indicate the total number of individuals evaluated.

to 83% in viable larvae that may survive into adulthood (on average across batches, 64.2%) in the high treatment group compared to unexposed controls. Furthermore, the results showed a repetitive and dose-dependent pattern across the three different egg batches, which support the significance and repeatability of the findings.

Reduction in length concomitant with an increase in the occurrence and severity of yolk sac alteration in oil-exposed larvae suggested that these individuals exhibited an energy deficit, although other mechanisms such as suppressed protein biosynthesis or impaired muscle development may be involved (Sokolova et al., 2012). These observations were different from previous studies that linked reduced length to premature hatching, i.e. smaller jaws, underdeveloped fins and increased yolk sac volume (Carls et al., 1999; Frantzen et al., 2012; Marty et al., 1997). The reduced length of larvae with dose might theoretically also be a result of a dose-dependent delayed hatching. Indeed, length data was not representative of length-at-hatch but of a group of larvae of different ages within a 10-days hatching window. Our experimental design did not allow testing a potential delayed hatching. Importantly, hatching started simultaneously in all incubators and there was a dose-dependent reduction in yolk sac alteration in all treatments (Fig. 2 and S3), indicating a reduced yolk mass. As the larvae are non-feeding, it is logical to assume that the yolk mass and length are correlated and thus that the dose-dependent reduction in larval length is likely an effect of reduced growth rather than delayed hatching. Similarly, Le Bihanic et al. (2014) did not find hatching delays in rainbow trout exposed to PAHs and PAH mixtures.

Because body mass is proportional to the cube of the length, these reductions ranging from 2% to 10%, also imply substantially impaired growth. Reduced length for polar cod larvae exposed to the toxic WSF may have important consequences for larval survival. Growing normally and increasing in size constitutes an essential advantage in larval and juvenile fish survival (Pepin et al., 2015; Sogard, 1997), which is also true for young-of-the-year polar cod (Bouchard and Fortier, 2008; Fortier et al., 2006; Thanassekos et al., 2012). It is important to note that the reduction in length observed in this study occurred for otherwise viable specimens (without spine curvature) suggesting that this adverse effect may impair even the most robust individuals. It has been shown through a modeling study that length-at-age of polar cod during the first 45 days after hatching is an important factor for survival to adulthood (Thanassekos et al., 2012). Consequently, reduced growth and developmental abnormalities during early life stages may result in reduced fitness and survival to the adult phase as reported for several fish species (Beamish and Mahnken, 2001; Cachot et al., 2007; Heintz et al., 2000; Houde, 1997; Thanassekos et al., 2012; Vignet et al., 2014).

The severity of the effects observed for spine malformations, yolk sac alterations and length reduction suggests that dose-dependent mortality would have occurred beyond the exposure period. Spine curvature may lead to a reduced swimming and thereby feeding capability (Carls et al., 1999). The amount of yolk available for self-sustained growth represents a crucial factor for larval survival, especially for cohorts hatching early in the season, when prey availability may show an important inter-annual variability (Fortier et al., 1995, 2006). At the rate of yolk resorption observed in the present study, i.e. between 5.9 ± 1.2% per day for batch C and 7.8 ± 0.7% per day for batch A (mean of all treatments) and assuming that the rate of resorption would remain stable until feeding begins (Aronovich et al., 1975), the yolk would be completely depleted within 15 days, giving only a narrow time window between hatching and onset of feeding. The increased incidence of yolk sac alterations (Fig. 2 and S3) in the medium and high treatments, which also implies a reduced yolk mass compared to control treatment, also suggests that a large proportion of larvae exposed to the crude oil WSF would have an even more reduced amount of time to rely on yolk reserves before depending on external food sources. One could speculate that increased temperature in a future Arctic might as well increase the rate of yolk depletion in polar cod larva through elevated metabolism (Martell et al., 2006; Peck et al., 2012). Although this slow-growing species may be relatively resilient to episodes of low food availability, as suggested by Pepin et al. (2015), early hatching cohorts may experience high mortality because of less favorable spring conditions and a potential mismatch between time of hatching and prey availability (Fortier et al., 1995, 2006). However, recent studies by Bouchard and Fortier (2008, 2011) also showed that earlier ice-break-up, more frequent polynyas and increased river discharges, as direct consequences of climate warming, may provide favorable conditions for early hatching of polar cod leading to larger pre-winter sizes and survival. Nevertheless, the result of the herein documented effects on developing larvae exposed to oil WSF (reduced length and yolk mass), may greatly reduce the likelihood of larvae successfully maturing to the adult phase.

Cardiotoxic effects, including the lack of pericardial and yolk sac edemas, suggested that the levels of crude oil WSF in the present study were too weak to cause any significant effects. Assuming that PAHs and in particular tricyclic PAHs found in the crude oil WSF are the main cause for cardiotoxic effects in fish (Incardona et al., 2004), our PAH levels (Table S2) were well below those reported in similar studies using crude oil WSF (e.g. Hicken et al., 2011; Incardona et al., 2015). Potential species differences in cardiovascular anatomy (Incardona and Scholz, 2016) may also cause differential sensitivities to cardiotoxicity. However, these cannot be discussed in light of our data and exposure levels.

Importantly, the observed effects were found at very low and decreasing concentrations of crude oil WSF with THC below the LOD and Σ26PAHs far below 2 μg/L. As a relevant comparison, the water quality criterion for ΣPAHs is set to 10 μg/L in the Alaska state, hence well above the range of hydrocarbons measured in our experiment. Assuming that the weathering process during the 10-days column flushing was similar among treatments, embryotoxicity could be expected to occur at concentrations of Σ26PAHs estimated below 0.2 μg/L and correspond to the low treatment when exposure commenced. The measured concentrations for the Σ26PAHs are environmentally realistic, and they are even lower than those estimated in brine water after encapsulation of this particular oil in ice (Faksness and Brandvik, 2008; Faksness et al., 2011). Only a few studies have shown significant developmental effects occurring at ΣPAHs below 1 μg/L in the crude oil WSF, including the Japanese medaka (*Oryzias latipes*) (González-Doncel et al., 2008) and herring (*Clupea pallasi*) (Incardona et al., 2012, 2015). Furthermore, slow degradation of oil due to sub-zero temperatures and low rates of dispersal/entrapment of oil in sea ice are factors that would further enhance the herein documented effects following an accidental release of hydrocarbons to the environment (Brandvik and Faksness, 2009).

Embryo developmental toxicity has been commonly related to PAHs (Barjhoux et al., 2014; Fallahtafti et al., 2012; Le Bihanic et al., 2014; Wu et al., 2012), which are often the only compounds quantified in crude oil WSF experiments (Carls et al., 2002; Frantzen et al., 2012; Hicken et al., 2011; Incardona et al., 2012; Marty et al., 1997). Although PAHs are known to cause developmental toxicity, we cannot rule out that some effects may be partially caused by other unknown substances in the WSF. The composition of the WSF is complex and composed of a large fraction (up to 98%, excluding the BTEX fraction) of unknown organic compounds that are designated as unresolved complex material (UCM) (Faksness and Brandvik, 2008; Melbye et al., 2009). An increasing body of literature shows that the UCM of the WSF may also contain toxic chemicals containing aryl hydrocarbon- and estrogen receptor agonists and metabolic activity disruptors (Booth et al., 2007, 2008; Melbye et al., 2009; Middaugh et al., 2002).

5. Concluding remarks

Our study shows significant sub-lethal effects on polar cod larvae when exposed to low levels of hydrocarbons in the WSF of crude oil. Our study is an environmentally realistic representation of potential oil spill scenarios occurring in ice-covered regions during the polar night, when epipelagic eggs from polar cod will aggregate under the ice. Polar cod populations may already be jeopardized as a result of climate warming impacts with fecundity levels reduced by one order of magnitude in regions influenced by warm water masses (Nahrgang et al., 2014). These additional stresses can only further impede their success of a complete life cycle. Additional work should evaluate the consequences of these observed effects on later developmental stages, as well as overall population sensitivity under various oil spill and climate scenarios.

The risk of accidental oil spills in the Arctic Ocean increases concomitantly with the opening of circumpolar shipping routes and exploration for new oil resources. Exploration of these resources in ice-covered regions of the Arctic is already occurring. In the Barents Sea, the Goliat oil field has started production in April 2016. In the USA, the Bureau of Ocean Energy Management (BOEM) approved a five-year offshore oil and gas-leasing program for 2012–2017 including Arctic regions. As a keystone species in arctic ecosystems, the high sensitivity of polar cod to crude oil exposure during its early life stages is therefore likely to have important and cascading effects on the entire food chain, not just limited to a single-species phenomenon. The results may also have ramifications for the viability of economically important fisheries that rely on stable ecosystem function in the Arctic.

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interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.07.044.

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