



Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvres

Effects of acute exposure to dispersed oil and burned oil residue on long-term survival, growth, and reproductive development in polar cod (*Boreogadus saida*)

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ARTICLE INFO

Keywords:

Arctic
Petroleum hydrocarbons
Growth
Reproductive cycle
Sublethal effects
Oil spill response

ABSTRACT

The present study investigates the potential long-term physiological effects on maturing polar cod (*Boreogadus saida*), an Arctic key species, after an acute exposure (48 h) to environmentally realistic concentrations of either mechanically dispersed oil (MDO), chemically dispersed oil (CDO) or burned oil residues (BO) (N = 58–60 per treatment). Following exposure, fish were monitored in a common tank supplied with clean water for a seven-month period coinciding with the period of reproductive development. Females exposed to BO residues were more frequently found in an earlier phase of gonadal maturation compared to unexposed females while no effects of different oil spill response (OSR) actions were seen in the reproductive development of males. Mechanically and chemically dispersed oil induced a transient short-term reduction in growth in the first week post-exposure. Overall, no significant long-term effects of exposure were seen in growth or mortality. Ultimately, this study provides information for the assessment of population consequences of different OSR actions as part of a net environmental benefit analysis.

1. Introduction

Increasing anthropogenic activities related to petroleum in the Arctic elevates the risk of acute and chronic oil spills. Conditions inherent in the Arctic, e.g. weather, sea ice, limited infrastructure, remoteness, and lack of precedent for responses in areas under multinational governance, place specific requirements on operations including eventual oil spill response (OSR) (Harsem et al., 2011). Recent OSR actions have stressed the need for OSR decision making that is site- and situation-specific to adequately consider physical factors and environmental and societal resources at risk (Beyer et al., 2016; Fingas, 2016).

Polycyclic aromatic hydrocarbons (PAHs) are one group of toxic compounds in petroleum, some of which are known to exhibit carcinogenic, genotoxic, and endocrine disrupting properties, even at low concentrations (Kime, 1995; Horng et al., 2010; Vignet et al., 2014). However, a large portion of the water-soluble fraction of crude oil is made up of poorly characterized mixtures of organic compounds, also classified as unresolved complex mixtures (UCM) (Melbye et al., 2009),

with toxicological effects difficult to discern (Booth et al., 2007, 2008).

Mechanical recovery of spilled oil is often the first choice in an OSR and risk mitigation, however, chemical dispersants and *in situ* burning may be better suited in ice infested waters of the Arctic (Fritt-Rasmussen et al., 2015). Use of chemical dispersants as an OSR action is intended to increase the biodegradation potential of petroleum by forming oil-surfactant micelles (Lessard and Demarco, 2000). The use of chemical dispersants thereby enhances the oil concentration in the water column for a time whilst reducing the surface slick, the risk for encapsulating the oil slick into the sea ice (Brandvik et al., 2006), and the exposure potential for birds and marine mammals (Fingas, 2011). Increased concentrations of small oil droplets in the water column may lead to increased bioavailability of PAHs (Ramachandran et al., 2004; Milinkovitch et al., 2012), which has been linked to adverse effects on behavior, growth, reproduction, and survival in several fish species (Gulec et al., 1997; Yamada et al., 2003; Milinkovitch et al., 2011; Wu et al., 2012; Yu et al., 2015; Nwaizuzu et al., 2016).

In situ burning is a countermeasure technology that was first implemented in 1958 along the ice-covered Mackenzie River in Northwest

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<https://doi.org/10.1016/j.marenvres.2018.09.005>

Received 30 October 2017; Received in revised form 23 March 2018; Accepted 3 September 2018

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Canada and since then has been used operationally in ice free areas during the 1989 Exxon Valdez oil spill in Alaska and extensively in the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (Beyer et al., 2016; Buist, 2004; Hunt, 2009). Burning is a quickly implemented OSR action that requires little infrastructure and, most significantly, is effective, leaving approximately 10% of the original hydrocarbon load (Guenette and Sveum, 1995; Buist, 2004; Buist et al., 2013; Fingas, 2016). Burning creates a burned oil residue (BO) that may sink depending on oil characteristics (Fritt-Rasmussen et al., 2015). A limited number of studies have investigated the potential effects of BO on biota and agreement on the low acute toxicity of BO applications has been reached (for reviews see Buist, 2004; Buist et al., 2013; Fingas, 2016). Compared to untreated and chemically dispersed oil, burned oil was found to be less acutely toxic in rainbow trout (*Onchoryncus mykiss*), three-spined stickleback (*Gasterosteus aculeatus*) (Blenkinsopp et al., 1996), Australian bass (*Macquaria novemaculeata*) (Cohen and Nugegoda, 2000, 2006), amphipods and snails (Gulec and Holdway, 1999), and in copepods (Faksness et al., 2012). However, the long-term effects of acute exposure to burned oil residues has yet to be examined.

Polar cod (*Boreogadus saida*) is an endemic key species with a pan Arctic distribution (Bradstreet and Cross, 1982) that has been used as a sentinel species in Arctic environments to understand the effects and mechanisms of petroleum exposure (Christiansen and George, 1995; Jonsson et al., 2009; Andersen et al., 2015; Nahrgang et al., 2010, 2016; Bender et al., 2016). The geographical distribution of polar cod overlaps with oil and gas fields and transport areas in all Arctic seas and fish can therefore encounter petroleum products (Bailly, 2008; Bird et al., 2008; David et al., 2015). Gonadal development in polar cod begins in early autumn and final maturation and spawning takes place from January to March (Nahrgang et al., 2014; Mueter et al., 2016). Gonadal investment in polar cod is high with up to 87% of liver reserves metabolized during reproductive development (Hop et al., 1995). Throughout the active gametogenesis period, male fish undergo more rapid development of gonadal tissue and an earlier increase in plasma sex steroid concentrations than female fish (Bender et al., 2016).

Acute toxicity data (e.g. LC₅₀) using model organisms represents the majority of existing data on the effects of OSR actions while actual oil spills present the challenge of confounding factors (e.g. other sources of pollution or climate and fishing driven pressure), indirect effects, and missing background data when interpreting effects on biota (Beyer et al., 2016; Peterson et al., 2003). Expanding potential effects from short-term responses of biomarkers after acute exposure to a predictive indicator of long-term effects is challenging (Forbes et al., 2006; Claireaux et al., 2013). The objective of this study was to investigate long-term resilience of adult polar cod exposed to mechanically dispersed oil (MDO), chemically dispersed oil (CDO) or BO. To link acute exposure to long-term effects, survival, growth, and reproductive investment in polar cod were monitored for seven months after an acute (48 h) exposure. Simulated wave and current energy in open exposure tanks ensured that acute exposures set up was dynamic whereby organisms were exposed to the whole dispersed oil solution (dissolved fractions and oil droplets) (Milinkovitch et al., 2011; Frantzen et al., 2015, 2016). We hypothesized that addition of chemical dispersants would increase the exposure of polar cod to crude oil resulting in reductions in growth and reduced investment in reproductive development compared to exposure to MDO and BO treatments. Growth and reproductive development are physiological endpoints that can provide an integrative measure of the effects of pollutants on whole organisms and are important to consider when evaluating the potential risks to populations. The aim of this study is to generate sound information on the sensitivity of adult polar cod to support the net environmental benefit analysis (NEBA) of OSR actions in the Arctic marine system.

2. Material and methods

2.1. Fish collection and husbandry

Wild polar cod were collected in Svalbard fjords in September 2014 by bottom trawl during a cruise aboard the RV Helmer Hanssen and thereafter transported to the Akvaplan-niva marine laboratory in Tromsø, Norway. The fish were reared in a single common 5000 L tank for an eight-month acclimation and maintenance period and hand fed twice a week on a commercial marine fish feed (ration equal to 4% body weight per feeding; Skretting, 3–4 mm dry pellets). The light regime was maintained on a simulated Svalbard light throughout acclimation, exposure and post-exposure periods. The seawater temperature in the tank followed the annual variation of Grøtsundet, the fjord outside the marine laboratory where seawater was collected from 50 m depth, with a high of 8.6 ± 0.1 °C in September and low of 3.74 ± 0.02 °C in February and yearly average of 6.2 ± 0.1 °C. Oxygen saturation was kept above 90% for acclimation and post-exposure period. On the 19th of May 2015, all fish (n = 310) were anesthetized (Metacaine at 0.08 g/L seawater) and received a passive integrated transponder tag (Trovan[®]) inserted intraperitoneally with no mortality or negative effects observed in the first weeks post tagging.

2.2. Exposure design

Exposure to MDO, CDO or BO commenced in late June 2015, and growth was followed over a seven-month period (i.e. until January 2016) coincident with the active vitellogenesis/spermatogenesis period of polar cod (Bender et al., 2016). Goliat (Kobbe) crude oil, a sweet light crude oil with a density of 0.83 kg/L, an °API gravity of 40.3 and a sulphur content of 0.14% wet weight (Eni Norge, 2015) was used for the MDO and CDO treatments. For preparing BO, 40 L of Goliat (Kobbe) crude oil was added at the surface of a tank containing approximately 200 L sea water. The oil was set on fire and burned for 3 min until the fire died out, leaving approximately 4 L burned residue in the tank (i.e. ~90% of the fresh oil volume was burned off). The set-up consisted of four treatments and three replicates per treatment; control (no oil, Ctrl), MDO or CDO (premixed with dispersant FINASOL[®]; 5% w/w) and BO residue. The BO concentration equaled 10% of the nominal oil concentration used in the mechanically and chemically dispersed oil treatments (i.e. 90% burned off in BO compares to MDO and CDO). To be able to directly compare between dispersed oil toxicity (MDO, CDO) and BO toxicity, the same exposure protocol was used for all treatments.

The dispersant mixtures were generated according to Frantzen et al. (2015, 2016) following the protocol developed by Cedre, France for the DISCOBIOL project (e.g. Milinkovitch et al., 2011). Briefly, the oil treatments (MDO and CDO; nominal concentration of 67 mg/L) or BO (nominal concentration of 6.7 mg/L) were introduced to individual 120 L exposure tanks through a funnel fixed at the surface. A pump in the bottom of each tank provided continuous mixing energy in all tanks. In order to ensure a homogeneous exposure mixture in the tanks and to allow some weathering of the oil to take place prior to exposure start, water and oil/oil premixed with dispersant/BO were mixed for 24 h before the introduction of fish to the system. The water system was static and oil exposures were conducted for 48 h after the introduction of the animals (water temperature 6.4 ± 0.3 °C; O₂ saturation was held > 80% with aide of aerators). In total, 236 specimens were exposed from the 26th to the 28th of June 2015 with each replicate exposure tank (n = 3 per treatment) containing 18–20 fish.

2.2.1. THC and PAHs in seawater

In order to monitor exposure concentrations, water samples (approximately 1 L) were taken from all exposure tanks (n = 3 per treatment) at the beginning of the experiment (t 0 h), after 24 h (t 24 h), and at the end of the 48 h exposure (t 48 h). Determination of total

hydrocarbon content (THC; n-C10 – n-C35) was performed on iso-octane extracts by Gas Chromatography-Flame Ionization Detector (GC-FID). Each sample extract was analyzed on the GC simultaneously with control solutions (reference oil EDC95/11; Norwegian Environment Agency M-408/2015) of three known concentrations of THC, covering the concentration range of the sample extracts (Frantzen et al., 2016). Analysis of 26 PAHs (16 Environmental Protection Agency [EPA] priority parent PAHs and C1–C3-alkylated naphthalenes, phenanthrenes and dibenzothiophenes) concentrations was performed by GC–Mass Spectrometry (GC–MS) operated in selected ion monitoring mode. Single PAH concentrations were calculated by quantification of altered deuterated standards added prior to extraction, and development of a pre-determined calibration curve of five PAH-standards at different concentrations (Frantzen et al., 2016). The measured THC and PAH concentrations represent dissolved components as well as oil droplets. In the determination of Σ 26 PAH concentrations, single components with values below the limit of detection (LOD) were assigned a value of zero. Due to a technical instrument failure, water samples from t0h at the start of the exposure gave unreliable results and were excluded from further analysis.

2.3. Post-exposure monitoring and final sampling

The 48 h exposure period was followed by a 48 h recovery period in 500 L flow-through tanks and subsequent growth registration (T1; see paragraph below) before the fish were transferred back to the common 5000 L rearing flow-through tank. The common tank ensured identical post-exposure rearing conditions for all treatment and replicate groups. The fish were fasted two days prior to exposure start, during the 48 h exposure period, and two days prior to every growth measurement. Fish were, however, offered food immediately following exposure in clean water tanks even though it was less than 48 h before the growth checkpoint.

Mortality was recorded daily over the entire experiment. Growth was recorded at monthly intervals by first anesthetizing, then measuring the total weight (± 0.01 g) and total length (± 0.1 cm) at the following time points: T0 (May 19th, pit tagging), T1 (June 30th, 2 days post-exposure), T2 (July 30th), T3 (September 3rd), T4 (October 5th), T5 (November 3rd), T6 (December 9th), and T7 (January 5th). An additional group of “unexposed” polar cod was included in the common rearing tank which consisted of the remaining acclimation fish that fell below (Unexp. 1) and above (Unexp. 2) the desired intermediate size range and were therefore not included in the exposure experiment ($n = 74$). These additional unexposed fish provided a control for experimental handling stress related to the exposure with growth measurements undertaken at T0, T2–T7 (excluded from T1 due to logistical limitations).

On the 5th of January, all remaining experimental fish and the unexposed fish were sacrificed by a sharp blow to the head and the following measurements were collected: total length (± 0.1 cm), total weight (± 0.01 g wet weight [wwt]), sex, gonad weight (± 0.01 g wwt), liver weight (± 0.01 g wwt) and somatic weight (empty carcass weight, ± 0.01 g wwt). The middle section of the testis and ovaries were fixed in a buffered formaldehyde solution (4%) for later histological analysis. Otoliths were collected for age determination and read under a dissection microscope (Leica M205C).

Specific growth rate (SGR) for individual fish for the entire experimental period was determined according to the equation:

$$SGR = [(\ln_t W_2 - \ln_t W_1) t^{-1}] 100$$

Where SRG is % increase in body weight per day. ${}_t W_1$ and ${}_t W_2$ are the total weights of the fish recorded at times 1 and 2 respectively, and t is the number of days between weighting events.

Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated according to the following equations:

$$GSI = (\text{gonad weight}/\text{somatic weight}) * 100$$

$$HSI = (\text{liver weight}/\text{somatic weight}) * 100$$

Condition factor for the different time points (T0–T7) was calculated:

$$CF = (W/L^3) * 100$$

Where W is total weight in g and L is the total length in cm.

2.4. Histological analysis

Briefly, gonad tissues were rinsed of buffered formalin, dehydrated in a series of 70% ethanol baths and embedded in paraffin wax (Aldrich, USA) overnight using Histo-clear[®] as a clearing agent in a Shandon Citadel 1000 (Micron AS, Moss, Norway). Tissues were then embedded into paraffin and sliced at 5 μ m (females) and 3 μ m (males) thickness, using a Leica RM 2255 microtome before being stained with haematoxylin and eosin. Two slides were prepared for each fish. Gonad maturity stages in females were classified using the development stage of oocytes within the respective categories of immature, resting, and early and advanced stages of maturation. Immature and resting females had only primary growth (PG) oocytes while maturing females had vitellogenic oocytes present. Resting females were identified by the presence of residual oocytes from previous spawning events with otherwise only PG oocytes. Maturing females exhibited different phases of oocyte development with varying extents of vitellogenin derived oil droplets in the oocyte cytoplasm (Fig. 4ab). Oil droplets were present but filling less than $\frac{1}{2}$ of the cytoplasm in early maturing females while advanced maturing females had oocytes completely filled or nearly filled with oil droplets. Abnormal oocyte development was noted with regard to the location of cortical alveolar vesicles and oil droplet within the oocyte. Oocyte diameter ($n \geq 6$ oocytes per female) was counted for oocytes in the most advanced cohort using the image processing software (Leica DFC 295 camera attached to a Leica DM 2000 LED microscope and Leica analysis software) and then averaged for each female. Oocyte stage frequency disruption was determined by classifying all oocytes with a nucleus in an area of 20 mm² placed randomly on the tissue slice. Frequency counts were averaged over both replicate slides. Presence of residue oocytes was noted and relative frequencies of atretic oocytes were semi-quantified using a 0–3 scale ranging from 0 (0% of oocytes were atretic); 1 (1–2%); 2 (3–20%); and 3 (20–30% of oocytes were atretic) for each female. Male testes were classified into the four different maturity stages of immature, resting, and maturing with either late spermatocytes stage I (Figure S1a) or with late spermatocytes stage II dominating (Figure S1b). Immature males were identified as those with testis containing only early stage spermatocytes (Figure S1c) and a low GSI while resting males had spent testis containing portions of early stage spermatocytes with otherwise empty lumen space (Figure S1d).

2.5. Statistical analysis

All statistical analyses were performed with R 3.1.1 (R Development Core Team, 2014). A Levene's test was used to test for normality and homogeneity of variance. When homogeneity criteria were met, a one-way analysis of variance (ANOVA) was run, and when a significant treatment effect was found, the Tukey's HSD post hoc for unequal sample sizes was used to distinguish differences between treatment groups. In cases where homogeneity criteria were not met, a nonparametric Kruskal Wallis ANOVA was used, followed by a multiple comparison of mean rank of all group tests. Difference in SGR variance was tested using an F-test. Maturity stage frequency distributions were tested using a Fishers exact test with the null hypothesis that all treatments have similar maturity stage distributions. With a significant Fishers exact test result, a *chi* squared test was run comparing all treatment groups and control against one another. A probability level of

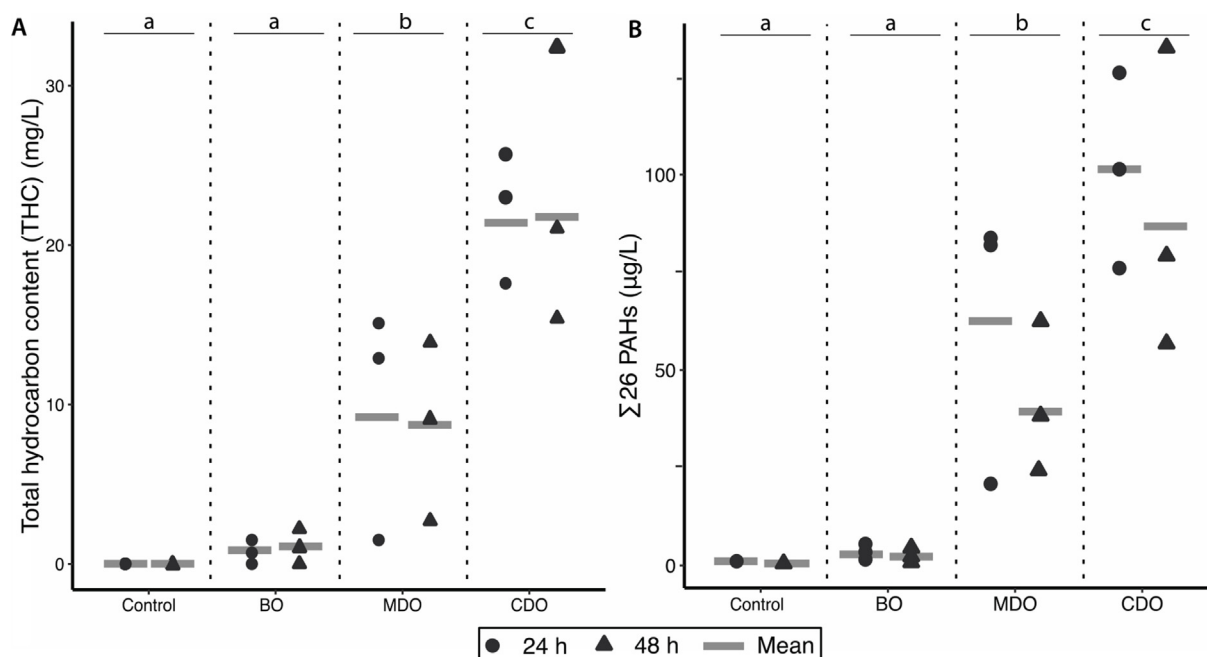


Fig. 1. (a) THC and (b) $\Sigma 26$ PAH concentrations at t24 (circles) and t48 (triangles) for all treatment groups with mean concentrations illustrated by black bars. Treatment mean concentrations that do not share a letter are significantly different ($p < 0.01$).

$p \leq 0.05$ was considered significant for all tests. All values are presented as mean \pm standard error of the mean (SE).

3. Results

3.1. Water chemistry

The total hydrocarbon content (THC) in water samples from the control treatment was not detectable throughout the exposure period, and remained relative stable throughout the last 24 h of exposure with values of 0.9 ± 0.5 , 9.2 ± 3.7 , 22.5 ± 3.7 mg/L in BO, MDO and CDO, respectively (Fig. 1a). Average $\Sigma 26$ PAH concentrations were highest after 24 h in all treatments and thereafter decreased by ca. 20% at 48 h (Fig. 1b, Table S1). Highest $\Sigma 26$ PAH concentrations were found the CDO treatment (101.5 ± 14.3 $\mu\text{g/L}$) at 24 h followed by the MDO (62.4 ± 20.7 $\mu\text{g/L}$), BO (3.5 ± 1.2 $\mu\text{g/L}$) and Ctrl (1.05 ± 0.0 $\mu\text{g/L}$) treatments. Dominating PAHs (> 98% of $\Sigma 26$ PAH) in all treatments (BO, MDO and CDO) were parent and alkylated naphthalenes, phenanthrene/anthracenes and dibenzothiophenes, whereas only parent and C1, C2-naphthalenes were detected in the Control. In BO treatment, the only high molecular weight PAHs measured above detection limits were benzo(b)fluoranthene (0.03 $\mu\text{g/L}$) and benzo(k)fluoranthene (0.01 $\mu\text{g/L}$), and these concentrations were comparable to measured concentrations in MDO and CDO (0.01–0.10 $\mu\text{g/L}$ and 0.01–0.013 $\mu\text{g/L}$, respectively; Table S1). Acenaphthylene was the only PAH with a higher concentration in BO (0.01 $\mu\text{g/L}$) compared to MDO and CDO (< 0.004–0.005 $\mu\text{g/L}$) (Table S1).

3.2. The initial fish population

Fish initially part of the exposure experiment ranged in size from 12.0 to 59.0 g total weight (mean 34.7 ± 0.6 SE), 12.0–22.0 cm length (mean 17.3 ± 0.1 SE) and age ranged between 2 and 6 years (mean 4.5 ± 0.1 SE) at T0 (Table 1). Fish used for the exposure experiment were all selected from the intermediate size group of the collected fish (size range 24.0–47.5 g) with no significant difference in size between any of the groups (Ctrl, BO, MDO, CDO). The remaining unexposed polar cod were not included in any of the treatment groups and were classified by size as they exhibited a bimodal size range that was

significantly smaller (Unexp1; size range 12–32 g) and bigger (Unexp2; size range 38–59 g) than the exposed fish.

3.3. Mortality

Mortality was observed after the first month post collection, and in the period February/March 2015 following the natural spawning period before exposure took place (data not shown), after which mortality subsided. Fish were otherwise in good condition throughout the acclimation, exposure and post-exposure monitoring period. No mortality was registered in any treatments tanks during the 48 h exposure period. Mortality was, however, observed during the post exposure period for all treatments independent of exposure. Mortality was most prevalent in the first month post-exposure (T1-T2 [June 30th – July 30th]) with 8–12% mortality occurring in all oil treatments and control. The mortality rate steadied to between 2 and 5% per month until the final sampling in January for all treatments and control with no statistical difference in cumulative mortality (Fig. 2). The group of larger unexposed fish (Unexp 2.) exhibited the highest cumulative mortality (32%).

3.4. Specific growth rate

In general, there was a great variation in SGR within all treatment groups throughout the experiment ranging from -2.5 to 3.5% change in body weight per day. Overall, growth rates (mean \pm SE) were lowest after tagging and during exposure (T0-T1 [May 19th - June 30th]), (-0.01 – 0.15% increase in body weight per day) and highest in the consecutive time period (T1-T2 [June 30th – July 30th]), 0.47 – 0.73% increase in body weight per day). In the period from tagging to immediately after exposure (T0-T1), significant treatment effects on growth rates were observed with high rates in the BO compared to lower growth rates in the MDO ($p < 0.01$) and CDO treatments ($p < 0.01$) (Fig. 3). In the following period (T1-T2) growth rates in the BO treatment were significantly reduced only when compared to the CDO treatment ($p < 0.01$). No significant differences in SGR were seen between any treatment groups or unexposed fish for the entire period (T0-T7 [May 19th – Jan 5th]) or for any other growth periods beyond the first two periods. Female and male SGR were not significantly

Table 1

Summary of polar cod sampled in January after a 7-month monitoring period following 48 h exposure to *in situ* burned oil residues [BO], mechanically dispersed oil [MDO], and chemically dispersed oil [CDO] treatment, and a control group. Unexposed fish have size distributions which fall outside the intermediate range included in the exposure experiment. Age, as determined by otoliths, total length, total weight, hepatosomatic index (HSI), and condition factor were calculated for all fish. All values are mean \pm SE.

Treatment	Number of fish sampled			Age	Length (cm)	Total Weight (g)	HSI (%)	Condition factor
	Females	Males	Total					
Control	27	20	48	4.7 \pm 0.1	20.0 \pm 0.2	56.5 \pm 2.0	9.3 \pm 0.3	0.53
BO	26	22	49	4.7 \pm 0.1	19.8 \pm 0.2	56.4 \pm 2.4	9.3 \pm 0.3	0.53
MDO	20	19	40	4.4 \pm 0.1	19.7 \pm 0.2	54.3 \pm 2.1	9.5 \pm 0.4	0.53
CDO	25	18	46	4.5 \pm 0.1	19.9 \pm 0.2	56.2 \pm 2.1	9.6 \pm 0.3	0.54
Unexp. 1	12	18	30	3.8 \pm 0.2	17.5 \pm 0.2	38.0 \pm 1.8	8.5 \pm 0.3	0.55
Unexp. 2	17	6	23	4.6 \pm 0.2	22.3 \pm 0.3	80.0 \pm 2.8	9.4 \pm 0.4	0.54

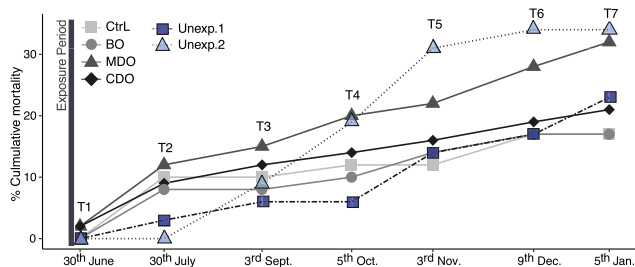


Fig. 2. Cumulative mortality (% of overall mortality) of polar cod during the course of the exposure and post exposure period (June 2015–January 2016) for each treatment group. No significant difference in % mortality was found between treatment groups, control or unexposed groups.

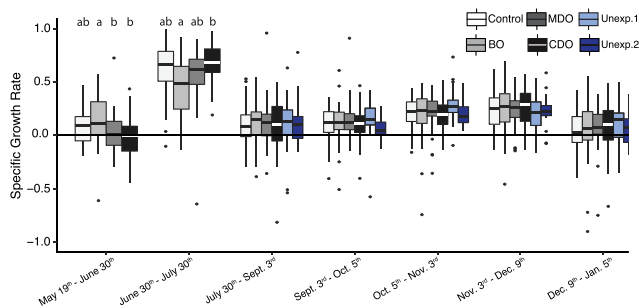


Fig. 3. Specific growth rate (SGR; % change in body weight per day) of different post-exposure time periods ($n = 40$ – 49 per treatment [Unexp. n is 23 – 30 fish]). The box ranges from the first (Q1) to the third quartile (Q3) of the data and represents the interquartile range (IQR). The line across the box indicates the median while the extreme data (outliers) outside Q1 $- 1.5 \times$ IQR and Q3 $+ 1.5 \times$ IQR are displayed as individual points. Limits of displayed data are only for SGR values from 1 to -1 excluding values outside that range. Different letters indicate significant differences between treatment groups.

different at any time period, therefore both sexes were pooled for statistical analysis.

3.5. Condition factor and hepatosomatic index

At T0 (May 19th), males (exposed and unexposed combined) had a significantly higher condition factor compared to females at 0.68 ± 0.0 and 0.64 ± 0.0 , respectively. At T1 (June 30th), the condition factor was significantly higher in females in the BO treatment (0.66 ± 0.0) compared to females in the other groups (control [0.62 ± 0.0], MDO [0.62 ± 0.0] and CDO [0.60 ± 0.0]). At no other time point were there significant differences found between any of the treatment groups (including control) or sex. Furthermore, no significant difference in age, HSI, or condition factor was seen between any treatment or sex at the end of the experimental period in January

(Table 1).

3.6. Reproductive development

3.6.1. Females

Histological analyses revealed that 56% of female fish had spawned previously as determined by presence of residual oocytes, while 22% exhibited first time maturation with no evidence of previous spawning and the remaining specimens were immature (6%) or resting (16%). From the maturing females, 68% revealed a leading oocyte cohort that had reached the vitellogenic stage II (Vtg II) and were categorized as advanced maturing with mean oocyte diameter of $547 \pm 8 \mu\text{m}$, a centrally placed nucleus and the cytoplasm filled with vitellogenin derived oil droplets (Fig. 4). In 32% of maturing females, however, the most advanced oocyte cohort was in an early vitellogenic stage (Vtg I) and was thus categorized as early maturing with an oocyte diameter of $446 \pm 11 \mu\text{m}$ and vitellogenin derived yolk droplets only at the periphery of the cytoplasm and persisting cortical alveolar vesicles, often in combination with atresia (Fig. 4). Abnormal oocyte development, characterized by partial inclusion of cortical alveolar vesicles into the cytoplasm, non-radial yolk globule orientation around nucleus, and few oocytes in the most advanced oocyte cohort, was observed in 35% of early maturing females with no statistical significance of treatment. Significant differences in gonadal maturity stage was observed in the BO exposed females exhibited by a lower percentage of advanced maturing (35%) and higher percentage of early maturing females (38%) compared to other treatment groups (mean percentage in advanced maturing stage was 61%) ($p = 0.042$) and when tested against the control group only, the significance increased (X-squared = 7.99, $df = 2$, p -value = 0.018) (Fig. 5a). No significant differences were found between treatments in mean oocyte diameter, the relative number of oocytes in the leading cohort, presence of residual oocytes or frequency of atresic oocytes. However, significantly greater variation in oocyte diameter was observed in early maturing females in the BO treatment ($443.5 \pm 42 \mu\text{m}$, $n = 7$) compared to the control ($409.0 \pm 10.7 \mu\text{m}$, $p = 0.015$, $n = 5$).

Gonadosomatic index (GSI) in females ranged between 0.6 and 11.5 with no significant difference between any of the treatments (Fig. 5b). GSI (Mean \pm SE) for immature, resting, advanced maturing and early maturing females was (0.9 ± 0.0), (2.6 ± 0.8), (5.7 ± 0.3) and (3.6 ± 0.3), respectively. Advanced maturing female GSI was significantly higher than all other maturity stages ($p < 0.001$).

3.6.2. Males

Testis development appeared normal for males in all treatments with no significant difference in the frequency of occurrence of different maturity stages among the treatments (Fig. 6a). GSI in males at the end of the experiment (T7) ranged between 0.0 and 33.3 with no significant difference between any of the treatments (Fig. 6b). Immature and resting fish made up 5.9% and 2.9% of the sampled males respectively

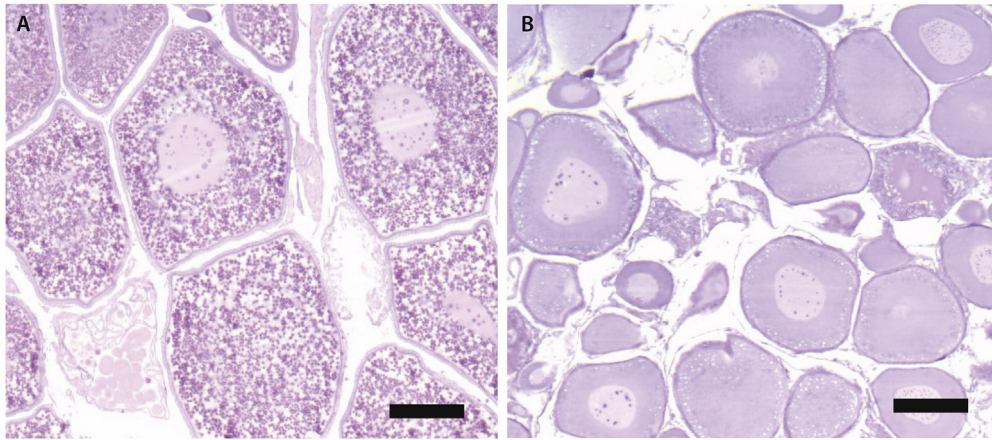


Fig. 4. Histological representation of an (a) early maturing female with cortical alveoli vesicles and early signs of vitellogenesis with yolk globules present in oocyte periphery; (b) advanced maturing female with vitellogenic oocytes. Scale bare is 200 μm in both pictures.

while 53.9% of males were in an early stage of maturation (late Sc I) and 37.3% of the males were in a later stage of development (late Sc II) (see Fig S1. for maturity stage representations). Immature and resting males had a low mean GSI (2.0 ± 1.6 and 2.0 ± 0.9 , respectively). Maturing males with late spermatocytes stage I had a lower GSI (15.8 ± 0.8) compared to those with more developed late spermatocytes stage II (22.6 ± 1.0).

4. Discussion

4.1. Exposure to dispersed oil and burned oil residue

The present study simulates conditions in which dispersant (CDO treatment) or *in situ* burning (BO treatment) might be used to combat an oil spill in Arctic waters in comparison to no action (MDO treatment). THC and PAH water concentrations in both MDO and CDO reflected environmentally realistic concentrations reported from experimental field trials and dispersant operations during actual oil spills (i.e. THC concentrations of 30–50 mg/L below the spill just after treatment before decreasing to < 1–10 mg/L, and ΣPAH concentrations of

6–115 $\mu\text{g/L}$ the first days or weeks after accidental oil spills) (Law, 1978; Humphrey et al., 1987; Lunel et al., 1995; Short and Harris, 1996; Kingston, 1999; Reddy and Quinn, 1999; Lessard and Demarco, 2000; Sammarco et al., 2013). Reports of hydrocarbon concentrations in seawater after *in situ* burning operations are scarce. PAH and THC levels in the present study are below seawater concentrations measured after experimentally spilled and burned oil in the Newfoundland Oil Burn Experiment (3.78 $\mu\text{g/L}$ $\Sigma 16$ EPA PAHs) (Daykin et al., 1994), and above THC concentration from an oil spill simulation and test burning experiment in the Barents Sea (13 $\mu\text{g/L}$) (Brandvik et al., 2010).

The overall THC and $\Sigma 26$ PAH concentrations in the Ctrl, MDO and CDO treatments were in agreement with previous experiments using the same nominal oil concentrations and exposure set-up as in the present study (Frantzen et al., 2015, 2016), and confirms that the addition of chemical dispersant increases the efficiency of the dispersion process leading to significantly elevated THC and PAH concentrations in CDO compared to MDO. Measured BO concentrations were $8 \pm 2\%$ of the measured MDO concentrations, indicating that mechanical dispersion of BO into the water column was equally efficient as for oil. In the present study, an identical exposure protocol was used for all

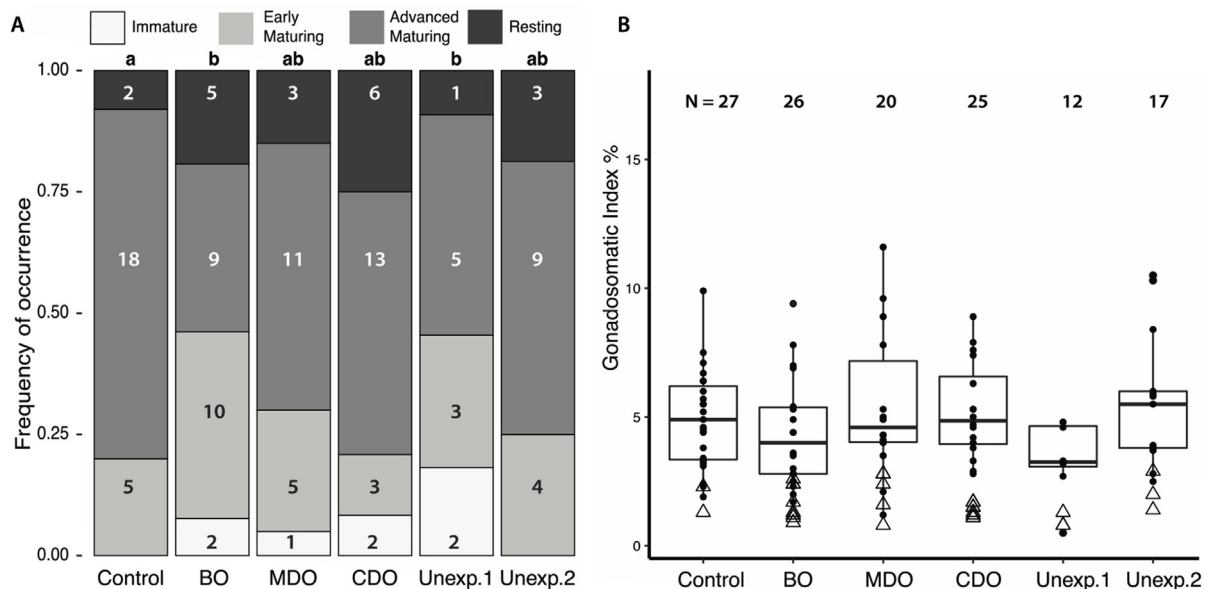


Fig. 5. (A) Maturity stage frequency distribution of females from all treatments; (B) boxplots of GSI of female fish in different treatment groups, maturing females are plotted in the boxplots and immature and resting females are indicated at triangles. Different letters above treatment groups indicate significant differences between treatment groups, those with letters in common are not significantly different from one another. The number in each box refers to the number of fish in that category.

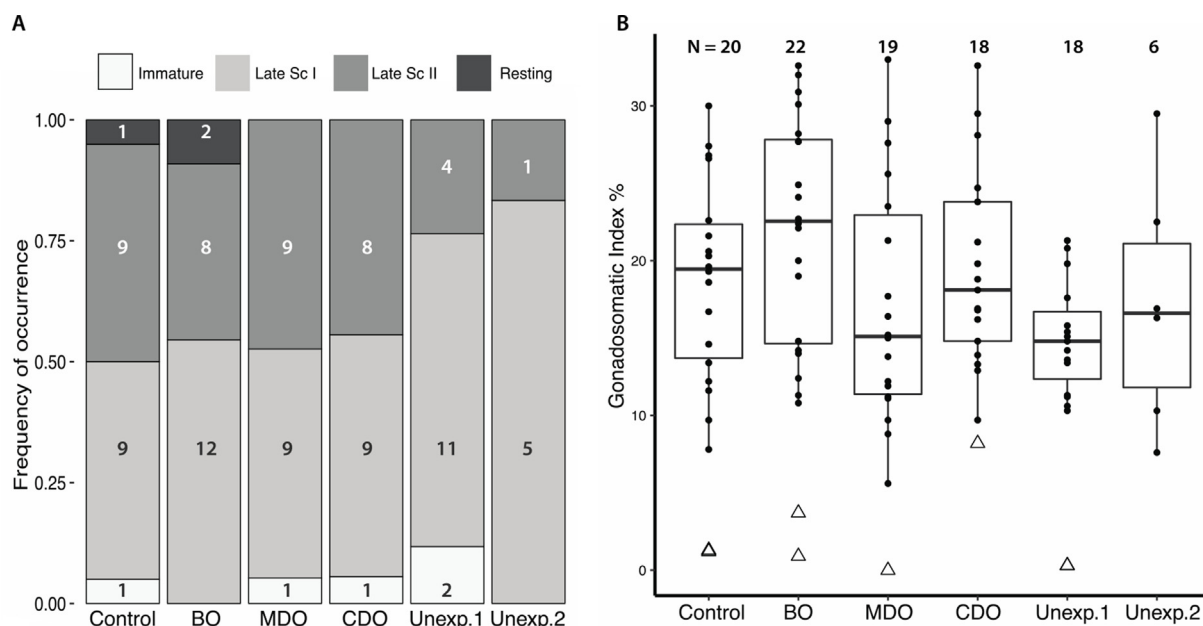


Fig. 6. (A) Maturity stage frequency distribution of males from all treatments; (B) boxplot of GSI of male fish in different treatment groups, maturing males are plotted in the boxplots and immature and resting males are indicated at triangles. No significant differences were found between treatments groups. The number in each box refers to the number of fish in that category.

treatments to allow for direct comparison of effects between the oil spill response measures investigated. Energy was added to the seawater to simulate a dynamic exposure with wave energy for the period of 4 tidal systems (48 h) (Merlin, 2005; Milinkovitch et al., 2011), and the measured concentrations of hydrocarbons represented both the water-soluble fraction as well as BO residue particles/dispersed oil droplets. Adding mixing energy to simulate wave action to the BO residue exposure dispersing it in the water column is, however, novel as previous studies have exposed organisms only to the burned oil WSF (Faksness et al., 2012; Gulec and Holdway, 1999), and reported measurements are taken only of seawater hydrocarbon concentrations underneath burned areas (Brandvik et al., 2010).

Forming of short-term temporary oil slicks, variation in oil adherence to equipment and mixing by fish movements between replicate tanks may be a source of the individual variability in THC and PAHs concentrations between replicate water samples, and the increased PAH/THC concentration at T48h compared to T24h observed in two individual tanks (one MDO and one CDO tank, respectively). Inter- and intra-tank variations did however not influence the overall significant difference in THC/PAH concentrations between the OSR actions investigated. Low concentrations of naphthalene measured in the control water may be considered elevated background levels with no potential toxic effects to biota (Molvær et al., 1997) and are evidence of the ubiquity of PAHs, especially naphthalene, one of the most abundant PAHs in the marine environment (Latimer and Zheng, 2003).

4.2. Physiological and reproductive effects

No relationship was found between treatment and mortality. The sustained mortality rate in seen in all groups (both exposed and unexposed) is most likely due to the post spawning physiological state of the mature fish as confirmed by the presence of residual oocytes in 56% of females. Handling stress at the beginning of the experiment could have induced higher mortality at this early time point. The mortality rate seen in this experiment (~24%) was lower than the mortality observed (~56%) in a long-term crude oil exposure on adult feral polar cod held in captivity (Bender et al., 2016). Fish were in a good state of health as evidenced by an unanimously high condition factor and HSI in

all treatment groups at the final sampling in January, although the HSI values reported for fish in the present study (8.5–9.6%) were lower than for polar cod of a similar size held in captivity at the same time of year (10.9–13.1%) (Bender et al., 2016). Fish in the latter study were fed a natural diet of *Calanus* sp. zooplankton whereas commercial feed was used in the present study and this difference in diet may have influenced the HSI. Higher condition factor in males compared to females at the start of the experiment is most likely due to the difference in the timing of reproductive investment, where males start gonadal investment earlier in the season than females (Hop et al., 1995; Nahrgang et al., 2014).

Growth rates observed in polar cod of the present study were within reported ranges from previous studies of mature fish (Jensen et al., 1991; Hop et al., 1997). Furthermore, the observed trends in growth rate did not indicate significant long-term effects by any of the OSR actions. The transient decrease in SGR for the MDO and the CDO treatments compared to BO treatment, may however, be due to a transient appetite depression in these two groups in the first days following the exposure. Low feeding activity was visually observed at this time. No effect of crude oil exposure on appetite has been observed in polar cod previously; however, exposure to crude oil contaminated food did lead to reduced growth in exposed fish (Christiansen and George, 1995). The SGR in July (T1-T2) was highest ($0.6 \pm 0.0\%$ body weight change per day) in all treatment groups compared to all other periods (0.1–0.2% change per day) and may reflect some compensatory growth following handling and fasting during exposure (Ali et al., 2003). Reduced feeding and growth was also observed in African catfish (*Claris gariepinus*) for two months following exposure to crude oil and dispersed crude oil (Nwaizuzu et al., 2016).

Females likely to spawn in the coming winter season were in the late maturing (Vg II) stage with a GSI around 5.7 ± 0.3 while it is unclear when or if the females in the early maturing stage would spawn. The timing of spawning from other laboratory polar cod populations in an analogous reproductive stage suggests that the late maturing females would be ready to spawn in March (Bender et al., 2016). The high frequency of early maturing females may be an evidence of stress resulting in reduced investment into reproductive development (Rideout et al., 2005; Kime, 1995). However, with only a single histological

sampling point it is not possible to resolve if the females in the early maturation stage initiated vitellogenesis at the same time as females in the late maturing phase and then paused further development or if vitellogenesis was ongoing at a reduced pace. Nevertheless, abnormal oocyte development observed in some early maturing females (i.e. nonconforming yolk globule orientation) may suggest that vitellogenesis was interrupted and that these oocytes may soon be reabsorbed through atresia (Rideout et al., 2005). Reabsorbing vitellogenic oocytes result in a lower fecundity and have been observed in Atlantic cod under environmental stressors like low temperature, poor nutritional, and pollution (Rideout et al., 2005). However, no increased incidence of atresia was observed in early maturing females at sampling. The increased frequency of early maturing females in the BO exposure group could indicate a reduced population fecundity compared to the unexposed and control groups. The large variation in oocyte size of early maturing females exposed to BO treatment may be early signs of reabsorption of vitellogenic oocytes or of some other disruption of oogenesis. PAHs have endocrine disrupting properties with potential to impair vitellogenesis in fish (Hylland, 2006; Aruwke and Goksøyr, 2003). Despite low tissue PAH concentrations, reproductive impairment was seen in Gulf killifish two months after the Deepwater Horizon oil spill (Whitehead et al., 2012). Similarly, depressed plasma 17 β -estradiol concentrations were seen in dolly varden and yellowfin sol after the Exxon Valdez oil spill (Sol et al., 2000).

Although the overall THC/PAH concentration in BO was an order of magnitude lower than in MDO and CDO, differences in physical characteristics of the BO may have altered the exposure route and time exposed to the BO treatment and thus enhanced the toxicity of the BO residue compared to MDO and CDO. Burned oil residues have increased viscosity and stickiness compared to crude oils (Fritt-Rasmussen et al., 2015; Fingas, 2016). The size of oil droplets and BO particles were not measured in this experiment; however, BO particles were most likely larger than MDO and CDO oil droplets as they could be observed with the naked eye as “black dots” in the water column during the exposure. In contrast to BO particles, mechanically and chemically dispersed oil droplets are generally found to be in the size of $\leq 100\ \mu\text{m}$ and 10–50 μm , respectively (Lessard and Demarco, 2000; Lewis and Daling, 2001), and could not be observed by eye. The BO residue may have clogged gills, adhered to skin, and/or been ingested and stick to the digestive tract of the fish and thereby increased the exposure time to PAHs and other compounds (including UCM) present in the BO residue compared to MDO and CDO. For instance, burned residues can be enriched in high molecular weight PAHs, pyrogenic PAHs, and metals (Buist, 2004; Shigenaka et al., 2015; Fingas, 2016). The UCM profiles of burned oil residues from the DWH burns exhibited an altered shape and lacked the more volatile *n*-alkanes when compared to the fresh unburned oil (Stout and Payne, 2016). These compounds were not quantified in the present study despite their potential contribution to adverse effects. Other studies investigating acute toxicity of BO residues have found non-toxic or little effects on snails and amphipods at concentrations below 1.46 mg/L THC or 5.83 $\mu\text{g/L}$ total PAHs when exposed for 24 h (Gulec and Holdway, 1999). No additional effect of the WSF after burning on *Calanus* spp. when exposed for 96 h at concentrations less than 1 mg/L THC compared to the WSF prior to burning (Faksness et al., 2012). Australian bass exposed to burned oil WAF for four days did express EROD activity levels, a biomarker of PAH exposure, similar to levels in fish exposed to mechanically dispersed oil WAF but significantly lower than fish exposed chemically dispersed WAF (Cohen et al., 2006). However, knowledge on the toxicokinetics of BO compared to other treatments is still lacking and there is a strong uncertainty when it comes to toxicity of UCM.

Gonadal investment occurred earlier in males compared to females in accordance with other studies investigating polar cod reproductive development (Bender et al., 2016; Nahrgang et al., 2014). No effect of any treatment on the timing, structure, or investment in male reproductive development indicated the relative resilience of this sex.

Male polar cod invest less energy in reproductive development compared to females (Hop et al., 1997), which may allow for greater tolerance to xenobiotic exposure during the reproductive development period. Inclusion of the unexposed fish into the experimental design provided additional information on background physiological change due to size differences. The smaller unexposed fish (Unexp. 1) were generally younger and less likely to mature in the current season, with an increased prevalence of immature individuals and lower HSI compared to their larger unexposed counterparts (Unexp. 2). Maturing individuals in Unexp. 1 had generally lower GSI values than maturing fish in larger size categories emphasising the importance of size in reproductive output (Nahrgang et al., 2014). The Unexp. 2 fish were of a similar age and larger size (both length and weight) than fish included in the exposure experiment but exhibited a higher mortality rate and no immature individuals further supporting the hypothesis that mortality is related to previous spawning events.

4.3. Conclusion and outlook

The transient effects observed on growth rate in the present study did not affect overall growth and survival of the polar cod during this 7-month experiment, demonstrating the robustness of adult polar cod. Early life stages of polar cod however have demonstrated increased sensitivity to crude oil (Nahrgang et al., 2016) and therefore are a major source of uncertainty when assessing population level impacts of a potential spill and response actions. The decreased frequency of maturing females exposed to the BO treatment is of importance with regard to potential reductions in population fecundity (Spromberg and Meador, 2006) and may reveal a sensitivity of polar cod when exposed to dispersed BO residues from this OSR countermeasure. This effect observed on the potential reproductive output of female polar cod exposed to BO is not explained by the relatively low THC and PAH levels measured in the BO treatment, therefore other hydrocarbon compounds, the UCM, physical properties and toxicokinetics of the BO residue warrants further investigation.

With increasing anthropogenic activity in the Arctic, polar cod are at risk for exposure to petroleum and OSR actions through accidental spills. The purpose of a NEBA is to aid in the decision making of an OSR and evaluate the environmental effects of an action or combination of actions *a priori*. However, no long-term effects on polar cod survival and growth were observed under acute dynamic exposure conditions to BO, MDO or CDO. Observed effects were overall limited. The physiological effects of BO need further investigation, including exposure method validation and additional chemical analysis. The reduction of overall oil by $\sim 90\%$ with *in-situ* burning will reduce the oil volume and the potential for organisms to come into contact with the oil and may still be a viable option despite the potential adverse effects observed in this study. Overall, this study demonstrates the robustness of the adult life stage of polar cod to a variety of OSR actions. The final endpoints of reproduction, such as fecundity, fertilization success and survival and fitness of offspring of exposed polar cod, were not included in the present study, however these endpoints would provide valuable information on ecosystem sensitive for the NEBA in the Arctic marine system. This study provides new evidence to aid in OSR decision making on the sensitivities of the Arctic key species polar cod.

Ethics statement

Permission to carry out this experiment was granted by the Norwegian Animal Welfare Authority in 2015 (ID 7851).

Acknowledgements

The authors would like to thank the members of the International Association of Oil and Gas Producers, Arctic Oil Spill Response Technology – Joint Industry Programme for funding this project. The

Principals of the JIP are: BP Exploration Operating Company, Chevron Canada Limited, ConocoPhillips Corporation, Eni E & P, ExxonMobil Joint Research Limited, North Caspian Operating Company, Shell International Exploration and Production BV, Statoil Petroleum AS and Total E & P Recherche et Développement. The authors greatly appreciate the help from the crew of the RV Helmer Hanssen for collecting and transporting the fish and the technical staff at the Akvaplan-niva marine laboratory in Tromsø, Norway for their polar cod husbandry expertise. Additional funding was allocated by the flagship research programme "Environmental impact of industrial development in the north (MIKON)" of the Fram Centre and the NRC projects EWMA (grant number 195160) and ARCEX (grant number 228107).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.marenvres.2018.09.005>.

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