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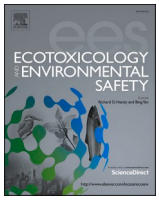

Effects of chronic exposure to the water-soluble fraction of crude oil and in situ burn residue of oil on egg-bearing Northern shrimp (*Pandalus borealis*)

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ABSTRACT

Oil spill clean-up measures using in situ burning can potentially result in seafloor contamination affecting benthic organisms. To mimic realistic exposure and measure effects, ovigerous Northern shrimp were continuously exposed for two weeks to the water-soluble fraction of oil coated on gravel followed by two weeks in clean seawater. North Sea crude oil (NSC) and field generated in situ burn residue (ISBR) of NSC were used (Low: 3 g/kg gravel, Medium: 6 g/kg gravel and High: 12 g/kg gravel). The concentrations of polyaromatic hydrocarbons (PAHs) in the water resulting from NSC were higher compared to ISBR. No mortality was observed in any treatment and overall moderate sublethal effects were found, mostly after exposure to NSC. Feeding was temporarily reduced at higher concentrations of NSC. PAH levels in hepatopancreas tissue were significantly elevated following exposure and still significantly higher at the end of the experiment in NSC_{High} and $ISBR_{High}$ compared to control. Mild inflammatory response reactions and tissue ultrastructural alterations in gill tissue were observed in both treatments. Signs of necrosis occurred in $ISBR_{High}$. No change in shrimp locomotory activity was noted from NSC exposure. However, ISBR exposure increased activity temporarily. Larvae exposed as pleopod-attached embryos showed significant delay in development from stage I to stage II after exposure to NSC_{High}. Based on this study, oil-contaminated seafloor resulting from in situ burning clean-up actions does not appear to cause serious effects on bottom-living shrimp.

1. Introduction

Oil exploration and production activities as well as ship traffic cause a risk of accidental oil spills at sea. In situ burning (ISB) of floating oil at or close to the site of the spill is one possible mitigation option (Fritt-Rasmussen et al., 2015). This spill clean-up method has the potential to rapidly remove large amounts of oil from the sea surface and, compared to other methods, can be easier to use in difficult to reach locations. The use of ISB is still contested due to potential health issues for operators (and human populations nearby) as well as to potential environmental effects both, above and under water. ISB produces smoke and often leaves a viscous residue. Fractions of light and heavy oil compounds are removed during burning, but depending on burning efficiency, a residue of these fractions remains. Since lighter compounds are removed more efficiently, heavier compounds concentrate in the residue (Fritt-Rasmussen et al., 2015). High-molecular weight PAHs have high chronic toxicity, but a low bioavailability in the residue matrix (Buist, 2004). The potential environmental impacts of ISB residues are related to the physical properties and oil composition, which vary with oil type and the burning efficiency (Buist, 2004). In an environmental as well as operative context, a key concern is the fate of the residue, whether it floats or sinks (Fritt-Rasmussen et al., 2015). If the residue sinks, it potentially affects benthic organisms. High molecular weight PAHs tend to bind readily to particulate matter and organic matter enriched sediments (Chiou et al., 1998; Barakat et al., 2011), and are degraded very slowly, potentially representing a long-term source of toxic compounds for benthic organisms (Environment Canada, 1999).

Several studies have recently investigated the potential toxic effects

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of oil and the ISB residue on pelagic organisms (Bender et al., 2018; Toxværd et al., 2018; Johann et al., 2020), concluding that ISB did not produce a burn residue that was more toxic than the weathered oil itself. Most recently, Keitel-Gröner et al. (2021) found that the water accumulated fraction of a crude oil (pre-weathered Oseberg blend) was acute toxic to Northern shrimp (*Pandalus borealis*) larvae whilst the ISB residue was not. Altogether, these results indicate that applying ISB potentially mitigates acute impacts of spilled oil on pelagic organisms. However, published work on the effects of long-term exposure on benthic marine organisms is scarce, although locally covered benthic habitats were identified as one of the most significant concerns when semi-solid or semi-liquid residues submerge (Buist, 2004). Blenkinsopp et al. (1996) compared the toxicity of weathered crude oil (Alberta sweet mixed blend) and the resultant burn residue from the Newfoundland offshore burn experiment on three-spine stickle back (*Gasterosteus aculeatus*) and white sea urchin (*Lytechinus pictus*) gametes and found all samples not to be toxic to the study organisms.

In the present study, adult shrimp (*P. borealis*), an epibenthic key species of the North Atlantic food web, was chosen as the study organism. *P. borealis* is widely distributed along the entire Norwegian coastline up to Svalbard at depths between 20 and 1330 m (Bergström, 2000). The shrimp spawn in the autumn and the eggs are carried on the pleopods of the females until spring, when the larvae hatch. Within a period of 2–3 months, the shrimp larvae pass through six developmental stages after which they settle to the bottom as post larvae (Shumway et al., 1985; Bergström, 2000).

The overarching hypothesis of the present study was that adverse effects of crude oil on *P. borealis* are more pronounced compared to ISB residue. Two sub-goals were established to confirm this hypothesis. The first goal was to determine acute and long-term effects of crude oil and ISB residue on ovigerous Northern shrimp. A gradient of field realistic oil concentrations was used, and ovigerous shrimp individuals were continuously exposed over two weeks to WSFs of both crude oil and ISB residue. We hypothesized no acute toxicity of ISB residue exposure and that crude oil exposure would result in more sublethal and dose-dependent effects. The second goal was to investigate the effects of embryonic exposure on shrimp larval development. Larvae exposed as eggs attached to the pleopods of the female shrimp were kept in clean seawater after hatching and delay in stage II development compared to control larvae was assessed. We hypothesized that larvae would be smaller, and development delayed when exposed to WSF of the crude oil compared to the ISB residue.

2. Material and methods

2.1. Shrimp sampling and maintenance

Ovigerous Northern shrimp (*Pandalus borealis*) were collected on January 27th, 2020 by bottom trawl from Kvitsøyfjorden (Rogaland County, Norway; N59.4, E5.34) at about 160 m depth and transported to the laboratory facilities within 2 h (see Bechmann et al., 2020 for more details). Shrimp were kept for a week in 500 L tanks in the laboratory with flow-through of sand filtered seawater pumped from a depth of 75 m from the adjacent fjord (Byfjord, ambient temperature of approx. 7 °C) before temperature was gradually reduced to 5 °C during one week of acclimatization. Salinity of the intake water was routinely checked and was stable around 34 psu. Shrimp were fed a mixture of 3 mm pellets of fish feed (Nutra Olympic, Skretting, Norway) and 1 mm shrimp feed (reference diet, Skretting, Norway) ad libitum during acclimatization. All experimental work was approved by the Norwegian Animal Research Authority (FOTS 22860).

2.2. Test oil and in situ burn residue

A pre-weathered North Sea crude oil (Oseberg 200°C+, representing oil at sea for 1–4 days; referred to as NSC in the following) and the ISB residue (referred to as ISBR) were provided by NOFO (Norwegian Clean Seas Association for Operating Companies). The oil is a light (API gravity: 39.6), intermediate low-sulfur (0.20%) oil (Leirvik and Myrhaug Resby, 2007). The ISBR sample was obtained during an ISB experiment conducted by NOFO and the Norwegian Coastal Administration in June 2018, where 6 m^3 of oil were released into a fire-resistant oil boom and ignited by a drone. Further details, including physical-chemical characterization can be found in Engen et al. (2018), Faksness and Krause (2018) and Faksness et al. (2021).

2.3. Experimental set-up and procedure (except for the locomotory activity recording)

All work was conducted in temperature and light controlled rooms (5 °C) with 8 h dimmed light and 16 h darkness. Ovigerous Northern shrimp (average length 8.0 ± 0.3 cm, average wet weight 6.3 ± 0.7 g) were exposed to the water-soluble fraction (WSF) of NSC or ISBR using oil-coated gravel columns (Carls et al., 1999; Nahrgang et al., 2010) to mimic seafloor exposure in addition to a seawater control. Gravel (approx. ø 1–5 mm) was washed, thoroughly air dried and mixed with NSC or ISBR at concentrations of 3, 6 and 12 g/kg gravel (Nahrgang et al., 2010) corresponding to Low, Medium and High treatment, respectively. Aliquots of ISBR were heated to 50 °C for about 20 min to ease fluidity and homogeneity prior to coating on gravel. A 10 mL syringe was used to transfer the required amount of ISBR, using 2 kg gravel at a time and mixing was performed by manual stirring on a warm plate (approx. 50 °C) until the gravel was evenly coated with oil (about 5 min). Then, the coated gravel was added to 1 m high PVC columns (10 kg per column, external column diameter 110 mm), with clean gravel added to a control column. One column provided exposure water to three replicate exposure tanks (60 L) containing 10 shrimp each (Fig. S1 A). Sand filtered seawater percolated upwards through the columns generating a WSF exposure with a water flow of approx. 450 mL min^{-1} to each exposure tank. During the first 2 h of water percolation, the water was discharged to waste drain to ensure that no large oil particles resulting from the initial washing of the coated oil with the flow accumulate in the exposure tanks. The shrimp were exposed for 2 weeks, before columns were removed and clean seawater was provided for another 2 weeks post-exposure at the same flow. Oxygen (87.6 ± 2.0%) and temperature (5.1 ± 0.03 °C) were recorded every other day in all exposure tanks. Flow rates were measured regularly and adjusted when necessary.

2.4. Chemical analyses

2.4.1. Oil compounds in the exposure water

Water samples (800 mL) were taken randomly from one exposure tank (replicate) per treatment when shrimp were transferred into exposure tanks at the start of the exposure as well as at the end of the exposure after two weeks. Two weeks post-exposure, one water sample was taken each from NSC_{High} and $ISBR_{High}$. Water samples were acidified (HCl, 15%) for conservation and chemical analyses were performed by SINTEF Ocean AS (Trondheim, Norway). Water samples were analyzed for semi-volatile organic compounds (decalins, PAHs and phenols) using GC/MS (Agilent Technologies 7890B GC system and 5977 A MSD). Surrogate internal standards (SIS, o-terphenyl, naphthalene-d8, phenanthrene-d10, chrysene-d12, phenol-d6, 4–methylphenol-d8) were added to the water samples prior to processing, and recovery internal standards (RIS, 5α-androstane, fluorene-d10, and acenaphthene-d10) were added prior to analysis on GC/MS. The water samples were spiked with the appropriate surrogate internal standards and serially extracted with dichloromethane (DCM), thereby following a modification of EPA method 3510C (US EPA, 1996). The combined extracts were dried with sodium sulphate and concentrated to approximately 1 mL using a Zymark Turbovap® 500 Concentrator. The final extract was spiked with the appropriate recovery internal standards and

analyzed on GC/MS. The list of target analytes includes the recommended analytes given by Singer et al. (2000) and is a typical standard list for the target compounds used during post-oil spill damage assessments. The semi-volatiles were quantified by modifications of EPA Method 8270D (US EPA, 2007). The mass spectrometer was operated in the selective ion monitoring mode to achieve optimum sensitivity and specificity. The quantification of target compounds was performed by the method of internal standards, using average response factors (RF) for the parent compounds. The PAH and phenol alkyl homologues were quantified using the straight baseline integration of each level of alkylation and the RF for the respective parent PAH compound. The response factors were generated for all targets and surrogates versus fluorene-d10. Details on the results can be found in the supplementary material (Figs. S2, S3 and Table S1).

2.4.2. Tissue concentration of PAHs

Samples were taken after two weeks exposure and at the end of the experiment. Shrimp were killed by decapitation. Total length, carapace length (±0.1 cm) and wet weight (±0.1 g) of all individuals were noted before dissection. After exposure, three shrimp per exposure tank (n = 9 per treatment) were sampled. Hepatopancreas tissue was dissected and pooled based on exposure tank, and frozen at −20 °C until analysis. At the end of the experiment, hepatopancreas tissue was sampled again from control, NSC_{High} and $ISBR_{High}$ (pooled tissue of three shrimp per replicate) to document post-exposure accumulation levels.

Pooled hepatopancreas samples (n = 3 per treatment) were sent to SINTEF Ocean AS (Trondheim, Norway) for analysis of 44 semi-volatile organic compounds. Tissue concentration of PAHs was also quantified by modifications of the EPA Method 8270D (US EPA, 2007). Accumulation of PAHs is used as a biomarker of oil exposure and can indicate the potential for transfer of PAHs in the food web. Data are presented as μg/g tissue wet weight.

Upon arrival at SINTEF Ocean AS, hepatopancreas samples were homogenized in the original sample tubes and an aliquot was subsampled for analysis. Surrogate internal standards (SIS, naphthalene-d8, phenanthrene-d10, chrysene-d12) were added to the samples prior to processing, and recovery internal standards (RIS, fluorene-d10 and acenaphthene-d10) were added prior to analysis on GC-MS/MS (gas chromatography/serial mass spectrometry).

For analyses of semi-volatile organic compounds (SVOC), samples were spiked with the appropriate surrogate internal standards and serially extracted with DCM:hexane (50:50). The extracts were dried with sodium sulphate and concentrated to approximately 1.5 mL using a gentle stream of N_2. The fat in the samples were removed using a HPLC fitted with a fraction collector and a GPC (Gel Permeation Chromatography) column. The extracts were concentrated to approximately 0.3 mL and spiked with the appropriate recovery internal standards and analyzed on GC-MS/MS.

The mass spectrometer was operated in MRM (Multiple Reaction Monitoring) mode to achieve optimum sensitivity and specificity. The quantification of target compounds was performed by the method of internal standards, using average response factors (RF) for the parent compounds. The PAH alkyl homologues were quantified using the baseline integration of each level of alkylation and the RF for the respective parent PAH compound. The response factors were generated for all targets and surrogates versus fluorene-d10.

2.5. Effects on adult shrimp

2.5.1. Mortality, behavior and feeding rate

Mortality and behavior of shrimp were monitored daily during the exposure and after 1 week and 2 weeks post-exposure. Each tank was observed (approx. 1 min) and individual shrimp behavior was categorized as normal (standing) or abnormal (stress swimming or lying immobilized on the side) according to Bechmann et al. (2019). Feeding behavior was examined during exposure (after 24 h and 96 h, 1 week and 2 weeks) as well as at the end of the experiment two weeks post-exposure. Therefore, 10 pellets were added to each tank and 24 h later uneaten feed was visually estimated. Daily feed consumption was then calculated as the total feed supplied minus residual feed and converted into consumed biomass. The feed biomass was based on average pellet dry weight. Feeding rates were then calculated as percentage body weight (based on total wet weight per tank) per day. The total wet weight did not differ significantly between tanks (Kruskal-Wallis test p = 0.43).

2.5.2. Gill histopathology

At the end of the exposure, anterior gill samples were dissected from two shrimp per exposure tank (n = 6 per treatment) from all treatments for histopathological examination. At the end of the post-exposure period, specimens from control as well as NSC_{High} and $ISBR_{High}$ were sampled again. After dissection, gill samples were fixed in Davidson's fixative for 48 h and transferred to formalin free Fine Fix® solution (Milestone Medical, Italy). The samples were then processed by serial alcohol dehydration, embedded in Technovit 7100, a plastic embedding system based on HEMA* (2-hydroxyethyl methacrylate), and cut into 8 μm transections by a Leica RM 2165 rotary microtome before being stained by toluidine blue staining. The degree of histological damage was scored using a light microscope according to Landers et al. (2020). Four fields were scored on each of 5 slides per individual per treatment. Scores were based on the number of fields in which histological changes were observed with class 0 = no histopathology in any field, class 1 = mild histopathology present in <25% of the fields, class 2 = moderate histopathology present in 25–75% of the fields, and class 3 = severe histopathology present in >75% of the field, following the scale suggested by Zodrow et al. (2004) and adapted to Northern shrimp by Bechmann et al. (2019).

2.5.3. Locomotory activity recording

In a supplementary, pilot scale experiment, oil-induced locomotory activity of adult shrimp was investigated using the infrared light beam system (Bamber and Westerlund, 2016) available at NORCE Mekjarvik, Norway. Adult shrimp typically stand upright, while they occasionally walk or swim towards food particles or periodically move short distances along the length of the tank. Increased activity above this basal level in the absence of feeding cues can be considered abnormal, indicating stress or potential avoidance response. Here, two questions were addressed: i) Do shrimp show oil-induced, immediate changes in swimming behavior? ii) Does oil exposure modify the swimming behavior response of shrimp to glycine, an established crustacean feeding stimulant (Heinen, 1980, Lim et al., 2021)?

Due to practicalities, water accommodated fractions (WAFs) of NSC and ISBR were prepared similar to Keitel-Gröner et al. (2021) for these recordings and similar PAH concentrations were assumed. No independent chemical analyses were conducted. Additionally, a dilution factor of 30 was introduced from the stock WAFs to exposure waters in the tanks, leading to estimated PAH concentrations of 7 μg/L in WAF_{NSC} and 0.8 μg/L in WAF_{ISBR}.

Briefly, the activity assay consisted of 4 test tanks (6.3 L volume), each provided with a continuous flow of filtered seawater (5 ± 0.5 °C) at a rate of 680 mL/min and equipped with four infrared light beams set across their width, each matched on the opposite side with phototransistors. Beams were stacked in pairs in order to detect movement throughout the tank. As shrimp moved through the beams, they interrupted the light to the phototransistor and the resulting drop in voltage was recorded as an activity event. Continuous recording of activity took place throughout the additions of the various test solutions to the tanks. Further details on technical specifications of sensors and data logging can be found in Bamber and Westerlund (2016). Shrimp (n = 4 for each treatment) were acclimatized for 24 h in the test tanks, before a glycine solution (giving a nominal concentration of 1 mM in the test tanks) was delivered through a peristaltic pump into each chamber for a period of 5

min. Preliminary tests had shown Northern shrimp responded to glycine at this concentration with increased swimming activity. Following this initial pulse of glycine, the test tanks received clean seawater for 55 min, before WAF of NSC or ISBR was added for one hour. The tanks were flushed with seawater for another hour before a final 5-minute delivery of glycine to compare the reaction before and after the WAF exposure. Locomotory activity of individual shrimp was plotted in activity plots (beam breaks per 5 min), allowing direct visual comparison of patterns in activity.

2.6. Effects on shrimp larvae after embryo exposure

After two weeks of exposure, two shrimp per tank (n = 6 per treatment) from control, NSC_{High} and $ISBR_{High}$ were transferred into separate hatching aquaria (18 L) to study carry-over effects on shrimp larvae exposed as embryos. Hatching aquaria were checked daily and larvae were removed until about 40 larvae had hatched within 24 h. The females were then removed. During the first week, larvae were fed freshly hatched *Artemia* nauplii enriched with *Thalassiosira weisslogii* algae (Arnberg et al., 2013), before *Artemia* nauplii only were provided ad libitum for the remaining time. Larvae were kept until 17 days post-hatch (dph) and staging was performed at 12, 14 and 17 dph according to Keitel-Gröner et al. (2020). Percentage of first instar (referred to as stage I larvae in the following) and second instar (referred to as stage II larvae) was documented. Total length as a proxy of larval growth was determined according to Rasmussen and Aschan (2011) in a subsample of larvae (n = 30 per treatment) at the end of the experiment (see Keitel-Gröner et al., 2020).

2.7. Statistics

Statistical analyses were performed using GraphPad Prism statistic software version 8.4.3 (GraphPad Software, San Diego, CA, USA). Data were tested for normal distribution using the Kolmogorov-Smirnov test. Differences in observed effects between the different treatments were tested using either one-way ANOVA followed by Dunnett's multiple comparisons test (normally distributed data) or Kruskal-Wallis followed by Dunn's multiple comparisons test when normality test failed. Statistical significance was tested at $p < 0.05$.

3. Results

3.1. Oil compounds in the exposure water

Initial PAH (sum 44 PAHs) concentrations measured in the water of NSC_{Low}, NSC_{Medium} and NSC_{High} were 15, 36 and 44 μg/L compared to 2, 4 and 8 μg/L in $ISBR_{Low}$, $ISBR_{Medium}$ and $ISBR_{High}$. In the control, 0.2 μg/L were found. All PAH concentrations declined over the time of the experiment (Fig. 1, Table S1) as was expected with the column gravel system. The predominant PAHs initially present were naphthalenes, mainly C1-C3 homologues (Fig. S2). However, the relative hydrocarbon abundances shifted towards C0-C5 phenols and 4–6 ring PAHs over the two weeks exposure period in all treatments (Fig. S3). At the end of the post-exposure period, PAH concentrations in NSC_{High} and $ISBR_{High}$ were comparable to control level.

3.2. Tissue concentration of PAHs

The concentration of sum PAHs was significantly higher in the hepatopancreas of NSC and ISBR exposed shrimp than in control shrimp (Table 1). The tissue concentrations in shrimp exposed to the various concentrations of NSC were approximately an order of magnitude higher than in shrimp exposed to the corresponding treatments of ISBR. Compared to the PAH concentrations in exposure waters at the start of the exposure, accumulation rates were comparable for NSC and ISBR. However, comparing tissue levels with concentrations in the exposure

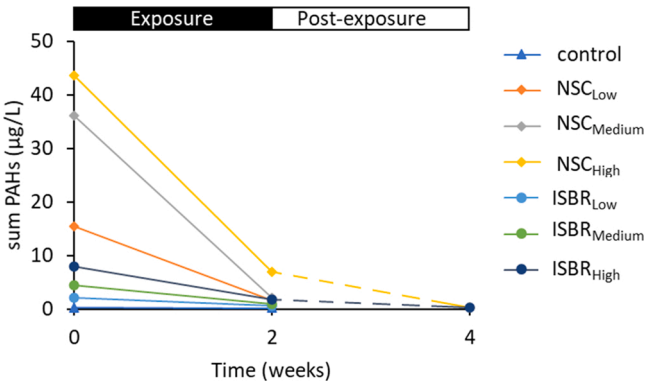

Fig. 1. Total concentration (μg/L) of polycyclic aromatic hydrocarbons (PAHs) in the water of the control (triangle), Low, Medium and High treatments of North Sea crude oil (NSC - diamond) as well as in situ burn residue (ISBR - circle) at start and end of the exposure. NSC_{High} and $ISBR_{High}$ were sampled again two weeks post-exposure.

waters at the end of the exposure, both NSC_{Medium} and NSC_{High} showed the greatest accumulation. Percentages of hydrocarbon fractions differed between NSC and ISBR treatments at the end of the exposure (Fig. S4). NSC treatments contained mainly naphthalenes (57–64%), followed by 2–3 ring PAHs (34–39%) and 4–6 ring PAHs (1–4%). In contrast, ISBR treatments mainly contained 2–3 ring PAHs (56–59%), followed by naphthalenes (32–37%) and 4–6 ring PAHs (6–12%). Two weeks after ending the exposure, the tissue concentrations of sum PAHs in shrimp exposed to NSC_{High} and $ISBR_{High}$ were still significantly higher than in the control shrimp, but significantly lower than in shrimp sampled at the end of the exposure period (Table 1).

3.3. Effects on adult shrimp

3.3.1. Mortality, behavior and feeding rate

No mortality occurred during the experiment and no significant differences in behavior were found between treatments in the exposure tanks. About 90–100% of shrimp showed normal behavior (standing) during the observation period in the different tanks throughout the experiment (data not shown).

Comparable feeding rates were found in control and ISBR treatments (Fig. 2), except for $ISBR_{Low}$ two weeks post-exposure. A dose-dependent decrease in feeding was found after NSC exposure, statistically significant for NSC_{Medium} and NSC_{High} compared to control (except for NSC_{Medium} 96 h exposure). After 96 h exposure, the feeding rate was reduced by half in NSC_{High} compared to control (1.6 ± 1% compared to 3.3 ± 0.3%). After one-week exposure, the feeding rate in NSC_{Medium} was reduced by 50% and in NSC_{High} by 75% compared to control feeding rate (control: 3.3 ± 0.3%; NSC Medium: 1.7 ± 0.9%; NSC High: 0.8 ± 0.6%). Two weeks post-exposure, no more significant differences were found between treatments (data not shown).

3.3.2. Gill histopathology

Variable level of alterations in gill histopathology were observed over the experimental time, mostly in NSC_{High} and $ISBR_{High}$ treatments. Overall, there were no severe histopathology alterations (score 3) in any shrimp or treatment in this study. Control shrimp showed mild histopathological changes (haemocytic infiltration and hyperplasia) (Fig. 3a and Fig. S5a) both at the end of exposure and post-exposure (Fig. 3b and Fig. S5b). NSC_{High} and $ISBR_{High}$ showed moderate (score 2) gill haemocytic infiltration (Fig. 3g–h – I and Fig. S5a) after the exposure with a greater occurrence (approx. 50%) in NSC_{High}, remaining significantly higher than control post-exposure (Table 2). After two weeks exposure to $ISBR_{High}$, mild but significant necrosis phenomena (Fig. 3h – II) were found that were not observed at the end of the post-exposure period

Table 1
Sum of polycyclic aromatic hydrocarbons (sumPAHs) in the exposure water (μg/L) and hepatopancreas tissue (μg/g wet weight) of *Pandalus borealis* after two weeks exposure to Low, Medium and High treatments of North Sea crude oil (NSC) and in situ burn residue (ISBR). Water samples (n = 1) were taken at the start and end of the exposure period in all treatments. Post-exposure, water samples of NSC_{High} and $ISBR_{High}$ were analyzed. Tissue samples (mean ± SD; n = 3) were taken after two weeks exposure in all treatments, and post-exposure only from control, NSC_{High} and $ISBR_{High}$. Asterisks indicate statistically significant differences between control and treatment groups (*p < 0.05, **p < 0.01, ***p < 0.001). Capital letters indicate statistically significant differences between exposure and post-exposure samples of the same treatment (A: p < 0.05, B: p < 0.01, C: p < 0.0001).

	Water (sum PAHs in μg/L)			Tissue (sum PAHs in μg/g wet weight)			
	Exposure		Post-exposure	Exposure		Post-exposure	
Treatment	Start	End		Mean	SD	Mean	SD
Control	0.22	0.21		0.50	0.06	0.24^{A}	0.13
NSC_{Low}	15.45	1.64		56.90	9.07		
NSC_{Medium}	36.08	2.06		214.9 *	133.1		
NSC_{High}	43.60	6.94	0.32	343.5 * *	88.75	60.24^{***B}	6.68
$ISBR_{Low}$	2.02	0.59		5.63	1.81		
$ISBR_{Medium}$	4.41	0.95		16.77	1.98		
$ISBR_{High}$	7.99	1.68	0.27	46.12 * *	3.06	4.76^{*C}	2.67

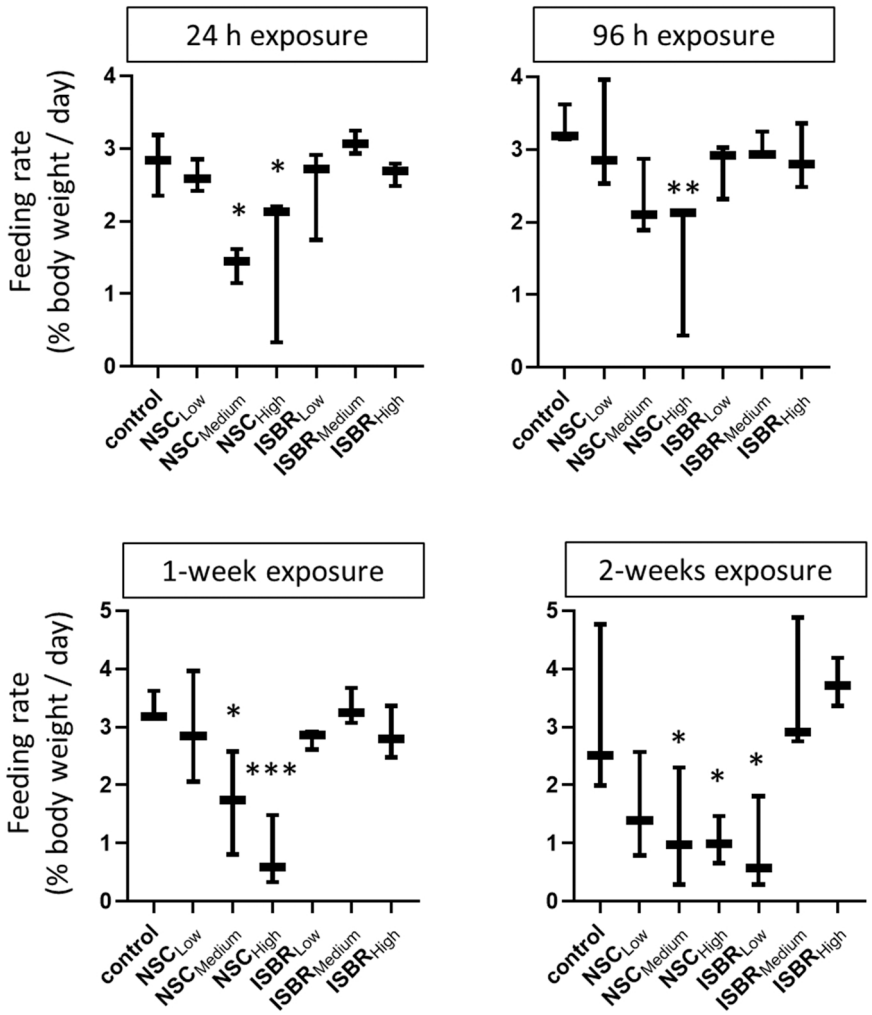

Fig. 2. Feeding rate (percentage body weight per day) of *Pandalus borealis* in control and exposed to Low, Medium and High levels of North Sea crude oil (NSC) or the in situ burn residue (ISBR). N = 3 exposure tanks per treatment with 10 shrimp in each tank. Asterisks indicate significant difference between exposed groups and control (*p < 0.05, **p < 0.01, ***p < 0.001).

(Fig. 3j). Mild, but highly significant observations of gill hyperplasia (Fig. 3g -III and Fig. S5a) were made after exposure to NSC_{High}, and this condition worsened post-exposure (Fig. 3i and Fig. S5b), mostly in NSC_{High} (30% occurrence of score 2). Swelling of gills was mostly observed at lower concentrations of NSC.

3.3.3. *Locomotory activity recordings*

Locomotory activity plots (Fig. 4) showed that shrimp were not active before receiving the first glycine pulse. The glycine addition (Fig. 4, red arrow) triggered a significant and drastic increase in locomotory activity that lasted for about half an hour in most shrimp. Upon the subsequent exposure to NSC (Fig. 4, top - blue arrows), only one

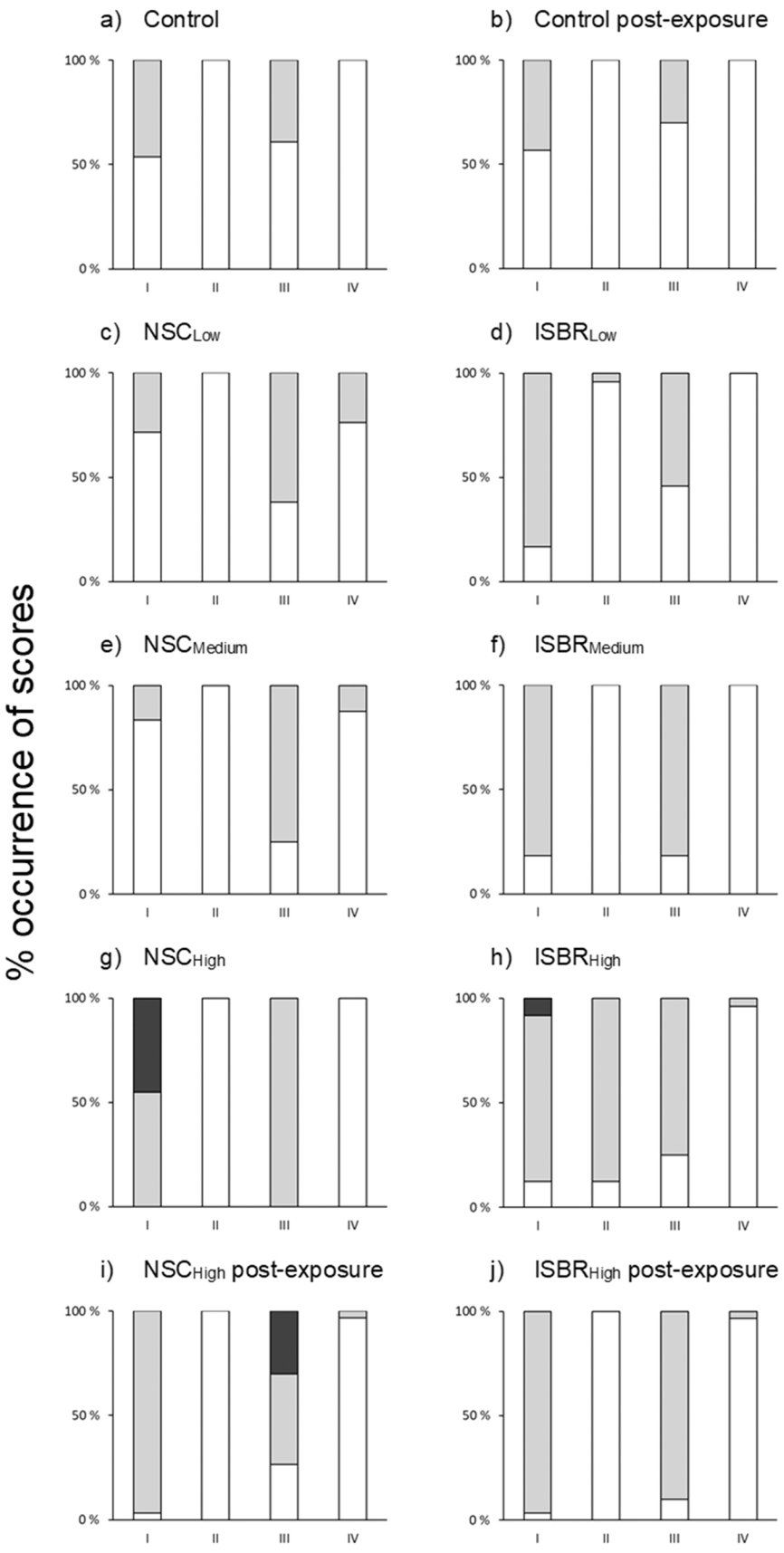

Fig. 3. Histopathological alterations in gills of control shrimp (a–b) and shrimp exposed to Low, Medium and High levels of North Sea crude oil (NSC) and the in situ burn residue (ISBR) (c–j) after two weeks of exposure (all treatments) and following two weeks in clean seawater post-exposure (Control, NSC_{High} and $ISBR_{High}$). Score 0: no histopathology in any field, score 1: mild histopathology present in <25% of the fields, score 2: moderate histopathology present in 25–75% of the fields. I: Haemocytic infiltration, II: Necrosis, III: Hyperplasia, IV: Swelling of gills. For statistical details, see Table 2.

Table 2
Overview of p-values from statistical analysis of histopathological changes in shrimp exposed to North Sea crude oil (NSC) or the in situ burn residue (ISBR) compared to control after exposure and post-exposure, respectively. I: Haemocytic infiltration, II: Necrosis, III: Hyperplasia, IV: Swelling of gills.

Histopathological alteration	Exposure	Post-exposure
I	NSC_{High} $p < 0.0001$	NSC_{High} $p = 0.003$
	$ISBR_{High}$ $p = 0.04$	$ISBR_{High}$ $p = 0.003$
II	$ISBR_{High}$ $p < 0.0001$	
III	NSC_{High} $p = 0.002$	NSC_{High} $p < 0.0001$
		$ISBR_{High}$ $p = 0.0002$
IV	NSC_{Low} $p = 0.004$	

shrimp (shrimp 4) remained active for approx. 35 min of the exposure. Following the first glycine addition, all shrimps in ISBR group came back to baseline activity and when ISBR was added (Fig. 4, bottom – blue arrows), locomotory activity of all shrimps increased again for a short-period (~15 min), yet at a lower amplitude (50%) compared to glycine. Upon the second glycine pulse, the locomotory activity in all shrimp increased again in both treatments to levels seen after the first pulse.

3.4. Sublethal effects on shrimp larvae after embryo exposure

Egg-carrying shrimp from control, NSC_{High} and $ISBR_{High}$ were transferred to clean seawater after exposure until larvae hatched. Hatching started 8 ± 9 days after transfer into clean seawater in the control, 6 ± 7 days in NSC_{High} and 4 ± 3 in $ISBR_{High}$. The time from the last day of exposure until hatching did not differ significantly between treatments. After hatching, larvae were kept until 100% of the control larvae had reached stage II. No differences in stage II larvae length were found after 17 days post-hatch (dph) in any of the treatments. Average

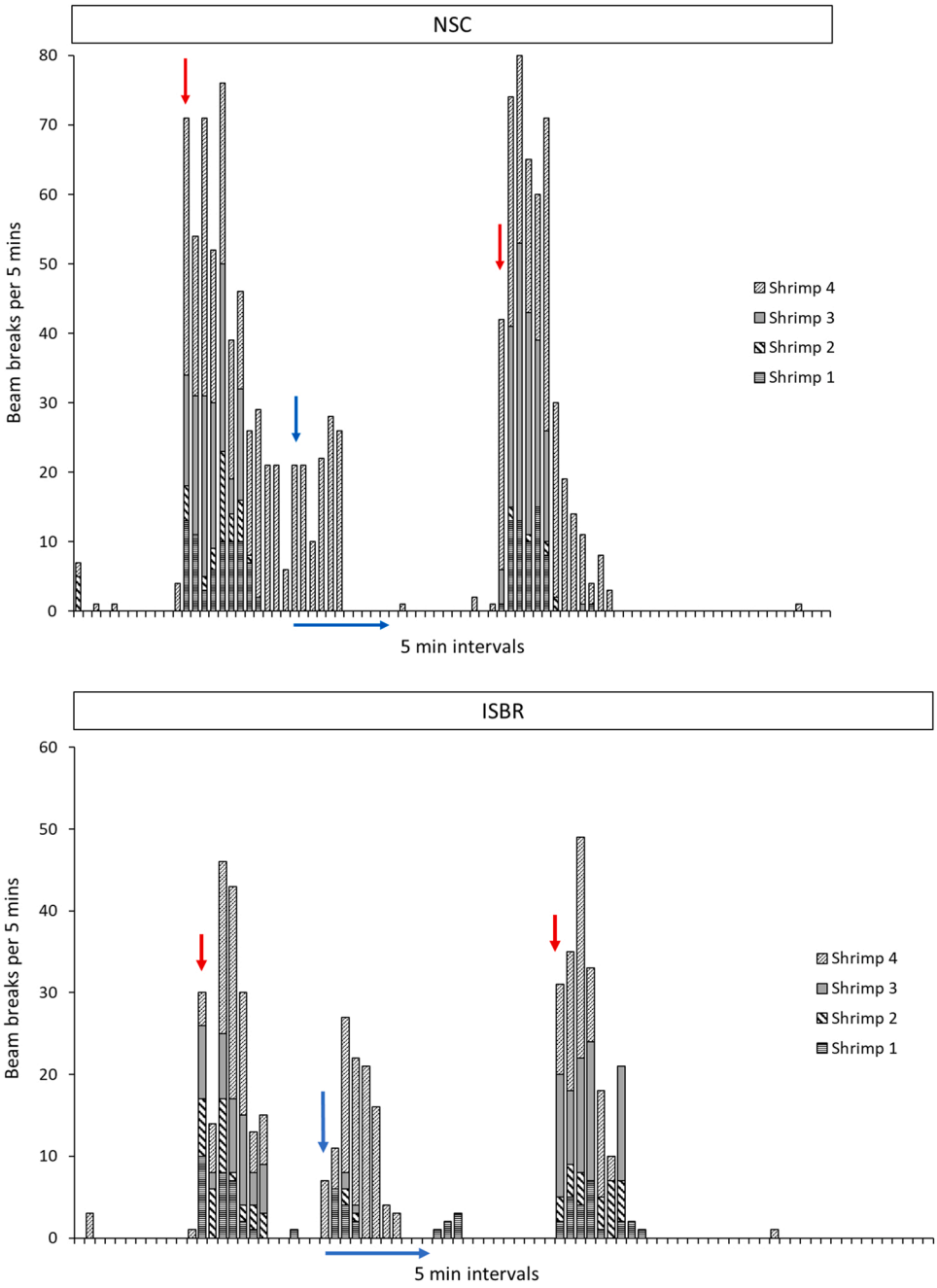

Fig. 4. Activity levels of shrimp (n = 4) expressed as number of beam breaks per 5 min intervals. Shrimp moving within the recording tank cause beam breaks by interrupting the infrared light beam from the source to the transmitter. Here, the number of passages of the beam during 5 min intervals is shown. Shrimp were exposed to two 5 min glycine deliveries (red arrow, nominal concentration 1 mM) before and after a 60-minute pulse (blue arrows) of the water accommodated fraction of North Sea crude oil (NSC, top) and the in situ burn residue (ISBR, bottom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

total length in the control group was 6.4 ± 0.4 mm compared to 6.5 ± 0.4 mm in NSC_{High} and 6.4 ± 0.3 mm in $ISBR_{High}$. However, exposure to NSC_{High} significantly reduced the percentage of larvae that reached stage II 14 and 17 dph compared to control (Fig. 5), but no effect was found upon ISBR exposure.

4. Discussion

4.1. Knowledge gap and relevance of the exposure

This work was conducted to increase knowledge on the acute and long-term effects of ISB residue on bottom living organisms. Despite identified research needs, knowledge of the consequences is still missing (Fritt-Rasmussen et al., 2015). The effects of ISB residue were evaluated compared to the original oil, assuming the residue would result in less environmental side effects than NSC. Low to no acute toxicity of burn residue WAF exposure were found in marine invertebrate species (Faksness et al., 2012; Keitel-Gröner et al., 2021) as well as several fish species (Cohen and Nugegoda, 2000; Bender et al., 2018; Johann et al., 2020). To our knowledge, this is the first study addressing the impact of ISB residue on the epibenthic (adult) stage of *Pandalus borealis*, and further toxicity studies on other benthic species are warranted to understand the actual consequences of this spill mitigation option on benthic life. This study indicates that ISB would have fewer toxic effects, hence be considered an oil spill mitigation option that would not impair significantly benthic organisms. However, this study did not take into account the other environmental trade-offs of ISB such as airborne components and heat energy, which also need to be considered to decide on the best oil spill response option.

The exposure levels (sum PAHs) of NSC in the present study were comparable to reported field measurements and similar to results published by Nahrgang et al. (2010). The concentrations at the start of the exposures were 15, 36 and 44 μg/L compared to 15, 18 and 40 μg/L (Nahrgang et al., 2010). Diercks et al. (2010) reported PAH concentrations in subsurface waters near the Deepwater Horizon oil well site to range from 29 μg/L to 189 μg/L three weeks after the sinking of the oil rig. Compared to lethal and sublethal effect concentrations found in the literature, the authors concluded that PAH concentrations reported could have led to toxicity effects of the subsurface PAH compounds at least as far as 13 km from the wellhead site at the time of data acquirement. Lower total concentrations and a shift towards larger PAHs were expected for the ISBR treatment due to the removal of mainly lighter compounds during the burning process. In a mesocosm based study with oil in ice, Toxværd et al. (2018) found that ISB resulted in the lowest PAH exposure concentration as a result of the removal of 80–90% crude oil volume during the incineration process. The low, declining PAH concentration in the exposure water found in this study, as well as a shift in the composition of the exposure water, is a likely exposure scenario of adult shrimp to contaminated sediment. The water-soluble fraction of crude oil is thought to cause the greatest toxicity in marine organisms due to its high bioavailability (Carls et al., 2008). Oiled rock columns have been used before to study oil spill effects on marine organisms to mimic exposure and consequences (Carls and Meador, 2009). Here, this approach was adopted to simulate exposure of shrimp to contaminated sediment (except for the locomotory activity recordings where separate WAFs were prepared).

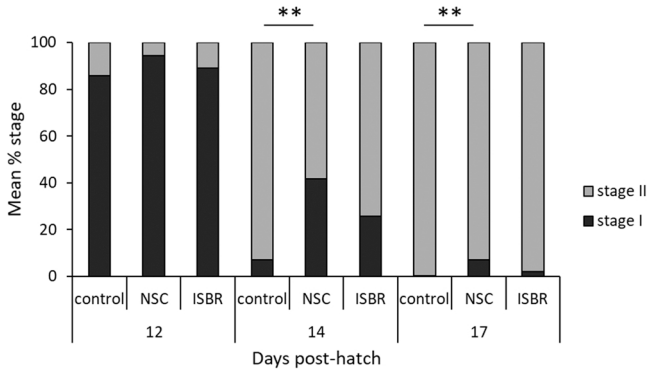

Fig. 5. Developmental stages (percentage) of *Pandalus borealis* larvae exposed as embryos to the high treatment concentration of North Sea Crude oil (NSC) and the in situ burn residue (ISBR) at selected days post-hatch (n = 6). Asterisks indicate significant differences between control and treatment (**$p < 0.01$).

The same amount of NSC and ISBR resulted in initial sum PAHs concentration differences of about 8-fold in Low and Medium and 5-fold in High, resulting from the burning process in ISB to reduce the oil amount. The exposure water composition was partly different between the two treatment groups. The proportion and actual concentration of naphthalenes was greatest in the NSC treatments, whilst larger PAHs were relatively more abundant in ISBR treatments. This could explain the differences in sublethal effects observed in the two treatments.

4.2. Effects on adult shrimp

Exposure concentrations did not cause mortality in adult shrimp, but sublethal effects were found. Much of the work on effects of oil spills on marine organisms has been conducted on different fish species and largely, no lethal effects on adults were found in those studies either. However, 28 days exposure of juvenile southern flounder (*Paralichthys lethostima*) to field collected Deepwater Horizon oil-contaminated sediments led to increase mortality, both with concentration and duration of the exposure (Brown-Peterson et al., 2017). Oil spills mainly kill fish at the egg or larval stage (Hjermann et al., 2007) with possible long-term population consequences (Langangen et al., 2017). In laboratory studies, adult fish were able to detect petroleum at very low concentrations (Hellstrøm and Døving, 1983; Beitinger, 1990; Farr et al., 1995) and juvenile and adult fish can avoid water with high hydrocarbon concentrations (Hjermann et al., 2007). Our results showed that the locomotory activity recordings of *P. borealis* under NSC exposure did not change whereas ISBR exposure resulted in an increase, indicating the shrimp could sense ISBR more than NSC. For both treatments, the estimated sum PAH concentrations in the locomotory activity study were approx. half of those in the column exposure at start in NSC_{Low} (7 μg/L compared to 15 μg/L) and $ISBR_{Low}$ (0.8 μg/L compared to 2 μg/L). In fathead minnows (*Pimephales promelas*), the lowest concentration of fluoranthene producing an avoidance response was found to be 14.7 μg/L and at 8.6 μg/L no avoidance response was produced (Farr et al., 1995). According to chemical composition, ISBR contained less of the soluble compounds that could trigger a behavioral response. The explanation for this response remains unclear. The response was short and did not seem to affect shrimp ability to sense food when glycine was added the second time. Our data support that exposure levels were not causing any de-sensibilization of the olfactory system of shrimp since swimming activities were similar after the first and second glycine pulse. Repeating the experiment with more replicates and higher exposure concentrations would be insightful to verify our observation. Overall, these data indicate that shrimp would not necessarily relocate from their living location after an oil contamination, which could lead to a long-term exposure to low concentrations of petroleum hydrocarbons.

Feeding behavior was found to be transiently altered in NSC treatments. Feeding was reduced dose-dependently during exposure, whereas after two weeks in clean seawater, feeding rates were similar to control again. In copepods, reduced feeding due to PAH exposure has been shown to be a very sensitive endpoint. Detrimental effects on feeding occurred at toxic concentrations ca. 2–3 fold lower than for narcotic effects in *Oithona davisae* (Saiz et al., 2009). The authors attributed this lower threshold for feeding effects to very subtle, preliminary drowsy effects disturbing feeding before full narcosis found at higher concentrations. Narcotic effects (lack of motility) on shrimp were not found in the present study, however sluggish effects could be a likely

explanation for the transient reduction in feeding.

Following oil exposure, accumulation of PAHs is commonly found in marine organisms, such as bivalves (Baussant et al., 2009) and crustaceans, including copepods (Almeda et al., 2013), krill (Moodley et al., 2018) and shrimp (Bechmann et al., 2010). Two major paths of PAH uptake, directly from the dissolved phase or via dietary intake have been identified (Wang and Wang, 2006). Here, PAHs were most likely passively taken up from the dissolved phase. The accumulation of PAHs in hepatopancreas tissue was documented for both NSC and ISBR and reflected to some extent the exposure water concentrations and composition of PAHs. The hepatopancreas is a major organ of decapods combining many functions of the liver, pancreas and intestine (Caceci et al., 1988), and is therefore greatly involved in the uptake and excretion of contaminants such as PAHs. Shrimp were shown to excrete PAHs over time. The PAH concentrations were much higher at the end of the exposure period than two weeks post-exposure but levels were still significantly higher in NSC and ISBR compared to the control. Bechmann et al. (2010) suggested that shrimp potentially metabolize and excrete more of the larger PAHs, while in the present study, the proportions of fractions of hydrocarbons in hepatopancreas tissue did not alter noticeably when comparing exposure and post-exposure conditions. Slow elimination and depuration of oil components can indicate a risk for transfer of oil components up the food web to pelagic fish, seabirds and marine mammals. Additionally, shrimp living in a region with contaminated sediment would also consume food potentially contaminated by PAHs, adding an additional route of exposure (Pie et al., 2015). Ultimately, the accumulation of PAHs in shrimp hepatopancreas tissue may allow the detection of previous exposures in shrimp sampled in the field, since after two weeks depuration, PAHs levels were still significantly increased in the tissues of shrimp exposed to oil in the lab.

In aquatic animals, the gills are generally the most delicate of the epithelia in direct contact with the environment and prone to damage by passive diffusion of pollutants through the membranes. Histopathological changes in target organs of shrimp have been shown to be relevant to characterize effects of chemical exposure (Chiodi Boudet et al., 2015; Bechmann et al., 2019). Control shrimp showed a well-organized structure (Bechmann et al., 2019; Soares et al., 2019). Only mild to moderate treatment effects on gills were mostly found in the highest exposure concentrations of both NSC and ISBR. The alterations found here in shrimp gill tissue were comparable to those found in fish species after oil exposure. However, histopathological changes in fish are often more diverse, including epithelial lifting and aneurysm (Khan, 1998; Brand et al., 2001; Simonato et al., 2008). Epithelium hyperplasia can be considered an adaptive defense mechanism that helps the organisms in preventing the entrance of xenobiotics (Simonato et al., 2008; Agamy, 2013). After two weeks post-exposure, the necrotic effects found in $ISBR_{High}$ were already reduced, while hyperplasia effects increased post-exposure. Histopathological examination revealed long-lasting effects also after ISBR exposure. Many adverse effects can be reversed by immune system reaction or tissue restructuration (Bechmann et al., 2019). A longer study would be needed to understand the meaning of the histopathological changes from NSC and ISBR exposure for shrimp population and if they can recover.

4.3. Sublethal effects on shrimp larvae after embryo exposure

During their development, shrimp embryos rely on yolk reserves for their nutritional needs (Brillon et al., 2005) and are carried in a mass attached to the pleopods of the females, who provide parental care by pleopod movements (Baeza and Fernández, 2002). Embryos of *P. borealis* develop over approx. five months at the latitude of shrimp capture for this study, whereas further north, development typically takes longer (Bergström, 2000). Embryos were exposed to decreasing WSF concentrations of NSC_{High} and $ISBR_{High}$ for two weeks in the late part of their embryonic development. The exposure ended on average 6 (±7) days before hatching started. Only minor effects on shrimp larvae development were found following exposure to NSC_{High}, with 93% of larvae reaching stage II 17 days post-hatch, compared to 100% in the control. ISBR exposure did not cause any significant effects. Delayed development associated with oil exposure has been observed in crustaceans (Almeda et al., 2013). Following a continuous exposure to a mechanically dispersed oil (0.015–0.25 mg/L) for 3 months as embryos, a dose dependent increase in mortality was found in larvae post-hatch in clean seawater, but only minor differences in larval developmental time (Bechmann et al., 2010). Direct exposure of shrimp larval resulted in significantly delayed development after short term exposure mimicking oil spill in water (~200 μg/L sum PAHs) (Arnberg et al., 2019; Keitel-Gröner et al., 2020). The uptake of PAHs is usually based on passive diffusion through external surface membranes or ingestion of contaminated food. As embryos, the egg membrane provides some barrier against pollutants, resulting in lower adverse effects, whilst larvae can be exposed via gills or food. Delayed development can cause a risk of decline in the pool of larvae for population recruitment due to mismatch with food availability, as well as predation and disease. However, a few days delay in development would be most critical for species with fairly short life spans, such as copepods, with possible consequence for population growth (Bejarano et al., 2006). The Northern shrimp can live up to eight years and therefore the actual significance of delayed larvae development on population growth can be considered minor for the population.

5. Conclusions

No acute toxicity was found in the present study, and only moderate sublethal effects were detected in adult shrimp and their larvae after embryonic exposure. The effects were more pronounced in NSC treatments compared to ISBR, confirming our original hypothesis. Overall, the results suggest that ISB does not represent a significant hazard to shrimp, should oil spill responders decide to use this method to remove oil from surface. These data can be used as inputs into population level impact models to predict responses based on different scenarios (e.g. with and without burning). These data are warranted for Spill Impact Mitigation Assessment (SIMA) (IPIECA-API-IOGP, 2017) to support oil spill responders choosing the best mitigation option with the lowest environmental side effects.

CRediT authorship contribution statement

Frederike Keitel-Gröner: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Shaw Bamber:** Activity assay – Formal analysis and Investigation, Writing – review & editing. **Renée K. Bechmann:** Conceptualization, Writing – original draft, Writing - review & editing. **Emily Lyng:** Investigation, Writing – review & editing. **Alessio Gomiero:** Histopathology – Investigation, Writing – review & editing. **Valentina Tronci**: Histopathology – Investigation and Resources. **Naouel Gharbi**: Histopathology – Investigation and Resources. **Frode Engen**: ISB field operations, Conceptualization, Writing – review & editing. **Ingrid C. Taban:** ISB field operations, Conceptualization, Resources, Writing – review & editing. **Thierry Baussant:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113013.

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