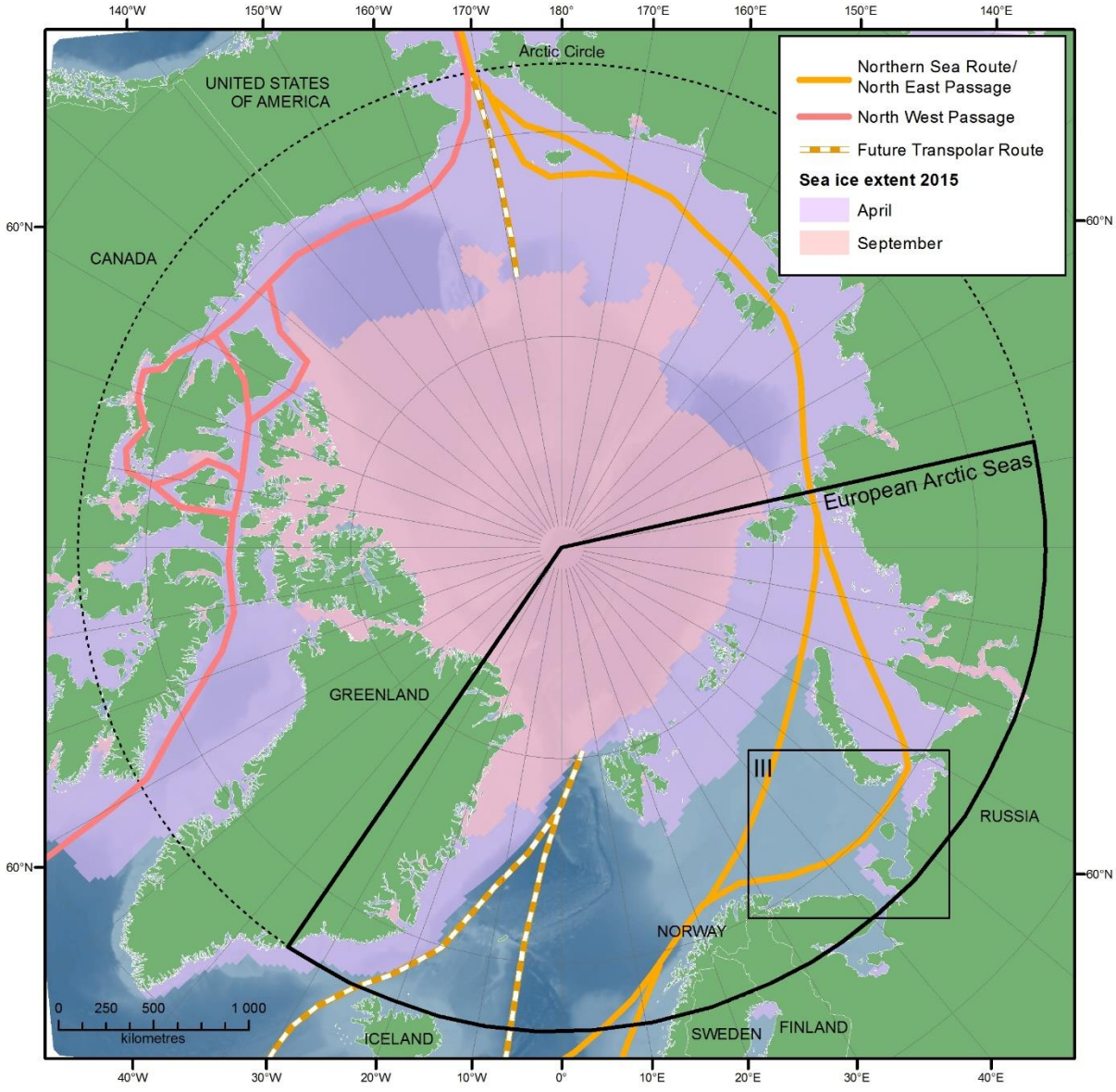

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Biological effects of marine diesel oil exposure in red king crab (*Paralithodes camtschaticus*) assessed through a water and foodborne exposure experiment

Kjetil Sagerup [a, *], Jasmine Nahrgang [b], Marianne Frantzen [a], Lars-Henrik Larsen [a, b], Perrine Geraudie [a]

[a] Akvaplan-niva, Fram Centre, Postboks 6606 Langnes, NO-9296 Tromsø, Norway
[b] UiT The Arctic University of Norway, Department of Arctic and Marine Biology, Postboks 6050 Langnes, NO-9037 Tromsø, Norway

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ABSTRACT

Shipping activities are expected to increase in the Arctic Seas. Today, the majority of vessels are using marine diesel oil (MDO) as propulsion fuel. However, there is a general lack of knowledge of how cold-water marine species respond to acute exposures to MDO. Arctic red king crabs (*Paralithodes camtschaticus*) were exposed to mechanically dispersed MDO in a flow-through exposure system for one week followed by three weeks of recovery. Observations of increased movements in exposed crabs were interpreted as avoidance behaviour. Further, glutathione peroxidase activity increased in high exposed crab, the catalase activity showed an insignificant increase with exposure, while no differences between groups were observed for lipid peroxidation and acetylcholinesterase activity. After three weeks of recovery in clean seawater, polycyclic aromatic hydrocarbons concentrations in the crabs were significantly reduced, with no specific biomarker responses in exposed groups compared to the control. The results suggest that effects from instantaneous MDO spill only will have short-term effects on the red king crab.

1. Introduction

Reduced ice cover in the Arctic leads to increased human presence and activities. Shipping, tourism and fishing are increasing in many parts of the Arctic region. Concomitant, an increasing number of ship operations, both within Arctic and in through Arctic transit will be enabled with the changing sea ice conditions (Smith and Stephenson, 2013). This intensification of ship traffic leads to increasing risk of accidents with hydrocarbon propulsion fuel being spilled.

To limit spills in case of an accident, ships produced after 2010 have to obey stricter rules for protection of- and capacity of their fuel tanks (DNV, 2006). Worldwide, most merchant ships use heavy fuel oil (HFO) as propulsion fuel, however a gradual reduction from an allowed maximum of 4.5% sulphur in HFO in 2011 to 0.5% towards 2020/2025 is expected to imply a shift towards lighter propulsion fuels, such as gasoline or marine diesel oil (MDO). Indeed, HFO has generally higher sulphur contents compared to MDO, and causes the release of extensive amounts of soot during combustion, leading to increased costs due to mandatory investments in cleansing of exhaust (Martinsen, 2013). Finally, HFO spilled into the sea has low evaporation rates compared to other petroleum products, therefore remaining in the water for a longer period and presenting a larger potential for soiling exposed littoral habitats. Based on this, the use of and potential spilling of HFO is considered a major threat to the Arctic marine environment, and alternation to MDO is suggested to reduce ecological impact. MDO has a higher content of short-chained hydrocarbons, and a larger part of the compounds in MDO compared to HFO evaporates in case of a spill (Fingas, 2011). Even at low temperatures MDO would exhibit low viscosity and thus a higher potential for dispersion and biodegradation compared to HFO (Fingas, 2011).

Despite lower prices of HFO, a review of satellite based Automatic Identification System (AIS) data of ship sailing in the Arctic during August–November 2010 showed that only 20% of the vessels were designed for using HFO as propulsion fuel (Martinsen et al., 2011). Vessels operating in the Arctic seas are mainly fishing boats, local community support vessels and smaller passenger

* Corresponding author.
E-mail address: kjetil.sagerup@akvaplan.niva.no (K. Sagerup).

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vessels, mainly running on distillate fuels (e.g. gasoline or MDO), while larger cargo vessels, tankers and cruise ships are mainly running on HFO.

The number of accidental oil spills along the Norwegian coast is recorded by the Norwegian coastal administration (NCA). Each year the NCA receive more than thousand reports of different types of spills (NCA, 2013). Most reported spills consist of a few tens of liters of diesel or gasoline, generally associated with overfilling of leisure- or fishing vessels. Despite the high frequency of small diesel spills, studies of impacts to marine biota are sparse and the need for more knowledge regarding biological impacts on cold water species and ecosystems are emphasized. A controlled experimental diesel spill in a subtropical estuary found acute effects, but also a quick recovery of the benthic macrofauna communities to pre-disturbance community structures (Egres et al., 2012). Similarly, the effects of diesel and bunker fuel spilled from the *Jessica* tanker at San Cristóbal island of the Galapagos archipelago in 2001 were moderate (Gelin et al., 2003), but the fuel from this spill did not strand as high temperatures favoured rapid evaporation and dispersion.

Biological effects of MDO are hypothesized to, at least partly, be triggered by polycyclic aromatic hydrocarbons (PAHs), known to induce reproductive impairment, behaviour alteration, immune and genetic toxicity in fish (Akcha et al., 2003; Frantzen et al., 2012; Rocha Monteiro et al., 2000) and invertebrates (Wootton et al., 2003). For example, naphthalene, a 2-ringed PAH compound numerously represented in MDO, has been shown to reduce larval growth in the fish species fathead minnow (*Pimephales promelas*), induce genotoxic damages in eel (*Anguilla anguilla*) (Degraeve et al., 1982; Teles et al., 2003), deoxyribonucleic acid (DNA) damages, ethoxyresorufin-O-deethylase (EROD) induction and acetylcholine esterase (AChE) inhibition in the littoral crab (*Carcinus aestuarii*) (Fossi et al., 2000).

MDO has in laboratory experiments been shown to induce deleterious effects in boreal and Arctic pelagic copepods (*Calanus finmarchicus* and *Calanus glacialis*) (Hansen et al., 2013) and in Icelandic scallop (*Chlamys islandica*) (Geraudie et al., 2016; Milinkovitch et al., 2015). Hansen et al. (2013) showed that the water-soluble fraction of MDO induced both acute- and genotoxic effects in both copepod species tested. The dispersed fraction of MDO, including both droplets and the water-soluble fraction, induced toxic effects on the nervous system, induced oxidative stress and altered swimming behaviour in Icelandic scallops (Geraudie et al., 2016; Milinkovitch et al., 2015).

The current experiment was designed to mimic a MDO leakage after a ship wreckage in shallow water of an Arctic coastal environment. It exhibited a scenario where red king crabs (*Paralithodes camtschaticus*) were exposed to the MDO from both its surrounding seawater and from its food items, the blue mussels (*Mytilus edulis*) and Icelandic scallops. The adult king crab normally live at depths of 100–300 m, but in late winter or spring they migrate to shallow areas to breed (Pedersen et al., 2006). The larvae settle and hatch at shallow water and the juvenile crabs (<120 mm in carapace length) generally remain in shallow water down to 50 m depth (Wallace et al., 1949) along the coast.

The aims of the study were to study the biological effects of MDO to red king crab as well as it's recovery potential. Uptake and elimination of PAHs from MDO associated with biomarkers of neurotoxicity and oxidative stress where therefore characterized both following a week of exposure and three weeks of recovery in uncontaminated water.

2. Material and methods

The experiment was conducted at Akvaplan-niva research laboratory in Tromsø, Northern Norway (69,75°N; 19,03°E) during November–December 2013. All sampling and handling were done in accordance with current regulations of the Norwegian Animal Welfare Act. The experiment was approved by the Norwegian Animal Research Authority (ID 5842).

In September 2013, twenty-four red king crabs caught off the coast of Finnmark, Northern Norway (71°N, 30°E) were transported to the laboratory and kept in a flow-through tank supplied with natural seawater of ambient temperature for acclimation and storage for 2.5 month. During this period, they were kept under natural light regime, and were daily cared for and fed on herring (*Clupea harengus*) chops until satiation. The crabs were all immature males with a carapace length of 86–118 mm and body weight of 400–870 g. In Norway, the red king crab is managed as an unwanted, invasive species in areas west of the 26° E longitudinal parallel (approximately at North Cape). Moving live female crabs west of this parallel is prohibited. Thus, only males were used in the study. Icelandic scallops were collected near Tromsø in October 2013 and were kept in a cage at sea for one month. Blue mussels were purchased from a commercial producer outside Trondheim, Norway (64°N, 10°E). The mussels and scallops were acclimatized to the experimental conditions for one week prior to the start of the experiment.

2.1. Experimental setup

Red king crab, Icelandic scallops and blue mussels were transferred to 110 L fiberglass tanks (550 × 550 × 400 mm). Organisms were placed in three groups; control, low exposure and high exposure. Each group consisted of 4 replicate tanks each holding 2 crabs, 10 Icelandic scallops and 30 blue mussels. The bivalves served as food for the crabs. To secure samples for contaminant analyses, 8 Icelandic scallops and 16 blue mussels were kept in a small shelter in each tank, inaccessible to the crabs (Fig. 1). The set-up consisted of a flow-through system of low sulphur (0.05% S) MDO, commercially purchased from Bunker Oil Ltd. Two peristaltic pumps, one for each exposure, supplied the tanks with MDO during the exposure, simulating a continuous leakage. Homogenous mixing was ensured by mechanical agitation as described in Milinkovitch et al. (2011). The nominal concentrations of MDO in our experiment were 5 mg L^{-1} and 20 mg L^{-1} in the low and high exposure groups, respectively, to achieve the desired non-lethal and representative concentration for a MDO spill. Indeed, the total hydrocarbon content (THC) of water during real spills and experimental field trials has shown maximum concentrations of 30–50 mg L^{-1} just below the slick and thereafter a rapidly decrease of the water concentrations (Humphrey et al., 1987; Law, 1978; Lunel et al., 1995). The lethal concentrations (LC_{50}) of chemically dispersed diesel was found to be 8 mg L^{-1} and the mechanically stirred diesel between 40 and 200 mg L^{-1} in juvenile rainbow trout (*Oncorhynchus mykiss*) (Schein et al., 2009).

The exposure lasted one week and was followed by three weeks of recovery in uncontaminated water. At the start of the recovery period, the red king crab were transferred to tanks with flow-through of natural clean seawater. Uncontaminated scallops and mussels served as food during recovery.

During the entire exposure, the seawater was pumped from 60 m depth, filtered through a 60 μm filter and the flow was regulated to 1 L per minute. The water temperature was 7.1 ± 0.2 °C, pH 8.5 ± 0, salinity 31 ± 0 parts per thousand and dissolved oxygen 93.6 ± 3.2% saturation. During the recovery period, the seawater temperature gradually decreased from 7 °C to 5.5 °C following the ambient temperature variation. The O_2 saturation was measured every second day in the tanks and remained between 95 and 100% during recovery. The organisms were kept at an 8:16 h light:dark regime, following the ambient light conditions at onset of the

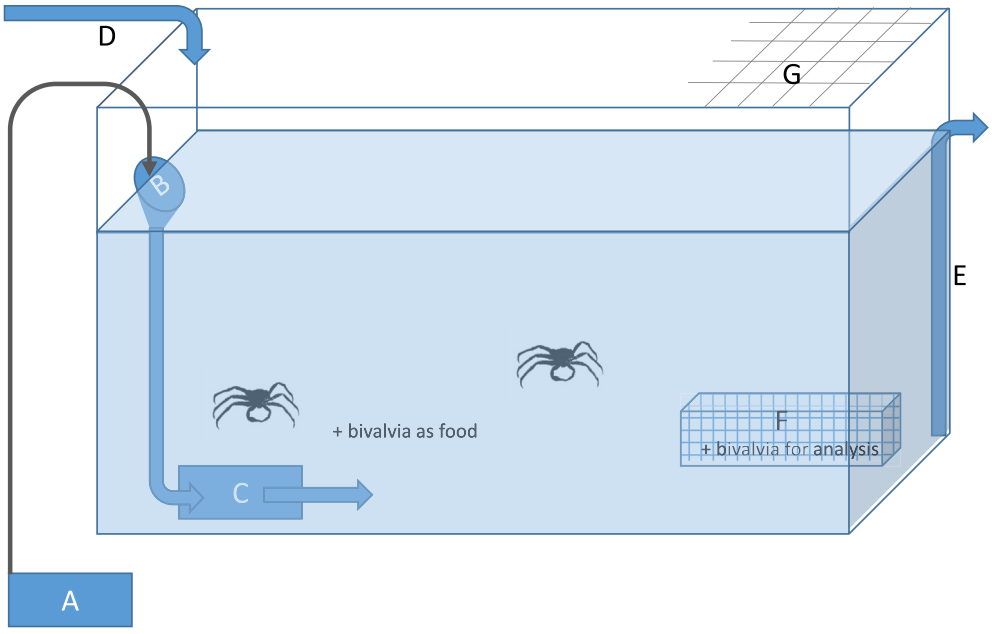

Fig. 1. Sketch of the experimental tanks. A) Peristaltic pump supplying marine diesel oil (MDO). B) Funnel adjusted to drain top water layer and the supplied MDO to the circulation pump. C) Circulation pump with a capacity of 7.5 L/min. D) Inflow of natural seawater 1 L/min. E) Drainage outlet from bottom of the tank. A siphon secure stable water level in the tank. F) Cage for blue mussel (*Mytilus edulis*) and Icelandic scallop (*Chlamys islandica*) inaccessible to the red king crab (*Paralithodes camtschaticus*). G) Top cover net to avoid the crabs escaping.

experiment (November).

At day four of the exposure, an unplanned power failure stopped the peristaltic pumps, causing a 36 hours' interruption of the MDO supply to both exposure groups. As seawater continually flowed through the tanks, the MDO concentration in the exposure decreased during these 36 h before being re-established for the last part of the exposure period (not measured).

2.2. Observations of crab's behaviour

The red king crabs were monitored each morning during the week of exposure for any unusual locomotion behaviours (e.g. escape behaviour). The observer also evaluated the feeding activity, walking speed, vertical and horizontal movements in the exposed groups compared to the controls. The observations were however no quantified. Even though these observations were subjective of nature, it provides a good indication that the crabs actively tried to avoid the oil.

2.3. Sampling and measurements

Water samples were collected from the middle part of the water column of each tank, 24 h after onset of exposure (T_{24h}) and stored at −20 °C prior to chemical analyses (THC and 16 Environmental protection Agency (EPA) PAHs).

Ten blue mussels were put aside prior to the experiment for chemical analysis. After the exposure, the soft tissue of the Icelandic scallops (n = 8 per tank) and the blue mussels (n = 16 per tank), respectively, were pooled into 4 composite samples per species/exposure and frozen at −20 °C for analyses of PAH contents. Half of the crabs (n = 4 per treatment), one from each replicate tank, were sacrificed immediately after the one-week exposure (T_1) and the other half were sacrificed following the three weeks recovery period (T_2). (Table 1). The wet weight (wt) of the crabs were recorded prior to the experiment (T_0) and before termination (T_1 or T_2). The hepatopancreas organ were dissected out from each individual and a part was stored at −20 °C for chemical analysis and parts were snap-frozen in liquid nitrogen and stored at −80 °C until biomarker analyses. Finally, a muscle sample, was taken from the second pereiopod of each crab, was snap frozen and stored at −80 °C for AChE analyses.

2.4. Chemical analyses

The contents of the 16 EPA PAHs (naphthalene, acenaphthylene, acenaphthene, fluorine, phenan-threne, antracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, benzo(ghi)perylene and dibenz[a,h]anthracene) were analysed in seawater, soft tissues of bivalves and hepatopancreas of crabs. In addition, THC was analysed in the water samples. For blue mussel and Icelandic scallops, the pooled samples from each tank were analysed, while individual samples from each crab were analysed. Finally, the relative PAH composition (16 EPA PAHs) and C1-C3-naphtalenes, C1-C3-phenanthrenes and C0-C3-dibenzothiophenes (NPD) of the diesel is given in the supplemental material (Table SM-1).

The analytical methods for PAH in tissue samples followed Nahrgang et al. (2013) and the analyses of PAH and THC in water followed the method described by Frantzen et al. (2016). Briefly, each tissue sample was ground and homogenised. The sample was weighed and potassium hydroxide-methanol solution and internal standards of deuterated PAHs were added. The solution was boiled, filtrated and extracted. The extract was purified with dichloromethane using gel permeation chromatography. The sample was further purified by filtration and solid phase extraction before analyses with a coupled gas chromatography − mass spectrometry (GC-MS with split/splitless injector and MS with EI ion source mass selective detector). Each water sample was mixed with an internal standard mixture of six deuterated PAHs, extracted with 3 × 60 ml dichloromethane, dried overnight with Na_2SO_4 and concentrated to 1 ml. The extracts were purified and concentrated. Thereafter

Table 1
Number of samples collected for analyses during the experiment. T_0: reference sample collected prior to the experiment. $T_{24\ h}$: Water sampling 24 h into exposure to establish exposure concentrations, T_1: biota sampling at the end of exposure, T_2: biota sampling after three weeks of recovery.

	Group	T_0	$T_{24\ h}$	$T_{1\ (one\ week)}$	$T_{2\ (3\ weeks\ recovery)}$
Water	Control	–	4	–	–
	Low	–	4	–	–
	High	–	4	–	–
Red king crab (Individual samples)	Control	–	–	4	4
	Low	–	–	4	4
	High	–	–	4	4
Blue mussel (Pool of soft tissue from 16 individuals)	Control	1	–	4	–
	Low	–	–	4	–
	High	–	–	4	–
Icelandic scallop (Pool of soft tissue from 8 individuals)	Control	–	–	4	–
	Low	–	–	4	–
	High	–	–	4	–

evaporated to dryness and re-solved in 100 ml isooctane. The THC (C10–C35) was analysed on a GC with an GC-flameIonization detector and the 16 EPA PAHs on a GC–MS operated in selected ion monitoring mode. Quality was assured by running blank, blind and proficiency test samples (Quasimeme, Netherlands). The limit of detection (LOD) was determined from analyses of a series of blank samples and calculated as: LOD = (blank average) + 3 × (blank standard deviation). As the concentrations of PAHs in all control samples were close to or under the LOD, the calculation of ∑16PAHs was made only for compounds with concentration above LOD.

The bioaccumulation factors (BAF) describes the net increase of an organic contaminant from water to biota due to uptake from all exposure routes (e.g. across skin, intestine or gills) (Mackay, 1982; Mackay and Boethling, 2000). The BAF was calculated for each PAH congener as; BAF = average concentration in soft tissue/average concentration in seawater (four replicate tanks) for all three species.

2.5. Biomarker analyses

2.5.1. Acetylcholinesterase (AChE) activity

Neurotoxicity of the MDO exposure was assessed in terms of inhibition of AChE in muscular tissues of crabs, and expressed as nmol min^{-1} mg^{-1} protein. Muscle samples were homogenised in cold 0.1 M phosphate buffer (Tris-buffer, pH 6.8) using a Potter-Elvehjem homogeniser and centrifuged (9000 g, 15 min, 4 °C). The enzyme activity was then determined in triplicate for each sample according to the colorimetric method initially developed by (Ellman et al., 1961). Briefly, the homogenate and acid 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) were added to a 96-well microtiter plate. The reaction was initiated by adding 7.5 μl of acetylthiocholine iodide solution (0.1 M). The reaction was followed at 412 nm for 10 min. Absorption kinetics were calculated in the linear range of the standard curve, then converted to nmol per minute, using the Beer-Lambert law and the molar extinction coefficient of DTNB ($\varepsilon = 1.36 \times 10^4$ $Lmol^{-1}$ cm^{-1}) (Ellman et al., 1961).

2.5.2. Oxidative stress and antioxidant enzymes

For analysis of the oxidative stress biomarkers, hepatopancreas tissue was homogenised with a Potter-Elvehjem type homogeniser in an ice-cooled potassium phosphate buffer (100 mM, pH 7.5) containing 2.5% NaCl. Homogenates were centrifuged (10,000 g, 4 °C) for 30 min and supernatants were subsequently stored at −80 °C. Glutathione peroxidase (GPx) activity was measured in accordance to the relative decrease in absorbance during 1 min at 340 nm ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) at 20 °C. Each sample was analysed in duplicate and expressed as μmol min^{-1} mg^{-1} protein (Livingstone et al., 1992). Briefly, 30 μl of homogenate diluted with a factor of 2.5 (final dilution factor of 118) was mixed with potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of NaN_3, GSH (1.5 mM) and 1U of glutathione reductase and incubated for 10 min at 20 °C. Then, the reaction was started by addition of 25 μl of nicotinamide adenine dinucleotide phosphate (NADPH) (0.12 mM) and cumene hydroperoxyde (4 mM, total GPx activity).

Catalase (CAT) activity was measured and expressed as μmol min^{-1} mg^{-1} protein. The decrease in absorbance at 240 nm ($\varepsilon = 40$ M^{-1} cm^{-1}) was recorded in quartz cuvettes during 1 min at room temperature after addition of 10 mM H_2O_2 to the sample using a spectrophotometer (Clairborne, 1985). For each sample the protein concentration was estimated in triplicates adapted to microplates, using bovine serum albumin as a standard.

Lipid peroxidation in hepatopancreas was measured as thiobarbituric reactive substances (TBARS) according to Buege and Aust (1978). Briefly, samples were homogenised in a Tris Buffer (0.1 M, pH 7.4) using a Precellys bead beater homogeniser (5000 rpm, 3 × 5 s). The homogenates (200 μl) were mixed with a reagent (800 μl) containing thiobarbituric acid (0.375%, w/v), trichloroacetic acid (15%, w/v) and HCl (0.25 N). The samples were incubated at 100 °C for 15 min, centrifuged at 1000 × g for 10 min and absorbance was measured at 532 nm, normalised to a background absorbance at 650 nm. Finally, TBARS (nmol TBARS g^{-1} tissue wet wt) were determined using a standard curve (1,1,3,3-tetramethoxypropane).

2.6. Statistics

Data were analysed for statistically significant differences between control and exposure groups at each sampling time using Statistica 7.1 and R version 3.1.2 (R Core Team, 2014). When requirements of normality and homogeneity of variances were fulfilled, a one-way ANOVA was used to compare different exposures. When requirements for normality were not met, the nonparametric Kruskal–Wallis test was used. The significance level was set to <0.05 for all analyses.

3. Results

The experiment was designed to mimic a near instantaneous release of MDO from a point source. The exposure was interrupted by an electricity failure stopping the MDO injection to the experimental tanks approximately from hour 120 to hour 156 of the exposure week (168 h). The exposure concentration was thus not constant. However, in case of for example a grounding of a vessel, the leaking pattern would most likely be non-linear and vary with

remediation, recovery efforts and the tidal cycle.

During the exposure period (1 week) and recovery period (3 weeks), no mortality in any of the species was recorded, except in bivalves due to predation from crabs.

3.1. THC in water and PAH concentrations in water and test organisms

The measured THC in water 24 h into the exposure reflected the desired nominal concentration with levels of THC below the detection limit for the controls, 7.4 and 19.0 mg L^{-1} in the low and high exposure groups, respectively (Table 2). The levels of ∑16PAH in the control crabs hepatopancreas were low (<88.6 ± 12.3 ng g^{-1}) at T_1 and T_2. The high exposure group had a ∑16PAH (22,251 ± 1563 ng g^{-1}) concentration reaching four times the concentration in the low exposure group (5401 ± 761 ng g^{-1}), thereby reflecting the almost four times higher water concentration (Table 2). The dominating PAHs in crab hepatopancreas were naphthalene > fluorene > phenanthrene > acenaphthene and reflect the composition of PAHs of MDO and measured in the seawater (Fig. 2).

After the recovery period (T_2), a significant decrease in ∑16PAH was found in both exposure groups (Table 2). The most volatile and water-soluble compound, naphthalene, showed the largest decrease by factors of 8 and 100 for the low and high exposure group, respectively. The mean concentration of the low exposure group decreased at a lower rate compared to the high exposure group. However, the result from the low exposure group at T_2 includes one extreme outlier with high concentrations of PAH. By removing this outlier, the mean naphthalene value of the low exposure group decrease by a factor 30. The ∑16PAH of this outlier crab was 29 times higher than the rest of the group, thereby influencing the mean and standard error (Table 2). The mean ∑16PAH from the low group at T_2 when excluding the outlier was 266 ± 43 ng g^{-1}.

Mussels and scallops also accumulated PAHs in their soft tissues (Table 3). Most of the control bivalves had non-detectable concentration of PAHs, but in one mussel pool and two scallop pools of the four control samples, low concentrations of fluorene, phenanthrene and chrysene were detected. The mussel sample collected prior to the exposure had non-detectable levels of PAHs. The soft tissue concentrations of PAHs were lower in the Icelandic scallops than in the blue mussels. The water concentration of ∑PAH was about three times higher in the high exposure group compare with the low group, but there was only a doubling of the PAH concentrations from the low to the high exposure group for both bivalve species.

The BAFs varied among species and PAH types. The crabs showed the highest BAFs for most of the PAHs with a BAF above 2000 for fluorene in the high exposure group (Table 4). Interestingly, the pattern of BAF differ between bivalves and the crab. The overall BAF slightly increased with the octanol-water partition coefficient (log Kow) in bivalves, while it increased from naphthalene to acenaphthene/fluorene and then decreased to phenanthrene and fluoranthene for the crab (Table 4). The acenaphtylene and anthracene could not be included here as their contribution to the ∑PAH only was 0.05 and 0.5%, respectively and that these substances were not detected in water of the low exposure group.

3.2. Biological effects of MDO on king crab

Both the control and exposed crabs had ingested the soft tissues of scallop and mussel through the exposure period. The number of eaten scallops and mussels did not differ significantly between groups (Kruskal-Wallis chi-squared = 1.9, p = 0.4 and chi-squared = 0.3, p = 0.9, respectively), indicating that the crabs did not reduce food consumption in an MDO contaminated environment.

Immediately upon onset of the exposure of crabs to MDO, a change in locomotion behaviour of the exposed crabs was observed. The crabs of both exposure groups showed an increased locomotive behaviour, with quick walking movements and escaping behaviour with climbing on the top cover grid. No such behaviour was observed in the control group, where the crabs crawled calmly on the bottom of the tank. The increased activity behaviour lasted during the whole week of exposure and stopped immediately as the crabs were transferred to the recovery tanks containing natural seawater.

The activity of the biomarker AChE did not differ significantly between the three groups (Kruskal-Wallis chi-squared = 5.1,

Table 2
The concentration (mean ± SE) of 16 polycyclic aromatic hydrocarbons (16PAHs, μg L^{-1}) and total hydrocarbon content (THC, mg L^{-1}) in water and hepatopancreas (ng g^{-1} wet weight) of red king crab (*Paralithodes camtschaticus*). Only levels above limits of detection are presented, concentrations below detection limit is presented as N.D. Time 1 (T_1) refer to samples collected after one week of exposure and T_2 following three weeks of recovery in uncontaminated water. Each group consists of four samples (Table 1).

Matrix	Contr			Low			High		
	Water	Red king crab		Water	Red king crab		Water	Red king crab	
Time	$T_{24\ h}$	T_1	T_2	$T_{24\ h}$	T_1	T_2	$T_{24\ h}$	T_1	T_2
Naphthalene	N.D.	56.7 ± 8.2	66.1 ± 5.9	1.9 ± 0.3	2412 ± 352	319 ± 207	9.7 ± 1.2	9994 ± 775	97 ± 16
Acenaphtylene	N.D.	0.5 ± 0	N.D.	N.D.	1.0 ± 0.02	3.4 ± 2.6	0.02 ± 0.01	1.7 ± 0.2	2.3 ± 0.3
Acenaphthene	N.D.	3.4 ± 0.2	3.0 ± 0.1	0.1 ± 0.02	256 ± 30.6	93.9 ± 54.7	0.5 ± 0.05	868 ± 55.4	112 ± 9.7
Fluorene	N.D.	12.1 ± 5.1	7 ± 1.1	0.9 ± 0.1	1537 ± 207.3	517 ± 372	3.1 ± 0.7	6186 ± 505	758 ± 106
Phenanthrene	N.D.	24 ± 10.5	6.4 ± 0	1.3 ± 0.2	1142 ± 172	1033 ± 849	4.0 ± 0.3	4956 ± 421	552 ± 136
Anthracene	N.D.	N.D.	N.D.	N.D.	12 ± 1.5	30.8 ± 19.3	0.06 ± 0.01	57.6 ± 4.9	11.2 ± 1.9
Fluoranthene	N.D.	N.D.	N.D.	0.1 ± 0	13 ± 2.4	141 ± 0	0.3 ± 0.01	58.5 ± 5.9	5.9 ± 0.2
Pyrene	N.D.	N.D.	N.D.	N.D.	25.4 ± 5.0	224 ± 0	N.D.	114 ± 11	11.2 ± 0.3
Benzo[a]anthracene	N.D.	N.D.	N.D.	N.D.	N.D	10.9 ± 0	N.D.	N.D.	N.D.
Chrysene	N.D.	N.D.	N.D.	N.D.	2.3 ± 0.3	161 ± 0	N.D.	14.1 ± 2.1	N.D.
Benzo[b]fluoranthene	N.D.	N.D.	N.D.	N.D.	N.D.	6.4 ± 0	N.D.	N.D.	N.D.
Benzo[k]fluoranthene	N.D.	N.D.	N.D.	N.D.	N.D.	2.8 ± 0	N.D.	N.D.	N.D.
Benzo[a]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	1.4 ± 0	N.D.	N.D.	N.D.
Indeno[1,2,3,c,d]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[g,h,i]perylene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenzo[a,h]anthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
∑16PAH	N.D.	88.6 ± 12.3	76.2 ± 7.3	4.2 ± 0.6	5401 ± 761	2127 ± 1622	17.4 ± 2.2	22,251 ± 1563	1543 ± 261
THC (only in water)	N.D.	–	–	7.4 ± 1.0	–	–	19 ± 0.3	–	–

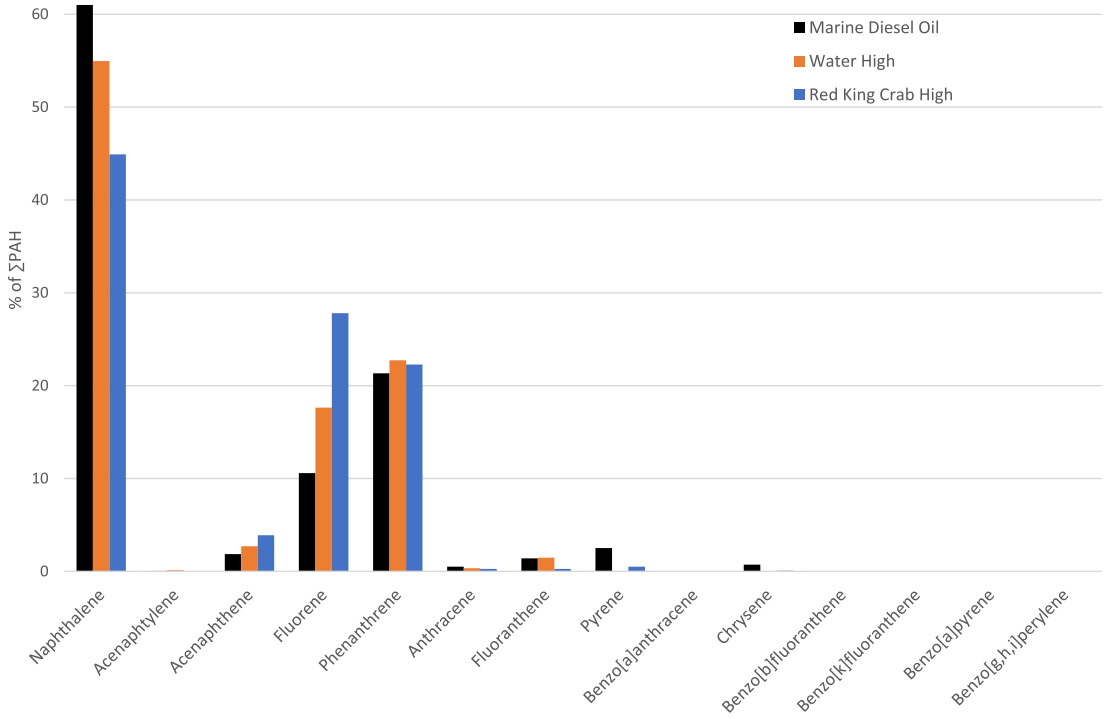

Fig. 2. The PAH fingerprint presented as % of ∑14PAHs of the marine diesel oil, water high exposure group (n = 4) and the hepatopancreas of the red king crab (*Paralithodes camtschaticus*) high group (n = 4).

Table 3
Concentration of polycyclic aromatic hydrocarbons (PAH) (mean ± SE, ng g^{-1} wet weight) in blue mussel (*Mytilus edulis*) and Icelandic scallops (*Chlamys islandica*) soft body tissue after one-week exposure to marine diesel oil. Each sample is a pool of 16 mussels or 8 scallops, respectively. Only levels above limits of detection are presented, concentrations below detection limit is presented as N.D. Each group, except the T_0 (prior to experiment) blue mussel pool, consists of four individual pool samples.

Species	T_0 (n = 1)	Blue mussel (*Mytilus edulis*)			Icelandic scallop (*Chlamys islandica*)		
Exposure		Contr	Low	High	Contr	Low	High
Naphthalene	N.D.	N.D.	132.7 ± 19	680 ± 40.4	N.D.	82.7 ± 10.5	404 ± 22.1
Acenaphtylene	N.D.	N.D.	2.28 ± 0.19	11.2 ± 7.01	N.D.	15.7 ± 2.72	26.9 ± 5.3
Acenaphthene	N.D.	N.D.	65.1 ± 5.86	171 ± 28.2	N.D.	55.6 ± 6.83	122 ± 14.7
Fluorene	N.D.	5.63 ± 0	500 ± 51.3	1325 ± 240	4.82 ± 0.91	451 ± 39.2	940 ± 138
Phenanthrene	N.D.	9.68 ± 0	1127 ± 127	1899 ± 65.7	8.91 ± 0	712 ± 61.7	1210 ± 73.1
Anthracene	N.D.	N.D.	19.8 ± 3.72	30.9 ± 2.32	N.D.	11.4 ± 0.89	17.4 ± 1.71
Fluoranthene	N.D.	N.D.	84.8 ± 8.75	107 ± 2.75	N.D.	56.1 ± 2.83	72.4 ± 6.78
Pyrene	N.D.	N.D.	140 ± 14	183 ± 3.24	N.D.	85.8 ± 4.52	118 ± 9.24
Benzo[a]anthracene	N.D.	N.D.	3.02 ± 0.4	3.24 ± 0.14	N.D.	1.9 ± 0.05	2.25 ± 0.13
Chrysene	N.D.	1.76 ± 0	54.2 ± 7.06	66.0 ± 3.92	1.48 ± 0	29 ± 1.35	39.1 ± 6.23
Benzo[b]fluoranthene	N.D.	N.D.	2.57 ± 0.14	2.69 ± 0.15	N.D.	2.19 ± 0.07	2.2 ± 0.15
Benzo[k]fluoranthene	N.D.	N.D.	0.72 ± 0	0.75 ± 0.04	N.D.	0.68 ± 0.02	0.78 ± 0
Benzo[a]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Indeno[1,2,3,c,d]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[g,h,i]perylene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenzo[a,h]anthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
∑16PAH	N.D.	5.1 ± 4.0	2131 ± 270	4479 ± 345	5.0 ± 3.6	1505 ± 141	2953 ± 140

p = 0.4, Fig. 3).

The CAT activities measured at T_1 and T_2 ranged from 0.2 to 28.0 μmol min^{-1} mg^{-1} protein (Fig. 4). Although non-significant, a concentration dependent increase in CAT activity was observed at T_1 (F = 2.5, p = 0.14). At T_2, the CAT activity in the low and high exposure groups were back to similar levels as the control group (F = 0.1, p = 0.9, Fig. 4).

At T_1, GPx activities of 556 ± 105 and 545 ± 93 μmol min^{-1} mg^{-1} protein was found in the control and low exposure crabs, respectively. A significantly higher activity was recorded in the high exposure group crabs (836 ± 121 μmol min^{-1} mg^{-1} protein, F = 3.0, p = 0.04) compared to the control and the low exposure groups. After the recovery period, GPx activities were similar in all groups (Fig. 5).

Lipid peroxidation, expressed as TBARS showed a high median and high variation in the control at T_1, while levels in the low and high exposure groups were significantly lower (F = 4.1, p = 0.01). Following the recovery, the average TBARS concentrations decreased in the control group and showed similar levels as the low and high exposed groups (Fig. 6).

4. Discussion

The THC and PAH concentrations in water and the PAH

Table 4
The bioaccumulation factors (BAF) of polycyclic aromatic hydrocarbons (PAHs) calculated for blue mussel (*Mytilus edulis*), Icelandic scallop (*Chlamys islandica*) and red king crab (*Paralithodes camtschaticus*) exposed to mechanically dispersed marine diesel oil for a week. The total hydrocarbon exposure was 7.4 mg L^{-1} in the low group and 19 mg L^{-1} in the high group (Table 2). All calculations made on wet weight basis.

	Log kow[a]	Low	High	Low	High	Low	High
		Blue mussel		Icelandic scallop		Red king crab	
Naphthalene	3.34	70	71	44	42	1281	1046
Acenaphtylene	4.1	–	501	–	1196	–	76
Acenaphthene	3.95	461	362	393	260	1812	1841
Fluorene	4.21	538	432	486	306	1654	2017
Phenanthrene	4.57	892	480	564	306	904	1254
Anthracene	4.58	–	510	–	287	–	950
Fluoranthene	5.1	792	415	524	282	122	228
Pyrene	5.1	–	–	–	–	–	–
Benzo[a]anthracene	5.67	–	–	–	–	–	–
Chrysene	5.71	–	–	–	–	–	–
Benzo[b]fluoranthene	6.4	–	–	–	–	–	–
Benzo[k]fluoranthene	6.5	–	–	–	–	–	–
Benzo[a]pyrene	6.3	–	–	–	–	–	–
Indeno[1,2,3,c,d]pyrene	6.92	–	–	–	–	–	–
Benzo[g,h,i]perylene	7	–	–	–	–	–	–
Dibenzo[a,h]anthracene	6.71	–	–	–	–	–	–
Sum 16 PAHs		439	250	310	165	1111	1240

[a] (Baussant et al., 2001).

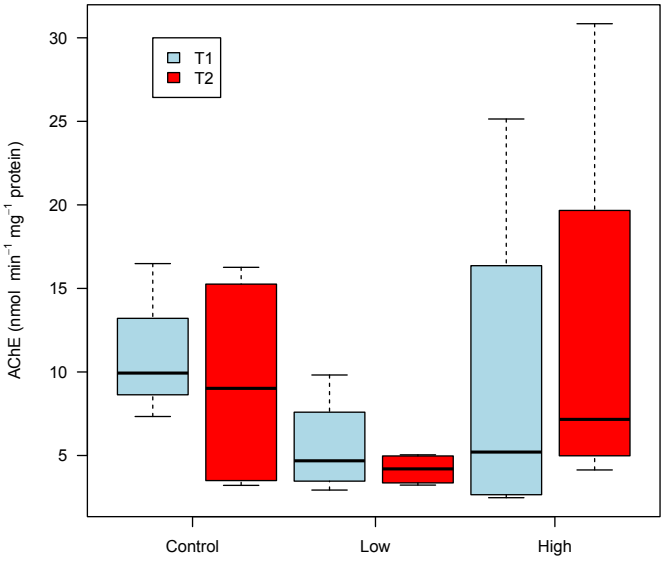

Fig. 3. Acetylcholine esterase (AChE) inhibition in nmol min^{-1} mg^{-1} protein in muscle tissue of red king crab (*Paralithodes camtschaticus*), after one week of exposure to marine diesel oil (T_1) and following three weeks of recovery (T_2) in natural seawater and uncontaminated food. In each group n = 4. Boxplots represent the median (horizontal line), 1st and 3rd quartile (box), and min-max range (whisker) of the data.

concentrations in the exposed organisms, confirmed the achievement of three levels of experimental exposure. The PAHs and THC concentration in water of the low exposure group was comparable to the concentrations measured in the water column five days after an acute mid-winter spill of 140 tons MDO to sea in Skjervøy, Northern-Norway (70°N; 21°E); ∑16PAH 10 μg L^{-1} and THC 3.5 mg L^{-1} (Sagerup, unpublished results). The concentration of PAHs in animal tissues confirmed the accumulation of MDO in a dose-dependent manner. One crab from the low exposure group showed relatively high levels of heavy molecular weight PAHs at T2. This might indicate a pre-exposure contamination of that

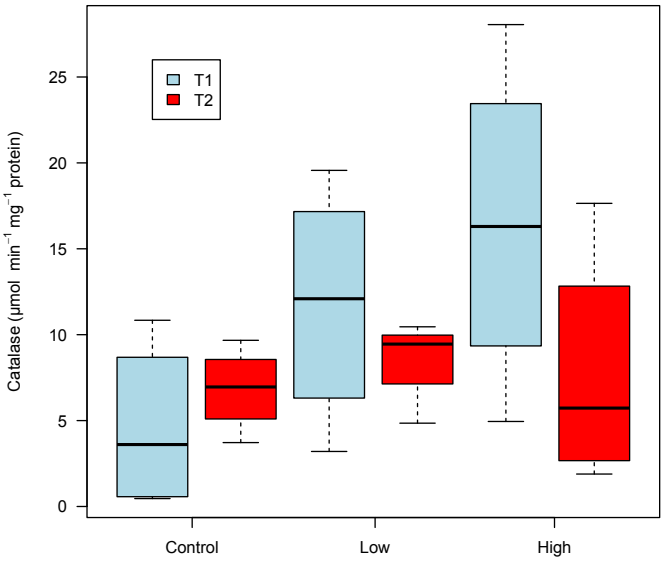

Fig. 4. The oxidative catalase activity expressed in μmol min^{-1} mg^{-1} protein in hepatopancreas of red king crab (*Paralithodes camtschaticus*), after one week of exposure to marine diesel oil (T1) and following three weeks of recovery (T_2) in natural seawater and uncontaminated food. In each group n = 4. Boxplots represent the median (horizontal line), 1st and 3rd quartile (box), and min-max range (whisker) of the data.

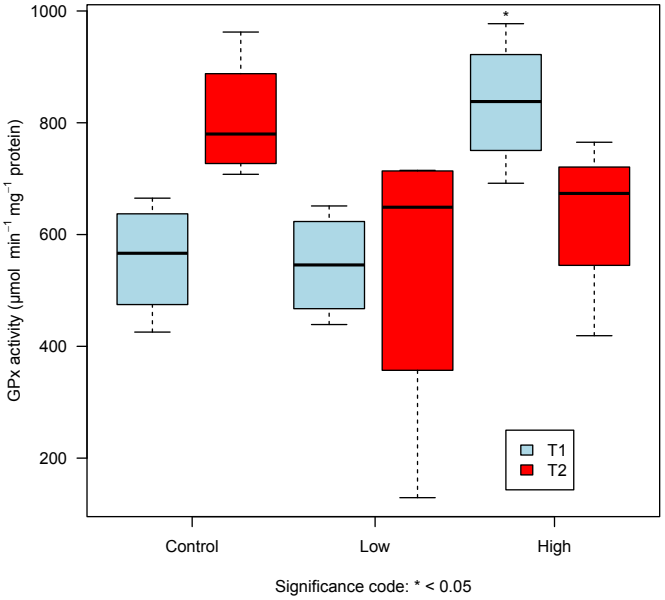

Fig. 5. The glutathione peroxidase (GPx) activity, μmol min^{-1} mg^{-1}, protein in hepatopancreas of red king crab (*Paralithodes camtschaticus*), after one week of exposure to marine diesel oil (T_1) and following three weeks of recovery (T_2) in natural seawater and uncontaminated food. In each group n = 4. Boxplots represent the median (horizontal line), 1st and 3rd quartile (box), and min-max range (whisker) of the data.

individual.

The elimination of PAHs from king crab tissues during the recovery period was rapid. The depuration appeared higher in crabs from the high exposed group, but this was only due to one animal exhibiting an outlier value. Excluding this outlier, the depuration rate did not differ significantly between the exposed groups. But, depuration varied for the different compounds, with naphthalene showing the largest ability to be eliminated quickly. After the recovery period, only 5% and 7% of the initial ∑16PAH concentrations

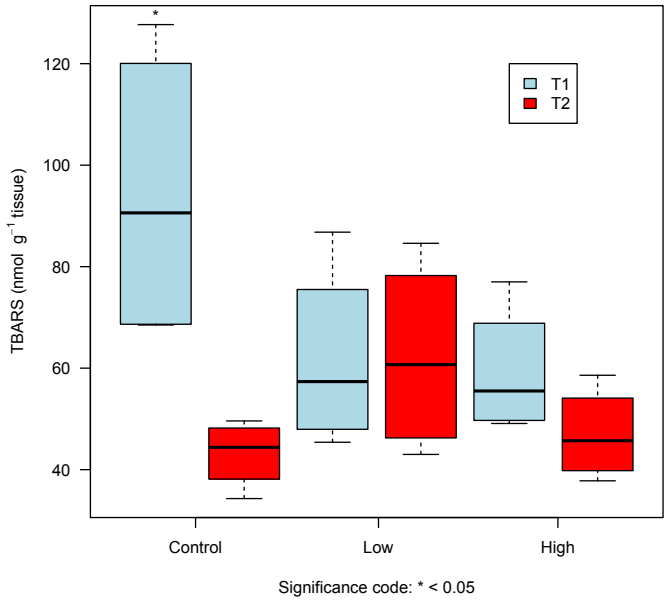

Fig. 6. The lipid peroxidation, expressed as thiobarbituric reactive substances (TBARS), nmol g^{-1}, in hepatopancreas of red king crab (*Paralithodes camtschaticus*), after one week of exposure to marine diesel oil (T_1) and following three weeks of recovery (T_2) in natural seawater and uncontaminated food. In each group n = 4. Boxplots represent the median (horizontal line), 1st and 3rd quartile (box), and min-max range (whisker) of the data.

were left in hepatopancreas tissue of the crabs in the low and the high group, respectively. This is comparable to the reported elimination efficiency of benzo(a)pyrene (BaP) in blue crab (*Callinectes sapidus*) that reduced its BaP to between 3 and 17% of its initial concentration in ten days at 23 °C (Hale, 1988). In our study, only a small proportion of the MDO was represented by BaP (Table SM-1) and only the outlier crab had detectable concentration of BaP. However, this large decrease of PAH concentrations showed that the red king crab had the capacity to eliminate PAHs. The PAHs are easily eliminated in fish and higher animals (van der Oost et al., 2003) through phase I and phase II cytochrome P450 enzymes in the liver. This system is also active in species of the phylum Arthropoda, to which the crustaceans belong (Rewitz et al., 2006), thereby producing PAH metabolites in crab (Koenig et al., 2009). In marine invertebrates a diversification within the P450-genes and thereby a variation in P450 enzymes capable of metabolising xenobiotics has been seen (Rewitz et al., 2006). There are limited information on the ability of crab to metabolise and excrete PAHs (Meador et al., 1995), but Rewitz et al. (2006) suggested that P450 enzymes which are present in the majority of marine invertebrates are involved in biotransformation of PAHs. Our results support this suggestion as the red king crab were able to eliminate most of the PAHs during three weeks of recovery in uncontaminated, cold water (5.5–7 °C). PAH metabolites have been found in crab urine providing evidences of internal metabolization capability of PAH (pyrene) into lighter metabolite compound (Watson et al., 2004).

Bivalves also accumulated PAHs dose-dependently. The blue mussels accumulated approximately 1.5 times the amount of PAHs compared to the Icelandic scallops. This could be related to the specific filtration rate which has been shown to be 3 times higher in mussels than in scallops (MacDonald and Ward, 2009). Blue mussels have lower activity of the cytochrome P450 enzymes involved in the breakdown and elimination of PAHs than crustaceans and fishes (Livingstone, 1998). In this species, bioaccumulated PAHs are mainly released by passive elimination (Rantamäki, 1997). However, biotransformation of organic contaminants by the cytochrome P450 has been reported in a wide range of invertebrate species including bivalves (Solé and Livingstone, 2005). Moreover, in the high exposure group, concentrations of Σ16PAHs were 2-fold higher than the low exposure group in both bivalve species. Crabs from the high exposure group accumulated PAHs with a 4-fold factor compared to the low exposure group. This difference might be explained by a more efficient uptake in the crab, or that the crabs, in addition to a direct uptake from water also accumulated PAHs from the bivalves. However, the experimental setup did not allow to distinguish whether the PAH contamination in exposed crabs is coming from the water or from contaminated food.

The THC and PAH levels in the water of the control tanks were below LOD, while the control animals had detectable concentrations of PAHs. The bivalve PAH concentrations of the controls were comparable to results reported by Nahrgang et al. (2013) who found ∑16PAH concentrations below or just above LOD within wild populations of blue mussels and Icelandic scallops close to Tromsø. The current control crabs showed low concentrations of five PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene) in their hepatopancreas. As MDO is known to be volatile, and as a cross-contamination through seawater in our setup was impossible, cross-contamination through air might explain the low PAH levels found in the control crabs. The five PAHs found in the control groups reflected the most abundant components of the used MDO (Table SM-1), thereby supporting the suspicion of cross-contamination via air because the tanks were kept uncovered during the exposure. However, the soft tissue concentrations of blue mussels, Icelandic scallops and the hepatopancreas concentrations of the low exposure groups were 420-, 300- and 60-fold higher, respectively, than the sum PAH concentration in tissues of crabs from the control groups.

The BAF for the crabs were generally higher than for the bivalves, except for fluoranthene which has the highest log Kow of the detected PAHs. The increasing trend of BAF for bivalves and the decreasing one for the crabs with the log Kow indicated a different pattern in accumulation or excretion of PAH in bivalves and crabs. The increased accumulation of PAH in bivalves are in agreement with Rantamäki (1997) who showed increased accumulation and an increased half-life with increasing Kow for the naphthalene, phenanthrene, fluoranthene and pyrene in blue mussels. The BAF value of the bivalves might be overestimated as the analyses of the whole soft tissue may include some mucus encapsulated oil droplets, thereby resulting in some overestimation of PAHs (Frantzen et al., 2016).

The behavioural observations showed that the crabs of both exposure group increased their movements when exposed to MDO. The crab obviously detected the MDO and the increased locomotor activity was interpreted as an avoidance response. Avoidance behaviour in crabs has been shown in the littoral crab exposed to pharmaceutical chemicals (Mesquita et al., 2011) and when exposed to water-borne copper (Hebel et al., 1997). A spill of MDO in shallow waters would therefore probably induce an avoidance reaction in the red king crab, and potentially a migration towards deeper areas.

Even though the crabs of our two exposure groups seemed to be able to detect hydrocarbons in the water, they cracked open and ate bivalves at the same rate as the controls. Thereby, the exposed groups became exposed both through water and food. As the crab were alive when weighted, the body cavity could have contained various amounts of water. Therefore, the small variance in weight changes is thought a natural variance due to the water content of the body cavity and not expressing various feeding behaviour.

Neurotoxicity of MDO, measured as inhibition of AChE activity, was not observed in our study. This is in contrast to an inhibition of AChE in muscle tissue seen in Icelandic scallops exposed to MDO

(Geraudie et al., 2016). However, the initial concentration of MDO in the exposure group that showed the AChE inhibition was more than three times higher than the current study's high exposure group. Further, the sensitivity to oil related chemicals including PAHs, has been reported to reduce the activity of AChE in *Astyanax* sp. (teleosti) exposed to the water-soluble fraction of Campos Bay's crude oil (Brazil) (Akaishi et al., 2004) and in eels exposed to seasonally variable concentrations of volatile PAHs (from naphthalene to chrysene) in the Vaccarès lagoon (France) (Roche et al., 2002). Further, lower AChE activities in blue mussels were seen for a whole year after exposure to oil from the wreckage of the tanker *Erika* on the Brittany coast (France) (Bocquené et al., 2004). Therefore, the AChE assay is one of the most used biomarkers for exposure to a wide range of pollutants and to assess neurotoxicity (Rodrigues and Pardal, 2014). However, in two studies conducted on a littoral crab, no correlation between AchE inhibition and BaP was reported (Fossi et al., 1996a, 1996b), while the third study only showed a weak reduction of AChE for the lowest BaP exposure group (Fossi et al., 2000). However, a concentration dependent decrease and a significantly lower activity of AchE with the two highest concentrations of fluoranthene were seen in the common shore crab *Carcinus maenas* (Rodrigues et al., 2013).

The antioxidant enzyme CAT showed an increasing trend with dose, corresponding to a similar effect seen in Icelandic scallops exposed to MDO (Geraudie et al., 2016). Similarly, the total GPx activity significantly increased in crabs of the high exposure group at T_1 suggesting that MDO induced pro-oxidant stress and an activation of antioxidant enzymes (Regoli et al., 2011). Lipid peroxidation, however, showed lower levels in the exposed groups at T_1 compared to the controls. Although widely used to quantify oxidative damage in the form of lipid peroxidation, the TBARS assay is, however, very unspecific and can react to many other compounds such as pyrimidines, biliverdin, sucrose, and amino acids, contributing to the overestimation of lipid peroxidation (Devasagayam et al., 2003; Lushchak and Semchuk, 2012). Furthermore, the assay may not detect all end products of lipid peroxidation, thereby increasing the complexity of its interpretation (Devasagayam et al., 2003). The significantly higher levels of TBARS in the control group at T_1 may have been caused by the unspecificity of the assay, as well as the low number of replicate individuals in each group. The weak dose-response patterns observed for CAT and GPx activities and the lack of TBARS response, suggests a weak induction of oxidative stress in red king crab exposed to the current MDO concentrations. Even so, further biomarker analyses should complement this study to draw more definite conclusions and provide a more holistic understanding of the effects of MDO to the red king crab (Regoli et al., 2011).

5. Conclusions

The study was designed to assess effects of an accidental spill of MDO in a low-temperature, shallow water Arctic marine ecosystem. The predatory red king crab was exposed to two concentrations (nominal 5 and 20 mg L^{-1}, but measured to 7.4 and 19 mg L^{-1}) of dispersed MDO through water and food for a short period (one week), a plausible scenario in case of a ship wreckage.

Both crabs, mussels and scallops showed significant and dose-dependent increase of PAHs in their tissues. Our observations indicated some degree of stress due to the MDO exposure, in the form of more movements and increased escape response. An increase in antioxidant enzymes in exposed organisms suggested some biological effects from the exposure, although only weakly. Following a recovery period of three weeks, the tissue concentration of PAHs decreased considerably and the biomarker responses returned to background levels in the exposed crabs.

MDO is currently a recommended hydrocarbon fuel over HFO in vessels operating in the Arctic. Our experiment indicates that impacts of a release of MDO to the marine ecosystems may be of a temporary character for benthic marine species such as the red king crab.

Conflicts of interest

No conflicts of interests have been identified for the present study.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2016.05.027.

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Supplemental materials

Biological effects of marine diesel oil exposure in red king crab (*Paralithodes camtschaticus*) assessed through a water and foodborne exposure experiment

Sagerup, Kjetil[a,*], Nahrgang, Jasmine[b], Frantzen, Marianne[a], Larsen, Lars-Henrik[a,b], Geraudie, Perrine[a]

[a] Akvaplan-niva, Fram Centre, Postboks 6606 Langnes, NO-9296 Tromsø, Norway

[b] UiT The Arctic University of Norway, Department of Arctic and Marine Biology, Postboks 6050 Langnes, NO-9037 Tromsø, Norway

Table SM-1: Distribution of the Environmental protection Agency (EPA) polycyclic aromatic hydrocarbons (PAHs) and the alkylated* homologs C1-C3-naphtalenes, C1-C3-phenanthrenes and C0-C3-dibenzothiophenes (NPD) in the marine diesel oil of current study. All sizes are relative GC-MS areas calculated as % of the total.

Target Compounds	Relative area GC-MS (% of total)
Naphthalene	5.30
* C1-Naphthalene	13.40
* C2-Naphthalene	11.66
* C3-Naphthalene	28.16
Acenaphthylene	0.00
Acenaphthene	0.11
Fluorene	0.52
Phenanthrene	3.64
* Dibenzothiophene	0.23
* C1-Anthr/Phenanthrene	11.51
* C1-Dibenzothiophene	1.32
Antracene	0.09
* C2-dibenzothiophene	1.81

* C2-Anthr/Phenanthrene	11.34
* C3-Anthr/Phenanthrene	5.64
* C3-dibenzothiophene	2.07
FLuoranthene	0.08
Pyrene	0.15
Benzo(a)anthracene	0.08
Chrysene	2.73
Benzo(b)fluoranthene	0.10
Benzo(k)fluoranthene	0.03
Benzo(a)pyrene	0.02
Benzo(ghi)perylene	0.00
∑ EPA-PAH	15
∑ NPD	85