**Title**

# Antioxidant defenses in polar cod (*Boreogadus saida*) and responsiveness toward dietary crude oil exposure

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**Abstract**

Increasing anthropogenic activities in the Arctic pose the risk for accidental oil spills but our knowledge of ecotoxicological effects is still limited for Arctic organisms. This study aimed to provide insights in baseline levels of antioxidants in polar cod (*Boreogadus saida*) from different Arctic locations and investigate the susceptibility of this species to oxidative stress during a 32 day dietary crude oil exposure. Baseline levels of individual antioxidants and total oxyradical scavenging capacity (TOSC) varied among different Arctic fjords. Upon crude oil exposure, dose- and time-dependent polycyclic aromatic hydrocarbons (PAHs) biotransformation was evident through hepatic ethoxyresorufine-O-deethylase activity, glutathione S-transferase mRNA expression and biliary PAH metabolites. Changes in the activity of individual antioxidants and TOSC was, however, slight and transient upon exposure. In conclusion, biotransformation was clearly induced by the sub-lethal levels of crude oil in polar cod, while the antioxidant defense responded with high variability.

Key words: Fish, Arctic, oil spills, oxidative stress, antioxidants, biotransformation, total oxyradical scavenging capacity, *Boreogadus saida*.

## 1. Introduction

Several studies have observed higher basal antioxidant levels in polar fish (Gieseg et al., 2000; Heise et al., 2007) and bivalves (Camus et al., 2005; Regoli et al., 2000) compared to temperate species, which is thought to be an adaption to an elevated pro-oxidant challenges in the polar marine environment. Polar marine species are exposed to a variety of oxidative stressor in their environment such as elevated levels of dissolved oxygen in the cold seawater, high UV radiation in Arctic summer and reduced respiration rates to compensate for low food supply and periods of starvation in winter (Abele and Puntarulo, 2004; Camus et al., 2005; Regoli et al., 2012a). Furthermore, cold-acclimated poikilotherms such as Antarctic fish and scallops (*Trematomus bernacchii* and *Adamussium colbecki*) (Acierno et al., 1996; Viarengo et al., 1995) have a different membrane lipid composition compared to temperate species, i.e. elevated levels of unsaturated fatty acids (FAs) in order to maintain membrane fluidity (Hazel, 1995). This adaption is thought to increase the organism´s susceptibility to reactive oxygen species (ROS) as polyunsaturated FAs are an exposed target of ROS driven oxidation (Abele and Puntarulo, 2004). Therefore, higher basal antioxidant levels are hypothesized in polar organisms. Although an explanations for higher antioxidant levels in polar species remain to be elucidated, there is a general consensus that abrupt changes in the pro-oxidant pressure as a consequence of, for instance, an acute pollution event such as an oil spill would strongly challenge the defense system of polar species (Abele and Puntarulo, 2004; Regoli et al., 2012a). Petroleum compounds released from natural seepages (Benedetti et al., 2014) or through a marine oil spill (Hannam et al., 2010) are shown to induce oxidative stress in marine organisms, where polycyclic aromatic hydrocarbons (PAHs) are regarded as the primary toxic compounds. PAHs can have deleterious effects in fish by enhancing the production of intracellular ROS levels during electron transfer and oxidative reactions (Livingstone, 2001). Another source for oxygen radical production during PAH exposure is represented by the redox cycling of PAH metabolites such as aromatic hydrocarbon quinones (Lemaire and Livingstone, 1997). In light of the increased global demand for petroleum resources and a global decrease of easily accessible exploration sites, petroleum resources in the Arctic are of economic interest and can lead to an increase in oil and gas exploitation in Arctic waters (Harsem et al., 2011). Along with the predicted increase in shipping traffic in Arctic waters (Eguíluz et al., 2016), these anthropogenic activities

increase the risk of potential oil discharges into the Arctic environment. To our knowledge, only few studies have examined the dynamics of the antioxidant defense systems in Arctic fish and the possibility to use such responses as biomarkers of natural or anthropogenic disturbance. Nahrgang and colleagues studied the seasonal fluctuation of selected antioxidant enzymes in polar cod as well as effects of petroleum compounds on these biomarkers (Nahrgang et al., 2009; 2010a; 2010b; 2010c). Polar cod (*Boreogadus saida*) is an important key species in the Arctic ecosystem (Bradstreet and Cross, 1982), playing a crucial role in channeling energy in the form of lipids from lower to higher trophic levels.

The aim of the present study was to provide further insights on baseline levels, natural variability and the response of antioxidants to crude oil exposure in polar cod, thus investigating the susceptibility of this species to oxidative stress. Basal levels in specimens from four different Arctic locations along a latitudinal gradient at the coast of Svalbard and Greenland were characterized. Furthermore, we investigated the susceptibility of polar cod to pro-oxidant pressure from crude oil contamination over a 4 week dietary exposure experiment. Crude oil compounds such as PAHs can be readily taken up through the diet and dietary exposure has been shown as an important exposure route for crude oil in polar cod (Nahrgang et al., 2010c; Bender et al., 2016). The biotransformation efficiency upon crude oil exposure was examined based on the cytochrome P4501A activity and levels of biliary PAH metabolites. Individual antioxidants, chosen as early and sensitive biomarkers of pro-oxidant challenge, were analyzed in parallel to the total oxyradical scavenging capacity (TOSC) in liver tissue, which is a quantifiable measure of the overall susceptibility of polar cod liver tissues to oxidative stress (Regoli and Winston, 1999). Furthermore, transcriptional variations were measured in order to compare the sensitivity of responses at mRNA level with the onset of corresponding functional effects at cellular level. Finally, the responses of all biomarkers were elaborated with the help of a quantitative hazard model in order to understand the overall effect of crude oil on polar cod and the sensitivity of this key species towards the pro-oxidant challenge from a potential crude oil contamination in Arctic waters.

## 2. Materials and methods

### 2.1 Baseline study of wild caught polar cod

Wild polar cod were caught by bottom trawl at 200 – 300 m depth during two research cruises: the first along the Northwest coast of Svalbard (September 2012) in Billefjorden (78°N), Hinlopen (79°N) and Rijpfjorden (80°N) and the second at the Northeast coast of Greenland (August 2013) in Tyrolerfjorden (74°N, Figure 1). At each location, the Seabird Electronics 911 CTD unit was deployed in parallel to the trawling procedure in order to obtain vertical profiles of temperature and salinity. Billefjorden and Tyrolerfjorden were determined as stations with sub-zero temperatures at the trawling depth (- 1 °C and between -2.2 and -1.9°C, respectively). These two fjords are both described as sill fjords and characterized by high freshwater influx in sea ice-free season (Arnkvaern et al., 2005; Christiansen et al., 2012). Tyrolerfjord is devoid of sea ice between late July to mid-October (Christiansen et al., 2012), while Billefjorden is covered by sea ice between December and June (Arnkvaern et al., 2005). Temperatures at trawling depth were similar (3.2 - 3.4) at the Hinlopen and Rijpfjorden station and mainly influenced by Arctic water masses (Howe et al., 2010). Fish were sacrificed on board and subsequently characterized by sex, total fish length (± 0.1 cm), total and somatic weight and gonad and liver weight (± 0.1 g, Table 1). All liver samples were snap frozen in liquid nitrogen and maintained at -80°C until further analyses.

### 2.2 Crude oil dietary exposure

#### 2.2.1 Sampling and acclimation

Polar cod for the experiment were caught along the Northwest coast of Svalbard in January 2013 (Billefjorden, Rijpfjorden, Figure 1). Fish were caught alive by trawl with the FISH-LIFT device attached to the trawl cod-end in order to minimize the damage to the caught fish, as described by Nahrgang et al (2010c). Subsequently fish were kept on deck of the ship in 500 L tanks with a constant seawater supply until their transfer to the research facilities in Kårvika (69°N, Norway). Fish were acclimated for 3 month to the laboratory conditions and transferred to the experiment tanks (300 L) one month prior to experiment start. In total, 153 polar cod were selected for the experiment based on similar size (mean total length 16.9 cm ± 0.1 mean somatic weight 18.0 g ± 0.3). The mean seawater

temperature in the tanks was 3.6 ºC ± 0.3 (±SD) with a mean dissolved oxygen levels of 91.7 % ± 5.2. The light regime in the experiment room simulated the *in situ* photoperiod at latitude 69º North between April and May, which is 24 hours daylight but with lower light intensities during night.

2.2.2 Experimental design

The experiment started in late April and fish were dietarily exposed to Kobbe crude oil at low, medium and high doses. The fish feed consisted of *Calanus sp.* blended with starch (20% wwt) to increase its consistency and allow force-feeding. Four different treatments (control, low, medium and high crude oil dose) were prepared by adding 0, 0.5, 2 and 4 mg of crude oil per feed, respectively and mixed with a magnetic stirrer for 5 minutes at room temperature. All fish were force fed with 0.6 g food by a 1 mL Tuberculin syringe every 4$^{th}$ day for 32 days (8 feeding events in total), corresponding to exposure doses of 0, 15.6 ± 3.4, 61.9 ± 12.2 and 105.3 ± 23.8 µg crude oil/ g fish/ feeding for the control, low, medium and high treatment respectively. After 0, 8, 16, 24 and 32 days, 10 fish of each treatment were anesthetized in a Finquel® bath (50 mg Finquel®/L seawater) and sacrificed by a sharp blow to the head. Fish sex, total length and weight, as well as liver- (HSI) and gonado-somatic indices (GSI) were recorded (Table 1). Liver and bile samples were collected, snap frozen in liquid nitrogen and kept at -80 °C until further analyses. Due to the small dimension of livers in post-spawning fish (unpublished results), biochemical analyses were not performed on all 153 exposed fish: for each parameter, at least 3 organisms from every experimental group were analyzed as indicated in respective tables and figures. The experiment was approved by the Norwegian Animals Research Authority (ID 5271) and performed by trained researches with the appropriate license (FELASA Category C).

2.3 Biochemical analyses

Triplicates of fish fed the four crude oil treatments (control, low, medium and high) were analyzed for PAH levels, including the 16 Environmental Protection Agency (EPA) PAHs and ten alkylated naphthalenes, phenanthrenes and dibenzothiophenes, as described in Nahrgang et al. (2010c).

The activity of 7- ethoxyresorufine-O-deethylase (EROD) was analyzed according to Nahrgang et al. (2010c). A subset of liver samples (n=80) were homogenized in 100 mM potassium phosphate buffer (pH 7.8), 150 mM KCl, 1mM DTT and 5% glycerol with the help of a Precellys homogenizer.

Following two centrifugation steps (9000 x *g* 30 min, 50 000 x *g* 120 min), the microsomal fraction of the samples was obtained. The EROD activity was measured in 230 µl final reaction mixture, containing 10 µl of diluted microsomal fraction (1:1000), 2 µM 7-ethoxyresorufine and 0.25 mM NADPH. Produced resorufine was quantified through fluorescence at 540/ 600 nm wavelength (excitation/ emission) in four replicates, using the plate reader Synergy H1 (BioTek®) and a resorufine standard curve (0-0.58 µM). Total microsomal protein concentrations were determined according to Bradford (1976) with bovine serum albumin (BSA) as standard. The EROD activity was expressed as pmol/ min/ mg of microsomal protein.

Aromatic metabolites in the polar cod bile were semi-quantitatively assessed by fixed fluorescence spectrofluorimetry according to Aas et al. (2000). Bile samples (n=81) were diluted in a range of 1:500 to 1:10 000 in 48 % ethanol in order to obtain linear responses in the fluorescence readings. Naphthalene-, pyrene- and benzo(a)pyrene (BaP) -type metabolites were determined based on excitation/ emission wavelengths at 290/335 nm, 341/383 nm and 380/430 nm (respectively), using the appropriate concentration of 1-naphthol for naphthalene-type metabolites and 1-OH-pyrene for pyrene- and BaP-type metabolites as reference standards (Gorbi et al., 2005). Results were expressed as metabolites-type in mg or µg per ml of bile.

Enzymatic antioxidants were measured in liver samples (n=50) homogenized (1:5 w/v ratio) in a 100 mM potassium phosphate buffer (pH 7.5), containing 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mg/ml bacitracin, 0.008 trypsin inhibitor unit/ml aprotinin, 1 ng/ml leupeptin, 5 ng/ml pepstatin and 1.8 % NaCl and centrifuged at 1000 000 x *g* for 1 h at 4°C (Regoli et al., 2012b). Measurements were made with a Varian (model Cary 3) spectrophotometer at constant temperature 18 ± 1°C and further assay conditions are described in Regoli et al. (2012b). Briefly, catalase (CAT) was assessed based on $H_2O_2$ consumption at 240 nm (ε = 0.04 mM/cm), while glutathione S-transferases (GST) was analyzed at 340 nm (ε = 9.6 mM/cm), measuring the degree of dehalogenation of reduced glutathione (GSH) with the substrate 1-chloro-2, 4-dinitrobenzene. Glutathione reductase (GR) was quantified by the loss of NADPH during the reduction of oxidized glutathione (GSSG) to GSH at 340 nm (ε =6.22 mM/cm). Glutathione peroxidases (GPx) were assayed in a coupled spectrometric assay, where NADPH is

consumed by GR to reconvert the formed GSSG to its reduced form. The decrease of NADPH absorbance was monitored at 340 nm (ε=6.22 mM/cm), using $H_2O_2$ or cumene hydroperoxide as substrates, respectively, for the selenium (Se)-dependent and for the sum of Se-dependent and Se-independent GPx forms. Levels of total glutathione concentration were measured in liver samples from wild specimens, which were homogenized (1:5 ratio, w:v ratio) in 5 % sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and subsequently centrifuged at 37 000 x *g* for 15 min. The resulting supernatants were enzymatically assayed (Regoli et al., 2012b).

The total oxyradical scavenging capacity (TOSC) was measured towards peroxyl radical (ROO), hydroxyl radical (OH) and peroxynitrite (HNOOH). Liver samples (n=77) were manually homogenized (1:5 w/v ratio) with a Potter-Elvehjem type homogenizer in 100 mM potassium phosphate buffer (pH 7.5), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, NaCl 1.8 %, subsequently centrifuged at 100 000 x *g* for 1h at 4°C. The artificially generated radicals (ROO·, ·OH, HNOOH) were obtained from the thermal homolysis of 2-2-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP), the iron (plus ascorbate)-driven Fenton reaction, and the thermal homolysis of 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), respectively. The absorption of artificially generated oxyradicals by cellular antioxidants was measured by the quantification of inhibited oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Regoli and Winston, 1999). Detailed assay conditions are described elsewhere (Regoli and Winston, 1999). Single antioxidants and TOSC values were calculated normalizing obtained values to protein content that was determined according to Lowry et al. (1951) with BSA as standard. Malondialdehyde (MDA) was quantified in livers of polar cod (n=48) homogenized (1:3 w/v ratio) in 20 mM Tris–HCl (pH 7.4), centrifuged at 3 000 x *g* for 20 min. A conjugation reaction was performed in 1 ml reaction mixture (45 °C, 40 min), containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3:1), 32% HCl, 100 µl water and 100 µl of sample or standard (standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl [pH 7.4]). Samples were finally cooled on ice, centrifuged at 15 000 x *g* for 10 min and spectrophotometrically analyzed at 586 nm. MDA concentrations were determined as a function of the 1, 1, 3, 3-tetramethoxypropane standard curve and expressed as nmol/g liver tissue (Benedetti et al., 2014).

## 2.4 Transcriptional analyses

### 2.4.1 RNA isolation and cDNA synthesis

Transcriptional analyses were performed on fish from day 8 and 32 in order to investigate the gene responses at the beginning and end of the exposure experiment. Total RNA was purified from liver samples and homogenized in Trizol® reagent (Sigma-Aldrich), according to the manufacturer's protocol. Following, the concentration of total RNA were measured using the Spectrophotometer (Nano-Drop ND-1000 UV-Visible Spectrophotometer) and the RNA quality was verified on an agarose-formaldehyde gel. Total cDNA was generated by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) from 1 µg of total RNA for each sample, using combined oligo (dT) and random hexamer primers (iScript cDNA Synthesis Kit, Bio-Rad).

### 2.4.2 Quantitative real-time PCR

Absolute quantitative real-time PCR with primer pairs (Table 2) was performed for evaluating the mRNA levels of the following target genes: *catalase* (*cat*), *glutathione peroxidase 1* (*gpx1*) and *phospholipid hydroperoxide glutathione peroxidase* (*gpx4*), *glutathione S-transferase pi* (*gstpi*), *cu/zn-superoxide dismutase* (*cu/zn-sod*) and *nuclear factor erythroid 2–related factor 2* (*nrf2*). The SYBR green method was performed in the StepOnePlus® Real-Time PCR System (Applied Biosystems). Each 15 µl DNA amplification reaction contained 7.5 µl of SYBR Select Master Mix (Life Technologies), 5 µl of total cDNA (1:5 in RNAse free water) and 200 nM of the respective forward and reverse primer (Table 2). The real-time PCR program included an enzyme activation step at 95 °C (2 min) and 40 amplification cycles of 15 s at 95 °C, 15 s at the respective annealing temperature (Table 2) and 1 min at 72 °C. The specificity of target cDNA amplification was checked by including controls lacking cDNA template and by a melting analysis (95 °C for 1 min, 65 °C for 10 s and fluorescence detection at increasing temperature between 65 and 95 °C). For each PCR run, serial dilutions of known amounts of plasmid containing the amplicon of interest were used as calibrator for each gene. Samples and calibrator were run in duplicates in the same run. Cycle threshold (Ct) values of unknown samples were converted into mRNA copy number by interpolating the standard curve of calibrator Ct versus log copy number. This absolute quantification method is a well validated procedure to calculate the absolute copy number

of the transcripts of interest, thus preventing a potential interference when housekeeping genes exhibit a parallel modulation pattern with experimental genes (Regoli et al., 2011; Arukwe, 2006).

### 2.5 Data analyses and statistical elaboration

After log transformation, fish biometrics (total length, somatic weight, GSI and HSI), enzyme activity, gene transcription levels, TOSC and MDA results were compiled and met the assumptions of normality and homogeneity of variance (Shapiro Wilk`s W test, Levene`s test, respectively). Hence, this data was analyzed by one-way ANOVA, testing in wild specimens differences among sampling areas and in exposed organisms differences among the exposure doses and time points, followed by a *post hoc* test (Tukey`s honest significant difference test). Data on PAH metabolite levels in bile violated the assumptions of normal distribution and homogeneity of variance and were therefore analyzed by Kruskal Wallis rank sum test followed by the *post hoc* test after Nemenyi (multiple comparisons of rank sum of independent samples). The correlation between variables was tested by Pearson`s product-moment correlation. The significance level for all statistical tests was set to $\alpha = 0.05$. Plots of the results and the statistical analysis of the data were generated with the R project language (R Core Team, 2014). Considering the low size of liver samples and the impossibility to perform all molecular and biochemical analyses in each fish, the effect of gender on analyzed responses could not be tested. All data are thus presented and statistically analyzed as mixed gender.

Biomarker results (CYP1A activity, bile metabolites, individual antioxidants, TOSC, MDA) in exposed polar cod were further elaborated within a quantitative model (Sediqualsoft), which summarizes a hazard index (Hazard Quotient, HQ) based on number, magnitude and toxicological relevance of observed variations. Details on whole calculations, flow-charts of the elaboration structure, rationale for weighting and thresholds are outlined in Piva et al. (2011). Briefly, the model assigns a 'weight' to each biomarker based on their toxicological importance and a 'threshold' for changes of biological relevance. The variation measured for each biomarker is compared to the respective threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. According to variations measured for the various biomarkers, the

model summarizes the level of cumulative hazard quotient (HQ) in one of five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011).

## 3. Results

### 3.1 Baseline study

Polar cod sampled from Billefjorden were significantly smaller in total length and had lower somatic weight than fish from the other Arctic locations, while polar cod from Tyrolerfjorden, Hinlopen and Rijpfjorden were comparable in body size (Table 1). Furthermore, polar cod from Tyrolerfjorden had HSI values almost two-fold higher than those of fish from the Svalbard locations (Table 1).

In general, analyses of antioxidant defenses revealed variation and significant differences among fish sampled in different geographical areas (Table 3). Compared to the Svalbard sites, polar cod from Tyrolerfjorden showed higher mean values for CAT, GST and TOSC towards HNOOH (Table 3), the TOSC value being significantly higher than in Hinlopen. Specimens from Billefjorden exhibited the lowest enzymatic activities in all antioxidants analyzed and at the same time the highest TOSC values toward ROO· and ·OH and the greatest content of MDA (Table 3).

### 3.2 Crude oil dietary exposure

#### 3.2.1 PAH levels in fish food

Analyses of Sum 26 PAH levels in the polar cod food showed a positive linear relationship with increasing nominal concentrations of crude oil added to the feed (Table 4). The total of 26 PAHs analyzed accounted for 1.1 -1.3 % of the crude oil, where alkylated naphthalenes (e.g. C1- to C3-naphthalene) dominated the overall PAH load (76 % of Sum 26 PAHs, Table 4). Based on these results, PAH doses in the food treatments were calculated: 0.08 (control), 6.5 (low), 22.9 (medium) and 48.2 μg PAHs/ g food (high) that equated to ingested PAH doses of 0, 0.05 ± 0.01, 0.18 ± 0.03 and 0.31 ± 0.08 μg PAHs/ g fish/ day, respectively.

#### 3.2.2 PAH metabolism in polar cod

The CYP1A activity showed an apparent dose- and time-dependent increase in polar cod exposed to medium and high doses of crude oil, with the highest activities measured at day 32 (Figure 2).

271 Variations in EROD activity were paralleled and significantly correlated with those of biliary PAH
272 metabolites, i.e. naphthalene-type (OH-Nap, $r=0.68$, d.f.=32, $p<0.001$), pyrene-type (OH-Pyr, $r=0.71$,
273 d.f.=32, $p<0.001$) and BaP-type metabolites (OH-BaP, $r=0.63$, d.f.=31, $p<0.001$). An evident increase
274 of OH-Nap and OH-Pyr metabolites related to crude oil exposure was observed at all time points,
275 when medium and high crude oil doses caused significant higher levels of metabolites compared to
276 control fish (Figure 3A and 3B). Similarly to the EROD activity, there was a significant increase in
277 bile metabolites over the course of the experiment with the highest levels of OH-Nap and OH-Pyr
278 metabolites at day 32 in the medium and high treatments compared to day 8 (Figure 3). A less clear
279 separation among crude oil doses was found for OH-BaP metabolites, nevertheless fish exposed to
280 high crude oil doses showed significantly higher levels than control fish at day 24 and day 32 (Figure
281 3C). OH-BaP metabolites increased over the course of the experiment in high crude oil-treated fish,
282 which had higher levels at day 32 compared to day 8 (Figure 3C).

283 The activity of GST tended to increase in crude oil exposed fish compared to controls during the first
284 two weeks of exposure with significantly higher levels in fish exposed to high crude oil doses
285 compared to control fish at day 16 (Table 5). Toward the end of the experiment, GST activity
286 remained more constant or even decreased in exposed compared to control fish. However, a
287 transcriptional up-regulation was observed for *gstpi* in the medium and high treatment at day 32
288 (Table 6).

289 3.2.3 Oxidative stress responses

290 The control fish of the crude oil experiment had comparable values of antioxidants to those measured
291 in wild fish from different populations but with slightly higher enzymatic activities for CAT, GR and
292 GPx (Table 3). GST, TOSC and MDA levels of control fish were within the range of values measured
293 for wild polar cod from the different sites.

294 Responses of antioxidant enzymes in exposed polar cod did not exhibit clear trends as opposite
295 relationships variations were seen in measured parameters over time and treatment (Table 5).
296 Furthermore, the magnitude of the effect was often within the range of variability observed in control
297 organisms throughout the exposure period. CAT, GR and GPx exhibited a biphasic and apparently

298 contradictory response, as both increased and decreased enzymatic activities were measured at various
299 time points (Table 5). A significant increase of GR activity was found in crude oil exposed fish
300 compared to control fish at day 16 (Table 5).$O_2$-

301 Similar to functional effects at the catalytic level, transcriptional responses were variable between the
302 different genes (Table 6). Transcription levels of the gene encoding catalase showed a biphasic change
303 at day 8, being induced and inhibited in the low and high treatments, respectively. While *gpx1* mRNA
304 expression was enhanced at day 32 in the low and high treatments, and reduced in the medium
305 treatment. Transcription levels of *Cu/ Zn superoxide dismutase* was also up-regulated in the high
306 treatment both at day 8 and day 32 (Table 6). The *nrf2* was down-regulated with a dose-dependent
307 trend at day 32 (Table 6).

308 Consistent with the limited variations of individual antioxidants, the total antioxidant capacity showed
309 weak dose- and time-dependent changes (Table 7). The capability to neutralize ·OH was generally
310 higher compared to those observed towards both ROO· and HOONO. Furthermore, significant dose-
311 dependent changes were found between day 16 and day 32 (Table 7). MDA levels were negatively
312 correlated with exposure time in all treatment groups ($r= -0.7$, $df=46$, $p<0.001$), showing significant
313 lower MDA levels at day 32 compared to day 8 and day 16 in the high treatment (Table 7). When
314 comparing results of the TOSC assay with MDA levels, a negative correlation between MDA and
315 TOSC level against ·OH was found ($r=-0.55$, $d.f.=17$, $p<0.001$).

316 The quantitative elaboration of biomarker results using weighted criteria summarized the biological
317 significance of cellular responses in the liver of polar cod exposed to different crude oil doses at
318 different time points into a single hazard index (HQ, Figure 4). In organisms treated with the highest
319 dose, the class of hazard was summarized as 'moderate' from the beginning to the end of exposure. An
320 intermediate situation was observed for medium oil concentration, with a 'slight'class of hazard at the
321 beginning (days 8 and 16) and 'moderate' in organisms exposed for 24 and 32 days. A temporal effect
322 was also evident for organisms exposed at the lowest dose with a 'slight' class of hazard for all time
323 points except at days 32 where it was raised to 'moderate'.

## 4. Discussion

### 4.1 Baseline study

Higher basal levels of CAT and GST were measured at Tyrolerfjorden compared to the other locations, which could indicate different pro-oxidant conditions in this fjord. In the baseline study, Tyrolerfjorden had the coldest (sub-zero) near bottom temperatures in comparison with the other fjords (above 0° C in Hinlopen and Rijpfjorden) and is characterized by the longest period of sea ice cover during the year (mid-October to mid-July) compared to the Svalbard locations (Christiansen et al., 2012). Low habitat temperature could be a relevant factor in explaining the higher antioxidant capacity of the Tyrolerfjorden fish and could confirm the adaptation mechanism suggested for polar fish species (Heise et al., 2007). Differences in antioxidant baseline levels among polar cod from various Arctic fjords could also be related to different type and abundance of food, which is known to modulate antioxidant status (Regoli et al., 2002a). Although all polar cod specimens were sampled during the same season (August), the investigated locations are characterized by a different timing in sea ice melting and associated spring phytoplanktonic bloom (Arnkvaern et al., 2005; Howe et al., 2010; Christiansen et al., 2012): in this respect, a delay of sea ice melting in Tyrolerfjorden could imply a lag of primary production in this fjord compared to the Svalbard locations. In contrast, Billefjorden fish had the lowest enzymatic activity of all antioxidants analyzed but combined with a higher TOSC capacity towards ROO· and ·OH and more elevated MDA levels. Such results would reflect a more elevated pro-oxidant pressure in these specimens. These fish were characterized by smaller body size and lower GSI levels than fish from the other Arctic location, which could indicate that these specimens were juvenile and immature. Although very few studies investigated the relationships between reproductive stages and oxidative pathway, a certain modulation of antioxidant parameters was suggested (Martínez-Álvarez et al., 2005).

The results of the present baseline study do not confirm the pattern of elevated basal antioxidant levels in Arctic fish compared to temperate species and support other comparative studies that did not find elevated oxidative defense mechanisms in polar compared to temperate organisms (Cassini et al., 1993; Speers-Roesch and Ballantyne, 2005; Witas et al., 1984). This polar-temperate comparison is,

however, often difficult to make because it is based on fish species of different genus and life history, inhabiting quite distinct habitats. Furthermore, the higher antioxidant levels found in polar fish are mainly based on differences in levels of non-enzymatic antioxidant such as vitamin E (Gieseg et al., 2000) and glutathione (Heise et al., 2007) that might be higher to protect against increased pro-oxidant challenge experienced in polar regions (Abele and Puntarulo, 2004; Camus et al., 2005; Regoli et al., 2012a).

## 4.2 Crude oil dietary exposure

### 4.2.1 Biotransformation

Dose- and exposure time-related increases in CYP1A activity demonstrated induced xenobiotic biotransformation in polar cod upon crude oil exposure. This is further confirmed by the increase of OH-Nap and OH-Pyr metabolites in fish bile. The biotransformation pathway has been previously induced in polar cod exposed to synthetic produced water (Geraudie et al., 2014) and crude oil (Andersen et al., 2015; George et al., 1995; Nahrgang et al., 2010b, 2010c). Compared to a similar dietary study by Nahrgang et al. (2010c), our results showed a less clear dose-dependent and time-related increase of OH-BaP metabolites and lower EROD activities between day 8 and day 24, but comparable in the medium and high treatment at day 32. A potential explanation could be the lower exposure levels in the fish food of the present study (0.05, 0.18, 0.31 µg PAHs/ g fish /day) compared to Nahrgang et al. (2010c) (0.6 and 1.3 µg PAH/ g fish/ day). In a study exposing polar cod to even lower PAH levels through the diet (0.8, 1.8, 7 and 13.1 ng PAH/ g fish/day) only a weak induction of EROD activity was observed with crude oil exposure (Bender et al., 2016). Furthermore, crude oil contains only to 1.6 -1.8 % of PAHs depending on the type of crude oil (Baussant et al., 2009) and several studies have shown that compounds other than PAH can induce biotransformation activity and contribute to the toxicity of crude oil in marine organisms (Booth et al., 2007; Melbye et al., 2009).

Another enzyme indicative of xenobiotic biotransformation in fish are the phase II enzyme glutathione S-transferases, which were shown as valuable biomarkers for water-soluble fraction of petroleum compound in polar cod (Nahrgang et al., 2009, 2010b), while dietary crude oil exposure induced GST activity first after 6 weeks exposure (Nahrgang et al., 2010c). In the present study, consistently higher

(4-fold) basal levels of GST might explain the limited response of this enzyme to crude oil exposure, although enhanced catalytic activity was seen during the first weeks in fish exposed to the high crude oil dose. The decrease of GST observed towards the end of the experiment opposed the increased CYP1A activity seen at day 32. An inverse relationship between GST and CYP1A activity has been described before in polar cod dietarily exposure to crude oil (Nahrgang et al., 2010c). High (BaP) and low molecular weight PAH compounds (anthracene) were shown to cause opposite responses with both induction and inhibition of GST activity, respectively, in common goby (*Pomatoscistus microps)* (Vieira et al., 2008). PAH composition in the food of polar cod was dominated by low molecular weight PAHs such as anthracene and naphthalene, which may have had an inhibiting effect on GST, thus providing a possible explanation for the decreased catalytic levels of in the high at treatment day 32. On the contrary, induced *gstpi* transcription at day 32 in the medium and high treatment indicated an effect of oil exposure at the molecular level and the involvement of GST in the PAH metabolism in polar cod. Previous studies on polar cod also observed *gstpi* induction after 2 weeks exposure to either water-soluble fraction or dietary crude oil (Nahrgang et al., 2010b, 2010c). The differential responsiveness of GST at molecular and catalytic level may be explained by the time discrepancy between transcriptional processes and their functional effect at cellular level or greater toxicological effects of crude oil on the protein than on the gene level (Regoli and Giuliani, 2014). However, this discrepancy may also be due to different methodological approaches that consider specific isoform (i.e. GSTpi) for molecular analyses and several isoforms for enzymatic assays (Regoli and Giuliani, 2014).

4.2.2. Antioxidant enzyme responses

Activity of antioxidants were generally higher in the control fish of the experiment compared to levels measured in wild polar cod from the baseline study. These values, however, were within the range of seasonal variability previously observed for antioxidant enzymes in polar cod (Nahrgang et al., 2010a) and the latter study suggested that antioxidant activity in polar cod varied in relation to food availability, lipid content and metabolic activity of fish (Nahrgang et al., 2010a). Hence, the different season between the baseline study (August) and the crude oil experiment (April/May) may explain the

differences observed in antioxidant activity. TOSC values and levels of MDA were comparable in control and wild-caught fish, indicating the absence of elevated pro-oxidant pressure due to experimental conditions.

Changes in antioxidant enzyme activities in marine organisms can be regarded as early warning signals of a varied oxidative challenge related to chemical exposure (Regoli et al., 2003). CAT and GR were identified as sensitive biomarkers in Mediterranean and Atlantic fish with increased activities after exposure to hydrocarbons in Atlantic cod (*Gadus morhua*) (Sturve et al., 2006), dab (*Limanda limanda*) (Livingstone et al., 1993), grey mullet (*Mugil* sp.) (Rodrígues-Ariza et al., 1993) and demersal fish species *Lepidorhombus boscii* and *Callionymus lyra* (Martinez-Gomez et al., 2006). Catalase activity can also exhibit a biphasic response and this was confirmed in the present study, where an initial increase of enzymatic activity was replaced by a progressive decrease in fish exposed to high crude oil doses. This could indicate that the pro-oxidant pressure was initially counteracted by higher antioxidant capacity at day 16 and 24 but finally overwhelmed at the end of the experiment. A decrease of CAT activities in polar cod was also observed in a dietary exposure study with higher doses of crude oil (Nahrgang et al., 2010c) than the present study, which indicates that lower exposure levels could have required a longer period of time to inhibit this antioxidant. The biphasic variation of CAT was also evident at the transcriptional level; low crude oil doses caused an up-regulation at day 8 and down-regulation at day 32, hence the *cat* mRNA expression pattern reflected the decreased catalytic activity at day 32. Such a biphasic variation to pro-oxidant pressure was also shown in European eel, which exhibited a down-regulation of *cat* mRNA expression after a longer exposure time (Giuliani and Regoli, 2014). The *cu/zn-sod* exhibited a different trend with an up-regulation of mRNA expression in fish exposed to high crude oil doses at day 8 and 32. This response was mirrored in another polar cod study, where intraperitoneally injected BaP induced a strong up-regulation *cu/zn-sod* after both short (16 hours) and long exposure (2 days) (Nahrgang et al., 2009). A clear dose-dependent modulation of oxidative response was seen for the *nrf2* gene in fish exposed to low and high crude oil doses, showing an up-regulation at day 8 compared to day 32. Few data is available for Nfr2 in marine organisms, even less for polar species, but initial studies suggest the involvement of

*nrf2* in the regulation of antioxidant enzyme genes such as *cat*, *gpx* and *gstpi* (Giuliani and Regoli, 2014; Regoli and Giuliani, 2014). Increased GR upon hydrocarbon exposure could be expected in polar cod as previously found in other marine fish (Martinez-Gomez et al., 2006; Regoli et al., 2003; Rodrígues-Ariza et al., 1993; Sturve et al., 2006). This response was observed at day 16, suggesting the need to regenerate reduced glutathione from its oxidized form GSSG (Di Giulio and Meyer, 2008). On the contrary, GPx levels were not clearly affected by the crude oil exposure, rather showing high variability that was not related to the exposure regime. Nevertheless, an up-regulation of *gpx1* and *gpx4* transcription levels at day 32 compared to day 8 indicate that an effect on this antioxidant might occur at a later time, as found in fish from chronically PAH-contaminated estuaries (Meyer et al., 2003; Rodrígues-Ariza et al., 1993). In summary, variable responses of antioxidant genes reflect the complexity of the oxidative network, thus supporting the non-synchronous response found in other marine organisms (Giuliani et al., 2013).

The TOSC assay provides an understanding of the overall antioxidant capacity of the liver cells by integrating the responses of enzymatic and non-enzymatic antioxidants with exposure to crude oil. The TOSC response in polar cod was highly variable among treatments, which could be explained by the rapid and transient formation of ROS in fish hepatocytes and by the complexity of antioxidant defenses (Regoli and Giuliani, 2014). In the present study, higher TOSC levels were found towards ·OH compared to other ROS (ROO· and HOONO), indicating that polar cod can better contrast pro-oxidant pressure primarily expressed by hydroxyl radicals. This relationship might explain the negative correlation of TOSC levels towards ·OH with lipid peroxidation levels (MDA), which in the present study decreased strongly over the course of the experiment. It might also indicate cellular changes in the hepatocytes upon the formation of ·OH radicals, which can either limit lipid peroxidation or increase membrane permeability and excretion of MDA from liver cells (Di Giulio and Meyer, 2008). Interestingly, environmentally relevant levels of North Sea oil showed no effect on lipid peroxidation in Atlantic cod, despite inducing significant effects on antioxidant enzymes levels (Sturve et al., 2006).

458 The combined results of endpoints related to biotransformation and antioxidant defense represent a
459 complex set of biological responses, which were elaborated through the quantitative hazard model
460 Sediqualsoft. The overall quantitative effects ranged between 'slight' to 'moderate' in the exposed fish
461 but with a different time course, depending on the exposure dose. The present study is the first to use
462 this model on data of an Arctic fish species with the aim to elaborate the bioavailability and hazard of
463 petroleum compounds for polar cod. The classification of crude oil treatments as 'slight' and 'moderate'
464 in the present study implies that petroleum compounds are bioavailable in the exposed fish, however
465 cellular effects related to the exposure depend on the exposure levels. The high crude oil dose, assign
466 with the 'moderate' HQ, induced significant cellular effects in biotransformation as indicated by the
467 exposure biomarkers EROD and PAH metabolite levels in the bile and transient effects on the
468 antioxidant defense system in polar cod. Hence, the high crude oil dose affect PAH biotransformation
469 but has only transient effects on the antioxidant defense in polar cod. The severity of these effects both
470 acute and on a longer time scale can, however, not be derived from the results of this model and more
471 data of polar cod would need to be analyzed with this model in order to integrate and understand the
472 relevance of these biological responses.

473 In conclusion, this study provides new data for basal levels of antioxidants in polar cod, an important
474 key species in the Arctic. The enzymatic activity of antioxidants (CAT, GST) were significantly
475 higher in Tyrolerfjorden compared to the Svalbard fjords, while Billefjorden fish had the lowest
476 enzymatic activities compared to the other locations. These spatial differences might be explained by
477 different factors such as the hydrography of the fjords or the metabolic activity of the fish.
478 Furthermore, the dietary exposure study showed that sub-lethal crude oil levels efficiently induced
479 PAH metabolization pathways in polar cod, highlighting the relevance of PAH biotransformation as
480 applicable biomarker of crude oil exposure in this fish species. In contrast, only minor fluctuating
481 variations in antioxidant defense were observed upon the chemical challenge of crude oil
482 contamination, which might be related to a naturally high environmental pro-oxidant pressure for polar
483 cod. In summary, our results indicate that adult polar cod cope well with the exposure to crude oil

484 levels used in the present experiment, presumably due to an efficient PAH biotransformation, while
485 the antioxidant defense system did not show a strong response upon the crude oil exposure.

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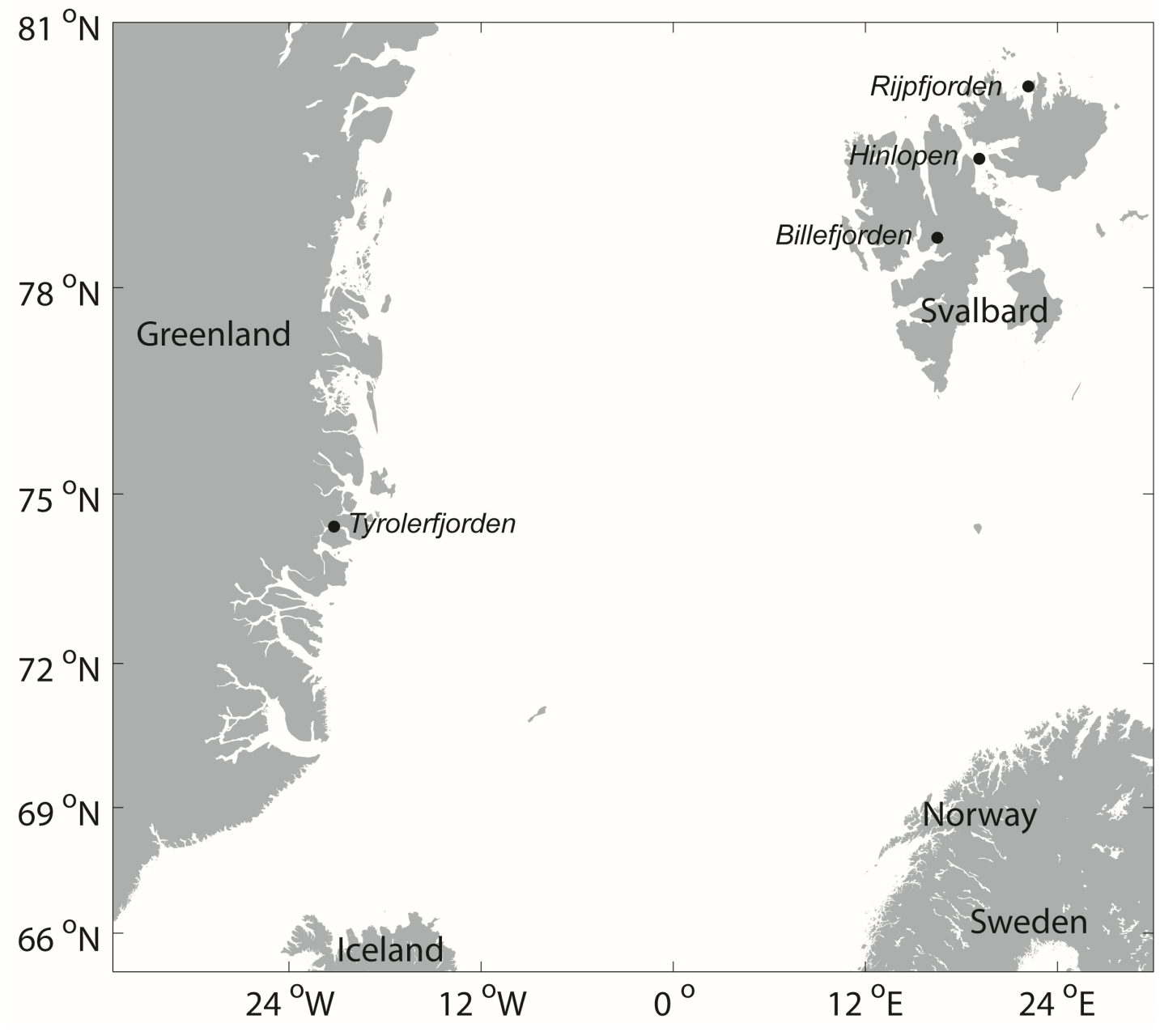
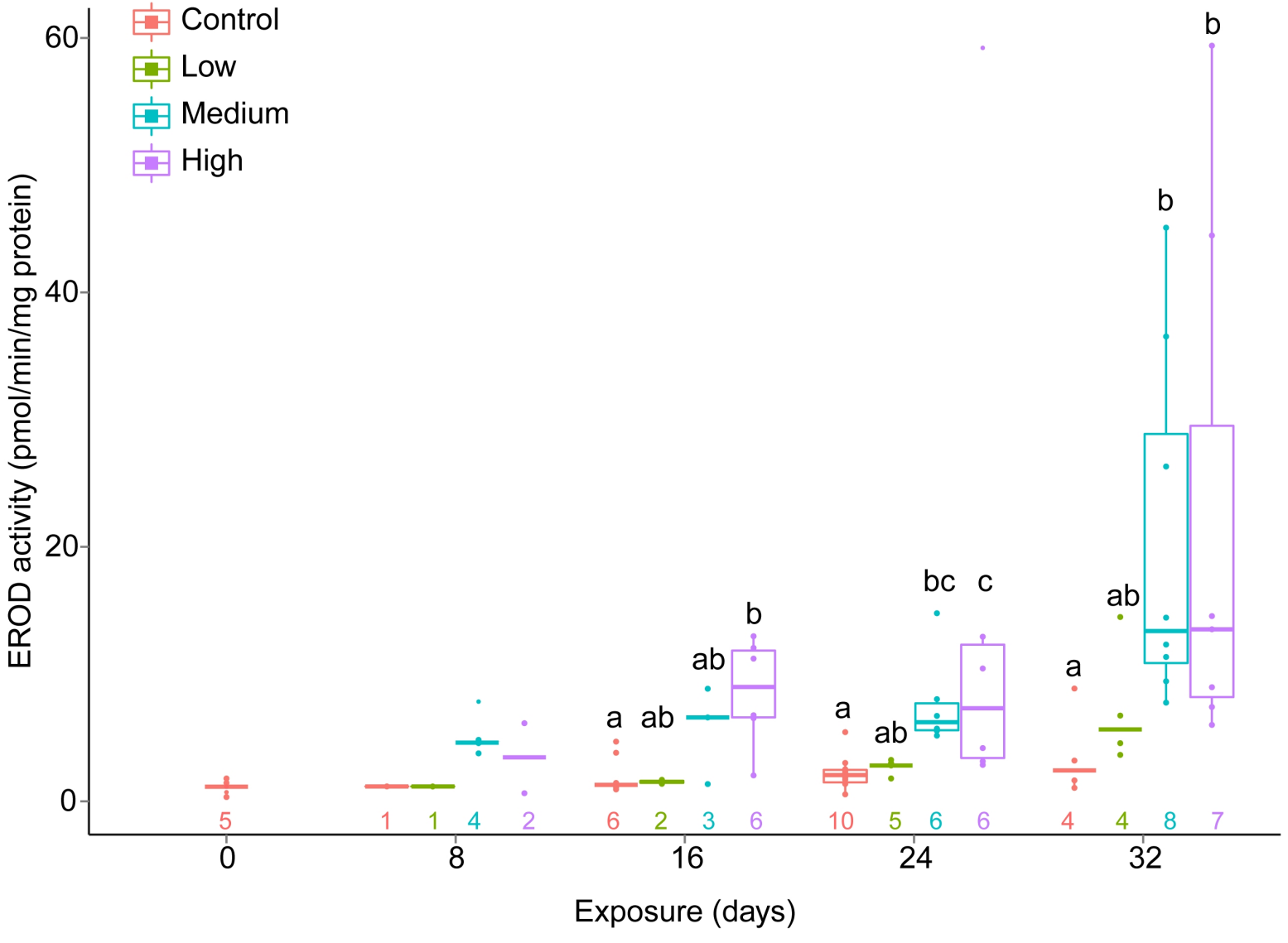
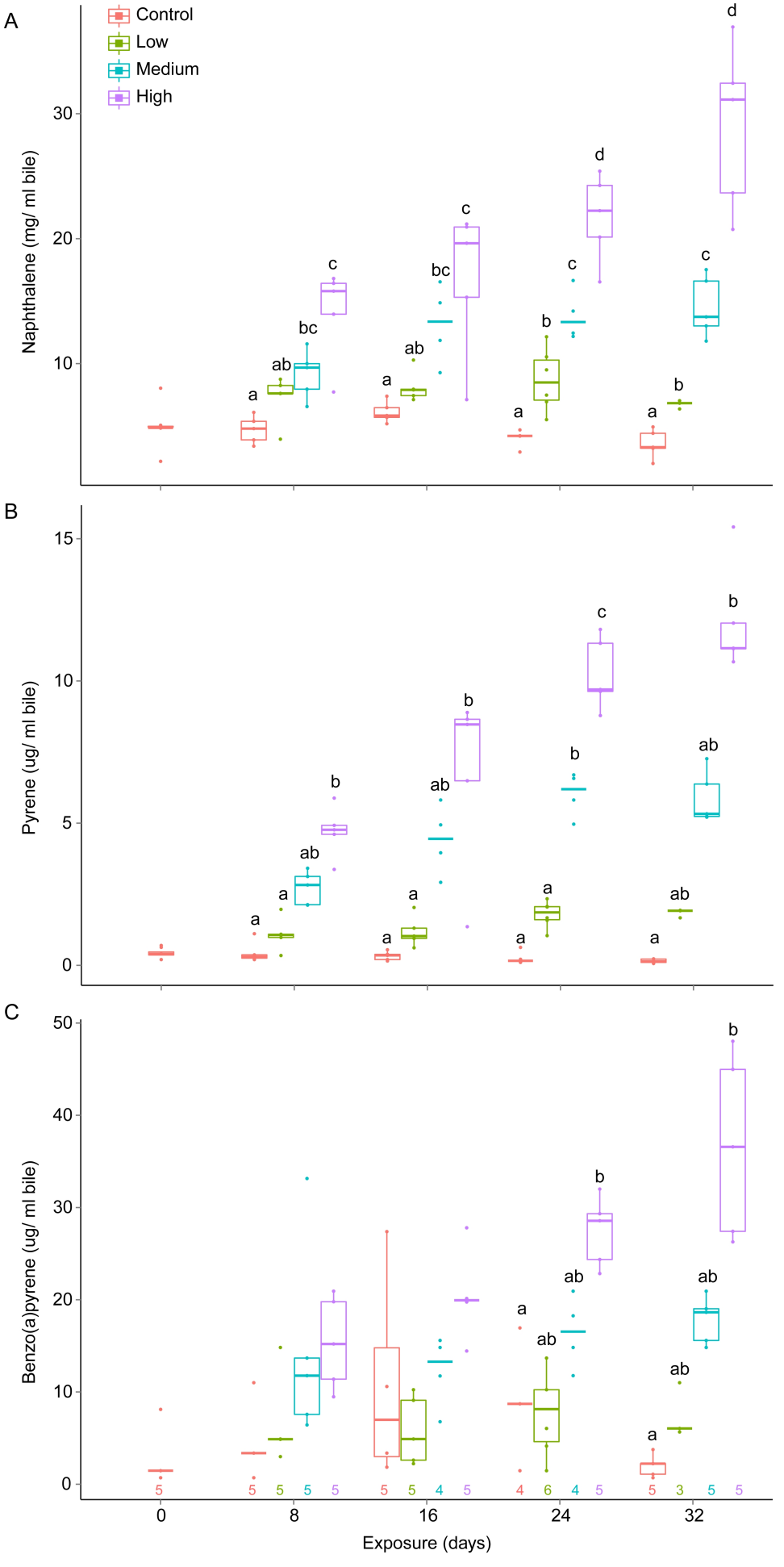
675 Figure 1. Map showing sampling areas for polar cod for the baseline study in Greenland
676 (Tyrolerfjorden) and Svalbard (Billefjorden, Hinlopen and Rijpfjorden).

677 Figure 2. Activity of 7- ethoxyresorufine O-deethylase (EROD) in liver of polar cod exposed to
678 control, low, medium and high doses of crude oil through their food. Boxplots represent the median
679 (horizontal line), 1. – 3.quartile (box), non-outlier range (whisker), outlier (points) of the data.
680 Significant differences among treatments are indicated by lowercase letters (one-way ANOVA, *post*
681 *hoc* Tukey`s honest significant difference test, $p<0.05$). Colored numbers indicate fish number per
682 treatment group.
683 Figure 3. Naphthalene-type (A), pyrene-type (B) and benzo(a)pyrene-type (C) metabolites in bile of
684 polar cod exposed to control, low, medium and high doses of crude oil. Significant differences among
685 treatments at each time point are indicated by lowercase letters (Kruskal Wallis rank sum test, *post hoc*
686 Nemenyi, $p<0.05$). Colored numbers indicate fish number per treatment group.

687 Figure 4. Classification of biomarker data in polar cod exposed to different crude oil doses (low,
688 medium, and high) by the quantitative model Sediqualsoft. The quantitative Hazard Quotient (HQ)
689 and the assigned class of hazard are given after the elaboration of biomarker data (7- ethoxyresorufine
690 O-deethylase activity, PAH metabolites in bile, individual antioxidants, total oxyradical scavenging
691 capacity and malondialdehyde).


81 °N
78 °N
75 °N
72 °N
69 °N
66 °N
24 °W
12 °W
0 °
12 °E
24 °E
Rijpfjorden
Hinlopen
Billefjorden
Svalbard
Greenland
Tyrolerfjorden
Norway
Sweden
Iceland


Control
Low
Medium
High
EROD activity (pmol/min/mg protein)
60
40
20
0
0
8
16
24
32
Exposure (days)
a
ab
ab
b
a
ab
bc
c
a
ab
b
b
5
1
1
4
2
6
2
3
6
10
5
6
6
4
4
8
7


A
Control
Low
Medium
High
Naphthalene (mg/ ml bile)
B
Pyrene (ug/ ml bile)
C
Benzo(a)pyrene (ug/ ml bile)
Exposure (days)
0
8
16
24
32

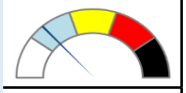
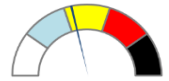
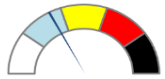
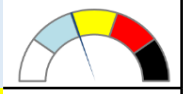
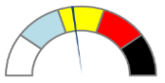
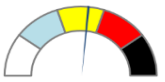
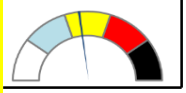
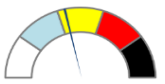
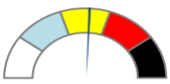
| Treatment | Day | HQ | Class of Hazard | |
|---|---|---|---|---|
| Low | 8 | 0 | slight |  |
| Low | 16 | 1.74 | slight |  |
| Low | 24 | 3.1 | slight |  |
| Low | 32 | 10.26 | moderate |  |
| Medium | 8 | 6.01 | slight |  |
| Medium | 16 | 11.73 | slight |  |
| Medium | 24 | 16.57 | moderate |  |
| Medium | 32 | 35.47 | moderate |  |
| High | 8 | 8.41 | moderate |  |
| High | 16 | 18.61 | moderate |  |
| High | 24 | 23.05 | moderate |  |
| High | 32 | 73.65 | moderate |  |

Table 1. Fish morphometrics (mean ± SE) of wild caught polar cod from different location within the European Arctic and polar cod exposed to crude oil through their food. Letters in brackets indicate significant differences among locations or crude oil treatments and asterisks indicate significant difference between sex (one-way ANOVA, *post hoc* Tukey`s honest significant difference test, $p<0.05$).

| | | Sex ratio | Length [a] | Weight [b] | GSI [c] | | HSI [c] | |
|---|---|---|---|---|---|---|---|---|
| Location | n | Females : Males | | | Females | Males | Females | Males |
| *Baseline study* | | | | | | | | |
| Billefjorden | 10 | 1 : 1 | 11.0 ± 0.3 (a) | 6.1 ± 0.5 (a) | 2.0 ± 0.3 | 5.6 ± 1.3* | 10.0 ± 1.0 (a) | 11.4 ± 0.8 (a) |
| Hinlopen | 9 | 1 : 1.3 | 20.2 ± 0.7 (b) | 40.0 ± 4.4 (b) | 3.6 ± 0.2 | 7.0 ± 0.8* | 12.7 ± 1.1 (a) | 10.2 ± 1.1 (a) |
| Rijpfjorden | 11 | 1 : 0.8 | 18.1 ± 0.8 (b) | 26.6 ± 4.1 (b) | 3.1 ± 0.6 | 7.8 ± 1.1* | 12.2 ± 0.8 (a) | 10.8 ± 1.5 (a) |
| Tyrolerfjorden | 9 | 1 : 0.5 | 18.4 ± 1.0 (b) | 40.1 ± 6.3 (b) | 3.0 ± 0.6 | 6.6 ± 1.3* | 21.3 ± 2.2 (b) | 24.3 ± 5.3 (b) |
| *Crude oil experiment* | | | | | | | | |
| Control | 48 | 1 : 1.4 | 16.9 ± 0.2 | 17.6 ± 0.5 | 2.6 ± 0.1 | 5.0 ± 1.1 (a) | 2.3 ± 0.2 | 2.9 ± 0.4 |
| Low | 35 | 1 : 1.1 | 16.8 ± 0.2 | 17.4 ± 0.6 | 2.4 ± 0.2 | 3.0 ± 0.7 (ab) | 2.2 ± 0.3 | 2.7 ± 0.3 |
| Medium | 37 | 1 : 3.1 | 16.7 ± 0.2 | 17.6 ± 0.6 | 2.3 ± 0.1 | 4.1 ±0.7 (b) | 2.4 ± 0.2 | 2.9 ± 0.3 |
| High | 33 | 1 : 0.9 | 17.5 ± 0.3 | 19.7 ± 0.9 | 2.8 ± 0.3 | 3.0 ± 0.6 (c) | 2.5 ± 0.3 | 3.1 ± 0.2* |

a Mean total fish length (cm)

b Mean somatic fish weight (g)

c Mean GSI and HSI (%)

GSI - gonadosomatic index; HSI - hepatosomatic index; n – number of fish

Table 2. Quantitative PCR conditions for the amplification of target genes in polar cod liver.

| Gene | Gene name | Primer sequences [a] | Amplicon [b] | Annealing [c] | Accession number |
|---|---|---|---|---|---|
| *cat* | *catalase* | FW: TTCCCGTCGTTCATCCACTC | 125 | 60 | EU682945 |
| | | RV: TCGCTGAACAGGAAGGACAC | | | |
| *gpx1* | *glutathione peroxidase 1* | FW: GGACATCAGGAGAACCTCAAGAA | 122 | 60 | LT601031 |
| | | RV: GCATCTTTCCCGTTCACATCAG | | | |
| *gpx4* | *phospholipid hydroperoxide glutathione peroxidase* | FW: CCCTGTGGAAGTGGCTGAAG | 129 | 60 | EX721840 [d] |
| | | RV: CATCCAAGGGTCCGTATCTCTT | | | |
| *gstpi* | *glutathione S-transferase pi* | FW: GTCCCCCTGCTGCCATTC | 126 | 60 | EX743626 [d] |
| | | RV: CCTCCATACACCGCCACCTA | | | |
| *Cu/Zn-sod* | *Cu/Zn-superoxide dismutase* | FW: CATGGCTTCCACGTCCATG | 133 | 60 | EX732302 [d] |
| | | RV: CGTTTCCCAGGTCTCCAACAT | | | |
| *nrf2* | *nuclear factor erythroid 2–related factor 2* | FW: ACAGAATGTCAGCTACACAGA | 105 | 57 | LT601032 |
| | | RV: GGTGTTCTCAGTTTCCTCTAC | | | |

a Primer sequence direction 5' - 3'

b Amplicon size in base pairs

c Annealing temperature (°C)

d Primer sequences designed for Atlantic cod and used in polar cod (Nahrgang et al., 2009)

FW - forward primer; RV - reverse primer

Table 3. Mean ± SE of biological responses including levels of total glutathione, individual antioxidants activity, levels of total scavenging capacity and lipid peroxidation (MDA) in polar cod liver from different location within the European Arctic (baseline study) and non-exposed polar cod (control treatment) from the crude oil exposure experiment. Letters in brackets indicate significant differences found among locations (one-way ANOVA, *post hoc* Tukey`s honest significant difference test, $p<0.05$).

| Location | n | GSH [a] | CAT [b] | GST [c] | GR [c] | GPx [c] | GPx_sum [c] | ROO [d] | OH [d] | HNOOH [d] | MDA [e] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Baseline study* | | | | | | | | | | | |
| Billefjorden | 3-6 | 0.7 | 143.8 ± 7.6 (a) | 1150.0 ± 131.2 (a) | 13.0 ± 0.7 (b) | 11.8 ± 1.5 (a) | 19.1 ± 1.3 | 488.4 ± 43.8 | 565.4 ± 74.7 (a) | 352.4 ± 23.8 (ab) | 36.8 ± 12.5 |
| Hinlopen | 3-5 | 0.3 ± 0.1 | 179.4 ± 11.7 (ab) | 1604.4 ± 152.8 (ab) | 15.9 ± 1.2 (ab) | 21.8 ± 2.4 (b) | 24.4 ± 2.1 | 392. 2 ± 47.9 | 342.5 ± 28.4 (b) | 258.9 ± 17.1 (a) | 21.5 ±7.5 |
| Rijpfjorden | 3-5 | 0.5 ± 0.3 | 151.1 ± 11.8 (ab) | 1320.1 ± 129.1 (ab) | 18.7 ± 0.9 (a) | 17.8 ± 2.4 (ab) | 26.7 ± 1.5 | 407.7 ± 27.1 | 378.1 ± 29.5 (ab) | 372.6 ± 28.4 (ab) | 24.5 ± 5.5 |
| Tyrolerfjorden | 3-7 | 0.3 ± 0.1 | 198.6 ± 16.3 (b) | 1961.8 ± 288.1 (b) | 13.5 ± 1.1 (b) | 18.5 ± 3.0 (ab) | 23.1 ± 3.0 | 427.6 ± 39.5 | 486.7 ± 37.2 (ab) | 444.2 ± 53.5 (b) | 14.2 ± 1.6 |
| *Experiment* | | | | | | | | | | | |
| Control fish [f] | 4 | - | 245.2 ± 14.4 | 1732.9 ± 229.1 | 32.8 ± 3.2 | 34.2 ± 1.7 | 33.9 ± 6.3 | 370.0 ± 40.8 | 495.8 ± 49.8 | 388.7 ± 26.0 | 28.0 ± 9.6 |

a µmol/ g liver tissue

b catalase activity in µmol/ min/ mg protein

c enzyme activity in nmol/ min/ mg protein

d TOSC unit/ mg protein

e nmol/ g liver tissue

f data measured in polar cod used as experiment control (day 0)

n – number of fish, GSH – total glutathione; CAT –catalase; GST – glutathione S-transferase; GR – glutathione reductase; GPx – glutathione peroxidase Se-dependent; GPx_sum - glutathione peroxidase Se-dependent and Se-independent; ROO – peroxyl radical; OH – hydroxyl radical; HNOOH – peroxynitrite; MDA – malondialdehyde

Table 4. Polycyclic aromatic hydrocarbons (PAHs) concentration in the *Calanus* sp. food used for polar cod exposure. Values are expressed as mean ± standard error ng/g wet weight.

| PAH composition | Control | Low | Medium | High |
|---|---|---|---|---|
| Naphthalene | < 15.2 [a] | 408 ± 47.2 | 1450 ± 22.3 | 2961 ± 36.1 |
| C1-Naphthalene | < 23.9 [a] | 977 ± 24.4 | 3630 ± 78.2 | 7432 ± 242 |
| C2-Naphthalene | < 27.0 [a] | 1476 ± 8.5 | 5366 ± 69.2 | 11500 ± 238 |
| C3-Naphthalene | < 67.4 [a] | 2501 ± 76.8 | 8427 ± 55.2 | 17881 ± 440 |
| Phenanthrene | 6.9 ± 1.7 | 105 ± 3.6 | 383 ± 8.0 | 811 ± 10.8 |
| Antracene | < 0.82 [a] | < 0.82 [a] | 1.9 ± 0.3 | 2.8 ± 0.1 |
| C1-Phenanthrene/anthracene | 16.7 ± 6.5 | 204 ± 4.4 | 720 ± 14.6 | 1515 ± 17.0 |
| C2-Phenanthrene/anthracene | 23.9 ± 15.7 | 319 ± 8.2 | 1102 ± 12.2 | 2332 ± 33.9 |
| C3-Phenanthrene/anthracene | 20.9 ± 13.0 | 240 ± 2.1 | 831 ± 6.8 | 1731 ± 28.9 |
| Dibenzothiophene | < 1.1 [a] | 17.2 ± 0.9 | 59.8 ± 0.7 | 125 ± 2.3 |
| C1-Dibenzothiophene | < 3.63 [a] | 27.2 ± 0.7 | 97.7 ± 1.3 | 201 ± 1.9 |
| C2-Dibenzothiophene | 6.7 ± 1.7 | 84.9 ± 2.3 | 299 ± 0.8 | 624 ± 10.3 |
| C3-Dibenzothiophene | 10.7 ± 6.1 | 83.8 ± 1.8 | 288 ± 3.2 | 612 ± 4.5 |
| Acenaphthylene | < 0.37 [a] | 0.4 ± 0.1 | 1.6 ± 0.2 | 2.8 ± 0.3 |
| Acenaphthene | < 2.55 [a] | 15.2 ± 3.1 | 39.4 ± 0.7 | 78.8 ± 0.4 |
| Fluorene | < 1.83 [a] | 35.5 ± 1.4 | 117 ± 0.8 | 248 ± 9.3 |
| Fluoranthene | 4.2 ± 1.9 | 9.2 ± 1.9 | 19.5 ± 0.9 | 40.7 ± 0.9 |
| Pyrene | 12 ± 7.9 | 10.7 ± 6.7 | 14.4 ± 3.1 | 29.7 ± 2.2 |
| Benzo(a)anthracene | 1.3 ± 0.7 | 1.9 ± 0.4 | 5.1 ± 0.5 | 9.8 ± 0.4 |
| Chrysene | 2.9 ± 1.7 | 6.8 ± 0.9 | 17.9 ± 0.4 | 34.3 ± 0.3 |

Table 4 continued.

| PAH composition | Control | Low | Medium | High |
|---|---|---|---|---|
| Benzo(b)fluoranthene | 2 .0 ± 1.1 | 2.0 ± 1.1 | 4.0 ± 0.5 | 8.0 ± 0.3 |
| Benzo(k)fluoranthene | 0.9 ± 0.6 | 1.3 ± 0.8 | 1.3 ± 0.2 | 2.2 ± 0.1 |
| Benzo(a)pyrene | 1.2 ± 0.8 | 1.4 ± 0.8 | 1.8 ± 0.3 | 3.4 ± 0.3 |
| Indeno(1,2,3-cd) pyrene | 1.6 ± 0.6 | 2.2 ± 1.3 | < 1.87 [a] | 1.3 ± 0.4 |
| Benzo(ghi)perylene | 1.7 ± 0.9 | 2.2 ± 1.4 | 1.3 ± 0.6 | 2.6 ± 0.4 |
| Dibenzo(a,h)anthracene | < 0.67 [a] | 0.6 ± 0.3 | 0.5 ± 0.1 | 1.0 ± 0.0 |
| Sum 26 PAH [b] | 186 | 6534 | 22880 | 48190 |

a Values below limit of detection (LOD)

b Values <LOD included as ½ of LOD

Table 5. Mean ±SE of individual antioxidant activity in the liver of crude oil exposed polar cod (low, medium and high) and control fish of the crude oil exposure experiment. Letters in brackets indicate significant differences found among treatments for each time point (one-way ANOVA, *post hoc* Tukey`s honest significant difference test, $p<0.05$).

| Time point [a] | Treatment | n | CAT [b] | GST [c] | GR [c] | GPx [c] | GPx_sum [c] |
|---|---|---|---|---|---|---|---|
| Day 0 | Control | 3 | 245.2 ± 14.4 | 1732.9 ± 229.1 | 32.8 ± 3.2 | 34.2 ± 1.7 | 33.9 ± 6.3 |
| Day 8 | Control | 3 | 244.5 ± 5.1 | 2293.6 ± 281.3 | 38.5 ± 5.2 | 35.6 ± 0.2 | 36.7 ± 3.1 |
| | Low | 3 | 267.5 ± 23.6 | 1698.4 ± 104.0 | 28.8 ± 1.0 | 28.8 ± 6.2 | 40.2 ± 6.4 |
| | Medium | 3 | 245.0 ± 29.0 | 2173.3 ± 457.7 | 30.7 ± 3.2 | 37.2 ± 4.5 | 56.4 ± 13.2 |
| | High | 2 | 219.5 ± 45.1 | 3191.3 ± 647.0 | 31.2 ± 4.5 | 44.4 ± 4.0 | 26.3 ± 3.9 |
| Day 16 | Control | 3 | 298.6 ± 29.7 | 1278.5 ± 180.3 (a) | 31.5 ± 2.0 (a) | 33.7 ± 8.1 | 36.8 ± 5.4 |
| | Low | 3 | 198.7 ± 37.5 | 1922.9 ± 286.3 (ab) | 40.8 ± 0.8 (b) | 34.1 ± 2.0 | 33.9 ± 2.7 |
| | Medium | 3 | 214.0 ± 27.4 | 1812.4 ± 146.0 (ab) | 39.2 ± 2.4 (b) | 28.7 ± 4.8 | 23.5 ± 9.2 |
| | High | 3 | 265.7 ± 58.8 | 2263.5 ± 151.8 (b) | 38.8 ± 1.0 (b) | 30.7 ± 2.0 | 23.6 ± 2.9 |
| Day 24 | Control | 3 | 200.1 ± 22.1 | 2226.0 ± 169.3 | 46.4 ± 4.0 | 22.4 ± 3.1 | 30.4 ± 3.6 |
| | Low | 2 | 230.0 ± 18.0 | 3272.8 ± 430.2 | 44.1 ± 1.1 | 23.0 ± 11.4 | 29.3 ± 5.5 |
| | Medium | 3 | 234.7 ± 43.4 | 1837.4 ± 361.7 | 32.9 ± 4.9 | 35.6 ± 4.5 | 32.1 ± 3.1 |
| | High | 4 | 305.5 ± 63.0 | 1565.1 ± 236.0 | 31.0 ± 4.0 | 31.8 ± 2.9 | 32.9 ± 2.0 |
| Day 32 | Control | 3 | 164.9 ± 21.0 | 1788.2 ± 52.8 | 43.2 ± 2.6 | 40.1 ± 5.7 | 42.9 ± 5.4 |
| | Low | 3 | 192.7 ± 26.5 | 1644.1 ± 88.5 | 34.3 ± 2.7 | 36.8 ± 1.2 | 35.9 ± 1.3 |
| | Medium | 3 | 309.1 ± 49.1 | 1479.8 ± 90.9 | 45.7 ± 2.8 | 33.1 ± 4.8 | 35.2 ± 5.3 |
| | High | 3 | 213.0 ± 28.8 | 1516.3 ± 215.2 | 46.8 ± 6.6 | 35.3 ± 9.5 | 39.2 ± 8.1 |

a time of exposure in days

b catalase activity in µmol/ min/ mg protein

c enzyme activity in nmol/ min/ mg protein

n – number of fish; CAT –catalase; GST – glutathione S-transferase; GR – glutathione reductase; GPx – glutathione peroxidase Se-dependent; GPx_sum - glutathione peroxidase Se-dependent and Se-independent

Table 6. Absolute mRNA expression levels of gene related to antioxidant defense in polar cod liver (n=3 per group) expressed as mRNA copy number per µg of total RNA ±SE.

| Gene name | Abbreviation | Time point [a] | Control | Low | Medium | High |
|---|---|---|---|---|---|---|
| *catalase* | *cat* [b] | 8 | 2.2 ± 0.2 | 3.7 ± 1.0 | 2.0 ± 0.7 | 1.3 ± 0.1 |
| | | 32 | 1.4 ± 0.3 | 1.4 ± 0.2 | 1.1 ± 0.3 | 1.4 ± 0.4 |
| *glutathione peroxidase 1* | *gpx1* [b] | 8 | 1.5 ± 0.8 | 1.1 ± 0.4 | 0.9 ± 0.1 | 1.1 ± 0.7 |
| | | 32 | 1.6 ± 1.0 | 4.2 ± 2.2 | 0.5 ± 0.3 | 3.0 ± 0.5 |
| *phospholipid hydroperoxide glutathione peroxidase* | *gpx4* [b] | 8 | 4858.3 ± 755.6 | 4978.8 ± 1357.8 | 4217.5 ± 474.2 | 5021.1 ± 1259.4 |
| | | 32 | 5741.9 ± 1586.3 | 6658.1 ± 898.1 | 5696.2 ± 1563.8 | 7853.7 ± 1840.0 |
| *glutathione S-transferase* | *gstpi* [b] | 8 | 7.8 ± 1.0 | 9.2 ± 3.8 | 10.2 ± 1.5 | 10.4 ± 3.7 |
| | | 32 | 4.5 ± 1.0 | 7.6 ± 0.6 | 12.9 ± 5.1 | 16.0 ± 0.8 |
| *nuclear factor erythroid 2–related factor 2* | *nrf2* [c] | 8 | 72.2 ± 18.8 | 110.6 ± 19.7 | 52.2 | 88.3 ± 2.4 |
| | | 32 | 115.9 ± 13.6 | 88.5 ± 7.4 | 71.4 ± 12.5 | 53.0 |
| *Cu/Zn-superoxide dismutase* | *cu/zn-sod* [b] | 8 | 22.8 ± 3.2 | 30.0 ± 10.5 | 22.4 ± 2.7 | 39.2 ± 10.6 |
| | | 32 | 25.8 ± 8.6 | 28.7 ± 7.4 | 21.4 ± 4.3 | 38.7 ± 2.5 |

a exposure time in days

b mean ± SE gene expression levels $*10^6$

c mean ± SE gene expression levels in total numbers

Table 7. Mean ± SE levels of total scavenging capacity (ROO·, ·OH and HNOOH) and lipid peroxidation (MDA) in the liver of crude oil exposed polar cod (low, medium and high) and control fish of the crude oil exposure experiment. Letters in brackets indicate significant differences found between treatments for each time point (one-way ANOVA, *post hoc* Tukey`s honest significant difference test, $p<0.05$).

| Time point [a] | Treatment | n | ROO· [b] | ·OH [b] | HOONO [b] | MDA [c] |
|---|---|---|---|---|---|---|
| Day 0 | Control | 4 | 370.0 ± 40.8 | 495.8 ± 49.8 | 388.7 ± 26.0 | 28.0 ± 9.6 |
| Day 8 | Control | 4 | 314.0 ± 22.6 | 588.5 ± 27.4 | 414.2 ± 38.6 | 20.7 ± 2.0 |
| | Low | 4 - 5 | 357.4 ± 17.8 | 465.1 ± 20.2 | 475.4 ± 50.2 | 29.0 ± 4.4 |
| | Medium | 4 | 314.8 ± 12.6 | 518.2 ± 62.7 | 440.1 ± 37.3 | 23.8 ± 2.3 |
| | High | 4 - 5 | 324.8 ± 11.4 | 619.0 ± 82.8 | 339.3 ± 71.0 | 20.3 ± 1.3 |
| Day 16 | Control | 4 - 5 | 360.2 ± 17.0 (ab) | 639.0 ± 81.3 | 544.2 ± 43.2 (b) | 21.7 ± 4.3 |
| | Low | 4 - 5 | 296.1 ± 27.8 (b) | 575.3 ± 50.5 | 500.5 ± 54.8 (b) | 16.4 ± 0.7 |
| | Medium | 4 | 431.3 ± 18.6 (a) | 535.7 ± 40.5 | 294.5 ± 21.7 (a) | 19.2 ± 3.3 |
| | High | 4 | 297.9 ± 16.5 (b) | 566.5 ± 93.5 | 368.0 ± 37.4 (ab) | 17.7 ± 2.3 |
| Day 24 | Control | 4 - 5 | 357.4 ± 50.6 | 541.2 ± 54.1 (ab) | 450.7 ± 60.1 | 18.4 ± 1.5 |
| | Low | 4 - 5 | 340.6 ± 25.6 | 683.5 ± 44.8 (a) | 401.2 ± 35.0 | 18.6 ± 2.1 |
| | Medium | 4 | 281.8 ± 17.1 | 438.5 ± 15.6 (b) | 522.5 ± 29.9 | 17.0 ± 2.1 |
| | High | 4 | 372.8 ± 60.1 | 578.1 ± 47.8 (ab) | 506.7 ± 41.8 | 13.9 ± 1.5 |
| Day 32 | Control | 4 - 5 | 355.8 ± 13.5 | 625.2 ± 36.3 (ab) | 403.4 ± 54.2 | 13.5 ± 2.7 |
| | Low | 4 - 5 | 355.0 ± 21.4 | 708.9 ± 20.3 (a) | 355.3 ± 24.0 | 13.2 ± 2.0 |
| | Medium | 4 - 5 | 336.6 ± 31.8 | 637.1 ± 36.2 (ab) | 463.1 ± 31.9 | 13.3 ± 0.1 |
| | High | 4 | 340.0 ± 29.0 | 512.2 ± 71.2 (b) | 389.0 ± 46.1 | 8.1 ± 1.6 |

a time of exposure in days

b TOSC unit/ mg protein

c nmol/ mg liver tissue

n – number of fish; ROO – peroxyl radical; OH – hydroxyl radical; HNOOH – peroxynitrite; MDA – malondialdehyde