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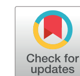
Oil-mediated oxidative-stress responses in a keystone zooplanktonic species, *Calanus finmarchicus*



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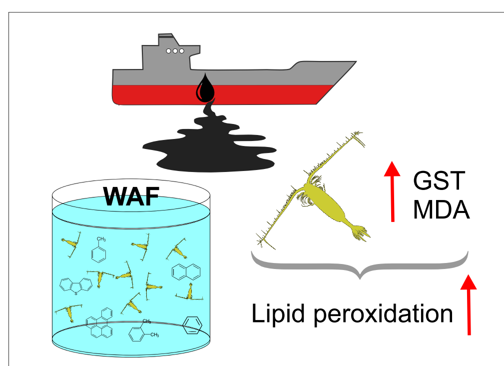
HIGHLIGHTS

- Oil derived pollution may pose a threat to ecologically important species in the North Atlantic.
- *C. finmarchicus* was exposed to WAF of a naphthenic North Atlantic crude oil.
- Oxidative stress, protein damage and lipid peroxidation were investigated.
- Increased GST enzymatic activity and MDA were found as main responses.
- Lipid peroxidation is suggested as the major mechanism of naphthenic oil WAF toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

The copepod *Calanus finmarchicus* is an ecologically important species in the North Atlantic, Norwegian and Barents seas. Accidental or continuous petroleum pollution from oil and gas production in these seas may pose a significant threat to this low trophic level keystone species.

Responses related to oxidative stress, protein damage and lipid peroxidation were investigated in *C. finmarchicus* exposed to a water-accommodated fraction (WAF) of a naphthenic North Atlantic crude oil. The exposure concentration corresponded to 50% of the 96 h LC_{50}, and samples were obtained at 0, 24, 48, 72 and 96 h after exposure initiation. Gene expressions (superoxide dismutase, catalase, glutathione S-transferase, glutathione synthetase, heat shock protein 70 and 90, ubiquitin and cytochrome P-450 330A1), enzyme activities (superoxide dismutase, catalase, glutathione S-transferase) and concentrations of total glutathione and malondialdehyde were analyzed. Gene expression analyses showed no differences between controls and the exposed animals, however significantly higher glutathione *S*-transferase activity and malondialdehyde concentrations were found in the exposed group, suggests lipid peroxidation as main toxic effect.

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1. Introduction

Oil and its by-products are regularly released in the marine environment from both natural and anthropogenic sources associated with

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extraction, transportation and petroleum use. Impacts of oil spills from vessels and platforms on marine biota have been extensively studied in the past decades due to their negative short- and long-term effects (Moore and Dwyer, 1974; Martínez-Gómez et al., 2010; White et al., 2012; Beyer et al., 2020). While the number of tank vessel spills has decreased significantly in the last decades, new shipping routes in the Arctic will most likely become operative in the near future due to climate change and consequent sea ice loss (ITORPF, 2019; Gunnarsson, 2021). Since most oil spills have occurred in temperate seas, limited knowledge on oil toxicity is available for species and populations inhabiting cold environments (De Hoop et al., 2011; Olsen et al., 2013a). Considerable concern also arises from produced water discharges, of which petroleum hydrocarbons represent the compounds of greatest environmental concern due to their potential bioaccumulation and toxic effects (Beyer et al., 2020). Toxicological studies on petrogenic oil have mostly focused on organisms at intermediate trophic levels, while less is known about species at the base of the marine food web, such as zooplankton (Almeda et al., 2013; Ozhan et al., 2014).

Following the initial phase of an oil spill, which may result in acute toxicity causing local mass mortality of marine organisms, zooplanktonic species have shown a high level of resilience (Abbriano et al., 2011; Ozhan et al., 2014; Hansen et al., 2015). However, delayed toxic effects of exposure to oil components have been shown in zooplankton (Jensen et al., 2008; Grenvald et al., 2013; Olsen et al., 2013b; Hansen et al., 2017b; Toxværd et al., 2018; Skottene et al., 2019). A study by Jiang et al. (2010) suggests that the sensitivity of zooplankton to water accommodated fraction of petrogenic oils is strongly related to the lipophilicity of the specific compounds in the oil. High lipid content may in fact temporarily protect against acute effects of exposure, as lipophilic contaminants may be sequestered in the lipid sacs of zooplankton (Øverjordet et al., 2018), and there are large variations in sensitivities among species and developmental stages, possibly due to differences in lipid content (Hansen et al., 2016; Jager et al., 2016).

From a toxicological perspective, monoaromatic hydrocarbons (MAH), such as benzene, toluene, ethylbenzene and xylene isomers (known as BTEX) and polyaromatic hydrocarbons (PAH) are generally considered as the petroleum hydrocarbons of most acute concern in the marine environment. Nevertheless, BTEX are volatile compounds hence they are rapidly absorbed but also rapidly released by marine organisms due to their low octanol-water partition coefficient (K_{ow}). Therefore, long-term bioaccumulation of BTEX is not expected (Popek, 2018). PAHs are less volatile and more lipophilic than BTEX. Their accumulation depends on the hydrophobicity and molecular mass of the specific compound, the habitat, trophic level, feeding behavior, exposure levels and the ability of organisms to metabolize and excrete these compounds (Meador et al., 1995; Baumard et al., 1998). High lipid content, common for cold-water zooplankton as a discrete lipid sac, also causes reduced elimination of lipophilic PAHs (Øverjordet et al., 2018).

Calanus finmarchicus is a planktonic copepod species that inhabits the subsurface layer and deep-sea basins of the North Atlantic, where seasonally it constitutes more than half of the copepod biomass (Planque and Batten, 2000). Besides being a keystone component of North Atlantic ecosystems, it has been frequently used as a test species in several toxicological studies related to marine oil pollution (Hansen et al., 2008a, 2008c, 2011, 2012, 2013). Several studies have investigated antioxidant responses in *C. finmarchicus* exposed to different petroleum components. Glutathione s-transferase (*gst*) transcription has been suggested as a biomarker in *C. finmarchicus* for exposure to naphthalene (Hansen et al., 2008a), crude oil (Hansen et al., 2008c, 2011) and refined oils (Hansen et al., 2013; Jager and Hansen, 2013). When compared to other marine species, *C. finmarchicus* appear very sensitive to H_2O_2 exposure, a recognized inducer of reactive oxygen species (ROS) that causes oxidative stress. However, exposure to H_2O_2 corresponding to the 96 h NOEC (no observed effect concentration), did not induce an antioxidant system response in exposed copepodite stage V (CV) or adult individuals (Hansen et al., 2017a). Thus, the role of oxidative stress in *C. finmarchicus* as a response to pollutants such as petroleum oil, and thus the capacity of pollutants to cause protein damage and lipid peroxidation in *C. finmarchicus* is still not fully understood.

To better understand oxidative stress, protein damage and lipid peroxidation responses to oil contamination in *C. finmarchicus*, adult individuals were exposed to a water accommodated fraction (WAF) of a naphthenic crude oil from the North Atlantic Ocean. Expression and activity of several genes, enzymes and by-products compounds involved in the putative detoxifying system (cytochrome P-450 330A1), oxidative stress (superoxide dismutase, catalase, glutathione S-transferase, glutathione synthetase), protein damage (heat shock protein 70 and 90 and ubiquitin) and lipid peroxidation (malondialdehyde) were analyzed.

2. Material and methods

2.1. Experimental organism

Adult females of *C. finmarchicus* from the continuous lab culture at SINTEF/NTNU Sealab (Trondheim, Norway) were used in the present exposure experiment. In the laboratory, cultures are kept in polyester containers (280 L) with running natural seawater at +8–10 °C. *C. finmarchicus* are fed with a mixture of the unicellular algae *Rhodomonas baltica*, *Isochrysis galbana* and *Dunaliella tertiolecta*. Generations are separated by transferring the nauplii into new containers in the first week after hatching. Details concerning the culturing have been previously described by Hansen et al. (2007).

2.2. Exposure medium

C. finmarchicus were exposed to a WAF generated from a naphthenic North Atlantic fresh crude oil of medium viscosity at SINTEF/NTNU Sealab. WAFs were prepared as recommended by the CROSERF guideline (Aurand and Coelho, 2005) with adjustments detailed in Faksness et al. (2015). Briefly, WAFs were prepared in a closed, low-energy mixing system at 10 °C for 72 h using an oil-to-water ratio of 1:40.

2.3. Experimental setup

The aim of the study was to investigate responses to crude oil-WAF exposure so it was necessary to use a concentration that (i) did not trigger considerable mortality but (ii) still resulted in toxic effects. The choice of a concentration corresponding to the 50% of the 96 h-LC_{50} allowed us to meet these two postulations. Thus, first, an acute toxicity test was performed to determine 96 h-LC_{50} concentrations in *C. finmarchicus*. This experiment was performed based on the modified standard tests of acute lethal toxicity on *Acartia tonsa* (ISO, 14669) (described in Supplementary S1). Based on the obtained 96 h-LC_{50}, the main experiment was designed. Here, individuals were exposed to the concentration corresponding to 50% of the 96 h-LC_{50} WAF concentration. This corresponded to 9.85% of the WAF stock solution diluted in filtered seawater (30 PSU). A control group was subjected to filtered seawater only. The exposure experiments lasted for 96 h. Exposures were conducted in closed 5 L borosilicate glass bottles (VWR International) containing 155 individuals each, with four replicates per sampling time point (0, 24, 48, 72 and 96 h). The exposure media was not exchanged during the experiment, the animals were not fed during the experiment, and the bottles were kept at a constant temperature of 10 °C and covered with a dark blanket to avoid light interference. At each sampling time, 10 specimens per replicate were pooled for gene expression analysis while the remaining individuals were sampled for analyses of enzymatic activities, total glutathione (tGSH) and malondialdehyde (MDA) determination. Throughout sampling, copepods were quickly dried and immediately snap-frozen in liquid nitrogen.

2.4. Chemical analysis

Concentrations of volatile organic compounds (VOC) and semi-volatile organic compounds (SVOC) were analyzed at the core facility for oil analyses at SINTEF Ocean using gas chromatography/flame ionization detector (for total extractable material) and gas chromatography/mass spectrometry (GC/MS) (for VOC and SVOC) using standard methodology published previously (Faksness et al., 2015) and are briefly described in Supplementary S2. VOC were analyzed in crude oil and in the WAF stock solution, while SVOC were analyzed on crude oil, WAF stock solution and in WAF test solution (the stock WAF solution diluted to 9.85%) at the beginning (0 h) and the end (96 h) of the experiment.

2.5. Gene expression

Gene expression analyses were performed at the Department of Biology at NTNU. mRNA extraction was performed using the RNeasy Plus Universal Mini Kit by QIAGEN following the producer's protocol (QIAGEN, HB-0391). Samples were homogenized in QIAzol Lysis Reagent using the TissueLyser II. gDNA Eliminator Solution and chloroform were added to the homogenate and aqueous and organic phases were separated by centrifugation. The aqueous phase was collected, mixed with ethanol, and RNA was purified using RNeasy spin columns. Total RNA, bonded to the spin column membrane, was eluted in RNase-free water and analyzed by spectrophotometry (NanoDrop 1000, Thermo Scientific, US) to ensure high RNA quality (260/280 and 260/230 ratios). cDNA synthesis was performed using the reverse transcription (RT) kit by QuantiTect (Qiagen, Hilden, Germany). RT-PCR was performed following the producer's protocol on 96-well plates (QuantiTect, 2009). For each plate, a negative control was performed (RT-PCR mastermix without reverse transcript enzyme) to ensure absence of gDNA contamination. qPCR was performed on a LightCycler 480 System (F. Hoffmann-La Roche AG, Basel, Switzerland) using the LightCycler 480 SYBR Green I Master kit from Roche according to available protocols (Roche, 2005). Primers sequence for housekeeping and target genes involved in the detoxifying system, antioxidant system and protein damage responses were chosen based on previous studies (Table 1) and ordered from Sigma-Genosys (The Woodlands, United States). To avoid plate-to-plate variation, each gene was analyzed on a single qPCR plate with two technical replicates. No gDNA contamination was detected.

Cycle threshold (Ct) values obtained from the LightCycler 480 software were imported into LinRegPCR software (Heart Failure Research Center, Netherlands) for baseline correction. Quantification cycle (Cq) values obtained by LinRegPCR were qualitatively analyzed. Outliers were identified and eliminated following the interquartile range method (IQR). Relative gene expression accounting for differences in primer efficiencies was calculated according to Pfaffl (2001) using 16S as reference gene. Gene expression results are presented as fold-change in gene expression levels relative to the expression at time 0 when none of the two groups were exposed to WAF.

2.6. Enzymatic activities, tGSH and MDA determination

Enzymatic assays and MDA and GSH analyses were performed at the Medical University of Gdańsk (Poland). Samples containing approximately 100 individuals were homogenized for 30 s using a MPW-309 instrument (Universal laboratory aid at Mechanika Precyzyjna, Warszawa). Homogenization was carried in ice-cold buffer containing 50 mM Tris-H_2SO_4, pH 7.6 with 0.1 mM EDTA, 1 mM PMSF, 2 mM DTT and 0.2% Triton® X, to obtain 20% homogenate. The homogenates were centrifuged for 30 min at 14000 RCF using a SIGMA 3 K18 centrifuge. The supernatant was then transferred into fresh tubes used for the analysis. All analytes were normalized to the cytosolic protein content determined following the Lowry et al.'s method (1951) with modification by Peterson (1977). tGSH was measured with a Glutathione Assay Kit (CS0260 Sigma-Aldrich) using GSH (Sigma-Aldrich) as the standard. MDA level was determined using the Lipid Peroxidation Assay Kit (MAK085 Sigma-Aldrich). Glutathione S-transferases activity (GST) was determined using a spectrophotometer (UV-VIS Beckman Coulter Spectrophotometer, USA) as described by Habig et al. (1974). Superoxide dismutase (SOD) activity was measured using the modified method of Sun et al. (1988) as the sum of its two main isoforms, CuZnSOD and MnSOD. Glutathione peroxidase (GPx) activity was measured using the Glutathione Peroxidase Cellular Activity Assay Kit (CGP1 Sigma - Aldrich) with 30 mM tert-butyl hydroperoxide as the substrate. Finally, catalase (CAT) activity was measured following the method described by Kankofer (2001). 0 h samples were not analyzed for enzymatic activities and tGSH and MDA concentrations due to logistical constraints.

2.7. Statistics

All statistical analyses were performed using R (3.6.1) and GraphPad Prism (8.0.2). Shapiro-Wilk test was used to assess normal distribution of residuals. Effects of time, treatment and their interaction, on variables showing normal distribution of model's residuals (*cat*, *cyp330a1*, *gss*, *gst-I*, *gst-III*, hsp70, hsp90, *ub*, and GST, CAT and GPX activity and tGSH and MDA concentrations) were analyzed using a two-way ANOVA. Model selection was based on Akaike information criterion (Supplementary S3). TukeyHSD was used as a post-hoc test. Unpaired *t*-test was used to detect differences at single time points, when the variable *time* was not included in the selected ANOVA model.

Data that showed no normal distribution of the residuals after log10 transformation (*sod*) were analyzed with Kruskal Wallis one-way analysis of variance, using Dunn's test of multiple comparisons as *post hoc* test.

Table 1
List of target genes and candidate reference genes selected for qPCR analyses.

Gene	Forward sequence	Reverse sequence	Accession no.
16s	CGTCTCTTCTAAGCTCCTGCAC	AAGCTCCTCTAGGGATAACAGC	GAXK01168561
act	CCATTGTCCGTCTTGATCTT	AAAGAGTAGCCACGCTCAGTG	ES387224
efa1α	AGGTTAAGTCCGTGGAGATG	ACTGGCTTGTTCTTGGAGTC	ES414812
cat	GTTGTACATGCAAAGGGAGC	AACAGTGGAGAACCTGACAG	EL965956
sod	GGAGATCTTGGCAATGTTCAG	CAGTAGCCTTGCTCAGTTCATG	ES237580
gst-I	CAACCCCCAGCACACTGTG	GGATAGACACAATCACCCATCC	JF825513*
gst-III	TCTTGCTCCCTGCTCAGAAT	TTGCGGGCTCTTTGTTAAGT	ES387262
gss	GAGAAGGCAAAGGACTATG	GGCAACCTTGTGCATCAAC	JF825516*
cyp330a1	ATTCACCCATTCAGGAAGCC	TTGCTCCTTCCAAGTGTGTC	EH666610
hsp70	GATCATAGTTGGTCTGGCAG	CATTAATGGTGACAGCGCTC	EH666705
hsp90	GTCTCGAAGAGAAGCATGAC	CTATATGGCGGCTAAGAAGC	ES414827
ub	TCCATCGAGAATGTCAAGGC	TGCTCTCCTTCTGGATGTTG	ES414814

Abbreviations: *16S*: 16S ribosomal RNA; *act*: actin; *cat*: catalase; efa1α: elongation factor A1α; *cyp330a1*: cytochrome P450 330A1; *gst*: glutathione S-transferase; *gss*: glutathione synthetase; *sod*: superoxide dismutase; *hsp70*: heat shock protein-70; *hsp90*: heat shock protein-90; *ub*: ubiquitin. * = from *C. helgolandicus*.

3. Results

3.1. Chemical analysis

The crude oil VOCs profile was dominated by methylcyclohexane, cyclohexane and methylcyclopentane, while naphthalene, decalin and their C1-C4-homologues were the most abundant SVOCs (Supplementary S4). In contrast, the WAF stock solution was dominated by BTEX compounds, as shown in Table 2. With respect to SVOC, naphthalene and its C1-C4-homologues accounted for more than 90% of all detected SVOCs (Table 2). SVOCs were also analyzed in the WAF test solution. At the termination of the experiment (96 h), the concentrations of all target compounds ranged from 12 to 0% of the initial exposure concentration (0 h). A complete list of all VOCs and SVOCs detected in the different samples analyzed is available in Supplementary S4.

3.2. Gene expression

16S was the only housekeeping gene used to normalize gene expression data following the method described by Pfaffl (2001), as *act* and *efa1α* showed unstable expressions across treatments (Supplementary S5).

Exposure time had no significant effect on *gst-III* expression. In the exposed group, *gst-III* was significantly up-regulated at 48 h (*t*-test, $p = 0.022$) and significantly down-regulated at 72 h (*t*-test, $p = 0.048$) when compared to the control group (Fig. 1A). Exposure time had a significant effect on *sod* expression (Kruskal-Wallis, *time* $p = 0.0035$), with expression levels at 96 h being significantly higher than at 72 h (*post hoc*, $p = 0.002$) and 48 h (*post hoc*, $p = 0.04$) (Fig. 1B). Furthermore, at 72 h the *sod* expression was also significantly down-regulated in the exposed group as compared to in the control group (*t*-test, $p = 0.03$) (Fig. 1B).

With respect to *cyp330 a1*, there was no difference in gene expression between the experimental groups. However, *cyp330a1* levels significantly varied between sampling points (ANOVA, *time* $p = 0.014$) in the exposed group, with cyp330*a1* levels being significantly higher at 72 h (*post hoc*, $p = 0.05$) and 96 h (*post hoc*, $p = 0.02$) as compared to 24 h (Fig. 1C).

Expressions of *cat* (Fig. 1D), *gss* (Fig. 1E), *hsp70* (Fig. 1F), *hsp90* (Fig. 1G) and *ub* (Fig. 1H) were not depending neither on time, nor on the treatment or their interaction.

Finally, it should be mentioned that *gst-I* showed multiple melting curves, and was therefore not considered in further analysis.

3.3. tGSH, MDA and enzymatic activities

GST enzymatic activity (Fig. 2A) was significantly higher in the treated group as compared to the control group (ANOVA, *group* $p < 0.0001$). Time did not have any significant effect on GST activity. At 24, 48, 72 and 96 h after initiation of the experiment, the GST activities in the exposed group were 2.7-fold (*t-test*, $p = 0.015$), 1.9-fold (*t*-test, $p > 0.05$), 2-fold (*t*-test, $p = 0.045$) and a 1.6-fold (*t*-test, $p > 0.05$) higher than in the control group, respectively (Fig. 2A).

The MDA concentrations (Fig. 2B) were also significantly higher in the treated group as compared to the control (ANOVA, *group* $p < 0.0001$), and were stable over time in both groups. At 24, 48, 72 and 96 h after initiation of the experiment, the MDA concentrations were 3.2-fold (*t*-test, $p = 0.015$), 2-fold (*t*-test, $p = 0.007$), 3-fold (*t*-test, $p = 0.004$) and 1.7-fold (*t*-test, $p > 0.05$) higher than in the control group, respectively (Fig. 2B).

With respect to tGSH (Fig. 2C), a significant effect of time (ANOVA, *time* $p = 0.034$), but not of treatment (ANOVA, *group* $p = 0.053$), was detected. tGSH concentrations were significantly higher at 96 h as compared to at 48 h after initiation of the experiment (*post hoc*, $p = 0.035$).

Regarding GPx (Fig. 2D) and CAT (Fig. 2E), neither time nor treatment had a significant effect on their activities. Nevertheless, CAT activity showed a non-significant but apparent decreasing trend (ANOVA, *time* $p = 0.056$). Results for SOD gene expression are not presented since in 24 of 30 the samples, SOD values were below the limit of detection.

Table 2
Concentrations of individual volatile compounds (VOC, *i.e.*, BTEX) and main semi volatile compound (SVOC) groups detected in the WAF stock solution. BTEX were analyzed in duplicates and values are presented as mean ± SD while SVOC concentrations were obtained from a single replicate. TEM: total extractable material.

Chemical composition	WAF stock solution (μg/L)
VOC	
Benzene	918.02 ± 96.55
Toluene	1611.76 ± 268.06
Ethylbenzene	400.61 ± 9.88
m-Xylene	1363.21 ± 92.12
p-Xylene	128.5 ± 16.49
o-Xylene	481.1 ± 31.97
∑BTEX	4903.2 ± 515.07
SVOC	
∑ Decalin and C1–C4- a.h	0.81
∑ Naphthalene and C1–C4- a.h	253.50
∑ Phenantrene/anthracene and C1–C4- a.h	3.09
∑ Dibenzothiophene and C1–C4- a.h	1.31
∑ PAH 2+ rings[a]	11.56
∑ Phenols and C1–C5- a.h	0
TEM	3088.96

a.h = alkylated homologues.
[a] ∑ PAH 2+ rings include benzothiophenes (C1–C4), acenaphthylene, acenaphthene, dibenzofuran, fluorenes (C1–C3), phenanthrenes (C1–C4), anthracenes (C1–C4), dibenzothiophenes (C1–C4), fluoranthenes (C1–C3), pyrenes (C1–C3), benz(*a*)anthracene, chrysenes (C1–C4), benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*e*)pyrene, benzo(*a*)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenz(*a*,*h*)anthracene and benzo(*g*,*h*,*i*)perylene.

4. Discussion

C. finmarchicus were exposed to the WAF of a naphthenic crude oil with a concentration corresponding to 50% of the 96 h LC_{50} to investigate for possible toxic responses related to oxidative stress, protein damage and lipid peroxidation. At the initiation of the experiment, the concentrations of VOC and SVOC were approximately 5000 and 3100 μg/L, respectively.

In the present study, the generated WAF was dominated by volatile components, like BTEXs and naphthalenes (Table 2). Given the high naphthalene concentration in the exposure medium (253.5 μg/L), it was expected to observe induction of glutathione *S*-transferases, as previously reported in *C. finmarchicus* (Hansen et al., 2008b, 2008c, 2011 and 2013). However, the involvement of single *gst* genes in responses to oil components contamination might lead to unequivocal conclusions in a species such as *C. finmarchicus*. A total of 39 putative *gst* genes have been suggested for *C. finmarchicus*, including members of the cytosolic, microsomal and mitochondrial classes (Roncalli et al., 2015). Responses of individual *gst* genes are rather specific and while many responds to toxicant exposure or specific stressors, others are involved in different cellular functions (Roncalli et al., 2016). *gst-III*, showing both significant up- and down-regulation in the exposed group (at 48 and 72 h), encodes for *Calfi-mGST-3-III*, a microsomal *gst* (subclass III). This specific *gst* appears to be sensitive to several toxicants such as petrogenic oil, diethanolamine and Ag nanoparticles (Hansen et al., 2010. Farkas et al., 2020), while it shows poor responses to other stressors, such as toxic dinoflagellates (Roncalli et al., 2016). It should be noted that this particular primer sequence for *gst-III* was applied by Hansen et al. (2008a, 2008b), where a time- and concentration dependent induction was reported following exposure of *C. finmarchicus* to naphthalene and to an artificially weathered North Sea oil dispersion

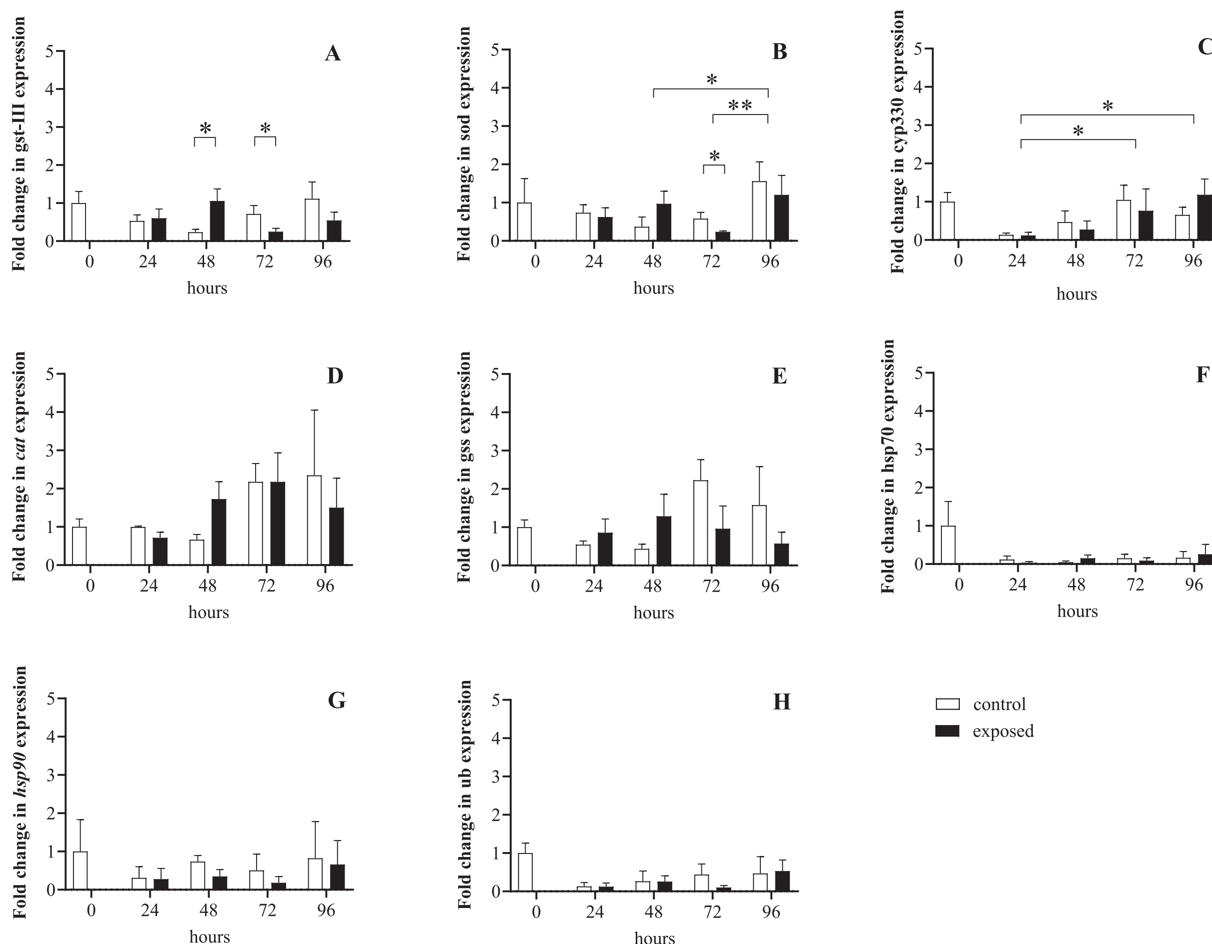

Fig. 1. Graph show the fold change in gene expression ratio every 24 h from the beginning (0 h) until the end (96 h) of the exposure experiment. Results are reported for the control and the WAF exposed group as mean ± standard error. * = $p < 0.05$ A = glutathione S-transferase III; B = superoxide dismutase; C = cyp330*a*1; D = catalase; E = glutathione synthetase; F = heat shock protein 70; G = heat shock protein 90; H = ubiquitin.

(Hansen et al., 2008c), respectively. While Hansen et al. (2008b, 2008c, 2011, 2013) suggested *gst* genes as fairly stable biomarkers following exposure to different types of oil and oil byproducts, our results do not support the use of *gst* expression as reliable oxidative stress biomarker of oil exposure in *C. finmarchicus*.

Potential factors contributing to the inconsistency in effects on the *gst-III* following exposure to petroleum hydrocarbons between the present study and the other studies on *C. finmarchicus* might be (i) differences in oil profile (ii) differences in life-stages or even (iii) the choice of reference genes. A detailed description of experimental set-ups, oil profiles and selected life-stages comparing our study to Hansen et al. (2008b, 2008c, 2011, 2013) can be found in Supplementary S6. Hansen et al. (2011, 2013) exposed CV *C. finmarchicus* to either marine diesel WSF or artificially weathered crude oil WAF, and showed an increase in *gst* mRNA levels at concentrations corresponding to 5% and 50% of the 96 h LC_{50} of each solution respectively. Those two test solutions were highly rich in phenols, molecules not detected in our WAF stock solution. Phenols have been shown to modulate *gst* expression and GST activity in several *in vivo* and *in vitro* studies (Fiander and Schneider, 2000; Santos et al., 2002; Krajka-Kuźniak et al., 2008; Mofeed and Abdel-Aal, 2005; Sun et al., 2019). Nevertheless, *gst* upregulation was also detected in adult *Calanus* exposed to naphthalene only and naphthalene rich North Sea oil WSF (Hansen et al., 2008b, 2008c). The differing responses between those reported in the present study and those reported by Hansen et al. (2008b, 2008c) might arise from the different experimental settings in the studies. In Hansen et al. (2008c) WSF exposed *Calanus* were fed throughout the experiment, which might have provided an additional route of exposure, in contrast to our study. Regarding *Calanus* exposed to naphthalene (Hansen et al., 2008b), the minimal concentration required to consistently upregulate *gst* expression was 583 μg/L, which is much higher than concentrations measured in our WAF stock solution (253 μg/L).

Regarding reference genes, in contrast to previous literature (Hansen et al., 2008a; Tarrant et al., 2008) where *actin* and *efa1α* were used, our results showed a stable expression across all treatments only for *16S*. This may also have affected the inconsistent results obtained between these studies and ours.

Well-known biomarkers for oxidative stress, such as *cat* and *sod* gene expressions, did not show a consistent modulation in *C. finmarchicus* exposed to oil-derived WAF. No differences in catalase gene expression (Fig. 1D) or enzymatic activity (Fig. 2E) were detected between the control and the exposed group, nor in *sod* gene expression (Fig. 1B) while its enzymatic activity was persistently below LOD. Hansen et al. (2008b) reported an up-regulation of *sod* and *cat* at low exposure concentrations of naphthalene (31 ng/L) 12 h after exposure, while after 48 h the up-regulation was no longer significant. Thus, the authors concluded that there was no clear effect of the naphthalene exposure on *sod* and cat gene expression (Hansen et al., 2008b), which is consistent with our study, where *sod* and *cat* expressions were not affected by WAF exposure.

Numerous PAHs are known peroxisomal proliferators in vertebrates; upon binding to the peroxisomal proliferator activated receptor α (PPARα), they cause a cascade of effects resulting in a potential increase of H_2O_2 and the release of transitional metals as Fe and Cu from

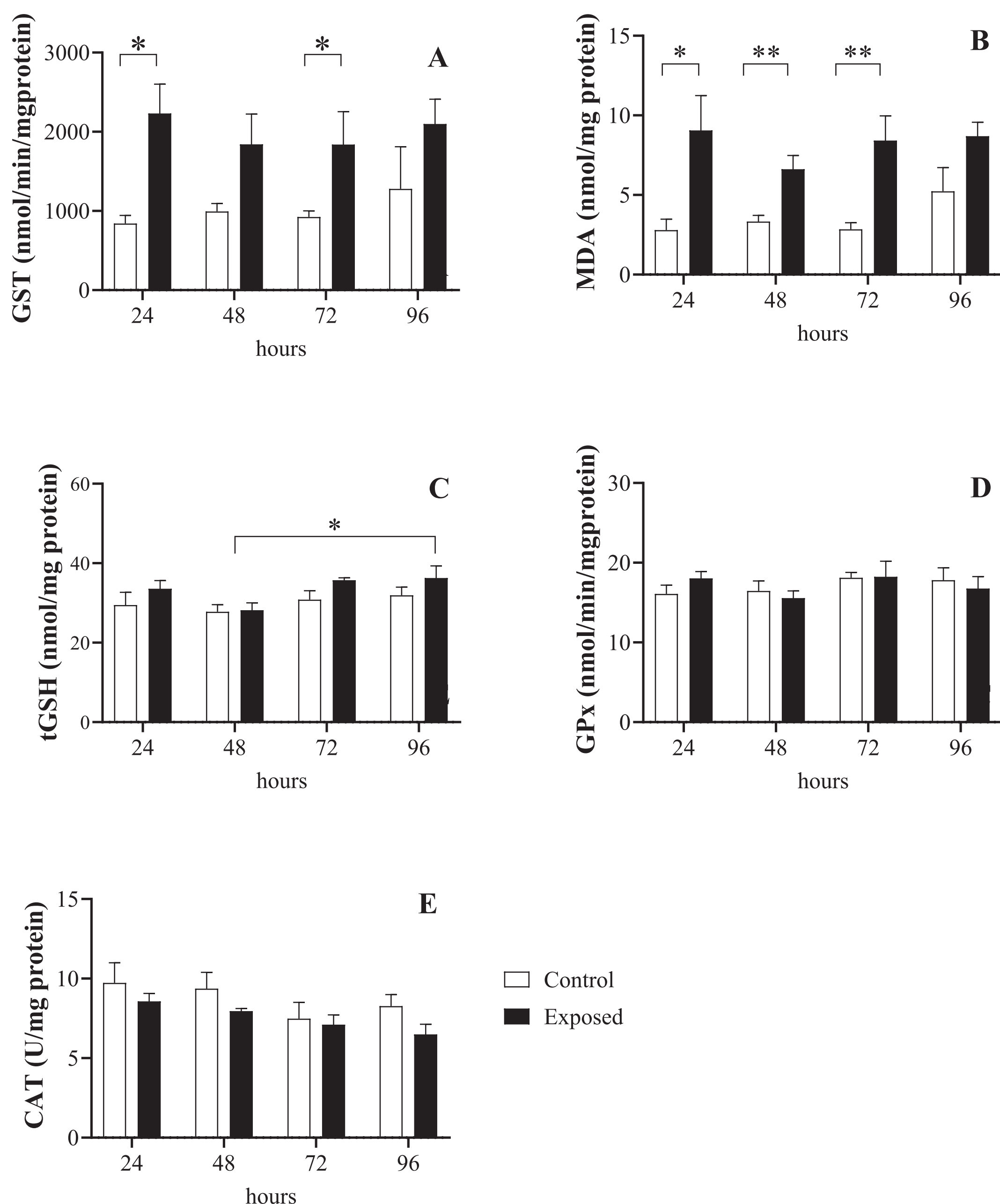

Fig. 2. Results from glutathione S-transferase (A), glutathione peroxidase (D) and catalase (E) activity, and malondialdehyde (B) and total glutathione (C) concentrations. Results are reported for the control and the WAF exposed group every 24 h from the beginning (0 h) until the end (96 h) of the exposure experiment. Results are reported as mean ± standard error. * = $p < 0.05$; ** = $p < 0.005$.

peroxisomes (Regoli et al., 2014). Some PAHs also bind to the aryl hydrocarbon receptor (AhR), which induces the cytochrome P450 enzymatic complex (CYP450). In vertebrates, P450 enzymes are responsible for the biotransformation of PAHs into more hydrophilic, hence easily excretable, metabolites (Pritchard and Bendt, 1991). However, induction of CYP450 enzyme activity has the potential to promote ROS production (Regoli et al., 2014). Although multiple CYP genes have been identified in copepods (Han et al., 2017), it is not yet clear whether these are involved in the detoxifying system in *C. finmarchicus* (Hansen et al., 2008b). While our study showed no induction of *cyp330a1* expression in the WAF exposed *C. finmarchicus*, Hansen et al. (2008b) reported that *cyp330a1* was significantly down-regulated in animals following exposure to naphthalene (approx. 2500 μg/L) as compared to controls. Furthermore, Hansen et al. (2008c) reported that *cyp330a1* was up-regulated in lipid-poor individuals and down-regulated in lipid-rich individuals exposed to the water-soluble fraction (WSF) of a weathered North Sea oil with concentrations of naphthalenes >1.33 μg/L (total hydrocarbons 2.9 μg/L). In the present study the initial concentration of naphthalene and C1-C4 alkylated homologue compounds in the WAF was 254 μg/L, but the WAF also contained other VOC and SVOC and the total extractable material was approx. 3100 μg/L (Table 2). The differing results among the studies may be due to differences in both composition and concentrations of the exposure solutions, and possibly also due to differing organismal lipid contents among the experiments. Expression of the cyp330*a*1 gene has also been shown to vary significantly between developmental stages in *C. finmarchicus*, being higher in post-ovulating females and in males (Hansen et al., 2008a). Together, these results suggest that effects of petroleum oil in *C. finmarchicus* on *cyp330a1* are dependent not only on the composition of exposure, but also on the developmental stage of the copepods.

In the present study, modulations of *hsp70*, *hsp90* and *ub* gene expression, which are indicators of increased cellular stress and protein

degradation, showed no relation with the treatment. This is in accordance with the results reported by Hansen et al. (2008b), where *C. finmarchicus* were exposed to naphthalene.

Interestingly, expressions of *cat*, *ub*, and *hsp 70* were high at 0 h, and were down-regulated 24 h into the exposure experiment. It is possible that these genes were up-regulated due to stress involved in the handling of the animals just prior to the initiation of the experiment (time 0), as shown by Aruda et al. (2011). It should also be noted that the animals were not fed during the experiment, and this may have resulted in a persistent down-regulation of these genes during the experiment due to starvation. Previous studies have shown a starvation-mediated reduction in (endogenous) metabolite concentrations in *C. finmarchicus*, a response also observed when exposed to crude oil dispersions (Hansen et al., 2017b). Nevertheless, in our experiment both the control and exposed groups received the same handling procedures and the high expressions of these genes were consistently detected only at 0 h (*i.e.*, before the starting time of exposure). Therefore, we can assume that if handling-induced up-regulation occurred, it was detectable only shortly after the handling procedure and did not act as a confounding factor across other time points during the exposure-experiment.

Commonly, variation in gene expression is linked with up- or down-regulation of enzymatic systems, but this assumption might be valid only for direct and tight substrate-receptor relationships (Regoli and Giuliani, 2014). Changes in mRNA abundance do not inevitably translate into changes in enzyme activity (Miyamoto et al., 2001; Glanemann et al., 2003). Considering the enzymatic activity and gene expression of key actors of the glutathione system analyzed in our study, contrasting patterns emerged. While GST activity was rather stable over time, *gst* gene expression varied considerably. Comparing *gss* and tGSH levels (Figs. 1E, 2C), a similar time-dependent pattern was observed in the control group, while a dissimilar trend was observed in the exposed group. Regarding catalase, although not significantly, in both groups *cat* transcription levels appeared to increase with time while catalase activity appeared to decrease, although also not significantly. These contrasting and incongruent results could be explained by the sequence of post-transcriptional events in regulation of genes which are involved in oxidative stress responses. In addition, mRNA stability, protein turnover, transcriptional and translational mechanisms, post-translational regulation of enzymatic kinetics, interactions and secondary non-genomic effects can mask the link between gene expression and enzymatic activities (Regoli et al., 2011).

In marine organisms, the mechanistic relationships between mRNA transcription and antioxidant enzymatic activities are not-well understood due to a lack of data. Therefore, studies often show contrasting trends for these two types of responses (Regoli et al., 2011). When considering the intricate pathways involved in antioxidant responses, from gene expression to enzymatic activity, it is reasonable to conclude that post-translational mechanisms and other antioxidants (*e.g.* astaxanthin) might play a major role in antioxidant responses in *C. finmarchicus* exposed to oxidative agents.

Our study shows markedly higher GST enzymatic activity and MDA concentrations in exposed copepods compared to controls (Fig. 2A, B), suggesting that WAF exposure induced intracellular oxidative stress. Even though the differences were not statistically significant at all-time points, GST and MDA levels detected in exposed individuals were consistently higher (1.6–2.7 and 1.7–3.2 times for GST and MDA, respectively) when compared to control condition at different sampling time points. Thus, although not consistent throughout the experiment, possibly due to low sample sizes, this indicates that WAF exposure induced lipid peroxidation intracellular oxidative stress in the exposed copepods. In *C. finmarchicus*, GST is hypothesized to handle lipid peroxidative end products and has therefore been suggested as potential biomarker for lipid peroxidation (Hansen et al., 2008b, 2008c). Malondialdehyde is generated by the oxidation of polyunsaturated fatty acids and by degradation of pre-existing oxidation products and it is also proposed as an indicator of lipid peroxidation (Draper and Hadley, 1990). Induction of GST activity and high MDA concentrations in the exposed group in the present study hence indicate that in *C. finmarchicus* lipid peroxidation is a major toxic endpoint following WAF exposure: This is in accordance with Hansen et al. (2008b, 2008c, 2011). Lipid peroxidation might be highly detrimental for *C. finmarchicus* survival and reproduction, considering the importance of lipids in its life cycle and their critical role in the regulation of the diapause timing (Irigoien, 2004; Hansen et al., 2008a; Skottene et al., 2020). Moreover, cholesterol depletion, induced by lipid peroxidation, could affect steroidogenesis, causing endocrine disruption (Hansen et al., 2008a).

Reproducing realistic environmental conditions of oil contamination in a laboratory experiment is a difficult task and the results obtained from laboratory studies must be treated with care when extrapolating to environmental or wild-life scenarios. The WAF in the present study was generated using low energy and is considered a droplet-free water-soluble (dissolved) fraction, thus containing no, or to a very limited extent, particulate oil droplets. Organisms subjected to a real oil spill/oil contamination, will be affected not only by water soluble components, but also by oil slicks, oil droplets, as well as other additional stressors, such as direct natural sunlight, and oil dispersants if applied (Duesterloh et al., 2002). The dissolved fraction is generally believed to be responsible for the toxicity of oil components due to the bioavailability of these components (Olsvik et al., 2011). However, ample evidence shows that copepods filter oil droplets, representing an additional route of exposure *via* ingestion (Hansen et al., 2008c, 2017b; Nordtug et al., 2015). In fact, modeling efforts have predicted that *C. finmarchicus*, due to its wide distribution and high filtration rates, can affect the total mass balance of oil spills (Nepstad et al., 2015). Considering the widespread distribution of *C. finmarchicus* across the northern North Atlantic (Hirche and Kosobokova, 2007), small oil spills are not likely to harm the entire NA population but can act as a local additional stressor. Our exposure setup, even though not representing a full spectrum of realistic environmental conditions, contributes to the understanding of zooplanktonic responses to oil exposure.

5. Conclusion

Our study aimed at examining specific responses to oil contamination in *C. finmarchicus*, a key species in North Atlantic, analyzing gene, enzymes and molecules involved in oxidative stress responses, the detoxifying system and the antioxidant system. Our results indicate increased GST enzymatic activity and MDA concentrations as main responses in *C. finmarchicus*, suggesting lipid peroxidation as the major mechanism of naphthenic oil WAF induced toxicity. Given the critical role lipids plays in *C. finmarchicus* life cycle, consequences of chronic oil contamination could be highly detrimental for this species. Gene expression results of *sod*, *cat*, *gst*, *gss*, *cyp330a1*, *hsp70*, *hsp90* and *ub* following exposure were inconsistent. The use of *gst* expression as a biomarker of oil contamination, as suggested by previous works, could not be supported. Field studies are needed to confirm our results in actual oil contaminated environments while more investigations are necessary to understand the role of lipids in oil-exposure responses in *C. finmarchicus*.

Declaration of competing interest

We hereby declare that none of the authors of manuscript 'Oil-mediated oxidative-stress responses in a keystone zooplanktonic species, *Calanus finmarchicus*' have conflict of interests whatsoever.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151365.

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