COVER PAGE

TITLE: Acute hydrogen peroxide exposure does not cause oxidative stress in late-copepodite stage of Calanus finmarchicus

RUNNING HEAD: Effects of H_2O_2 on copepod

AUTHORS AND AFFILIATIONS:

Bjørn Henrik Hansen

SINTEF Ocean AS, 7465 Trondheim

E-mail: bjorn.h.hansen@sintef.no. Phone: +47 98283892.

Anna Hallmann

Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Dębinki 1, Gdańsk 80-211, Poland

E-mail: hallmann@gumed.edu.pl.

Dag Altin

BioTrix, 7022 Trondheim, Norway

E-mail: biotrix@frisurf.no. Phone: +47 92643500.

Bjørn Munro Jenssen

Norwegian University of Science and Technology, Department of Biology, 7491 Trondheim, Norway

E-mail: bjorn.munro.jenssen@ntnu.no. Phone: +47 91897120.

Tomasz M. Ciesielski

Norwegian University of Science and Technology, Department of Biology, 7491 Trondheim, Norway

E-mail: tomasz.m.ciesielski@ntnu.no. Phone: +47 40465552.

Acute hydrogen peroxide exposure does not cause oxidative stress in late-copepodite stage of *Calanus finmarchicus*

Bjørn Henrik Hansen[1,*], Anna Hallmann[2], Dag Altin[3] Bjørn Munro Jenssen[4] and Tomasz M. Ciesielski[4]

[1]SINTEF Ocean AS, 7465 Trondheim

[2]Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Dębinki 1, Gdańsk 80-211, Poland

[3]BioTrix, 7022 Trondheim, Norway

[4]Norwegian University of Science and Technology, Department of Biology, 7491 Trondheim, Norway

*Corresponding author: E-mail: bjorn.h.hansen@sintef.no. Phone: +47 98283892.

Abstract

Use of hydrogen peroxide (H_2O_2) for removal of salmon lice in the aquaculture industry has been met with a concern that non-target organisms may be affected during treatment scenarios. In the present work, we evaluated the potential for H_2O_2 to cause reduced survival and oxidative stress in one of the most abundant zooplankton species in Norwegian coastal areas, the copepod *Calanus finmarchicus*. We subjected the copepod to two 96-hour tests: An acute toxicity test where mortality was determined, and a second experiment where we treated copepods to an exposure concentration below the No Observed Effect Concentration (0.75 mg L^{-1}) and analyzed for antioxidant enzymes, glutathione (GSH) and malondialdehyde (MDA). Compared to available and comparable LC_{50}-values from the literature, our results suggests that *C. finmarchicus* is highly sensitive to H_2O_2. However, 96

hour exposure of *C. finmarchicus* to 0.75 mg H_2O_2 L^{-1} did not cause responses on the antioxidant systems even though the concentration is just below the level where mortality is expected. This suggests that aqueous H_2O_2 exposure does not cause cellular accumulation with associated cellular oxidative stress, but rather cause acute effects on copepod surface (carapace). This certainly needs further investigation in order to assure that aqueous exposure during H_2O_2 treatment in salmon fish farms does not have adverse effects on local non-target crustacean species and populations. Particularly, studies on copepod developmental stages with a more permeable carapace is warranted.

Key words: Superoxide dismutase; catalase; glutathione peroxidase; glutathione; glutathione S-transferase; lipid peroxidation; H_2O_2

1. Introduction

In order to reduce the devastating impacts of salmon lice *Lepeophtheirus salmonis* (Copepoda: Caligidae) on the marine salmon production and farming, several pesticides have been used over the years, i.e. organophosphates, pyrethroids, avermectins, chitin synthesis inhibitors and topical disinfectants (Denholm et al., 2002). The extensive use has resulted in genetic resistance in salmon lice to some of these, mainly the organophosphates, carbamates and pyrethroids. Hydrogen peroxide (H_2O_2), which was introduced in Norway as a treatment in the 90s, is by far the most widely used salmon lice pesticide in Norway (31,577 tons used in 2014). H_2O_2 is a prooxidant and functions as a pest control by being a topic disinfectant, meaning it is administered directly through water to remove attached lice from the fish skin. Because it has an acute effect and is readily broken down to oxygen and water through abiotic processes, it is considered less environmentally harmful than many of the other pesticides which have much larger acute-to-chronic ratios (ACRs>300,000) due to their specific (and delayed) modes of toxic action (Lillicrap et al., 2015). However, high H_2O_2 concentrations are necessary for lice removal causing concern for acute effects on the treated fish as well as for organisms in the environment surrounding the fish farms. Atlantic salmon (*Salmo salar*) post-smolts exposed to 1230 mg L^{-1} H_2O_2 for 20 min at 13.5°C suffered an acute toxicity resulting in a 35% mortality within 2 hours. Thirty-three per cent of adult and pre-adult sea lice (*L. salmonis*) were immobilized or killed following exposure to 500 mg L^{-1} H_2O_2 at 10°C, rising to 98% at 2000 mg L^{-1} (Bruno&Raynard, 1994).

Due to the large quantities used, and the manner in which application is performed, there is a concern about acute effects of H_2O_2 on non-target species like zooplankton. Acute toxicity levels of H_2O_2 to crustaceans have been shown to be in the concentration range used for application in fish farms with 1 hour-LC_{50}s of 973, 1637 and 3182 mg L^{-1} for *Mysis* sp., American lobster (*Homarus americanus,* stage 1) and sand shrimp (*Crangon septemspinosa*), respectively (Burridge et al., 2014). Other types of effects have also been reported at lower exposure concentrations, e.g. inhibited feeding activity in the copepod *Acartia hudsonica* (Van Geest et al., 2014), reduced metabolic rate and muscle intracellular

pH in shrimp (*Crangon crangon*), and oxidative stress in the copepod *Tigriopus japonicus* (Lee&Raisuddin, 2008).

Oxidative stress, i.e. the production of reactive oxygen species (ROS) like superoxide anion ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\bullet}$) may occur naturally during oxidative phosphorylation in mitochondria or induced by redox cycling chemicals, transition metals and many other compounds (Stohs&Bagchi, 1995; Livingstone, 2001). To reduce oxidative damage by ROS (e.g. lipid peroxidation, DNA damage), organisms have antioxidant defense systems in the form of enzymes, vitamins and pigments. Important antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9), and the latter two detoxifies H_2O_2 and organic hydroperoxides resulting from oxidative stress. The tripeptide glutathione (GSH) also functions as an antioxidant through conjugation to electrophilic substances often catalyzed by the enzyme glutathione S-transferase (GST) (Di Giulio et al., 1995; Halliwell&Gutteridge, 2015).

The main aim of this work was to evaluate the potential of acute aqueous H_2O_2 exposure to cause oxidative stress in the copepod *Calanus finmarchicus* (Copepoda: Calanoida). This copepod is the most abundant zooplankton species in the Norwegian Sea and serves a crucial role in the marine food web, as it is an important transfer route for energy between primary producers and fish. Late developmental stages consist of large lipid reservoirs, which are utilized for seasonal periods of diapause and reproduction. In this work, we performed an acute toxicity test for H_2O_2 to determine a no-effect concentration based on copepod mortality. Thereafter, copepods were treated with in a sub-lethal H_2O_2 concentration where analyses of a battery of antioxidant enzymes was performed.

2. Materials and Methods

2.1. Copepod culture

Copepods (*C. finmarchicus*) from the continuous lab culture at SINTEF/NTNU Sealab were used for the experiments on this species. They are routinely kept at 10 °C, and the details regarding the culturing have previously been described (Hansen et al., 2007).

2.2. Preparation of exposure medium

The substance tested in this study was H_2O_2 (30% pro analysis Perhydrol®, Merck, Darmstadt, Germany). Although we did not verify the H_2O_2 concentrations in the different treatment, the substance was considered fully soluble in seawater at the respective desired concentrations. As aeration and high temperatures increases the degradation of H_2O_2 (Bruno&Raynard, 1994; Burridge et al., 2010), relatively low temperatures as well as capped bottles were used during treatment to limit degradation during treatment. Stock solution was prepared by diluting a test substance in a deionized water, and the stock solution was then dissolved in seawater in a 2L borosilicate glass bottle by automatic pipettes to give the desired exposure concentrations.

2.2. Acute toxicity test

The acute toxicity test design applied for *C. finmarchicus* was adopted from standard tests on *Acartia tonsa* (ISO) with adaptations to temperature (10±2°C), exposure volume (500 mL) and exposure time (96 hours). Briefly, 7 copepods (CV or adults) were transferred to exposure bottles. A total of seven exposure concentrations were used based on nominal dilutions of stock solution covering the range of expected no effect to full immobilization after 96 hours based on a preliminary assay. At each concentration, four replicates (N=4) were set up. Negative controls with seawater only was also prepared (N=8). To reduce the loss of the active compound during exposure the seawater was filtered to 0.22 μm (Sterivex™, Millipore) to reduce the level of organic particles. The animals were not fed

during exposure, and the test was performed in darkness except for when the survival was scored at 24, 48, 72 and 96 hours exposure.

2.3. Sub-lethal experiment

Two treatment groups were used in this experiment; Negative controls containing only filtered seawater, and a 'treated group' exposed to a sub-lethal concentration of 0.75 mg L^{-1} H_2O_2 based on the 96 hours-NOEC from the acute toxicity test. Both groups with five bottles (borosilicate, 2L) were stocked with 70 copepods in each and a total volume of 2 L exposure solution. As for the acute test the water used was filtered to 0.22 µm (Sterivex™, Millipore), the animals were not fed during exposure, and the exposure was performed in darkness at 10±2°C.

2.4. Determination of enzyme activities, concentrations of GSH and levels of MDA.

The samples (50 individuals per sample) were homogenized using a MPW-309 universal laboratory aid (Mechanika Precyzyjna, Warszawa) for 30 s. in ice-cold buffer (to obtain 20% homogenate) 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. The homogenates were centrifuged at 14 000 RCF for 30 minutes at 4°C using Centrifuge SIGMA 3K18. The supernatants were transferred into fresh tubes and used for analysis. Cytosolic protein was determined by Lowry method (Lowry et al., 1951) with modification of Peterson (1977).

The enzymatic methods used for analyses of all oxidative stress markers described below were all based on established methods utilized in a copepod species (*Limnocalanus macrurus*) from the northern Baltic Sea (Vuori et al., 2015), and are therefore expected to work well for *C. finmarchicus* as well.

Samples for the GSH determination were made from the protein supernatant via deproteinization by adding a 1:1 volume of 5% sulfosalicylic acid (SSA). The sample was incubated with SSA on ice for 5 min

and centrifuged for 2 min at 10 000 × g. The total glutathione (totGSH) concentration, which includes reduced (GSH) and oxidized glutathione (GSSG) species was measured with Glutathione Assay Kit (CS0260, Sigma-Aldrich) using GSH as the standard (Sigma-Aldrich). Samples were pipetted on a 96-well microplate (Thermo Scientific), and working mixture reagent was added. The plate was then incubated for 5 min at room temperature and kinetic read of absorbance was measured at 412 nm in Synergy 2 Multi-Mode Reader (BioTek) to determine the leveltotGSH. The totGSH were normalized to the protein content of the samples.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined spectrophotometrically (UV-VIS Spectrophotometer, Beckman Coulter) by the method of Habig et al. (1974). The reaction mixture contained 100 mM phosphate-buffered saline buffer (pH 6.5), 100 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and the reaction was started by the addition of supernatant and 100 mM glutathione as substrate. The absorbance was followed for 5 min at 340 nm. Total GST activity was expressed in nmol/min/mg of total protein concentration.

Catalase (CAT; EC 1.11.1.6) activity was measured following the method described by Kankofer (2001). To supernatant cold 6 mM H_2O_2 was added and vortexed. After incubation in ice, the reaction was stopped by 3 mM H_2SO_4. Then 2 mM $KMnO_4$ was added, vortexed and the absorbance was read at 480 nm (UV-VIS Spectrophotometer, Beckman Coulter). The enzyme activity was determined by measurement of H_2O_2 reacting with a standard excess of $KMnO_4$ and the detection of the residual $KMnO_4$ spectrophotometrically. Catalase activity was expressed in U mg^{-1} of total protein concentration.

Superoxide Dismutase (SOD; EC 1.15.1.1) activity was measured using the modified method of Sun et al.(Sun et al., 1988). There are several isoforms of SOD. Two main forms occurring in cells are CuZnSOD, which is present primarily in the cytoplasm and the MnSOD form, which can be found in mitochondria. In this experiment the sum of two isoforms of SOD were detected. SODs activity involved inhibition of nitroblue tetrazolium reduction, with xanthine-xanthine oxidase used as a superoxide generator. The

reaction mixture contained 50 mM Na_2CO_3, 3 mM xanthine, 3 mM EDTA, 0.75 mM NBT (nitro blue tetrazolium), 15% BSA (bovine serum albumin) and 0.05 mU/ml xanthine oxidase. The absorbance was measured at a wavelength of 560 nm. The total activity was expressed in units per mg protein where 1 U of SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

MDA level was detected using the Lipid Peroxidation (MDA) Assay Kit (MAK085, Sigma-Aldrich). In this kit, lipid peroxidation was determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present. Samples were pipetted on a 96 - well microplate and absorbance was measured at 532 nm in Synergy 2 Multi-Mode Reader (BioTek). The amount of MDA was normalized to the protein content of the samples and expressed in nmol/mg of total protein concentration.

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was measured using the Glutathione Peroxidase Cellular Activity Assay Kit (CGP1, Sigma - Aldrich) with 30 mM *tert*-butyl hydroperoxide as the substrate. The assay system consisted of: 50 mM Tris-HCl, pH 8.0 with 0.5 mM EDTA, 5 mM NADPH, 42 mM GSH, 10 U/ml of glutathione reductase. The product of GPx catalyzed reaction: GSSG (glutathione disulfide) was recycled to GSH using glutathione reductase and NADPH. Oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm wavelength in Synergy 2 Multi-Mode Reader (BioTek) and was proportional to GPx activity in the sample. Total GPx activity was expressed in nmol/min/mg of total protein concentration. For this assay, limited sample was available, so two pooled samples from each of the treatments were analyzed. Although not enough analytical data for statistical analyses were obtained, the data is still included for reporting enzyme activity range values for *C. finmarchicus*.

2.5. Statistical analyses

For the acute toxicity test, the number of immobilized copepods in replicate bottles in each concentration of the exposure media was pooled before calculation, and normalized numbers of

immobilized copepods were calculated as percentage of control. The LC-values were calculated by a non-linear model based on a sigmoidal dose-response model with variable slope (four-parameter logistic equation) using GraphPad Prism version 6.00 for Macintosh (GraphPad Software, La Jolla, California). When performing the calculations constraints on the exposure concentration-response curve was placed both on top and bottom forcing the effect to be calculated within the interval 0-100% effects. To compare the enzyme activities between the two groups in the sub-lethal experiment, the non-parametric Mann-Whitney test was also performed using GraphPad Prism.

3. Results and Discussion

3.1. Acute toxicity

Despite being considered a relatively environmentally friendly pesticide to remove salmon lice, very little information exists on the ecotoxicity of H_2O_2 in the literature. Acute toxicity tests performed on H_2O_2 have been done almost exclusively on salmon lice, and the exposures have utilized high concentrations and very short exposure times, typically 20 min to 5 hours (Burridge et al., 2014; Abele-Oeschger et al., 1997; Bruno&Raynard, 1994). This because rapid dilution and degradation is expected during real applications. High concentrations are used because acute mortality of salmon lice is the desired outcome of the application, and fast dilution is expected when H_2O_2-containing water is dumped into the sea after bath treatment. When concerned with exposures of non-target species, more diffuse exposure regimes characterized by lower concentrations and longer exposure times may be expected. Our acute toxicity tests on *C. finmarchicus* was performed over a period of 96 hours and the highest concentration being 6 mg L^{-1}. Survival is plotted as a function of exposure concentrations in Fig. 1. The figure displays the curves for 24, 48, 72 and 96 hours exposure. Our acute test included readings from 24 hours and up to 96 hours because exposures of *C. finmarchicus* in the environment will be expected to occur for longer periods and at much lower concentrations than typically found

inside aquaculture cages during application. This, however, makes our data less comparable to available data from other species. LC_{50} values decline exponentially with time as observed in our acute experiment (Table 1). At 24 hours, the calculated LC_{50} value was approx. 6 mg L^{-1} declining to 2.5 mg L^{-1} after 96 hours of exposure. Comparing our results to the few available and comparable acute toxicity data from other species, it seems that *C. finmarchicus* is a much more sensitive species than a number of other marine species. Substantially higher LC_{50}-values have been reported for brine shrimp (*Artemia salina*, 24 hour-LC_{50} of 800 mg L^{-1}) (Matthews, 1995), amphipod (*Corophium volutator*, 96 hour LC_{50} of 46 mg L^{-1}) (Smit et al., 2008), zebra mussel (*Dreissena polymorpha*, 72 hour-LC_{50} of 30 mg L^{-1}) (Martin et al., 1993), rabbitfish (*Siganus fuscescens*, 24 hour-LC_{50} of 224 mg L^{-1}), striped goby (*Tridentiger trigonocephalus*, 24 hour-LC_{50} of 155 mg L^{-1}) and jack mackerel (*Trachurus japonicus*, 24 hour-LC_{50} of 89 mg L^{-1}) (Kanda et al., 1989). For mysids, lobster and shrimps, LC_{50}s in the range 1000 - 3000 mg L^{-1} has been reported following 1 hour of exposure with a subsequent 95 hours recovery (Burridge et al., 2014). Five hours exposure to 680 mg L^{-1} caused altered metabolic rate and decreased intracellular pH in the sand shrimp (Abele-Oeschger et al., 1997).

3.2. Sub-lethal experiment – design

The final experiment was designed to cause no mortality, as the concentration used (0.75 mg L^{-1}) did not result in any mortality in the 96 hours acute toxicity test. The concentration may also be considered more environmentally relevant as it is well below normal treatment concentrations for salmon lice (1200-1500 mg L^{-1}; (Kiemer&Black, 1997)) which is expected to be rapidly diluted in the sea. The exposure time used (96 hours) was chosen in order to simulate an environmental exposure scenario, but also to allow time for enzyme activation through gene expression. In *C. finmarchicus*, increased stress gene expression has previously been reported already after 24 hours exposure to ionic mercury (Øverjordet et al., 2014), polycyclic aromatic hydrocarbons (Hansen et al., 2008) and oil (Hansen et al., 2011). No mortality was observed during this treatment.

3.3. Antioxidant enzymes

Microcrustaceans do possess antioxidant enzymes and the ability to detoxify prooxidants and deal with environmentally stressful situations through enzymatic activity (Barata et al., 2005; Cailleaud et al., 2007; Cailleaud et al., 2009). However, in the present experiment no significant responses on antioxidant enzymatic activities were observed as a function of H_2O_2 exposure (Fig. 2).

Although the antioxidant systems are not well described for *C. finmarchicus*, it is expected to be somewhat similar to vertebrates. In addition, other copepods, like *Tigriopus japonicus*, express SOD (Kim et al., 2011), CAT (Han et al., 2015), GPx (Kim et al., 2015) and GSTs (Lee et al., 2008; Lee et al., 2007) in response to oxidative stress-inducing pollutants. Importantly however, most studies on antioxidants in microcrustaceans have involved gene expression analyses alone and not being complemented with enzyme activity measurements, but there are exceptions, e.g. in the study on *Daphnia magna* transplanted to polluted areas (Rivetti et al., 2015).

No significant differences between treatments were observed for SOD and CAT, however, a weak negative relationship ($p=0.548$, $R2=0.39$, linear regression, slope= -1.6) between their activities in the samples was observed. This may be explained by the fact that the substrate of SOD inhibits CAT activity (Kono&Fridovich, 1982). SOD converts the superoxide anion $\bullet O2^-$ to H_2O_2, and as such provides the substrate for CAT, which reduces H_2O_2 to water. GPX also detoxifies H_2O_2 and organic hydroperoxides produced during oxidative stress, for example, by lipid peroxidation (Di Giulio et al., 1995; Halliwell&Gutteridge, 2015). Lipid peroxidation is considered a major toxic mechanism by which radicals cause tissue damage resulting in altered cell membranes and thereby disrupts vital cell functions (Rikans&Hornbrook, 1997). Lipid peroxides are known to cause production of a variety of substances, the most important of which is malondialdehyde (MDA) (Leibovitz&Siegel, 1980). Particularly for lipid-storing copepods like *C. finmarchicus*, lipid peroxidation may have adverse outcomes, as their discrete lipid storage is the basis for longer periods of starvation during winter

(diapause), and subsequently utilized during reproduction to produce eggs (Marshall&Orr, 1972). It has been shown that the expression of ferritin was greater in diapausing copepodite V (CV) with large oil sacs, consistent with a role of ferritin in chelating metals to protect cells from oxidative stress suggesting that the integrity of lipids is of importance (Tarrant et al., 2008). However, evidence of lipid peroxidation caused by exposure to 0.75 mg H_2O_2 L^{-1} in the present exposure of CV copepods was not provided, since MDA levels were unaltered by the exposure (Fig. 3B).

In *C. finmarchicus*, GST gene expression has shown to be responsive to alkanolamines (Hansen et al., 2010), PAHs (Hansen et al., 2008), oil (Hansen et al., 2009; Hansen et al., 2011), marine fuels (Hansen et al., 2013) and mercury (Øverjordet et al., 2014). However, the gene expression data have never been complemented by enzyme activity analyses. In the present experiment, increased GST enzyme activity was observed as a function of exposure, however not significant (p=0.07). In the copepod *Limnocalanus macrurus,* enzymatic GST activities have been shown between different polluted sites, and the enzyme activity range (0.56-4.58 µmol/min/mg protein) were comparable to our study (Vuori et al., 2015). In addition, the copepod *Eurytemora affinis* has displayed GST enzyme responses to environmental stress, however, for this species, enzyme activity range was much higher (600-800 mol/min/mg protein) (Cailleaud et al., 2009; Cailleaud et al., 2007). GST catalyzes the conjugation of glutathione (GSH) with various electrophilic substances and as such plays an important role preventing oxidative damage. Analyses of GSH in H_2O_2-treated *C. finmarchicus* were also in line with the responses on GST enzyme activity, displaying no significant response (Fig. 3A). Measurements of total GSH may be less sensitive to stress than using the ratio between reduced (GSH) and oxidized glutathione (GSSG), however, our study only included the totGSH. The totGSH concentration rage observed for C. finmarchicus in our study was in the low end of what was observed for *Limnocalanus macrurus* (Vuori et al., 2015), supporting low level of oxidative stress during our sublethal H2O2 experiment.

4. Conclusion

Exposure of *C. finmarchicus* to hydrogen peroxide (H_2O_2) does not appear to cause cellular oxidative stress during the sub-lethal exposure concentration used in the present experiment (0.75 mg L^{-1}) even though the concentration is just below concentrations causing mortality after 96-hours exposure. A lack of antioxidant enzymatic response and no alterations in GSH and MDA levels to a 96-hour treatment to waterborne H_2O_2 suggests that cellular oxidative stress is not a primary mode of action for this chemical. This does not necessarily provides evidence for reduced concern for H_2O_2 exposure on this species; as the sensitivity measured as 24 hour-LC_{50} is 10 - 100-fold lower than most other reported sensitivity levels in other species. As H_2O_2 is a very reactive compound, there is reason to believe aqueous exposure does not cause cellular accumulation and associated cellular toxicity of this chemical, but rather cause acute effects on copepods directly through effects on copepod surface (carapace). This certainly needs further investigation in order to assure that aqueous exposure during H_2O_2 application in salmon fish farms does not have health effects on non-targeted local crustacean species and populations. Currently, H_2O_2 applications involves using bath treatments and subsequent dumping of H_2O_2-containing water in more open water. Future studies should also involve investigations of earlier life stages of copepods, which are less mobile as well as having a less protective exoskeleton. Studies on commercially important species such as lobster support this suggestion as early developmental stages have displayed higher sensitivity than older (Burridge et al., 2014). Also in the few cases where *L. salmonis* has displayed resistance to H_2O_2, a mechanism related to reduced carapace permeability has been proposed (Treasurer et al., 2000).

Acknowledgements

This work was funded internally by SINTEF, BioTrix, NTNU, Medical University of Gdańsk (ST-40 project), and externally through European Union's Horizon 2020 GRACE project (grant agreement No 679266).

References

Abele-Oeschger, D., Sartoris, F.J., and Pörtner, H.-O. 1997. Hydrogen peroxide causes a decrease in aerobic metabolic rate and in intracellular pH in the shrimp Crangon crangon. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 117 (2):123-129.

Barata, C., Varo, I., Navarro, J.C., Arun, S., and Porte, C. 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran Daphnia magna exposed to redox cycling compounds. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 140 (2):175-186.

Bruno, D., and Raynard, R. 1994. Studies on the use of hydrogen peroxide as a method for the control of sea lice on Atlantic salmon. *Aquaculture International* 2 (1):10-18.

Burridge, L., Lyons, M., Wong, D., MacKeigan, K., and VanGeest, J. 2014. The acute lethality of three anti-sea lice formulations: AlphaMax®, Salmosan®, and Interox® Paramove™ 50 to lobster and shrimp. *Aquaculture* 420:180-186.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., and Bostick, K. 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture* 306 (1–4):7-23.

Cailleaud, K., Forget-Leray, J., Peuhiet, L., LeMenach, K., Souissi, S., and Budzinski, H. 2009. Tidal influence on the distribution of hydrophobic organic contaminants in the Seine Estuary and biomarker responses on the copepod Eurytemora affinis. *Environmental Pollution* 157 (1):64-71.

Cailleaud, K., Maillet, G., Budzinski, H., Souissi, S., and Forget-Leray, J. 2007. Effects of salinity and temperature on the expression of enzymatic biomarkers in Eurytemora affinis (Calanoida, Copepoda). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 147 (4):841-849.

Denholm, I., Devine, G.J., Horsberg, T.E., Sevatdal, S., Fallang, A., Nolan, D.V., and Powell, R. 2002. Analysis and management of resistance to chemotherapeutants in salmon lice, Lepeophtheirus salmonis (Copepoda: Caligidae). *Pest Management Science* 58 (6):528-536.

Di Giulio, R., Benson, W., Sanders, B., and Van Veld, P. 1995. Biochemical mechanisms: metabolism, adaptation, and toxicity. *Fundamentals of aquatic toxicology* 2:523e560.

Habig, W.H., Pabst, M.J., and Jakoby, W.B. 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* 249 (22):7130-7139.

Halliwell, B., and Gutteridge, J.M. 2015. *Free radicals in biology and medicine*: Oxford University Press, USA.

Han, J., Won, E.J., Lee, M.C., Seo, J.S., Lee, S.J., and Lee, J.S. 2015. Developmental retardation, reduced fecundity, and modulated expression of the defensome in the intertidal copepod Tigriopus japonicus exposed to BDE-47 and PFOS. *Aquatic Toxicology* 165:136-143.

Hansen, B.H., Altin, D., Booth, A., Vang, S.-H., Frenzel, M., Sørheim, K.R., Brakstad, O.G., and Størseth, T.R. 2010. Molecular effects of diethanolamine exposure on Calanus finmarchicus (Crustacea: Copepoda). *Aquatic Toxicology* 99 (2):212-222.

Hansen, B.H., Altin, D., Nordtug, T., and Olsen, A.J. 2007. Suppression subtractive hybridization library prepared from the copepod Calanus finmarchicus exposed to a sublethal mixture of environmental stressors. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 2 (3):250-256.

Hansen, B.H., Altin, D., Overjordet, I.B., Jager, T., and Nordtug, T. 2013. Acute exposure of water soluble fractions of marine diesel on Arctic Calanus glacialis and boreal Calanus finmarchicus: Effects on survival and biomarker response. *Science of The Total Environment* 449:276-284.

Hansen, B.H., Altin, D., Rorvik, S.F., Overjordet, I.B., Olsen, A.J., and Nordtug, T. 2011. Comparative study on acute effects of water accommodated fractions of an artificially weathered crude oil on Calanus finmarchicus and Calanus glacialis (Crustacea: Copepoda). *Science of the Total Environment* 409 (4):704-709.

375 Hansen, B.H., Altin, D., Vang, S.-H., Nordtug, T., and Olsen, A.J. 2008. Effects of naphthalene on gene
376 transcription in Calanus finmarchicus (Crustacea: Copepoda). *Aquatic Toxicology* 86 (2):157-
377 165.
378 Hansen, B.H., Nordtug, T., Altin, D., Booth, A., Hessen, K.M., and Olsen, A.J. 2009. Gene Expression of
379 GST and CYP330A1 in Lipid-Rich and Lipid-Poor Female Calanus finmarchicus (Copepoda:
380 Crustacea) Exposed to Dispersed Oil. *Journal of Toxicology and Environmental Health-Part a-*
381 *Current Issues* 72 (3-4):131-139.
382 Kanda, T., Murata, H., and Kuroki, A. 1989. Toxicity of Removal Agents of Red Tide Plankton to the
383 Fishes with Special Reference to the Toxicity of Hydrogen Peroxide, Iron Sulfate (II) and Iron
384 Chloride (III). *Aquaculture Science* 37 (3):221-224.
385 Kankofer, M. 2001. Catalase activity in bovine placenta: spectrophotometric and electrophoretic
386 analysis. *WIENER TIERARZTLICHE MONATSSCHRIFT* 88 (9):272-275.
387 Kiemer, M.C., and Black, K.D. 1997. The effects of hydrogen peroxide on the gill tissues of Atlantic
388 salmon, Salmo solar L. *Aquaculture* 153 (3):181-189.
389 Kim, B.M., Rhee, J.S., Lee, K.W., Kim, M.J., Shin, K.H., Lee, S.J., Lee, Y.M., and Lee, J.S. 2015. UV-B
390 radiation-induced oxidative stress and p38 signaling pathway involvement in the benthic
391 copepod Tigriopus japonicus. *Comparative Biochemistry and Physiology Part - C: Toxicology*
392 *and Pharmacology* 167:15-23.
393 Kim, B.M., Rhee, J.S., Park, G.S., Lee, J., Lee, Y.M., and Lee, J.S. 2011. Cu/Zn- and Mn-superoxide
394 dismutase (SOD) from the copepod Tigriopus japonicus: Molecular cloning and expression in
395 response to environmental pollutants. *Chemosphere* 84 (10):1467-1475.
396 Kono, Y., and Fridovich, I. 1982. Superoxide radical inhibits catalase. *Journal of Biological Chemistry*
397 257 (10):5751-5754.
398 Lee, J.-S., and Raisuddin, S. 2008. Modulation of expression of oxidative stress genes of the intertidal
399 copepod Tigriopus japonicus after exposure to environmental chemicals. *Interdisciplinary*
400 *studies on environmental chemistry-biological responses to chemical pollutants. TERRAPUB,*
401 *Tokyo*:95-105.
402 Lee, K.W., Raisuddin, S., Rhee, J.S., Hwang, D.S., Yu, I.T., Lee, Y.M., Park, H.G., and Lee, J.S. 2008.
403 Expression of glutathione S-transferase (GST) genes in the marine copepod Tigriopus japonicus
404 exposed to trace metals. *Aquatic Toxicology* 89 (3):158-166.
405 Lee, Y.M., Lee, K.W., Park, H., Park, H.G., Raisuddin, S., Ahn, I.Y., and Lee, J.S. 2007. Sequence,
406 biochemical characteristics and expression of a novel Sigma-class of glutathione S-transferase
407 from the intertidal copepod, Tigriopus japonicus with a possible role in antioxidant defense.
408 *Chemosphere* 69 (6):893-902.
409 Leibovitz, B.E., and Siegel, B.V. 1980. Aspects of free radical reactions in biological systems: aging.
410 *Journal of gerontology* 35 (1):45-56.
411 Lillicrap, A., Macken, A., and Thomas, K.V. 2015. Recommendations for the inclusion of targeted testing
412 to improve the regulatory environmental risk assessment of veterinary medicines used in
413 aquaculture. *Environment International* 85:1-4.
414 Livingstone, D.R. 2001. Contaminant-stimulated reactive oxygen species production and oxidative
415 damage in aquatic organisms. *Marine Pollution Bulletin* 42 (8):656-666.
416 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin
417 phenol reagent. *J biol Chem* 193 (1):265-275.
418 Marshall, S., and Orr, A.P. 1972. *The Biology of a Marine Copepod*. Vol. Reprint. New York: Springer-
419 Verlag.
420 Martin, M., Mackie, G., and Baker, M. 1993. Acute toxicity tests and pulsed-dose delayed mortality at
421 12 and 22 C in the zebra mussel (Dreissena polymorpha). *Archives of Environmental*
422 *Contamination and Toxicology* 24 (3):389-398.
423 Matthews, R.S. 1995. Artemia salina as a test organism for measuring superoxide-mediated toxicity.
424 *Free Radical Biology and Medicine* 18 (5):919-922.
425 Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more
426 generally applicable. *Analytical Biochemistry* 83 (2):346-356.

Rikans, L.E., and Hornbrook, K.R. 1997. Lipid peroxidation, antioxidant protection and aging. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1362 (2):116-127.

Rivetti, C., Campos, B., Faria, M., De Castro Català, N., Malik, A., Muñoz, I., Tauler, R., Soares, A.M.V.M., Osorio, V., Pérez, S., Gorga, M., Petrovic, M., Mastroianni, N., de Alda, M.L., Masiá, A., Campo, J., Picó, Y., Guasc, H., Barceló, D., and Barata, C. 2015. Transcriptomic, biochemical and individual markers in transplanted Daphnia magna to characterize impacts in the field. *Science of The Total Environment* 503-504:200-212.

Smit, M.G., Ebbens, E., Jak, R.G., and Huijbregts, M.A. 2008. Time and concentration dependency in the potentially affected fraction of species: the case of hydrogen peroxide treatment of ballast water. *Environmental Toxicology and Chemistry* 27 (3):746-753.

Stohs, S.J., and Bagchi, D. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine* 18 (2):321-336.

Sun, Y., Oberley, L.W., and Li, Y. 1988. A simple method for clinical assay of superoxide dismutase. *Clinical chemistry* 34 (3):497-500.

Tarrant, A.M., Baumgartner, M.F., Verslycke, T., and Johnson, C.L. 2008. Differential gene expression in diapausing and active Calanus finmarchicus (Copepoda). *Marine Ecology-Progress Series* 355:193-207.

Treasurer, J., Wadsworth, S., and Grant, A. 2000. Resistance of sea lice, Lepeophtheirus salmonis (Krøyer), to hydrogen peroxide on farmed Atlantic salmon, Salmo salar L. *Aquaculture Research* 31 (11):855-860.

Van Geest, J.L., Burridge, L.E., Fife, F.J., and Kidd, K.A. 2014. Feeding response in marine copepods as a measure of acute toxicity of four anti-sea lice pesticides. *Marine Environmental Research* 101 (1):145-152.

Vuori, K.A., Lehtonen, K.K., Kanerva, M., Peltonen, H., Nikinmaa, M., Berezina, N.A., and Boikova, E. 2015. Oxidative stress biomarkers in the copepod Limnocalanus macrurus from the northern Baltic Sea: Effects of hydrographic factors and chemical contamination. *Marine Ecology Progress Series* 538:131-144.

Øverjordet, I.B., Altin, D., Berg, T., Jenssen, B.M., Gabrielsen, G.W., and Hansen, B.H. 2014. Acute and sub-lethal response to mercury in Arctic and borealcalanoid copepods. *Aquatic Toxicology* 155:160-165.

460 **Tables**

461

462 Table 1: Calculated LC_{50}-values for 24, 48, 72 and 96 hours of exposure of *Calanus finmarchicus* to
463 hydrogen peroxide. The values are listed as the nominal concentrations in mg H_2O_2 L^{-1} causing 50%
464 effect with 95 % confidence intervals (CI).

Effect concentration	**24 hour**	**48 hour**	**72 hour**	**96 hour**
H_2O_2 mg/L	5.992*	3.912	3.824	2.540
95 % CI	NC**	3.187-4.801	3.536-4-136	2.356-2.738

465 *Calculated value ambiguous. **Not calculated

466