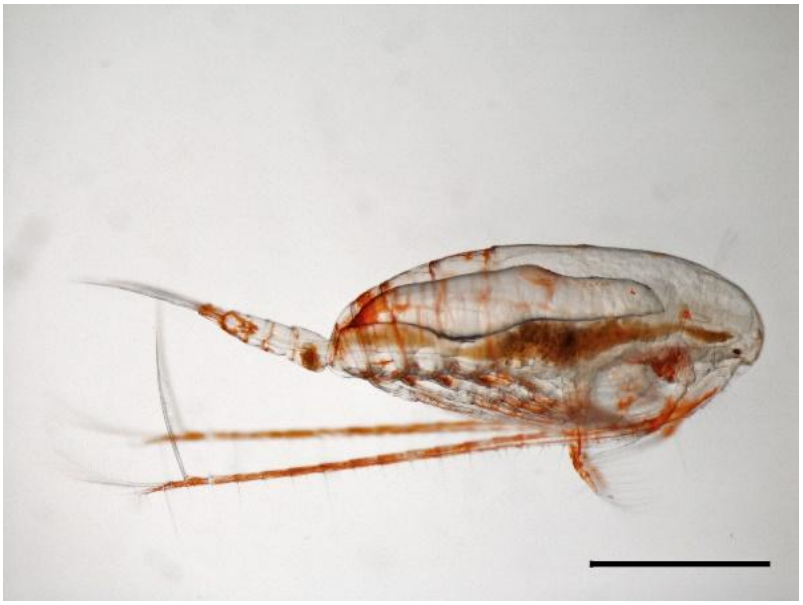
GRACE grant no 679266

# Effects on zooplankton

D3.14

WP3: Determination of oil and dispersant impacts on biota using effect-based tools and ecological risk assessment



Prepared under contract from the European Commission
Contract n° 679266
Research and Innovation Action
Innovation and Networks Executive Agency
Horizon 2020 BG-2014-2015/BG2015-2

| | |
|---|---|
| Project acronym: | GRACE |
| Project full title: | Integrated oil spill response actions and environmental effects |
| Start of the project: | 01 March 2016 |
| Duration: | 42 months |
| Project coordinator: | Finnish Environment Institute (SYKE) |
| Project website | http://www.grace-oil-project.eu |

| | |
|---|---|
| Deliverable title: | Effects on zooplankton |
| Deliverable n°: | D3.14 |
| Nature of the deliverable: | R |
| Dissemination level: | PU |

| | |
|---|---|
| WP responsible: | WP3 |
| Lead beneficiary: | NTNU |

| | |
|---|---|
| Due date of deliverable: | Feb 28 2019 |
| Actual submission date: | March 29 2019 |

Deliverable status:

| Version | Status | Date | Author | Approved by |
|---|---|---|---|---|
| 1.0 | Draft | 18.03.2019 | Tomasz Ciesielski, Bjørn Munro Jenssen (NTNU), Dag Altin (BioTrix), Kari Lehtonen, Raisa Turja, Aino Ahvo (SYKE) | WP 3 27.03.2019 |
| 2.0 | Final | 28.03.2019 | Tomasz Ciesielski, Bjørn Munro Jenssen (NTNU), Dag Altin (BioTrix), Kari Lehtonen, Raisa Turja, Aino Ahvo (SYKE) | Steering group 28.03.2019 |

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# Executive Summary

The aim of D.3.14 is to evaluate the effects of petroleum oils versus their chemically treated (with dispersant addition) counterparts on *Calanus finmarchicus*, and in relation to effects in *Arcartia tonsa* and Arctic and Baltic zooplankton species.

Experiments on the North-Atlantic copepod *C. finmarchicus* were conducted at NTNU-SeaLab (Norway) and experiments on the Baltic copepod *Limnocalanus macrurus* were conducted at SYKE (Finland). For acute toxicity testing, *C. finmarchicus* were exposed to the low energy water accommodated fraction (LEWAF) of three oil types, a North Sea (NS) naphthenic crude oil, a marine gas oil (MGO), and a marine bunker oil (IFO180), and to the chemically enhanced water accommodated fraction (CEWAF) of these oil-types following treatment with a commercially available chemical dispersant (Finasol OSR® 52). In *C. finmarchicus* acute $LC_{50}$ levels to the LEWAF and CEWAF treatments were compared, and sub-acute effects on established and novel biomarkers (gene expression, enzymatic activity and metabolomic study) were investigated following exposure to NS naphthenic crude oil. In *L. macrurus*, sub-acute effects on a range of established biomarkers were investigated. Comparisons of sensitivities among calanoids were conducted based on the results from the present studies and literature data.

In the acute toxicity studies, *C. finmarchicus* displayed a higher sensitivity to the naphthenic North Sea (NS) crude oil than to the marine gas oil (MGO) and the IFO180. Both the MGO and IFO180 were found to be ca. 2-3 times times less toxic than the naphthenic NS crude oil. The experiments showed that the dispersant enhanced the toxicity of the oil by 4.7, 18.3 and 13.0 times for the naphthenic NS crude oil, the MGO and the IFO180), respectively. Thus, for the chemically treated oils, the toxicity of the MGO was highest, whereas the toxicities of treated naphthenic NS and IFO180 oils were similar. Finasol OSR® 52 alone had a much higher lethality to *C. finmarchicus* than the oil-types and the chemically treated oil-types. However, due to the dynamic complexity of oil/water dispersions and subsequent changes in the toxicity of both oil and the dispersant in the laboratory static systems, it is be concluded that the direct toxicity comparison of chemically dispersed oil versus naturally dispersed oil should not be used to make recommendations for or against using dispersants. Therefore, before the final recommendations on oil toxicity on application of dispersant after oils spill in the Arctic can be made, the results should be evaluated in the Net Environmental Benefit Analysis (NEBA) process.

In the sub-acute studies, gene expression results were inconsistent. However, glutathione S-transferase (GST) enzymatic activity and malondialdehyde (MDA) concentrations appear to be reliable biomarkers of oxidative stress in *C. finmarchicus* exposed to oil, Moreover, lipid peroxidation was confirmed as a major toxic endpoint following exposure. Metabolome changes suggested that exposure to the naphthenic North Sea oil might impair energy balance in *C. finmarchicus*. Malonate and proline concentrations seems to be promising novel biomarkers for detecting sub-lethal effects of oil exposure in *C. finmarchicus*. In *L. macrurus* increased SOD and decreased GPx enzymatic activities, and indications of an increase in the GSH/GSSG ratio, were identified following exposure to the oils and the chemically treated oil-types. This confirms that exposure to sub-lethal concentrations caused oxidative stress in both in *L. macrurus* and *C. finmarchicus*, and that enzyme activities related to oxidative stress responses may be suitable biomarkers for identifying sublethal effects of oil pollution in calanoids.

A review of sensitivities (i.e. $LC_{50}$ values) of *A. tonsa* and other Arctic copepods revealed an almost coherent lack of comparable data. However, based on the available studies, it seems that the sensitivity of *C. finmarchicus* to the WAF of petroleum oil may be higher than that of the Arctic *Calanus glacialis*. This could be attributed to temporal immobilization of oil components in the large lipid reservoir in *C. glacialis* in comparison to in the smaller lipid stores of the more sub-Arctic *C. finmarchicus*. Although the literature data indicates that chemical treatment of crude oil enhances the toxicity of the oil in *A. tonsa*, as shown for *C. finmarchicus* in the present study, it was not possible to retrieve data in the scientific literature that would provide a meaningful comparison of species differences in toxicity to oil and chemically treated oil in these two species.

# 1 Introduction

Being at the bottom of the food chain, planktonic species play a key role in marine ecosystem since their lipids regulates the overall energy balance (Fenchel, 1988). Calanoid copepods are of major importance in marine ecosystems, feeding on the phytoplankton and forming a direct link between primary producers and fish and whales. In addition, pelagic copepods dominate the number of planktonic organisms, representing 55-95% of the total plankton biomass. *C. finmarchicus* (Fig. 1) is a representative planktonic copepod inhabiting the subsurface layer and deep-sea basins of the North Atlantic. *C. finmarchicus* is an ecologically important species in the North-Sea, Norwegian Sea and Barents Sea, periodically constituting up to 90% of the standing stock of zooplankton. Due to continued development of areas for oil and gas production, as well as increased shipping, the risk of oil discharges will increase, and thus respective exposure of *C. finmarchicus* to oil constituents.

**Figure 1**: *C. finmarchicus* adult female with large lipid storage. Scale bar = 1mm (Photo: Dag Altin, BioTrix)

*C. finmarchicus* feeds on phytoplankton and stores large amounts of high-energy lipids which are transferred up to higher trophic levels; dry mass lipid level increase from 10-20 % in phytoplankton to 50 -70 % in herbivorous *C. finmarchicus* (Falk-Petersen et al., 2007). The importance of Calanus in the Arctic ecosystem has been described by Falk-Petersen et al. (1990; 2002; 2007). The calanoid copepod *Limnocalanus macrurus* is a brackish water, coldwater stenotherm species with a wide geographical distribution (Vanderploeg et al. 1998 and references therein). In the northern Baltic Sea it is the dominant zooplankton species in the Bothnian Bay and the Bothnian Sea during most of the year (Dahlgren et al. 2010), and is also abundant in the Gulf of Finland (Ojaveer et al. 1998, Peltonen et al. 2014). The Baltic Sea is a semi-enclosed large brackish water basin in northern Europe. It receives freshwater from a catchment area from 9 different countries inhabited by approximately 80 million people. Its position and hydrodynamic properties make it especially vulnerable to both human impacts and natural changes. Animals and plants inhabiting the Baltic Sea are subjected to multiple stressors, from considerable spatial, seasonal and vertical variability in hydrography to human-generated loads of harmful substances and nutrients (Voipio 1981). The Baltic Sea has one of the longest recorded histories of contamination and is often described as the most polluted sea in the world. In the Baltic Sea tides are negligible and water exchange with the North Sea is limited.

Oil and its by-products are regularly released in the marine environment from both natural and anthropogenic sources associated with 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 et al., 1974; O'Brien et al., 1976; Engelhardt, 1983 ; Brannon, 1996; Kingston, 2002; Couillard et al., 2005 ; Martínez-Gómez et al.,

2010; Abbriano et al., 2011; White et al., 2012; Dupuis et al., 2015). The number of tank vessel spills has considerably decreased in the last decades owing to new regulations and better technologies. On the other hand, new shipping routes in the Arctic will most likely become operative in the near future due to climate change and consequent sea ice loss (Marigomez et al., 2017). While a considerable amount of knowledge exists about effects of oil exposure on key-species in temperate seas, fewer data are available for colder environments (De Hoop et al. 2011, Olsen et al., 2013, Marigomez et al., 2017). Regarding planktonic species, after an initial phase of acute toxicity resulting in local mass mortality, they have shown a high level of resilience following oil spills (Abbriano et al., 2011, Hansen et al., 2015). However, delayed toxic effects of exposure to oil components have also been shown (Toxwærd et al., 2018). Toxicological studies suggest that zooplankton sensitivity to the water accommodated fraction (WAF) of the oil is strongly related with the lipophilicity of its compounds but largely varies among species, growth and developmental stages (Jiang et al., 2010). The WAF include the dissolved fraction and the smaller, neutrally buoyant oil droplets.

To remove oil pollution slicks at sea, the oil can be treated with chemicals, so called dispersants, that causes the oil to break into small droplets following an oil spill. These small droplets disperse throughout the water volume, and are biodegraded by microbes in the water. Dispersant use involves a trade-off between exposing coastal life to surface oil and exposing aquatic life to dispersed oil. Whereas treating the oil with dispersants reduces exposure of marine life on the surface, it temporarily increases exposure for animals in the water column below the oil-slick. A dispersant can be added to the WAF preparation to create a dispersed WAF, also termed a chemically-enhanced WAF (CEWAF)
The aim of D3.14 was to evaluate the effects of untreated versus oil treated with dispersant on *C. finmarchicus*, and in relation to effects in *Acartia tonsa* and Arctic and Baltic zooplankton species.

Given the lack of suitable biomarkers for oil toxicity for *C. finmarchicus*, a key species in the North Atlantic, studies were performed at NTNU (Norway) using a laboratory cultivated population to investigate and validate lethal concentrations ($LC_{50}$). Although this culture may have diverged genetically from wild populations, it represents a good laboratory model for performing controlled and comparable laboratory studies due to the similar genetic diversity within this population. In addition, responses of specific genes, enzymatic activities and molecules as potential biomarker candidates of oil exposure in calanoids were recorded. Similar studies were performed on the Baltic Sea *Limnocalanus macrurus* at SYKE (Finland). The temperate copepod (*Acartia tonsa*) is a species widely cultivated in research laboratories and used as a standard model species for toxicological testing, including oil pollution. Thus, a comparison of the sensitivity of *C. finmarchicus*, Arctic copepods and *A. tonsa* was made based on literature data.

# 2 Materials and Methods

## 2.1 Oil and oil dispersant characterization

Three different petroleum products were selected for the experiments: a naphthenic North Sea (NS) crude oil, a commercially available marine gas oil (MGO) and an intermediate fuel oil (IFO 180). The naphthenic NS crude oil was selected as the crude and untreated petroleum sample. It is a light crude oil with low viscosity and characterized by a high proportion of low molecular weight saturates and aromatics. The MGO used as a fuel in ships engines is obtained from crude oils after a complex refining process involving atmospheric distillation and the refining of distillates. MGO is considered as a light gas oil due to its high content (~60%) of aromatic hydrocarbons. The intermediate fuel oil (IFO 180), which is a blend of heavy fuel oil and gas oil, was selected as an intermediate stage of petroleum products purity between crude and marine gas oil. The IFO is

characterized by a high viscosity (maximum viscosity = 180 centistokes) and a sulphur content of less than 3.5%.

The commercially available dispersants FinaSol OSR® 51 and FinaSol OSR® 52 (Total Fluides, Paris-La Defense, France) were selected to test their effect on oil toxicity. Following an oil spill, dispersants can be applied to combat the oil spill by alteration of the distribution of the oil in the water column. Dispersants lower surface tension and disperse oil into particulate-sized droplets. Smaller droplets of oil contain a higher surface area, allowing hydrocarbon-degrading bacteria to breakdown the oil more quickly. In spite the fact that the application of dispersants may reduce the overall impact of an oil spill dispersing oil into water may result in an increase of chemical load of oil components into marine organisms. Both dispersants are relevant in the study region of the GRACE project and the treatment of the selected oil types. The selected third-generation dispersants have slightly different chemical composition (Table 1). Finasol OSR® 51 contains 15-30 % non-ionic and 0.2-0.5 % anionic surfactants, while Finasol OSR® 52 contains >30 % non-ionic surfactants and 15-30 % anionic surfactants. Finasol® OSR 52 is compliant with all the three regulations on the market (EPA, MMO, CEDRE), while Finasol OSR 51 is compliant with two of them (MMO, CEDRE).

**Table 1.** Ingredients and composition of the dispersants Finasol OSR® 51 and Finasol OSR® 52.

| **Finasol OSR 51** | | **Finasol OSR 52** | |
|---|---|---|---|
| **chemical name** | **weight %** | **chemical name** | **weight %** |
| hydrocarbons, C11-C14, n-alkanes, isoalkanes, cyclics, <2% aromatics | 60 - 70 | hydrocarbons, C11-C14, n-alkanes, isoalkanes, cyclics, <2% aromatics | 15 - 20 |
| docusate sodium | 0.2 - 5 | docusate sodium | 20 - 25 |
| | | (2-methoxymethylethoxy)propanol | 15 - 20 |
| | | carboxylic acids, di, C6-12 cmpds, with ethanolamine, boric acid cmpd with ethanolamine | 0 - 2 |
| | | ethanolamine | 0 - 1 |
| non-ionic surfactants | 15 - 30 % | non-ionic surfactants | > 30 % |
| anionic surfactants | 0.2 5 % | anionic surfactants | 15 - 30 % |

## 2.2 Description of the experiments

Experiments on *C. finmarchicus* were performed at NTNU-SeaLab (Norway) using animals from the continuous lab culture. Experiments on *L. macrurus* were performed at SYKE (Finland) aboard the research vessel Aranda.

### *2.2.1 Calanus finmarchicus*

*Preparation of water-accommodated fractions (WAFs)*

A detailed chemical profile of the naphthenic North Sea crude oil was generated in the GRACE project, and the results are presented in the next section of the report (Results and Discussion). For *C. finmarchicus* exposures, low-energy water accommodated fractions (LEWAF) were prepared for the respective oil exposure only, chemically enhanced water accommodated fractions (CEWAF) were prepared for the combined exposure of oil and dispersant and high energy water accommodated fraction (HEWAF) was generated for testing the toxicity of the dispersant FINASOL OSR 52 alone, according to the GRACE protocol from the selected oil and petroleum products at NTNU Sealab (Trondheim, Norway). The LEWAFs were prepared in a closed aspirator glass flask (10L), under low-energy mixing conditions (no vortex), as recommended by the CROSERF guideline with a 1:40 oil-to-water ratio. For CEWAFs 10 % by weight of the dispersant (Finasol OSR 52) was carefully added to the oil resting on the water before the stirring was increased to create a 25 % vortex in the water phase. The HEWAF of the dispersant only, was prepared as described for the CEWAF stock solution with dispersant loadings corresponding to the amounts added for the CEWAF production to ensure the comparability of the resulting stock solution. Due to the higher toxicity of the HEWAF of the dispersant, this was generated in a 2 L aspiration bottle. LEWAFs, CEWAFs and HEWAFs were all generated at 10 °C in darkness for 72 h, followed by 1 h settling time for the HE- and CEWAFs while the LEWAFs were harvested without any settling. Afterwards, water fractions were carefully drained off from the bottom of the flasks.

*Acute toxicity in C. finmarchicus*

To determine $LC_{50}$ concentrations in *C. finmarchicus*, sets of experiments were performed based on the modified standard tests of acute lethal toxicity on *Acartia tonsa* (ISO, 14669) adopted to 10±2 °C. *C. finmarchicus* from the continuous lab culture at SINTEF/NTNU Sealab were used for the exposure experiment. Details concerning the culturing have been previously described (Hansen et al., 2007). The selected age stage of the animals for the experiment was non-ovulating adult females. *C. finmarchicus* were exposed to a WAF of the selected oil types alone (naphthenic NS, MGO and IF) and in combination with the dispersant Finasol OSR-52, and the dispersant only, for 96 h in darkness without feeding. For each exposure, 7 individuals in each experimental bottle (0.5 L, 4 replicates) were placed. Seven exposure concentrations (diluted with filtered sea water) and one control were applied. Control individuals were exposed to filtered seawater only.

*Sub-lethal effects in C. finmarchicus*

Exposure of *C. finmarchicus* to the WAF solutions were performed in 5 L glass bottles, containing 155 individuals each. The animals were not fed during the experiment and the glass bottles were kept at 10 °C, covered with a dark blanket to avoid light interference. *C. finmarchicus* individuals were sampled each 24 hours until the 4th day of exposure (0, 24, 48, 72 and 96 hours), and four biological replicates were set for each treatment (Table 2).

The following end-points were analysed: CYP1A2: cytochrome P450 1A2, CYP330A1: cytochrome P450 330A1, GST: glutathione S-transferase, GSS: glutathione synthetase SOD: superoxide dismutase, CAT: catalase, HSP70: heat shock protein-70, HSP90: heat shock protein-90, UB: ubiquitin, GPx: glutathione peroxidase, MDA: malondialdehyde and GSH: glutathione.

***Table 2***: Design of the experimental set-up. Each treatment consists of four biological replicates (1-4), containing 155 individuals each. The figures in the brackets indicate each of the replicates.

| Group | Sampling time points | | | | |
|---|---|---|---|---|---|
| | 0h | 24 h | 48 h | 72 h | 96 h |
| Exposed | | [1] [2] [3] [4] | [1] [2] [3] [4] | [1] [2] [3] [4] | [1] [2] [3] [4] |
| Control | [1] [2] [3] [4] | [1] [2] [3] [4] | [1] [2] [3] [4] | [1] [2] [3] [4] | [1] [2] [3] [4] |

To avoid exposure time variation within a single exposure group, all experimental bottles were prepared and sampled following the exposure order. 10 specimens were collected for qPCR analysis and 100 for enzymatic activities and GSH and MDA determination. The animals were quickly dried, placed in a cryotube and immediately snap-frozen in liquid nitrogen.

Several biomarkers of oxidative stress, lipid peroxidation and protein damage were determined using qPCR (CYP1A2, CYP330A1, GST, GSH, SOD, CAT, HSP70, HSP90 and UB) and enzymatic activities analysis (SOD, CAT, GST, GPx). Oxidative stress-related molecules MDA and GSH were analysed by colorimetry and UV-VIS spectrophotometry. In addition, samples (25 copepods per sample) were collected to study the effects of WAF exposure on the metabolome profile using proton nuclear magnetic resonance (1H NMR) spectroscopy technique.

Water samples were subjected to standard gas chromatography/mass spectrometry (GC/MS) analyses of semi-volatile organic compounds (SVOC) and volatile organic compounds (VOC) at the core facility for oil analyses at SINTEF Ocean using standard methodology described previously (Faksness et al., 2015). Analyses were performed on three different WAF samples: WAF (1:40 oil-to-water ratio), WAF exposure medium (WAF dilution corresponding to 50% of the 96 h $LC_{50}$ dilution), and WAF medium after 96 h exposure.

### *2.2.2 Limnocalanus macrurus*

*Preparation of water-accommodated fractions (WAFs)*

For the *L. macrurus* exposures, crude oil WAF and CEWAF were prepared according to the GRACE protocol. Briefly, for WAF, a stock solution of 5g crude oil in one liter of artificial sea water was mixed for 72 hours, left to set for 3 hours and drained carefully to new bottles without disturbing the surface oil layer. CEWAF was prepared similar way except crude oil was first mixed with dispersant Finasol OSR® 51 (amount of dispersant was equal to 10% of the crude oil volume). The cruise for *L. macrurus* sampling started one week before the actual *Limnocalanus* sampling. Therefore, WAF and CEWAF used in the experiment were prepared before the cruise started to prevent handling crude oil in the laboratories aboard Aranda. WAF and CEWAF were stored a week in the climate room (5°C), in darkness, filled to the top of the bottle, closed well and sealed with plastic to prevent contact with air.

*Acute toxicity and sub-lethal effects in L. macrurus*

*L. macrurus* sampled from the Baltic Sea were exposed to control and crude oil treatments (WAF and CEWAF) for 96 h in order to test the species for biomarker methods. *L. macrurus* were sampled by towing with closing WP2 net from the deepwater layer. To collect sufficient individuals for the experiment three zooplankton hauls from bottom to under thermocline were taken. Water for the animals was collected with 30 L Jussi sampler under the thermocline before the zooplankton haul. Animals were acclimated to laboratory conditions for 72 hours in 30 L bucket filled with ambient sea water at climate room (5 °C) with aeration, in darkness. Experimental set-up consisted of eight replicate 1 L glass vials per treatment (control, WAF and CEWAF). Fifty individuals were handpicked into each vial with 500 mL of sea water (0.2 µm filtered) on 4$^{th}$ of June and their viability was checked before starting the experiment next day. Experiment started 5$^{th}$ of June and lasted for 96 h until 9$^{th}$ of June, 2017. Final concentrations in the exposure media were 20 % for the WAF and 1 % for the CEWAF. Based on chemical analysis done with similarly prepared WAF and CEWAF, the latter contained 560 times higher mineral oil concentration. Therefore 1 % CEWAF concentration was decided for this experiment aiming at sublethal concentrations of toxic compounds in the exposure media. After adding WAF and CEWAF to the experimental vials on top of the existing 500 mL of sea water, sea water was added to reach the exposure volume of 900 mL in each vial. Control treatment was sea water only. Vials were closed with parafilm and the experiment was run in the climate room at 5 °C, without aeration, 12 h light/dark cycle. Viability of the organisms was checked daily. There was no mortality during the experiment. At the end of the experiment animals in each vial were quickly divided into two Eppendorf tubes, 30 for enzymatic and glutathione assays and 20 for lipid peroxidation assay, snap frozen to liquid nitrogen and stored to -80 °C until used for analysis. Enzymatic assays (catalase [CAT], glutathione-S-transferase [GST], glutathione reductase [GR], glutathione peroxidase [GPx] and superoxide dismutase [SOD]), glutathione assay (reduced and oxidized glutathione [GSH, GSSG]) and lipid peroxidation assay [LPx] were performed according to established protocols.

## 3 Results and discussion

### 3.1 Chemical analyses

The naphthenic NS crude oil chemical profile was largely dominated by VOCs, such as methylcyclohexane, cyclohexane and methylcyclopentane and SVOCs, such as C1-C4-naphthalenes. Individual BTEX compounds, such as m-xylene and toluene were found to be the major constituents of the crude oil. The LEWAF solution was dominated by BTEX compounds, followed by VOCs, such as methylcyclopentane, cyclopentane and methylcyclohexane while naphthalene and its C1-C4-homologues accounted for more than 90 % of all detected SVOCs (Table 3).

Aromatic compounds, such as benzene, toluene, ethylbenzene and xylene isomers, known as BTEX, and polycyclic aromatic hydrocarbons (PAHs), are generally considered the most important petroleum hydrocarbon (HCs) from a toxicological perspective detected in the marine environment. BTEX have moderate affinity for partitioning into lipid-rich tissues, but due to their volatility they are rapidly removed from the marine environment and are seldom recorded at high concentrations in marine organisms (Boyles, 1980; Neff, 2002). On the other hand, PAHs are ubiquitous pollutants in coastal sediments worldwide (Neff, 1979; 2002). They are hydrophobic, and once released in the marine environment tend to sorb to any organic particle colloids, sediment or tissue of marine organisms (Knezovich et al., 1987). Copepods were exposed to a relatively high concentration, corresponding to 50 % of the 96 h-$LC_{50}$ concentration. The LEWAF was dominated by volatile components, like BTEXs and naphthalenes, not surprisingly considering a fresh crude was used for generating WAF.

***Table 3*:** Concentrations of individual BTEX (µg/kg) and main SVOC groups (µg/L) detected in the in the LEWAF non-diluted medium. BTEX were analysed 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 medium** |
|---|---|
| *VOC* | *(µg/kg)* |
| 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* | *(µg/L)* |
| ∑ All identifiable compounds | 269.95 |
| ∑ Decalin and C1–C4-alkylated homologues. | 0.81 |
| ∑ Naphthalene and C1–C4-alkylated homologues | 253.50 |
| ∑ Phenantrene/anthracene and C1–C4-alkylated homologues | 3.09 |
| ∑ Dibenzothiophene and C1–C4-alkylated homologues | 1.31 |
| ∑ PAH 2+ rings* | 11.56 |
| ∑ Phenols and C1–C5-alkylated homologues. | 0 |
| TEM | 3088.96 |

*∑ 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. exp. med: exposure medium.

## 3.2 Acute toxicity

### *3.2.1 Calanus finmarchicus*

The results of the acute toxicity study for selected oil types, dispersant and their mixtures are given in Table 4. To determine the $LC_{50}$ values the animals were exposed to seven different concentrations (dilutions: 4-100% WAF) of the stock LEWAF (low energy water accommodated fraction) and CEWAF (chemically enhanced water accommodated fraction) of the different oils. All dilutions were made using filtered seawater (30 psu), and the $LC_{50}$ was determined using a sigmoidal concentration response curve. In the HEWAF exposure to Finasol OSR® 52, the dispersant was added in a mass ratio corresponding to that which was used in the CEWAF experiments. To test for stability of the exposure condition for CEWAFs we measured particle size and distribution at the beginning and at the end of the experiment with a Coulter Counter (Multisizer 3; Beckman Coulter). The results were processed and plotted with the Beckman Coulter particle characterization software (Beckman Coulter, Ver 3.51, 2002 and Ver 4.01, 2008).

***Table 4:*** The acute toxicity results values ($LC_{50}$) of LEWAF and CEWAF of naphthenic NS crude oil, marine oil gas, bunker oil and the dispersant alone in *Calanus finmarchicus.*

| WAF System vs. *C. finmarchicus* (Adult pre-ovulating females) | 96 h $LC_{50}$ % WAF | 95%CI |
|---|---|---|
| Naphth. North Sea fresh 1:40 OWR | | |
| CEWAF (10% FINASOL OSR52) | 4.23 | 3.76 to 4.75 |
| LEWAF | 19.7 | 18.5 to 20.9 |
| Marine Gas Oil 1:40 OWR | | |
| CEWAF (10% FINASOL OSR52) | 1.55 | 1.33 to 1.82 |
| LEWAF | 43.9 | 37.9 to 50.9 |
| IFO180 Bunker Fuel 1:40 OWR | | |
| CEWAF (10% FINASOL OSR52) | 4.56 | 4.28 to 4.86 |
| LEWAF | 59.35 | 51.2 to 68.9 |
| FinaSol OSR52 1:400* DWR | | |
| HEWAF | 0.640 | 0.546 to 0.749 |

* Corresponding to 10% by mass in 1:40 of Oil:SeaWater, that is the same mass ratio as in the CEWAF

The results show that *C. finmarchicus* displayed a higher sensitivity to the naphthenic North Sea crude oil than to both MGO and IFO180. Naphthenic North Sea crude oil was ca. 2.2 and 3.0 times more toxic to MGO and IFO180, respectively. The CEWAF experiments showed that the dispersant enhanced the toxicity of the oil by 4.7, 18.3 and 13.0 times for the NA naphthenic oil, the MGO and the IFO180, respectively. Thus, the toxicity of the CEWAF of the MGO was highest, whereas the toxicities of NA naphthenic and IFO180 oils were similar. Finasol OSR® 52 alone had a much higher lethality to *C. finmarchicus* than the oils in LEWAF and CEWAF systems. The results for CEWAF oil droplets distribution (e.g. MGO) show that although the total number of particles did not change considerably during the exposures (Figure 2), the oil droplet mass (Figure 3) was substantially different between the beginning and end of the experiment.

It is already known that the addition of chemical dispersant can increase the toxicity of oil to aquatic organisms (Hansen et al., 2012; Coelho et al., 2013). However, it is difficult to quantitatively answer the question, if the increase of toxicity is due to the intrinsic dispersant toxicity, the higher dissolution of hydrocarbons (through faster partitioning kinetics of oil components in dispersed oil), or higher availability of oil droplets to organisms following chemical treatment. In CEWAF the dispersant itself seems to be less toxic than the dispersant alone, which could be the result of its lower availability to the organism when combined with oil. In addition, the application of static system for CEWAF results in a formation of oil dispersion which is unstable as can be seen for MGO CEWAF (Figure 2 and 3). Therefore, comparing the toxicity of chemically dispersed oil versus naturally dispersed oil should NOT be used to make recommendations for or against using dispersants

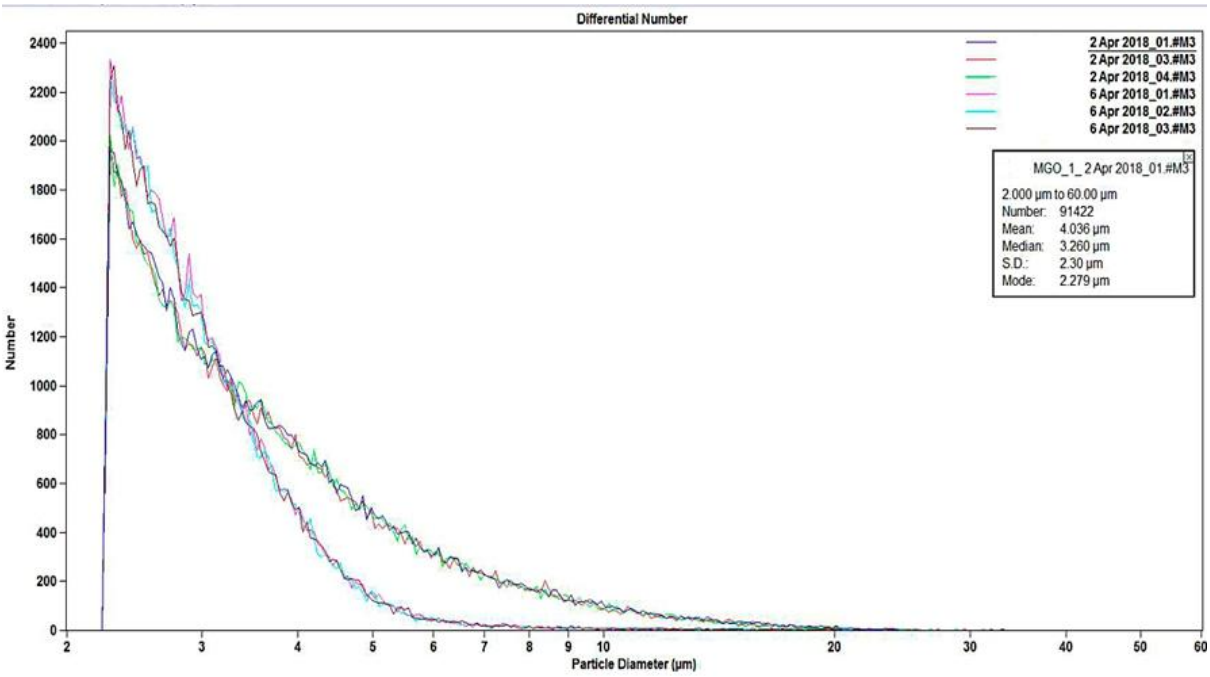


***Figure 2***. Oil droplet size distribution (volumetric).

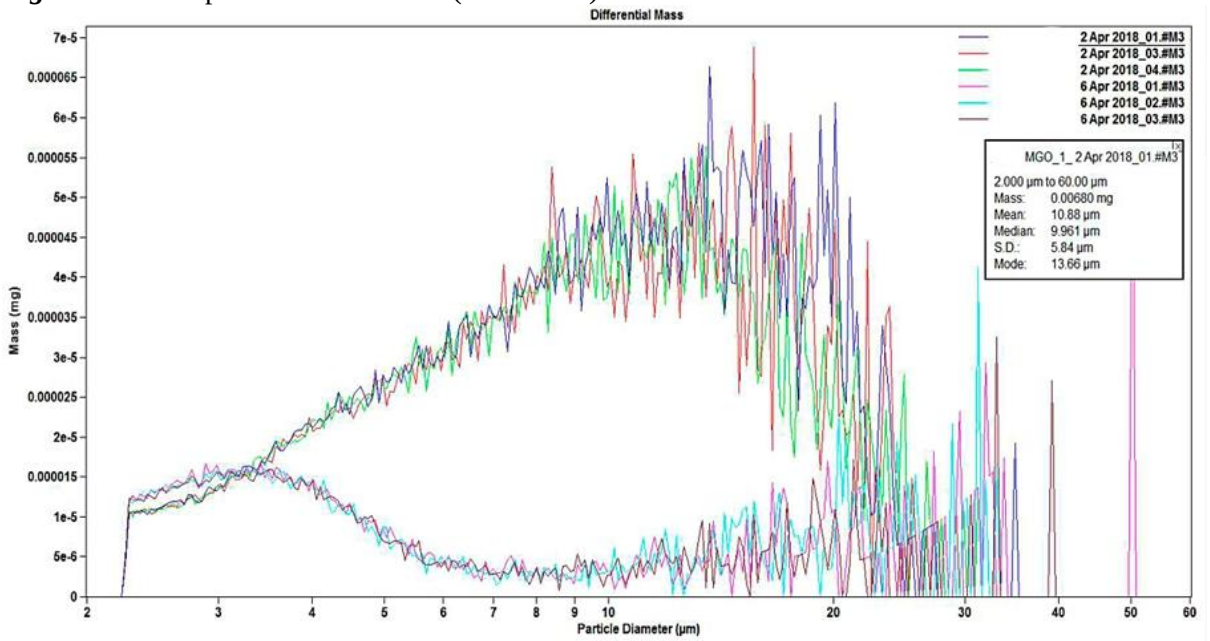


***Figure 3***. Oil droplet size distribution (volumetric).

In addition, while the dispersant will temporarily increase exposure and effects of oil to the organisms in the water column, it will also reduce exposure and effects at the water surface and potentially at the coastlines. Therefore, before the final recommendations on oil toxicity on application of dispersant after oils spill in the Arctic can be made, the results should be evaluated in the Net Environmental Benefit Analysis (NEBA) process.

### 3.3 Sub-lethal effects

Although lethality is important for comparing direct oil toxicity with respect to lethal effects of different types, organisms in the environment are also be exposed to concentrations that are sub-lethal, and can affect their long-term survival and fecundity. Thus, to evaluate the ecological relevant total effects of oilspills, there is also need for knowledge on biomarkers that can provide information about sub-lethal effects in key marine species. Thus, sub-lethal effects were

investigated in order to identify biological response parameters that can provide information on such effects in *C. finmarchicus* and *L. macurus.*

### *3.3.1 Calanus finmarchicus*

So-called biomarkers, which are quantifiable molecular or physiological variables in organisms that respond to stress, are applied to assess effects of oil pollution in marine invertebrates. In the last decades oxidative stress has been widely used as a biomarker for xenobiotic exposure in aquatic organisms (Valavanidis et al., 2006). Despite importance of planktonic species in the marine ecosystem, little is known about oxidative stress pathways in these organisms. Thus, to investigate stress responses as biomarkers in *C. finmarchicus* for environmental risk assessment, time-dependent responses of well-known biomarkers and additional novel biomarkers of oil exposure and oxidative stress in copepods were evaluated in *C. finmarchicus* exposed to the WAF of a naphthenic crude oil. The animals were exposed to concentrations corresponding to 50% of the $LC_{50}$ value for 96h.

*Gene expression studies in C. finmarchicus*

The gene expression results showed some significant, however inconsistent responses with both up- and downregulation of GST-2 at 48 and 72 hours and downregulation of SOD at 72 hours (Figure 4). Significant differences in gene expression among time points were also detected for *cat* and *cyp330*, although no significant differences were observed between control and exposed groups (data not shown). *ub, hsp70, hsp90, gss, gst-I and gst-II* modulation did not appear to be related neither with time, nor with the treatment. Thus, the results from the present study indicate that gene expressions of all investigated stress-related biomarkers were not suitable for assessing stress-related effects in *C. finmarchicus*. As discussed below, interestingly this contrasts findings in some previous studies.

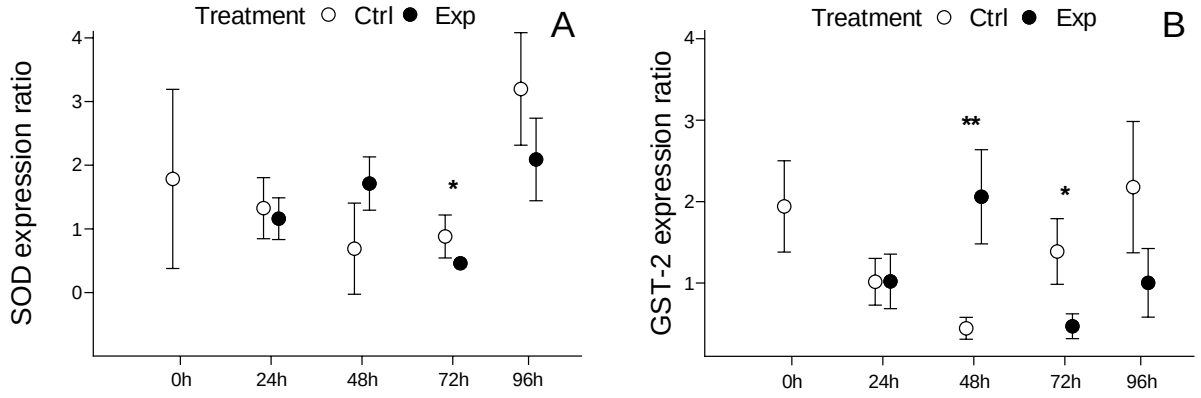


***Figure 4*:** Pfaffl expression ratio of (A) SOD (*sod-I*) and (B) GST (*gst-III*) in the exposed and control group over time. Values are presented as the mean ± SE for each sampling time point. * = $p<0.05$; ** = $p<0.005$

***Table 5***. Gene expression (GST, SOD, CAT, CYP330A1) responses in *C. finmarchicus* exposed to different crude oil-derived medium. The main experimental parameters are listed for each study and only significant results are presented.

| Exposure medium | Dominating HCs | Experimental set-up | Life-stage | Responses | Ref. |
|---|---|---|---|---|---|
| Naphthalene | | static; starvation; 0.5, 5, and 50% of $LC_{50}$ | adult | **GST**: concentration-dependent ↑ at 24 and 48 h; **CYP330**: ↓ at all time points; **SOD, CAT**: ↑ only at 12 h at *lc*; **CYP1A2**, **HSP70**, **HSP90**, **UB**: modulation not related with the treatment | *1* |
| WSF North Sea oil | C1-C4 naphthalene | flow-through; feeding; dilution series | adult | **GST**: ↑ in both poor and lipid rich individuals; **CYP330A**: ↑ in lipid rich and ↓ in lipid poor individuals | *2* |
| WAF artificially weathered crude oil | C1-C4 naphthalenes, phenols | static; starvation; 0.5, 5, and 50% of $LC_{50}$ | CV | **GST**: ↑ only at hc | *3* |
| WSF marine diesel | C1-C4 naphthalenes, phenols | static; starvation; 0.5, 5, and 50% of $LC_{50}$ | CV | **GST**: ↑ at 12 h at *hc*, ↑ at 24 at *hc* and *mc*, ↑ at 48 h at all concentrations | *4* |
| WAF naphthenic North Atlantic crude oil | C1-C4 naphthalenes | static; starvation; 50% of 96 h $LC_{50}$ | non-ovulating adult | **GST:** ↑ at 48 h and ↓ at 72 h; **SOD**: ↓ at 72 h; **CAT, CYP1A2, GST, CYP330, HSP70, GSH, HSP90, UB:** modulation not related with the treatment | *5* |

CV: fifth copepodite stage; *lc, mc, hc*: low, medium and high concentrations respectively. ↑: upregulation; ↓: downregulation; 1: Hansen et al., 2008; 2: Hansen et al., 2009; 3: Hansen et al., 2011; 4: Hansen et al., 2013.a; 5: Present study

Different previous studies exposing *C. finmarchicus* to oil or oil compounds have indicated that oxidative stress and lipid peroxidation are probable toxic endpoint, but only GST gene expression was found to be fairly reliable biomarker (Hansen et al., 2008; 2009; 2011; 2013). Contrarily, experimental exposure to hydrogen peroxide, a well-known ROS, did not cause oxidative stress in late-copepodite stage of *C. finmarchicus* (Hansen et al., 2017). Several studies have investigated oil-induced modulation of stress gene expression in *C. finmarchicus* (Table 5), and consistently shown increased GST and reduced CYP330A1 transcription in this species (Hansen et al 2008, 2009, 2011 and 2013). Despite the other studies (Hansen et al. 2008 and 2013) reported GST transcription being up-regulated in a concentration-dependent manner, based on our results it can be concluded that gene expression cannot unequivocally support the use of GST as reliable biomarker of oxidative stress in *C. finmarchicus*.

*Enzymatic activities, tGSH and MDA determination C. finmarchicus*

An increased tGSH level in the exposed group was detected at 24, 72 and 96 h, but due to the large variation of tGSH in control groups the only significant differences were found at 96 h (Figure 5B). The increase in MDA concentrations (Figure 5C) and the induction of GST (Figure 5A) in the exposed groups seem more reliable and consistent throughout time, even though, due to large variation within single groups, differences were not significant at 96 h for MDA and at 72 h for tGSH.

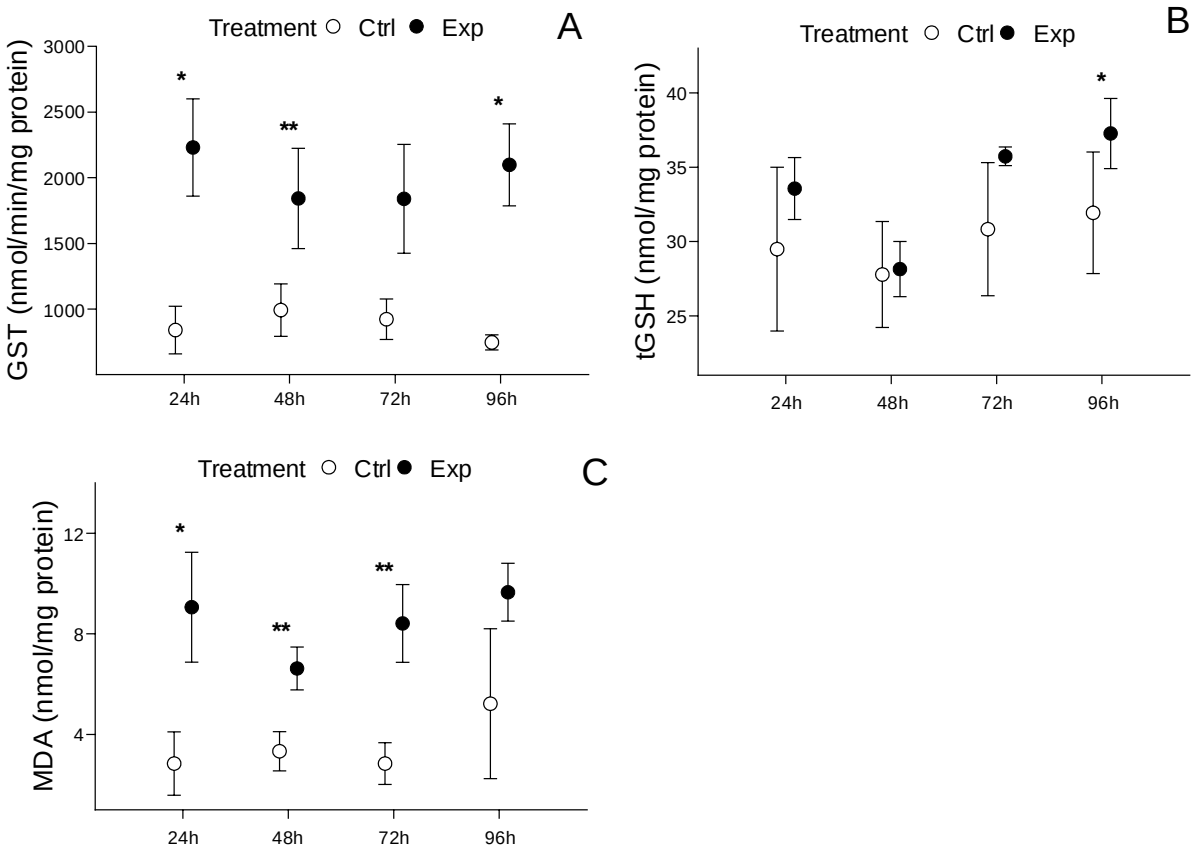


***Figure 5***: Enzymatic activity of (A) GST, (B) tGSH and C (MDA) expressed as nmol/min/mg protein, in the exposed and control group over time. Values are presented as the mean ± SE for each sampling time point. * = $p<0.05$; ** = $p<0.005$

Nevertheless, the induction of GST and increase in MDA and GSH levels clearly indicates the occurrence of WAF induced oxidative stress. Glutathione contributes to the regulation of cell cycles and in addition is involved in reduction and conjugation reactions which are crucial in neutralization of ROS and other xenobiotic electrophiles (Meister, 1992). The conjugation of reduced GSH molecules with electrophilic compounds is catalysed by different enzymes belonging to a family of gluthatione-S-transferases (GSTs) (Sharma et al., 2004). In *C. finmarchicus*, GST is thought to handle lipid peroxidative end products and therefore it has been suggested as potential biomarker for lipid peroxidation (Hansen et al., 2008; 2009). Malondialdehyde (MDA) is however generated by the oxidation of polyunsaturated fatty acids and by degradation of pre-existing oxidation products. Therefore, MDA has been proposed as an indicator of lipid peroxidation (Draper et al., 1990). Given the induction of GST and high concentrations of MDA detected in the exposed group in this study, lipid peroxidation is confirmed as a major toxic endpoint following WAF exposure, in accordance with Hansen et al. (2008, 2009, 2011). Lipid peroxidation might be highly detrimental

for *C. finmarchicus* survival and reproduction, considering the importance of lipids in its life cycle (Irigoien, 2004; Hansen et al., 2008). For instance, cholesterol depletion induced by lipid peroxidation, could affect steroidogenesis, causing endocrine disruption (Hansen et al., 2008).

The contrasting results obtained from gene expression compared to enzymatic activity analyses and GSH concentration 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. In marine organisms, these mechanisms are not-well understood due to a lack of data and comparisons between mRNA transcription and antioxidant enzymatic activities and therefore studies often show contrasting trends (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 antioxidants (e.g. astaxantine) might play a major role in antioxidant responses in *C. finmarchicus* exposed to oxidative agents.

Reproducing realistic environmental conditions of oil spill in a laboratory experiment is a difficult task and the results obtained from laboratory must be treated with care when extrapolating to real oil spill scenarios. The LEWAF (generated using low energy), as applied in the present work, is considered a droplet-free water-soluble (dissolved) fraction containing no, or to a very limited extent, particulate oil droplets. Organisms subjected to a real oil spill will be affected not only by water soluble components, but also by oil slicks, oil droplets, oil dispersants as well as other additional stressors e.g. direct natural sunlight (Duesterloh et al., 2002). The dissolved fraction is generally believed to be responsible for the toxicity of oil components due to its bioavailability. However, ample evidence exists that copepods filter oil droplets, suggesting an additional route of exposure (Hansen et al., 2009, 2017, Nordtug et al., 2015). In fact, modelling efforts have predicted that *C. finmarchicus*, due to their wide distribution and high filtration rates, can affect the total mass balance of oil spills (Nepstad et al., 2015). Taking into account the widespread distribution of *C finmarchicus* across the northern North Atlantic (Hirche, 2007), small oil spills are not likely to harm the entire NA population, but can act as local additional stressor. Nevertheless, the existence of reliable biomarkers of oil exposure is fundamental for monitoring programs and decision-making processes in realistic scenario of environmental oil spill or continuous and ongoing discharges of oil (e.g. produced water).
The results from the present study suggest that GST enzymatic activity and MDA concentrations are reliable biomarkers of oxidative stress in *C. finmarchicus* exposed to a WAF of a naphthenic NS oil, while gene expression results were inconsistent. Moreover, lipid peroxidation is confirmed as a major toxic endpoint. Our exposure setup, even though not representing a full spectrum of realistic environmental conditions, allows a high degree of repeatability and control of confounding factors in laboratory conditions, to detect direct effects of oil exposure. Moreover, this project provides new data contributing to the understanding of zooplanktonic responses to oil exposure.

*Metabolomic biomarkers in C. finmarchicus*

A total of 27 metabolites were quantified with the $^{1}$H-NMR spectroscopy technique. Sixteen out of the total 27 detected metabolites were detected with statistically significant changes in the concentration after the exposure. The concentrations of some metabolites, such as proline, were significantly lower in the WAF exposed animals than in the control animals (Figure 6A). Similarly, the organic acid malonate concentration decreased during the experiment in control animals, but increased in the WAF exposed animals (Figure 6B). These changes could be explained by the combined effects of oil and starvation. Some aminoacids, like proline, can be selectively metabolised and used as an energy source during starvations. Malonate is a competitive inhibitor of

cellular respiration since it binds to the active site of the succinate dehydrogenase in the citric acid cycle. Therefore, observed changes suggests that the exposure to the WAF most likely affected the citric acid cycle related to energy metabolism.

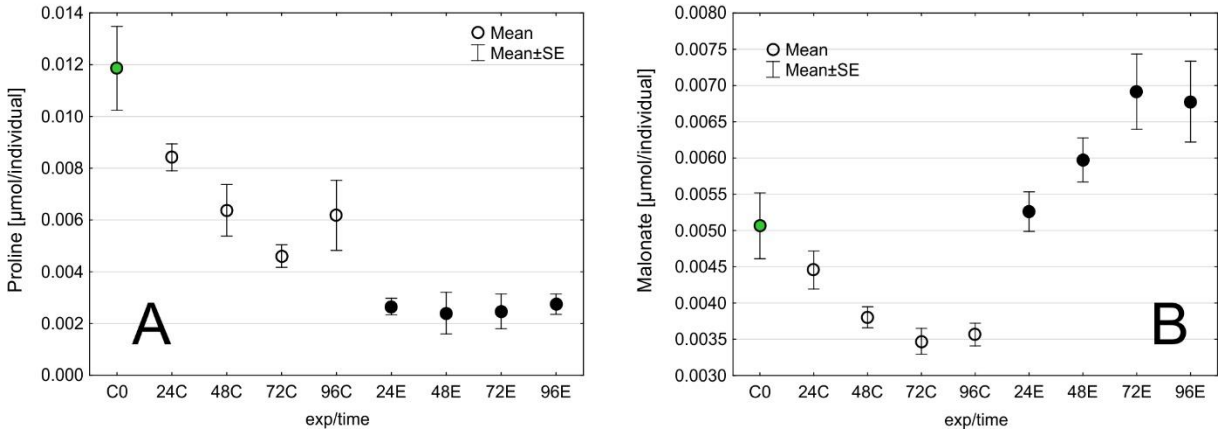


***Figure 6***: Change in concentration of proline (A) and malonate (B) in *C. finmarchicus* in the control (open circles) and exposed (closed circles) groups over time. Values are presented as the mean ± SE for each sampling time point. Green circle (C0) indicates control samples collected at the beginning of the experiment (that is not exposed and fed).

Metabolome changes suggest that exposure to naphthenic North Sea oil WAF might impair energy balance in *C. finmarchicus* and, e.g. malonate and proline concentrations seem to be a promising biomarker of oil exposure. Field studies are needed to confirm and test the proposed biomarkers and metabolome changes in the real scenerio of an oil spill.

### *3.3.2 Limnocalanus macrurus*

*Sub-lethal effects*

Increased SOD activity and decreased GPx activity in WAF and CEWAF treatments compared to control treatment indicate exposure-mediated effects to the antioxidant system. However, no change in CAT, GST or GR activities was observed. There were no differences in the LPx between treatments and small increase in the GSH/GSSG ratio was seen in the WAF treated organisms, both of which indicate that organisms were able to compensate the possible ongoing oxidative challenge.

Chemical analysis showed that the concentrations of polycyclic aromatic hydrocarbons (PAHs) were lower than expected; at the beginning the total PAH concentration in the exposure media was 0.109 µg/L in WAF and 0.311 µg/L in CEWAF treatments. After 96h the PAH concentrations were 0.091 and 0.038 µg/L respectively.

All applied methods worked well for *L. macrurus*, also the sampling and running the experiment went according to plan. Most likely the exposure concentrations were too low to induce clear differences between treatments in most of the measured biomarkers.

## 4 Comparison of acute toxicity of crude oils, refined products and chemically treated petroleum oils in boreal and Arctic zooplankton species and *Acartia tonsa*

Oil released into the sea during an oil spill will form surface oil slicks and/or be dispersed in small oil droplets with dissolved water-soluble oil components in the water column. The proportion of the phases varies with time, the type of oil, the size and depth of the release, and physical parameters such as temperature, light conditions, wind and currents (Nordtug et al. 2011). In general, much

attention has been focused on the oil itself due to its intrinsic physical properties, and the dissolved fraction because of its bioavailability. For research on the dissolved components of oil fraction, the WAF is commonly used to study toxicity in marine organisms. WAF is defined as a laboratory-prepared medium derived from low energy mixing of a poorly soluble test material (e.g., an oil or petroleum product) which is essentially free of particles of bulk material. Therefore, WAF represents the water-soluble oil compounds (e.g. BTEX, naphthalenes, PAHs, phenols and polar components) with high bioavailability for marine organisms and is relevant for studying acute oil spills toxicity.

For chemically dispersed oils, the term CEWAF is used, also to describe the dissolved fraction of the chemically treated oil. Thus, as for WAF, CEWAF is a laboratory-prepared medium. Enhancing the dispersion process chemically will increase the oil concentration temporarily and, as shown for the oil-types investigated in the present study result in higher toxicity, caused by faster increasing concentrations of bioavailable BTEX compounds in addition to possible toxic compounds in the chemical dispersant, for pelagic organisms. The toxicity of dispersed oil to pelagic organisms is a critical component in evaluating the net environmental consequences of dispersant use or non-use in open waters. Therefore, the CEWAF is used to study the effect of dispersed oil.

A review of sensitivities (i.e. $LC_{50}$ values) of *A. tonsa*, boral and Arctic copepods revealed an almost coherent lack of comparable data (Table 6). This because previous studies have used a variety of methods to assess the acute and chronic toxicity of crude oil WAF by applying various water and oil combinations to produce the WAF. Moreover, different exposure systems e.g. static vs. flow-through systems were applied. Also, in spite of the importance of reporting information on acute toxicity of petroleum oil and chemically treated oil in copepods, there are very limited numbers of acute toxicity ($LC_{50}$) values for Arctic and boreal marine zooplankton species reported in scientific literature. This makes comparisons between animal species and oil types difficult. Nevertheless, based on reported science-based data, some indications on species differences can be made, and thus some valuable information related to the relative sensitivity of Arctic copepods can be provided.

***Table 6***. Comparison of acute toxicity data ($LC_{50}$) for *Acartia tonsa* (temperate coastal and estuarine species) and Arctic zooplankton species. The upper part of the table presents data on $LC_{50}$ in low energy WAF (LEWAF) experiments, whereas the bottom part of the table presents data on CEWAF experiments on chemically treated oil types.

| Species | Exposure medium | WAF o:w | Life-stage | $LC_{50}$ | Ref. |
|---|---|---|---|---|---|
| *A. tonsa* (culture) | LEWAF<br>Argentinean light crude oil (HYDRA oil) | 1:10 o:w ratio | adult females and males | <<br>69.5 µg/L (24 h)<br>48.0 µg/L (48 h)<br>males<br>84.4 µg/L (24 h)<br>70.1 µg/L (44 h) | *1* |
| *A. tonsa* | OSPAR method and<br>continuous flow system (CFS) WAFs<br>Blended Arabian Light*<br>Topped at 150 ºC | various o:w rations | - | OSPAR: No toxicity for oil loads up to 10 000 mg/L (48 h)**<br>CFS (100 mg/L)** | *2* |
| *C. finmarchicus* (culture) | LEWAF<br>naphthenic crude oil from the North Sea<br>weathered 200 ºC | 1:40 o:w ratio | copepodite V | 0.817 µg/L (96 h) THC | *4* |
| *C. finmarchicus* (culture) | LEWAF<br>naphthenic crude oil from the North Sea<br>weathered 200 ºC | 0.25 mg oil/L,<br>1.79 mg oil/L 12.5 mg oil/L | mainly copepodite V | 801.4 µg/L (96 h) THC | *3* |
| *C. finmarchicus* (culture) | LEWAF<br>marine diesel | 1:40 o:w ratio<br>1: 10 000 o:w ratio | copepodite V | 1600 µg/L (96 h) THC<br>799 µg/L (96 h) THC | *5* |
| *C. glacialis* (Kongsfjord, Ny-Ålesund, Svalbard) | LEWAF<br>naphthenic crude oil from the North Sea<br>weathered 200 ºC | 1:40 o:w ratio | copepodite V | 1.037 µg/L (96 h) THC | *4* |
| *C. glacialis* (Kongsfjord, Ny-Ålesund, Svalbard) | LEWAF<br>marine diesel | 1:40 o:w ratio<br>1: 10 000 o:w ratio | copepodite V | 9630[a] µg/L (96 h) THC<br>2270[b] µg/L (96 h) THC | *5* |
| *A. tonsa* | Continuous flow system CEWAF<br>Blended Arabian Light<br>Topped at 150ºC<br>+ dispersant Arkopal N-060 | various o:w rations<br>5% dispersant | - | 17-46 mg/L (48 h)** | *2* |
| *A. tonsa* | Continuous flow system CEWAF<br>Blended Arabian Light<br>Topped at 150ºC<br>+ dispersant Sophorolipid SO 93 12 F | 5% dispersant | - | 120 µg/L (48 h)** | *2* |
| *C. finmarchicus* (culture) | CEWAF<br>naphthenic crude oil from the North Sea<br>weathered 200 ºC<br>dispersant Dasic NS | 0.25 mg oil/L,<br>1.79 mg oil/L 12.5 mg oil/L 4% w/w dispersant | mainly copepodite V | 490 µg/L (96 h) THC | *3* |

1: Avila et al., 2010; 2: Skadsheim et al., 1996, 3: Hansen et al., 2012; 4: Hansen et al., 2011; 5: Hansen et al., 2013; *light oil comparable to some North Sea crudes, **the concentrations refer to WAF load (the amount of oil mixed into the sea water), [a]extrapolated as the LC concentrations were above 100% WSF concentration

[b]almost no mortality was observed in this test, so heavily extrapolated and unreliable data

The reported marine zooplankton $LC_{50}$ values for WAFs (Table 6) showed a wide range of toxicity, dependent on different oil types and their treatment, species, test period, and experimental setup. For instance, previously reported 96 h $LC_{50}$ values for *C. finmarchicus* exposed to weathered naphthenic NS oil varies from 0.817 to 801.4 µg/L (Hansen et al. 2011, 2012). Nevertheless, in *C. finmarchicus* the $LC_{50}$ concentrations of the CEWAF of weathered naphthenic NS oil were lower (490 µg/L) than the $LC_{50}$ concentrations of the LEWAF of weathered naphthenic NS oil (801.4 µg/L) (Hansen et al. 2012). This confirms the results from the present study that chemical treatment of naphthenic NS cude oil enhances the toxicity of oil in *C. finmarchicus* in laboratory exposure scenario. Furthermore, a comparative study indicated that *C. finmarchicus* gave a somewhat lower $LC_{50}$ when exposed to the LEWAF of weathered naphthenic NS crude oil as compared to the Arctic *Calanus glacials,* of 0.817 and 1.037 µg/L, respectively (Hansen et al. 2011). Also, when comparing exposures to the LEWAF of a marine diesel oil in *C. finmarchicus* and *C. glacialis*, the results showed that the $LC_{50}$ for *C. finmarchicus* were lower than that for *C. glacialis* (799-1600 vs. 2270-9630 µg/L, respectively). Thus, the data reported in the literature indicates that the sensitivity of *C. finmarchicus* to the WAF of petroleum oil may be higher than that of the Arctic *C. glacialis.* This could be related to the differences between *C. finmarchicus* and *C. glacialis* in storage of lipids

and subsequent variation in accumulated oil substances and their release from the lipid sack and also increased internal load when lipids are mobilized during periods when there is no food. A large lipid reservoir in Arctic species like *C. glacialis*, may protect against short-term acute toxicity if the oil compounds accumulates directly in the lipid reservoir, and thus became temporarily immobilized.

The literature data on $LC_{50}$ values for *A. tonsia* are highly divergent, which probably mainly is due to the exposure of highly differing age stages (Table 6). It should also be noted that $LC_{50}$ values for *A. tonsia* are reported based on 24-48h exposure experiments, as opposed to 96h experiments in the studies on *C. finmarchicus* and *C. glacialis*. This makes trustful comparisons to *C. finmarchicus* and *C. glacialis* impossible. Nevertheless, it seems that also for *A. tonsia* exposure to CEWAF increases the toxicity of the oil (Avilia et al. 2010, Skadsheim etl. 1996).

In summary the data reported in the literature indicates that the sensitivity of *C. finmarchicus* to the WAF of petroleum oil may be higher than that of the Arctic *C. glacialis*. Although the literature data indicates that chemical treatment of crude oil enhances the toxicity of crude oil in *A. tonsa*, as shown for *C. finmarchicus* in the present study, it was not possible to retrieve data in the scientific literature that would provide a meaningful comparison of species differences in toxicity to oil and chemically treated oil in these two species.

# 5 Conclusions

- *C. finmarchicus* displayed a higher sensitivity to the NS crude oil than to the MGO and the IFO180. Both the MGO and IFO180 were found to be ca. 2-3 times less toxic than the NS crude oil. The CEWAF experiments showed that the dispersant enhanced the toxicity of the oil by 4.7, 18.3 and 13.0 times for the naphthenic NS oil, the MGO and the IFO180), respectively. Thus, the toxicity of the CEWAF of the MGO was highest, whereas the toxicities of naphthenic NS crude oil and IFO180 oils were similar. Finasol OSR® 52 alone had a much higher lethality to *C. finmarchicus* than the oils. However, due to the dynamic complexity of oil/water dispersions and subsequent changes in the toxicity of both oil and the dispersant in laboratory static systems, it can be concluded that the direct toxicity comparison of chemically dispersed oil versus naturally dispersed oil should not be used to make recommendations for or against using dispersants. Therefore, before the final recommendations on oil toxicity on application of dispersant after oils spill in the Arctic can be made, the results should be evaluated in the Net Environmental Benefit Analysis (NEBA) process. It should also be noted that the present study was performed using a laboratory culture of *C. finmarchicus*. Thus, care should be taken to directly extrapolate the results to populations in the wild.

- While gene expression results were inconsistent, GST enzymatic activity and MDA concentrations appear to be reliable biomarkers of oxidative stress in *C. finmarchicus* exposed to oil. Moreover, lipid peroxidation is confirmed as a major toxic endpoint following exposure. Metabolome changes suggest that exposure to naphthenic North Sea oil WAF might impair energy balance in *C. finmarchicus*. Malonate and proline concentrations seem to be promising novel biomarkers for detecting sub-lethal effects of oil exposure in *C. finmarchicus*. In *L. macrurus* increased SOD and decreased GPx enzymatic activities and indications of an increase in the GSH/GSSG ratio were identified following exposure to the WAF and the CEWAF of the oil types. This confirms that exposure to sublethal concentrations caused oxidative stress in both *L. macrurus* and *C. finmarchicus*, and that enzyme activities related to oxidative stress responses may be suitable biomarkers for identifying sublethal effects of oil pollution in calanoids.

- A review of sensitivities (i.e. $LC_{50}$ values) of *A. tonsa* and other Arctic copepods revealed an almost coherent lack of comparable data. However, based on the available studies it seems that the sensitivity of *C. finmarchicus* to the WAF of petroleum oil may be higher than that of the Arctic *Calanus glacialis*. Although the literature data indicates that chemical treatment of crude oil enhances the toxicity of the oil in *A. tonsa*, as shown for *C. finmarchicus* in the present study, it was not possible to retrieve data in the scientific literature that would provide a meaningful comparison of species differences in toxicity to oil and chemically treated oil in these two species.

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