NTNU

Norwegian University of
Science and Technology

Biomarkers of oxidative stress in Calanus finmarchicus exposed to a water accommodated fraction of a naphthenic North Atlantic crude oil

Sofia Soloperto

Environmental Toxicology and Chemistry
Submission date: May 2018
Supervisor: Bjørn Munro Jenssen, IBI

Norwegian University of Science and Technology
Department of Biology

Sofia Soloperto

Biomarkers of oxidative stress in *Calanus finmarchicus* exposed to a water accommodated fraction of a naphthenic North Atlantic crude oil

Master's thesis in Environmental Toxicology and Chemistry

Trondheim, May 2018

Supervisor: Bjørn Munro Jessen
Co-supervisor: Tomasz Maciej Ciesielski
Co-supervisor: Elise Skottene

Norwegian University of Science and Technology
Faculty of Natural Science
Department of Biology

Acknowledgments

This master thesis was written at the Department of Biology, Norwegian University of Science and Technology (NTNU) in Trondheim, 2018, and was financed by GRACE project (Integrated Oil Spill Response Action and Environmental Effect), supported by the EU Program Horizon 2020 under the grant agreement no. 679266.

I would like to thank my main supervisor, Bjørn Jessen, for his exhaustive help in the final stages of my master thesis. A big thanks to Elise Skottene for guiding me through the lab work and for being always present and helpful. Thanks to Dag Altin for his priceless help during the experimental phase, and to Torfinn Sparstad and all the Post Doc and PhDs students working in his lab for listening to my thousands questions and helping me during the lab work. A special thanks goes to Tomasz Maciej Ciesielski for his endless support and trust; you made the whole journey a lot funnier! A big thanks to Berta and Ieva for our late-night, semi-scientific discussions that made us feel like real scientists.

Finally, a big thanks to my family and all my friends, near and far, for always being there!

Abstract

Calanus finmarchicus is an ecologically important species in the North, Norwegian and Barents Seas, periodically constituting up to 90% of the standing stock of zooplankton. Due to continued development of areas for oil and gas production, there are environmental discharges of contaminants occurring both continuously and accidentally in these areas. Existence of reliable biomarkers for oil exposure are fundamental for monitoring programs and decision-making processes in case of environmental discharges and oil spills. To assess the potential negative effects of oil exposure on keystone component of marine ecosystems, adult females of *Calanus finmarchicus* were exposed to a water-accommodated fraction (WAF) of a naphthenic North Atlantic crude oil. Adult non-ovulating females from the continuous lab culture at SINTEF/NTNU Sealab, were exposed without feeding to sub-lethal concentration of WAF in seawater and collected at 5 different time points: 0, 24, 48, 72 and 96 hours. Several oxidative stress biomarkers were tested with gene expression (qPCR), enzymatic activity analyses, determination of glutathione (GSH) and lipid peroxidation assay for malondialdehyde (MDA) concentration. Our results demonstrated induction of glutathione *S*-transferases (GST) and increase of GSH and MDA concentrations in the exposed group at each time point sampled. Gene expression results showed inconsistent responses with both up- and downregulation of GST at 48 and 72 hours and downregulation of superoxide dismutase (SOD) at 72 hours. Our study indicates that GST enzymatic activity, and GSH and MDA concentrations can be applied as effective biomarkers of oxidative stress in *C. finmarchicus*, while gene expression of SOD and GST are less suitable.

Table of Content

List of Figures

List of Tables

Abbreviations

Act	Actine
AhR	Aryl Hydrocarbon Receptor
BTEX	Benzene, Toluene, Ethylbenzene And Xylene
CAT	Catalase
CYP1A2	Cytochrome P450 Family 1 Subfam. A Member 2,
CYP330A1	Cytochrome P450 Family 330 Subfam. A Member 1
EFA1α	Elongation factor A1α
GPx	Glutathione Peroxidases
GSSG	Glutathione Disulfide
GSH	Glutathione
GST	Glutathione *S*-Transferase
GST-2	Glutathione *S*-Transferase Subfamily-2
HO_2	Hydroperoxyl
HSP70	Heat Schock Protein 70
HSP90	Heat Schock Protein 90
LC_{10}	10% Lethal Concentration
LC_{50}	50% Lethal Concentration
LC_{90}	90% Lethal Concentration
MDA	Malondialdehyde
MM	Master Mix
NA	North Atlantic Ocean
O_2	Superoxide
$^{\bullet}OH$	Hydroxyl
PAHs	Polycyclic Aromatic Hydrocarbons
PP	Peroxisomal Proliferators
PPARα	Peroxisomal Proliferator Activated Receptor Alfa
RO_2	Peroxyl
ROS	Reactive Ossigen Species
SOD	Superoxide Dismutase
SVOC	Semi-Volatile Orgnic Compound
UB	Ubiquitin
VOC	Volatile Organic Compound
WAF	Water-Accomodated Fraction
WSF	Water-Soluble Fraction

1 Introduction

The marine environment is subjected to several anthropogenic threats among which, global warming, ocean acidification, unsustainable fishing and chemical pollution are of major importance. All together, these factors act as potent stressors to marine ecosystems, degrading a wide range of habitats and ecosystem services. Regarding pollution, the main source, accounting for the 44% of the total load, is runoff and discharges from land, followed by airborne emission and shipping and accidental spills (Potter, 2013).

The marine ecosystem provides to humans a vast range of *free* services as carbon sequestration, pollution mitigation, shoreline stabilization, and recreational areas. The biotic compartment, moreover, is an essential source of human food and countless economic activities and enterprises worldwide depend on fish and shellfish stock. The marine ecosystem hence, is not only of importance *per se,* but its deterioration implies huge economic and social repercussions. The North Atlantic is one of the most productive oceanic areas and is severely affected by anthropogenic activities (UNEP, 2008). Due to global warming and consequent sea ice loss, shipping and oil field's explorations in the circumpolar region are expected to increase, becoming additional stressors to the arctic and sub-arctic marine environment (Borgerson, 2008). 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 to marine biota have been deeply studied in the past decades due to their destructive short-term effects. Although much is known about acute and short-time effects on mammals, seabirds and mussels in temperate seas, fewer data are available regarding potential chronic effects on other organisms and spill consequences in colder environments (Marigomez et al., 2017).

1.1 Marine oil pollution

Oil industry boomed after the development of the internal combustion engine at the beginning of the 20th century, leading to the onset of offshore oil explorations. Since then, petroleum demand kept on increasing dramatically, and today it represents one of the most problematic environmental challenges of our time. In 2015, consumption and production of crude oil globally reached 95,345 and 96,707 thousand barrels per day respectively (1 barrel = 158.98 L) (EIA, 2017). Anthropogenic sources of petroleum release add up to the naturally occurring seeps increasing the amount of oil discharged in the ocean. For example, natural seeps occur in

coastal regions in south Alaska, where they represent a minor release source, and in the Gulf of Mexico and California, where offshore seeps are a major source of oil release (Transportation Research Board and National Research Council, 2003). The benthic community seems to have acclimated to those natural oil seeps, eventually utilizing hydrocarbons as a source of energy (Spies et al., 1983; Montagna et al., 1985, 1989). Extraction of petroleum generates unwanted oil release throughout platform accidental spills and produced water (90%of total petroleum input) but it accounts only for the 2% of the total amount released by natural seeps (Transportation Research Board and National Research Council, 2003). Table 1 shows an estimation of the amount of petroleum released annually in the oceans, considering data from 1990 until 1999. Accounting for the increase in oil consumption and production in the last decades (EIA, 2017) the total amount of oil released at present day is likely to be much higher. In addition to this data is worth mentioning that intentional illegal discharges of oil from ships is thought to account for the 46% of total oil release in the ocean, representing a problematic source to manage and control (Transportation Research Board and National Research Council, 2003).

Table 1: Average annual releases (1990-1999) of petroleum in thousand of tonnes. After: Transportation Research Board and National Research Council, 2003.

Release sources worldwide	**Best Estimates**	**Minimum**	**Maximum**
	(thousand of tonnes)		
Natural seeps	600	200	2000
Extraction of petroleum	38	20	62
Transportation of petroleum	150	120	260
Consumption of petroleum	480	130	6000
Total	**1300**	**470**	**8300**

The number of tank vessel spills has decreased since the late ‘90s thanks to the introduction of new regulations and the development of new technologies (Marigomez et al., 2017), potentially decreasing the overall amount of oil released by transportation. On the other hand, new shipping routes in the Arctic will most likely become operative in a close future due to climate change. Sea ice shipping and potential oil spills in cold or ice-covered environments represent therefore a new environmental challenge (Marigomez et al., 2017). Moreover, the magnitude of an oil spill appears to depend more on its location rather than its size. Most spills occurred in coastal areas, which represent sensitive ecosystems, providing different habitats to a wide range of

mammals, birds, fishes and benthic species. To determine and interpret toxicological responses in marine organisms exposed to oil, it is essential to understand the physical-chemical properties of oil components and products, as well as their fate in the environment.

1.1.1 Petroleum chemical components

Petroleum, literally rock oil, comprises non-hydrocarbon and hydrocarbon compounds. Non-hydrocarbon constituents entail sulphur, nitrogen, oxygen and metallic compounds. Hydrocarbons are usually divided into saturates, olefins, aromatics, and polar compounds. The proportion of different hydrocarbon largely varies among types of oil and determines the specific physical-chemical properties of the oil mixture. Aromatics is a class of main concern; all components include at least one benzene ring which is very stable and persistent in the environment. The most volatile compounds, consisting of one single benzene ring, are classified as BTEX (benzene, toluene, ethylbenzene, and xylene). BTEX compounds are moderately soluble and seldom detected at high concentration in marine organisms (Neff, 2002). They have moderate affinity for partitioning into lipid-rich tissues (Neff, 2002) and can cause nonspecific narcosis (Boyles, 1980) and alter the permeability of cell membranes (Meyerhoff, 1975). BTEX are readily uptaken by marine organisms but can be rapidly released when individuals are placed in hydrocarbon-free environment (Nunes et al., 1979; Herman et al., 1991; Heras et al., 1992). Most laboratory studies, though, use non-environmentally relevant concentrations; in a realistic scenario, BTEX would readily volatilize in the atmosphere, exiting the marine environment. Studies on sublethal concentrations of BTEX showed an increase in the time needed to complete a molt cycle in exposed juveniles blue crabs, *Callinectes sapidus* (Cristini et al., 1984) as well as production of abnormal larvae and delay in egg's development in pacific herring (*Clupea pallasii*) and northern anchovy (*Engraulis mordax*) (Struhsaker et al., 1974). Nevertheless, BTEX seem to be more of concern in terrestrial habitat (i.e. in soils), near local sources of contaminations as leakages from gasoline stations (An, 2004) than for the marine environment. High concentrations of dissolved BTEX are generally detected in oilfield produced water (Neff, 2002).

Polycyclic aromatic hydrocarbons (PAHs) constitute another class of chemicals of main concern, including the most toxic oil compounds. PAHs are rather hydrophobic and, once released in the marine environment, tend to sorb to any organic particles, colloids, sediments or tissues of marine organisms (Knezovich et al., 1987). PAHs are not easily degradable under natural conditions and their persistence increase with molecular weight. (Haritash et al., 2009).

Indeed, they are almost ubiquitous in marine sediments worldwide, mainly in intertidal sediments, which can retain and subsequently release PAHs, acting as secondary sources of contaminations (Irvine et al., 1999). Specific PAHs have been proved to induce the cytochrome P450 (CYP450), after binding to the AhR receptor. Even though the sensitivity to PAHs varies among different species, AhR's properties and the developmental signaling pathways in the vertebrate taxa are well conserved (Billiard et al., 2008). CYP450 is responsible for the biotransformation of PAHs into more hydrophilic, hence easily excretable, compounds (Pritchard et al., 1991). Some of these metabolites are unfortunately more toxic than the parental compounds themselves. Uptake, metabolism pathways, bioaccumulation and excretion anyhow appear to be species-specific (Brummelen et al., 1998). PAHs are readily uptaken and accumulated by marine organisms but do not appear to biomagnify (Reed et al., 1995; Takeuchi et al., 2009). Concentrations of PAHs appeared to be negatively correlated with an increase in the trophic level (Takeuchi et al., 2009) and the highest PAHs concentrations are usually reported in benthic organisms inhabiting contaminated sediments (Burns et al., 1993; Kingston, 2002). PAHs toxic effects on marine organisms are the most diverse. Metabolically activated PAHs, as benzo(a)pyrene, are known to be DNA adductor, therefore, possible carcinogens (Levin et al., 1976; Denissenko et al., 2011). A positive correlation between mutagenic PAHs congeners and the occurrence of gonadic neoplastic disorders was detected in exposed mussel *Mytilus galloprovincialis* (Ruiz et al., 2011). Increased mortality, deformities and edemas were recorded in fish embryos exposed to a PAHs mixture, proving noxious effects during early vertebrate development (Barron et al., 2003).

1.1.2 Petroleum physical properties

The proportion of hydrocarbon groups does not only give an insight on the possible toxicity of the oil mixture, but also define its physical properties. High percentage of light compounds, and low amount of asphaltenes, entail low viscosity. Heavy viscous oils are more persistent since they tend to weather slower than light viscous oils, which spreads into slicks (Transportation Research Board and National Research Council, 2003). Density is commonly used to classify oils into light or heavy. Heavy oils contain high-molecule weight hydrocarbons and tend to sink in the water, while light oils, containing mainly volatile and low-weight compounds, are more likely to float on the surface and be easily weathered. Solubility is an important parameter to estimate oil toxicity since the water-soluble fraction can be readily uptaken by marine organisms.

The main concern regarding heavy oils and weathered residues is organism's smothering and coating while toxic effects appear to be of minor importance. Light oils instead have higher water solubility hence oil compounds are more bioavailable to organisms, shifting the concern towards possible chemically-induced toxic effects (ITOPF, 2011).

1.1.3 Petroleum faith in the marine environment

The fate and weathering degree of oil in the marine environment is primarily determined by weather conditions, oceanic currents and oil composition. Evaporation is the main process responsible for mass loss, and it is speeded up in warm environments rather than at higher latitudes. Emulsification processes (or water-in-oil ratio) result in density changes and increased viscosity and volume. An increase in viscosity and emulsifications increase the persistence of oil in the environment (McLean et al., 1998). Dissolution happens at a lower rate than evaporation and emulsifications due to the hydrophobicity of oil compounds, and strongly depends on the oil composition. Dissolved compounds, mainly the small aromatics, are of great importance since they are toxic to marine organism and easily bioavailable. The last main weathering process is oxidation, which results in the generation of new compounds by oxidative processes. Microbial community adaptation to heavily contaminated water results in faster hydrocarbons' biodegradation (Aamand et al., 1989), and it is considered to be the main removal mechanism in aquatic environment. Being oceans a dynamic environment, oil released in the marine environment is subjected to different transport mechanisms shown in Figure 1.

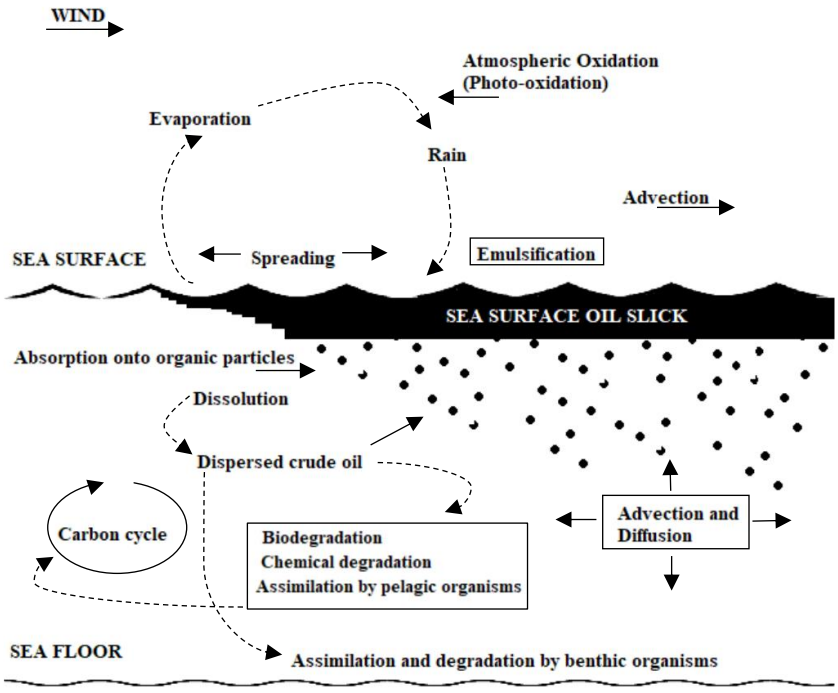

Figure 1: Open-ocean oil fate and major transport processes.

Transport mechanisms can be dived into horizontal transport, horizontal dispersion and vertical dispersion. In Figure 1, only the main parameters and mechanisms responsible for oil's fate in the environment have been reported, but it is clear how arduous is to predict oil's fate in the ocean. Regarding potential toxic effects, only dissolved hydrocarbons can cross cell membranes, while oil droplets or hydrocarbon associated with particles are more likely to be ingested (Menon et al., 1999).

As mentioned in the previous paragraph, some petroleum hydrocarbons, and some of their metabolites, have been proved highly toxic to marine biota (1.1.1 Petroleum chemical compounds). The extent of possible toxic effects depends, among other parameters, on oil composition, oil weathering, bioavailability of toxic compounds, and source's characteristics. A more detailed analysis of oil spills' behaviour and harmful effects to the environment is presented in the next chapter.

1.2 Oil Spills

1.2.1 Overview

Oil spills are accidental release of oil into the marine environment from tankers, offshore drilling rings or underwater pipelines. Massive tanker spills are of major concern due to the large amount of oil released in open waters or coastal environments and their hazardous repercussions on the marine ecosystem. Despite an increase in oil production over the last 50 years, tanker spills have dramatically decreased, as well as the consequent amount of oil released in the marine environment (Figure 2).

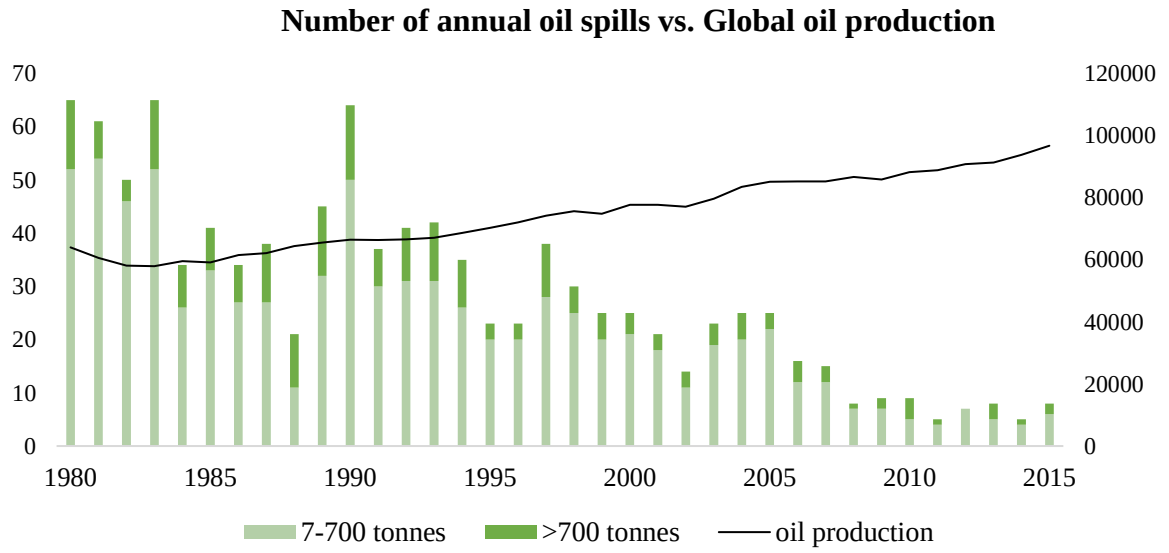

Figure 2: Number of large (>700 tonnes) and medium (7-700 tonnes) spills recorded from 1980 to 2015. vs. Global oil production. Data Source: U.S. Energy Information Administration, April 2017 Units: Thousand Barrels Per Day (ITOPF, 2016).

Large spills (<7000 tonnes) account for a high percentage of total oil spilt. In 2000, for example, the 75% of total oil spilt was released in only 10 accidents out of the 181 recorded (ITOPF, 2017). 50% of the major oil spills from 1970 until 2017 occurred in open waters mainly because of collision, allisions or groundings. Despite a considerable decrease in tank spills and an improvement in preventive measures, the effects of large and medium spills in open waters or enclosed systems remain catastrophic. An overview on oil spill's effects to the marine environment is discussed in the following paragraph.

1.2.2 Acute and chronic toxic effects

Oil spills have physically and chemically induced effects on the marine and costal environment. Floating oil can smother animals or coat their fur and plumage reducing their ability to float and thermoregulate (Loughlin, 1992; Transportation Research Board and National Research Council, 2003). Mass mortality was reported after the Exxon Valdez spill (Alaska, 1989) in marine mammals, seabirds, benthic organisms and macro algae (Loughlin, 1992; Garrott et al., 1993; Paine et al., 1996; Piatt et al., 1996). Long-term impacts are usually related with oil persistence in specific habitats as marshals and sediments (Wolfe et al., 1994). Elevated mortality was detected in incubated pink salmon eggs for at least 4 years (Seeb et al., 1998) while the sea otters population from Knight Island did not show any recovery in number of individuals 11 years after the spill (Bodkin et al., 2003). Chronic exposure was detected using biomarkers in sea otters and sea ducks decades after the oil spill occurred (Trust et al., 2000; Bodkin et al., 2003). Harlequin ducks population showed high sensitivity to oil exposure, and induction of the CYP1A detoxification enzyme, used as exposure biomarker, demonstrated ongoing exposure 9 years after the spill (Esler et al., 2002). Species feeding on passive filtration and sediment-affiliated organisms showed the highest levels of chronical exposure and toxic effects, proving the persistence of oil in those environments (Garrott et al., 1993; Sharp et al., 1996). The Deepwater Horizon deep sea spill (Gulf of Mexico, 2011), showed peculiar characteristics as the formation of a surface oil slick and a deeper plume, which resulted in high deep-water sedimentation via marine snow (Marigomez et al., 2017). Macro- and meio-faunal abundance and diversity in the sediment was the lowest in prossimity of the well (Baguley et al., 2015). Salt marshal fiddler crab abundance recovered in 2011, to decline again in 2013 showing the importance of annual and seasonal variation (Zengel et al., 2016). PAHs metabolites, found in higher concentrations in fish populations, were associated to parental compounds detected at the Deepwater Horizon well-head and correlated with an increase of skin lesions (Murawski et al., 2014). The majority of the studies on oil spill focuses on concentrations and effects on marine mammals, seabirds or economic-relevant species. Due to the possible high concentration of dissolved hydrocarbons in the water column is of interest to determine oil spill effects in the subsurface planktonic ecosystem.

1.2.3 Effects of oil pollution on planktonic community

Planktonic species play a key role in the aquatic ecosystems; being at the bottom of the food chain, their supply in the pelagic zone is regulating the energy balance in oceanic ecosystems.

After *Tsesis* spill (Baltic Sea, 1977) a significant decrease in zooplankton population was detected within 1 km from the spill, most likely due to avoidance of the area or narcotic effects. Changes in zooplankton abundance led to an increase in the abundance of both phytoplankton and planktonic bacteria, because of decreased grazing pressure (Johannson et al., 1980). Another study conducted after the Prestige shipwreck (Galicia, 2002) reported no significant changes in planktonic community structure and abundance (Varela et al., 2006). Abbriano et al. (2011) speculated that, on a long term perspective, zoo- and phytoplankton may have been only minimally affected following the Deepwater Horizon spill thanks to the rapid reproduction rates of planktonic organisms and dispersion and degradation of surface oil. It can therefore be assumed that, after an initial phase of acute toxicity resulting in local mass mortality, zooplankton communities generally rapidly recover.

Bioaccumulation of hydrocarbons at a lower trophic level might increase the hydrocarbon exposure at higher trophic levels (Wolfe et al., 1998) but no biomagnification was observed for toxic oil compounds as PAHs (Takeuchi et al., 2009). Concerning sublethal effects, exposure to crude oil emulsion caused a decrease in egg production, faecal pellet production and egg hatching in copepods *Acartia tonsa* and *Temora turbinats* (Almeda et al., 2014). Decreased ingestion rates and decreased egg viability were reported in *Centropages hamatus* exposed to crude oil/seawater dispersion at 10-80 ppb concentration (Cowles, 1983). *Calanus finmarchicus* exposure to naphthalene (levels well below LC_{50}) resulted in the upregulation of glutathione *S*-transferase (GST), a common biomarker for oxidative stress (Hansen et al., 2008). Biomarkers as CYP450 induction and oxidative stress, have been widely used since the beginning of this century to assess the impact of pollution in coastal and marine environments (Livingstone, 1993; Cajaraville et al., 2000). However, there is a lack of knowledge regarding effects and responses in planktonic species exposed to oil.

1.3 Oxidative stress

Oxidative stress is defined as *"a disturbance in the balance between the production of reactive oxygen species and antioxidants defences"* (Betteridge, 2000). Reactive oxygen species, ROS, are all those oxygen species that contain one or more unpaired electrons, as superoxide (O_2), hydroxyl ($^{\bullet}OH$), peroxyl (RO_2), and hydroperoxyl (HO_2) radicals. ROS are natural by-products of oxygen metabolism in aerobic organisms and the main cellular sources are mitochondrial electron transport chains and the microsomal cytochrome P450 and relative enzymes (Turrens, 2003; Hrycay et al., 2015). However, only little is known about these mechanisms in planktonic

species. Aerobic organisms have developed an antioxidant system able to overcome the natural amount of ROS produced (Birben et al., 2012). The antioxidant system includes antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and low molecular weight scavengers as glutathione (GSH) and vitamin C, which prevent free radicals-mediated cellular damages by maintaining a reducing intracellular environment (Halliwell et al., 1999). ROS are unstable molecules and can form covalent bonds with DNA, proteins and lipids. Beside H_2O_2 which do not bind with DNA, $^{\bullet}OH$ can attack any DNA components while O_2 has high affinity to bind to guanine (O'Neill, 1987; Aruoma et al., 1991). DNA attacks can result in single base damages, single strand breaks, DNA-protein cross links or apurinic sites (Bayir, 2005). Proteins containing -SH groups, or bounded with transition metal ions are a main target for several ROS (Stadtman et al., 1991). The cellular repairing system works efficiently recognizing DNA abnormalities or damaged proteins and repairing or eliminating the damages (Brot et al., 1982; Aruoma et al., 1991). ROS moreover, excluding O_2 and H_2O_2, can initiate lipid peroxidation; specific chain-breaking antioxidant and glutathione peroxidase can remove peroxides from membranes (Burton et al., 1986; Schuckelt et al., 1991).

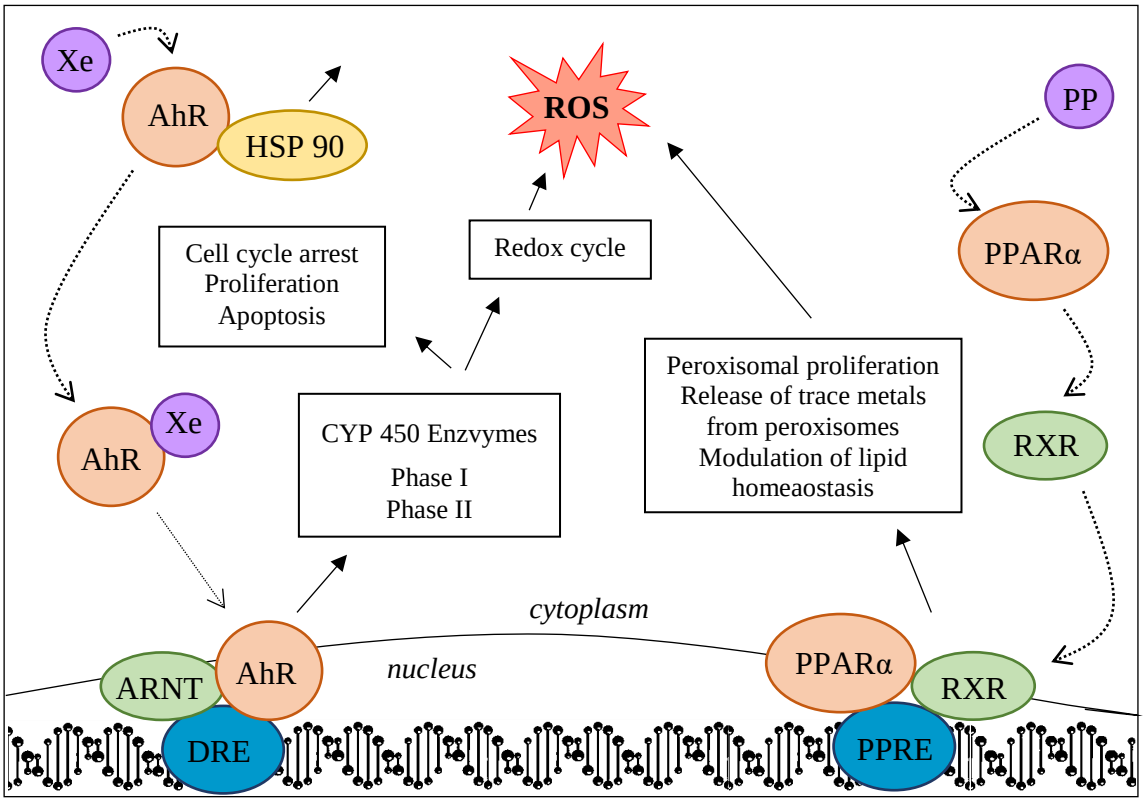

Figure 3: Main oxidative interaction between PAHs and cellular compartments, leading to production of ROS. Xe: Xenobiotic (PAHs) activating the Ahr pathway leading to ROS production. PP: peroxisomal proliferators (PAHs) pathway leading to increased ROS production.

Trace metals and organic xenobitics are known to increase the productions of ROS throughout different and multiple pathways, possibly overcoming antioxidant capacities. (Regoli et al., 2014). Some PAHs are able to activate the "redox-cycle", a source of chemically generated ROS (Livingstone, 1993). Other PAHs, instead, are known peroxisomal proliferators (PPs); upon binding to the peroxisomal proliferator activated receptor α (PPARα) they escalate a cascade of effects resulting in potential increase of H_2O_2 and release of transitional metals as Fe and Cu from peroxisomes, which, in turn, can enhance generation of •OH (Regoli et al., 2014). The main oxidative interaction between PAHs and cellular compartments, leading to production of ROS, is shown in Figure 3. In the last decades, oxidative stress has been widely used in the marine environment as biomarker for xenobiotic exposure (Valavanidis et al., 2006).

1.3.1 Biomarkers of oxidative stress

Several molecules and by-products of cellular damages have been approved as successful biomarkers for oxidative stress (Table 2).

Table 2: Categories and examples of molecular biomarkers of oxidative stress (Valavanidis et al., 2006).

Molecular biomarkers of oxidative stress	
Category	**Examples**
Enzymatic antioxidant defenses	SOD, CAT, GP_X, GST
Non-enzymatic antioxidant defences	Glutathione (GSH), β-carotene, vitamin E
Lipid peroxidation	Malondialdehyde (MDA) formation
Oxidative damage to DNA	Double-strand breaks, hydroxylation by HO' of the nucleobase guanosine (8-OHdG)
Protein oxidation	Phenylalanine and tyrosine amino acids

The detection of cellular damages usually focuses on the three main targets of ROS: DNA, proteins and lipids. Lipid peroxidation, which represents one of the main oxidative stress-induced damage, is commonly determined by quantification of a secondary lipid peroxidation product, malondialdehyde (MDA) (Janero, 1990). Determination of gene expression and detection of enzymatic activity are commonly used to assess enzymatic antioxidant responses. In marine organisms, though, mechanisms involved from a transcriptional event to a functional response of antioxidants is poorly understood. Moreover, compensatory mechanisms in

response to antioxidant depletion and other non-genomic effects can create discrepancies between gene expression and catalytic activities results (Regoli et al., 2014). When considering using enzymatic antioxidant defences as a biomarker for oxidative stress in marine organisms is hence, essential, to investigate responses at both levels.

1.4 Calanus finmarchicus

Calanus finmarchicus is a planktonic copepod inhabiting the subsurface layer and deep-sea basins of the northern North Atlantic, where it constitutes more than half of the copepod biomass (Planque et al., 2000). *C. finmarchicus* development and survival rates are tightly related with the presence of geomorphological features, as deep basins, for overwintering (Melle et al., 2014), and its life cycle is regulated by temperature, chlorophyll abundance (Bryant et al., 1997), and potentially lipid content (Irigoien, 2004). In early spring, eggs arise to surface waters and nauplii larvae (N) emerge within 72 hours from spawning. Nauplii stage is characterized by consecutive moulting leading to an increase in larvae size and complexity. Nauplii VI metamorphosis into CI determine the beginning of the copepodite stage, defined by 6 consecutive moults generating an adult individual (Figure 4). Once *Calanus finmarchicus* reaches CV stage it either allocates fat to mature into an adult and ovulate, under favourable conditions, or stores fat and prepare for overwintering (Skaret et al., 2014). A complete life cycle, from egg to adult, lasts approximately one month. Copepodite developing in late summer will not reach adulthood before the onset of cold temperatures and will therefore, enter diapause, migrating at depths of 300-1500 m (Heath et al., 2004). This strategy allows individuals to overcome rigid temperatures and food shortage. The survivals re-emerge to shallow water in late winter and start reproducing in early spring. *Calanus finmarchicus* distribution and abundance is of great importance in cold-water food chains, being a key-species between primary producers and secondary consumers. Cod, herring, mackerel, blue whiting are examples of *Calanus finmarchicus* dependent species (Prokopchuk et al., 2006; Heath et al., 2007; Espinasse et al.,

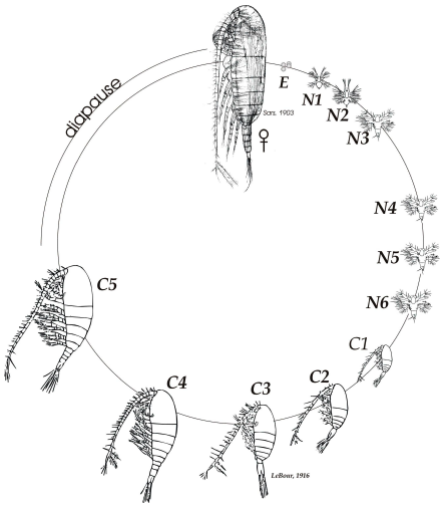

Figure 4: *Calanus finmarchicus* life cycle from egg until adult stage. E: egg; N: nauplii; C: copepodite. Credit: Mark Baumgartner at Woods Hole Oceanographic Institution.

2016). Calanus feeds on phytoplankton and stores huge amounts of high-energy lipids which are quickly transferred up to higher trophic levels; dry mass lipid level increase from 10-20% in phytoplankton to 50 -70% in herbivorous Calanus (Falk-Petersen et al., 2007). Lipid content and quality is therefore an essential parameter regulating the energy flux in cold-environment food chains. Planktivorous fishes directly shape *Calanus finmarchicus* stock (Kaartvedt, 2000; Utne et al., 2012) and in return, its abundance and energy content are key-aspects in the food web structure. Beside natural fluctuations, anthropogenic factors as climate change (temperature rise, shifts in oceanic currents etc.) and acute or chronic sources of pollution can negatively influence *Calanus finmarchicus* population dynamics, potentially leading to changes in ecological communities' structure. The importance of this species in the arctic food web has been deeply studied by Stig Falk-Petersen et al. (1990; 2007) strongly supporting the use of *Calanus finmarchicus* as a bioindicator. *C. finmarchicus* has been recently used as a test species in toxicological studies related to marine oil pollution (Hansen et al., 2008; 2009; 2011; 2013).

1.5 Aim of the present study

Given the opportunity to use *C. finmarchicus* as a test species, and its key role in North Atlantic food chains, the present study aims to detect and validate specific oxidative stress biomarkers in *Calanus finmarchicus* exposed to a water accommodated fraction (WAF) of a naphthenic crude oil from the North Atlantic Ocean (NA). A WAF of oil is considered as the laboratory analogous of the water-soluble oil compounds that enter the water column as a consequence of oil weathering (SINTEF, 2015). A possible relationship between treatment and exposure time was investigated. Existence of reliable biomarkers of oil exposure is fundamental for monitoring programs and decision-making processes in case of environmental oil discharges or oil spills.

2 Material and Methods

2.1 Test species and experimental set-up

According to previous similar studies (Hansen et al., 2011; 2013) it was decided to use *Calanus finmarchicus* at CV stage (last copepodite stage prior adulthood). Due to a delay in the experimental set-up, at the start of the experiment individuals had already entered adulthood and were therefore classified as "non-ovulating adult". Individuals were exposed in a static system at a final dilution of 9.85% WAF in filtered seawater (salinity= 30%). To determine variation in *Calanus finmarchicus* responses following exposure to WAF, individuals were sampled each 24 hours, until the 4th day of exposure (0, 24, 48, 72 and 96 hours). To ensure strong statistical power, using a reasonable number of individuals, four biological replicates were set for each treatment. The experimental set-up is shown in Table 3.

Table 3: Experimental set-up. Treatment groups: control-exposed. Sampling time points: 0, 24, 48, 72 and 96 hours. 1-4: biological replicates, containing 155 individuals each.

Treatment	**Sampling time points**				
	0h	**24h**	**48h**	**72h**	**96h**
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]

Thirty-six 5 L glass bottles were used in the experiment, 20 filled with filtered seawater for the control groups and 16 with the WAF medium for the exposed groups. Around 5600 copepods were collected from the continuous culture at SINTEF/NTNU Sealab (Trondheim, Norway), and groups of 155 individuals were placed into each glass bottle. To extract sufficient mRNA to analyse an acceptable number of genes, 10 individuals per biological replicates were devoted to qPCR analysis. 100 animals were used for the enzymatic assays and MDA and GSH determination, and 39 were distributed to other projects (4: comet assay, 25: metabolomics, 10: lipid analysis). Animals were not fed during the experiment and the experimental bottles were kept in a 10 °C room covered with a dark blanket to avoid light interference with the medium (Figure 5).

Figure 5: Naphthenic crude oil WAF exposure. 20 bottles were filled with filtered seawater for the control groups and 16 with the WAF medium for the exposed groups. Bottles were kept at 10 °C and covered with a dark blanket to avoid light interference with the medium.

2.2 Exposure medium

WAF was generated from a naphthenic North Atlantic crude oil at SINTEF (Trondheim, Norway) in a closed, low-energy mixing system for 72h as recommended by the CROSERF guideline (Chemical Response to Oil Spills – Ecological effects Research Forum) with a 1:40 oil-to-water ratio. The WAF was diluted to correspond to 50% of the 96h LC_{50} dilution (WAF exposure medium). 96h LC_{50} was obtained from a previous experiment performed at SINTEF/NTNU Sealab (Trondheim, Norway) where *Calanus finmarchicus* were exposed to different concentrations of WAF of the same naphthenic North Atlantic crude oil. For more details about the LC_{50} experiment and results see Appendix 1.

2.3 Sampling

A pilot sampling, to familiarize with the technique and to record sampling timing, was carried before the start of the main experiment. 20 minutes were needed for each bottle to be sampled, hence, the exposure bottles were prepared with a 30 minutes interval with the following order C1-E1-C2-E2-C3-E3-C4-E4. A difference in sampling time of 1-3 hours among bottles belonging to the same time point would generate unwanted variation within a single time-group. Sampling was carried as fast as possible to reduce at minimum the handling stress (Figure 6).

The whole content of the experimental bottle was divided into two glass bowls (1A-2A) directly into a plastic cylinder with an opening on the top and a 300 nm-sieve at the bottom. 14 individuals from 1A were devoted to lipid analysis and comet assay. Using a plastic spoon, 10 individuals, for gene expression analysis, were put from 1A into the cylinder of a second bowl (1B) containing the same experimental medium. The 1B-cylinder was removed from the medium, the sieve quickly dried with tissue-paper and the animals drugged with a laboratory spatula into a labelled cryotube and put into liquid nitrogen. The same procedure was carried out in the 2A-bowl with 25 animals for metabolomics. The remaining individuals from 1A and 2A were pulled together using the same procedure into labelled tubes and put into liquid nitrogen. This procedure was followed for each bottle and at the end of the whole sampling process *gene expression* and *enzymatic assay* samples were stored at -80 °C until further analysis.

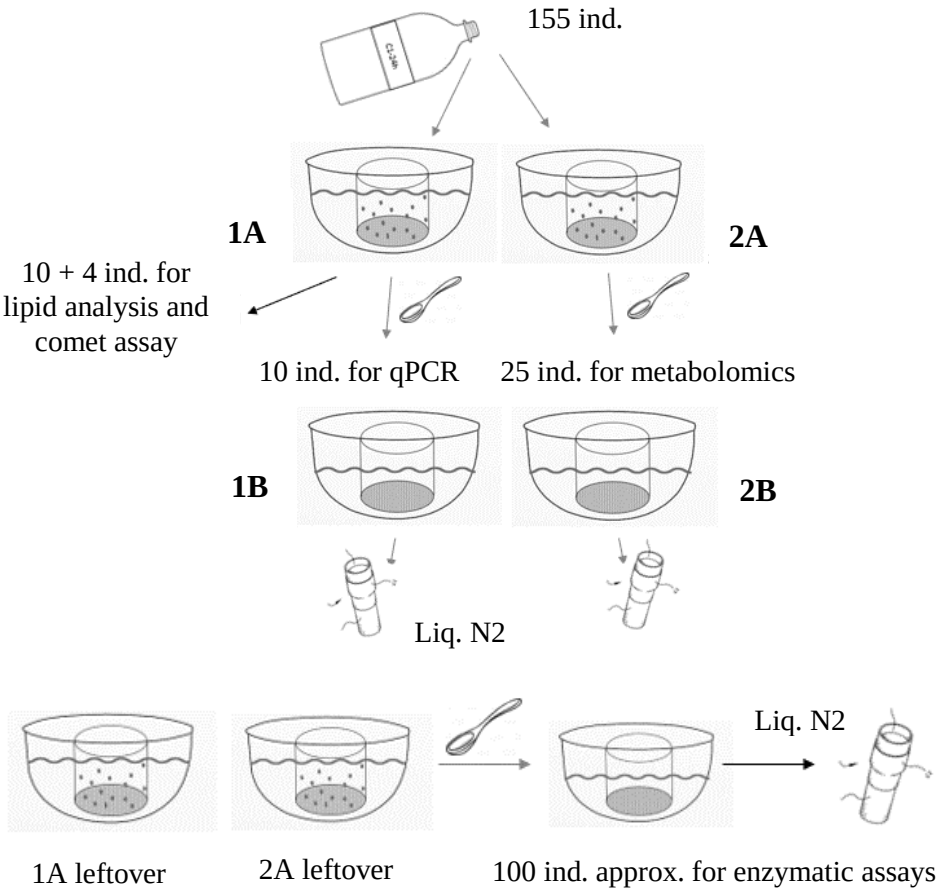

Figure 6: Sampling procedure. ind: individuals; Liq. N2: liquid nitrogen

2.4 Chemical analysis

Standard analyses of semi-volatile organic compounds (SVOC) and volatile organic compounds (VOC) were performed at SINTEF Ocean, on both crude oil and WAF. SVOC were analysed using a GC-MS (gas chromatography – mass spectrometry) while VOC analysis was performed using a Purge and Trap GC-MS. For VOC analysis, crude oil was run in triplicates and WAF (1:40 oil-to-water ratio) in duplicates. SVOC analyses instead were performed on a single crude oil sample and on three different WAF samples: WAF (1:40 oil-to-water ratio), WAF exposure medium (9.85% of WAF corresponding to 50% of the 96h LC_{50} dilution), and WAF medium after 96h.

2.5 Gene expression

2.5.1 RNA extraction

"RNeasy Plus Universal Mini Kit" by QIAGEN was used for mRNA extraction. The kit contains 50 RNeasy mini spin columns, 50 1.5 mL collection tubes, 50 2 ml collection cubes, 8 ml gDNA eliminator solution, 15 mL buffer RWT concentrated solution, 11 mL buffer RPE concentrated solution, 10 mL of RNase-free water and the relative handbook. In addition, chloroform (EMSURE, Merck), ethanol 70% and QIAzol lysis reagent were used. RNA extraction was performed according to the producer protocol. Each sample was weighted on an analytical balance and quickly put on ice to avoid defrosting. Disruption and homogenization of the tissues was performed using the TissueLyser II (QIAGEN). Eight 2 mL microcentrifuge tubes were labelled, and a single steel bead added in each tube. The pool of 10 individuals was placed in the relative tube and 900 µL of QIAzol lysis reagent immediately added. Tubes were placed in the TissueLyser II Adapter 2x24 and operated for 2 min at 20 Hz. After 2 min the adapter set was reassembled so that the outermost tubes were then the closest; this step allows an even homogenization. The lysate was carefully pipet into new tubes and let resting for 5 min to promote dissociation of nucleoprotein complex. Subsequently, 100 µL of gDNA eliminator solution were added in each tube followed by 15 sec of intense shaking. 180 µL of chloroform were then added and the vigorous shake repeated. Tubes containing the homogenate were let resting for 2-3 min prior centrifugation. The centrifuge (BIOFUGE fresco, Hareaus) was set at 12,000 x g for 15 min at 4 °C. Centrifugation resulted into the separation of the sample in 3 distinct phases: an upper liquid one containing RNA, about 600µL, a white lipid-interphase and a red organic phase (Figure 7).

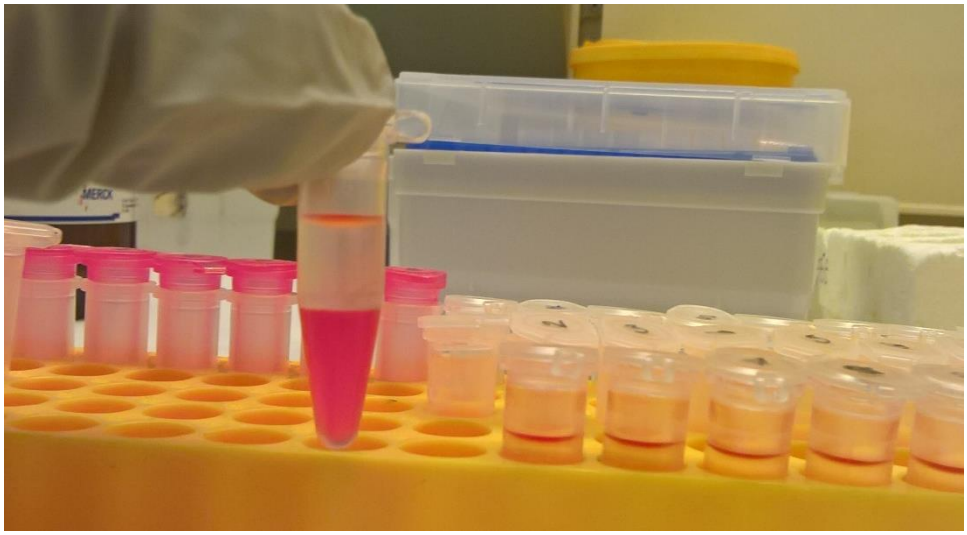

Figure 7: Phase separation resulting from centrifugation. Samples separates into 3 distinct phases: an upper liquid one containing RNA, a white lipid-interphase and a red organic phase.

The aqueous phase was transferred into a new microcentrifuge tube using a micropipet set on 600 µL and an equal volume of ethanol 70% was added. RNA-phase volumes were varying among samples, and an estimation of the actual volume was interfered for each sample. Mixing was obtained by pipetting up and down in the tubes. 700 µL from each sample were transferred to the RNeasy Mini spin column placed into a 2 mL collection tube and centrifuged for 15 sec at more than 10,000 rpm at room temperature. mRNA binds with the spin column membrane and contaminants are efficiently washed away afterwards using different buffers. The flow-through obtained from centrifugation was discarded and the previous step repeated with the remaining volume of sample. 700 µL of Buffer RWT, a stringent washing buffer, was added, and the tubes centrifuged for 15 sec at more than 10,000 rpm at room temperature. The flow-through was discarded. 500 µL of Buffer RPE were added twice and the samples centrifuged for 15 sec and then for 2 min at more than 10,000 rpm at room temperature to wash the membrane. The flow-through was discarded and the spin columns placed in new 2 mL collection tubes and centrifuged for 1 min to eliminate any possible carryover of Buffer RPE. The spin columns were placed in new 1.5 mL collection tubes and 30 µL of RNase-free water was added to elute the RNA from the spin membrane. The tubes were centrifuged for 1 min at more than 10,000 rpm. Another volume of RNase-free water was added, and the tubes centrifuged. The spin columns were discarded and the tubes containing 60 µL of mRNA solution were kept on ice and analysed by spectrophotometer (NanoDrop by BioNordika); 1.5

µL for each sample was analysed twice to check for RNA quality (260/280 and 260/230 ratio) and to calculate RNA concentration. RNA extracts were stored at -80 °C until PCR (polymerase chain reaction).

The advantage of using this kit is that no DNAase treatment is required since gDNA eliminator solution removes efficiently most of the genomic DNA contamination. Prolonged frozen lysate incubation or sample defrosting prior QIAzol Lysis addition can compromise RNA integrity. For these reasons the whole procedure was carried at once and RNA samples were frozen at -80° immediately after the spectrophotometer analysis. Steps prior QIAzol lysis reagent addition were carried as fast as possible and no more than 8 samples were processed at the tame; samples were constantly kept on ice until tissue disruption and homogenization. Additional information about the procedure can be found in the "RNeasy Plus Universal Mini Kit" handbook by QIAGEN.

2.5.2 cDNA synthesis

The "Reverse Transcription kit" by QuantiTect® was used to synthetize cDNA from RNA samples (PCR). The kit provides gDNA wipeout buffer (gDNA WB), to eliminate gDNA contamination, reverse transcriptase (RT) Quantiscript, reverse transcriptase buffer, reverse transcriptase primer mix and RNase-free water. All reagents were stored at -20 °C and let to thaw on ice right before analysis. cDNA synthesis was performed according to the protocol.

To obtain a concentration of 1 µg of mRNA in 21 µL, as required by protocol, the appropriate starting volumes were calculated from the samples' concentrations obtained by spectrophotometer analysis (NanoDrop by BioNordika) (Table 4).

Table 4: RNA concentration (ng/µL), RNA volume (µL) to obtain 1 µg of RNA in 21 µL, and 260/280 and 260/230 ratio detected for each biological replicate.

Sample ID	RNA conc. (ng/µL)	RNA (µL) for 1 µg/21 µL	260/280 ratio	260/230 ratio
C1-0	509.51	1.96	2.23	2.24
C2-0	533.74	1.87	2.24	1.94
C3-0	663.85	1.51	2.23	2.32
C4-0	656.31	1.52	2.25	2.53
C1-24	574.24	1.74	2.22	1.72
C2-24	280.44	3.57	2.2	1.32
C3-24	480.44	2.08	2.21	1.56

C4-24	358.02	2.79	2.17	1.66
E1-24	498.5	2.01	2.2	1.79
E2-24	480.29	2.08	2.21	1.5
E3-24	460.51	2.17	2.13	1.68
E4-24	461.06	2.17	2.11	1.76
C1-48	313.96	3.19	2.05	1.9
C2-48	371.83	2.69	2.13	2.46
C3-48	500.37	2	2.2	1.55
C4-48	570.95	1.75	2.21	1.48
E1-48	388.57	2.57	2.13	1.94
E2-48	449.31	2.23	2.46	2.35
E3-48	350.75	2.85	2.15	0.93
E4-48	447.42	2.24	2.12	1.23
C1-72	470.63	2.12	2.16	2.33
C2-72	580.99	1.72	2.16	2.3
C3-72	328.86	3.04	2.18	1.17
C4-72	479.1	2.09	2.2	1.15
E1-72	335.79	2.98	2.18	0.9
E2-72	426.53	2.34	2.13	2.22
E3-72	313.74	3.19	2.19	1.04
E4-72	366.83	2.73	2.16	1.75
C1-96	404.28	2.47	2.12	2.49
C2-96	368.58	2.71	2.16	1.22
C3-96	390.92	2.56	2.15	1.53
C4-96	414.66	2.41	2.12	2.45
E1-96	269.89	3.71	2.18	1.8
E2-96	244.23	4.09	2.18	2.55
E3-96	235.17	4.25	2.17	0.96
E4-96	279.33	3.58	2.17	1.11

Table 4 shows the RNA concentrations detected and the relative RNA quality values. All samples show a 260/280 ratio about 2, which is considered as an indicator of “pure” RNA (Wilfinger et al., 1997). 260/230 ratio it’s a secondary quality indicator and values around 2 indicate “pure” nucleic acid (Wilfinger et al., 1997); 260/230 ratios are lower than 2 for most samples. Low 260/230 ratio suggests contamination from guanidine thiocyanate contained in the Qiazol or RLT buffer, but it’s not clear yet how or whether it may influence further analysis. QIAGEN, moreover, affirms that “Pure RNA should yield a 260/230 ratio of around 2 or

slightly above; however, there is no consensus on the acceptable lower limit of this ratio" (QIAGEN, 2010). Given the satisfying 260/280 ratios, the few samples with significantly low 260/230 values and the absence of proofs that low 260/230 ratio can affect downstream analysis, all samples were considered suitable for cDNA synthesis.

1.5 µL of gDNA WB, RNA calculated volume (Table 4) and RNA-free water were respectively transferred into each well to obtain the final required dilution (1 µg of RNA in 21 µL); the plate was kept on ice during the whole procedure. After 2 min incubation at 42 °C to allow gDNA elimination, the plate was put immediately on ice and 14 µL from each well were transferred in second a microplate, "cDNA plate", using a multipipette. The former 96-wells microplate containing only 7 µL was used as a -RT plate, or negative control, to check for gDNA contamination. 6 µL of RT master mix and 3 µL of -RT control reverse-transcription master mix were added in each well on the relative plates using a multi dispenser pipette. To fasten the procedure the 2 master-mix were prepared at the beginning of the procedure according to the protocol. The plates were incubated for 15 min at 42 °C to allow cDNA synthesis, and subsequently at 95 °C for 3 min to inactivate the reverse transcriptase reaction.

2.5.3 qPCR

qPCR was performed using the LightCycler 480 System and "LightCycler 480 SYBR Green I Master kit" from Roche. The kit provides H_2O PCR-grade, to adjust the final reaction volume, and a "Master" containing a FastStart Taq DNA Polymerase, reaction buffer, Dntp MIX, SYBR Green I dye, and $MgCl_2$. LightCycler 480 system provides the LightCycler 480 Instrument (qPCR machine), LightCycler 480 Multiwell Plates 348 and LightCycler 480 Sealing Foil. A single multiwell plate was used for each gene analysed. qPCR was performed according with protocols. Data obtained by the LightCycler 480 Instrument were then uploaded into the LightCycle 480 Software and LinRegPCR to check for analysis' quality.

2.5.3.1 Primers selection

Primers for both oxidative stress involved genes and housekeeping genes were ordered from Sigma® based on previous studies (Hansen et al., 2008; Tarrant et al., 2008; Lauritano et al., 2012; Rhee et al., 2013). Housekeeping genes are defined as those genes that are not affected by the treatment and are therefore equally expressed in both control and exposed groups. 16S and actin were selected as housekeeping genes, while SOD, CAT, GST, and GSH as oxidative stress related genes. The primers' sequences are shown in Table 5.

Table 5: Primers sequences used in the first qPCR series.

Gene	Sequence	Gene Type
16S-F	AAGCTCCTCTAGGGATAACAGC	Housekeeping
16S-R	CGTCTCTTCTAAGCTCCTGCAC	
Act-F	CCATTGTCCGTCTTGATCTT	Housekeeping
Act-R	AAAGAGTAGCCACGCTCAGTG	
EFA1α-F	AGGTTAAGTCCGTGGAGATG	Housekeeping
EFA1α-R	ACTGGCTTGTTCTTGGAGTC	
CAT-F	TGTACATGCAAAGGGAGCTG	Antioxidant system
CAT-R	GGTGTCTGTTTGCCCACTTT	
SOD-F	GGAGATCTTGGCAATGTTCAG	Antioxidant system
SOD-R	CAGTAGCCTTGCTCAGTTCATG	
GST-F	CAACCCCCAGCACACTGTG	Antioxidant system
GST-R	GGATAGACACAATCACCCATCC	
GSH-F	GAGAAGGCAAAGGACTATG	Antioxidant system
GSH-R	GGCAACCTTGTGCATCAAC	

Abbreviations: 16S: mitochondrial 16S, Act: actin, EFA1α: elongation factor A1α, CAT: catalase, SOD: superoxide dismutase, GST: glutathione *S*-transferase. GSH: glutathione. (F): forward primer (R): reverse primer

Amplification curves obtained by LightCycle 480 software were analysed with LinRegPCR to calculate qPCR efficiency for each plate. During each qPCR cycle the amount of DNA theoretically doubles, i.e., qPCR efficiency is equal to 2. Generally, values between 1.8 and 2.2 are considered very good efficiency; any of our primers exceeded this range. Examining further the data obtained by the LightCycle 480 Software, GST and GSH showed unregular melting curves, as in Figure 8. Specific primers produce specific amplicons, which have a precise melting temperature. Multi-picks graphs for melting temperature are indicators of unspecific primers, meaning that during qPCR not only the gene of interest was amplified.

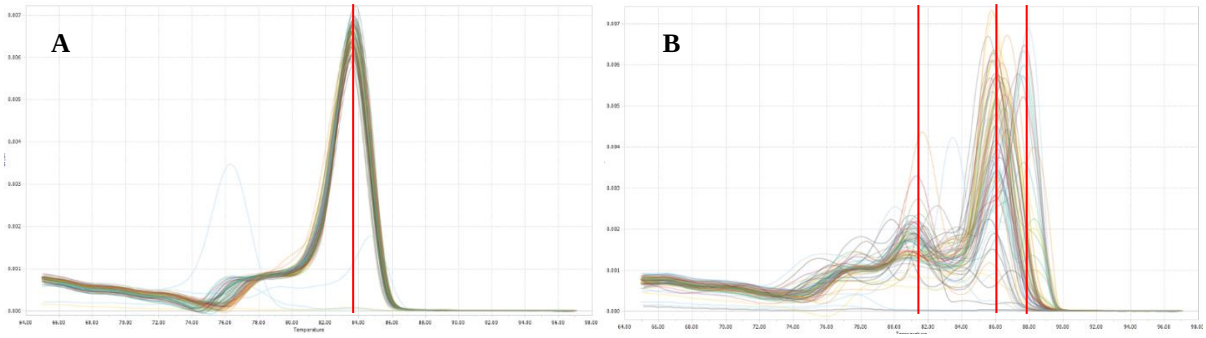

Figure 8: Melting curves obtained from a specific (Actin) **(A)** and a non-specific primer (GST[1]) **(B)**. Graphs obtained from LyghtCycler 480 Software. [1]: first order of primers.

To broaden the dataset and correct for primers with unregular melting curves a new set of primers was ordered from Sigma® based on relevant genes presented in (Farkas et al., in preparation). New primers' sequences are shown in Table 6.

Table 6: Primers sequences used in the second qPCR series.

Gene	Sequence	Gene type
Act-F	TCATACTGTGCCTTGGTGTG	Housekeeping
Act-R	AGCCTATTGAGGTTCAGGTG	
CYP1A2-F	TGGTCCTCTTGACCCAAAAG	Detoxifying system
CYP1A2-R	ATAGCTTGGTGGAACTTGGC	
CYP330A1-F	ATTCACCCATTCAGGAAGCC	Detoxifying system
CYP330A1-R	TTGCTCCTTCCAAGTGTGTC	
GST-F	TTCTTCTGACTCCTCTCTCG	Antioxidant system
GST-R	ATGTCATGATGACCAAGGCC	
SOD-F	TGTTGTTCTGGGTATCCAGG	Antioxidant system
SOD-R	GTATAGAGATCTTCCCTCCG	
CAT-F	GTTGTACATGCAAAGGGAGC	Antioxidant system
CAT-R	AACAGTGGAGAACCTGACAG	
HSP70-F	GATCATAGTTGGTCTGGCAG	Heat shock protein Protein damage
HSP70-R	CATTAATGGTGACAGCGCTC	
HSP90-F	GTCTCGAAGAGAAGCATGAC	Heat shock protein Protein damage
HSP90-R	CTATATGGCGGCTAAGAAGC	
UB-F	TCCATCGAGAATGTCAAGGC	Protein damage
UB-R	TGCTCTCCTTCTGGATGTTG	

Abbreviations: Act: actin, CYP1A2: cytochrome P450 1A2, CYP330A1: cytochrome P450 330A1, GST: glutathione *S*-transferase, SOD: superoxide dismutase, CAT: catalase, HSP70: heat shock protein-70, HSP90: heat shock protein-90, UB: ubiquitin. (F): forward (R): reverse

A second PCR was run to synthetize more cDNA to analyse the new primers, following the exact same protocol. All primers showed regular melting curves and qPCR efficiency between 1.8 and 2.2.

2.5.3.2 Procedure

To run qPCR our cDNA and -RT plates were diluted 1:10 with autoclaved water. Both diluted plates were sealed with adhesive aluminium foil after each use, and stored at -20 °C.

Using a multi dispenser pipette, 15 µL of qPCR master mix (Table 7) were added into the predefined wells of the LightCycler 480 Multiwell Plates 348 (plates' set-up in Appendix II). Subsequently, 5 µL from the "cDNA-" or "-RT-" diluted sample were transferred and mixed with the master mix. The multiwell plate was immediately sealed with LightCycler 480 Sealing Foil and spin for 2 min at 1500 g to allow mixing. While only one replicate was used for the –RT plate, the cDNA plate was run in duplicates. The plate was hence transferred into the plate holder of the LightCycler 480 Instrument and the program started (45 cycles). -RT samples were run only for the housekeeping genes and one target gene, once no gDNA contamination was detected, only the cDNA plate was analysed.

Table 7: Volumes of reagents used to prepare qPCR primer mix and qPCR master mix.

qPCR primer mix		**qPCR master mix**	
Reagents	**Volume (µL)**	**Reagents**	**Volume (µL)**
Forward primer	10	H2O	237.6
Reverse primer	10	PCR primer, (1:10)	158.4
H2O	180	Light Cycle 480 Probes Master	792
Total volume	200	**Total volume**	1188

2.5.4 Data handling

Ct (cycle threshold) values obtain from the LightCycler 480 software were imported into LinRegPCR software for baseline correction. Cq (quantification cycle) values obtain by LinRegPCR were qualitatively analysed. Technical replicates were accepted only when $\Delta Ct < 1.6$; based this criteria, sample "24hE1 – *Hsp70*" was discarded, showing $\Delta Ct > 6$. The presence of possible outliers among biological replicates was detected using the modified *z*-score

method; biological replicates with *z* values greater than 3.5 were labelled as "possible outliers" but not directly excluded from the analysis. Pflaff method (Pfaffl, 2001) was implemented to calculate relative gene expression, since it accounts for differences in qPCR efficiency between primers (Equations 1).

Equation 1: Gene expression ratio obtained implementing Pflaff method. GOI: gene of interest; HKG: housekeeping gene; E: primer efficiency

$$\text{Gene expression ratio} = \frac{(E_{\text{GOI}})^{\Delta Ct\ GOI}}{(E_{\text{HKG}})^{\Delta Ct\ HKG}}$$

2.6 Enzymatic assays and MDA and GSH concentrations

Enzymatic assays and MDA and GSH analyses were performed at the Medical University of Gdańsk (Poland). Samples containing about 100 individuals were homogenized for 30 seconds using a MPW-309 (Universal laboratory aid at Mechanika Precyzyjna, Warszawa). Homogenization was carried in ice-cold buffer containing 50 mM Tris-H_2SO_4, pH 7.6 with 0.1 mM EDTA, 1 mM PMSF, 2 mM DTT and 0.2% Triton® X, to obtain 20% homogenate. The homogenates were centrifuged for 30 minutes at 14000 RCF using a SIGMA 3K18 centrifuge. The supernatant was then transferred into fresh tubes used for the analysis. Unfortunately, "Control-0h" samples were not analyzed for enzymatic activities and MDA and GSH concentrations.

2.6.1 GSH determination

1:1 volume of 5% sulfosalicylic acid (SSA) was added to the protein supernatant for deproteinization on ice for 5 min and centrifuged for 2 min at 10000 xg. GSH was measured with Glutathione Assay Kit (CS0260 Sigma-Aldrich) using GSH (Sigma-Aldrich) as the standard. Samples were pipetted into a Thermo Scientific 96-well microplate and the Working Mixture reagent was added. The plates were incubated at room temperature for 5 min and absorbance was measured in Synergy 2 Multi-Mode Reader (BioTek). Total GSH was determined as ∑GSSG+GSH, oxidized and reduced glutathione species respectively. tGSH was normalized to the protein sample content. Cytosolic protein was determined following Lowry method (1951) with modification of Peterson (1977).

2.6.2 MDA level

MDA level was determined using the Lipid Peroxidation (MDA) Assay Kit (MAK085 Sigma-Aldrich). Lipid peroxidation was detected by MDA reaction with thiobarbituric acid (TBA) which forms a colorimetric product proportional to MDA level. Samples were pipetted into a 96-well microplate and absorbance was read by Synergy 2 Multi-Mode Reader (BioTek) at 532 nm. MDA level was normalized to the protein content of the sample and expressed as nmol/mg of total protein concentration.

2.6.3 Enzymatic activities

GST activity was determined using a spectrophotometer (UV-VIS Beckman Coulter Spectrophotometer, USA) as described by 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). The reaction was started adding 100 nM of glutathione as substrate and the supernatant; absorbance was followed for 5 min at 340 nm. GST activity was expressed as nmol/min/mg of total protein concentration.

SOD activity was measured using the modified method of Sun et al. (1988). In this experiment the sum of the two main isoforms of SOD were detected, CuZnSOD and MnSOD. SOD 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 1U of SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

GPx activity was measured using the Glutathione Peroxidase Cellular Activity Assay Kit (CGP1 Sigma - Aldrich) with 30mM 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+, proportional to the GPx activity in the sample, was monitored spectrophotometrically at 340 nm wavelength in Synergy 2 Multi-Mode Reader (BioTek). Total GPx activity was expressed in nmol/min/mg of total protein concentration.

CAT activity was measured following the method described by Kankofer (2001). 6 mM of cold H_2O_2 were added to the supernatant and vortexed. After incubation on ice the reaction was stopped by the addition of 3 mM of H_2SO_4; 2mM of $KMnO_4$ were added, vortexed and the absorbance read at 480 nm (UV-VIS Beckman Coulter Spectrophotometer, USA). The CAT 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 of total protein concentration.

2.7 Statistics

To obtain normal distribution, data were Log-transformed (Log_{10}) and normality was checked with Shapiro-Wilk test (Appendix VIII). Two-way ANOVA was performed to check if one of the two independent variables (time and treatment) and/or their interaction were significant. Data that showed no normal distribution following Log_{10} transformation (CAT, CYP1A2, CYP330A1, GST[1] GST-2 and SOD[2] for gene expression, and GST for enzymatic activity) were analysed with Mann-Whitney test and Kruskal-Wallis test. All statistical analyses were performed using IBM SPSS statistic 25 software (see Appendix IX).

3 Results

3.1 Oil and WAF profile

3.1.1 Volatile Organic Compounds

Crude oil VOC profile was dominated by methylcyclohexane (20.31 g/kg), cyclohexane (9.38 g/kg) and methylcyclopentane (6.36 g/kg) while, in the WAF samples, toluene **(**1.61 g/kg), m-xylene (1.36 g/kg) and benzene (0.92 g/kg) were the major volatile compounds (Table 8). BTEX (mixture of benzene, toluene, and the three xylene isomers) are well-known toxicants for terrestrial and aquatic organisms (Meyerhoff, 1975; Headley et al., 2001), and were the most prominent VOC in the WAF matrix. For more details regarding VOC profiles see Appendix III.

Table 8: Concentrations of individual BTEX compounds in oil and WAF (g/Kg).

BTEX	**Water solubility (mg/L)**	**Log(K_{ow})**	**Oil**		**WAF**	
			Average (g/Kg)	SD	Average (µg/Kg)	SD
Benzene	1700 [a]	2.13 [b]	0.5	0	918.02	96.55
Toluene	515 [a]	2.69 [b]	2.9	0.02	1611.76	268.06
Ethylbenzene	534.8 [a]	3.15 [b]	1.57	0.01	400.61	9.88
m-Xylene	175 [a]	3.2[c]	5.38	0.05	1363.21	92.12
p-Xylene	130 [a]	3.15[c]	1.73	0.02	128.5	16.49
o-Xylene	152 [a]	3.12 [c]	1.52	0.01	481.1	31.97
Sum BTEX			13.61	0.11	4903.2	515.07

a. Njobuenwu et al. (2017); b.Chesnaux (2008) c. Eom et al. (2011)

3.1.2 Semi-Volatile Organic Compounds

The oil sample was largely dominated by C1-C4-naphthalenes (7.55g/Kg) and C1-C4 decalins (5.5g/Kg), while naphthalene and C1–C4-alkylated homologues were extremely dominant in all WAF samples. Differences in the water solubility of chemical compounds is reflected in the contrasting SVOC profiles of oil and WAF (Appendix IV). Phenols were not detected in any samples while naphthalene and homologues comprise about 90% of all detectable WAF compounds (Table 9).

Table 9*:* Main classes of SVOC detected in WAF, WAF exposure medium, and WAF after 96h. Concentrations from a single replicate are given in µg/L.TEM: Total extractable material.

Compound groups	**Mass (µg/L)**		
	WAF	WAF exp. med.	WAF after 96h
TEM	3088.96	2786.59	198.63
∑ All identifiable compounds	269.95	218.2	21.67
∑ Decalin and C1–C4-alkylated homologues.	0.81	0.7	0.02
∑ Naphthalene and C1–C4-alkylated homologues	253.5	207.42	19.9
∑ Phenantrene/anthracene and C1–C4-alkylated homologues	3.09	2.68	0.18
∑ Dibenzothiophene and C1–C4-alkylated homologues	1.31	1.14	0.11
∑ PAH 2+ rings*	10.92	9.63	0.84
∑ Phenols and C1–C5-alkylated homologues.	0	0	0

* ∑ 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

Table 9 shows the concentrations of the main semi-volatile compounds groups for WAF, WAF exposure medium, and WAF after 96h. After 4 days all semi-volatile compounds shown a marked decrease, accounting from 88 up to 100% of the initial load (Figure 9). The only exception, probably resulting from an instrumental error, is biphenyl, which was detected at a concentration of 4.08 µg/L in "WAF" and 0.88 µg/L in "WAF after 96h" but not in the "WAF exposure medium". For more details about SVOC profiles see Appendix IV.

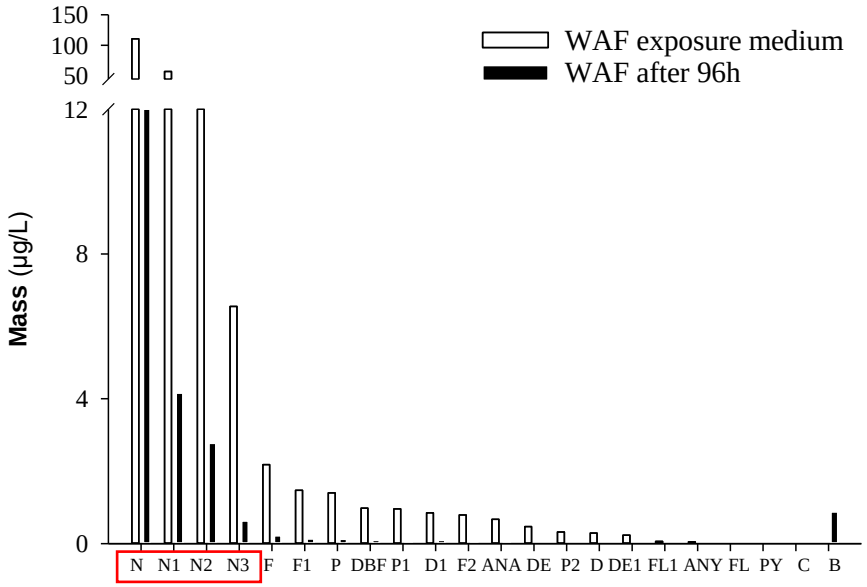

Figure 9: Total SVOC compounds detected in the WAF exposure medium and in WAF after 96h. The X axis shows all the individual compounds above limit of detection. In red, the 4 main SVOC compounds.

3.2 qPCR

No significant differences in Ct values were observed among treatment or time groups ($p > 0.05$) in the housekeeping gene 16S (Figure 10) while actin and EFA1α showed irregular expression across samples (see Appendix V). Since Pflaff formula requires only one reference gene, the housekeeping gene 16S was selected to normalize the data, while actin and EFA1α were discarded. "Control-0h" was surprisingly high in all target gene while no significant differences between 0h and the other time points was detected for 16S (Figure 10). This result suggests that elevated RNA ratios detected in "Control-0h" in all target genes could be a biological response to handling stress (sampling, counting and bottle's allocation), onset of starvation, or a technical error.

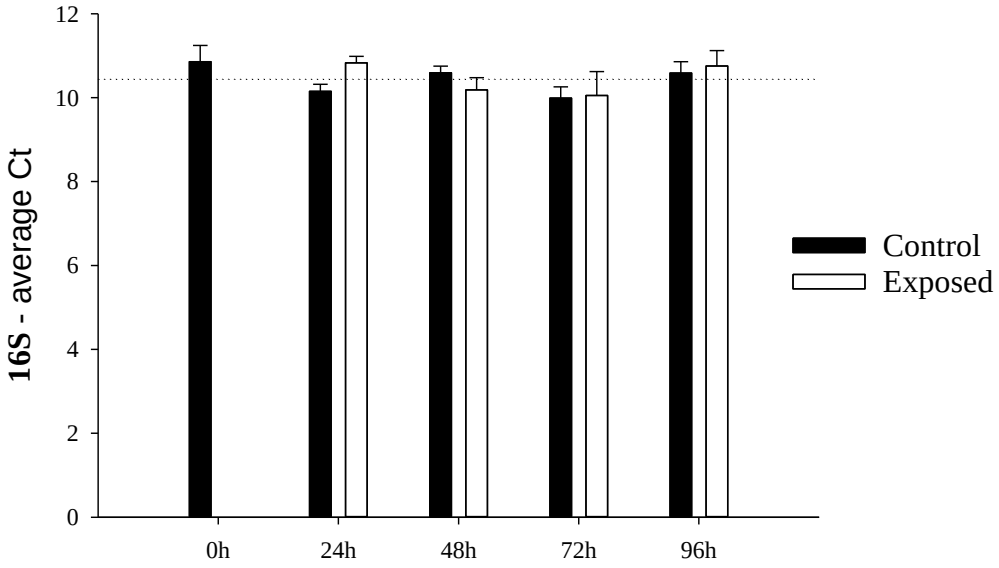

Figure 10: Ct values of the candidate reference gene 16S in the WAF-exposed and control groups. Values are presented as the mean ± standard deviation for each sampling time point. No significant differences were observed between the two groups (p=0.821).

A significant increase over time was detected in CAT (p=0.023), CYP330 (p=0.047) (Figure 11), CYP1A2 (p=0.042) and SOD (p=0.023) expression ratio. However, no differences were detected between control and exposed groups.

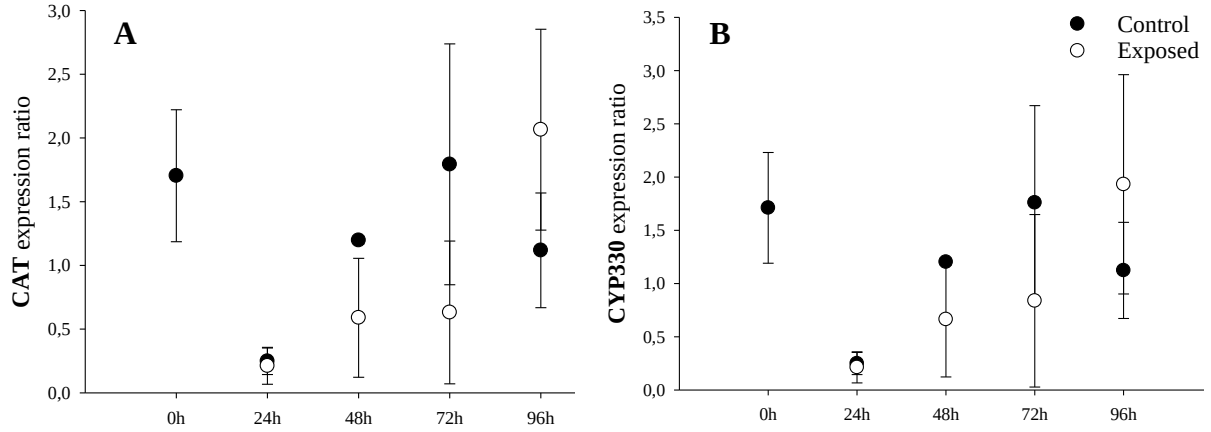

Figure 11: Expression ratio (Pflaff method) of the target gene CAT (A) and CYP330 (B) for the exposed and control groups. Values are presented as the mean ± standard deviation for each sampling time point. A significant upregulation over time was detected in both genes (CAT p=0.023; CYP330 p=0.047), but no significant difference was detected between the control and exposed groups (CAT p=0.222; CYP330 p=0.275).

Significant differences at single sampling time point were detected for SOD[1] (Independent T-test) and GST-2 (Mann-Whitney test). At 48h GST-2 showed a significant upregulation (p=0.021) while at 72h both GST-2 (p=0.034) and SOD[1] (p=0.033) were significantly downregulated (Figure 12). For SOD[1], the interaction between time and treatment was significant (p=0.031) but treatment alone was not (p=0.776).

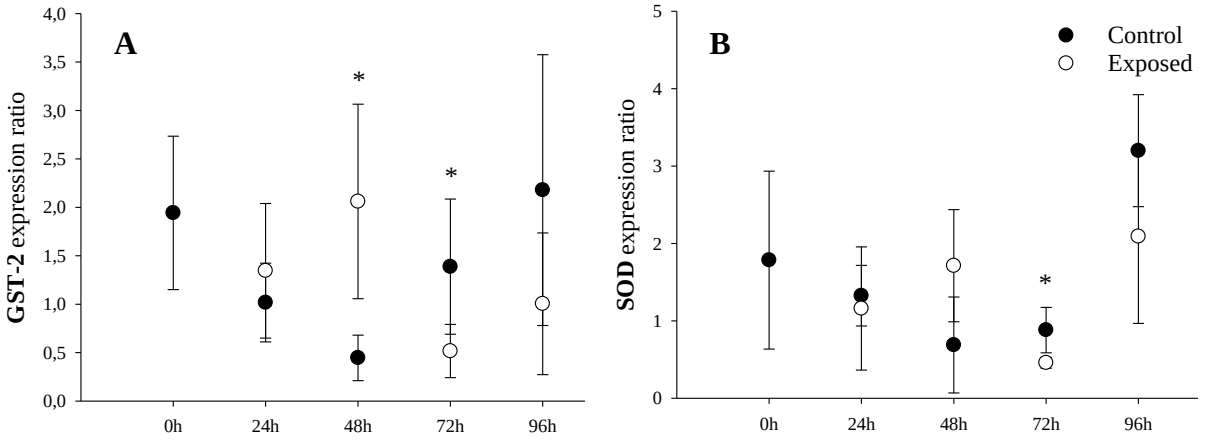

Figure 12: Expression ratio (Pflaff method) of target genes GST-2 **(A)** and SOD[1] **(B)** in the exposed and control groups. Values are presented as the mean ± standard deviation for each sampling time point. Upregulation is detected for GST-2 at 48h (p=0.021). A significant downregulation was detected at 72h for both GST-2 (p=0.034) and SOD[1] (p=0.033). (*): (p< 0.05).

For more details about the results obtained from all target genes analysed see Appendix VI.

3.3 Enzymatic assays and MDA and GSH concentrations

Analysis of CAT and GPx enzymatic expression showed no significant differences between the exposed and control groups (Figure 13). GPx activity appear to increase over time, but no significant differences among sampling points were detected (p=0.333).

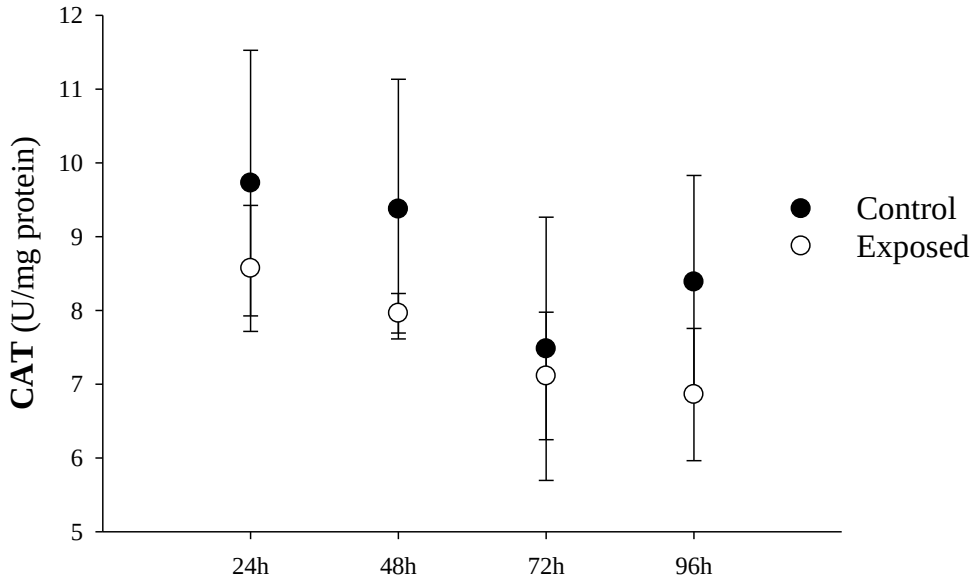

Figure 13: Enzymatic activity of CAT in the exposed and control groups detected as nmol/min/mg protein. CAT activity increased over time (p=0.047) but CAT enzymatic activities between the control and exposed groups (p=0.056).

The CAT activity (Figure 13) decreased significantly over time (p=0.047), but no differences between experimental groups were observed (p=0.056). Time had no effect on GST enzymatic activity (p=0.928) but exposed and control groups were significantly different (p=0.001). The exposed group shows a 3.1-fold increase at 24h, a 2.2-fold increase at 48h, a 2.4-fold increase at 72h and a 3.1-fold increase at 96h when compared to the control group (Figure 14).

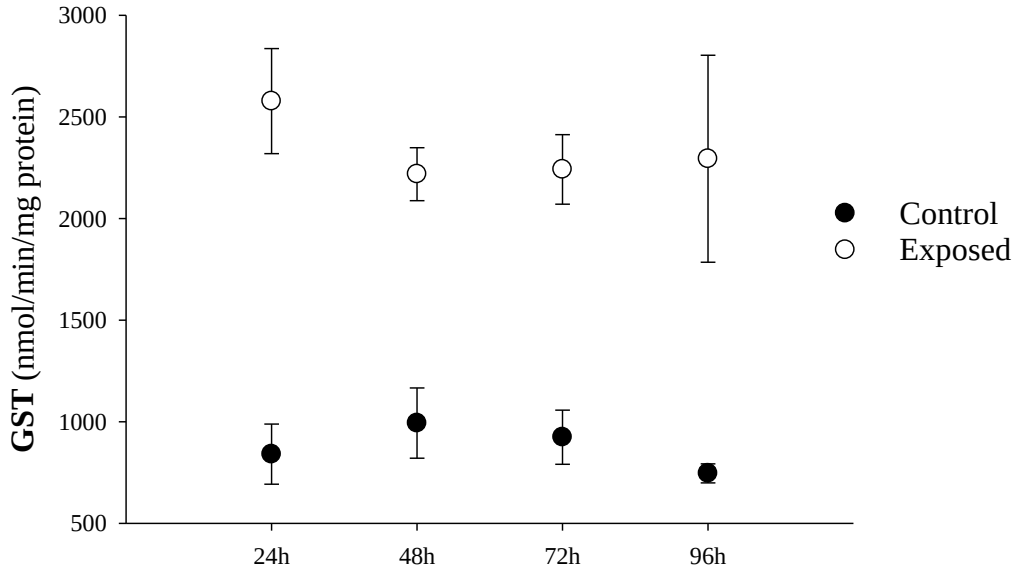

Figure 14: Enzymatic activity of GST in the exposed and control groups detected as nmol/min/mg protein. GST enzymatic activity is significantly induced in the WAF-exposed group (p=0.001) but no time-dependent modulation was determined (p=0.928).

Glutathione concentration smoothly increased over time in both treatment groups with a significant increase detected between 48h and 96h time points (p=0.024). GSH concentrations in the exposed groups were significantly higher than in the control groups at each time point (Figure 15) with an average of approx. 1.1-fold increase (p=0.028). However, the interaction between time and treatment, did not show any significant effect on GSH concentration (p=0.073). MDA concentration was significantly higher in the exposed group (Figure 15) when compared to the control (p=0.0000005). A 3.2-fold increase was detected at 24h, a 2-fold increase at 48h, a 3-fold increase at 72h and a 2.5-fold increase at 96h. As in GST, time had no effect on MDA concentrations (p=0.475) hence, they appear to be valuable biomarkers of oxidative stress in *Calanus finmarchicus*.

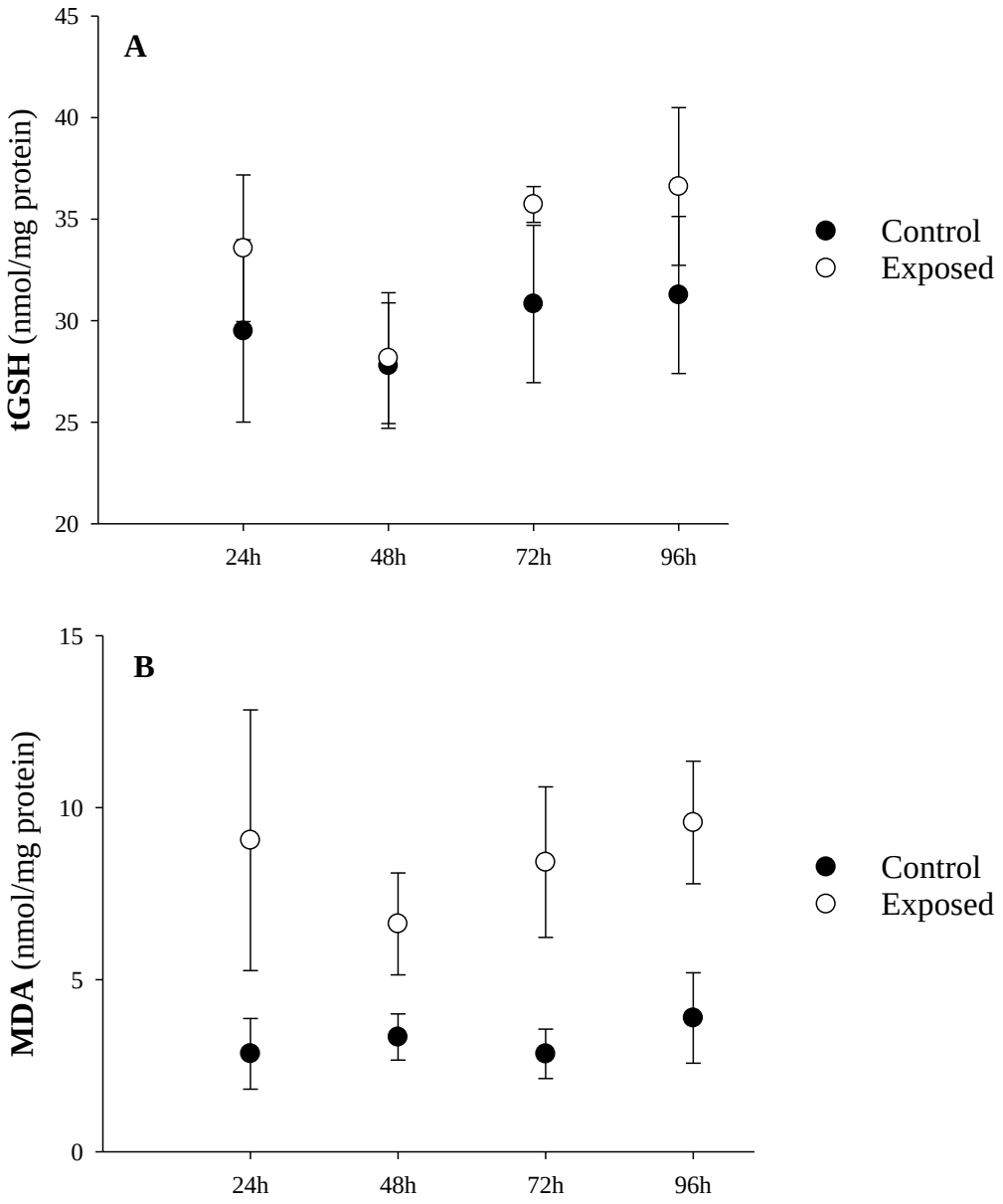

Figure 15: Concentrations of tGSH (**A**) and MDA (**B**) in the exposed and control groups detected as nmol/mg protein. A moderate but significant time-dependent increase in tGSH concentration was detected in the WAF-exposed group (treatment p=0.028; time p=0.024). A significant increase was also detected in MDA concentration (p=0.0000005).

One more analysis was performed to investigate the SOD enzymatic activity. For 24 samples out of 30, levels of SOD were below the limit of detection (Table 20), therefore, it was not considered in further statistical analysis. The complete results tables regarding enzymatic activities and MDA and GSH concentrations are presented in Appendix VII.

4 Discussion

Our study aimed at validating biomarkers for oxidative stress in *Calanus finmarchicus* exposed to a WAF of a naphthenic NA crude oil. Several target genes (CYP1A2, CYP330A1, GST, GSH, SOD, CAT, HSP70, HSP90 and UB), enzymes (SOD, CAT, GST, GPx) and oxidative stress-related molecules (MDA and GSH) were analysed. Considering our results, GST enzymatic activity, and GSH and MDA concentrations appear to be successful biomarkers of oxidative stress in *Calanus finmarchicus* while gene expression results were inconsistent.

4.1 WAF toxicity

The main contributors in the WAF chemical profile are BTEX, and for the semi-volatile compounds, naphthalene (Table 10). The majority of compounds detected is volatile, while naphthalene (SVOC) accounts only for 1.7% of the total amount of petroleum hydrocarbons. For the experiment to be reproducible and comparable, experimental bottles were sealed with minimum headspace to prevent uncontrolled loss of volatile chemicals. Under realistic oil spill conditions, VOC would quickly evaporate, exiting the marine environment.

Table 10: Concentration of the major chemical compounds detected in the WAF of a naphthenic North Atlantic oil (µg/L). All major compounds are volatile organic compound beside naphthalene (SVOC).

Compounds	**(µg/L)**	**Class**
Toluene	1611.8	VOC
m-Xylene	1363.2	VOC
Benzene	918	VOC
Cyclohexane	735.4	VOC
o-Xylene	481.1	VOC
Ethylbenzene	400.6	VOC
Methylcyclopentane	375.5	VOC
Cyclopentane	361.1	VOC
Methylcyclohexane	322.4	VOC
Isopentane	228	VOC
1,2,4-Trimethylbenzene	185.9	VOC
1-Methyl-3-ethylbenzene	162.7	VOC
Naphthalene	137.8	SVOC

p-Xylene	128.5	VOC

Headley et al. (2001) published a toxicity screening of BTEX including bacterial luminescence using *Vibrio fischeri* (Microtox@), *Daphnia magna* and fathead minnow (*Pimephales promelas*) mortality test and lettuce seedling emergence. Concentrations of BTEX above 5 g/L showed high toxicity, where population mortality reached about 90% for both daphnia and fathead minnow. WAF concentrations shown in Table 10 are well below this threshold. BTEX LC_{50} however, largely varies among species (Meyerhoff, 1975). Poor knowledge about the toxic potential of BTEX on aquatic organisms is a mere consequence of the difficulty to perform toxicity tests on volatile compounds in water environment. BTEX's K_{ow} values indicate that they have a moderate tendency to accumulate in lipid tissues (Table 8) but their toxicity in the marine environment is limited by their tendency to evaporate.

Regarding naphthalene, it has been proved to cause glutathione *S*-transferase induction in *Calanus finmarchicus* well below LC_{50} concentration, while no effect was detected for common oxidative stress biomarkers as SOD and CAT (Hansen et al., 2008). GST is thought to be involved in lipid peroxidation which is likely to represent the major mode of naphthalene toxicity (Hansen et al., 2008). Detrimental effect on feeding and narcotic effects were reported on copepod *Oithona davisae* after a 24h exposure to naphthalene and 1,2-dimethylnaphtalene (Saiz et al., 2009) whilst Ott et al. demonstrated reduced rates of egg production in copepod *Eurytemora affinis* exposed to 10µg/L of naphthalene and its alkylated derivatives (Ott et al., 1978). Phenantrene is one of the most common PAHs in the marine ecosystem and has be shown to stimulate the production of hydroxyl radical and to provoke changes in antioxidant enzymes' activities in *Carassius auratu*s (Sun et al., 2006).

However, WAF toxicity, investigated as a mixture of various compounds, has been widely reported to cause increased mortality rates, and modulation of gene expression, enzymatic activity and developmental rhythms in copepods (Jiang et al., 2010).

4.2 Gene expression of GST-2 and SOD as biomarkers

A total of 15 primers were tested with qPCR. Housekeeping genes are rarely universal, they must be equally expressed in all experimental groups and they must be validated for each study to avoid further input of error in the final dataset (Dheda et al., 2004). They are used as reference genes to normalize the data obtained from the genes of interest by qPCR. Different scientific

publications were examined to find housekeeping genes' candidates for *Calanus finmarchicus*. Ann Tarrant et al. (2008) published a study where they examined and approved the stability of actin and 16S as housekeeping genes in *Calanus finmarchicus* (Tarrant et al., 2008). Hansen et al. (2008) working on naphthalene's modulation on gene transcription in Calanus finmarchicus successfully used actin (Act) and elongation factor A1α (EFA1α) as reference genes (Hansen et al., 2008). In contrast to previous literature (Hansen et al., 2008; Tarrant et al., 2008), only 16S showed standard expression across all treatments (mean Ct: 9.8, SD: 0.3). Actin and EFA1α, instead, showed variations among and within treatments corresponding to > 2 Ct points (see Appendix V).

No target gene showed significant differences in expression rate between control and exposed groups in more than one sampling time point. Upregulation was detected at 48h for GST-2, while both SOD and GST-2 showed downregulation at 72h. Considering our results, no gene could be validated as a successful biomarker for oxidative stress.

Unexpectedly, in our study the control group showed a marked upregulation of all target genes at time 0h, to decrease then at 24h (Appendix VI). Upregulation of stress-related genes in the control groups may occurred as a consequence of handling. A pilot study conducted by Aruda et al. (2011) detected the induction of three *Hsp* transcripts (Hsp70A, Hsp21 and Hsp22) by handling stress in *Calanus finmarchicus*. A positive correlation was found between gene upregulation and waiting time before sampling (Aruda et al., 2011). During handling, test individuals were exposed to additional unwanted stressors as direct light and short-term temperature shift, hence control individuals sampled right after collection and counting processes (0h) are likely to reflect the relative short time stress experienced. Both control and exposed groups underwent the same handling procedures and upregulation of target genes was consistently detected only at 0h. Considering these assumptions, we can assume that whether handling-induced upregulation occurred, it was detectable only shortly after the handling procedure and did not act as a confounding factor across other time points. Starvation might have also act as a confounding factor in our study. Despite a lack of knowledge about modulation of gene expression in starved *Calanus finmarchicus*, starvation was associated with reduced protein body content, increased rate of immature gonads and decreased egg production and clutch size (Niehoff, 2000; Helland et al., 2003). Another possible explanation is that higher values detected at 0h derive from instrumental errors or human mistake during samples' preparation. However, care was taken during the analysis and this hypothesis seems not likely. Control samples were collected at each time point allowing to compare responses of WAF

exposed individuals with baseline expression and to standardize against possible additional confounding stressor as starvation.

Few studies have investigated modulation of gene expression following oil exposure in *Calanus finmarchicus*, and results are not consistent. Hansen et al. (2009) separately exposed lipid-rich and lipid-poor females to the water soluble fraction (WSF) from a North Sea oil. All individuals showed elevated transcription of GST, while CYP330A1 was upregulated in lipid-poor copepods and downregulated in lipid-rich individuals. During a second experiment by Hansen et al. (2013) *C. finmarchicus* at CV stage were exposed to sublethal concentration of WSF of a marine diesel in a static system. Upregulation of GST was detected after 12h, 24h and 48h at 50% of LC_{50} WSF concentration, while for low and medium concentrations (0.5 and 5% of LC_{50}, respectively), GST upregulation was detected at 24h and 48h only. In another study by Hansen et al. (2008) *C. finmarchicus* were exposed to three different concentration of naphthalene, 0.5, 5 and 50% of the LC_{50}. At the lowest exposure concentration, SOD and CAT were upregulated, while upregulation of GST was detected at 24h and 48h in medium and high concentration, and at 12h and 24h for the lowest concentration. The authors concluded that no clear evidence of oxidative stress could be assessed following exposure and they suggested lipid peroxidation as major mode of naphthalene toxicity (Hansen et al., 2008). No clear modulation of CYP1A2 and CYP330A1 was detected, suggesting that cytochrome P450 do not take part in the detoxification enzyme system (Hansen et al., 2008). Hansen et al. (2011) detected upregulation of GST in *C. finmarchicus* exposed to high concentration of WAF of an artificially weathered crude oil (50% of the LC_{50}), but no increase was detected at low of medium concentrations (0.5% and 5% of LC_{50} respectively). A summary of data obtained regarding gene modulation following oil exposure in *Calanus finmarchicus* is presented below (Table 11).

Table 11: Gene expression (GST, SOD, CAT, and CYP330A1) responses in *Calanus finmarchicus* exposed to different crude oil-derived medium. The main experimental parameters are listed for each study and only significant results are presented. CV: fifth copepodite stage; *l*: low concentration; *m*: medium concentration; *h*: high concentration.

Exposure medium	Experimental set-up	Diet	Life-stage	Responses	Reference
WSF North Sea oil	flow-through	*Rhodomonas baltica*	adult	Lipid-poor: ↑ CYP330A1; ↑ GST Lipid-rich: ↓CYP330A1; ↑ GST	*Hansen et al., 2009*

WSF marine diesel	static 0.5, 5, and 50% of LC_{50}	starvation	CV	*h*: ↑ GST at 12, 24 and 48h *l-m*: ↑ GST at 24 and 48h	*Hansen et al., 2013*
Naphthalene	static 0.5, 5, and 50% of LC_{50}	starvation	adult	*l*: ↑SOD; ↑CAT; ↑GST at 12 and 24h *h-m*: ↑GST at 24 and 48h	*Hansen et al., 2008*
WAF artificially weathered crude oil	static 0.5, 5, and 50% of LC_{50}	starvation	CV	*h*: ↑ GST	*Hansen et al., 2011*
WAF naphthenic North Atlantic crude oil	static 50% of LC_{50}	starvation	non-ovulating adult	↑ GST-2 at 48h ↓ GST-2 at 72h ↓ SOD at 72h	*Present study*

Considering our results, cytochrome P450 does not seem to be involved in the detoxification system according to CYP1A2 and CYP330A1 expression, as previously observed in *Calanus finmarchicus* exposed to naphthalene (Hansen et al., 2008). CYP330A1 was upregulated in lipid-poor individuals and downregulated in rich-lipid individuals exposed to the WSF of a North Sea oil, highlighting the significance of lipid content in responses mechanisms. Results are anyhow hardly comparable since no lipid-distinction was considered in our study. Moreover, Hansen et al. (2009) used adult individuals and exposed them in a flow-through system adjusting algal level to support growth and development.

Modulation of GST expression occurred in all studies presented in Table 11 but upregulation is seldom concentration dependent. Our study showed upregulation at 48h, followed by downregulation after 72h from exposure. Inconsistent responses among studies might come from different experimental designs and set-ups. Hansen et al., 2011 and Hansen et al., 2013 used CV copepods while in our study we used non-ovulating adults. The marine diesel WSF chemical composition (Hansen et al., 2013) differ significantly from our WAF; phenols for instance were a major SVOC component group (168 μg/L in the 1:40 WSF matrix). *Calanus finmarchicus* CV exposed to a WAF of an artificially weathered crude oil showed GST upregulation only at high concentration (50% of LC_{50}) (Hansen et al., 2011). Once again, the WAF chemical profile differ from the one determined in this study, with a major contribution

of phenols (110 μg/L of WAF exposure medium). GST expression, therefore, does not seem a reliable biomarker of oxidative stress in *C. finmarchicus* exposed to oil.

Well-established biomarkers for oxidative stress as CAT or SOD also showed poor efficiency as biomarkers for oxidative stress. *Calanus finmarchicus* exposed to a low concentration of naphthalene showed upregulation of SOD and CAT (Hansen et al., 2008) but no significant differences between exposed and control group were detected at medium or high concentrations. In the present study, a significant downregulation of SOD was detected 72h after exposure, but no significant differences were detected at any other sampling time point; regarding CAT, no differences between the control and exposed groups was detected at any time point following exposure.

Moreover, heat shock protein 70 and 90 (HSP70, HSP90) and ubiquitin (UB) were analyzed to investigate for cellular stress and protein degradation but neither time nor treatment had any effect on their expression ratios.

Due to the high concentration used in our experiment (50% of 96h LC_{50}) it was expected to detect upregulations of target genes as GST, CAT and SOD. Our results though failed to detect any consistent modulation in target genes expression, hence, no gene could be validated as reliable biomarker of oxidative stress in *Calanus finmarchicus*.

4.3 Enzymatic activity of GST and concentration of GSH and MDA as biomarkers

A significant increase in MDA and GSH concentrations and GST activity were detected in the exposed group at all sampling time points. Commonly, variation in gene expression is directly linked with up- or down-regulation of enzymatic systems, but this assumption might be valid only for direct and tight substrate-receptor relationships (Regoli et al., 2014). As previously remarked, compensatory mechanisms in response to antioxidant depletion, protein turnover, transcriptional and translational mechanisms, and other non-genomic effects can create discrepancies between gene expression and catalytic activities results (Regoli et al., 2014). It is of importance to compare results obtained from gene expression and enzymatic activity in order to understand mechanisms of toxicity and cellular responses. Even though qPCR did not detect any significant modulations in gene expression related with WAF exposure, enzymatic assays' and MDA and GSH determination's results showed a different scenario.

Catalase is involved in decomposition of hydrogen peroxide to water and oxygen and its activity in *Calanus finmarchicus* exposed to WAF was lower, but not significantly ($p>0.05$). A decrease in the antioxidant level can occur under cellular oxidative stress (Janero, 1990). Glutathione does not only contribute to the regulation of cell cycles, but contains a sulfidric group involved in reduction and conjugation reactions providing the means for removal of ROS and other xenobiotic electrophiles (Meister, 1992). The conjugation of reduced GSH molecules with electrophilic compounds is catalysed by different isoenzymes termed GST. GST isoenzymes are involved in several GSH-dependent reactions as reduction of hydroperoxides (Sharma et al., 2004) and isomerization of unsaturated compounds (Benson et al. 1977). The increase in GSH and induction of GST isoenzymes in the exposed group suggest an increase in intracellular oxidative compounds caused by WAF exposure. Malondialdehyde, which is significantly higher in our exposed groups, is generated by oxidation of polyunsaturated fatty acids (PFUA) and by degradation of pre-existing oxidation products and it is a well-establish indicator of lipid peroxidation (Draper et al., 1990).

Two studies conducted by Sawicki et al. (2003) and Agianian et al (2003) on *Drosophila melanogaster* indicated GST-2 subfamily to be a major antioxidant enzyme involved in conjugation of lipid peroxidative end products. On these bases, Hansen et al. (2008) concluded that increased GST transcription following *Calanus finmarchicus* exposure to naphthalene might indicate lipid peroxidation as possible toxic endpoint. Lipid peroxidation appears to be the main toxic endpoint in *Calanus finmarchicus* exposed to oil or oil compounds (Hansen et al., 2008; 2009; present study) and it seems to represent an appropriate indicator of oil exposure in this species.

Concluding, GSH and MDA concentrations and GST activity appear to be successful biomarkers of oxidative stress, while SOD, CAT and GPx activities, instead, showed inconsistent responses. Moreover, considering our results, lipid peroxidation is suggested as a main toxic endpoint in *C. finmarchicus* exposed to a WAF of a naphthenic crude oil.

4.4 Gene expression vs. enzymatic activities and MDA and GSH concentrations

The contrasting results obtained from gene expression compared to enzymatic activity analyses and GSH concentrations can be interpreted by two different approaches. The first hypothesis concerns mRNA degradation. Gene expression levels might be disguised by the deterioration

of the starting RNA, and therefore, expression ratios detected would not reflect the actual cellular responses to WAF exposure. Single stranded RNA is naturally unstable inside the cell; aberrant or unwanted mRNA degradation is strictly regulated by surveillance mechanisms (Garneau et al., 2007). Sampling, storage, extraction and handling of samples can lead to single stranded RNA degradation (Becker et al., 2010). No degradation, however, was detected in purified RNA stored at -20 or -70 °C in RNase-free water for at least one year when isolated using the QIAGEN system (QIAGEN, 2018). Although possible, this hypothesis seems rather weak.

The second hypothesis suggests that post-transcriptional events are involved in oxidative stress responses. Natural-occurring mRNA-decay rates account as a major driving component of gene regulations (Garneau et al., 2007). 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 ratios and enzymatic activities. Regarding marine organisms, those mechanisms are not-well understood due to a lack of investigations and the comparison between mRNA transcription and antioxidant enzymatic responses generally shows contrasting trends (Regoli et al., 2011). Antioxidant responses can therefore be detected at an enzymatic level while no clear upregulation is shown by qPCR analysis. Considering the intricate pathways involved in antioxidant responses, from gene expression to enzymatic activity (Figure 3), is reasonable to conclude that post-translational mechanisms might play a major role in antioxidant responses of *Calanus finmarchicus* exposed to oxidative agents. Thus, gene expression appear to be a poor indicator for oil exposure in *Calanus finmarchicus*.

4.5 Experimental design and analyses limitations

Immediately after the occurrence of an oil spill, the physical and chemical properties of the oil released in the marine environment are altered by weathering processes. Being oil a complex mixture, biological responses detected following exposure are specific for the type of oil studied and extendable to oil showing similar chemical profiles.

Reproducing realistic environmental conditions in a laboratory experiment is seldom achievable, and the results obtained must be considered with regard to the experiment's limitations. Organisms subjected to a real oil spill will not be affected by soluble chemicals only, but also by oil slicks and oil particles. Different studies investigated the possible interactions and effects of oil droplets to planktonic species. Oil droplets <70 μm tent to

accumulate in the water column (Li et al., 2011) and resemble the size range of diatoms chain actively uptaken by filter-feeding organisms (Conover, 1971; Herbert et al., 1980; Hansen et al., 2009). Filter-feeding organisms can switch their feeding preferences according to the most abundant particles in seawater and therefore dietary intake can represent the more important source of contamination under certain condition (Herbert et al., 1980). The presence of oil droplets in the exposure medium can decrease the uptake of oil compounds as a consequence of reduced filtering rates or increase bioavailability of chemical compounds throughout ingested small particles (Hansen et al., 2009). A comparative study between WSF and dispersed oil anyhow, indicates that oil droplets seem to have little effect on gene transcription (GST-2) (Hansen et al., 2011).

Another main factor affecting the extent of toxic responses in the environment is natural sunlight. Photo-enhanced toxicity has been proved in a laboratory experiment using two calanoid copepods, *Calanus marshallae* and *Metridia okhotensis*. Interaction between UV-light and aqueous polycyclic aromatic compounds (PAC) was significant for both test species leading to increased morbidity and mortality (Duesterloh et al., 2002). To overcome possible alteration in *Calanus finmarchicus* responses due to light interference with our experimental medium, the exposure was conducted in darkness.

C. finmarchicus sensitivity to the WSF of fresh and weathered oil, moreover, appears to be both stage and sex dependent (Jager et al., 2016). Males and early copepodite stages appear to be more sensitive than nauplii and older individuals. Also lipid content and body size seem to act as confounding factors (Hansen et al., 2009; Jiang et al., 2012). The overall effect of oil contamination on *Calanus finmarchicus* wild populations therefore, might differ from the one detected in our study. Our exposure set-up is not representative of realistic environmental conditions but allows high degree of control of confounding factors and strong comparability.

Regarding the analytical approach, 60 µL of RNA were obtained per sample (10 individuals), allowing to analyse up to 1000 primers at 1:10 cDNA-dilution. On the other hand, 100 individuals per sample were needed to analyse CAT, GPx, SOD and GST enzymatic activities and GSH and MDA concentrations only. To avoid unwanted discrepancies in the gene expression results, possibly deriving from running the same primer in two different plates, cDNA samples were run only in duplicates (Appendix II). This choice, however, made it impossible to detect outliers since only two values were available per samples. Considering the large variation occasionally detected within the same sample, we suggest for future studies, to run cDNA samples in triplicates to obtain a more precise and meaningful dataset. Based on the

results of the present study, despite the high amount of animals required for the analyses, GSH and MDA concentrations and GST activity are suitable biomarkers for oil exposure in *Calanus finmarchicus*.

4.6 Implication of the present study

MDA, and to a certain extent GST, are involved in lipid peroxidation events (Sawicki et al., 2003; Agianian et al., 2003; Hansen et al., 2008) and PAHs are known to act as peroxisomal proliferators (Regoli et al., 2014). Lipids play a major role in *Calanus finmarchicus* life cycle and the greatest amount of lipid stored is detected in adults and fifth copepodite stage females (Niehoff et al., 1999). Only 5% is consumed during overwintering while lipids are heavily used for egg and sperm production when food level is low (Irigoien, 2004; Hansen et al., 2008). Cholesterol depletion, induced by lipid peroxidation, could affect steroidogenesis, causing hormonal modulation (Hansen et al., 2008). Lipids moreover, appear to be involved in triggering diapause (cholesterol and fatty acid derived hormones), determining overwintering depth and regulating adaptation to hydrological basin conditions (Irigoien, 2004). Lipids degradation hence, could be highly detrimental for *Calanus finmarchicus*. Beside the several chain-effects related with lipid peroxidation and oxidative stress, modulation in ROS cellular content can affect different cellular processes. ROS indeed act as effector molecules regulating cell growth, induction and maintenance of the transformed state, programmed cell death and cellular senescence (Finkel, 2003). At low concentration, ROS act as regulators in signalling cellular processes for intended biological responses (Dröge, 2002) but at high intracellular concentrations, they are highly harmful, damaging all major cellular compartments and interfering with the normal cellular signalling pathways. The increase in GSH and MDA concentrations and the induction of GST in the exposed groups suggest the occurrence of WAF-induced oxidative stress, which could elicit a cascade of sublethal and potentially lethal effects.

5 Conclusion

Given the lack of suitable biomarkers for a key species as *Calanus finmarchicus,* our study aimed at investigating and validating appropriate biomarkers of oil exposure. Considering our results, GST enzymatic activity, and GSH and MDA concentrations appear to be successful biomarkers of oxidative stress in *Calanus finmarchicus* exposed to a WAF of a naphthenic NA crude oil. Moreover, lipid peroxidation is suggested as major toxic endpoint. Contrary to gene expression analyses, determination of GST activity and MDA and GSH concentrations require a rather high number of individuals (≈100 individuals per sample), possibly posing some limitations to their use as biomarkers. Gene expression results, anyhow, appear inconsistent. Field studies are needed to confirm our results in actual oil contaminated environments while more investigations are necessary to understand the role of lipids during oil exposure and to identify specific PAHs toxic pathways in *Calanus finmarchicus*.

References

Aamand, J., Jørgensen, C., Arvin, E., & Jensen, B. K. (1989). Microbial adaptation to degradation of hydrocarbons in polluted and unpolluted groundwater. *Journal of Contaminant Hydrology*, *4*(4), 299–312. https://doi.org/10.1016/0169-7722(89)90030-2.

Abbriano, R.M., Carranza, M.M. , Hogle, S.L., Levin, R.A., Netburn, A.N., Seto, K.L., Snyder, S.M., & Franks, P.J.S. (2011) Deepwater Horizon oil spill: A review of the planktonic response. *Oceanography 24*(3):294–301, http://dx.doi.org/10.5670/oceanog.2011.80.

Agianian, B., Tucker, P. A., Schouten, A., Leonard, K., Bullard, B., & Gros, P. (2003). Structure of a Drosophila sigma class glutathione S-transferase reveals a novel active site topography suited for lipid peroxidation products. *Journal of Molecular Biology*, *326*, 151–165. https://doi.org/10.1016/S0022-2836(02)01327-X.

Almeda, R., Baca, S., Hyatt, C., & Buskey, E. J. (2014). Ingestion and sublethal effects of physically and chemically dispersed crude oil on marine planktonic copepods. *Ecotoxicology*, *23*, 988–1003. https://doi.org/10.1007/s10646-014-1242-6.

An, Y.-J. (2004). Toxicity of Benzene, Toluene, Ethylbenzene, and Xylene (BTEX) Mixtures to Sorghum bicolor and Cucumis sativus. *Bulletin of Environmental Contamination and Toxicology*, *72*, 1006–1011. https://doi.org/10.1007/s00128-004-0343-y.

Aruda, A. M., Baumgartner, M. F., Reitzel, A. M., & Tarrant, A. M. (2011). Heat shock protein expression during stress and diapause in the marine copepod Calanus finmarchicus. *Journal of Insect Physiology*, *57*, 665–675. https://doi.org/10.1016/j.jinsphys.2011.03.007.

Aruoma, O., Kaur, H., & Halliwell, B. (1991). Oxygen free radicals and human diseases. *Journal of the Royal Society of Health*, *111*(5), 172-177.

Baguley, J. G., Montagna, P. A., Cooksey, C., Hyland, J. L., Bang, H. W., Morrison, C., Ricci, M. (2015). Community response of deep-sea soft- sediment metazoan meiofauna to the Deepwater Horizon blowout and oil spill. *Marine Ecology Progress Series*, *528*, 127–140. https://doi.org/10.3354/meps11290.

Barron, M. G., Carls, M. G., Heintz, R., Rice, & S.D. (2004). Evaluation of Fish Early Life-Stage Toxicity Models of Chronic Embryonic Exposures to Complex Polycyclic Aromatic Hydrocarbon Mixtures. *Toxicological Sciences*, *78*(1), 60–67.

Bayir, H. (2005). Reactive oxygen species. *Critical Care Medicine*. *33*(12), 498-501 https://doi.org/10.1097/01.CCM.0000186787.64500.12.

Becker, C., Hammerle-Fickinger, A., Riedmaier, I., & Pfaffl, M. W. (2010). mRNA and microRNA quality control for RT-qPCR analysis. *Methods*, *50*, 237–243. https://doi.org/10.1016/j.ymeth.2010.01.010.

Benson, A. M., Talalay, P., Keenf, J. H., & Jakobyf, W. B. (1977). Relationship between the soluble glutathione-dependent A5-3-ketosteroid isomerase and the glutathione S-transferases of the liver (human and rat liver/ligandin/isoelectric focusing). *Biochemistry*, *74*(1), 158–162.

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism: Clinical and Experimental*, *49*(2), 3–8. https://doi.org/10.1016/S0026-0495(00)80077-3.

Billiard, S. M., Meyer, J. N., Wassenberg, D. M., Hodson, P. V., & Giulio, R. T. Di. (2008). Nonadditive effects of PAHs on Early Vertebrate Development: Mechanisms and Implications for Risk Assessment. *Toxicological Sciences*, *105*(1), 5–23. https://doi.org/10.1093/toxsci/kfm303.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative Stress and Antioxidant Defense. *The World Allergy Organization Journal*, *5*(1), 9–19. http://doi.org/10.1097/WOX.0b013e3182439613.

Bodkin, J. L., Ballachey, B. E., Dean, T. A., Fukuyama, A. K., Jewett, S. C., Mcdonald, L., … Vanblaricom, G. R. (2003). Sea otter population status and the process of recovery from the 1989 'Exxon Valdez' oil spill. *Marine Ecology Progress Series*, *241*, 237–253.

Borgerson, S. G. (2008). Arctic Meltdown - The economy and security implication of global warming. *Foreign Affair*, *87*(2), 63–77.

Boyles, D. (1980). Toxicity of Hydrocarbons and Their Halogenated Derivatives in an Aqueous Environment. In *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment.* (pp. 545–557). Boston, MA: Environmental Science Research.

Brot, N., & Weissbach, H. (1982). The biochemistry of methionine sulfoxide residues in proteins. *Trends in Biochemical Sciences*. *7*(4); 137-139. https://doi.org/10.1016/0968-0004(82)90204-3.

Bryant, A. D., Heath, M., Gurney, W. S. G., Beare, D. J., & Robertson, W. (1997). The seasonal dynamics of Calanus finmarchicus: development of a three-dimensional structured population model and application to the northern North Sea. *Journal of Sea Research*, *38*(3–4), 361–379. https://doi.org/10.1016/S1385-1101(97)00046-4.

Brummelen, T.C., Hattum, A.G.M., Crommentuijn T., Kalf, D.F. (1998). Bioavailability and ecotoxicity of PAHs. *The Handbook of Environmental Chemistry*, Springer-Verlag. Part J: PAHs and Related Compounds, 203-263.

Burns, K. A., Ehrhardt, M. G., Howes, B. L., & Taylor, C. D. (1993). Subtidal Benthic Community Respiration and Production near the Heavily Oiled Gulf Coast of Saudi Arabia. *Marine Pollution Bulletin*, *27*, 199–205.

Burton, G. W., & Ingold, K. U. (1986). Vitamin E: Application of the Principles of Physical Organic Chemistry to the Exploration of Its Structure and Function. *Accounts of Chemical Research*, *19*(7), 194–201. https://doi.org/10.1021/ar00127a001.

Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C., & Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: A practical approach. In *Science of the Total Environment* (Vol. 247, pp. 295–311). https://doi.org/10.1016/S0048-9697(99)00499-4.

Chesnaux, R. (2008). Analytical closed-form solutions for assessing pumping cycles, times, and costs required for NAPL remediation. *Environmental Geology*, *55*, 1381-1388. 10.1007/s00254-007-1088-9.

Conover, R. J. (1971). Some relations between zooplankton and bunker C oil in Chedabucto Bay following the wreck of the tanker Arrow. *Journal of the Fisheries Research Board of Canada*, *28*, 1327–1330.

Cowles, T. J. (1983). Effects of exposure to sublethal concentrations of crude oil on the copepod Centropages hamatus - II. Activity patterns. *Marine Biology*, *78*(1), 53–57. https://doi.org/10.1007/BF00392971.

Cristini, A., & Saiff, E. (1984). The Effects of Benzene on the Histopathology of the Midgut Gland of Juvenile Blue Crabs, Callinectes sapidus. *Annals of the New York Academy of Sciences*, *435*(1), 248–253. https://doi.org/10.1111/j.1749-6632.1984.tb13785.x.

Denissenko, M. F., Pao, A., Tang, M., & Pfeifer, G. P. (2011). Preferential Formation of Benzo[a]pyrene Adducts at Lung Cancer Mutational Hotspots in P53. *American Association for the Advancement of Science*, *274*(5286), 430–432.

Dheda, K., Huggett, J. F., Bustin, S. A., Johnson, M. A., Rook, G., & Zumla, A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *BioTechniques*, *37*(1), 112–119. https://doi.org/http://dx.doi.org/10.1016/j.ymeth.2010.01.003.

Draper, H. & Hadley, M. (1990). Malondialdehyde determination as index of lipid Peroxidation. *Methods in Enzymology*, *186*, 421–431.

Dröge, W. (2002). Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews*, *82*, 47–95. https://doi.org/10.1152/physrev.00018.2001.

Duesterloh, S., Short, J.W., Barron, M.G. (2002). Photoenhanced Toxicity of Weathered Alaska North Slope Crude Oil to the Calanoid Copepods Calanus marshallae and Metridia okhotensis. *Environmental Science and Technology*, *36*(18), 3953-3959ager. https://doi.org/10.1152/physrev.00018.2001.

EIA. (2017) *Energy Information Administration, International Energy Statistics*. https://www.eia.gov/beta/international/data/browser#/?pa=0000001001vg00000000000000000000g00000000000g&c=4100000002000060000000000000g00020000000000000000001&tl_id=5-A&vs=INTL.53-1-AFRC-TBPD.A&vo=0&v=H&end=2017 (Visited 13.05.2017).

Eom, I.Y. (2011). Estimation of Partition Coefficients of Benzene, Toluene, Ethylbenzene, and p-Xylene by Consecutive Extraction with Solid Phase Microextraction. *Bulletin of the Korean Chemical Society*, *32*. 10.5012/bkcs.2011.32.5.1463.

Esler, D., Bowman, T. D., Trust, K. A., Ballachey, B. E., Dean, T. A., & Jewett, S. C. (2002). Harlequin duck population recovery following the 'Exxon Valdez' oil spill: progress, process and constraints. *Marine Ecology Progress Series*, *241*, 271–286.

Espinasse, B., Basedow, S. L., Tverberg, V., Hattermann, T., & Eiane, K. (2016). A major Calanus finmarchicus overwintering population inside a deep fjord in northern Norway: implications for cod larvae recruitment success. *Journal of Plankton Research*, *38*(3), 604–609. https://doi.org/10.1093/plankt/fbw024.

Falk-Petersen, S., Hopkins, C., & Sargent, J. (1990). Trophic relationships in the pelagic arctic food web. In *Barnes M, Gibson RN (eds) Trophic relationships in the marine environment. Proc 24th Eur Mar Biol Symp. Aberdeen University Press, Aberdeen.* (315 –333).

Falk-Petersen, S., Pavlov, V., Timofeev, S., & Sargent, J. R. (2007). Climate variability and possible effects on arctic food chains: The role of Calanus. In *Arctic Alpine Ecosystems and People in a Changing Environment* (pp. 147–166). https://doi.org/10.1007/978-3-540-48514-8_9.

Farkas, J., Capadonna, V., Olsen, A. J., Posch, W., Wilflingseder, D., Blatzer, M., Altin, D. Jenssen, B.M. & Ciesielski T. M. Exposures to engineered silver nanoparticles and crude oil water soluble fraction cause oxidative stress in the marine copepod Calanus finmarchicus. *Manuscript in preparation.*

Finkel, T. (2003). Oxidant signals and oxidative stress. *Current Opinion in Cell Biology*, *15*, 247–254. https://doi.org/10.1016/S0955-0674(03)00002-4.

Garneau, N. L., Wilusz, J., & Wilusz, C. J. (2007). The highways and byways of mRNA decay. *Nature Reviews Molecular Cell Biology*, *8*, 113–126. https://doi.org/10.1038/nrm2104.

Garrott, R., Eberhardt, L., & Burn, D. (1993). Mortality of sea otters in Prince William Sound following the Exxon Valdez oil spill. *Marine Mammal Science*, *9*(4), 343–359.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-Transferases The First Enzymatic Step In Mercapturic Acid Formation*. *The Journal of Biological Chemistry*, *249*(22), 7130–7139.

Halliwell, B., & Gutteridge, J. M. C. (1999). *Free radicals in biology and medicine*. *Oxford: Oxford University Press.* Fifth edition.

Hansen, B. H., Altin, D., Vang, S. H., Nordtug, T., & Olsen, A. J. (2008). Effects of naphthalene on gene transcription in Calanus finmarchicus (Crustacea: Copepoda). *Aquatic Toxicology*, *86*(2), 157–165. https://doi.org/10.1016/j.aquatox.2007.10.009.

Hansen, B. H., Nordtug, T., Altin, D., Booth, A., Hessen, K. M., & Olsen, A. J. (2009). Gene expression of GST and CYP330A1 in lipid-rich and lipid-poor female Calanus finmarchicus (Copepoda: Crustacea) exposed to dispersed oil. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *72*(3–4), 131–139. https://doi.org/10.1080/15287390802537313.

Hansen, B. H., Altin, D., Rørvik, S. F., Øverjordet, I. B., Olsen, A. J., & 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. https://doi.org/10.1016/j.scitotenv. 2010.10.035.

Hansen, B. H., Altin, D., Olsen, A. J., & Nordtug, T. (2012). Acute toxicity of naturally and chemically dispersed oil on the filter-feeding copepod Calanus finmarchicus. *Ecotoxicology and Environmental Safety*, *86*, 38–46. https://doi.org/10.1016/j.ecoenv.2012.09.009.

Hansen, B. H., Altin, D., Øverjordet, I. B., Jager, T., & 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. https://doi.org/10.1016/j.scitotenv.2013.01.020.

Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials*, *169*, 1–15. https://doi.org/10.1016/j.jhazmat.2009.03.137.

Headley, J. V., Goudey, S., Birkholz, D., Linton, L. R., & Dickson, L. C. (2001). Toxicity Screening of Benzene, Toluene, Ethylbenzene and Xylene (BTEX) Hydrocarbons in Groundwater at Sour- Gas Plants. *Canadian Water Resources Journal / Revue Canadienne Des Ressources Hydriques*, *23*(3), 345–358. https://doi.org/10.4296/cwrj2603345.

Heath, M. R., & Lough, R. G. (2007). A synthesis of large-scale patterns in the planktonic prey of larval and juvenile cod (Gadus morhua). *Fisheries Oceanography*, *16*(2), 169–185. https://doi.org/10.1111/j.1365-2419.2006.00423.x.

Heath, M.R., Boyle, P.R., Gislason, A., Gurney W.S.C., Hay, S.J., Head, E.J.H., Holmes, S., Ingvarsdóttir, A., Jónasdóttir, S.H., Lindeque, P., Pollard, R.T., Rasmussen, J., Richards, K., Richardson, K., Smerdon, G., & Speirs, D. (2004) omparative ecology of over-wintering *Calanus finmarchicus* in the northern North Atlantic, and implications for life-cycle patterns , *ICES Journal of Marine Science*, 61(4), Pages 698-708

Helland, S., Nejstgaard, J.C., Fyhn, H.J., Egge, J.K., & Båmstedt, U. (2003). Effects of starvation, season, and diet on the free amino acid and protein content of Calanus finmarchicus females. *Marine Biology*, *143*, 297-306. doi:http://dx.doi.org/10.1007/s00227-003-1092-x.

Heras, H., Ackman, R. G., & Macpherson, E. J. (1992). Tainting of Atlantic salmon (Salmo salar) by petroleum hydrocarbons during a short term exposure. *Marine Pollution Bulletin*, *24*(6), 310–315. Retrieved from wos:A1992JC40200013.

Herbert, R., & Poulet, S. A. (1980). Effect of modification of particle size of emulsions of venezuelan crude oil on feeding, survival and growth of marine zooplankton. *Marine Environmental Research*, *4*(2), 121–134.

Herman, D. C., Mayfield, C. I., & Inniss, W. E. (1991). The relationship between toxicity and bioconcentration of volatile aromatic hydrocarbons by the alga Selenastrum capricornutum. *Chemosphere*, *22*(7), 665–676. https://doi.org/10.1016/0045-6535(91)90294-N.

Hrycay, E.G., Bandiera, S.M. (2015). Involvement of Cytochrome P450 in Reactive Oxygen Species Formation and Cancer. *Advances in Pharmacology, 74*, 35-84. https://doi.org/10.1016/bs.apha.2015.03.003.

Irigoien, X. (2004). Some ideas about the role of lipids in the life cycle of Calanus finmarchicus. *Journal of Plankton Research*, *26*(3), 259–264. https://doi.org/10.1093/plankt/fbh030.

Irvine, G., mann, D, H., & Short, J. (1999). Multi-year Persistence of Oil Mousse on High Energy Beaches Distant from the Exxon Valdez Spill Origin. *Marine Pollution Bulletin*, *38*(7), 572–584. http://www.sciencedirect.com/science/article/pii/S0025326X98001155.

ITOPF. (2016). *Oil Tanker Spill Statistics 2016*. https://www.itopf.com/fileadmin/data/Photos /Publications/Oil_Spill_Stats_2016_ low.pdf.

ITOPF. (2017). *Oil Tanker Spill Statistics 2017*. https://www.itopf.com/fileadmin/data/ Photos/ Publications/Oil_Spill_Stats_2016_low.pdf.

ITOPF. (2011). *Effects of Oil Pollutionon the Marine Environment*. http://www.itopf.com/ fileadmin/data/Documents/TIPS%20TAPS/TIP13EffectsofOilPollutionontheMarineEnvironment.pdf.

Jager, T., Altin, D., Miljeteig, C., & Hansen, B. (2016). Stage-dependent and sex-dependent sensitivity to water-soluble fractions of fresh and weathered oil in the marine copepod Calanus finmarchicus. *Environmental Toxicology and Chemistry*, *35*(3), 728–735.

Janero, D. R. (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology and Medicine*. https://doi.org/10.1016/0891-5849(90)90131-2.

Jiang, Z., Huang, Y., Xu, X., Liao, Y., Shou, L., Liu, J., Zeng, J. (2010). Advance in the toxic effects of petroleum water accommodated fraction on marine plankton. *Acta Ecologica Sinica*, *30*(1), 8–15. https://doi.org/10.1016/j.chnaes.2009.12.002.

Jiang, Z., Huang, Y., Chen, Q., Zeng, J., & Xu, X. (2012). Acute toxicity of crude oil water accommodated fraction on marine copepods: The relative importance of acclimatization temperature and body size. *Marine Environmental Research*, (81), 12–17. https://doi.org/10.1016/j.marenvres.2012.08.003.

Johannson, S., Larsson, O., & P. Boehm. (1980). The Tsesis oil spill. *Marine Pollution Bullettin*, *11*, 284–293.

Kaartvedt, S. (2000). Life history of Calanus finmarchicus in the Norwegian Sea in relation to planktivorous fish. In *ICES Journal of Marine Science* (Vol. 57, pp. 1819–1824). https://doi.org/10.1006/jmsc.2000.0964.

Kankofer M. (2001) Antioxidative defence mechanisms against reactive oxygen species in bovine retained and not-retained placenta: activity of glutathione peroxidase, glutathione transferase, catalase and superoxide dismutase. *Placenta*, *22heah*466-72.

Kingston, P. F. (2002). Long-term Environmental Impact of Oil Spills. *Spill Science & Technology Bulletin*, *7*(1–2), 53–61.

Knezovich, J. P., Harrison, F. L., & Wilhelm, R. G. (1987). The bioavailability of sediment-sorbed organic chemicals: A review. *Water, Air, and Soil Pollution*, *32*(1–2), 233–245.

Lauritano, C., Procaccini, G., & Ianora, A. (2012). Gene expression patterns and stress response in marine copepods. *Marine Environmental Research*, *76*, 22–31. https://doi.org/10.1016/j.marenvres.2011.09.015.

Levin, W., Wood, A. W., Yagi, H., Dansette, P. M., Jernia, D. M., & Conney, A. H. (1976). Carcinogenicity of benzo[a]pyrene 4,5-, 7,8-, and 9,10-oxides on mouse skin. *Proceedings of the National Academy of Sciences of the United States of America*, *73*(1), 243–247.

LightCycler® 480 SYBR Green I Master - Easy-to-use hot-start reaction mix for PCR using the LightCycler® 480 System (2005) Roche Applied Science. https://biochimie.umontreal.ca /wp-content/uploads/sites/37/2015/11/LC480SYBR Masterguide.pdf.

Livingstone, D. R. (1993). Review Biotechnology and Pollution Monitoring : Use of Molecular Biomarkers in the Aquatic Environment. *Journal of Chemical Technology & Biotechnology*, *57*, 195–211. https://doi.org/10.1002/jctb.280570302.

Loughlin, T. R. (1994). *Marine Mammals and the "Exxon Valdez*. San Diego: Elsevier.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT. *The Journal of Biological Chemistry*, *193*(1), 265–275.

Marigomez, I., Lehtonen, K., Johann, S., Nusser, L., Jenseen, B. & Ciesielski, T., Part, S. (2017). State-of-art in biological monitoring - Literature review report on the monitoring of biological effects of oil spills and oil spill responses in the northern Atlantic and Baltic Sea. GRACE, Deliverables D3.1 http://www.grace-oil-project.eu/en-US/About/Deliverables.

McLean, J. D., Spiecker, P. M., Sullivan, A. P., & Kilpatrick, P. K. (1998). The Role of Petroleum Asphaltenes in the Stabilization of Water-in-Oil Emulsions. *Structures and Dynamics of Asphaltenes*, 377–422.

Meister, A. (1992). Biosynthesis and Functions of Glutathione, an Essential Biofactor. *Journal of Nutritional Science and Vitaminology*, *38*(special), 1–6.

Melle, W., Runge, J., Head, E., Castellani, C., Licandro, P., Pierson, J. & Chust, G. (2014). The North Atlantic Ocean as habitat for Calanus finmarchicus: Environmental factors

and life history traits. *Progress in Oceanography*, *129*, 244–284. https://doi.org/10.1016/j.pocean.2014.04.026.

Menon, N. N., & Menon, N. R. (1999). Uptake of polycyclic aromatic hydrocarbons from suspended oil borne sediments by the marine bivalve. *Aquatic Toxicology*, *45*, 63–69.

Meyerhoff, R. D. (1975). Acute toxicity of benzene, a component of crude oil, to juvenile striped bass (Morone saxatilis). *Journal of the Fisheries Research Board of Canada*, *32*(10), 1864–1866. https://doi.org/10.1139/f75-223.

Montagna, P. A., & Spies, R. B. (1985). Meiofauna and Chlorophyll Associated with. *Marine Environmental Research*, *16*, 231–242.

Montagna, P. A., Bauer, J. E., Hardin, D., & Spies, R. B. (1989). Vertical distribution of microbial and meiofaunal populations in sediments of a natural coastal hydrocarbon seep. *Journal of Marine Research*, *47*, 657–680.

Murawski, S. A., Hogarth, W. T., Peebles, E. B., & Barbeiri, L. (2014). Prevalence of External Skin Lesions and Polycyclic Aromatic Hydrocarbon Concentrations in Gulf of Mexico Fishes, Post-Deepwater Horizon. *Transactions of the American Fisheries Society*, *143*(4), 1084–1097. https://doi.org/10.1080/00028487.2014.911205.

Neff, J. M. (2002). *Bioaccumulation in Marine Organisms: Effect of Contaminants from Oil Well Produced Water*. Elsevier.

Njobuenwu, D.A., Amadi, S., & Ukpaka, C. (2008). Dissolution Rates of BTEX Contaminants in Water. *The Canadian Journal of Chemical Engineering*, *83*, 985 - 989. 10.1002/cjce.5450830608.

Niehoff, B. (2000). Effect of starvation on the reproductive potential of Calanus finmarchicus. *ICES Journal of Marine Science 57*, 1764-1772. doi:10.1006/jmsc.2000.0971.

Niehoff, B., Klenke, U., Hirche, H. J., Irigoien, X., Head, R. N. and Harris, R. P. (1999) A high frequency time series at Weathership M, Norwegian Sea, during the 1997 spring bloom: the reproductive biology of Calanus finmarchicus. *Marine Ecology Progress* Series, *176*, 81–92.

Nunes, P., & Benville Jr, P. (1979). Uptake and depuration of petroleum hydrocarbons in the manila clam,Tapes semidecussata reeve. *Bulletin of Environmental Contamination and Toxicology*, *21*(1), 719–726.

O'Neill, P. (1987). The chemical basis of radiation biology. *International Journal of Radiation Biology*, *52*(6), 976–976. https://doi.org/10.1080/09553008714552571.

Ott, F. S., Harris, R. P., & O'Hara, S. C. M. (1978). Acute and sublethal toxicity of naphthalene and three methylated derivatives to the estuarine copepod, Eurytemora affinis. *Marine Environmental Research*, *1*(1), 49–58. https://doi.org/10.1016/0141-1136(78)90013-2.

Paine, R. T., Ruesink, J. L., Sun, A., Soulanille, E. L., Wonham, M. J., Harley, C. D. G., … Secord, D. L. (1996). TROUBLE ON OILED WATERS: Lessons from the Exxon Valdez Oil Spill. *Annual Review of Ecology and Systematics*, *27*, 197–235.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical Biochemistry*, *83*, 346–356. https://doi.org/10.1016/0003-2697(77)90043-4.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*(9), 16–21.

Piatt, J. F., & Ford, R. G. (1996). How Many Seabirds Were Killed by the Exxon Valdez Oil Spill? *American Fisheries Society Symposium*, *18*, 712–719.

Planque, B., & Batten, S. D. (2000). Calanus finmarchicus in the North Atlantic: The year of calanus in the context of interdecadal change. In *ICES Journal of Marine Science* (Vol. 57, pp. 1528–1535). https://doi.org/10.1006/jmsc.2000.0970.

Potter, G. (2013). *Marine Pollution*. Retrieved from http://bookboon.com/en/marine-pollution-ebook.

Pritchard, J. B., & Bendt, J. R. (1991). Relative Roles of Metabolism and Renal Excretory Mechanisms in Xenobiotic Elimination by Fish. *Environmental Health Perspective*, *90*, 85–92.

Prokopchuk, I., & Sentyabov, E. (2006). Diets of herring, mackerel, and blue whiting in the Norwegian Sea in relation to Calanus finmarchicus distribution and temperature conditions. *ICES Journal of Marine Science*, *63*, 117–127. https://doi.org/10.1016/j.icesjms.2005.08.005.

QIAGEN. (2018). How long can I store my purified RNA? https://www.qiagen.com/dk/resources/faq?id=d28e72d8-ee39-41ea-8eb8-222ffec4007e&lang=en. (Visited 11.05.2018).

QIAGEN. (2010). Gene Expression and Function Studies. *Qiagen Newsletter*, (15), 1–8. https://doi.org/10.1097/INF.0b013e31825d0ebc. (Visited 11.05.2018).

QuantiTect® Reverse Transcription Handbook - For cDNA synthesis with integrated removal of genomic DNA contamination - For use in real-time two-step RT-PCR (2009) https://www.qiagen.com/ch/resources/resourcedetail?id=f0de5533-3dd1-4835-8820-1f5c088dd800&lang=en.

Reed, M. (1995) Regulation of Produced Water by the U.S. Environmental Protection Agency. In M. Reed & S. Johnsen (Eds.), *Produced water 2*.

Regoli, F., & Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Marine Environmental Research*, *93*, 106–117. https://doi.org/10.1016/j.marenvres.2013.07.006.

Rhee, J. S., Yu, I. T., Kim, B. M., Jeong, C. B., Lee, K. W., Kim, M. J., Lee, J. S. (2013). Copper induces apoptotic cell death through reactive oxygen species-triggered oxidative stress in the intertidal copepod Tigriopus japonicus. *Aquatic Toxicology*, *132–133*, 182–189. https://doi.org/10.1016/j.aquatox.2013.02.013.

RNeasy® Plus Universal Handbook - For purification of total RNA from all types of tissue (2014) https://www.qiagen.com/es/resources/resourcedetail?id=a8f31442-95db-4cbd-9f9d-9e6a63b4af58&lang=en.

Ruiz, Y., Suarez, P., Alonso, A., Longo, E., Villaverde, A., & San Juan, F. (2011). Environmental quality of mussel farms in the Vigo estuary: Pollution by PAHs, origin and effects on reproduction. *Environmental Pollution*, *159*(1), 250–265. https://doi.org/10.1016/J.ENVPOL.2010.08.031.

Saiz, E., Movilla, J., Yebra, L., Barata, C., & Calbet, A. (2009). Lethal and sublethal effects of naphthalene and 1,2-dimethylnaphthalene on naupliar and adult stages of the marine cyclopoid copepod Oithona davisae. *Environmental Pollution*, *157*(4), 1219–1226. https://doi.org/10.1016/j.envpol.2008.12.011.

Sawicki, R., Singh, S. P., Mondal, A. K., Beneš, H., & Zimniak, P. (2003). Cloning, expression and biochemical characterization of one Epsilon-class (GST-3) and ten Delta-class (GST-1) glutathione S-transferases from Drosophila melanogaster, and identification of additional nine members of the Epsilon class. *Biochemical Journal*, *370*, 661–669. https://doi.org/10.1042/bj20021287.

Schuckelt, R., Brigelius-Flohé, R., Maiorino, M., Roveri, A., Reumkens, J., Strabburger, W., Flohé, L. (1991). Phospholipid hydroperoxide glutathione peroxidase is a seleno-enzyme distinct from the classical glutathione peroxidase as evident from cdna and amino acid sequencing. *Free Radical Research*, *14*(5–6), 343–361. https://doi.org/10.3109/10715769109093424.

Seeb, J., & Habicht, C. (1998). Laboratory Examination of Oil-Related Embryo Mortalities that Persist in Pink Salmon Populations in Prince William Sound. *Transactions of the American Fisheries Society*, *127*, 35–43.

Sharma, R., Yang, Y., Sharma, A., Awasthi, S., & Awasthi, Y. (2004) Antioxidant Role of Glutathione S-Transferases: Protection Against Oxidant Toxicity and Regulation of Stress-Mediated Apoptosis. *Antioxidants and Redox Signalling*, *6*(2).

Sharp, B., Cody, M., & Turner, R. (1996). Effects of the Exxon Valdez oil spill on the black oystercatcher. In *Proceedings Of The Exxon Valdez Oil Spill Symposium* (pp. 748–758).

SINTEF. (2015). Chemical and toxicological characterization of water accommodated fraction (WAF) of crude oils.

Skaret, G., Dalpadado, P., Hjøllo, S. S., Skogen, M. D., & Strand, E. (2014). Calanus finmarchicus abundance, production and population dynamics in the Barents Sea in a future climate. *Progress in Oceanography*, *125*, 26–39. https://doi.org/10.1016/j.pocean .2014 .04 .008.

Spies, R. B., Livermore, L., & Field, M. (1983). Natural Isotope Study of Trophic Enrichment of Marine Benthic Communities by Petroleum Seepage. *Marine Biology*, *71*, 67–71.

Stadtman, E. R., & Oliver, C. N. (1991). Metal-catalyzed oxidation of proteins: Physiological consequences. *Journal of Biological Chemistry*. https://doi.org/10.2144/000113869.

Struhsaker, J. W., Eldbridge, M. B., & Echewerria, T. (1974). Benzene (a water-soluble component of crude oil) on eggs and larvae of pacific herring and northern anchovy. *Pollution and Physiology of Marine Organisms*, 253–284.

Sun Y.I., Larry W.O. & Ying Li. (1988) A simple method for clinical assay of Superoxide Dismutase. *Clinical Chemistry, 34*, 497-500.

Sun, Y., Yu, H., Zhang, J., Yin, Y., Shi, H., & Wang, X. (2006). Bioaccumulation, depuration and oxidative stress in fish Carassius auratus under phenanthrene exposure. *Chemosphere*, *63*(8), 1319–1327. https://doi.org/10.1016/j.chemosphere.2005.09.032.

Takeuchi, I., Miyoshi, N., Mizukawa, K., Takada, H., Ikemoto, T., Omori, K., & Tsuchiya, K. (2009). Biomagnification profiles of polycyclic aromatic hydrocarbons, alkylphenols and polychlorinated biphenyls in Tokyo Bay elucidated by δ13C and δ15N isotope ratios as guides to trophic web structure. *Marine Pollution Bulletin*, *58*, 663–671. https://doi.org/10.1016/j.marpolbul.2008.12.022.

Tarrant, A. M., Baumgartner, M. F., Verslycke, T., & Johnson, C. L. (2008). Differential gene expression in diapausing and active Calanus finmarchicus (Copepoda). *Marine Ecology Progress Series*, *355*, 193–207. https://doi.org/10.3354/meps07207.

Transportation Research Board and National Research Council, Y. (2003). *Oil in the Sea III: Inputs, Fates, and Effects*. (T. N. A. Press, Ed.). Washington, DC. Retrieved from https://doi.org/10.17226/10388.

Trust, K., Esler, D., Woodin, B., & Stegeman, J. (2000). Cytochrome P450 1A Induction in Sea Ducks Inhabiting Nearshore Areas of Prince William Sound, Alaska. *Marine Pollution Bulletin*, *40*(5), 397–403.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*, *552*(2), 335–344. https://doi.org/10.1111/j.1469-7793.2003.00335.x.

UNEP. (2008). *In dead water - climate change, pollution, over-harvest, and invasive species in the world's fishing grounds.*

Utne, K. R., Hjøllo, S. S., Huse, G., & Skogen, M. (2012). Estimating the consumption of Calanus finmarchicus by planktivorous fish in the Norwegian Sea using a fully coupled 3D model system. *Marine Biology Research*, *8*(5–6), 527–547. https://doi.org/10.1080/17451000. 2011.642804.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, *64*(2), 178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013.

Varela, M., Bode, A., Lorenzo, J., Viesca, L., Miranda, A., Patrocinio, T., … Groom, S. (2006). The effect of the “Prestige” oil spill on the plankton of the N–NW Spanish coast ´. *Marine Pollution Bullettin*, *53*, 272–286. https://doi.org/10.1016/j.marpolbul.2005.10.005.

Wilfinger, W. W., Mackey, K., & Chomczynski, P. (1997). 260/280 and 260/230 Ratios NanoDrop ® ND-1000 and ND-8000 8-Sample Spectrophotometers. *BioTechniques.*, *22*, 474–481. https://doi.org/10.7860/JCDR/2015/11821.5896.

Wolfe, D. A., Hameedi, M. J., Galt, J. ., Watabayashi, G., Short, J., O’Claire, C., … Sale, H. (1994). The Fate of the Oil Spilled from the Exxon Valdez. *Environmental Science and Technology*, *28*(13), 560–568.

Wolfe, M., Schlosser, J. ., Schwartz, G. J. ., Singaram, S., Mielbrecht, E. ., Tjeerdema, R. ., & Sowby, M. . (1998). Influence of dispersants on the bioavailability and trophic transfer of petroleum hydrocarbons to primary levels of a marine food chain. *Aquatic Toxicology*, *42*(3), 211–227.

Zengel, S., Pennings, S. C., Silliman, B., Montague, C., Weaver, J., Deis, D. R., Nixon, Z. (2016). Deepwater Horizon Oil Spill Impacts on Salt Marsh Fiddler Crabs (Uca spp.). *Estuaries and Coasts*, *39*(4), 1154–1163. https://doi.org/10.1007/s12237-016-0072-6.

Appendices

Appendix I: Determination of LC_{50} concentrations of naphthenic North Atlantic crude oil WAF in *C. finmarchicus*

The LC*x* test based on standard tests on *Acartia tonsa* (ISO) with adopted to temperature (10±2 °C) was performed at SINTEF/NTNU Sealab (Trondheim, Norway). 7 individuals in each experimental bottle (0.5 L), 4 replicates, 7 exposure concentrations (diluted with filtered sea water) plus one control. Starved *Calanus finmarchicus* were exposed to a WAF of a naphthenic North Atlantic crude oil for 96h in darkness. 96h LC_{50} corresponds to the concentrations of WAF that kills 50% of the test animals during 96 hours of exposure.

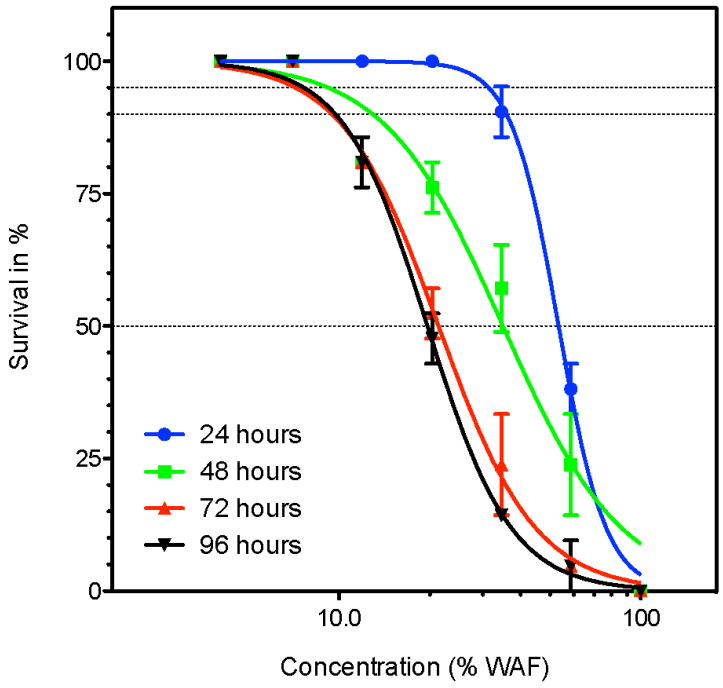

Figure 16: Survival percentage of Calanus finmarchicus exposed to WAF of a naphthenic North Atlantic crude oil. 7 exposure concentrations and 4 sampling time point (24, 48,72 and 96 hours).

50% of the 96h LC_{50} dilution corresponded to 9.85% WAF (1:10 oil-to-water ratio) in seawater.

Appendix II: qPCR set-up

Table 12&13: Plates set-up for real time qPCR. Samples' allocation was semi-randomized to account for "room effect" in the LightCycler 480 Instrument. H_2O: negative control sample to detect possible contamination during plates preparation. –RT: negative control plate to determine possible carryover of genomic DNA. The cDNA plate was run in duplicates.

cDNA	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0hC1	0h01	48hC1	48hC1	96hC1	96hC1			48hC3	48hC3	96hC3	96hC3
B	0hC2	0h02	48hC2	48hC2	96hC2	96hC2			48hC4	48hC4	96hC4	96hC4
C	0hC3	0h03	48hE1	48hE1	96hE1	96hE1			48hE3	48hE3	96hE3	96hE3
D	0hC4	0h04	48hE2	48hE2	96hE2	96hE2			48hE4	48hE4	96hE4	96hE4
E	24hC1	24hC1	72hC1	72hC1	H_2O		24hC3	24hC3	72hC3	72hC3		
F	24hC2	24hC2	72hC2	72hC2	H_2O		24hC4	24hC4	72hC4	72hC4		
G	24hE1	24hE1	72hE1	72hE1			24hE3	24hE3	72hE3	72hE3		
H	24hE2	24hE2	72hE2	72hE2			24hE4	24hE4	72hE4	72hE4		

-RT	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0hC1		48hC1		96hC1				48hC3		96hC3	
B	0hC2		48hC2		96hC2				48hC4		96hC4	
C	0hC3		48hE1		96hE1				48hE3		96hE3	
D	0hC4		48hE2		96hE2				48hE4		96hE4	
E	24hC1		72hC1		H_2O		24hC3		72hC3			
F	24hC2		72hC2		H_2O		24hC4		72hC4			
G	24hE1		72hE1				24hE3		72hE3			
H	24hE2		72hE2				24hE4		72hE4			

Appendix III: Oil and WAF Profile – VOC

Table 14&15: Concentrations of individual volatile organic compounds in oil and WAF (g/Kg). The crude oil sample was run in triplicates while the WAF in duplicates.

OIL PROFILE - VOC			
Compound	**g/Kg**	**Compound**	**g/Kg**
Isopentane	1.84 ± 0.06	Ethylbenzene	1.57 ± 0.01
n-C5 (Pentane)	0.54 ± 0.02	m-Xylene	5.38 ± 0.05
Cyclopentane	1.28 ± 0.08	p-Xylene	1.73 ± 0.02
2-methylpentane	2.16 ± 0.07	o-Xylene	1.52 ± 0.01
3-Methylpentane	1.41 ± 0.03	n-C9 (Nonane)	0
n-C6 (Hexane)	0.3 ± 0.01	Propylbenzene	0.52
Methylcyclopentane	6.36 ± 0.07	1-Methyl-3-ethylbenzene	1.58 ± 0.01
Benzene	0.5	1-Methyl-4-ethylbenzene	0.64 ± 0.01
Cyclohexane	9.38 ± 0.16	1,3,5-Trimethylbenzene	1.12 ± 0.01
2,3-Dimethylpentane	0.96 ± 0.02	1-Methyl-2-ethylbenzene	0.62 ± 0.01
3-methylhexane	1.11 ± 0.03	1,2,4-Trimethylbenzene	2.32 ± 0.03
n-C7 (Heptane)	0.03	n-C10 (Decane)	0.05 ± 0.07
Methylcyclohexane	20.31 ± 0.26	1,2,3-Trimethylbenzene	0.8 ± 0.01
Toluene	2.9 ± 0.02	n-Butylbenzene	0.18 ± 0.01
2,4 diethylhexane	0.09	1,2,4,5-Tetramethylbenzene	0.28 ± 0.01
2-Methylheptane	0.03 ± 0.02	n-Pentylbenzene	0
n-C8 (Octane)	0	C4-Benzenes	4.53 ± 0.06
		C5-Benzenes	4.43 ± 0.07
Sum BTEX	13.61 ± 0.11	Sum C3-benzene	7.6±0.08

WAF – VOC			
Compound	**µg/L**	**Compound**	**µg/L**
Isopentane	227.96 ± 14.66	Ethylbenzene	400.61 ± 9.88
n-C5 (Pentane)	38.45 ± 2.46	m-Xylene	1363.21 ± 92.12
Cyclopentane	361.12 ± 29.1	p-Xylene	128.5 ± 16.49
2-methylpentane	36.45 ± 1.03	o-Xylene	481.1 ± 31.97
3-Methylpentane	28.32 ± 5.77	n-C9 (Nonane)	0
n-C6 (Hexane)	1.85 ± 0.29	Propylbenzene	41.63 ± 4.55
Methylcyclopentane	375.55 ± 43.97	1-Methyl-3-ethylbenzene	162.69 ± 19.25
Benzene	918.02 ± 96.55	1-Methyl-4-ethylbenzene	54.65 ± 6.66
Cyclohexane	735.44 ± 9.21	1,3,5-Trimethylbenzene	84.42 ± 10.34
2,3-Dimethylpentane	21.66 ± 4.06	1-Methyl-2-ethylbenzene	74.07 ± 9.03
3-methylhexane	2.04 ± 0.25	1,2,4-Trimethylbenzene	185.9 ± 25.15
n-C7 (Heptane)	0	n-C10 (Decane)	0.05 ± 0.05
Methylcyclohexane	322.42 ± 30.62	1,2,3-Trimethylbenzene	84.64 ± 12.14
Toluene	1611.76 ± 268.06	n-Butylbenzene	1.97 ± 0.35
2,4 diethylhexane	0.14 ± 0.14	1,2,4,5-Tetramethylbenzene	6.96 ± 0.92
2-Methylheptane	0	n-Pentylbenzene	4.68 ± 0.44
n-C8 (Octane)	0	C4-Benzenes	102.26 ± 14.19
		C5-Benzenes	14.88 ± 1.4
Sum BTEX	4903.2 ± 515.07		

Appendix IV: Oil and WAF Profile – SVOC

Table 16&17: Concentrations of individual semi-volatile organic compounds in oil and WAF (g/Kg). SVOC analyses were performed on a single crude oil sample and on three different WAF samples: WAF, WAF exposure medium and WAF after 96h.

OIL PROFILE - SVOC			
Compound	**g/Kg**	**Compound**	**g/Kg**
Decalin	0.99	C3-dibenzothiophenes	0.1
C1-decalins	1.68	C4-dibenzothiophenes	0.05
C2-decalins	1.29	Fluoranthene	0.01
C3-decalins	0.9	Pyrene	0.02
C4-decalins	0.65	C1-fluoranthrenes/pyrenes	0.13
Benzo(b)thiophene	0	C2-fluoranthenes/pyrenes	0.15
Naphthalene	0.95	C3-fluoranthenes/pyrenes	0.13
C1-naphthalenes	1.89	Benz(a)anthracene	0
C2-naphthalenes	2.25	Chrysene	0.01
C3-naphthalenes	1.61	C1-chrysenes	0.08
C4-naphthalenes	0.85	C2-chrysenes	0.08
Biphenyl	0.28	C3-chrysenes	0.05
Acenaphthylene	0.02	C4-chrysenes	0
Acenaphthene	0.03	Benzo(b)fluoranthene	0.01
Dibenzofuran	0.04	Benzo(k)fluoranthene	0
Fluorene	0.13	Benzo(e)pyrene	0.01
C1-fluorenes	0.32	Benzo(a)pyrene	0
C2-fluorenes	0.41	Perylene	0
C3-fluorenes	0.3	Indeno(1,2,3-c,d)pyrene	0
Phenanthrene	0.2	Dibenz(a,h)anthracene	0
Anthracene	0	Benzo(g,h,i)perylene	0
C1-phenanthrenes/anthracenes	0.45	Phenol	0
C2-phenanthrenes/anthracenes	0.49	C1-Phenols (o- og p-cresol)	0
C3-phenanthrenes/anthracenes	0.49	C2-Phenols	0
C4-phenanthrenes/anthracenes	0.28	C3-Phenols	0
Dibenzothiophene	0.03	C4-Phenols	0
C1-dibenzothiophenes	0.09	C5-Phenols	0
C2-dibenzothiophenes	0.12	30 ab hopane	0.18

WAF - SVOC			
Compounds	**WAF µg/L**	**WAF exposure medium µg/L**	**WAF after 96h µg/L**
Decalin	0.57	0.47	0.02
C1-decalins	0.24	0.23	0
C2-decalins	0	0	0
C3-decalins	0	0	0
C4-decalins	0	0	0
Benzo(b)thiophene	0	0	0
Naphthalene	137.84	110.55	12.34
C1-naphthalenes	71.63	57.32	4.16
C2-naphthalenes	36.23	33	2.77
C3-naphthalenes	7.8	6.55	0.63
C4-naphthalenes	0	0	0
Biphenyl	4.08	0	0.88
Acenaphthylene	0.06	0.05	0
Acenaphthene	0.78	0.67	0.06
Dibenzofuran	1.15	0.98	0.09
Fluorene	2.5	2.18	0.22
C1-fluorenes	1.69	1.47	0.13
C2-fluorenes	0.84	0.79	0.06
C3-fluorenes	0	0	0
Phenanthrene	1.59	1.4	0.12
Anthracene	0	0	0
C1-phenanthrenes/anthracenes	1.11	0.96	0.06
C2-phenanthrenes/anthracenes	0.39	0.32	0
C3-phenanthrenes/anthracenes	0	0	0
C4-phenanthrenes/anthracenes	0	0	0
Dibenzothiophene	0.46	0.29	0.02
C1-dibenzothiophenes	0.85	0.85	0.09
C2-dibenzothiophenes	0	0	0
C3-dibenzothiophenes	0	0	0
C4-dibenzothiophenes	0	0	0
Fluoranthene	0.02	0.02	0
Pyrene	0.02	0.02	0
C1-fluoranthrenes/pyrenes	0.07	0.06	0
C2-fluoranthenes/pyrenes	0	0	0
C3-fluoranthenes/pyrenes	0	0	0
Benz(a)anthracene	0	0	0

Chrysene	0.01	0.01	0
C1-chrysenes	0	0	0
C2-chrysenes	0	0	0
C3-chrysenes	0	0	0
C4-chrysenes	0	0	0
Benzo(b)fluoranthene	0	0	0
Benzo(k)fluoranthene	0	0	0
Benzo(e)pyrene	0	0	0
Benzo(a)pyrene	0	0	0
Perylene	0	0	0
Indeno(1,2,3-c,d)pyrene	0	0	0
Dibenz(a,h)anthracene	0	0	0
Benzo(g,h,i)perylene	0	0	0
Phenol	0	0	0
C1-Phenols (o- og p-cresol)	0	0	0
C2-Phenols	0	0	0
C3-Phenols	0	0	0
C4-Phenols	0	0	0
C5-Phenols	0	0	0
30 ab hopane	0	0	0
TEM	3088.96	2786.59	198.63

Appendix V: Average Ct values for actin and EFA1α

Table 18: Average Ct values for the two discarded housekeeping genes actin and EFA1α. Ct values were obtained from the LightCycler 480 software and baseline-corrected with LinReg.

Sample ID	Average Ct - actin	Average Ct – EFA1α
0h01	20.31	19.17
0h02	21.55	21.7
0h03	20.94	17.19
0h04	18.67	-
24hC1	21.03	19.86
24hC2	25.08	-
24hC3	19.73	18.66
24hC4	21.5	20.18
24hE1	-	43.52
24hE2	21.34	22.27
24hE3	21.94	20.16
24hE4	-	19.57
48hC1	27.48	26.74
48hC2	27.44	28.97
48hC3	21.38	19.4
48hC4	21.44	19.9
48hE1	20.54	18.84
48hE2	19.55	18.43
48hE3	23.46	21.62
48hE4	22.99	21.61
72hC1	19.43	18.26
72hC2	20.26	18.15
72hC3	20.52	20.69
72hC4	20.62	20.85
72hE1	22.23	21.34
72hE2	20.32	21.54
72hE3	24.45	25.47
72hE4	23.37	21.48
96hC1	20.05	18.25
96hC2	20.67	20.02
96hC3	23.66	20.04
96hC4	26.14	28.05
96hE1	20.61	18.13
96hE2	19.88	19.53
96hE3	22.07	19.17
96hE4	21.42	20.09

"-": not detected.

Appendix VI: Expression ratio of target genes

Table 19: Gene expression ratio, following Pfaffl method, for each target gene analysed. 16S was selected as reference gene to normalize gene expression levels. Values are presented as average ratio (from the 4 biological replicates) and relative standard deviation.

Sample ID	Cat[1]		Cat[2]		Cyp1a2		Cyp330	
	Average	SD	Average	SD	Average	SD	Average	SD
C 0h	0.93	0.29	1.704	0.518	5.606	1.533	2.08	0.632
C 24h	0.94	0.04	0.248	0.104	1.607	1.05	0.303	0.127
C 48h	0.59	0.22	1.197	0.030	0.891	0.887	1.462	0.036
C 72h	2.2	0.89	1.793	0.945	3.335	1.438	2.14	1.108
C 96h	2.5	3.2	1.118	0.450	2.116	2.059	1.446	0.495
E 24h	0.44	0.26	0.212	0.145	1.068	0.345	0.259	0.177
E 48h	1.71	0.79	0.589	0.467	1.075	0.912	0.806	0.657
E 72h	2.29	1.4	0.631	0.560	1.535	1.638	1.019	0.985
E 96h	1.35	1.47	2.065	0.788	1.85	1.186	2.42	1.133

Sample ID	Hsp70		Gst[1]		Gst-2		Gst[2]	
	Average	SD	Average	SD	Average	SD	Average	SD
C 0h	14.049	9.312	2.866	1.594	1.942	0.792	4.812	2.678
C 24h	1.865	1.566	0.735	0.432	1.017	0.407	1.235	0.725
C 48h	0.837	0.81	0.855	0.474	0.446	0.235	1.078	0.927
C 72h	2.33	2.035	1.532	0.975	1.387	0.698	2.573	1.637
C 96h	3.349	3.336	1.163	0.822	2.178	1.398	1.49	1.439
E 24h	1.028	0.257	0.422	1.455	1.152	0.747	1.155	0.137
E 48h	2.329	1.787	0.796	0.739	2.06	1.004	1.337	1.241
E 72h	1.448	1.64	0.551	0.363	0.52	0.28	0.925	0.609
E 96h	3.728	4.982	1.564	0.652	1.004	0.731	3.357	1.803

Sample ID	Hsp90		Sod[1]		Sod[2]		Ub	
	Average	SD	Average	SD	Average	SD	Average	SD
C 0h	2.269	1.67	1.731	1.11	3.057	0.299	6.392	3.801
C 24h	0.731	0.611	1.286	0.378	0.493	0.249	0.782	0.652
C 48h	1.455	0.366	0.67	0.602	0.924	0.203	1.862	1.922
C 72h	0.53	0.116	0.965	0.245	1.175	0.787	2.59	2.121
C 96h	2.464	2.431	3.091	0.697	1.17	1.142	2.416	3.463
E 24h	0.992	1.316	1.129	0.769	0.675	1.242	1.207	3.391
E 48h	0.722	0.361	1.66	0.699	0.83	0.682	1.522	1.164
E 72h	0.392	0.371	0.447	0.067	0.575	0.127	0.627	0.389
E 96h	1.404	1.621	2.003	1.106	2.806	2.683	3.095	2.24

Sample ID	Gsh	
	Average	SD
C 0h	1.142	0.325
C 24h	0.604	0.161

C 48h	0.469	0.229
C 72h	2.807	1.243
C 96h	2.674	2.222
E 24h	0.969	0.454
E 48h	1.556	1.244
E 72h	1.141	1.244
E 96h	0.629	0.617

“-1”: first order of primers; “-2”: second order of primers

Appendix VII: Enzymatic assays and MDA and GSH concentrations

Table 20: Results obtained from the enzymatic assays. For each samples is presented the weight (mg), total cytosolic protein content (mg/mL) and enzymatic activity (nmol/min/mg protein) of GST, GPx, CAT and SOD.

Sample ID	**mg**	**Protein in cytosol** (mg/mL)	**GST** (nmol/min/mg protein)	**GPx**	**SOD**	**CAT** (U/mg protein)
24hC1	170	9.8	645.73	14.07	-	8.13
24hC2	181	6.9	871.83	16.34	-	12.24
24hC3	175	8.2	1003.24	17.87	6.52	8.8
24hE1	200	6.5	2550.08	16.95	1.54	7.25
24hE2	150	8.2	2275.47	17.44	-	8.9
24hE3	157	8	2906.9	17.23	-	9.6
24hE4	214	7.1	1190.58	20.58	-	8.52
48h E1	127	7.8	2178.49	15.13	-	7.74
48h E3	128	8.8	2081.85	15.66	-	8.23
48hC1	166	9.3	853.49	14.67	-	7.76
48hC2	145	8.5	790.44	15.2	1.53	12.31
48hC3	160	9.6	1145.83	15.77	1.07	8.31
48hC4	130	7.6	1182.15	20.2	-	9.11
48hE2	134	10.2	2393.54	13.56	-	7.65
48hE4	153	8.8	715.55	17.93	-	8.22
72h E3	185	6.8	2412.68	16.04	-	7.75
72hC1	172	8.4	1034.23	17.25	-	6.62
72hC2	201	7.5	1072.92	18.99	1.33	7.08
72hC3	180	8.8	830.08	16.64	-	5.76
72hC4	201	6.5	757.21	19.52	4.23	10.46
72hE1	190	7.1	2070.5	16.54	-	5.89
72hE4	183	6.5	1036.06	22.14	-	7.69
96h E3	132	8	2696.94	13.74	-	7.61
96hC1	171	9.1	769.23	13.95	-	8.62
96hC2	186	7	2877.6	18.01	-	7.92
96hC3	185	6.8	788.14	21.45	3.44	6.51
96hC4	192	7	680.8	17.88	-	10.02
96hE1	190	6.3	1647.24	18.01	-	5.38
96hE2	154	6.1	1953.13	18.52	-	6.48
96hE4	186	5.8	1074.89	22.59	-	6.9

"-": below limit of detection.

Table 21: Results obtained from determination of MDA and GSH concentration (nmol/mg protein). Weight (mg) and total cytosolic protein content (mg/mL) is presented for each sample.

Sample ID	**mg**	**Protein in cytosol** (mg/ml)	**MDA** (nmol/mg protein)	**GSH**
24hC1	170	9.8	1.76	24.36
24hC2	181	6.9	2.57	35.29
24hC3	175	8.2	4.07	28.82
24hE1	200	6.5	15.52	39.62
24hE2	150	8.2	7.44	31.23
24hE3	157	8	5.85	30.46
24hE4	214	7.1	7.41	32.94

48h E1	127	7.8	8.95	33.47
48h E3	128	8.8	6.85	27.62
48hC1	166	9.3	2.73	25.31
48hC2	145	8.5	2.64	28.95
48hC3	160	9.6	4.22	24.58
48hC4	130	7.6	3.75	32.3
48hE2	134	10.2	5.19	24.88
48hE4	153	8.8	5.5	26.63
72h E3	185	6.8	7.26	34.56
72hC1	172	8.4	2.44	28.07
72hC2	201	7.5	2.05	31.91
72hC3	180	8.8	3.97	26.65
72hC4	201	6.5	2.92	36.65
72hE1	190	7.1	11.48	36.7
72hE4	183	6.5	6.51	35.91
96h E3	132	8	7.89	30.24
96hC1	171	9.1	5.73	25.81
96hC2	186	7	9.24	33.94
96hC3	185	6.8	2.75	34.3
96hC4	192	7	3.19	33.69
96hE1	190	6.3	10.44	39.6
96hE2	154	6.1	7.74	39.05
96hE4	186	5.8	12.53	40.19

Appendix VIII: Normality test

Table 22: Normality test (Shapiro-Wilk) performed on untransformed data. For Sig.>0.05, the data is normal.

	Shapiro-Wilk – Untransformed data	
	Statistic	**Sig.**
Cat[2]	.901	.013
Cat[1]	.729	.000
Cyp1a2	.896	.004
Cyp330	.906	.008
Gsh	.853	.000
Gst[1]	.890	.000
Gst-2	.908	.005
Gst[2]	.890	.000
Hsp70	.756	.000
Hsp90	.646	.000
Sod[1]	.903	.003
Sod[2]	.633	.000
Ub	.769	.000
GST	.872	.001
GPX	.968	.383
CAT	.913	.069
MDA	.933	.026
GSH	.965	.200

Table 23: Normality test (Shapiro-Wilk) performed on Log_{10}-transformed data. For Sig.>0.05, the data is normal.

	Shapiro-Wilk – Log_{10} transformed data	
	Statistic	**Sig.**
Cat[2]	.927	.021
Cat[1]	.986	.930
Cyp1a2	.894	.000
Cyp330	.915	.017
Gsh	.966	.323
Gst[1]	.965	.000
Gst-2	.945	.000
Gst[2]	.965	.468
Hsp70	.948	.729
Hsp90	.938	.377
Sod[1]	.964	.006

Sod[2]	.973	.000
Ub	.925	.226
GST	.859	.006
GPX	.968	.674
CAT	.960	.644
MDA	.906	.529
GSH	.963	.182

Appendix IX: Results from statistical analysis

Table 24: T-test for equality of means performed on Ct values from the reference gene 16S.

	t	df	Sig. (2-tailed)
Equal variances assumed	-.229	31	.821
Equal variances not assumed	-.226	28.351	.823

Table 25: Results from Mann-Whitney Test performed on non-normal data, grouped by treatment.

	Cat[2]	Cyp1a2	Cyp330	GST (e.a.)
Mann-Whitney U	77.000	80.000	80.000	28.000
Wilcoxon W	182.000	185.000	185.000	148.000
Z	-1.222	-1.548	-1.091	-3.361
Asymp. Sig. (2-tailed)	.222	.122	.275	.001

	Gst[1]	Gst-2	Sod[2]
Mann-Whitney U	87.000	124.000	80.000
Wilcoxon W	192.000	244.000	171.000
Z	-1.039	-.398	-.806
Asymp. Sig. (2-tailed)	.299	.691	.420

e.a. enzymatic activity

Table 26. Results from Kruskal-Wallis test on non-normal data, grouped by time. (non-parametric one-way Anova).

	Cat[2]		Cyp1a2		Cyp330	
	With C0h	Without C0h	With C0h	Without C0h	With C0h	Without C0h
Kruskal-Wallis H	11.293	9.717	9.927	3.904	9.629	8.248
df	4	3	4	3	4	3
Asymp. Sig.	.023	.021	.042	.272	.047	0.41

	Sod[2]		Gst-2		GST (e.a)	
	With C0h	Without C0h	With C0h	Without C0h	With C0h	Without C0h
Kruskal-Wallis H	7.356	1.492	2.939	.928		.459
df	4	3	4	3		3
Asymp. Sig.	.118	.684	.568	.819		.928

e.a. enzymatic activity

Table 27: Results from two-way ANOVA analyses performed on Log_{10}-transformed data. Treatment treated as between-subjects factor and time as within-subjects factor.

Gsh	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.359[a]	8	.295	1.414	.243
Intercept	4.305E-5	1	4.305E-5	.000	.989
treatment	.189	1	.189	.906	.351
time	.400	4	.100	.480	.750
treatment * time	1.653	3	.551	2.642	.073
Error	4.796	23	.209		
Total	7.155	32			
Corrected Total	7.155	31			

Gst[2]	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.485a	8	.186	1.031	.443
Intercept	.007	1	.007	.037	.849
treatment	.044	1	.044	.244	.627
time	.938	4	.235	1.303	.300
treatment * time	.491	3	.164	.908	.453
Error	3.959	22	.180		
Total	5.452	31			
Corrected Total	5.444	30			

Hsp70	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.236a	8	.655	1.484	.219
Intercept	.640	1	.640	1.451	.241
treatment	.020	1	.020	.044	.835
time	3.646	4	.912	2.067	.120
treatment * time	1.433	3	.478	1.083	.377

Error	9.702	22	.441		
Total	15.120	31			
Corrected Total	14.938	30			

Hsp90	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.112a	8	.264	1.040	.439
Intercept	.575	1	.575	2.265	.147
treatment	.305	1	.305	1.202	.285
time	.920	4	.230	.906	.479
treatment * time	.429	3	.143	.564	.645
Error	5.332	21	.254		
Total	8.234	30			
Corrected Total	7.444	29			

Sod[1]	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.862a	8	.358	2.982	.019
Intercept	.040	1	.040	.331	.571
treatment	.010	1	.010	.083	.776
time	1.663	4	.416	3.466	.023
treatment * time	1.262	3	.421	3.508	.031
Error	2.759	23	.120		
Total	5.623	32			
Corrected Total	5.621	31			

Ub	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.926a	8	.366	1.595	.183
Intercept	.624	1	.624	2.722	.113
treatment	.022	1	.022	.095	.760
time	1.530	4	.382	1.668	.193
treatment * time	1.154	3	.385	1.678	.201
Error	5.043	22	.229		
Total	8.397	31			
Corrected Total	7.968	30			

Cat[1]	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.809	8	.226	1.377	.256
Intercept	.030	1	.030	.181	.675
treatment	.028	1	.028	.172	.682
time	1.078	4	.269	1.640	.197

treatment * time	.729	3	.243	1.480	.245
Error	3.941	24	.164		
Total	5.756	33			
Corrected Total	5.751	32			

GPx(e.a)	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.020a	7	.003	.714	.661
Intercept	45.010	1	45.010	11500.664	.000
treatment	.001	1	.001	.146	.706
time	.014	3	.005	1.199	.333
treatment * time	.005	3	.002	.435	.730
Error	.086	22	.004		
Total	45.838	30			
Corrected Total	.106	29			

CAT(e.a)	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.083a	7	.012	2.054	.093
Intercept	24.034	1	24.034	4179.666	.000
treatment	.023	1	.023	4.060	.056
time	.054	3	.018	3.116	.047
treatment * time	.006	3	.002	.373	.773
Error	.127	22	.006		
Total	24.573	30			
Corrected Total	.209	29			

MDA	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.330a	7	.190	8.045	.000
Intercept	14.815	1	14.815	627.201	.000
treatment	1.152	1	1.152	48.778	.00000052
time	.134	3	.045	1.891	.161
treatment * time	.064	3	.021	.900	.457
Error	.520	22	.024		
Total	17.015	30			
Corrected Total	1.850	29			

GSH	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.059a	7	.008	2.567	.043
Intercept	66.288	1	66.288	20227.406	.000
treatment	.018	1	.018	5.572	.028

time	.037	3	.012	3.806	.024
treatment * time	.005	3	.002	.525	.670
Error	.072	22	.003		
Total	67.391	30			
Corrected Total	.131	29			

e.a: enzymatic activity

Table 28: Significant differences at single sampling time point. Data grouped by treatment. Untransformed data were used for the non-parametric analysis (Mann-Whitney test) and Log_{10}-transformed data for T-test.

Mann-Whitney Test		
Gst-2	**48h**	**72h**
Mann-Whitney U	.000	.000
Wilcoxon W	10.000	6.000
Z	-2.309	-2.121
Asymp. Sig. (2-tailed)	.021	.034

	T-test for equality of Means		
Sod[1] 72h	**t**	**df**	**Sig. (2-tailed)**
Equal variances assumed	2.765	6	.033
Equal variances not assumed	2.765	4.104	.049