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Acute Toxicity and Stress Gene Transcription in Two Copepod Species (*Calanus finmarchicus* and *Calanus glacialis*) Exposed to Fresh Crude Oil WSF

Siv Fiske Rørvik

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Supervisor: Anders Johny Olsen, IBI
Co-supervisor: Bjørn Henrik Hansen, Sintef Materialer og kjemi

Norwegian University of Science and Technology
Department of Biology

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[1] Picture on the front page: Net hauling in Kings Bay (Photo: BHH)

Abstract

Increasing oil activities from the gas and oil industry may constitute a hazard towards the marine environment due to potential risks for accidents, such as oil spills. This study examine acute effects on mortality (LC_{50}) and stress gene expressions in two closely related copepod species, *Calanus finmarchicus* (temperate-boreal species) and *Calanus glacialis* (Arctic species), adapted to different temperatures 10 °C and 2 °C, respectively. The copepod species were exposed to water soluble hydrocarbon fractions (WSFs) of fresh crude oil. In addition, an experiment was conducted in order to investigate if lipid rich specimen survived a longer exposure of WSFs than lipid poor specimen. The results from the acute toxicity tests indicated that the Arctic species is more tolerated to WSF exposure than the temperate species. Gene expression analysis revealed that both species induced glutathione S-transferase (GST) mRNA levels in a concentration- and time related manner. *Calanus glacialis* showed a greater and faster induction of the GST transcription compared to *Calanus finmarchicus*. The results of gene expression analysis of superoxide dismutase (SOD), cytochrome 330A1 (CYP330A1) and γ-glutmylcystein synthase (γGCS) did not show any concentration- and time related trends following WSF exposure. Biometric measurements of the specimen, and analyzing by a linear multi variable regression model showed that neither of the variables, including the lipid content, explained the differences in surviving a WSFs exposure. The study support the use of the cultured *Calanus* species as a model species when testing for acute effects in the marine environment.

Abbreviations

ATP	Adenoine triphosphate
bp	Base par
BTEXs	Benzene, toulene, ethylbenzene and xylenes
cDNA	Complementary deoxyribonucleic acid
C. finmarchicus	*Calanus finmarchicus*
C. glacialis	*Calanus glacialis*
C. hyperboreus	*Calanus hyperboreus*
CAT	Catalase
CYP 450	Cytochrome P450
DNA	Deoxyribonucleic acid
EF1α	Elongation factor 1α
γGCS	γ-Glutmylcystein synthase
GC-FID	Gas chromatograph-flame ionization detector
GC-MS	Gas chromatograph-mass spectrometry
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-Transferase
Gsynt	Glutathione synthase
H_2O_2	Hydrogen peroxide
LC_{20}	Lethal concentration were 20 % of the organisms are killed
LC_{50}	Lethal concentration were 50% of the organisms are killed
mRNA	Messenger ribonucleic acid
MNE	Mean normalized expression
MSD	Mass selective detector
NADPH	Nicotinamide adenine dinucleotide phosphate
O_2	Molecular oxygen
$O_2 \cdot^-$	Superoxide anion
$OH \cdot$	Hydroxyl radical
PAH	Polyaromatic hydrocarbons
P&T GC-MS	Purge & Trap GC-MS
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative real time polymerase chain reaction
SE	Standard error
SIM	Selected ion monitoring
SOD	Superoxide dismutase
SVOC	Semi-volatile organic compounds
TEOC	Total extractable organic compounds
THC	Total hydrocarbon concentration
VOC	Volatile organic compounds
WSF	Water soluble hydrocarbon fraction

Contents

1 Introduction

The gas and oil industry is always searching for new areas for oil production. With increasing oil activities, there are potential risks for accidents, e.g. oil spills. Shipwreck incidence such as Exxon Valdez (1989) in Alaska, and the more recent Deepwater Horizon (2010) accident in the Gulf of Mexico, have shown that oil production activities may be a serious threat to the marine environment.

The *Calanus* species are an important contributor to the great zooplankton biomass of the coastal waters of the Atlantic Ocean, the Pacific Ocean and the Arctic Ocean (Marshall and Orr, 1972), and hence, an important linkage between primary producers and the higher trophic level species that feed on the *Calanus* species.

There is little knowledge to what extend an oil spill will effect the zooplankton biomass, and the potential effects on the marine food web. Investigating these effects may answer some of the questions concerning the environmental impacts of an oil spill.

1.1 Oil production and composition

The worlds energy demand is increasing with increasing global population. To this date the greatest source of energy is fossils fuels (Demirbas et al., 2004). To satisfy the increasing oil demand the Norwegian oil production industry needs to expand the activities along the Norwegian coast and further up to the Barents Sea (OLF, 2011).

Opening of new areas in the Arctic due to the retreat of the sea ice will make it possible to enhance the oil and gas exploitation, which in turn may increase ship traffic through the Baring Strait, and between the mainland

and the oil rigs. The activities may not only increase the oil production, but also the risk of accidents, as oil spills, and hence threatening the fauna and flora that inhabit the area (Miller et al., 2010).

Oil composition, temperature, time of year, clean-up actions and spill location are important factors influencing the extent and consequences of an oil spill. These factors are especially important in the Arctic, since e.g. formation of sea ice may cause problems with the oil clean-up methodologies (DeCola et al., 2006).

Components in crude oil are basically divided into hydrocarbons and non-hydrocarbons (Figure 1). Crude oil is the basis for a wide range of refined products including gasoline, diesel oils, waxes and asphalts. In refined products, the major components are alkanes, naphthalenes, aromatics and alkenes. The hydrocarbons of the greatest environmental concern in crude oil are the aromatics, and especially the polyaromatic hydrocarbons (PAHs) (Miller and Connell, 1982).

1.1.1 Oil compounds as potential metabolic disturbers

Once the PAHs are in the water, they can be absorbed to organic matter in the sediments and made bioavailable for the marine food web by bottom living organisms (Neff, 2002). PAHs have received big environmental concerns because of the effects they are shown to cause in marine invertebrates; reduced feeding (Jensen and Carroll, 2010), growth (Foss and Forbes, 2007) and reproduction (Street et al., 1998), increased respiration (Kim et al., 2007; Olsen et al., 2007), lipid peroxidation and oxidative stress (Hannam et al., 2010).

One way to eliminate lipophilic and large compounds that have the

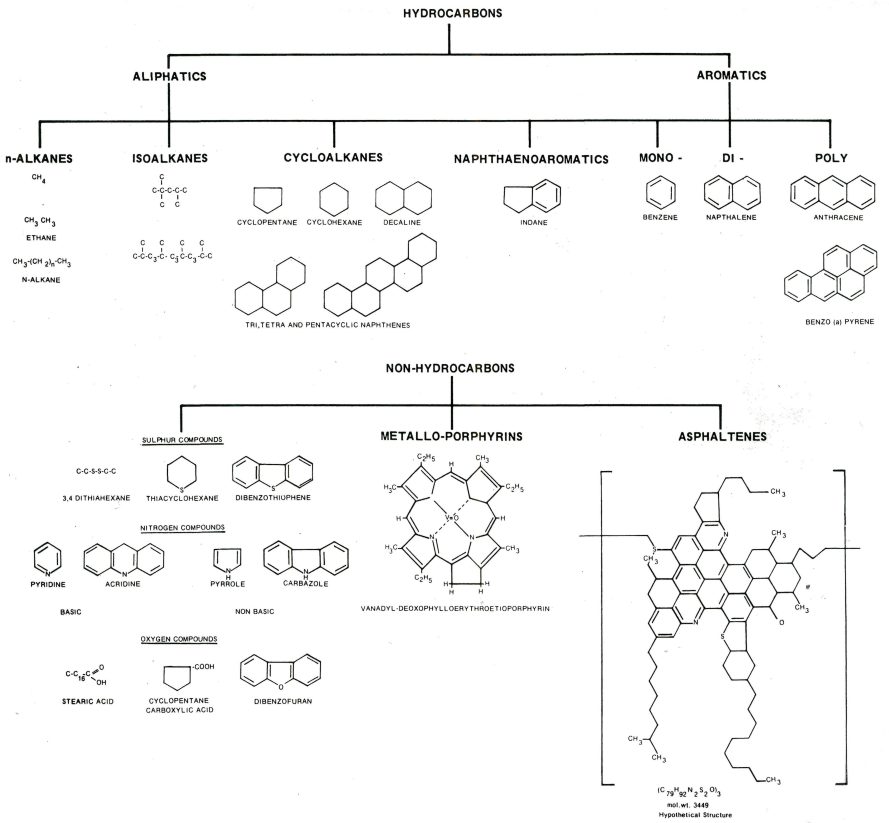

Figure 1: Chemical structure of some of the more common components in crude oil (Miller and Connell, 1982).

potential to cause harm is through biotransformation, an enzymatically converting process. The compounds are transformed to more hydrophilic species, either by an addition/cleavage of a functional group (e.g. hydroxide ion (OH)), or through a conjugation of an endogenous substrate (e.g. GST). The biotransformation make the originally lipophilic compound more hydrophilic, which make excretion through the urine possible (Bolesterli, 2009).

Arctic mammals are known to have good metabolizing systems (Jenssen, 2006). A theoretic disadvantage with a good metabolizing system is the

possibility to produce toxic intermediate metabolites instead of complete mineralization of the mother compound (Bolesterli, 2009).

Invertebrates are shown to have low or variable ability to metabolize PAHs. A consequence of this may be an accumulation of the PAHs in membranes or fatty tissue. Alternatively the PAHs may pass the species internal systems without causing any harms (Rust et al., 2004). The toxicity of PAHs may also have secondary effects; lower feeding might contribute to decreased egg production and hatching (Jensen and Carroll, 2010).

1.2 Arctic organisms

Arctic organisms, as a rule, have a higher lipid content than similar temperated or tropic species, this make them more tolerable to survive long periods of limiting food resources (Lee, 1974).

Long and simple food webs dominate the Arctic, hence a loss of a single species may significantly affect the other species in the food web due to the low species diversity (Borgå et al., 2004). Further, the Arctic organisms have generally a longer life span than their southern siblings and may therefore have a longer exposure time to contaminants (Review by Chapman and Riddle, 2005; Koszteyn et al., 1995).

1.3 Calanoid copepods - *Calanus*.

Herbivorius copepods are the main link of matter and energy between primary producers (micro algae) and higher trophic level species such as fish species (herring and capelin), sea birds and whales (Sastri and Dower, 2009; Loeng and Drinwater, 2007). Calanoid copepods are the predominant group of crustacean-plankton worldwide, and include among others the genus

Calanus (Tande, 1991).

The predominant species in the North Atlantic and the Arctic are *C. finmarchicus* (Gunnerus, 1765), *C. glacialis* (Jaschov, 1955) and *C. hyperboreus* (Krøyer, 1838). Each of the three species have their distinct areas where they dominate. However, the areas overlap so we can find all three species in one area (Mauchline, 1998).

The *Calanus* species accumulate large amounts of lipid in the form of wax esters. They transfer the wax esters into a lipid sac which function as an energy storage. Their huge lipid content combined with their relatively large size, makes the *Calanus* species a valuable fish prey, and hence, an important linkage in the marine food web (Lee et al., 1971a,b; Navenzel, 1970).

Development of *Calanus* is divided in two main periods before they reach the adult stage (CVI), as either female or male; characterized by naupliar stages (NI-NVI) and copepodite stages (CI-CV) (Speirs et al., 2006). During the CIII, CIV and CV stages *Calanus* species transfer a large portion of the accumulated energy into lipids. The lipid store is an important contributor to the final development into sexually maturate adults, and the size of the lipid sac is largely dependent on the amount of food present. Further, the energy stored as lipids is used to produce egg, so the lipid sac is the basis for reproduction (Marshall and Orr, 1972; Lee et al., 1971b).

Calanus species survive the winter as CIV or CV, when the lipid store is at its largest size. During the overwintering period the *Calanus* species descend into deeper water to depths between $200 - 4000$ m, depending on the location (Marshall and Orr, 1972). They stay at these depths until the next spring and phytoplankton bloom, when they enter the surface to

mature into adults and reproduce (Heath et al., 2004). The over-wintering period is characterized by arrested development, low metabolism and low activity (diapause). During the diapause the lipid sac supplies the copepods with energy (Pepin and Head, 2009; Heath et al., 2004).

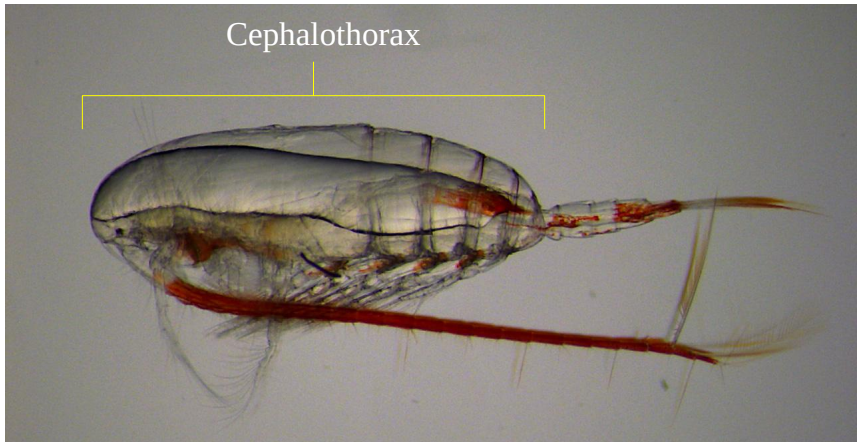

Figure 2: *Calanus glacialis* stage CV (Photo by Dag Altin).

1.3.1 *Calanus finmarchicus.*

C. finmarchicus is widely distributed in The Northern Atlantic Ocean, where it is the major food resource for several fish species and therefore represents a key species in the marine food web. The distribution area identifies *C. finmarchicus* as a temperate species (Sakshaug et al., 1992; McConnaughay, 1978).

C. finmarchicus is the smallest copepod compared to *C. glacialis* and *C. hyperboreus*. The CV cephalothoraxes length (Figure 2) is smaller or equal to 2.8 mm (Marshall and Orr, 1972). The species have at least one annual generation in the southern part of the distribution area (Pedersen et al., 2001)

1.3.2 *Calanus glacialis.*

C. glacialis is considered an Arctic species because of its distribution in the Barents Sea, through the Canadian islands and along the North West coast of North America. *C. glacialis* is believed to have a two year life cycle and reproduce during the spring of its third year. The spawning is dependent on the availability of food , i.e. the spring bloom of phytoplankton (Falk-Petersen et al., 2009).

C. glacialis was recognized as a distinct species in the middle of the 20^{th} century, before this discovery the species was considered a bigger form of *C. finmarchicus*. This indicates that these two species are difficult to separate morphologically. *C. glacialis* have a larger CV cephalothoraxes length (Figure 2), compared to *C. finmarchicus*, generally between 2.7 and 4.1 mm (Madsen et al., 2001).

1.4 Stress gene transcription

Gene expression is the transcription process of a specific gene in producing a messenger ribonucleic acid (mRNA). The mRNA can further be processed into a functional protein through mRNA translation and posttranslational processing (Berg et al., 2006). Exposing a species for a toxic exposure solution will almost always give a directly or indirectly alteration of gene expression. The gene expression can therefore be used to evaluate stress responses induced by toxic components (Nuwaysir et al., 1999). Stress gene expressions could also be used as biomarkers for endocrine disrupting chemicals (Mortensen et al., 2006).

1.4.1 Glutathione S-transferase

Glutathione S-transferase (GST) belongs to a group of phase II xenobiotic detoxification enzymes (Blanchette et al., 2007), and play an important role in oxidative stress regulation (Lee et al., 2008). The crucial role of GST is to defend chemical induced toxicity through inactivation of the chemical. The inactivation is committed by catalyzing the conjugation of a reduced glutathione (GSH) sulfur group to an electrophilic substrate, which may make the substrate more water-soluble and easier to excrete (Bolesterli, 2009).

Previous studies conducted on *C. finmarchicus* have shown that exposure to naphthalene (Hansen et al., 2008b) dispersed oil droplets and water soluble fractions (WSFs) of weathered crude oil increases the GST gene expression (Hansen et al., 2009). The elevated level of GST is an indication of oxidative stress (Hansen et al., 2008b; Lee et al., 2008).

Hansen et al. (2008b) suggest that the increased level of GST mRNA after exposure to naphthalene indicates a lipid peroxidation in *C. finmarchicus*. Since the lipid stores are primarily used for egg and sperm production, this may have an effect on the reproduction. Also, a reduction in cholesterol, the substrate for steroid production, followed by lipid peroxidation, could affect the steroidogenesis and the reproduction.

1.4.2 Cytochrome P450

CYP 450 super family is considered a phase I reaction enzyme in the biotransformation system, also called the functionalization step of the compound. They are important for oxidative, reductive and peroxidative biotransformation of both endogenous and exogenous compounds, where the

overall function is to insert one oxygen into the substrate for making it more available for elimination (Bolesterli, 2009).

The CYP1 and CYP2 families function as possible biomarkers for environmental pollutants, while the CYP4 family is important in metabolizing fatty acids in vertebrates (Bolesterli, 2009; Casarett and Doull, 2008). Invertebrates have been shown to have a lower CYP induction when exposed to PAHs compared to vertebrates. A lower metabolizing capacity is thought to be the reasons for the low CYP induction in invertebrates (Hahn, 1998). However, induction of CYP enzymes after exposure to toxic compounds have been reported in marine crabs and polychaetes (Lee et al., 1981), a crayfish (*Pacifastacus leniusculus*) (Ashley et al., 1996), a water flee (*Daphnia Magna*) and spider crabs (*Maja crispata*) (Snyder, 2000).

There have not been many studies reporting CYP4 enzyme activity in invertebrates, though Rewitz et al. (2004) found significant up-regulation of a CYP4 isogene in the marine polychaeta *Nereis virens* after exposure to crude oil and PAHs. This study may indicate that CYP4 has a function in metabolization of PAHs in some crustaceans. Reduction of CYP4 enzyme activities has been reported in the mollusc *Mytilus galloprovincialis* after exposure to β-naphthoflavone (Snyder, 1998).

Hansen et al. (2008a), showed that CV of *C. finmarchicus* had a higher expression of CYP330A1 than females and males. This is consistent with observation of the production of ecdysteroids, a group of polyhydroxylated ketosteroids serving the primary function to induce production of molting and reproduction hormones. The ecdysteroids are believed to be catalyzed by CYP330A1 (Rewitz et al., 2003; Subramoniam, 2000). The crustacean CYP 300 family is closely related to the vertebrate CYP 2 family (Hansen

et al., 2008a; Rewitz et al., 2003). Hansen et al. (2008b), showed that CYP330A1 was down-regulated after exposure to naphthalene.

There are ongoing studies on the functions of the CYP 450 family system in crustacean, and there are still many unanswered questions (Rewitz et al., 2004).

1.4.3 Antioxidant enzymes

Production of partially reduced O_2 may cause the formation of potential toxic reactive oxygen species (ROS), such as superoxid anion ($O_2 \cdot^-$), hydroxyl radical ($OH \cdot$), and hydrogen peroxide (H_2O_2). ROS are continually produced in biological systems as undesirable bi-products of normal metabolism from various endogenous sources and processes (Livingstone et al., 2001). Production of oxyradicals may be increased by intake of natural and anthropogenic xenobiotics and through a change in the oxygen tension (Gamble et al., 1995).

In order to control the ROS species, organisms produce a variety of antioxidant defenses. Among these are superoxide dismutase (SOD; converts $O_2 \cdot^-$ to H_2O_2), catalase (CAT; converts H_2O_2 to water) and glutathione peroxidase (GPx; detoxifies H_2O_2 and organic hydroperoxides produced, for example, by lipid peroxidase) (Giulio et al., 1995).

Induced production of antioxidant enzymes have been reported after exposure to pollution, as well as variation in diet (Peters et al., 1994), age (Arun and Subramaniam, 1998; Hole et al., 1993), hypoxia (Abele-Oeschger and Oeschger, 1995), temperature (Abele et al., 1998) and seasonality/reproduction cycle (Ringwood and Conners, 2000; Viarengo et al., 1991).

Normally there is a balance between ROS production and antioxidant defense. However, sometimes the balance is switched towards the ROS production and the level increases beyond the capacity of the antioxidant system (Livingstone et al., 2001). This increase the possibility of the ROS species to cause mutations in the deoxyribonucleic acid (DNA), destruction of protein function and structure, and peroxidation of lipids (Dixon et al., 2002).

Increased SOD activity has been reported in fruit fly (*Drosophila melanogaster*) exposed to benzene, toulene and xylene (Singh et al., 2009) and in a fresh water shrimp (*Macrobrachium borelli*) exposed to WSF of petroleum (Lavarías et al., 2011). Correia et al. (2003) reported age variation of SOD mRNA levels in an amphipode (*Gammarus locusta*).

GSH is an ubiquitous tripeptide consisting of glutamate, cysteine and glycine and is one of the most important molecules in antioxidant defense. GSH has multiple functions ranging from antioxidant defense to cell proliferation, and is an important substrate for many enzymatic reactions. It interacts with hydroxyl radicals, peroxinitrit, hydroperoxides and reactive electrophiles, and transform them to less toxic and more hydrophilic compounds (Andrews, 2001).

γ-glutmylcystein synthase (γGCS) and glutathione synthase (Gsynt) catalyzes the adenoine triphosphate (ATP) dependent synthesis of GSH. The maximum production of GSH is regulated by a negative feedback inhibition of γGCS (Soltaninassab et al., 2000; Anderson, 1998). Enzyme systems like amino acid transporters, GPx and glutathione reductase (GR) are contributing to control the cellular levels of GSH (Cnubben et al., 2001).

GSH acts either as a chemical antioxidant in reducing ROS or as a

co-factor in GPx mediated reduction of peroxides. In both cases GSH is oxidized to glutathione disulphide (GSSG), and then reduced back by GR under consumption of nikotinamide adenine dinucleotide phosphate (NADPH). An elevated level of intracellular GSSG indicates oxidative stress, while the GSSG/GSH ratio reflex the cellular redox status (Cnubben et al., 2001; Kearns and Hall, 1998). The activity of γGCS and glutathione synthase may give an indication of the production of GSH (Bolesterli, 2009).

Induction of γGCS is shown in *C. finmarchicus* after exposure to diethanolamine (Hansen et al., 2010). GSH induction has been reported in in clams (*Ruditapes philippinarum*) (Luca-Abbot et al., 2005) and in green-lipped mussel (*Perna viridis*) after exposure to PAHs and organochlorine pesticides (Richardson et al., 2008; Luca-Abbot et al., 2005), while there was reported a reduced GSH level in a freshwater bivalves (*Unio tumidus* (Cossu et al., 1997). A change in the GSH level may be correlated with a similar change of γGCS, because GSH production is regulated by negative feedback of γGCS.

Figure 3 shows how the measured antioxidant systems may function together when detoxifying ROS species.

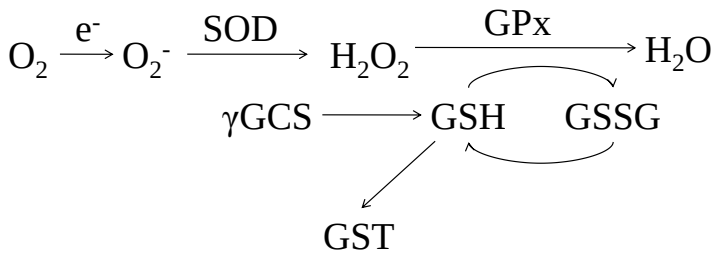

Figure 3: The relationship between the measured enzymes in a detoxification process.

1.4.4 Measuring stress gene transcription

Measuring stress gene transcription can be performed by quantitative real time polymerase chain reaction (RT-qPCR). RT-qPCR allows real time quantification of transcript levels, including an evaluation of the effect a chemical may cause on specific genes. RT-qPCR conducts the amplification and detection of the transcript level simultaneously, which provide a rapid and automated detection of the amount of the target gene mRNA (Tang et al., 2007).

Housekeeping genes are genes that code for products that are required all along, and are expressed more or less at a constant level. Examples of such products are enzymes central in metabolic pathways (Berg et al., 2006). Housekeeping genes are commonly used as reference genes in RT-qPCR. Normalizing the RT-qPCR product against a housekeeping gene will indicate potential alterations in the target genes. When choosing a reference gene, it is important to use one that does not change during developmental changes or before and after experimental manipulation (Curtis et al., 2010; Tang et al., 2007).

Elongation factor 1 alpha (EF1α) is one of the most common housekeeping genes with lowest transcriptional variation (Curtis et al., 2010), because of its involvement in the synthesis of proteins (Berg et al., 2006). Earlier studies of gene expression on *C. finmarchicus* and *C. glacialis* have confirmed that EF1α is suitable for the use as a reference gene also in these species (Hansen et al., 2009, 2008a,b).

1.5 Present study

1.5.1 Background

Numerous studies (Hansen et al., 2011, 2010, 2008b, 2007; Jensen et al., 2008; Magnusson et al., 2007; Durbin et al., 2002; Fisk et al., 2001) have used *Calanus* species when investigating how chemicals may effect the marine environment. The *Calanus* species may therefore be considered an important model species for marine environment related experiments.

Reports on LC_{50} values for marine invertebrates are varying depending on the exposure media, the species and exposure time. For single oil-related compounds, Hansen et al. (2008b) reported that *C. finmarchicus* had a 96 h-LC_{50} of 7 mg/L when exposed to naphthalene. Resent experiments have shown a 96 h-LC_{50} for *C. finmarchicus* and *C. glacialis* exposed to weathered crude oil WSF to be 0.817 and 1.037 µgTHC/L, respectively (Hansen et al., 2011). Similarly, a water flee (*Daphnia magna*) exposed to crude oil WSF had a 48 h-LC_{50} between $0.25 - 0.65$ g/L (Martínez-Jeronimo et al., 2005). Even more sensitive were two subspecies of the rotifer genus *Brachionus Plicatilis Muller* exposed to crude oil WSF. That gave a 24 h-LC_{50} of 0.13 mg/L and 48 h-LC_{50} of 0.04 mg/L for *B. plicatilis rotundiformis*, and 24 h-LC_{50} of 0.23 mg/L and 48 h-LC_{50} of 0.05 mg/L for *B. plicatilis hepatotomus* (Alayo and Innacone, 2002).

Fresh crude oil is expected to be even more toxic than weathered crude oil (Altin, 2007), and experiments using fresh crude oil WSF would be the next step in looking for oil differences in marine invertebrates including the *Calanus* species.

GST mRNA has been induced in *Calanus* species when exposed to naph-

thalene (Hansen et al., 2008b), WSF of weathered crude oil (Hansen et al., 2011, 2009) and dispersed oil (Hansen et al., 2009). GST mRNA is therefore considered as a potential biomarker for oxidative stress and lipid peroxidation in *Calanus*. An increase of the GST mRNA in *Calanus* exposed to fresh crude oil WSF would further legitimate the use of GST mRNA as a biomarker.

The CYP 450 system is not as well developed in invertebrates as in vertebrates. It is therefore believed that each family in the CYP 450 system in invertebrates has a wider function than in the CYP 450 system of vertebrates. CYP330A1 has a function both in the detoxifying exogenous compounds and in the fatty acid metabolism (Rewitz et al., 2004). A reduced expression of CYP330A1 mRNA levels in *C. finmarchicus* have been found when exposed to naphthalene (Hansen et al., 2008b), dispersed oil (Hansen et al., 2009) and WSF of weathered crude oil (Hansen et al., 2011).

Experiments on the CYP mRNA responses are important for understanding their functions in invertebrates. Exposing *Calanus* species for a range of different components may hence contribute to increase the knowledge of the functions of the CYP enzymes, in copepods, and their biological and ecological significance.

The SOD- and γGCS enzymes are important in the antioxidant defense system through detoxifying ROS (SOD) and producing GSH (γGCS) that is further used in the antioxidant defense (Giulio et al., 1995). A clear relationship between SOD- and γGCS mRNA transcription levels and chemical pollutant exposure has, to the authors knowledge, not been reported for *C. finmarchicus*. However, Hansen et al. (2010) found an increased production

of SOD- and γ mRNA in *C. finmarchicus* exposed to diethanolamine. The antioxidant defense system is important for tolerating chemical exposures, and experiments should be carried out to reveal potential effects of the chemicals on the antioxidant system of the species. Also, since there are few studies that show a clear relationship between the antioxidant enzymes and chemical exposure, further investigations are needed to reveal if this is generally the case.

1.5.2 Aims of the study

The main purpose of this thesis is to do a comparative study of *C. finmarchicus* and *C. glacialis* to investigate potential differences in tolerating crude oil exposure. The sub-aims are as following: First, evaluate how adaption to different temperatures impact tolerance. Second, use gene expression analysis to investigate potential differences between the two species in time- and concentration-dependent responses. And finally, investigate how important varying lipid content is for tolerating crude oil exposure.

The following hypotheses may be postulated based on the current understanding that species adapted to different temperatures are different in terms of metabolism, uptake rates and lipid content:

- *C. glacialis* is more tolerant (i.e. higher LC_{50} concentration) towards oil exposure than *C. finmarchicus*.
- The concentration- and time relationships are different between the two species.
- The acute effects of oil exposure is dependent on lipid content of the copepods ("survival of the fattest").

2 Material and Method

2.1 Experimental organisms

C. finmarchicus was collected from the lab culture at SINTEF/NTNU Sealab, kept at 10 °C. The organisms used in the experiment were starved for 2-3 days prior to the start of the experiment. *C. glacialis* was carefully collected by a plankton net in the Kings Bay of Ny-Ålesund at Svalbard. They were brought to the Marine laboratory, where the CVs were sorted out using a stereo microscope, and kept in flow-through tanks at $2-3$ °C prior to the exposure period.

2.2 Generation of water soluble hydrocarbon fractions (WSFs) of fresh crude oil

Troll B was chosen as the representative of the North Sea fresh crude oil of the study. Preparation of WSF was made as recommended by Singer (2001), with some modifications; 10 L baked bottles were filled with 9.25 L sea water and 225 g oil to make a 1:40 loading. The oil was carefully poured along a glass tube to the water surface, in order to prevent formation of oil droplets in the water column. The water-oil system was stirred with the help of a stir bar and a stirring plate for 72 hours at 13±2 °C, before the water phase was tapped for chemical analysis and the experiments (Figure 4). Finally, the WSFs were cooled down to the respective experimental temperatures for the two copepods; 10±2 °C for *C. finmarchicus* and 2±1 °C for *C. glacialis*.

Figure 4: Setup for making WSF from Troll B fresh crude oil. A stirring magnet at the bottom of the flask enhance equilibrium between the oil components dissolved in the water and the crude oil. The glass tube inside the bottle is used for tapping of the WSF after the stirring is finished. The whole system is closed with a Teflon-lined cap.

2.3 Chemical analysis

The WSFs were chemically analyzed at SINTEF Material and Chemistry. Gas chromatography-mass spectrometry (GC-MS) was used to measure the amount of semi-volatile organic compounds (SVOCs), the total amount of volatile organic compounds (VOCs) was determined by Purge and Trap Gas Chromatography-Mass Spectrometry (P&T GC-MS), while gas chromatograph-flame ionization detector (GC-FID) was used to measure the total extractable organic compounds (TEOC). The analysis show which compounds that are dominating the WSFs.

Two samples ($\sim$ 1 L each) of the WSFs were sampled prior to the experiments and acidified with diluted hydrochloric acid for chemical analysis of SVOCs; including phenols, naphthalenes, and three- to five- ring PAHs. The GC-MS was programmed to operate in a single-monitoring (SIM) mode,

which mean that the GC-MS will only detect specific components. The GC-MS was constituted of an ion source (quadrupole mass spectrometer ion source temperature was 230 °C), an analyzer (a HP6890N gas chromatograph fitted with a Hewlett-Packard HP7683B series auto sampler) and a detector (HP5975B quadrupole mass-selective detector). A phenomenex ZB-5MS fused silica capillary column (30 m x 0.25 mm id x 0.25 μm film thickness) provided optimal separation of the target compounds. Helium, the carrier gas, had a constant flow of 1 mL/min. 1 μL of each WSF samples were injected into a 310 °C splittless injector, with a programmed temperature of 40 °C for 1 min, followed by 315 °C at 6 min and held for 15 min. The data and chromatograms were recored and monitored by MSD ChemStation software (version D.03.00.611).

The principle of GC-FID is that the electrical conductibility of a gas is proportional with the concentration of the charged particles in the gas. The amount of organic compounds are measured by an electrical signal from the burning of the compounds (Greibrokk et al., 1994). A HP-5 fused silica capillary column (30 m x 0.32 mm ID x 0.25 μm film thickness) was required for analyses with GC-FID. Gas chromatograph equipment and program were the same as for the GC-MS.

Three samples of 40 mL each were taken out to be analyzed for VOCs. A total of 34 target VOCs in the C5 to C10 area were determined by P&T GC-MS, using a modified EPA-Method 8260, with a 50 m (0, 20 mm id, 0, 50 μm film thickness) Supelco Petrocol capillary column. Target compounds were detected with a Agilent 5973B mass selective detector (MSD), and the data was analyzed by Agilent EnviroQuant Chemstation software.

2.4 Acute toxicity

The acute toxicity test was carried out in accordance with the modified ISO-guideline (ISO 14669, 1999) for assessing acute toxicity of WSF of oils in copepods. As the ISO-guideline was described for smaller warm water copepods (*Acartia tonsa, Tisbe battaglia, Nitocra spinipes*) there were some modifications, i.e. the exposure time was increased from 46 to 96 hours, the temperature was decreased from 20 °C to 10 °C for *C. finnmarchicus* and to 2 °C for *C. glacialis*, and larger bottles had to be used (1 L). Seven concentrations of WSF were used; 4,1 %, 7,0 %, 12 %, 20,4 %, 34,6 %, 58,8 % and 100 %. For each concentration three parallels were included, with seven copepods in each bottle. The bottles were checked for dead copepods every 24 h. These observations made the basis for a non-linear regression that calculated the lethal concentration (LC) using the software package GraphPad Prism version 5.00 for Macintosh (GraphPad software, San Diego California, USA). The 24, 48, 72, 96, 120 and 144 h LC_{50} concentration, which is the concentration where 50 % of the organisms are dead after the given time exposure, were determined from the calculations.

The LC_{50} concentration was used for deciding the proper exposure for the gene expression analysis. The LC_{20} concentration was used for testing how the varying lipid content affected the crude oil exposure tolerance.

2.5 Gene transcription analysis

In order to detect changes in the gene transcriptions of GST, CYP330A1, γGCS and SOD after exposure to fresh crude oil, the copepods were first exposed to different WSF concentrations as described below. The gene transcription analysis were then performed using RT-qPCR. This method uses

complementary DNA (cDNA), which is made from the copepods mRNA, in analyzing up-/down regulations of the gene transcription.

2.5.1 Experimental design

The organisms were exposed to four different WSF concentrations; 0 % (control), 0.5 % , 5 % and 50 % of the 96 h LC_{50} concentration. For each concentration there were three parallels, each consisting of a 2 L bottles containing $\sim$ 75 experimental copepods. The sampling of the copepods was done 12, 24 and 48 h after the start of the experiment, in order to see if there were any time dependent changes in the gene expression. After sampling, the $\sim$ 75 *Calanus* specimens in each bottle were equally distributed on three eppendorph tubes (1.5 mL) containing 500 µL RNAlater, in order to preserver the RNA. The transfer of the copepods from the bottles to the eppendorph tubes were carefully done with a filter and a pincette to prevent any damage on the specimens. All tubes were stored at -80 °C.

2.5.2 Ribo Nucleic Acid (RNA) isolation

To isolate total RNA from the copepod samples the TRIZOL®Reagent (category number 15596-018) method was used. The TRIZOL®Reagent maintains the integrity of the RNA, during the disturbance of the cells and the dissolving of the cell components in the homogenization of the samples. Adding chloroform separates the solution into different phases following centrifugation. The upper aquatic phase contains the RNA, while lower organic phase contain among others deoxyribonuckleic acids (DNAs) and proteins. Adding isopropanol and ethanol precipitates and washes the RNA pellet.

Empty eppendorph tubes (1.5 mL) were filled with 500 µL TRIZOL® Reagent. The samples stored in RNAlater were transfered to the TRIZOL® Reagent filled tubes, before they were homogenized with a manual pestle and added additional 500 µL TRIZOL®Reagent. The samples were then incubated for 5 min at room temperature, to complete dissociation of the RNA complexes. Then 200 µL of chloroform was added, and the tubes were shaked vigorously by hand for 15 s, before they again were incubated at room temperature for 2 to 3 min. Following centrifugation at 12,000 $x\ G$ for 15 min at 4 °C the samples separated in three phases; an organic lower phase, a fatty middle phase and an aqueous upper phase with the RNA. 500 µL of the aqueous phase was then transfered to a new eppendorph tube and mixed with 500 µL isopropanol. Now the samples were incubated for 10 min at room temperature before being centrifuged at 12,000 $x\ G$ for 10 min at 4 °C. The RNA was now precipitated and formed a "gel-like" pellet on one side in the tube. Then the supernatant was removed using water suction, and 1 mL 75 % ethanol was added to wash the RNA. Vortexing loosened the RNA pellet from the wall, and the samples were finally centrifuged at 7,500 $x\ G$ for 5 min at 4 °C. As for the isopropanol step, the ethanol was then removed using water suction. When rest-ethanol had evaporated from the eppendorph tubes, 50 µL of nucleotide free water was added and the samples were then stored at −80 °C.

The isolated RNA samples were treated with DNase 1 (Ambion, Austin, Tx, USA) to remove potentially damaging DNases that could affect the final product. Further, 5 µL sodium acetate (NaAc) and 150 µL ethanol were added to the RNA samples and shaken vigorously before stored at −80 °C over night. Next, the samples was centrifuged at 12,000 $x\ G$ for 25 min at

4 °C, followed by a water suction removal of the supernatant. the samples were then washed with 1000 µL 75 % ethanol and centrifuged at 7,500 x G for 5 min at 4 °C. Finally, all the ethanol was removed and the RNA pellet was dissolved in nucleotide free water and stored at −80 °C.

2.5.3 Complementary deoxyribonuckleic acid (cDNA)

A NanoDrop®ND-1000 machine was used to quantify and measure the purity if the RNA samples. Nucleic acids (RNA and DNA) absorb light at 260 nm. At 280 nm RNA absorbs light with half of the efficiency as at 260 nm. An A260/280 absorbency ratio of 2.0 indicated pure RNA product (NanoDrop, 2010).

cDNA synthesis converts the mRNA sequences to a double stranded cDNA, which is a more stable molecule, and make it possible to perform a RT-qPCR analysis of the mRNA expression. The synthesis was performed by using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). The cDNA synthesis reaction is carried out by reverse transcriptase, which copies the mRNA template into single-stranded cDNA. Then a new single-stranded cDNA is produced from the single-stranded cDNA:mRNA complex by DNA polymerase I. The two single-stranded cDNAs are making the complete double-stranded cDNA molecule (Avison, 2007).

One of the purified RNA samples was used to make an appointed series of diluted cDNA concentrations (15.7, 31.3, 62.5, 125, 250, 500 and 1000 ng RNA). This series was used to make the standard curve for all target genes that were chosen to analyze. All the cDNA samples were diluted (1:5) with 5 µL nucleotide free water. The nucleotide free water inactivated potential RNases in the samples, and therefore, decreased potential synthesis

inhibitors. 5 µL of the diluted cDNA samples were finally added to a 96 well plate and stored at −80 °C.

2.5.4 Quantitative real time PCR

RT-qPCR was performed on a Stratagene Mx4005P RT-PCR system (La Jolla, CA, USA) to quantify GST-, CYP330A1-, γGCS- and SOD gene transcriptions. Each gene was quantified on separate plates, to prevent plate-to-plate variations. Table 1 shows the different thermal profile setup for the measured genes. The primer sequences are listed in Hansen et al. (2010, 2008b).

Table 1: RT-qPCR thermal profile setup for the measured genes

Temperature	Time	Cycles
95 °C	30 s	1 cycle
95 °C	30 s	53 cycles
58 °C*	30 s	
95 °C	1.0 min	
55 °C	30 s	1 cycle
95 °C	30 s	

* 55 °C for SOD.

To each well the in 96 well plates 5 µl cDNA template, 15 µl of master-mix (containing SYBR Green Supermix with ROX (1060 µl) (BioRad)), forward and reverse primers (53 µL of each primer), and water (424 µL)), were added to obtain a total volume of 20 µL. During the RT-qPCR analysis the master mix provided the cDNA template with the necessary components for potential up regulations of the gene expressions. The concentrations of the gene expressions were normalized against a reference gene (EF1α) using the software program Q-Gene (Microsoft® Excel®-based software application coded in Visual Basic for Applications) (Muller et al., 2002; Miller et al.,

1998).

The mean normalized expression (MNE) levels of each individual target genes were converted to fold expression levels compared to the corresponding controls. The controls for the time related gene transcriptions were merged, and increased the n from 3-9 (*C. finmarchicus*) and from 2-8 (*C. glacialis*). A consequence of the increased n was a change in the standard error (SE) of the controls. Since the conversion did not affect the concentration related gene transcriptions, there were no changes in the n and SE. The different n in the time and concentration related mRNA transcriptions contributed to a differences in the fold expression and SE of the exposed groups.

Induction of the gene transcriptions should exceed a two fold induction compared to the corresponding controls, in order to verify the use of RT-qPCR as method for describing possible changes in the gene transcription.

The fold expression levels were used to make the graphs showing the potential concentration/time dependent trends as a function of WSF concentration, while the MNE levels were used to make the graph showing the natural level of each target genes in the two copepod species.

2.6 Qualitative analysis of a RT-qPCR product

Agarose gel was used to validate the quality of the PCR product, and secured that the same target gene had been analyzed for both of the copepod species. Preparation of the gel was performed according to the Biorad protocol: 100 mL (10x) TBE-buffer diluted with distilled water to 2 L. 160 mL of the diluted solution was added to 2 g agarose and heated in the microwave oven for 4 min. The solution was heated to the boiling point in order to

make a complete reaction of the solutions. After the heating, the solution was cooled down in a water bath to 5 °C. When the solution had reached the desired temperature, 50 µL of Sybr safe (Sybr safe DNA gel stain, 10 000 x concentration in DMSO, Invitrogen Molecular Probes^{TM}, Eugene, Oregon, USA) was added and the mixture was transferred to tray. All air bubbles were removed and the gel was placed in room temperature for 15 min to stiffen. When ready, the steeping tank with the gel was transferred to a horizontal electrophoresis basin and the TBE-buffer solution was added until it was completely covering the gel.

23 µL of specific PCR-products (3 µL gel-loading and 20 µL PCR-product) was added to separate wells. 6 µL of 50 base pair (bp) DNA ladder ((2 µL gel-loading and 4 µL standard) (Invitrogen, cat. no. 10416-014 Lot nr. 586280, Calsbad, CA 92008 USA)) was added in the well prior to the products. The PCR-products for *C. glacialis* were placed on the left side of the gel, while the products for *C. finmarcicus* were placed on the right side. A lid was put on the electrophoresis basin and a constant voltage of 150 V was turned on.

After 1.5 h the gel was transferred to a GEL-DOC instrument and visualized by UV-light. The bonds of interest would lie between 100 and 200 bp.

The agarose gel confirmed that the correct amplicon sites for individual target genes were found for *C. glacialis* (left) and *C. finmarchicus* (right) (Figure 5). All target genes were in the expected area of 100-150 bp. The target gene parallels were at the same level. This indicated that the same target gene had been detected in both species and that the gene transcription could be compared.

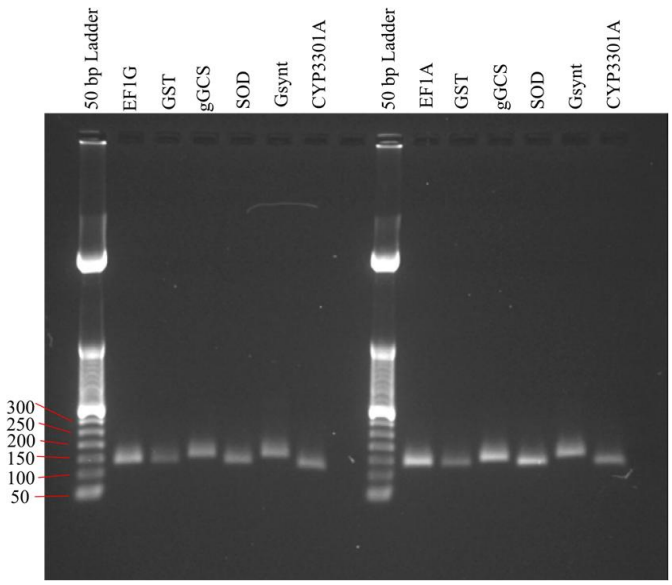

Figure 5: Agarose gel representation of the genes that have been tested for *C. glacialis* (left) and *C. finmarchicus* (right). The numbers on the left are indicating the base par (bp) for the ladder.

2.7 Crude oil tolerance and biometry of copepod lipid content

To investigate if lipid rich copepods would tolerate a higher and longer exposure than lipid poor copepods, a duplicate of $\sim$ 75 copepods each were exposed to a LC_{20} concentration corresponding to 21.16 % and 57.13 % WAF for *C. finmarchicus* and *C. glacialis*, respectively. Each bottle were checked for dead copepods every 24 h throughout the whole experimental time of 120 h. The dead copepods were picked out and observed in a Leika MZ125 (*C. finmarchicus*) or in a Leika MZ6 microscope (*C. glacialis*). In order to investigate how the lipid content affected survival, all the copepods were photographed by a digital still-video camera (Sony DFW-SX900, color digital camera, Japan) operated Fire-i software (Unibrain, Inc., San

Ramon, CA, USA). The copepods that survived the 120 h exposure were anesthetized with Tricane Methansulfonate (Finquel, Argent Chemical Laboratories, Redmond, WA, USA, 1.5 g/L stock solution) before being photographed.

The software program ImageJ (v. 1.4.2q, National Institutes of Health, Bethesda MD, USA) and a graphical tablet (Wacom Intous3, Wacom Co., Ltd., Saitama, Japan) were used to measure the area and length of the lipid content and the whole animal from the pictures (Figure 6). A method developed by Miller et al. (1998) was used to calculate the percentage value of fat in the copepods.

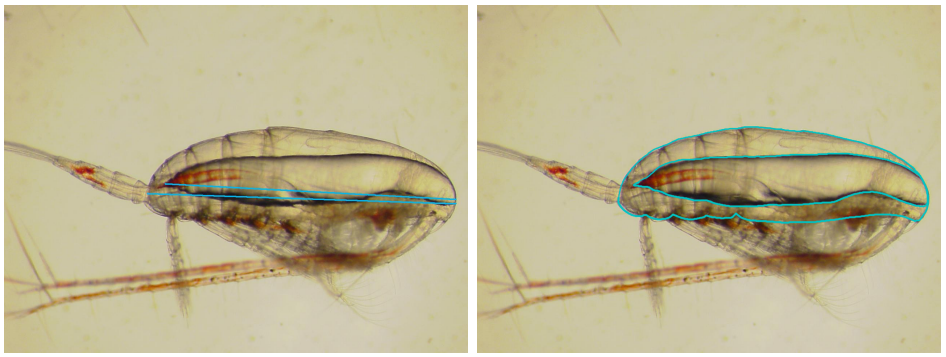

Figure 6: Defined length (left) and areal (right) of lipid sac and whole body (blue lines). Measured in ImageJ.

If the lipid sac was split into different parts, each part was measured and then added together. However, if the lipid sac was divided into several different parts the lipid sac was considered as "exploded" and no measurements were done. Also, the damaged ones were eliminated from the analysis. Examples of discharged specimens are shown in the figure 7.

Figure 7: Examples of two female copepods that are not included in the measurements. Left; an exploded lipid sac. Right; a damaged copepod.

2.8 Statistical analysis

For the target gene transcriptions the significant level was set at $\alpha = 0.05$, and all statistical analysis were one-tailed. Due to small n, non-parametric analysis were chosen. Mann-Whitney t-test was used to test for significant differences between the exposed groups and the controls of the gene transcriptions. A Kruskal-Wallis test analyzed for differences in the gene expressions in the controls of target genes and between the specimens. Both tests used GraphPad Prism version 5.00 for Windows (GraphPad software, San Diego California, USA). Nonlinear-regression test (regression wizard, 2 parameter power test) was performed using SigmaPlot 11.0 (Systat system Inc. 2009) to test for significant trends in the gene transcriptions.

The LC-graphs were made in GraphPad Prism version 5.00 for Macintosh (GraphPad software, San Diego California, USA), while the gene transcription graphs were made in SigmaPlot 11.0 (Systat system Inc. 2009).

In order to find the variable that had the highest significance for survival, a linear multi variable regression model was adapted. The measured variables were; body areal, body length, lipid sac areal, lipid sac length

and percentage lipid fat. The model also considered the effects of gonadal development (egg production) and gender.

The R^2 is a measure of the fit of the data (observations) to the regression model, and has a value between 0.0 and 1.0, and has no units. In a linear regression model R^2 may explain the relationship between the x- and y-values, and a value of 1.0 is a perfect relationship, also called a perfect fit. Perfect fit means that all the observations lie on a straight line. The R^2 value will indicate how close the regression line is a perfect fit (Motulsky, 2007). Compared to a linear regression model, a multi variable linear regression model try to fit a plane instead of a line. A plane is an extension of a line into higher dimensions, and is based on several measured variables. The principle is the same in both models, the only difference is that the multi variable linear regression model can combine several variables in order to find the best-fit regression plane in the dataset. The analysis were done using R 2.12.2 (R Development Core Team, 2011). The analysis tries first to find the single variable that gives the best fit, then more variables are included to increase the R^2 value. If the model needs to combine all the variables, and the R^2-value is still low, it may be an indication that none of the variables explain the differences that has been observed, e.g. the ability to survive, and the regression model is considered a poor model.

3 Results

3.1 Generation of water soluble hydrocarbon fractions (WSFs) of fresh crude oil

The results from the chemical analysis of the WSFs conducted by SINTEF Materials and Chemistry are summarized in Table 2. The WSFs were generated at 13 °C before they were cooled down to the appropriate exposure temperature. The standardization of the WSF generation procedure ensured that the total hydrocarbon concentration (THC) and the composition of the exposure solution would be comparable for both species. Table 2 shows that fresh crude oil from Troll B was rich in BTEXs (benzene, toulene, ethylbenzene and xylenes) and naphthalenes.

3.2 Acute toxicity

The LC_{50} and LC_{20} values at different exposure times are summarized in Table 3 (*C. finmarchicus*) and Table 4 (*C. glacialis*). The LC-values are based on Figure 8, which shows the interaction between the different WSF exposure time series and the percentage survival in the two species. In addition to the difference between the species with regard to the LC-values, the mortality response curve for *C. glacialis* appears to be steeper than the corresponding curve for *C. finmarchicus* up to 120 hours. This may indicate that *C. glacialis* tolerates the exposure up to a certain level. When crossing this level, only an insignificant further increase in the concentration seems to be lethal.

The 144 h LC curves of the two species show that with longer time exposure the response patterns are more similar compared to shorter time

exposure. Also, for both species the LC_{50} and LC_{20} values are clearly both time- and concentration related.

Table 2: Summary of the chemical composition in the WSFs analyzed with GS-MS, P&T GC-MS and GC-FID (THC). Concentrations are given as average (µg/L) and standard deviations of two replicate WSFs generated for each species in this experiment.

Compound groups	*C. finmarchicus* (µg/L)	*C. glacialis* (µg/L)
THC (GC-FID)	4514 ± 1295	5494 ± 499
$\sum$ **VOCs**	5080 ± 119	5133 ± 187
Benzene	137 ± 5	139 ± 4
Toulene	134 ± 10	128 ± 1
Ethylbenzene	410 ± 12	432 ± 2
Xylenes	1627 ± 67	1712 ± 48
$\sum$ **SVOCs** (excluded phenols)	292 ± 43	308 ± 2
$\sum$ Naphthalenes	234 ± 46	255 ± 1
$\sum$ 2-3 ring PAH *	23 ± 4	28 ± 1
$\sum$ 4-6 ring PAH **	0.5 ± 0.193	0.8 ± 0.034
$\sum$ C0-C5 phenols	28 ± 4.82	21 ± 1.9
$\sum$ Decalins	4.9 ± 3.1	2 ± 0.93
$\sum$ **THC (for LC_{50})**	9594 ± 1415	10627 ± 312

* $\sum$ 2-3 ring PAH include biphenyl, acenaphthylene, acenaphthene, dibenzofuran, fluorene (C0-C3), phenanthrene, anthracene, phenanthrenes/anthracenes (C1-C4), dibenzothiophenes (C0-C4). ** $\sum$ 4-6 ring PAH include fluoranthene, pyrene, fluoranthene/pyrene (C1-C3), benz(a)anthracene, chrysene.

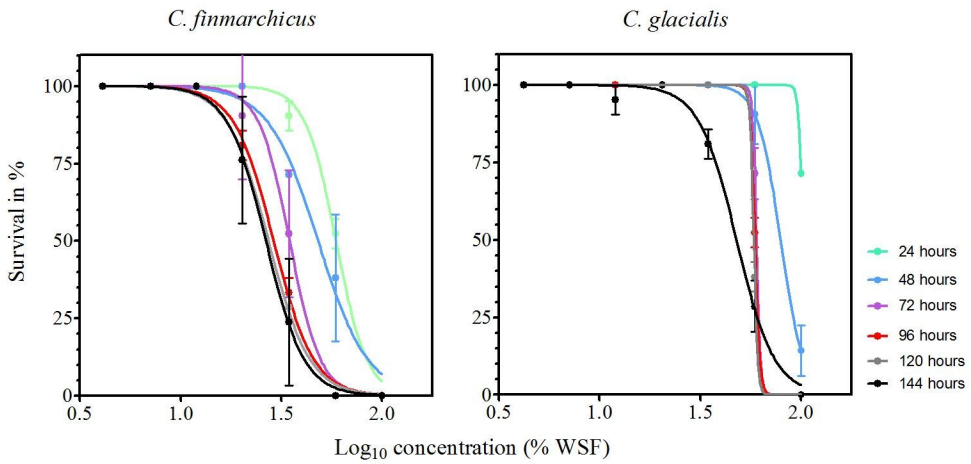

Figure 8: Relationship between percentage survival of *C. finmarchicus* (left) and *C. glacialis* (right), and Log_{10} concentration of WSF.

Table 3: LC_{50} and LC_{20} values in % WSF and in µgTHC/L for *C. finmarchicus*.

	% WSF		µgTHC/L	
Time (h)	LC_{50}	LC_{20}	LC_{50}	LC_{20}
24	59.07	46.31	5076	3979
48	48.12	32.50	4135	2739
72	34.76	27.26	2987	2342
96	29.00	21.16	2492	1818
120	27.32	19.72	2347	1694
144	26.50	19.60	2277	1684

Table 4: LC_{50} and LC_{20} values in % WSF and in µgTHC/L for *C. glacialis*.

	% WSF		µgTHC/L	
Time (h)	LC_{50}	LC_{20}	LC_{50}	LC_{20}
24	102.20	98.93	11087	10732
48	79.09	65.97	8580	7156
72	60.11	58.97	6521	6397
96	59.05	57.13	6406	6197
120	58.26	56.37	6320	6115
144	47.85	35.53	5191	3854

3.3 Gene transcription analysis

3.3.1 Comparing the control groups

The results from the comparison of the control groups (Figure 9) showed that *C. glacialis* (white dots) had a higher basal MNE level of GST mRNA than *C. finmarchicus* (black dots). The levels for CYP330A1 are almost the same for both species, while *C. finmarchicus* had a higher basal transcription of γGCS and SOD.

The Kruskal-Wallis test showed that all control groups were significantly different from each other ($p < 0.0001$). The test based the analysis on the variations in between the replicates, and not on the level of the gene expressions.

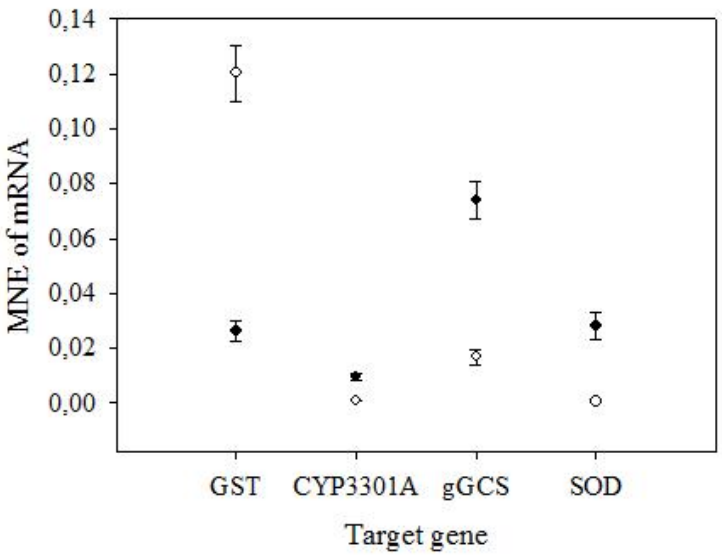

Figure 9: Basal gene levels in *C. finmarchicus* (black dots, n= 9) and *C. glacialis* (white dots, n= 8). The target genes were significant different from each other with a $p < 0.0001$.

3.3.2 GST gene transcription

Concentration- and time related relationships between WSF exposure and GST mRNA transcription are shown for both species in Figure 10 and Figure 11. The highest transcriptional response was shown after 48 h exposure time for both *C. finmarchicus* (black dots) and *C. glacialis* (white dots), with a 2.5 and 3.5 fold expression of the GST mRNA levels, respectively (Figure 10(C)). A ~ 2.5 fold expression response was shown for both species when exposed to a high WSF concentration (Figure 11(C)).

Only *C. glacialis* GST mRNA showed a significant correlation with concentration when exposed for 48 h, and with time for the highest concentration (Table 5). In general, *C. glacialis* had a higher gene transcription towards the WSF concentration compared to *C. finmarchicus*.

Table 5: Significant correlation in GST expression in*C. glacialis* with WSF concentration (48 h) and time (high exposure concentration).

Exposure	species	p-value
48 h	*C. glacialis*	< 0.0001
High	*C. glacialis*	0.0075

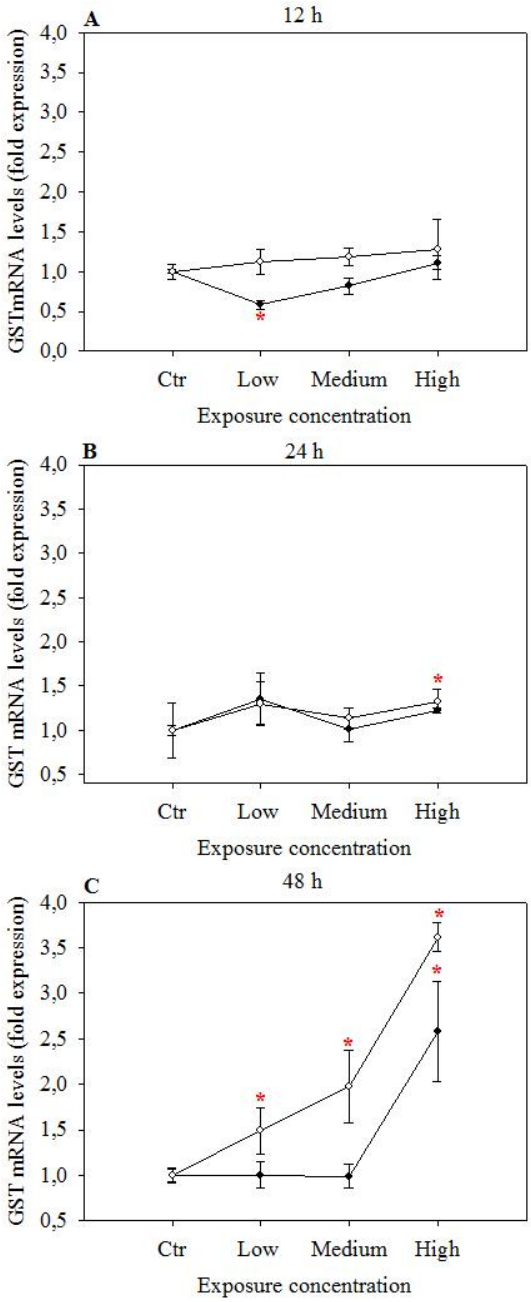

Figure 10: Concentration related GST mRNA transcription in *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed for three time periods (A-C). GST mRNA levels are given as mean±SE. Significant differences between exposed groups and the corresponding controls are marked *$= p < 0.05$. $n = 3$ for all groups except the control group for *C. glacialis* in (A) that has a $n = 2$.

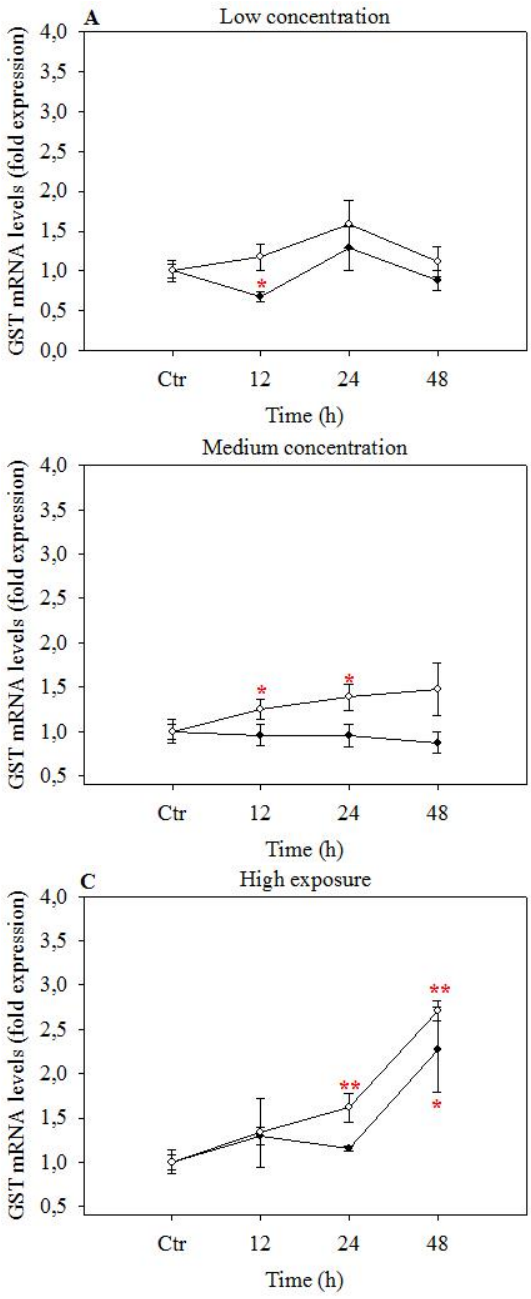

Figure 11: Time related changes in GST mRNA levels of *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed to three WSF concentrations (A-C). GST mRNA levels are given as mean±SE. Significant differences between exposed groups and the corresponding controls are marked *= $p < 0.05$ and **= $p < 0.001$. The control groups have a n of 9 (*C. finmarchicus*) and 8 (*C. glacialis*), while the exposed groups have a n= 3.

3.3.3 CYP330A1 gene transcription

Concentration- and time related CYP330A1 mRNA transcription in response to fresh crude oil WSF are shown for both species in Figure 12 and Figure 13. No significant correlation between CYP330A1 mRNA transcription and WSF concentration, and between CYP330A1 mRNA transcription and time was found for any of the species. Nevertheless there are some exposure groups that show significant differences compared to the controls.

The CYP330A1 mRNA levels for *C. finmarchicus* (black dots) are not showing a high response towards the WSF exposure (Figure 13), even though there is almost a 2.0 fold induction when exposed to the medium concentration for 12 h (Figure 13(B)). However, the response was back to the basal level after 24 h exposure.

C. glacialis (white dots) did not induced a high CYP330A1 mRNA transcription when exposed to WSF (Figure 12 and Figure 13). However, a significant response was evident after 48 h exposed to a high WSF concentration (Figure 12(C) and Figure 13(C)).

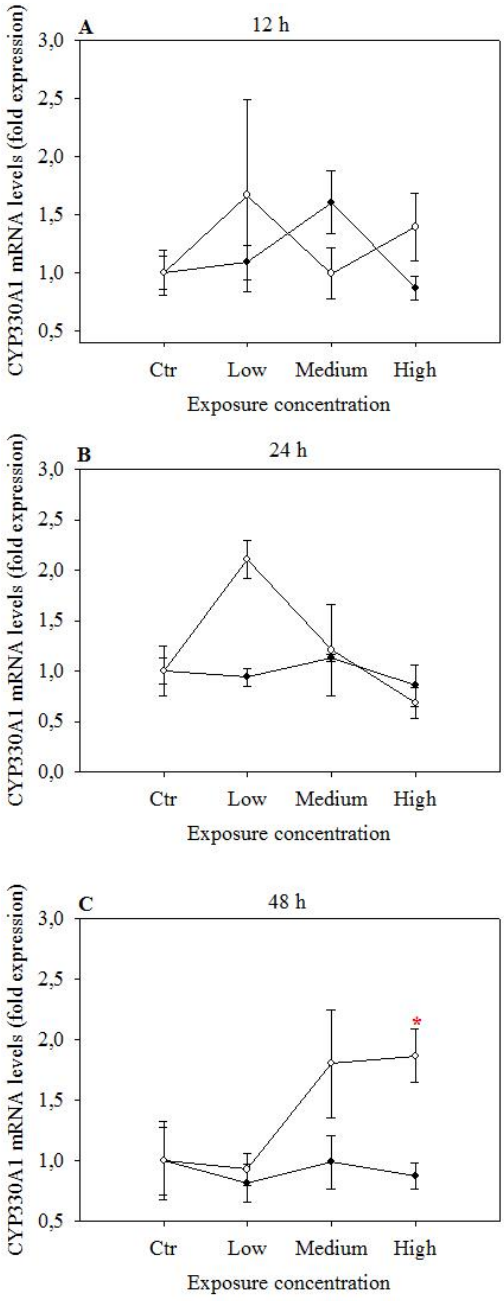

Figure 12: Concentration related CYP330A1 mRNA transcriptions in *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed for three time periods (A-C). The mRNA transcriptions are given as mean±SE. Significant differences between exposed groups and the corresponding controls are marked * ($p<0.05$). $n=3$ for all groups except for the control group in A and the low exposure in B, for *C. glacialis*.

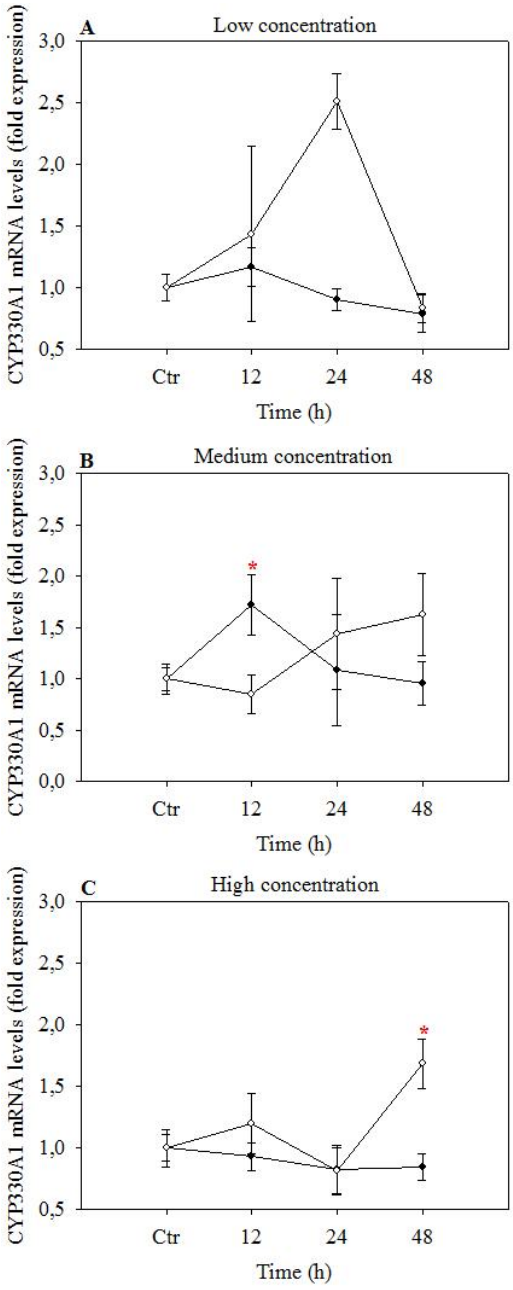

Figure 13: Time related CYP330A1 mRNA transcriptions in *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed to three different WSF concentrations (A-C) are given as mean±SE. Significant differences between exposed group and its corresponding control are marked *= $p < 0.05$. The controls have a n of 9 (*C. finmarchicus*) and 8 (*C. glacialis*). The exposed groups have a n of 3, except for the 24 h group of *C. glacialis* that has a n= 2.

3.3.4 γGCS gene transcription

Concentration- and time related transcriptions between γGCS mRNA level and WSF exposure are shown for both species in Figure 14 and Figure 15. No significant correlations between γGCS mRNA levels and for exposure concentration- or time were observed.

A significant reduction compared to the control is shown for both species when exposed to the high WSF concentration for 24 h (Figure 14(B)). The same pattern is evident in Figure 15(C), although the reduction is only significant in the *C. finmarchicus* (black dots) γGCS mRNA level. For both species the transcriptional levels are going back to the control level with longer exposure time.

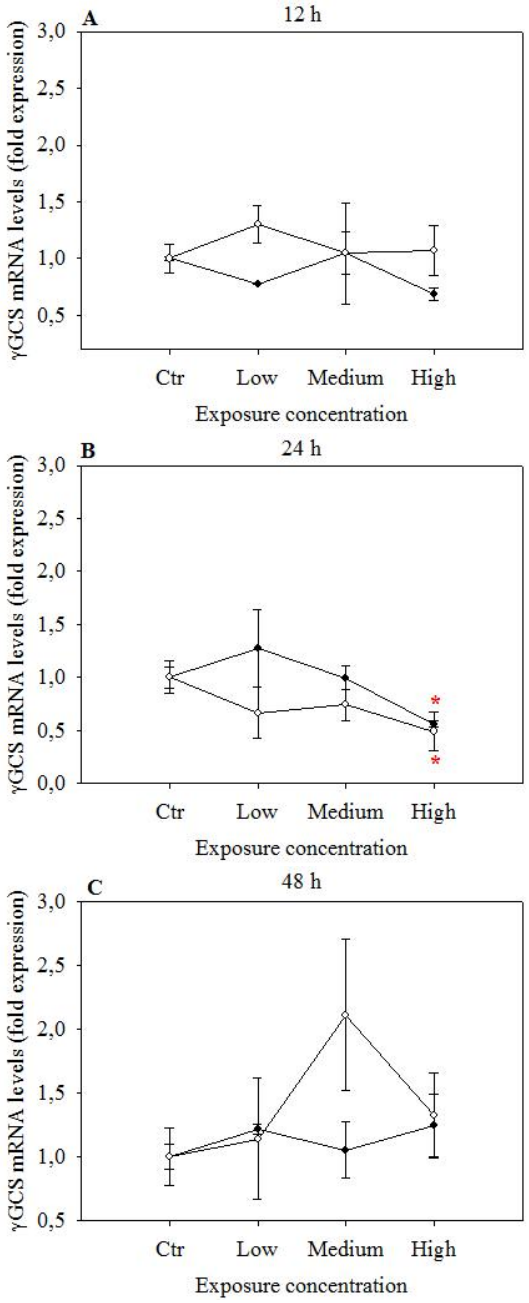

Figure 14: Concentration related γGCS mRNA transcriptions in *C. finmarchicus* (black dots) and *C. glacialis*(white dots) exposed to three different time periods (A-C). Transcription levels are given as mean±SE. Significant differences between exposed groups and the corresponding controls are marked *= $p < 0.05$. All groups have a n= 3, except for the control group of *C. glacialis* in A (n= 2).

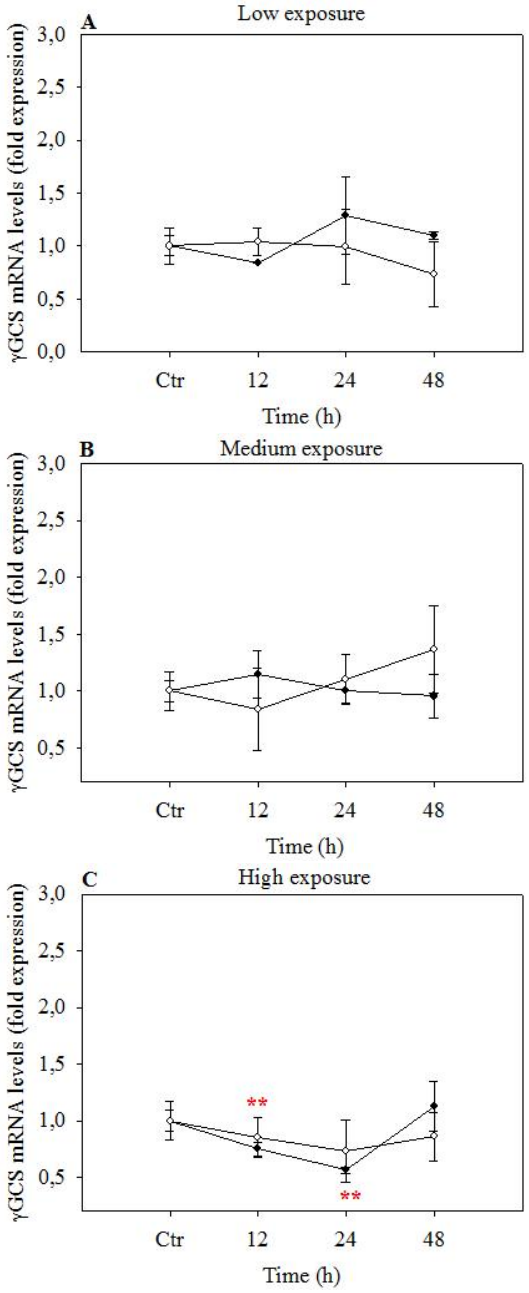

Figure 15: Time related γGCS mRNA transcriptions in *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed to three different WSF concentrations (A-C). Significant differences between exposed groups and the corresponding controls are marked **$= p < 0.001$. The control groups have a n of 9 (*C. finmarchicus*) and 8 (*C. glacialis*). The exposed groups have a n of 3.

3.3.5 SOD gene transcription

Concentration- and time related transcriptions between fresh crude oil WSF exposure and SOD mRNA levels are shown in Figure 16 and Figure 17, respectively. No significant correlation between SOD mRNA levels and WSF concentration or time were found.

C. glacialis (white dots) shows a mean four fold transcriptional expression when exposed to a medium WSF concentration for 12 h (Figure 16(A) and Figure 17(B)), but the increase was not statistically significant.

C. finmarchicus (black dots) had a two fold induction of SOD mRNA when exposed to a low concentration for 24 h (Figure 16(B) and Figure 17(A)). For both species there were some variations in the mRNA levels, but they were not statistically significant.

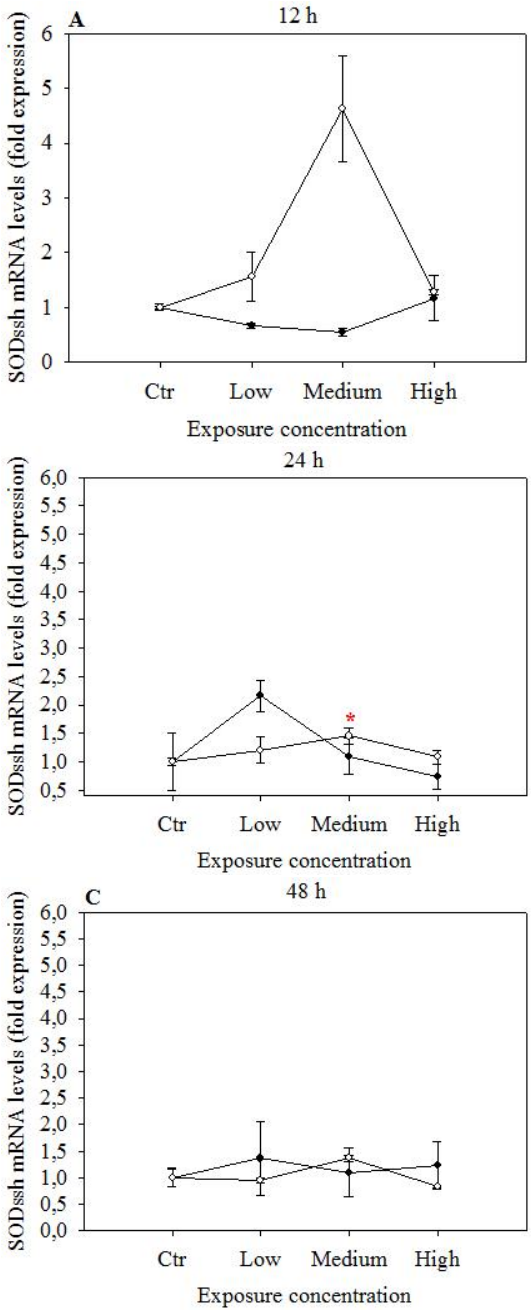

Figure 16: Concentration related SOD mRNA responses in *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed to three different time periods (A-C). Significant differences between exposed groups and the corresponding controls are marked *$= p < 0.05$. All groups have a n of 3, except for the control (A), medium- (A) and low concentration (B), belonging to *C. glacialis*, that have a n of 2.

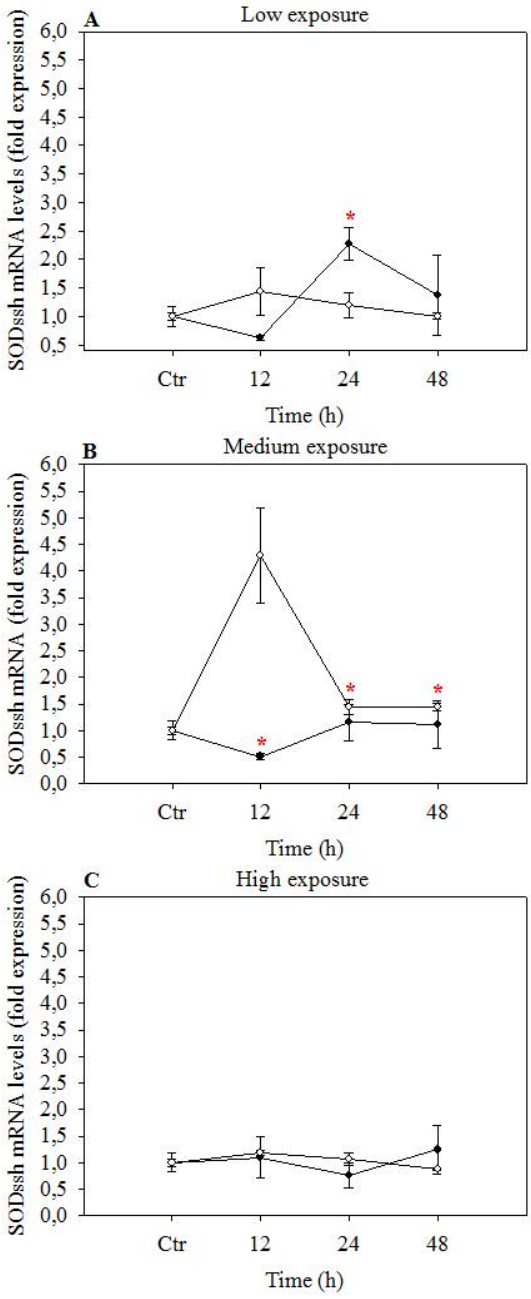

Figure 17: Time related SOD mRNA changes in *C. finmarchicus* (black dots) and *C. glacialis* (white dots) exposed to three different WSF concentrations. Significant differences between exposed groups and the corresponding controls are marked *= $p < 0.05$. The control groups have a n of 9 (*C. finmarchicus*) and 8 (*C. glacialis*). All the exposed groups have a n of 3, except for the 24 h (A) and 12 h (B) in *C. glacialis* that have a n= 2.

3.4 Crude oil tolerance and biometry of the copepod species

Figure 18 shows the results from the twelve *C. finmarchicus* who did not survive the 120 hour exposure. According to the regression analysis, the body areal was the most important variable. With $R^2 = 0.17$ it was the variable that gave the best fit when represented alone. In order to achieve a R^2 equal to 0.9, the model needed to consider all variables.

Figure 19 demonstrates that percentage lipid sac was the most significant variable ($R^2 = 0.14$) in the model for *C. glacialis*. When combing all the variables, there was a value of R^2 equal to 0.29.

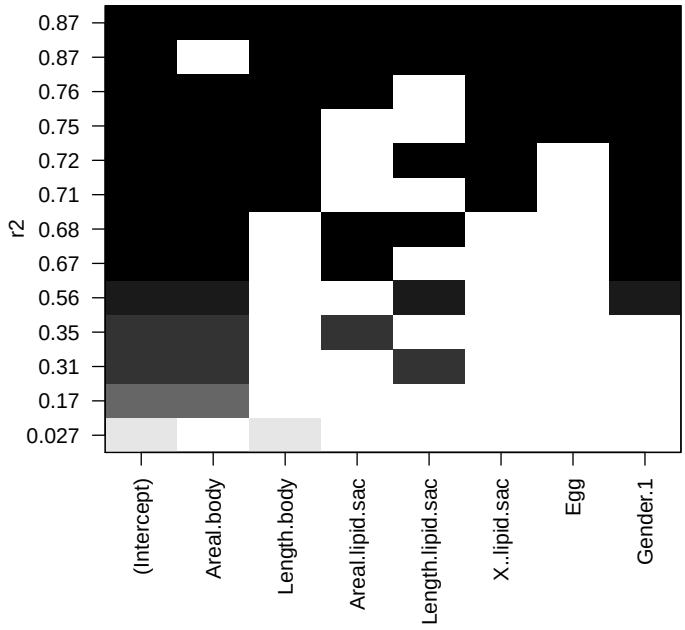

Figure 18: The variables with the largest significance on the survival of *C. finmarchicus* ($n = 11$) after 120 h exposure of WAF. The "X.lipid.sac" denotes % lipid content.

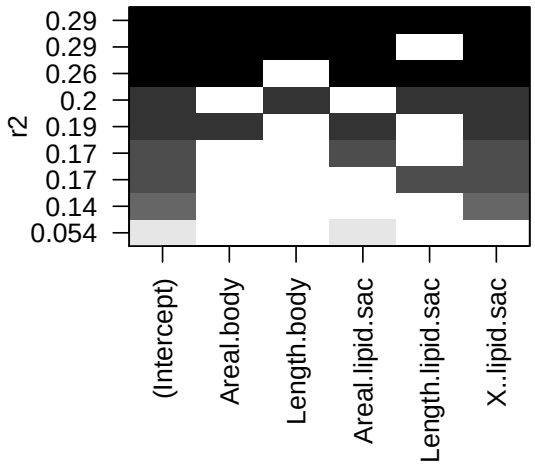

Figure 19: The variables with the largest significance on the survival of the *C. galcialis* ($n = 15$) after 120 h exposure of WAF. The "X.lipid.sac" denotes % lipid content.

4 Discussion

4.1 Acute toxicity

The results in Figure 8 show that *C. glacialis* has a delayed response to the WSF exposure compared to *C. finmarchicus*. In addition, the 96 h-LC_{50} curve for *C. glacialis* is much steeper than the curve for *C. finmarchicus*. This may indicate that *C. glacialis* was able to compensate for the exposure until a certain limit where they quickly died, and may also explain why there were no big differences in the percentage survival between the different exposure times. Since the curve for *C. finmarchicus* has a less steep slope it may signify that these copepods did not have the same capacity to compensate the impact of WSF exposure. Instead, the percentage survival reduced gradually with increasing WSF concentration and exposure time. Other factors that may explain the differences in the acute response of the two copepod species are exposure temperature and lipid content. Chemical reactions are known to act slower at lower temperatures (Berg et al., 2006), and this may also be applied to metabolism of xenobiotics in cold-adapted species (Landrum, 1988; Jimenez et al., 1987). *C. glacialis*, which is an Arctic species, may hence exhibit a slower response compared to the more southern *C. finmarchicus*. Further, a higher lipid content may give the copepods a short-time protection if the crude oil components are accumulated directly to-, and immobilized in the lipid reservoir. *C. glacialis* has a higher lipid content than *C. finmarchicus* and may therefore have a slower response to the acute acting toxicants (Hansen et al., 2011).

The LC_{50} values in THC concentration (Table 3 and Table 4) verify that *C. glacialis* was less sensitive to the fresh crude oil WSF compared to *C.*

finmarchicus. The Arctic species tolerated an exposure concentration up to three times more than the temperated species. These results also indicate that the *Calanus* species are less sensitive to fresh crude oil WSF compared to two subspecies of rotifera genus *B. pilcatilis hepatotomus* (48 h-LC_{50} of $0.04 -$ and 0.05 mg/L) (Alayo and Innacone, 2002), and more sensitive to the WSF exposure compared to a water flee, *Daphnia magna* (48 h-LC_{50} between 0.25-0.26 g/L) (Martínez-Jeronimo et al., 2005). (Hansen et al., 2011) exposed *C. finmarchicus* and *C. glacialis* to weathered crude oil WSF, and found a higher sensitivity (9g h-LC_{50} of 0.817 and 1.037 µgTHC /L, respectively) compared to the fresh crude oil WSF that gave 96 h-LC_{50} of 2492 and 6406 µg/L. This may indicate that unresolved complex materials and the naphthalenes in the WSF from weathered crude oil (Altin, 2007) are more toxic than the more volatile compounds in the fresh crude oil WSF.

There is a general correlation between lethal concentration, exposure time and WSF exposure concentration. With increasing exposure time the sigmoidal parts of the LC-curves are shifted towards left; implying that a lower concentration of WSF is sufficient to affect the specimens. Also, it is interesting to see that with longer exposure time the two species are showing the same shape of the 144 h LC-curve, indicating a fatiguing of the proposed resistance mechanisms in *C. glacialis*.

4.2 Gene transcriptions

All the gene transcription results are presented as average ±SE in a line/scatter graph, in order to visualize possible changes in the gene transcriptions. The reason was that the results may be somewhat biased due to low n and possible pipetting errors. All gene transcriptions raw data for making the graphs

are presented in the Appendix.

The Mann-Whitney t-test used to test deviations of exposed groups from the corresponding control groups was carefully chosen according to the challenge of the material (low n). This is a non-parametric test for comparing two groups with $n \geq 3$. All groups with a $n = 2$ could not be statistically tested. As a rule a small n will limit the possibility of revealing statistically significance, even where differences exist.

A nonlinear regression test (regression wizard, 2 parameter power test (SigmaPlot 11.0)) was chosen to test for any significant trends in the gene transcription data. The gene transcription results indicated an exponential response, and the chosen test had the shape that corresponded best with these responses.

4.2.1 Comparing the control groups

The specimens in the control groups did not show any indications of discomfort during the experiments which could affect the gene transcription. The results in Figure 9 can therefore be considered as representative for the basal gene transcription in *C. finmarchicus* and *C. glacialis*.

C. finmarchicus expressed a notable higher level of γGCS- and SOD mRNA, while *C. glacialis* had higher expression of GST mRNA. The species expressed almost the same level of CYP330A1 mRNA. Higher gene transcription levels may indicate that the species are naturally exposed to compounds that induces the gene transcription. Another reason may be that the different genes have a function in the species that is not related to detoxification of oil compounds. However, at the moment this has not been confirmed by any researchers.

4.2.2 GST gene expressions

Figure 10 and Figure 11 show that the production of GST mRNA in *C. finmarchicus* is both concentration- and time related. However, no significant dependency trends were shown, neither with regard to concentration nor time of the results. The results are corresponding with the findings of Hansen et al. (2011) who exposed *C. finmarchicus* to weathered crude oil WSF. They found that *C. finmarchicus* only expressed high levels of GST mRNA when they were exposed to a high WSF concentration for a fairly long time.

Also for *C. glacialis* the GST mRNA only exceeded a two fold induction when the copepods were exposed to a high WSF concentration for a longer period of time (Figure 10 and Figure 11). But in contrast to the results from *C. finmarchicus*, the results for *C. glacialis* also showed significant concentration- and time dependently correlation. A time related response is also probable for medium exposure (Figure 11(B)), but the levels do not exceed a two fold expression and are not considered a reliable response here.

C. glacialis had a delayed response toward the acute acting toxicants compared to *C. finmarchicus* (Figure 8). If this was because of a slower uptake of oil compounds, a similar response should be expected with regard to gene transcription. However, no such responses are present in these results. On the contrary, *C. glacialis* had a faster response compared to *C. finmarchicus*. These findings are opposite to the findings by Hansen et al. (2011), who found a faster response in GST gene transcription in *C. finmarchicus* compared to *C. glacialis* when exposed to weathered crude oil. They assumed this was correspondent to the delayed response they saw in the acute toxicity test.

The fresh crude oil WSF used in the current experiments had a high concentration of VOCs (Table 2). These small and toxic substances (Berg et al., 2006) may act to fast for the species' molecular systems and bind to specific receptors, which may be the reason for the opposite GST mRNA transcription. Also, Jimenez et al. (1987) found a greater number of metabolites in colder-temperature experiments than comparable higher-temperature experiments when fish was exposed to benzo(a)pyrene. The faster response of GST mRNA in *C. glacialis* may therefor be due to a higher production of metabolites.

Hansen et al. (2008b) exposed *c. finmarchicus* to three different concentrations (between $31 - 2505$ µg/L) of naphthalene, and found significant induction of GST mRNA transcription for all exposure concentrations. The naphthalene concentration in these experiments was between the low and medium exposure concentration in Hansen et al. (2008b). The induction of the GST mRNA transcription seen in *C. finmarchicus* and *C. glacialis* (Figure 10 and Figure 11) may therefore be a respond to the naphthalenes in the WSF.

The MNE control levels were compared between the two species, and showed that *C. glacialis* had a much higher natural expression of GST than *C. finmarchicus* (Figure 9). This may be another reason for the faster transcription of GST mRNA in *C. glacialis*. GST is involved in detoxifying of exogenous compounds, so when *C. glacialis* was exposed to crude oil WSF there may be an initial use of the GST. When the exposure increases with time and concentration, the molecular system has to increase the transcription in order to metabolize the WSF compounds.

Hansen et al. (2011, 2008a,b) and Barata et al. (2005) have suggested

that GST could be used as a biomarker for lipid peroxidation, since GST mRNA is thought to have an antioxidant function through conjugation of lipid peroxidases. GST mRNA has been induced in *C. finmarchicus* when exposed to naphthalene, weathered crude oil WSF and dispersed oil. Based on the present results, fresh crude oil WSF can also be included in the list.

4.2.3 CYP330A1 gene transcriptions

Figure 12 shows the CYP330A1 mRNA transcription of *C. finmarchicus* to the WSF exposure. There are some variations in the CYP330A1 level, with some indications of a reduced level after prolonged exposure.

C. glacialis had a concentration related two fold induction of CYP330A1 mRNA when exposed for 24 h (Figure 12(B)) and 48 h (Figure 13(C)). The clearest response was shown after 48 h exposure time.

Time related-relationship between WSF exposure and CYP330A1 is shown in Figure 13. These results show the same responses as the concentration related results, but have a much higher variation compared to the concentration-related responses. *C. finmarchicus* did not seem to respond towards the WSF, while *C. glacialis* appears to induce CYP 330A1 when exposed for longer periods, however, there are some variations here as well.

Hansen et al. (2009) saw that lipid-poor *C. finmarchicus* expressed elevated levels of CYP330A1, while lipid-rich specimens had a reduced level when exposed to WSF of crude oil. Since the copepods in this experiments are not selected based on the amount of lipids, and neither was checked for lipid content, the exposure groups may consist of copepods with different lipid content which may at least partly explain the variations in the

CYP330A1 responses. Based on the results from Hansen et al. (2009) one may expect that *C. finmarchicus* had a higher CYP330A1 mRNA induction than *C. glacialis*, since *C. glacialis* has a higher lipid content than *C. finmarchicus*. In stead, *C. glacialis* showed the highest responses. The results from Hansen et al. (2009) may therefore only be applicable when looking at individual differences within a species, and not when comparing two different species.

CYP330A1 mRNA levels has also been found to correlate with the amount of ecdysteroids found in *C. finmarchicus*, where CV had a higher CYP330A1 mRNA level than females and males (Hansen et al., 2008a). Since the primary function of ecdysteroids is to induce production of molting and reproduction hormones, one may expect different levels of ecdysteroids at different developmental times during the CV stage. As Figure 9 shows, the CYP330A1 mRNA levels are almost the same in the two species. Based on these results, one would suggest that *C. finmarchicus* and *C. glacialis* have reached approximately the same developmental level in the CV stage.

4.2.4 Antioxidant enzymes

Two of the main enzymes in the antioxidant system, γGCS and SOD, were measured to investigate potential changes in the the antioxidant system of *C. finmarchicus* and *C. glacialis* exposed to fresh crude oil WSF.

γGCS and Gsynt catalyzes *de novo* synthesis of GSH (Bolesterli, 2009). GSH is used by GST when detoxifying compounds, hence a covariation of the GST- and γGCS mRNA results should be present. However, as shown in Figure 14 and Figure 15 there are no clear response in the γGCS mRNA transcription according to the WSF concentration. There was not a clear

relationship between the γGCS mRNA levels and the GST mRNA levels (Figure 10 and Figure 11) in these experiments. Even though there are no changes in the γGCS transcription, there may be changes in the γGCS activity, which may be related to the GSH production. A possible change in the GSH production may also correspond to the changes seen in the GST mRNA transcriptions.

Figure 16 and Figure 17 show that there are no concentration- or time related production of SOD mRNA in *C. finmarchicus*. The results are correspondent with the findings of Hansen et al. (2008b) who only found a short increase in the SOD mRNA level of *C. finmarchicus* exposed to naphthalene for 12 h. Previous experiments have shown an increased SOD mRNA levels in fresh water prawn (*Macrobrachium borellii*) exposed to WSFs of crude oil (Lavarías et al., 2011), and in spot (*Leiostomus xanthurus*) when exposed to PAHs (Winston and Giulio, 1991). The low level of PAHs in the WSFs in these experiments (Table 2) may explain the low SOD mRNA transcriptions.

An increased SOD mRNA transcription was found by Singh et al. (2009) when exposing a fruit fly (*Drosophila melanogaster*) to benzene, toulen and xylene. *C. glacialis* showed a concentration and time related SOD mRNA response when exposed to the medium concentration for 24 h (Figure 16(A) and Figure 17(B)). The increased gene transcription may be due to the high levels of BTEXs in the WSFs (Table 2).

Figure 9 shows that the natural level of SOD mRNA is very low in *C. glacialis* compared to *C. finmarchicus*. Also, the LC-results (Figure 8) show that there is a delayed response in *C. glacialis* according to time. These two observations may explain the inductions seen in Figure 16(B) and Fig-

ure 17(B). Since *C. glacialis* had a low basal level of SOD mRNA the WSF exposure may trigger the metabolic system to increase the gene transcription. However, due to the delayed response, the SOD mRNA induction is first seen after 24 h exposure time. Also, with only two replicates in the group, there was a higher uncertainty with regard to the interpretation of the induction results.

4.3 Wild living copepods compared to copepods living in artificial environments

C. finmarchicus used in these experiments originates from a lab culture, and not from the wild as *C. glacialis*, and this may contribute to the low gene transcription seen in *C. finmarchicus* compared to *C. glacialis*. Wild living species are constantly exposed to exogenous compounds and may therefore induce enzymes faster than species that live in an artificial environment. Since the measured enzymes are thought to have a wide range of functions, e.g. detoxification activities, they may act differently in species dependent on where the species live. These assumptions should be further investigated through experiments looking at biological and genetic differences between free living and lab cultured *C. finmarchicus* specimens. Also, there may be a greater production of metabolites in *C. glacialis*, and hence a higher gene transcription.

4.4 Evaluation of the gene transcription method

Statistical tests are based on models that normally will give the best results when the n is large. In these experiments the samples were pooled down from 25 species to 3 replicates for each concentration and time. Finding

a test suitable for these experiments was not easy. The consequence of a small n was the difficulties of proving significant trends and variations. The statistical significant level was set to $\alpha = 0.05$, however, one cannot conclude that a results is insignificant just because the p-value is larger than 0.05. Even though there is a general assumption that an α of 0.05 is the level that is giving the best evaluation of statistical tests, this does not have to be an absolute answer.

One way to satisfy the statistical criteria is to increase the number of replicates for each exposure group, hence, increasing the n. However, due to expensive analysis this could not be considered for the present investigation. Also, in experiments with live animals there may be large individual differences between the specimens that may obscure the results. And, even though the biological systems responses cannot be proved to be statistical significant, we cannot conclude that there are no responses at all.

Further, experiments with whole animals may give a different result than experiments with one specific organ or tissue. Specific organs and tissues express genes at different levels based on what function they have in the animal. For example, CYP 4 is an important enzyme in the fatty acid metabolism in vertebrates (Bolesterli, 2009) and would be highly expressed in lipid tissues. If the same enzyme had been analyzed in the skin tissue the responses may have been different since there are other metabolic systems that are dominating here.

The RNA used in these gene expression experiments are extracted from the whole animal, and may be obscured by the dominance of different types of tissues. This may explain at least some of the variation in the responses between the exposed groups. Unfortunately we cannot extract only one

tissue from the copepods, so at the moment this is the best way of testing gene expressions.

The changes in the antioxidant system could be further investigated by measuring the protein concentration, e.g. by western blot. Also, measuring the GSH level could give an indication of oxidative stress.

When the exposure media consist of several types of components, like fresh crude oil, there is the possibility of having additive, synergistic or antagonistic effects between the components. If there is an additive effect, there may be a high induction of the gene transcription. An induction may also be seen when exposing for chemicals that have a synergistic effect. On the contrary, chemicals that are acting as antagonists may inhibit the response that one of the chemicals can induce. This may inhibit an induction of mRNA transcription (Casarett and Doull, 2008). Further, the LC_{50} results (Figure 8) for *C. glacialis* showed that there are small differences between the area that are giving a response and the area of no response. There might be a possibility that the concentrations that are used in these experiments are to low to induce a response in the gene transcription.

4.5 Crude oil tolerance and biometry measurement of lipid content

Earlier studies focusing on lipid content after exposure for weathered crude oil (Hansen et al., 2011) showed that copepods with a higher percentage of lipids survived longer than copepods with a lower lipid content. A similar pattern was expected to be present in the present experiment. However, this was not the case. There was no clear relationship between time of death and percentage lipid content. The exposure medium in this experiment was

WSF from fresh crude oil. In contrast to weathered crude oil, fresh crude oil contain both heavy and light hydrocarbons (Miller and Connell, 1982). This probably made the WSF from fresh crude oil more toxic than WSF from weathered crude oil. Since more components were present in fresh crude oil there could be additional processes that contributed to the death of the copepods, not only the lipid content. A linear multi variable regression model was used to test if there were biometric variables, other than the lipid content, that were significant for surviving the WSF exposure.

Figure 18 shows that the body areal is the variable with the best fit for *C. finmarchicus*. When combining the body areal with the areal of the lipid sac the fit is doubled (see section 2. 8). It was postulated that the lipid sac content was the most important variable in order to survive an exposure to WSF from fresh crude oil. This cannot be confirmed for *C. finmarchicus* since the model showed that the body areal is the most important variable, with a $R^2 = 0.17$ compared to the percentage lipid sac that had a poor fit when presented alone. Since the next best variable, body length, had a $R^2 = 0.027$ the percentage lipid sac had a fit less than 0.027. In comparison, the results from *C. glacialis* in Figure 19 showed that the percentage lipid content was the variable with most significance for survival. This is consistent with the hypothesis, but we cannot conclude that the lipid content really was an important variable since the fit was not good ($R^2 = 0.14$). However, the lipid content may have an important role in the defense system of the copepods, since it was selected as significant for both species.

The statistical models were only based on the dead copepods. The same test was run on the survived copepods to unveil potential differences

compared to the results from the dead copepods (the results were not shown in this thesis). The difficulties with this approach was that the time of death of the copepods had to be known, in order to achieve the correct answer. Since we did not know how long the copepods would survive a WSF exposure, the time of death had to be assumed, and was set to 300 h. Based on the assumptions the R^2 was higher for each model, and signifies that the variables contributed in a higher degree for the surveillance of the copepods. However, since these results were based on assumptions and not on facts, the uncertainties were to high for consider them as significant results and were therefore excluded from this paper.

5 Conclusions and further work

Was *C. glacialis* less sensitive to the WSF exposure compared to *C. finmarchicus*?

This study revealed that the arctic species, *C. glacialis*, tolerated a higher WSF concentration than its more southern sibling, *C. finmarchicus*. However, *C. glacialis* had a steeper dose-response relationships than *C. finmarchicus*.

The lower tolerance of *C. finmarchicus* in the acute toxicity test showed that the species may be used as a model species for evaluating potential toxic effects an exposure has on *C. glacialis*. Since they tolerate less than *C. glacialis*, a recommended maximum exposure based on results from *C. finmarchicus* will in most cases be tolerable for *C. glacialis*.

In terms of gene expressions, where there differences in concentration- and time relationships?

Greater and faster responses in *C. glacialis* may signify that they have a better developed metabolic system to handle oil components. This may be an artifact due to the use of bred *C. finmarchicus*, and should be further investigated with the use of free living *C. finmarchicus*. The need for a continually development of the genetic library of *Calanus* specimens is important in order to understand what function the different genes have in the specimen.

Did the lipid content in specimens make them less sensitive to the WSF exposure?

The results from the linear multi variable regression model revealed that there were several variables that were important for tolerating fresh crude oil exposure, not only the lipid content.

The method used in these experiments cannot be concluded as a preferable method in analyzing how the crude oil sensitivity is dependent on varying lipid content.

Further work

As suggestion for elaborating these experiments, there could be planned a long term experiment where only generation I was exposed to crude oil droplets in a flow through system. The copepod system would be kept operating until generation III reached stage CV. Samples of each generation would be collected to perform gene transcription analysis. With this experiment gene transcription changes between the generations could be revealed. Also, an evaluation of how the crude oil affects the reproduction would be possible through observations of expanding or decreasing population size. This kind of experiment is time consuming, but it would give a more accurate answer to the question of how crude oil affect the *Calanus* population.

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Appendix

The chemical analysis of the WSFs were done at SINTEF Material and Chemistry. All the components that were detected are presented in Table A.1-A.2. Table A.3 shows the summarized values used in the results.

The raw data of the gene expression calculations are presented in Table A.4-A.5.

Table A.6-A.7 present the biometrically measurements and calculations in ImageJ and Excel. The results were used in the linear multi variable regression analysis for both *C. finmarchicus* (A) and *C. glacialis* (B).

Table A.1: The VOCs compounds that were found in fresh crude oil WSFs

	Calanus finmarchicus		**Calanus glacialis**	
	LC50	Matrise	Matrise	LC-50
VOC	ug/L	µg/L	µg/L	µg/L
Isopentane	180.9082383	230.6769116	238.6104082	210.3355186
n-C5 (Pentane)	46.4334768	43.31749146	38.76629041	32.38353528
Cyclopentane	436.3856252	471.3979269	486.2229265	462.3295832
2-methylpentane	53.37121541	36.06938631	30.73292066	22.80322004
3-Methylpentane	44.96519359	26.55669398	24.00702156	17.56654684
n-C6 (Hexane)	58.56708046	6.934486315	3.443815309	2.494407933
Methylcyclopentane	343.9167132	376.4683154	399.9072464	341.3271123
Benzene	140.6592535	133.3948793	142.059798	136.2881188
Cyclohexane	736.8385925	827.3038355	875.9774583	788.7017983
2,3-Dimethylpentane	0	0	0	0
3-methylhexane	0	0	2.003290031	0
n-C7 (Heptane)	5.567471264	0	0	0
Methylcyclohexane	323.3991302	322.5802943	346.5402368	257.630832
2,4-dimethylhexane	0	0	0	0
Toluene	141.6746495	126.3755673	129.2612935	127.0105299
2-Methylheptane	0	0	0	0
n-C8 (Octane)	0	0	0	0
Ethylbenzene	401.8697742	418.9774262	433.8490868	430.8603614
m-Xylene	994.3015015	1089.362308	1084.694622	1134.706021
p-Xylene	330.666812	331.8018168	342.9153666	351.2083269
o-Xylene	254.853035	253.5987278	250.9667298	261.276472
n-C9 (Nonane)	6.88	2.783485632	0	0
Propylbenzene	27.31841089	20.60099889	20.03413443	17.37810301
1-Methyl-3-ethylbenzene	110.745645	107.4624867	107.6841325	102.1428816
1-Methyl-4-ethylbenzene	32.8124802	31.60442389	30.57880796	29.65117047
1,3,5-Trimethylbenzene	58.05431802	55.05537446	54.45261837	52.75029985
1-Methyl-2-ethylbenzene	47.91585974	42.34018949	41.12375825	40.3961017
1,2,4-Trimethylbenzene	148.8702964	140.4025257	132.9408595	132.9512343
1,2,3-Trimethylbenzene	58.76908269	51.2743523	45.63483257	45.92006147
n-C10 (Decane)	3.146543543	14.27212668	0	0
n-Butylbenzene	2.411978378	1.216191503	0.894181761	0.53133299
1,2,4,5-Tetramethylbenzen	0.6554181	0.459187103	0.405437465	0.35184691
n-Pentylbenzene	3.596277724	2.677970426	1.854495813	1.687818193
C4-Benzenes	35.89560587	24.14611057	21.29799993	17.26848989
C5-Benzenes	2.432525347	0	0.120991722	0

Table A.2: The SVOCs that were found in fresh crude oil WSFs

	C. finmarchicus		*C. glacialis*	
	LC50	**Matrise**	**Matrise**	**LC50**
SVOC	ug/L	µg/L	µg/L	µg/L
Decalin	2.351143	0.497212	0.108852	1.089741
C1-decalins	2.626169	0.356461	0.250195	0.570687
C2-decalins	1.22522	0.428735	0.413239	0.500487
C3-decalins	0.993688	0.809746	0.81277	0.745601
C4-decalins	0	0.675803	0	0
Benzo(b)thiophene	0.077718	0	0.053205	0
Naphthalene	103.4233	147.7314	137.1386	135.6919
C1-naphthalenes	70.39497	90.02945	87.56004	90.21271
C2-naphthalenes	22.6847	21.5273	20.91645	21.35951
C3-naphthalenes	5.383231	5.361253	5.375193	5.280656
C4-naphthalenes	0	2.954783	3.51737	3.381787
Biphenyl	6.899874	6.662046	6.494124	6.531534
Acenaphthylene	0.054827	0.017096	0.022124	0.022052
Acenaphthene	0.597542	0.579223	0.552983	0.547332
Dibenzofuran	0.576856	0.557364	0.540483	0.525043
Fluorene	2.090396	2.067986	2.008431	1.939629
C1-fluorenes	1.633379	2.671541	3.587876	2.911515
C2-fluorenes	2.233444	2.505903	2.897373	2.535058
C3-fluorenes	1.212325	1.40153	1.905134	1.593153
Phenanthrene	1.26308	1.285902	1.247554	1.183322
Anthracene	0.396285	0.474996	0.480791	0.431771
C1-phenanthrenes/anthracene	1.167479	1.298378	1.313869	1.18915
C2-phenanthrenes/anthracene	0.689172	1.430658	1.573979	1.273878
C3-phenanthrenes/anthracene	0.192564	0.654816	0.785826	0.832272
C4-phenanthrenes/anthracene	0	0.798324	0.976739	0.837999
Dibenzothiophene	0.264897	0.251969	0.252297	0.249019
C1-dibenzothiophenes	1.031549	1.161648	1.428452	1.286775
C2-dibenzothiophenes	0	1.397003	1.580076	1.63445
C3-dibenzothiophenes	0	1.14476	1.074169	1.408559
C4-dibenzothiophenes	0	0.660085	0.687054	0.65083
Fluoranthene	0.041018	0.037527	0.043931	0.046135
Pyrene	0.021139	0.019918	0.019051	0.017729
C1-fluoranthrenes/pyrenes	0.176266	0.229884	0.277343	0.229955
C2-fluoranthenes/pyrenes	0.136866	0.23947	0.291351	0.303868
C3-fluoranthenes/pyrenes	0	0.132888	0.185347	0.178248
Benz(a)anthracene	0.000772	0	0.00763	0
Chrysene	0.011319	0	0	0
C1-chrysenes	0	0	0	0
C2-chrysenes	0	0	0	0
C3-chrysenes	0	0	0	0
C4-chrysenes	0	0	0	0
Benzo(b)fluoranthene	0	0	0	0
Benzo(k)fluoranthene	0	0	0	0
Benzo(e)pyrene	0	0	0	0
Benzo(a)pyrene	0	0	0	0
Perylene	0	0	0	0
Indeno(1,2,3-c,d)pyrene	0	0	0	0
Dibenz(a,h)anthracene	0	0	0	0
Benzo(g,h,i)perylene	0	0	0	0
Phenol	1.258716	0.619534	0.773959	0.421027
C1-Phenols (o- og p-cresol)	3.490352	2.929669	2.646654	3.856221
C2-Phenols	14.4985	9.708187	7.279272	8.798698
C3-Phenols	5.45445	5.625611	4.412216	4.665842
C4-Phenols	4.219146	3.235421	2.84966	3.034972
C5-Phenols	2.581145	2.565487	2.641754	2.5232

Table A.3: The summarized concentration of the compounds making the basic for the chemical results presented in the result section

	C. finmarchicus		***C. glacialis***	
	LC50	Matrise	Matrise	LC50
THC (GC-FID)	3598.56255	5430.86081	5140.98325	5847.96709
SUM VOC	4995.55407	5164.96538	5265.56177	5000.68324
Benzene	140.659254	133.394879	142.059798	136.288119
Toluene	141.67465	126.375567	129.261294	127.01053
Ethylbenzene	401.869774	418.977426	433.849087	430.860361
Xylenes	1579.82135	1674.76285	1678.57672	1747.19082
SUM BTEX	2264.02503	2353.51073	2383.7469	2441.34983
Sum C4-C5-benzenes				
SUM THC (for LC50)	8594.11662	10595.8262	10406.545	10848.6503
Sum SVOC (excl phenols)	261.353506	322.736971	306.98343	310.492337
Naphthalenes	201.886215	267.604193	254.507666	255.926583
2-3 ring PAH	20.3036699	27.0212274	29.4093335	27.5833447
4-6 ring PAH	0.38737914	0.65968599	0.82465376	0.77593461
C0-C5 phenols	31.5023046	24.683908	20.6035149	23.2999593
Decalins	7.1962197	2.76795622	1.5850563	2.90651536

Table A.4: The MNE results for *C. finmarchicus* obtain from Q-Gene

	CYP330A1	gGCS	GST	SOD
CFT-1-12	0.006569965	0.048490002	0.021246162	0.021448233
CFT-2-12	0.010165496	0.073291505	0.022883515	0.017568529
CFT-3-12	0.013957675	0.119848656	0.047893222	0.040669408
LFT-1-12	0.008274212	0.061685133	0.016519732	0.020260323
LFT-2-12	0.011623405	0.062353231	0.015864771	0.016031033
LFT-3-12	0.013490595	0.062976098	0.021107505	0.016387788
MFT-1-12	0.021889817	0.113657726	0.030470273	0.018320063
MFT-2-12	0.014679838	0.061938695	0.019550639	0.011848468
MFT-3-12	0.01269043	0.078106842	0.025211347	0.013162447
HFT-1-12	0.010417805	0.052474885	0.030132506	0.029922913
HFT-2-12	0.006815235	0.049562456	0.033280915	0.012607924
HFT-3-12	0.009405474	0.064156619	0.038772846	0.050396256
CFT-1-24	0.006828303	0.059221589	0.013784216	0.010323648
CFT-2-24	0.009925894	0.080976707	0.021278296	0.019801052
CFT-3-24	0.010688765	0.083386561	0.039493055	0.058831241
LFT-1-24	0.008486459	0.144611884	0.033409206	0.04824413
LFT-2-24	0.007190526	0.051824511	0.021214048	0.069587996
LFT-3-24	0.010087357	0.088489929	0.046241878	0.074935783
MFT-1-24	0.01094738	0.058737634	0.019916259	0.050193276
MFT-2-24	0.010090061	0.076617785	0.023998951	0.028723848
MFT-3-24	0.010011929	0.086933202	0.031395574	0.018445931
HFT-1-24	0.009642502	0.041793608	0.029179619	0.02782393
HFT-2-24	0.003994874	0.038243755	0.030007707	0.008422021
HFT-3-24	0.009858505	0.045380012	0.031643376	0.028700402
CFT-1-48	0.010314565	0.071715321	0.019908116	0.026042172
CFT-2-48	0.013006869	0.075231165	0.025765408	0.037329876
CFT-3-48	0.004284108	0.053902269	0.023700842	0.022236413
LFT-1-48	0.006328092	0.086391861	0.016938485	0.013430817
LFT-2-48	0.010412722	0.080256163	0.024646668	0.025038868
LFT-3-48	0.005851965	0.077870602	0.027982838	0.078124525
MFT-1-48	0.006086961	0.044339128	0.017965379	0.020028122
MFT-2-48	0.012865923	0.073027886	0.022418016	0.016908237
MFT-3-48	0.008336573	0.094277214	0.028360885	0.057228093
HFT-1-48	0.006714038	0.050556531	0.038892218	0.028174717
HFT-2-48	0.010012133	0.101184928	0.082812412	0.016922495
HFT-3-48	0.007451897	0.098467336	0.056906949	0.060254465

Table A.5: The MNE results for *C. glacialis* obtain from Q-Gene

	CYP330A1	gGCS	GST	SOD
Troll-12-C1	0.00072018	0.0136946	0.11374864	0.00049397
Troll-12-C2	0.00048305	0.0132964	0.1381385	0.00047147
Troll-12-C3				
Troll-12-L1	0.0003805	0.01787069	0.10819815	0.00049246
Troll-12-L2	0.0006434	0.01352913	0.13730722	0.00057465
Troll-12-L3	0.00198617	0.02125982	0.17805694	0.00118378
Troll-12-M1	0.00055213	0.00985675	0.16442179	0.00270174
Troll-12-M2	0.00039782	0.02603953	0.16303114	
Troll-12-M3	0.00084593	0.00650577	0.12204225	0.00176264
Troll-12-H1	0.00055155	0.01977351	0.25016597	0.00057342
Troll-12-H2	0.00081477	0.01411369	0.14103678	0.00064561
Troll-12-H3	0.00114792	0.00949854	0.09007618	0.00064658
Troll-24-C1	0.00050298	0.03028034	0.15580887	0.00059591
Troll-24-C2	0.00120408	0.02729163	0.15530141	0.00047611
Troll-24-C3	0.00079647	0.01756799	0.12896546	0.00048285
Troll-24-L1	0.00156536	0.02244237	0.11820977	0.00051019
Troll-24-L2	0.00194774	0.02282867	0.22081135	0.00074081
Troll-24-L3		0.00477065	0.23346629	
Troll-24-M1	0.00165933	0.01560286	0.13566475	0.00059985
Troll-24-M2	0.00101242	0.01414478	0.16875666	0.00080098
Troll-24-M3	0.00034989	0.02599189	0.19588547	0.00085548
Troll-24-H1	0.0008241	0.00319586	0.23346799	0.00059276
Troll-24-H2	0.0004793	0.0175108	0.17232665	0.00064965
Troll-24-H3	0.00040868	0.01623738	0.1782009	0.00043964
Troll-48-C1	0.00025354	0.01389013	0.08010108	0.00040744
Troll-48-C2	0.00095798	0.00604889	0.10381945	0.00073541
Troll-48-C3	0.00068455	0.01286112	0.08680476	0.00050641
Troll-48-L1	0.00049438	0.02200592	0.1163497	0.00047855
Troll-48-L2	0.00051403	0.01117906	0.10747846	0.00051058
Troll-48-L3	0.00075312	0.00420941	0.17961664	0.00057501
Troll-48-M1	0.00095248	0.01244058	0.11398567	0.00068781
Troll-48-M2	0.00077379	0.03484768	0.23763482	0.00076957
Troll-48-M3	0.00169689	0.02194696	0.18256965	0.00079397
Troll-48-H1	0.0009649	0.012741	0.35258669	0.00046395
Troll-48-H2	0.00144412	0.00929604	0.30442028	0.00042779
Troll-48-H3	0.00113299	0.02148673	0.32184965	0.0004858

Table A.6: The measured and calculated values for *C. finmarchicus*.

Exposure time	Photo nr	Gender	Comments	A body	L body	A lipid sac	L lipid sac	V lipid sac	V body	% lipid sac
48	fersk_007	CV		0.619	1.67	0	0	0	0.18010861	0
48	fersk_008	female	egg	1.782	2.703	0.662	1.921	0.17908461	0.9222295	19.4186601
48	fersk_001	male		1.381	2.381	0	0	0	0.62877841	0
48	fersk_002	male		1.42	2.401	0	0	0	0.65925614	0
48	fersk_003	female	egg	1.709	2.639	0.6	2.384	0.11854027	0.86878916	13.644308
48	fersk_004	female	egg	1.807	2.662	0.488	1.118	0.16721202	0.96289274	17.3655917
48	fersk_005	female		1.671	2.575	0.705	1.949	0.20018708	0.85122687	23.517477
72	fersk_003	male		1.535	2.464	0.002	0.044	7.1364E-05	0.75066421	0.00950673
72	fersk_004	female		1.68	2.58	0.35	1.142	0.08420534	0.85875349	9.80553123
72	fersk_002	female	egg	1.732	5.595	0.542	1.725	0.13368391	0.42088683	31.762435
96	fersk_001	female	egg	1.811	2.635	0.703	2.17	0.17878068	0.97707058	18.2976213

Table A.7: The measured and calculated values for *C. glacialis*.

B

Exposure time	Photo nr	stage	comments	A body	L body	A lipid sac	L lipid sac	V lipid sac	V body	% lipid sac
24	Frame_$F1	CV		1.516	2.605	0.351	2.393	0.04041487	0.692564668	5.835537434
24	Frame_$F1.jpg	CV		1.69	2.811	0.26	2.182	0.02431989	0.797594628	3.049154188
48	48_1_$F1	CV		2.68	3.441	0.411	2.462	0.053859864	1.63853066	3.287083071
48	48-2_001.jpg	CV		3.532	3.916	1.02	3.711	0.220079224	2.500738979	8.800567585
48	48-2_002.jpg	CV		3.025	3.645	1.048	3.374	0.255533088	1.970710734	12.96654471
72	Troll_72_hrs_Dyr1	CV		2.809	3.327	1.473	3.233	0.526828724	1.861745592	28.29756796
96	96_1_001	CV		2.447	3.107	1.257	2.989	0.414967703	1.512851646	27.42950403
96	96_1_002	CV		1.874	2.915	0.632	2.753	0.113893149	0.945736762	12.04279604
96	96_1_003	CV	split lipid sac	2.76	1.019	0.695	0.311	1.219211013	5.868317959	20.77615803
96	96-2_008	CV		2.122	2.983	0.922	2.875	0.232109892	1.184969474	19.58783727
96	96-2_009	CV		2.551	3.101	1.246	3.023	0.403150863	1.647361104	24.47252532
96	96-2_006	CV		1.82	2.931	0.548	2.749	0.085754325	0.887149096	9.66628108
120	120_1_001	CV		1.669	2.75	0.412	2.19	0.060844311	0.795151049	7.651918534
120	120_1_002	CV		2.177	3.067	0.891	2.993	0.208218037	1.213033344	17.1650712
120	120-2_001	CV		3.465	3.662	0.713	2.592	0.153962062	2.573699242	5.982131074