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# Effects of Crude Oil Water Accommodated Fractions (WAF) on the Escape Behaviour in *Calanus finmarchicus* Gunnerus (Copepoda)

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Ida Fines

## Abstract

The calanoid copepod *Calanus finmarchicus* is the most common zooplankton species in the North Atlantic Ocean and the Barents Sea. It has a key position in the marine food web and has the ability to store large amounts of lipids, which may be the main transfer route of lipophilic contaminants to higher trophic levels. *C. finmarchicus* is therefore considered to be an ecological relevant test species.

As inappropriate behavioural responses to environmental and physiological stimuli due to toxic effects of aquatic contaminants can have severe implication for survival even at sub-lethal concentrations, it is important to examine behavioural indicators for aquatic toxicity.

In total four oil exposure series were conducted, where the exposure media were WAFs based on weathered and fresh crude oil. The exposure time was 24, 48, 72 and 96 hours. Escape behavioural alterations as a response to oil exposure induced narcosis were investigated. The parameters measured were escape response frequencies, escape response latency time and fatiguing effects of a repetitive hydrodynamic disturbance.

Weathered and fresh crude oil exposures were found to cause a decrease in escape response frequencies at sub-lethal concentrations, at all exposure times. There were not found any clear correlation between crude oil exposure and alteration in response latencies and fatiguing effects of a repetitive hydrodynamic disturbance.

The presence of significant reduction in escape response frequency in *C. finmarchicus* indicates that escape behavioural alterations can be measured as endpoints for sub-lethal toxicity, and may be used as ecologically relevant indicators of pollution.

# Content

# 1 Introduction

The world's energy demand is rapidly increasing with the growth of the world's human population and economic growth of emerging markets, especially China and India. Fossil fuels are to this date the greatest source of energy, crude oil being the largest contributor in this group. As the energy demand is increasing and the world's petroleum reserves are being depleted, the oil industry is searching for new areas for oil production (Ødegård Hansen et al., 2011). However, increasing search and transport of crude oil makes the environment susceptible to operational and accidental spills. There are several examples of oil production activities resulting in severe damages to the marine environment, like the Deepwater Horizon incident in 2010 in the Gulf of Mexico and the Exxon Valdez tanker incident in Alaska in 1989 (DeLaune and Wright, 2011; Harwell and Gentile, 2006).

*C. finmarchicus* is considered to be an ecologically relevant test species when assessing the impact of environmental contaminants in the marine ecosystem. Its relevance is based on its key position in the marine food web, and the ability to store large amount of lipids, which may be the main transfer route of lipophilic contaminants to higher trophic levels in Arctic and Sub-Arctic food chains (Magnusson et al., 2007; Sormo et al., 2006) . A short generation time permits investigation of effects from contaminants in life-cycle studies (Hansen et al., 2008).

The results of numerous studies have shown that certain fish species and other organisms may be influenced by organic pollutants, like oil residues, that may interfere with endocrine and physiological mechanisms (Brian et al., 2006; Clotfelter et al., 2004; Jensen and Carroll, 2010). This again may influence behaviour, and potentially reduce or put down survival. Little is known about how, or if, oil residues interfere with neural or endocrine signals in copepods, but in a study by Wibe et al. (2001) three groups of threespine sticklebacks (*Gasterosteus aculeatus L.*) where exposed to an organotin compound and anti-predator behaviour was compared to a control group. The organotin exposure caused a considerable change in response to predator attack, recovery time and latency time. It was suggested that behaviour as a response to pollution may be used as an ecological relevant integrative

biomarker. Further it is known that when *C. finmarchicus* get exposed to oil this exposure will result in an unspecified narcotic effect on the *C. finmarchicus*, and this may decrease their survival and reproduction ability. Behaviour response to pollution in *C. finmarchicus* has not been elucidated thoroughly, but because of results from previous studies with other species, it is likely to believe that escape response in *C. finmarchicus* may be altered when exposed to oil compounds.

## 1.1 Oil compounds

Crude oil is a complex organic mixture of hydrocarbons and is naturally occurring, generated by geological and geochemical processes. Various oil types can have different characteristics, such as density, viscosity and colour, depending on the composition. The main components of crude oils are alkanes, naphthalenes, aromatics and alkenes (National, 2003). Of the hydrocarbon compounds common in crude oil polyaromatic hydrocarbons (PAHs) are of greatest concern for the marine biota, with genotoxic, carcinogenic or reproductive effects, and may biotransfere in the marine food web (Corner et al., 1976; Manahan, 1994; Miller and Connell, 1982).

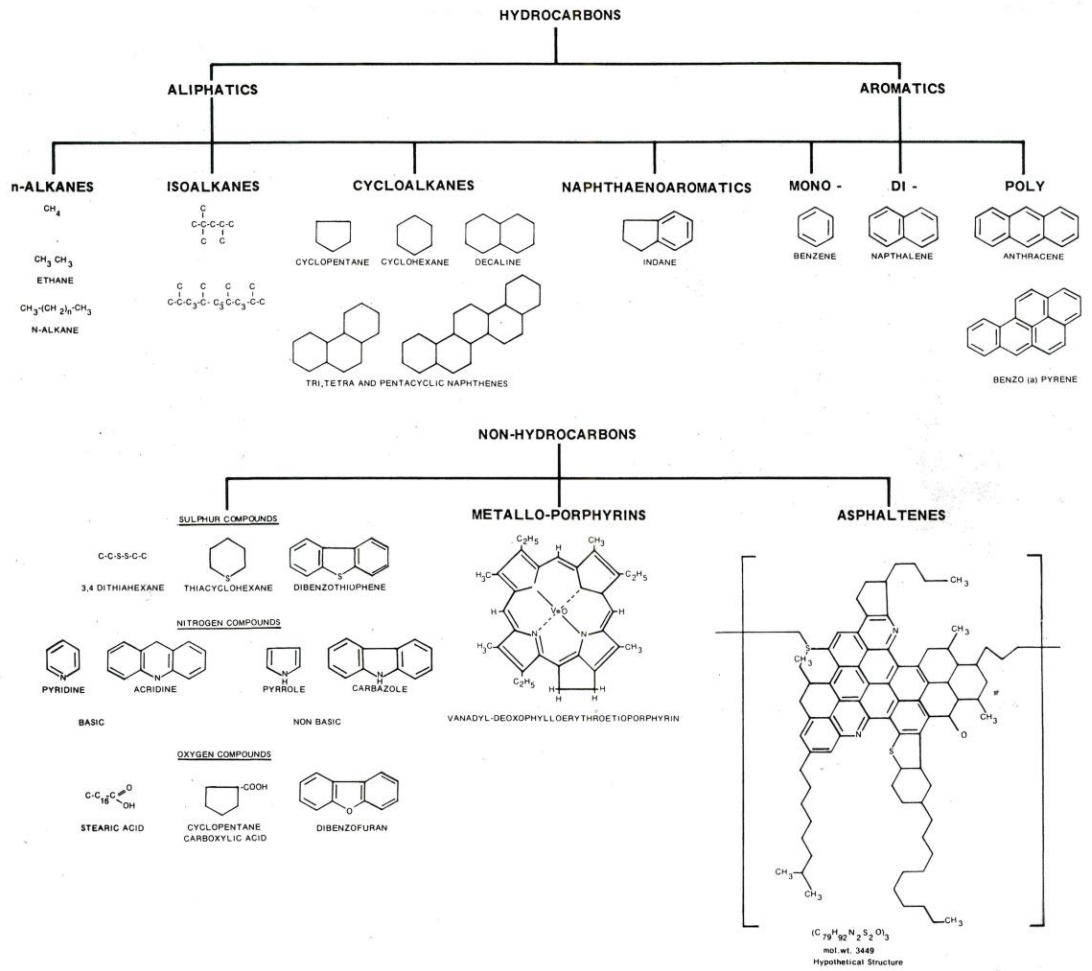



**Figure 1. Some of the common hydrocarbons and non-hydrocarbons present in crude oil(Miller and Connell, 1982).**

### 1.1.1 Octanol/water partition coefficient ($K_{OW}$) and route of uptake in organisms

The octanol-water partition coefficient is described by Walker et al. (2006) as an index of hydrophobicity of a chemical component, and is used in the prediction of the environmental distribution of a compound. $K_{OW}$ is computed simply as the ratio between the concentrations of a component in the octanol phase to its concentration in the aqueous phase, after mixing and equilibration in a two-phase system made up of the two solvents (Rand, 1995):

$$K_{OW} = \frac{Conc.\ in\ octanol}{Conc.\ in\ water}$$

Octanol represents, in this context, fat in all living beings, assuming only minor disparities in component kinetics between different fat compounds (Rand, 1995). When oil components get distributed in water the route and magnitude of uptake in organisms are therefore

thought to largely depend of the octanol-water partition coefficient. For hydrophobic compounds with log $K_{OW}$>5 intestinal uptake from contaminated food is especially of concern (De Laender et al., 2008; Smitkova et al., 2005). However, for components less hydrophobic the main route of uptake is considered to be from water (De Laender et al., 2011).

### 1.1.2 Potential effects of exposure on marine invertebrates

When water-soluble oil compounds, such as naphthalenes, PAH, thiophenes and phenols, dissolve in the ocean water they may become bioavailable to marine organisms. The compounds may then be absorbed over gill surfaces or other membranes (Megharaj et al., 2011). Compound may also be associated with particulate material and accumulate in sediments and as a consequence be bioavailable to bottom living organisms (Boehm et al., 2007). The limited mobility of phyto- and zooplankton severely reduces their ability to actively escape unfavoured conditions such as oil-contaminated water following an oil spill incident, rendering them vulnerable to exposure (Fleeger et al., 2003). Primary producing marine microalgae have been shown to rapidly accumulate high concentrations of petroleum hydrocarbons (PH) during exposure to low concentrations, making bioaccumulation from the feed possible in plankton grazers (Wolfe et al., 2001).

When absorbed into the bloodstream or lymphatic system of an organism a xenobiotic (a chemical or substance that is foreign to an organism or biological system) may be distributed throughout the body. The chemical may cause damage to several organs or tissues (target organs/tissues), but it may also be stored in fat deposits before eventually reaching the target organ or tissue (Van Wezel and Opperhuizen, 1995). Xenobiotic chemicals are generally excreted from the body through urine and faeces, and biotransformation to more water-soluble (hydrophilic) products is usually a prerequisite to excretion. Xenobiotic biotransformation is a series of enzyme-catalyzed processes that alter the physiochemical properties of a chemical from lipophilic to hydrophilic through hydrolyse, reduction, oxidation or conjugation(Klaassen, 2008). If not excreted the chemical may bring about a change at the target site, disturbing the functioning of the organism. The effects of the chemical exposure are manifested in the absence of a repair mechanism or adaptation by the organism (Van Wezel and Opperhuizen, 1995).

Different oil components have been shown to cause several effects in marine invertebrates. Adult *C. finmarchicus* exposed to naphthalene was found to exhibit increased glutathione S-transferase (GST) induction, indicating oxidative stress and lipid peroxidation as the major mode of naphthalene toxicity (Hansen et al., 2008).Furthermore, when exposed to water-soluble fractions (WSF) in an experiment that was set to mimic a natural oil spill, unexposed *C. finmarchicus* had higher feeding rates than those exposed to WSF (Jensen and Carroll, 2010). Also, when investigating effects of PAH on offspring production in the estuarine copepod *Schizopera knabeni*, Lotufo (1997) found decreased nauplii and copepodid production at sub-lethal sediment concentrations.

#### 1.1.2.1 Narcotic effects of oil exposure

Most oil-associated compounds (aromatic hydrocarbons and alkanes) are expected to exhibit a narcotic toxicity mode of action (De Laender et al., 2011). Narcosis as an effect of exposure to pollutants in aquatic organisms is defined as a non specific reversible disturbance of the membrane function, caused by the accumulation of the pollutants in hydrophobic phases within the organism (Van Wezel and Opperhuizen, 1995).Further, Van Wezel and Opperhuizen (1995) describe that narcotic chemicals exert their effects in all fatty tissues. However, because the lipid membrane seems to be more sensitive it is known to be the primary site of toxic action, where the components penetrate the lipid bilayer region of membranes. An effect of this is alteration of the lipid properties, such as fluidity, thickness, surface tension and fatty acid composition. Disturbance of the membrane function results in decreased activity and a diminished ability to react to stimuli, and can ultimately lead to death of the organism (De Hoop et al., 2011; Van Wezel and Opperhuizen, 1995).

Several mechanisms underlying narcosis is discussed by Van Wezel and Opperhuizen (1995) . The presence of narcotic chemicals increases the fluidity of phospholipids in the membrane by lowering the transition temperature between gel phase and the liquid-crystalline phase of phospholipids. Disturbance of the equilibrium in the membrane may potentially affect all biological processes that depend on this equilibrium, one of them being the functioning of the membrane proteins. The disturbance of the lipid-protein interaction have been explained by several mechanisms, one hypothesis suggested is that increased fluidity of the lipids surrounding the sodium channel disturbs the regulation of the channel. Further, it has been hypothesised that absorption of narcotic chemicals in the membrane alters the

thickness of the membrane, and thereby reducing the electric field at a given potential difference over the membrane. As a consequence more $Na^+$-channels are inactivated, leading to disturbance of the nerve function by preventing the transmission of nerve impulses.

## 1.2 Weathering of oil in the marine environment

Crude oil may enter the oceans from natural seeps, runoffs or spills from oil production activity, and about 47 % of the oil that end up in the oceans is from natural seeps (Prince and Clark, 2004). Natural weathering of the crude oil will occur when, and after entering the ocean water, and includes a series of changes in chemical and physical properties (National, 2003). The degree of weathering depends on factors such as temperature, wind, waves and the characteristic of the oil (Sebastiao and Soares, 1995).

Oil spilled onto the ocean surface will start spreading as a continuous slick, viscous oil spread more slowly than less viscous oil, and the whole slick will drift powered by wind and waves (Brandvik and Daling, 1998). A considerable part of the oil will evaporate in the first days, and the slick also loses water-soluble hydrocarbons to the water (Wang and Fingas, 1995). Oil is slightly less dense than water and is mostly hydrophobic, and will therefore not easily blend with water (National, 2003). However, natural dispersion will be accomplished by wind and currents, and dispersed oil in the form of droplets in various sizes may be transported down, and spread in the water column by vertical currents (Brandvik and Daling, 1998) .

In the marine environment water may be taken up by the oil, called water-in-oil emulsification, to form a stable “mousse”. The degree of oil emulsification depends on oil composition and the turbulence of the water masses, and heavy oils and strong winds will create the most stabile mousses (French-McCay, 2004).

Evaporation is regarded a main process of mass loss from surface crude oil spills, and may significantly modify the physical and chemical properties of the oil. During the period of evaporation, the more volatile, low-weight compounds are primarily lost, reducing the fraction of these substances in the remaining oil and thereby altering the density and viscosity of the oil (Stiver and Mackay, 1984).

Transformation of the oil by photo-oxidation may take place in oil on, or near the water surface. This is a process where components in the oil react with oxygen under the influence of the ultraviolet radiation in the sunlight (Garrett et al., 1998).

Sedimentation of the oil may occur when oil is adsorbed on suspended material, and then deposited on the bottom. The heavier fractions of the oil sediments first, and the process is more rapid and comprehensive in waters rich on particulates. This makes sedimentation in deep open waters a particularly slow process compared to sedimentation in shallow and coastal waters (Brandvik, 1997).

Microbial degradation is a process where microorganisms degrade oil for nutritional purposes, and plays a major role in the weathering process (Atlas, 1981). Degradation rate primarily depends on the degree of oil dispersion in the water column, giving higher rates when the droplets are small due to their relative larger surfaces. Hence, dispersed oil droplets are much more biodegradable than emulsified oil nuggets (Brandvik and Daling, 1998; Moran et al., 2000).

### 1.2.1 Weathering of oil reduces toxic potential

The toxicity of an oil component depends both of its log ($K_{OW}$) value and its solubility, a concept known as toxic potential. As the toxicity increases with increasing log ($K_{OW}$) values, the solubility decreases more rapidly, reducing the toxic potential of the oil components. Thus, weathering of a crude oil reduces toxicity by removing the chemicals with greater toxic potential and leaving the residual oil with components with lower toxic potential (Di Toro et al., 2007).

## 1.3 *Calanus finmarchicus*

The copepod *C. finmarchicus* occurs throughout the North Atlantic, and plays a crucial role in the transfer of energy between primary producers and a number of planktivorous fish and fish larvae.

*C. finmarchicus* stores energy mainly as wax esters, and has a lipid reserve that is composed of fatty acids and wax esters. The lipid reserve is used for energy utilization and egg production, and will therefore decrease during food deprivation and reproduction (Mayor et

al., 2009). The complex life cycle of copepods starts with six naupliar stages, from NI to NVI, followed through a main metamorphosis by the first copepodid stage (CI). Then there are five successive copepodid stages, from CI to CV. The adult stage is CVI, the sixth copepodid stage (Marshall and Orr, 1972).

In general *C. finmarchicus* hibernates in deep waters from late summer and fall, to late winter or early spring. During this stage of dormancy no diel vertical migration or feeding will occur (Conover, 1988). However, a small number of *C. finmarchicus* is always found in surface waters even during winter. The reason for this is not clear for the moment, but it is known that most of the *C. finmarchicus* that hibernate belongs to copepodid stage 4 or 5 (Hirche, 1983). In January and February the following year the overwintering population of *C. finmarchicus* migrates to surface water, where they moult to adults and the females are fertilized (Marshall and Orr, 1972).

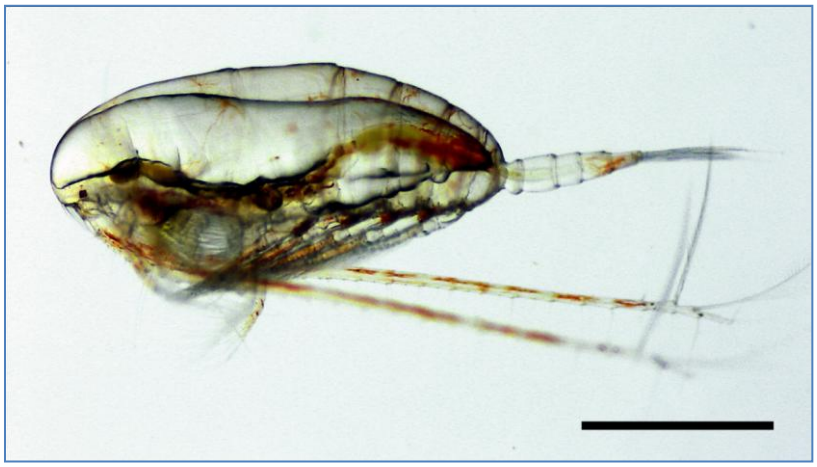


**Figure 2. *Calanus finmarchicus* stage CV. Note the large fat sac extending along the entire body. The bar in the picture is 1 mm (Photo by Dag Altin).**

### 1.3.1 Escape reactions

To reduce predation pressure, copepods have developed several strategies like diel vertical migration, transparency, small size and a rapid escape mechanism (Hamner, 1996). This rapid escape mechanism is triggered by hydrodynamic disturbance created by approaching predators. The first antennulae has innervation setae that detect hydrodynamic disturbances, and when these setae are bent by a sufficient disturbance a depolarization of innervating neurons will occur. Neurophysical signals will be sent from the site of depolarizing and will trigger a motor response that brings about the escape response. The escape response have been triggered and measured for several species, and it has been

recorded that copepods can propel itself more than 100 body lengths per second (Mauchline, 1998).

The kinematics during an escape reaction in *C. finmarchicus* and two other copepods species were described by Kiorboe et al. (2010). The sequences of events during the escape reactions is initiated by the copepod re-orienteering by bending the urosome (tail) and folding back one or both of the first antennulae. Subsequently, the posterior pair of swimming legs strikes backward at very high speed followed by one pair of swimming legs after the other striking back. This results in the copepod accelerating to its maximum velocity over a period of a few milliseconds. A full beat cycle is completed when the swimming legs are simultaneously recovered and the copepod coasts forward with decelerating velocity. An escape reaction may consist of several successive beat cycles and the last beat cycle in a jump is completed when the antennulae unfolds and the copepods stops.

## 1.4 Behaviour as a biomarker in ecotoxicology

When screening chemicals as a part of environmental risk assessment the criteria are based on standard laboratory toxicity test that have effects endpoints such as reduced survival, growth and reproduction (Atchison et al., 1987). The reasons for the initial focus on these criteria in toxicity test may be the ease and expense of performing them, and the need to implement regulations based on acquisition of irrefutable proof of harm (Hellou, 2011). Behaviour toxicology is slowly gaining more recognition as alterations in behaviour from sub-lethal exposures are considered to have a far greater sensitivity than the conventional $LC_{50}$ (lethal concentration) test (Hellou, 2011; Robinson, 2009). Behavioural effects have been documented at concentrations orders of magnitude below those observed to elicit mortality (Robinson, 2009). Behaviour is defined as the action, reaction or functioning by an organism under a set of specific circumstances, and is the physical manifestation of an organisms integrated physiological response to its environment (Clotfelter et al., 2004; Hellou, 2011). Behavioural effects can consist of a variety of activities as alteration in activity, feeding, anti-predator behaviour, communication, courtship, parental or social behaviour or learning (Clotfelter et al., 2004). Hellou (2011) suggested that these behavioural endpoints could

potentially be ranked on their “early warning” relativity, the time from exposure to alteration in behaviour become apparent. Avoidance and escape behaviour were examples on rapid responses that would be expected to be displayed readily by the organism, before succumbing to less rapid symptoms as impaired balance and righting response. These symptoms can progress further to decreased locomotor performance and alteration in respiration as a response to longer exposure, and were classified as behaviour endpoints with worse expected consequences.

Behaviour can be controlled by hormones or by the nervous system. Hormones have no effect until they reach the target where they affect all sensitive cells, on the contrary the nervous system is responsible for rapid and more precise communication (Alcock, 2005a; Schmidt-Nielsen, 1997a). The nervous system acquire environmental information from sensory neurons, which produces action potentials propagated by nerve fibres and signal to each other at the synapses by chemical transmitters, providing appropriate motor responses to the acquired information (Alcock, 2005a; Schmidt-Nielsen, 1997b). As discussed in section 1.1.2.1 a narcotic chemical may interfere with the nervous system by increasing membrane fluidity or interfacial area which may lead to a peak in ion permeability, and cause difficulties in maintaining the membrane potential (Van Wezel and Opperhuizen, 1995).

Hormones function within those mechanisms that establish behavioural priorities. Changes in the physical environment or social environment are detected by neural mechanisms and translated into hormonal messages. As a consequence behaviour like mating, nesting etc. are a top priority at times when it is most likely to translate into production of surviving offspring (Alcock, 2005b). Endocrine disrupting chemicals can interfere with biosynthesis, transportation, metabolism or binding of hormones and thereby lead to behavioural changes (Clotfelter et al., 2004).

## 1.5 Aims of this study

The main aim of this study is to assess whether behaviour in response to a hydrodynamic stimulus in *C. finmarchicus* is altered when exposed to artificially weathered and fresh crude oil WAFs. Main sub-goals are to reveal behavioural parameters including:

- Escape response frequency in non-exposed *C. finmarchicus* compared to exposed *C. finmarchicus*
- Escape response latency time (ms) in non-exposed *C. finmarchicus* compared to exposed *C. finmarchicus*
- Fatiguing effects of a repetitive hydrodynamic disturbance in non-exposed *C. finmarchicus* compared to exposed *C. finmarchicus*. The fatiguing effects measured were escape response frequency and escape response latency

Since there has not been developed a good standardized method to quantify unspecified narcotic effects due to oil exposure in *C. finmarchicus*, the aim of this thesis was also to develop a potential method using behaviour as a response to oil exposure as a relevant biomarker.

Based on findings in previous studies the hypothesis was that exposure to crude oil WAFs would result in a narcotic effect that could affect the escape response frequencies and response latencies.

# 2 Materials and methods

The experiment was carried out at NTNU Centre for Fisheries and Aquaculture ( SeaLab) in May 2011. *C. finmarchicus* stage five (CV) copepodids were exposed to water accommodated fractions (WAF) of both fresh and artificially weathered crude oil, from the Troll oil production platform in the North Sea. The exposed copepodids escape response to hydrodynamic stimuli was recorded. A total of 4 exposure series, two replicates for each of the oil qualities, were conducted. All series lasted five days and testing of response to hydrodynamic stimuli was carried out after 24, 48, 72 and 96 hours exposure, with three replicates each time. For every replicate the escape stimulus was repeated 5 times, with 10 minutes intervals to allow recovery to pre-stressed conditions. All testing included controls in three replicates treated identically to the exposed animals.

## 2.1 Experimental organisms

*C. finmarchicus* material was collected from the continuous lab culture run at SINTEF/NTNU SeaLab. In May 2011 the culture had been run for 29 successive generations. The culture was established primarily from copepodid stage V (CV) collected from Trondheimsfjorden in the late fall of 2004 (Hansen et al., 2007), and is maintained in containers (280 L) with running natural seawater at ~10 °C, and fed a mixture of the unicellular algae *Rhodomonas baltica*, *Isochrysis galbana* and *Dunaliella tertiolecta* (Hansen et al 2008). The culture is maintained in a conditioned room with a set light regime to adjust the circadian rhythm with 6 hours light and 18 hours darkness, the transition from light to darkness is eased with a “from dusk to dawn” computer-aided routine.

## 2.2 Experimental facilities

The experiment was conducted in a conditioning room at ~ 10 ° C. To prevent stray light in the hallway from reaching the experimental setup when opening the door, the room had an encased custom made light-blocking entry passage, made in an optical dense material. At the long side of the light-blocking entry passage, facing the experimental area, there was a

zipper door, giving easy access to the rest of the room. This entry passage was large enough to contain an illuminated workstation for use during experiments.

## 2.3 Exposure media

The WAFs were based on crude oil from the Troll oil exploration field, and had a loading of 1:10 000 oil:water ratio. An exposure concentration corresponding 25 % of the median lethal concentration after 96 hours exposure, $LC_{50}$ was chosen. This concentration would most likely give an effect on escape behaviour, but not cause lethality. The $LC_{50}$ data was acquired from acute toxicity testing with *C. finmarchicus* and fresh and artificially crude oil WAF. To achieve these levels the exposure media concentration had to be a dilution to 40 % of maximum WAF concentration when based on artificially weathered crude oil, and a dilution to 16 % of maximum WAF concentration when based on fresh crude oil.

Crude oil from the Troll field was chosen because it is the main representative used in research with *C. finmarchicus* and oil exposure at SINTEF/NTNU SeaLab. Furthermore a full set of $LC_{50}$ values are available for *C. finmarchicus*, thus allowing the qualified choice of exposure concentrations for the current experiment.

### 2.3.1 Generation of exposure media of fresh crude oil and weathered crude oil

WAF was made from both fresh crude oil and artificially weathered crude oil. To prepare an artificially weathered oil the lightest compounds were removed by heating to 200°C by the method of Stiver and Mackay (1984). The residue was collected and used for the generation of WAF. Weathering of crude oil gives a loss of volatile, low-molecular weight components, these components are also the most water soluble components (mainly BTEXs) in crude oil. This gives WAFs based on weathered crude oil a lower total concentration of total hydrocarbon which should reduce the toxicity of the WAF. However, some of the remaining heavier components (as PAH and the “unresolved complex material” UCM ) can be potent toxicants (Hokstad et al., 1999).

The chosen method to generate WAF was Singer et al. (2000). Depending on the different amounts of the two types of oil (fresh or weathered) needed, one or two 10 L baked bottles (Schott, Germany) were applied for the weekly WAF generation. The different volume

needed was related to the agreed exposure media dilution, which again was adjusted according to the $LC_{50}$ values of the two oil types. The flasks were filled with filtrated seawater and oil to make the desired loading (1:10 000). To prevent formation of oil droplets the oil was gently poured along the side of the bottle with a syringe to form a slick on the water surface. Magnetic stirrers were used to facilitate the equilibration of the system for about 72 hours at ~13°C, but gently to avoid disturbance of the oil-water interface. To collect the water phase (WAF) from the bottles a siphoning device was arranged by connecting a glass tube inside the bottle to a Viton rubber hose. When two 10 L bottles were used the water phase were siphoned off and mixed in a 20 L Florence flask (Schott, Germany). Samples to chemical analysis were tapped from the flask before the remaining WAF was transferred to 2 L bottles, diluted with clean seawater to the desired concentration, closed with a Teflon-lined screw cap, and then cooled down to ~10°C prior to addition of the test specimens.

## 2.4 Exposure system

The exposure system consisted of 2 L bottles (Schott, Germany), filled with the desired amount of WAF –in-seawater fraction. To close of the exposure system and prevent evaporation of chemical components a Teflon-lined cap was used (Schott, Germany). 24 bottles were required for each exposure series.

## 2.5 Escape response measurement system

The escape response measurement system was composed of an aquarium placed on a movable table top, which had a tactile transducer attached to the underside. The tactile transducer was connected to an amplifier which again was controlled by a computer. The computer also communicated with an IR-diode attached to a corner in the aquarium, through a USB-unit. A video camera mounted above the aquarium recorded the escape response. The measurements of escape response were executed in infrared (IR) light from two lamps illuminating the aquarium laterally from two sides.

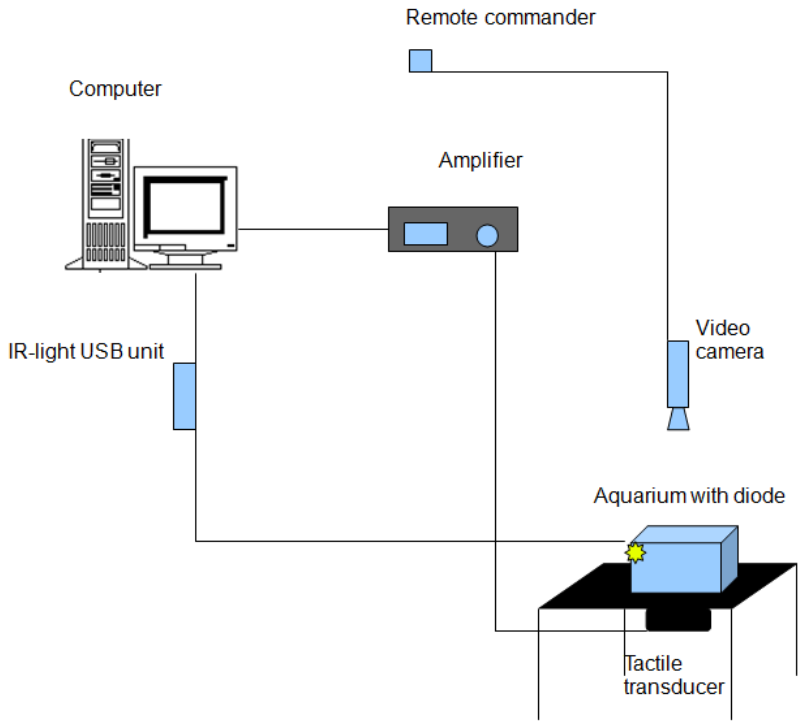



**Figure 3 .The escape response measuring setup, including a computer, IR-light USB unit, IR-diode, amplifier, tactile transducer, video camera, remote commander and an aquarium. A computer-generated sound wave and a light signal are generated simultaneously according to a routine programmed in LabView 8.6. The sound wave is amplified and sent to the tactile transducer that vibrates through the table top, and thus generates a hydrodynamic disturbance in the experimental aquarium. The IR-diode flash signals the start of a hydrodynamic disturbance in the experimental aquarium. A remote commander was used to start the video recording of escape reactions.**

The experimental chamber applied in the experiment was a custom-made clear acrylic plastic aquarium (machined by Mymek AS, Norway), the inner measures of the aquarium were 25 x 15 x 10 cm. To transfer the copepods to the aquarium a large glass bowl, a sieve and a small ladle were used. In order to have the optimal visibility in the aquarium without stressing the copepods due to a low water level, approximately 1650 ml of water was applied.

Preliminary tests showed that light and reflections in the aquarium were reducing the copepods response to stimuli and that video recording of the copepods in visible light gave poor visibility of the copepods on the recordings. It was therefore decided to perform the

experiments in darkness. The reduction in escape response when preliminary tests were performed in a conditioned room with visible light was not quantified with video recordings, only assumed based on the poor visibility of the copepods on video when captured in light.

The IR illumination was delivered by two 850 nm IR- lamps (Eneo, Germany), one placed at one short side and the other long side of the aquarium. The IR-lamps were powered by an Iso-Tech power supply (dual tracking with 5V fixed, England). The power supply also allowed the power to the lamps to be adjusted to give the best visibility (Ampere ~1 and Volt ~11).

The aquarium was placed on a movable table top (50 x50 cm) made of a black polyethylene plate (2 cm thick). The table top had a circle hole cut out in each corner so that it could rest on four tennis balls placed between the table top and its heavy steel undercarriage. This arrangement aided to direct the vibration from the transducer to the aquarium.

The tactile transducer (Viasaton BS 130 4 OHM, Germany) was pressed against the underside of the table top with its mounting plate, and secured in place with four screws. The two main components in the transducer are an oscillating mass and the mounting plate. When hooked up to the amplifier the oscillating mass will start vibrating with the frequency of the applied signal. The vibration is then transmitted to the mounting plate and then to the surface it is attached to.

The amplifier was a Pioneer stereo amplifier (A-441, Japan).

The computer routines were programmed in LabView 8.6 (National Instruments, USA), and the output signal connected to the stereo amplifier via an USB-unit (Multifunction Data Acquisition-box). The synchronously of the wave pulse (50 ms, 50 hz) and the light signal was accomplished by using the NI LabView SignalExpress Development System.

The video camera (Sony HDR-XR550, Japan) was mounted on a tripod hanging from the ceiling, and the whole setup covered with a custom made drape that prevented light from the computer monitor from entering the experimental setup (figure 4b). Latency and response to hydrodynamic stimuli were documented by the video camera recording in smooth slow record mode. This function captures 3 seconds of fast motion with a record rate of 100 frames per second (fps). The video recording of the escape reactions were started manually with a remote commander (Sony RM-AV2, Japan).

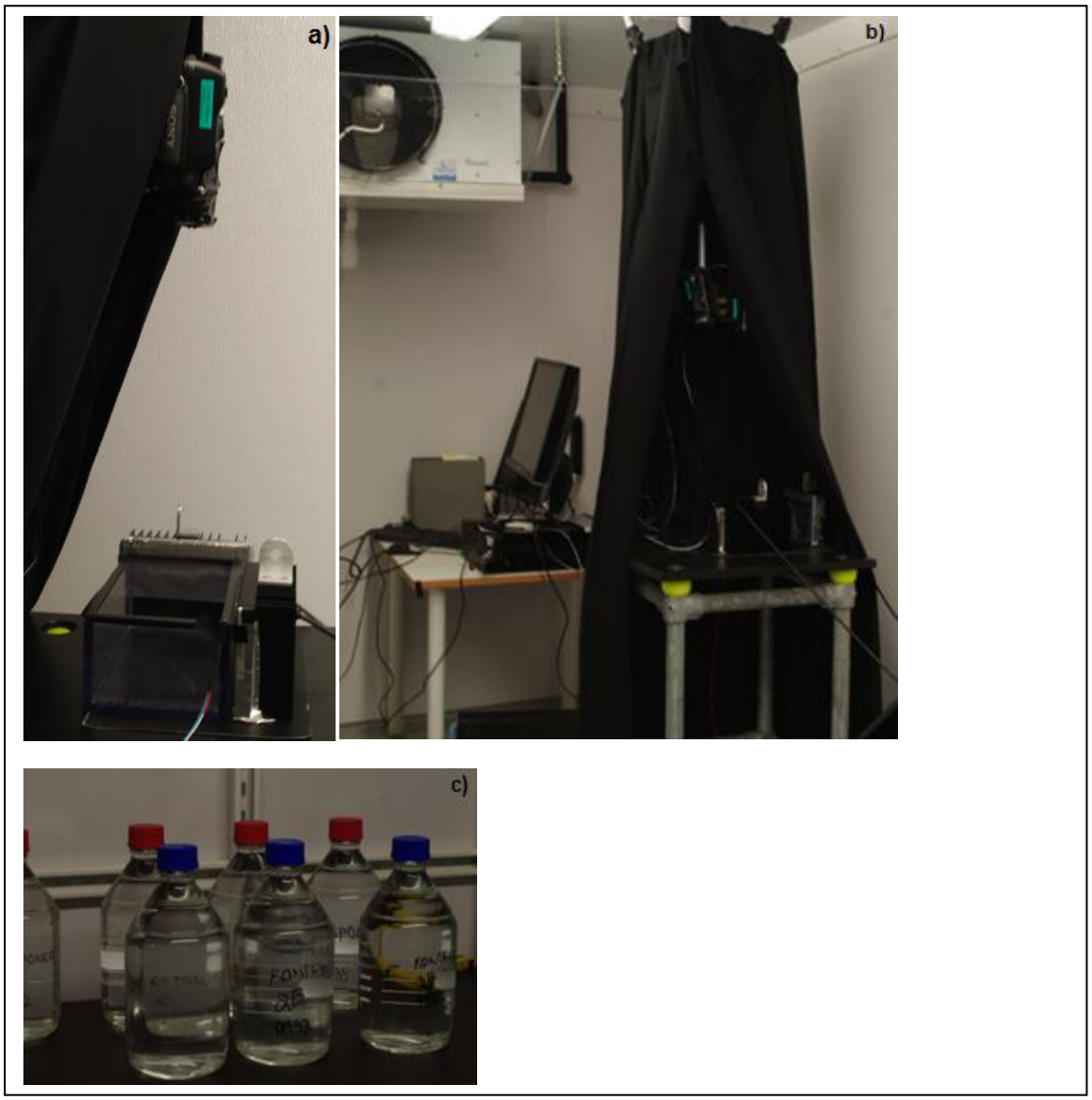



**Figure 4. Selected pictures from the experimental setup. a) The aquarium and the IR-lamps. To prevent reflections in the aquarium the IR-lamps were adjusted so that their light field would only illuminate the water column. b) The entire setup for recording escape response. The camera was mounting on a tripod hanging from the ceiling. In the background the computer and amplifier can be seen. c) Some of the exposure bottles containing the copepods, from start of an exposure experiment until transfer to the experimental aquarium. The bottles were closed with a Teflon cap, red cap for exposure bottles and blue for control bottles.**

## 2.6 Experimental procedure

Each of the four exposure series lasted 5 days. For each exposure series 240 CV copepodids were used, distributed on the 24 bottles with 10 individuals in each. Half of the bottles contained exposure medium, the other half clean seawater for control. Testing of response to a hydrodynamic disturbance was performed at four exposure times within each exposure series, were each testing comprised three replicates and the corresponding controls.

Before stimulating started the *C. finmarchicus* copepodids cohort from the relevant flask had to acclimate in the aquarium for 30 minutes before stimulation started. Then the animals were repeatedly stimulated five times, with 10 minutes apart, giving a stimulus session about 40 minutes for each replicate. This meant that a day with measuring escape response consisted of measuring response of 6 cohorts of copepodids, where 3 of them were control.

### 2.6.1 Treatment of experimental organisms

240 stage CV copepods were collected from the running culture the evening before an oil exposure experiment started. They were kept in a 5 L bucket next to the culture tanks so they all would experience the same light and temperature conditions as the mother culture. The subsequent morning ~ 50 of the animals from the bucket were randomly collected and the stage of the copepods was examined using a stereo microscope. If this test confirmed that all ~ 50 animals were stage CV all the animals collected the previous evening were considered stage CV. However, if non-CV copepods were present, all the 240 copepods were sorted under the stereo microscope to secure a homogenous material.

The copepods were starved from the night before each oil exposure experiment started and until the experiment ended (~36-108 hours).

To get exposure times as close as possible to 24, 48, 72 and 96 hours a strict schedule for the measurements was made and followed. Approximately 45 minutes before the desired exposure-time was up, the copepods and ~ 1650 ml of the exposure medium were transferred to the test aquarium. To acclimate to aquarium conditions the copepods were then kept in the aquarium in darkness without any form of disturbance for the next 30 minutes.

### 2.6.2 Handling of exposure media

All the 24 2-litre bottles were filled with cold filtrated seawater (~10°C) the evening before the start of an exposure series, and kept in a conditioned room at ~ 10°C. The control bottles were filled up to the bottle cap mark with seawater and the rest of the bottles were filled up corresponding to their WAF-in-seawater dilution. The following morning the exposure bottles were filled up with WAF and a cohort of 10 randomly collected individuals were added to each flask.

### 2.6.3 Sampling for chemical analysis

Two samples (~0,8L) of the primary WAF were taken prior to each exposure series, and acidified with diluted hydrochloric acid, in order to measure the amount of semi-volatile organic compounds (SVOCs). To determine the total amount of volatile organic compounds (VOCs) three samples of 40 ml WAF were also taken. At the end of an exposure series three samples of 40 ml were taken from the 96 hours exposure media. This was done one time for each type of oil, to determine the evaporation of VOCs during the experimental periode.

## 2.7 Chemical analysis

The chemical analysis of the WAFs were performed at SINTEF Material and Chemistry, and the protocol followed is found in Hansen et al. (2011).

To measure the amount of SVOCS a gas chromatography-mass spectrometry (GC-MS) was used. To detect specific components the GC-MS operated in the selected ion monitoring (SIM) mode. The GC-MS comprised of an HP6890N gas chromatograph fitted with a Hewlett-Packard HP7683B Series autosampler and a HP5975B quadrupole mass-selective detector. The column was a Phenomenex ZB-5MS fused silica capillary column (30 m x 0.25 mm id × 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1 ml/min. A 1 µl sample was injected into a 310 °C split-less injector. The oven temperature was programmed from 40 °C for 1 min, then to 315 °C at 6 °C/min and maintained for 15 min. Data and chromatograms were monitored and recorded using the MSD ChemStation (version D.03.00.611) software. The quadrupole mass spectrometer ion source temperature was 230 °C.

Determination of the total amount of volatile organic compounds (VOCs) were done by purge and trap gas chromatography-mass spectrometry (P&T GC-MS). The P&T GC-MS used

a modified EPA-Method 8260 with a 50 m (0.20 mm id, 0.50 µm film thickness) Supelco Petrocol capillary column. An Agilent 5973B mass selective detector (MSD) was used to detect target compounds, and data were analyzed with Agilent EnviroQuant Chemstation software. With this method a total of 34 target VOCs (C5 – C10) were determined.

To determine the total extractable organic compounds (TEOC) a gas chromatograph-flame ionization detector (GC-FID) was used. The GC-FID used to determine TEOC used the same gas chromatograph equipment and programs as for the GC-MS.

## 2.8 Analyzing video

A graphic interface was created in the Java programming language, which contained preset Ffmpeg (FFmpeg, France) command lines, exported the videos as individual frames. Ffmpeg is a complete, cross platform solution to record, convert and stream audio and video. Each video was turned into 299 frames.

ImageJ 1,43u (National Institutes of Health, USA) and the plug-in Particle tracker 2D and 3D, were used to analyze the videos. Image J allows you to import your frames into the program in image stacks, a series of images that share the same window. When imported the frames can be analysed and edited.

The frames from an escape response video where imported into ImageJ, and contrasts in the frame where enhanced and background removed if necessary. The particle tracker gives information about the movement of the particles from frame 1 to 299 and in which frame the movement starts. However the particle tracker does not work optimally when copepods move vertically in the aquarium or along the sides of the aquarium. All videos where therefore also checked manually, registering the copepods responding to the stimulus, and making a noting in which frame the escape movement started. By this method it was confirmed that responding copepods in the aquarium were visible in the video, where non-responding individuals were hardly tracked. As we knew for certain that ten seemingly healthy copepods were present in the aquarium, and all the reacting individuals were tracked and could be counted, we had a sound basis for calculations and statistical analyses.

The response latency time was calculated from the first frame were the diode blink was visible, to the first frame where the detection of the characteristic escape reaction was observed.

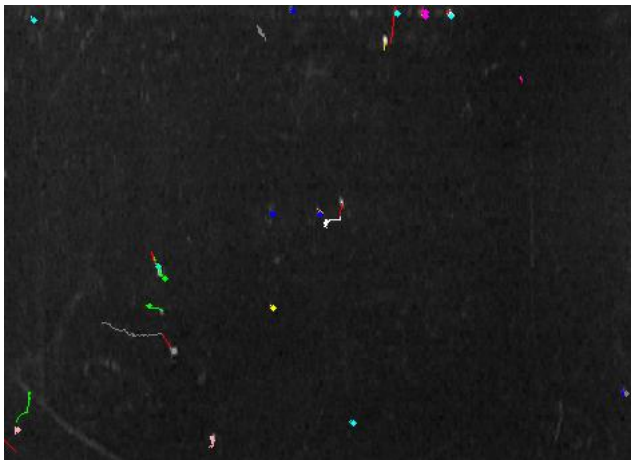


**Figure 5. Screenshot from ImageJ with the particle tracker. The coloured dots and lines are detected particles and their trajectory.**

## 2.9 Statistical analysis

Normality was tested by a Shapiro-Wilk´s test (n<100) and a Kolmogrov-Smirnov test (n>100). The dataset was not normally distributed, and failed to be transformed, thus non-parametric tests were used. Differences between control and exposed groups at the applied exposure times were pair-wise tested with Mann-Whitney U tests. A Kruskal-Wallis test with a pair-wise Mann-Whitney U test was used to test for differences in frequencies, and latency times in regards to fatiguing effects of a repetitive hydrodynamic disturbance. When testing for differences between data based on repeated measures of the same animals, the mean of the measurements for each hydrodynamic disturbance were included. Hence, applying the same n in statistical test as in the actual study, and thereby limiting errors from the statistical analysis. A significance level of p=0.05 was adopted in all analysis.

When not significantly different from each other groups exposed to the same crude oil type with the same exposure time, and their corresponding controls, where combined to get a larger n. This was pair-wise tested with Mann-Whitney U tests.

All graphs and statistical analysis were made in PASW Statistics v 19.0 (SPSS Inc., USA) for PC.

# 3 Results

## 3.1 Chemical content of water accommodated fractions (WAFs) of fresh and weathered crude oil

The results of the analyses of the WAFs are summarized in table 1. The WAFs based on fresh crude oil were significantly richer in BTEXs (benzene, toluene, ethylbenzene and xylenes) than the WAFs based on weathered crude oil.

**Table1. Summary of the chemical composition in the WAFs (before dilution in the experimental bottles) analyzed with GS-MS, P&T GC-MS and GC-FID. Concentrations are given as mean (µg/L) ± standard deviations based on two replicate WAFs generated for each treatment in this experiment.**

| Compound groups | Weathered crude oil (µg/L) | Fresh crude oil (µg/L) |
|---|---|---|
| **THC (GC-FID)** | 379 ± 8 | 403 ± 9 |
| **∑ VOCs** | 511 ± 13 | 1506 ± 35 |
| Benzene | 0.104 ± 0,011 | 3.535 ± 0.053 |
| Toulene | 1.242 ± 0.114 | 12.123 ± 0.218 |
| Ethylbenzene | 38 ± 0.9 | 114 ± 3 |
| Xylenes | 183 ± 2 | 472 ± 13.5 |
| **∑ SVOCs** (excluded phenols) | 444 ± 6 | 460 ± 5 |
| ∑ Naphthalenes | 425 ± 18 | 449 ± 4 |
| ∑ 2-3 ring PAH* | 18 ± 12 | 9.136 ± 4.764 |
| ∑ 4-6 ring PAH** | 0.150 ± 0.002 | 0.151 ± 0.008 |
| ∑ C0-C5 phenols | 3.198 ± 3.682 | 0.648 ± 0.329 |
| ∑ Decalins | 0.540 ± 0.461 | 1.141 ± 0.129 |

* ∑ 2-3 ring PAH include biphenyl, acenaphthylene, acenaphthene, dibenzofuran, fluorine (C0-C3), phenanthrene, anthracene, phenanthrenes/anthracenes (C1-C4), dibenzothiophenes (C0-C4).

**∑ 4-6 ring PAH include fluoranthene, pyrene, fluoranthenre/pyrene (C1-C3), benz(a)anthracene, chrysene.

## 3.2 Hydrodynamic disturbance signal

The hydrodynamic disturbance signal was measured on the experimental aquarium by an accelerometer, and the dominant frequency measured was ~ 330 Hz. The acceleration ($m/s^2$) from the signal is presented in figure 6, and show that the signal lasted for about 60 ms. This shows that the actual frequency and duration of the hydrodynamic disturbance signal measured were different than the theoretical signal which were set to last 50 ms with a frequency of 50 Hz). However, the signal is well within the limits of hydrodynamic disturbance perception in copepods (Fields and Yen, 1997).

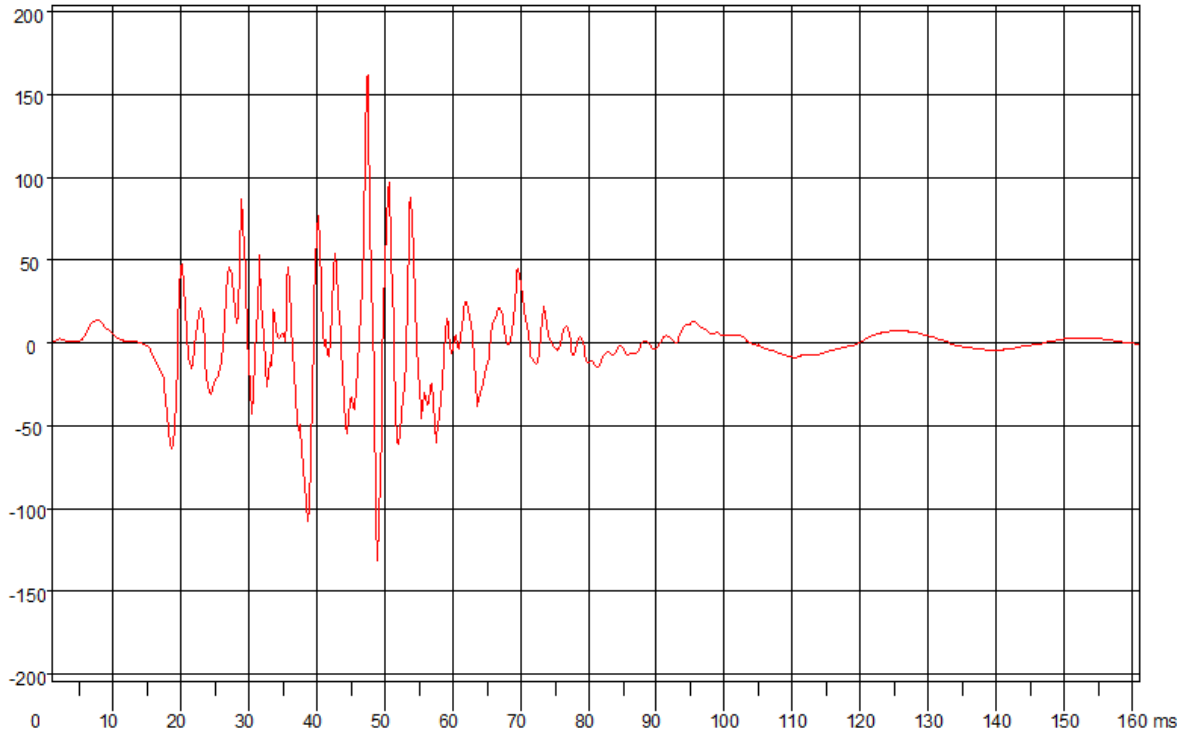



**Figure 6. Acceleration ($m/s^2$) (y-axes) and duration (x-axes) of the hydrodynamic signal measured on the experimental aquarium.**

## 3.3 Frequency of escape reactions in response to hydrodynamic disturbance

The data indicated an overall negative correlation between escape response frequencies (no/replica) and exposure to crude oil (figure 7). Significant lower frequencies for exposed groups compared to controls were found after 24, 48 and 72 hours exposure to weathered crude oil and after all exposures (24, 48, 72 and 96 hours) for exposure to fresh crude oil (Mann-Whitney U test).

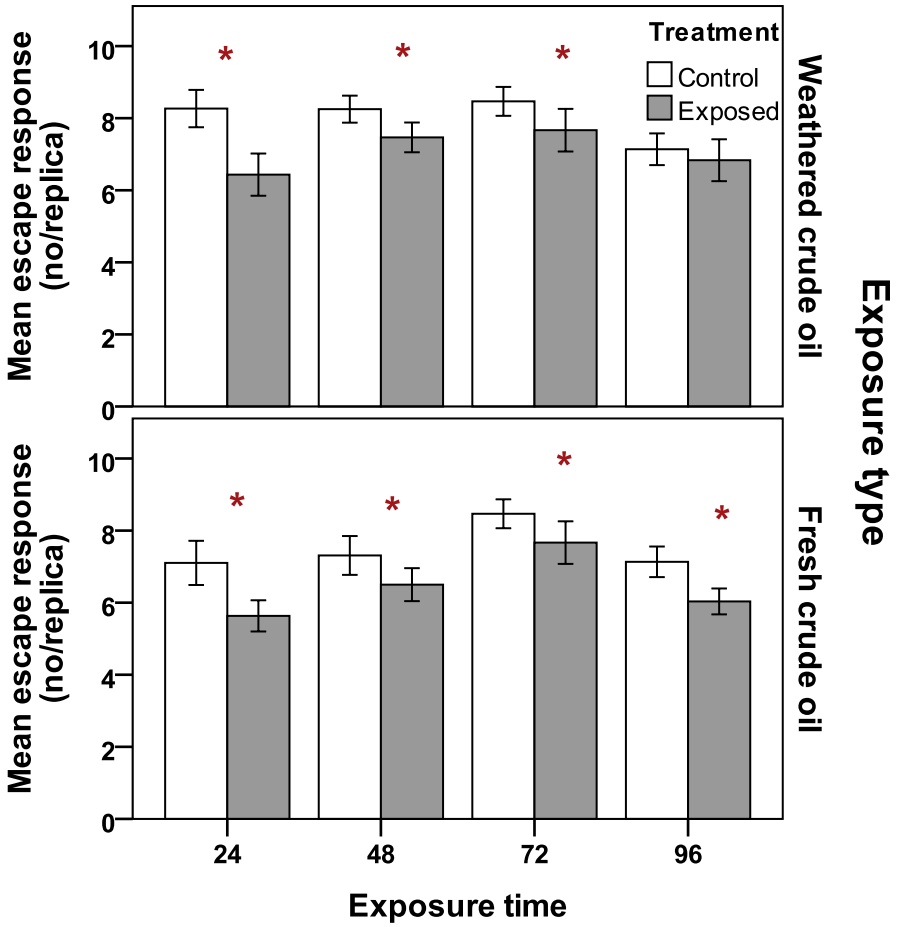



**Figure 7. Mean escape response (no/replica) for control and exposed groups after 24, 48, 72 and 96 hours. Significant differences between control and exposed groups are marked with * in the graph, $p < 0.05$. Replica N=6 and every replicate contains 10 *C. finmarchicus* which were stimulated 5 times each. Error bars represent 95 % confidence interval (CI).**

### 3.3.1 Fatiguing effects by a repetitive hydrodynamic disturbance on escape response frequency

No general correlation between escape responses frequency and the order of the hydrodynamic disturbance was demonstrated (figure 7). However, for controls mean escape response frequency at the 5. hydrodynamic disturbance was generally reduced compared to the 1. disturbance, which could point to a frequency reduction with increasing disturbance numbers. A similar trend was less evident in exposed groups.

There were however found significant differences between control and exposed replicates in several points in a hydrodynamic disturbance series with both exposure to weathered and fresh crude oil. 24 and 48 exposure time, and all exposure times, respectively.

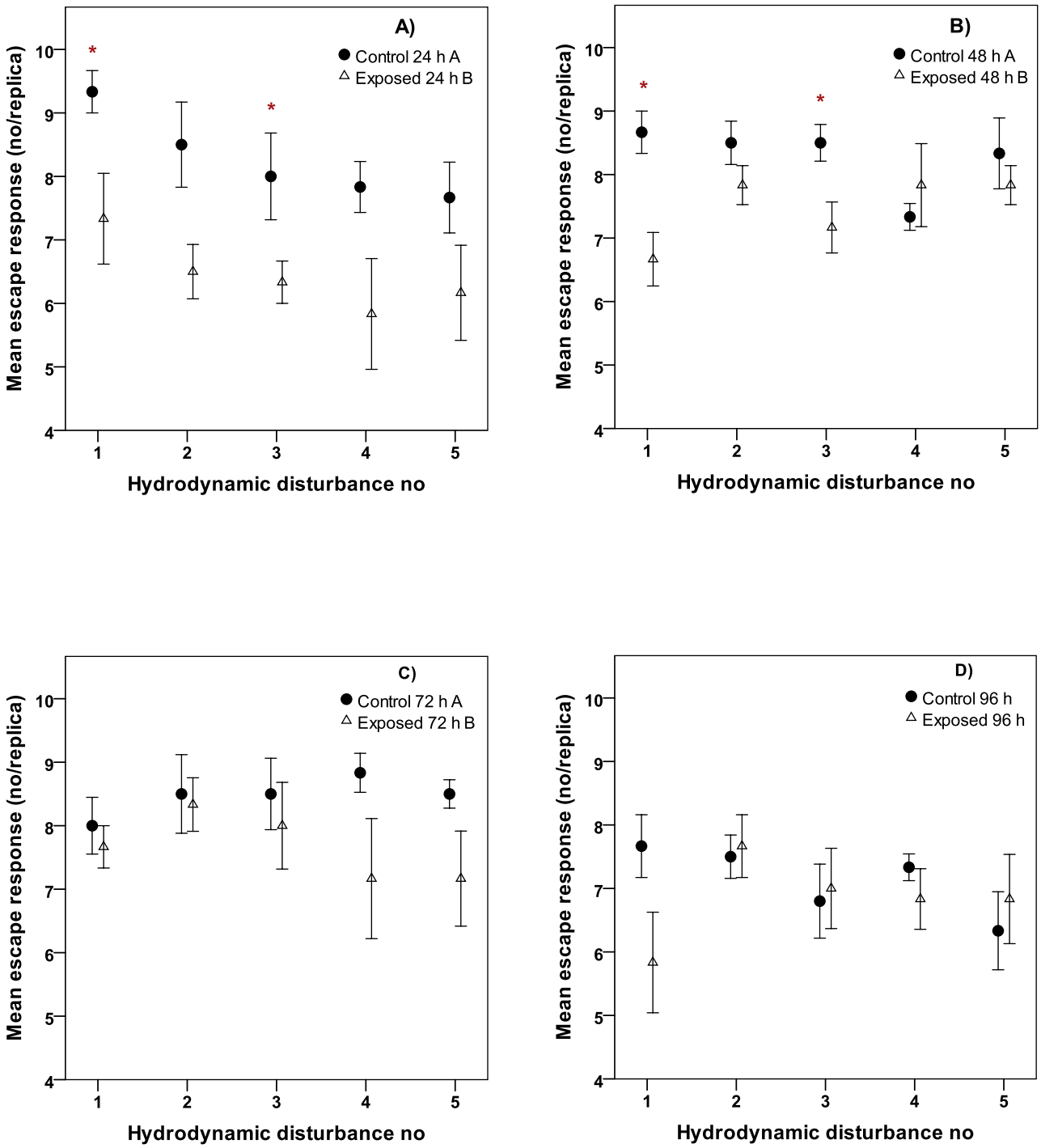



**Figure 8. Weathered crude oil: Portion of *C. finmarchicus* responding to repeated hydrodynamic disturbance in control and exposed groups, after 24, 48, 72 and 96 hours. Interval between one disturbance and the next is 10 min. Significant difference between controls and corresponding exposed groups are marked with different letters (A or B) after the appurtenant legend, $p < 0.05$. Significant difference between control and exposed groups at disturbance no 1, 2 etc. is marked * in the graph (above the dots), $p < 0.05$. Replica N=6, except for disturbance no 3 control 48 h (N=4) and disturbance no 3 control 72 h (N=5), and every replica contains 10 *C. finmarchicus*. Error bars indicate ± 1 standard error (SE).**

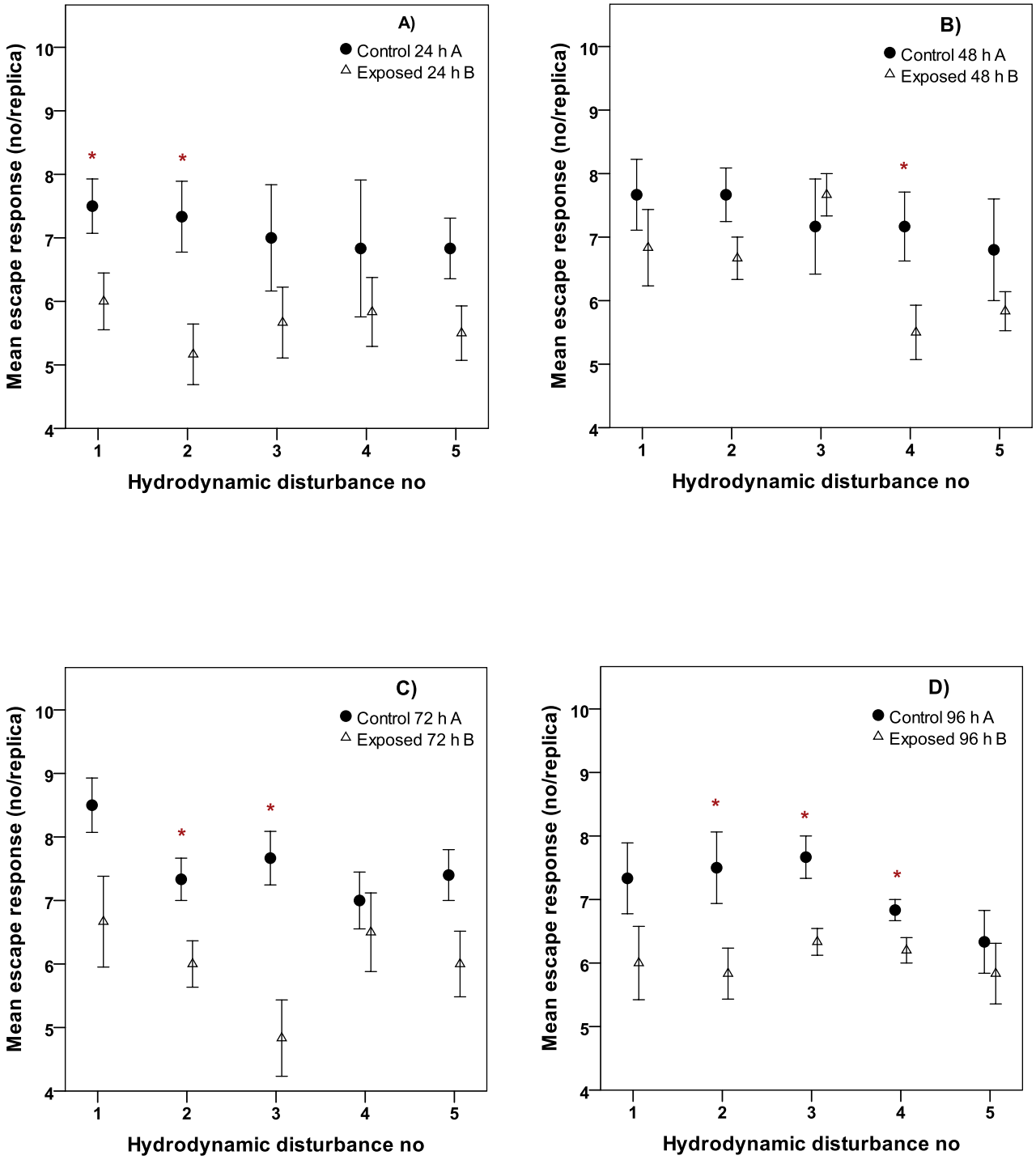



**Figure 9. Fresh crude oil: Portion of *C. finmarchicus* responding to repeated hydrodynamic disturbance in control and exposed groups, after 24, 48, 72 and 96 hours. Interval between one disturbance and the next is 10 min. Significant difference between controls and corresponding exposed groups are marked with different letters (A or B) after the appurtenant legend, $p < 0.05$. Significant difference between control and exposed groups at disturbance no 1, 2 etc. is marked * in the graph (above the dots), $p< 0.05$. Replica N=6, except for disturbance no 3 control 24 h (N=5) and disturbance no 3 exposed 96 h (N=5), and every replica contains 10 *C. finmarchicus*. Error bars indicate ± 1 standard error (SE).**

## 3.4 Response latency

### 3.4.1 Relationship between response latency and exposure to crude oil

Mean response latency, as measured from the 1. hydrodynamic disturbance, varied considerably for both controls and exposed groups, but no clear correlation between latency, exposure and time was found (figure 10). The only significant difference between control and exposed groups was recorded after 72 hours exposure for weathered crude oil.

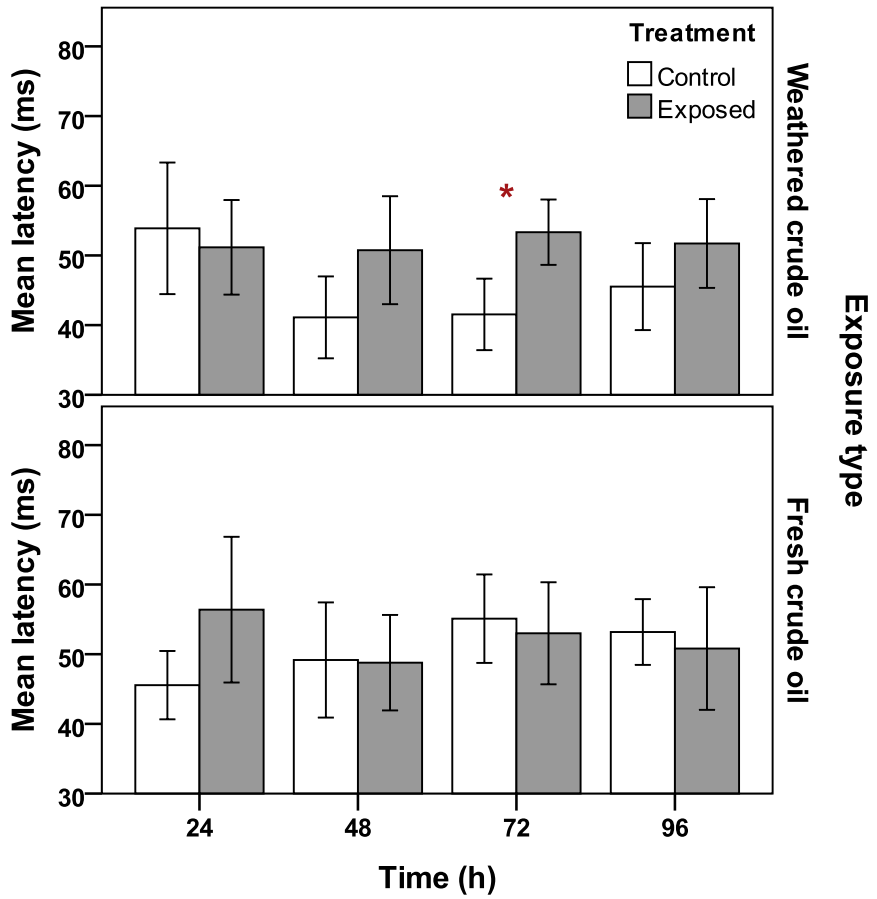



**Figure 10. Mean response latency (ms) for the 1. hydrodynamic disturbance with time (h) for control and exposed replicates. Significant differences between control and exposed groups are marked * in the graph (above the bars), p< 0.05. N=6, and every replica contains 3-10 measurements. Error bars indicate 95% confidence interval.**

### 3.4.2 Fatiguing effects by repetitive hydrodynamic disturbance on response latency

Generally there was not found a significant correlation between response latency and the order of the disturbance. However, significant differences between latency of exposed *C. finmarchicus* and controls at some points within the hydrodynamic disturbance series were revealed for the 24 and 72 hours weathered crude oil exposure (figure 11), and for the 24 and 96 hours fresh crude oil exposure (figure 12 ). For the 72 hours weathered crude oil exposure there was also a significant difference within the hydrodynamic disturbance series of both the control and the exposed groups. Only two significantly differences were found between latency of controls and *C. finmarchicus* exposed to fresh crude oil, at the 24 and 96 hours exposure series, respectively.

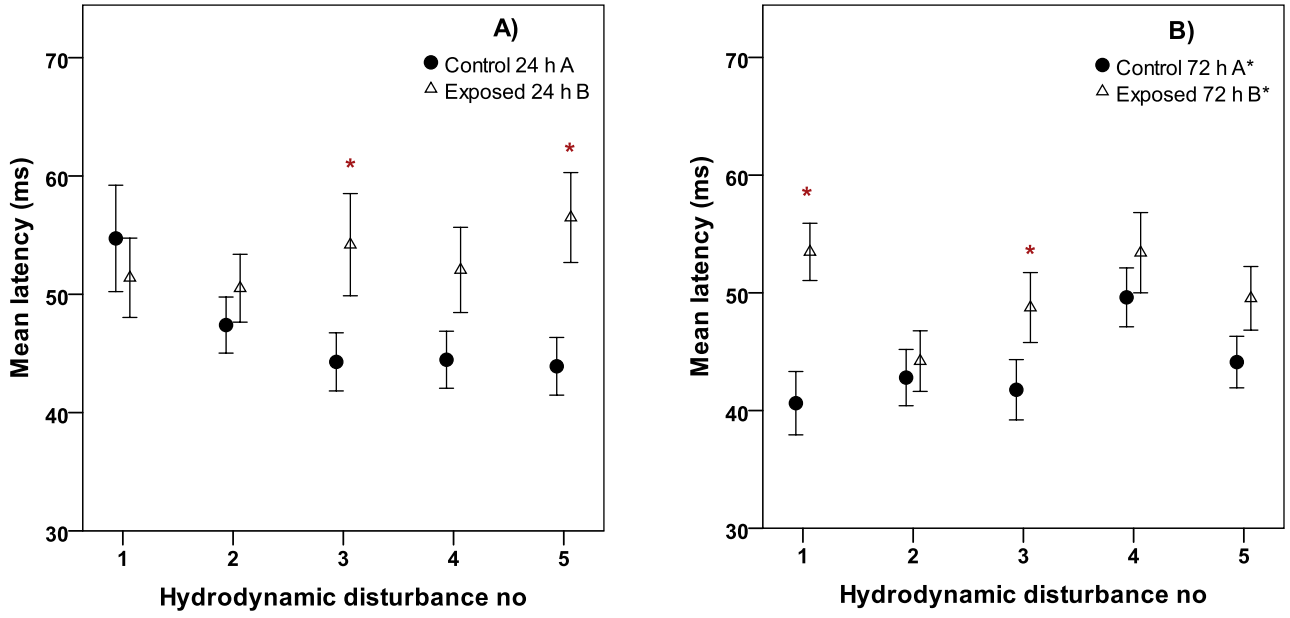
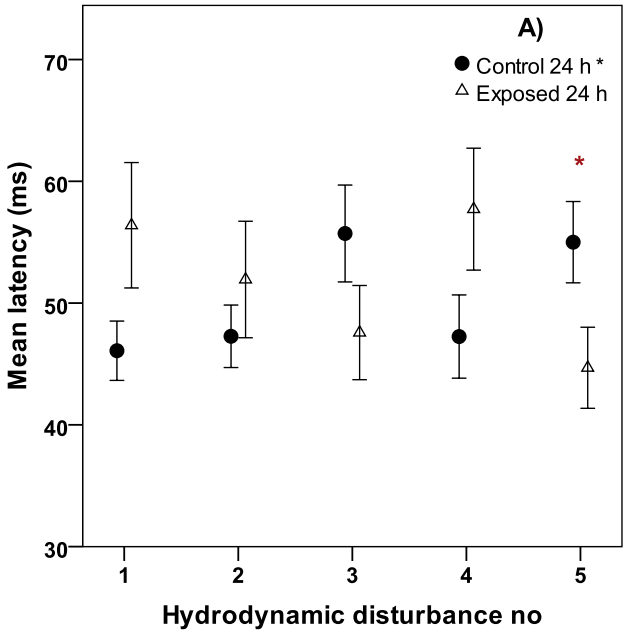



**Figure 11. Relationship between mean response latencies (ms) and the order of a hydrodynamic disturbance for controls and exposures of weathered crude oil for: A) 24 h and B) 72 h. Significant difference between controls and corresponding exposed groups are marked with different letters (A or B) after the appurtenant legend, further significant differences within a disturbance series are marked * after the letter or appurtenant legend, p < 0.05. Significant differences between control and exposed groups at disturbance no 1, 2 etc. are marked * in the graph (above the dots), p< 0.05. Replica N=6 , and every replica has 3-10 measurements.**



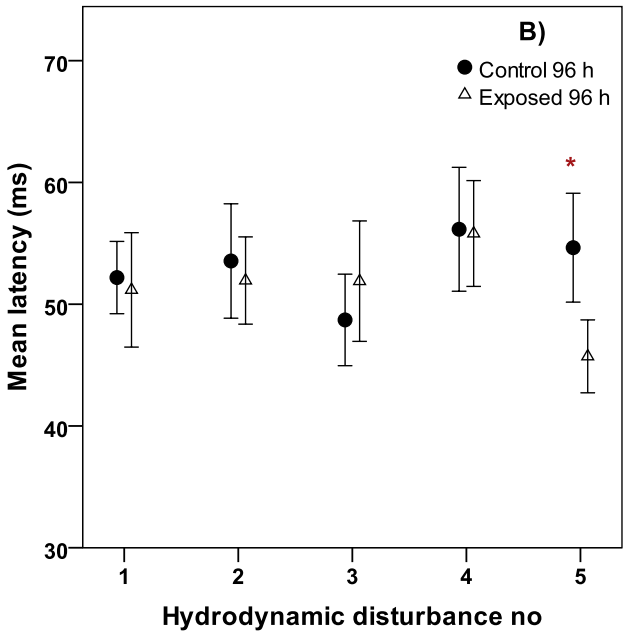




**Figure 12. Relationship between mean response latencies (ms) and the order of a hydrodynamic disturbance, for control and exposure of fresh crude oil for 96 h. No significant differences were found between control and exposed groups, the full disturbance series taken together. Significant differences within a disturbance series are marked * after the appurtenant legend, significant difference between control and exposed groups at disturbance no 1, 2 etc. are marked * in the graph (above the dots), p< 0.05. Replica N=6, except for disturbance no 3 control 24 h (N=5) and disturbance no 3 exposed 96 h (N=5) and every replica has 3-10 measurements.**

Figure 13 and 14 show selected examples of the frequency distribution of response latencies in the cases where differences between control and exposed groups were found. The control individuals have shorter and a less spread response latencies compared to the individuals exposed to weathered crude oil (figure 13). On the contrary, the few significant differences between controls and groups exposed to fresh crude oil, show a decrease in the response latency (figure 14).

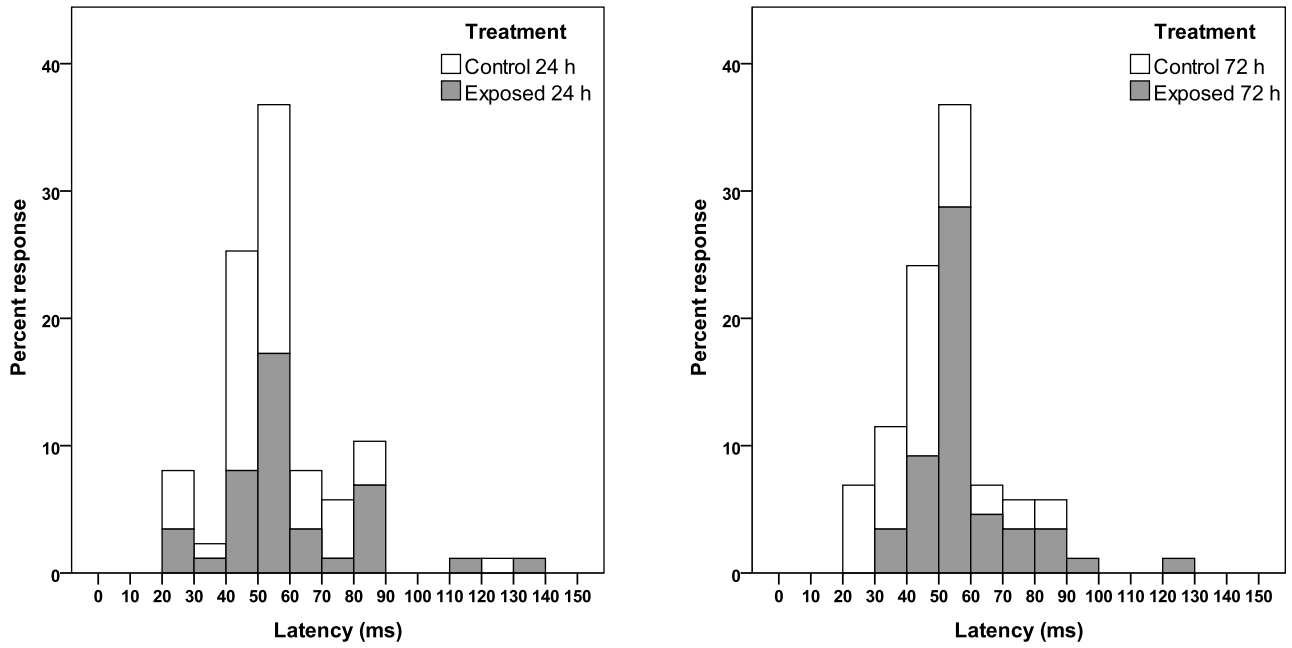
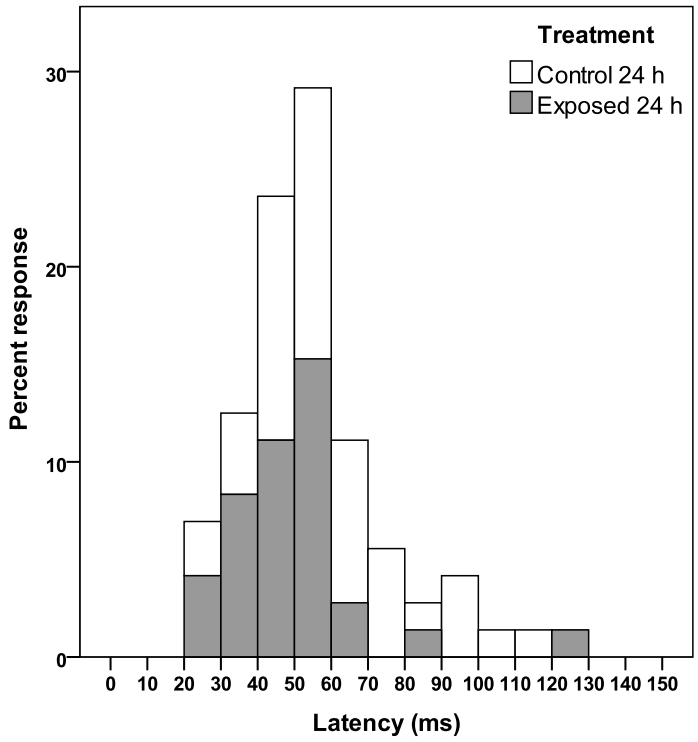



**Figure 13. Frequency distribution of response latencies for *C. finmarchicus.* Exposure type is weathered crude oil, and the histogram presents a point in the hydrodynamic disturbance series where control and exposed groups are significantly different, p< 0.05. Replica N=6, and every replica contains 3-10 measurements. Bin width is 10 ms.**



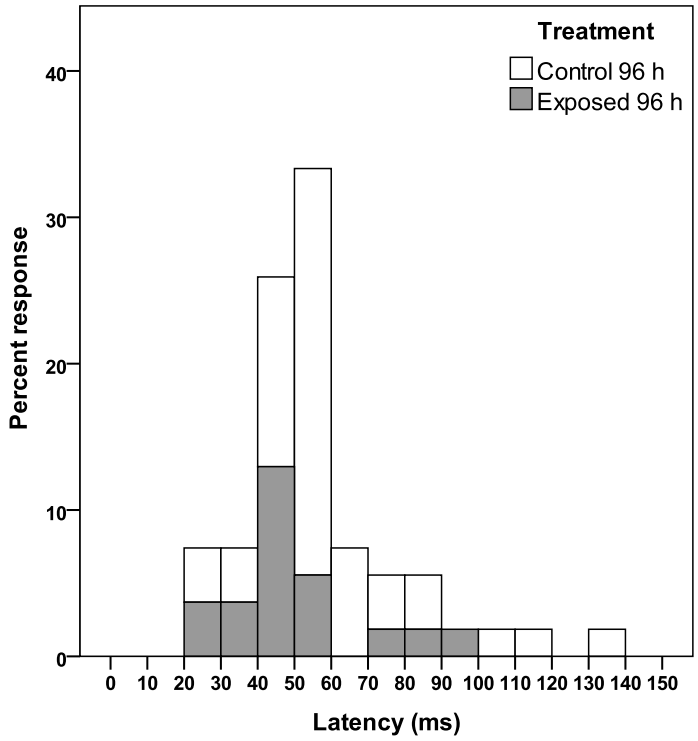




**Figure 14. Frequency distribution of response latencies for *C. finmarchicus.* Exposure type is fresh crude oil, and the histogram presents a point in the hydrodynamic disturbance series where control and exposed groups are significantly different, p< 0.05. Replica N=6, and every replica contains 3-10 measurements. Bin width is 10 ms.**

# 4 Discussion

Several previous studies have investigated the kinetics, length, latency time and threshold in regards to escape behaviour in copepods (Kiorboe and Visser, 1999; Lenz and Hartline, 1999; Waggett and Buskey, 2007). To elicit an escape response in laboratory experiments several mechanisms have been used, the most common ones have been to create a hydrodynamic disturbance by vibration or a siphon flow. However, alteration in copepod escape behaviour in response to chemical exposure has not earlier been examined. Because of this, information to support or contradict findings in this study is not always available. Further, response latency times are available for several copepod species, making it easy to compare species specific latencies between studies. There are several reasons to not directly compare result from the same species when it comes to response latency in copepods, mainly because the studies have different hydrodynamic setups. Different hydrodynamic signals will create their own specific fluid disturbance, and since copepods have numerous types of sensors different signals may be perceived by different sensory systems, which may result in differences in latency and thresholds (Fields and Yen, 1997; Kiorboe et al., 2010). Also, it has been found big differences in response latencies between copepod species (Waggett and Buskey, 2008), and between different sizes of the copepods of the same species (Kiorboe et al., 1999). Further, it has been reported that the calanoid copepod *Centropages hamatus* can habituate to different levels of fluid disturbance, and thereby altering its threshold for an escape response, indicating that there will be variation between species, and between individuals from the same species (Fields and Yen, 1997).

## 4.1 Relationship between exposure and frequency of escape reactions in response to hydrodynamic disturbance

In the present study the frequency of escape reactions was clearly affected by exposure to crude oil, regardless of oil condition (fresh or weathered). Decreasing escape response frequency after 24, 48 and 72 hours exposure to weathered crude oil was found. On the contrary no significant differences in escape response frequencies were found between exposed and control groups after 96 hours exposure. An explanation for the lack of significant differences at 96 hours exposure time can be a temporarily compensatory

response in *C. finmarchicus* triggered by the oil exposure. It is well known that exposure to certain oil-related chemicals may lead to stress that alters physical parameters, and this is often followed by compensatory biochemical and physiological responses that can prevent further negative effects on health status(Zachariassen et al., 1991). With higher pollutant concentration or longer exposure time for a pollutant there will be a point where the organism is no longer is able to compensate for the stress (Walker et al., 2006; Zachariassen et al., 1991). For more long-term exposure to sub-lethal concentrations of weathered crude oil compensatory mechanisms in *C. finmarchicus* may be displaced beyond tolerated limits, which then could lead to further alteration of escape response. However, the association could also merely reflect that control replicates had suffered a decrease in escape response ability with time. Kiorboe et al. (2010) suggested that for escaping animals, natural selection may be for speed and distance rather than energy efficiency during escape. However, as the energetic cost of an escape response in a copepod can consume up to 400 times the normal energetic expenditure (Fields and Yen, 1997) this cost may be too high for starved copepods with reduced vitality. Also, unnecessary escape reactions have been shown to attract predators (Fields and Yen, 1997), and when low on energy non-responsiveness may be an advantage. Exposed and non-exposed *C. finmarchicus* endured the same conditions, and it would be expected that exposure would be an additional stress to starvation, and that this would result in significant differences between exposed and non-exposed groups. Little is known about the mechanisms behind oil exposure induced narcosis in *C. finmarchicus* so the reason for the lack of data supporting this theory is unclear. A possible explanation could be that compensatory mechanisms in *C. finmarchicus* are strong enough to keep the escape response at a level that is lower than under optimal conditions, but at the same level as non-vital control animals. Most likely the lack of a significant difference between control and exposed groups at a 96 hours exposure, can be explained by both reduction of vitality for control animals and a compensatory response in exposed *C. finmarchicus*.

All groups exposed to fresh crude oil demonstrated reduced escape response frequencies compared to the concurrent controls. The significant difference between control and exposed groups at 96 hours exposure, which were not present for weathered crude oil exposure, may indicate that components in fresh crude oil affects the escape response more than the decrease in vitality due to starvation. Fresh crude oil are considered more toxic

than weathered crude oil because weathering removes some of the lower-log ($K_{OW}$) chemicals with greater toxic potential, like BTEXs, and thereby leaving the higher log ($K_{OW}$)chemicals with lower toxic potential (Di Toro et al., 2007). From the chemical analysis in this study it is evident that the WAFs based on fresh crude oil have a higher content of chemicals considered to have significant toxic potential then the WAFs based on weathered crude oil, and this can explain why the compensatory response discussed earlier was less pronounced in this exposure series.

Previous studies have reported that some narcotic chemicals lower the minimum stimulus required to generate an action potential (Van Wezel and Opperhuizen, 1995). By exposing the axon to these narcotics spontaneous firing of the action potential occurs. A consequence of this may be an adaptation of the neuron or receptor receiving the action potential, leading to non-responsiveness of the receiving neurons. This may explain the decrease in escape response frequency found in this study, and for future studies it would be interesting to assess if non-exposed *C. finmarchicus* responds to lower hydrodynamic stimulus than exposed *C. finmarchicus*.

### 4.1.1 Differences in escape response frequencies for weathered and fresh crude oil controls

When comparing mean escape response frequencies between the control series from the weathered crude oil exposure series to the fresh crude oil, it is evident that the mean response is higher in controls for weathered crude oil. The reason for this is not clear. However, parameters like nutritional status and how far into the CV stage the copepods have reached may be contributing factors.

The *C. finmarchicus* individuals applied for the weathered crude oil exposure series were collected from sub-cultures where the copepods had recently reached the CV stage, and it is therefore likely that most copepods remained as CV throughout the exposure series. The fresh crude oil exposure series were performed after the weathered crude oil exposure series, and because the copepods were taken from the same sub-cultures as before, they had now come further in the stage CV development. This meant that it was a higher

possibility for copepods to moult to the adult stage before the exposure series were ended. Copepod excuvia were found in the exposure bottles when transferring *C. finmarchicus* from the exposure bottles to the experimental aquarium, which proves that at least some of the copepodids had moulted. However, this could not be quantified, so it is uncertain whether there were significant more adult *C. finmarchicus* in the fresh crude oil exposure series than the weathered one. Also, no information is available on whether adult *C. finmarchicus* respond more or less to a hydrodynamic disturbance than CV copepodids.

Nutritional status and lipid content in *C. finmarchicus* may also be factors that affect escape response frequency, where lipid-poor copepods could be more affected by starvation and stress, resulting in a reduced ability to respond to a hydrodynamic disturbance. The opposite is also possible, because lipid-rich copepods would have a reduced need to graze in the surface water layer, and thereby reducing their predation pressure and responsiveness. As most of the *C. finmarchicus* that hibernates during winter belong to copepodid stage CIV or CV (Hirche, 1983), and the lipid storage is important for energy utilization, egg production and possibly to achieve neutrally buoyancy (Visser and Jonasdottir, 1999), the lipid storage will generally increase during the CV stage. However, the lipid storage may also be dependent of the density of *C. finmarchicus* in the culture. Since the lipid content of each *C. finmarchicus* not was quantified in this study, it is not known if the lipid content were higher in animals applied in the fresh crude oil exposure series than the weathered, but it is expected because they have had the possibility to graze for a longer period.

### 4.1.2 Fatiguing effects of a repetitive hydrodynamic disturbance

In the majority of the hydrodynamic repeating disturbance series the mean of the first disturbance is higher than the last disturbance, nevertheless this is possibly not consistent enough to claim that there is a general declining trend in escape response frequencies. Further, these findings are less pronounced in exposed groups, and it may seem as exposed *C. finmarchicus* fatigues or responds differently to repetitive hydrodynamic disturbance than non-exposed *C. finmarchicus*.

It is possible that a time interval of 10 minutes between each disturbance is long enough for the copepods to recover more or less completely and hence masking any fatiguing effect, especially when the disturbance is only repeated 5 times. Waggett and Buskey (2008)

recorded escape reactions in response to hydrodynamic disturbance in calanoid copepods from nine families: Metridinidae, Acartiidae, Centropagidae, Pontellidae, Temoridae, Calanidae , Paracalanidae, Eucalanidae and Euchaetidae, none of the species were *C. finmarchicus*. The copepods were exposed to the hydrodynamic disturbance 3 to 10 times, and were only allowed a 2 minute time interval between each disturbance. They reported that the copepods showed no habituation to the disturbances.

## 4.2 Response latency

The latency period (from stimulus to response) was measured over a time period 0 – 3000 ms. However, the majority of escape reactions (~90%) occurred within 150 ms, followed by a few similar reactions randomly spread throughout the remaining time interval. Because it is unlikely that the primary escape response in copepods may have such a broad response range (Waggett and Buskey, 2008), we interpreted the delayed reactions as secondary or random responses. These values were therefore excluded from the dataset.

Even with extreme values excluded from the dataset escape response latencies were highly variable among *C. finmarchicus* in this study, from 10 to 150 ms, with a median of 40 to 50 ms. One reason for this may be the duration and structure of the hydrodynamic disturbance signal. The signal lasted for about 60 ms, and although the nominal frequency was 50 Hz, the actual frequency as measured on the aquarium proved to be much higher, in the range of 300 Hz. Because the excitation level for escape behaviour will probably vary, the escape reaction of some individuals may have been triggered already at the start of the signal, while the reaction in others has been initiated later. It is also possible that apparently long escape latencies can in fact be secondary responses by individuals responding to the disturbance of the others copepod escaping, not the artificially hydrodynamic disturbance itself.

A previous study by Waggett and Buskey (2008), which looked at escape reaction performance of myelinated and non-myelinated calanoid copepods, triggered escape reactions with a hydrodynamic disturbance that resembles the one used in this study. One of the species was *Temora turbinate,* and the study showed that the species, in accordance with *C. finmarchicus*, had a wide range of escape latencies. They discussed the possibility of the hydrodynamic disturbance activating multiple neural pathways for initiating an escape response. This may in fact be a possibility because copepods have numerous sensors and

types of sensors, which all are integrated on some level prior to eliciting a behaviour response (Fields and Yen, 1997). Also, it has not always been clear which part of the fluid disturbance a copepod responds to (Kiorboe and Visser, 1999).

Another species from the same study by Waggett and Buskey (2008), *Pleuromamma xiphias* had relatively long response latencies compared to the other species in the study. The authors concluded that the species habitat and migration patterns may explain the observed variations. *P. xiphias* is associated with open sea environments and has diel vertical migration patterns, just as *C. finmarchicus*. As diel vertical migration in associated with avoidance of surface-dwelling predators (Lenz and Hartline, 1999), shortened and consistent response latencies may not be that vital as it is for copepods that do not have this migration patterns. This theory is further supported by Fields and Yen (1997), which stated that in situation were copepods suffer a high risk of predation, because of the lack of migration ability, evolutionary selection clearly favours successful escapes over conserving the energy with an unnecessary escape.

### 4.2.1 Relationship between exposure and escape latencies

Although some significant differences in escape response latencies were found between control and exposed groups at some points for both types of oil exposure, we suggest there is not enough evidence to state that exposure to fresh or weathered crude oil WAF at the applied concentrations affects escape latencies in *C. finmarchicus*. The general trend in this study seems to be that the effect of exposure is greater on the fraction of *C. finmarchicus* responding than on latency response time.

The two significant differences between control and exposed groups found, after exposure to fresh crude oil, indicated a higher response latency time for the non-exposed *C. finmarchicus*. As most of the results in this study and other studies contradict this, a plausible explanation may be that these findings are a result of coincidence or statistical flaws.

The lack of demonstrated effect of crude oil exposure on response latencies contradicts the hypothesis that oil exposure will increase response latencies because of its proven narcotic effects (Van Wezel and Opperhuizen, 1995). Since the mechanisms behind oil exposure induced narcosis and the neurophysiology in *C. finmarchicus* is unclear the reasons for this is

not known. However, the lack of findings supporting that oil exposure increase latencies may indicate that evolutionary selection have favoured escape behaviour in *C. finmarchicus*, and a factor defining successful escape from a predator is latency.

## 4.3 Cultured copepods compared to wild living copepods

Previous studies have shown that it is possible that wild living copepods are more habituated to a certain level of hydrodynamic noise, and will therefore need a stronger hydrodynamic disturbance to respond than cultured copepods (Fields and Yen, 1997; Kiorboe and Visser, 1999; Waggett and Buskey, 2007). The culturing process may also have selected for copepods that are more sensitive to a hydrodynamic disturbance.

## 4.4 Quality of data

The sample sizes in the study were generally restricted. For each exposure time and exposure type (oil quality or control) the sample contained 6 replicates only. However, as the experiment was time consuming and required a great number of CV *C. finmarchicus*, it was not practically feasible to expand the experiment further. Nevertheless, despite off the small sample sizes, significant differences between control and exposed *C. finmarchicus* in escape response frequencies were demonstrated.

All *C. finmarchicus* included in this experiment were of stage CV when sampled. However, it was possible that moulting to adult stage could take place during an exposure series. It was not feasible to determine the stage of the responding *C. finmarchicus* from the video material, so the importance of this parameter in is not known in the present study. Further, the general health condition is unknown, and injuries e.g. due to handling may have affected the escape response behaviour to some extent. Also, since narcosis-inducing chemicals are believed to exert their effects in all fatty tissues, but do most harm to the lipid bilayer in cell membranes (Van Wezel and Opperhuizen, 1995), copepods with a large lipid storage are thought to have an advantage. Larger lipid storage may “dilute” uptaken narcosis-inducing chemicals to lower concentrations, and hence reduce possible effects on cell membranes.

### 4.4.1 Chemical content in WAFs after 96 hours exposure

In an open static exposure system there are expected some loss of volatile chemicals due to evaporation, this is also evident from the chemical analysis of the WAF based on fresh crude oil after 96 hours exposure (Appendix A). There is also a possibility that some oil residues will stick to the inside of the experimental aquarium. Nevertheless, in this study the chemical content of the samples taken before the escape behaviour testing began are the most relevant. This is based on that the exposure system was closed during the applied exposure time, up until escape behaviour testing began. Also, since no trend of increasing escape response frequency with time spent in the experimental aquarium was found, the time spent in the aquarium was most likely too short for the copepods to experience any recovery from loss of volatile chemicals in the exposure media.

## 4.5 Evaluation of the method

The main aim of this study was to assess behaviour alterations in *C. finmarchicus* due to oil exposure induced narcosis. Based on previous studies the hypothesis was that exposure with the concentrations applied in this study would decrease escape response frequency and increase escape response latency time. Findings in this study indicate that exposure to the selected sub-lethal WAF concentrations do indeed affect escape response frequencies, however our hypothesis of increasing latencies was not confirmed. These findings indicate that the exposures applied in this study affect escape response frequencies more than latencies, which suggests that the method can be modified before further use.

However, the demonstrated effects on escape response frequencies indicate that a simplified version of the method may be developed further, for future uses, e.g. within ecotoxicity testing. The most time consuming part of the present methodology was the executing of the experiment followed by analysing all the video material. So if video analyses for latency times could be omitted, this would save time. Further it seems that the time intervals between a repetitive hydrodynamic disturbance were either too long, too few, or both to see a fatiguing effect. Increasing the number of disturbances would not necessary increase the time used on each exposure series, but it would increase the time used for analysing and handling the video material considerably.

An even more simplified and less time-consuming version of the method would then be to just measure escape response one time for each replicate. This would also mean that the person executing the experiment could spend less time in darkness.

The advantage of a toxicity tests that use behaviour as an endpoint compared to acute toxicity testing were death is the endpoint is that these tests reveal effects that often occur at sub-lethal concentrations, but could still possess a serious risk to the success of the population. Each test has their specific advantages, and as the acute toxicity tests are often used in the purpose to determine the concentration of a chemical that causes severe biological effects, a behavioural toxicity test may contribute to a better understanding of the effects of the tested chemical, and give information on effects from sub-lethal concentrations.

# 5 Conclusion

This study showed that sub-lethal concentrations of weathered and crude oil alter escape behaviour in *C. finmarchicus*, at exposure times 24, 48, 72 and 24, 48, 72 and 96, respectively. The most affected parameter is escape response frequencies. Further, this study indicates that escape behaviour may be used as a biomarker for oil exposure induced narcosis. However, all behaviour parameters used in this study need standardization before used as a quality parameter in toxicity testing.

Behaviour as a biomarker in ecotoxicology has been illuminated through several studies, and is slowly gaining more attention. As behaviour is considered a more sensitive biomarker than the ones used in standardized acute toxicity tests (Clotfelter et al., 2004), it is a biomarker worth investigating further. A possible behaviour parameter used in a future experiment with *C. finmarchicus* and oil exposure could be the threshold eliciting an escape response.

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# Appendix A

**Table A. Summary of the chemical composition in the exposure media after the exposure series were ended (~96 h) analyzed with GS-MS, P&T GC-MS and GC-FID. Concentrations are given as (µg/L) based chemical samples from the exposure media generated for each treatment in this experiment. The exposure media concentration were a dilution to 40 % of maximum WAF concentration when based on artificially weathered crude oil, and a dilution of 16 % of maximum WAF concentration when based on fresh crude oil.**

| Compound groups | Weathered crude oil (µg/L) | Fresh crude oil (µg/L) |
|---|---|---|
| **THC (GC-FID)** | 80.44 | 126.51 |
| **∑ VOCs** | 110.87 | 146.76 |
| Benzene | 0.028 | 0.358 |
| Toulene | 0.263 | 1.034 |
| Ethylbenzene | 8.88 | 12.05 |
| Xylenes | 46.19 | 56.63 |
| **∑ SVOCs** (excluded phenols) | 74.51 | 149.60 |
| ∑ Naphthalenes | 72.94 | 146.25 |
| ∑ 2-3 ring PAH* | 1.41 | 3.113 |
| ∑ 4-6 ring PAH** | 0.021 | 0.038 |
| ∑ C0-C5 phenols | 0.729 | 0.638 |
| ∑ Decalins | 0.125 | 0.203 |

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

# Appendix B

Mean escape response frequencies.

| Treatment | Exposure time (h) | Mean | Standard deviation | Total N |
|---|---|---|---|---|
| Control 1 | 24 | 8.1 | 1.44 | 15 |
| | 48 | 8.1 | 1.06 | 15 |
| | 72 | 8.2 | 0.94 | 15 |
| | 96 | 7.3 | 1.03 | 15 |
| Weathered crude oil 1 | 24 | 6.0 | 1.93 | 15 |
| | 48 | 7.4 | 1.24 | 15 |
| | 72 | 6.9 | 1.46 | 15 |
| | 96 | 5.9 | 1.28 | 15 |

| Treatment | Exposure time (h) | Mean | Standard deviation | Total N |
|---|---|---|---|---|
| Control 2 | 24 | 8.4 | 1.25 | 15 |
| | 48 | 8.4 | 0.87 | 13 |
| | 72 | 8.7 | 1.16 | 15 |
| | 96 | 7.0 | 1.30 | 14 |
| Weathered crude oil 2 | 24 | 6.9 | 0.99 | 15 |
| | 48 | 7.5 | 0.99 | 15 |
| | 72 | 8.6 | 1.18 | 15 |
| | 96 | 7.7 | 1.28 | 15 |

| Treatment | Exposure time (h) | Mean | Standard deviation | Total N |
|---|---|---|---|---|
| Control 1 | 24 | 6.6 | 1.99 | 14 |
| | 48 | 6.9 | 1.49 | 15 |
| | 72 | 8.2 | 1.16 | 15 |
| | 96 | 7.6 | 1.18 | 15 |
| Fresh crude oil 1 | 24 | 5.9 | 1.30 | 15 |
| | 48 | 6,5 | 1.41 | 15 |
| | 72 | 8.5 | 1.25 | 15 |
| | 96 | 6.4 | 0.94 | 14 |

| Treatment | Exposure time (h) | Mean | Standard deviation | Total N |
|---|---|---|---|---|
| Control 1 | 24 | 7.6 | 0.99 | 15 |
| | 48 | 7.71 | 1.27 | 14 |
| | 72 | 8.7 | 1.16 | 15 |
| | 96 | 6.7 | 0.9 | 15 |
| Fresh crude oil 1 | 24 | 5.4 | 0.99 | 15 |
| | 48 | 6.5 | 0.94 | 15 |
| | 72 | 8.45 | 1.25 | 15 |
| | 96 | 5.7 | 0.82 | 15 |

# Appendix C

Mean latency time for the first hydrodynamic disturbance in a series.

| Treatment | Exposure time (h) | Mean | Standard deviation | Week |
|---|---|---|---|---|
| Control | 24 | 48.8 | 21.76 | 1 |
| | 48 | 42.5 | 19.16 | 1 |
| | 72 | 41.0 | 16.16 | 1 |
| | 96 | 43.1 | 14.37 | 1 |
| Weathered crude oil | 24 | 55.2 | 25.02 | 1 |
| | 48 | 55.5 | 30.35 | 1 |
| | 72 | 56.1 | 18.52 | 1 |
| | 96 | 51.4 | 13.51 | 1 |

| Treatment | Exposure time (h) | Mean | Standard deviation | Week |
|---|---|---|---|---|
| Control | 24 | 58.4 | 32.36 | 2 |
| | 48 | 39.4 | 15.26 | 2 |
| | 72 | 41.8 | 15.93 | 2 |
| | 96 | 48.6 | 21.16 | 2 |
| Weathered crude oil | 24 | 46.5 | 17.56 | 2 |
| | 48 | 46.0 | 15.36 | 2 |
| | 72 | 50.8 | 13.52 | 2 |
| | 96 | 51.9 | 21.60 | 2 |

| Treatment | Exposure time (h) | Mean | Standard deviation | Week |
|---|---|---|---|---|
| Control | 24 | 42.9 | 15.21 | 3 |
| | 48 | 48.6 | 27.48 | 3 |
| | 72 | 55.1 | 18.81 | 3 |
| | 96 | 52.8 | 18.15 | 3 |
| Fresh crude oil | 24 | 63.7 | 35.62 | 3 |
| | 48 | 51.5 | 24.77 | 3 |
| | 72 | 47.1 | 20.24 | 3 |
| | 96 | 46.2 | 26.39 | 3 |

| Treatment | Exposure time (h) | Mean | Standard deviation | Week |
|---|---|---|---|---|
| Control | 24 | 47.9 | 17.19 | 4 |
| | 48 | 50.0 | 19.61 | 4 |
| | 72 | 58.2 | 25.43 | 4 |
| | 96 | 53.7 | 11.65 | 4 |
| Fresh crude oil | 24 | 48.2 | 22.98 | 4 |
| | 48 | 46.2 | 18.57 | 4 |
| | 72 | 57.4 | 24,16 | 4 |
| | 96 | 56.9 | 28.22 | 4 |