Acknowledgments

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Abstract

The oil exploration and search for new oil production fields is expanding further north and has reached the Arctic. Oil drilling activities release large amounts of produced water (PW) to the marine environment and sub-lethal effects on biota cannot be excluded. The polycyclic aromatic hydrocarbons (PAHs) present in PW have previously been shown to exhibit negative effects on growth, development and survival of aquatic organisms. The Arctic copepod *Calanus glacialis* is an abundant zooplankton species and holds a key position in the energy transfer from primary production to higher trophic levels. *C. glacialis* accumulates large volumes of lipids during late developmental stages which makes it prone to the uptake of lipophilic oil components. This study assesses the potential impact of PW-related PAHs on the metabolism of *C. glacialis*. In a semi-static setup, stage V *C. glacialis* copepodites from Kongsfjorden, Svalbard were exposed to the water soluble fraction of 11 selected PAHs (ΣPAH 7,90 µg L^{-1}). The copepods were sampled at equal intervals during the exposure (120 h) and the following recovery (120 h). Oxygen consumption was measured in temperature-controlled glass chambers containing three *C. glacialis* each, using a fiber-optic oxygen meter. Dry weight, carbon and nitrogen content of the specimens were determined and body and lipid sac volumes were calculated based on biometrical measurements obtained from pictures of the *C. glacialis* individuals. Additionally, the PAH concentrations in the exposure media and the body burden in copepods were analyzed, in order to validate the exposure and assess the uptake and depuration of the components. No effects on *C. glacialis* respiration rate or body mass after PAH exposure were observed in the study. Findings revealed large variations in the measured endpoints between the specimens. The body size variations between individuals in the samples might be a consequence of depth-related difference in body size, since the copepods were collected with vertical hauls using an open net, and hence were sampled from the entire water column. Several of the PAHs showed modest or no decrease in body concentration after 5 days of recovery. The slow depuration of PAHs in *C. glacialis* confirmed in this experiment indicates that especially components with a high log K_{ow} (octanol-water coefficient) may be more available for transfer to progeny, and for chronic exposure to species of higher trophic levels. These findings warrant for exposure studies with longer recovery periods to examine *C. glacialis* depuration rates of heavy PW components and for studies including the potential for trophic transfer. Further, this study expands the limited knowledge on possible effects on lipid-rich Arctic copepods resulting from exposure to PW components.

Sammendrag

Oljeindustrien flytter seg nærmere Arktis. Produsert vann (PV) er et biprodukt fra oljeproduksjonen som slippes ut i havet i store mengder og skadelige effekter på akvatiske organismer kan ikke utelukkes. PV inneholder polysykliske aromatiske hydrokarboner (PAHer) som i tidligere studier har vist negative effekter på vekst, utvikling og overlevelser hos flere marine arter. Den arktiske kopepoden (hoppekreps) *Calanus glacialis* har en nøkkelrolle når det gjelder energioverføring fra primærproduksjon til høyre trofiske nivåer i sitt område. Under de siste utviklingsstadiene akkumulerer *C. glacialis* store mengder lipider hvilket gjør den utsatt for opptak av lipofile oljekomponenter. I denne studien ble det undersøkt om PV-relaterte PAHer kan ha en effekt på metabolismen til *C. glacialis*. I et semi-statisk oppsett ble *C. glacialis* i copepodittstadium V, fra Kongsfjorden på Svalbard, eksponert for den vannløselige fraksjonen av 11 utvalgte PAH (ΣPAH 7,90 µg L^{-1}). Kopepoder ble tatt ut for analyse med jevne mellomrom under eksponering (120 h) og påfølgende utskillingsfase (120 h). Oksygenopptaket ble målt med en fiberoptisk oksygenmåler i små temperaturregulerte glassbeholdere som hver inneholdt tre *C. glacialis* individer. Tørrvekt, karbon- og nitrogeninnhold i hvert individ ble også analysert. Volumet av kroppen og lipidsekken ble beregnet utfra biometrimålinger gjort på bilder tatt av enkeltindivider. I tillegg ble PAH-konsentrasjonen i eksponeringsløsningen og i kroppen til kopepodene analysert, for å validere eksponering og vurdere opptak og utskilling av komponentene. Det ble ikke observert noen effekt på oksygenopptak eller kroppsmasse til *C. glacialis* som følge av PAH eksponering i dette eksperiment. Derimot avdekket dataene store variasjoner i de målte endepunktene mellom individene som gjorde resultatene delvis vanskelige å tyde. Store variasjoner i kroppsstørrelse mellom enkeltindivider i materialet kan muligens være en konsekvens av at *C. glacialis* på forskjellige dybder i fjorden hadde ulik størrelse. Håvtrekkene ble utført som vertikaltrekk fra bunn til overflaten og samlet derfor kopepoder fra hele vannsøylen. PAHer med høy log K$_{ow}$ (oktanol-vann koeffisient) viste moderat eller ingen reduksjon i kroppskonsentrasjonen til kopepodene hele fem dager etter avsluttet eksponering. Langsom utskilling øker risikoen for overføring av de kjemiske komponentene til neste generasjon. I tillegg kan langsom utskilling av oljekomponenter i zooplankton føre til kronisk eksponering av arter på høyere trofisk nivå. Utskillingskinetikken hos kopepoder er ikke godt studert, og trenger mer forskning. Resultatene i denne studien utvider et begrenset kunnskapsområde om mulige effekter på fettrike arktiske kopepoder som følge av eksponering for PV komponenter.

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Abbreviations

ANOVA   Analysis of Variance

BCF   Bioconcentration factor

C   Carbon

CTD   Conductivity, temperature and density

C I-VI   Copepodite stages I-VI

CYP   Cytochrome P450

DW   Dry weight

GC-MS   Chromatography-mass spectrometry

GSH   Glutathione

GST   Glutathione S-transferase

N   Nitrogen

PAH   Polycyclic aromatic hydrocarbons

PEC   Predicted environmental concentration

PNEC   Predicted no effect concentration

PW   Produced water

ROS   Reactive oxygen species

SD   Standard deviation

SPE   Solid-phase extraction

V_B Body volume

V_L Lipid sac volume

V_R Respiration volume

WSF   Water soluble fraction

1 Introduction

Receding sea ice and an increasing global demand for energy have turned the search for new oil fields on the Norwegian continental shelf further north towards the Arctic. While the worlds existing oil reservoirs are being depleted, the Arctic region (north of the Arctic Circle) is suggested to contain large petroleum hydrocarbon resources (AMAP, 2007). Exploration activity in the Barents Sea has been high in the last years, and several new findings of oil and gas have been made. As an example the Goliat field in the Barents Sea started producing oil in 2015 (The Norwegian Oil and Gas Association, 2015).

An introduction of oil production operations are expected to increase the emission of petroleum compounds in Arctic waters. During oil drilling activity, produced water (PW) is generated and released into the marine environment in large quantities. Produced water contains oil components (hydrocarbons) and sub-lethal effects on biota cannot be excluded (Hylland, 2006; Neff et al., 2011; Peterson et al., 2003).

1.1 Produced water

Produced water (PW) is a byproduct, associated with the oil exploration and production, which accompanies the oil and gas from the reservoir. PW consists of formation water already present in the reservoirs, and water that has been injected into the well to enhance recovery (Neff et al., 2011). When an oil field ages, a larger amount of water has to be injected into the well to maintain reservoir pressure. Hence, the quantity of PW increases over the operation period of the production unit (The Norwegian Oil and Gas Association, 2016).

In addition to water, the PW also holds oil residuals from the reservoir and production chemicals. Therefore, PW is a complex mixture of chemicals which composition differs considerably depending on the region and the age of the oil field (Neff et al., 2011). Typically it contains various organic compounds (mainly hydrocarbons) and inorganic substances including ions, metals and radioisotopes. Petroleum hydrocarbons in PW are of primary environmental concern, because of their toxicity and ability to accumulate in aquatic organisms. These compounds will appear both as dispersed oil droplets and dissolved in the water. The solubility of the hydrocarbons in water increases with decreased molecular weight (Hylland, 2006; Neff et al., 2011).

In order to reduce the environmental impact of PW there are technologies implemented in the production process to separate the oil from the water (Faksness et al., 2004). The treatments are generally only efficient in removing particulate oil (oil droplets) but not the dissolved hydrocarbons (Neff et al., 2011). However, even the treated PW will still hold some dispersed oil (droplet size ranging from 1-10 µm) (Johnsen et al., 2004). In Norway, legislation practice based on The Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention) places restrictions on the concentration of petroleum (dispersed oil) that is allowed to be present in the PW discharge to the ocean. Since 2007 the average concentration of oil permitted in PW is 30 mg L^{-1} (DNV GL, 2015; Yang, 2011).

Today PW represents the most comprehensive discharges of petroleum hydrocarbons to the sea from the oil and gas production industry (Neff et al., 2011; The Norwegian Oil and Gas Association, 2016). In 2015, 148 million standard cubic meters of PW was discharged into the sea on the Norwegian continental shelf, which is a slight increase compared to the previous year (The Norwegian Oil and Gas Association, 2016).

1.1.1 Polycyclic aromatic hydrocarbons

In a toxicological context the petroleum hydrocarbons of highest concern are the polycyclic aromatic hydrocarbons (PAHs) (Hylland, 2006; Neff et al., 2011). These are lipophilic compounds with the potential to accumulate in biota (Almeda et al., 2013; Norregaard et al., 2015). Several toxic effects, such as carcinogenicity, immunotoxicity and endocrine disruption after exposure to PAH have been reported in a large span of marine organisms (Balk et al., 2011; Hylland, 2006; Neff et al., 2011; Peterson et al., 2003). The common structure for all PAHs are two or more fused aromatic rings. The dissolved fraction of PW consists mainly of 2- and 3-ring PAH. Higher molecular weight (4- through 6-ring) congeners are, due to their low solubility in water, primarily associated with the dispersed oil droplets (Neff et al., 2011). Hence, they are less detectable in properly treated PW where the dispersed oil partly has been removed (Faksness et al., 2004). Normally, the concentrations of total PAH in PW range from 0,040 to 3 mg L^{-1} (Neff et al., 2011).

1.1.2 Environmental risk of produced water discharge

The rapid dilution of discharged PW and its low PAH content makes an acute biological effect on biota unlikely. However, the marine ecosystems are continuously receiving large amounts of PW and chronic exposure may cause sub-lethal effects which can possibly harm populations

and communities by affecting reproduction, growth and development of the exposed organism (Balk et al., 2011; Neff et al., 2011).

The Norwegian Environmental Agency evaluates the condition of the marine ecosystem by requiring regional monitoring of the water column since 1997 around offshore installations (Nilssen and Bakke, 2011). According to results from monitoring, the long-term impact on marine populations and communities is considered low and the effects on individual organisms seems to be limited to an area within a couple of kilometers off the discharges (Bakke et al., 2013; Brooks et al., 2011; Johnsen et al., 2004). However, it has been shown that biomarker response and PAH-metabolites occurs in natural populations of haddock and Atlantic cod in North Sea areas with widespread oil production (Balk et al., 2011). These observations indicate exposure to PAHs, and chronic effects on the ecosystem may first become evident after observing several life stages, generations of keystone species or taking into account other stressors affecting the ocean, such as climate change or fisheries (Neff et al., 2011).

In order to reduce the ecological risk of PW discharges the environmental management in Norway is based on a "zero impact" policy, with the purpose to eliminate all potential harmful emissions. To implement this an Environmental Impact Factor (EIF) was introduced in 2000-2005, as a part of a Dose-related Risk and Effect Assessment Model (DREAM) (Johnsen and Frost, 2011). The EIF (described in Johnsen et al. (2000)) provides a conservative quantitative risk assessment based on a ratio between the predicted environmental concentration (PEC) and the predicted no effect concentration (PNEC) (Johnsen et al., 2000). Due to knowledge gaps about chronic effects on biota, PNEC sometimes has to be calculated from acute toxicity values in combination with a safety factor. Additionally, mainly temperate living species have been used in toxicity tests, and there is less information on the sensitivity of Arctic species. Hence, when the offshore oil and gas activities is expanding further north the risk assessment procedure has to be adjusted to Arctic environments (Olsen et al., 2011).

1.2 Produce water components in the Arctic

The Arctic region consists of several seas with a great biodiversity. The high primary production in the Barents Sea supports large commercial fish stocks, which is the second most important resource in Norway (Olsen et al., 2011; Sakshaug and Kovacs, 2009). At high latitudes, low water temperatures and seasonal fluctuations in primary production have important influence on the associated marine biota. Hence, the Arctic ecosystems and its

organisms have some physiological characteristics that differ from those in temperate areas, and that are likely to influence their susceptibility to xenobiotics (Chapman and Riddle, 2005; Olsen et al., 2007).

One important characteristic is the energy transfer through the Arctic food web, which is lipid based. High lipid content of arctic species is an adaptation to cold temperatures and a large seasonal variation in food availability. In general, lipid-rich organisms are thought to accumulate more lipophilic contaminants, such as PAHs (Chapman and Riddle, 2005; Olsen et al., 2011). However it has also been suggested that the binding of lipophilic toxicants in adipose tissue may act like a buffer, leading to a smaller amount of free PAHs that are able to interfere with cell membranes (Jensen and Carroll, 2010). Nevertheless, during starvation, often associated with the winter period in Arctic areas, the lipid storage is mobilized and the oil components might become available to induce a toxic effect at the target tissue (Chapman and Riddle, 2005). Other traits among Arctic species are slow metabolic rate and the tendency towards gigantism, which causes a lower surface-area-to-volume ratio. These are features usually associated with decreased accumulation rate of contaminants. On the other hand, lower energy usage might lead to slower detoxification and elimination processes (Chapman and Riddle, 2005).

It is not yet clear if the adaptations of Arctic organisms to the harsh conditions of the polar environment make them more vulnerable to contaminants than temperate species. Recent studies on PW toxicity did not find that Arctic species were more vulnerable than related organisms further south (Camus et al., 2015; de Hoop et al., 2011; Hansen et al., 2013; Hansen et al., 2011; Hjorth and Nielsen, 2011). However, it has been suggested that the sensitivity of individual species from standard toxicity tests might not reflect whether the Arctic ecosystem react in the same way as temperate ecosystems. Olsen et al. (2007) observed enhanced respiration rates as a response to oil components in benthic communities from the Barents Sea but not in benthic communities from the temperate Oslo fjord.

There are several other factors in the Arctic environment that will interact and influence on the effect of pollutants on populations, communities and ecosystems (Camus et al., 2015; Olsen et al., 2007). The low water temperature, for instance, prolongs the persistence of hydrocarbons and slows down their evaporation rate. Hence, the biota might experience long-term exposures and, in addition, exposure to a larger fraction of volatile hydrocarbons (AMAP, 2007; Camus et al., 2015; Grenvald et al., 2013). The long development time of many Arctic species entails

they have to spend a longer time in the early life stages, which has been shown to be more susceptible to oil components (Olsen et al., 2011; Short, 2003). Moreover, polar ecosystems typically consist of relatively long and simple food chains and elimination of a single species can cause severe consequences and change the structure or functionality of the food web (Borgå et al., 2004; Chapman and Riddle, 2005; Olsen et al., 2007; Peterson et al., 2003).

1.3 *Calanus glacialis*

The marine calanoid copepod *Calanus glacialis* (Jaschnov, 1955) is an important contributor to the biomass of zooplankton north of the polar front, and possesses a key role in the Arctic pelagic food web (Falk-Petersen et al., 2004; Slagstag and Tande, 1990). Arctic *Calanus* species are predominantly herbivores that can convert low-energy carbohydrates and proteins into high-energy lipids. The lipids, mainly in the form of wax esters, are accumulated into a lipid sac which functions as an energy storage. In *C. glacialis*, the amount of lipids can contribute to 70% of their total dry weight (Scott et al., 2000; Tande and Henderson, 1988). Therefore, they are essential prey for many species. The lipid compounds are transferred in the Arctic food chain, which supply fish stocks, seabirds and marine mammals with energy (Falk-Petersen et al., 2004; Gabrielsen, 2009).

The development of *Calanus* spp. is divided into three distinct periods; the egg stage, the naupliar stages (NI-NVI) and the copepodite stages (CI-CVI). In the last copepodite stage (CVI) the copepod has turned into an adult female or male (Marshall and Orr, 1972). The energy reserves in the lipid sac can be used in diapause to survive long periods without food during winter season (CIV and CV) or to produce eggs (CVI). Therefore, these three copepodite stages are the most lipid-rich (Scott et al., 2000; Tande and Henderson, 1988).

Two other important *Calanus* species that co-existing with *C. glacialis* in the European Arctic are *Calanus hyperboreus* (Krøyer 1838) and *Calanus finmarchicus* (Gunnerus 1765). Although the three species are closely related they possess different life strategies due to their adaptations to different environmental conditions. *C. hyperboreus* is a deep-water species, most abundant in areas such as the Arctic Ocean Basin. *C. hyperboreus* is considerably larger and contains more lipids per individual than the other two (Falk-Petersen et al., 2009; Scott et al., 2000). The *C. finmarchicus* belongs mainly to boreal areas but is similar to *C. glacialis* in morphology (Grainger, 1961). However, *C. glacialis* is a true Arctic-shelf species, well adapted to cold water (temperature optimum: 2 ºC, (Hansen et al., 2013)) and large variations in timing and length of

the annual phytoplankton bloom. Also, *C. glacialis* is bigger in size and contains more lipid reserves than *C. finmarchicus* (Falk-Petersen et al., 2009; Grainger, 1961; Hansen et al., 2013; Scott et al., 2000). Because of the large amount of energy necessary for *C. glacialis* to develop from stage CIV to stage CV it can take up to three years to fulfill its life-cycle (Falk-Petersen et al., 2009).

1.4   Bioconcentration and depuration of PAH

The PAHs in PW are taken up by *Calanus* species by passive diffusion directly from water or through their diet (Jensen and Carroll, 2010; Jensen et al., 2008). The accumulation through non-dietary routes is called bioconcentration. The potential of a compound to increase in concentration in biota can be estimated by the bioconcentration factor (BCF), which is the ratio between the chemical concentration in an organism to that in the surrounding water at equilibrium (steady state) (Nendza and Müller, 2007). Relatively small, nonpolar and moderate lipophilic molecules tend to easily move across biological membranes by passive diffusion. These compounds are characterized by an optimal n-octanol-water partition coefficient (K_{ow}). K_{ow} is usually determined experimentally, by measuring the concentration of the substance in the lipid and aqueous phases after partitioning between the phases in an oil/water system. By convention octanol is used as a lipid proxy (Walker et al., 2006). There is generally a confirmed correlation between the K_{ow} of a compound and its bioconcentration potential (Nendza and Müller, 2007). PAHs found in PW have a log K_{ow} ranging from 3,3 to about 6, and are considered to be highly bioavailable for marine organisms (Hylland, 2006).

The internal body burden of a PAH in marine biota might, mainly due to bioconcentration, reach high enough levels to cause a toxic effect even though its concentration in the ambient water is much lower. (Hylland, 2006; Nendza and Müller, 2007). However, there are generally processes in marine organisms counteracting and sometimes reducing the accumulation of chemicals. These are eliminating processes, such as enzymatic detoxification, excretion and maternal transfer to offspring. In addition dilution by growth may be relevant in lowering the internal body concentration. Hence, the BCF may vary between individuals due to differences in physiological parameters such as lipid content, size, age and sex (Nendza and Müller, 2007).

 Toxicokinetic models can be used to study the rates of uptake and depuration processes. The organism can be divided into compartments represented by different organs or tissues. In ecotoxicology it is common to apply first-order kinetics models where the whole organism is

treated as one compartment. For an aquatic organism continuously exposed to a dissolved organic pollutant, the concentration of the compound in its body will initially increase rapidly until steady state is reach. When the exposure stops the depuration rate of the pollutant, following first-order kinetics, should be proportional to the body burden (Walker et al., 2006).

1.5   PAH toxicity mechanisms in *Calanus* spp.

Calanoid copepods have been used in numerous toxicology studies (e.g. Gardiner et al., 2013; Grenvald et al., 2013; Hansen et al., 2012; Hansen et al., 2011; Hansen et al., 2015; Jager et al., 2016; Jensen et al., 2012; Olsen et al., 2013) and may be considered as an important model species in assessing the effects of oil components in the marine environment. Although decades with offshore oil industry in the North Sea has resulted in a main focus on the boreal *C. finmarchicus*, the knowledge about Arctic copepods has increased in recent years.

One of the basic modes of action of PAH reported in *Calanus* spp. is narcotization. The condition is defined as non-specific disturbance of the cell membrane function caused by lipophilic compounds (Van Wezel and Opperhuizen, 1995). Disturbance of the membrane function may result in diminished activity and a reduced ability to react to stimuli (Barata et al., 2005). Jensen et al. (2008) suggested that reduced grazing rate of *C. finmarchicus* after exposure to pyrene was caused by narcosis and disturbed feeding pattern in *Calanus* spp. following PAH exposure has been confirmed by several other studies (Hjorth and Nielsen, 2011; Jensen and Carroll, 2010; Norregaard et al., 2014).

Another important concern of PW exposure is oxidative stress. The PAH-mediated production of reactive oxygen species (ROS) may injure the organism by oxidizing DNA, proteins or lipids. In many organisms, a cellular defense system might be activated in order to reduce the effects of ROS. This biotransformation system is usually divided in two phases, phase I and phase II, including different enzyme pathways (Walker et al., 2006). The capacity of metabolizing toxic compounds varies widely between species of marine invertebrates. In general, vertebrates possesses a higher activity of phase I enzymes, where the cytochrome P450 (CYP) family are of most importance (Hylland, 2006). Calanoid copepods most likely do have enzymes in the CYP 450 family. However, there have not been any clear evidence that these enzymes take part in a detoxification system (Hansen et al., 2008).

Glutathione S-transferase (GST) belongs to the phase II xenobiotic detoxification enzymes, and has an important role in oxidative stress regulation in vertebrates (Walker et al., 2006). These enzymes facilitate excretion of electrophilic substrates by catalyzing conjugation with the reduced antioxidant glutathione (GSH), and thereby prevent lipid peroxidation. Recent studies indicate that GST has a similar function in copepods (Hansen et al., 2008; Hansen et al., 2009). A normal defense response by exposed organisms is to increase the levels of enzymes, participating in the biotransformation of the contaminant (Walker et al., 2006). Elevated levels of GST in *C. glacialis* and *C. finmarchicus* exposed to single components and mixtures of PAHs have been suggested to indicate a protection against oxidative stress (Hansen et al., 2013; Hansen et al., 2008; Hansen et al., 2009).

1.6 Respiration rate and biochemical changes as biomarkers

Like other living organisms, *C. glacialis* needs energy in order to grow, reproduce and survive. Animals satisfy their energy requirement through the oxidation of energy rich molecules, originally derived from their food, in a series of metabolic reactions called cellular respiration or simply respiration (Schmidt-Nielsen, 1997). When an organism is not feeding or growing its total metabolism can be explained by the energy used for maintenance of body functions (basal metabolism) and activity (movements) (Maps et al., 2013).

The chemical pathways participating in the metabolism are catalyzed by different enzymes at different locations in the cell, however, the overall (unbalanced) formula for respiration is:

$$O_2 + \text{organic nutrient} \rightarrow CO_2 + H_2O + \text{energy} \tag{1}$$
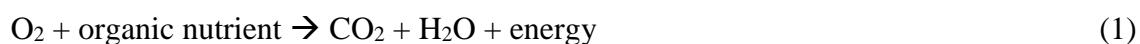
During starvation, lipid-rich copepods, utilize their internal organic nutrients, mainly in the terms of lipids and proteins (Mayzaud, 1976). Since the cellular respiration (equation 1) requires oxygen, the determination of oxygen consumption per unit time (respiration rate) can be used as an estimation of metabolic rate (Schmidt-Nielsen, 1997).

Contaminants can change biochemical processes in exposed organisms and thereby change their energy requirement (Walker et al., 2006). The responses to PAH exposure, described in previous section (1.5) may alter the metabolism of the animals. For example exposure to a water soluble fraction (WSF) of oil has been shown to increase the respiration rate of the arctic amphipod *Gammarus wilkitzkii* (Hatlen et al., 2009). Therefore, measurement of oxygen

consumption can be used as a biomarker to evaluate stress responses, induced by toxic components.

The consumption of internal lipids and protein can also be used as an estimate of energy expenditure. Lipid molecules consist of long carbon chains (Nelson et al., 2008). When these are oxidized, the carbon atoms are released from the body as carbon dioxide and feces (Jensen et al., 2008; Schmidt-Nielsen, 1997). Proteins are constructed from amino acids, containing nitrogen (Nelson et al., 2008). During cellular respiration the carbon chain of amino acids can be used as metabolic fuel while the nitrogen is excreted as ammonia (NH_3) (Tande, 1988). Hence, carbon and nitrogen are commonly used as proxies for lipids and protein, respectively (Forest et al., 2011; Mayzaud, 1976).

1.7 Aim of study

The highly productive marine ecosystems in the Arctic requires special considerations with respect to anthropogenic contamination (Olsen et al., 2011). Harmful effects of PW components on the zooplankton communities might have an influence on the entire pelagic production system (Almeda et al., 2013; Bakke et al., 2012). There is still an incomplete understanding about potential consequences of PW exposure on the Arctic copepod *C. glacialis,* which high lipid content most likely makes it prone to the uptake of lipophilic oil components (Chapman and Riddle, 2005). The aim of the present study was to demonstrate whether exposure to the WSF of PW-related PAHs may affect oxygen consumption or the carbon and nitrogen content of *C. glacialis*. In addition, the objective was to study the uptake and depuration of the selected PAHs for respectively 5 days. To the authors' knowledge this has not been examined before, and hopefully the results will add on to the limited information of possible ecotoxicological impacts of PW on the marine environment.

2 Materials and Methods

2.1 Experimental animals

Calanus glacialis were collected (28.08.2015 and 29.08.2015) by slow vertical hauls (0,3 m sek^{-1}) from 275 m and 150 m depth in Kongsfjorden, Svalbard (78°N, 11°E), using a WP3 plankton net (1000 µm mesh size) with a closed cod-end. The environmental conditions were monitored by a conductivity, temperature and density profile sampler (CTD), conducted (05.09.2015) in the same area. The copepods were transported to the Kings Bay Marine Laboratory in plastic containers (50 L) and subsequently transferred to larger tanks (~100 L) with a flow through seawater setup. The average temperature in the tanks was maintained at 1,5±0,7 °C. In the laboratory the animals were gently captured with plastic scoops in order to determine the species and developmental stage using a stereo microscope (Leica MZ 12$_5$). C. *glacialis* individuals of copepodite stage V (CV) showing an intact appearance (antennas and tails) were selected for the experiment, damaged copepods were discarded. The copepodite stage V was identified based on the number of segments of the urosome ("tail segments), as described by Mauchline (1998). Total acclimation time for the animals was 3-4 days prior to relocation to the exposure chambers.

2.2 Experimental setup

C. glacialis CV copepodites were exposed to a water-solved mixture of PAHs for five days (120 h) followed by five day (120 h) depuration in clean seawater. The nominal exposure and recovery regimes are shown in Figure 1. The entire experiment was performed in a flow-through exposure system, the MiniRig (Figure 1). The MiniRig is custom-made for exposure of planktonic organisms to PW compounds and comprises 64 glass bottles (0,5 L) receiving water by teflon tubes, to provide equal flow of exposure media/clean seawater to all chambers. Twenty *C. glacialis* CVs were incubated in each bottle. The exposure was performed according to a semi-static protocol where 0,3 L (60 %) of the volume was renewed daily. After 120 h, the exposure solution was replaced by a continuous flow (flux of 1,9±0,6 mL·min^{-1}) of clean filtered seawater (Cuno, 1 µm, salinity 34 ‰), thereby initiating the 120 h recovery period. The MiniRig was installed in a temperature controlled room kept at 1,7±0,1 °C. Only artificial light sources were used throughout the experimental period, in order to reduce light impact on

the toxicity of PAH or the respiration rate of the copepods (Hylland, 2006; Marshall and Orr, 1972). The copepods did not receive any feed during the experiment.
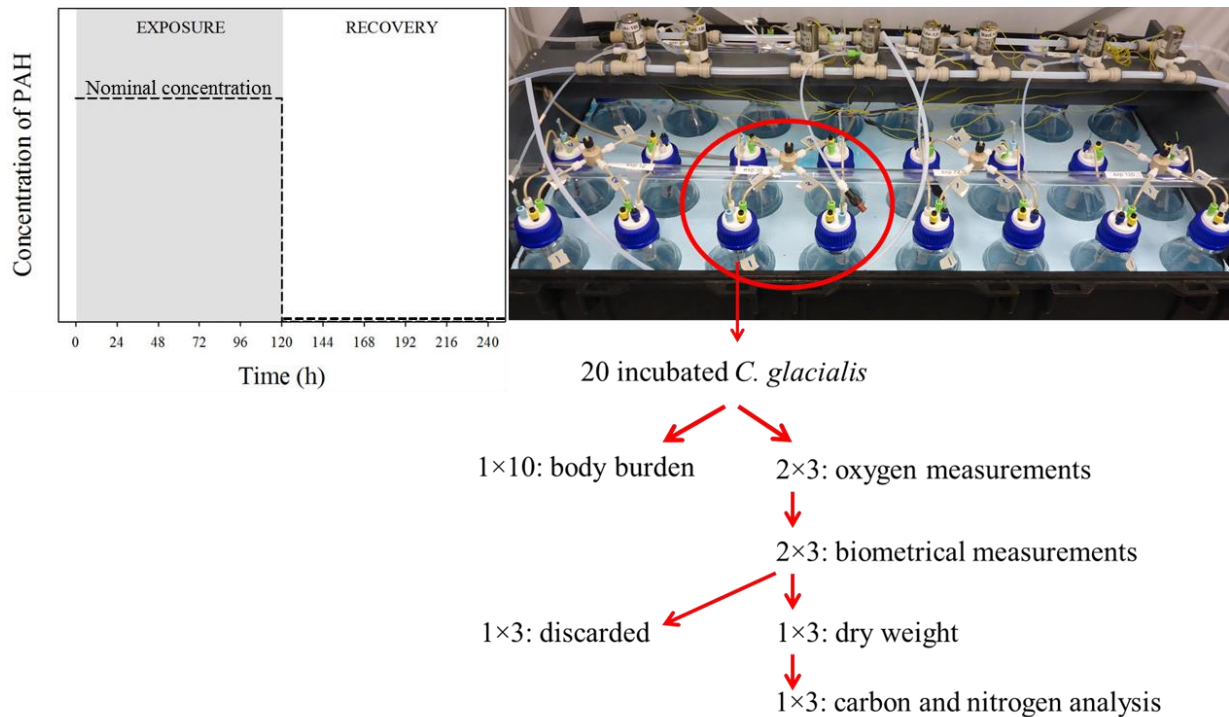
Figure 1. The nominal exposure regime; the dashed line represents a constant concentration of PAH exposure during 120 h, followed by a recovery period in clean seawater for another 120 h (left). The experiment was carried out in a flow-through set-up (MiniRig) with 64 bottles (0,5 L) each containing 20 C. glacialis copepodite V (right). Copepods from four parallel bottles were sampled at regular intervals. From each bottle; 10 individuals were used for PAH body burden analysis and 6 individuals used for biological endpoints (dry weight, carbon and nitrogen were only measured for 3 copepods per bottle).

Throughout the exposure period, *C. glacialis* specimens were sampled for analysis at regular intervals: 12, 24, 36, 48, 72, 96 and 120 h. Likewise, during the depuration phase copepod samples were taken at: 132, 144, 156, 168, 192, 216 and 240 h from the start of the exposure experiment. Each sample comprised four replicates, each taken from a different, but similarly treated incubation bottle. Non-exposed *C. glacialis*, used as control group, were sampled after 0 h (prior incubation in the MiniRig), at 97 h of exposure and 97 h of recovery (217 h total incubation). These were also obtained in four replicates. Sampling included emptying the entire content (copepods and solution) of an incubation bottle into a plastic bucket (1 L). From the bucket, the 10 largest copepods were first selected for body residue analysis while 6 of the

remaining 10 individuals were used to assess the effect of exposure on physiological processes (Figure 1). All animals were carefully captured with a dipper. The ones that did not respond to physical disturbance were recorded as dead and were not used in further analysis (Appendix A).

2.2.1 Exposure solution

The exposure medium was an aqueous solution of a mixture of 11 selected PAHs at sub-solubility concentrations. The exposure medium was generated inline from seawater and a synthetic N-alkane oil spiked with the PAHs (Table 1). The PAH-spiked oil was produced by the Department of Chemistry and Biochemistry, Florida International University, USA. The N-alkane oil itself has a very low solubility in seawater and was not expected to have any biological effect on the animals. The chosen PAHs are all common components in PW and represent a broad array of lipophilicities, which determines the aqueous solubility and bioaccumulation characteristics of the components (here given by their *n*-octanol-water partition coefficient, K_{ow} (Table 1)).

Table 1. The PAHs used to expose C. glacialis, and their corresponding log K_{ow} and molecular formula. The log K_{ow} values were obtained from SINTEF Materials and Chemistry (Environmental Technology department).

ID	Name	Log K_{ow}	Molecular formula
N	Naphthalene	3,30	$C_{10}H_8$
N1	2-Methylnaphthalene	3,79	$C_{11}H_{10}$
N2	2,6-Dimethylnaphthalene	4,24	$C_{12}H_{12}$
F	Fluorene	3,93	$C_{13}H_{10}$
P	Phenanthrene	4,58	$C_{14}H_{10}$
D	Dibenzothiophene	4,37	$C_{12}H_8S$
D1	4-Methyldibenzothiophene	4,86	$C_{13}H_{10}S$
D2	4,6-Dimethyldibenzothiophene	5,33	$C_{14}H_{12}S$
Fl	Fluoranthene	5,19	$C_{16}H_{10}$
Py	Pyrene	5,13	$C_{16}H_{10}$
C	Chrysene	5,78	$C_{18}H_{12}$

The first step in the production of the exposure solution was to produce a dispersion of the N-alkane oil using a droplet generator system (Figure 2, shows the setup). The droplet generator (Nordtug et al., 2011) consists of four hollows in series with connecting nozzles (diameter 0,5 mm). Filtered seawater (Cuno, 1 μm) was pumped through the generator at a flowrate of 160

mL min^{-1} using a Q-SAN metering pump (Fluid Metering Inc., Syosset NY, USA), producing high shear forces downstream the nozzle in each hollow sufficient for breaking the oil down to micro droplets. The oil was added from a glass syringe (2,5 mL, SEG, Australia), operated by an Aladdin syringe pump (WPI, Sarasota FL, USA), through a teflon capillary into the generator at a flowrate of 0,889 µL min^{-1}, generating an oil dispersion of nominally 5 mg L^{-1} (5 ppm). The dispersion was led into an overflow chamber holding ca. 4,5 L to allow sufficient residence time to assume equilibrium between the PAHs in the droplets and the PAHs dissolved in the water. The next step was to remove the droplets from the dispersion, allowing only the dissolved components in the exposure. The dispersion was sucked (again with a Q-SAN metering pump) through a filter-unit consisting of fine glass wool, to remove the biggest droplets, on top of a Whatman Grade GF/C and GF/F Glass Microfiber Filter (Whatman Ltd., Maidstone, UK; 1,2 µm and 0,7 µm respectively) to ensure only the water soluble fraction (WSF) to come through.
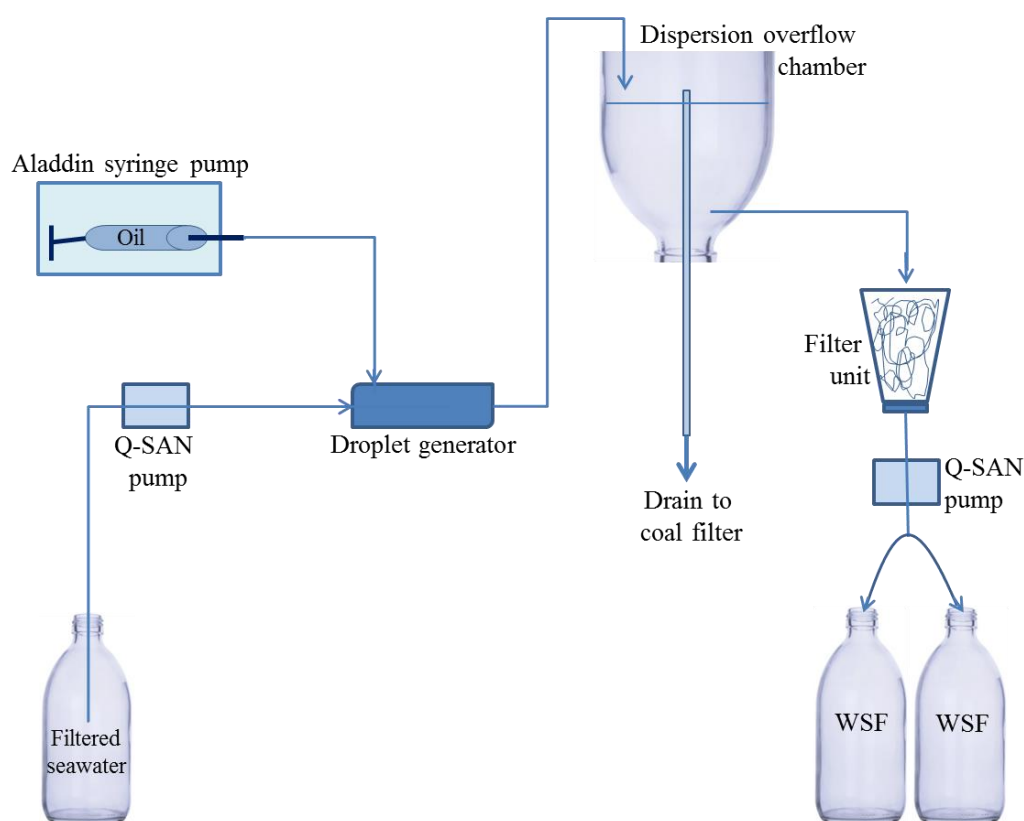
Figure 2. The setup of the water soluble fraction (WSF) generation system. The oil (N-alkane oil, spiked with 11 different PAHs) was pumped from a glass syringe to the droplet generator where it was forced together with filtered seawater, generating a dispersion. The dispersion was kept in the overflow chamber to allow sufficient residence time to assume equilibrium between the PAH in the droplets and the PAH dissolved in the water. Finally, the dispersion was sucked through a filter-unit to ensure only the WSF to come through.

In order to avoid crystal formation of the oil at low temperatures, the WSF was produced at 19±0,8 °C. Filtered seawater from the fjord (~1 °C) was stored in 30 L buckets overnight to increase its temperature (13±2 °C). Subsequently, the final exposure solution (WSF) was collected in two glass bottles (2 L) simultaneously, and cooled down to 0±0,1 °C prior distribution to the MiniRig chambers. The water temperature in all bottles of the MiniRig was measured once (12.09.2015).

Water samples of the dispersion (n=4) and the newly made WSF (n=21) were collected daily for analysis of the PAH concentration. Moreover, exposure solution from selected bottles in the MiniRig (n=8) were taken after the exposure period, in order to validate the exposure. Blank samples (n=10) of the filtered seawater were also collected.

2.3 Experimental measurements and analysis

2.3.1 Respiration rate

The respiration rate of *C. glacialis* was measured as oxygen (O_2) consumption over time using a fiber-optic oxygen meter (FireStingO2, PyroScience GmbH, Germany) to determine the dissolved oxygen concentration (mg L^{-1}) inside a closed glass chamber with the incubated copepods. The instrument is based on REDFLASH technology, where a fiber-optic cable emits light signals (orange-red: λ = 610-630 nm) which creates an oxygen-dependent luminescence by a REDFLASH indicator, in the near infrared band (NIR, λ = 760-790 nm). Collision between oxygen molecules and the REDFLASH indicator impairs the NIR luminescence. Thus, when the oxygen content in the water is reduced due to respiration of the animals the luminescence is increased (FireSting O2, user manual).

C. glacialis individuals sampled from the MiniRig were carefully transferred to the glass vials (6,9 mL) using a plastic pipette with a wide opening. Oxygen measurements, were carried out in two parallels with three *C. glacialis* incubated in each chamber. The chambers were filled up with filtered seawater (sterivex, 0,22 µm, 0,1±0,1 °C) to avoid the occurrence of any other oxygen consuming organisms. In order to achieve reliable results it was necessary to mount the chamber cap onto a positive meniscus and cautiously eliminate all air bubbles. An external temperature sensor recorded water temperature in a third glass vial (11 mL) to allow automatic temperature compensation of the oxygen measurements. This chamber was also filled with filtered seawater (sterivex, 0,22 µm, 0,1±0,1 °C) but did not receive any copepods. The

measurements were initiated shortly after the caps had been attached. All chambers were submersed into a customized aluminum cooling block which was placed onto a temperature-controlling plate (Cole-Parmer, IC22, USA, set at 3 °C) and isolated using polystyrene to minimize temperature variations.

The fiber-optic sensor (Retractable Oxygen Minisensor, Item No. OXR230) was retracted into a needle tip, before the measurement started. An adjustable tripod kept the sensor in a stable position while the needle was pressed through a septum in the chamber cap into the chamber water. The fiber-optic sensor was then extended by a sliding step mechanism to come into contact with the water and start monitoring the oxygen level. The signal from the instrument was recorded in the Pyro Oxygen Logger software every 1 to 3 seconds for a minimum of 40 minutes to monitor changes in oxygen concentration. The temperature in the third chamber was constantly registered during the same period. The salinity was determined to be 34 ‰ in the respiration chambers, hence, the same as in the MiniRig.

The measurements were conducted by different channels for the two parallel chambers and two different fiber-optic sensors were used (channel 1: sensor code; ZA7-515-202, channel 2: sensor code; ZB7-533-202). During the measurement of the control group, sampled after 96 h, an error occurred, due to a low signal from the REDFLASH indicator and both sensors were replaced by new ones (channel 1: sensor code; ZA7-527-203, channel 2: sensor code; ZA7-518-193). The instrument was calibrated daily with air-saturated distilled water (Milli-Q) using a one-point calibration mode according to the user manual (FireSting O2, user manual). The air-saturated water was prepared by a few minutes intense shaking of a bottle (1L) containing 50% water and 50% air. Between all measurements the needles and the fiber-optic sensors were rinsed in distilled water (Milli-Q). A preliminary test of the instrument, without animals in the chambers revealed almost no decrease in water oxygen content.

The oxygen concentration was plotted as a function of time by the Pyro Oxygen Logger software, where the last 20 minutes showed a linear decrease for all samples. The oxygen consumption of *C. glacialis* was then determined as change in oxygen concentration (mg L^{-1}) over time (the last 20 minutes of measuring) based upon least square regression according the linear equation:

$$y = ax + b \tag{2}$$

where x is the time in seconds, y is the oxygen concentration in mg L^{-1} and b is the intercept. The regression coefficient a, indicates the oxygen reduction rate in the respiration chamber, which is the oxygen consumption given as mg O_2 L^{-1} s^{-1}. Since a represents the slope of the line it has a negative value. All a values were therefore multiplied by -1 prior use in further calculations (see all a values in Appendix B).

The units were converted to µg O_2 consumption per individual *C. glacialis* per hour using the formula:

$$\mu g O_2 h^{-1} = \frac{a \cdot V \cdot 1000 \cdot 3600}{n} \tag{3}$$

where a is the regression coefficient from equation 2, V is the volume of the respiration chamber (0,0069 L) and n is the number of animals in the chamber (n=3). The results were multiplied by 1000 to convert the units into µg L^{-1} and by 3600 to achieve oxygen consumption per hour (h).

2.3.2 Biometric measurements

All *C. glacialis* used to assess the respiration rate were also photographed for biometric measurements and subsequently for calculations of the body (prosome) and lipid sac volume. Copepods were placed individually in a concave Petri dish containing only a few droplets of water to prevent it from moving around. Digital images were taken from a lateral view through a stereo microscope (Leica MZ 12$_5$) with a Unibrain firewire camera (Fire-i 785c color: 4605) operated by Fire-i software (Unibrain S.A., Athens, Greece), calibrated at magnification 0,8. Every picture was given a unique ID-name and stored on a personal computer. In this process two picture of *C. glacialis* from the start control (0 h) chambers were mistakenly deleted.

The perimeter and length of the lipid sac and the prosome of the copepods were outlined manually on a graphical tablet (Wacom CintiQ 12WX, Wacom Co., Ltd., Saitama, Japan) and analyzed by the ImageJ software (National Institutes of Health, Bethesda MD, USA). ImageJ

was calibrated, based on the known magnification (0,8) of the pictures, to count 132 pixels per millimeter. The program then derived the marked length (L) and area (A) by converting pixels into millimeters and square millimeters respectively. The length of the prosome was measured by drawing a line from the anterior dome of the head to the rearmost part of the fifth metasome segment. To check reproducibility of the method, the outlining was done twice per dimension in the first few pictures. However, the variation between the repeated measurements was so small that one measurement per picture was decided to be enough. Several of the *C. glacialis* had none or close to empty lipid sac. In these cases the area and the length was given the number 0,001 instead of zero to enable continued calculations. Area and length measurements were utilized for volume calculations, assuming that both lipid sac and prosome were ellipsoids according to the formula:

$$V = \frac{8A^2}{3\pi L} \qquad (4)$$

where A is the area (mm^2) based on the perimeter line, L is the length (mm) and V is the volume (mm^3).

The assumption that the lipid sac and prosome of *Calanus* spp. approaches ellipsoid shape has been applied in previous works, for example by Plourde and Runge (1993) or Arashkevich et al. (2004). However, these studies use the length and width (measured at the largest respective distance) to calculate the area used in equation 4. Vogedes et al. (2010) suggested this would lead to an overestimation of the lipid content. Referring to their analysis, the area calculated by length × width could be up to 75% larger than calculated by the perimeter line.

The body volume (V$_B$) and lipid sac volume (V$_L$) were used to derive a respiration volume (V$_R$), by subtracting the lipid sac volume from the body volume:

$$V_R = V_B - V_L \qquad (5)$$

2.3.3 Dry weight and carbon and nitrogen analysis

Only one half of the copepods were used in further analysis of dry weight (DW) and carbon (C) and nitrogen (N) content. Individual *C. glacialis* were carefully lifted up with a pair of tweezers, rinsed in distilled water (Milli-Q) to avoid the formation of salt crystals and then transferred into pre-weighed tin capsules (5x9 mm, average empty weight; 30,5 µg) placed within a 96 well

plate. The samples were dried in a heating cabinet at 60 °C for a minimum of 24 hours. The dry weight was determined by subtracting the weight of the empty tin capsules from the weight of the tin capsules containing the dried sample. The same micro-scale weight (Mettler Toledo) was used for all measurements and was calibrated daily at 2 g with maximum acceptable deviation ±0,04 mg.

Once weighed, the samples were sent to SINTEF Fisheries and Aquaculture for quantification of the individual carbon and nitrogen content, using an Elemental Combustion System CHNS-O (Costech ECS, model 4010, Costech International, Firenze, Italy).

2.3.4 PAH body burden

Body residuals of PAHs were analyzed in pooled soft tissue samples, consisting of 10 *C. glacialis* individuals. As the micro-extraction procedure (Hansen et al., 2015) requires a certain amount of biological tissue to determine the PAH content of *C. glacialis* the biggest individuals were chosen. *C. glacialis* from the MiniRig were gently collected by a sieve, transferred by tweezers to Eppendorf tubes and frozen at -20 °C prior transportation to Trondheim. SINTEF Material and Chemistry (Environmental Technology department), processed all samples using a micro-extraction procedure followed by chromatography-mass spectrometry (GC-MS) (Hansen et al., 2015; Nordtug et al., 2015). The results of PAH body burden from each replicate were divided by 10 in order to obtain the average PAH concentration per individual copepod.

2.3.5 Chemical analysis of water samples

Water samples (400 mL) were extracted by solid-phase extraction (SPE) using an Agilent Bond Elut PPL SPE column following a SINTEF method for collecting water samples in the field. The columns containing the samples were kept at -20 °C until analyzed by SINTEF Material and Chemistry (Environmental Technology department) in Trondheim. The analysis were conducted with GC-MS using a method based on the US EPA 8270D method to quantify the PAH components (US EPA Method 8270D, 2014).

2.4 Statistics

Calculations and sorting of data were completed in Microsoft Excel (version 2013) while statistical analysis and generation of graphs were performed using the software Sigmaplot 13.0 (Systat Software). One-way Analysis of Variance (ANOVA) was used for statistical comparison of differences between the samples. The significance level (α) was set to 0,05 for all tests. If the null hypothesis, implying that all samples have the same mean was rejected ($p<0,05$) a Holm-Sidak post-hoc test (multiple comparison) was performed to identify the groups that differed. The carbon and nitrogen data were tested for bottle effects, i.e. differences between the four replicates (One-way ANOVA). However, all observations were in the range of the controls and the three individuals from the each bottle were pooled (n=12).

One-way ANOVA assumes that the data is approximately normally distributed and have equal variance (homoscedasticity) i.e. that different groups have a standard deviation within the same range (Townend, 2002). Even though ANOVA is not particularly sensitive to either non-normality or heteroscedasticity when applied to a balanced design, the analysis may result in too many false positives (type I error) if the requirements are not fulfilled (McDonald, 2009). The data sets were assessed for normality using Shapiro-Wilk test and, in addition, visually considered by examining frequency histograms. A Brown-Forsythe test (also called The Modified Levene's test) was used to test for equal variance. In the few cases the data was not normally distributed or homoscedastic, the data set was log transformed ($\log_{10}$) before being analyzed.

Correlations (Pearson's product moment correlation) and regressions (linear and nonlinear) were used to study the relationship between different variables and to test for significant trends in respiration rate over time. The correlation/regression coefficient (r^2) indicates how well the data fit to a straight line (correlation) or the regression model (Townend, 2002). The r^2 has a value between 0-1, where 1 states a perfect fit.

3 Results

3.1 Exposure concentration

Results from the chemical analysis of the water samples are summarized in Table 2. *C. glacialis* were exposed to the PAH concentrations showed in the MiniRig column, where the ΣPAH was measured to 7,90 µg L^{-1}. The dispersion which contains both oil droplets and the dissolved fraction (WSF) had the highest PAH concentrations (ΣPAH 28,75 µg L^{-1}). After the dispersion was filtered only the WSF remained (Figure 2), and as expected its concentration of PAH was lower (ΣPAH 12,80 µg L^{-1}). The PAH concentrations in the WSF represents the solution before it was transferred to the bottles in the MiniRig. However the newly made WSF had a higher concentration of naphthalene than comparable samples from the MiniRig. Naphthalene has a relatively high vapor pressure (Neff et al., 2011) and probably evaporated to some extent from the MiniRig system. Evaporation and spreading via the air might also be the reason why naphthalene could be detected in the blank samples of filtered seawater.

Table 2. The measured PAH composition and concentration (μg L^{-1}, mean ± SD) in water samples collected from the raw dispersion (n=4), the newly made WSF (n=21) and the MiniRig bottles (n=8). The blank samples (n=10) were taken from the clean filtered sea water before entering the droplet generator. Refer to Table 1 for identification of PAH.

PAH	Concentration (μg L^{-1})			
	Dispersion	WSF	MiniRig	Blank
N	9,09 ± 0,98	4,05 ± 2,65	0,24 ± 0,06	0,02 ± 0,00
N1	7,48 ± 0,83	3,68 ± 1,71	3,30 ± 0,21	0,01 ± 0,00
N2	4,41 ± 0,47	2,02 ± 0,73	1,75 ± 0,12	0,01 ± 0,01
F	0,91 ± 0,16	0,39 ± 0,18	0,38 ± 0,02	0,00 ± 0,00
P	4,47 ± 0,70	2,00 ± 0,78	1,74 ± 0,11	0,01 ± 0,00
D	0,42 ± 0,07	0,19 ± 0,08	0,17 ± 0,01	0,00 ± 0,00
D1	0,64 ± 0,09	0,24 ± 0,08	0,17 ± 0,01	0,00 ± 0,00
D2	0,65 ± 0,07	0,13 ± 0,05	0,07 ± 0,01	0,00 ± 0,00
Fl	0,05 ± 0,01	0,01 ± 0,01	0,01 ± 0,00	0,00 ± 0,00
Py	0,17 ± 0,03	0,04 ± 0,02	0,04 ± 0,01	0,00 ± 0,00
C	0,47 ± 0,08	0,06 ± 0,03	0,04 ± 0,01	0,00 ± 0,00
Σ PAH	28,75 ± 3,42	12,80 ± 6,14	7,90 ± 0,50	0,05 ± 0,01

3.2 Temperature and salinity

The CTD measurements of water column parameters in the middle of Kongsfjorden are presented in Figure 3. Salinity and temperature were 34,9 ‰ and 3,3 ºC respectively at 140 m depth. Salinity in the seawater used at the laboratory was 34 ‰. Temperature registered in the different MiniRig bottles and in the respiratory chambers during the oxygen measurements are shown in Figure 4. The average temperature in the exposure (n=28), recovery (n=28) and the control (n=8) bottles from the MiniRig were 0,00±0,07, 0,41±0,10 and 0,23±0,43 ºC, respectively (mean±SD).
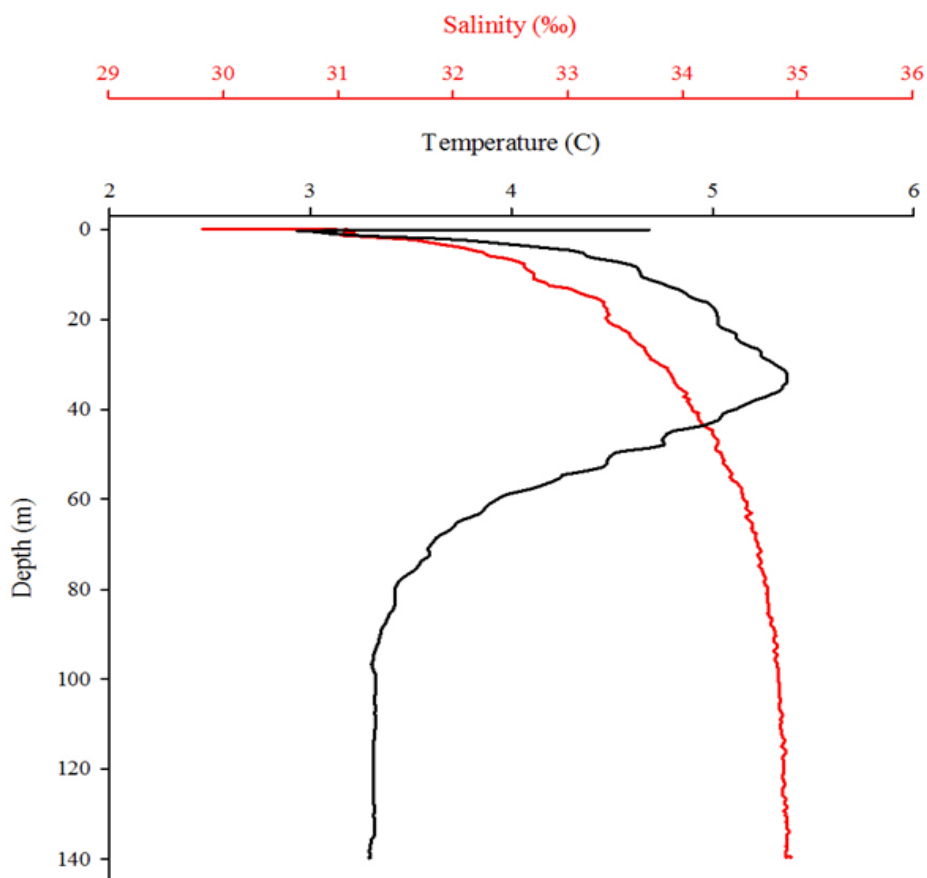

Figure 3. Vertical CTD profile showing temperature and salinity in the water column down to 140 m depth in Kongsfjorden, Svalbard. The data were collected 05.09.2015.
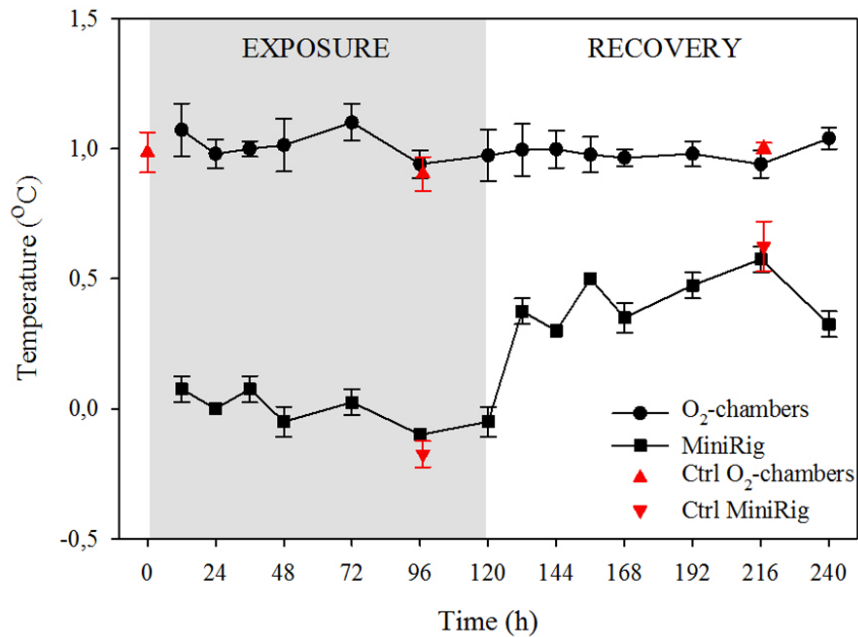
Figure 4. The average water temperature (mean±SD) in the oxygen chambers were derived from the Pyro Oxygen Logger, which registered the temperature every 3 seconds for 20 minutes during oxygen measurements. Average water temperatures (mean ± SD, n=4) in the MiniRig bottles measured once 12.09.2015. The exposure period was conducted as a semi-static setup while the recovery had a constant flow of water through the bottles. The red symbols are the control groups. The start control (0 h) was sampled before incubation in the MiniRig, hence, the temperature is missing for this group.

3.3 Stage composition and body characteristics of *C. glacialis*

The *C. glacialis* captured in Kongsfjorden mostly consisted of copepodite stages IV and V but a few adult females were also present. Many individuals were damaged during the first haul (28.08.2015). Therefore, a second collection in the same area was conducted the following day (29.08.2015). The additional animals were only slightly less damaged and extensive work had to be carried out in order to sort out unharmed individuals of the correct developmental stage. Pictures of the copepods did not reveal any damage among the individuals used in the experiment. However, as evident from Figure 5 the sampled *C. glacialis* were in different conditions. Out of 1360 *C. glacialis* individuals used in the experiment only 7 were found to be dead, none of these were sampled for further analysis (Appendix A).

3.3.1 Body mass, carbon and nitrogen content

There were large variations in size, weight and biochemical composition between the *C. glacialis* specimens sampled for biological endpoints (Table 3 and Figure 5). The individual with highest carbon content had almost 26 times more carbon than the one with least. The largest body volume (V_B) was 7 times bigger than the smallest (Table 3). It should be mentioned

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that the water in the large tanks where the copepods were acclimated was not filtered. However, the presence of phytoplankton in the water was assumed to be negligible. Therefore, it is unlikely that any of the *C. glacialis* contained carbon or nitrogen residuals in their stomach or intestine which could have influenced the result of the analysis.

Table 3. C. glacialis. Body dry weight (mg), total carbon and nitrogen content (µg), carbon:nitrogen ratio, body volume, lipid sac volume and respiratory volume (mm^3) for individuals sampled in August 2015 in Kongsfjorden, Svalbard. Most of these copepods had been exposed to low concentrations of PAH at different time ranges prior to the analyses.

	DW (mg)	C (µg)	N (µg)	C:N ratio	V_B (mm^3)	V_L (mm^3)	V_R (mm^3)
mean±SD	$0,32 \pm 0,18$	$162,16 \pm 106,07$	$23,27 \pm 10,29$	$6,50 \pm 1,62$	$1,22 \pm 0,38$	$0,15 \pm 0,15$	$1,07 \pm 0,26$
Maximum value	1,11	603,32	69,23	9,12	2,87	0,96	2,20
Minimum value	0,07	23,65	5,54	3,71	0,41	0,00	0,38
Number of *C. glacialis*	204	203	204	203	406	406	406

Even though the body volume varies greatly within the sample it was normally distributed. The lipid sac size and carbon content however were skewed towards the left implying that most of the animals had a low lipid content while few ad a high lipid (see histograms in Appendix C). Fifteen animals had a carbon content between 300 and 600 µg while 188 individuals had a range of carbon from near 0 to 300 µg. One of the carbon values was shown to be surprisingly low (18,17 µg) compared with the other measurements for the same individual (DW=0,36 mg, N=26,44 µg, V_B=1,21 mm^3 and V_L=0,2 mm^3). Most likely something went wrong during the analysis of this specimen and the carbon content was removed from the data set.
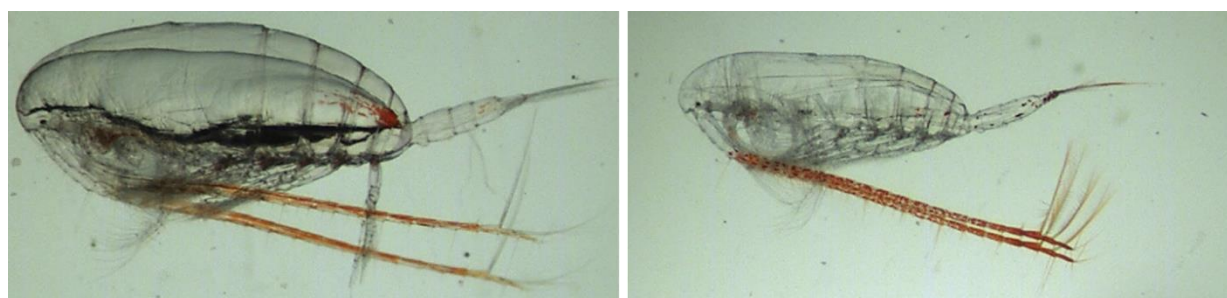

Figure 5. Two examples of wild-caught C. glacialis in development stage CV collected in Kongsfjorden, Svalbard, August 2015. Both individuals were used in the experiment. The copepod to the left had a dry weight of 1,01 mg and was sampled after 12 h of exposure. The small copepod (right), was sampled after 72 h of exposure and had a dry weight of 0,09 mg.

3.3.2 Theoretical calculations of dry weight, carbon and nitrogen

The dry weight, carbon and nitrogen were only analyzed for one half of the sampled *C. glacialis* (n = 204). Since the body volume has been measured biometrically for all the copepods (n = 406), theoretical values of these parameters can be calculated for the remaining copepods based on relationships with the measured body volume. Nonlinear regression analysis (quadratic equation, constraints: $y_0 = 0$, see graphs in Appendix D) between the measured dry weight, carbon and nitrogen and the corresponding body volume from the same copepod ($r^2 = 0,88$, $r^2 = 0,83$ and $r^2 = 0,90$, respectively, n=204, P<0,0001) gave the applied formulas:

$$DW = 0,097 \cdot V_B + 0,121 \cdot V_B^2 \tag{6}$$
$$C = 20,844 \cdot V_B + 82,341 \cdot V_B^2 \tag{7}$$
$$N = 11,628 \cdot V_B + 5,326 \cdot V_B^2 \tag{8}$$

Where DW is the dry weight in mg, C is the carbon content in µg, N the nitrogen content in µg and V_B the body volume in mm^3. Obviously the theoretical values have a perfect fit to the regression lines and will show some deviation from the real (unmeasured) values.

3.4 Uptake and depuration

Uptake and depuration curves for the 11 PAHs in exposed *C. glacialis* are shown in Figure 6. The ID of each graph refers to a specific PAH identified in Table 1.
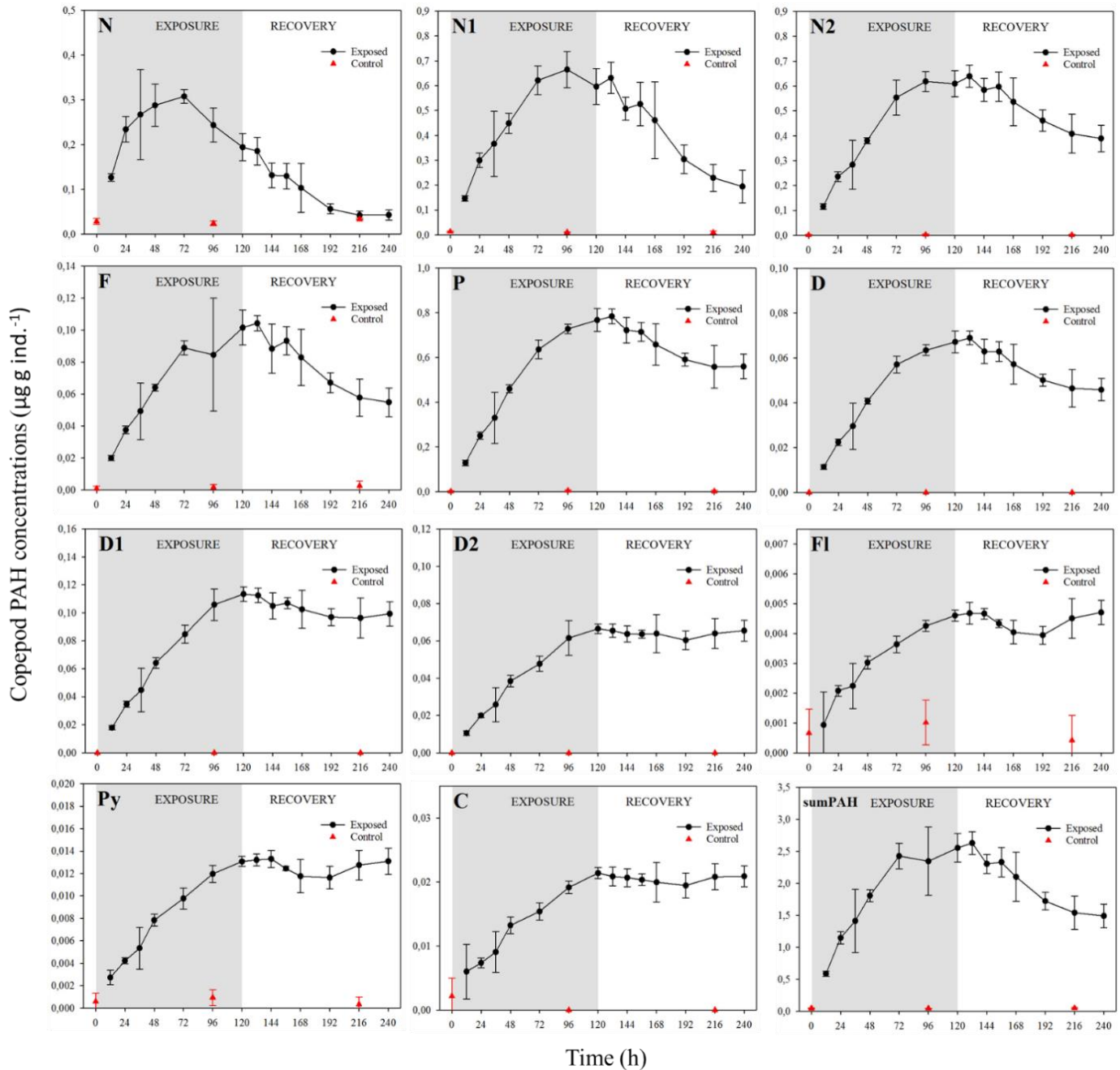

Figure 6. Uptake and excretion curves for 11 PAHs in exposed C. glacialis. Each data point represents the mean of four replicates (n=4), ±SD. The value derived from each replicate is the average PAH concentration per individual copepod in the sample (n=10). Refer to Table 1 for identification of PAH.

Several of the PAHs, showed modest or no decrease in body concentration after 5 days of recovery (Figure 6). Especially the heavier molecules with a high log K_{ow} (Table 1) such as the methylated dibenzothiophene (D1 and D2), fluoranthene (Fl), pyrene (Py) and chrysene (C) seemed to be fully retained in the copepods over the period. On the other hand, depuration of naphthalene (N) apparently started while the animals were still exposed to the WSF of PAHs. However, this was consistent with the drop in water concentration of naphthalene (Table 2, WSF vs. MiniRig).

3.5   Metabolism

Oxygen consumption (respiration rate) and carbon and nitrogen content (proxies for lipid and protein) are parameters representing or revealing main functions of metabolism, and were measured to assess possible effects of PAH on the metabolism of *C. glacialis* copepods.

3.5.1   Respiration rate

The average oxygen consumption (μg O_2 h^{-1}) of *C. glacialis* individuals is presented in Figure 7. There was large variation in oxygen consumption between the animals after correction for individual size differences (dry weight) or nitrogen content.

Since 50 % of the copepods only have theoretical values of dry weight and nitrogen content (section 3.3.2, equation 6 and 8), all results were separated in two parallel graphs with the measured nitrogen and dry weight per copepod to the left (graph C and E) and the theoretical calculated nitrogen and dry weigh to the right (graph D and F). Figure 7A and B shows the average oxygen consumption per individual without correction for size differences.

An increase in oxygen consumption was observed for exposed copepods sampled at 12 h compared to the control groups sampled at 0 h. In addition, a similar increase was observed at the initiating recovery from 120 h of exposure to 132 h, particularly evident in Figure 7C and E. These results could have been interpreted as if *C. glacialis* responded with an increased respiration rate when the condition changed first from the introduction of PAH exposure and then again when the clean seawater was turned on. In between those events the oxygen consumption was to some extent stabilized back at a lower level. However, no statistical significant effect was found on the respiration rates of exposed *C. glacialis* compared to the control groups (one-way ANOVA, p>0,05). In contrast there were differences between the control groups 0 h and 217 h (p=0,006) and 97 h and 217 h (p=0,009) in Figure 7B (Holm-

Sidak). These control groups showed a significant increase in oxygen consumption over time (linear regression, $r^2=0,40$, p=0,03, n=12).

The 192 h sample from Figure 7D and F had a significantly higher respiration rate compared to the controls (Holm-Sidak, p<0,05). Nevertheless, the linear regression showes a decrease in oxygen consumption when corrected for nitrogen content (Figure 7D, $r^2=0,14$, p=0,049, n=28) and dry weight (Figure 7F, $r^2=0,16$, p=0,04, n= 28). None of the graphs on the left in Figure 7 revealed any trends or significant differences between the observations. The discrepancy between the control groups and the large standard deviation for all samples indicate there was large individual variation in respiration rate between the copepods of this population.

The background oxygen concentration in chambers without any copepods were measured once during the experiment. The average decrease ($\pm$SD) of oxygen in the two empty chambers recorded by different fiber-optic sensors was $0,25\pm0,11$ μgO_2 h^{-1} (Appendix B).

Generally it was observed that it was easier to capture the copepods and keep them still for photography the longer exposure time lasted. Especially the specimens sampled after 72 h was noted to react very slowly to physical stimuli. After 36 h of recovery (i.e. 156 h) the individuals appeared to be more active again.
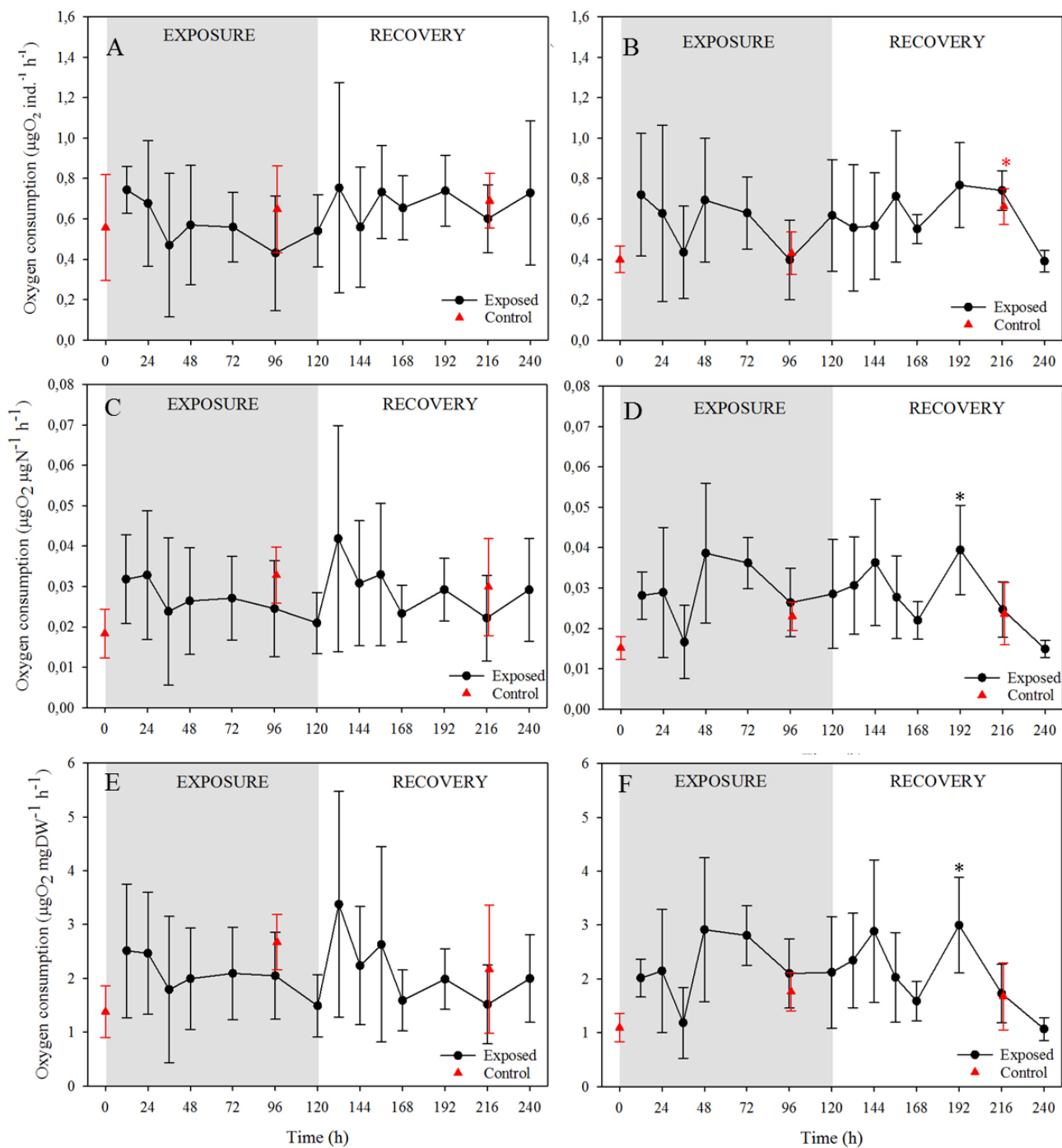
Figure 7. Average oxygen consumption per individual C. glacialis (graph A and B) at different sample time during exposure and recovery, presented as mean±SD of the four replicate bottles. Red triangles represent the control groups. The oxygen consumption was normalized against nitrogen content (graph C and D) and dry weight (graph E and F). The nitrogen content and dry weight used in graph C and E were measured values, while the nitrogen and dry weight used in graph D and F were theoretically calculated. Significant difference between samples and the pooled controls are marked with asterisks (*), red asterisks indicates difference between control groups (*), (one-way ANOVA, Holm-Sidak p<0,05).

3.5.2 Biochemical changes

The amount of carbon and nitrogen per body volume sampled over time is presented in Figure 8. The data in the graphs represent the mean±SD of the 12 *C. glacialis* sampled simultaneously. Again the results were splitted in separate graphs with measured values (Figure 8, A and C) and theoretically calculated values (Figure 8, B and D) of nitrogen and carbon. The body volumes per specimen were included (Figure 8E and F) to evaluate its influence on fluctuations in carbon and nitrogen content.

Overall there were no effects of PAH exposure or the following recovery on carbon or nitrogen content of the copepods even though a few significant differences were discovered. The copepods sampled after 96 h (Figure 8B) of exposure had significantly lower carbon content per unit volume then the start control and the last control group sampled at 217 h ($p<0,05$, Holm-Sidak). However, there were no significant difference between the 96 h of exposure and the control group sampled at 97 h. In addition the variation between the control groups was significant (Figure 8B, $p<0,05$, Holm-Sidak). Although, the copepods did not receive any food throughout the experiment carbon and nitrogen content occasionally varied significantly during the incubation time. In Figure 8A for instance the carbon content was significantly higher in copepods sampled after 168 h compared to those sampled at 132 h ($p=0,03$, Holm-Sidak). However, they did not differ significantly from the controls. In addition, both carbon and nitrogen content was significantly increased during the recovery in Figure 8B and D (linear regression $p<0,05$).
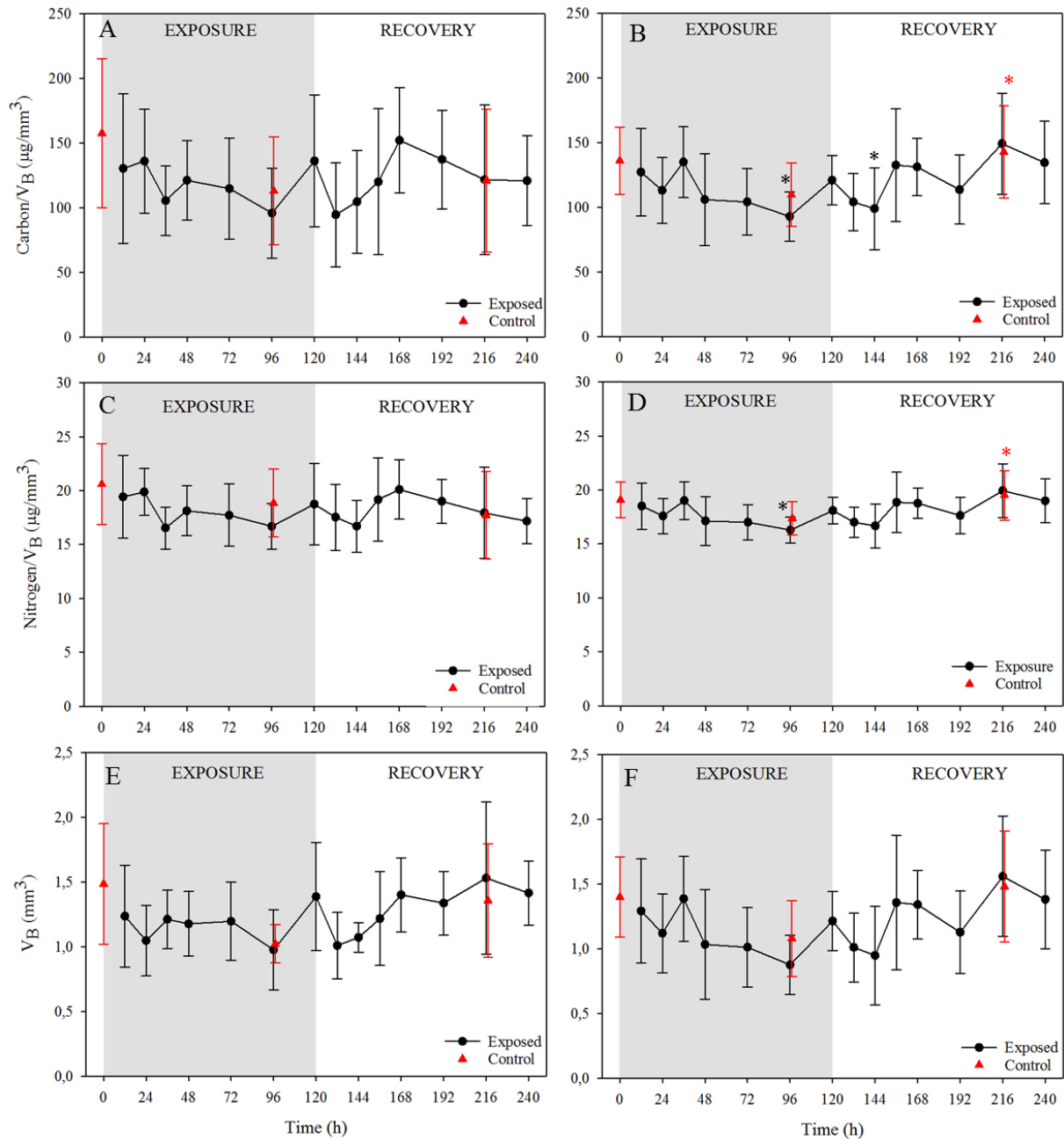
Figure 8. The average (mean±SD) carbon (A and B) and nitrogen content (C and D) per body volume (V$_B$) in C. glacialis at different sampling times during exposure and recovery. The average (mean±SD) body volume (E and F) was included to evaluate its influence on fluctuations in carbon and nitrogen content. Red triangles represent the control groups. The carbon and nitrogen in graph A and C are measured values, while B and D are calculated from the correlation with V$_B$ (section 3.4.1). The number of individuals (n) at each sampling is 12, except the control sampled at 0 h in graph B, D, E and F and the measured carbon (A) after 156 h where n=11. Significant difference between samples and the pooled controls are marked with asterisks (*), red asterisks indicates difference between control groups (*), (one-way ANOVA, Holm-Sidak p<0,05).

The C:N ratios in Figure 9 followed a similar pattern as the carbon and nitrogen content in Figure 8. The ratio was significantly increased during the recovery period (linear regression, p=0,02 Figure 9A, p=0,0008 Figure 9B). There was a significant increase in C:N ratio between sampling at 132 h and 168 h in Figure 9A but C:N did not differ from that of the pooled controls (p<0,05, Holm-Sidak). For the theoretically calculated values (Figure 9B) the *C. glacialis* sampled after 96 h of exposure had a significantly lower C:N ratio than the controls sampled at 0 h and 217 h, and C:N of the copepods sampled at 144 h differed significantly from that of the 217 h control group. However, there were no significant differences between C:N after 96 h exposure and that of the control from approximately the same sampling time (97 h). In addition, significant differences were observed between the controls (Figure 9B, p<0,05, Holm-Sidak).
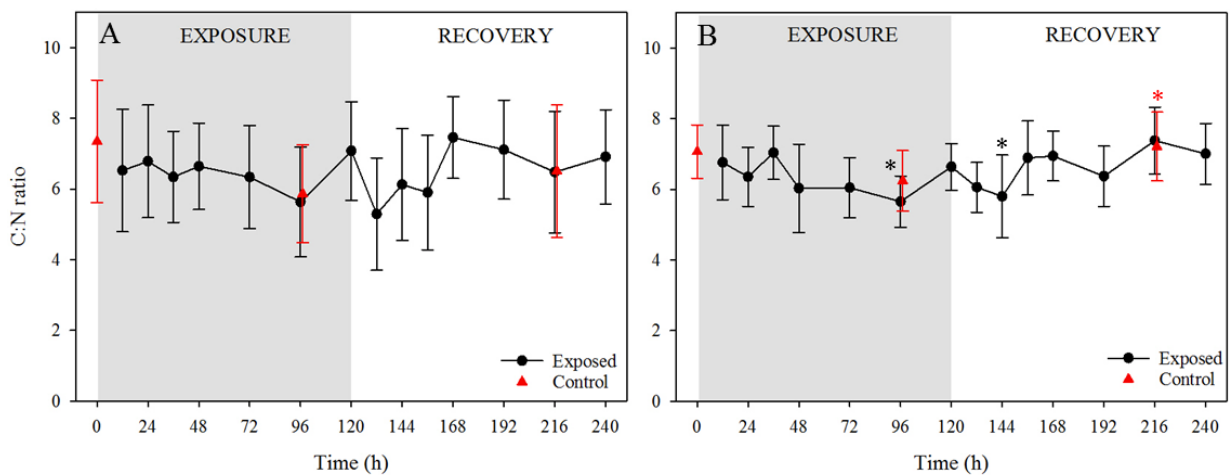
Figure 9. Carbon:nitrogen ratio in C. glacialis over time, presented as the mean of 12 individuals (n=12) ±SD (n=12, except at 156 h sampling (graph A) and the 0 h control sampling (graph B) where n=11). The red triangles represent the control groups. Graph A shows the measured values while graph B are theoretical calculated values as described in section 3.3.2.

3.6 Structural and biochemical correlations of *C. glacialis*

The measured carbon and nitrogen content in *C. glacialis* of the present study were highly correlated with the dry weight and different volume compartments described in Figure 10 (Pearson's product moment correlation). Both the nitrogen and carbon content increased with the body size of *C. glacialis* (correlation with DW (Figure 10) and V_B (Appendix D)), but the copepods contained approximately ten times more carbon than nitrogen. Interestingly, a weaker relationship was found between nitrogen content and the respiration volume (r^2=0,79) than between nitrogen content and lipid sac volume (r^2=0,94).
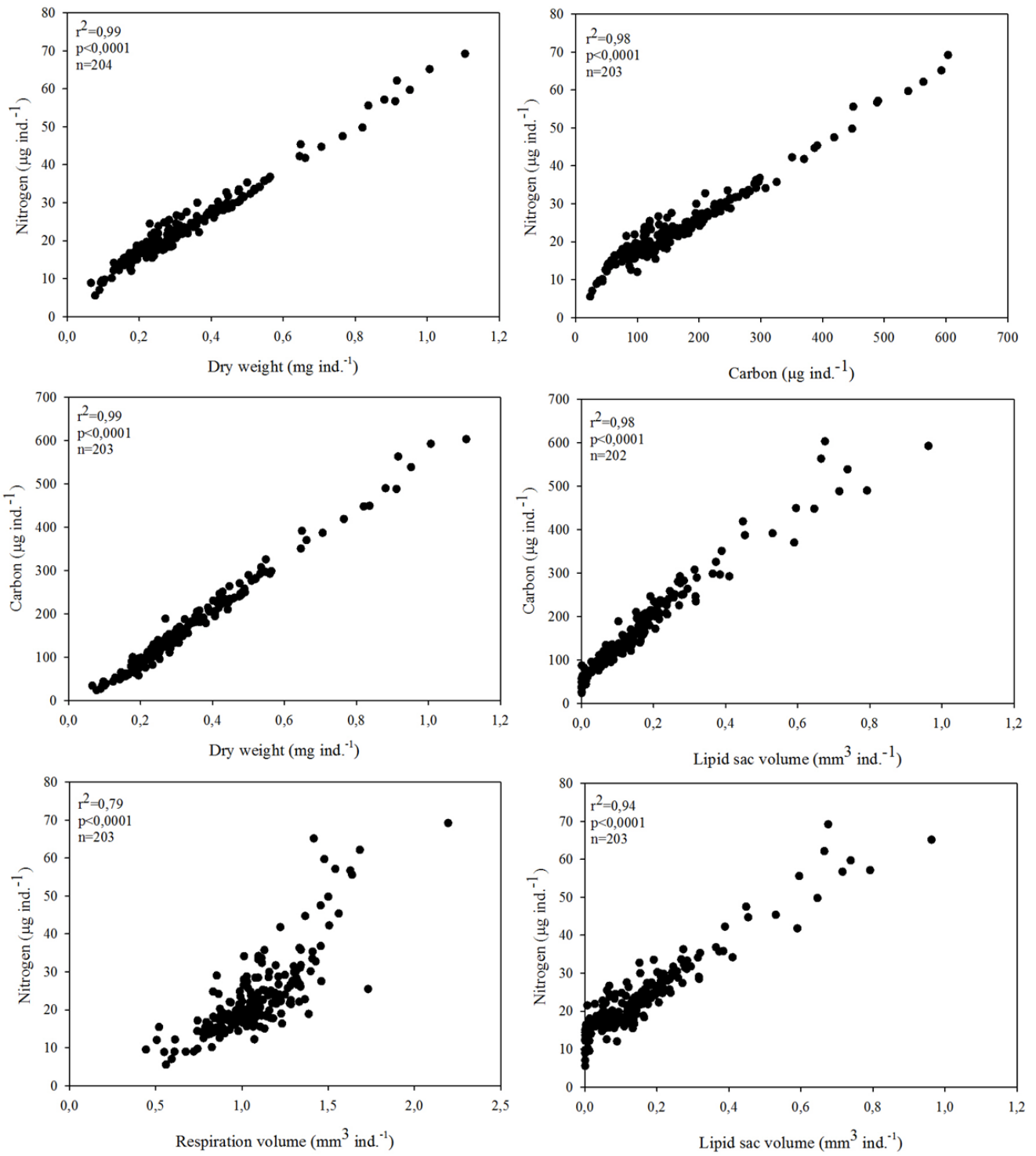
Figure 10. Different correlations (Pearson's product moment) between the dry weight, lipid sac volume, respiration volume, carbon and nitrogen content of the C. glacialis specimen used in the present study.

The concentration of nitrogen in the respiration volume increased as the lipid sac volume increased (% of body volume, Figure 11). This may partly relate to displacement of water by the expanding lipid sac leading to increased concentration of nitrogen in the remaining volume. Any further increase in nitrogen concentration with increase in lipid sac size might indicate co-accumulation of nitrogen and lipid in the copepods.
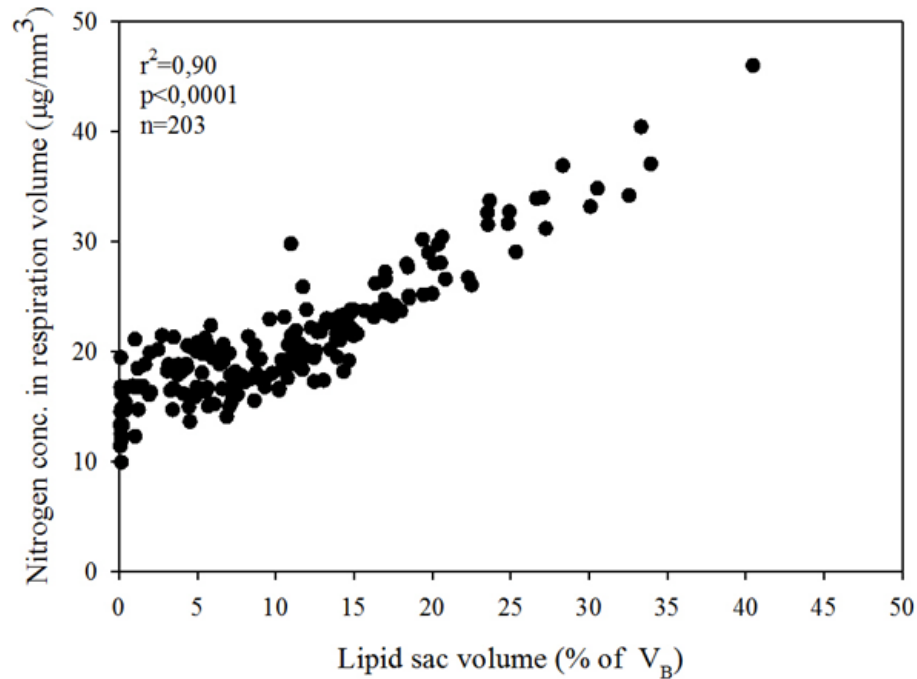
Figure 11. The nitrogen concentration in the respiration volume correlated (Pearson product moment) with the lipid sac volume (% of body volume) in C. glacialis.

Figure 12 shows the lipid sac volume (% of body volume) in relation to the body volume. This correlation may indicate that the amount of lipids often is greater in larger *C. glacialis*.
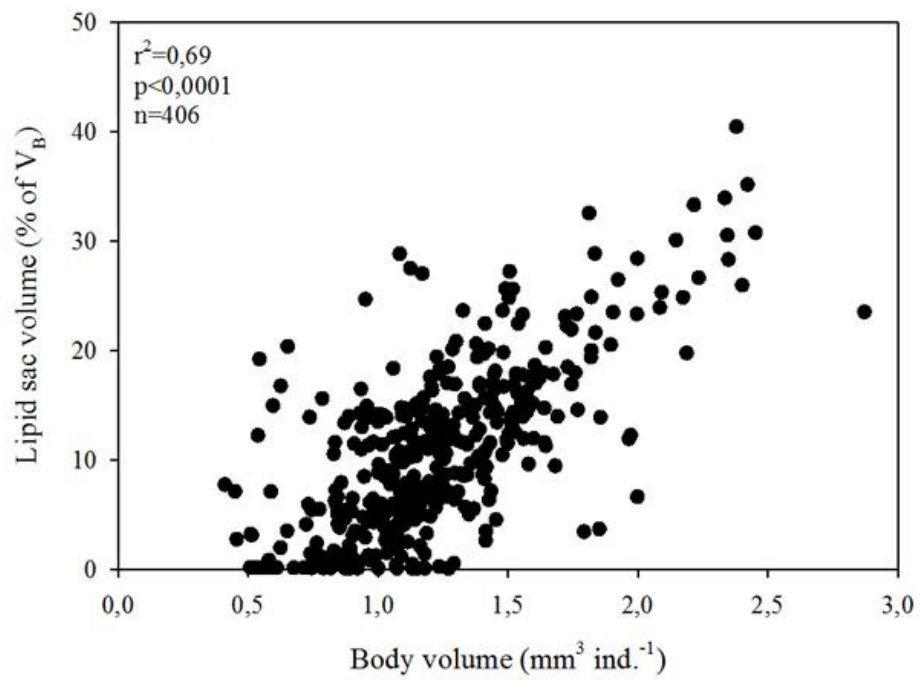

Figure 12. Correlation (Pearson product moment) between the lipid sac volume (% of body volume) and the body volume of C. glacialis.

4 Discussion

The present discussion starts with considering the exposure methodology applied, the model components and the selected concentrations, all in an environmental perspective. Next topic is a discussion of the uptake and depuration of PAHs in *C. glacialis*. The oxygen consumption will be examined in association with the PAH exposure, and subsequently be discussed in relation to size differences and body compartments of the copepods. The effect on energy expenditure, in terms of carbon and nitrogen, during PAH exposure will also be assessed. Finally, an evaluation of the methodology is given in a separate section.

4.1 Environmental relevant exposure to PAH

In the present study *C. glacialis* cohorts were exposed to a constant, low concentration of the WSF of 11 PAHs (Table 2) in order to simulate a realistic exposure to PW in the Arctic. The smaller PAH compounds with low log K_{ow} (Table 1) such as the naphthalenes (N, N1, N2) are more easily dissolved in water compared to PAHs with higher log K_{ow}, and were accordingly found in higher concentrations in the exposure solution. As pointed out in the results section, naphthalene concentrations in the exposure containers of the MiniRig system were lower compared to the newly made WSF, probably due to evaporation. Some of the heavier compounds such as, the dibenzothiophenes (D, D1, D2) and chrysese were also found in lower concentrations in the MiniRig compared to the freshly made WSF. A possible explanation may be adsorption onto wetted surface of the system, i.e. the glass walls of the bottles, however this was not investigated further (Nendza and Müller, 2007).

The total PAH concentration of the exposure solution was on average 7,90 µg L^{-1} (measured values). Most previous studies that investigated sub-lethal effects of PAHs on *C. glacialis* have used higher exposure concentrations, probably more realistic for an oil spill accident. In addition, it has become commonplace to use only one PAH as a proxy for the total oil content in exposure studies (Hansen et al., 2008; Hjorth and Nielsen, 2011; Jensen et al., 2008). However, exposure to a WSF of a mixture of several carefully selected PAHs allows the examination of potential additive toxic effects that may arise from PW emission where a large number of PAHs will be present (Barata et al., 2005).

The environmental concentrations of PW in the North Sea have been monitored and predicted since the 90s as a part of the quantitative risk assessment in the DREAM model (Johnsen and Frost, 2011). The concentrations of total PAH in PW emissions may vary from 40 to 3000 µg L^{-1} (Neff et al., 2011) which are considerably above the total PAH concentration used in the present experiment. However, it has been shown that the dilution of PW discharges in the sea is usually high and relatively fast. Actually, the total PAH concentration in the surface water approximately 1 km outside the offshore installations in the North Sea has been estimated to range from 0,025 to 0,35 µg L^{-1} (Durell et al., 2006; Neff et al., 2011). The PAH concentration used to expose the *C. glacialis* in this study was not unrealistically high and is within the range of exposure concentrations which can be experienced by wild-populations of Arctic copepods in an oil production area. To the authors' knowledge, it is not known how the PW plume will spread or dilute in Arctic waters, where the temperature is lower and the PAH may resist longer in the water column than at lower latitudes (AMAP, 2007).

4.2 Uptake and depuration of PAH

The body burden analysis indicated that all PAHs in the exposure solution accumulated in *C. glacialis* (Figure 6). Since the copepods were not fed during the experiment, uptake was limited to passive diffusion through the biological membranes (boiconcentration) (Jensen and Carroll, 2010). The accumulation ability demonstrated in *Calanus* species is probably mediated by their high internal lipid content and low capacity for degrading and active elimination of PAHs (Jensen et al., 2012; Norregaard et al., 2015).

Even though large, hydrophobic compounds are associated with a higher bioconcentration potential in aquatic organisms i.e. a higher BCF, such compounds may also show similar or slower uptake kinetics than less hydrophobic ones (Durell et al., 2006; Walker et al., 2006). This is in accordance with the present data (Figure 6) where the uptake curve of the heaviest compound chrysene (log K_{ow} 5,78) was linear during the 120 h exposure indicating the uptake never left the initial phase. On the other hand the uptake of the lighter compounds (log K_{ow} 4,37-5,13) seemed to level off towards an asymptotic value in the last part of the period, showing they were approaching, but did not reach equilibrium after 120 h of exposure.

In Jensen et al. (2012), phenanthrene (log K_{ow} 4,58) reached equilibrium within 96 h whereas in the present study the body burden seemed to be still increasing after 120 h (Figure 6). There are however essential differences between their study and the present one and the discrepancy

may be related to the use of *C. finmarchicus* as a model species, and higher exposure temperature in their experiment. On the other hand, the body burden of the slightly less hydrophobic 2,6-dimethylnaphthalene (log K_{ow} 4,24) and fluorene (log K_{ow} 3,93) seemed to have reached equilibrium within the 120 h of exposure in the present experiment, while the body level of even lighter compounds naphthalene (log K_{ow} 3,30) and 2-methylnaphthalene (log K_{ow} 3,79) dropped toward the end of the period. The lightest compounds are however rather volatile, and the reduction may be explained by decreasing water concentrations of these compounds in the last days of exposure (Table 2).

The uptake kinetics of all the tested PAHs seemed to follow first-order uptake kinetics (Walker et al., 2006), and it could be expected that the depuration of the compounds also would follow this model. When the exposure stopped the body concentration of the lighter components began to fall, in agreement with first-order kinetics. However, with increasing lipophilicity properties of the PAHs the depuration gradually deviates from the model, and an important discovery was that the heavier PAHs with a high log K_{ow} (Table 1) were not excreted from the copepods during the 5 days of recovery in clean seawater (Figure 6). This is in accordance with findings reported by authors working with other copepod species. Norregaard et al. (2015) found that phenanthrene and pyrene were still present in *C. hyperboreus* after 77 days into the depuration period. The mechanisms behind the slow elimination are not yet fully understood, and were not investigated in this experiment.

4.3 Respiratory rate

4.3.1 Responses in respiration rate after PAH exposure

No significant effect of PAH on the respiration rate of *C. glacialis* was found during the exposure in this experiment. Concerning the low concentrations of PAH, and the relatively short exposure time this result is consistent with previous studies showing that, generally, oil exposure in low concentrations does not severely affect vital physiological processes in *C. glacialis* (Hjorth and Nielsen, 2011; Jensen and Carroll, 2010; Jensen et al., 2008). Additionally, Gilfillan et al. (1986) did not observe any differences in oxygen consumption of *C. hyperboreus* captured in waters contaminated by oil seeps, contra individuals from control sites. However, none of these studies investigated the oxygen consumption of *C. glacialis* followed exposure to a mixture of PAHs.

The low (7,90 µg L^{-}1) total PAH concentration in the present exposure solution may add to explain the present non-effect. However, there were quite large differences in oxygen consumption both within and between the samples even after the normalization against dry weight and nitrogen (Figure 7). This may in part be due to methodological flaws (oxygen gradients, temperature fluctuations etc.) but large individual variation in activity or other energy demanding processes cannot be ruled out. Large non-target variations may mask any low-level response on the PAHs, especially if there are large variations in responsiveness between individuals. The intra-species variation in sensitivity to artificial PW has previously been suggested to be particularly high for *C. glacialis* (Camus et al., 2015).

Intrinsic differences, such as lipid content or the ability to metabolize PAHs are thought to have a vital influence on both inter- and intra-species differences in toxicity (Camus et al., 2015; Hylland, 2006). However, there are conflicting theories about whether high lipid content will protect against a potential toxic effect or not. Copepods with a higher lipid content will most likely experience a higher body burden of PAH in steady state since these are assumed to accumulate in the lipid sac (Hansen et al., 2009). At the same time a large lipid reservoir could act like a sink for PAHs preventing them from binding to molecular receptors and exhibit a toxic effect (Camus et al., 2015; Hansen et al., 2009). Chemicals reaching the site of (toxic) action, where the interaction with endogenous macromolecules or structures takes place (Walker et al., 2006), triggers a variety of cellular responses such as alteration in the level of enzymes, creation of reactive intermediates or modifications in DNA etc. These processes often requires energy initially derived from cellular respiration (equation 1) (Walker et al., 2006).

A lipid-rich, healthy copepod might have a higher capacity to handle these energy demanding processes, which ultimately could come at expense of survival, growth or reproduction for a copepod without enough energy storages. Gene expression in lipid-rich and lipid-poor female *C. finmarcicus* exposed to the WSF of a North Sea oil (concentrations as low as 5,4 µg L^{-1} total hydrocarbon) was compared by Hansen et al. (2009). A slightly higher level of GST mRNA indicating oxidative stress and lipid peroxidation, was found in the lipid-poor compared to the lipid-rich individuals. This would possible increase their metabolism in order to answer the energy requirements needed to facilitate the detoxification or cellular repair systems. Additionally, an elevated transcription of CYP330A1 was observed only in the lipid-poor copepods. Hansen et al. (2009) suggested that the lack of protective lipids may have triggered defence mechanisms (e.g. CYP330A1 detoxification) in the lipid-poor females. Whether the *C. glacialis* with low lipid content in present study exhibited higher oxygen consumption due to a

more extensive upregulation of enzymes can only be speculated since this was not the scope of the study. Besides, the differences found by Hansen et al. (2009) were small, and in general their results did not fully support the hypothesis that copepods with low content of lipids are more susceptible to oil toxicity.

If the lipid content of individual *Calanus* spp. functions as a sink for lipophilic compounds, such storage may be protective in the short term. However, eventually the copepods have to mobilize their lipids in order to use the energy e.g. for metabolism during winter starvation, molting or spawning. While the lipids are being depleted the xenobiotics can be released and may lead to delayed toxic effects (Walker et al., 2006). This concern has been addressed before (Grenvald et al., 2013; Hjorth and Nielsen, 2011), yet, to the authors' knowledge it has not been investigated in *C. glacialis* so far.

In this study the only copepods showing a significant difference in oxygen consumption compared to the controls were sampled during recovery (192 h, Figure 7D and F). It has to be assumed that the body burden of PAHs were consistent with the data showed in Figure 6, even though the uptake and respiration experiments were done on different individuals. From the uptake experiment it was shown that heavier PAHs were still present in the copepods after the exposure period was ended and throughout the entire depuration period. Additionally, at the end of the recovery the copepods had been starving for a longer time. If the specimens showing high respiration rate at 192 h had started consuming their internal lipid reservoirs the results could have indicated a delayed effect. However, no significant increase was found in the other half of the studied *C. glacialis* (Figure 7C and E), and after the increase at 192 h the oxygen consumption drops again, suggesting a decreasing trend in total. In addition, the starvation did not seem to have an effect on the lipid content (Figure 8A and B) making the delayed effect a doubtful explanation.

It has also been suggested that starvation is an important factor for causing a decrease in physiological rates (Ikeda, 1977). An alternative theory for the data presented in Figure 7D and F is that PAH exposure increased the respiration rate and therefore the lower oxygen demand became evident first during the recovery. However, there was no reduced oxygen consumption in the control groups over time, and for the same reason as described above, it seems therefore unlikely.

Another hypothesis for the lack of significant effects on the oxygen consumption, and the individual large variation in oxygen consumption observed in this experiment is that the 11

PAHs did not exhibit a common mode of action. PAHs can elicit toxicity through several mechanisms. It is possible that *C. glacialis* suffered from narcosis, in addition to oxidative stress. Hence, the outcome of these two PAH-mediated physiological responses could be predicted to have an opposite effect on the metabolic rate, where the respiration rate might increase as a consequence of oxidative stress, and on the other hand, decrease as a consequence of the lower activity while weakened of narcosis. Furthermore, it was noted that the *C. glacialis* sampled after 120 h of exposure had a lower ability to react to stimuli, suggested to be an indication of narcosis (Barata et al., 2005), and that they seemed to slightly recover after 36 h in clean seawater. However, this observation was not followed up, and whether this response deviated from the control groups is not known. On the other hand, it seems fair to raise the question whether the respiration rate is the most sensitive endpoint to use when investigating the stress response of PAH exposure.

4.3.2 Correction for size difference

The first graphs in Figure 7 (A and B) show the oxygen consumption rate per individual (μgO_2 ind.$^{-1}$ h^{-1}) without considering the size difference between the animals. Size is, however, a key factor affecting individual respiration rate (Schmidt-Nielsen, 1997). Since the variation in individual size in the material was high (Table 3) a potential variation in size within the groups selected for oxygen consumption measurements (group size n=3) could obscure a modest difference in the oxygen consumption between exposed and unexposed animals. In order to, at least partly compensate for size the rate of oxygen consumption was normalized against body mass on a dry weight basis (Figure 7E and F).

Normalizing against body mass is a common procedure for all kinds of animals when examining the respiration rate (Schmidt-Nielsen, 1997). However, *Calanus* spp. differ from many other species because of their lipid sac which can hold a large percent of their dry weight (Scott et al., 2000). The lipid sac mainly contains wax ester (Swalethorp et al., 2011) which are not expected to exert a metabolic demand. Harris (1983) proposed that the body of calanoid copepods should be considered as two distinct parts or compartments. The first compartment was referred to as the lipid sac, while the second was called the "structural" compartment. Hence, only the second compartment was considered to be a part of the respiratory structure. Harris (1983) suggested that the respiratory rate might relate more to the structural weight than to the total dry weight of the copepod. In a study by Campbell et al. (2001) the nitrogen weight of *C. finmarchicus* was used as a proxy for their structural weight. Accordingly, the oxygen consumption in present experiment was calculated per nitrogen weight unit of the individual *C.*

glacialis as an alternative interpretation of the results (Figure 7C and D). Unexpectedly, the nitrogen based oxygen consumption gave a very similar pattern as the data derived on a dry weight basis. Furthermore, a correlation between dry weight and nitrogen was shown to have a very good fit (Figure 10, r^2=0,99), indicating that either measurement could be used to correct the oxygen consumption for individual size differences. However, correcting for the structural weight seems like a more reasonable way of presenting the data.

Considering the discussion above a relationship between oxygen consumption and the nitrogen content per copepod could be expected, but although the correlation was significant, it had a surprisingly bad fit (r^2=0,26, p=0,03, n=68). These results may indicate that there were factors other than size, having an effect on the measured oxygen consumption.

4.3.3 Comparison with other studies

As mentioned above there is presently no other study which has investigated the respiration rate in calanoid copepods after PAH exposure. However, there are some papers which have reported oxygen consumption of unexposed *C. glacialis* e.g. Båmstedt and Tande (1985), Hirche (1987), Ikeda et al. (2001) and Hildebrandt et al. (2014). Unfortunately, these studies mainly used different experimental setup (time, location, temperature, copepodite stage etc.) or methods to measure the oxygen concentration, hence, the results are hard to compare. In addition some of the techniques used to measure the oxygen concentration in previous studies might not have the same accuracy as the REDFLASH technology used in this experiment. Another challenge is the unit in which the oxygen concentration was measured. Here the oxygen content was recorded in weight (gram) while other studies have documented oxygen concentration as volume (liter). The ideal gas law states that the amount of gas (in mole) is determined by its pressure, volume and temperature. The papers cited did not specify the pressure during their oxygen measurements, making the recalculations and comparisons difficult.

However, one study carried out by Ingvarsdóttir et al. (1999) examined the respiration rate of *C. finmarchicus* in μmol O_2 gC^{-1} h^{-1}. They report an oxygen consumption in the range of 151-219 μmol O_2 gC^{-1} h^{-1} (n=3) for CV *C. finmarchicus* captured west of Scotland in June 1995 incubated at 12,5 ^oC. In order to compare the oxygen measurements, the data presented in Figure 7A were divided by the individual *C. glacialis* carbon content and the oxygen molar mass (32 g mol^{-1}), hence, μg O_2 ind^{-1} h^{-1} was converted to μmol O_2 gC^{-1} h^{-1}. The range of average oxygen consumption between the samples in the present study was found to be 79-241 μmol O_2 gC^{-1} h^{-1} (n=17). This is a larger spread in the data set compared to Ingvarsdóttir et al.

(1999), but the rate of oxygen consumption did not differ extensively which somewhat validates the measurements presented here.

4.4 Responses in consumption of lipid and protein

When animals do not receive any food they have to rely on internal energy resources in order to maintain essential physiological processes necessary to survive. The last *C. glacialis* sampled during this experiment had starved for at least 10 days. However, the control groups in Figure 8 did not show a decreasing trend in neither carbon nor nitrogen content. This species depends on its stored energy through the entire winter, and even after months of starvation *Calanus* spp. can be found to possess large sacs filled with lipids (Marshall and Orr, 1972). Hence, after only 10 days without food a reduction in carbon and nitrogen was probably too small to measure in the present restricted material.

Weight (C or N in µg) per unit volume may be used to evaluate the nutritional status of copepods (Campbell et al., 2001). Since a larger copepod can be expected to hold a higher total nutrient content (Figure 10 and Appendix D) total carbon and nitrogen per individual were divided by corresponding body volume. Remarkably, the carbon and nitrogen content sometimes seemed to rise over time (e.g. Figure 8B, significant increase of the last control). Maybe this could be interpreted as a consequence of heterogeneity in body conditions within the specimens collected from the fjord. In addition, the increase in carbon and nitrogen despite correction for size, might be a result of a greater amount of carbon or nitrogen per volume in larger *C. glacialis*. Which is consistent with Figure 12 suggesting the lipid sac volume holds a higher % of the body volume in larger copepods. Consequently it may be hard to observe the expected decrease in the energy storage, at least over the relatively short period of time and when the copepods showed a great variation in size. It would of course have been preferable to measure the content of nutrition in the same individuals over time, but this cannot be done since the animal do not survive the required analysis.

Ten days of starvation might not significantly reduce the lipids or proteins in *C. glacialis* (Figure 8, control groups). However the objective was to test if an additional stressor in this case selected PAHs, would have an effect on energy consumption in exposed copepods. It is known that contaminants can trigger energy demanding possesses in exposed organisms (Walker et al., 2006). Nauplii of *C. glacialis* have been shown to experience reduced growth and delayed development when exposed to pyrene (Grenvald et al., 2013) and reduced energy status has been shown in shrimp embryos after exposure to dispersed oil (Bechmann et al.,

2010). Nevertheless, it can be assumed that there is a relationship between the oxygen consumption and the molecules which are oxidized to produce energy (equation 1) (Marshall and Orr, 1972). Considering this the lack of a significant decrease in energy status during the exposure in the present experiment (Figure 8) is consistent with the lacking effect on the oxygen consumption (Figure 7).

Although no effect on the levels of lipids or proteins was shown it is possible that PAH exposure had an effect on the biochemical pathways involved in metabolism, for example whether fat or proteins were preferable catabolized. Changes in energy storage composition would reflect the C:N ratio (Figure 9). A significant increasing trend in the C:N ratio during the recovery period was found which may indicate a protein dominated metabolism. This result is, however, in conflict with a previous study of *C. finmarchicus* where a declining C:N ratio during 10 days of starvation was found suggesting the copepods primarily consumed lipids (Mayzaud, 1976). Importantly, the study by Mayzaud (1976) differed from this study in many aspects, such as the use of a different model species and the copepods had not been exposed to any contaminants. However, as shown in Figure 9 the C:N ratio seemed to be slightly decreasing (although not significantly) during the first 96 h. Therefore, it is possible that *C. glacialis* in the present study also mainly utilized its lipids in the beginning of the experiment. During the exposure the body burden of PAH increased (Figure 6), and after 120 h the concentration peaked. An explanation to why *C. glacialis* chose to use its proteins could be that the lipids were partly damaged by lipid peroxidation caused by PAH-mediated ROS. But the controls also seem to follow a similar pattern and the fluctuations in C:N ratio can therefore not be associated with PAH exposure. Additionally, an indication of a largely protein based metabolism in *C. hyperboreus,* independent of oil seep exposure was found by Gilfillan et al. (1986).

Generally, it appears like the data presented in Figure 8 and Figure 9 are displaying randomly fluctuations over time, and that neither the exposure nor starvation had a measurable effect on body content parameters of the copepods. Compared with previous studies the values presented in Table 3 were slightly lower and in addition, the standard deviations were somewhat greater (Hildebrandt et al., 2014; Scott et al., 2000). As an example, Hildebrandt et al. (2014) analyzed 149 CV *C. glacialis* capture in June/July 2010, and reported a dry weight of 0,52±0,24 mg ind.$^{-1}$, a carbon content of 329±126 µg ind.$^{-1}$ and a nitrogen content of 42±12 µg ind.$^{-1}$.

Considering the differences in size and fat content the *C. glacialis* of the present study probably had experienced a very different food supply during the summer before being captured. When

inspecting the pictures of the copepods used in this experiment it is hard to believe that all of them (e.g. Figure 5, right) would have been able to survive through the coming winter without additional food supply. A thinkable reason for this diversity in condition could be that they were in different phases of their life cycle. *C. glacialis* may have a life span of 1-3 years (Falk-Petersen et al., 2009). It is possible that some of the sampled copepods had recently developed into stage CV while others already had been CV for one year and soon would be ready to turn into the adult stage. Scott et al. (2000) presented data of *C. glacialis* captured in Kongsfjorden in August/September 1998. They reported the dry weight of CIV and CV to be 0,09 mg ind^{-1} and 0,62 mg ind^{-1} respectively. The mean dry weight of the CV measured in present study was 0,32 mg ind^{-1} and the lowest was 0,07 mg ind^{-1}. Hence, great variability in size of wild-caught *C. glacialis* has been reported previously, although the issue of different ages of development stages has, to the author's knowledge not been discussed.

4.5 *C. glacialis* biochemical budgets and the respiratory volume

As discussed earlier, it is assumed that the lipid sac of *C. glacialis* mainly consists of wax esters with no or negligible protein content (nitrogen). However, in the present study total nitrogen correlated better with lipid sac volume (r^2=0,94, Figure 10) than with respiration volume (V_R, equation 5, r^2=0,79, Figure 10). In addition, the concentration of nitrogen in the respiratory volume increased when lipid sac volume increased as percent of total volume (r^2=0,90, Figure 11). It has been suggested previously that *C. glacialis* can assimilate protein simultaneously with the lipid from their food (Swalethorp et al., 2011), which may explain the relationship between nitrogen and lipid sac volume.

In order to better understand this process a linear regression was used shown in Figure 13 (Black slope) to receive the equation:

$$y = 0{,}645 \cdot x + 14{,}046 \tag{9}$$

where, y is the nitrogen concentration in the respiration volume and x is the lipid sac volume as % of the body volume.

Equation 9 suggests that the nitrogen concentration in the respiration volume was approximately 14,05 µg/mm^3, when the lipid sac volume utilized zero % of the copepod body volume. This also means that an individual consisting of 100% respiration volume would have ca. 14,05 µg nitrogen per mm^3. If the amount of nitrogen was independent of the lipid sac volume and the lipid sac volume was expanding, the new nitrogen concentration of the respiration volume can be calculated by:

$$y = \frac{14,046}{1 - (\frac{x}{100})} \tag{10}$$

where again, y is the nitrogen concentration in the respiration volume and x is the lipid sac volume as % of the body volume.

By the use of equation 10 the nitrogen concentration (y) in a copepod with a lipid sac volume of 25 % was calculated to be 18,73 µg/mm^3. However, this theoretical nitrogen concentration represented by a red triangle and a red line in Figure 13, was shown to be lower than the measured concentration (30,17 µg/mm^3, black line). The red line indicates a dilution effect i.e. how much the nitrogen concentration increased only as a result of the lipid sac displacing water from respiratory tissue. The difference between this dilution effect and the measured value might be explained by an additional accumulation of nitrogen when the copepods are building up their lipid reservoirs.
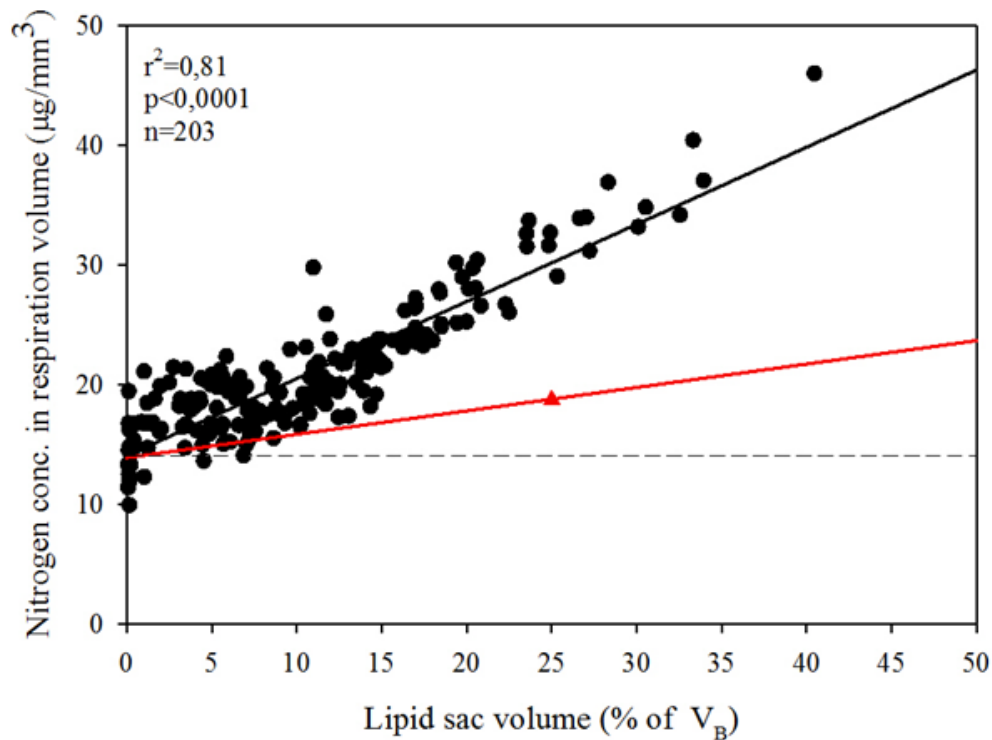
Figure 13. Correlation (black dots) between the nitrogen concentration in the respiration volume and the lipid sac volume (in percent of the body volume) of C. glacialis. The black slope is the linear regression of the measured values. The red slope represents the dilution effect (displacement of water as the lipid sac expands). The red triangle is symbolizing the theoretical nitrogen concentration in the respiration volume when the lipid sac volume is 25% of the total body volume.

Another interesting observation from the biometry measurements was that the amount of lipids (in % of V_B) to some extent increases with the body volume (Figure 12). PAHs are hydrophobic contaminants expected to accumulate in the lipid sac of *C. glacialis*. As a consequence these contaminants might bioconcentrate to a relatively higher level in a large copepod compared to a smaller copepod.

In some cases the obtained data from this study did not comply with the assumptions. Especially two cases were hard to explain. The first was the poor correlation between nitrogen content and the respiration volume. The second was the oxygen demand which was expected to correlate closely with nitrogen content (structural weight). However, this relationship was not particularly strong ($r^2=0,26$, $p=0,03$, n=68, Pearson's product moment correlation). It might be possible that the copepods contained different amounts of water. In that case it must be most likely that the water was accumulated in the respiration volume and not in the lipid sac. Hence, the respiration volume may vary more independently of the nitrogen content than assumed here. Unfortunately, the wet weight could not be measured in this experiment. The reason why the

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relationship between oxygen consumption and nitrogen was not stronger is not known. In general, there is still a huge lack of knowledge when it comes to the physiological properties of *C. glacialis*.

4.6   The ecosystem approach

Expansion of petroleum activity into the Arctic creates a need to evaluate the environmental risks and possible impacts of anthropogenic discharges on Arctic organisms and ecosystems. The findings from the present study can be important for environmental risk assessment and monitoring. Today the basis for predicting the EIF is derived from the PEC/PNEC ratio. However, toxicity tests on *C. glacialis* may not generate enough information to evaluate the impact of PAHs on the ecosystem. In accordance with other studies, the CV *C. glacialis* used in the present experiment appears to be relatively tolerant to low concentrations of PAH. On the other hand the slow depuration of PAHs in Arctic copepods demonstrated in this experiment indicates that heavier PAHs may be available for maternal transfer (Jensen and Carroll, 2010) and for transfer to higher trophic levels.

It is known that vertebrates, including fish, efficiently can metabolize PAHs which therefore will not biomagnify in the food chains (Hylland, 2006). However, *C. glacialis* may be a link for chronic exposure of PAH, which may lead to effects on species that preferentially feeds on them (Lønne and Gulliksen, 1989; Peterson et al., 2003). Developing fish are among the organisms of biggest concern since these are expected to be vulnerable to contaminants (Brette et al., 2014). Aas et al. (2000) found formation of DNA adducts, a covalent binding of xenobiotics to DNA (Walker et al., 2006) in juvenile Atlantic cod following chronic exposure to dispersed oil with a total PAH concentration as low as 0,3 μg L^{-1}.

The findings from the present study suggest that the use of PAH body burden data and surveys of uptake kinetics for lipophilic compounds in zooplankton is a relevant approach for estimating exposure risk and fate of PW discharges (Almeda et al., 2013). However, caution is required when extrapolating laboratory results to the field and natural conditions.

4.7 Methodological reflections

4.7.1 Capture stress and individual condition

The wild-caught *C. glacialis* used in this experiment was brought from 275 m depth up to the surface at a speed of 0,3 m sek^{-1}. Some marine invertebrates are sensitive to hydrostatic pressure shifts (Schmidt-Nielsen, 1997) and concerns about metabolic stress shortly after capture have been addressed (Skjoldal and Båmstedt, 1977). In the present study the copepods were acclimated 3-4 days prior the start of the experiment. In addition, Pearcy and Small (1968) did not find a significant effect of pressure on respiratory rate in pelagic crustaceans. Indeed, a natural behaviour of *Calanus* spp. is vertical migration, where the copepods travel from deep water to the surface and back again in 24 h. Hence, the difference in pressure experienced by the copepods was not considered to be an issue for the reliability of the results. However, a problem might be the handling during the experiment. Even though the objective was to only incubate the intact *C. glacialis*, some of them might have been damaged or hurt. Actually, many of the copepods were injured from the first haul, which was the reason a second haul was conducted the next day. The mortality during the experiment was low, however, the reason for these deaths is unknown.

Another issue that relates to the collection of wild copepods is the method of using an open net. As described above, the copepods of the present study were captured from 275 m depth and up to the surface, thereby integrating the entire water column. *C. glacialis* spend the winters in diapause at 200-300 m depth (Falk-Petersen et al., 2009). In a study conducted by Søreide et al. (2010), the seasonal decent of *C. glacialis* in Rijpfjorden, Svalbard, started already in August. They also found that, between August and October, CV *C. glacialis* captured from the deep water (100-140 m) contained more lipids compared to specimens from the surface water (upper 50 m). The metabolic rate of copepods from high latitudes could be expected to be reduced during the diapause (Ikeda et al., 2001; Maps et al., 2013). Ingvarsdóttir et al. (1999) found that the oxygen consumption in *C. finmarchicus* was significantly lower during the winter months than in summer.

The field work in the present study was conducted in late August only a couple of days before the midnight sun period ended. There is still a lack of knowledge about the factors initiating the diapause but the seasonal fluctuations in illumination are believed to be an important trigger (Ingvarsdóttir et al., 1999). A speculation is that the lipid-rich copepods, found in the data presented here might have dived deep (collected at 275 m) to hibernate, while the thin

specimens were still higher up in the water column foraging or waiting to die. Hence, if some of the copepods were ready to go into diapause while others were not, this may have influenced the respiration rate despite the correction for size.

Open nets are, without doubt, the most common method to use when collecting copepods for experimental purpose. It has been suggested by Swalethorp et al. (2011) that sampling of different meta-populations can be a problem in *in situ* studies. It might be better to collect all *C. glacialis* from the same depth to obtain a more homogenous sample, when the aim is to investigate effects of low PAH exposure.

4.7.2 Temperature

Temperature is a confounding factor affecting the metabolism of animals. The oxygen consumption of *C. glacialis* (CV) has previously been shown to increase by almost 40 % when the water temperature raised from -1,7 ºC to 1 ºC (Båmstedt and Tande, 1985; Tande, 1988). Hirche (1987), also reports that the respiration rate increased by approximately 40% in *C. glacialis* CV when the temperature increased from 0 to 3,2 ºC. This is consistent with the concept that, within a manageable range, an increase in temperature will accelerate the metabolic processes in the body (Schmidt-Nielsen, 1997).

Another consequence is that the solubility of oxygen decreases with increasing temperature (Schmidt-Nielsen, 1997). However, the fiber-optic oxygen meter used to measure the oxygen concentration in this experiment automatically corrected the measurements for changes in temperature and, hence, the solubility of oxygen. Therefore, the issue is not whether a change in temperature affected the reliability of measurements but if it had an effect the respiration rate of the copepods.

It should be noted that the *in situ* water temperature (Figure 3) was about 3 ºC warmer than the water used at the laboratory. According to Marshall and Orr (1972), the alteration in respiration is most likely an immediate response by poikilothermic species like *Calanus* spp. to an experienced change in temperature. The copepods were acclimated for 3-4 days in tanks kept at 1,5±0,7 °C prior relocation to the MiniRig. Hence, if temperature had an influence on the results it was probably due to different temperatures in the MiniRig or in the chambers during the oxygen measurements.

From Figure 4 it is apparent that the cooling block ensured a rather constant temperature during the incubation in the small chambers where the oxygen was measured. However, the

temperature in the MiniRig was slightly lower (down to -0,2 ºC), especially evident during the semi-static exposure period. The MiniRig was installed in a refrigerated room (kept at 1,7±0,1 ºC) and it looks like the solution in the bottles during the semi-static setup tend to be chilled faster than the seawater in a flow-through setup. Even if the temperature in the MiniRig only was measured once, the room temperature was rather stable and probably that of the exposure solutions and seawater as well.

An interesting observation when comparing the MiniRig temperature in Figure 4 with the oxygen consumption in Figure 7 is that the increase in temperature from 120 h to 132 h occurs simultaneously with an increase in oxygen consumption. The oxygen measurements conducted right after the temperature increase (132 h) had the largest standard deviation of all which could indicate that the individual copepods responded differently to the change. However, this observation is only true for one half of the results (Figure 7A, C and E), and in addition a decreasing trend in oxygen was noticed during the total recovery in Figure 7D and F. If the differences in lipid and protein content between the copepods had an influence on the potential sensitivity to changes in temperature and subsequently affected the metabolic rate cannot be determined within the scope of this study.

4.7.3   Study design considerations

It has to be assumed that the haul in Kongsfjorden gave a random sample of the wild population of *C. glacialis*. However, in order to obtain sufficient biomass for the PAH body burden analysis the 10 biggest copepods were always picked out first. Although the natural population probably was morphologically rather heterogeneous, the lack of randomization during sampling may have influenced the large variation in biometry and chemical content between copepods.

In the present experiment the oxygen consumption of three *C. glacialis* individuals had to be measured simultaneously in order to receive a measurable decrease in the oxygen concentration. It is conceivable that the measured respiration rate was affected by a crowding effect. Movements and activity level of the incubated copepods were not studied. However, the influence of crowding on *C. glacialis* specimens oxygen consumption were assumed to be negligible in previous studies (Båmstedt and Tande, 1985; Ingvarsdóttir et al., 1999). At lower temperatures the viscosity of water increases, which may impede mixing of the water, and hence, the dissolved oxygen (Sakshaug et al., 2009). The advantage of having three copepods incubated at the same time was that the circulation of water was assumed to be sufficient to avoid oxygen gradient formation.

A suggested challenge with the study design is that altered respiration rate is a nonspecific response to a variety of stress factors (Olsen et al., 2007; Schmidt-Nielsen, 1997). Considering the variabilities in the results presented here, it may be proposed that the use of a more specific method should be chosen for further experiments when investigating the effects of low PAH exposure.

5 Conclusions and future perspectives

In the present study, the metabolism of *C. glacialis* did not seem to be affected by PAH exposure for 5 days. However, large variations between body mass and size of the specimens made the analysis challenging and the results more difficult to interpret. The size difference was suggested to be a consequence of collecting copepods of different size at different depths when taking vertical hauls using an open net. This study further showed that several of the PAHs were fully retained in body of the *C glacialis* individuals even after 5 days of recovery in clean seawater. The slow depuration increases the concerns that PW-related PAHs with a high log K_{ow} may be available for transfer to progeny, and for chronic exposure to species of higher trophic levels.

These findings may expand the limited knowledge about uptake and depuration of PW components in Arctic copepods. In addition, the new data will contribute to the understanding of the potential effects on *C. glacialis* from the ongoing expansion of human activities in Arctic waters, resulting in increased emissions of PW. The present results warrant for exposure studies with longer recovery periods to quantify *C. glacialis* depuration rates of heavy PAHs, and studies including the potential for trophic transfer. Future studies on long-term exposure assessing the cocktail effect of PW on *Calanus* spp. are also needed.

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Appendix A

Table A. Summary of experimental setup, showing the incubation time in the MiniRig, the date C. glacialis individuals were sampled and the number of copepods used in the different analyses. The 7 copepods found dead were not used in the analyses.

Treatment	Incubation time	Sample date	Mark	Numbers of individual *C. glacialis*:					
				Incubated in MiniRig	Oxygen measurement	Biometry	DW, C and N	Theoretical DW, C and N	Body Burden
Control	0 h	03.09.2015		80	24	22	12	11	40
Exposure	12 h	03.09.2015		80	24	24	12	12	40
Exposure	24 h	04.09.2015		80	24	24	12	12	40
Exposure	36 h	04.09.2015	1 dead	80	24	24	12	12	40
Exposure	48 h	05.09.2015		80	24	24	12	12	40
Exposure	72 h	06.09.2015		81	24	24	12	12	40
Exposure	96 h	07.09.2015		81	24	24	12	12	40
Control	97 h	07.09.2015		80	24	24	12	12	40
Exposure	120 h	08.09.2015		80	24	24	12	12	40
Recovery	132 h	08.09.2015	4 dead	81	24	24	12	12	40
Recovery	144 h	09.09.2015		80	24	24	12	12	40
Recovery	156 h	09.09.2015		79	24	24	12	12	40
Recovery	168 h	10.09.2015		83	24	24	12	12	40
Recovery	192 h	11.09.2015		81	24	24	12	12	40
Recovery	216 h	12.09.2015	1 dead	84	24	24	12	12	40
Recovery	240 h	13.09.2015		81	24	24	12	12	40
Control	217 h	12.09.2015	1 dead	82	24	24	12	12	40
Sum				1373	408	406	204	203	680

Appendix B

Table B. Regression coefficients (a) for estimated oxygen consumption (mg O_2 L^{-1} s^{-1}) of three incubated *C. glacialis*. All treatments were measured by two different fiber-optic sensors simultaneously. Ctrl without animals represents the background consumption of oxygen in the filtered seawater.

Treatment refers to: time of incubation.exposure/recovery/control.replicate bottle, (e.g. 12.exp.F1)

| Treatment	a (10^6)	a (10^6)	Treatment	a (10^6)	a (10^6)	Treatment	a (10^6)	a (10^6)
12.exp.F1	66,26	70,196	12.rec.F1	104	73	0.ctrl.F1	45	99
12.exp.F2	132	89	12.rec.F2	45	23	0.ctrl.F2	44	51
12.exp.F3	49,461	100	12.rec.F3	66	33	0.ctrl.F3	44	88
12.exp.F4	100	100	12.rec.F4	118	171	0.ctrl.F4	60	31
24.exp.F1	130	129	24.rec.F1	112	116	97.ctrl.exp.F1	105	48
24.exp.F2	29	82	24.rec.F2	37	73	97.ctrl.exp.F2	52	96
24.exp.F3	32	37	24.rec.F3	68	39	97.ctrl.exp.F3	39	59
24.exp.F4	112	79	24.rec.F4	56	42	97.ctrl.exp.F4	69	53
36.exp.F1	81	77	36.rec.F1	135	105	97.ctrl.rec.F1	85	100
36.exp.F2	69	106	36.rec.F2	83	118	97.ctrl.rec.F2	84	80
36.exp.F3	20	34	36.rec.F3	39	58	97.ctrl.rec.F3	87	91
36.exp.F4	40	10	36.rec.F4	87	73	97.ctrl.rec.F4	64	62
48.exp.F1	54	73	48.rec.F1	74	69	ctrl with out animals	7	13
48.exp.F2	83	65	48.rec.F2	69	93			
48.exp.F3	137	101	48.rec.F3	69	97			
48.exp.F4	79	18	48.rec.F4	54	57			
72.exp.F1	103	81	72.rec.F1	88	104			
72.exp.F2	84	81	72.rec.F2	122	106			
72.exp.F3	58	71	72.rec.F3	100	87			
72.exp.F4	59	37	72.rec.F4	61	60			
96.exp.F1	77	98	96.rec.F1	104	84			
96.exp.F2	54	48	96.rec.F2	81	42			
96.exp.F3	48	39	96.rec.F3	94	80			
96.exp.F4	19	17	96.rec.F4	79	84			
120.exp.F1	91	95	120.rec.F1	53	44			
120.exp.F2	81	51	120.rec.F2	49	103			
120.exp.F3	26	48	120.rec.F3	49	64			
120.exp.F4	100	67	120.rec.F4	38	141			

Appendix C
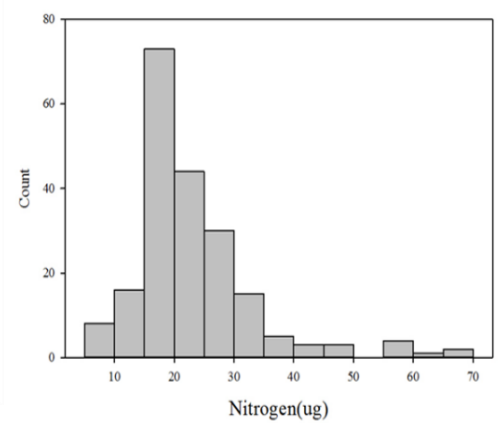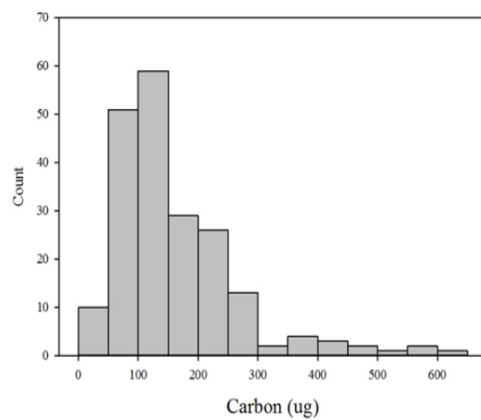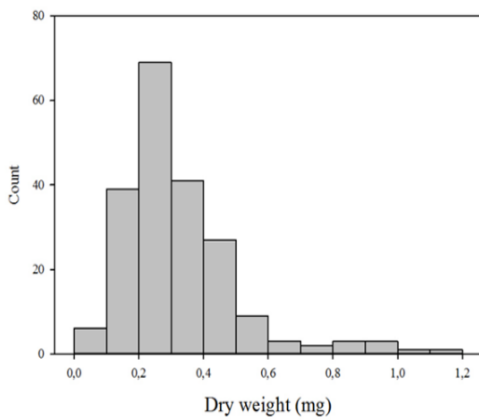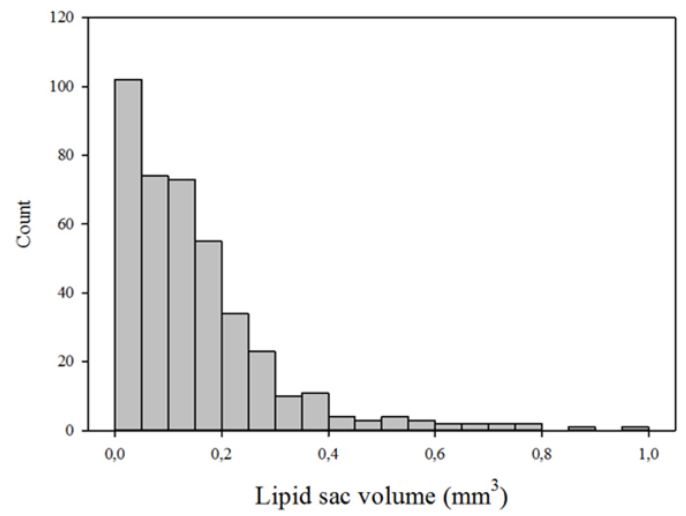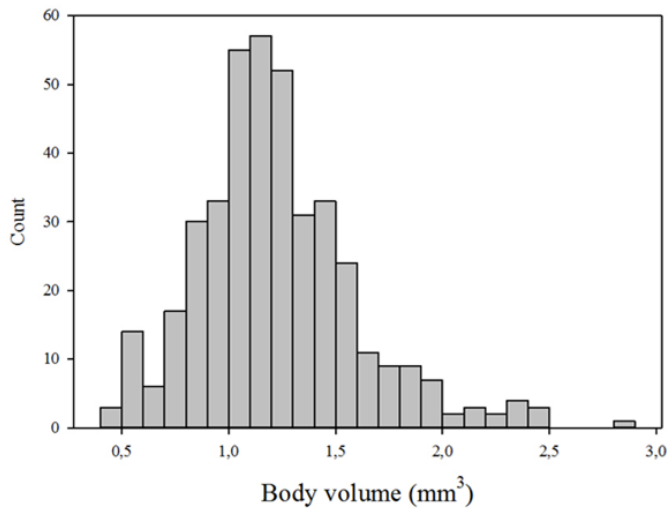
Figure C. Histograms showing the distribution of measured parameters of C. glacialis used in the present study. The "count" at the y-axis represents number of copepods.

Appendix D

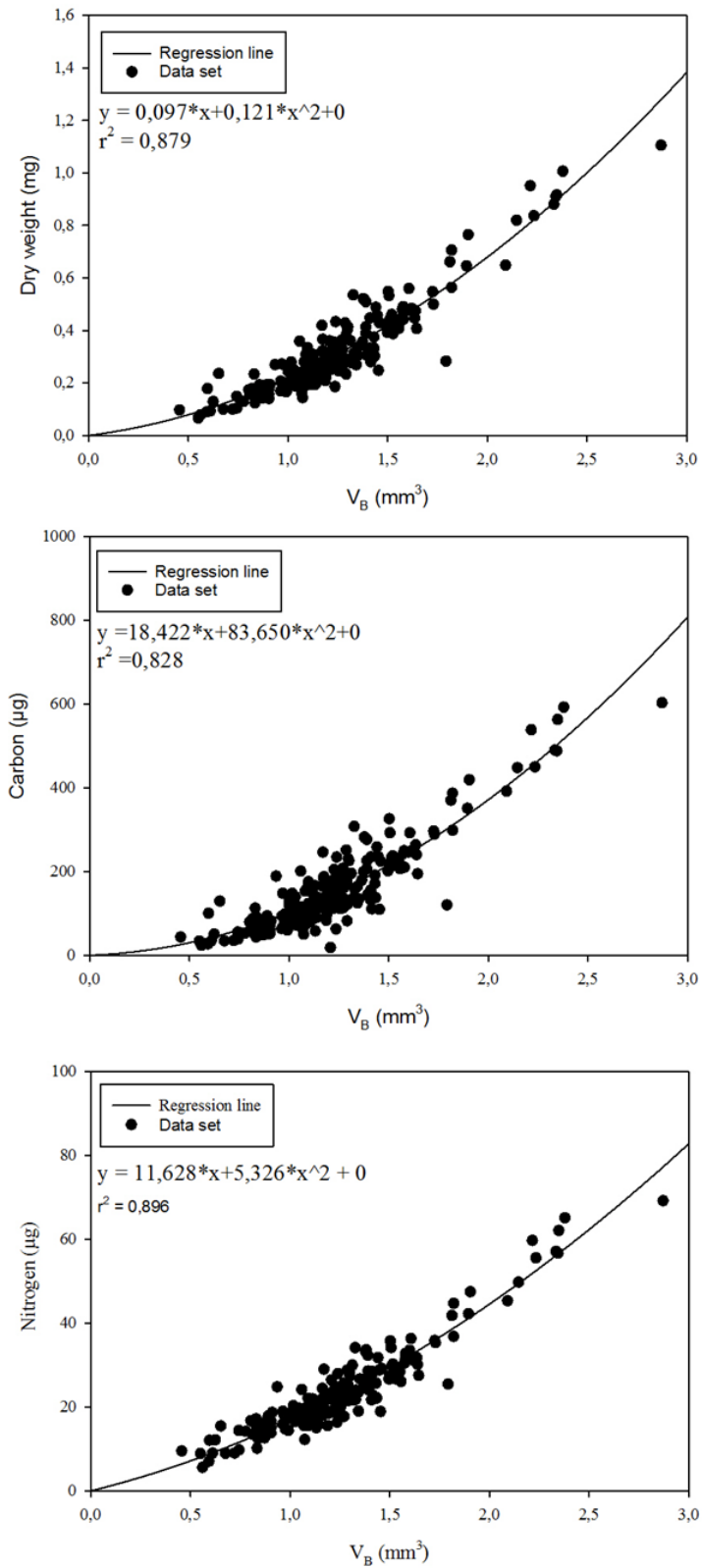
Figure D. Nonlinear regression analysis (quadratic equation, constraints: $y_0 = 0$) between the measured dry weight, carbon and nitrogen and the corresponding body volume for C. glacialis.