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The role of the copepod
Calanus finmarchicus in
affecting the fate of marine
oil spills

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PREFACE AND ACKNOWLEDGEMENTS

I am grateful to Anders and Bjørn for supervising me through my PhD and for giving me the possibility to work with marine environmental research. Thank you for sharing of your expertise in experimental biology and marine research. My PhD projects visits multiple aspects of marine oil pollution and I was fortunate to have access to excellent research communities at SINTEF and at NTNU where people have willingly shared of their knowledge. I am equally grateful to all of you who have participated in and contributed to my PhD: Odd Gunnar, Raymond, Bjørn Henrik, Roman, Trond, Ida Beate, Iurgi, Dag, Liv-Guri, Andy, Emlyn, Petter, Tor, Jørgen, Joakim, Kristin, Ute, Kari, Synnøve, Karen, Maria, Cecilie, Tora, Maren, Berit, Tu, Keshuai, Arne, Kjersti, Kristin, Anette, Marian, Henrik, Liv-Mari, and the many more employees and students at SINTEF Environmental Technology and NTNU Department of Biology. Especially, I want to thank Odd Gunnar for the contribution to papers IV and V and for guiding me into scientific writing. Thank you to Raymond for discussions of research, for constructive feedback on my experimental ideas, and for input to the summary of my thesis. I will miss talking about interesting science stuff with you!

The solvent-solvent extraction of oil dispersions may be shortly described in the materials and methods parts of the papers, but this is a tedious task in the laboratory. Appreciated support and socialization have been provided by the laboratory staff at SINTEF: Marianne, Kjersti, Marianne, Kristin, Kaja, Lisbet, Thor-Arne and Marius (Sorry for that phone call that Saturday morning! I should have cross-checked the pour point of the Statfjord oil and the temperature requirements of *Calanus finmarchicus*!).

I also want to thank the industry partners in NRC project: Statoil, Eni, Shell and BP, for the interest you showed for my PhD work. It has given me valuable insight to attend the steering committee meetings with you. I have my heart set on applied research, and I am passionate about communicating research to generate knowledge, and using it to build

solid foundations for sustainable management of marine environments. I am looking forward to develop my qualifications within these areas in future employments.

I also want to thank my fellow PhD students, Berit and Ingunn, for sharing the ups and downs of PhD life. Thank you to Ingunn for providing input to the summary of my thesis. Your experience and knowledge on environmental modelling in marine environments provided valuable perspective!

To my dear friends, you are now apparently free from the monologues on the amazing feeding apparatus of the *Calanus finmarchicus* and the fascination over the complexity of different oils and how different spills can progress. Thank you for the PhD-survival kit, the gigantic Easter-egg, the wine and dine, and fun that we had during these four years.

At last, I want to thank my family for support and encouragement: Marius, Ann Iren, Guro, Bendik, Berit and Frode. You are a super all-star team to be a part of! This PhD would have been harder to complete without you guys.

Trondheim, 15.07.15

Ingvild Fladvad Størdal

SUMMARY

Background:
Oil spills in marine environments are subject to biological, physical and chemical weathering processes, including entrainment of oil as droplets in the water column. The oil droplets with diameter < 100 μm are within the size range of particles ingested by marine filter-feeders. Ingestion of oil droplets has been reported for several species of zooplankton, including the calanoid copepod *Calanus finmarchicus*. *C. finmarchicus* is ubiquitous in the North Sea, the Norwegian Sea, and the Barents Sea. Based on their high abundance, high feeding activity, and indiscriminate feeding strategy, copepods have been suggested to contribute to weathering and transport processes of oil spills.

Aim:
The aim of this thesis was to investigate how the abundant marine filter-feeder *C. finmarchicus* influence transport and weathering of oil dispersions.

The work included:
1. A modelling approach using the oil spill contingency and response model OSCAR with a filter-feeder module implemented, determining the quantity of an oil spill that can be removed by ingestion by *C. finmarchicus*.
2. Laboratory studies determining:
   a. Concentration of oil in *C. finmarchicus* feeding in dilute oil dispersions
   b. Feeding activity of *C. finmarchicus* in dilute oil dispersions
   c. Accumulation of oil compounds to *C. finmarchicus* from dilute oil dispersions and the corresponding water soluble fraction (WSF)
   d. Viable and total microbial communities in clean and oil-containing feces from *C. finmarchicus*
   e. Biodegradation of dilute oil dispersions in the presence of feces from *C. finmarchicus*

Results and discussion:
The modeling approach estimated that *C. finmarchicus* may ingest between 1 and 40% of an oil spill. The estimates in the lower ranges ($\leq 2\%$) were suggested to be realistic, since the high range estimates combined extreme values for several input parameters. The input parameters that had highest impact on the quantity of oil removed by *C. finmarchicus* were the size limit for droplets ingested, and the population density.

The laboratory studies showed that at fixed density (50 ind. L^{-1}) and oil droplet size (diameter < 40 μm), the concentrations of oil in *C. finmarchicus* biomass were ranging between 3 and 14 mg oil kg^{-1} (exposure concentration 5.5-0.3 mg L^{-1}). Both the

concentration of oil in the biomass and the feeding activity of the copepods were low at the high concentration of oil. The feeding activity were rapidly significantly reduced at low concentrations of oil (17 h, 1.4 µL L^{-1},) indicating that *C. finmarchicus* have largest impact on oil spills at an early stage and at low concentrations. Ingestion of oil droplets contributed to rapid accumulation of all oil compounds. Accumulation from the WSF reached steady state for the low lipophilic (log K_{ow} < 5) compounds within 24 hours, while the high lipophilic compounds (log K_{ow} > 5) did not reach steady state within the 96 hour exposure. Over time, lower concentrations of the low lipophilic compounds were observed in oil dispersion exposed *C. finmarchicus* compared to WSF-exposed. This indicated elimination to the water, and may cause redistribution of these compounds during oil spills. Since the concentration of the high lipophilic compounds not was affected similarly, *C. finmarchicus* biomass may act as a sink for high lipophilic oil compounds.

The oil-containing feces from *C. finmarchicus* feeding in dilute oil dispersions contained significantly higher concentrations of viable oil-degrading microorganisms. The total microbial communities were similar between the clean and oil-containing feces, and the oil-degrading activity was suggested to be mediated by indigenous feces bacteria. The presence of oil-containing feces resulted in higher biodegradation of the n-alkanes in a dilute oil dispersion, while the presence of clean copepod feces resulted in lower biodegradation of the n-alkanes. This supported the suggestion that the indigenous feces bacteria were mediating the oil-degrading activity. These bacteria may have preferred carbon in feces prior to the carbon in the n-alkanes. The oil and copepod feces also formed large agglomerates. These may increase the sedimentation of relatively un-weathered oil towards the seabed during oil spills, depending on their effective density. The presence of clean *C. finmarchicus* feces resulted in higher biodegradation of the aromatic fraction, suggested to be caused by leaking of nutrients from the copepod feces. The presence of *C. finmarchicus* feces can thus increase the biodegradation of the dissolved fraction of an oil spill.

Conclusion:
The results indicated that a substantial concentration of oil can be contained in the *C. finmarchicus* biomass during oil spills. Further, *C. finmarchicus* biomass can contribute to redistribution of the low lipophilic oil compounds and function as a sink for dissolved high lipophilic compounds. The excretion of oil in feces increased the concentration of viable oil-degrading microorganisms in the feces, mediated by the indigenous feces bacteria. Biodegradation of the n-alkanes was dependent on the quantity of feces present, while the biodegradation of the aromatic compounds was increased in the presence of copepod feces.

TABLE OF CONTENTS

LIST OF PAPERS

I. Nepstad, R., Størdal, I.F., Brönner, U., Nordtug, T., Hansen, B.H. 2015. Modeling filtration of dispersed crude oil droplets by the copepod *Calanus finmarchicus*. Marine Environmental Research 105, 1-7. [1]

II. Nordtug, T., Olsen, A.J., Salaberria, I., Øverjordet, I.B., Altin, D., Størdal, I.F., Hansen, B.H. 2015. Oil droplet ingestion and oil fouling in the copepod *Calanus finmarchicus* exposed to mechanically and chemically dispersed crude oil. Environmental Toxicolocy and Chemistry 9999, 1-8. [2]

III. Størdal, I.F., Jenssen, B.M. Uptake of PAHs in *Calanus finmarchicus* from seawater petroleum oil dispersions and the water soluble fraction. *Manuscript.* [3]

IV. Størdal, I.F., Olsen, A.J., Jenssen, B.M., Netzer, R., Hansen, B.H., Altin, D., Brakstad, O.G. 2015. Concentrations of viable oil-degrading microorganisms are increased in feces from *Calanus finmarchicus* feeding in petroleum oil dispersions. Marine Pollution Bulletin 98, 69-77. [4]

V. Størdal, I.F., Olsen, A.J., Jenssen, B.M., Netzer, R., Altin, D., Brakstad, O.G. Biotransformation of hydrocarbons and microbial communities in seawater with small droplet oil dispersion and copepod feces. Marine Pollution Bulletin, doi: 10.1016/j.marpolbul.2015.10.029. [5]

Contributions:

[1] Paper I was initiated and planned by RN, UB, IFS, TN and BHH. RN and UB designed the model and performed the modeling exercise. The writing was performed by RN and IFS with comments BHH, TN and UB.

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[3] Paper III was initiated and planned by IFS, experiments were organized and run by IFS, the analytical work and writing was performed by IFS with comments from BMJ.

[4] Paper IV was initiated by IFS and planned by IFS in collaboration with OGB and RN. Experiments were organized and run by IFS, the analytical work, statistical analyses, and writing was performed by IFS with comments from OGB, RN, BMJ, BHH, AJO and DA.

[5] Paper V was initiated by IFS and planned by IFS in collaboration with OGB and RN. Experiments were organized and run by IFS, the analytical work, statistical analyses, and writing was performed by IFS with comments from OGB, RN, BMJ, AJO and DA.

ABBREVIATIONS

Abbreviation	Description
C5	*Calanus finmarchicus* fifth copepodite stage
BTEX	Benzene, toluene, ethylbenzene, xylenes
DVM	Diel vertical migration
FID	Flame ionization
GC	Gas chromatography
HC	Hydrocarbons
HM	Heterotrophic microorganisms
MPN	Most probable number method
MS	Mass spectrometry
ODM	Oil-degrading microorganisms
OSCAR model	Oil Spill Contingency And Response model
PAH	Polycyclic aromatic hydrocarbons
TPH	Total extractable Petroleum Hydrocarbons with carbon numbers from C10 to C36
WSF	Water soluble fraction

PhD thesis by Ingvild Fladvad Størdal

1 INTRODUCTION

The sources of crude oil in marine environments are both anthropogenic and natural. Worldwide, accidental oil spills constitutes approximately a quarter of the oil discharged each year. The remaining volume of oil is from natural seeps, or from intentional release of oil and oil-containing water (Wang and Stout, 2007). When crude oil is spilt in marine environments it is subject to transport and weathering processes, as oil dispersion formation and biodegradation (NRC, 1985). The transport and weathering of oil can be predicted using numerical models (Reed et al., 1995a; Reed et al., 1999a). These predictions form the basis for contingency and response operations during oil spill situations (Aamo et al., 1997). They should therefore be as precise as possible. The oil droplets that are entrained in the water in oil dispersions have been reported to be ingested by various marine filter feeders (Almeda et al., 2014; Conover, 1971; Hansen et al., 2012; Lee et al., 2012; Olsen et al., 2013). Therefore, the aim of this thesis was to investigate how the abundant marine filter-feeder *Calanus finmarchicus* influences transport and weathering of oil dispersions. (Fig. 1).
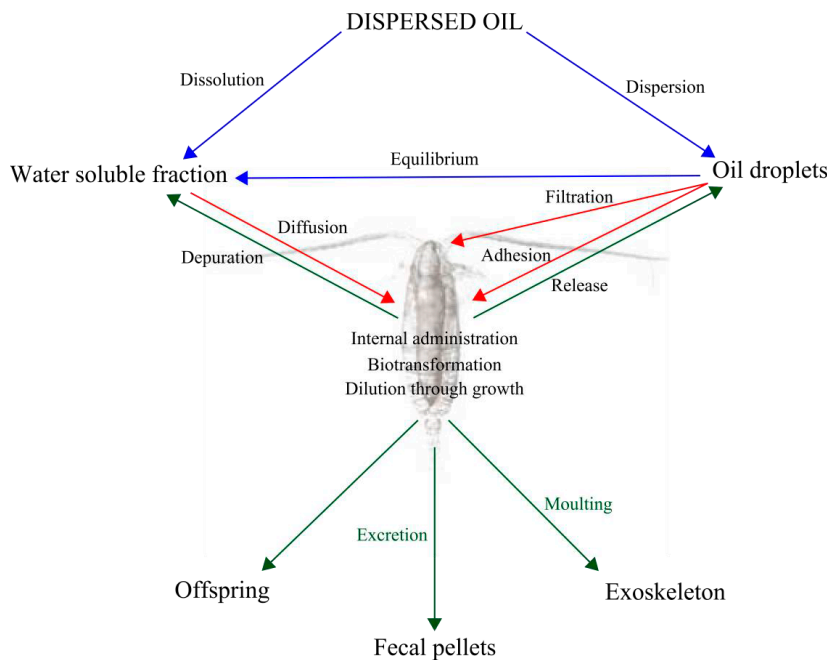

Fig.1. Potential interactions between the copepod *Calanus finmarchicus* and oil dispersions.
Figure credits: Bjørn Henrik Hansen, SINTEF/Environmental technology.

C. finmarchicus are ubiquitous in the North Sea, Norwegian Sea and Barents Sea and have a relative indiscriminate feeding behavior in addition to high feeding activity. Ingestion of oil may cause a significant amount of the particulate oil to be located in the *C. finmarchicus* biomass and feces. However, oil compounds may also accumulate from the dissolved fraction. Further, the transport and weathering of the accumulated oil compounds may be altered by redistribution within the copepods, by metabolization, and by eliminated to the water when they are located in the gut or in the tissue of the copepods compared to freely suspended in the water. Excretion of oil in copepod feces may influence biodegradation of the oil compounds and the location of biodegradation. The presence of copepods together with oil dispersions may also enhance biodegradation of oil dispersions, as activity of oil-degrading microorganisms may be limited in seawater by the availability of inorganic nutrients and copepods release nutrients into their surrounding by sloppy feeding and through feces.

1.1   Crude oil spills in marine environments

Crude oils are complex mixtures consisting of both hydrocarbon (HC) and non-HC compounds (Wang and Stout, 2007). The HC compounds in crude oil contain only carbon and hydrogen, while non-HC compounds also contains nitrogen, sulfur and oxygen (NSO compounds). The HC compounds can be classified based on structure as aliphatic and aromatic HCs (Wang and Stout, 2007). The major aliphatic HC compound groups in crude oil are n- and iso-alkanes, waxes (alkanes > 20 C), and naphthenes. The wax content of selected North Sea crude oils were found to vary between 0 and 18 wt.% (Rønningsen et al., 1991). The aromatic HC compounds present in crude oils are the mono-aromatic compounds (the BTEX compounds: Benzene, toluene, ethylbenzene, and xylene) and the polycyclic aromatic hydrocarbons (PAHs). The BTEX and PAHs are regarded as the toxicologically significant compounds (Page et al., 2002).

The composition and therefore also the physical and chemical properties of different crude oils are highly variable. Crude oils produced in the North Sea can be grouped into four categories: Naphthenic, paraffinic, waxy, or asphaltenic (Wang and Stout, 2007). Crude oils produced from the North Sea oil fields Gullfaks, Åsgard, Grane, and Norne, respectively, are examples of these four categories of crude oil. In this thesis, the naphthenic crude oil from the Troll oil field in the North Sea was used. The alkane content of the Troll oil has been found to be approximately 3.5% (Skaare et al., 2007). The physical and chemical weathering and transport processes is partly determined by the composition of the oil that is spilled to the environment (Daling et al., 1990).

1.1.1 Physical and chemical processes influencing an oil spill

The physical and chemical processes influencing oil spills in marine environments include spreading, drifting, sedimentation, stranding, evaporation, formation of oil-in-water (oil dispersions) and water-in-oil (emulsions) dispersions, dissolution and photo-oxidation (NRC, 1985). Dissolution of oil compounds from oil droplets is dependent on several factors, amongst others the concentration of the oil dispersion and the solubility of the compounds in the water and oil compartment (Nordtug et al., 2011; Redman et al., 2012). The oil compounds that dissolve to the water phase constitute the water soluble fraction (WSF) of the oil. The relative importance of weathering processes over time is illustrated in Fig. 2. However, their reciprocal importance is dependent on properties of the oil and environmental parameters, such as wind and waves (Daling et al., 2003). For instance, during the spill of 85 000 tonnes of the naphthenic oil Gullfaks after the grounding of the Braer outside Shetland in 1993, the formation of oil dispersions dominated (Ritchie, 1993). The formation of surface emulsions and evaporation of oil compounds are reduced when the formation of dispersions is rapid, however, dissolution to the water phase is increased (Daling et al., 2003).
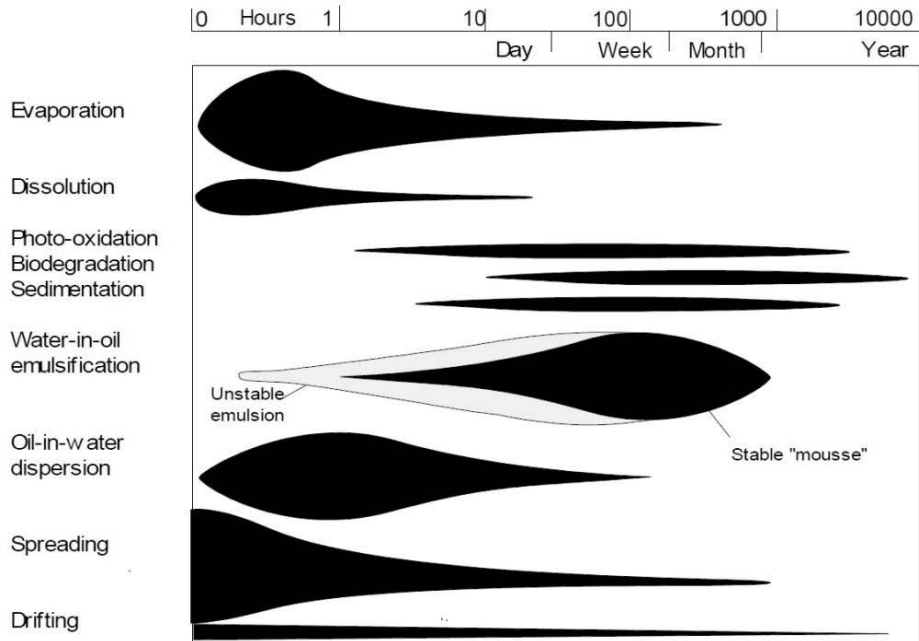

Fig. 2. The relative importance of weathering processes of oil over time (Strøm-Kristiansen and Daling, 1994).

Oil dispersions are formed in the water column when surface oil slicks are subjected to breaking waves (Delvigne and Sweeney, 1988). The entrainment of small droplets is enhanced by the use of dispersants (Brandvik, 1997; Li et al., 2009). Oil dispersed into the water column is rapidly diluted by turbulent movement of the sea (Lee et al., 2013). While large oil droplets resurface and form surface slicks, small droplets (diameters < 50-70 µm) have low surfacing rate and therefore remain entrained in the water column (Delvigne, 1993; Valentine et al., 2014). Oil dispersion exposures studies in laboratories should therefore include small oil droplets at low concentrations (Lee et al., 2013). The entrainment of oil into the water column relocates the oil to a different environmental compartment, for complete removal of oil from marine environments, biodegradation by microorganisms is fundamental (NRC, 1985).

1.1.2 Biodegradation of oil in marine environments

Oil is highly reduced carbon and it is a rich source of energy for the organisms that are capable of utilizing it. Oil-degrading microorganisms are ubiquitous in marine environments. When oil is introduced, their activity and quantity are rapidly increased, e.g. as shown in beach sands, temperate estuarine waters and during the Deepwater Horizon accident (Coulon et al., 2007; MacNaughton et al., 1999; Redmond and Valentine, 2012; Venosa et al., 1996). Active oil-degrading bacteria need bioavailable HCs, an electron acceptor, such as oxygen, and inorganic nutrients. Since oil has a high content of carbon, oil degradation can be limited by the available concentrations of inorganic nutrients, such as nitrogen and phosphorus (Atlas and Bartha, 1972; NRC, 1985). Supplying an oil spill area with inorganic nutrients may thus enhance the degradation of oil compounds (Atlas, 1995; Röling et al., 2002; Venosa et al., 1996). However, as discussed by Dell'Anno et al. (2012), and others (Head and Swannell, 1999; McKew et al., 2007b; Smith et al., 1998), the extent of biodegradation of petroleum HC depends on the interplay between several factors; the presence of oil-degrading bacteria, the interaction and competition over resources between oil-degrading bacteria and other bacteria, and biotransformation through co-metabolism. Co-metabolism oxidizes an oil compound without harvesting the energy derived from the oxidation (Bouchez et al., 1995). Studies have shown that very different oil-degrading bacterial communities develop under similar conditions, and that these communities are capable of degrading oil to the same extent (Röling et al., 2002).

Bacteria capable of degrading oil are identified within the classes Alphaproteobacteria, Gammaproteobacteria, and Flexibacter-Cytophaga-Bacteroides (Head et al., 2006; Prince, 2005; Yakimov et al., 2007). After oil spills, a limited number of oil-degrading bacteria have been observed to bloom in succession (Dubinsky et al., 2013). The bacteria abundance corresponded approximately with the biodegradation rates of the oil

compounds. The biodegradation rates have the following order: n-alkanes > branched alkanes > low-molecular weight aromatics > cyclic alkanes (Leahy and Colwell, 1990). High lipophilic oil compounds are biodegraded at the oil-water interphase, while low lipophilic compounds are degraded in the water phase (Brakstad et al., 2004). The lipophilic properties of oil compounds can be quantified as their partitioning between the two phases water and octanol, expressed as their log K_{ow} value.

1.2   Predicting oil spill weathering and transport

Oil weathering and transport can be forecasted using numerical models to provide a foundation for contingency and response operation decisions during oil spills. One such model is the Oil Spill Contingency and Response (OSCAR) software (Reed et al., 1995a; Reed et al., 1999a). The model includes description of the physical environment, as coastline, currents and waves, and the physical and biological weathering and transport processes of spilled oil, including advection, spreading, dissolution, volatilization, dispersion of oil into the water, and biodegradation. As input to the OSCAR software, the chemical composition and properties of numerous crude oils are provided from laboratory experiments (Daling et al., 2003). The model thus accounts for the behaviors of different crude oils. The distribution of oil is calculated in three physical dimensions, plus time. The distribution of oil is predicted for water surface, along shorelines, in the water column and in the sediments. Decay and transformation processes are calculated separately for pseudo-components in numerical particles. Biodegradation is accounted for by first-order rate constant kinetics of these oil compound groups in oil droplets, for dissolved components in the water, in surface oil and in oil in sediment.

1.3   Crude oil in zooplankton biomass

Oil compounds may accumulate to zooplankton by ingestion of particulate oil and by passive partitioning over body surfaces. Various species of zooplankton have been reported to ingest oil (Almeda et al., 2014; Conover, 1971; Hansen et al., 2012; Lee et al., 2012). Therefore, already in 1971, zooplankton were suggested as biotic contributors to weathering of oil, after observations of ingestion of bunker C oil by zooplankton after an oil spill in Nova Scotia (Conover, 1971). Also, passive partitioning over body surfaces of copepods contributes to the concentration of oil compounds in zooplankton (Jensen et al., 2012). Reports on extensive passive elimination of oil compounds indicate that the cycling of oil compounds between zooplankton and water are important for the transport of these compounds in marine environments (Almeda et al., 2013; Berrojalbiz et al., 2009).

1.3.1 The calanoid copepod *Calanus finmarchicus*

The species used as a model pelagic filter-feeder in the present thesis was *C. finmarchicus* (Gunnerus). These copepods are ubiquitous in the North Sea, the Norwegian Sea, and the Barents Sea. In the North Atlantic, the copepod contributes to between 50 and 90% of the total copepod biomass (Planque and Batten, 2000). Population densities may be as high as 3 000 *C. finmarchicus* m^{-3} in the upper 60 m of the water column (Helle, 2000). The densities are highly irregular across copepodite and nauplii stage, geographical area and season (Helle, 2000; Unstad and Tande, 1991).

C. finmarchicus matures from egg to the adult, reproductive stage through six naupliar stages (N1-6) and five copepodite stages (C1-5) (Fig. 3) (Marshall and Orr, 1972). The duration of the life cycle varies from 2-3 months up to 1-2 years, depending on the temperature of the water (Falk-Petersen et al., 2009). During its development, *C. finmarchicus* generally performs seasonal migration to deeper waters from fall until early spring. After diapause the copepods ascend to surface waters, molts into mature, reproducing adults and spawn (Marshall and Orr, 1972).
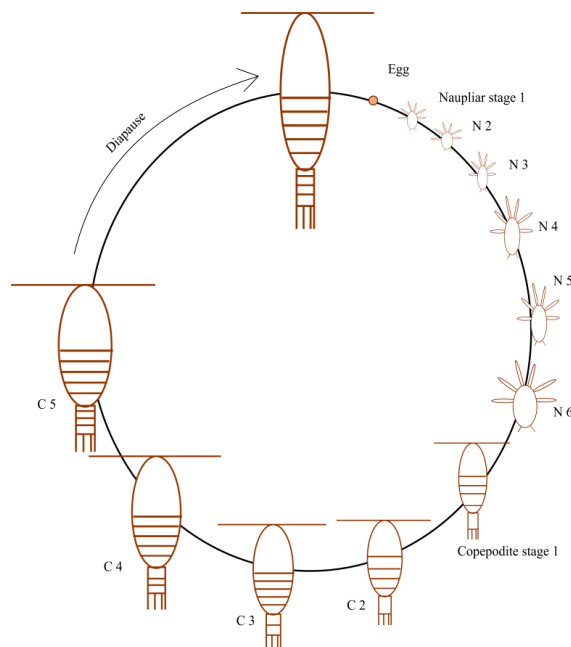

Fig. 3. *Calanus finmarchicus* life cycle consists of six naupliar (N1-6) stages and five copepodite (C1-5) stages. As stage C5 the copepods migrates to deeper waters for diapause. The figure is modified from description given in Marshall and Orr (1972).

In addition to seasonal migration, *C. finmarchicus* perform diel vertical migration (DVM). This is regarded as a anti-predator behavior, and involves active feeding in the surface layers at night while migrating to deeper waters (50-150 m depth) during the day (Baumgartner et al., 2011; Hays, 2003). However, the DVM is not unwavering, as actively feeding *C. finmarchicus* have been reported in surface waters during daytime (Baumgartner et al., 2011). The depth which they migrates to depends on the light attenuation in the water column (Miljeteig, 2013). Recently, *C. finmarchicus* exposed to WSF displayed enhanced movement towards light, i.e. positive phototaxis (Miljeteig et al., 2013). This indicates that oil spills may increase the presence of copepods in surface waters.

A characteristic feature of *C. finmarchicus* is its large lipid reserve, mainly composed of wax esters. These are contained within the lipid sac positioned dorsally on the animal (Falk-Petersen et al., 2009). The lipid sac constitutes as much as 34% of the body volume (Falk-Petersen et al., 2009). *C. finmarchicus* is rich in energy and is a keystone species in the North Atlantic, transferring energy from primary production to higher trophic levels (Sakshaug et al., 1994). The lipid sac of *C. finmarchicus* may also be a potential site for accumulation of oil compounds, as was suggested by Hansen et al. (2009) after studies with lipid-rich and lipid-poor female *C. finmarchicus*.

1.3.2   Feeding by *C. finmarchicus*

The feeding behavior of *C. finmarchicus* can be divided into non-selective suspension feeding and raptorial predator feeding on single particles (Kiørboe, 2011). Non-selective suspension feeding takes place by generating two vortexes of moving water perpendicular on each side of the prosome of the copepods and subsequently using a sieve-like appendage to collect particles from the water moving in the vortex. The mechanism for non-selective suspension feeding was first described by Cannon (1928). Particles that are collected on the sieve-like appendage are scraped off by a third appendage, passed forward to the mandibles and ingested. The animal-to-animal variability in feeding strategy has been reported to be significant under identical conditions, after observing female *C. finmarchicus* (Turner et al., 1993).

Feeding activity of *C. finmarchicus* can be quantified from the number of particles removed from a volume of water with a known concentration of particles. This can be converted to the volume of water swept clear of feed particles per copepod per unit time, the clearance rate of copepods (Frost, 1972). Feeding can also be quantified by other methods, such as by fecal pellet production (Spooner and Corkett, 1979). Clearance rates for C 5 *C. finmarchicus* varies from 40 to 500 mL copepod^{-1} day^{-1} (Irigoien et al., 1998; Nejstgaard et al., 1995). Feeding activity has been reported to depend on e.g. algae type, particle concentration, and oil exposure (Hansen et al., 2012; Nejstgaard et al., 1995; Spooner and Corkett, 1979). The algae composition in *C.*

finmarchicus feces have been found to fairly accurately reflect the algae composition in the water column, the feeding on small particles are suggested to be by non-selective suspension feeding (Leiknes et al., 2014; Urban et al., 1993). The size of the particles ingested by *C. finmarchicus* is downwards restricted by the distance between the projections, called setae, used for collecting particles from the feeding vortex, and upwards by the size of the mouthparts. A proposed demarcation for the size of particles ingested by calanoid copepods is 5 to 100 μm diameter (Boyd, 1976), while Urban et al., (1993) stated that *C. finmarchicus* preferred particles between 20 and 60 μm. However, *C. finmarchicus* is assumed to be capable of ingesting larger particles, as *Calanus* copepods are observed to behave as cannibals and ingest nauplii of all stages (Basedow and Tande, 2006; Bonnet et al., 2004). The size of N1 and N6 are approximately 200 and 700 μm, respectively (Marshall and Orr, 1972).

1.3.3 Copepod feces in marine environments

The degradation and transport processes of *C. finmarchicus* feces in marine environments are important for the weathering and transport of oil in the presence of *C. finmarchicus* since ingested oil is excreted in the copepod feces (Conover, 1971; Olsen et al., 2013; Spooner and Corkett, 1979).

The sedimentation of copepod feces has been reported to be minor across very different marine environments; the Norwegian Sea, the Humboldt Current, and the Southern Indian Ocean (Bathmann et al., 1987; Gonzalez et al., 2000; Møller et al., 2011). In both the Baltic Sea and the Indian Ocean, > 99% of copepod fecal material was remineralized within the upper mixed water layer (Møller et al., 2011; Viitasalo et al., 1999). The low degree of sedimentation of copepod feces is generally caused by high rates of degradation in the upper water column. However, both density and degradation of copepod feces varies with the composition of the fecal pellets, and the composition of the fecal pellets depends on the phytoplankton community that the copepods are grazing on (Small et al., 1979; Thor et al., 2003; Urban et al., 1993). Urban et al. (1993) showed that fecal pellets from *C. finmarchicus* feeding on diatoms and dinoflagellates had a density of 1.11 g cm^{-3}, while those produced on a nanoflagellate diet had a density of 1.19 g cm^{-3}. Nanoflagellates can be packed tighter compared to diatoms and dinoflagellates, which enables production of denser, more compact fecal pellets. Fecal pellets produced by *Acartia tonsa* feeding on *Rhodomonas baltica* have been reported to be degraded faster compared to those produced on a diatom or dinoflagellate diet (Hansen et al., 1996; Thor et al., 2003).

The degradation of copepod feces in the water column is mediated by a diverse community of heterotrophic organisms: Cyclopoid copepods, the copepods themselves, ciliates, dinoflagellate, and microorganisms, as bacteria (Gonzalez and Smetacek, 1994; Iversen and Poulsen, 2007; Møller et al., 2011; Poulsen and Kiørboe, 2006; Svensen et

al., 2012). The release of material from copepod fecal pellets has been reported to increase the activity of free-living bacteria in the surrounding sea water (Carman, 1994). From these results, Carman (1994) suggested that fecal pellets are important sources of inorganic and organic carbon, nitrogen and silica for microbial communities in the water column. In addition, the copepod feces have higher density of bacteria compared to the more dilute surrounding seawater and they are regarded as microbial hotspots (Tang, 2005). The sources of the bacteria in copepod feces are the gut of the copepods, the surrounding seawater, and the ingested particles (Hansen and Bech, 1996; Poulsen and Iversen, 2008; Tang, 2005). The bacterial communities colonizing copepod feces are generally different from bacterial communities in seawater (Delille and Razouls, 1994; Jing et al., 2012).

1. Ingestion of oil by *C. finmarchicus*

2. Accumulation of oil compounds in *C. finmarchicus*

1 mm

3. Microbial communities in *C. finmarchicus* feces and biodegradation of oil in the presence of feces
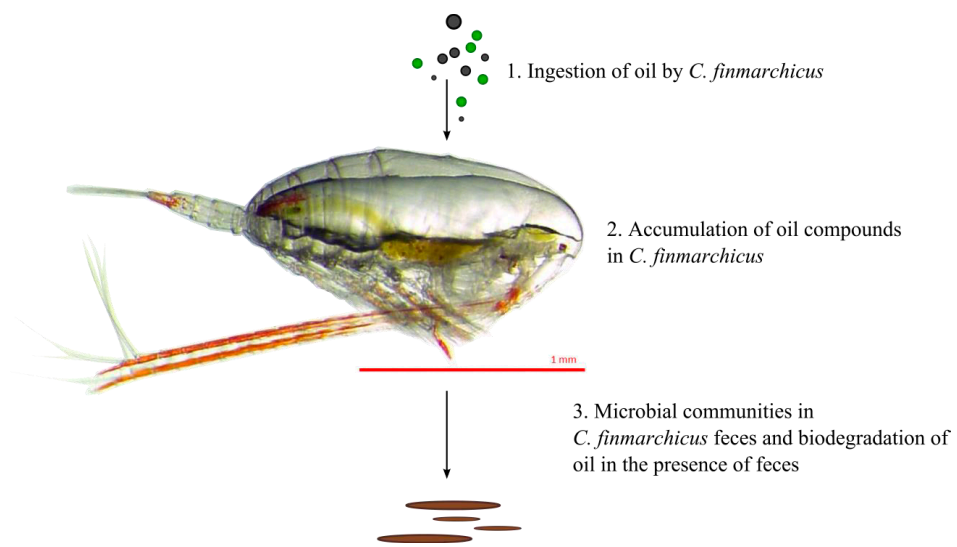
Fig. 4. The aim of this thesis was to investigate how the abundant marine filter-feeder *Calanus finmarchicus* influence transport and weathering of oil dispersions. To achieve this aim, the three numbered focus areas with subsidiary research objectives were addressed.

2   AIM OF THE THESIS

The aim of this thesis was to investigate how the abundant marine filter-feeder *C. finmarchicus* influence transport and weathering of oil dispersions. *C. finmarchicus* is ubiquitous in the North Sea, the Norwegian Sea and North in the Atlantic Ocean where it is recognized as a keystone species. It has been extensively studied for decades and the physiology and ecology of the species is well known. Due to high population densities, high feeding activity and indiscriminate feeding, *C. finmarchicus* were expected to influence oil spill transport and weathering.

To achieve the aim of this thesis, the following three focus areas (illustrated in Fig. 4, page 10), with subsidiary research objectives were addressed:

Ingestion of oil by *C. finmarchicus* (1).

    i.   Determine the fraction of an oil spill that can be ingested by *C. finmarchicus* (Paper I)

    ii.   Determine the concentration of oil in *C. finmarchicus* biomass feeding in oil dispersions (Paper II).

    iii.   Determine the effect of oil dispersions on feeding activity of *C. finmarchicus* (Paper II and Paper IV).

Accumulation of oil compounds in *C. finmarchicus* (2).

    iv.   Determine accumulation of oil compounds to *C. finmarchicus* from oil dispersions (Paper III).

    v.   Determine accumulation of oil compounds to *C. finmarchicus* from the WSF of oil dispersions (Paper III).

Microbial communities in *C. finmarchicus* feces and biodegradation of oil in the presence of feces (3).

    vi.   Characterize microbial communities in *C. finmarchicus* feces with and without oil (Paper IV).

    vii.   Determine biodegradation of oil compounds in crude oil droplets in the presence of clean and oil-containing feces from *C. finmarchicus* (Paper V).

    viii.   Characterize microbial communities in crude oil dispersions in the presence of clean and oil-containing feces from *C. finmarchicus* (Paper V).

3   METHODS

3.1   OSCAR software and filter-feeder module description

To estimate the fraction of an oil spill due that is removed due to ingestion of droplets by *C. finmarchicus*, the OSCAR software was run with a filter-feeder module implemented.

3.1.1   The OSCAR model

The OSCAR software model has been thoroughly described (Reed et al., 2004; Reed et al., 1999a; Reed et al., 1995b; Reed et al., 1999b). Briefly, the model is based on a transport-reaction equation (shown in Paper I) accounting for physical, chemical and biological processes affecting an oil spill, which calculates the concentration of a number of specified pseudo-components as they evolve over time. Generally, 25 pseudo-components are included, and each of these components are separately subjected to decay and transformation processes. Advection and turbulent diffusion in the water column is also accounted for in the model since ocean currents and wind fields are provided as input to the OSCAR software. These inputs drive the transport of the oil in three dimensions. The transport-reaction equation accounting for the weathering processes of the oil is solved numerically using a particle model. Each numerical particle contains a certain amount of oil. Each particle is treated separately, and has a spatial location within the model area.

3.1.2   The filter-feeder module

To calculate the removal of oil from an oil spill by *C. finmarchicus*, a rate equation (shown in Paper I) was implemented into the OSCAR software. The equation calculated removal of oil over time, and included the following parameters: Population density of *C. finmarchicus*, feeding activity quantified as clearance rate, reduction in clearance rate based on oil concentration, location of actively feeding *C. finmarchicus* in the water column, and the size of oil droplets ingested.

3.1.3   Model testing

To test the filter-feeder module, two oil spill scenarios were simulated using OSCAR with the module implemented. The scenarios considered were two accidental releases of 25 000 tonnes of Troll crude oil being released either at the sea surface or at the seafloor. The scenarios specifies the oil spill simulation by location, release rate, oil type and amount, release and simulation duration, and the boundary conditions which includes the model area and the environmental driving forces. The OSCAR software calculates the transport and weathering of the oil, and the filter-feeder module considers

removal of oil by *C. finmarchicus*. For each release scenario, the simulations were run with a base case, and five additional cases to explore the importance of the input parameters for removal of oil. The size of oil droplets ingested was explored in two cases, while the population density, reduction in clearance rate caused by the oil concentration, and the location of actively feeding copepods in the water column were explored in one case each. To see how population density and size of the oil droplets scaled with total oil removed, a scan was performed for each of these parameters.

3.2 Experimental set-up

3.2.1 Exposure solutions

3.2.1.1 Seawater
Seawater used for all experimental work was supplied to the laboratory facilities from 80 m depth in Trondheimsfjorden, Norway (63°26′N, 10°23′E) through a pipeline system. The seawater was collected from below the thermocline and is considered to be non-polluted and not to be influenced by seasonal variations. The seawater has previously been found to have a salinity of 34‰, a temperature of 6-8 °C, and dissolved oxygen of approximately 8 mg L^{-1} (Brakstad et al., 2004). Prior to use, the seawater was sand-filtered to remove coarse particles and adjusted to ambient atmospheric conditions.

3.2.1.2 Small droplet oil dispersions
Oil dispersions were generated for exposure of *C. finmarchicus* (Paper II, III, IV and V), and to study biotransformation of oil in the presence of feces (Paper V). Oil dispersions were made using the oil droplet generator, according to Nordtug et al. (2011). The method enables tight control of the oil dispersion parameters, and implies that a small flow of oil is directed perpendicular to a larger flow of water (Fig. 5). The flow of the oil was controlled by a syringe pump (Aladin AL-1000, WPI Precision Intruments, UK) and the flow of the water by a metering pump (Fluid Metering Inc., NY, USA). The force of the water tears the oil stream apart and generates small droplets of oil. The oil and water mixture is further forced through three narrow nozzles (inner diameter 0.5 mm) generating repeated turbulence in the oil-water mixture. To simulate real oil spills, oil dispersions with small droplets (diameter < 40 μm) at low concentrations (< 5.5 mg L^{-1}) were generated (Lee et al., 2013).

3.2.1.3 Water soluble fraction
Oil dispersions and the corresponding WSF were generated to study the accumulation of oil compounds from the two oil fractions (Paper III). The WSF was produced as described in Nordtug et al. (2011) by filtration of the oil dispersion using glass wool

(approximately 15 g) and a GF/C filter (pore size 1.2 µm, Whatman Ltd., Maidstone, UK) mounted in a filter cartridge. This procedure removes the particulate oil efficiently, but leaves the dissolved oil components. To compensate for the increased flow resistance introduced by the glass wool and the GF/C filter, a metering pump (Fluid Metering Inc., NY, USA) was positioned inline between the filtering cartridge and the exposure tanks. The flow of the inline metering pump was adjusted to half of the flow of the metering pump feeding seawater to the oil droplet generator. The remaining half of the flow from the oil droplet generator was passed passively into the oil dispersion exposure tanks.
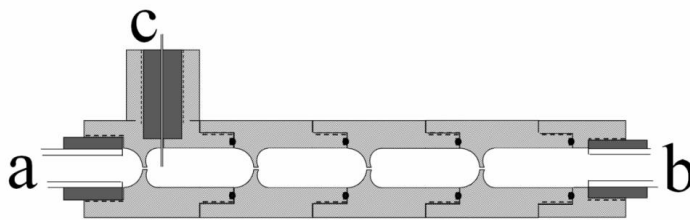
Fig. 5. The oil droplet generator produces oil droplet dispersions with a defined and restricted size of the droplets. The seawater inflow (a) and the inlet capillary for oil (c) are directed perpendicular to each other. The oil-water mixture is forced through three narrow nozzles creating an oil dispersion (b) with defined size distribution of the droplets.

Figure credits: Nordtug et al. (2011).

3.2.2   *C. finmarchicus* culture

C. finmarchicus for the experimental work (Paper II-V) were obtained from the continuous culture kept at NTNU/SINTEF Sealab (Trondheim, Norway). The culture was established in October 2004 (Hansen et al., 2007) and had been running for 34 (Paper II and III) and 38 (Paper IV and V) generations when used. The culture was kept in polyester containers (280 L) at ~10 °C in continuously running seawater in a climate room at 8-10 °C with a light:dark regime of 18:6 hours. The cultures were regularly fed a diet of microalgae (*R. baltica* Karsten, *Dunaliella tertiolecta* Butcher, *Isochrysis galbana* Parke).

C. finmarchicus C 5 were used in all laboratory exposure studies.

3.2.3 *C. finmarchicus* **exposure**

All experiments were performed with flow-through of exposure solution. The set-up used in Paper II has been described in detail in Nordtug et al. (2011). Briefly, it consists of 5 L borosilicate flasks (Schott AG, Mainz, Germany) with the bottoms removed and mounted up-side down in a rig. Two rigs with 14 flaks each were deployed resulting in a total of 28 exposure tanks. Four of the tanks were run with clean seawater and served as control. The exposure treatment included three oil concentrations with four biological replicates each, for both oil dispersed with and without chemical dispersant. Three additional tanks were used as algae control without copepods.

The set-up used in Paper III, IV and V consists of 20 L round bottom flasks with detachable lids (Fig. 6). The total exposure volume of the tanks was approximately 18L. Exposure solutions were supplied to the exposure tanks through teflon capillary tubing. The flow of the exposure solutions was regulated by the height of the water column upstream of the exposure tanks, and was restricted by the length of the teflon tubes going into the exposure tanks, as described by Nordtug and Olsen (1993). Feed algae were supplied by a stricture pump (Cole-Parmer Instrument Co. Chicago IL, USA) also through teflon tubes.

For all experiments the *R. baltica* was used as feed algae. *R. baltica* have an equivalent mean spherical diameter of approximately 6.9 μm.
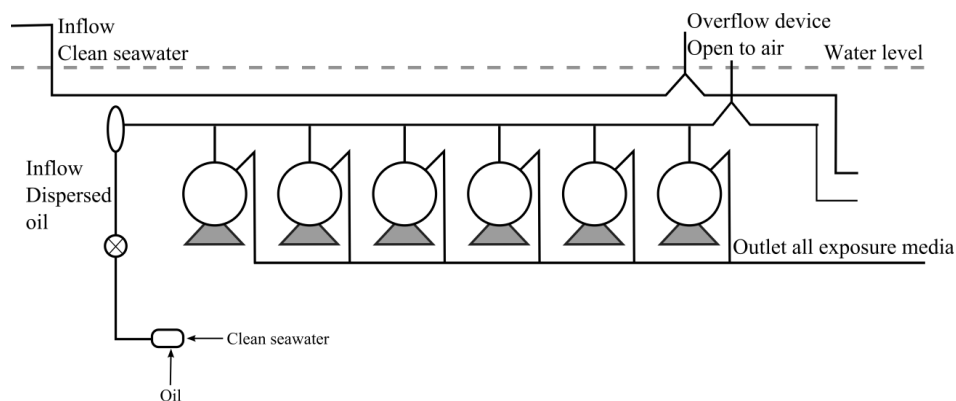

Fig. 6. Schematical representation of exposure set-up used for exposing *Calanus finmarchicus* to oil dispersion, dissolved oil compounds, and clean seawater.

3.2.4 Handling of *C. finmarchicus* and feces

3.2.4.1 C. finmarchicus

At the start of each exposure of *C. finmarchicus* for Paper II, III, IV and V, copepods were carefully introduced to the tanks using a plastic scoop. A custom made scoop was used for sampling of copepods from the experimental set-up used in Paper III. Following the entire circumference of the wall in the scoop, a strip of mesh (20 μm pore size) was inserted halfway up the wall to allow drainage of water without losing *C. finmarchicus*. Sampling was performed carefully not to damage the copepods.

3.2.4.2 Feces

Feces from *C. finmarchicus* were collected to study the microbial communities (Paper IV), and to study biodegradation of oil in the presence of feces (Paper V). The sampling was performed using an acrylic suction pipe, a 500 mL borosilicate flask (Schott AG, Mainz, Germany) filled with seawater, and a peristaltic pump (Watson-Marlow, Falmouth, Cornwell, UK). The peristaltic pump and the suction pipe were both connected to the flask and the pump created suction at the distal end of the suction pipe. The suction was sufficient to transport fecal pellets from the bottom of the tank and into the borosilicate flask. The inflow at the distal end of the suction pipe was supplied with a mesh sieve (200 μm pore size) to keep *C. finmarchicus* from entering, and the outflow from the flask was supplied with a fine mesh (15 μm pore size) to detain feces within the flask. After removal of copepods, the water volume of the exposure tanks was filtered using a bowl with a mesh (20 μm pore size) inserted at the bottom to collect feces suspended in the water. The total volume of the feces suspension in the flask used for collecting the feces was reduced using a bowl with a filter (20 μm pore size) bottom. The bowls were washed with detergent (Neodisher® LaboClean A 8) and rinsed using ethanol (96%) to sterilize them prior to use. In these bowls, the collected copepod feces were thoroughly rinsed with filtered and autoclaved seawater to minimize the amount of oil adhering to the external surfaces of the fecal pellets. When sampling feces for the experiments, each exposure tank had its own dedicated flask and suction pipe to minimize contamination between the samples.

3.2.5 Incubation of *C. finmarchicus* feces and oil dispersions

Incubation was performed with clean and oil-containing *C. finmarchicus* feces (Paper IV), and oil dispersions and feces (Paper V) to study microbial communities and biodegradation of oil, respectively. The incubations was performed in 2 L borosilicate flasks (Schott AG, Mainz, Germany) completely filled (no headspace), and mounted on a carousel system (Fig. 7). The carousel system rotated in the vertical plane at 0.75 rounds per minute by a gear motor (SEW Eurodrive, Moss, Norway). Each carousel system had double sets of wheels, and each wheel could hold 8 flasks. Each carousel system could thus hold a total of 16 flasks. This carousel system has previously been

found to maintain oil dispersions with oil droplet size < 30 µm in 2 L flasks (Brakstad et al., 2015).
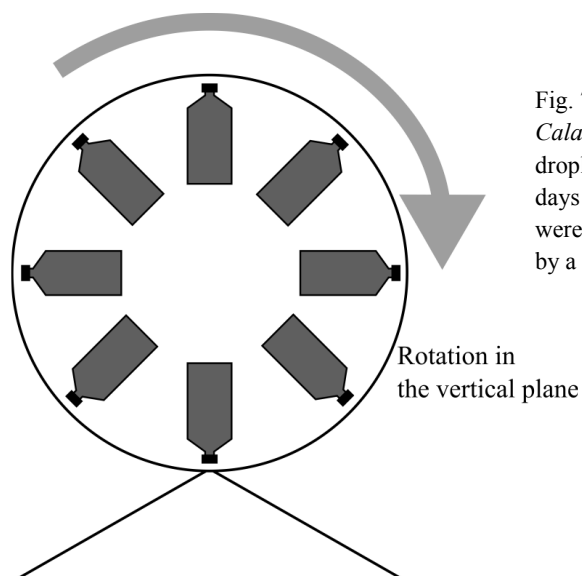
Fig. 7. Carousel system used for holding *Calanus finmarchicus* feces and oil droplets in suspension during the 14 days of incubation in seawater. Flasks were rotated at 0.75 rounds per minute by a gear motor in the vertical plane.

Rotation in the vertical plane

3.3 Experimental methods

3.3.1 Particle count analyses

Concentration and particle size of algae and oil droplets were monitored by a Coulter Counter (Multisizer 3, Beckman) during all experiments. When samples contained both algae and oil droplets, the volume of alga particles were separated from the volume of oil particles by extrapolating the curve for the oil droplets in the region of the algae peak (approximately between 5 and 9 µm, see Hansen et al. (2012)). The volume of algae particles was calculated by subtracting the volume of the oil particles from the total particle volume.

3.3.2 Quantification of feces

To quantify feces collected from exposed *C. finmarchicus*, three methods were used.

a) In Paper III, feces were sampled from all exposure tanks at 24, 48, 72 and 96 hours. Feces were concentrated using bowls with mesh (20 µm pore size) in the bottom, and subsamples (30 µL/600 µL) were transferred to pre-weighed tin cups and dried (80 °C, 48 hours). For each sampling time and each exposure tank, two parallel samples were included, resulting in a total of eight quantifications of feces for each sampling time

from each treatment. The two parallel sample series were treated separately. When the weights of the two parallels were compared there was a systematic increase between the first and second parallel. This is suggested to have been caused by absorption of water from the air by the salt left when the seawater evaporated. In addition, the weight of the tin cups was large compared to the weight of the seawater/feces, which further reduced the accuracy of the weighing. Data on feces quantity were not included in Paper III since the data were considered to be inaccurate.

b) In Paper IV, feces were sampled from exposure tanks after 48 hours of exposure to clean seawater or oil dispersions. Feces were concentrated using bowls with a mesh (20 μm pore size) in the bottom, and subsamples (0.5 mL/16 mL) were transferred to a custom made sieve with a grid and photographed using a Leika MZ125 dissecting microscope (Leica Microsystems, Wetzlar, Germany) with a digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo) operated by Fire-I software (Unibrain, Inc., San Ramon, CA). The length and width of fecal pellets from each treatment were determined on scaled pictures. The volume of one fecal pellet was estimated assuming that one fecal pellet was represented by a cylinder and two half sections of one sphere on each end of the cylinder. The total volume of feces in the samples was calculated from counting of fecal pellets. When counting, the fecal pellets were assigned to three categories: Intact pellets, fragments with one rounded end, and fragments with two blunt ends. The total number of fecal pellets in each subsample was assigned from all intact fecal pellets enumerated plus half the number of fragments where one rounded end clearly could be seen. Fragments with one rounded end were considered to be the ends of fragmented fecal pellets and two ends represent one fecal pellet.

c) In Paper V, feces were sampled from exposure tanks after 48 hours of exposure to clean seawater or oil dispersion. Feces were concentrated using bowls with a mesh (20 μm pore size) inserted in the bottom, and subsamples (0.5 mL/16 mL) of feces and seawater were sieved through a custom made set-up with pre-weighed, dried miniature mesh sieves (mesh size 20 μm). The use of sieves was assumed to minimize the quantity of salt from seawater after drying. The filters with feces were dried and weighed to determine dry-weight of the feces. When sampling the 16 mL glass containers, a clear difference in feces produced by oil-exposed and control *C. finmarchicus* was observed (Supplementary Information, Paper V). However, the weights of feces from the different treatments were similar and the data were therefore disregarded. From these results, we interpreted that the dry-weight of the feces in the subsample was not sufficient to quantify differences between the two treatments.

The results from the second method (b) gave the most precise results, and it is recommended that this method is used in further quantifications of copepod feces.

3.3.3 Quantifying oil concentration in *C. finmarchicus* and seawater

Gas chromatography (GC) with a Flame Ionization Detector (FID) or a mass spectrometry (GC-MS) operated in selected ion monitoring mode was used for chemical analyses of exposure solution or *C. finmarchicus* biomass. Quantification was based upon the use of a suite of reference compounds.

Samples of exposure solution (approximately 800 mL) were acidified using dilute hydrochloric acid (HCl, 15%) upon sampling (pH < 2) and solvent-solvent extracted using dichloromethane (DCM). The extracts were dried using anhydrous sodium sulphate concentrated to 1 mL under nitrogen gas in a heating block (35 °C).

C. finmarchicus biomass (21 ind.) was sampled directly into sterilized glass-vials and frozen prior to extraction. To obtain saponification of the samples, the vials were added potassium hydroxide (3 mL, 6.5%) in methanol (80%) and internal standards and heated (2 hours, 80 °C) in an ultrasonic bath. The samples were filtered to remove coarse material and extracted three times using hexane (3 x 3 ml). The extracts were dried using Na_2SO_4 and concentrated to approximately 0.5 ml using a Zymark Turbovap® 500 Concentrator. The concentrated extracts were cleaned using solid phase extraction with 3 ml columns containing 0.5 g normal phase silica packing (Superclean LC-Si, Supelco). The columns were eluted using 3 x 2 ml of DCM:hexane (1:3). Finally, the extracts were concentrated to 90 µl under nitrogen gas in a heating block.

For analyses of oil-containing feces in seawater (Paper IV) and of oil dispersions with and without feces (Paper V), the samples were treated as exposure solution samples. The final volume of the solvent extract was 100 µL.

Oil concentration in exposure solution and biomass were quantified by different methods in the papers included in this thesis and were therefore reported using different units (Table 1). In Paper IV and V, the oil concentrations in the exposure solution were determined using particle counting. This analysis produces volume concentrations, reported as ppm or µL L^{-1}. Paper II quantified the oil exposure concentration by both particle count analyses and GC-FID analyses of the extractable fraction of the crude oil. Concentration from GC-FID analyses were reported as mg Total extractable Petroleum Hydrocarbons with carbon numbers from C10 to C36 (TPH) L^{-1}. The particle count analyeses and the GC-FID analyeses produced similar oil concentrations (Paper II), showing that results from these two methods are comparable. Oil concentration in *C. finmarchicus* biomass was found by GC-MS analyses of compounds expected to remain exclusively in the oil phase. The concentration of oil was calculated from the concentration of these compounds in the biomass compared ot their concentration in the source oil (Paper II). This oil concentration was reported as mL kg^{-1} copepod.

Table 1. Denomination and method of detection for oil concentration in seawater and biota.

	Definition	Quantity	Method of detection
Oil exposure concentrations			
µL /L	$\dfrac{\text{µL oil}}{\text{L seawater}}$	Volume concentration	Coulter counter analyses
ppm	Parts per million	Volume concentration	Coulter counter analyses
mg TPH (C5-C36)/L	$\dfrac{\text{mg TPH}}{\text{L seawater}}$	Mass concentration	GC-FID
Oil concentration in biomass			
mL/kg	$\dfrac{\text{mL oil}}{\text{kg copepod}}$	Mass concentration	GC-MS

3.3.4 Quantification of oil in feces

In Paper III, the concentration of oil compounds in *C. finmarchicus* feces were tentatively quantified. Samples with feces in seawater (540 µL) were treated in the same way as *C. finmarchicus* biomass, with saponification and removal of biogenic material. Chemical analysis using the GC-MS was performed with similarly conditions as when body burden in *C. finmarchicus* biomass are analysed. Contrary to what was expected, high lipophilic compounds were detected in feces from the WSF exposure. The WSF exposure solution did not contain these compounds and they were therefore not expected to be present in WSF feces. For this reason, the analyses of the feces were assumed to be incorrect, and the results were not included in Paper III.

For determination of the oil compounds in seawater with oil-containing feces (Paper IV) and for quantification of biodegradation of target oil compounds (Paper V), samples were treated as water samples. When performing GC-MS analyses, the mass spectrometer was operated with extractor ion source (etune) which enabled higher sensitivity by transporting more ions to the mass filter. In addition, the samples were concentrated to 100 µL. This second approach produced reliable results.

3.3.5 Characterization of microorganisms

The microbial communities in samples with clean and oil-containing *C. finmarchicus* feces (Paper IV), and in samples with oil dispersions and feces (Paper IV) were characterized by the most probable number method (MPN) detecting the concentration of viable heterotrophic (HM) and oil-degrading (ODM) microorganisms, and by 16S rRNA gene amplicon library analyses detecting relative abundance of all bacteria present in the samples. The total numbers of cells in the samples were enumerated using 4', 6'-diamidino-2-phenylindole (DAPI) staining and oil immersion fluorescence microscopy

3.3.5.1 Total number of cells

Total cell counts were enumerated in samples stained with nucleic acid DAPI stain using oil immersion fluorescence microscopy at 1,250 times magnification (Nikon Eclipse 80i) (Porter and Feig, 1980). Samples were diluted in sterile particle-free water, incubated in DAPI (0.06 mg mL^{-1}) for 10 min, and filtered (0.2 μm pore size, black polycarbonate filters, Nucleopore, Costar, Cambridge, USA).

3.3.5.2 Concentrations of viable microorganisms

Concentrations of viable HM and ODM were analysed in 24-well tissue culture plates (Costar). HM were enumerated in Marine Broth 2216 medium (Difco), and ODM with Bushnell-Haas Broth (Brown and Braddock, 1990) supplemented with 30 g L^{-1} NaCl and 0.01% (v/v) artificially weathered Troll oil (+200 °C fraction). The plates were incubated (10 °C) for 10 days for HM and for 14 days for ODM determination. Actively respiring ODM were detected using fluorescein diacetate as indicator (Chrzanowski et al., 1984).

3.3.5.3 Characterization of total bacterial communities

For characterization of total bacterial communities, DNA was extracted and a 16S rRNA gene amplicon library was generated for sequencing using Illumina MiSeq®. Material from samples was collected by filtration (0.45 μm pore size, Millipore, 47 mm diameter HAWG type). Filters with material were stored in sterile tubes at -20 °C until DNA-extraction. Cells were lysed by adding 4.5 mL lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, and 1 M NaCl), 0.5 mL lysozyme (20 mg mL^{-1}), 0.125 mL proteinase K (20 mg mL^{-1}), lauryl-sarkosyl (1%) and sodium-dodecyl-sulfate (1%) to each tube. The mixture was vigorously shaken for 1 hour at 37 °C. Extraction of DNA in the lysis buffer was performed using hot phenol:chlorophorm:isoamylalcohol (25:24:1), pH 8.0 (Sambrook and Russel, 2001). Extracted DNA was dissolved in 100 μL ultrapure water (MolBio grade, 5prime) and stored at -20 °C until shipment for 16S rRNA gene amplicon library analyses.

16S rRNA gene amplicon library analyses of DNA extracted from the samples were performed by GATC Biotech (Constance, Germany) using Illumina MiSeq®. An amplicon library was generated of the bacterial 16S rRNA gene by polymerase chain reaction (PCR) with the forward primer 27F (AGAGTTTGATCCTGGCTCAG) and the reverse primer 534R (ATTACCGCGGCTGCTGG) generating a 471 base pair product. Taxonomic classification was based on National Center for Biotechnology (NCBI) taxonomy. All hits per cluster with > 97% similarity were used to calculate relative abundance of bacterial genera. Abundancies of bacterial species are summed at genus level, and genera with a total abundance < 2.5% were grouped as others.

4 SUMMARY OF RELEVANT RESULTS

4.1 Paper I

Modeling filtration of dispersed crude oil droplets by the copepod *Calanus finmarchicus*.

Marine Environmental Research 105, 1-7.

Small oil droplets have been reported to be ingested by *C. finmarchicus*. The aim of Paper I was to use a modeling approach to estimate the fraction of an oil spill that can be removed by ingestion by *C. finmarchicus*. Two scenarios were specified, a surface and a subsurface oil spill. In five cases for each scenario, the input parameters in the filter-feeder module were varied over a span that was assumed to be realistic based on available peer-reviewed data. This was done to determine the importance of the copepod parameters for the fraction of oil removed.

For the conditions specified by the scenarios, the model estimated that between 1 and 40% of the oil can be removed by ingestion by *C. finmarchicus*. The size limit for oil droplets ingested by the copepods was the most important parameter for the fraction of oil removed by *C. finmarchicus*. The second most important parameter was the population density. The fraction of oil ingested by the copepods was linearly dependent on the population density of the copepods, while it leveled off at higher oil droplet sizes. For all cases, a larger fraction of the oil spill was ingested from the subsea scenario.

4.2 Paper II

Oil droplet ingestion and oil fouling in the copepod *Calanus finmarchicus* exposed to mechanically and chemically dispersed crude oil.

Environmental Toxicology and Chemistry 9999, 1-8.

During oil spill, surface oil slicks may be treated with dispersants to increase the entrainment of small-sized droplets into the water column. The aim of Paper II was to provide quantitative data on the concentration of oil in the biomass and the feeding activity of *C. finmarchicus* in oil dispersions with or without chemical dispersant. The concentration of oil in *C. finmarchicus* biomass were 14 mL kg^{-1} for low and medium

concentrations of both mechanically and chemically dispersed oil (low: 0.3 and 0.2 mg L^{-1}, medium: 1.24 and 0.88 mg L^{-1}, respectively), and approximately 3 mL kg^{-1} at the high concentrations (5.5 mg L^{-1}). The predicted oil concentration in biomass was significantly (ANOVA, $p < 0.05$-0.0001) lower at the high concentrations compared to the corresponding medium and low oil concentrations. At the low concentration of chemically dispersed oil, the oil concentration in the *C. finmarchicus* biomass was significantly (ANOVA, $p < 0.01$) higher compared to the oil dispersed without. Feeding activity, quantified as clearance rate of algae, of *C. finmarchicus* exposed to oil dispersed both with and without chemical dispersant was reduced in a concentration dependent manner for all treatments expect the high concentration of the mechanically dispersed oil. It varied from approximately 30 to 8 mL $copepod^{-1}$ day^{-1} for oil-exposed *C. finmarchicus*. Control clearance rate was 41.4 mL $copepod^{-1}$ day^{-1}. In chemically dispersed oil, the clearance rate of *C. finmarchicus* was significantly (ANOVA, $p < 0.0001$ and $p < 0.001$, respectively) higher for low and medium concentrations, compared to the corresponding concentration of mechanically dispersed oil.

4.3 Paper III

Uptake of PAHs in *Calanus finmarchicus* from seawater petroleum oil dispersions and the water soluble fraction.

Manuscript.

The oil in the *C. finmarchicus* biomass is accumulated by passive partitioning of oil compounds over body surfaces and by ingestion of oil droplets. The aim of Paper III was to evaluate the accumulation of PAHs over time from the WSF and from the particulate fraction. The accumulation of PAHs from the WSF reached steady state for low lipophilic (log $K_{ow} < 5$) oil compounds within 24 hours, while the high lipophilic (log $K_{ow} > 5$) oil compounds did not reach steady state within the duration of the exposure (96 hours). The accumulation of oil compounds in oil dispersion exposed *C. finmarchicus* was rapid and reached higher concentrations compared to the WSF-exposed. However, over time the concentration of the low lipophilic compounds in the oil dispersion exposed *C. finmarchicus* was lower relative to the concentration in the WSF-exposed *C. finmarchicus*. This lower concentration was observed concomitant with a lower volume of the lipid sac of the oil dispersions exposed *C. finmarchicus* relative to the lipid sac in the WSF-exposed.

4.4 Paper IV

Concentrations of viable oil-degrading microorganisms are increased in feces from *Calanus finmarchicus* feeding in petroleum oil dispersions.

Marine Pollution Bulletin 98, 69-77.

The ultimate removal of oil in marine environments is dependent on biodegradation of oil by microorganisms. The aim of Paper IV was to study feeding activity of *C. finmarchicus* in oil dispersions and microbial communities in *C. finmarchicus* feces with and without oil. Feeding activity, quantified as clearance rate of algae was significantly (t-test, $p < 0.05$) lower for oil-exposed *C. finmarchicus* compared to control copepods, approximately 40 and 8 mL copepod^{-1} day^{-1}, respectively. The volume of feces produced by oil-exposed *C. finmarchicus* was also significantly (t-test, $p < 0.0001$) lower compared to the control. The concentration of viable ODM determined using the MPN method was significantly (t-test, $p = 0.008$) higher in feces with oil compared to clean feces. The total bacterial communities were similar between feces with and without oil, and dominated by bacteria of the Alphaproteobacteria class and the family *Rhodobacteraceae*. The relative abundance of known specialist oil-degrading bacteria was low. The species *Phaeobacter* had significantly (t-test, $p = 0.021$) higher abundance in feces with oil, 27 and 28%, compared to control feces, 18 and 20%.

4.5 Paper V

Biotransformation of petroleum hydrocarbons and microbial communities in seawater with oil dispersions and copepod feces.

Marine Pollution Bulletin, doi: 10.1016/j.marpolbul.2015.10.029.

Biotransformation of oil in seawater can be limited by available inorganic nutrients. The concentrations of these were assumed to be increased by the presence of *C. finmarchicus* feces. Oil-containing *C. finmarchicus* feces were also shown to contain increased concentrations of viable ODMs (Paper IV). The aim of Paper V was to study biotransformation, and also activity and relative abundance of bacteria in oil dispersions in the presence of *C. finmarchicus* feces with and without oil and compare this with results from oil dispersion controls (no feces). The presence of clean feces resulted in significantly (One-way ANOVA, $p < 0.01$) higher biotransformation of aromatic HCs compared to the oil dispersion control. The biotransformation of n-alkanes was > 85% in all three treatments. However, it was ambiguous when compared to the oil dispersion

control; significantly (One way ANOVA, p = 0.009 and p = 0.027) higher for nC19- and C20-alkanes in the presence of oil-containing feces, and significantly (One way ANOVA, p < 0.016) lower in the presence of clean feces. The concentration of viable ODM as quantified using MPN was highest in oil dispersions with oil-containing feces. The bacterial communities in oil dispersions where *C. finmarchicus* feces were present showed a dominance of *Rhodobacteraceae* (class Alphaproteobacteria) bacteria. The bacterial communities in the oil dispersion control showed a dominance of Gammaproteobacteria. Large aggregates, assumed to contain feces and oil formed in the oil dispersions with feces.

5 DISCUSSION OF MAIN FINDINGS

The aim of this thesis was to investigate how the abundant marine filter-feeder *C. finmarchicus* influence transport and weathering of oil dispersions. Ingestion of oil droplets contributed to rapid accumulation of oil compounds in the *C. finmarchicus* biomass (Paper III). The modelling showed that ingestion of oil may be relevant for between 1 and 40% of an oil spill (Paper I). The two parameters that had most influence on the quantity of oil ingested were the upper size limit determined for ingested particles and the *C. finmarchicus* population density (Paper I). The concentration of oil in the *C. finmarchicus* biomass were dependent on the oil concentration and varied between approximately 14 and 3 mL kg^{-1} for dilute oil dispersions (0.3 to 5.5 mg L^{-1}, respectively) (Paper II). The concentration of low lipophilic oil compounds (log $K_{ow} < 5$) were rapidly equilibrated between water phase and the *C. finmarchicus* biomass, while high lipophilic compounds (log $K_{ow} > 5$) continued to accumulate in the biomass over the duration of the 96 hour exposure (Paper III). Further, the results indicate that the low lipophilic compounds were eliminated from oil-dispersion exposed *C. finmarchicus* after exposure ≥ 72 hours (Paper III). Oil-containing *C. finmarchicus* feces had higher concentrations of viable ODMs (Paper IV). However, in the presence of *C. finmarchicus* feces, degradation of oil compounds was ambiguous; increased for the aromatic compounds, but both increased and decrease for the n-alkanes compared to the oil dispersion control (no feces) (Paper V).

Since the transport and weathering of an oil spill are complex processes, they are usually predicted using numerical models. The interactions between oil spills and *C. finmarchicus* should be accounted for in such models if they are significant, either for oil spill weathering and transport, or for quantifying harmful effects in ecosystems. The output from oil spill models can be useful information during oil spills, for optimizing oil spill mitigation techniques.

5.1 Oil in *C. finmarchicus* biomass

To investigate the accumulation of oil compounds, *C. finmarchicus* were exposed to oil dispersions and the corresponding WSF for 96 hours (Paper III). Samples were collected for chemical analyses using GC-MS every 24 hours. Compared to the WSF-exposed, the oil dispersion exposed *C. finmarchicus* rapidly accumulated higher concentrations of all oil compounds (Paper III). The results were consistent with ingestion of oil droplets by the copepods, as the rapid accumulation also was observed for the high lipophilic oil compounds that are expected to be exclusively located in the oil droplets (Redman et al., 2012). Ingestion of oil droplets have previously been

reported after several laboratory studies with *C. finmarchicus* (Hansen et al., 2012; Olsen et al., 2013). This shows that the ingestion of droplets by *C. finmarchicus* can contribute to the oil concentration in the biomass.

5.1.1 Parameters important for ingestion of oil by *C. finmarchicus*

To estimate the fraction of an oil spill that can be removed by ingestion by *C. finmarchicus*, the OSCAR software was run with a filter-feeder module implemented (Paper I). The OSCAR software calculated weathering and transport processes of the oil spill, including the size, concentration and location of the droplets. The filter-feeder module calculated the total oil ingested by *C. finmarchicus*, and explored the importance of the input parameters (Paper I). The results showed that *C. finmarchicus* removed between 1 and 40% of an oil spill (Paper I). The 40% removal combined extreme values for several of the input parameters and was suggested to be less likely than the lower ranges of the estimate (Paper I).

Highest influence on the total removal of oil was observed for the parameter value set for the maximum size of droplets ingested by the copepods. Highest removal, 40%, was observed when this parameter was set to 300 µm. This was because a large volume of the oil spill was contained within droplets with diameter ≤ 300 µm, compared to when to smaller values were set for this parameter. The size of the particles ingested by *C. finmarchicus* is upwards restricted by the size of their mouthparts and downwards restricted by the distance between the setae on the sieve-like feeding appendage used for collecting particles (Boyd, 1976). *C. finmarchicus* have been observed to ingest their own nauplii, which can be as large as 700 µm (Basedow and Tande, 2006; Bonnet et al., 2004; Marshall and Orr, 1972). Particles are ingested by at least two different strategies in *C. finmarchicus*, by non-selective suspension feeding and raptorial predator feeding on single particles (Cannon, 1928; Kiørboe, 2011; Turner et al., 1993). The feeding rate used in the filter-feeder module was obtained from experiments with small feed particles, where the feeding mechanism was assumed to be non-selective suspension feeding (Paper I). As feeding activity of *Calanus* copepods is decreased when the size of the feed particles is increased, this rate may not be directly transferable to the filtration rate when the particles ingested have diameter between 100 and 300 µm (Frost, 1972; Kiørboe, 2011). This relationship between particles ingested and filtration rate is illustrated in Fig. 8. Based on the arguments presented above, the estimated removal of 40% was suggested to be unrealistic.

The maximum value set for particles ingested by *C. finmarchicus* was set to approximately half the size of the nauplii to explore the significance of this input parameter for removal of oil. The feeding strategy of *C. finmarchicus* on oil droplets was not investigated in this thesis, but was assumed to be by non-selective suspension feeding (Paper II, III and IV). Non-selective suspension feeding was assumed to be

relevant for particles with diameter < 35 μm, based on available literature (Cannon, 1928; Leiknes et al., 2014; Urban et al., 1993). The default value in the filter-feeder module for the size of the particles ingested were ≤ 50 μm (Paper I). In the modeled cases where the default value was used, a removal of ≤ 2% of the total oil was observed. Previous studies have estimated a removal by *Calanus* spp. of 10% of the total oil after a spill of bunker C oil in Nova Scotia in 1971 (Conover, 1971). The estimates of a ≤ 2% removal of the total oil were therefore suggested to be realistic for the scenario specified in the modelling (Paper I).
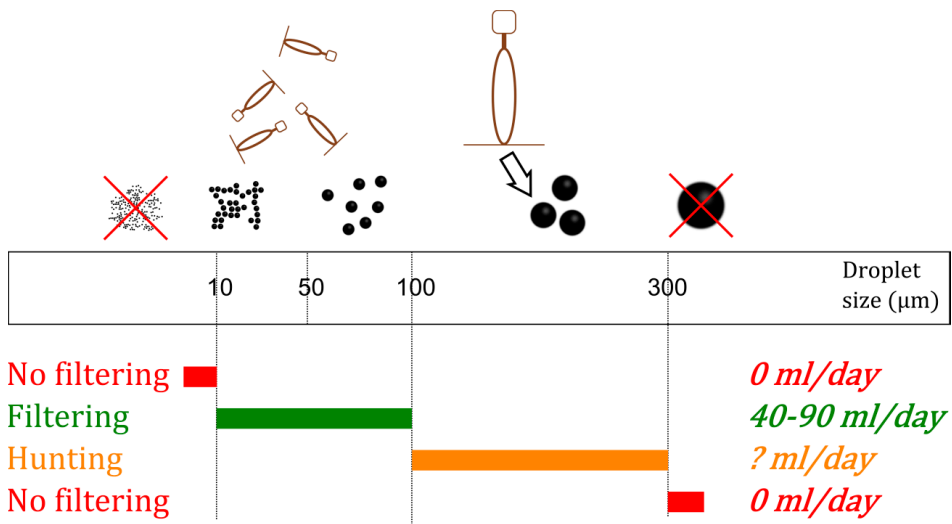
Fig. 8. Illustration of the how the relationship between particle size and feeding activity of *Calanus finmarchicus* quantified as clearance rate (mL copepod[-1] day^{-1}) where considered in the filter-feeder module implemented in the oil spill model OSCAR.

Figure credits: Raymond Nepstad, SINTEF Environmental Technology.

For all cases modeled in Paper I, the subsea scenario resulted in a higher removal compared to the surface scenario. This was caused by the discharge conditions for the two scenarios; the subsurface scenario produced more small droplets and a larger fraction of the oil spill was available for the copepods to ingest. These results show that the removal of oil by *C. finmarchicus* was dependent on parameters of the oil spill, in this case oil droplet size, in addition to parameters of the *C. finmarchicus*, as feeding activity and size of the oil droplets ingested (Paper I).

The second most important parameter in the filter-feeder module for total oil removed by *C. finmarchicus* was the population density (Paper I). The default value for this parameter was 3000 copepods m^{-3}, based on data reported by Helle (2000). When a scan was performed over relevant population densities, it scaled approximately linearly with the total oil removed (Paper I). The results from the modeling show that it is necessary to know the biomass of *C. finmarchicus* for predicting the total oil removed by copepods (Paper I). *C. finmarchicus* performs both seasonal and dial vertical migration, and the variability in population density are large (Marshall and Orr, 1972; Sakshaug et al., 1994). Also in the horizontal direction the population density is patchy, and mainly dependent on the ocean currents (Helle, 2000; Unstad and Tande, 1991). To improve the model estimates of total oil removed by the *C. finmarchicus* population, *Calanus* population density can be accurately predicted using a biomass model, e.g. as the SINMOD model (Slagstad and McClimans, 2005). The SINMOD model is a coupled 3D hydrodynamic chemical and biological model system, accounting for diapause and the development through the copepodite stages.

5.1.2   Oil concentration in *C. finmarchicus* biomass

C. finmarchicus was exposed over 96 hours to dilute (5.5-0.3 mg TPH L^{-1}) oil dispersion with small oil droplets (diameter < 40µm) to quantify the concentration of oil in the biomass (Paper II). The oil compounds quantified in the *C. finmarchicus* biomass were due to particulate oil ingested by the copepods and oil compounds accumulated from the WSF, as was shown in Paper III. The oil concentration in the biomass was quantified from compounds located exclusively in the particulate phase of the oil, with log K_{ow} > approximately 6 (EPISuite, 2012; Redman et al., 2012). Fouling of external surfaces (Fig. 9) was suggested to be minimal (Paper II).

At low (0.3 mg TPH L^{-1}) and medium (1.24 mg TPH L^{-1}) oil dispersion concentrations, the concentration of oil in the *C. finmarchicus* biomass at 96 hours were approximately 14 mL oil kg^{-1} copepod^{-1} (Paper II). The oil concentration in the biomass at the high oil dispersion concentration (5.5 mg TPH L^{-1}) was 3 mL kg^{-1} (Paper II). These results show that the oil concentration in the biomass were dependent on the oil dispersion concentration, and were lower for higher oil concentrations. The decrease in oil concentration in the *C. finmarchicus* biomass was observed concomitant with a decrease in feeding activity of the oil-exposed copepods (Paper II). Conover (1971) found a concentration of oil in a mixed zooplankton community of maximum 4.76×10^{-5} mL oil kg^{-1} zooplankton^{-1} (reported as 46.2 µg m^{-3} zooplankton^{-1}, the density of bunker C is approximately 0.97 kg L^{-1}, density of the zooplankton assumed to be 1). Conover (1971) regarded droplets with diameter < 100 µm as ingestible by the zooplankton community, and the concentration of oil droplets < 100 µm was approximately 1.55×10^{-6} mL L^{-1}. The magnitude difference between the oil dispersion concentration in the water and the oil associated with the zooplankton/*Calanus* biomass between Conovers (1971) field

data and the data from the laboratory exposure studies with low oil dispersion concentration (0.3 mg L^{-1}) (Paper II) were 20 000 and 30 000 times lower in the field, respectively. The correspondence between the field data obtained by Conover (1971) and the data obtained from the laboratory exposures indicates that the oil in the zooplankton biomass during oil spills may be estimated from the oil dispersion concentration in the water for lower concentrations < approximately 1 mg L^{-1}. However, as indicated in Paper III, repeating oil dispersion exposure studies using several zooplankton species and different feeding regimes to quantify oil concentration in biomass may be necessary before extrapolating oil concentration across several species.
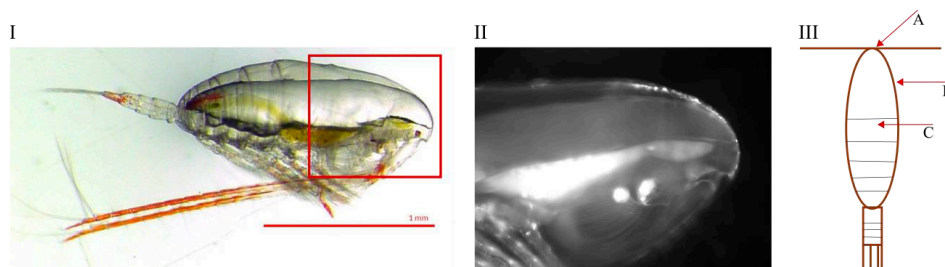
Fig. 9. I) *Calanus finmarchicus* photographed through a dissecting microscope showing the section of the copepod captured with II) fluorescence microscopy (Photo credits: Dag Altin, Biotrix) visualizing crude oil droplets in the intestine and adhered to copepod external surfaces and the feeding apparatus. III) The concentration of oil in *C. finmarchicus* are mainly oil in the gut (C), but can also include oil adhered to the filtering apparatus (A) and to external surfaces of the copepods (B).

5.1.3 Feeding activity of *C. finmarchicus* in dilute oil dispersions

To determine the effect of oil dispersions on feeding activity, clearance rates of *C. finmarchicus* were quantified in clean seawater and in oil dispersions (Paper II and IV). Average clearance rate was quantified over 96 hours, determined as a function of dispersion concentration (5.5-0.3 mg TPH L^{-1}) (Paper II), and over time at similar exposure concentrations as the medium concentration in Paper II (1.24 µL L^{-1}) (Paper IV). The clearance rate of *C. finmarchicus* feeding on *R. baltica* in clean seawater was approximately 40 mL copepod^{-1} day^{-1} (Paper II and IV). These results showed good consistency, and were also in accordance with clearance rates previously reported for *C. finmarchicus* feeding at similar concentrations of *R. baltica* (Nejstgaard et al., 1995).

The clearance rates recorded for oil-exposed *C. finmarchicus* varied from 30 to 8 mL copepod^{-1} day^{-1} (Paper II). The clearance rate was lowest for the highest oil concentrations (Paper II). This was consistent with a low concentration of oil in the biomass at the high oil dispersion concentrations (Paper II), and show that the oil concentration in the biomass was mainly due to ingested oil (Fig. 9). The clearance rate of *C. finmarchicus* exposed to the medium concentration of dispersed oil in Paper II and the clearance rate of oil-exposed copepods in Paper IV. The oil types, degree of weathering and the exposure concentration were similar between these two exposures (Troll/+200 °C fraction, 1.24 mg L^{-1} and 1.37 µL L^{-1}).The results thus indicates that the clearance rate of *C. finmarchicus* feeding in oil dispersions may be predicted from the type of oil spilled, degree of weathering and the concentration of the oil dispersion.

Over time, the clearance rate of exposed *C. finmarchicus* was significantly (t-test, $p < 0.02$) lower already after 17 hours of exposure to dilute oil dispersion (1.37 µL L^{-1}) compared to the control (Paper IV). The early significant reduction shows that the feeding activity of *C. finmarchicus* was rapidly deteriorated in oil dispersions. Reduced feeding activity of copepods exposed to oil dispersions has previously been reported (Hansen et al., 2012; Spooner and Corkett, 1979). Oil dispersions are dissolved compounds and particulate oil, and the reduced feeding activity may be due to a combined effect of oil droplets and the WSF. Exposure to the WSF has previously been observed to reduce feeding by *C. finmarchicus*, suggested to be due to a general narcotic effect in the copepods (Jensen and Carroll, 2010). The oil droplets are suggested to reduce the feeding activity by obstructing the functionality of the feeding apparatus of *C. finmarchicus* (Paper II and IV). This was suggested to be due to the sticky surface properties of crude oil which may cause agglutination of the fine setae of the feeding appendages of the copepod. This is in line with suggestions made by Conover (1971), who observed large particles caught in the filtering apparatus of the *Calanus* copepods.

5.1.4 Oil surface properties are important for feeding activity and oil concentration in biomass

Since the sticky surface properties of oil droplets was suggested to be partially responsible for the reduced feeding of oil-exposed copepods (Paper II and IV), crude oil types may affect feeding activity and accumulation of oil compounds differently. Feeding activity and accumulation of oil compounds in *C. finmarchicus* were determined after exposure to oil dispersed both with and without chemical dispersant under otherwise similar exposure conditions (Paper II). Since oil type, concentration and droplet size were similar, the only difference between the two exposures was the presence of chemical dispersants in one of the series. Surfactants in chemical dispersants position themselves at the oil-surface and change the surface properties of the oil droplets (Brandvik, 1997). The clearance rates of *C. finmarchicus* were significantly (ANOVA, $p < 0.0001$ and $p < 0.001$, respectively) higher at the low and

medium concentrations of oil dispersed with chemical dispersant compared to the oil dispersed without (Paper II). This also resulted in significantly (ANOVA, $p < 0.01$) higher concentration of oil in the biomass exposed to the low concentration of oil dispersed with chemical dispersant compared to oil without (Paper II). These results indicate that crude oils with different surface properties may affect clearance rate and therefore the oil concentration in the *C. finmarchicus* biomass differently. These results strengthens the discussion in Paper I, where the concentration of oil in the biomass of *C. finmarchicus* after an oil spill was suggested to be dependent on the parameters of both the oil spill and of *C. finmarchicus*. However, in addition to oil droplet size, oil type and degree of weathering are suggested to be important parameters.

5.1.5 *C. finmarchicus* biomass can redistribute low lipophilic oil compounds and act as a sink for dissolved high lipophilic oil compounds

To determine the contribution to the *C. finmarchicus* biomass from the WSF fraction and from the particulate oil fraction of an oil spill, *C. finmarchicus* were exposed to oil dispersions for 96 hours and samples collected every 24 hours (Paper III). In the WSF-exposed *C. finmarchicus,* the concentration of low lipophilic (log $K_{ow} < 5$) oil compounds reached steady state within 24 hours, while the high lipophilic (log $K_{ow} > 5$) oil compounds did not reached steady state during the 96 hour exposure. Accumulation of phenanthrene and benzo(a)pyrene have previously been quantified in non-fed *C. finmarchicus* (Jensen et al., 2012). In their study, phenanthrene (log K_{ow} 4.3) reached steady state after approximately 50 hours, while benzo(a)pyrene (log K_{ow} 6.1) did not reach steady state during the 192 hours of exposure. Since high lipophilic oil compounds continued to accumulate over a prolonged exposure, it was suggested that *C. finmarchicus* biomass may function as a sink for high lipophilic oil compounds dissolved in the water phase.

The experiment was run in two consecutive series, with two populations of C5 assumed to be identical. However, in the second series the low lipophilic compounds were accumulated to lower levels during the first 24 hours (Paper III). The levels of oil contaminants in biomass are known to depend on the lipid levels (De Hoop et al., 2013; Hendriks et al., 2001). Lower levels of low-lipophilic compounds were therefore suggested to be caused by lower lipid levels in the second C5 population (Paper III). The results show that there may be differences in the oil concentration accumulated by different populations of *C. finmarchicus*.

After 72 hour exposure, a lower concentration of low lipophilic oil compounds was observed in the oil dispersion exposed compared to the WSF-exposed *C. finmarchicus* (Paper III). These results may show the onset of elimination of accumulated oil compounds after prolonged oil dispersion exposure and were suggested to be caused by a lower volume of the lipid sac at the end of the 96 hour exposure of the oil dispersion

exposed relative to the WSF-exposed *C. finmarchicus* (Paper III). It may be speculated that the reduced volume of the lipid sac is a consequence of the reduced feeding activity observed in oil dispersion exposed *C. finmarchicus*. However, no data was obtained on feeding activity of WSF-exposed *C. finmarchicus* in this thesis and the cause for the observed lower volume of the lipid sac of the oil dispersed *C. finmarchicus* relative to the WSF-exposed was not determined. However, starvation of *Calanus* copepods has previously been reported to result in mobilization of both the triacylglycerols and the wax esters in the lipid sac (Lee et al., 2006). In starved female *C. finmarchicus*, changes have been reported in metabolization already after 3 days of starvation (Pasternak et al., 2013). Regardless of the cause, the results indicate that the *C. finmarchicus* biomass can contribute to redistribution of accumulated oil compounds to the water phase during oil spill.

5.1.6   Summary: Total oil removed by *C. finmarchicus* biomass

The total oil removed by *C. finmarchicus* during an oil spill (Fig. 10) was dependent on the size of the droplets ingested by the copepods and the droplet size distribution of the oil spill dispersion (Paper I). The total oil removed after an oil spill was also linearly dependent on the population density (Paper I). Oil concentration in biomass and the feeding activity of oil dispersion exposed *C. finmarchicus* were both dependent on the oil dispersion concentration, and were lower for higher oil dispersion concentrations (Paper II). Both parameters were also dependent on the oil surface properties (Paper II). Lower levels of low lipophilic oil compounds over time in the oil dispersion exposed compared to the WSF-exposed *C. finmarchicus* may show that the accumulated oil compounds are eliminated to water phase after prolonged exposure (Paper III). There were differences in the accumulation between populations of *C. finmarchicus* which may have been caused by lower lipid content (Paper III). The results show that the total oil removed by *C. finmarchicus* will be dependent on both oil spill parameters and *C. finmarchicus* parameters.

5.2   Microbial communities in feces from *C. finmarchicus*

For the ultimate removal of oil from marine environments, biodegradation and mineralization by microorganisms are fundamental. When *C. finmarchicus* were exposed to oil dispersions, oil was excreted in feces (Paper IV). Excretion of oil in feces of *C. finmarchicus* has been shown in previous field and laboratory studies (Conover, 1971; Hansen et al., 2012; Olsen et al., 2013). To study if the presence of oil in feces may increase the activity of microorganisms capable of oil-degradation, the concentrations of viable HM and ODM were analysed by the MPN method in seawater with clean and oil-containing feces after 14 days of incubation (Paper IV). The results show a significant increase in the concentration of viable ODMs (t-test, $p = 0.008$) in
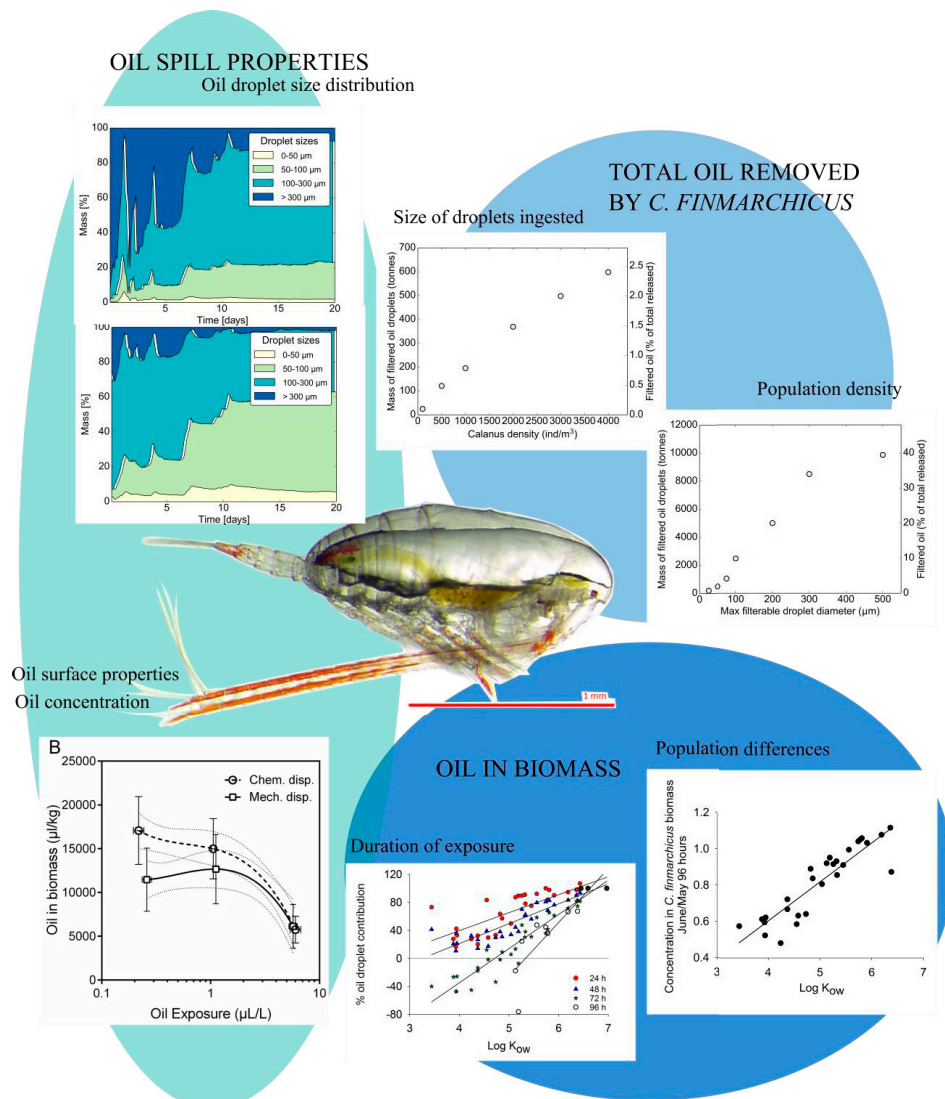
Fig. 10. Summary of interaction processes between an oil dispersion and *Calanus finmarchicus*. The total oil removed from an oil spill were dependent on the oil droplet size distribution (Paper I) and the volume of the oil spill contained within the fraction of droplets that the copepods ingest by non-selective suspension feeding (Paper I), as well as the *Calanus finmarchicus* population density (Paper I). The oil concentration in the biomass was dependent on oil surface properties (Paper II), concentration of the oil dispersion (Paper II), duration of exposure (Paper III) and population specific variation (Paper III).

seawater with oil-containing feces compared to clean feces (Paper IV). Oil-degrading bacteria are ubiquitous in marine environment, and the abundance of bacteria capable of assimilating carbon from oil has been observed to be rapidly increased in the presence of oil (Head et al., 2006).The concentrations of viable HM were similar between the oil-containing feces and the clean feces (Paper IV). This showed that the increased concentration of viable ODM did not decrease the activity of the heterotrophic microorganisms, as has previously been reported (Smith et al., 1998). The results show that the ingestion of oil by *C. finmarchicus*, and the excretion of oil in feces results in a higher potential for oil-degradation by the bacterial communities in the feces.

The total bacterial community from clean and oil-containing feces from *C. finmarchicus* was characterized by 16S rRNA gene amplicon library analyses determining the relative abundance of all bacteria. The bacterial genera *Colwellia* and *Pseudoalteromonas* were detected at 3 and 4% relative abundance, respectively, in oil-containing feces (Paper IV). These two bacterial genera were reported at high abundance after the shut-in of the well during the Deepwater Horizon accident and were suggested to be correlated with an increase in the relative concentration of the aromatic HCs (Dubinsky et al., 2013). *Pseudoalteromonas* has in numerous studies been indicated in the degradation of aromatic HC compounds (Hedlund and Staley, 2006; McKew et al., 2007a; Melcher et al., 2002). While *Colwellia* has been associated with degradation of many classes of HC compounds, e.g. gaseous and small aromatic HCs (Bælum et al., 2012; Redmond and Valentine, 2012). The presence of these bacteria in the *C. finmarchicus* feces were therefore assumed to be due the presence of the oil (Paper IV).

In oil-containing *C. finmarchicus* feces, the relative abundance of the bacterial genera *Phaeobacter* was significantly (t-test, p = 0.021) higher (27 and 28%) compared to control feces (18 and 20%) (Paper IV). The results show that the activity and proliferation of this genus thus were high in the presence of oil. Bacteria affiliated with *Phaeobacter* have previously been detected at high abundance in alkane-enriched microcosms (McKew et al., 2007a). The high relative abundance of this genus corresponds with the close to total loss of n-alkanes from the oil droplets in the feces after 14 days of incubation in seawater (Paper IV).

Except for the differences noted above, the bacterial communities detected in oil-containing feces and clean feces were similar. *Rhodobacteracea* family bacteria were detected at high relative abundance in all microbial communities. Of genera with relative abundance > 2.5%, *Rhodobacteracea* family bacteria represented 76 and 79% in the two replicates of clean feces, and 77 and 78% in the two replicates of oil-containing feces (Paper IV). However, the increased concentration of viable ODMs shows that the bacterial communities in the oil-containing feces were capable of oil degradation (Paper IV). This increased oil-degrading activity was suggested to have been mediated by

bacteria indigenous to the copepod feces (Paper IV). *Rhodomonas* sp. have been indicated to release straight-chain HCs during lysis, and thus the bacteria metabolizing copepod feces consisting of partially digested *Rhodomonas* sp. may already possess enzymatic systems for degradation of straight-chain HC compounds (Kameyama et al., 2009; Suzuki et al., 2009). Also, the three *Rhodobacteraceae* genera *Sulfitobacter*, *Roseovarius*, and *Rugeria* found at similar abundances in both control and oil-containing feces from *C. finmarchicus* have previously been detected at high abundance when petroleum HC compounds are present, or found capable of assimilating carbon from HC compounds (Harwati et al., 2007; McKew et al., 2007a). The results indicated that the higher concentration of viable ODM in *C. finmarchicus* feces with oil was due to indigenous feces bacteria (Paper IV).

5.3 Biodegradation of oil in the presence of feces from *C. finmarchicus*

Oil-degrading bacteria utilize oil as a source of carbon, source of energy, and as an electron donor, and in addition they generally also need an electron acceptor, and inorganic nutrients, as nitrogen and phosphorus. Oil has a high quantity of carbon, and low or no inorganic nutrients and during oil spills, the carbon:nitrogen (C:N) and carbon:phosphorus (C:P) ratios are locally increased (NRC, 1985). Biotransformation of HCs by bacteria can therefore be limited by the concentrations of inorganic nutrients at an oil spill site (Atlas and Bartha, 1972; Leahy and Colwell, 1990). Copepod feces has a higher content of nitrogen relative to carbon compared to other organic particles suspended in the water column (Bathmann et al., 1987; Claustre et al., 1992). High abundance of *C. finmarchicus* feces was thus suggested to increase concentration of inorganic nutrients and therefore the activity of oil-degrading bacteria during oil spills (Paper V). In addition, as the concentration of viable ODM was increased in oil-containing *C. finmarchicus* feces (Paper IV), the presence of oil-containing feces was expected to further increase biodegradation (Paper V). To study biotransformation in the presence of *C. finmarchicus* feces, dilute oil dispersions were incubated with clean and oil-containing feces and the concentration of target HC compounds were quantified after 14 days using GC-MS. In the oil dispersions incubated with clean copepod feces, the biotransformation of n-alkanes were significantly (One-way ANOVA, $p < 0.016$) lower compared to the oil dispersion control (no feces), while the biotransformation of the aromatic HC compounds were significantly (One-way ANOVA, $p < 0.01$) higher (Paper V). In oil dispersions incubated with oil-containing feces, the biotransformation of n-alkanes were significantly (One-way ANOVA, $p = 0.009$ and $p = 0.027$) higher, while there were no significant (One-way ANOVA, $p > 0.5$) differences for aromatic compounds compared to oil dispersion control (Paper V). The oil dispersion exposed *C. finmarchicus* produced less feces compared to the control copepods (Paper V). Since all

feces from one copepod exposure tank was transferred to one flask with oil dispersion, the oil dispersion incubated with oil-containing feces contained less feces.

The ambiguity in the biodegradation of the n-alkanes was suggested to be due to their location, the type of bacteria that were degrading the n-alkanes and the quantity of feces present (Paper V). The increase in the concentration of viable ODM reported in oil-containing *C. finmarchicus* feces was suggested to be caused by the indigenous feces bacteria (Paper IV). These bacteria were then capable of assimilating carbon both from feces and from the oil, and the ambiguity of the degradation of n-alkanes can be caused by a preference for carbon in feces prior to the carbon in the n-alkanes (Paper V). The n-alkanes may also have been kept in close proximity to the feces, as large agglomerates assumed to contain feces and oil droplets with n-alkanes were observed visually in the flasks with oil dispersions and clean copepod feces. The n-alkanes quantified in the study were highly lipophilic (log K_{ow} > 7.2) (EPI Suite, 2012). These compounds were expected to not partition to the water phase (Redman et al., 2012). Degradation of n-alkanes mainly takes place at the oil-water interphase (Brakstad et al., 2004). Based on the results it was suggested that in the presence of large quantities of *C. finmarchicus* feces, the formation of agglomerates of feces and oil may enhance sedimentation of relatively un-weathered oil (Paper V). The extent of sedimentation will depend on the effective density of these agglomerates in the seawater.

The increased biotransformation of aromatic compounds in the presence of clean feces was suggested to be caused by higher concentrations of inorganic nutrients in the water in the presence of feces (Paper V). The ratios of carbon to nitrogen (in $NO_3^-+NO_2^-$) and phosphorus (in PO_4^{3-}) in the oil dispersion control (no feces) were calculated to be 100:3:0.5 (elemental concentrations, C:N:P), based on concentrations measured in a parallel experiment in water from Trondheimsfjorden by Brakstad et al. (2015). The oil added to the seawater increased the ratios of C:N:P above the ratio of 100:10:1 (C:N:P), regarded as optimal for bacterial growth (Bouchez et al., 1995; Obbard et al., 2004). The higher biotransformation of the aromatic HC compounds in the presence of feces was suggested to show that the activity of oil-degrading bacteria in the water was increased in the presence of *C. finmarchicus* during oil spills (Paper V).

The total microbial communities were characterized using 16S rRNA gene amplicon library analyses. The bacterial communities of oil dispersions in seawater with feces (Paper V) were dominated by the same bacteria as the clean feces (Paper IV). There was a high abundance of *Rhodobacteraceae* family bacteria (84 and 87% oil dispersion with oil-containing feces, and 62 and 84% oil dispersion and clean feces), and the bacterial genera *Sulfitobacter, Phaeobacter,* and *Leisingera* dominated. The *Rhodobacteraceae* bacteria were suggested to be indigenous to the copepod feces (Paper IV), but also to be capable of degrading oil (Paper IV and V). Further, the high dominance of these

bacteria when feces were present in seawater with oil dispersions shows that the feces were a strong factor in the selection of the bacterial communities. During oil spills in the presence of large populations of copepods, the presence of feces may determine which bacterial species that degrade the oil.

5.4 Sedimentation of oil with feces of *C. finmarchicus*

5.4.1 Sedimentation of oil with feces

Ingestion of oil by *C. finmarchicus* and sedimentation of oil in feces has been suggested as a transport route for oil to the seabed (Conover, 1971; Johansson et al., 1980). Sedimentation of oil with *C. finmarchicus* feces may alter the site of degradation for an oil spill. Oxygen is limited in sediment habitats and biodegradation of oil compounds is reduced when oil is entrained in beach sand or sediments (Atlas and Hazen, 2011). Sedimentation of oil with *C. finmarchicus* feces may thus reduce the overall biodegradation of an oil spill. The extent of sedimentation of *C. finmarchicus* feces were not quantified in the present thesis. However, sedimentation is dependent on the rate of biodegradation of feces in surface waters compared to the sinking rate, which both varies with the particles ingested by the copepods (Thor et al., 2003; Urban et al., 1993). Quantification of sedimentation of feces in field, have shown that the remineralization of copepod fecal pellets in the photic zone is high (Møller et al., 2011; Viitasalo et al., 1999). The oil-containing *C. finmarchicus* feces were shorter and less wide compared to the clean feces (Paper IV). Smaller fecal pellets sediment slower compared to the larger fecal pellets (Poulsen and Kiørboe, 2006; Small et al., 1979). Crude oil also generally has lower density than water and the presence of oil droplets in feces may decrease the overall density of the fecal pellets. The total number of cells per mg feces was higher in oil-containing feces compared to control feces, and the concentrations of HM were similar between oil-containing feces and clean feces (Paper IV). These results suggest that the bacterial activity was maintained in the presence of the oil (Paper IV). From the results, it is indicated that the extensive biodegradation previously observed for *C. finmarchicus* feces in surface waters will be maintained. Based on this, the sedimentation of oil with *C. finmarchicus* feces is suggested to be low.

5.4.2 Quantity of oil in *C. finmarchicus* feces

It was not possible to obtain reliable measurements on the quantity of oil in the feces excreted from oil dispersion and WSF-exposed *C. finmarchicus* (Paper III). Oil has previously been observed in the feces of *C. finmarchicus* after exposure to oil dispersions (Hansen et al., 2012; Olsen et al., 2013). As is evident from the microscope pictures presented in Olsen et al. (2013), there are large differences in the quantity of oil

per fecal pellet. Determining the dynamics of excretion of oil in *C. finmarchicus* feces is important for fully understanding the comprehensive interaction processes between oil dispersions and *C. finmarchicus* and should be a topic for further studies.

5.5 Future perspectives on modelling of oil transport and weathering in the presence of zooplankton

The aim of this thesis was to investigate how the abundant marine filter-feeder *C. finmarchicus* influence transport and weathering of oil dispersions. Transport and weathering of an oil spill are complex processes and these can to some extent be predicted using numerical models. The interactions between oil spills and *C. finmarchicus* may be accounted for in such models if they are of significance, either for oil spill weathering and transport, or for quantifying harmful effects in ecosystems. Oil spill mitigation techniques can then be optimized during oil spills.

Based on the results from this thesis, and the above discussions, the following suggestions are put forward on how to include zooplankton in the modelling of transport and weathering of oil.

During oil spills, the OSCAR model will predict the concentration, location and size of the oil droplets in the water column (Paper I). The work included in this thesis has contributed with data on concentrations of oil in the *C. finmarchicus* biomass for dilute oil dispersions (Paper II). From these results and their consistency with field data reported by Conover (1971), it is suggested that the oil concentration predicted by OSCAR can be used to estimate the oil concentration in the biomass. This data may, however, be relevant only for dilute oil dispersions (< 5 mg L^{-1}) since the concentration in the biomass was rapidly reduced at the high oil dispersion exposure concentration (5.5 mg TPH L^{-1}).

The total quantity of oil removed during an oil spill was linearly dependent on the *C. finmarchicus* population density (Paper I). It is therefore beneficial to include information on the biomass of *C. finmarchicus* in the filter-feeder module. The SINMOD model is a *Calanus* biomass model which also includes information on seasonal migration and copepodite stages (Slagstad and McClimans, 2005). Combining the information retrieved from the *Calanus* biomass model with the information from the OSCAR software and the filter-feeder module, is thus suggested to significantly improve the model estimates of total oil removed by *C. finmarchicus*.

The two populations of C5 *C. finmarchicus* accumulated oil compounds to different extent (Paper III). Investigating populations and different stages for their feeding

activity and accumulation of oil may beneficial information before the filter-feeder module is implemented into OSCAR.

The oil in the biomass may be subject to different transport and weathering processes than the oil suspended in the water column. *C. finmarchicus* are hotspots for microbial activity (Tang, 2005; Tang et al., 2010), and studying both the microbial communities and the biodegradation of oil in *C. finmarchicus* is interesting topics for further work. Further, the *C. finmarchicus* ingesting oil were rapidly deteriorated (Paper III). Dead and dying *C. finmarchicus* may be an easy food source for predators (Daase et al., 2014). Ingestion of oil contained in *C. finmarchicus* can enhance the accumulation of oil to higher trophic levels in the ecosystems. Investigating the mechanism and significance of this route of transfer of oil in ecosystems is valuable contribution to understanding the effect of oil spills on ecosystems.

C. finmarchicus is one of many filter-feeding pelagic organisms, and other species have also been indicated to ingest oil droplets (Almeda et al., 2014; Almeda et al., 2013; Lee et al., 2012). Almeda et al. (2013) performed experiments with oil and *A. tonsa*, and found that the oil concentration in the biomass was reduced when protozoans were introduced as feed source. This was explained as scavenging of oil by the protozoan. However, an alternative explanation may be that *A. tonsa* switched from non-selective suspension feeding to raptorial predator feeding when the protozoans were introduced, therefore reducing the quantity of oil ingested. The feeding behavior of filter-feeders is therefore suggested to be significant for estimating the oil in the biomass. Studying the feeding behavior of *C. finmarchicus* in the presence of oil can contribute with detailed information of the maximum size of the droplets that can be ingested, which was a crucial parameter in the filter-feeder module (Paper I), and the feeding strategy in the presence of different food sources. A preliminary experiment was performed during Easter 2014, organized by Emlyn Davis at SINTEF Environmental Technology where we looked at feeding and swimming behavior of *C. finmarchicus*. With the system used, it was possible to observe the swimming and feeding in detail. Performing further work using this camera system on the feeding behavior of *C. finmarchicus* both in clean seawater with different feed sources and also in oil dispersions would provide valuable information.

The concentration of oil in the biomass increased when the oil was treated with dispersants (Paper II). Crude oil properties can vary substantially (Daling et al., 1990). To fully be able to predict the oil in the *C. finmarchicus* biomass using OSCAR with the filter-feeder module, it is important to repeat some of the exposure studies with other crude oils. Ideally, oils representing the four crude oil groups (Naphthenic, paraffinic, asphaltenic and waxy) should be included.

When dilute oil dispersions were incubated with feces from *C. finmarchicus* visual observations were made of large agglomerates assumed to contain feces and oil (Paper V). Interaction between oil and other particles in the water column are known to cause formation of marine snow containing oil (Fu et al., 2014; Passow et al., 2012). Marin snow is large aggregates (≥ 0.5 mm) of organic particles that plays an important role for transport of materials towards the ocean seafloor (Alldredge and Silver, 1988). If formed during oil spills, such aggregates of copepod fecal matter and oil droplets may sediment to the sea floor, depending on their effective density. These aggregates may also scavenge the small oil droplets efficiently and potentially relocate large quantities of an oil spill (Hill et al., 1990). They may also scavenge sediment particles, which will contribute to increasing their density and therefore sedimentation. Since this has potential for affecting a large volume of oil, further investigations of these processes are necessary.

6 CONCLUSIONS

The results from this thesis suggest that *C. finmarchicus* can influence the transport and weathering of the oil in oil dispersions. The extent of the effect is dependent on parameters of the oil spill governing the concentration of oil and the size of the droplets, and also on parameters of the *C. finmarchicus* biomass, such as population density, feeding activity, and lipid content. For a comprehensive understanding of transport and weathering in the presence of *C. finmarchicus*, the oil excreted in the feces of *C. finmarchicus* has to be quantified. The main results relating to the research questions put forward in this thesis are summarized below.

Ingestion of oil by *C. finmarchicus* (1).

i. Between 1 and 40% of an oil spill was estimated to be removed by ingestion by *C. finmarchicus*. The lower range of estimates, $\leq 2\%$, were assumed to be realistic. The important parameters for total oil removed by ingestion by *C. finmarchicus* were the size of the droplets ingested and the population density.

ii. The concentrations of oil in the *C. finmarchicus* biomass were ranging from 14 to 3 mL kg^{-1} (oil dispersion concentration 5.5-0.3 mg TPH L^{-1}). From the results and their close correspondence with previously reported field data, it was suggested that the oil concentration in biomass can be estimated from the oil concentration in the water column for dilute oil dispersions (< 5 mg L^{-1}).

iii. The feeding activity was significantly reduced in oil dispersions compared to in clean seawater (approximately 40 mL copepod day $^{-1}$) and varied between 30 and 8 mL copepod day $^{-1}$ (oil exposure concentration 5.5-0.3 mg TPH L^{-1}). Feeding activity was rapidly reduced after onset of exposure (17h). The feeding activity was consistent between experiments with similar exposure conditions (oil type, concentration, degree of weathering and droplet size) and it was suggested that feeding activity can be estimated from oil concentration in the water. The removal of oil due to ingestion of droplets by *C. finmarchicus* is suggested to be largest early (< 24h) and at low oil dispersion concentrations (< 1 µL L^{-1}).

Accumulation of oil compounds in *C. finmarchicus* (2).

i. Rapid accumulation of all oil compounds were observed in oil dispersion exposed *C. finmarchicus*. A lower concentration in the low lipophilic (log K$_{ow} <$ 5) oil compounds were observed over time compared to the WSF-exposed *C. finmarchicus*, indicating eliminating and redistribution of these compounds to the water phase.

ii. Accumulation of low lipophilic compounds rapidly reached a steady state in WSF-exposed *C. finmarchicus*, while the high lipophilic (log K$_{ow} > 5$) oil compounds did not reach steady state over the 96 hour exposure. The results

indicate that *C. finmarchicus* biomass may function as a sink for high lipophilic oil compounds dissolved in the water during oil spills.

Microbial communities in *C. finmarchicus* feces and biodegradation of oil in the presence of feces from *C. finmarchicus* (3).

i. The concentrations of viable oil-degrading microorganisms were increased in oil-containing feces from *C. finmarchicus*. The relative abundancies of bacteria were similar between feces with and without oil, indicating that the activity was mediated by indigenous feces bacteria.

ii. Biodegradation of n-alkanes in the presence of clean and oil-containing feces from *C. finmarchicus* were ambiguous and were suggested to be dependent on the quantity of feces present. Biodegradation of aromatic oil compounds was high in the presence of large quantities of feces, suggesting that the biodegradation of the dissolved fraction of an oil spill may be enhanced due to leakage of nutrients from *C. finmarchicus* feces.

iii. Concentrations of oil-degrading microorganisms were highest in oil dispersions with oil-containing feces. The relative abundancies of bacteria were similar to the bacterial communities in clean feces. The bacteria that degrade oil were therefore suggested to be determined by the presence of feces.

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Paper I

Modeling filtration of dispersed crude oil droplets by the copepod *Calanus finmarchicus*

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ABSTRACT

Oil droplets may form and disperse in the water column after an accidental spill of crude oil or petroleum products at sea. Micro-sized oil droplets may be available for filter feeding organisms, such as the copepod *Calanus finmarchicus*, which has been shown to filter oil droplets. In the present paper, a modeling approach was used to estimate potential ingestion amounts by copepod filtration of oil droplets. The new model was implemented in the OSCAR (Oil Spill Contingency and Response) software suite, and tested for a series of oil spill scenarios and key parameters. Among these, the size of the filtered droplets was found to be the most important factor influencing the model results. Given the assumptions and simplifications of the model, filtration of dispersed crude oil by *C. finmarchicus* was predicted to affect the fate of 1–40% of the total released oil mass, depending on the release scenario and parameter values used, with the lower end of that range being more probable in an actual spill situation.

1. Introduction

When oil is spilled into the marine environment, it will be affected by abiotic (e.g. dispersion, dissolution, evaporation etc.) and biotic (biodegradation) processes which are collectively called weathering. Generation of micro-sized oil droplets occurs naturally in the marine environment following oil spills at the water surface as a function of breaking waves and wind (National Research Council (US), 2005). The concentration and size distribution of oil droplets in the water after an oil spill is dependent on oil type, sea state and temperature, e.g. (Daling et al., 2003). In a laboratory experiment, concentrations of oil under an oil slick was measured between 5 and 18 mg/L (Li et al., 2009), while the fraction of oil droplets considered to be permanently dispersed in the water column have a diameter <100 μm (Lunel, 1993; Delvigne and Sweemey, 1988; Lee et al., 2013a; Li et al., 2011). During sub-

surface spills, e.g. during the Deepwater Horizon incident in 2010, oil droplets are formed as oil enters the water column from the release site, and the droplet size distribution is a function of the turbulence depending on rate, size of the orifice and the gas-to-oil ratio of the release. Oil droplet formation from the sea surface into the water column may as well be facilitated by application of chemical dispersants that cause a decrease in the interfacial tension between oil and water resulting in dispersion of small oil droplets.

Oil spill models, such as the Oil Spill Contingency and Response (OSCAR) model (Reed et al., 1995, 1999a) are used to predict the overall fate and effects of oil spills, which serve as input to Net Environment Benefit Analyses for oil spill response planning and decision making. Such decision support models depend on available input data for oil weathering processes. Biotic effects on spilled oil usually only comprise biodegradation, while the potential implications of filtration by filter feeding organisms to the overall fate of oil spills are not considered in existing models.

Filter feeding organisms ingest particles present in the water column and can be found in the pelagic zone (e.g. copepods) as well as in the littoral zone (e.g. mussels). In the present paper we focus on sub-adult copepods (*Calanus finmarchicus* copepodite V; CV)

because in most cases the overall mass of oil from offshore oil spills will be present in the pelagic zone. *C. finmarchicus* is the dominating copepod species in the Norwegian and Barents Sea (Planque and Batten, 2000). The filtration rate for *C. finmarchicus* has been estimated at 40–98 ml/(day × ind.), representing an ability to filter a water volume of 100 000 times of its body volume per day (Nordtug et al., 2015; Meyer et al., 2002). Their abundance in the pelagic environment is high (annual production of 300 million tons) with of up to 3000 copepods per m^3 (Helle, 2000) in the upper pelagic zone (¡50 m depth (Unstad and Tande, 1991)) where they filter algae. *C. finmarchicus* have been shown to ingest particles up to 300 μm, as they prey on their own offspring (naupliar stages) (Basedow and Tande, 2006; Bonnet et al., 2004). During an oil spill copepods may be exposed to dissolved oil components and dispersed oil droplets in the water column. Oil components will be taken up through diffusion over body surface from the dissolved oil phase, while oil from dispersed oil droplets may be taken up through different routes: over gut lining from ingested oil droplets, or diffusion over body surfaces from oil droplets adhered to external surfaces (Berrojalbiz et al., 2009; Almeda et al., 2013; Gyllenberg, 1981).

Adhesion of oil droplets to copepod body surface and filtering apparatus has been shown, but more importantly copepods have been shown to filter and ingest oil droplets during exposure to dispersed oil (Nordtug et al., 2015; Gyllenberg, 1981; Hansen et al., 2009; Hansen et al., 2012; Conover, 1971; Spooner and Corkett, 1979). The size distribution of oil droplets from a spill incident may in some cases have significant overlap with the corresponding distribution of natural copepod food particles, and oil droplets will then be ingested as food, as found experimentally by Hebert and Poulet (1980) (Hebert and Poulet, 1980), as well as others (Conover, 1971; Lee et al., 2012; Almeda et al., 2014a, 2014b). While the ultimate fate of the ingested oil remains uncertain, it will mostly end up in the fecal pellets produced by the copepods (Spooner and Corkett, 1979).

If the oil concentration to which copepods are exposed is sufficiently high, accumulated oil components may cause toxic effects (Almeda et al., 2013; Hansen et al., 2012; Hebert and Poulet, 1980; Cowles, 1983; Hansen et al., 2008, 2011, 2013; Lee et al., 2013b). Furthermore, exposure to oil in droplet form has been shown to decrease filtration activity in copepods (Nordtug et al., 2015; Hansen et al., 2009; Hansen et al., 2012; Cowles and Remillard, 1983). Thus, such lethal and/or sub-lethal/narcotic responses may prevent the copepods from filtering dispersed oil droplets and lower their contribution to the overall removal of dispersed oil droplets from the water column. A concentration-dependent reduction in algae filtration rates was shown during exposure of *C. finmarchicus* to dispersed oil with concentrations ranging from 0.25–5 mg oil/l, however, at the lower end of that range the filtration of oil was substantial (Nordtug et al., 2015). Sustained food uptake has been shown in other copepods (*Calanus helgolandicus, Pseudocalanus elongatus, Temora longicornis*) during exposure to oil concentrations up to 10 ppm (Spooner and Corkett, 1979).

The main aim of the present paper is to estimate the potential contribution of pelagic copepods to the overall mass balance of oil spilled to the marine environment. Data from peer-reviewed literature on copepod filtration (filtration rates, particle sizes filtered, filtration depths), abundance and sensitivity to oil exposure were used as input, and as a basis for prediction of filtered oil mass following two typical spill scenarios: one with oil spilled at the sea surface and one sub-surface spill. A filter feeder model was developed and integrated with OSCAR, in order to assess filtration potential, and the importance of individual model parameters to the overall mass balance of an oil spill were explored.

2. Materials and methods

2.1. Model description

The present filter feeder model, sketched in Fig. 1, has been implemented as a new module for the oil spill model OSCAR; the latter has been described elsewhere (Reed et al., 1995, 1999a, 1999b, 1999c, 2004). For the benefit of the reader, we summarize the main capabilities of OSCAR in the next section, before discussing the details of the filter feeder model.

2.1.1. The OSCAR model

OSCAR is based on the three-dimensional (plus time) transport-reaction equation, formulated in the Lagrangian frame of reference. The concentration of species i (that is, oil pseudocomponent i) thus evolves in time according to

$$\frac{DC_i}{Dt} = \nabla \cdot \bar{D} \nabla C_i + \sum_{j=1}^{m} r_j C_i + \sum_{k} \sum_{l} t_{kl} C_i + \sum_{m} q_m. \tag{1}$$

Here, C_i is the concentrations of pseudocomponent i, the total number n of which can be tailored to the problem at hand, but $n = 25$ is commonly used. These 25 pseudocomponents, mainly separated by boiling points, also capture important differences in physical, chemical and toxicological properties of groups of actual oil fractions. Each component is subject to a number of decay (r_j) and transformation (t_{kl}) processes, which include evaporation, natural dispersion and droplet formation, biodegradation, and emulsification (see e.g. Reed 1999 (Reed et al., 1999c)). Three-dimensional ocean currents and two-dimensional wind fields, provided as input to the model, will drive the transport of oil, as well as several of the decay and transformation processes. In addition, a set of customizable time-dependent source terms q_m provide influx of oil into the model domain. Since the Lagrangian frame of reference is used, advection is included in the material derivative (D/Dt). Turbulent diffusion, characterized by the diffusion tensor $\bar{D}$ appearing in the diffusion term, is computed by a Monte-Carlo procedure.

The set of Equation (1) is solved numerically using a standard particle method (see e.g. Csanady (1973) (Csanady, 1973) or De Dominicis et al. (2013) (De Dominicis et al., 2013)). Each numerical particle represents a certain amount of oil fractioned into pseudocomponents, and is further characterized by a set of attached chemical and physical properties. Each particle is advected and undergoes reaction processes separately, while the Eulerian concentrations are computed as a sum over all particles (by pseudocomponent). The particles have an associated spatial distribution, $\omega_j(\mathbf{x})$ for the *j*'th particle. Denoting the mass of pseudocomponent i for particle j by M_j^i, the concentration in a cell or control volume $\delta V = \delta x \times \delta y \times \delta z$ is computed from,

$$C_i(\mathbf{x}, t) = \frac{1}{\delta V} \sum_j M_j^i \int_{\delta V} \omega_j(\mathbf{x}') dV' \tag{2}$$

The Eulerian concentrations are computed on regular 3D grids ($n_x \times n_y \times n_z$); in the present case with $n_x = 850$, $n_y = 970$ and $n_z = 25$.

2.2. The filter feeder model and its parameter data

In order to model the filtration process of oil droplets from the water column by copepods, we consider a simple rate equation for an oil mass m
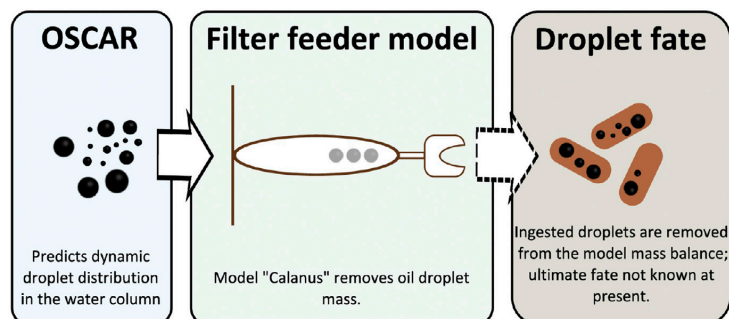
Fig. 1. The filter feeder model and its connection to OSCAR.

$$\frac{dm}{dt} = F(m, t), \qquad (3)$$

where

$$F(m, t) = -\gamma \cdot \rho_{cop} \cdot f_{thc}(C_T) \cdot f_{vert}(z) \cdot f_{ds}(d). \qquad (4)$$

In this equation we find the copepod density ρ_{cop} (ind./m^3), copepod filtration rate γ (ml/day/ind.), filtering reduction f_{thc} due to ambient total hydrocarbon concentration $C_T(t)$ (i.e. concentration of dissolved oil and droplets), filtration rate dependence on water depth f_{vert}, and a term f_{ds} depending on oil droplet diameter. The terms γ, f_{thc} and f_{ds} are determined from either experimental data or literature values, see Table 1.

From experimental studies it is known that the filtration rate will diminish due to narcotic effects as the total hydrocarbon concentration (THC) increases (Nordtug et al., 2015). The effect is insignificant at low concentrations (<0.1 ppm), while at high concentrations (>5 ppm) filtration activity ceases completely. The model approximately accounts for this change in filtration rate through the term f_{thc}, a linearly decreasing function over a finite interval, being either 1 or 0 outside this interval:

$$f_{thc}(x) = \begin{cases} \dfrac{x - C_{min}}{C_{max} - C_{min}} & C_{min} \le x \le C_{min}, \\ 1 & x \le C_{min}, \\ 0 & \text{otherwise}. \end{cases} \qquad (5)$$

Reasonable values for C_{max} and C_{min} are determined from experimental data (Nordtug et al., 2015), see Table 1. This

simplification of the underlying mechanisms of toxicity, while crude, nevertheless captures its most important feature: a decrease in filtration as ambient concentration of oil increases. Below C_{min}, filtration activity is unaffected, and ingestion rate will increase with the concentration of oil droplets.

The vertical distribution of copepods is accounted for by f_{vert}. We use a simple rectangular function, i.e. a constant distribution inside the interval [z_{min}, z_{max}], and zero elsewhere. The same approach is used for the term f_{ds}, which determines the droplet sizes that are filtered in the model; only droplets with diameters in the interval [d_{min}, d_{max}] are filtered.

We add the model function F as a new decay term in the OSCAR Equation (1), in order to be able to assess the potential amount for oil removal through filtering for different environmental conditions and release scenarios. In practice, the model is applied separately to each pseudocomponent in each numerical (droplet) particle. The term f_{thc} introduces a nonlinearity into the equation, since it depends on the ambient total hydrocarbon concentration, which in turn is computed from the mass of the numerical particles, cf. Equation (2). The horizontal distribution of copepods within the model domain is set at a uniform but configurable value (ρ_{cop}). This is done both to keep the overall model complexity at a suitable level for the present investigation, and to more clearly demonstrate the possible impact on the overall oil mass balance. Through a systematic adjustment of the ρ_{cop} parameter, we can get a better understanding of how changes in copepod density affects the oil mass balance.

2.2.1. Oil spill scenarios for model testing

In order to test the filter feeder model, and to explore the

Table 1
Model parameters with sources for nominal values.

Model parameter	Symbol	Nominal value	Reference	Comment
Calanus density	ρ_{cop}	3000 ind./m^3	Helle (2000) (Helle, 2000)	Maximum reported value
Filtration rate	γ	40 ml/ (day × ind.)	Nordtug (submitted) (Nordtug et al., 2015)	
Min feeding depth	z_{min}	0 m		
Max feeding depth	z_{max}	50 m	Unstad (1991) (Unstad and Tande, 1991)	Bulk biomass found between 0 and 50 m
Max tolerable concentration	C_{max}	5 ppm	Nordtug (submitted) (Nordtug et al., 2015)	
No effect concentration	C_{min}	0.1 ppm	Nordtug (submitted) (Nordtug et al., 2015)	
Smallest filterable droplet diameter	d_{min}	1 μm		Exact value not known
Largest filterable droplet diameter	d_{max}	50 μm	Herbert (1980) (Hebert and Poulet, 1980)	Calanus can filter even larger particles (Basedow and Tande, 2006; Bonnet et al., 2004)

potential impact on the mass balance of the spilled oil, we have simulated variations of two scenarios with a version of OSCAR containing the filter feeder module. A scenario specifies the oil spill simulation by location, release rate, composition (oil type) and amount, release and simulation duration and boundary conditions such as the model area and environmental driving forces (winds and currents). OSCAR computes the fate of the released oil and the resulting oil masses in different compartments, including sediments, sea surface, air (evaporated) etc. This is referred to as the mass balance.

The scenarios considered were two accidental releases of 25 000 tonnes of Troll crude oil being either released from the sea surface or from the sea floor, at a location in the Norwegian Sea (see Table 2). Surface releases may generate oil droplets in the water column due to turbulence and vertical mixing caused by wind and wave energy. For sub-surface releases, a Lagrangian element near-field sub-model (Johansen, 2000) is used to account for turbulence and oil droplet size distribution from the release parameters, i.e. release orifice, rate and oil-to-gas ratio.

We selected Troll crude oil (Daling et al., 1997) for two reasons: a set of weathering data is available as input for the OSCAR model, and this oil type was used in earlier experiments on filtration of oil droplets by copepods (Nordtug et al., 2015; Hansen et al., 2012). All simulations were run for a duration of 20 days, with the oil released at a continuous rate during the first 5 days. The OSCAR model area in the Norwegian Sea is shown in Fig. 2.

To assess the effect of the different filter feeder model parameters on the amount of filtered oil, we have run several simulations with different parameter sets (see Table 3). Five cases were simulated for the surface and sub-surface scenarios, and the resulting amounts of filtered oil is shown in Fig. 3. In addition we performed a scan over two of the model parameters that were found to be most important, namely copepod density and maximum droplet size for filtration. The results are shown in Fig. 6, and discussed in the following section.

3. Results and discussion

Based on our current understanding of the processes involved, and a simplified model for filtration using available literature data on selected parameters, we have demonstrated by the use of the OSCAR model that filter feeding organisms may under certain conditions contribute substantially to the overall fate of oil spilled into the marine environment. A base case was designed using a realistic spill scenario with input data available from the literature, and we tested the model by adjusting the data to their reported extreme values. This resulted in an amount of filtered oil between 1–40% of the total spilled oil mass (see Figs. 3–5). However, the high end of this range combines extreme values of several model parameters, and is thus less likely to occur during a real spill situation compared with the lower end of the range. The estimated amount of oil droplets filtered by copepods is highly dependent on the data values used for input to the model and spill characteristics (surface or sub-surface spill). Filter feeders have been mentioned by several authors as a potentially important contributor to the fate of oil (Gyllenberg, 1981; Hansen et al., 2009; Hansen et al., 2012; Conover, 1971; Lee et al., 2012; Lee, 1975), however, no similar

Table 2
Key OSCAR parameters for the surface and sub-surface release scenarios.

Parameter	Surface release	Sub-surface release
Release depth	Sea surface	Sea floor
Release rate	5000 tonnes/day	5000 tonnes/day
Released oil	Troll	Troll
Release diameter	–	5 cm
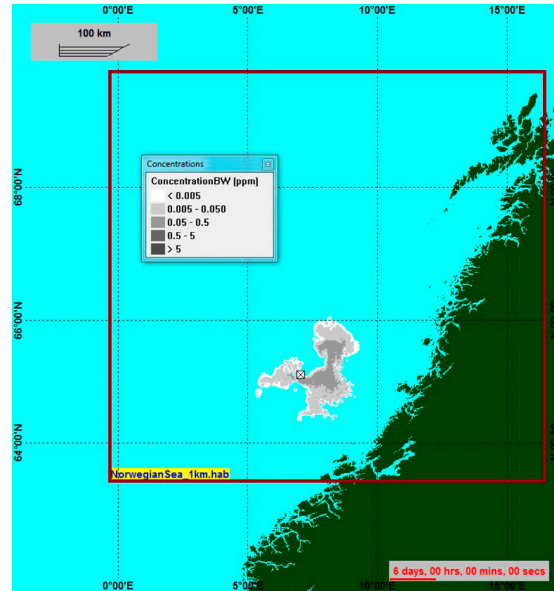
Fig. 2. Overview of the OSCAR model area, also showing total hydrocarbon concentrations in the water column (depth average) for the base case surface scenario.

Table 3
Different simulations exploring the various model parameters. Where blanks occur, the Basecase value is used.

Parameter	Basecase	Case I	Case II	Case III	Case IV	Case V
Max droplet size (μm)	50	100	300			
Max THC (ppm)	5			1.0		
Calanus density (ind/m^3)	3000				1000	
Max filtering depth (m)	50					25
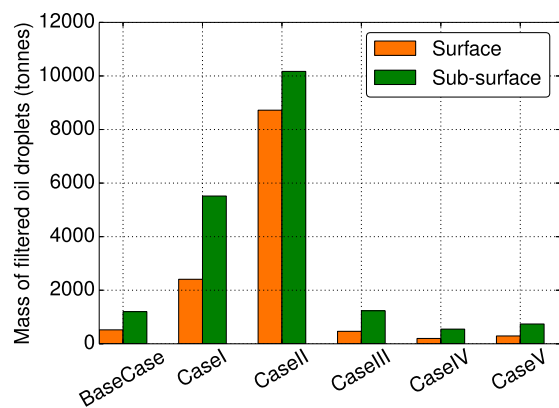
Fig. 3. Total filtered mass at the end of each simulation.

model-based estimations appears to have been reported previously.

Fig. 3 shows the total amount of filtered oil for each simulation case given in Table 3, for both the surface and sub-surface OSCAR scenarios. Fig. 4 shows filtered oil as a function of days after
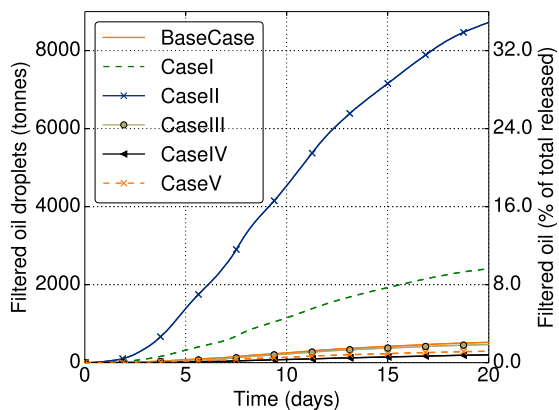
Fig. 4. Simulated filtered oil droplet mass as a function of time for the surface release scenarios.

Fig. 5. Predicted droplet size classes as a function of time for the base case surface release (top panel) and sub-surface release (bottom panel).

simulation start. All scenarios had certain parameters in common. The spill magnitude and rate of release were identical. The geographical location (Norwegian Sea) and timing (March) were also identical between scenarios, chosen based on the optimal

situation for copepod abundance, and suitable data sets for wind and current data for the year 2000 were used. We chose to consider surface spills as well as sub-surface blowouts. When comparing surface and sub-surface spill cases, sub-surface blowouts resulted in higher filtered mass of oil. This is due to the fact that a larger fraction of small droplets is generated by turbulence caused by the small diameter of the release point, as can be seen in Fig. 5, where the droplet diameters as a function of time are shown for the base case. We note that the amount of droplets in each size category changes with time, illustrating the dynamic process whereby surfaced oil is being broken up and entrained by wind and breaking waves, before surfacing yet again. It is also apparent, from Fig. 4, that filtration will continue past day 20 (when the simulation ends), with a corresponding increase in total filtered oil. However, at that point oil in the simulation began to spread beyond the selected model area, which is why 20 days was chosen as simulation duration.

In the base case the abundance of copepods was 3000 ind/m^3, which was the maximum record from Helle (2000) (Helle, 2000). Testing of a lower abundance of copepods (1000 ind/m^3) resulted as expected in lower filtered oil mass (values). As the density of copepods varies significantly throughout the year (Sakshaug et al., 2009), this is an important factor to consider. High copepod abundance corresponds well with the algal spring bloom as mentioned above. Thus, a spill scenario e.g. during the winter time

Fig. 6. Oil filtered as a function of a) the Calanus density parameter (top panel) and b) maximum filterable droplet diameter (bottom panel). Otherwise base case values were used.

will likely result in significantly lower mass of oil filtered by copepods. The sensitivity of the model to changes in copepod density is explored in Fig. 6, where mass of filtered oil scales approximately linearly with density. Scaling with maximum filterable droplet diameter (bottom panel in the same figure) on the other hand displays a more interesting behavior. For droplet diameters less than 100 μm, the increase is less than the corresponding increase in the interval 100—300 μm, while above this threshold the mass of filtered oil levels off. This is due to the dynamic nature of oil mass droplet diameters, illustrated by Fig. 5. The oil mass available for ingestion by copepods, given a maximum filter feeder model droplet diameter d_{max}, varies strongly with the diameter of oil droplets present in the water column at any given time. For instance, a rather small mass fraction of oil droplets is associated with the interval 300—500 μm, which explains why increasing d_{max} in this interval only causes a small increase in filtered mass, cf. Fig. 6. Conversely, the interval 100—300 μm contains a large mass fraction of the total oil, and thus the filtered mass grows strongly with corresponding d_{max} values in this interval.
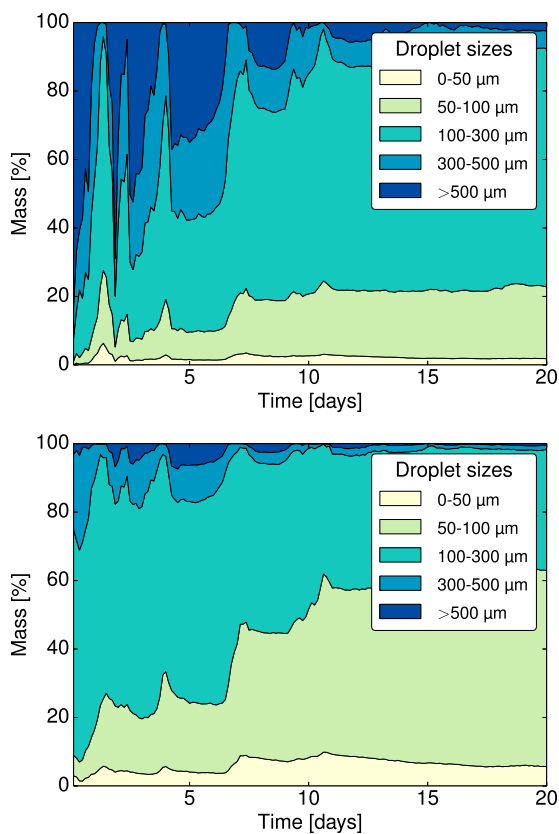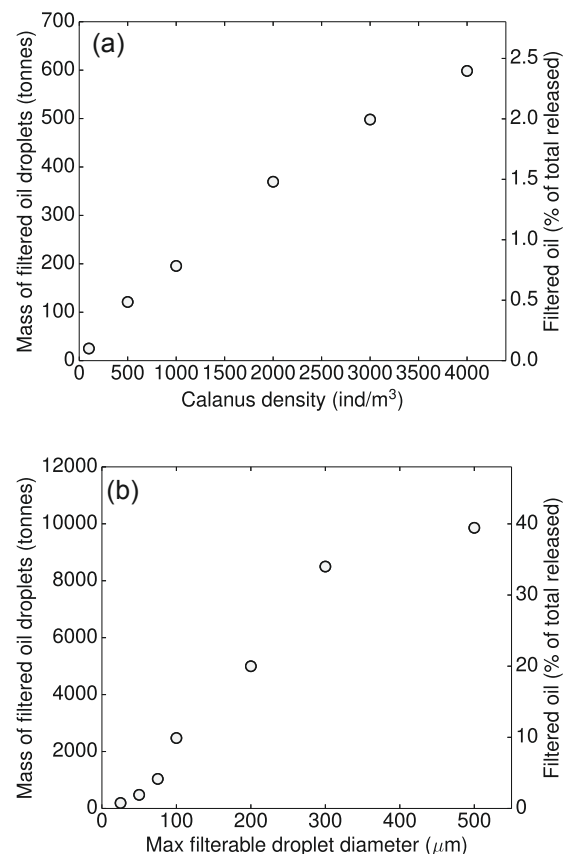
Vertical distribution of actively feeding zooplankton in the water column is determined by diel vertical migration (Hays, 2003), where upward migration of *C. finmarchicus* is triggered by light and food availability, and downward migration is expected to be an anti-predator behavior (Baumgartner et al., 2011). Recently, it was also found that positive phototaxis is enhanced by oil exposure (Miljeteig et al., 2014). We have chosen a uniform distribution of active filtering *C. finmarchicus* between 0 and 50 m, which seems a realistic demarcation based on studies performed in the Irminger Sea (Bonnet et al., 2007), Gulf of Maine (Baumgartner et al., 2011) and in Atlantic and Arctic waters (Unstad and Tande, 1991). In addition to the seasonal fluctuations and vertical distributions, zooplankton distribution in pelagic environments are generally considered to be patchy both on spatial and temporal scale (Unstad and Tande, 1991). A reasonably accurate representation of temporal/spatial variation could be achieved by incorporating predicted copepod distributions from a Calanus population model (for instance SINMOD (Slagstad and McClimans, 2005)), thus enhancing the realism of estimates of copepod oil filtration. As discussed above, we have not included this in the present version of the model, but it will be considered for future iterations.

The mass of oil predicted to be filtered by *C. finmarchicus* in the model is strongly influenced by the droplet size range model parameter. Therefore the amount of filtered mass is large when the maximum filterable size of droplets is set to 300 μm (Case II), which causes a significant fraction of the dispersed oil at any given time to be available for filtering (see Fig. 5). Copepods possess a wide and varied repertoire of feeding strategies. Filtering of larger particles (e.g. nauplii) by copepods is suggested to not occur by passive filter feeding, but by active detection and capturing of prey (Kiørboe, 2011). Active detection and capturing of larger prey particles requires a different modeling approach as both feeding strategy and filtering rates may be different. *C. finmarchicus* is observed to select particles based on size (Meyer et al., 2002), which is a good strategy for optimizing quantity and quality of food (Kiørboe, 2011). Whether active detection and capturing of individual particles is a relevant grazing strategy for the observed filtration of oil particles is not known. It is suggested that larger particles are detected via sensors responding to hydrodynamic cues given off by moving prey (Kiørboe, 2011), but also nutritional value is indicated to be of significance for selective grazing (Leiknes et al., 2014). If selective grazing by copepods is purely based on size it may be expected that larger oil droplets ascending in the water column will transmit similar hydrodynamic cues as large feed particles, and hence be grazed upon in the same manner. Mass of oil filtered in Case II, may thus overestimate an actual filtration due to the lack of knowledge concerning filtering of larger sized oil droplets.

At present, the ultimate fate of filtered oil is not treated in the model. Experiments show that filtered oil is excreted with feces (Nordtug et al., 2015; Olsen et al., 2013), and, at minimum, two paths are possible: 1) Fecal pellets with oil droplets may sediment and deposit on the seabed, or 2) Fecal pellets and oil droplets may be degraded in the water column. Copepod feces is extensively degraded in the photic zone (Viitasalo et al., 1999; Bathmann et al., 1987). Degradation of fecal pellets are likely dependent on multiple factors (Svensen et al., 2012), including the activity of bacteria (Hansen and Bech, 1996; Jing et al., 2012) and other heterotrophic organisms feeding on feces (Svensen et al., 2012; Poulsen and Iversen, 2008), and also composition and compaction of the fecal pellets (Urban et al., 1993). Feces may provide inorganic nutrients (Møller and Nielsen, 2001) and a diverse bacterial community, enhancing the degradation of oil. However, fecal pellets have higher density than seawater (ranging from 1.11 to 1.19 g/cm^3 (Urban et al., 1993) vs 1.020—1.029 g/cm^3) and fecal particles sink in the water column. The extent of sedimentation of feces is dependent on the degradation rate and how the presence of oil influence e.g. bacterial activity may be important for further fate of oil in calanus feces. Sedimentation of calanus feces to seabed and/or higher degradation of oil in feces may contribute to the removal of oil from surface waters via ingestion of oil by calanus copepods. The fate of oil droplets incorporated into feces is not well studied, and further investigation is needed to determine its potential for altering the fate of oil spills.

4. Conclusion and future work

This work provides an initial attempt at quantifying the potential contribution of pelagic filter feeders to the overall fate of spilled oil in the marine environment based on a combination of models and data. Given a time of year with high copepod abundance, and conditions of an oil spill resulting in a large fraction of small dispersed droplets, we have shown that copepods may filter a significant portion of the spilled oil, between 1 and 40% depending on the spill conditions and model parameters. The high end of that range corresponds to combinations of extreme model parameter values, and is thus less likely to be found in an actual spill situation compared with the lower end. Nevertheless, the contribution of filter feeding copepods to the fate of spilled oil should be considered as a component in environmental models. However, before such a model component can be reliably used for predictions of specific real-world spill scenarios, some issues must be addressed: accounting for spatial and temporal variations in copepod abundance in the model, and inclusion of data regarding the ultimate fate of filtered oil. In addition, experimental studies should be performed to test and validate our initial findings.

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Paper II

Environmental Toxicology

OIL DROPLET INGESTION AND OIL FOULING IN THE COPEPOD *CALANUS FINMARCHICUS* EXPOSED TO MECHANICALLY AND CHEMICALLY DISPERSED CRUDE OIL

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Abstract: The rates of ingestion of oil microdroplets and oil fouling were investigated in the zooplankton filter-feeder *Calanus finmarchicus* (Gunnerus, 1770) at 3 concentrations of oil dispersions ranging from 0.25 mg/L to 5.6 mg/L. To compare responses to mechanically and chemically dispersed oil, the copepods were exposed to comparable dispersions of micron-sized oil droplets made with and without the use of a chemical dispersant (similar oil droplet size range and oil concentrations) together with a constant supply of microalgae for a period of 4 d. The filtration rates as well as accumulation of oil droplets decreased with increasing exposure concentration. Thus the estimated total amount of oil associated with the copepod biomass for the 2 lowest exposures in the range 11 mL/kg to 17 mL/kg was significantly higher than the approximately 6 mL/kg found in the highest exposure. For the 2 lowest concentrations the filtration rates were significantly higher in the presence of chemical dispersant. Furthermore, a significant increase in the amount of accumulated oil in the presence of dispersant was observed in the low exposure group. *Environ Toxicol Chem* 2015;9999:1–8. © 2015 SETAC

INTRODUCTION

Following oil spills, micron-sized oil droplets are formed naturally in the marine environment through the turbulence created by breaking waves (mechanical dispersion). These droplets are mixed into the water column by vertical turbulence [1]. The larger droplets will resurface and coalesce to reform thin oil films, whereas oil droplets with diameters less than 50 μm to 70 μm have a much slower resurfacing velocity, will accumulate in the water column, and will passively drift with the currents [2,3]. Chemical dispersants contain surfactants that reduce the interfacial tension between oil and water, thereby reducing the energy needed for breaking the oil into small droplets. This increases the fraction of small oil droplets compared with untreated oil and thus increases the amount of oil in the water column [4,5]. Dispersants may therefore be used to reduce the amount of oil on the sea surface, to protect marine birds and mammals and to keep the oil from reaching sensitive shoreline areas.

Several studies indicate that the use of chemical dispersant increases the bioavailability of polycyclic aromatic hydrocarbons (PAHs), resulting in higher toxicity to organisms [6,7]. This is a consequence of a higher proportion of small-sized droplets with low surfacing velocity causing an increased concentration of oil in the water. In addition, the increased concentration of small oil droplets may increase the uptake of oil components in pelagic organisms by oil fouling and oil droplet filtration [8–13]. Recent studies have suggested that the contribution of oil droplets to the acute toxicity to fish eggs [14], and fish larvae [15–17], and shrimps [18] is low.

However, filter-feeding organisms, such as copepods and mussels, actively filter particles in the same size range as the smallest fractions of oil droplets (<50 μm); thus, the oil droplets may be ingested and made bioavailable through the digestive system [8–10,18,19].

The calanoid copepod *Calanus finmarchicus* (Gunnerus) is the dominating zooplankton species of the Barents Sea during the most productive season and is an important food source for many fish and bird species [20,21]. It can be found in densities well above 3000 individuals/m³ during summer in the photic zone of the northeastern Atlantic Ocean and Barents Sea [20], and its ubiquitous distribution and large biomass in this area make this species a relevant model organism for investigating the interactions between dispersed oil and planktonic organisms. *Calanus finmarchicus* has previously been shown to actively filter oil droplets [9,11], but there are currently no data available on the quantitative relationship between exposure and oil associated with the copepod biomass. If active uptake of oil occurs on a large scale, the fate of the oil can be affected, rendering it more available to predators through feeding [22,23].

The processes affecting the fate and effects of oil in the marine environment are complex, and the exposure patterns for organisms are dynamic because of the continuous weathering of spilled oil [4]. As a result of this complexity, it is virtually impossible to predict the fate and effects of a large oil spill without the use of environmental modeling [23–25]. Current models can predict the fate of the oil in time and space including 3-dimensional concentration fields of dispersed oil, droplet size distributions, and composition of dissolved components [25,26]. These models can be linked to distributions of biological resources to predict interactions between oil and biota [27,28]. Copepods have high filtration rates [29], constitute large biomasses [20,21], and may therefore contribute to the overall fate of the smallest fractions of oil droplets [22,23].

The objective of the present study is to provide quantitative data on oil and oil components associated with the biomass of the copepod *C. finmarchicus* when exposed to crude oil microdroplets. In particular, we focus on filtration and accumulation of oil droplets and whether the presence of a chemical dispersant affects the processes involved in accumulation of oil in the copepod biomass. The data are intended for use in environmental modeling of fate and effects of oil spills.

MATERIALS AND METHODS

Calanus finmarchicus were exposed to 3 concentrations (low, medium, high) of mechanically dispersed oil without dispersant (MD) or to 3 concentrations (low, medium, high) of chemically dispersed oil (CD). The copepods were exposed in a flow-through system in which oil droplet size ranges and concentrations were kept comparable between parallel MD and CD treatments, and they were fed continuously with algae throughout the exposure period.

Copepods and husbandry

Marine copepods (*C. finmarchicus*) were obtained from an in-house culture reared at SINTEF/NTNU Sealab (Trondheim, Norway) routinely kept in flowing seawater at $8\,^{\circ}C$ to $10\,^{\circ}C$ as described elsewhere [30]. Two hundred and fifty adult copepods were carefully introduced into each exposure chamber; into which live feed algae (*Rhodomonas baltica* Karsten, nominal cell diameter 6.9 µm) were added continuously. The average concentration of algae based on wet weight was 1 mg/L (8.8×10^6 cells/L), corresponding to 224 µg C/L. The flow-through in all exposure units was kept constant at 15.7 ± 1.6 mL/min, corresponding to a mean residence time in the water of approximately 5.5 h. Water temperature ($10 \pm 1\,^{\circ}C$) was controlled by submerging all exposure chambers into a water bath.

Exposure

A naphthenic crude oil (Troll) from the North Sea was chosen for the present study. The oil was artificially weathered by heating to $200\,^{\circ}C$ [31], and the $+200\,^{\circ}C$ residue was collected and used for the generation of oil dispersions. The chemical dispersant used was Dasic NS, which is the dispersant with the largest stockpile in Norway.

Chemical and mechanical dispersion of oil was generated according to the method described by Nordtug et al. [32]. Three nominal concentrations were used for both chemically and mechanically dispersed oil; 0.4 mg oil/L seawater, 2 mg oil/L seawater, and 10 mg oil/L seawater. Based on previous experiments [33], the highest concentration was in the high sublethal range for acute toxicity, and the lowest concentration was limited by the need to obtain reliable chemical data. For the chemically dispersed oil, the dispersant Dasic NS was premixed into the oil before the oil was dispersed at a dispersant to oil rate of 1:25, which is the recommended application rate. To achieve similar oil droplet size distributions, the energy input for generating the dispersion with Dasic NS was lower than for the purely mechanically generated dispersion [32].

The exposure system has been previously described by Nordtug et al. [32]; in the present study, we used 28 exposure containers—4 control groups and 12 each for chemically and mechanically dispersed oil, respectively. Three additional containers were used as an algae control without copepods. To achieve biological replicates, 4 parallel exposure chambers were used for each exposure concentration. The copepods were exposed for 96 h.

Particle counting

Concentrations and size distribution of oil droplets and algae in the exposure chambers were analyzed daily using a particle analyzer (Coulter Counter Multisizer 3; Beckman). The results from day 2, day 3, and day 4 were used for further analysis. Water samples were collected from the outlets of the exposure chambers and contained both algae and oil droplets. Total volume and size distribution of oil droplets were separated from those of the algae by conventional baseline correction and subtracting the algae counts from the total particle counts. The concentration of algae entering the exposure vessels was determined from particle counts in the exposure vessels 1 d before the start of the present study, when the system was run with algae only. The stability of the supply of algae during the present study was verified by daily particle counting in the algae controls.

Chemical analysis of water and copepod samples

Samples of the exposure media for chemical analysis (~800 mL) were collected on day 1 and day 3 of the exposure period and acidified with diluted hydrochloric acid. Acidified water samples were extracted with dichloromethane, dried over Na_2SO_4, and concentrated to 1 mL. Water samples for volatile organic compounds (carbon numbers 5–9) were sampled in 40-mL vials with added hydrochloric acid for acidification, capped without headspace, and analyzed using Purge & Trap gas chromatography/mass spectrometry (GC/MS). Determination of the total extractable organic compounds (total hydrocarbon content with carbon numbers from 10 to 36; [C10–C36]) was made on dichloromethane extracts by using a GC–flame ionization detector (GC-FID). Further details of the analytical procedures are given in the Supplemental Data.

For the analysis of PAHs in biological samples, 20 copepods (~18 mg wet wt) from each exposure group were sampled after 96 h of exposure directly from exposure solutions into sterilized glass vials (10 mL) and frozen prior to extraction and analysis. The PAHs analyzed in copepods included gut content and external fouling of oil. Surrogate internal standards (naphthalene-d8, phenanthrene-d10, chrysene-d12, phenol-d6, 4-methylphenol-d8) were added to the copepod samples prior to microextraction. The copepods were weighed into screw-capped glass reaction vials (10 mL) with replaceable Teflon septa. Details of the analytical procedures are given in the Supplemental Data.

Fluorescence microscopy and image capture

The feeding activity of the copepods after 4 d of exposure was assessed as gut filling visualized by the autofluorescence of algal chlorophyll and its degradation products. Prior to examination, 6 copepods from each exposure group were randomly chosen from the exposure units and irreversibly anesthetized with metacaine. Examination of the anesthetized copepods was performed using an inverted microscope (Nikon TE2000) with a 10× planapochromatic objective (Nikon). Fluorescence in the gut was induced by illuminating the specimen with a 120-W mercury arc lamp (x-cite 120; EXFO) passing through a B-2A filtercube (Nikon) in the microscope. Images were captured with a Peltier cooled CCD camera (DS 5Mc; Nikon) controlled from a computer running NIS Elements F (Nikon).

Data treatment and calculations

Algae clearance volume. The water volume cleared of algae by *C. finmarchicus* was calculated in each of the 28 groups for

day 2, day 3, and day 4 of the exposure period based on the difference between algae concentration of the supply water and the water of the exposure chambers and the water flow-through [34,35]

$$CV_A = [(A_s - A_{ch})/A_s] \times F \qquad (1)$$

where CV_A is the clearance volume of algae in L/d, A_s is the average algae concentration in the supply water (μl/L), A_{ch} is the measured algae concentration in the water of the exposure chamber (μl/L), and F is the average water flow through each exposure chamber (L/d). To convert this to clearance per individual, the total volume was divided by the number of individuals in the group during the exposure period, ranging from 232 to 249. All data from similar exposures ($n = 4$; 3 sampling d) were then pooled to obtain the average values for each exposure group.

Partitioning between exposure solution and copepod biomass. The partitioning between dissolved chemicals and the tissue of aquatic organisms is referred to as the bioconcentration factor (BCF), which is the equilibrium concentration in biological tissue divided by the water concentration. Correspondingly, the accumulation of any defined constituent in the water may be expressed in same way as a bioaccumulation factor (BAF)

$$BAF = C_{bio}/C_w \qquad (2)$$

where C_{bio} is the concentration in the biomass, and C_w is the concentration of the same substance in the water. To estimate the partitioning of oil droplets between water and biomass, we either have to quantify the oil or use a marker that is assumed to be found exclusively in the oil. Because of the very limited biomass samples available in the present study (<20 mg) common biomarkers for oil such as hopane were below the detection limit for some of the groups. Analysis by GC-FID of total hydrocarbons has limited sensitivity, is also affected by biogenic molecules, and is therefore not suited for biological samples at very low concentrations.

Three analyte groups (C3-decalins, and C1- and C2-chrysenes), all with octanol-water partition coefficient (log K_{OW}) values above approximately 6 (low solubility) and a sufficiently high concentration, were therefore selected as markers for the oil in further calculations on oil partitioning. To increase the robustness of the calculations, the results are based on the average of all 3 analyte groups (Supplemental Data, Figure S1). According to the initial assumption that a component i is found exclusively in the oil phase, the bioaccumulation factor will be the same as for the oil ($BAF_i = BAF_{oil}$). The BAF_{oil} is the fraction between the oil in the biomass ($C_{oil\ bio}$) and oil in the water ($C_{oil\ w}$)

$$BAF_{oil} = C_{oil\ bio}/C_{oil\ w} \qquad (3)$$

Rearranging this equation and replacing BAF_{oil} with BAF_i, the concentration of oil in the biomass will be

$$C_{oil\ bio} = C_{oil\ w} \times BAF_i \qquad (4)$$

Clearance volume for oil droplets. Because of the relatively large span in oil droplet concentration and the tendency of oil droplets to drift toward the water surface or attach to surfaces, it was impossible to measure the clearance rate of oil. However, the clearance volume for oil can be calculated from oil

associated with the biomass (Equation 4), oil concentration in the exposure solution, and biomass per liter (Equation 5)

$$CV_O = C_{oil\ water}/(C_{oil\ bio} \times B_m) \qquad (5)$$

where CV_O is the clearance volume of oil (L); C_{water} and C_{bio} are the total oil droplet concentrations in water (μL/L) and biological sample (μL/kg), respectively; and B_m is the copepod biomass (kg). The weight per individual is assumed to be 0.9 mg, and the individual clearance volume was calculated by dividing the total clearance by the average number of copepods per liter.

The residence time of the oil in the digestive system of *C. finmarchicus* (R_O in days) can be estimated by the clearance volume of oil associated with the biomass (CV_O; L) and the clearance rate of algae (CV_A; L/d)

$$R_i = CV_i/CV_A \qquad (6)$$

Data analysis. Statistical analysis and data visualization were carried out using GraphPad Prism 6. Unless otherwise stated, measures of variation are given as standard deviation and comparison between data and testing of statistical significance done by analysis of variance followed by Tukey's multiple comparisons test.

RESULTS

Physicochemical properties of the oil dispersions

Samples taken from the high exposure groups showed similar volumetric oil droplet size distributions in the presence (chemically dispersed oil, high concentration [CDH]) and absence (mechanically dispersed oil, high concentration [MDH]) of chemical dispersant (Figure 1). The presence of algae is seen as a peak superimposed on the oil droplet spectrum around 7 μm. When the concentration of algae and oil droplets were evaluated, the 2 spectra were separated by baseline correction for the algae peak by extrapolating the curve for oil
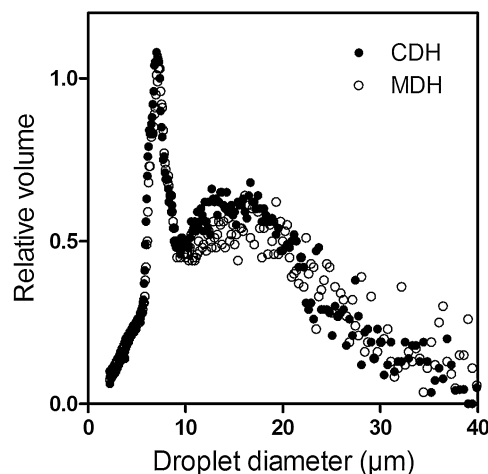

Figure 1. Comparison of droplet sizes in the high exposure groups. The peak superimposed on the oil droplet size spectrum represents the algae used for feeding. CDH = chemically dispersed oil, high concentration; MDH = mechanically dispersed oil, high concentration.

droplet peak in the region of the algae peak (~5–9 μm; see Hansen et al. [11]). Mean size of the droplets was approximately 14 μm in both exposure solutions and was maintained for all exposure concentrations throughout the experimental period.

Chemical analysis of the exposure solutions showed that the 2 parallel experiments with CD and MD generated nearly identical concentrations of total petroleum hydrocarbons (Figure 2A). The highest concentration of CD and MD was approximately 5.5 mg total petroleum hydrocarbons (C5–C36) L^{-1} and the concentrations were similar between parallel treatments. However, the low and medium exposure concentrations were found to be significantly lower for CD compared with MD: 0.20 mg/L versus 0.30 mg/L ($p < 0.01$; t test) and 0.88 mg/L versus 1.24 mg/L ($p < 0.01$), respectively (Figure 2A).

Particle counting showed a maximum volume fraction of oil droplets of the high exposure groups of approximately 6 ppm (Figure 2B), which corresponds to an oil droplet concentration of 5.4 mg/L (oil density: 0.9 kg/L). The oil droplets fractions for both chemically and mechanically dispersed oil were 17%, 45%, and 83% of the total particulate matter (algae and oil droplets) in the low, medium, and high exposures, respectively.

Filtering activity and algae clearance rate

The control animals extracted on average 45% of the algae from the water, which was significantly more than percentages for the exposed groups, which were in the range of 6% to 33% (Figure 3A). Thus, the average individual clearance volume of the control of 41.4 mL/copepod/d was also significantly higher than that of the exposed groups, which, except for the MDH group, decreased in a concentration–dependent manner. Compared with the low and medium MD groups, there were significantly higher clearance rates for the copepods exposed to low concentrations of chemically dispersed oil (CDL; $p < 0.0001$) and medium concentrations of chemically dispersed oil (CDM; $p < 0.001$; Figure 3B). No significant difference was observed between CD and MD for the high concentration. Fluorescence microscopy used to visualize algae

in the gut of the copepods showed some individual variance within the groups, but generally there was a trend of reduced algae content in copepod guts as a function of increased exposure (Supplemental Data, Figure S2).

Bioaccumulation of oil and oil components

The average BAF for selected high log K_{OW} components (C3-decalines, and C1- and C2- chrysenes) increased significantly ($p < 0.0001$) for each step of dilution (decreasing exposure concentration) for both MD and CD (Figure 4A). Compared with the highest concentrations, the BAF increased by factors of 13.1 ± 3.0 and 71.8 ± 16.3 for the CDM and CDL groups and 10.6 ± 3.3 and 41.1 ± 12.9 for the mechanically dispersed oil, medium concentration (MDM) and mechanically dispersed oil, low concentration (MDL) groups, respectively. There were no significant differences between chemically and mechanically dispersed oil for the medium and high exposure groups, whereas the BAF of the CDL groups was significantly higher than that of the MDL groups ($p < 0.001$).

The oil content in samples of *C. finmarchicus* was estimated from Equation 4 based on the bioaccumulation of selected components with high log K_{OW}. The resulting estimates were approximately 11 mL/kg and 17 mL/kg for copepods exposed to the low and medium oil concentrations, respectively, and approximately half this value for copepods exposed to the high concentration (Figure 4B). Predicted concentrations at the highest oil droplet exposure for both MDH and CDH were significantly lower than for all other groups ($p < 0.05$–0.0001). No differences were found between MD and CD or between high and medium exposure, whereas CDL was significantly higher than MDL ($p < 0.01$).

Clearance of oil by copepods

Because of the technical challenges associated with working with oil droplets, we were not able to accurately determine the inlet oil droplet concentration, so clearance rates for oil could not be calculated. However, the clearance volume corresponding to the amount of oil associated with the copepods was
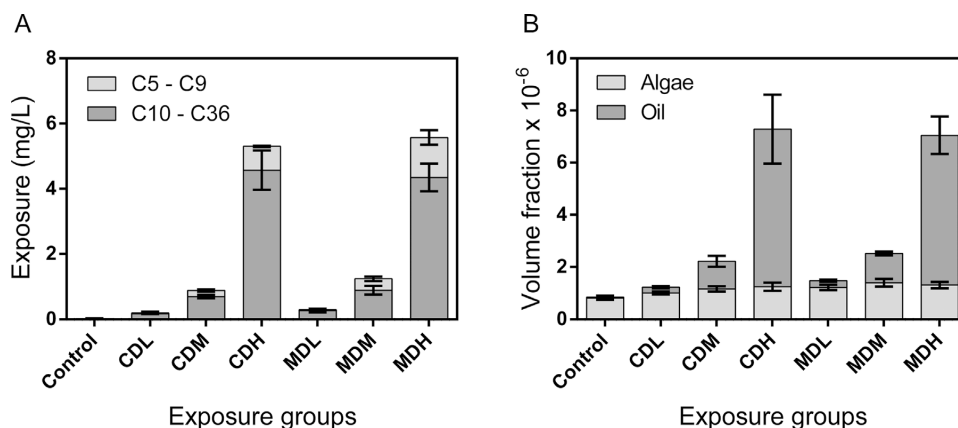
Figure 2. Composition of exposure solutions. (**A**) Concentrations of oil components with carbon numbers from 5 to 36 (C5–C36) separated into volatiles (C5–C9) and nonvolatiles (gas chromatrography–flame ionization detector analysis of C10–C36). Samples were taken on day 1 and day 3 of exposure for each group. Vertical bars indicate mean concentration and whiskers standard deviation (SD); $n = 8$ for each group. (**B**) Volume of particle content. Individual bars show the mean of a total of 12 replicates from exposure day 2, day 3, and day 4 separated in algae and oil droplets with standard deviation. CDL = oil dispersed with the chemical dispersant Dasic NS, low concentration; CDM = oil dispersed with the chemical dispersant Dasic NS, medium concentration; CDH = oil dispersed with the chemical dispersant Dasic NS, high concentration; MDL = mechanically dispersed oil, low concentration; MDM = mechanically dispersed oil, medium concentration; MDH = mechanically dispersed oil, high concentration.
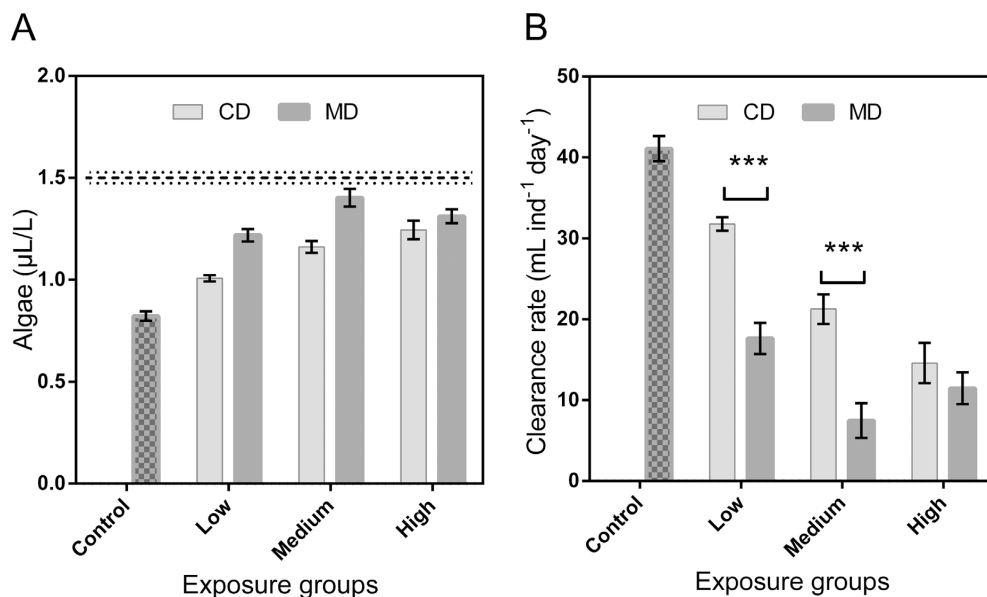
Figure 3. Average depletion of algae and corresponding clearance rates during the last 3 d of a 4-d exposure. (**A**) Recorded concentrations of algae in exposure containers. Bars indicate average algae concentrations for each treatment. Horizontal line shows average algae concentration in inlet water with standard error of the mean (SEM) indicated. (**B**) Algae clearance rates for copepods during exposure to chemically or mechanically dispersed oil for 4 d. Asterisks indicate significant differences between groups (***$p < 0.001$). Whiskers indicate SEM; $n = 12$ for all groups. CD = chemically dispersed oil; MD = mechanically dispersed oil.

calculated from Equation 5 (Figure 5A). The oil clearance volume decrease with increasing concentration in the same manner as the clearance rate for algae, and the volume was significantly different between MD and CD for the 2 lowest concentrations. The residence time for the oil in the gut for the low exposure group was estimated at approximately 40 h based on oil clearance volume and the algal filtration rate for the low

exposure group (Equation 6), indicating no difference between the CD and MD groups (Figure 5B).

DISCUSSION

The experimental system used [32] made it possible to compare crude oil droplets made from untreated and chemically treated oil that were similar in terms of oil concentrations and
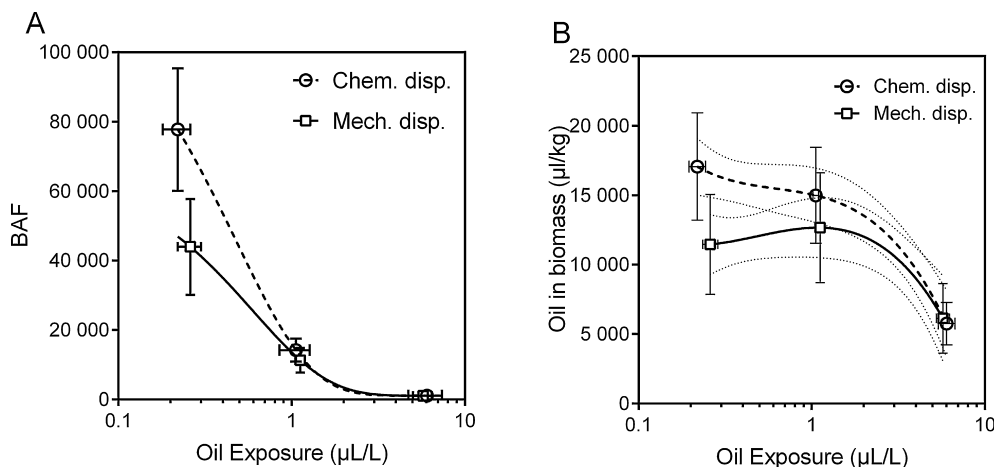
Figure 4. (**A**) Accumulation factors ($C_{bio} \times C_{water}$-1) for oil in biomass of *C. finmarchicus* as a function of exposure concentration of oil dispersion with standard deviation. (**B**) Oil associated with biomass with 95% confidence interval and standard deviation indicated. For both (**A**) and (**B**), curves are generated by second-order polynomial (quadratic) fit with interval indicated. $n = 12$ except for the highest exposure groups (chemically dispersed oil, high concentration: $n = 6$; mechanically dispersed oil, high concentration: $n = 9$). BAF = bioaccumulation factor.

A

B
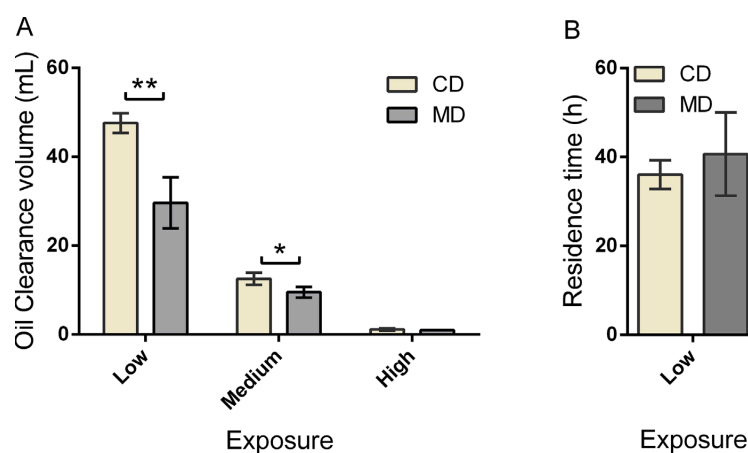
Figure 5. (**A**) Oil clearance volumes (mL) calculated from measured body residues of oil, based on average filtration rates for 3 d in 4 exposure groups ($n = 4$) except for the highest exposure groups (chemically dispersed oil, high concentration, $n = 2$; mechanically dispersed oil, high concentration, $n = 3$) . Asterisks indicate significant differences between parallel treatments of CD/MD (*$p = 0.017$; **$p < 0.01$). (**B**) Residence time of oil in the gut for the low exposure group expressed as body oil clearance volume (mL) divided by the clearance rate for algae (mL/ind/d, $n = 4$). CD = chemically dispersed oil; MD = mechanically dispersed oil.

droplet size and chemical composition. It should be noted that the experimental design in the present study did not intend to simulate a real oil spill scenario, where dynamic changes in droplet size distribution, droplet concentration, and dissolved hydrocarbon concentration are expected to occur. Furthermore, in a field situation the effect of the dispersant on oil droplet size distribution and oil concentrations in the water is highly dependent on the physiochemical properties of the oil and the turbulence created by wave action [4].

The oil concentrations were stable throughout the 4-d experiment, and both oil droplet concentrations and mean droplet sizes were similar between MD and CD treated oil. Thus, the major difference between the 2 exposure series generated was the presence of chemical dispersant in 1 of the series (CD). The exposure concentrations used in the present study are in the high range of what is expected in large water volumes in a real oil spill situation. The most extensive dataset of field data on dispersed oil microdroplets is from the Deepwater Horizon incident: approximately 20% of the samples had a concentration of small oil droplet small particles (d ≤ 70 μm) above the detection limit of 0.3 μL of the method used, and most of the values detected were below 0.5 μL/L to 1 μL/L, with a few peak values up to 15 μL in the surface layers [2].

Both the chemically and mechanically dispersed oil caused a reduction in the filtration activity of the copepods that appeared to be concentration dependent. These findings were in line with observations using fluorescence microscopy imaging where a reduced stomach content of algae was observed in copepods exposed to oil concentrations above approximately 1 mg/L. The main candidates for causing a concentration-dependent reduction in filtration are partial narcosis because of uptake oil components and/or mechanical obstruction and clogging of the feeding apparatus. The observed differences in filtration rates and bioaccumulation between mechanically and chemically dispersed droplets in spite of the similar chemical exposure may indicate that the mechanical properties of the droplets are involved. Chemical dispersants are known to change the interfacial tension between oil and water and thus reduce the stickiness of the oil; therefore chemically dispersed oil may

have a reduced ability to obstruct the feeding apparatus of filter feeders.

Reduced algae filtration has previously been reported for copepods exposed to oil dispersions (1–10 ppm) [11,36]. Spooner and Corkett [36] observed only marginal effects at concentrations of 1 ppm to 2 ppm Kuwait crude oil dispersion, but feeding rates and fecal pellet production was significantly reduced at 10 ppm exposure. Hansen et al. [11] found reduced algae stomach content in copepods exposed to chemically and mechanically dispersed oil at concentrations ranging from 0.05 mg to 3 mg total hydrocarbon content/L, and reduced filtration rates were observed for concentrations ≥0.3 mg total hydrocarbon content/L.

The high copepod densities, in combination with relatively high particle concentrations used in the present study, would be expected to negatively affect the filtered volume per individual, and the clearance rate of the controls (41 mL/individual/d) was considerably less than the maximum values of up to 500 mL/d reported from experiments with low algal densities [37]. However, the clearance volumes observed for our control group were in the exact same range as previously recorded for the same algae at comparable concentrations [38]. The corresponding clearance rate reported by the same authors [38] increased to 60 mL/ind/d when the algal concentration was reduced to approximately 20% of the concentration used in the present study.

To calculate the body residue of oil, we assumed that the target analytes used as markers for the oil were completely contained in the oil droplets. However, there are other potential sources for uptake of these components in the copepods, and the maximum value of 17 mL oil/kg is probably overestimated. Possible candidates for uptake are passive partitioning from dissolved fractions and uptake from contaminated algae. We know from previous studies with the same oil that the analytes used in the present study have a dissolved fraction in water of less than 3% at oil-in-water dispersion concentrations of approximately 1 mg/L [32]. In a study with the copepod *Paracartia grani* [39], correlations between log K_{OW} values were established both for BCFs and for BAFs for PAHs in the

presence of contaminated algae. Using these regressions, the expected BCF for a component with a log K_{OW} of 6 is in the range 4.08 to 4.42 ($K_{OW} = 12\,000–26\,300$). In the present study, the lowest BAF found at the highest exposure was approximately 1000, and assuming a dissolved fraction of 3% and a BCF of 26 300, it can be shown that the contribution of uptake from the water phase will be 55% of the BAF. Berrojalbiz et al. [39] also showed that feeding contaminated algae did not have a significant effect on BAF. Thus, the estimate for oil associated with the highest concentration may be twice as high as the actual value. At the lower exposure concentration the contribution of the uptake from water is expected to decrease primarily because the BAF increases, whereas the BCF is expected to be independent of exposure concentration. Because the contribution of the passive uptake to body residue at the lower concentrations is expected to decrease rather than increase, the potential contribution of passive diffusion does not affect the conclusion that uptake of oil is significantly higher at the lowest concentrations. If we assume as a highest estimate that the uptake from the dissolved fraction is the same for the lower concentrations as for the high concentration, the actual oil concentration in the biomass is expected to be approximately 14 mL/kg for the 2 lowest concentrations and approximately 3 mL/kg for the highest exposure.

By comparing the estimated clearance rate for algae and the corresponding clearance volumes for oil, it is evident that the proportional reduction in clearance volumes is much higher for oil than for algae in the high exposure groups. One potential explanation is that there is a loss of algae either because of intoxication and settling or adhesion to oil droplets in the high exposure, causing an overestimation of the filtration rate. We tested this in a separate experiment with algae and oil without copepods and found no indication that this is the case. Alternatively, there may be different residence times for oil in the gut in the high exposure group; however, to explain the observed differences, the residence time for the MDH group would have to be 2 h, which we find rather unlikely in comparison with the values of 40 h estimated for the low exposure group. Selective feeding, which has been described previously in *C. finmarchicus* [40], could also explain the observed differences but is unlikely to appear exclusively in the high exposure group. Some undetected technical failure in the supply of algae can also not be excluded, but is equally unlikely. Thus, we are not at present in a positon to explain the apparent differences observed between the estimated values for clearance of algae and oil droplets; this should be a topic for further investigation.

We have no quantitative measure of the relative fraction of oil that adhered to the surface of the copepods compared with oil in the digestive system. However, external fouling is expected to vary in proportion to the oil exposure and will thus contribute most to the estimated oil clearance volume at high exposure concentrations, where we obtained the smallest values for clearance volume. Thus, with the possible exception of the highest exposures (with an increased amount of oil attached to the surfaces of the animals), fouling is most likely of minor importance compared with the fraction ingested in association with normal feeding behavior.

To achieve good quantitative data, the densities of copepods were kept unrealistically high in the present study. The densities used (~50 individuals/L) by far exceed the densities found in nature, which rarely exceed 5 individuals/L on average in the upper 50 m to 60 m of the water column [21]. In parts of the Atlantic water of the Barents Sea, average densities of stage V copepods may be expected to reach 1000 to 3000 individuals/m^3 or more during late summer [21]. In spite of the high copepod densities used in the present study we expect that the observed effects of oil on filtration rates and partitioning of oil components between oil and copepods will also occur at lower and more environmentally realistic copepod densities.

The high abundance, ubiquitous distribution, and high biomass concentrations of oil in copepods exposed to low dispersion concentrations suggest that copepods may have an impact on the overall fate of dispersed crude oil. The potential impact of mass transport of oil on pelagic filter-feeders has also been described by others [12], and the process of oil filtration by filter-feeders might be considered to be included in oil fate and transport models (e.g., the OSCAR model) [25,41]. Implementation of this mechanism is, however, not straightforward, as different conditions will affect the mass proportion of oil that will be filtered by copepods. Simulations of oil spill scenarios including the effect of copepod filtration of oil droplets have recently been published [23]. Not surprisingly, the results show that the fraction of the total oil mass processed by copepods is highly dependent on the release conditions and the particle size preferences of the copepods.

CONCLUSIONS

The present study was conducted to assess how much oil can be associated with copepod biomass following exposure to dispersed oil and to evaluate how chemical dispersants affect the interaction between particulate oil and copepods (*C. finmarchicus*). The results indicate that the copepods may accumulate more than 10 mL oil/kg biomass through active filtration of microdroplets of crude oil. Because of a concentration-dependent decrease in filtration rates toward higher oil droplet concentrations, the highest potential for accumulation appears at oil concentrations below approximately 1 mg/L. At equally low concentrations of similar sized mechanically and chemically dispersed oil droplets, accumulation of chemically dispersed oil is slightly higher than for mechanically dispersed oil. This may be associated with changes in surface properties of the droplets as a result of the dispersant, making the oil less likely to obstruct the feeding apparatus of the copepods.

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SUPPLEMENTAL DATA

OIL DROPLET INGESTION AND OIL FOULING IN THE COPEPOD *CALANUS FINMARCHICUS*

EXPOSED TO MECHANICALLY AND CHEMICALLY DISPERSED CRUDE OIL

Content of supplemental data:

- Analysis of water samples (method).

- Analysis of Biological samples (method).

- Figure S1. Oil associated with biomass in copepods.

- Figure S2. Fluorescence pictures copepods.

Analysis of water samples

Determination of the total extractable organic compounds (total hydrocarbon content with carbon numbers from 10 to 36; THC (C10-C36)) was performed on dichloromethane (DCM) extracts by Gas Chromatography–Flame Ionization Detector (GC-FID). The system comprised of an Agilent6890N GC fitted with an Agilent 7683B Series autosampler. The column was an Agilent J&W HP-5 fused silica capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1.5 ml x min^{-1}. 1 µl samples were injected into a 310 °C split/splitless injector. The oven temperature was heated to 40 °C for 1 min, then heated to 315 °C at 6 °C/min and held at 315 °C for 15 min.

Volatile organic compounds (C5-C10) were quantifed by Purge and Trap Gas Chromatography/Mass Spectrometry, using a modified US Environmental Protection Agency EPA-Method 8260, with a 50 m (0.20 mm ID, 0.50 µm film thickness) Supelco Petrocol capillary column. Target analyses were detected with an Agilent 5973B MSD and the data were acquired using the Agilent EnviroQuant Chemstation software.

Analysis for semi-volatile organic compounds including decalins, naphthalenes, and 3 to 5-ring polycyclic aromatic hydrocarbons were performed by Gas Chromatography–Mass Spectrometry (GC-MS) operated in selected ion monitoring mode. The system comprised of an Agilent 6890N GC with an Agilent 5975B quadrupole Mass Selective Detector. The column was an Agilent J&W HP-5MS fused silica capillary column (60 m x 0.25 mm ID × 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1.2 ml x min^{-1}. A 1 µl sample was injected into a 310 °C split/splitless injector. The oven temperature was heated to 40°C for 1 min, then heated to 315 °C at 6 °C x min^{-1} and held for 15 min. Data and chromatograms

were monitored and recorded using MSD ChemStation (version D.03.00.611) software. The

MSD ion source temperature was 230 °C.

Analysis of Biological samples

To each 10 ml vial containing 20 copepods 3 ml of potassium hydroxide (6.5%) in methanol (80%) and internal standards (SIS) were added. The mixture was heated for two hours in an ultrasonic bath at 80 °C to achieve saponification, followed by filtration and serial extraction with hexane (3 x 3 ml). The combined extracts were dried with anhydrous sodium sulphate and concentrated to approximately 0.5 ml using a Zymark Turbovap® 500 Concentrator. Cleanup of the extracts was performed by solid phase extraction using 3 ml columns containing 0.5 g normal phase silica packing (Superclean LC-Si, Supelco). The samples were eluted through the column with 3 x 2 ml of DCM:hexane (1:3). The purified extracts were concentrated to 90 µl in an insert GC vial insert and recovery internal standards (RIS; fluorene-d10, and acenaphthene-d10) were added in 10 µl of DCM immediately prior to GC-MS analysis. The following target oil compounds were quantified: decalins (C0-C3), naphthalenes (C0-C3), biphenyl, acenaphtylene, acenaphthene, dibenzofuran, fluorenes (C0-C3), phenanthrenes/anthracenes (C0-C4), dibenzothiophenes (C0-C4), fluoranthenes/pyrenes (C0-C3), benz(a)anthracene, chrysenes (C0-C3), benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenz(a,h)anthracene and benzo(g,h,i)perylene.
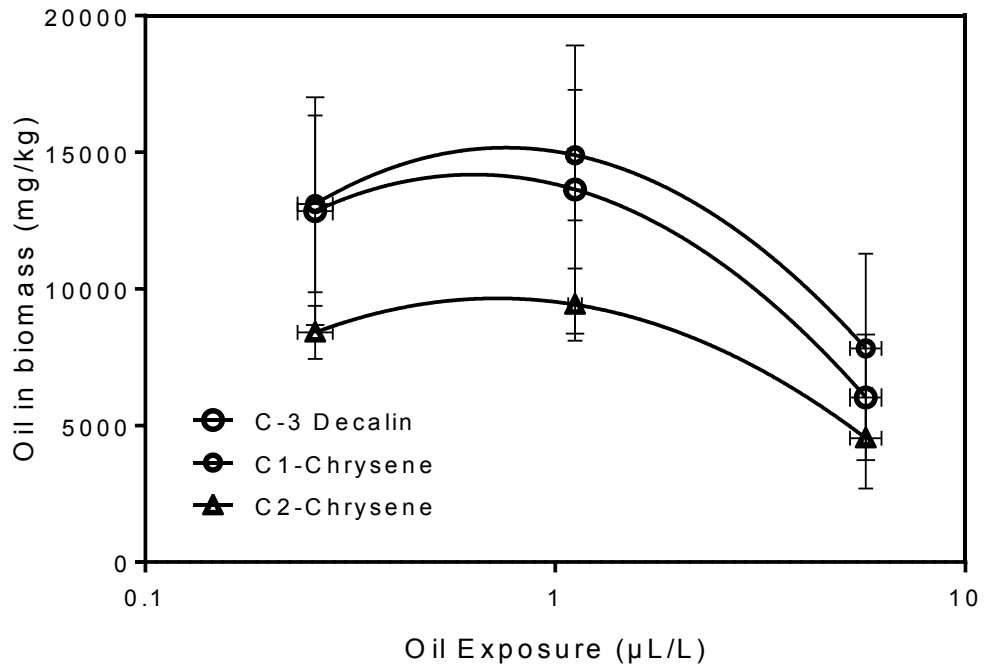
Figure S1. Oil associated with biomass in copepods exposed to mechanically dispersed oil droplets. Based on three different components with high Log Kow (> ~6). Curves generated by second order polynomial (quadratic) fit, N= 2 and 3 for the high exposure groups and 4 for the other groups, SD indicated.
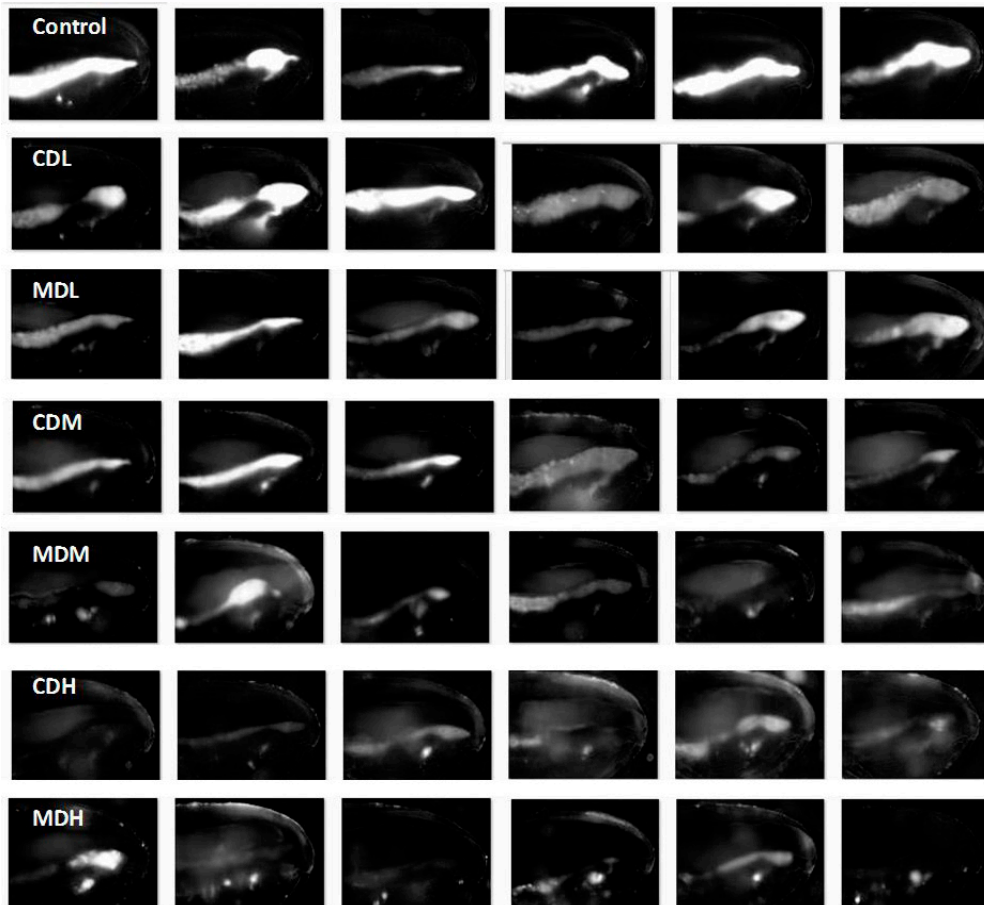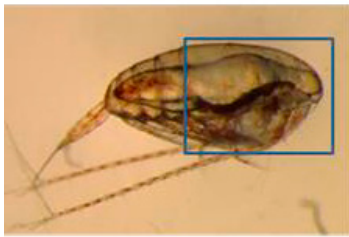
Figure S2. Fluorescence pictures of the frontal part (indicated in the picture on top) of randomly selected individuals exposed to various concentrations of oil dispersions. Fluorescence from algae is seen as white areas. N = 6 for all exposure groups; CD = chemically dispersed; MD = mechanically dispersed; L = low exposure concentration; M = medium exposure concentration; H = high exposure concentration.

Paper III

Uptake of PAHs in *Calanus finmarchicus* from seawater petroleum oil dispersions and the water soluble fraction

Ingvild Fladvad Størdal [*] and Bjørn Munro Jenssen

Abstract

Oil dispersions are formed in the water during oil spills. The accumulation of oil compounds in pelagic filter feeders may impact oil spill transport and weathering. In the present experiment, we quantified accumulation of polycyclic aromatic hydrocarbons (PAHs) from the oil dispersion and the water soluble fraction (WSF) during 96 hour exposure to the ubiquitous copepod *Calanus finmarchicus*. When exposed only to the WSF, the low lipophilic compounds (log K_{ow} < 5) reached a steady state within 24 hours, while the high lipophilic compounds (log K_{ow} > 5) did not reached steady state during the 96 hour exposure. The oil dispersion exposed *C. finmarchicus* accumulated highest concentration of oil compounds. Elimination of low lipophilic compounds was observed.. The results show that *C. finmarchicus* can impact oil spills and affect transport and weathering of oil by sequestering oil and eliminating oil compounds after exposure.

Keywords

Dispersed crude oil, Water soluble fraction, *Calanus finmarchicus,* Bioaccumulation, PAHs, Oil fate

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Introduction

Oil spills in marine environments are subject to weathering processes, such as spreading, evaporation, emulsification, dispersion, dissolution and biodegradation. Dispersion of oil droplets into the water column occurs through the action of breaking waves and is enhanced by the application of chemical dispersants (Brandvik, 1997). Dispersion of small droplets (< 50 µm) into the water accelerate dissolution and biodegradation of oil components (NRC, 2005). Compounds partition to the water depending on several factors, amongst others the concentration of the oil dispersion and the solubility of the compounds in the water and the oil compartment (Nordtug et al., 2011; Redman et al., 2012). The oil compounds that dissolved to the water phase constitute the water soluble fraction (WSF) of an oil spill. The weathering and transport processes affecting an oil spill are complex and interdependent and may be predicted using numerical oil spill models (Reed et al., 1999; Reed et al., 1995).

Petroleum polycyclic aromatic hydrocarbons (PAHs) and other crude oil components accumulate in marine plankton after exposure to oil and oil compounds (Berrojalbiz et al., 2009; Jensen et al., 2012; Nordtug et al., 2015). The main focus has traditionally been on accumulation of dissolved components through passive diffusion processes, where components distribute and partition between the water and the animal body according to the polarity of the two compartments and the lipophilicity (often expressed as log K_{ow}) of the components (De Hoop et al., 2013; Hendriks et al., 2001) However, ingestion of oil has been reported for various marine filter feeders (Almeda et al., 2014; Hansen et al., 2012; Lee et al., 2012; Nordtug et al., 2015; Olsen et al., 2013). Ingestion of oil is an additional route of accumulation of oil components to biomass, potentially contributing in addition to the passive accumulation of oil compounds over body surfaces. Due to their indiscriminate feeding strategy, high feeding activity and high numbers, copepods may impact transport and weathering of oil and oil compounds in the water column during oil spills (Nepstad et al., 2015). Uptake, elimination dynamics, in-body redistribution and/or degradation of oil compounds are processes that may have an impact on the transport and weathering processes of an oil spill. Improving the knowledge on the interaction between oil dispersions and zooplankton may therefore be mandatory to fully understand the interaction between zooplankton and oil spills (Nepstad et al., 2015).

During the last approximately 10 years, a number of studies have used *Calanus* copepods as model marine filter-feeders to understand interaction processes between biota and oil (Hansen et al., 2012; Jensen et al., 2012; Jensen et al., 2008; Nordtug et al., 2015). In North Sea, Norwegian Sea and Barents Sea, the *C. finmarchicus* is generally recognized as a keystone species serving as the main food, at least during juvenility, for a number of commercial fish species (Sakshaug et al., 2009). The species is an omnivorous filter-feeder, and its value as fish feed is largely dependent on its ability to

accumulate energy in the lipid storages, which mostly consist of wax-esters. The large fat content additionally makes the species prone to accumulation to high concentrations of lipophilic contaminants, such as PAHs.

The aim of the present work was to study PAH uptake from the WSF and from the particulate (oil droplets) fraction of dilute oil dispersions by exposing *C. finmarchicus* to two parallel sub-lethal treatments; i) Oil dispersion (with oil droplets) and ii) the corresponding water soluble fraction (WSF; filtered dispersion without droplets), for 96 hours. Samples of *C. finmarchicus* were collected every 24 hours and analysed using gas chromatography-mass spectrometry (GC-MS). To our knowledge, little information is available on the relative contribution to accumulation of oil compounds from the particulate fraction of an oil spill and the corresponding WSF in marine filter-feeders.

Materials and methods

<u>Copepods, seawater, oil, and generation of oil dispersion and WSF</u>
C. finmarchicus (Gunnerus) were obtained from the continuous culture running at NTNU/ SINTEF Sealab (Trondheim, Norway). The copepods were cultured in flowing natural seawater at 8-10 °C and regularly fed a diet of the microalgae (*Rhodomonas baltica* Karsten, *Dunaliella tertiolecta* Butcher and *Isochrysis galbana* Parke). The culture has previously been described in Hansen et al. (2007). Natural seawater was used in the experiment, continuously supplied to the NTNU/SINTEF Sealab laboratory facilities through pipelines from 80 m depth in Trondheimsfjorden, Norway (63°26´N, 10°23´E). The seawater was sand-filtered to remove coarse particles, adjusted to ambient atmospheric conditions and filtered (5 μm exclusion limit, Cuno Aqua-Pure water filter system) before use.

The oil used in the experiment was the naphthenic North Sea crude oil from the Troll exploration field. The oil was artificially weathered by evaporation to 200 °C (Stiver and Mackay, 1984). The +200 °C residue was filtered (VWR syringe teflon filter, 0.2 μM pore size) to remove particles and used for generating the oil dispersion and the WSF, as described in Nordtug et al. (2011). The method enables tight control of the oil droplet size range and concentration. In the present experiment, a dilute oil dispersion with small droplets was generated to realistically represent oil spill scenarios (Lee et al., 2013). The oil dispersion was generated with a nominal concentration of 2 μL L^{-1} and a droplet size distribution < 40 μm, using water and oil flow rates of 160 mL min^{-1} and 0.35 μL min^{-1}, respectively.

<u>Exposure of copepods</u>
The copepods were exposed to the oil dispersion and corresponding WSF for 96 hours in a exposure set-up previously described in Størdal et al. (2015). The setup also included a control series (copepods and feed algae only). The average flow through each exposure tank was 20.6 ± 1.8 mL min^{-1}. All exposure tanks were supplied with live *R. baltica* microalgae (nominal cell diameter 6.9 μm) during the whole exposure period at a nominal concentration of 400 μg C L^{-1}. For both exposures and the control treatment four replicates were included.

Due to limitations in the capacity of the set-up, the experiment had to be run in two series; one in May and one in June. Each series included two replicates for each treatment.

At the start of each exposure series 400 copepodite stage 5 *C. finmarchicus* were carefully transferred to each exposure tank. The oil exposure was started concomitantly with the transfer of the copepods.

Particle counting
Concentration of algae supplied to the exposure vessels were quantified by particle counting of the algae stock solution (Coulter Counter Multisizer 3; Beckman). The concentration of oil and size of droplets in the oil dispersion were also controlled using the Coulter Counter.

Concentration in the oil dispersion exposure tanks
During the first day of exposure, the concentration of the exposure solutions (oil dispersion and WSF) approached the maximum level in the exposure tanks logarithmically (Fig. 1). A curve calculated as exponential rise to maximum was fitted to the oil concentration quantified in the outflow tubes of one of the oil dispersion tanks to illustrate the increase in oil concentration over the first 24 hours:

$$f(x) = a \times (1 - e^{-bx}) \qquad (1)$$

Analyses of PAHs in C. finmarchicus and in the exposure media
The analyses of PAHs in *C. finmarchicus* biomass and exposure media was performed as previously described by Nordtug et al. (2015). For the analysis of PAHs in *C. finmarchicus* biomass, samples of 21 randomly selected copepods were collected at 24, 48, 72 and 96 hours of exposure. Samples for analysis of PAHs in the exposure media were collected from the outflow tubes of all exposure tanks at 48 and 96 hours of the exposure.

The following target components were quantified: Naphthalenes (C0-C4), biphenyl, acenaphthylene, acenaphthene, dibenzofuran, fluorenes (C0-C3), phenanthrenes/anthracenes (C0-C4), dibenzothiophenes (C0-C4), fluoranthenes/pyrenes (C0-C3), benz(a)anthracene, chrysenes (C0-C3), benzo(a)pyrene, benzo(e)pyrene and perylene. Benzo(a)pyrene was also detected in most samples, but close to detection limit and therefore not quantifiable. Approximately the same set of components were quantified in the *C. finmarchicus* biomass as in the exposure media samples, but the very small samples and low concentrations caused problems that reduced the number of components. The components missing from the *C. finmarchicus* biomass analyses were C0-, and C1-naphthene, across all samples. In addition the analyses of same samples and of some components in a few of the other samples were compromised and had to be removed (see Table 1). C4-naphthalene was not analyzed in the *C. finmarchicus* biomass.

The accumulation of components to *C. finmarchicus* over time (Fig. 2) was represented by fitting lines calculated as exponential rise to a maximum to the concentration of each component separately for the two treatments:

$$f(x) = y_0 + a(1-b^x) \qquad\qquad (2)$$

Stability of the exposure system

The chemical analyses of the exposure media showed similar concentration values for the selected PAHs both in replicate samples and at the two sampling occasions (48 and 96 hours exposure)(Supplementary Information, Table S1). No significant differences were found when comparing single components (t-test, $p > 0.05$) or average concentrations (Mann-Whitney Rank Sum Test, $p > 0.05$) of PAHs between the two exposures in May and June (N = 6-8). The concentrations of components with high K_{ow} (e.g. C3 chrysene, Perylene) were low in the WSF exposure and not significantly different from control values (t-test, $p > 0.05$), showing that the filtration of the oil dispersion was successful.

Biometric measurements

Images for biometric measurements of the lipid sac were captured under a dissecting microscope (Leica MZ125, Leica Microsystems, Wetzlar, Germany) with a digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo) operated by Fire-I software (Unibrain, Inc., San Ramon, CA) on *C. finmarchicus* randomly collected after 96 hours exposure t the end of the June exposure. The measurements and calculations of body and lipid sac volume of *C. finmarchicus* were performed on scaled images.

Calculations

The percent share of the accumulated PAHs in *C. finmarchicus* biomass assumed to be due to ingestion of oil droplets was calculated by subtracting the concentration in the *C. finmarchicus* exposed to WSF from the oil dispersion data. The data were presented as percent of the total oil concentration in the oil dispersion exposed *C. finmarchicus* (Fig. 3) as function of the lipophilic properties of the compounds (log K_{ow}). A curve was fitted to the data using linear regression:

$$f(x) = y_0 + ax \qquad\qquad (3)$$

To illustrate differences in the accumulation between the two populations of *C. finmarchicus* used for the two experimental runs in May and June, the concentration in the *C. finmarchicus* biomass in June was presented as fraction of the concentration in the May biomass (Fig. 4). A curve was fitted to the data using linear regression, as shown above, Equation 3.

Results and discussion

<u>Accumulation of oil compounds in C. finmarchicus</u>
To determine the accumulation of oil compounds in *C. finmarchicus* from the WSF and from the oil dispersion, samples of *C. finmarchicus* were collected every 24 hours during a 96 hour exposure and analyzed using GC-MS. The accumulation over time in the *C. finmarchicus* exposed to WSF and oil dispersion were presented for all oil compounds individually in Table 1. During the exposure, all compounds reached higher concentrations in *C. finmarchicus* exposed to the oil dispersions compared to in those exposed to the WSF. Towards the end of the exposure, lower concentrations were observed in the *C. finmarchicus* exposed to the oil dispersion compared to those exposed to the WSF (Table 1).

To illustrate the differences in accumulation to *C. finmarchicus* exposed to WSF and to the oil dispersion, the concentrations of four selected oil compounds with increasing lipophilic properties (quantified as log K_{ow}) were presented as a function of exposure time (Fig. 2). The dissolution of oil compounds from oil droplets is dependent on several factors, amongst others the concentration of the oil dispersion and the solubility of the compounds in the water and oil compartment (Nordtug et al., 2011; Redman et al., 2012). The partitioning from the WSF and the oil dispersion to the *C. finmarchicus* was also expected to be governed by the same parameters.

Accumulation of oil compounds from the WSF
The concentration of PAHs in *C. finmarchicus* exposed to the WSF ($\mu g\ g^{-1}$) increased over time (Table 1) for all compounds except for the most lipophilic compounds (e.g. C2- and C3-chrysene, and perylene). As these compounds were not quantified in the WSF exposure solution (Supplementary Table S1) they were not expected to accumulate in the biomass. Biphenyl (log K_{ow} 3.9) reached a steady state already after approximately 24 hours of exposure, while the compounds with high lipophilic properties (log $K_{ow} \geq 4.9$), the uptake to *C. finmarchicus* appeared to be close to linear from 24 hours exposure and throughout the duration of the exposure (Fig. 2). It is assumed that uptake from the WSF mainly reflects passive partitioning over the body surfaces of *C. finmarchicus* from the water phase, with the possible addition of components adhered to-, or absorbed by the feed algae ingested by the copepods. Recently, Jensen et al. (2012) quantified passive accumulation of the two PAHs phenanthrene and benzo(a)pyrene in non-fed *C. finmarchicus* and found that the accumulation of phenanthrene (log K_{ow} 4.3) reached steady state already after approximately 50 hours of exposure, while steady state was not reached for benzo(a)pyrene (log K_{ow} 6.1) over the 192 hours of exposure. The results from the present study are in accordance with those reported by Jensen et al. (2012).

The high lipophilic compounds (log $K_{ow} \geq 4.9$) showed no or little evidence of reaching steady state in the biomass during the 96 hour WSF exposure (Fig. 2), and the accumulation of these compounds still remained in the linear phase of uptake. We suggest that this indicates that *C. finmarchicus* may be a sink for high lipophilic compounds dissolved in the water phase during oil spills.

Accumulation of oil compounds from the particulate oil droplets

The accumulation of PAHs in *C. finmarchicus* biomass exposed to the oil dispersion was similar to the accumulation observed in the *C. finmarchicus* exposed to the WSF in that it increased over time (Fig 2). However, unlike for the WSF exposure, a rapid accumulation of all PAHs was observed in the oil dispersion exposure. This higher uptake of PAHs to the oil-dispersion exposed *C. finmarchicus* is consistent with ingestion of oil droplets, since it was most pronounced for the high lipophilic compounds (Fig. 2). High lipophilic (log $K_{ow} > 6$) compounds are expected be mainly be located in the oil droplets (Redman et al., 2012). Ingestion of oil droplets have been reported in several laboratory studies with dilute oil dispersions and *C. finmarchicus* (Hansen et al., 2012; Nordtug et al., 2015; Olsen et al., 2013). In one of these studies, feeding activity were quantified for increasing concentrations of oil dispersions, showing that the feeding activity was low at high concentrations of oil (Nordtug et al., 2015). Further, the concentration of oil in the biomass was low at high concentrations of oil (Nordtug et al., 2015). The feeding activity and the concentration of oil in the biomass thus corresponded well, and the accumulated oil was assumed to mainly be a result of ingested oil (Nordtug et al., 2015). The results presented from the present study show that ingestion of oil by the *C. finmarchicus* rapidly contributes to a substantial accumulation of oil compounds in the biomass, compared to the WSF. The higher accumulation was most important for the high lipophilic oil compounds.

The experimental design of the present experiment was concerned with the transport and weathering of oil in the presence of *C. finmarchicus*. The location of the accumulated PAHs in the *C. finmarchicus* biomass could not be determined. Previous studies have predicted low or insignificant contribution from ingested oil droplets to the accumulation of PAHs in tissue of marine filter feeders (Viaene et al., 2014). Further, a low accumulation of oil compounds from the oil in the gut to the copepod tissue is in accordance with the observed negligible effect of oil droplets on toxicological endpoints in *C. finmarchicus* (Hansen et al., 2009). The concentration of PAHs quantified in *C. finmarchicus* biomass in the present experiment represents the sum of compounds contained within the oil in the gut and compounds accumulated to the tissue. Accumulation to copepod tissue may have taken place both from the oil droplets in the gut and from the WSF.

It may be speculated that the relative large body surface of the *C. finmarchicus* and the small distance between the lipid sac and the seawater facilitate a rapid dissolution of oil

compounds to and from the lipid sac. Any additional body burden load accumulated over the gut lining from the ingested oil droplets may have been rapidly eliminated over the body surfaces to surrounding water. In *Acartia grani* exposed to a mixture of PAHs passive elimination over body surfaces were the dominating route of excretion (Berrojalbiz et al., 2009). However, this experiment did not quantify elimination in the presence of oil droplets, and it may therefore only be valid for WSF exposure.

The results from this experiment show that *C. finmarchicus* rapidly accumulated all oil compounds when exposed to oil dispersions, showing that the copepod can impact the particulate fraction of an oil spill by ingesting oil. The results from the present study supports previous suggesting that zooplankton can impact transport and weathering of oil spills (Almeda et al., 2014; Almeda et al., 2013a; Almeda et al., 2013b; Conover, 1971). Conover (1971) made his suggestions after observing a mixed zooplankton community after an oil spill, while Almeda et al. (2013b) quantified concentration of oil compounds in *Acartia tonsa* in a controlled laboratory set-up. In their set-up Almeda et al. (2013b) found lower concentrations in the *A. tonsa* biomass when the feed particles were changed from nanoflagellates *Rhodomonas* sp. to the protozoan *Oxyrrhis marina*. This was suggested by the authors to be due to scavenging of oil by the protozoans. Zooplankton feeds by different strategies, which can be classified as non-selective suspension feeding or raptorial predator feeding on single particles (Kiørboe, 2011). The reduced concentration of oil compounds in the *A. tonsa* biomass in the presence of the protozoan may also have been caused by a switch from non-selective filter-feeding on *Rhodomonas* sp. where oil droplets accidentally are ingested, to raptorial feeding selectively on protozoans. Studying more zooplankton species under different feed regimes are therefore suggested to be necessary before the oil concentration in *C. finmarchicus* biomass can be extrapolated across different zooplankton species.

Elimination of accumulated oil compounds may affect weathering of oil

To illustrate differences observe in the accumulation patterns for all oil compounds between the two exposures, the concentration in *C. finmarchicus* exposed to the WSF was subtracted from the concentration in *C. finmarchicus* exposed to the oil dispersion (Fig. 3). This is an indication on the percent concentration caused by ingestion of oil droplets and were presented separately for the four sampling times, as function of lipophilic properties of the oil compounds. The figure shows that the correlations between the contribution to the concentration due to ingestion of oil droplets and the lipophilic properties of the compounds were more or less linear at all sampling times. However, the slope of the regression became steeper over time. For samples collected $\leq$ 48 hours, the regression did not cross the x-axis, showing that the concentrations were higher for all oil compounds in the oil dispersion exposed *C. finmarchicus* (Fig. 3). For samples collected $\geq$ 72 hours of exposure, the regression crosses the x-axis. The concentration of oil compounds with log K_{ow} < approximately 5 were lower in the *C. finmarchicus* exposed to the oil dispersion compared to the WSF-exposed. This relative

lower concentration of oil compounds in oil dispersion exposed *C. finmarchicus* was most pronounced at 96 hours of exposure.

During the May experiment, visual observations, such as cloudiness of the interior, reduction in the lipid sac and lethargic behavior, indicated deterioration of the *C. finmarchicus*. Quantification of body volume and volume of the lipid sac was subsequently performed after 96 hours of exposure after the June experiment. The lipid sac of the oil dispersion exposed *C. finmarchicus* constituted on average 89% of the volume of the lipid sac in the WSF-exposed *C. finmarchicus*. The individual variation was large and the samples relatively small (n = 8-10), and the difference between the *C. finmarchicus* exposed to oil dispersion and WSF was not statistically significant when tested (One Way ANOVA, $p > 0.05$). The concentration of oil compounds is known to vary with the lipid content of the exposed biota (De Hoop et al., 2013; Hendriks et al., 2001). We thus suggest that the lower levels of lipid in the oil dispersion exposed *C. finmarchicus* were the cause for the observed lower concentration of low lipophilic compounds compared to WSF-exposed *C. finmarchicus*. Previously, exposure to oil dispersions have been reported to reduce the ingestion of feed particles by *C. finmarchicus*, suggested to be due to a combined mechanical obstruction of the feeding apparatus by the oil droplets and narcotic action of the oil compounds (Hansen et al., 2011; Nordtug et al., 2015; Størdal et al., 2015). Reduced capability to acquire energy by oil dispersion exposed *C. finmarchicus* may have caused starvation in the copepods and subsequent mobilization of the storage lipids (Lee et al., 2006). Starved female *C. finmarchicus* have been observed to change their metabolism already after three days of starvation (Pasternak et al., 2013). Mobilization of the storage lipids would result in mobilization of the oil compounds accumulated in the lipid sac. These compounds are suggested to be eliminated to the water. The results therefore indicate that *C. finmarchicus* may be contributing to redistribution of accumulated oil compounds during oil spills. Biodegradation of the dissolved fraction of an oil spill has been observed to be enhanced in the presence of *C. finmarchicus* feces (Størdal et al., In Prep.). A elimination of the low lipophilic compounds accumulated to *C. finmarchicus* may therefore increase their biodegradation.

Deviations in bioconcentration between series
Although the uptake pattern for each component remained similar for the two exposure series both for WSF exposed and oil dispersion exposed *C. finmarchicus* (Fig 2. and Fig. 3), differences were observed in the concentration levels (Fig. 4). During the June experiment almost all oil compounds reached lower concentrations compared to the May series. The concentration of the most lipophilic (log K_{ow}) components were slightly higher in the June exposure compared to the May exposure (Fig. 4). The data presented were from the 96 hour sampling. The difference between the concentration in May and June was linearly dependent on the lipophilicity of the compounds, and the

concentrations in the June series varied from about 50% to about 110% of the levels observed in the May series. We suggest that this was caused by lower levels of lipids in the June population. Since the low lipophilic oil compounds rapidly attained steady state with surrounding seawater (Fig. 2), the total concentration in the biomass of these may be dependent on the total lipid content.

Pictures for biometric measurements were only collected after the last exposure series, and comparison of the lipid content of the *C. finmarchicus* between the two consecutive series were not possible. However, the lipid content of *C. finmarchicus* collected from our laboratory culture of *C. finmarchicus* has previously been shown to vary between approximately 8 and 28% for individuals at the same stage collected concomitantly (Hansen et al., 2011). The lipid levels of *C. finmarchicus* at the same developmental stage thus have potential to vary within this span. A variation between the high and low lipid level within this span between the series may have caused the accumulated levels to differ with 70%, if the concentration in the biomass is linearly dependent on the lipid content. These explanations are tentative as we have no data to support them. However, what is shown is that the accumulation and elimination of oil compounds from zooplankton are dependent on parameters of the copepods, such as lipid content, feeding strategy and feed regime, in addition to factors of the oil spill and the oil dispersion.

Conclusion

The accumulation of oil compounds from the dilute oil dispersion and the corresponding WSF show that *C. finmarchicus* influences weathering and transport processes of an oil spill by sequestering oil compounds. The results show ingestion of oil droplets by the copepods, resulting in rapid accumulation of all oil compounds. Further, the accumulation of high lipophilic compounds from the water phase to the *C. finmarchicus* did not reach steady state during the 96 hour exposure and the copepods may thus function as a sink for dissolved high lipophilic compounds. At the end of the exposure, a reduction in the low lipophilic oil compounds were observed in the oil dispersion exposed *C. finmarchicus* suggested to be caused by a mobilization of the lipid reserves of the copepods. Elimination and dissolution of oil compounds to the water phase is expected to increase the biodegradation of oil compounds.

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Tables

Table 1. Accumulated levels of PAHs in *Calanus finmarchicus* after 24, 48, 72 and 96 hours of exposure during the May and June exposure series (µg g^{-1}). Each number represents the average of two samples from parallel exposure chambers if valid analyses from both are available (numbers based on one parallel only is shaded grey).

	Oil dispersion exposure								WSF exposure							
	May series				June series				May series				June series			
C2-naphthalenes	6.381	9.419	10.64	12.91	3.165	7.776	5.641		3.973	9.804	15.18	17.76	2.289	5.262	8.185	8.375
C3-naphthalenes	4.275	8.060	9.539	12.78	2.685	7.178	6.097		2.603	7.373	12.20	15.41	1.793	5.048	8.146	9.881
Biphenyl	0.565	0.649	0.707	0.783	0.313	0.520	0.381		0.322	0.721	1.007	1.175	0.180	0.333	0.561	0.599
Acenaphthylene	0.034	0.061	0.073	0.092	0.027	0.050	0.039		0.020	0.052	0.098	0.116	0.007	0.029	0.055	0.067
Acenaphthene	0.065	0.106	0.121	0.134	0.040	0.088	0.085		0.040	0.112	0.161	0.206	0.029	0.070	0.108	0.124
Dibenzofuran	0.046	0.081	0.097	0.097	0.035	0.069	0.075		0.033	0.095	0.128	0.158	0.023	0.054	0.094	0.093
Fluorene	0.233	0.440	0.612	0.696	0.178	0.414	0.367		0.190	0.507	0.734	1.101	0.148	0.370	0.539	0.636
C1-fluorenes	0.682	1.402	2.013	2.569	0.465	1.332	1.382		0.449	1.548	2.174	3.320	0.314	0.977	1.629	2.275
C2-fluorenes	4.516	8.990	12.37	16.87	3.594	8.575	8.989		1.997	7.273	11.78	15.63	1.314	5.312	9.151	14.15
C3-fluorenes	0.800	1.698	2.040	2.757	0.737	1.531	1.645		0.262	0.858	1.419	1.833	0.067	0.595	1.144	1.841
Phenanthrene	0.646	1.337	1.728	2.210	0.443	1.205	1.469		0.494	1.406	2.221	2.808	0.311	0.996	1.494	1.778
Anthracene	0.041	0.091	0.121	0.166	0.028	0.076	0.103		0.022	0.083	0.152	0.174	0.005	0.046	0.091	0.129
C1-phenanthrenes/anthracenes	1.655	3.335	4.069	5.662	1.213	3.164	3.675		0.872	2.849	4.700	5.913	0.609	2.089	3.463	4.817
C2-phenanthrenes/anthracenes	1.825	3.771	4.206	5.782	1.304	3.263	3.517		0.518	1.739	3.138	3.986	0.325	1.431	2.429	3.865
C3-phenanthrenes/anthracenes	1.276	2.497	2.609	3.615	0.954	2.004	2.088		0.166	0.586	1.066	1.269	0.094	0.475	0.809	1.483
C4-phenanthrenes/anthracenes	0.740	1.422	1.523	2.044	0.525	1.093	1.134		0.017	0.122	0.302	0.380	0.011	0.105	0.208	0.502
Dibenzothiophene	0.052	0.105	0.125	0.164	0.030	0.093	0.106		0.041	0.114	0.175	0.224	0.024	0.080	0.120	0.150
C1-dibenzothiophenes	0.189	0.408	0.503	0.684	0.143	0.365	0.460		0.095	0.314	0.567	0.721	0.061	0.252	0.418	0.612
C2-dibenzothiophenes	0.266	0.556	0.616	0.893	0.200	0.481	0.506		0.059	0.242	0.462	0.606	0.044	0.181	0.313	0.584
C3-dibenzothiophenes	0.225	0.424	0.463	0.624	0.199	0.359	0.385		0.011	0.083	0.151	0.199	0.005	0.062	0.134	0.228
C4-dibenzothiophenes	0.095	0.153	0.150	0.214	0.092	0.153	0.145		0.003	0.025	0.032	0.037	-	0.010	0.027	0.083
Fluoranthene	0.047	0.087	0.119	0.145	0.037	0.087	0.103	0.091	0.014	0.074	0.133	0.169	0.004	0.054	0.111	0.160
Pyrene	0.078	0.153	0.177	0.226	0.055	0.123	0.146	0.163	0.021	0.093	0.162	0.204	0.007	0.068	0.126	0.192
C1-fluoranthrenes/pyrenes	0.181	0.627	0.731	0.928	0.223	0.531	0.566	0.679	0.048	0.211	0.386	0.530	0.023	0.159	0.297	0.512
C2-fluoranthenes/pyrenes	0.324	0.660	0.728	0.888	0.274	0.516	0.535	0.672	0.038	0.139	0.267	0.332	0.021	0.105	0.222	0.352
C3-fluoranthenes/pyrenes	0.257	0.535	0.573	0.669	0.215	0.360	0.401	0.485	0.014	0.060	0.136	0.150	0.004	0.062	0.102	0.159
Benz(a)anthracene	0.016	0.027	0.035	0.040	0.010	0.022	0.023	0.034	0.001	0.008	0.013	0.018	0.000	0.004	0.015	0.019
Chrysene	0.108	0.213	0.245	0.300	0.082	0.182	0.197	0.274	0.023	0.077	0.131	0.165	0.013	0.057	0.114	0.176
C1-chrysenes	0.183	0.368	0.386	0.450	0.140	0.273	0.275	0.393	0.015	0.045	0.092	0.105	0.008	0.046	0.077	0.131
C2-chrysenes	0.141	0.298	0.295	0.371	0.110	0.206	0.218	0.298	0.001	0.003	0.060	0.033	-	0.008	0.016	0.059
C3-chrysenes	0.048	0.119	0.112	0.177	0.092	0.089	0.157	0.142	-	-	0.029	-	-	-	-	0.012
Benzo(e)pyrene	0.010	0.021	0.022	0.024	0.011	0.015	0.016	0.021	0.000	0.001	0.003	0.004	-	0.001	0.004	0.006
Perylene	0.005	0.012	0.012	0.015	0.005	0.009	0.013	0.014	0.000	0.001	0.002	0.001	-	0.000	0.003	0.003

Figures

Fig. 1. Exposure concentration in outflow tubes from one of the oil dispersion tank (May experiment) showing the increase in oil exposure concentration over the first 24 hours. Curve fitted to data was calculated as exponential rise to maximum, $R^2 = 0.97$.

Uptake of PAHs from oil dispersions in copepods
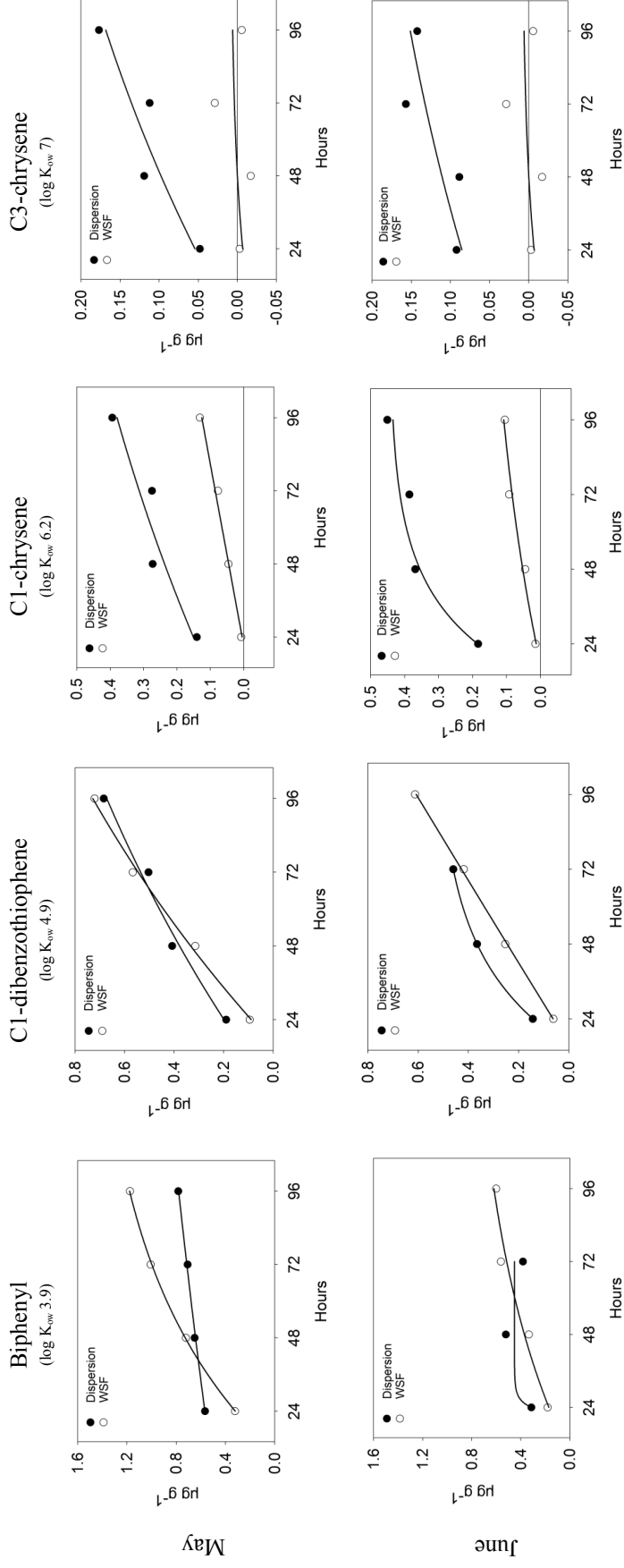
Fig. 2. Accumulation of PAHs in *Calanus finmarchicus* exposed to oil dispersion and the corresponding WSF (μg g^{-1}) as a function of duration of exposure. Upper panel: May series, lower panel: June series. Each data point represents the average of two parallel exposure groups. Curves fitted to the data calculated as exponential rise to maximum, $R^2 > 0.90$.
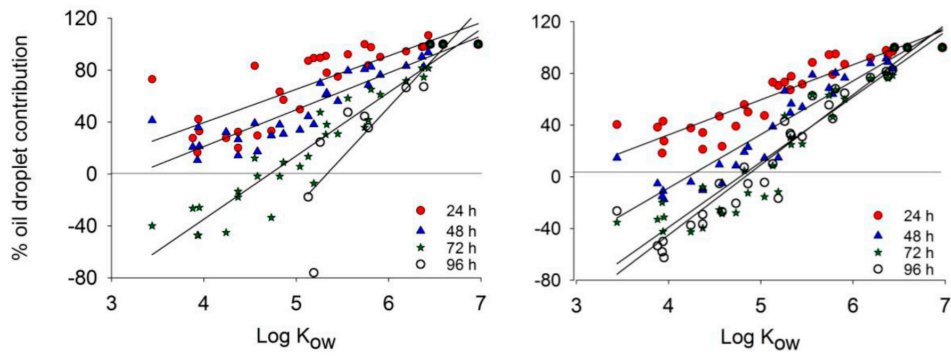
Fig. 3. The percentage share of accumulated PAHs in *Calanus finmarchicus* assumed to originate from ingested oil was calculated by subtracting the concentration in WSF-exposed biomass. This was done for four separate time points during a 96 hour exposure, at 24, 48, 72 and 96 hours. The percentage share was presented as function of log K_{ow}. Curves fitted to the data calculated as linear regression, $R^2 > 0.68$.
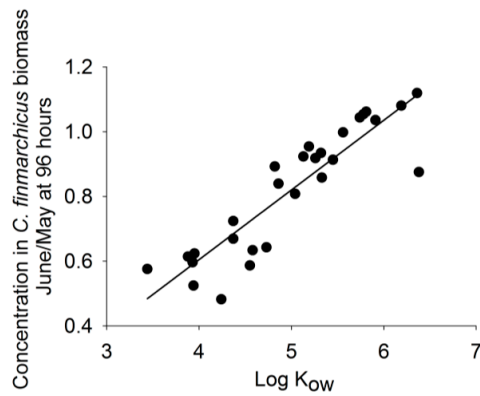
Fig. 4. Fraction difference in concentration of PAHs between two populations of *Calanus finmarchicus* at the end of a 96 hour exposure presented as a function of log K_{ow}. Curve fitted to the data calculated as linear regression, $R^2 > 0.80$.

SUPPLEMENTARY INFORMATION

**UPTAKE OF PAHS IN C*ALANUS FINMARCHICUS* FROM SEAWATER
PETROLEUM OIL DISPERSIONS AND THE WATER SOLUBLE FRACTION**

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Table S1. Concentration (µg L^{-1}) of oil compounds in control exposure, oil dispersion exposure and the corresponding water soluble fraction (WSF) of the oil dispersion generated to study accumulation of oil compounds in the copepod *Calanus finmarchicus*. Oil dispersion concentration was generated with the +200 °C fraction of Troll crude oil at 2 µl L^{-1} and with a oil droplet size < 40 µm.

	Clean seawater control							Oil dispersion exposure								WSF exposure						
	May			June				May				June				May			June			
	48h	96h		48h		96h		48h		96h		48h		96h		48h		96h	48h		96h	
Component	AVG	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	AVG	SD	AVG	SD
Naphtalenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	1.6	0.1	1.5	0.1	1.6	0.0	1.5	0.1	1.6	1.4	0.1	1.5	0.6
C1-naphthalenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0	3.2	0.3	2.8	0.2	3.1	0.1	2.8	0.2	2.9	2.6	0.1	2.8	1.1
C2-naphthalenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7	0.1	2.6	0.4	2.3	0.3	2.3	0.2	2.3	0.1	2.4	2.1	0.0	2.2	0.7
C3-naphthalenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	1.5	0.3	1.4	0.2	1.4	0.1	1.2	0.1	1.3	1.1	0.0	1.2	0.2
C4-naphthalenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.7	0.1	0.7	0.1	0.7	0.0	0.4	0.0	0.4	0.3	0.0	0.4	0.0
Biphenyl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.3	0.0	0.3	0.1
Acenaphthylene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acenaphthene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dibenzofuran	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fluorene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.2	0.0	0.2	0.0
C1-fluorenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.4	0.1	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.3	0.0	0.3	0.0
C2-fluorenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.4	0.1	0.4	0.0	0.4	0.0	0.2	0.0	0.3	0.2	0.0	0.2	0.0
C3-fluorenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.1	0.3	0.0	0.3	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.0
Phenanthrene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.1	0.3	0.0	0.3	0.0	0.2	0.0	0.2	0.2	0.0	0.2	0.1
Anthracene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C1-phen./anth.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5	0.1	0.4	0.0	0.4	0.0	0.3	0.0	0.3	0.3	0.0	0.3	0.1
C2-phen./anth.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.4	0.1	0.4	0.0	0.4	0.0	0.2	0.0	0.2	0.1	0.0	0.2	0.0
C3-phen./anth.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C4-phen./anth.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dibenzothiophene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C1-dibenzothio.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C2-dibenzothio.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C3-dibenzothio.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C4-dibenzothio.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fluoranthene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pyrene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C1-fluor./pyrenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C2-fluor./pyrenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C3-fluor./pyrenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Benz(a)anthracene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chrysene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C1-chrysenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C2-chrysenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C3-chrysenes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Benzo(e)pyrene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Benzo(a)pyrene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Perylene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Paper IV

Concentrations of viable oil-degrading microorganisms are increased in feces from *Calanus finmarchicus* feeding in petroleum oil dispersions

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ABSTRACT

Zooplankton are suggested to be biotic contributors to the transport and weathering of oil in marine environments due to their ingestion of oil. In the present experiment, feeding activity and microbial communities in feces from *Calanus finmarchicus* feeding in oil dispersions were characterized. Feeding activity was significantly reduced in oil dispersions. The microbial communities in clean and oil-containing copepod feces were dominated by *Rhodobacteraceae* family bacteria (*Lesingera*, *Phaeobacter*, *Rugeria*, and *Sulfitobacter*), which were suggested to be indigenous to copepod feces. The results also indicated that these bacteria were metabolizing oil compounds, as a significant increase in the concentrations of viable oil degrading microorganisms was observed in oil-containing feces. This study shows that bacteria in feces from copepods feeding in dilute oil dispersions have capacity for degradation of oil. Zooplankton may therefore contribute to weathering of oil by excreting feces with microbial communities already adapted to degradation of oil.

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1. Introduction

When oil is spilt in marine environments it is subject to physical, chemical and biological weathering processes. One of the physical weathering processes is dispersion of oil as droplets into the water column. This process can relocate large masses of the oil from the sea surface to the water column (Ritchie, 1993). Dispersion of oil into the water is enhanced by the use of dispersants (Brandvik, 1997). Small oil droplets (diameter <50–70 μm) have slow surfacing velocity and accumulate in the water column based on the turbulent motion in the water (Delvigne, 1993). Oil droplets with diameter <100 μm are within the size range of particles ingested by suspension feeding by calanoid copepods (Boyd, 1976; Cannon, 1928). Ingestion of oil droplets has been documented for the calanoid copepod *Calanus finmarchicus* and other zooplankton in laboratory and field experiments (Conover, 1971; Lee et al., 2012; Nordtug et al., 2015). The copepod *C. finmarchicus* is ubiquitous in the northern North Sea and in the Norwegian Sea (Helle, 2000; Sakshaug et al., 1994). Based on their high abundance, high feeding activity, and ingestion of oil,

copepods have been suggested to be an important biotic contributor to weathering of oil spills (Conover, 1971; Nepstad et al., 2015). The purpose of this study was to characterize microbial communities in oil-containing *C. finmarchicus* feces, with the aim of understanding the contribution of copepods to weathering of oil spills.

Since *C. finmarchicus* contribution to weathering of oil spills is based on its ingestion of oil, its feeding activity in oil dispersions is of importance. The feeding activity of *C. finmarchicus* is dependent on feed algae type, particle concentration, and oil exposure, which can be determined from quantification of clearance rate or from estimates of feces production (Frost, 1972; Nejstgaard et al., 1995; Nordtug et al., 2015; Spooner and Corkett, 1979). Clearance rate is specified by the number of algae particles removed from suspension over time (Frost, 1972). The oil droplets that are ingested by the copepods are excreted in feces (Conover, 1971; Olsen et al., 2013).

For ultimate removal of oil from marine environments, biotransformation and mineralization of the hydrocarbon (HC) compounds by microorganisms are fundamental. Bacteria capable of degrading oil are diverse and found within several phyla (Alpha- and Gammaproteobacteria, Actinobacter, Flexibacter-Cytophaga-B acteroides) (Prince, 2005). These bacteria may be either obligate hydrocarbonoclastic bacteria, or capable of degrading a range of

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organic compounds (Kostka et al., 2011; Yakimov et al., 2007). Oil-degrading bacteria require inorganic nutrients in addition to bioavailable HCs and an electron acceptor. Petroleum oil has a high content of carbon and little or no inorganic nutrients, and the availability of inorganic nutrients at the oil spill site may therefore restrict the activity of oil-degrading bacteria (Atlas and Bartha, 1972; Röling et al., 2002). The activity and proliferation of bacterial species in a community are determined by their ability to compete for their necessary resources (Smith et al., 1998; Tilman, 2004). Copepod feces are high in nutrients and have a high content of nitrogen relative to carbon in comparison to other particles in the marine environment (Bathmann et al., 1987; Claustre et al., 1992). The oil excreted in the copepod feces is in close proximity to this nutrient and since activity and proliferation of oil-degrading bacteria are generally rapidly increased when oil is introduced to an environment, the ingestion of oil by copepods and excretion of oil in copepod feces may facilitate rapid proliferation of oil-degrading bacteria in the feces. Furthermore, since defecation by copepods is important for bacterial communities in seawater, the excretion of oil-containing feces during oil spills may introduce bacterial communities adapted to degradation of oil to the seawater together with the nutrient-dense feces (Tang, 2005; Thor et al., 2003).

The aims of this study were to quantify feeding activity of *C. finmarchicus* feeding in clean seawater and oil dispersions, and to characterize viable and total microbial communities in the feces collected from control and oil-exposed copepods. In the present experiment, copepods were exposed to oil dispersions at concentrations and droplet sizes designed to mimic dilute oil dispersions (Lee et al., 2013). Feeding activity of the copepods was quantified as clearance rates and feces production. Clearance rates were quantified during the copepod exposure, from particle count analyses of exposure solution in inflow and outflow tubes of the exposure tanks. Feces were sampled from the copepod exposure tanks after 48 h of exposure and incubated (14 days, 10 °C) in darkness in filtered and autoclaved seawater to determine the bacterial communities in the feces. The maximum residence time for the feces in the exposure tanks was 48 h, and the bacteria colonizing the fecal pellets originated from the particles ingested by the copepods, the gut of the copepods, and from the seawater in the copepod exposure tanks (Hansen and Bech, 1996; Poulsen and Iversen, 2008; Tang, 2005). The volumes of feces produced by the copepods were determined prior to incubation of the feces. After the incubation period, concentrations of total and viable microorganisms were evaluated in seawater with feces using epifluorescence microscopy and the nucleic acid stain 4′,6′-diami dino-2-phenylindole (DAPI) and most probable number method (MPN; oil-degrading microorganisms [ODM] and heterotrophic microorganisms [HM]), respectively. The relative abundance of bacteria in the total microbial communities in seawater with clean and oil-containing feces was determined using 16S rRNA gene amplicon library analyses. Selected target n-alkanes and aromatic HC compounds were analyzed in seawater with oil-containing feces to establish ingestion of oil by the copepods and the excretion in feces. The target oil compounds were detected using gas chromatography–mass spectrometry (GC–MS).

2. Materials and methods

2.1. Copepod husbandry, seawater, crude oil, and exposure of copepods

C. finmarchicus (Gunnerus) were obtained from a continuous culture running at NTNU/SINTEF Sealab (Trondheim/Norway). The cultured copepods were kept in flow-through natural seawater at 8–10 °C and regularly fed a diet of microalgae (*Rhodomonas*

baltica Karsten, *Dunaliella tertiolecta* Butcher and *Isochrysis galbana* Parke). The culture is further described in Hansen et al. (2007). Natural seawater was utilized in the experiment. This was continuously supplied to the NTNU/SINTEF Sealab laboratory facilities through pipelines from 80 m depth in Trondheimsfjorden, Norway (63°26′N, 10°23′E). The seawater was sand-filtered to remove coarse particles, adjusted to ambient atmospheric conditions and filtered (5 μm exclusion limit, Cuno Aqua-Pure water filter system) before use.

The oil used for exposure of the copepods was a naphthenic crude oil, artificially weathered by heating to 200 °C (Stiver and Mackay, 1984). The +200 °C fraction was collected and filtered (VWR syringe Teflon filter, 0.2 μM) and utilized for generation of the oil dispersion according to Nordtug et al. (2011). The method enables tight control of oil droplet size range and concentration. In the present experiment, an oil dispersion with a nominal concentration of 2 μL L^{-1} and a droplet sizes <40 μm was produced using water and oil flow rates of 160 mL min^{-1} and 0.35 μL min^{-1}, respectively.

At the start of the copepod exposure experiment, 400 *C. finmarchicus* in their fifth developmental stage (copepodite 5, C5) were carefully introduced into exposure tanks with round bottoms and detachable lids. The exposure tanks were prefilled with clean seawater (control copepods) or oil dispersions in seawater (oil-exposed copepods). The set-up consists of six exposure tanks. The experiment was run twice with three + two replicates for control and three + three replicates for oil-exposed copepods. This resulted in N = 5 for the control copepods and N = 6 for the oil-exposed copepods. The exposure lasted for 48 h in a climate controlled room at 10 °C under continuous low light. The flows of clean seawater and oil dispersion were passively regulated as described by Nordtug and Olsen (1993) to 27 mL min^{-1}. The exposure solution volume of the tanks was approximately 18 L, while the total volume of the tanks was 20 L. All exposure tanks were supplied with live *R. baltica* at a nominal concentration of 400 μg C L^{-1}. The concentration and size of oil droplets in the oil dispersion and of the algae cells in the algae suspension were monitored using a Coulter Counter (Multisizer 3; Beckman) equipped with a 100 μm aperture tube with the lower threshold set to 2 μm, measuring particles with diameter between 2 and 60 μm.

2.2. Collection of clean and oil-containing copepod feces

Copepod feces were collected from oil-exposed copepods (oil-containing feces) and control copepods (clean feces) at the end of the 48 h exposure using a borosilicate bottle (Schott, 500 mL) filled with filtered (0.22 μm pore size, Sterivex GV filters, Millipore Corp., Bedford, MA, USA) and autoclaved (120 °C, 20 min) seawater, a peristaltic pump, and an acrylic suction pipe. The peristaltic pump and the suction pipe were connected to the flask and the pump thus created suction at the distal end of the suction pipe that transported fecal pellets from the bottom of the exposure tanks and into the borosilicate flask. The outflow from the flask to the pump was supplied with a mesh sieve (15 μm) to retain feces within the flask, while the distal end of the suction pipe was equipped with a 200 μm mesh to prevent copepods from being sucked in. After removal of copepods, the water volume of the exposure tanks was filtered (20 μm) to collect feces suspended in the water. The total volume of the feces suspension was reduced using a bowl with a filter (mesh size 20 μm) bottom. The bowls were washed with detergent (Neodisher® LaboClean A 8) and rinsed using ethanol (96%) to sterilize them prior to use. In these bowls, the collected copepod feces were thoroughly rinsed with filtered and autoclaved seawater to minimize the amount of oil adhering to the external surfaces of the fecal pellets. The feces were then transferred to 20 mL containers with filtered and

autoclaved seawater (total volume 16 mL) using sterile plastic pipettes. Subsamples (4 × 0.5 mL) were taken from these containers for analyses of feces volume.

From defecation and until collection, the maximum residence time of fecal pellets in copepod exposure tanks was 48 h. Each exposure tank had a dedicated flask, suction pipe and tubing for collection of feces to avoid carryover between samples. The seawater used when collecting the feces was filtered and autoclaved to avoid contamination.

2.3. Incubation of clean and oil-containing copepod feces

Seawater suspensions with clean and oil-containing copepod feces (14 mL) were transferred to borosilicate flasks (2 L; Scott) capped with Teflon inserts (VWR International). The flasks were filled with filtered and autoclaved seawater, and filled to the brim (no headspace, total volume about 2.3 L) after the feces suspension was added. Incubation was performed in darkness for 14 days at 10 °C and the flaks were mounted on a carousel system, rotating in the vertical orientation at 0.75 rounds per minute to keep the fecal pellets in suspension (Brakstad et al., 2015). A single flask mounted on the carousel system contained all feces from a single copepod exposure tank. To minimize adherence of organic material to the inside walls of the flask during the incubation period, flasks were washed (Neodisher® LaboClean A 8) and rinsed, heated to 450 °C for 3 h, soaked in deconex (10%, 11 Universal, VWR International) for a minimum of 24 h, and again washed (Neodisher® LaboClean A 8) and rinsed. The flasks were autoclaved (120 °C, 20 min) prior to use.

2.4. Feeding activity quantified as clearance rate and feces volume of the copepods

Feeding activity was calculated as clearance rate determined from the loss of algae particles between the inflow and the outflow tubes of the copepod tanks. Particles in the inflow and outflow tubes of the exposure tanks were monitored approximately every 24 h during the experiment using the Coulter Counter. Water samples collected from the outflow of the exposure tanks contained both algae and oil droplets which have overlapping size distribution. The volume of the algae particles was separated from the total volume of particles by estimating the volume of the oil droplets by extrapolating the curve for the oil droplet peak in the region of the algae peak (approximately between 5 and 9 μm, see Hansen et al. (2012)). The volume of the oil droplets was then subtracted from the total particle volume to obtain the volume of algae particles per mL seawater. Clearance rate was calculated separately for each tank:

$$\text{Clearance rate (mL day}^{-1}) = \left(\frac{AV_{inflow} - AV_{outflow}}{AV_{inflow}} \right) \times F \qquad (1)$$

AV_{inflow} is the inflow algae volume ($\mu m^3\ mL^{-1}$), $AV_{outflow}$ the outflow algae volume ($\mu m^3\ mL^{-1}$), and F the flow rate through the tanks (mL day^{-1}). To calculate clearance rate per individual, the total clearance rate was divided by the initial number of individuals corrected for the death rate (ind. h^{-1}).

The total volume of feces produced over the 48 h exposure period by 400 C5 C. finmarchicus was calculated from the volume found for single fecal pellets from control and oil-exposed copepods and enumeration of the total number of fecal pellets in each tank. Scaled pictures of fecal pellets in subsamples (4 × 0.5 mL) from all replicate samples were captured under a dissecting microscope (Leica MZ125, Leica Microsystems, Wetzlar, Germany) with a digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo) operated by Fire-I software (Unibrain, Inc., San Ramon,

CA). Measurements of length and width of 30 fecal pellets in each subsample were subsequently performed using ImageJ and a graphical tablet, and the volume of single fecal pellets was calculated from the average length and width of clean and oil-containing copepod feces:

$$\text{Volume one fecal pellet} = \pi \times B^2 \times \frac{3L - B}{12} \qquad (2)$$

where B is the width and L is the length of the pellet.

The total number of fecal pellets in a subsample was calculated as the sum of all intact fecal pellets plus half the number of pellets with one rounded end. Fragments with two blunt ends were counted, but disregarded from the calculations.

2.5. Chemical analyses of seawater with oil feces

After 14 days of incubation, subsamples were collected of the filtered and autoclaved seawater with clean and oil-containing copepod feces. Analyses of target oil compounds were performed in all replicates using gas chromatography–mass spectrometry (GC–MS) operated in selected ion monitoring mode (Agilent 7890B GC with an Agilent 5977A inert XL electron impact mass selective detector), as described by Nordtug et al. (2015). Seawater with oil feces (approximately 1 L) was acidified with HCl (pH < 2) and solvent–solvent extracted (dichloromethane; DCM). After the flasks were emptied, the inside walls were extracted using DCM. The extracts were pooled and concentrated to 100 μL. Chromatograms were monitored and data recorded using MSD ChemStation software (version F.01.00.1903). The analysis included determination of the response area of the following target oil compounds, with a signal-to-noise ratio of 5 as the lower limit of detection (LOD): decalins (C0–C4), naphthalenes (C0–C4), biphenyl, acenaphthylene, acenaphthene, dibenzofuran, fluorenes (C0–C3), phenanthrenes/anthracenes (C0–C4), dibenzothiophenes (C0–C4), fluoranthenes/pyrenes (C0–C3), benz(a)anthracene, chrysenes (C0–C3), benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, alkanes from nC$_{10}$–nC$_{36}$ and 17α(H),21β(H)-hopane (30ab hopane). The compounds: Decalins (C0–C4), biphenyl, acenaphthylene, acenaphthene, dibenzofuran, benz(a)anthracene, chrysenes (C0), benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, alkanes <16 C and >30 C, were not included in evaluation of biotransformation due to a response area < LOD in the seawater with oil-containing feces in seawater or in the source oil.

The oil compounds detected in seawater with oil-containing feces were presented as the ratio of the response area of the target oil compounds normalized against the response area of 30ab hopane. These ratios were presented as percent of the ratio in the source oil used for preparing the oil dispersion, as modified from CEN (2010):

$$\text{Remaining target analyte (\%)} = 100 \times \frac{C_{i\,sample}/C_{30ab\,sample}}{C_{i\,source\,oil}/C_{30ab\,source\,oil}} \qquad (3)$$

where C_i and C_{30ab} are the response area of the target analyte and 30ab hopane, respectively, in either the sample or the source oil. 30ab hopane is a recalcitrant biomarker commonly used to normalizing the quantity of single oil compounds in environmental samples (Aeppli et al., 2014; Brakstad et al., 2015; Mills et al., 1999).

2.6. Characterization of microbial communities in seawater with clean and oil-containing feces

The remaining volume of seawater with copepod feces was subsampled for analyses of total cell enumeration and concentration

of viable HM and ODM (20 mL) and for determination of relative abundance of bacteria using 16S rRNA gene amplicon library analyses (approximately 1.2 L).

2.6.1. Enumeration of total cells

Enumeration of total cells was performed in all replicates using epifluorescence microscopy (1250 times magnification; Nikon Eclipse 80i). Samples (1 mL) were stained by the nucleic acid stain DAPI (0.06 mg mL^{-1}) for 10 min and filtered (0.2 μm black polycarbonate filters, Nucleopore, Costar, Cambridge, USA) prior to manual counting (Porter and Feig, 1980).

2.6.2. Determination of concentration of viable heterotrophic and oil-degrading microorganisms

Concentrations of viable HM and ODM were determined in triplicates for each replicate of seawater with clean and oil-containing copepod feces by MPN analysis in 24-well tissue culture plates (Costar), as described for ODM by Brown and Braddock (1990). Determination of concentration of viable HM performed in Marine Broth 2216 medium (Difco). Concentration of ODM was determined in Bushnell-Haas Broth supplemented with 30 g L^{-1} NaCl and 0.01% (v/v) artificially weathered Troll oil (+200 °C fraction). The plates were incubated (10 °C) for 10 days for HM and for 14 days for ODM determination. Actively respiring ODM were detected using fluorescein diacetate as indicator (Chrzanowski et al., 1984).

2.6.3. Extraction of nucleic acids and 16S rRNA gene amplicon library analyses

For determination of total microbial communities, seawater was filtered (0.45 μm pore size, Millipore, 47 mm diameter HAWG type) and total DNA extracted from the filters with material from seawater with clean and oil-containing feces. Prior to DNA extraction cells were lysed adding 4.5 mL lysis buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, and 1 M NaCl), 0.5 mL lysozyme (20 mg mL^{-1}), 0.125 mL proteinase K (20 mg mL^{-1}), lauryl-sarkosyl (1%) and sodium-dodecyl-sulfate (1%) to each tube containing filters from one flask. The mixture was vigorously shaken for 1 h at 37 °C. Extraction of DNA in the lysis buffer was subsequently performed using hot phenol:chloroform:isoamylalcohol (25:24:1), pH 8.0 (Sambrook and Russel, 2001). Extracted DNA was dissolved in 100 μL ultrapure water (MolBio grade, 5prime) and stored at −20 °C until shipment for 16S rRNA gene amplicon library analyses.

16S rRNA gene amplicon library analyses of DNA extracted from the samples were performed by GATC Biotech (Constance, Germany) using Illumina MiSeq®. An amplicon library was generated of the bacterial 16S rRNA gene by polymerase chain reaction (PCR) with the forward primer 27F (AGAGTTTGATCCTGGCTCAG) and the reverse primer 534R (ATTACCGCGGCTGCTGG) generating a 471 base pair product. Taxonomic classification was based on National Center for Biotechnology (NCBI) taxonomy. All hits per cluster with >97% similarity were used to calculate relative abundance of bacterial genera. 16S rRNA gene amplicon library analyses were performed for two replicates of seawater with clean copepod feces and two replicates of seawater with oil-containing copepod feces. Replicates with DNA with high purity (NanoDrop ND-1000, Thermo Fisher Scientific) and high quantity (separation of DNA on agarose gel using electrophoresis at 150 V for 1.5–2 h) were selected for further analyses. When presenting the results, the abundancies of bacterial species were summed at genus level, and genera with a total abundance <2.5% were grouped as others. The total bacterial communities determined in the individual four samples are presented as Supplementary Information A, Figs. S1–S4.

2.7. Statistical analyses

The results were presented as mean ± standard deviation (SD). Statistical analyses (*t*-test) were performed at a confidence level of 95% ($p < 0.05$) and a significance level of 0.05.

3. Results and discussion

3.1. Copepod feeding and ingestion of oil droplets

To determine the effect of oil-exposure on the feeding activity of the copepods, the clearance rates of control and oil-exposed copepods were calculated from coulter count analyses of particles in inflow and outflow tubes of control and oil-exposure tanks (Frost, 1972). Clearance rates for control copepods were between 19 and 50 mL copepod^{-1} day^{-1} (Fig. 1), which corresponds to previously reported rates (Nejstgaard et al., 1995; Nordtug et al., 2015). The clearance rates for oil-exposed copepods were between 8 and 14 mL copepod^{-1} day^{-1} (Fig. 1). Clearance rates for the oil-exposed copepods were significantly lower compared to control copepods at 17 and 26 h (*t*-test, $p = 0.009$ and $p = 0.02$, respectively). These results show that the feeding activity of the copepods was significantly reduced when they were exposed to dilute oil dispersions. Furthermore, the reduction was rapid after the onset of the experiment, indicating early effects of oil spills on copepod feeding. Previous studies at a concentration of 1.4 μL L^{-1} have reported a clearance rate for oil-exposed *C. finmarchicus* of 8 mL copepod^{-1} day^{-1} (Nordtug et al., 2015). The oil dispersion concentration in the exposure tanks of the oil-exposed copepods was 1.37 ± 0.24 μL L^{-1} ($N = 6$, ±SD). The close correspondence, both for control and oil-exposed copepods, between previously reported clearance rates and clearance rates reported here (Fig. 1), indicate that it may be possible to predict the feeding activity of copepods from the oil and algae concentration in the water column during oil spills. The reduced feeding activity by copepods exposed to oil dispersions have been suggested to be caused by mechanical obstruction of the feeding apparatus of the copepods due to oil droplets sticking to the fine setules of the feeding appendages, or by general narcotic action of the oil compounds (Nordtug et al., 2015).

Feces production can be used as a proxy to quantify feeding activity of the copepods. For clean copepod feces, the measured lengths and widths were: 0.41 ± 0.025 and 0.07 ± 0.01 mm ($N = 6$,

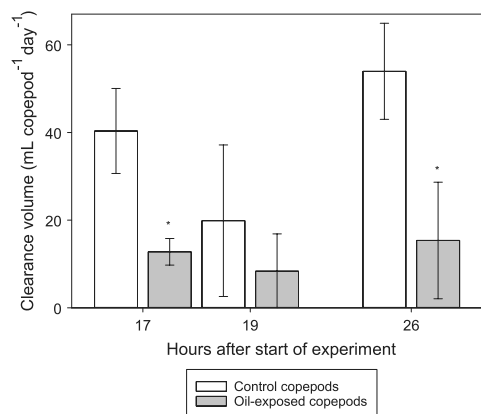
Fig. 1. Clearance rate (mL copepod^{-1} day^{-1}) for *Calanus finmarchicus* feeding on algae (*Rhodomonas baltica*) in clean seawater (control copepods, $N = 3$, ±SD) and on algae in oil dispersions (oil-exposed copepods, $N = 3$, ±SD).

±SD), respectively, and for oil-containing feces: 0.36 ± 0.04 and 0.06 ± 0.01 mm (N = 6, ±SD), respectively. The average length and width of fecal pellets excreted by oil-exposed copepods were significantly reduced compared to the average length and width of control copepod fecal pellets (t-test, $p < 0.0001$). Several mechanisms may result in reduced size of fecal pellets by the oil-exposed *C. finmarchicus*, including efficient metabolization of oil compounds, or reduced feeding activity. Effective metabolization of energy-rich oil may result in a lower volume of the excreted fecal pellets compared to ingestion of algae only. Fecal pellets from *C. finmarchicus* collected from waters outside New Foundland have been found to have an average length between 0.54 ± 0.17 mm and 0.31 ± 0.11 mm, depending on the algae ingested by the copepods (Urban et al., 1993). The lengths of both clean and oil containing feces were within this size range. The average total volume of feces produced by 400 control C5 *C. finmarchicus* over 48 h was 2.30 ± 0.73 mm^3 (N = 4, ±SD), while the average volume of feces produced by oil-exposed copepods was 0.44 ± 0.23 mm^3 (N = 6, ±SD). The feces volume produced by the oil exposed copepods was significantly (t-test, $p < 0.001$) lower compared to the feces volume produced by the control copepods. Significantly lower feces production was reported for *Calanus* sp. after exposure to Kuwait oil residue at a nominal concentration of 10 ppm (Spooner and Corkett, 1979). The results show that the feeding activity of copepods exposed to diluted oil-dispersion was sufficient to produce oil-containing feces.

To mimic diluted oil dispersions, the oil dispersions used when exposing *C. finmarchicus* was produced with droplets smaller than 40 μm (Fig. 2A) (Delvigne, 1993; Lee et al., 2013). These droplet diameters overlap with the diameter of the feed algae (*R. baltica*, ~7 μm, Fig. 2B) of *C. finmarchicus*, which feed non-selectively on particles <~35 μm (Cannon, 1928; Leiknes et al., 2014; Meyer et al., 2002). The copepods were thus expected to feed on both algae and oil droplets (Fig. 2C). Ingestion of oil by *C. finmarchicus* and excretion in feces were confirmed by detection of HC compounds in the seawater at the end of the 14 days incubation period (Fig. 3), as it also has been shown in previous studies (Nordtug et al., 2015; Olsen et al., 2013). To minimize the amount of oil adhering to external surfaces, the fecal pellets were thoroughly rinsed in filtered and autoclaved seawater prior to incubation. This procedure has previously minimized fouling by oil on external surfaces of *C. finmarchicus* feeding in oil dispersions (Nordtug et al., 2015). The HC compounds detected in the seawater with the oil-containing feces were thus assumed to originate from the oil droplets ingested by the copepods.

3.2. Oil compounds in copepod feces

GC–MS analyses performed on seawater with oil-containing feces (after 14 days of incubation) confirmed excretion of oil in *C. finmarchicus* feces, and quantified the loss of oil compounds in relation to the Troll source oil used in the oil dispersion. Loss of oil compounds occurred at three stages in this study: (1) By dissolution from oil droplets prior to ingestion by the copepods; (2) by partitioning from oil droplets in the gut of the copepods to copepod body tissue; (3) by dissolution and biotransformation from the oil in the feces during the 14 days of incubation. The responses of n-alkanes and PAHs were normalized against the response of the recalcitrant compound 30ab hopane and presented as percent of the corresponding ratio in the source oil, Fig. 3A and B.

All n-alkanes in seawater with oil-containing feces were depleted by >85% compared to their ratio in the source oil (Fig. 3A). The n-alkanes in Fig. 3A are lipophilic (log K_{ow} > 8.2), thus partitioning from oil droplets to the water phase was assumed to be low and the n-alkanes were expected to mainly be located in the oil droplets in the feces (EPI Suite, 2012). Biodegradation is generally higher for short-chain n-alkanes compared to long-chain n-alkanes (Douglas et al., 1996; Leahy and Colwell, 1990; Miralles et al., 2007). The extensive loss of the long-chain n-alkanes observed in this study shows that degradation of n-alkanes in oil droplets in feces of copepods can be substantial.

For the aromatic HC compounds, the total loss of compounds were higher for non-alkylated homologues compared to the alkylated homologous of naphthalenes, fluorenes, phenanthrenes, dibenzothiophenes, and the pyrenes/fluoranthenes (Fig. 3B). Decreasing biotransformation with increasing alkyl substitution has been observed for naphthalene and 3- and 4-ring aromatic HC compounds (Douglas et al., 1996; Venosa and Holder, 2007). The loss of aromatic compounds was in accordance with previous biodegradation studies of small droplets (Brakstad et al., 2015). However, the depletion of C0- and C1-dibenzothiophenes was higher compared to the depletion of C0- and C1-phenanthrenes (Fig. 3B). The rates of biotransformation of equally alkylated congeners of phenanthrene and dibenzothiophene are identical (Douglas et al., 1996; Wang et al., 1998). Since aromatic HC compounds mainly are degraded in the water phase after dissolution from the oil phase, the results may indicate that the dissolution of aromatic HC compounds from oil embedded in copepod feces was different (Brakstad et al., 2004). However, dibenzothiophene was not detected in seawater with oil-containing feces. We further suggest that degradation of aromatic compounds dissolved in the water phase in the presence of copepod and copepod feces can be substantial.

### 3.3. Concentration of total and viable microorganisms

To characterize the microbial communities in seawater with clean and oil-containing copepod feces, total cells were enumerated using epifluorescence microscopy (DAPI staining) and concentrations of viable HM and ODM were quantified by MPN analysis. The total cell count per mL seawater was higher in seawater with control feces compared to seawater with oil-containing feces (Table 1). However, since the oil-exposed copepods produced significantly less feces, there was less growth substrate for bacteria in seawater with oil-containing feces. The total number of cells was thus also calculated per mm^{-3} feces. For clean copepod feces, the total number of cells was $4 \times 10^8 \pm 10^8$ mm^{-3}, and for oil feces $8 \times 10^8 \pm 10^8$ mm^{-3}. This was twice as high for oil-containing feces compared to clean feces. The oil-containing fecal pellets were smaller than the clean fecal pellets, and they thus have a high surface-to-volume ratio. The higher number of cells in seawater with oil-containing feces may be due to a higher surface area relative to the volume of the oil-containing fecal pellets, which enables attachment of more bacteria per volume of feces.

The concentrations of viable HM were similar between oil-containing feces and clean feces (Table 1). The concentration of viable HM is suggested to represent the active indigenous feces bacteria, and the results thus show that the population was consistent between the clean and oil-containing copepod feces.

The concentration of viable ODM was significantly (t-test, p = 0.008) higher in seawater with oil-containing feces compared to the concentration in seawater with clean feces (Table 1). The results show that ingestion of oil droplets by copepods and excretion of oil in feces induced a capacity for degradation of oil by the bacteria present in the copepod feces. Increased activity and proliferation of the ODM were not at the expense of the concentration of viable HMs, which was similar between oil-containing and clean copepod feces (Table 1). The oil in feces thus represented new niches for the bacteria to exploit (Tilman, 2004). These results show that the feeding of copepods in oil dispersions induced oil-degrading capacity in the feces of the copepods.
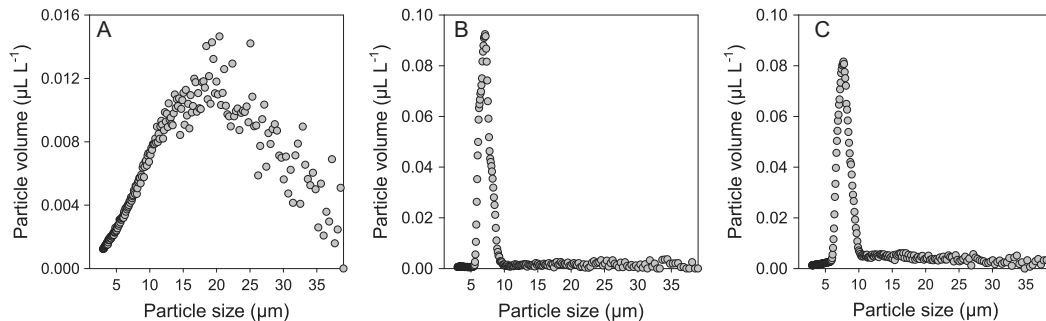
Fig. 2. Size distribution of oil dispersions in seawater inflow tubes of copepod oil exposure tanks (A), alga (*Rhodomonas baltica*) in alga inflow tubes to copepod oil exposure and control tanks (B), and algae and oil droplets in outflow tubes of copepod oil exposure tanks (C).
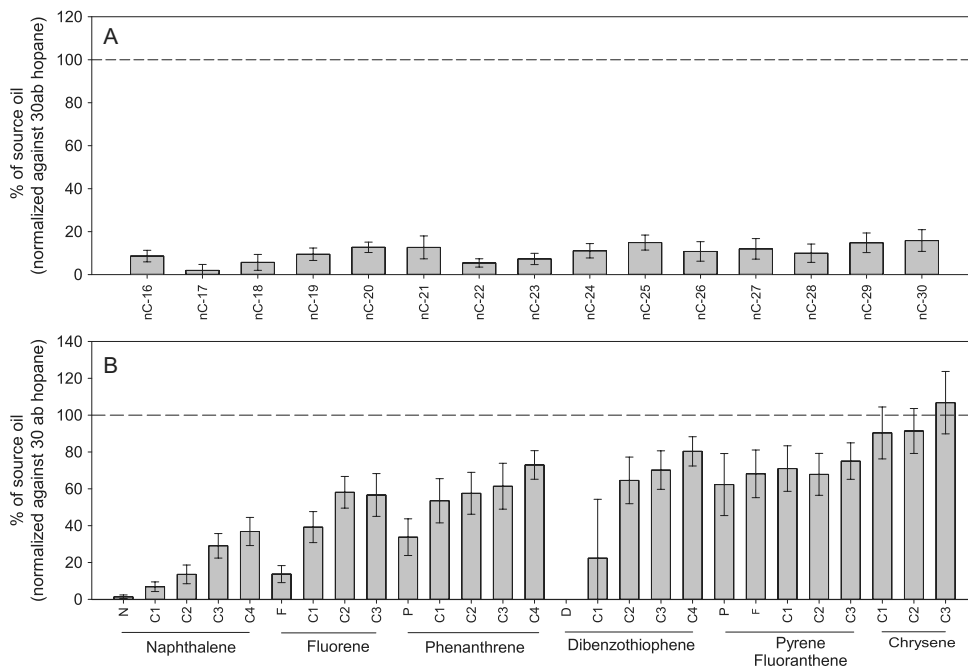
Fig. 3. The response of target oil compounds normalized against the response of 30ab hopane presented as percent of the corresponding ration in the source oil (Troll + 200 °C fraction) used in the oil dispersion determined in seawater incubated (14 days, 10 °C, dark) with oil-containing feces from *Calanus finmarchicus* feeding in oil dispersions for 48 h ($N = 5$, ±SD). Upper panel (A) show alkanes, lower panel (B) shows aromatic compounds.

Table 1
Total number of cells (counted using DAPI stain and epifluorescence microscopy), and concentration of viable heterotrophic microorganisms (HM) and oil-degrading microorganisms (ODM) analyzed by MPN (marine Broth medium or marine Bushnell-Haas Broth with Troll + 200 °C fraction as carbon source, respectively) in seawater with feces from control copepods (*Calanus finmarchicus*) feeding on alga (*Rhodomonas baltica*) only (control feces, $N = 4$) or from oil-exposed copepods feeding on alga in oil dispersions (oil feces, $N = 5$).

	Total number of cells (mL^{-1})		HM (mL^{-1})		ODM (mL^{-1})	
	AVG	SD	AVG	SD	AVG	SD
Control feces	628 587	±247 130	217 965	±618 681	8	±7
Oil feces	247 080	±100 850	226 000	±15 166	255	±131

3.4. Total bacterial communities

To characterize the bacteria of the total microbial communities, the relative abundance of bacteria species were determined in seawater with oil-containing and clean copepod feces by 16S rRNA gene amplicon library analyses. The relative abundance of bacteria was summed at genus level, and bacteria present <2.5% were grouped as other. The genera *Colwellia* (3% relative abundance, oil feces 2) and *Pseudoalteromonas* (4% relative abundance, oil feces 1) were detected only in seawater with oil-containing feces (Fig. 4). *Colwellia* and *Pseudoalteromonas* degrade HC compounds and the presence of these bacterial genera in seawater with oil-containing feces was therefore ascribed to the presence of oil in the copepod feces (Dubinsky et al., 2013; Hedlund and Staley,
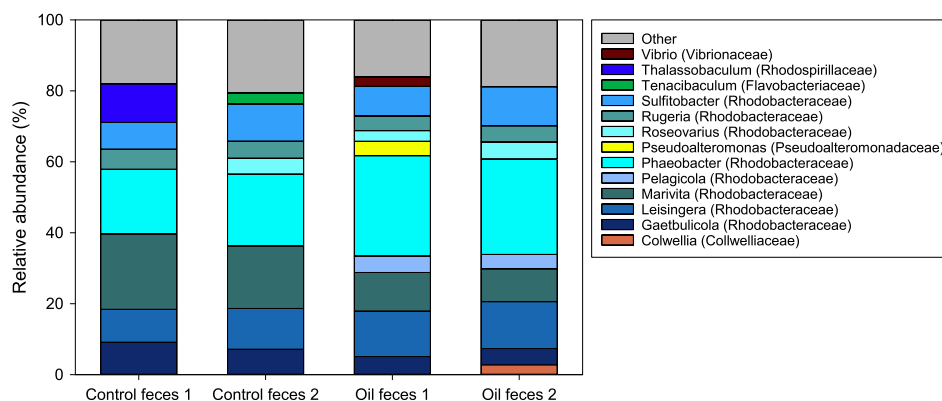
Fig. 4. Relative abundance of bacterial genera determined by 16S rRNA gene amplicon library analysis in seawater incubated (14 days, 10 °C, dark) with control feces from *Calanus finmarchicus* feeding on algae (*Rhodomonas baltica*) in clean seawater or in oil dispersions (oil feces) using 16S rRNA gene amplicon analysis. Genera with <2.5% abundance were grouped as other.

2006; Melcher et al., 2002). These bacteria were also likely metabolizing the HC compounds (Fig. 3). The genus *Phaeobacter* was detected at significantly (t-test, $P = 0.021$) higher relative abundance in seawater with oil-containing feces compared to seawater with clean feces. This indicates that proliferation and activity of *Phaeobacter* were stimulated by the presence of oil in the copepod feces. Bacteria of this genus have previously been identified at high abundance in alkane-enriched microcosms (McKew et al., 2007). Thus, the high relative abundance of *Phaeobacter* in the seawater with oil-containing feces was suggested to correspond with the extensive loss of n-alkanes (Fig. 3A).

Except for the differences noted above, the bacterial communities in oil feces and control feces were similar. The genera *Thalassobaculum*, *Sulfitobacter*, *Rugeria*, *Roseovarius*, *Phaeobacter*, *Leisinegra*, *Gaetbulicola* were detected both in seawater with clean feces and seawater with oil-containing feces. Of these genera, all except *Thalassobaculum* belong to the family *Rhodobacteraceae*. Of the genera with relative abundance >2.5%, those affiliated with the family *Rhodobacteraceae* represented total relative abundances of 77% and 78% in seawater with oil-containing feces (oil feces 1 and 2, respectively) and 76% and 79% in seawater with clean feces (control feces 1 and 2, respectively) (Fig. 4). The family *Rhodobacteraceae* is from the results suggested to be indigenous to copepod feces under the conditions in this study. Bacteria affiliated with this family are abundant in marine environments, and are associated with degradation of complex organic material (Dong et al., 2014; Eilers et al., 2001; McCarren et al., 2010). This family of bacteria is therefore also likely to be indigenious to copepod feces in marine environments.

The high similarity between bacterial communities in seawater with clean and oil-containing copepod feces shows that the feces were more important for the relative abundance of bacteria than the ingested oil droplets (Smith et al., 1998; Tilman, 2004). Thus, the bacterial communities in seawater with oil-containing feces were also different from the bacterial communities generally observed in seawater during degradation of oil (Dubinsky et al., 2013; Redmond and Valentine, 2012). However, a high concentration of viable ODM was detected in seawater with oil-containing feces compared to seawater with clean feces, which suggest that the bacteria present in these samples were capable of degrading oil. In line with this, bacteria belonging to the family *Rhodobacteraceae* represented a relatively large fraction of the bacteria that were increasing their metabolic activity after the Deepwater Horizon accident and the family was thus indicated

to be of significance during oil spill degradation (Kostka et al., 2011). During an oil spill incident in marine environments, a simultaneous distribution of feeding *C. finmarchicus* can introduce fecal pellets with a diverse heterotrophic bacterial community capable of HC degradation. The presence of a highly diverse microbial community in close proximity of inorganic nutrients in the copepod feces may enhance the mineralization of oil in the water column during oil spills. The results thus indicate that *C. finmarchicus* can be a biotic factor contributing in weathering of oil spills.

4. Conclusion

The potential of the copepod *C. finmarchicus* to contribute to weathering of oil spills in marine environments was studied, for the first time, by characterizing feeding activity of copepods in oil dispersions and the microbial communities in oil-containing and clean copepod feces. The clearance rate of the oil-exposed copepods was significantly reduced compared to copepods feeding in clean seawater. However, the feeding of the oil-exposed copepods was sufficient to produce oil-containing feces. The results show that the presence of the oil in the copepod feces induced a significantly higher concentration of viable oil-degrading microorganisms. The indigenous feces bacteria were assumed to be capable of oil degradation, as the total bacterial communities were similar between the oil-containing copepod feces and the clean copepod feces. Bacterial communities were dominated by the family *Rhodobacteraceae*, and the genera *Lesingera*, *Phaeobacter*, *Rugeria*, and *Sulfitobacter*. This indicates that copepods can act as a biotic factor contributing in the weathering of oil spills in marine environments by excreting feces containing microbial communities adapted to degradation of oil to the water column.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2015.07.011.

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SUPPLEMENTARY INFORMATION A. Bacterial communities in seawater with copepod feces

Figure S1-4. Percent composition of bacteria operational taxonomic units (OTUs) determined with 16S rRNA gene amplicon library analyses in feces from copepods (*Calanus finmarchicus*) ingesting algae (*Rhodomonas baltica*, control feces) and in feces from copepods ingesting algae and oil droplets (oil feces). The taxonomic assignment of OTUs of unique clusters were based in National Center for Biotechnology (NCBI) taxonomy and the results presented are all hits from clusters assigend with >97% similarity.

Microbial communities in copepod feces with oil

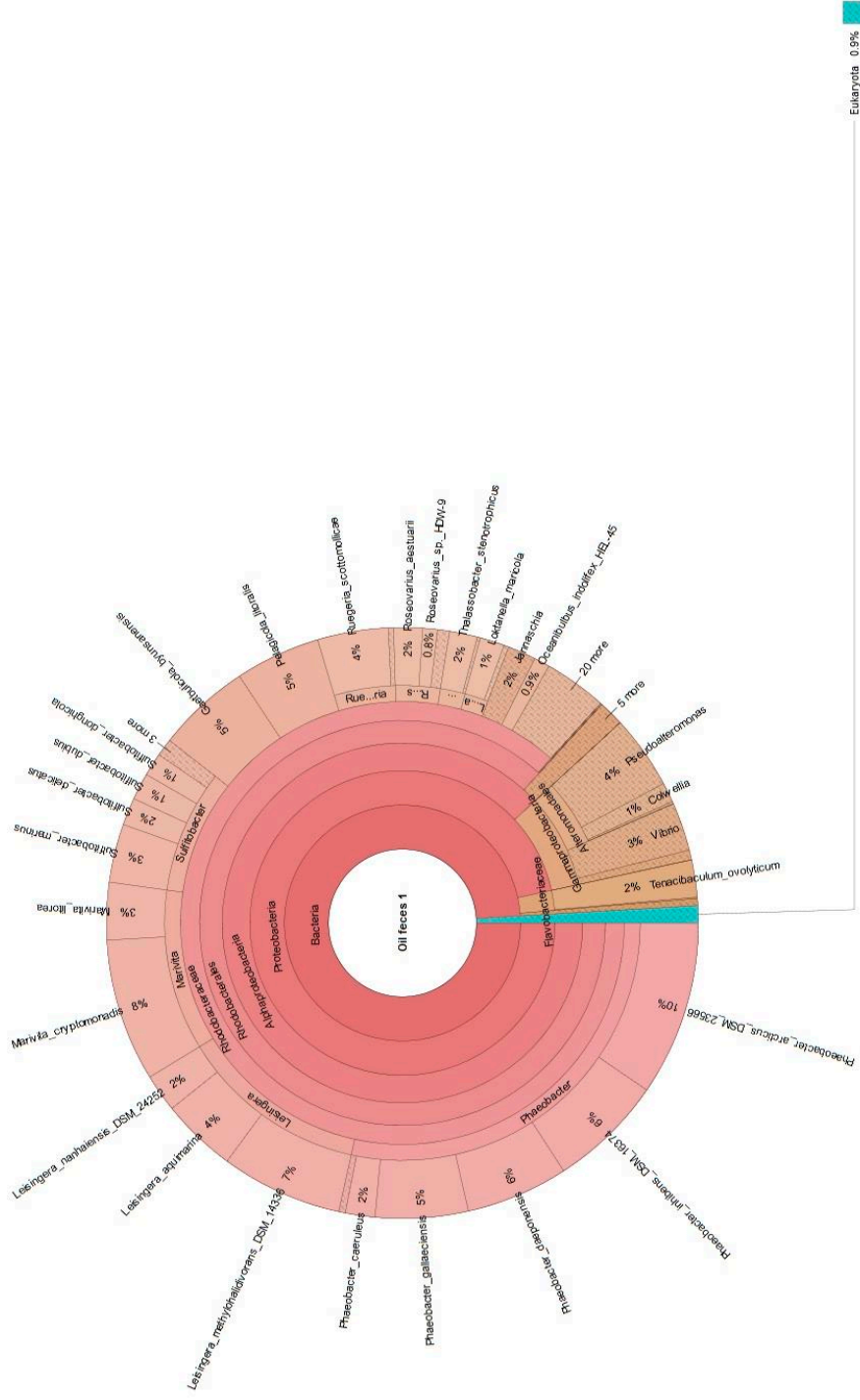Fig. S1. Control feces 1.

Microbial communities in copepod feces with oil

Fig. S2. Control feces 2

Microbial communities in copepod feces with oil

Fig S3. Oil feces 1.

Microbial communities in copepod feces with oil

Fig S3. Oil feces 2.

Paper V

Biotransformation of petroleum hydrocarbons and microbial communities in seawater with oil dispersions and copepod feces

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ABSTRACT

To determine biotransformation of components in crude oil dispersions in the presence of feces from marine copepods, dispersed oil was incubated alone, with the addition of clean or oil-containing feces. We hypothesized that the feces would contribute with nutrients to bacteria, and higher concentrations of oil-degrading bacteria, respectively. Presence of clean feces resulted in higher degradation of aromatic oil compounds, but lower degradation of n-alkanes. Presence of oil-containing feces resulted in higher degradation of n-alkanes. The effect of clean feces on aromatic compounds are suggested to be due to higher concentrations of nutrients in the seawater where aromatic degradation takes place, while the lower degradation of n-alkanes are suggested to be due to a preference by bacteria for feces over these compounds. Large aggregates were observed in oil dispersions with clean feces, which may cause sedimentation of un-weathered lipophilic oil compounds towards the seafloor if formed during oil spills.

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1. Introduction

Dispersion of oil into the water column is one of several weathering processes an oil spill in marine environments is subjected to. This process is driven by mechanical action on surface oil slicks, and the concentration and size of the oil droplets therefore depends on sea state, in addition to properties of the oil (Daling et al., 1990; Delvigne, 1993). During oil spills, chemical dispersants can be used to reduce the surface tension between the oil and the water, to enhance the formation of small oil droplets in the water (Brandvik, 1997). The small oil droplets (diameter < 100 μm) have slow surfacing velocity in seawater and accumulate in the water column (Lee et al., 2013). These droplets may drift passively with underwater ocean currents (Camilli et al., 2010). Suspended oil droplets can interact with organic particles, and may also interact with copepod feces (Muschenheim and Lee, 2002). The interaction between oil, and oil and organic particles in the water column has been shown to cause formation of large oil-containing aggregates, classified as marine snow (Fu et al., 2014; Passow et al., 2012). Marine snow is a large aggregate (≥0.5 mm) of organic particles which contribute substantially to the transport of organic materials towards the ocean seafloor (Alldredge and Silver, 1988).

The dispersion of oil into the water alters the location of the oil from the surface to the water column. However, for ultimate removal of petroleum hydrocarbon (HC) compounds from marine environments, biological weathering of oil is fundamental. Biological weathering includes biotransformation and mineralization of HC by microorganisms (Head et al., 2006). Oil-degrading bacteria are ubiquitous in marine environments, and these bacteria have been identified within several phyla (Alpha- and Gammaproteobacteria, Actinobacter, Flexibacter-Cytophaga-Bacteroides) (Harwati et al., 2007; Prince, 2005). Oil-degrading bacteria may be obligate hydrocarbonoclastic bacteria or capable of utilizing a range of organic compounds (Ward and Brock, 1976; Yakimov et al., 2007). For the utilization of bioavailable HC, bacteria need an electron acceptor and inorganic nutrients. Since oil has a high content of carbon, but contains low or no inorganic nutrients, biodegradation of oil spills by bacteria can be enhanced by the addition of nutrients to areas affected by oil spills (Atlas, 1995; Röling et al., 2002). Especially the ratios of carbon to nitrogen (C:N) and carbon to phosphorus (C:P) are of importance for bacterial oil degradation (Atlas and Bartha, 1972). A ratio of C:N:P of 100:10:1 has generally been regarded as optimal for bacterial activity (e.g. Bouchez et al., 1995; Obbard et al., 2004). Feces from copepods have higher nitrogen concentrations relative to carbon concentrations when compared to other organic particles in the water (Bathmann et al., 1987; Paffenhöfer and Köster, 2005). Copepod fecal pellets are in addition abundant, and constitute up to 90% of the total suspended material in

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the surface waters of the Norwegian Sea (Bathmann et al., 1987). The presence of copepod feces during an oil spill incident may thus increase microbial biotransformation of HC by supplying oil-degrading microorganisms with inorganic nutrients. During oil spills, this effect may be reduced, as oil-exposed *Calanus finmarchicus* defecate less than control copepods (Spooner and Corkett, 1979; Størdal et al., 2015). However, oil-containing feces from oil-exposed copepods have been shown to contain high concentrations of viable oil-degrading bacteria (Størdal et al., 2015). The presence of oil-containing feces may thus also enhance the degradation of oil compounds.

The purpose of this study was to investigate the influence of copepod feces on biodegradation of oil spills, and microbial communities in oil dispersions in a laboratory set-up. The two main aims were to investigate if biotransformation of target oil compounds was increased by the presence of copepod feces, which were assumed to contribute with nutrients, and if biotransformation also was increased by the presence of oil-containing feces, which have increased concentrations of viable oil-degrading microorganisms (Størdal et al., 2015). In this experiment, feces from the ubiquitous calanoid copepod *C. finmarchicus*, was used as a model matrix for copepod feces. This copepod is found at high densities in the North Sea, the Norwegian Sea, and the Barents Sea (Helle, 2000). Three different oil dispersions were included: I) Dispersed oil in seawater (no feces, oil dispersion control); II) Dispersed oil with the addition of clean copepod feces; and III) Dispersed oil with the addition of oil-containing feces. Copepod feces were collected from two groups of *C. finmarchicus*; one group that was kept in clean seawater and one group that was kept in seawater with dispersed oil. To evaluate reduction in feces production due to oil exposure, feces quantity from oil-exposed copepods was related to feces quantity from control copepods.

2. Materials and methods

2.1. Experimental design

Fig. 1 shows a schematic overview of the experiment. The laboratory set-up included: 1) collection of feces after 48 h of feeding of control copepods in clean seawater (clean feces) and from oil-exposed copepods feeding in oil dispersions (oil-containing feces), 2) incubation of the copepod feces from the two exposure separately in oil dispersions (nominal oil concentration $2\,\mu L\,L^{-1}$). To evaluate the extent of biotransformation in the oil dispersions, petroleum HC were quantified using gas chromatography-mass spectrometry (GC-MS) in the crude source oil before the experiment and after 14 days of incubation of the dispersion. Concentrations of viable microorganisms (heterotrophic microorganisms [HM] and oil-degrading microorganisms [ODM]) were determined in the samples by cultivation, using most probable number method (MPN; Brown and Braddock, 1990). The total numbers of microorganisms were also determined, by enumeration using 4′, 6′-

diamidino-2-phenylindole (DAPI) staining and oil immersion fluorescence microscopy. The relative abundances of bacteria in the total microbial communities were determined using 16S rRNA gene amplicon library analysis.

2.1.1. Oil droplet dispersions and seawater

The petroleum oil used in this experiment was a naphthenic North Sea crude oil from the Troll exploration field. The oil was artificially weathered to 200 °C, which corresponds to approximately 24 h of weathering at sea as described by Stiver and Mackay (1984). The weathered oil was filtered (VWR, polytetrafluoreten filter, 0.2 μm pore size), and used for producing oil dispersions as described by Nordtug et al. (2011). This method includes forcing a water/oil-mixture through several nozzles with diameter of 0.5 mm, and enable tight control of the size distribution of the droplets and the concentration of the oil in seawater by adjusting the flows of water and oil, respectively. The oil dispersions produced for exposure of copepods and the oil droplet dispersions incubated with clean and oil-containing copepod feces were generated under identical conditions. The flow of water was 160 mL min^{-1} and the flow of oil was 0.35 μL min^{-1}. This produced oil dispersions with nominal concentration of $2\,\mu L\,L^{-1}$, and oil droplets with diameter < 40 μm.

Seawater used in the experiment is continuousl y supplied to our laboratory facilities from 80 m depth in Trondheimsfjorden (63°26′N, 10°23′E), sand filtered to remove coarse particles, and filtered (5 μm exclusion limit) prior to use.

2.1.2. Copepod husbandry and exposure of copepods

To obtain copepod feces to incubate with the oil dispersions, feces were collected from the copepod *C. finmarchicus* (Gunnerus) feeding in clean seawater and in oil dispersions in clean seawater. *C. finmarchicus* was obtained from the culture running at NTNU/SINTEF Sealab (Trondheim, Norway). The culture copepods were kept in continuously running seawater at 8–10 °C and were regularly fed a diet of microalgae (*Rhodomonas baltica* Karsten, *Dunaliella tertiolecta* Butcher and *Isochrysis galbana* Parke). Further details on the culture have been described by Hansen et al. (2007).

The copepods were exposed in glass exposure tanks with round bottoms and detachable lids. Clean seawater or oil dispersions were supplied to the exposure tanks through teflon capillary tubing at an average flow rate of 27 mL min^{-1}. The copepod exposure solution was supplied at the bottom of the exposure tanks, while the outlet was positioned close to the surface. This generated a gentle mixing of the exposure solution. *C. finmarchicus*, 400 copepodite stage 5 (C 5) were carefully introduced to the exposure tanks using a ladle, and exposed to clean seawater or oil dispersions under dim light conditions for 48 h in a climate controlled room (10 °C). Copepods were fed with live *R. baltica*. (nominal concentration 400 μg C L^{-1}).
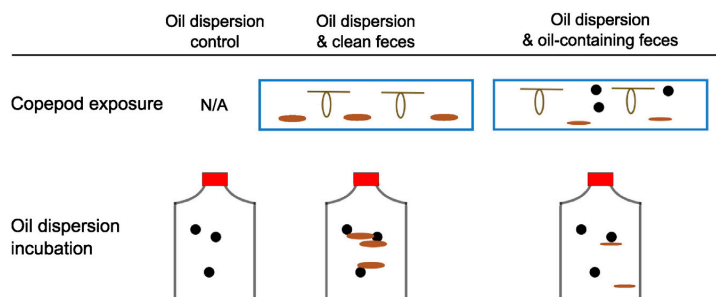

Fig. 1. Schematic layout of the experiment. Oil dispersions were incubated (14 days, 10 °C, dark) with clean copepod feces collected from copepods feeding in clean seawater and with oil-containing copepod feces collected from copepods feeding in oil dispersions. After the 14 day incubation, biodegradation of oil compounds and microbial communities in the oil dispersions was examined.

2.1.3. Collection of clean and oil-containing copepod feces

After 48 h of exposure, feces were collected from each control and oil dispersion copepod exposure tank by removing the detachable lid and maneuvering an acrylic suction pipe over the bottom of the tank. The suction pipe was connected to a borosilicate flask (Schott, 500 mL) detaining the collected feces. The flask was pre-filled with seawater and suction was provided to the system by a peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL, USA) connected to the flask. The outlet from the flask to the pump was supplied with a fine mesh (15 μm), keeping feces within the flask. The distal opening of the acrylic suction pipe was supplied with a mesh sieve (200 μm) to prevent copepods from entering together with the feces. Each exposure tank had a dedicated flask and a suction pipe with its own tubing. The entire volume of the exposure tank was also filtered (20 μm) to collect feces suspended in the water. The total volume of each feces suspension was reduced (mesh size 20 μm) to 16 mL, before transfer of all feces collected from one exposure tank to one individual flask containing oil dispersion.

2.1.4. Incubation of oil dispersions and copepod feces

Borosilicate glass flasks (Schott, 2 L) were completely filled (no headspace, total volume of 2.3 L) with oil dispersions (no feces), oil dispersions and clean copepod feces, or oil dispersions and oil-containing copepod feces, and incubated for 14 days, in the dark, on a carousel system slowly rotating in the vertical direction (0.75 rounds per minute). The incubation was performed in a climate room at 10 °C. The flasks with oil dispersions and feces were rotated to keep oil droplets and feces in suspension. The carousel system has previously been described by Brakstad et al. (2015). To minimize adherence of organic material to the inside walls of the flasks, the flask were baked (400 °C, 3 h), soaked in deconex over night (10%, 11 Universal, VWR International), washed (Neodisher® LaboClean A 8) and thoroughly rinsed with water.

After incubation, subsamples of seawater with oil dispersions and feces were collected for chemical analysis (1 L), enumeration of cells and determination of HM and ODM (20 mL), and nucleic acid extraction for bacterial community analysis (1.2 L). All flasks were vigorously shaken prior to collection of subsamples to obtain a homogeneous distribution of feces and oil.

2.2. Analyses

2.2.1. Particle count analysis

During the exposure of the copepods and preparation of the oil dispersions, particle count analysis was performed to verify size and concentrations of the algae particles and oil droplets. Particle count analysis was also performed on the oil dispersions incubated with feces to verify size and concentrations of the oil droplets. The particle counting and sizing were performed using a Coulter Counter (Multisizer 3; Beckman) equipped with a 100 μm aperture tube with the lower threshold set to 2 μm.

2.2.2. Chemical analysis

Analysis of oil compounds were performed with GC-MS operated in selected ion monitoring mode (Agilent 7890B GC with an Agilent 5977A inert XL electron impact mass selective detector). The analysis was performed as described by Nordtug et al. (2015). All replicate samples of oil dispersion controls, oil dispersions and clean feces, and oil dispersions and oil-containing feces were acidified with hydrochloric acid (HCl, pH < 2) and solvent-solvent extracted (dichloromethane; DCM). The inside walls of the flasks were extracted with DCM and pooled with the sample extract. The pooled extracts were concentrated to 100 μL volume. The analysis included determination of the response area of the following target oil compounds, with a signal-to-noise ratio of 5 as the lower limit of detection (LOD): decalins (C0-C4), naphthalenes (C0-C4), biphenyl, acenaphtylene, acenaphthene,

dibenzofuran, fluorenes (C0-C3), phenanthrenes/anthracenes (C0-C4), dibenzothiophenes (C0-C4), fluoranthenes/pyrenes (C0-C3), benz(a)anthracene, chrysenes (C0-C3), benzo(b)fluoranthene benzo(k)fluoranthene, benzo(a)pyrene, benzo(e)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, alkanes from nC$_{10}$-nC$_{36}$ and 17α(H),21β(H)-hopane (30ab hopane). The biotransformation of the compounds acenaphtylene, acenaphthene, dibenzofuran, chrysene, benzo(b)fluoranthene benzo(k)fluoranthene, benzo(a)pyrene, benzo(e)pyrene, perylene, indeno(1,2,3-c,d)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and n-alkanes < 14 C and > 30 C were not evaluated due to a response < LOD. Biotransformation of the compounds decalins (C0-C4) and naphthalenes (C0-C3) were not included in the results due to presence of these compounds in laboratory blanks at levels which interfered with calculations. However, the small response of these compounds detected when analyzing samples with oil dispersions and feces show that they were present at negligible levels, indicating extensive biotransformation over the 14 day incubation. This is in line with previous studies showing extensive loss of these compounds after 16 days, from oil dispersions incubated at 5 °C (Brakstad et al., 2015).

2.2.3. Concentrations of microorganisms

Enumeration of cells and determination of concentrations of viable microorganisms were performed in all replicate samples for the three scenarios.

2.3. Enumeration of cells

Enumeration of total cells was performed by oil immersion fluorescence microscopy (Nikon Eclipse 80i) at 1 250 times magnification on samples stained with the nucleic acid stain DAPI (0.06 mg mL^{-1}, 10 min) and filtered through 0.2 μm black polycarbonate filters (Nucleopore, Costar, Cambridge, USA) (Porter and Feig, 1980).

2.4. Concentration of viable heterotrophic and oil-degrading microorganisms

Determination of concentrations of HM and ODM were performed in triplicates for each replicate sample by MPN analysis in 24-well tissue culture plates (Costar), as described for ODM by Brown and Braddock (1990). Concentration of HM was determined in Marine Broth medium (Difco). Concentration of ODM was determined in Bushnell-Haas Broth supplemented with 30 g L^{-1} NaCl and 0.01% (v/v) artificially weathered Troll oil (+200 °C residue). The plates were incubated at 10 °C for 10 days for the HM and for 14 days for the ODM. Actively respiring ODM were detected using fluorescein diacetate (Chrzanowski et al., 1984).

2.4.1. Extraction of nucleic acids and 16S rRNA gene amplicon library analysis of bacterial communities

Total deoxyribonucleic acids (DNA) were extracted from replicate samples of seawater with oil dispersion controls, oil dispersions with clean feces, and oil dispersions with oil-containing feces by filtration of suspended material (0.45 μm pore size, 47 mm diameter, Millipore, HAWG filter). Filters with material were transferred to sterile tubes and stored at −20 °C until extraction. To the tubes containing the filters with fecal material and oil, 4.5 mL lysis buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, and 1 M NaCl) with 0.5 mL lysozyme (20 mg mL^{-1}), 0.125 mL proteinase K (20 mg mL^{-1}), lauryl-sarkosyl (1%) and sodium-dodecyl-sulfate (1%) were added to lyse the cells. The tubes were then shaken for 1 h at 37 °C using a vortex mixer. The DNA extraction was performed using a mixture of hot phenol:chloroform:isoamylalcohol (25:24:1, pH 8.0) (Sambrook and Russel, 2001). Extracted DNA was dissolved in 100 μL ultrapure water (MolBio grade, 5prime) and stored at −20 °C until subsamples were shipped for 16S rRNA gene amplicon library analyses.

16S rRNA gene amplicon library analysis of DNA extracted from the samples were performed by GATC Biotech (Constance, Germany) using the Illumina MiSeq®. Bacterial 16S rRNA amplicon libraries were generated by polymerase chain reaction (PCR) using the forward primer 27 F (AGAGTTTGATCCTGGCTCAG) and the reverse primer 534R (ATTA CCGCGGCTGCTGG) resulting in a 471 base pair product. Taxonomic classification was based on National Center for Biotechnology (NCBI) taxonomy. The results presented in on the total bacterial communities were calculated from operational taxonomic units (OTUs) and their percent composition of clusters assigned from all hits obtained with >97% similarity. Analysis of total bacterial communities were performed for two replicates for each scenario, and samples with DNA of high purity (NanoDrop ND-1000, Thermo Fisher Scientific) and quantity (separation of DNA on agarose gel using electrophoresis at 150 V for 1.5–2 h) were selected.

2.5. Calculations

2.5.1. Feces produced by copepods

Mean gray value of identically sized squares positioned on a photographic picture of the feces in containers was used as proxy for feces quantity. The photographic picture was taken of the 20 mL containers with 16 mL of seawater with feces. The containers were left for the feces to sediment before the picture was taken and the squares were positioned close to the bottom of the containers. The mean gray value of the squares was calculated by converting each pixel to a grayscale using ImageJ (National Institute of Health, Bethesda MD, USA) without considering weighting of red, green and blue pixels. A value of 0 corresponds to black and 256 to white. The photographic picture of the containers with the feces is shown in Supplementary Information A, Fig. S1.

2.5.2. Biotransformation of HC compounds in oil dispersions

To assess loss of single oil compounds from the oil dispersion incubated in closed flasks for 14 days ($C_{i\ sample}$), the response area of target oil compounds were calculated as a ratio using the 30ab hopane response area ($C_{hop\ sample}$) as a reference. 30ab hopane is a recalcitrant biomarker in oil (Aeppli et al., 2014; Mills et al., 1999). 30ab hopane has been applied for normalization of the quantity of single petroleum compounds to evaluate biodegradation in numerous studies, (e.g. Brakstad et al., 2015; McFarlin et al., 2014). The target compound remaining in the oil dispersions incubated for 14 days was calculated as percent of the corresponding ratio in the source oil ($C_{i\ source\ oil}$/$C_{hop\ source\ oil}$), as modified from CEN (2010):

$$\text{Remaining target compound } (\%) = 100 \times \left(\frac{C_{i\ sample}}{C_{hop\ sample}} \bigg/ \frac{C_{i\ source\ oil}}{C_{hop\ source\ oil}} \right) \tag{1}$$

2.5.3. Statistical analysis and data treatment

Mean ± standard deviation (SD) is presented. Statistical analysis (One-way Analysis of Variance [One-way ANOVA]) were performed at a confidence level of 95% ($p < 0.05$) with pairwise multiple comparison of all treatments using the Holm-Sidak method. The One-way ANOVA includes an initial check of normality using the Shapiro–Wilk test ($p < 0.05$).

3. Results and discussion

Petroleum oil has a high content of carbon, and during oil spills the ratio of carbon to inorganic nutrients are locally increased. Oil-degrading bacteria require inorganic nutrients and biotransformation of oil spills can therefore be enhanced by adding these at an oil spill site. Copepod feces are abundant, and also high in nutrients. The aim of this study was to investigate if the presence of copepod feces may enhance biotransformation of HC compounds. This was done by incubation oil dispersions with copepod feces in seawater for 14 days. Oil dispersions incubated in clean seawater served as control. Biotransformation of HC compounds was quantified using GC-MS, concentrations of microorganisms (HM and ODM) were determined using MPN and DAPI, and total microbial communities were found by 16S rRNA gene amplicon library analyses.

3.1. Oil dispersions and feces production by control copepods and oil-exposed copepods

To verify the size and concentration of oil droplets and algae, the particle volume in the copepod exposure solutions and in the oil dispersions incubated with the feces was monitored using particle count analyses. The majority of the volume of the oil in the oil dispersions (97%, N = 10) was found in droplets with diameter <40 μm (Fig. 2A). Thus, the size of the oil droplets correspond to the size observed in dilute oil dispersions observed after oil spills (Camilli et al., 2010; Lee et al., 2013). The oil dispersion supplied to the copepod exposure tanks and the oil dispersions incubated with the copepod feces were similar (Fig. 2A and B). The incubation of oil dispersions with oil-containing feces was aimed at mimicking a situation with copepods feeding and defecating in dilute oil dispersions.

The volume of feces produced by the oil-exposed copepods was not sufficient for chemical analysis to be performed prior to incubation with oil dispersions. However, previous studies at our laboratory have shown excretion of oil droplets in feces when C. finmarcicus were feeding in an oil dispersion with a nominal concentration of 2 μL L^{-1}, with oil droplet diameters < 40 μm (Olsen et al., 2013; Størdal et al., 2015). C. finmarchicus has been documented to feed non-selectively by suspension feeding on particles with diameter < 35 μm (Leiknes et al., 2014). The size distributions of the feed algae R. baltica and of the oil droplets supplied to the copepods were overlapping (Fig. 2C). We thus expected non-selective suspension feeding on algae and oil droplets by the copepods feeding in oil dispersions, and further, excretion of oil and high concentrations of oil-degrading microorganisms in copepod feces.

Mean gray values quantified from a photographic picture of the feces in glass containers (20 mL) were used to estimate feces quantity. The feces produced by the control copepods corresponded to a mean gray value of 50 ± 5, while the mean gray value for feces produced by copepods feeding in the oil dispersion was 139 ± 9 (high values indicate less feces). These results confirm previously reported reduction in feces production by copepods feeding in oil dispersions (Spooner and Corkett, 1979; Størdal et al., 2015). The oil dispersion concentration in the present experiment was 1.56 ± 0.24 μL × L^{-1} (N = 10, ± SD). The results indicate an approximate three-fold reduction in feces production by oil exposed copepods at a low concentration of oil.

3.2. Biotransformation of oil compounds in dispersions

The response values of n-alkanes and aromatic HC were normalized against 30ab hopane and presented as percent of the corresponding ratio in the source oil to determine loss of HC compounds due to biotransformation in the oil dispersions (Fig. 3). The oil dispersions were incubated in closed flasks with no headspace and in the dark, minimizing loss due to evaporation and photo-degradation. The depletion of n-alkanes was >85% in all three scenarios. In the total biotransformation, there were significant differences over the 14 days of incubation between the scenarios. In the oil dispersions incubated with clean copepod feces, the biotransformation of n-alkanes was significantly lower (One-way ANOVA, $p < 0.016$) compared to the oil dispersion control. This included nC14-, nC19-, nC20-, nC22- to nC25-, and nC27- to nC30-alkanes. In oil dispersions incubated with oil-containing feces, the biotransformation of nC19- and nC20-alkanes were significantly higher compared to the oil dispersion control (One-way ANOVA, $p = 0.009$ and $p = 0.027$) (Fig. 3A). The latter results
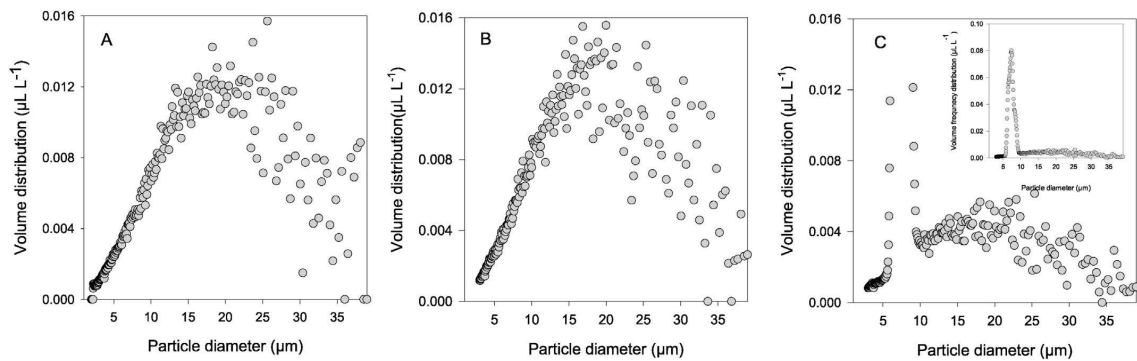
Fig. 2. Size distribution of oil droplets in oil dispersions incubated with feces (A), oil droplets in oil dispersions in inflow tubes to *Calanus finmarchcicus* exposure tanks (B), and exposure media mixture with oil droplets and the algae *Rhodomonas baltica* in outflow tubes of copepod oil dispersion exposure tanks (C). The figure C insert shows the whole algae peak.
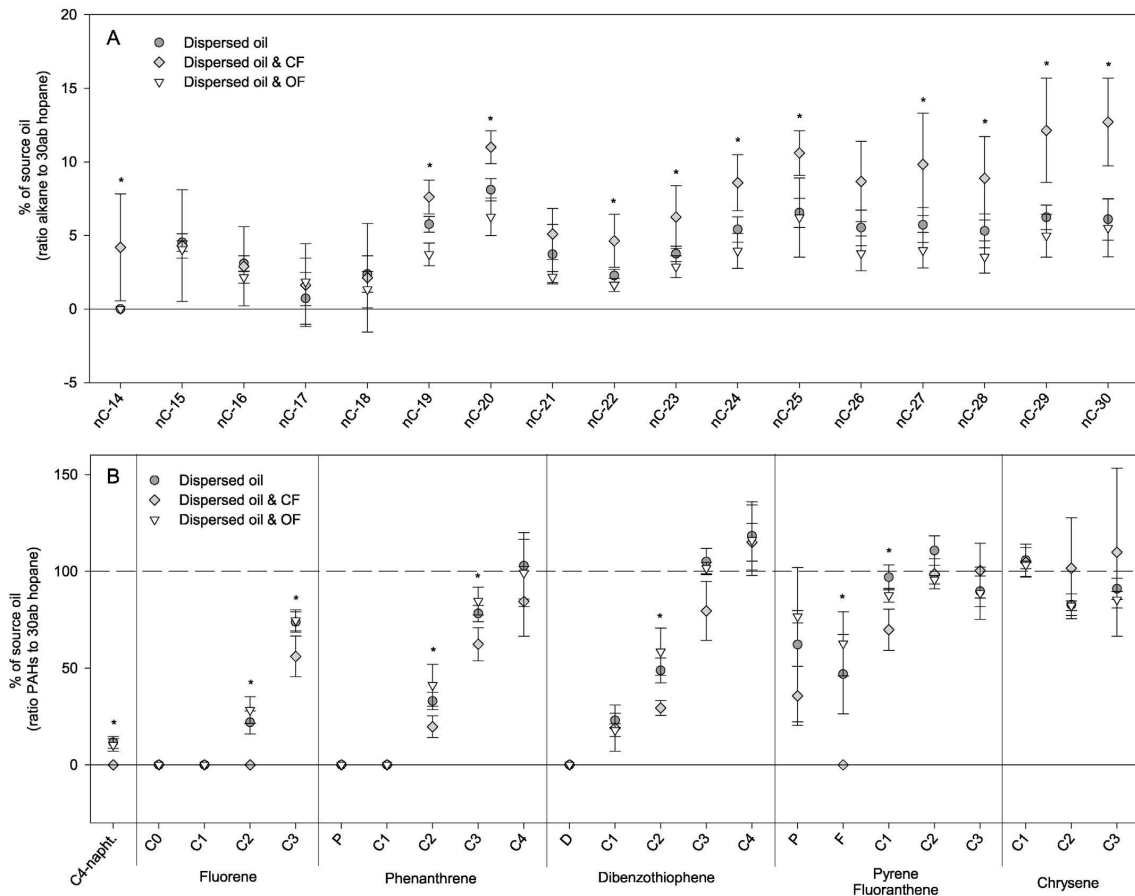
Fig. 3. The response of target oil compounds normalized against the response of 30ab hopane presented as percent of the corresponding ratio in the source oil (Troll + 200 °C fraction) in oil dispersions incubated (14 days, 10 °C, dark) in seawater with clean copepod feces (CF, N = 3), or with oil-containing copepod feces (OF, N = 3). Oil dispersions incubated (N = 6) in clean seawater served as control. Asterisk denotes biotransformation in Oil dispersion + CF significantly different from Oil dispersion control and Oil dispersion + OF. Feces were obtained from *Calanus finmarchicus* feeding on *Rhodomonas baltica* in clean seawater or in seawater with oil dispersions. Upper panel (A) shows alkanes, while lower panel (B) shows aromatic HC compounds.

show that the presence of feces from copepods feeding in oil dispersions resulted in higher biodegradation of n-alkanes as expected.

Contrary to what was expected, the presence of clean copepod feces resulted in significantly lower biotransformation of only the n-alkanes compared to the oil dispersion control. We suggest that this was caused by the bacteria present in the samples with feces were preferring feces as their carbon and energy source prior to n-alkanes. The compounds in the copepod feces may have similar structure as the n-alkanes, since a decaying bloom of the *Rhodomonas* sp. have been indicated as the source of short-chain alkanes (Kameyama et al., 2009; Suzuki et al., 2009). Previous studies have shown low mineralization of the HC compound hexadecane when labile carbon and glucose were present, suggested to be caused by assimilation of these compounds by bacteria rather than assimilation of HC compounds (Ward and Brock, 1976).

Furthermore, large agglomerates assumed to contain feces and oil droplets with n-alkanes were observed visually in the flasks with oil dispersions and clean copepod feces. This indicates that the n-alkanes may have been kept in close proximity to the feces. The n-alkanes included in this study were highly lipophilic (log $K_{ow} > 7.2$) (EPI Suite, 2012). These compounds were expected not to partition to the water phase and to be degraded at the oil-water interphase. Degradation of n-alkanes mainly at the oil-water interphase has been experimentally shown by Brakstad et al. (2004). If formed during oil spills, such aggregates of copepod fecal matter and oil droplets may sediment to the sea floor as marine snow (Alldredge and Silver, 1988; Fu et al., 2014; Turner, 2002). Large aggregates may scavenge the small oil droplets efficiently and can accumulate a substantial fraction of an oil spill (Hill et al., 1990). The results presented here indicate that the biodegradation of lipophilic oil compounds primarily contained within the oil phase may be low in such aggregates and that the sedimentation therefore will be of less weathered oil. However, it should be noted that the sedimentation rate of these agglomerates will depend on their effective density in the seawater.

The biodegradation of oil was also evaluated for the aromatic HC compounds (Fig. 3B). The biotransformation of C4-naphthalene ranged from 88 to 100% depletion across the three scenarios and was close to complete in both the oil dispersion control and in the oil dispersions incubated with copepod feces. Biotransformation of the 3-ring aromatic HC ranged from 25–100% for fluorenes and 0–100% for both phenanthrenes and dibenzothiophenes. The 4-ring aromatic HC were biotransformed to a lesser degree than the 3-ring aromatic HC (Fig. 3B), ranging from 11–64% and 0–15% for fluoranthenes/pyrenes and chrysenes, respectively. In oil dispersions incubated with clean copepod feces, the biotransformations were significantly higher for C4-naphthalene, C2- and C3-fluorene, C2- and C3-phenanthrene, C2-dibenzothiophene, fluoranthene and C1-fluoranthene (One-way ANOVA, $p < 0.01$) compared to the oil dispersion control. There were no significant differences (One-way ANOVA) between oil dispersions incubated with oil-containing feces and oil dispersion control. The difference in response between biotransformation of n-alkanes and aromatic HCs in the presence of clean copepod feces was suggested to be caused by their location during degradation. n-alkanes have been shown to be degraded at the oil/water-interphase, while light-weight aromatic HC compounds dissolve to the water phase and are degraded there (Brakstad et al., 2004). Also, the two compound classes are degraded by different species of bacteria, which are suggested to be affected differently by the presence of copepod feces (Head et al., 2006; Smith et al., 1998).

Although inorganic nutrients were not quantified in this study, we suggest that the higher biotransformation of aromatic compounds observed in oil dispersions incubated with clean feces was due to a high activity of bacteria in the water phase due to high concentrations of inorganic nutrients. In seawater with the oil dispersion control (no feces) the ratios of carbon to nitrogen (in $NO_3^- + NO_2^-$) and phosphorus (in PO_4^{3-}) were calculated to be 100:3:0.5 (elemental concentrations, C:N:P), based on the nominal oil dispersion concentration and

concentrations of nutrients measured in the seawater supplied from the Trondheimsfjord at the same time as the present experiment was performed (Brakstad et al., 2015). The C:N:P ratios in the oil dispersion control were thus different from the optimal ratio of 100:10:1 for bacterial activity, indicating that the oil-degrading bacteria could have been limited by access to inorganic nutrients (Atlas and Bartha, 1972; Bouchez et al., 1995; Obbard et al., 2004; Röling et al., 2002). Copepod feces have a higher content of nitrogen relative to carbon compared to other organic particles, and the activity of bacteria have previously been reported to be increased in the presence of copepod feces (Bathmann et al., 1987; Paffenhöfer and Köster, 2005; Thor et al., 2003). The clean copepod feces were thus suggested to supply inorganic nutrients to the bacteria degrading oil compounds in the water phase.

3.3. Total and viable microorganisms in oil dispersions

Total cell concentrations and concentrations of viable HM and ODM were determined in all replicate samples from the three scenarios. Microbial communities were determined by 16S rRNA gene amplicon library analysis in two replicate samples from each scenario.

3.3.1. Cell concentration, heterotrophic and oil-degrading microorganisms

The microbial communities were characterized by DAPI staining using fluorescence microscopy and by the MPN method. The total numbers of cells enumerated using DAPI staining were highest in the oil dispersion control and lowest in the oil dispersion incubated with oil-containing feces (Table 1). The differences observed between the three scenarios were not significant (One-way ANOVA) and the number of cells were within the same range as previous total cell numbers reported in seawater from the Trondheimsfjord using DAPI, $7.2 \times 10^5 \pm 5.4 \times 10^4$ cells mL^{-1} (Brakstad et al., 2004). The results show that the total numbers of cells in seawater were not influenced by the presence of the oil dispersions or copepod feces.

Compared to the oil dispersion control, the average value for viable concentrations of HM (Table 1) were higher both in seawater with clean and oil-containing copepod feces. However, due to large variation in the results, the higher concentration was not significantly different (One-way ANOVA) from the concentrations in the oil dispersion control.

The concentrations of viable ODM (Table 1) were higher in seawater when clean and oil-containing feces were present, compared to the oil dispersion control. The differences were not significant (One-way ANOVA). However, we suggest that the increase in concentration of viable ODM between oil dispersion control and oil dispersion with clean copepod feces show that the clean feces increased the number of active oil-degrading bacteria. The highest concentration of viable ODM was observed in oil dispersions with oil-containing feces, confirming that ingestion and excretion of oil in copepod feces cause higher oil-degrading activity. This activity has previously been observed also in oil-containing copepod feces and was suggested to be mediated by indigenous feces bacteria (Størdal et al., 2015). The higher

Table 1
Total number of cells (counted using DAPI stain and epifluorescence microscopy), and concentrations of viable heterotrophic microorganisms (HM) and viable oil degrading microorganisms (ODM) analyzed by MPN (marine Broth medium or marine Bushnell-Haas Broth with Troll 200+ fraction as carbon source, respectively) in oil dispersions incubated in seawater with clean copepod feces (CF, N = 3) or with oil-containing feces (OF, N = 3) for 14 days. Oil dispersions incubated alone (N = 6) in seawater served as control. Feces were obtained from *Calanus finmarchicus* feeding on *Rhodomonas baltica* in clean seawater or in seawater with oil dispersions.

	Total counts (mL^{-1})		HM (mL^{-1})		ODM (mL^{-1})	
	AVG	SD	AVG	SD	AVG	SD
Dispersed oil	1,084,008	±226,159	19,517	±23,734	274	±220
Dispersed oil & CF	902,248	±291,572	64,000	±35,791	375	±628
Dispersed oil & OF	727,115	±475,294	211,333	±224,378	1,767	±2,456
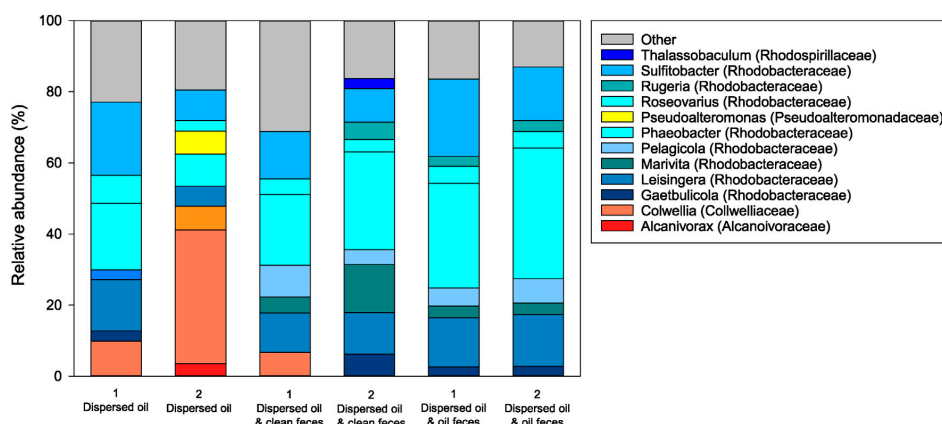
Fig. 4. Relative abundances of bacterial genera determined by 16S rRNA gene amplicon library analysis in oil dispersions incubated (14 days, 10 °C, dark) in seawater with clean copepod feces, or with oil-containing copepod feces. Oil dispersions incubated in clean seawater served as control. Feces were obtained from *Calanus finmarchicus* feeding on *Rhodomonas baltica* in clean seawater or in seawater with oil dispersions. Genera with < 2.5% abundance were grouped as other.

concentration of ODM in oil dispersions incubated with oil-containing feces was suggested to be the cause for the higher biotransformation of n-alkanes observed in these samples (Fig. 3A).

3.3.2. Total bacterial communities

For analysis of the bacterial communities using 16S rRNA gene amplicon library analyses, abundance of bacterial species was summed at genus level (Fig. 4). All samples except one of the oil dispersion controls (termed 2 Dispersed oil in Fig. 4) were dominated by bacteria in the class Alphaproteobacteria, ranging from 86 to 62% relative abundance. The 2 Dispersed oil was dominated by the class Gammaproteobacteria (54%), while the dominant genera were *Colwellia* (45%), *Cycloclasticus* (8%), *Alcanivorax* (4%) and *Pseudoalteromonas* (4%). These bacterial genera have all been shown to be involved in biodegradation of oil (Dubinsky et al., 2013; Kostka et al., 2011). The presence of these genera was thus attributed to the presence of the oil. The Alphaproteobacteria genera *Sulfitobacter, Roseovarius, Phaeobacter* were present in all samples. The relative abundances of these genera ranged from 9 to 22%, from 4 to 8%, and from 9 to 37%, respectively (Fig. 4). These genera were present at higher relative abundance in samples with feces. Bacteria of the genera *Pelagicola* were only present in oil dispersions incubated with clean and oil containing copepod feces (Fig. 4). From the results, we suggest that the presence of copepod feces resulted in a higher abundance of bacteria of the family *Rhodobacteraceae* of the class Alphaproteobacteria. The results show that the copepod feces exerted a high influence on the bacteria species present in the seawater compared to the oil dispersions.

The previously presented results show high concentration of viable ODM in the presence of copepod feces (Table 1), high biotransformation of n-alkanes in the oil dispersion incubated with oil-containing feces (Fig. 3A), together with the high biotransformation of aromatic HC compounds in oil dispersions incubated with clean feces (Fig. 3B). The bacteria present in oil dispersions incubated with copepod feces thus had a larger capacity for degradation of oil compounds compared to the bacterial communities in the oil dispersion control (Fig. 4). In line with this, the genera *Phaeobacter, Roseovarius, Rugeria* and *Sulfitobacter* have previously been associated with biodegradation of HC compounds, or with degradation of HC metabolites (Gertler et al., 2009; Harwati et al., 2007; Kostka et al., 2011). Kostka et al. (2011) identified the *Rhodobacteraceae* family as an important contributor to the active bacteria population during oil contamination of beach sand. The present results indicate that the indigenous copepod feces bacteria have the capacity to degrade oil compounds. Also, in another study, a similar

rate and extent of biodegradation of oil for very different microbial communities have previously been found in beach microcosms contaminated with oil and amended with inorganic nutrients (Röling et al., 2002). From the results, we suggest that different bacterial genera were mediating the oil-degrading activity in oil dispersion in clean seawater compared to oil dispersion incubated with copepod feces.

4. Conclusion

The presence of clean feces from control copepods resulted in higher biotransformation of aromatic HC compounds compared to the oil dispersion control (no feces). This was attributed to increased bacterial activity due to high concentrations of inorganic nutrients in the presence of copepod feces. The effect of the presence of feces on biotransformation of n-alkanes was ambiguous. Biotransformation of n-alkanes was lower in the presence of clean feces and higher in the presence of oil-containing feces. Lower biotransformation is suggested to be due to a preference of bacteria for feces rather than n-alkanes, while higher biotransformation was attributed to an adaptation of the same bacteria to degradation of oil. The data indicate that the presence of a large population of actively feeding copepods concomitantly with an oil spill may enhance biotransformation of the dissolved fraction of an oil spill. The presence of large quantities of copepod feces may also increase agglomeration and thus potentially sedimentation of the lipophilic compounds in a relatively un-weathered state towards the seafloor. The total bacterial community in oil dispersions incubated with feces is of particular interest, as the results indicate that the indigenous feces bacteria were capable of oil degradation. Further studies of these bacterial communities is warranted, as the mineralization rate of oil over an extended period of time may be different in the presence of the diverse bacterial communities present with copepod feces.

for valuable input on the chemical analyses, and Kari Attramadal for fruitful discussions on bacterial community dynamics.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2015.10.029.

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BIOTRANSFORMATION OF PETROLEUM HYDROCARBONS AND MICROBIAL
COMMUNITIES IN SEAWATER WITH OIL DISPERSIONS AND COPEPOD FECES

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Fig. S1. Total feces produced over 48 hour by 400 *Calanus finmarchicus* copepodite stage 5 feeding in clean seawater (N = 3 to the left) or in oil dispersion (N = 3 to the right) at a nominal 2 µL L^{-1} concentration and oil droplet size < 40 µm (Troll crude oil, +200 °C fraction). Algae (*Rhodomonas baltica,* 400 µg C L^{-1}) were supplied to all exposure tanks continuously during the 48 hour exposure. The copepods were exposed in glass exposure tanks with a exposure volume of 18 L. The exposure was performed in a climate room at 10 °C under dim light conditions. Natural seawater was utilized for the exposure, collected through pipelines from 80 m depth in Trondheimsfjorden.

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1995	Svein Håkon Lorentsen	Dr. scient Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition
1995	Chris Jørgen Jensen	Dr. scient Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995	Vidar Moen	Dr. scient Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations
1995	Hans Haavardsholm Blom	Dr. philos Botany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae
1996	Ola Ugedal	Dr. scient Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines
1996	Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996	Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
1996	Gunnar Henriksen	Dr. scient Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region
1997	Gunvor Øie	Dr. scient Botany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters

1997	Ole Reitan	Dr. scient Zoology	Responses of birds to habitat disturbance due to damming
1997	Jon Arne Grøttum	Dr. scient Zoology	Physiological effects of reduced water quality on fish in aquaculture
1997	Per Gustav Thingstad	Dr. scient Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997	Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as
1997	Signe Nybø	Dr. scient Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997	Atle Wibe	Dr. scient Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry
1997	Rolv Lundheim	Dr. scient Zoology	Adaptive and incidental biological ice nucleators
1997	Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997	Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998	Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach
1998	Bente Gunnveig Berg	Dr. scient Zoology	Encoding of pheromone information in two related moth species

1999	Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999	Hans Kristen Stenøien	Dr. scient Botany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway
1999	Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis
1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families:
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Zoology	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)

2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient Zoology	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr. philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana

2003	Marit Stranden	Dr. scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr. scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr. scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr. scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr. scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr. scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr. scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr. scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr. scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr. scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr. scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr. scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr. scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr. scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr. scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr. scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr. scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr. scient Biology	Studies on antifreeze proteins

2005	Eugen G. Sørmo	Dr. scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr. scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr. scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds, Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr. scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr. philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr. scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	ph.d Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine

2007	Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007	Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008	Katarina Mariann Jørgensen	Dr. scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Biology	*Arabidopsis thaliana* Responses to Aphid Infestation

2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease
2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments - Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters

2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	ph.d Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in Arabidopsis thaliana
2011	Grethe Robertsen	ph.d Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011	Line-Kristin Larsen	ph.d Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011	Maxim A. K. Teichert	ph.d Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011	Torunn Beate Hancke	ph.d Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology
2011	Sajeda Begum	ph.d Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh
2011	Kari J. K. Attramadal	ph.d Biology	Water treatment as an approach to increase microbial control in the culture of cold water marine larvae
2011	Camilla Kalvatn Egset	ph.d Biology	The Evolvability of Static Allometry: A Case Study
2011	AHM Raihan Sarker	ph.d Biology	Conflict over the conservation of the Asian elephant (*Elephas maximus*) in Bangladesh
2011	Gro Dehli Villanger	ph.d Biology	Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals
2011	Kari Bjørneraas	ph.d Biology	Spatiotemporal variation in resource utilisation by a large herbivore, the moose
2011	John Odden	ph.d Biology	The ecology of a conflict: Eurasian lynx depredation on domestic sheep
2011	Simen Pedersen	ph.d Biology	Effects of native and introduced cervids on small mammals and birds
2011	Mohsen Falahati-Anbaran	ph.d Biology	Evolutionary consequences of seed banks and seed dispersal in *Arabidopsis*
2012	Jakob Hønborg Hansen	ph.d Biology	Shift work in the offshore vessel fleet: circadian rhythms and cognitive performance
2012	Elin Noreen	ph.d Biology	Consequences of diet quality and age on life-history traits in a small passerine bird
2012	Irja Ida Ratikainen	ph.d Biology	Theoretical and empirical approaches to studying foraging decisions: the past and future of behavioural ecology

2012	Aleksander Handå	ph.d Biology	Cultivation of mussels (*Mytilus edulis*): Feed requirements, storage and integration with salmon (*Salmo salar*) farming
2012	Morten Kraabøl	ph.d Biology	Reproductive and migratory challenges inflicted on migrant brown trour (*Salmo trutta* L) in a heavily modified river
2012	Jisca Huisman	ph.d Biology	Gene flow and natural selection in Atlantic salmon
	Maria Bergvik	ph.d Biology	Lipid and astaxanthin contents and biochemical post-harvest stability in *Calanus finmarchicus*
2012	Bjarte Bye Løfaldli	ph.d Biology	Functional and morphological characterization of central olfactory neurons in the model insect *Heliothis virescens*.
2012	Karen Marie Hammer	ph.d Biology	Acid-base regulation and metabolite responses in shallow- and deep-living marine invertebrates during environmental hypercapnia
2012	Øystein Nordrum Wiggen	ph.d Biology	Optimal performance in the cold
2012	Robert Dominikus Fyumagwa	Dr. Philos Biology	Anthropogenic and natural influence on disease prevalence at the human –livestock-wildlife interface in the Serengeti ecosystem, Tanzania
2012	Jenny Bytingsvik	ph.d Biology	Organohalogenated contaminants (OHCs) in polar bear mother-cub pairs from Svalbard, Norway. Maternal transfer, exposure assessment and thyroid hormone disruptive effects in polar bear cubs
2012	Christer Moe Rolandsen	ph.d Biology	The ecological significance of space use and movement patterns of moose in a variable environment
2012	Erlend Kjeldsberg Hovland	ph.d Biology	Bio-optics and Ecology in *Emiliania huxleyi* Blooms: Field and Remote Sensing Studies in Norwegian Waters
2012	Lise Cats Myhre	ph.d Biology	Effects of the social and physical environment on mating behaviour in a marine fish
2012	Tonje Aronsen	ph.d Biology	Demographic, environmental and evolutionary aspects of sexual selection
	Bin Liu	ph.d Biology	Molecular genetic investigation of cell separation and cell death regulation in *Arabidopsis thaliana*
2013	Jørgen Rosvold	ph.d Biology	Ungulates in a dynamic and increasingly human dominated landscape – A millennia-scale perspective
2013	Pankaj Barah	ph.d Biology	Integrated Systems Approaches to Study Plant Stress Responses
2013	Marit Linnerud	ph.d Biology	Patterns in spatial and temporal variation in population abundances of vertebrates
2013	Xinxin Wang	ph.d Biology	Integrated multi-trophic aquaculture driven by nutrient wastes released from Atlantic salmon (*Salmo salar*) farming
2013	Ingrid Ertshus Mathisen	ph.d Biology	Structure, dynamics, and regeneration capacity at the sub-arctic forest-tundra ecotone of northern Norway and Kola Peninsula, NW Russia
2013	Anders Foldvik	ph.d Biology	Spatial distributions and productivity in salmonid populations
2013	Anna Marie Holand	ph.d Biology	Statistical methods for estimating intra- and inter-population variation in genetic diversity
2013	Anna Solvang Båtnes	ph.d Biology	Light in the dark – the role of irradiance in the high Arctic marine ecosystem during polar night
2013	Sebastian Wacker	ph.d Biology	The dynamics of sexual selection: effects of OSR, density and resource competition in a fish

2013	Cecilie Miljeteig	ph.d Biology	Phototaxis in *Calanus finmarchicus* – light sensitivity and the influence of energy reserves and oil exposure
2013	Ane Kjersti Vie	ph.d Biology	Molecular and functional characterisation of the IDA family of signalling peptides in *Arabidopsis thaliana*
2013	Marianne Nymark	ph.d Biology	Light responses in the marine diatom *Phaeodactylum tricornutum*
2014	Jannik Schultner	ph.d Biology	Resource Allocation under Stress - Mechanisms and Strategies in a Long-Lived Bird
2014	Craig Ryan Jackson	ph.d Biology	Factors influencing African wild dog *(Lycaon pictus)* habitat selection and ranging behaviour: conservation and management implications
2014	Aravind Venkatesan	ph.d Biology	Application of Semantic Web Technology to establish knowledge management and discovery in the Life Sciences
2014	Kristin Collier Valle	ph.d Biology	Photoacclimation mechanisms and light responses in marine micro- and macroalgae
2014	Michael Puffer	ph.d Biology	Factors influencing African wild dog *(Lycaon pictus)* habitat selection and ranging behaviour: conservation and management implications
2014	Gundula S. Bartzke	ph.d Biology	Effects of power lines on moose *(Alces alces)* habitat selection, movements and feeding activity
2014	Eirin Marie Bjørkvoll	ph.d Biology	Life-history variation and stochastic population dynamics in vertebrates
2014	Håkon Holand	ph.d Biology	The parasite *Syngamus trachea* in a metapopulation of house sparrows
2014	Randi Magnus Sommerfelt	ph.d Biology	Molecular mechanisms of inflammation – a central role for cytosolic phospholiphase A2
2014	Espen Lie Dahl	ph.d Biology	Population demographics in white-tailed eagle at an on-shore wind farm area in coastal Norway
2014	Anders Øverby	ph.d Biology	Functional analysis of the action of plant isothiocyanates: cellular mechanisms and in vivo role in plants, and anticancer activity
2014	Kamal Prasad Acharya	ph.d Biology	Invasive species: Genetics, characteristics and trait variation along a latitudinal gradient.
2014	Ida Beathe Øverjordet	ph.d Biology	Element accumulation and oxidative stress variables in Arctic pelagic food chains: Calanus, little auks (alle alle) and black-legged kittiwakes (Rissa tridactyla)
2014	Kristin Møller Gabrielsen	ph.d Biology	Target tissue toxicity of the thyroid hormone system in two species of arctic mammals carrying high loads of organohalogen contaminants
2015	Gine Roll Skjervø	dr. philos Biology	Testing behavioral ecology models with historical individual-based human demographic data from Norway
2015	Nils Erik Gustaf Forsberg	ph.d Biology	Spatial and Temporal Genetic Structure in Landrace Cereals
2015	Leila Alipanah	ph.d Biology	Integrated analyses of nitrogen and phosphorus deprivation in the diatoms *Phaeodactylum tricornutum* and *Seminavis robusta*
2015	Javad Najafi	ph.d Biology	Molecular investigation of signaling components in sugar sensing and defense in *Arabidopsis thaliana*
2015	Bjørnar Sporsheim	ph.d Biology	Quantitative confocal laser scanning microscopy: optimization of in vivo and in vitro analysis of intracellular transport

| 2015 | Magni Olsen Kyrkjeeide | ph.d Biology | Genetic variation and structure in peatmosses (*Sphagnum*) |
| 2015 | Keshuai Li | ph.d Biology | Phospholipids in Atlantic cod (*Gadus morhua* L.) larvae rearing: Incorporation of DHA in live feed and larval phospholipids and the metabolic capabilities of larvae for the de novo synthesis |