TITLE: Maternal PAH transfer and effects on offspring of copepods exposed to dispersed oil with and without oil droplets

RUNNING HEAD: Maternal PAH transfer and effect in copepods

AUTHORS AND AFFILIATIONS:

Bjørn Henrik Hansen
SINTEF Ocean AS, 7465 Trondheim
E-mail: bjorn.h.hansen@sintef.no, Phone: +47 98283892.

Ann M. Tarrant
Woods Hole Oceanographic Institution, Biology Department, Woods Hole, USA
E-mail: atarrant@whoi.edu

Iurgi Salaberria
Norwegian University of Science and Technology, Department of Biology, 7469 Trondheim, Norway
E-mail: iurgi.salaverria@ntnu.no

Dag Altin
BioTrix, 7022 Trondheim, Norway
E-mail: biotrix@frisurf.no, Phone: +47 92643500.

Trond Nordtug
SINTEF Ocean AS, 7465 Trondheim
E-mail: trond.nordtug@sintef.no

Ida Beathe Øverjordet
SINTEF Ocean AS, 7465 Trondheim
E-mail: Ida.Beathe.Overjordet@sintef.no
Maternal PAH transfer and effects on offspring of copepods exposed to dispersed oil with and without oil droplets

Bjørn Henrik Hansen¹, *, Ann M. Tarrant², Iurgi Salaberria³, Dag Altin⁴, Trond Nordtug¹, Ida Beathe Øverjordet¹

¹ SINTEF Ocean AS, Environmental Technology, 7465 Trondheim, Norway
² Woods Hole Oceanographic Institution, Biology Department, Woods Hole, USA
³ Norwegian University of Science and Technology, Department of Biology, 7469 Trondheim, Norway
⁴ BioTrix, 7022 Trondheim, Norway

Abstract

Copepods of the genus *Calanus* have the potential for accumulating lipophilic oil components due to their high lipid content, and they have been shown to filter and ingest oil droplets during exposure. As female copepods produce eggs at the expense of their lipid storage, there is a concern for transfer of lipophilic contaminants to offspring. To assess the potential for maternal transfer of oil components, we exposed ovigerous female copepods (*Calanus finmarchicus*) to filtered and unfiltered oil dispersions for 4 days, collected and kept their eggs in clean seawater and studied hatching and gene expression in hatched nauplii. Oil droplet exposure contributed to PAH uptake in dispersion-treated adult copepods, as displayed through PAH body residue analyses and fluorescence microscopy. Applying the latter methodology, transfer of heavy PAH from copepod mothers to offspring was
strongly supported. Subtle effects were observed in offspring with a temporal reduction in hatching success, which appears to be occurring only when mothers were exposed to the unfiltered oil dispersions. Offspring were reared in clean water through to late naupliar stages, collected for RNA extraction and preparation of libraries for high-throughput transcriptome sequencing. Differentially expressed genes were identified through pairwise comparisons between treatments. Among these, several have known roles in responses to chemical stress including xenobiotic metabolism enzymes, antioxidants, chaperones and components of the inflammatory response. While the gene expression results suggest a transgenerational activation of stress responses, the relatively small number of differentially expressed genes suggest a mild long-term effect on offspring following maternal exposure.

**Key words:** Copepod; dispersion; micro-droplets; maternal transfer; ecotoxicity; oil; petroleum; Arctic

1. Introduction

When oil is released into the marine environment it may disperse into the water, i.e. break up into small oil droplets. This process occurs when surface slicks experience turbulence, such as through breaking waves and/or the use of chemical dispersants, which facilitates the oil dispersion process by reducing the interfacial tension between oil and water. During the Deepwater Horizon incident in 2010 in the Gulf of Mexico, oil was released at the sea floor and dispersed naturally due to the turbulence caused by high pressure and concomitant release of gas. Chemical dispersants were also added during the release to facilitate the process and cause even smaller droplets to form and avoid oil surfacing and impacting shorelines. Also regular discharges of produced water from offshore oil production platforms release dispersed oil droplets, and these are usually regulated and monitored. On the
Norwegian continental shelf, the regulations allow dispersed oil concentrations in raw effluents of produced water of up to 30 mg/L.

Hence, pelagic organisms may be exposed to dispersed oil during both regular as well as accidental discharges of oil. Due to their higher bioavailability, the dissolved components are expected to be the main driver for toxicity. However, the majority of the mass of the oil is contained in the particulate phase of the dispersions, i.e. the oil droplets. Several studies, focusing mainly on fish eggs (Carls et al., 2008) and fish larvae (Nordtug et al., 2011b; Olsvik et al., 2011; Olsvik et al., 2010), have concluded that oil droplets are not the main driver for toxicity. These assumptions have been challenged recently as fish eggs from some species like Atlantic haddock have displayed a high susceptibility to oil droplet adhesion (Sørhus et al., 2015). In addition, filter-feeding organisms, which filter large volumes of water to acquire food, may be exposed to oil droplets through direct filtration and ingestion (Hansen et al., 2012; Nordtug et al., 2015; Almeda et al., 2014; Almeda et al., 2015; Conover, 1971). Recent studies have shown that exposure to oil dispersions cause up-regulation of stress-associated genes (Hansen et al., 2009), decreased survival (Hansen et al., 2012), reduced food uptake (Hansen et al., 2012) and altered reproduction (Hansen et al., 2015; Olsen et al., 2013) in copepods, however, these publications provide limited insight into the potential contribution of oil droplets to these effects.

Sub-Arctic and Arctic copepods from the Calanus genus produce and store a large reservoir of lipids, mainly as wax esters, during their copepodite stages serving as an energy reserve for use during overwintering (diapause) and reproduction as adults (Miller et al., 1998; Madsen et al., 2008). Owing to their high lipid content, these copepods have the potential to accumulate large amounts of lipophilic organic toxicants like polycyclic aromatic hydrocarbons (PAHs), as low elimination rates are expected (Hansen et al., 2016). In addition to the passive uptake of dissolved oil components, ingestion of oil droplets may potentially increase the uptake of PAHs further increasing the accumulation in the lipid reservoir. At the expense of the lipid reservoir, eggs and spermatophores are produced in female and male copepods, respectively, during the reproduction period. Hence, theoretically any PAHs
accumulated in the lipid reservoir may be transferred into offspring. To the authors' knowledge, transfer of PAHs from parent to offspring has never been studied in copepods before.

In the present work, we wanted to investigate the potential for PAH transfer from ovigerous female copepods to their offspring and the potential delayed effects this may have on the offspring during development. We also wanted to isolate and assess the potential contribution of oil droplets to uptake and effects. In order to do this, we exposed ovigerous female copepods to an oil dispersion concentration (2 mg oil/L nominally) that previously has been shown to affect reproductive success, and a filtered dispersion, containing only the dissolved oil components, i.e. water soluble fraction (WSF), of the dispersion. After a 4-day exposure to these treatments, the adult copepods were transferred to clean seawater for egg production. The offspring were monitored for short-term hatching success as well as potential delayed effects by monitoring the differential gene transcription between negative controls (treated with seawater), oil dispersion and WSF. The main aims of the current work were to assess if i) uptake of PAHs was higher in adult copepods when treated with oil dispersions than the corresponding WSF, ii) oil components were transferred between adults and offspring, and finally iii) if potential impacts (based on gene expression) were more severe on offspring from dispersion-treated copepods than WSF-treated copepods.

2. Materials and Methods

2.1. Experimental organisms

Copepods (Calanus finmarchicus) from the continuous lab culture at SINTEF/NTNU Sealab were used for the experiments described here. The culture is routinely maintained at approximately 8-10°C, and details regarding culturing were described previously (Hansen et al., 2007).
2.2. Choice of oil

A naphthenic crude oil from the Troll reservoir in the northern part of the North Sea was selected as the representative oil for the studies. The oil was artificially weathered by heating to 200°C (Stiver & Mackay, 1984), and the resulting oil residue, which corresponds to approximately 0.5–1 days of weathering on the sea surface, was collected and used to generate the oil dispersions and filtered oil dispersion (water-soluble fractions (WSF)) (Nordtug et al., 2011a).

2.3. Exposure and recovery experiment

An overview of the whole experiment is given in Error! Reference source not found.. Ovigerous female copepods (250 individuals in each replicate) were exposed in 5L bottles continuously for 96 h to dispersion or filtered dispersions (WSFs) at 9 ± 1°C. The dispersion had a nominal concentration of 2 mg oil/L, and the WSF was a filtered dispersion removing the oil droplets but keeping the water-soluble components (Nordtug et al., 2011a). Females kept in clean seawater were used as negative controls. All three treatments were done in triplicates and had a flow of approximately 15 ml/min. The copepods were continuously fed the unicellular algae *Rhodomonas baltica* at a level of 400 µg C L\(^{-1}\) by tubing that pumped the algal culture from a common reservoir. The algae in the reservoir was renewed on a daily basis supplying a fresh stock. After exposure, 200 copepods were transferred to clean seawater in 50L tanks with flow-through seawater (temperature 10 ± 0.5°C) for recovery. During recovery the feeding was maintained as for the exposure period, but due to lower copepod density, food concentration was reduced to 150 µg C L\(^{-1}\). The remaining copepods were either sampled for body burden analyses (25 individuals) or photographed for biometry (23-25 specimens per replicate). 7 days after exposure, eggs were harvested from recovery tanks from all treatments/replicates and transferred to PET bottles (1.5 L) for hatching and maintenance with food (150 µg C L\(^{-1}\)). Nauplii (N5-N6) were sampled 13 days later using a filter (mesh 63 µm) and transferred immediately into RNAlater (Ambion). The samples were
flash-frozen in liquid nitrogen and kept at -20°C or colder until they were thawed, extracted and analyzed for gene expression.

2.4. Hatching success

Two days after transfer of females to egg laying chambers, a subset of eggs harvested from each tank was transferred to glass vials (40 ml) and kept in darkness at 8°C for hatching. They were terminated after 48, 72 and 96 hours by adding 6 droplets of Lugol's solution to the vial. A Leica MZ125 dissecting microscope (Leica Microsystems, Wetzlar, Germany) was used to image eggs and nauplii, and these images were analyzed using automated classifications using ImageJ (National Institute of Health, Bethesda MD) to quantify the number of eggs and nauplii in each sample. These data formed the basis for determination of time- and exposure-dependent hatching success.

2.5. Chemical analyses of water samples

After 24 and 72 h of exposure water samples were taken in 1L glass bottles for analysis of semi-volatile organic components (SVOC; 800 mL) and in sealed glass vials without headspace for analysis of volatile organic components (VOC; 40 mL). All water samples were acidified at sampling. Semi-volatile organic components (SVOC) and volatile organic components (VOC) samples were preserved by acidification with 15% hydrochloric acid. Prior to processing, surrogate internal standards (SIS, o-terphenyl, naphthalene-$d_9$, phenanthrene-$d_{10}$, chrysene-$d_{12}$, phenol-$d_6$, 4-methylphenol-$d_8$) were added to the water samples. Prior to gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS), analysis recovery internal standards (RIS, 5α-androstane, fluorene-$d_{10}$ and acenaphthene-$d_{10}$) were added. Prior to SVOC and total petroleum hydrocarbon (TPH) measurements, water samples were spiked with the appropriate surrogate internal standards and serially extracted with dichloromethane (DCM) using a modification of US EPA method
The combined extracts were dried over sodium sulphate and concentrated to approximately 1 mL using a Zymark Turboprep® 500 Concentrator. The final extract was spiked with the appropriate recovery internal standards and analyzed.

Water samples were analyzed for SVOC content (decalins, polycyclic aromatic hydrocarbons (PAHs) and phenols) using GC/MS with a modification of US EPA Method 8270D (USEPA, 2007). The mass spectrometer was operated in the selective ion monitoring (SIM) mode to achieve optimum sensitivity and specificity. The quantification of target compounds was performed by the method of internal standards, using average response factors (RF) for the parent compounds. The PAH and phenol alkyl homologues were quantified using the straight baseline integration of each level of alkylation and the RF for the respective parent PAH compound. The response factors were generated for all targets and surrogates versus fluorene-\textsubscript{d\textsubscript{10}}. As an objective way of excluding samples corrupted by the extraction process, all values deviating more than 1.3 \times \text{STDEV} from the average value were excluded as outliers (corresponding to 80 % or more chance of the values being outliers). The majority of excluded values were from defined samples where irregularities such as excessive evaporation of solvent or low recovery of standards could be identified.

Concentrations of TPH in water samples were measured using GC/FID according to a modification of US EPA Method 8100 (USEPA, 1986). Resolved and unresolved TPH (C\textsubscript{10}-C\textsubscript{36}) were quantified by the method of internal standards using the baseline corrected total area of the chromatogram and the average response factor for the individual C\textsubscript{10} to C\textsubscript{36} n-alkanes. Water levels of 35 target VOC (C\textsubscript{5}-C\textsubscript{10}) including BTEX (benzene, toluene, ethylbenzene, and xylenes) were determined by Purge and Trap GC/MS (P&T GC/MS) using a modification of US EPA method 8260C (USEPA, 2006) after spiking the samples with SIS (toluene-\textsubscript{d\textsubscript{6}} and ethylbenzene-\textsubscript{d\textsubscript{8}}) and RIS (chlorobenzene-\textsubscript{d\textsubscript{5}}). Quantification of individual compounds was accomplished using the RFs of the individual compounds relative to the internal standards. All standards and samples were analyzed in a full scan mode. Total hydrocarbon
content (THC, C₅-C₃₆) of water samples was calculated by adding VOC (C₅-C₉) and TPH (C₁₀-C₃₆) concentrations.

2.6. PAH body burden analyses

After exposure, all copepods were counted, assessed for viability and dead individuals removed. Live copepods (25 individuals) were pooled and sampled from each exposure group and frozen in liquid nitrogen for body burden analysis. Surrogate internal standards (SIS; naphthalene-d₈, phenanthrene-d₁₀, chrysene-d₁₂, phenol-d₆, and 4-methylphenol-d₈) were added to the samples, and the samples were then processed using a microextraction procedure. Recovery internal standards (RIS; fluorene-d₁₀ and acenaphthene-d₁₀) were added prior to analysis. The copepods were weighed into conical, screw-capped sample vials (10 mL) with replaceable Teflon septa, and 3 mL of potassium hydroxide (6.5%) in methanol (80%) and SIS were added to each vial. The mixture was treated for 2 hours in an ultrasonic bath at 80 °C to achieve saponification, followed by filtration and serial extraction with 4 mL of Milli-Q water/2 x 3 mL of hexane/4 mL of Milli-Q water and 0.5 mL of saturated NaCl. The combined organic extracts were dried with sodium sulfate 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 of normal-phase silica packing (Superclean LCSi, Supelco Bond Elut, SI, Agilent). 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, spiked with RIS components (to a total volume of 100 μL) and analyzed on GC-MS as described above for the water samples.

2.7. Sampling and biometry of females

Copepod survival was determined at the end of the 96 hour exposure period, and a sample of surviving copepods (23-25 individuals from each chamber) was photographed during observation in a Leica
MZ125 dissecting microscope (Leica Microsystems, Wetzlar, Germany). Pictures were captured with a
digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo, Japan) operated by Fire-i
software (Unibrain, Inc., San Ramon CA, US). Measurements of biometry were performed manually by
the use of the software ImageJ (National Institute of Health, Bethesda MD, US) on scaled captured
images. 2D projected areas of the lipid storage and the prosome were measured using a graphical
tablet (Wacom Cintiq 12wx, Wacom Co., Ltd., Saitama, Japan).

2.8. Fluorescence microscopy

Copepods (N=6) from each treatment as well as eggs from females from each treatment group were
subjected to fluorescence microscope imaging as described in Hansen et al. (Hansen et al., 2012)
(2012). To achieve simultaneous identification of oil droplets either ingested or adhered to the
copepod surface, ingested algae as well as accumulated fluoranthene, the reported B-2A filter cube
(Nikon Corp., Tokyo, Japan) was replaced with a triband filter cube (D/F/Tr, Nikon Corp., Tokyo, Japan)
producing a better resolution of the fluorescence-signatures of interest.

2.9. Transcriptional profiling

Eggs from exposed females were collected over the following 3-6 days in recovery after the exposure
period, hatched and reared in clean seawater through to the late naupliar stages (N5-6) before they
were harvested and stored in RNAlater (Invitrogen) at -20°C. For RNA extraction, nauplii were
recovered onto filter paper (Whatman Qualitative Grade 2) using a vacuum pump. The collected nauplii
were then rinsed into a glass Potter Elvehjem tissue grinder with 1 ml of Purezol Reagent (Bio-Rad) and
homogenized using a PFTE pestle. Total RNA was extracted from the homogenate using the Aurum
Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). RNA samples were sent to the Genomic Services
Laboratory at HudsonAlpha (Huntsville AL, US), where RNA quality was evaluated using a Bioanalyzer
and libraries were synthesized for high-throughput sequencing using Illumina TruSeq Reagents. Three
replicate libraries were prepared from each treatment. For the control and dispersion treatments these were derived from independently-reared batches of nauplii. For the WSF treatment, one group of nauplii was lost due to a technical error during the grow-out phase; therefore, the two of the libraries were synthesized from subsamples of the same RNA sample and serve as technical replicates. The libraries were sequenced as 100 basepair paired-end reads across two lanes of an Illumina HiSeq2000. All raw sequences are archived within the NCBI database as BioProject PRJNA358233.

Adapter sequences and low-quality portions of reads (< phred 20) were trimmed using Trimmomatic (Lohse et al., 2012). In exploratory data analysis, reads were assembled into a transcriptome de novo using Trinity 2.0.3 with default parameters (Grabherr et al., 2011). Differential gene expression analysis was conducted using scripts bundled with Trinity. Sequences were mapped to the new transcriptome as well as two previously published transcriptomes (Lenz et al., 2014; Tarrant et al., 2014) using RSEM (Li&Dewey, 2011). The highest mapping success was obtained by using a published transcriptome derived from multiple copepod life stages (Lenz et al., 2014), so these results were used in subsequent analyses, except where noted. Differentially expressed genes were identified using EdgeR software (Robinson et al., 2010) and annotated through Blastx-based queries of the Swiss-Prot database. The distribution of gene ontology (GO) terms in differentially expressed genes was assessed using WEGO (Ye et al., 2006). Within WEGO, Pearson Chi-Square tests were conducted to compare GO terms specifically associated with a particular oil exposure (dispersion vs. WSF) or direction of regulation (up-regulated vs. down-regulated within a treatment).

3. Results and Discussion

3.1. Exposure concentrations – description, partitioning and relevance

Based on the chemical analyses of water samples, the experimental conditions appear to have caused PAH loading as anticipated (
The concentrations of volatile organic components (VOC) were comparable between dispersions and WSFs reflecting their solubility in water, i.e. they were not removed by filtration of the dispersion to generate the WSF. The PAH concentrations were higher in the dispersion compared to the corresponding WSF due to the presence of oil droplets in the dispersions. The filtration of dispersion caused a decrease in PAH concentration in WSFs depending on the water solubility of the PAH, i.e. the fraction of heavier 4-6-ring PAHs associated with droplets were higher than for the lighter naphthalenes. The naphthalenes were found at comparable concentrations in the dispersions and corresponding WSFs displaying their higher water solubility. In terms of environmental relevance, the T-PAH concentrations in the experiment (approximately 20 µg/L) are in the high range of the concentrations observed in the field following oil spills. The same applies for the total oil concentrations (approx. 1 mg oil/L) (Li et al., 2011). After the Deepwater Horizon incident in the Gulf of Mexico in 2010, most water samples taken displayed T-PAH concentrations below 10 µg/L (Sandoval et al., 2017). Keeping a steady exposure concentration over 4-days is also not environmentally relevant, however, our design was chosen primarily to isolate the contribution of oil droplets to chosen effect endpoints by comparing observed effects after treatments with and without oil droplets present (Nordtug et al., 2011a; Nordtug et al., 2011b).

3.2. Uptake of polycyclic aromatic hydrocarbons in adult copepods

The exposure was well below the LC$_{50}$ recorded for *C. finmarchicus* for the current oil and weathering (Hansen et al., 2012), and very low mortality was observed in the experiment with only three dead (of 250) in dispersion, one dead in WSF and none in the negative controls. The concentrations of PAHs associated with the copepods clearly was higher in animals exposed to dispersion than those exposed to the corresponding WSF (Fig. 2). The difference between the two exposures is related to the size of the PAHs with differences increasing from approximately 20% in naphthalenes to 80% in 4-6 ring PAHs. Previous studies have shown that *C. finmarchicus* may accumulate oil micro-droplets through active
filtration of and through adhesion onto carapace surface (Nordtug et al., 2015). Thus, it is expected that PAHs contained in oil associated with the copepods contribute to the measured body residue of copepods exposed to dispersions.

In Figure 3 the fraction between body residue (Cb) and water concentration (Cw) of individual PAHs represented by their octanol water partitioning coefficient (Kow). The Figure show that below approximately Log Kow = 6, the fraction between water and tissue is similar for the two exposures. Based on the assumption that octanol is a suitable proxy for lipids in organisms the bioconcentration factor (BCF) for exposure to dissolved lipophilic chemicals is generally assumed to be related to the octanol water partitioning coeffiecient (Kow). Thus, the linear relationship observed between the bioconcentration and Kow of the dissolved components in the WSF is expected (Figure 3). Components with Log Kow > 6 were below the analytical detection limit for the animals exposed to WSF. In the individuals exposed to oil dispersions, components with Log Kow up to above 7 could be detected, and the relation with Kow was best fitted by a bilinear model (Kubinyi, 1977) where the initial part up to curve is similar to the WSF exposure. This indicate that these components are largely dissolved in the water phase of the dispersion. The fall-off from a linear correlation at higher Kow values is consistent with studies on fish and several other aquatic organisms (Chiou, 1985; Banerjee&Baughman, 1991) exposed to dissolved components. However, in the current experiment the components with high Log Kow (> 6) could also be contained in oil droplets in the digestive system and on the surface of the copepods. If droplets were sole source of components above log Kow = 6, the bioaccumulation for these components should be equal and represented by a straight horizontal line (slope = 0) in Figure 3. The fact that the slope is negative above log Kow = 5.5 strongly indicates that a portion of the larger PAHs is also accumulated in the tissue of the animals.

3.3. Effects of exposure on adult female copepods
Biometric analyses of female copepods after exposure revealed that there were no differences in prosome length and projected 2D lipid sac area on the images, however, the area of the lipid sac was significantly lower in copepods treated with oil dispersions compared to control (p<0.0001) and WSF-treated copepods (p<0.001) (Supplemental 1 Figure S1). The underlying reason for this is unknown. Exposure to oil dispersions may be more costly to recover from, and reduced lipid content may be a function of increased demand for energy to recover and uphold maintenance during exposure. It is unknown whether a relatively short (4-day) exposure could cause a measurable decrease in lipid sac size. These copepods are adapted to withstand long periods of starvation during diapause (Hirche, 1996), but diapause is associated with a substantial metabolic depression. While the metabolic rate of copepods within the experiment was not known, exposure to oil dispersions could have resulted in a sustained or elevated metabolic rate, and/or a complete stop in filtration and a subsequent depletion of lipid reserves. Lower mortality of lipid-poor copepods than lipid-rich copepods is also unlikely as previous studies have shown the opposite; that lipid-poor copepods are more susceptible to oil exposure than lipid-rich copepods (Hansen et al., 2011; Hansen et al., 2016; Hansen et al., 2013). Whatever the mechanistic basis for the dispersion-induced low lipid content is, it may be expected that low lipid content in adult females will result in reduced fecundity and/or reduced quality of offspring.

3.4. PAH transfer between adults and offspring

Due to the small biomass of copepod eggs, we did not attempt to measure PAH concentrations in eggs of reproducing females after exposure. Instead, fluorescence microscopy was used to visualize PAHs (tuned to fluoranthene) in both eggs and adult females. In adult females, when comparing a negative control (Error! Reference source not found.A) against a dispersion-treated copepod (Error! Reference source not found.B), a clear blue area is apparent in the exposed specimen. This represents the lipid sac of the copepod, and strongly suggests lipid-accumulation of PAHs. The dispersion-treated copepod
also displays a greenish stain in the filtration apparatus, which is produced by oil droplets stuck there. In addition, a red-orange color is evident for both treatments representing the digestive system with partly degraded algae (Hansen et al., accepted). The eggs from the negative control, WSF- and dispersion-exposed copepods are displayed in Error! Reference source not found.C, D and E, respectively. The eggs from females exposed with oil dispersion are blue in color (E) compared to both the controls (C) and the WSF-treated eggs (D), suggesting accumulation of heavy PAHs. Although this is not a quantitative measure of PAH content in eggs, it strongly indicates that maternal transfer of PAHs to eggs occurs, and that this process is more pronounced in copepods exposed to oil droplets. To the authors' knowledge, PAH transfer has not been reported previously between mother and offspring in copepods. In vertebrates, transfer of PAHs between parent and offspring has been shown. For example, maternal PAH exposure has been shown to affect endocrine function and behavioral traits in both F1 and F2 generations in zebrafish (Danio rerio) (Vignet et al., 2015). Other organic contaminants have been shown to transfer between crustacean parents and offspring. Notably, in the cladoceran Daphnia magna, mercury (ionic as well as methyl-mercury) is transferred between mothers and neonates. In fact, as much as 41% of accumulated methyl-mercury in adult daphnids was transferred to their neonates (Tsui&Wang, 2004).

### 3.5. Implications of PAH-transfer on hatching success and development

After transfer to clean seawater for recovery the female copepods started producing eggs. Hatching success was monitored in eggs collected after 48 hours of recovery, and was monitored at 48, 72 and 96 hours of incubation of the test. (Error! Reference source not found.). Significantly, lower hatching was observed in the eggs after 48 and 72 hours in eggs from the females exposed to dispersion compared to the negative control, whereas no significant differences between treatments were observed after 96 hours of incubation. No significant differences in hatching were observed between the groups exposed for dispersion or WSF, or between the negative control and the WSF-exposure.
Thus, if maternal exposure to oil droplets has an effect on the hatching success of the offspring, the effect is subtle. Several studies have reported reduced hatching rates or hatching success of copepods following oil exposure. Han et al. (2014) reported reduced hatching rate in response to a 10-day WSF exposure in the harpacticoid copepod *Tigriopus japonicus*. Jensen & Carroll (2010) found reduced hatching success in *Calanus glacialis* females treated with WSF (10.4 µg 16 EPA PAH/L), which was argued by the authors to have resulted from passive uptake by the eggs or transfer from the mothers. They did not observe differences in the cumulative egg production. For *C. finmarchicus* lower hatching success has also been shown after female exposure to pyrene, however, interestingly direct exposure of eggs to pyrene did not cause reduced hatching success (Jensen et al., 2008), suggesting the importance of maternal exposure and transfer on this endpoint.

Delay in initiation of reproduction after treatment with dispersed oil (5.5 mg oil/L) was observed in *C. finmarchicus* in a previous study (Hansen et al., 2015), but the copepods were able to fully recover and produce offspring at the rate of (and even above) untreated copepods over a period of 25 days. Similarly, in a study by Olsen et al., (2013), *C. finmarchicus* were exposed for 120 hours to concentrations ranging 0.02 to 16.5 mg oil/L followed by recovery in clean seawater. 13 days post exposure, no significant differences were observed in egg production rates or hatching success, however, a significantly lower portion of the surviving females from the highest exposure participated in the egg production.

### 3.6. Maternal exposure causes altered nauplii gene expression

Differentially expressed genes were identified through pairwise comparisons between treatments (Error! Reference source not found.). Using a significance cut-off of 4-fold change in expression and 1% false discovery rate, expression of 173 genes was altered in response to the dispersion, 184 in response to the WSF, and 35 genes were affected by both treatments. In each group, a larger number of genes was downregulated following exposure compared to the number of genes upregulated.
Approximately 2/3 of the differentially identified genes could be annotated according to their similarity to known genes in the SwissProt database. The complete list of differentially expressed genes is contained in Supplemental File 2.

Overall, the differentially expressed genes were associated with a total of 339 level 2 and level 3 GO terms. Given the relatively small number of differentially expressed genes, many terms were not sufficiently represented to enable statistical comparisons. Of the categories that could be compared, several GO terms were enriched in one of the two exposure treatments or according to the direction of regulation within an exposure (Table 2). For example, following exposure to the dispersion, several GO terms associated with cellular metabolism were more frequently associated with up-regulated genes than with down-regulated genes (e.g., cellular metabolic process, primary metabolic process, cellular process, negative regulation of cellular process). Of particular interest, within the WSF treatment the catalytic activity GO term was more frequently found among the up-regulated genes.

These included several genes with known roles in responses to chemical stressors (Table 3 and Supplemental File 2), such as Phase I oxidizing enzymes (Cyp2J-like), Phase 3 exporting enzymes (multidrug resistance proteins), oxidoreductases (thioredoxin reductase, xanthine dehydrogenase, quinone oxidoreductase, and 2-oxoglutarate dehydrogenase-like), and apoptosis regulators (mitogen-activated protein kinase). While the specific role of Cyp2J-like enzymes in copepods is not known, many enzymes in cytochrome P450 family 2 are involved in oxidative detoxification of contaminants and metabolism of endogenous lipids; upregulation of this gene in nauplii from exposed mothers supports the assumption that PAH transfer to nauplii occurs. Thioredoxin reductase and xanthine dehydrogenase were up-regulated by both the WSF and dispersion treatments suggesting that the nauplii are subjected to oxidative stress as crustacean thioredoxin is regarded a potent antioxidant protein (Aispuro-Hernandez et al., 2008). It may also be suggested that the nauplii are sensitive to pathogen challenge as well, as expression of thioredoxin has been observed following exposure to
pathogens *Vibrio alginolyticus, Micrococcus luteus* and *Pichia pastoris* in swimming crab (*Portunus triberculatus*) (Song et al., 2012).

Based on previous studies demonstrating robust up-regulation of glutathione-S-transferase (GST, a Phase 2 conjugating enzyme) in response to oil exposure in copepodites and adult *C. finmarchicus* and *C. glacialis* (Hansen et al., 2009; Hansen et al., 2011), we specifically investigated expression of this gene within our data set. No GST sequences were included in the set of differentially expressed genes, and we did not detect this GST sequence through blast-based searches of the transcriptome used for these analyses (i.e., published by Lenz et al (2014)). However, this sequence is present in the transcriptomes we assembled within this study (c163217_g1 within Supplemental File 3) and previously ((Tarrant et al., 2014), comp257357_c0 within PRJNA231164). When differential expression analysis was conducted on reads mapped to the transcriptome assembled within this study, the GST showed a trend toward higher expression with both exposures, but this difference was modest in magnitude (<2-fold) and not statistically significant (Supplemental File 1 Figure S2). Overall the gene expression analyses indicate that exposure of females to both the dispersion and the WSF produced mild changes in gene expression in the offspring consistent with responses to oil exposure. While most of the differentially expressed genes were unique to each treatment, we did not see evidence for increased toxicity of the dispersion relative to the WSF. This is somewhat surprising and contrasts with the visual observations (Fig. 4) that suggest increased PAH exposure in eggs produced by females exposed to oil-dispersion. One possible explanation is that the effects on gene expression primarily result from exposure to the smaller more water soluble PAHs.

4. Conclusions and suggestions for future work

Oil droplet exposure contributes to PAH uptake in dispersion-treated copepods as displayed by PAH body residue analyses and visualized in copepods using fluorescence microscopy. Utilizing the latter methodology, transfer of heavy PAH from copepod mothers to offspring was strongly supported. Only
subtle effects were observed in offspring, namely a temporary reduction in hatching success, which appears to be relevant only when mothers were exposed to the oil dispersion that included droplets. Dispersion-treated adult copepods also displayed lower lipid levels than WSF-treated copepods and controls, suggesting droplets caused alterations in copepod energy budgets. Gene expression analyses of offspring during the last naupliar stage suggest affected expression of genes that were toxicologically relevant. However, the small number of differentially expressed genes suggest a relatively mild long-term effect of maternal exposure on the offspring.

Acknowledgements

This project was funded by the Research Council of Norway (the ENERGYBAR project no. 225314/E40).

References


USEPA. 2006. Method 8260C: Volatile organic compounds by gas chromatography/mass spectrometry (GC/MS).


**FIGURE CAPTIONS**

**Figure 1:** Schematic of experimental design. Female copepods were exposed to clean sea water (negative control), oil dispersion or WSF for 4 days, after which they were allowed to recover in clean seawater where eggs were harvested for assessment of hatching success and development of nauplii until the first copepodites appeared. For each treatment, there were four replicates.

**Figure 2:** PAH body residue in the copepod *Calanus finmarchicus* exposed to seawater (negative control), oil dispersion and WSF. Data are given as average ± STDEV (N=3).

**Figure 3:** Bioaccumulation in *Calanus finmarchicus* plotted as a function of Kow of individual PAH components. Data are given as average ± STDEV (N=3). Both datasets fitted by a bilinear equation (y=A*\log(x)-B*\log(C*x+1)+D).

**Figure 4:** Fluorescence microscopy. Example of female copepod from the negative control group (A, seawater) and dispersed oil (B) as well as eggs from females from the negative control group (C, seawater), WSF (D) and dispersion (E). In the top images the red fluorescence represents partly digested algae in their intestine. The dispersion treated copepod (B) displays a blue lipid sac indicating uptake of heavy PAHs in the lipid sac, whereas the control copepod (A) does not display the same coloration. The eggs) from females treated with oil dispersion are blue in color (E) compared to both the negative controls (C) and the eggs from WSF-exposed copepods (D), suggesting transfer of heavier PAHs from the dispersion. Photo: Dag Altin, BioTrix.

**Figure 5:** Hatching success of eggs from female copepods treated with clean seawater (negative control), dispersion and WSF. Bars display average ± STDEV (N=4 throughout). Asterisks * denote significant differences (p<0.05) between treatments determined using 2-way ANOVA with Tukey's multiple comparison test.

**Figure 6:** Summary of gene expression results for the offspring (nauplii) of mothers exposed to oil dispersion. (A) Heat map indicating normalized expression patterns of genes differing in expression among treatments. Columns indicate replicate samples clustered into treatments (colored bars at top). Rows represent individual differentially expressed genes. (B) Venn diagram indicating the number of differentially expressed genes in the WSF and dispersion treatments relative to control. Initial numbers indicate results using a significance threshold of 4-fold change in expression and 1% false discovery rate (FDR). Numbers indicated parenthetically indicate a 0.1% false discovery rate.
Table 1: Concentrations of oil components in the water samples taken during exposure. For each treatment, three replicates were used, so results are presented as mean ± STDEV (N=3).

<table>
<thead>
<tr>
<th></th>
<th>Control (µg/L)</th>
<th>WSF (µg/L)</th>
<th>Dispersion (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sum VOC</strong></td>
<td>0,42 ± 0.10</td>
<td>16,53 ± 1.10</td>
<td>16,89 ± 0.51</td>
</tr>
<tr>
<td>Sum BTEX</td>
<td>0,36 ± 0.05</td>
<td>4,72 ± 0.25</td>
<td>5,15 ± 0.13</td>
</tr>
<tr>
<td><strong>Sum SVOC</strong></td>
<td>0,11 ± 0.00</td>
<td>16,44 ± 0.84</td>
<td>24,22 ± 0.42</td>
</tr>
<tr>
<td>Sum Naphthalenes</td>
<td>0,07 ± 0.01</td>
<td>11,85 ± 0.72</td>
<td>12,62 ± 0.55</td>
</tr>
<tr>
<td>Sum 2-3-ring PAH</td>
<td>0,04 ± 0.00</td>
<td>3,28 ± 0.15</td>
<td>6,51 ± 0.15</td>
</tr>
<tr>
<td>Sum 4-6-ring PAH</td>
<td>ND</td>
<td>ND</td>
<td>1,26 ± 0.06</td>
</tr>
<tr>
<td>Sum Phenols</td>
<td>ND</td>
<td>0,93 ± 0.17</td>
<td>1,13 ± 0.06</td>
</tr>
<tr>
<td>Hopane</td>
<td>ND</td>
<td>ND</td>
<td>0,21 ± 0.01</td>
</tr>
</tbody>
</table>
Table 2: GO terms exhibiting significant enrichment in pairwise comparisons between groups of differentially expressed genes (Chi square, \( p < 0.05 \)).

<table>
<thead>
<tr>
<th>Dispersion vs. WSF</th>
<th>Up-regulated in dispersion</th>
<th>Up-regulated in WSF</th>
<th>Down-regulated in dispersion</th>
<th>Down-regulated in WSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO terms</td>
<td></td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Dispersion: Up vs. Down

<table>
<thead>
<tr>
<th>Primarily up-regulated</th>
<th>GO:0006996 organelle organization; GO:0007017 microtubule-based process; GO:0043473 pigmentation; GO:0048519 negative regulation of biological process; GO:0050794 regulation of cellular process; GO:0065007 biological regulation; GO:0050789 regulation of biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primarily down-regulated</td>
<td>None</td>
</tr>
</tbody>
</table>

WSF: Up vs. Down

<table>
<thead>
<tr>
<th>Primarily up-regulated</th>
<th>GO:0003824 catalytic activity; GO:0001882 nucleoside binding GO:0006996 organelle organization; GO:0048646 anatomical structure formation involved in morphogenesis; GO:0009605 response to external stimulus; GO:0005515 protein binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primarily down-regulated</td>
<td>None</td>
</tr>
</tbody>
</table>
A: Naphthalenes

B: 2-3-ring PAH

C: 4-6-ring PAHs

D: T-PAHs
The bar chart shows the percentage hatching of eggs over different hours after they were laid. The chart compares three groups: Control, Dispersion, and WSF. The percentage hatching is measured on the y-axis, with values ranging from 80 to 100. The x-axis represents hours after the eggs were laid, with three time points: 48, 72, and 96 hours.

Notable differences are indicated by asterisks above the bars. The chart suggests that the WSF group has a higher percentage hatching compared to the Control group at certain time points, as indicated by the asterisks.
### Table 3: Examples of differentially expressed genes with known roles in responses to chemical stressors.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Transcript</th>
<th>Expression relative to Control</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia upregulated protein 1</td>
<td>GAXK01175378.1</td>
<td>Up WSF</td>
<td>Hsp70 family member (endoplasmic reticulum)</td>
</tr>
<tr>
<td>Cyp2J</td>
<td>GAXK01019268.1</td>
<td>Up WSF</td>
<td>Phase 1 metabolism, eicosanoid metabolism</td>
</tr>
<tr>
<td>Thioredoxin 3</td>
<td>GAXK01014080</td>
<td>Up Dispersion and WSF</td>
<td>Antioxidant response</td>
</tr>
<tr>
<td>Xanthine dehydrogenase</td>
<td>GAXK01025498.1</td>
<td>Up Dispersion and WSF</td>
<td>Antioxidant response</td>
</tr>
<tr>
<td>Phospholipase A2 activating protein</td>
<td>GAXK01017887</td>
<td>Down Dispersion and WSF</td>
<td>Inflammatory response, involved in cell death pathways</td>
</tr>
</tbody>
</table>
Control Dispersion WSF
−2 0 2
Median-centered Expression log₂(fpkm)

A.

B.

WSF Dispersion Control

Total
Up 183 (96) 35 (16) 173 (76)
Down 84 (41) 24 (12) 73 (35)
99 (55) 11 (4) 100 (41)

1% FDR (0.1% FDR)