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Rapport

Effects of produced water on pelagic marine organisms

Focus on the copepod Calanus finmarchicus

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SAMMENDRAG
The main aim of this project was to provide a basis for assessing the potential health effects of produced water (PW) on the marine pelagic zooplankton Calanus finmarchicus in the field. This report describes three experiments assessing the impact of reconstituted PW extracts on copepod PAH body residue, stress gene expression and metabolic profiles. The project established relationships between produced water exposure, PAH uptake and biological responses in a laboratory settings, in order to be used as a basis for sampling and analyses of copepods exposed to environmentally realistic scenarios in the field. A selection of endpoints are suggested for analyses of field-collected copepods in the water column monitoring in 2017.

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1 MAIN AIMS AND APPROCH

The main aim of this project was provide a basis for assessing the possibility of produced water to cause health effects on the marine pelagic zooplankton *Calanus finmarchicus* in the field. To do this, we wanted in the present project to establish relationships between produced water exposure, PAH uptake and biological responses in a laboratory settings, so that these end points can be measured and provide information about the health of copepods in the environment. To do this we exposed copepods to extracts of produced water from the Statfjord A platform in three different experiments assessing effects on survival, PAH uptake, metabolite profiles and stress gene expression.

Fig. 1: The copepod *Calanus finmarchicus* at the CV stage.

2 MATERIALS AND METHODS

2.1 Choice of produced water

Upon arrival of produced water (PW) from Statfjord A (SFA), filtered and unfiltered samples were taken for extraction and chemical analyses (see section 2.3) as well as particle analyses using the Beckman Coulter Counter. Sub-samples were stored in smaller volumes (Fig. 2), but larger volumes were extracted using DCM after acidification and store for analyses and reconstitution.

Fig. 2: Picture of samples of PW.

2.2 Experimental animals

C. finmarchicus (Figure 1) from the continuous laboratory culture at SINTEF/NTNU Sealab was used for the experiments described below. The culture is routinely kept at approximately +10°C, and the details regarding the culturing have been described previously (Hansen, Altin et al. 2007). For all tests copepodite stage V (CV) was used.

2.3 Chemical characterization

Samples of PW and water samples from experiments were taken in baked glass bottles for analysis of semi-volatile organic components (SVOC; 800 mL sample volumes) and in sealed glass vials without headspace for analysis of volatile organic components (VOC; 40 mL). Water samples were preserved by acidification with 15% hydrochloric acid.

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 3510C (USEPA 1996). The combined extracts were dried over sodium sulphate and concentrated to approximately 1 mL using a Zymark Turbovap® 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 using 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-d_{10}. As an objective way of excluding samples corrupted by the extraction process all values deviating more than 1.3 x SD 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_{10}-C_{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_{10} to C_{36} n-alkanes. Water levels of 35 target VOC (C_5-C_{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-d_8 and ethylbenzene-d_8) and RIS (chlorobenzene-d_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, C_5-C_{36}) of water samples was calculated by adding VOC (C_5-C_9) and TPH (C_{10}-C_{36}) concentrations.

2.4 Reconstitution of PW

Due to very high microbial activity in the PW, we used reconstituted PW extracts for most of the experiments with copepods. DCM extracts were added at a predefined volume based on THC mass into a large glass flask (10L). It was flushed for two hours with nitrogen gas in order to remove the DCM. Afterwards, filtered seawater was added and the solution was ultra-sonicated for 0.5 hours to dissolve the PW components. The solution was used as a basis for the individual experiments and diluted with filtered seawater to the appropriate concentration.

2.5 Acute toxicity testing

The exposure solutions (filtered/unfiltered effluent and reconstituted PW) were diluted to a series of seven concentrations with filtered seawater, and 0.5 L borosilicate glass bottles with Teflon lined screw caps were used as exposure vessels. Triplicates were used for each exposure concentration, and six groups were used as controls (containing filtered seawater only). The exposure vessels were filled close to the rim with exposure solution before seven copepodites were carefully added to each bottle in a small amount of seawater with a wide bore pipette. The bottles were then capped leaving virtually no headspace. Mortality was monitored at 24, 48, 72, 96, 120 and 144 h, and these data formed the basis for estimating LC50 values. Temperature was monitored throughout the exposure, and the saturation of oxygen was measured at end of exposure. The animals were not fed during exposure. Water samples were taken from the highest and lowest concentration for chemical analyses.

2.6 Time- and concentration-dependent responses

In this experiment, 60 bottles (2L) were prepared with reconstituted PW at three nominal concentrations corresponding to 0 (ctrl), 0.5 (low), 5 (medium) and 50% (high) of the LC50-level from the acute toxicity experiment. In addition, we included a DCM control where the same amount of DCM as used for the highest reconstituted PW concentration was used to make sure that any potential traces of DCM did not cause any toxicity. All treatments were performed with N=4 (see Table 1 below for experimental design).

Table 1: Experimental design for the time- and concentration-dependent experiment.

	Ctrl (0%)	Low (0.5%)	Med (5%)	High (50%)	DCM-ctrl
0 hrs	4 reps	-	-	-	-
12 hrs	4 reps	4 reps	4 reps	4 reps	4 reps
24 hrs	4 reps	4 reps	4 reps	4 reps	4 reps
48 hrs	4 reps	4 reps	4 reps	4 reps	4 reps

Each bottle was added 50 copepods, and at sampling they were split to analyses of PAH body burden (20 individuals), metabolomics (15 individuals) and gene expression (15 individuals).

2.7 Realistic PW exposure

Based on a scenario-realistic estimate, a 10,000x dilution is expected for a PW plume over a period of 4 days.

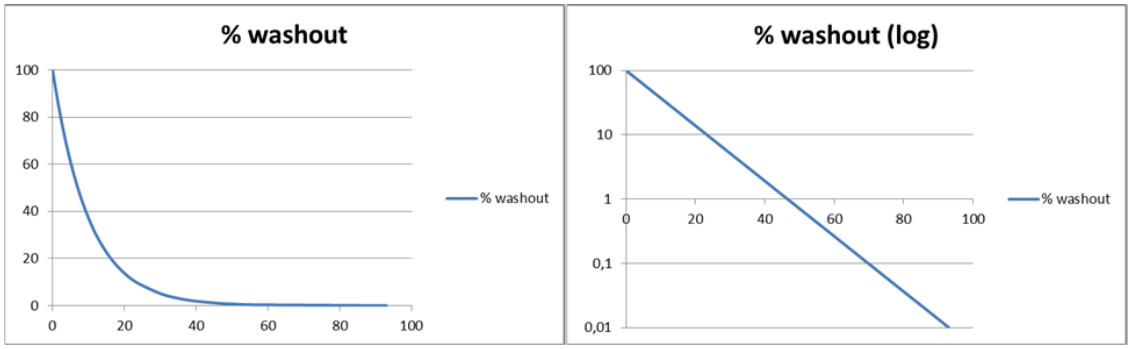

Fig. 3: Theoretical dilution curve for the bottles based on flow rate and exposure vessel volume used in the flow experiment.

In the present experiment, we wanted to start with a high concentration (6.4 mg/L based on C9-C40-THC) and simulate an exponential decline over time (96 h) (
Fig. 3). This was performed in 5L bottles placed upside down with their bottoms removed (
Fig. 4). This was a flow-through system, which continuously diluted the original reconstituted PW according to specifications given by the scenario. Each of the bottles (N=4 for PW-treatment and N=4 for control with sea water only) were added 150 individuals, and sampled for analyses of PAH body burden, metabolomics and gene expression at t=24 and t=96.

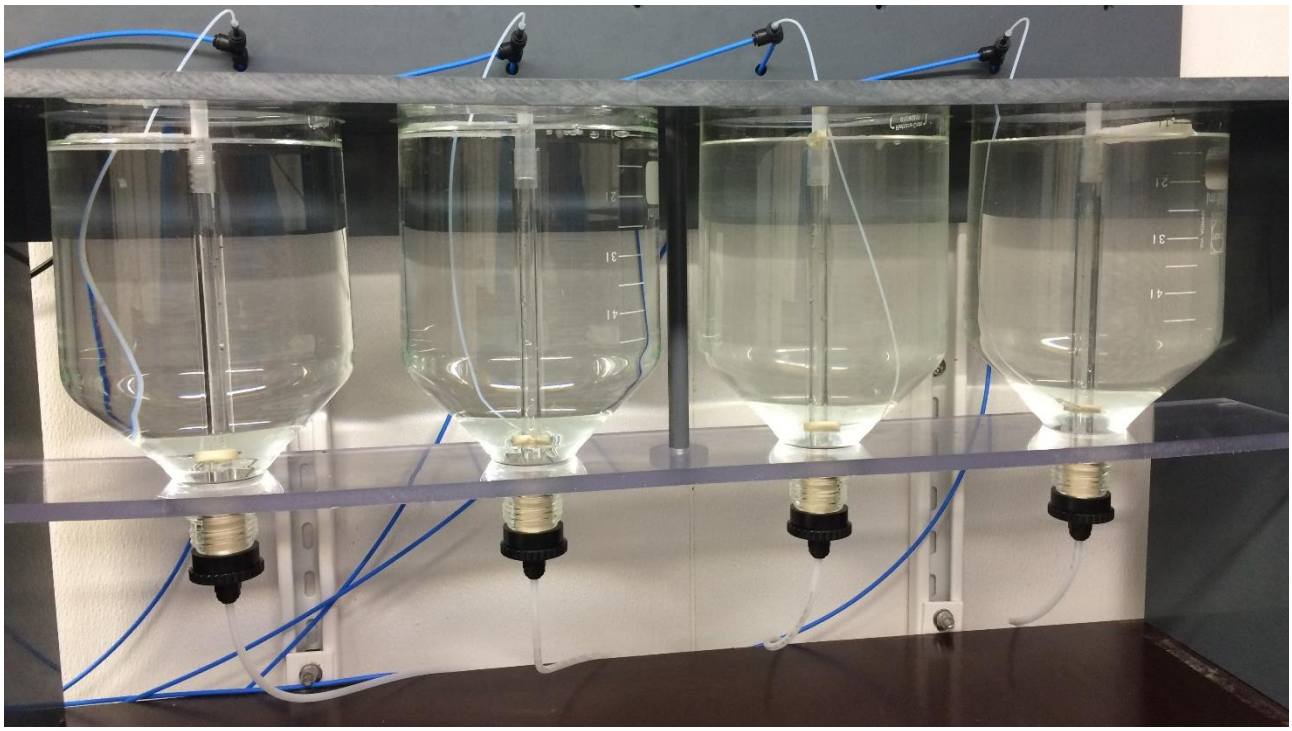

Fig. 4: The rig system used for the flow-through experiment.

2.8 Sampling and extraction

2.8.1 Sampling

Copepods were sampled for analyses of PAH body burden, metabolic profiling and gene expression. Pools (n=15) of copepods were sampled in tubes and frozen at -80°C for PAH body burden and metabolic profiling. For gene expression analyses pools of 15 copepods were sampled from each treatment/replicate in RNAlater (Ambion) and frozen at -80°C.

2.8.2 Extraction for PAH body burden and metabolomics

The copepods were extracted and analyzed as previously described (Hansen, Degnes et al. 2013) by the method described by Wu et al. (Wu, Southam et al. 2008) which is a modified Bligh and Dyer extraction method (Bligh and Dyer 1959).

2.8.3 RNA extraction for gene expression analyses

RNA has been extracted from 7 individuals each using the ZymoResearch Tissue & Insect RNA MicroPrep kit according to manufacturer's protocol. *In-column* DNase I treatment has been performed using DNase I Set (ZymoResearch). Quantification of RNA has been performed on a Qubit 3.0 fluorometer using the RNA high sensitivity assay kit (Life technologies).

5 µl of each isolated RNA sample was used for cDNA synthesis using the cDNA Supermix (qScript) according to manufacturers' protocol. cDNA quantification was performed using the dsDNA high sensitivity kit (Life technologies). Samples were diluted to 1 ng cDNA/µl prior to gene expression analyses using ddPCR (see section 2.12).

2.9 Microscopy analyses

2.9.1 Biometry

A sub-sample (N=60) of copepods from the culture used for the experiments was sampled and photographed using a Leica MZ125 dissecting microscope (Leica Microsystems, Wetzlar, Germany) and pictures of 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, USA). The pictures were used for analyses of biometry manually by the use of the software ImageJ (National Institute of Health, Bethesda MD, USA) on scaled captured images. Areas of the lipid storage and area of the prosome were measured on the computer screen with the aid of a graphical tablet (Wacom Intous3, Wacom Co., Ltd., Saitama, Japan). Volume of lipid storage size and prosome were calculated according to Miller et al. (1998) based on the area and length of the lipid storage or prosome, respectively, and the lipid content were presented as % volume of lipid storage compared to volume of prosome (Hansen, Altin et al. 2008; Hansen, Nordtug et al. 2009).

2.9.2 Fluorescence microscopy

Copepods (N=6) from each treatment of the acute toxicity experiment were subjected to fluorescence microscope imaging. Examples of such images are shown in Fig. 5.

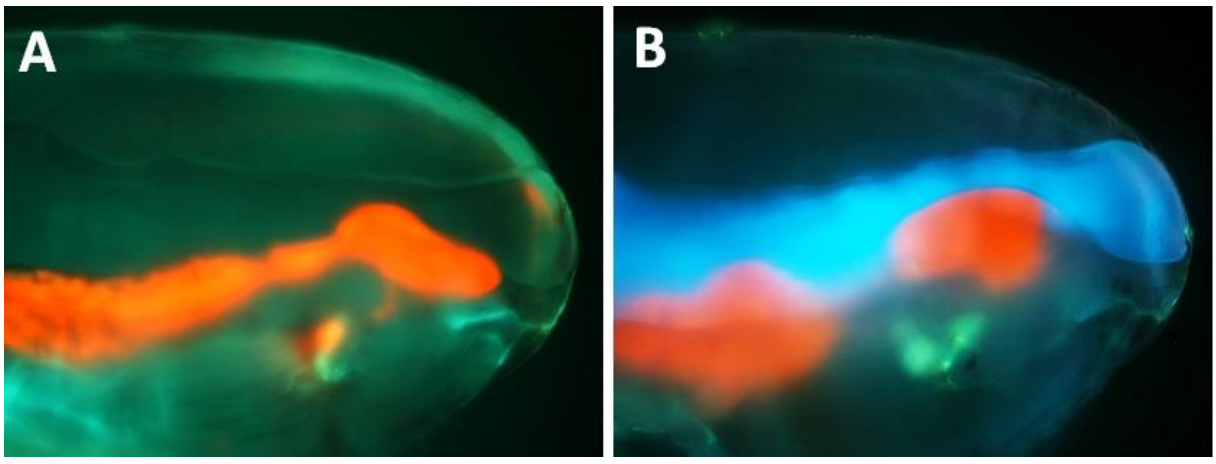

Fig. 5: Fluorescence photograph (using 10xPA B2 001 F6 ND4 HYBR) of a female copepod treated with seawater (A) and dispersed oil (B). Photo: Dag Altin, BioTrix.

2.10 PAH body burden analyses

After exposure, all copepods were counted and dead individuals removed. Live copepods (25 individuals) were pooled and sampled from each exposure group for body burden analysis and frozen in liquid nitrogen. 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 h in an ultrasonic bath at 80 °C to achieve saponification, followed by filtration and serial extraction with 4 mL of Milli-Q water/2 × 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 × 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.

2.11 Metabolic profiling

^{1}H-NMR spectra recorded on a 600 MHz NMR Bruker DRU spectrometer. The Spectra were processed and integrated using MestReNova (v8.1.4, Mestrelab Research S.L., Santiago de Compostela). Multivariate analysis was performed using PLS-Toolbox (Eigenvector Research) in Matlab (R2012a, MathWorks, Natick, Massachusetts).

2.12 Gene expression analyses

Droplet digital PCR (ddPCR) allows absolute quantification without the need of external calibrators and has been proposed as the method of choice to overcome limitations of qPCR, such as detection of low copy numbers and copy number variations. Digital PCR is an endpoint measurement relying on partitioning (mimicking limiting dilution) and Poisson statistics. ddPCR is a type of dPCR that generates tens of thousands of individual droplets in a water-oil emulsion. The total number of targets in a sample is the fraction of positive droplets after amplification to the terminal plateau phase of PCR. ddPCR was performed on the QX100™ Droplet Digital™ PCR system (Bio-Rad) with the ddPCR™ Supermix for Probes with no dUTP kit (Bio-Rad) and ddPCR™ Supermix for Residual DNA Quantification. Reactions were set up in a final volume of 20 µl at 500 nM for each primer (primer sequences in Table 2) and 200 nM for the probe according to the manufacturer's instructions. The reaction mixture was loaded into a sample well of the cartridge (Bio-Rad). Seventy microliters of oil droplet generation was added in the appropriate well. Then, the cartridge was placed in the Droplet Generator (Bio-Rad). Following this step, the droplets generated were transferred to a standard 96-well PCR plate. The PCR plate was heat-sealed with a foil plate seal (Bio-Rad) and placed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) for PCR amplification, using the following thermal conditions (ramping rate 2 °C s^{-1}): 10 min at 95 °C, followed by 45 cycles of 30 s at 95 °C and 60 s at 50 °C for *qnr*B or 58 °C for *sul*1. Upon completion of PCR, the plate was transferred to a Droplet Reader (Bio-Rad) for data acquisition. Automatic measurement of fluorescence in each droplet and in each well was recorded and analyzed by the QuantaSoft™ Software 1.7.4. (Bio-Rad). Quality controls including no amplification in NTC wells, exclusion of wells with less than 10,000 accepted droplets, distribution of the number of accepted droplets per reaction, fluorescence amplitude of positive and negative droplets, and Poisson mean estimates were checked. ddPCR assays were performed at least in quadruplicate with one "no template control" (NTC) per microplate line.

Table 2: Primer sequences used for gene expression analyses.

Gene name	Gene	Forward (5'-3')	Reverse (5'-3')
Glutathione S-transferase	GST-2	TTGCGGGCTCTTTGTTAAGT	TCTTGCTCCCTGCTCAGAAT
Cytochrome P450-4	CYP4	CTGATCACTCCAACTTTTCACTTC	CCATTGCAGTCTCACAGATTATG
retinoid X receptor	RXR	GAACTGGCACCTGTTCCTCT	GGGTTGTAAGGGGTTCTTCA
A-type allatostatins	AST	AACAACAGTAATGGCTTGCACTATGA	TGTCAGTATCAGGTCCATCTTCTCC
Ecdysteroid Receptor	EcR	GACATTGCTGCTAAGAATTGTGCTA	TCACACTTGGATGCTCAAACTCTC
Fatty acid binding protein	FABR	GGTCATTGTCATGGTCAAACC	CCAACCAGAAGGCTGTCAAG
Elongation of very long chain fatty acid; elongase	ELOV	GTCTGGTGGTGTTTCTTCTCC	CACATGCAGAGAGGTAAGTTGG
gamma-glutamylcysteine synthase.	GGCS	ACAAACGCACACTGGATGAG	CAAAAAGGGAGGGGTCTTTC
Glutathione Synthase	GSH-S	GAGAAGGCAAAGGACTATGCTC	GGCAACCTTGTGCATCAAC
Cellular Apoptosis Susceptibility Protein	CAS	CTACAACCACTACCTGTTCGAGT	CAGGGACATGATCTGGAACAC
Elongation factor	EFA	CTCCGACTCCAAGAACAAGC	AATATGGGCGGTGTGACAAT
Cytochrome P450 1A2	CYP1A2	TCTGTCTCGCTCTTTGGACA	AACCGGACGATGATTGAAAG
Cytochrome P450 330A1	CYP330A1	CAGGTCCTTTCTCCCCTCTT	CAAAGGCCATCCTTTTGGTA
Ubiquitin	UBQ	GACCAGCAGAGGCTCATCTT	CTTCTTCTTGCGCTTCTTGG
Phospholipase	PL	TCGCTCATGTCTCAAGATGG	GACCCCACCAGTTACGAGAA
trifunctional hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase beta subunit	Tp-beta	TAACAGGATGATCCGGGAAG	CAAGGCAAGGACCCAGATAG
Glutathione S-Transferase	GST	CAACCCCCAGCACACTGTG	GGATAGACACAATCACCCATCC
Catalase	CAT	TGTACATGCAAAGGGAGCTG	GGTGTCTGTTTGCCCACTTT
Superoxide Dismutase	SOD	GGAGATCTTGGCAATGTTCAG	CAGTAGCCTTGCTCAGTTCATG
Cell Cycle and Apoptosis Regulatory 1 Protein	CARP	GCCAAGAGTGGGAAGTTTGAC	GAACATTTCATTGAACAATTCTGC
Inibitor of Apoptosis Protein	IAP	CAGGATTCTTCTACACAGGCAG	CCATTTCTTGTGTTCTCCCC
Heat shock protein 70A	HSP70A	CGAAACAGCAGGAGGAGTGATG	TGACAGCAGGTTGGTTGTCTTG
Heat shock protein 70B	HSP70B	TGGAGGGAAAGGCAGCTAAAG	CATCGCTGGAACTAACCCAAGC
Heat shock protein 70D	HSP70D	GGGTGGAGGTGATCCCTAATG	TGCACCACTTCATCAGTCCAC
Xanthine dehydrogenase	XAD	TGCTGCTTCAGTCTTCTTTGC	CGAATTCTTTCTGCTGTAGCC
Neuronal calcium sensor; hippocalcin	NCS	AAGATCTACAGCCAGTGCTTCC	TCCCATCAGTGTCAAAAGTCC
Barnacle cyprid specific gene 2	BCS2	CGTGAATGAGCAGACTGAGG	CTGCTTGTTGATCATGTTGTCC
thioredoxin-2	trx-2	TGGTTGACTTCCACGCTACC	GGAACACCACCTCCTCCATG
Glutamate dehydrogenase	GHD	GGTAGTTGCTCTCCCTCTCG	GACATGTACATCAACGCGGG
nucleosome assembly protein 1-like 1	nap111	GTTCACCCTGACCTTCCACT	GAGCTCGTACTCCTTGGTCA
Reductase/dehydrogenase	RDH	CTAGCCAGGTTGCTGATGAAG	TCTTGGAGATGGTGAGGTCTG
Ferritin	Ferritin	AATATCAGACCAAGCGTGGAG	AGCTTCCATTGCCTGAATAGG
Heat shock protein 90	HSP90	TCATCCGGATTCAGCTTGGAG	GGTGGCATGTCGCTGTCATC
Heat shock protein 22	HSP22	GGCTACAAGCCAAGTGAGCTG	GAGACCATGGTGTGGCCTTC
Heat shock protein 21	HSP21	TGCAAACACAGCAACAAGCTG	GCCTCGGAAAGAGCATTCTTC
Small heat shock protein 26	p26	CTTGCCAAGCATGAGACCAAG	GGATTGACCCCAGATGGTAATG
16S	16S	AAGCTCCTCTAGGGATAACAGC	CGTCTCTTCTAAGCTCCTGCAC
Actin	Actin	CCATTGTCCGTCTTGATCTTG	AAAGAGTAGCCACGCTCAGTG
GAPDH	GAPDH	CACCTGATGTGTCTGTGGTTG	CTTGAGCTTGGCACAGATTTC
Heat shock protein 40 (dnaja)	DNAJA1	GCTCCTGCCTTTCCTCCAAT	ACAACGGGACAGTCAGGAAG

3 RESULTS AND DISCUSSION

3.1 Characterization of Statfjord produced water

Upon arrival, it was evident that the PW contained particulate material, so samples were characterized for particle content using the Coulter Counter. Mean size of the particles was 3.2 µm and 7.2 µm based on particle number and particle volume, respectively (Fig. 6).

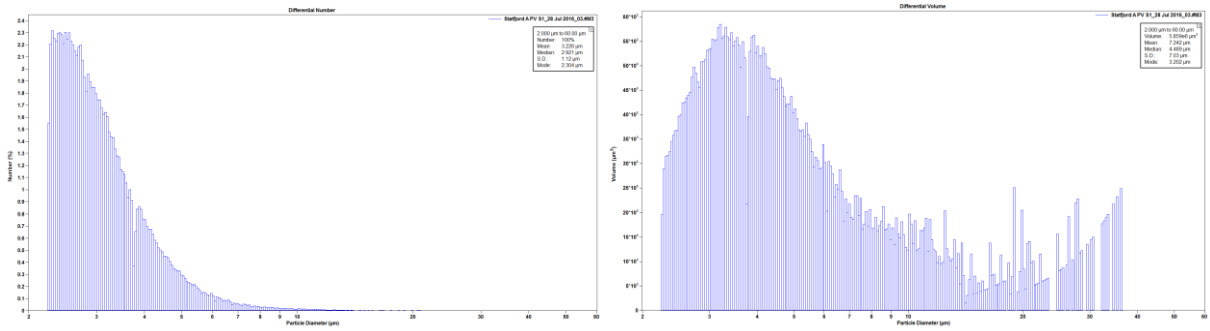

Fig. 6: Particle analyses of produced water from Statfjord A. Top: Number of particles as a function of particle diameter (µm). Bottom: Volume (µm^3) of particles as a function of particle diameter (µm).

The mineral particles also appeared to contain oil components as they displayed fluorescence under the fluorescence microscope (
Fig. 7). To verify this, the PW volume was split into two, where one was filtered and the other kept unfiltered. DCM-extraction and GC-FID and GC-MS analyses was performed. Clearly, filtration removed parts of the higher PAHs and the alkanes (Fig. 8).

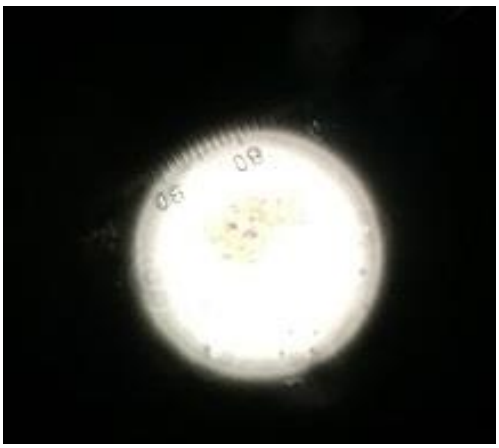

Fig. 7: Mineral particle covered with oil photographed under a fluorescence microscope.

GC-MS analyses also revealed the presence of more extractable material from the unfiltered PW, including PAHs, the biomarker hopane and unresolved complex material (UCM) which is estimated by subtracting the concentration of identified SVOC-components from TEM. Hopane is often used as a biomarker for petrogenic oil as it is inert to biodegradation and is insoluble in water (Table 3).

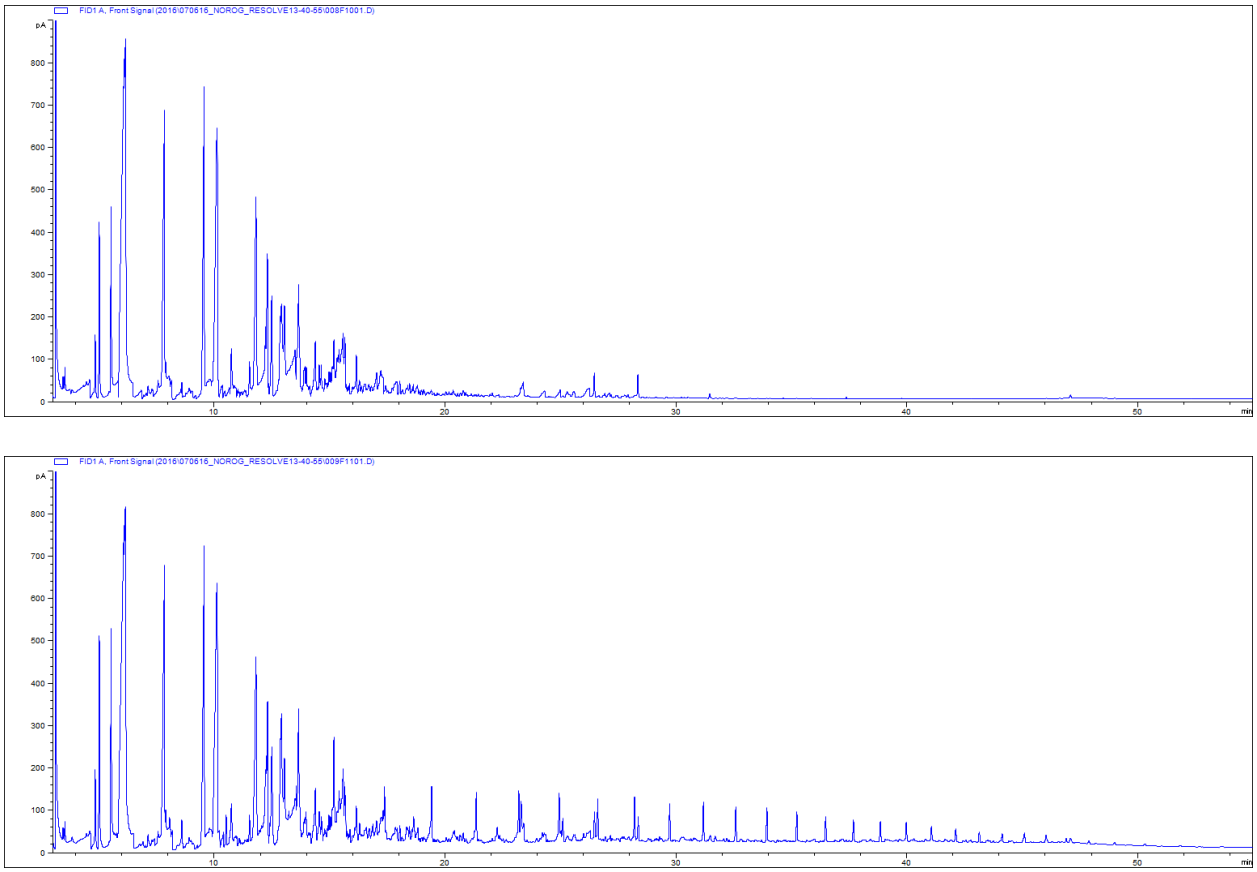

Fig. 8: GC-FID chromatograms of PW from SFA (sample MC-2016-183). Top: Filtered SFA PW. Bottom: Unfiltered SFA PW.

Table 3: GC-MS analyses of PW. Concentrations of individual groups of components.

	Filtered PW	**Unfiltered PW**
TEM (C9-C40)	5633,56	9651,56
VOC (C5-C9)	8691,34	11829,48
SVOC	4150,97	4188,44
Total (C5-C40)	14324,89	21481,04
Sum decalins (C0-C4)	0,03	5,76
Sum naphthalenes (C0-C4)	89,18	103,22
Sum 2-3 ring PAHs	5,37	17,51
Sum 4-6 ring PAHs	0,00	3,20
Sum C0-C5 phenols	4056,40	4057,17
Hopane	0,00	1,58
UCM	1482,58	5463,13

3.1.1 Toxicity predictions using EU-TGD QSAR

As raw PW has not previously been tested on *Calanus finmarchicus* in our laboratory, we wanted to assess the theoretical toxicity of the PW. Based on component concentrations from the GC-MS analyses predictions of acute toxicity was performed using the EU-TGD QSAR. This was done for all components (including volatile and semi-volatile components) and for semi-volatiles alone (Table 4). According to the predictions, a filtrate of the PW (including VOC) would be able to cause 50% mortality (in a *Daphnia magna* acute toxicity test, 24 hrs), as the total concentration is comparable to the predicted LC50. For unfiltered (including VOC), the ratio between the sum of components and the LC50 was 2.1 indicating that the unfiltered PW is more toxic than the filtered. These calculations were also performed without including the VOC. Here only the unfiltered PW displayed a concentration comparable to the predicted LC50.

Table 4: Predicted LC50s using the EU TGD QSAR and the component speciation based on GC-MS analyses.

Predicted all components	LC50	LC50-min	LC50-max	Sum all/LC50
MC-2016-183 filtered	11559,9	9182,3	14553,0	1,1
MC-2016-183 unfiltered	7694,5	6112,0	9686,8	2,1

Predicted without VOC (C9-C40)	LC50	LC50-min	LC50-max	Sum all/LC50
MC-2016-183 filtered	8927,91	7091,69	11239,58	0,458294457
MC-2016-183 unfiltered	3195,77	2538,49	4023,24	1,290119358

3.2 Copepod stage and biometry

All individuals (N=60) photographed were developmental stage CV (Fig. 9), except from one adult female. This is satisfactory for the experiments performed. It was impossible to visually inspect all individuals used in the experiments, so having 98% of the same developmental stage is very good.

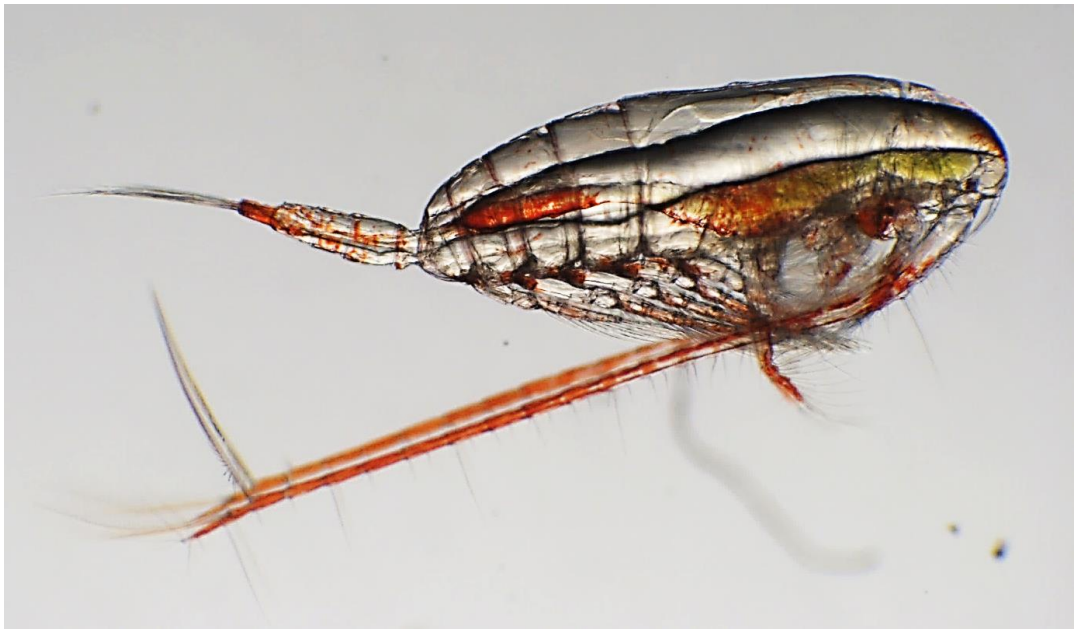

Fig. 9: Image of a copepodite stage CV. Photo: Bjørn Henrik Hansen, SINTEF.

The biometric measures of the 60 individuals display natural ranges in prosome area, lipid area and prosome length (1.847-2.375 mm) (Fig. 10). The length was 2.0 ± 0.1 (mean ± STDEV) mm, which is a bit in the low end for this developmental stage. They were however, relatively lipid rich as expected for this stage.

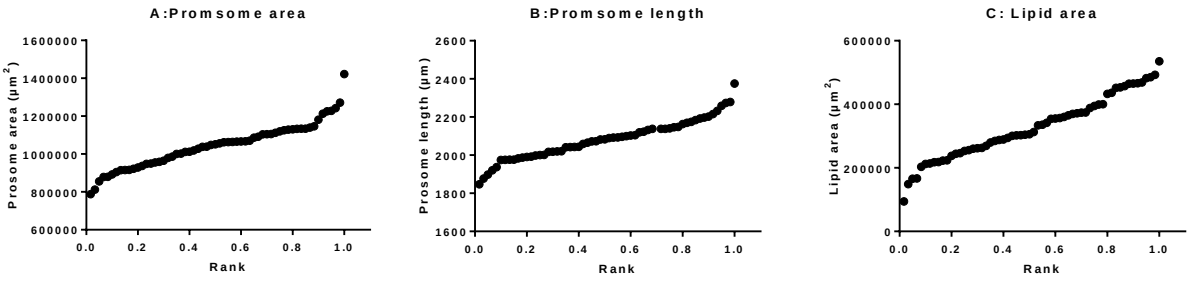

Fig. 10: Biometric measures of copepods used for the experiments. The one last individual to the right in all three plots is the same female copepod.

3.3 Acute toxicity testing

3.3.1 Acute toxicity on raw extracts

Acute toxicity tests were performed on raw extracts (filtered and unfiltered) as well as on reconstituted PW. The raw extracts were run twice, and none of them produced reliable acute toxicity data, as the tests did not meet criteria (>80%) for oxygen concentrations at the end of the tests (Table 5). The PW degraded during the 96 h exposure period as can be seen from the low oxygen concentrations in the exposure vessels. Even at very high dilutions (0.7%), there was a significant drop in oxygen concentration. Thus, using raw PW as a basis for assessing acute toxicity of PW was not considered valid. Thus, we needed to come up with an alternative approach to expose copepods to PW, and this approach was to reconstitute PW extracts and use as a basis for the remaining tests.

Table 5: Oxygen concentrations (mg/L) in exposure vessels at the end of the acute toxicity tests using raw PW at different dilutions.

% dilution	Unfiltered T1	Filtered T1	% dilution	Unfiltered T2	Filtered T2
Control	8,07	8,07	**Control**	8,07	8,07
Control	8,18	8,18	**Control**	8,26	8,26
4,1	0,12	0,04	**0,4**	7,51	7,22
7,0	0,10	0,03	**0,7**	6,61	6,57
12,0	0,04	0,03	**1,2**	5,57	5,53
20,4	0,04	0,07	**2,0**	3,24	2,87
34,6	0,09	0,00	**3,5**	0,03	0,08
58,8	0,04	0,00	**5,9**	0,00	0,02
100,0	3,84	3,96	**10,0**	0,00	0,00

3.3.2 Acute toxicity testing of reconstituted extracts

The 100% solution generated through reconstitution of PW extracts were 6.4 mg/L based on C9-C40 THC, and for the acute toxicity tests 7 dilutions were used for generating LC50-data (Fig. 11). Concentrations obtained in the solutions were measured analytically and were lower than nominal (1.6 mg/L for the highest concentration, 100%). This is caused by the fact that not all of the components in the extract will dissolve back into the water.

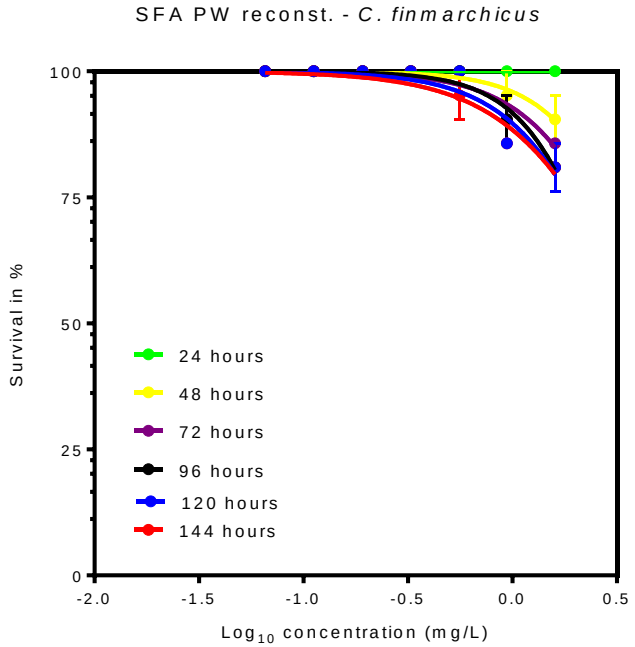

Fig. 11: Survival (percentage) plotted as a function of nominal exposure concentrations (Log mg/L).

Low mortality was observed in the test; however, we were able to estimate a 96 h-LC20 of 1.62 mg/L and extrapolate a 144 h-LC50 of 4.1 mg/L based on C9-C40 THC.

3.3.3 Fluorescence microscopy

Seven individuals from the acute toxicity test of reconstituted PW were sampled and analyzed using fluorescence microscopy. Representative images for all treatments (control and seven concentrations) are given in Fig. 12. These copepods were exposed for 144 hours to the concentration series, and blue color is visible in all treatments (except controls). The blue colored lipid sac (also shown in Fig. 5) seems to be visible even for the lowest exposure concentrations, and also appear to display a concentration-dependent increase in response. These aspects clearly suggest that this marker can be valuable for field assessments as well.

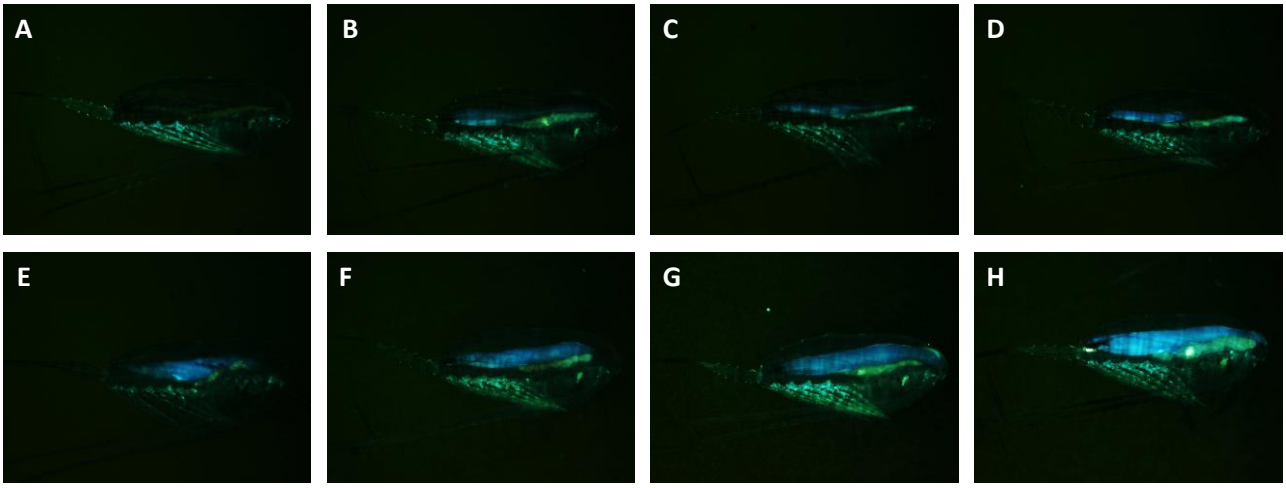

Fig. 12: Fluorescence microscope images of copepods exposed for 144 hours to seven concentrations of reconstituted PW. A: Controls. B: 4.1%. C: 7%. D: 12%. E: 20.4%. F: 34.9%. G: 58.8%. H: 100% reconstituted PW.

3.4 Time- and concentration-dependent biomarker responses

Three concentrations of reconstituted PW were made for this experiment (low, medium, high), as well as negative controls (seawater only) and solvent controls (DCM).

3.4.1 Exposure verification

The concentrations obtained in the stocks of reconstituted PWs are provided in and in Fig. 13. A nice 10-fold concentration series were made based on PAHs, however, for the phenols this series is not as expected with very high concentrations of alkylated phenols in the highest treatment. This is unexpected and may be a result of release of particulate extract material being released during ultrasound treatment when generating the solution.

Table 6: Concentrations of different component groups in the stock solutions prepared for low, medium and high PW concentrations.

	Stock-Low	Stock-Medium	Stock-High
TEM	62,23864045	116,260524	1619,492925
Sum SVOC	0,575156868	7,051567772	899,1059405
Sum Naphthalenes	0,229025473	0,202931931	10,41883657
Sum 2-3-ring PAH	0,186330486	1,278205472	19,213311
Sum Fluorenes	0,021356348	0,024092364	2,904350649
Sum Phenanthrenes	0,087126688	0,14388329	4,664367795
Sum Dibenzothiophenes	0,060679003	1,089702917	10,76198769
Sum 4-6-ring PAH	0,058722239	0,969508421	28,02275504
30 ab hopane	0	0	0
Phenol	0	0	0
Sum C1-phenols	0	0	1,015910285
Sum C2-phenols	0,013879606	0,197548564	1,872387673
Sum C3-phenols	0,02126789	0,072365267	2,57648878
Sum C4-phenols	0	0,041204152	25,82566722
Sum C5-phenols	0	0,075352233	142,6333929
Sum C6-phenols	0	0,090929006	118,4712598
Sum C7-phenols	0	0,248357222	15,98586814
Sum C8-phenols	0	0,037479624	1,445094046
Sum C9-phenols	0	0	0,80294881
Sum phenols	0,035147496	0,763236067	310,6290177

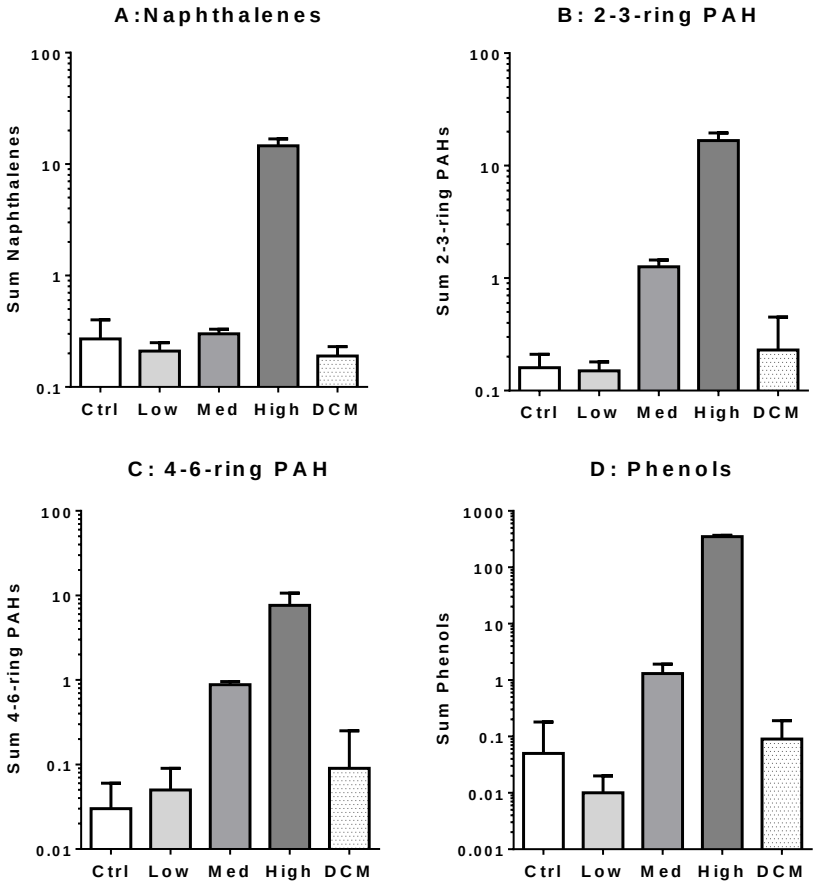

Fig. 13: Concentrations of different component groups in water samples taken from the exposure vessels at sampling. Data displayed are average concentrations ± SD (N=6 throughout).

3.4.2 PAH body burden

Uptake of PAHs was observed above background level for the medium and high concentrations used. The PAH body burden levels were very low compared to other experiments performed in our lab (Hansen, Salaberria et al. 2015; Nordtug, Olsen et al. 2015). Nevertheless, a temporal increase in body burden was observed (Fig. 14).

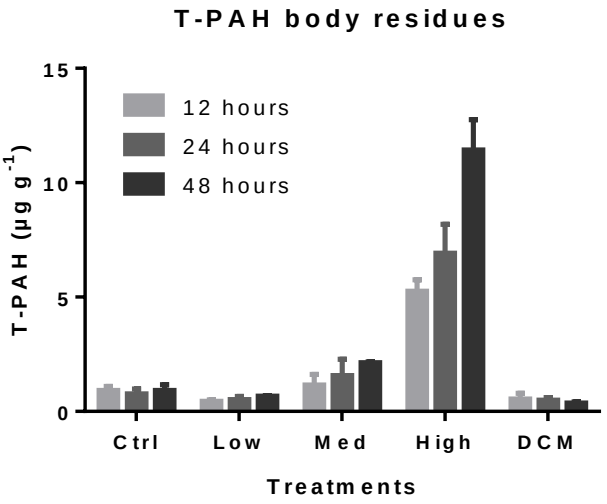

Fig. 14: Concentrations of T-PAH in copepods after exposure to three concentrations of reconstituted PW at three times (12, 24 and 48 hours). Data displayed are average concentrations ± SD (N=4 throughout).

3.4.3 Gene expression

Expression of a total of 23 different genes were analyzed, and only a handful displayed dose-responses (Fig. 15). GST displayed a good dose-response even after 12 hours of exposure in line with previous findings in our lab (Hansen, Nordtug et al. 2009; Hansen, Altin et al. 2011; Hansen, Altin et al. 2013). An oil-mediated reduction in the expression of CYP330A1 has also been shown previously (Hansen, Nordtug et al. 2009). Based on this experiment and depending on the availability of homogenous samples (based on developmental stage), the following genes should be analyzed: GST-2, CYP330A1, HSP70B, FABP, PLA2 and EcdR. EcdR and CYP330A1 is highly dependent on developmental stage and lipid content, respectively, and should for environmental monitoring be used with caution in field-sampled copepods.

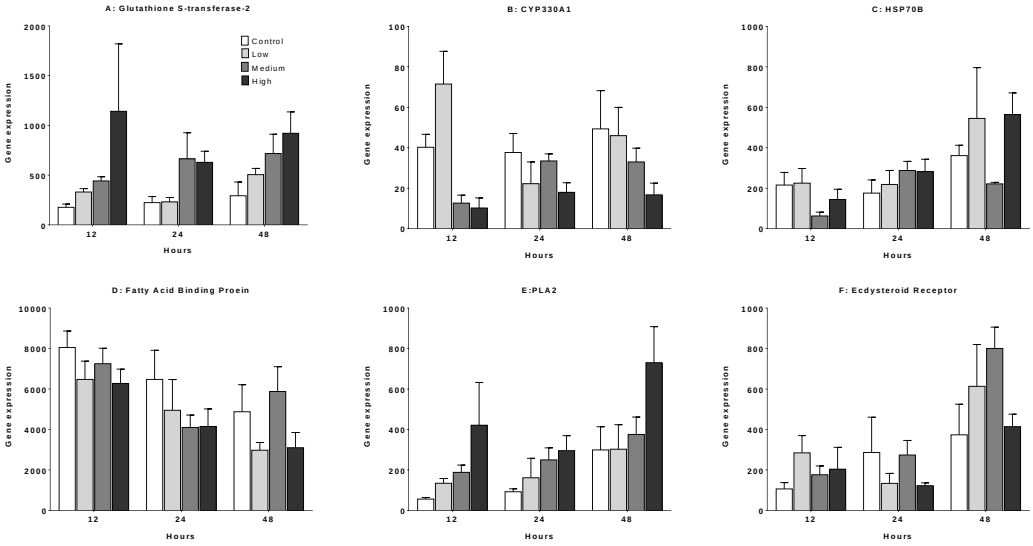

Fig. 15: Selected genes analyzed for expression in copepods exposed to produced water. A: Glutathione S-transferase. B: Cytochrome P450 330A1. C. Heat-shock protein 70B. D: Fatty acid binding protein. E: Phospholipase A2. F: Ecdysteroid Receptor.

3.4.4 Metabolite profile and expression

Analysing copepod extracts using NMR provided information about metabolite concentrations in copepods as a response to exposure over time (Fig. 16). Six amino acids were observed to vary in expression as a function of exposure (alanine, leucine, isoleucine, phenylalanine, tyrosine and valine). In general, these amino acids were upregulated at the highest concentration after 12 hours and upregulated at the two highest exposure concentrations after 48 hours. Interestingly, the middle time point resulted in no significant responses. Lack of significant differences may be caused by one of the samples (one replicate in the medium 24 hours) being an outlier.

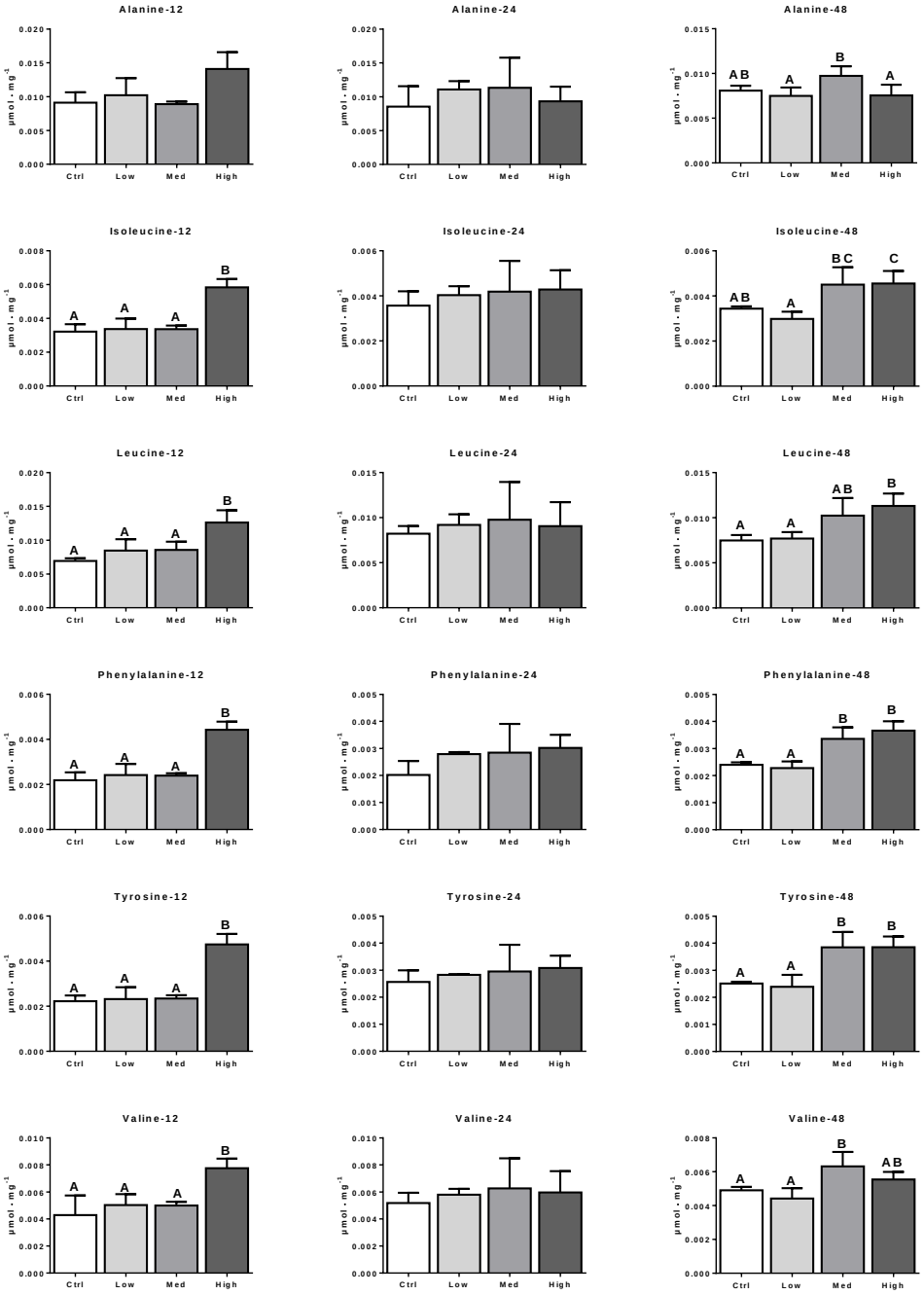

Fig. 16: Concentrations of selected amino acids in copepods after exposure to PW extracts over time (12, 24 and 48 hours) and at different concentrations (low, medium and high).

3.5 'Realistic' produced water exposure scenario

3.5.1 Exposure medium

Reconstituted PW extracts were analysed using GC-MS and provided a component profile. As described for the other experiments, the majority of components were alkylated phenols, specifically C3-C6-phenols (Fig. 17). Since we observed measurable concentrations of very low-solubility components in the solution, we can be relatively certain that the initial solution contained particle fractions of the dried extract. This was probably broken off from the bottom of the bottle during ultrasound treatment. Concentrations of these components are rapidly reduced, e.g. hopane is not detected at the first water sampling point (24 hours).

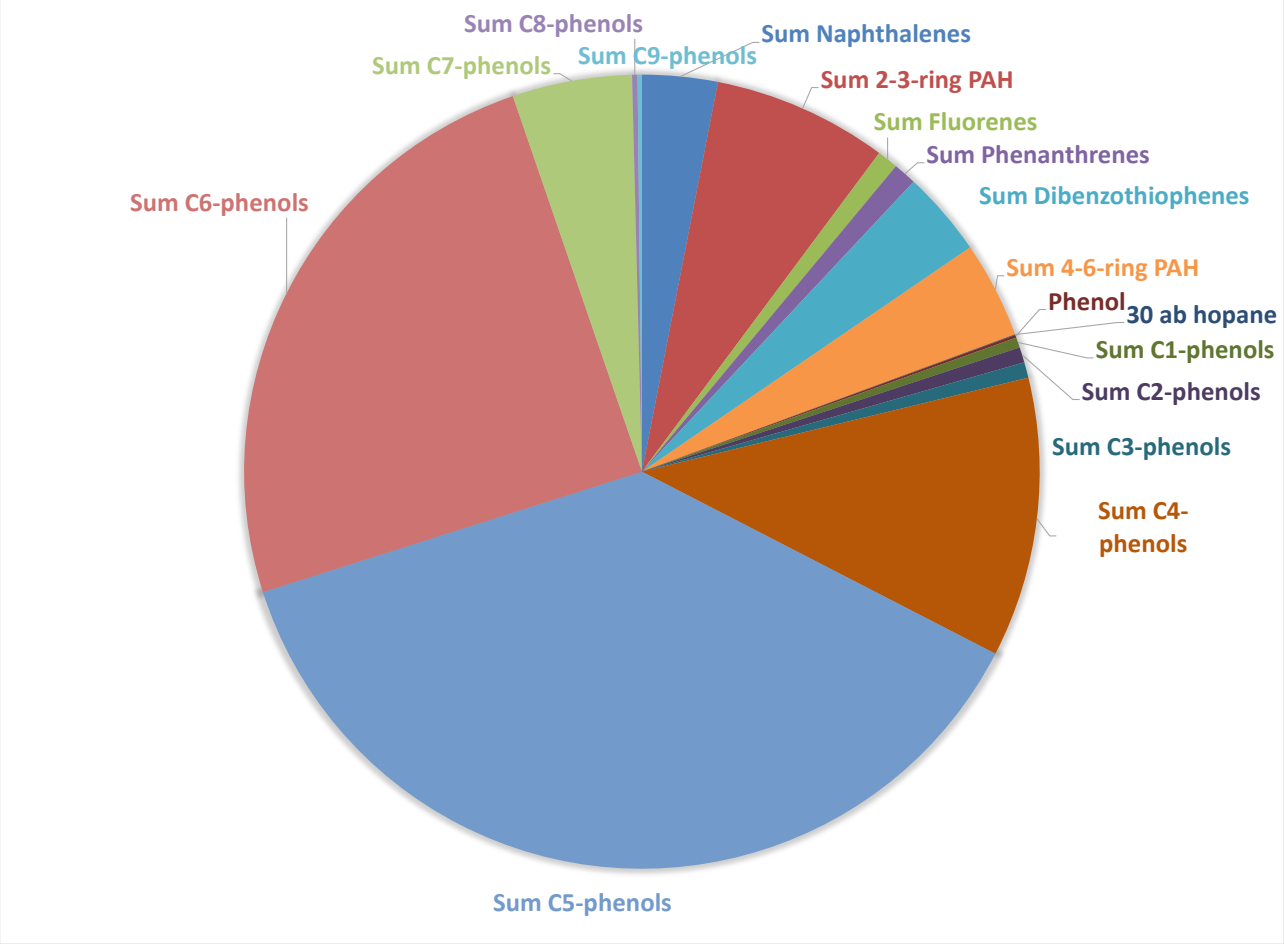

Fig. 17: Component composition in the reconstituted extract used for the 'realistic exposure scenario'.

We also observed a very nice exponential decrease in all components during the experiment (Fig. 18). This was expected due to the constant dilution of the initial solution with clean seawater.

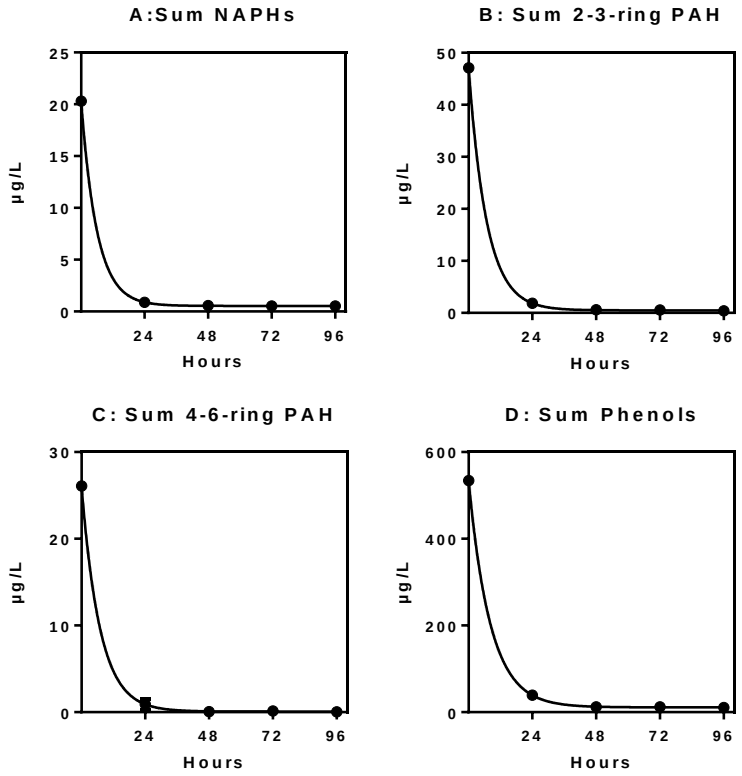

Fig. 18: Concentrations of different component groups in the water at different time points during the 'realistic PW exposure scenario'. Results are displayed as mean ± SDEV (N=4).

3.5.2 PAH body residues

PAH body burden was analyzed in copepods after 24 and 96 hours exposure; both times displaying concentrations above background level (Fig. 19).

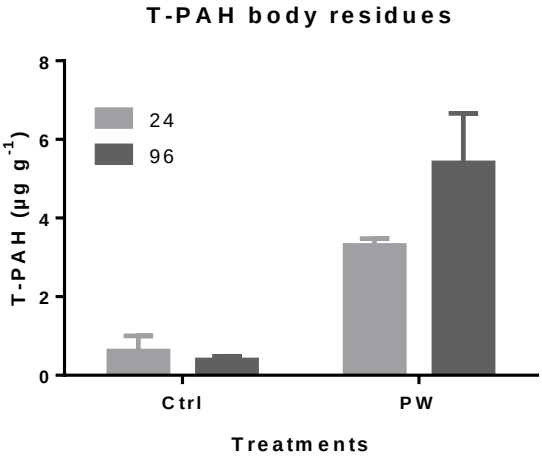

Fig. 19: T-PAH concentrations in copepods sampled from controls and PW-treatment 24 and 96 hours into the exposure scenario. Results are displayed as mean ± SDEV (N=4).

3.5.3 Gene expression

Expression of a total of 23 different genes were analyzed, and a selection of the responsive genes are plotted in Fig. 20. GST-2 was responsive after 24 hours, but decreased to control-levels at 96 hours. Both lipid metabolism-related genes FABP and ELOV displayed lower expression in exposed copepods compared to controls.

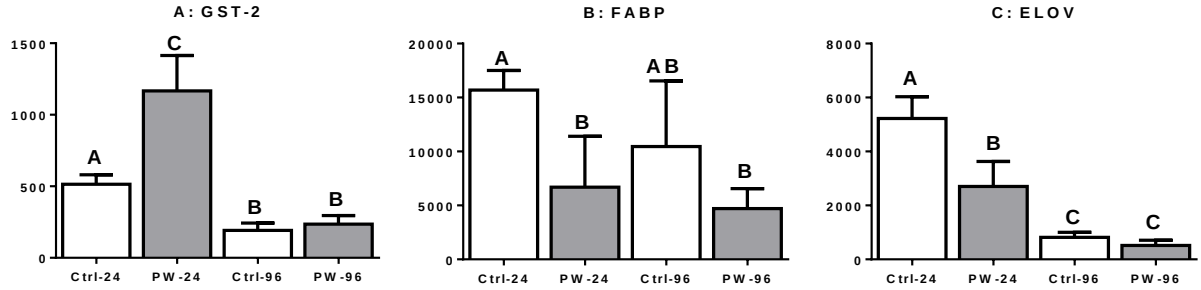

Fig. 20: Expression of GST-2, FABP and ELOV in copepods treated to a decreasing concentration of PW over a period of 96 hours. Different letters denote significant differences in expression between treatments.

3.5.4 Metabolite profiling

Very few metabolites deciphered from the NMR spectra displayed any change as a function of exposure and/or time. In fact, only sarcosine displayed significant differences between controls and exposed copepods (Fig. 21).

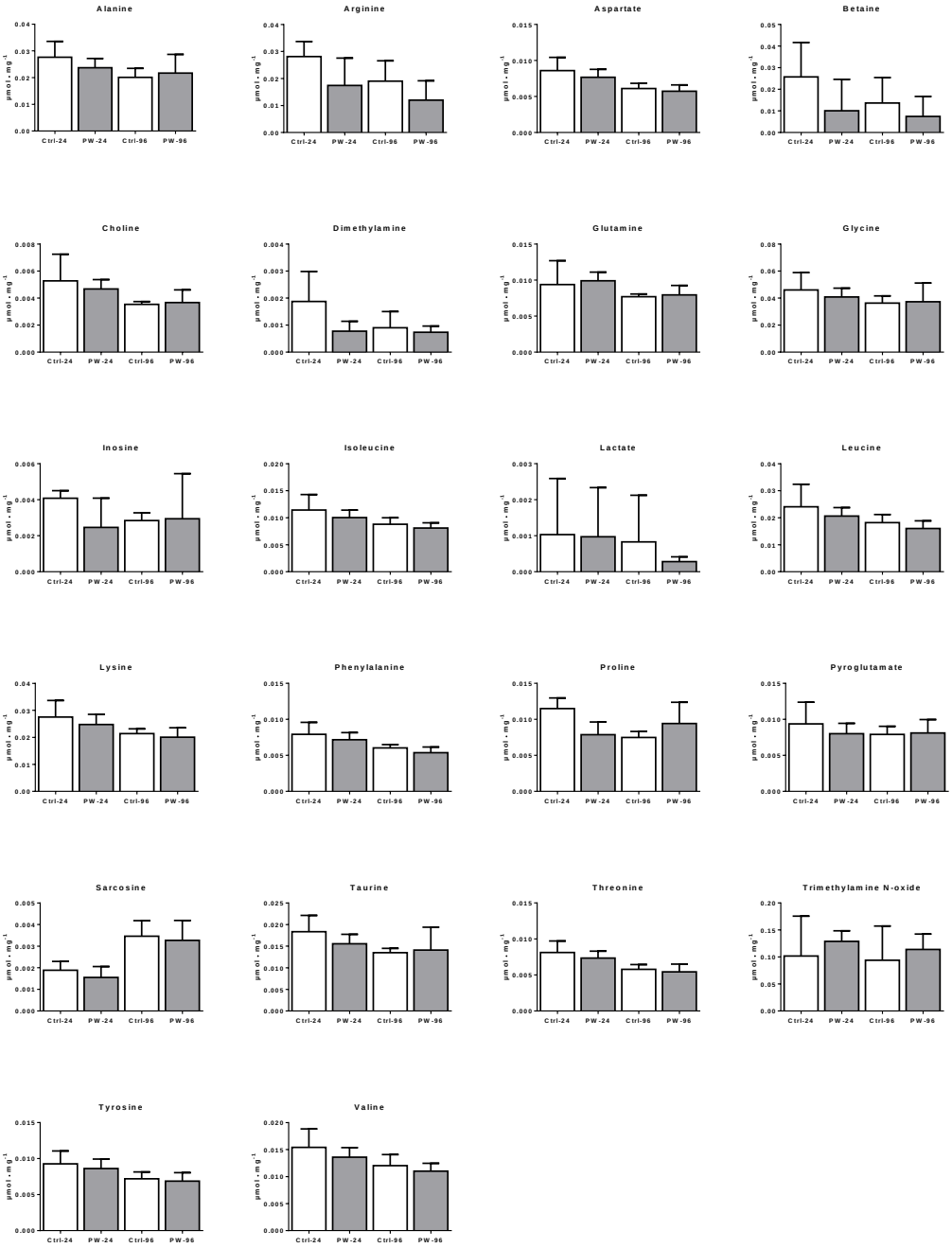

Fig. 21: Metabolite concentrations in copepods sampled after 24 and 96 hours of exposure to an exponentially declining concentration of reconstituted PW extract. Results are displayed as mean ± SDEV (N=4).

4 Recommendations for field sampling during WCM-2017

This project aimed at establishing methods for evaluation of potential effects of produced water on copepods in a field situation to be used during water column monitoring in 2017. Below is suggested tasks for performing field work in 2017.

Task 1: Fieldwork
Zooplankton samples should be collected by the use of a 1000 µm standard mesh size in 4 different stations (3 in the Tampen area and 1 reference site). Live copepods from net hauls will be studied under a microscope in order to distinguish between species and developmental stages. Samples of copepods will be separated based on species/stage so that the samples will be as homogenous as possible. The aim is to collected individuals at stage CV/CVI of *Calanus* spp. based on size. A pool of 50 copepods will be used for PAH body burden and metabolomics analyses. These samples will be frozen immediately in liquid nitrogen and transported to the lab for analyses. In addition, 20 copepods will be pooled and used for gene expression analysis; they will be stored in RNA later at +4 °C or frozen directly in liquid nitrogen. A batch of sample from each station will be fixed in ethanol for determination of species and stage. A batch of copepods from each site will also be studied for PAH exposure using fluorescence microscopy tuned for fluoranthene. A subset of sample will be transferred in cooling container live to SINTEF Sealab if fluorescence microscopy is proven impossible to do on the boat.

Task 2: Copepod analyses
Analyses of sampled copepods are summarized in Table 7, and a more detailed methodology is given in Section 2.

Table 7: Zooplankton analysis.

Parameter	**Method**	**Number of individuals/samples,**
PAH exposure	Fluorescence microscopy	24 individuals from each station (4 stations)
Extraction and PAH body burden	Microextraction and GC-MS, to determine presence and concentration of >40 PAHs in biota	12 samples from each station. Each sample containing 50 pooled copepods of homogenous stage/species
Metabolite profile analyses	Nuclear magnetic resonance (NMR)	12 samples from each station. Each sample containing 50 pooled copepods of homogenous stage/species
Gene expression	Extraction, cDNA-syntheses, dd-PCR , 5 stress genes will be analysed in RAN-extracts from copepods using Digital Droplet PCR	12 samples from each station. Each sample containing 25 pooled copepods of homogenous stage/species
Species and stage determination	Light microscope analyses	1 sample from each station containing a pool of individuals

Task 3: Data treatment and reporting
Results obtained in the WCM 2017 will be compared to data from controlled laboratory experiments where defined effects have been studied. Stress gene expression data from ddPCR will be subjected to normalization procedures and compared to data from laboratory experiments to predict effects on adverse outcome pathways, e.g. impacts on growth, development, endocrine system and lipid metabolism. These data will also be combined with data on endogenous metabolites from the metabolomics-analyses using NMR and/or MS. Multivariate statistics will be performed on datasets where appropriate.

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