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Report

Body burdens of PAHs and alkylated phenols in copepods in the Ekofisk region

Water column monitoring 2021

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ABSTRACT
This report summarizes the findings from the Water Column Monitoring in April 2021 where zooplankton (Calanus finmarchicus) were collected and sampled around the Ekofisk region in the North Sea. Extraction and analyses of polycyclic aromatic hydrocarbons and alkylated phenols in copepod were determined to provide data for comparison to DREAM-MER simulations.

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1 Background and objective

To assess potential impacts of produced water discharges on zooplankton communities, the water column monitoring (WCM) campaign in 2017 conducted field sampling of the copepod *Calanus finmarchicus* in the Tampen region. Subsequent analyses of body burden of polycyclic aromatic hydrocarbons (PAHs) complemented with a suite of biomarkers was performed. Significant differences between study sites were found for PAH body residue profiles and several biological endpoints (Hansen et al. 2020). *Calanus* copepods are ubiquitously distributed pelagic organisms with a complex life history, and although they perform active migrations (diel vertical migrations and overwintering in the deep) (Hirche 1996), they are also transported with currents. This means that they may be subjected to diluting produced water plumes *in situ*. Limited data exists on PAH body burdens in copepods sampled in the marine environment, but some information exist from studies where copepods have been exposed to produced water components in controlled laboratory conditions (Hansen et al. 2017).

The DREAM-MER tool can dynamically predict body burdens of produced water components in organisms surrounding point sources of produced water discharges (Nepstad et al. 2021). Comparing data from such simulated discharge scenarios and field samples is necessary input to discuss model results. The aim of this project was to provide data to compare field samples with model simulations. Sampling of copepods from the Ekofisk area was performed during the WCM survey in 2021, and copepods were analysed for body burdens of produced water components (PAHs and alkylated phenols), lipid content and species determination.

2 Methodology

2.1 The cruise and stations sampled

The sampling stations were chosen in a transect from the Ekofisk installation based on DREAM simulations (ST1-ST5). A map is given in in Figure 1, and the specific coordinates are given in Table 1. In addition, two additional sampling sites were selected. Egersundbanken (ST6) was chosen as reference station as this is far away from oil fields with active production. Vikingbanken (ST7) was chosen as a potential contaminated site as it is downstream the Tampen region which has extensive oil and gas activities.

Table 1: Stations sampled during the Water Column Monitoring 2021.

Station	Location	Sample	startTime	endTime	startLat	startLon	endLat	endLon
ST1	Ekofisk	435	2021-05-12 T18:34:10.059Z	2021-05-12 T19:00:10.661Z	56.59896	3.502252	56.59955	3.506553
ST2	Ekofisk	434	2021-05-12 T14:25:48.423Z	2021-05-12 T14:59:54.053Z	56.55303	3.084343	56.55427	3.083233
ST3	Ekofisk	433	2021-05-12 T12:03:27.866Z	2021-05-12 T12:34:33.589Z	56.6002	2.99925	56.60227	2.99635
ST4	Ekofisk	432	2021-05-12 T04:43:39.676Z	2021-05-12 T05:12:25.096Z	56.70175	2.896542	56.70284	2.891887
ST5	Ekofisk	431	2021-05-12 T02:38:11.818Z	2021-05-12 T02:45:48.067Z	56.89978	2.698598	56.89908	2.697328
ST6	Egersundbanken	436	2021-05-13 T08:34:44.395Z	2021-05-13 T09:23:11.828Z	57.85674	5.527285	57.84409	5.408518
ST7	Vikingbanken	437	2021-05-14 T11:05:12.056Z	2021-05-14 T11:33:50.864Z	60.41564	2.541578	60.41565	2.536008

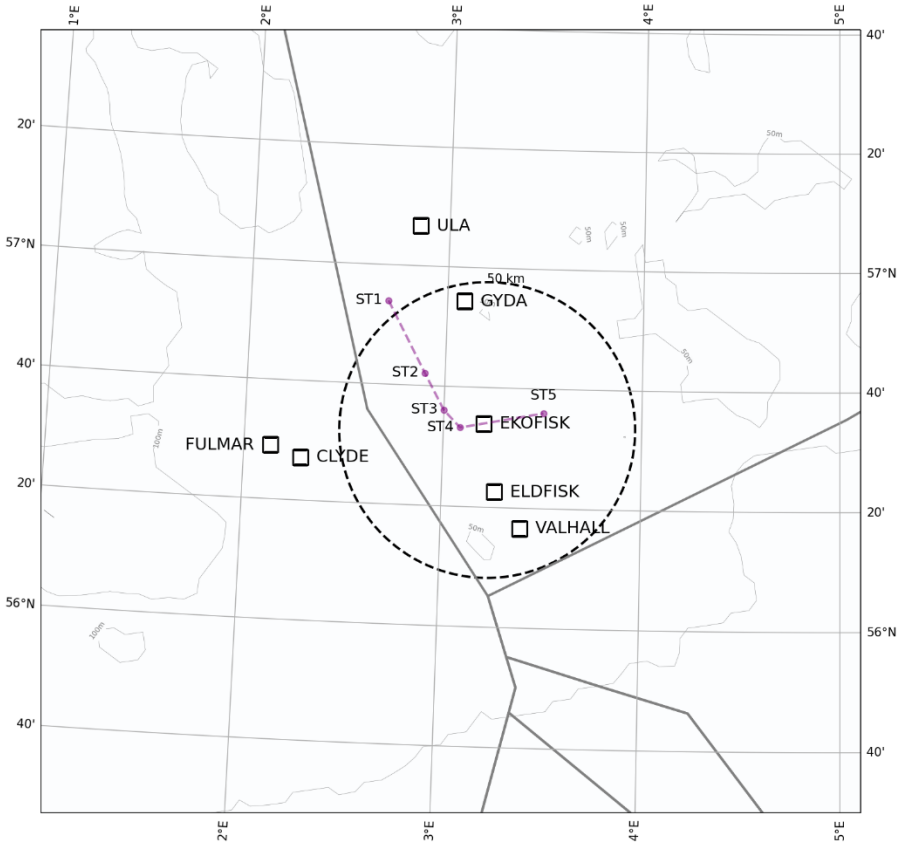

Figure 1: Map of the sampling sites close to the Ekofisk platform (ST1-5).

2.2 Copepod sampling

Zooplankton samples were collected from the upper 100 m using vertical net hauls with a 180µm mesh size WP2 plankton net with a sealed cod end at five stations in the vicinity of the Ekofisk installations (ST1-5), at Egersundbanken (ST6) and at Vikingbanken (ST7). Live copepods from net hauls were studied under a dissecting microscope onboard to provide homogenous samples for analyses of *Calanus* spp. The aim was to collect individuals at stage CV/CVI of *Calanus* spp. A pool of 50 individuals were used for produced water component (PAHs and alkyl-phenol) body burden and lipid analysis, respectively, and 8 replicate samples (of 50 individuals) for each analysis were collected from each site. Unfortunately, few copepods were collected from net hauls in ST7, and samples for PAH and alkyl-phenol analyses were prioritized (no lipid content analyses, and species determination were possible at this site). Samples were frozen immediately on dry ice and transported to the lab for analyses. A representative batch of live copepods from each site were also transported to the laboratory for image documentation and species determination. Table 2 gives an overview of samples and analyses.

Table 2: Summary of the analysis performed on the collected copepod samples.

Parameter	Method	Number of individuals or samples
PAH and alkyl phenol body burden	GC-MS/MS	8 samples (pool of 50 individuals) from each station
Lipid content	Folch method	8 samples (pool of 50 individuals) from each station
Species determination	PCR method	108 individuals from each station

2.3 Species determination

Live copepods were transported alive to the laboratory at SINTEF Sealab where they were imaged individually, labelled and shipped to Nord University for species determination. 108 copepods were imaged from each of stations ST1, ST3, ST4, ST5 and ST6. Unfortunately, due to limited amounts of copepods found at ST2 and ST7, no individuals were available for species determination at these locations. Species identification of each *Calanus* specimen was performed following the protocol described in detail by Choquet et al. (2017). In short, DNA was extracted from the two antennules of each individual (544 individuals in total) using a HotShot-based protocol (Montero-Pau et al. 2008). DNA was then used as a template for the amplification of six molecular markers of the type Insertion-Deletion (Smolina et al. 2014), multiplexed in one PCR reaction per individual. The resulting amplified fragments were sized using a 3500xL Genetic Analyzer (Applied Biosystems) to establish the genotype of each individual and determine the species.

2.4 Lipid analyses

Total lipid content was determined in pooled samples (50 copepods) following a modified Folch extraction (Folch et al. 1957). Samples (50 copepods per sample) were homogenized in chloroform-methanol (2:1 v/v, 4 mL) using UltraTurax, centrifuged (2,000 rpm, 10 minutes) and the supernatant was collected. NaCl (0.9 % in MilliQ-water, 1 mL) was added, and the sample centrifuged (2,000 rpm, 5 minutes). The organic phase was isolated and evaporated to dryness. The weight of the total extracted lipid was recorded.

2.5 Extraction and PAH and alkyl-phenol analyses

Extraction of tissue samples and analyses of PAHs and alkyl-phenols was performed as previously described (Sørensen et al. 2016; Sørensen et al. 2017; Oppegård et al. 2020), with minor modifications. The biota samples were transferred to Kimax tubes, and the accurate wet weight was registered and used for quantification. Dichloromethane (DCM)-*n*-hexane (1:1, v/v, 4 mL) were added for extraction and sodium sulphate (Na_2SO_4) was added to remove water. Surrogate internal standards (25.22 ng naphthalene-*d*8, 4.8 ng phenanthrene-*d*10, 5.0 ng chrysene-*d*12, 5.08 ng perylene-*d12* and 2.554 µg phenol-*d*6) were added. An IKA T10 basic Ultra Turrax fitted with an IKA S10N-5GA stainless steel knife was used for homogenisation followed by centrifugation (2,000 rpm, 2 minutes). The supernatant was transferred to a new Kimax tube and reduced to approximately 1 mL at 35°C under a gentle stream of N_2. Lipids were removed by Gel Permeation Chromatography (GPC). Samples (0.5 mL) were injected on an Agilent 1260 infinity system fitted with an Agilent 1260 Fraction Collector using constant flow (5 mL/min) of 100% DCM. A Waters Envirogel™ GPC Clean-up column (19 x 300mm) fitted with a Waters Envirogel Guard column (15µm, 4.6 x 30mm) was used for separation, and the analyte fraction was collected from 10.5-15 minutes. The cleaned extract was reduced at 35°C under gentle stream of N_2. Recovery internal standard (10 ng fluorene-*d*10) was added prior to analysis.

Analysis of the samples was performed using an Agilent 7890 B GC gas chromatograph (GC) coupled to an Agilent 7010 B triple quadrupole MS. Samples (1 µL) were introduced at 310°C in pulsed splitless mode. Separation was achieved using two serial coupled Agilent HP-5MS UI columns (30 m length, 0.25 µm film thickness and 0.25 mm internal diameter). The carrier gas was helium at a constant flow of 1.2 mL/min.

For analysis of the PAHs the column oven temperature was programmed at 40 °C (1.5 min hold), ramped by 40 °C/min until 110 °C, 6 °C/min until 220 °C and 4 °C until 325 °C (5 min hold). For analysis of phenols the column oven temperature was programmed at 40 °C (1.5 min hold), ramped by 40 °C/min until 110 °C, 6 °C/min until 300°C and 40 °C/min until 325 °C (1 min hold). The transfer line temperature was 300 °C, the ion source temperature 230 °C and both quadrupoles at temperature 150 °C. The ion source was operated in MRM mode at 70 eV, with a solvent delay of 9 minutes.

Analytes were identified by two representative MRM transitions (Sørensen et al. 2016; Sørensen et al. 2017; Oppegård et al. 2020), and quantified using the most abundant response. After normalization to the response of internal standard (fluorene-*d*10), parent compounds were quantified using quadratic regression in Masshunter Quantititative Software, while alkylated PAHs were quantified using the relative response factor of the corresponding methyl PAH.

The following target analytes were quantified: Benzo(b)thiophene, C0-C4-naphthalenes, biphenyl, acenaphthylene, acenaphthene, dibenzofuran, C0-C3-fluorenes, C0-C4-phenanthrenes/anthracenes, C0-C4-dibenzothiophenes, C0-C3-fluoranthenes/pyrenes, benz(a)anthracene, C0-C3-chrysenes, 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, 2-methylphenol, 4-methylphenol, 4-ethylphenol, 2,4-dimethylphenol, 3,5-dimethylphenol, 4-n-propylphenol, 2,4,6-trimethylphenol, 2,3,5-trimethylphenol, 4-n-butylphenol, 4-tert-butylphenol, 4-isopropyl-3-methylphenol, 2-tert-butyl-4-methylphenol, 4-n-pentylphenol, 4-tert-butyl-2-methylphenol, 4-n-hexylphenol, 2,5-diisopropylphenol, 2,6-diisopropylphenol, 2-tert-butyl-4-ethylphenol, 6-tert-butyl-2,4-dimethylphenol, 4-n-heptylphenol, 2,6-dimethyl-4-(1,1-dimethylpropyl)phenol, 4-(1-ethyl-1-methylpropyl)-2-methylphenol, 4-n-octylphenol, 4-tert-octylphenol, 2,4-di-sec-butylphenol, 2.6-di-tert-butylphenol, 4-n-nonylphenol, 2-methyl-4-tert-octylphenol, 2,6-di-tert-butyl-4-methylphenol and 4,6-di-tert-butyl-2-methylphenol

2.6 Statistical analyses

Statistical analyses comparing data on obtained variables (copepod lipid content and body burdens) for different locations were conducted using One-way ANOVA followed by Dunnett's multiple comparisons test (for data showing normal distribution) and with Kruskal-Wallis followed by Tukey's multiple comparisons test (for data not showing normal distribution) using GraphPad Prism version 9.4.1 for Windows, GraphPad Software, San Diego, California USA.

3 Results and Discussion

3.1 Species determination

Of the 544 copepods sampled for species determination, all were *Calanus finmarchicus* except for one single copepod, which was *C. helgolandicus*. This copepod was sampled at station ST05 and represents less than 1% of the sampled copepods (total 108 copepods) at this station *C. helgolandicus* is very similar in size and lipid content to *C. finmarchicus* (Figure 2), so it is not expected that a small portion of this species in the pooled samples should interfere with results observed on PAH and alkyl-phenol body burden. Approximately 90 % of the sampled copepods were at the copepodite V (CV) stage.

Figure 2: Images of *C. helgolandicus* (left) and *C. finmarchicus* (right) sampled at station ST05.

3.2 Total lipid content

Total lipids (TL) were analysed on a wet weight basis on copepod samples taken from ST1-ST6. Unfortunately, we were not able to find enough copepods in the net hauls in ST7, so no information on total lipids could be determined at this station. The highest TL content was found in sample ST1 (16.2 ± 0.8 %), and the lowest in ST2 (10.2 ± 1.9 %). TL content in sample ST1 was significantly higher (One-way ANOVA, $p<0.05$) than all other stations, and ST2 was also significantly lower ($p<0.05$) in TL content compared to all stations except ST5 (Figure 3).

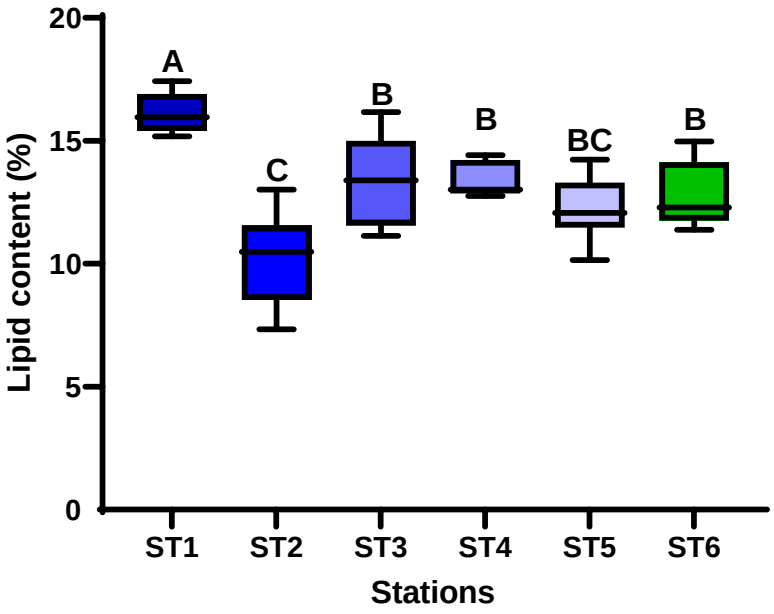

Figure 3: Total lipid content (% of wet weight) in copepods sampled at six different stations in the Ekofisk region in the North Sea. Significant differences (One-way ANOVA, Tukeys multiple comparison test, $p<0.05$) between stations are displayed as different letters.

3.3 Body burdens of PAHs

PAH body burden data are given in Figure 4A, where we have plotted the sum of 43 PAHs for all stations. Due to loss of one sample from ST5, only 7 samples were available from this station, yielding a total of 55 samples for analyses. No significant differences ($p>0.05$) were found between copepods sampled at the five Ekofisk stations (ST1-ST5), and we only found significant differences (Kruskal-Wallis test, $p<0.05$) between ST3 (Ekofisk) and ST7 (Vikingbanken), where the latter, considered a location heavily contaminated by produced water discharges from the Tampen region, was elevated.

The concentrations in individual samples ranged 56.8-934.5 ng/g, and the coefficient of variation (CoV) was large within stations, ranging from 28.27 % (ST4) to 82.78 % (ST5). Due to high variation within stations, we found no clear pattern in PAH body burdens in relation to the location vicinity to the Ekofisk platforms.

Compared to the 2017 campaign where copepods were sampled around the Statfjord platforms (Hansen et al. 2020), the PAH body burdens at Ekofisk were lower (Figure 4B). Data from the two water column monitoring campaigns in 2017 (Statfjord) and 2021 (Ekofisk) represent the only existing data sets available where extensive PAH profiles have been determined in copepods sampled in the environment. They are still limited data sets, so it is difficult to draw strong conclusions regarding trends in relation to produced water exposure. The PAH body burden levels observed may be within the range of natural background for copepods, but more data is needed on PAH body burdens in copepods from different locations to establish a natural background level.

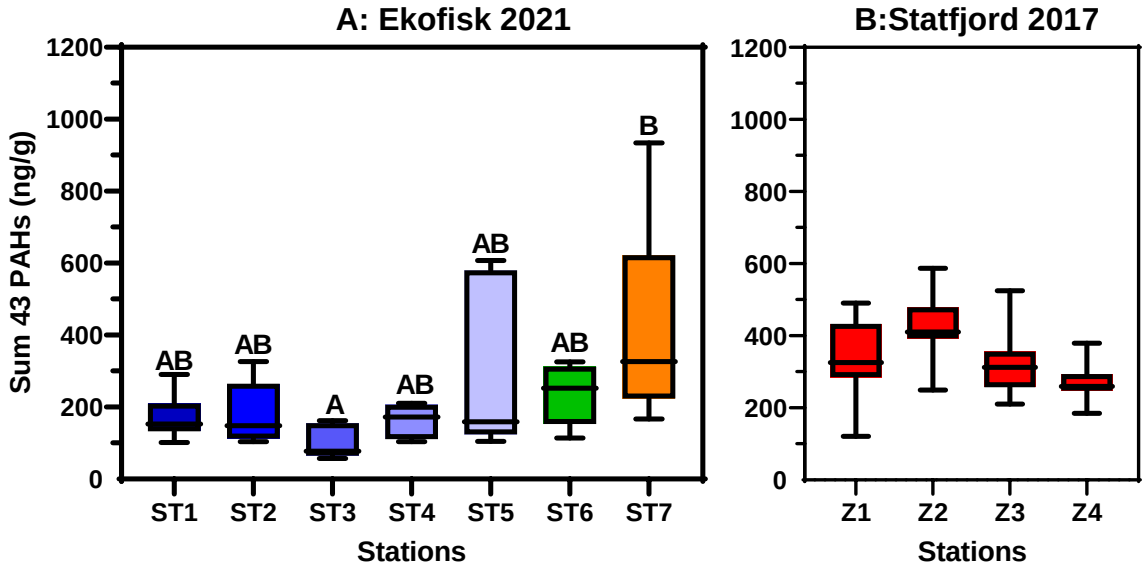

Figure 4: Body burdens of 43 PAHs in copepod samples sampled close to A) Ekofisk (ST1-ST5, blue), at the reference station (ST6, Egersundbanken, green) and Vikingbanken (ST7, orange) during the water column monitoring campaign in 2021, and during the 2017 water column monitoring at Statfjord. Significant differences (Kruskal-Wallis followed by Tukey's multiple comparisons test, $p<0.05$) between stations from the 2021 campaign are displayed as different letters.

In a field survey where lumpfish embryos were used for environmental monitoring (Hansen et al. 2022), PAH body burdens were found within a range of 10–752 ng/g, which is within the range found in copepods in the current work. In the lumpfish study, lumpfish exposed in a contaminated harbour area for 17 days displayed PAH body burdens of 523 ± 165 ng/g (Brattørkaia) and 291 ± 79.2 ng/g (Nyhavna). Embryos exposed in Brattørkaia also displayed higher fractions of developmental deformations. In the same study,

measured PAH body burdens at reference stations from Trondheimsfjorden and at the coast of Trøndelag were lower, with 84.1 ± 75.7 ng/g and 13.6 ± 3.3 ng/g, respectively. The PAH body burdens found in zooplankton in the current study are higher than the levels observed in lumpfish from the reference stations. Comparisons between copepods and lumpfish embryos should, however, be done carefully. The lumpfish embryos contain lower lipid levels than *C. finmarchicus*, and vertebrates are also more effective in biotransformation of PAHs than invertebrates (Hahn et al. 2017). Studies have shown that even short-term petrogenic exposure of copepods result in long-term PAH accumulation due to distribution to their internal lipid storage causing slow depuration and subsequent prolonged internal exposure to oil components (Hansen et al. 2016; Hansen et al. 2018; Øverjordet et al. 2018). The lipid rich properties of copepods (discrete lipid reservoir) appear to reduce their sensitivity to acute effects like narcosis and mortality (Hansen et al. 2011; Hansen et al. 2013). No biomarkers of effect were measured in the sampled copepods in the 2021 campaign, but in the 2017 campaign, lipidomics analyses showed site-specific profiles for acyl-glycerols and wax esters indicating a potential for produced water components interfering with lipid metabolism in copepods (Hansen et al. 2020).

Laboratory studies have shown toxicity to copepods at PAH body burden several orders higher than the range observed in field sampled copepods. Impacts on copepod feeding activity and reproduction was observed at PAH body burden levels in the range 100–300 µg/g wet weight following a 4-day exposure to dispersed crude oil (Hansen et al. 2015; Nordtug et al. 2015). In these experiments, oil droplet exposure probably caused high PAH body burdens as they filter oil droplets and oil droplets also adhere to copepod surfaces. Approximately 1/3 of the PAH body burden in dispersion-exposed copepods were attributed to oil droplet adhesion and filtration (Hansen et al. 2018). Body burdens of approx. 130 µg PAH/g in female ovulating copepods did not cause effects on the offspring in terms of development and stress gene expression, whereas a concentration of 210 µg/g did (Hansen et al. 2017). It therefore seems unlikely that the much lower body burdens found after produced water exposure in the field should cause reproductive effects and influence recruitment of copepod populations, but this needs to be verified. It is also important to emphasize that when these copepods accumulate lipophilic produced water components, the lack of biotransformation ability and slow depuration increases the likelihood of transfer to predator species (Agersted et al. 2018).

The composition of PAHs in copepods may indicate timing of exposure. 43 PAHs were analysed in a total of 55 samples during the 2021 water column monitoring campaign, and naphthalene, biphenyl, C_0-C_2-fluorenes, phenanthrene, C_3-phenanthrene, dibenzothiophene, C_0-C_2-fluoranthenes/pyrenes and C_0-C_1-chrysenes were detected in all samples analysed. Figure 5 shows PAH body burdens separated into ring numbers. These PAHs represents a wide range in partitioning coefficient (K_{ow}). Low K_{ow} compounds, like parent 2-rings (as naphthalene and biphenyl), indicate that exposure was recent as these components are rapidly eliminated through passive diffusion in clean sea water. High Low K_{ow} compounds, like fluoranthenes and chrysenes (4-rings), tend to bioconcentrate as they eliminate at a lower rate, suggesting prolonged exposure time. 4-ring PAH levels were somewhat lower than 2-3 ring PAHs, but still suggest prolonged exposure to these PAHs. The levels of 5-ring PAHs were orders of magnitude lower than most of the 2-4 ring PAHs, and 6-ring PAHs were detected only in a few samples (Figure 5). Individual PAH composition in copepods from the different locations is given in Appendix A (Figure S1).

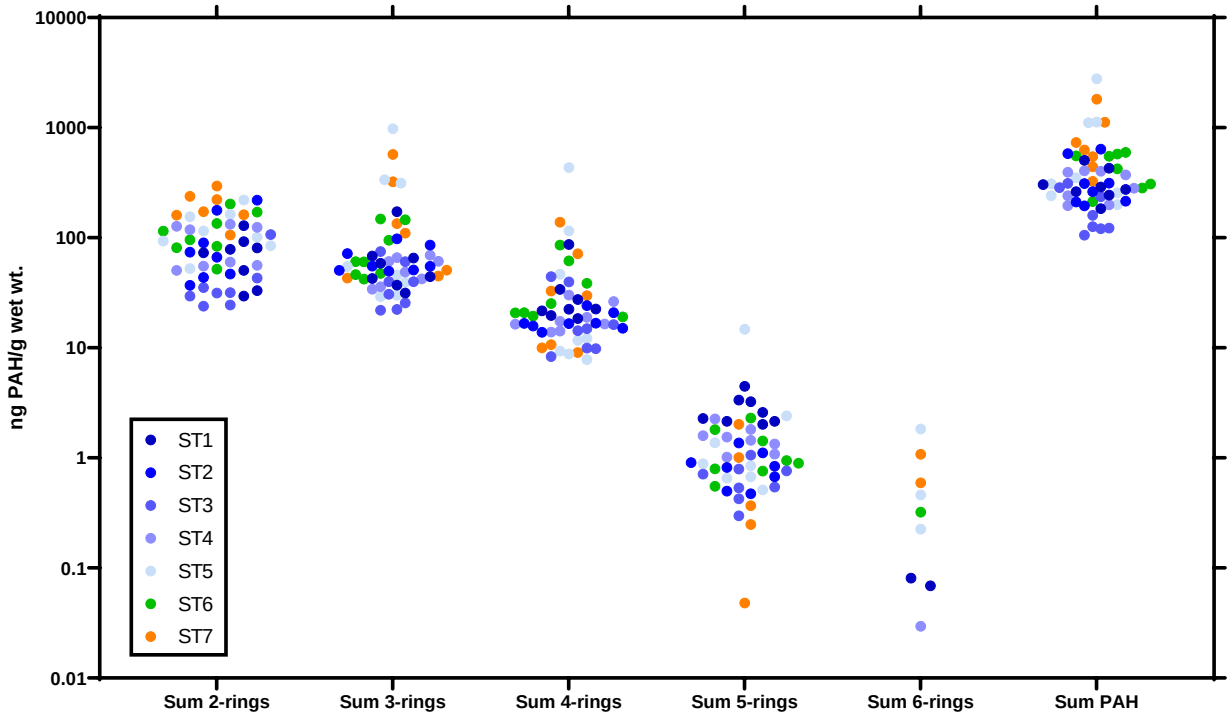

Figure 5: Summed concentrations of 2-6-ring PAHs in copepods sampled close to Ekofisk (ST1-ST5, blue), at the reference station (ST6, Egersundbanken, green) and Vikingbanken (ST7, orange).

3.4 Body burdens of alkylated phenols

The levels of alkylated phenols (sum of 30 alkyl-phenols) were much lower than for the sum of PAHs, ranging 0-299.2 ng/g, with average values ranging from 7.4 to 32.6 ng/g at stations ST2 and ST5, respectively (Figure 6). There were no clear trends in the data in relation to exposure to produced water discharges from Ekofisk. Copepods sampled at ST3 (Ekofisk) had in fact significantly lower body burden than the reference station (ST6) (Kruskal-Wallis test, $p<0.05$) and these two groups were the only ones displaying significant differences. The variation within location was very high for some replicates (as high as 230 % for ST2), suggesting highly variable exposure history for copepods sampled even at the same station. Of the 55 samples analysed for alkyl-phenol body burdens, the most abundant alkyl-phenols were 4-*n*-butylphenol (detected in 47 samples) and 4-*n*-pentylphenol (detected in 30 samples).

The water column campaign of 2021 was the first time alkylated phenols were measured in *C. finmarchicus* sampled *in situ*, so there is no data to compare with, but the facts that the body burden levels of alkylated phenols were generally low, displaying large within-station variations, and that the highest levels were found at the reference station, indicate that uptake of these components may not be associated with produced water exposure. To the authors knowledge, the only comparable data set showing detailed alkyl-phenol body burdens in marine animals collected in the wild is from lumpfish embryos exposed in different locations in the Trøndelag region (Hansen et al. 2022). In that study, sum alkyl-phenol body burdens after 17-19 days exposure at different locations ranged from 37.4 ± 29.8 ng/g to 165.3 ± 64.0 ng/g. The highest concentrations were found in lumpfish embryos incubated in Trondheimsfjorden which may have been subjected to riverine runoff or wastewater from a nearby wastewater treatment plant. Fish embryos incubated in the contaminated

harbour areas, where high PAH levels were found, did not show elevated alkyl-phenol body burdens (20.6 ± 3.7 ng/g).

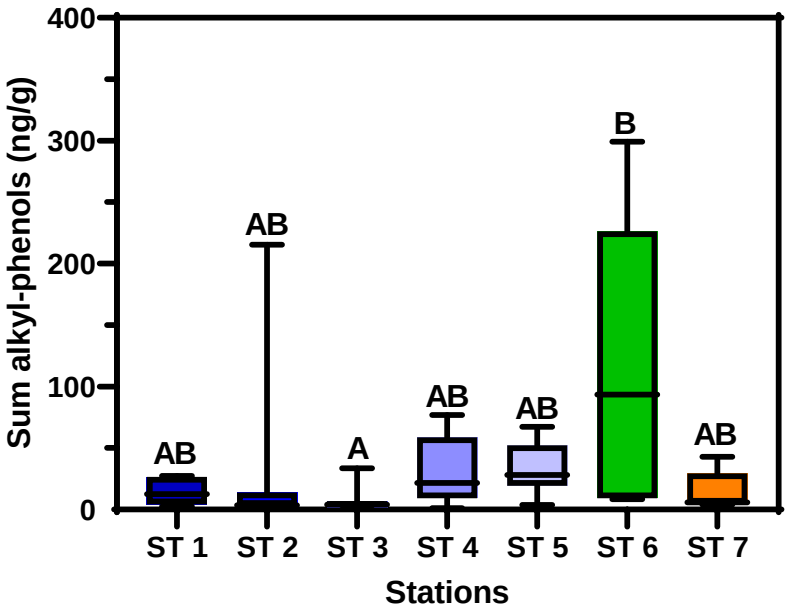

Figure 6: Body burdens of 30 alkylated phenols in copepod samples sampled close to Ekofisk (ST1-ST5, blue), at the reference station (ST6, Egersundbanken, green) and Vikingbanken (ST7, orange) during the water column monitoring campaign in 2021. Significant differences ($p<0.05$) between stations from the 2021 campaign are displayed as different letters.

4 Conclusions

Concentrations of 43 PAHs and 30 alkylated phenols were measured in copepods sampled at seven different stations. 5 stations were in the vicinity of the Ekofisk platform, one station presumed to be a reference station (Egersundbanken) and one station potentially influenced by produced water from other platforms in the Tampen region (Vikingbanken). As *C. finmarchicus* are lipid rich copepods with a low ability to metabolize PAHs, they can accumulate lipophilic contaminants over time, which may facilitate food chain transfer. No clear pattern in body burdens in copepods were observed at sampling stations around Ekofisk. The PAH body burden was higher in copepods sampled at Vikingbanken, but significantly lower concentrations were observed only for one of the Ekofisk stations. Body burdens of alkyl phenols were lower than for PAHs, and the highest body burden of alkylated phenols were found at the reference station. There is reason to believe that there are additional sources of PAHs and alkylated phenols exposing copepods collected in the selected stations. Copepods are pelagic and will be transported in the water column over large areas, and it needs to be verified if the levels detected in the sampled copepods can be considered natural background levels. This is needed to enable a proper assessment of the potential contribution from produced water discharges to copepod body burdens. Potential toxicological impacts of exposure (e.g. biomarkers) were not assessed in the present work, but the PAH body burdens found were lower than body burdens associated with toxicity in laboratory studies.

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Appendix A: Individual PAH concentrations in copepods sampled during 2021 water column monitoring

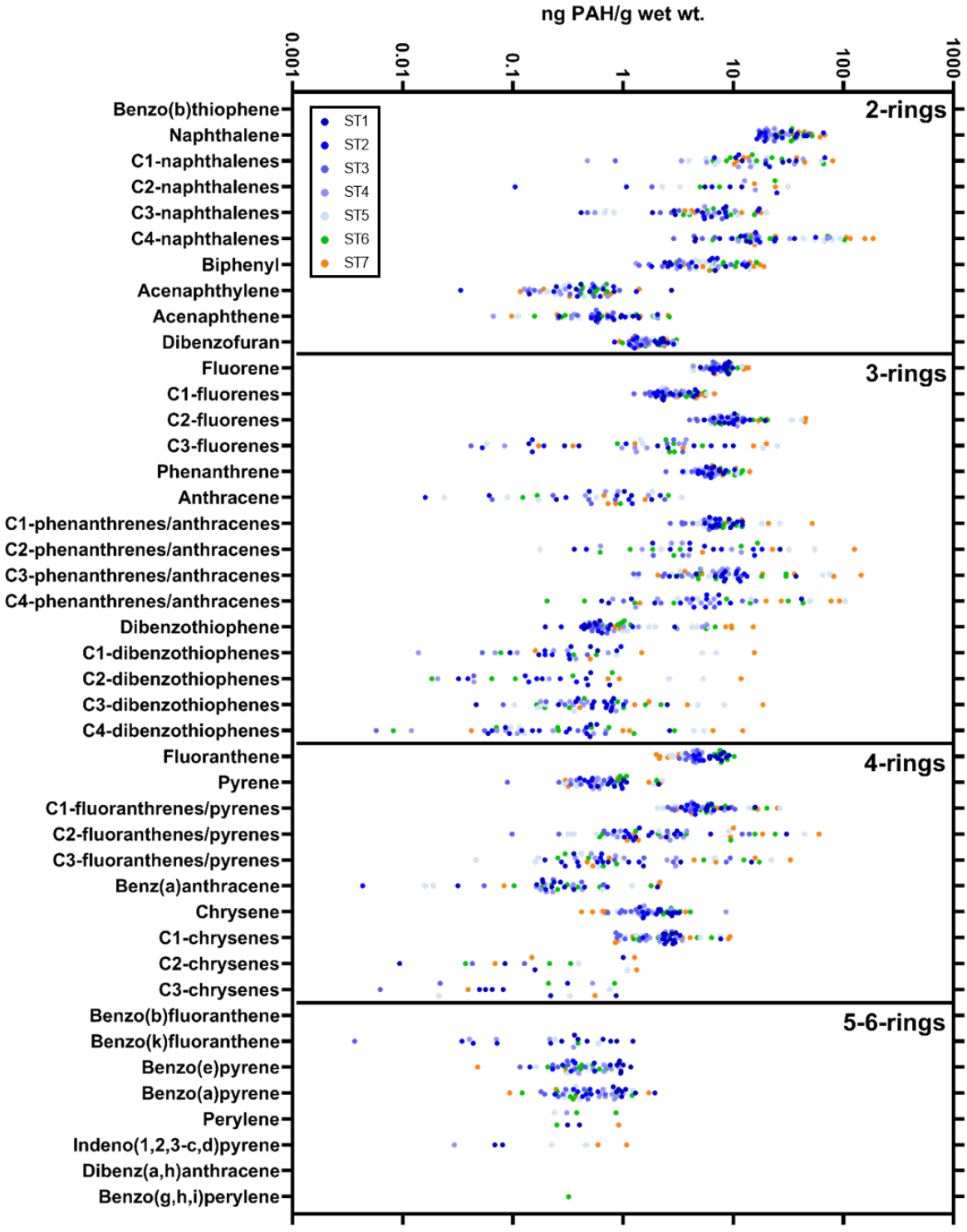

Figure S1: Individual PAH concentrations in copepods sampled close to Ekofisk (ST1-ST5, blue), at the reference station (ST6, Egersundbanken, green) and Vikingbanken (ST7, orange).