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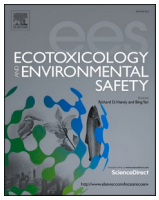

The impact of exposure timing on embryo mortality and the partitioning of PAHs when cod eggs are exposed to dispersed and dissolved crude oil

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ABSTRACT

During sub-sea oil spills to the marine environment, oil droplets will rise towards the sea surface at a rate determined by their density and diameter as well as the vertical turbulence in the water. Micro-droplets (< 50 μm) are expected to have prolonged residence times in the water column. If present, pelagic fish eggs may thus be exposed to dispersed oil from subsurface oil spills for days, and the contribution of these micro-droplets to toxicity is not well known. The purpose of this work was to investigate to what extent timing of exposure and the presence of oil micro droplets affects PAH uptake and survival of pelagic Atlantic cod eggs. A single batch of eggs was separated in two groups and exposed to dispersions and corresponding water-soluble fraction at 3–7 days (Early exposure) and 9–13 days (Late exposure) post fertilization. Partitioning of PAHs between crude oil microdroplets, water and eggs was estimated as well as the contribution of oil droplets to PAH body residue and acute and delayed mortality. Timing of oil exposure clearly affects both the mortality rate and the timing of mortality. Even though the body residue of PAHs were lower when embryos were exposed in the later embryonic stage, mortality rate increased relative to the early exposure indicating that critical body residue threshold is stage specific. Although our results suggest that the dissolved fraction is the dominating driver for toxicity in cod embryos exposed to oil dispersions, crude oil micro droplets contribute to increased mortality as well.

1. Introduction

During the Deepwater Horizon incident in 2010, oil was released from the sea floor for a prolonged period of time (Camilli et al., 2012, 2010; Spier et al., 2013). The oil dispersed into the water column from the release site at the wellhead, and periodically this process was enhanced using chemical dispersants with the aim to disperse, dilute and prevent oil from reaching the surface and sensitive shoreline habitats along the Gulf of Mexico (GOM). Subsequently there was a concern for adverse effects on developing fish embryos potentially exposed to oil dispersions rising through the water column. Many pelagic fish species were spawning in the northern GOM in the months of April-July before containment of the damaged Mississippi Canyon 252 (MC252) wellhead (Muhling et al., 2012). The uniqueness of the spill scenario with oil dispersion plumes continuously rising towards the surface resulted in an exposure scenario where a spatial sub-surface overlap between developing fish embryos and rising oil droplets may have caused uptake of polycyclic aromatic hydrocarbons (PAHs) and subsequent toxicity to embryos inside the eggs.

From a multitude of studies during recent years it has become evident that the embryonic stage is the most oil-sensitive stage for fish, and even low oil exposure concentrations during the embryonic period can have devastating effects after hatch, suggesting delayed toxic responses to exposure (Carls et al., 2005; Carls and Rice, 1990; Sørhus et al., 2015). Exposure of early life stages of fish to oil components alter gene transcription connected of a wide range of potential adverse outcome pathways (Sørhus et al., 2017), cause cardiotoxicity (Carls et al., 2008; Hicken et al., 2011; Incardona et al., 2004; Nelson et al., 2016), physiological and morphological alterations (Carls and Rice, 1990; Kirby et al., 2019; Laurel et al., 2019; Mager et al., 2018; Pasparakis et al., 2019), altered lipid metabolism (Laurel et al., 2019; McGruer et al., 2019), developmental retardation (Carls and Thedinga,

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2010; Greer et al., 2019; McGruer et al., 2019), altered swimming behavior (Mager et al., 2018) and increased mortality rates (Carls et al., 1999; Nordtug et al., 2011b). These documented effects have primarily been correlated to dissolved oil components (PAHs), and only a limited number of studies have isolated the potential contribution from dispersed oil droplets to toxicity in early fish life stages (Carls et al., 2008; Hansen et al., 2019b; Nordtug et al., 2011b; Olsvik et al., 2011, 2010). Transcriptional profiling of exposed cod larvae suggested low contribution from oil droplets to induction of genes responsive to oil exposure (Olsvik et al., 2011, 2010). Exposure of zebrafish (*Danio rerio*) embryos suggested that the contribution of oil droplets to embryo toxicity was limited (Carls et al., 2008). Survival probability and food assimilation in first-feeding Atlantic cod (*Gadus morhua*) larvae was not significantly impacted by particulate oil droplets (Nordtug et al., 2011b).

Developing cod embryos inside the eggs are hypoosmotic to sea water and thus dependent on the integrity and low permeability of the chorion to preserve water and remain in osmolytic balance. Possibly due to the impermeability of the chorion of the egg, fish embryos bioaccumulate dissolved oil components significantly less than hatched fish larvae, likely leading to higher hydrocarbon body residue in the latter when exposed to dissolved oil components (Carls and Rice, 1988). Fish embryos develop impermeability due to a cortical reaction within 60 min after fertilization (Davenport et al., 1981), suggesting that for a short period of time, embryos may be more susceptible to PAH bioaccumulation, but this has, to the authors' knowledge, never been studied. Furthermore, adhesion of oil droplets to the surface of fish eggs have been demonstrated, but the potential toxic implications of these oil droplets remain controversial. This may be due to species differences in egg surface properties affecting the amount of oil associated with the eggs (Brannon et al., 2006; Hansen et al., 2018; Sørhus et al., 2015).

Laboratory testing to characterize oil toxicity is poorly standardized and often suffer from limited chemical characterization of exposure medium (Hodson et al., 2019; Nordtug and Hansen, 2021). In the current study we applied an oil droplet generator to produce oil dispersions with a defined concentration and droplet size range connected to a flow-through exposure system where cod eggs were continuously exposed to stable concentrations of dispersions or filtered dispersions (droplet-free WSF). Using this system to expose cod embryos at two different developmental stages, we assessed partitioning of PAHs between oil, water and cod eggs and determined the differential contribution of dissolved and particulate (micro-droplets) oil phases to observed toxicity in fish embryos exposed at different stages of embryogenesis.

2. Methods

2.1. Cod embryos

Fertilized eggs from Atlantic cod (*Gadus morhua*) were collected from breeding tanks at the National breeding center for cod (Tromsø, Norway) and sent by courier in a temperature-stabilized container within 24 h after fertilization. After arriving at the test laboratory, the eggs were temperature acclimated before transferred to an aerated 100 L tank with natural sea water (exchange rate approx. 200 ml/min) in a temperature-controlled room (5.2 °C ± 0.4). No disinfectant treatment was applied during the handling of the embryos. Seawater used in the experiment was continuously supplied from 70 m depth in the Trondheimsfjord, filtered through a sand filter and matured for approximately 2 days. Sea water entering the culture system and the exposure system was further temperature controlled with heat exchangers and air equilibrated in a gas exchange system.

2.2. Experimental design

The oil used for the experiments was a naphthenic crude oil (Troll) artificially weathered using one step distillation procedure at 150 °C (Stiver and Mackay, 1984). The method for generating oil dispersions and the technical details are given in Nordtug et al. (2011a). A "stock" dispersion of nominal 10 mg oil/L with mean volumetric oil droplet size 10–14 μm (Supporting information Fig. S5) was diluted in-line with seawater at dispersion-to-water ratios of 1:1 (5 mg/L), 1:5 (1 mg/L) and 1:25 (0.2 mg/L) by computer-controlled pulsing of 3-way solenoid valves. At each dilution step, 50% (average 15.7 ml/min) of the diluted dispersion was fed directly to 4.5 L exposure containers and the remaining 50% was filtered to separate the WSF into a parallel series of identical exposure containers (Supporting Information, Fig. S1). The filtration device consisted of a custom-made filter inside a glass container (250 ml) containing loosely packed fine glass wool (10 g) on top of a Whatman Grade GF/C and GF/F Glass Microfiber Filters (Whatman Ltd., Maidstone, UK) with particle retention 1.6 and 0.7 μm, respectively (Supporting Information, Fig. S2).

Results from this experiment has already published previously, focusing primarily on sub-lethal effects related to fish development, thus, the experiment has been described in detail before (Hansen et al., 2019b), but it is rewritten here for coherency. One single batch of cod eggs were divided into two groups, and the groups were subjected to 4 days of exposure at two different developmental stages. The first group (Early exposure) was exposed during the gastrula stage (3–7 days post fertilization - dpf), when the first cell differentiation, tissue layers, and embryo axis is formed. The second group (Late exposure) was exposed after the gastrulation and closure of blastopore (9–13 dpf), when major organogenesis and neurulation occur in the embryo (Gorodilov et al., 2008) (exposure timeline given in Supporting Information, Fig. S3). During both exposure periods, embryos were exposed to a concentration series of oil dispersions or to the corresponding water-soluble fractions (WSFs) obtained by removing the oil droplets by filtering at each concentration step. To simulate exposure in the water column, a net (300 μm Nitrex mesh) was positioned horizontally in each exposure container just below the water surface to keep the slightly positively buoyant cod eggs below the water surface layer preventing them from getting in contact with any surfaced oil droplets.

After exposure, the eggs were transferred to clean seawater and kept until 2–3 days post hatch when the experiment was terminated. Survival was measured at the end of the exposure (96 h) and after the recovery period (2–3 days post hatch), and PAH body residues were analyzed only after exposure (96 h). All treatments and negative controls (seawater only) were performed using four replicates (Supporting Information: Fig. S1). After the exposure period, the embryos were transferred to a recovery system consisting of a 0.5 L glass bottles with flow-through (approximately 25 ml/min), mean residence time 20 min) sea water containing a large (300 μm, 10 cm^2) mesh at the outlet and limited head space. The embryos/larvae were kept in this system until 2–3 days post hatch when the experiment was terminated.

2.3. Documentation of exposure

2.3.1. Chemical analyses

Water samples for chemical analyses of the exposure media were collected on day 1 and day 3 of the exposure period. Samples for chemical analysis (approximately 800 ml each) were collected at the outlet of the exposure containers and acidified with diluted hydrochloric acid. The water samples were liquid-liquid extracted with dichloromethane (DCM), dried over Na_2SO_4, and concentrated to 1 ml. Analyzes of total hydrocarbon content with carbon numbers from 10 to 36 (THC (C10-C36)) were performed on dichloromethane (DCM) extracts by Gas Chromatography–Flame Ionization Detector (GC-FID). Semi-volatile organic compounds (SVOC) including decalins, naphthalenes, and 3–5-ring PAHs in the same samples were performed by Gas Chromatography–Mass Spectrometry (GC-MS) operated in selected ion monitoring mode. Samples for analyses of volatile organic compounds (C5 -C9, VOC) were collected in 40 ml vials pre-added hydrochloric acid for

acidification and capped without headspace. The samples were analyzed by Purge and Trap Gas Chromatography/Mass Spectrometry, using a modified US Environmental Protection Agency EPA-Method 8260, with a 50 m (0.20 mm ID, 0.50 μm film thickness) Supelco Petrocol capillary column. Further details on all procedures for chemical analyses of water samples and equipment used are given in Supporting Material (S9).

2.3.2. Particle counting

Particle characterization of the exposure solutions were performed daily on a particle analyzer (Coulter counter Multisizer 3, Beckman Coulter Life Sciences, US) for monitoring of oil droplet concentration and size range in the exposure tanks. The Multisizer 3 was equipped with a tube with an aperture of 100 μm and a fixed sample volume of 1 ml was analyzed. All samples for measurements were collected in 25 ml polystyrene vials (Kartell) and the samples were analyzed immediately after sampling to avoid loss of oil droplets in the dispersions due to surfacing. The results were processed and plotted with the Beckman Coulter particle characterization software (Beckman Coulter inc. ver. 3.51, 2002 and ver. 4.01, 2008).

2.4. Sampling of cod eggs and larvae

Following exposure and recovery, eggs with dead embryos and dead hatched larvae were counted, respectively. From each exposure chamber, 20 eggs with living embryos from each replicate of every treatment were collected and pooled using a sieve and gently transferred into sterilized glass vials (10 ml). These were snap-frozen in liquid nitrogen and kept at − 20 °C until further extraction and analyses of PAH body residue.

2.5. Analyses of PAH body residue

Surrogate internal standards (SIS; naphthalene-d8, phenanthrene-d10, chrysene-d12, phenol-d6, 4-methylphenol-d8) were added to the samples prior to micro-extraction. The samples were weighed into screw-capped glass reaction vials (10 ml) with replaceable Teflon septa. To each vial 3 ml of potassium hydroxide (6.5%) in methanol (80%) and internal standards (SIS) were added. The mixture was heated for two hours in an ultrasonic bath at 80 °C to achieve saponification, followed by filtration and serial extraction with hexane (3 ×3 ml). The combined extracts were dried with anhydrous sodium sulphate and concentrated to approximately 0.5 ml using a Zymark Turbovap® 500 Concentrator. Cleanup of the extracts was performed by solid phase extraction using 3 ml columns containing 0.5 g normal phase silica packing (Superclean LC-Si, Supelco). The samples were eluted through the column with 3 × 2 ml of DCM:hexane (1:3). The purified extracts were concentrated to 90 μl in an insert GC vial insert and recovery internal standards (RIS; fluorene-d10, and acenaphthene-d10) were added in 10 μl of DCM immediately prior to GC-MS analysis. The target oil compounds that were included is listed in Supporting Material (S10).

2.6. Data processing and statistics

Mortality rates were calculated as the fraction of dead individuals per day relative to the initial population. Individuals sampled during the test period were assigned the same survival probability as the rest of the population. Statistical tests were made by GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) and indicated statistical variation of fractions (e. g. Fig. 5C) is calculated by error propagation (Ku, 1966).

3. Results and discussion

3.1. Composition of exposure solutions

The exposure concentrations of the dispersions ranged from approximately 0.15 mg THC/L in the low exposure to approximately 3 mg THC/L in the highest exposure for both experiments (Supporting Information: Fig. S4A (early exp.) and S4B (late exp.)). The THC of the WSF of the highest exposure groups were about 11% of the concentration of the corresponding dispersion. The average exposure concentrations based on oil volume were approximately 25% higher in Late exposure (Supplementary Information: Fig. S4C). The oil droplet size distribution based on volume remained the same in both experiments (10.97 ± 0.39 μm and 10.81 ± 0.27 μm, [avg. ± SD] Supplementary Information, Fig. 5). The average particle volumes recorded from the dispersion exposure ranged from 0.012 to 3.53 ppm and 0.012 – 4.34 ppm for Early exposure and Late exposure, respectively (Supplementary Information, Fig. S4C). Particle concentrations in the WSFs ranged from 0.09% to 0.26% of that of the highest dispersion concentrations and were not significantly different from the controls. Furthermore, there was no correlation with the concentration gradient of the parent dispersions, indicating that the oil droplets were retained by the filtering process.

The distribution of oil components in the exposure solutions was similar in the two experiments (early and late exposure), and the recorded values from Late exposure are shown in Fig. 1 for dispersions and WSFs (absolute concentrations in Fig. 1A and C; relative composition in Fig. 1B and D). The dominating fractions in the oil mass of the dispersions were unresolved material of THC (C5–C36) and mass (UCM) from the particle analyses measurements not accounted for in the GC-FID analysis (Fig. 1B). The analytically resolved fractions (VOC and SVOC) constitute 2–5% of the oil mass in the dispersions (Fig. 1B). The fraction of WSF relative to the parent dispersions increased with increasing dilution from about 10% of the THC at the highest concentration to about 25% in the lowest concentration (Supporting Material Fig. S4) corresponding to 7.5–15% of the total particle mass (Fig. 1C). The WSFs were dominated by volatiles at the highest concentration and the unresolved THC fraction (C5–C36) at the lowest concentration (Fig. 1D).

Fig. 2 displays the profile of the dominating resolved semi-volatile components excluding decalins. When the resolved PAHs of dispersions at different concentrations are scaled by the nominal dilution factor, it was evident that the PAH profiles are virtually identical at the three dispersion concentrations (Fig. 2A). This was expected since it reflects the chemical profile in the added crude oil and the sampling does not discriminate between particulate and dissolved oil components. A similar pattern was also observed for the more soluble components with low $LogK_{ow}$ such as naphthalene, biphenyl, acenaphthene and dibenzofuran, in the corresponding WSFs. However, for the WSFs, less soluble components with higher high K_{ow}, such as the alkylated phenanthrenes/anthracenes, fluoranthenes, dibenzothiophenes and fluorenes, increased in relative concentration in the highest dilutions. This is a direct consequence of depletion of lighter components in the oil phase as the dispersion dilutes and can be described by the principle of Raoults Law (Guggenheim, 1937) as a reduction of the mole fraction of the components in the oil (Sterling et al., 2003). This is a successive process where the most soluble components become depleted first as the oil: water ratio decreases because most of their mass is being transferred to the water compartment (Fig. 3). The depletion of the heavier components occurs at higher dilutions simply because they are less soluble so that their mole fraction in the oil is maintained at higher dilutions (oil: water ratio) due to their lower solubility. The low K_{ow} components in the WSF, such as naphthalene (N) and biphenyl (B), were diluted according to the measured dilution factor (Fig. 2B) whereas the concentrations of the high K_{ow} components, such as alkylated phenanthrenes (P2, P3), were almost the same in the three dilutions. This indicates that a new equilibrium between oil and water was established at each step in the dilution series.

3.2. PAH body residue and oil droplet adhesion to chorion

For comparisons of body residues, only PAHs recorded above the

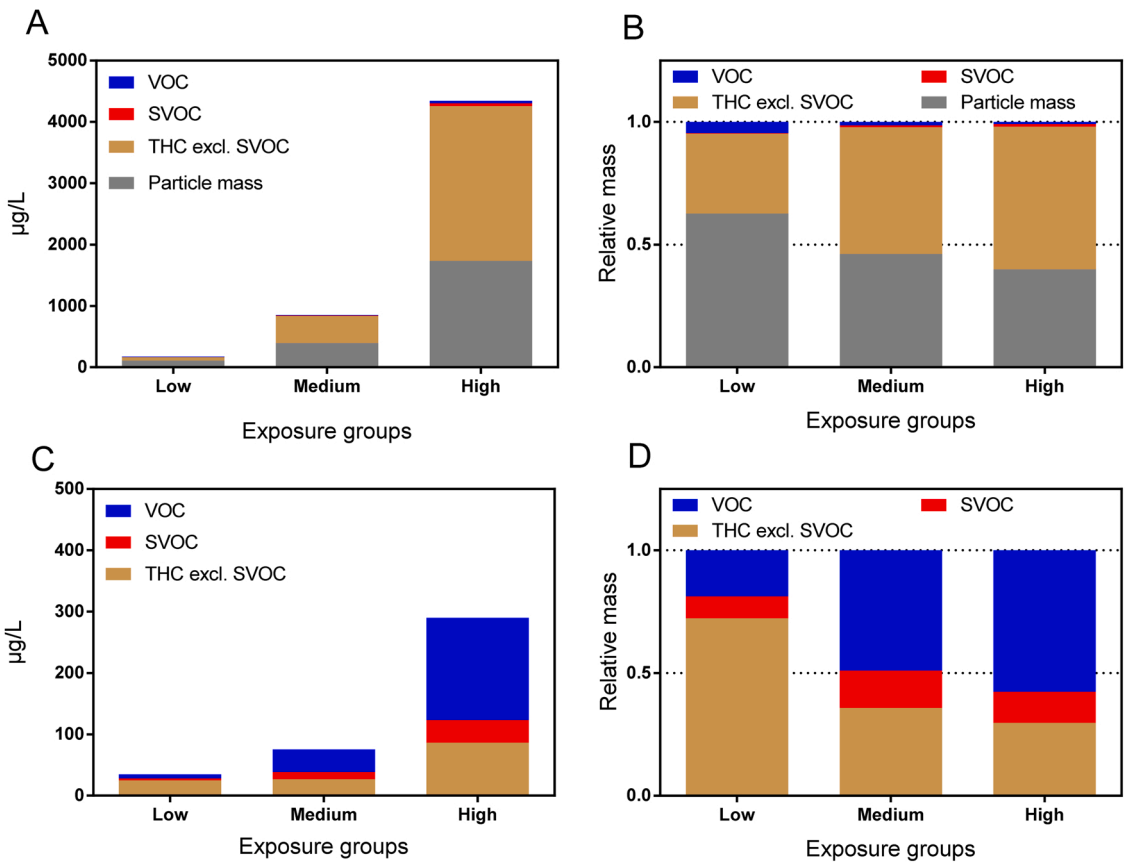

Fig. 1. Average concentrations and compositions of the exposure media (late exposure) sub-divided into VOC, SVOC, THC and unresolved mass. A) Concentrations in dispersions, B) relative composition of dispersions, C) concentrations in WSFs, and D) relative composition of WSFs. Note the 10-fold difference in the scale of the x-axis between A and C.

limit of quantification (25% of lowest standard calibration point of 0.01 or 0.02 µg/L) in all parallel groups were included. Thus, the sum of three groups of PAHs (C4-Napthalenes, C1- and C2-Fluorene) were selected for comparing body residue between exposure groups. The summed molar concentrations of these components are shown in Fig. 4A and clearly indicate that the body residue after the late exposure is lower than after the early exposure in the two groups. This was confirmed by comparing the body residue relative to water concentrations of components for the high exposure groups. In the late exposure to WSF, bioconcentrations was on average 70% (± SD = 15, range 41–87%) compared to the early exposure to WSF (Fig. 4). After exposure to dispersions a reduction of body residue to 58% (± SD = 10, range 42% and 70%) was observed for the late exposure groups compared to the corresponding early exposure groups.

Oil droplets attach to the surface of the embryos, as indicated by fluorescence images (Supplementary Fig. S6). The droplets appeared to be evenly distributed over the egg surface of the late exposure group whereas during the early exposure group only occasional droplets were observed at the highest exposures. According to fluorescence analysis, the oil content on the surface of eggs from the late exposure group at medium and high exposure concentrations is significantly higher, and in the range 5–30 times that of the early exposure group (Supplementary Fig. S6). Thus, the contribution from attached oil droplets to body residue is expected to be largest in dispersions of the late exposure groups, and this will inevitably cause an increase in the measured bioconcentration. From the current results, we conclude that there is a significant decrease in bioaccumulation rates of PAHs from the early to the late embryo stage in cod.

Potential explanations for the apparent reduced accumulation of PAHs from early to late embryo stages may be related to closure of the embryo membranes, reduced lipid content due to consumption of the developing embryo, or onset of enzymatic degradation of PAHs by biotransformation systems (Sørensen et al., 2017). Cod embryos contain about 1–2% of lipid based on wet weight (Finn et al., 1995; Tocher and Sargent, 1984) and a reduction in lipid content between the two developmental stages used in the exposure studies could be expected to affect the measured body residue. However, according to Finn et al. (1995) there is only a minor reduction in lipids between the two exposure periods and this alone cannot account for the measured reduction in uptake.

3.3. Partitioning between oil, water, and biota

Based on the basic assumption that the water-soluble fraction in the dispersion is comparable to the filtered WSF in the experiment, we can calculate the mass distribution of individual components between oil, water, and fish eggs at the end of the experiment. The content in the oil is the difference between the total concentrations of a component in the dispersion and the WSF, which should be constant throughout the experiment. Concentration of the accumulated component in the eggs increases with time and were analyzed only at the end of the exposure period. We do not expect the larger PAH body residues to be in equilibrium with the water within the 96 h exposure period. The biomass per liter of water is approximately 200 mg/L which is the biomass used in Fig. 5 that shows the relative mass in the three compartments in the dispersion exposure and correspondingly the distribution between water and egg biomass in the WSF exposure. The figure shows that in the dispersion, the relative proportion of the selected PAH in the biomass increases at higher dilutions. In the WSFs, the relative distribution between water and biomass is fairly constant, which is consistent with

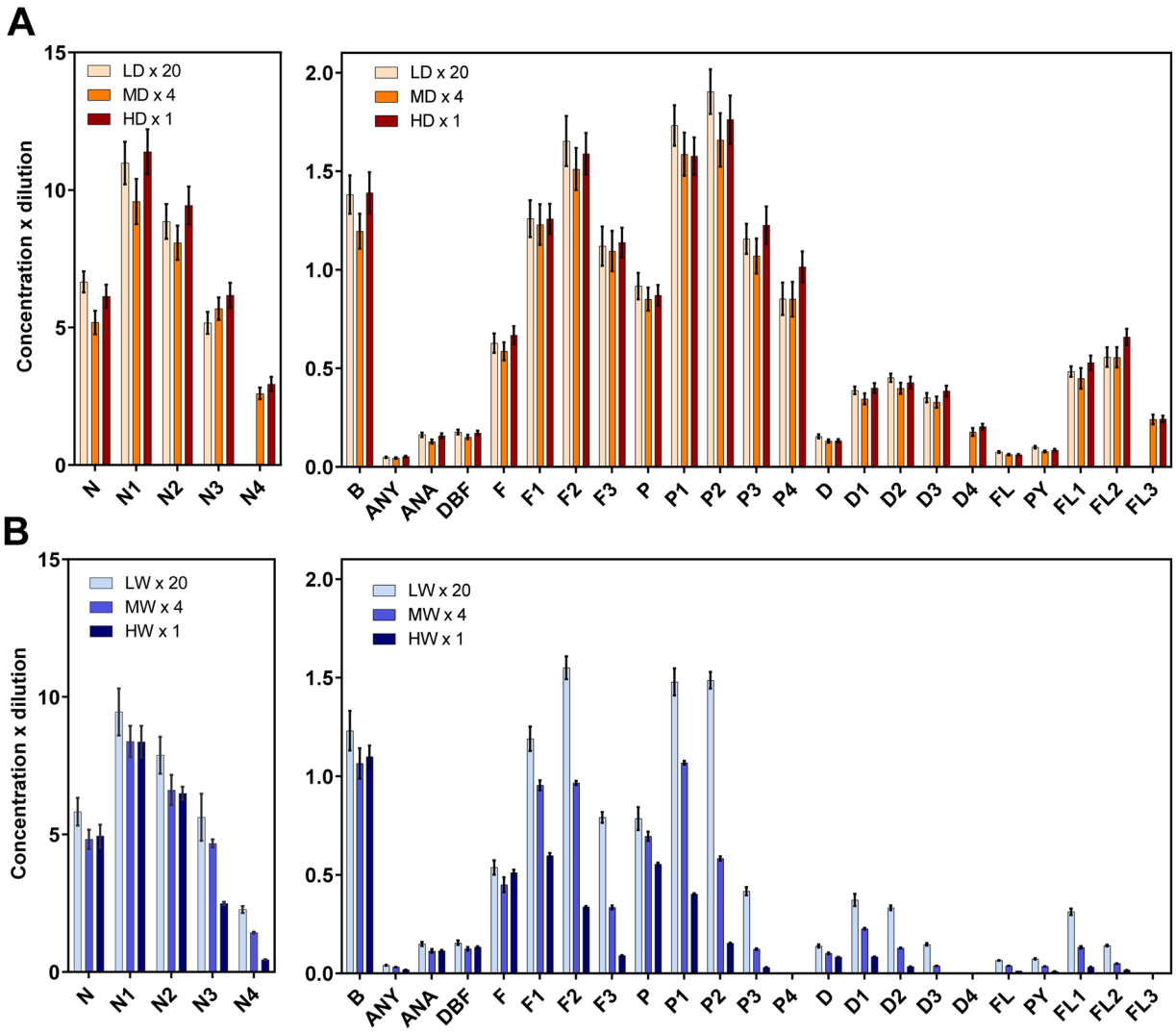

Fig. 2. PAH composition of the exposure solutions. A) Dispersions, B) Water soluble fractions. Y-axis shows actual concentration of the high exposure groups (HD, HW) whereas the other groups are scaled by multiplying with the dilution factor. Each column shows average value of 7–8 parallel samples with SEM indicated. (N–N4; C0–C4 Naphthalene, B; Biphenyl, ANY; Acenaphthylene, ANA; Acenaphthene, DBF; Dibenzofuran, F - F3; C0 – C3 Fluorene, P – P4; C0 – C4 Phenanthrenes/anthracenes, D – D4; C0 – C4 Dibenzothiophenes, FL; Fluoranthene, PY; Pyrene, FL1 – FL3; C1 – C3 Fluoranthenes/pyrenes.

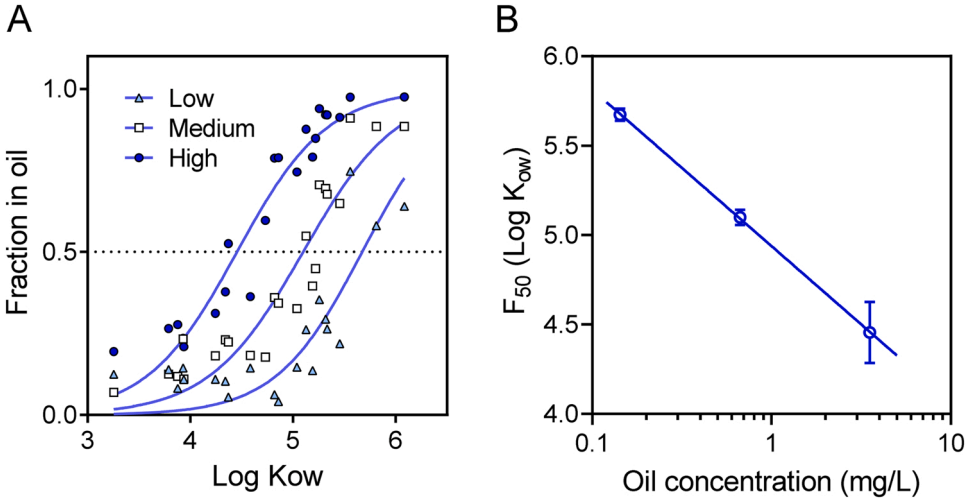

Fig. 3. Partitioning of PAHs between oil and water at different oil concentrations A) Fraction of PAHs associated with the oil phase as a function of their Log K_{ow}. B) Changes the Log K_{ow} corresponding to a 50/50 distribution between oil and water (F_{50}) related to oil concentration (THC C5 – C36).

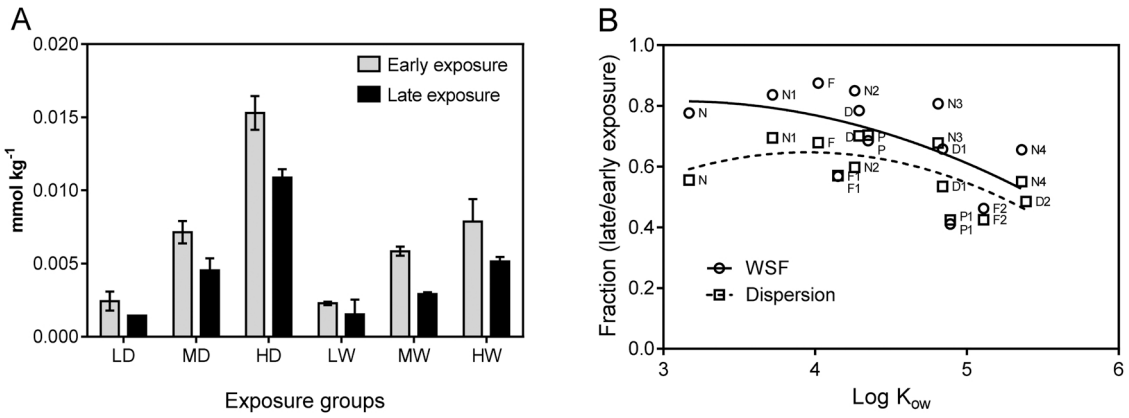

Fig. 4. A) Comparison of body residue of selected components (Sum of N4, F1 and F2 with SD indicated, N = 4) B) Accumulated fractions of selected PAH's during late exposure (High exposure) compared to early exposure (= 1) as a function of log K_{ow} for WSF (HW) and corresponding dispersions (HD). N; Naphthalene, N1; C1-Naphthalene, N2; C2-Naphthalene, N3; C3-Naphthalene, N4; C4-Naphthalene, F; Fluorene, F1; C1-Fluorene, F2; C2-Fluorene, F3; C3-Fluorene, P; Phenanthrene, P1; C1-Phenanthrenes/Anthracenes, P2; C2-Phenanthrenes/Anthracenes, P3; C3-Phenanthrenes/Anthracenes, D; Dibenzothiophene, D1; C1-Dibenzothiophenes, D2; C2-Dibenzothiophenes.

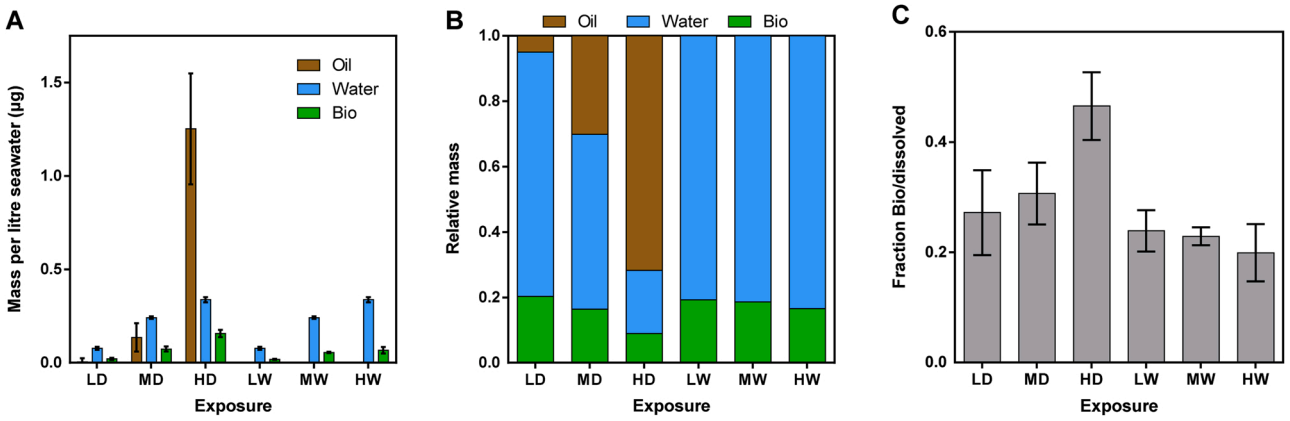

Fig. 5. Partitioning of C2-Fluorene (log Kow = 5.2) at the end of the 4-day exposure period with 200 mg cod eggs per litre. A) Actual mass in 1 litre of exposure solution. B) relative mass distribution. C) Fraction of oil components in biomass relative to dissolved mass.

uptake kinetics being independent of concentration. It is also evident that for the dispersions, the distribution between oil and water shifts towards the water phase at lower concentrations. This is consistent with the increased relative concentration of the heavier components in the WSF seen in Fig. 1 at higher dilutions. Fig. 5C shows that the bio-concentration, when related to the water-soluble fraction, is slightly higher in the groups exposed to dispersion. This indicate that the presence of droplets increases PAH uptake.

3.4. The effect of timing of exposure on mortality

In the early exposure group, the average 96-hr mortality increased significantly relative to the control at the highest concentration for both dispersion ($p < 0.0001$) and WSF ($p = 0.0019$) (Fig. 6). Corresponding mortality in the late exposure group was very low for all groups (0.3–5%), and not significantly different from the control group (Fig. 6C). The average mortality rate of the negative control group during the recovery period was approximately 0.05 fraction per day (Supplementary Information: Fig. S7). There was no correlation between exposure concentration and mortality during recovery for the early exposure group (Fig. 6B). In contrast there was a concentration-dependent increase in mortality during recovery for the late exposure group, which was significantly different from the control for the highest exposures to both WSF ($p = 0.0035$) and dispersion ($p = 0.0003$), and at the medium exposure to dispersion ($p = 0.015$) (Fig. 6D). Thus, there is a clear difference in the response to exposure between the early and late exposure groups. Similar results have been shown for another cold-water marine fish, Atlantic haddock (*Melanogrammus aeglefinus*), where early exposure to oil dispersions caused more severe effects than late exposure (Sørhus et al., 2021).

These differences may be attributed to differences in embryonic PAH body residues caused by higher PAH permeability in embryos from the early exposure group as also suggested as an explanation for differences in bioconcentration above. Lower PAH biotransformation activity may also be expected in embryos exposed in the early exposure group. Teleost embryo transition from maternal to zygotic transcription occur during the mid-blastula transition (Kimmel et al., 1995), meaning that the embryo exposed early in the development rely at least partly on maternal RNA for protein expression. Maternal RNA is not expected to contain biotransformation enzyme-encoding RNA unless maternal exposure has occurred. Thus, embryos exposed early in the development may have a restricted capacity to synthesize and activate enzymes required for PAH biotransformation and this may be the cause of the observed acute mortality during exposure. Lack of cytochrome P450 1 A (*cyp1a*) expression has been linked to higher PAH uptake and toxicity in early embryonic stages (2.5–5.5 dpf) of Atlantic haddock exposed to crude oil dispersions (Sørhus et al., 2021). For the late exposure group, the lack of acute mortality after exposure may be attributed to higher biotransformation activity because the embryo at this stage is capable of zygotic transcription (Skjærven et al., 2011). Cod embryos exposed to

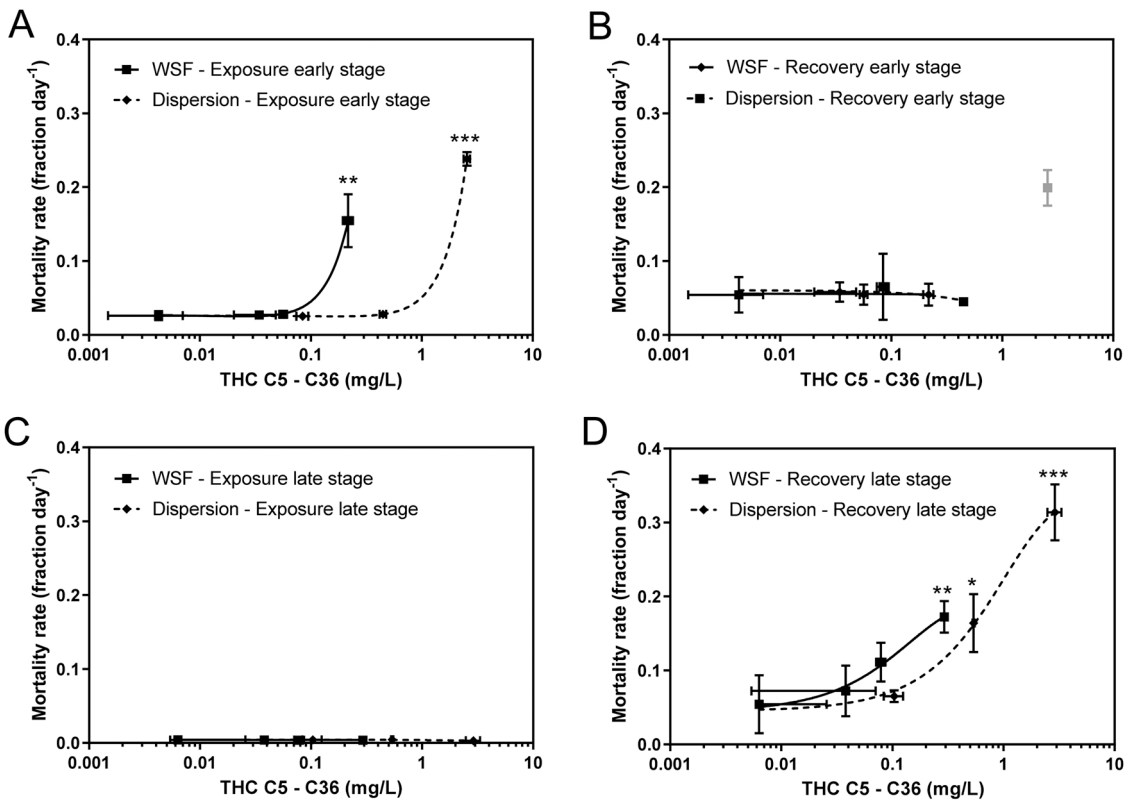

Fig. 6. Mortality rates until 4 days post hatch as a function of exposure for early and late embryo exposure A) early exposure group B) recovery from early exposure, C) late exposure group, and D) recovery from late exposure. Dots indicate average of four parallel groups with SEM. Asterisks indicate significant difference from controls (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0001$). The grey point to the right in Fig. B is based on very few individuals (average survival of 1.3 individuals).

benzo(a)pyrene displayed increased transcription of aryl hydrocarbon receptor (AHR) and *cyp1a* in embryos at 8 dpf (Aranguren-Abadía et al., 2020), so it is reasonable to assume some degree of biotransformation in our late exposure group. Similarly, exposure of late embryonic stages of haddock (7.5–11.5 dpf) to oil dispersions caused a rapid increase in *cyp1a* expression (Sørhus et al., 2021). Furthermore, the delayed mortality observed for the late exposure may be attributed to impact on embryo and larvae development, e.g. deformations and cardiotoxicity (Hansen et al., 2019b; Incardona et al., 2011), often associated and mediated by AHR activation (Incardona et al., 2011; Van Tiem and Di Giulio, 2011).

3.5. Contribution from oil droplets to embryo mortality

If we assume that the observed mortality is exclusively caused by the water-soluble fraction of the oil (Carls et al., 2008; Nordtug et al., 2011b), there should be no significant difference in mortality between the dispersion exposure and exposure to the WSF (filtered dispersion). Furthermore, the LC_{50} of the two exposures should be similar when relating mortality in both exposures (dispersion and WSF) to the WSF concentration. If this is the case, the oil droplets in the dispersion are only acting as an inert mass contributing to an increase in LC_{50} in proportion to the mass present. When plotting the survival after the early exposure against the extractable oil mass (THC C5 - C36) of the exposure solution, the dispersion appears to be less toxic than the WSF as

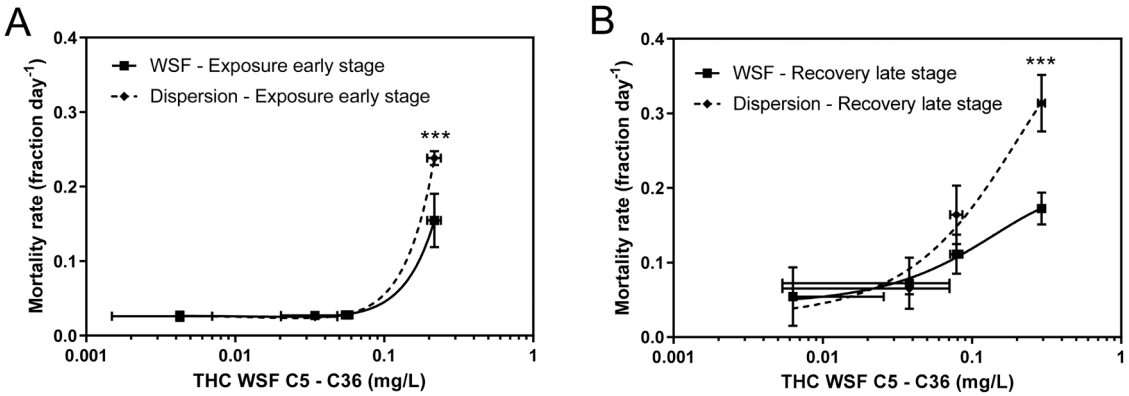

Fig. 7. Comparison of average mortality rates until 4 days post hatch of cod early stages exposed dispersions and their corresponding WSFs rates related to the WSF only. A) During exposure at early embryonic stage (3 – 7 dpf). B) During recovery from late exposure (9–13 dpf). Horizontal and vertical bars represent standard deviation of 4 replicates for mortality and 8 replicates for THC concentration. Asterisks indicate significant differences between WSF and dispersion exposure (*** = $p < 0001$).

indicated by Figs. 6A and 5D. However, when relating survival in both dispersions and WSFs to the concentrations in WSFs, it is evident that the mortality rates for both early and late dispersion exposures are significantly higher than for the corresponding WSF exposures (Fig. 7). The average mortality rate is significantly elevated in the presence of oil droplet by a factor of 1.54 (± SD = 0,23, $p < 0001$) in the early exposure group and 1.82 (± SD = 0.17, $p < 0001$) during recovery from exposure for the late exposure group.

Using a conventional LC_{50} approach during early exposure group, the dispersion treatment displayed a LC_{50} about 8 times higher than the corresponding WSF for the early experiment (Fig. S8; Table S8.1). However, if relating both datasets to WSF concentration, the LC_{50} for the dispersion is significantly lower ($p = 0.025$) (Supporting Table S8.1).

For the delayed mortality observed in the exposure of the late development group, the estimated LC_{50} of the dispersion was also significantly higher ($p = 0.03$) than that of the WSF exposed group, but the difference in this case was only a factor of 3 (LC_{50}-curves available in Supporting Information: Fig. S8. When relating the mortality of the group exposed to dispersion to WSF-concentration, the obtained LC_{50} is significantly lower than that of the WSF-exposure ($p = 0.037$) (Supporting Fig. S8D and Table S8.1).

In a study by Sørensen et al. (2019), chemical characterizations of whole egg, chorion and embryos were performed in Atlantic haddock (*Melanogrammus aeglefinus*) after exposure to oil dispersions, showing that although the major part of the oil-related compounds were associated with the chorion, some components do in fact penetrate the chorion. Although attachment of droplets to cod eggs are shown to be less than for haddock (Hansen et al., 2018; Sørhus et al., 2015), our results indicate that oil droplets attached to the egg surface also contribute to the toxicity of oil in cod embryos.

From a methodological point of view, the observed droplet-associated toxicity could be explained by retainment of water-soluble components during the filtering procedure used to generate the WSF, i.e., the WSF composition before and after filtration are not identical. If this was the case, however, we would expect to see changes in the composition of WSF over time as the filter became saturated. The chemical analyses do not indicate that this was the case. Alternatively, the water phase of the dispersions may not have been in equilibrium before the separation of the WSF through filtration (Hansen et al., 2019a). This is plausible for the heavier components, however, for the analyzed components it is evident from Fig. 2 that the relative contribution of the heavier components increases in a pattern expected from partitioning theory. The third possibility is that even in a dispersion at equilibrium, the oil droplet will act as a reservoir or buffer constantly replacing components that are bioaccumulated to maintain a slightly higher WSF over time. Finally, the droplets attached to the eggs may cause uptake directly from the oil into the tissue, as seen in the study by Sørensen et al. (2019). All these factors will contribute to an overestimation of the toxic effect of oil droplets. As we are not able to quantify the potential impacts of these confounding factors, we conclude that it is plausible that the oil droplets contribute to the oil toxicity observed in the current experiment, but the mass-related contribution is much lower than for the water-soluble fraction.

4. Conclusions

Due to the varying chemical complexity and physical properties of crude oils, it is crucial that the exposure conditions in toxicity tests are well described and documented. Without detailed documentation of exposure it is nearly impossible to relate toxicity data to actual oil spill scenarios. From the differences observed in the toxicity responses from exposure at different developmental stages it is evident that short acute toxicity studies without additional follow up of the exposed groups may lead to erroneous conclusions. The timing of oil exposure during the embryonic phase of Atlantic cod clearly affects both the mortality rate and the timing of mortality. Even though the body residue of PAHs were lower when embryos were exposed in the later embryonic stage, mortality rate increased relative to the early exposure indicating that critical body residue threshold is stage specific. Furthermore, our results indicate that oil droplets contribute to increased mortality, but the dissolved fraction is the dominating contributor to toxicity in cod embryos exposed to oil dispersions.

CRediT authorship contribution statement

Trond Nordtug: Conceptualization, Formal analysis, Validation, Investigation, Data curation, Writing – original draft, Visualization, Writing – review & editing, Supervision, **Anders J. Olsen:** Conceptualization, Investigation, Data curation, Writing – review & editing, Supervision, **Per-Arvid Wold:** Formal analysis, Investigation, Writing – review & editing, **Iurgi Salaberria:** Formal analysis, Investigation, Writing – review & editing, **Ida Beathe Øverjordet:** Formal analysis, Investigation, Writing – review & editing, **Dag Altin:** Formal analysis, Investigation, Writing – review & editing, **Elin Kjørsvik:** onceptualization, Writing – review & editing, Supervision, **Bjørn Henrik Hansen**: Conceptualization, Formal analysis, Validation, Investigation, Data curation, Writing – original draft, Visualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113100.

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