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Embryonic exposure to produced water can cause cardiac toxicity and deformations in Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) larvae

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Abstract

Regular discharges of produced water from the oil and gas industry represents the largest direct discharge of effluent into the marine environment worldwide. Organic compound classes typically reported in produced water include saturated hydrocarbons, monoaromatic and polyaromatic hydrocarbons (MAHs, PAHs) as well as oxygenated compounds, such as phenols, acids and ketones. This forms a cocktail of known and suspect toxicants, but limited knowledge is yet available on the sub-lethal toxicity of produced water to cold-water marine fish species. In the present work, we conducted a 4-day exposure of embryos of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) to produced water extracts equivalent to 1:50, 1:500 and 1:5000 times dilutions of raw effluent. No significant reduction in survival or hatching success was observed, however, for cod, hatching was initiated earlier for exposed embryos in a concentration-dependent manner. During recovery, significantly reduced embryonic heart rate was observed for both species. After hatch, larvae subjected to embryonic exposure to produced water extracts were smaller, and displayed signs of cardiotoxicity, jaw and craniofacial deformations. In order to improve risk assessment and regulation of produced water discharges, it is important to identify which produced water components contribute to these effects.

Key words: Petroleum; fish embryo; Arctic; cardiotoxicity; deformations; produced water

1. Introduction

Regular discharges of produced water (PW) from the oil and gas industry represent the largest direct discharge of effluent into the marine environment worldwide (Lee and Neff, 2011). Approximately 1.3 x 10^8 m^3 PW is released on the Norwegian continental shelf annually from offshore production platforms (NOROG, 2017). PW contains an aqueous mix of formation water, oil and/or gas from the reservoir, injected freshwater or brine water and added production chemicals. The chemical composition of PW is therefore very complex and comprises a mixture of dissolved and particulate, organic and inorganic compounds. Organic compound classes typically reported in PW include saturated hydrocarbons, monoaromatic and polyaromatic hydrocarbons (MAHs, PAHs) as well as oxygenated compounds, such as phenols, acids and ketones (Faksness et al., 2004; Lee and Neff, 2011). Total 2016 PW releases from activities on the Norwegian continental shelf was estimated to include 1 600 tons of crude oil, 2221 tons BTEX, 576 tons phenols, 28 438 tons organic acids and 126 tons PAHs (NOROG, 2017). This forms a cocktail of known and suspected toxicants, but limited knowledge is yet available on the sub-lethal toxicity of produced water to marine cold-water species.

Emissions of produced water (PW) to the marine environment in the North Atlantic and Barents Sea are regulated by the authorities with the overall aim of producing no harmful environmental effect using estimations of the ratio between 'predicted environmental concentration' (PEC) and 'predicted no effect concentration' (PNEC), called the Environmental Impact Factor (EIF), as a proxy (Johnsen et al., 2000). Typically, PNECs are determined based on acute toxicity thresholds, and uncertainty factors are included to account for sub-lethal/chronic toxicity (Neff et al., 2006).

Developing fish embryos and yolk sac larvae are especially vulnerable to crude oil-derived pollutants (Hodson, 2017; Incardona et al., 2004; Pasparakis et al., 2016; Sørhus et al., 2015). In these early life stages of fish, cardiotoxicity has been identified as the most prominent effect of crude oil exposure, typically in association with craniofacial and jaw malformation (Incardona et al., 2004). Cardiotoxicity, manifested as pericardial edema, bradycardia, arrhythmia, reduced stroke volume, reduced contractility, poor looping, and failed ventricular cardiomyocyte proliferation, has been shown following low crude oil exposures (Incardona, 2017; Incardona and Scholz, 2016; Khursigara et al., 2017; Sørhus et al., 2017; Sørhus et al., 2016). Cardiotoxicity has also been linked to other developmental abnormalities in larvae including reduced swimming activity which ultimately may affect predator avoidance behavior and long-term survival (Hicken et al., 2011). Limited knowledge exists on the potential for produced water to cause cardiotoxic effects, particularly in cold water species. Early life stages of Atlantic cod (*Gadus morhua*) exposed to diluted produced water effluents (maximum 1%) displayed no effects on survival and hatching success, but displayed deformations and a transient lack of pigmentation (Meier et al., 2010).

The main aim of the present work was to determine the potential for produced water to cause pericardial edema, deformations and other associated effects in developing fish embryos and larvae. To investigate this, embryos of the cold-water fish species Atlantic cod (*G. morhua*) and haddock (*Melanogrammus aeglefinus*) were exposed to three concentrations of reconstituted produced water for four days during embryogenesis. Acute and sub-lethal effects were studied throughout the embryonic phase until 2 days post hatch.

2. Materials and Methods

2.1. Produced water sampling, extraction and characterization

The produced water sample (~28 L) was collected at an offshore platform in the Norwegian Sea during a period of normal operation, transferred to Teflon lined bags and transported to the SINTEF Sealab laboratory by air freight the same day. Upon arrival in the onshore laboratory, the samples were immediately acidified (HCl, pH <2) and extracted within four days. The PW sample was serially extracted using dichloromethane (DCM) following a modification of EPA method 3510C (USEPA, 1996). A sub-sample of the PW (0.5 L) was extracted with surrogate internal standards (naphthalene-*d*8, acenaphthene-d10, phenanthrene-*d*10, chrysene-*d*12, perylene-*d*12, phenol-*d*6, *p*-cresol-*d*8, 4-*n*-propylphenol-*d*12 and 5α-androstane) to account for target analyte loss in the extraction step. The remaining volume of each PW was extracted without internal standard in batches of ~2 L and the final extracts combined to a "total PW extract" to be used for toxicity testing. The extracts were dried over sodium sulfate and concentrated by solvent evaporation (Zymark TurboVap® 500). Prior to analysis, recovery internal standards (fluorene-*d*10 and *o*-terphenyl) were added. Analysis of semi-volatile organic components (SVOC) including decalins, PAHs, alkylated PAHs and C0-C9 phenols was performed using gas chromatography mass spectrometry (GC-MS), and for GC-amenable total extractable matter (TEM) using gas chromatography flame ionization detection (GC-FID). For GC-FID analysis, an Agilent 7890A GC was used. The GC-column was a HP-5MS UI (30 m × 0.25 mm x 0.25 µm), and the carrier gas was helium at a constant flow of 1.5 mL/min. Samples (1µL) were injected at 330 °C by pulsed splitless injection. The oven temperature was held at 40 °C for 1 min, then ramped to 315 °C by 6 °C /min and held at this temperature for 15 min. For GC-MS analysis an Agilent 7890B GC coupled with an Agilent 5977A quadrupole MS was used. The GC-column was a HP-5MS UI (60 m × 0.25 mm x 0.25 µm), and the carrier gas was helium at a constant flow of 1 mL/min. Samples (1µL) were injected at 325 °C by pulsed splitless injection. The oven was programmed to 40 °C (1 min hold) then ramped to 220 °C by 6 °C /min and further ramped to 325 °C by 4 °C /min (15 min hold). The transfer line temperature was 300 °C, the ion source temperature was 300 °C and the quadrupole temperatures were 165 °C. The EI source was operated at 70 eV. Analysis was performed in both full scan (50-500 amu) and selective ion monitoring (SIM) mode. A list of all target analytes for the GC-MS analysis is shown in Supporting Information (SIA: Table S1). Quantification of target compounds was performed using average response factors (RF) of the parent PAH or phenol compounds.

2.2. Preparation of exposure media

Based on GC-FID analyses of the initial extract, extract volumes equivalent to 50-, 500- and 5000-times dilution of the initial PW effluent were reconstituted into seawater to generate the exposure solutions. The appropriate volume of total extract to make the exposure stock solution was supplied in a pre-cleaned and water de-activated glass bottle by a gas tight syringe. DCM was removed by evaporation to dryness at 35 °C under a very gentle flush with N2 gas (10 min). Once dry, the flasks were filled with sterile filtered (0.22 µm Sterivex® cartridges) seawater at room temperature, and re-dissolution of the dried extract was assisted by immersion in a sonication bath (3x10 minutes). Solvent controls (DCM) were also prepared. The temperature of the resulting exposure solutions was adjusted passively to 6 °C followed by aeration of the solution with filtered air for 10 min to increase oxygen tension. Exposure

solutions (200 ml) were transferred into 0.5 L-glass jars for exposure of fish embryos. Sub-samples of the reconstituted PW solutions were analyzed as described above for exposure characterization.

2.3. Fish exposure

Fertilized Atlantic cod (*G. morhua*) and haddock (*M. aeglefinus*) eggs were collected from spawning brood stocks kept in 7000 L tanks at Austevoll Research Station at the Institute of Marine Research (IMR). Eggs (300 ml) were collected early in the morning from overnight spawning, transferred to sea water in closed bottles which were insulated with bubble wrap, placed on ice in a styrofoam container and sent to SINTEF Sealab in Trondheim using airfreight. At arrival, less than 12 hours after fertilization, eggs were transferred to 50 L tanks with flow-through of filtered (1 µm) seawater (6 ± 1°C) delivering one volume exchange of seawater per day. Natural sea water, collected from a depth of 80 m (below thermocline) in a nonpolluted Norwegian fjord (Trondheimsfjord; 63°26' N, 10°23' E), was supplied by a pipeline system from the source to our laboratories (salinity of 34 ‰, pH 7.6). Gentle air bubbling kept embryos moving continuously in the tanks. Dead and unfertilized eggs were removed from the tank daily. The embryos were acclimated for 10 days until being transferred to glass jars for exposure. Three concentrations of PW extract were used, in addition to a negative control containing seawater only. Approximately 200 fish eggs with embryos (11 dpf) were transferred to glass jars consisting of 200 mL exposure medium. Images of 11 dpf embryos of both species are given in Supporting Information (SIB, Fig. S1). All treatments were run with four replicates (N=4), and eggs were exposed for 4 days (11-15 dpf). During this time an extra 200 ml exposure solution was added to the glass jars after 2 days to maintain the exposure concentration. After 4 days exposure, dead eggs were counted and removed, and the surviving eggs were transferred to glass bowls (2 L) containing clean sea water (1 L) and maintained at 6 ± 1°C until 2 days post hatch (2 dph). Survival and hatching were monitored throughout the recovery period. Identical experiments were performed for cod and haddock eggs. A complete time line of the exposure experiment is given in Supporting Information (SIB: Table S2).

2.4. Microscopy, heart rate analyses and biometry

Images and videos of 10-20 embryos (14 dpf) and individual larvae (2 dph) were taken through a microscope (Eclipse 80i, Nikon Inc., Japan) equipped with Nikon PlanApo objectives (2x for egg videos and whole larvae images and 10x for close-up larvae images and videos), a 0.5x videoadaptor and a CMOS camera (MC170HD, Leica Microsystems, Germany). Videos were used as a basis for heart rate (HR) analyses in individual embryos/larvae using automated video analyses. Briefly, this method identifies the heart tissue region in the video through pixel intensity difference between frames. Then, the time sequence of mean value of the intensity in that region is extracted. This signal tends to oscillate in concert with heart contraction and expansion. After normalization and smoothing the signal, the number of peaks is counted, which is interpreted as the number of heart beats, providing an estimate of the heart rate. The method also performs an analysis of the video and signal quality, which is used to indicate potential outliers (e.g. non-beating hearts, strong larval motion) (Nepstad et al., 2017). Larvae images were used for biometric analyses using Image J (Schneider et al., 2012) and blinded deformation ranking analysis adopted from Sørhus et al (2015). All larvae were analyzed for standard length, yolk sac area, body area, eye diameter, jaw length and eye-to-forehead distance. Representative images of larvae with highlighted traces of distances/areas are given in Supporting

Information (SID, Figures SI2-SI6). Morphological abnormalities (jaw deformations, craniofacial deformations, pericardial edema and spine deformations) were determined for larvae (2 dph) according to a severity degree scale (0-3 where 0 is normal, 1 is minor deformation, 2 is moderate deformation and 3 is severe deformation) (Sørhus et al., 2015). Positioning of the marginal finfold was also investigated, but not ranked in the same manner as the other deformations. Examples of control and deformed larvae (2 dph) are given in Figure 1, where the main observed deformations are indicated. Additional examples of larvae with different deformation ranking is provided in Supporting Information (SIE, Fig. S7).

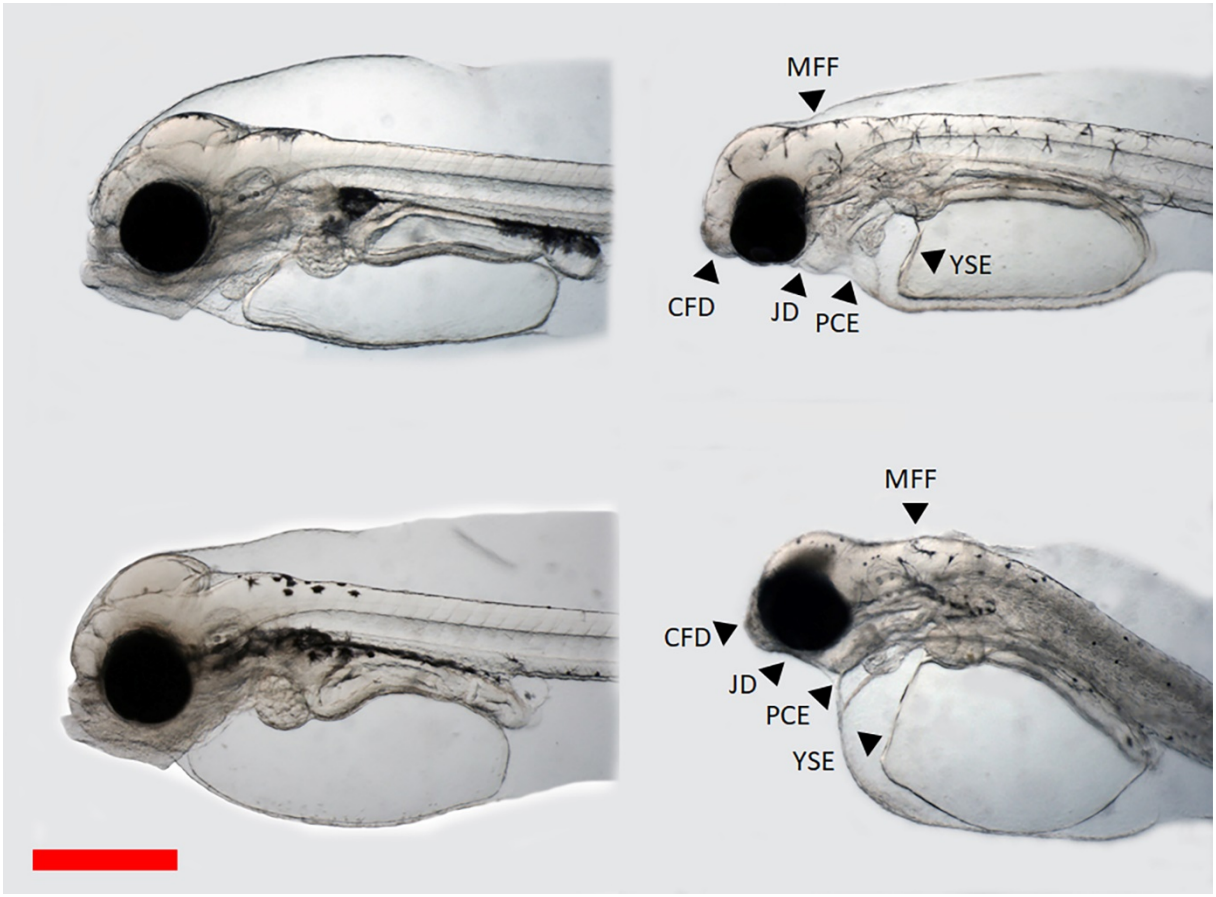


Figure 1: Examples of normal (control) and deformed larvae 2 days post hatch. Top left: Control cod. Top right: Deformed cod. Bottom left: Control haddock. Bottom right: Deformed haddock. MFF = Marginal finfold. CFD = Craniofacial deformation. JD = Jaw deformation. PCE = Pericardial edema. YSE = Yolk sac edema. Both deformed larvae were characterized to have severity degree 3 for CFD and JD and severity degree 2 for PCE and YSE. The red scale bar indicates 0.5 mm.

2.5. Statistical analyses

Statistical analyses were conducted using GraphPad Prism statistic software, V6.00 (GraphPad Software, Inc., CA, USA). Comparisons between treatments were done using one-way ANOVA followed by Tukey's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparison test. The latter was used on data sets not passing the D'Agostino & Pearson omnibus normality test. Significance level was set at $p<0.05$ unless otherwise stated. Nonlinear curve fit (third-order

polynomial) was used in figures displaying measured parameters plotted as a function of exposure concentrations.

3. Results and Discussion

3.1. Chemical characterization of produced water

The total extractable material (TEM) of the whole effluent was 22 mg/L containing primarily PAH (mostly naphthalenes) and phenols (Table 1). During reconstitution some loss of decalins and naphthalenes was expected, but a good concentration series was obtained for all analyzed components. Exposure solutions were prepared to be a dilution of the original produced water starting at a concentration expected to be in a 50x dilution (high exposure) of whole effluent, and then 10- and 100-fold dilutions for the medium and low exposures, respectively. There was an apparent loss of C0-C1-naphthalenes and phenols during reconstitution, probably due to evaporation during DCM removal. Toxicity was estimated based on T-PAH concentrations (45 PAHs and alkylated homologues) in the individual treatments. Importantly, this does not mean that PAHs are the only component group in produced water responsible for eliciting the studied toxic effects (Hansen et al., 2018a), but provides a basis for comparison to other studies.

Table 1: Chemical characterization of exposure solutions and the whole effluent used as a basis to generate the exposure solutions. All concentrations are given in µg/L.

Compound group	**Control (sea water)**	**Low**	**Medium**	**High**	**Raw effluent**
Total Extractable Material (TEM)	**17**	**18**	**21**	**105**	**22090**
Sum SVOC	0.15	0.42	4.6	4.7	8098
Sum decalins	ND	0.0080	0.015	ND	33.0
SUM PAH	0.062	0.31	2.5	3.4	3197
Naphthalenes	0.059	0.12	0.26	3.9	2731
2-3 ring PAHs	0.0035	0.18	2.0	2.7	42.4
4-6 ring PAHs	ND	0.012	0.19	3.3	4.2
C0-C5 Phenols	0.083	0.11	2.1	1.3	4869

SVOC: Semi-volatile organic components quantified by GC-MS. ND: Not detected.

3.2. Acute toxicity, hatching success and larvae condition

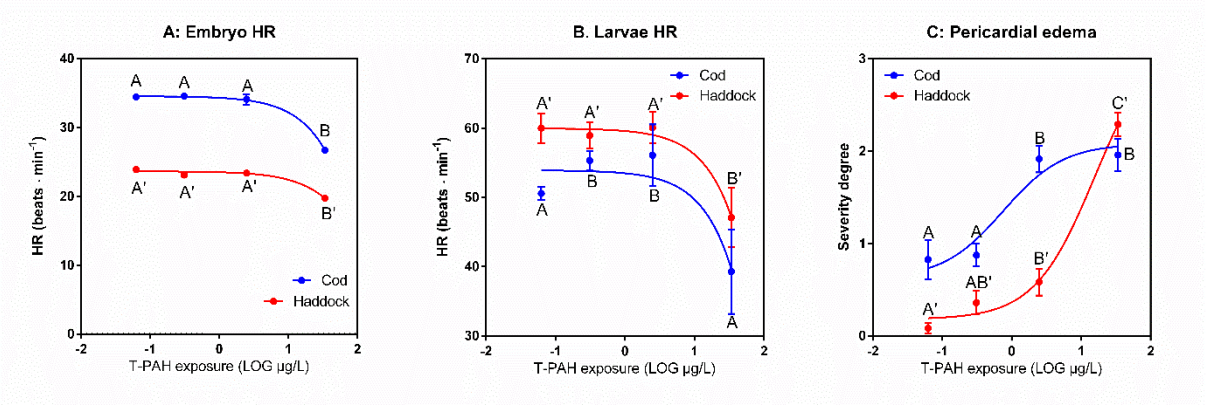
At the end of exposure, survival was not significantly reduced in PW-treated fish compared to controls (Supporting Information SIF, Fig. S8). Lack of acute effects of produced water have been shown for cod previously. Meier et al (2010) displayed no acute mortality even at high concentrations (1 % diluted effluent), however, delayed mortality was observed during first-feeding as the larvae were unable to feed, possibly due by severe jaw deformations.

In our experiment, hatching success was comparable between treatments and controls for both species. For cod, hatching was initiated earlier for exposed embryos in a concentration-dependent manner (Supporting Information SIG, Fig. S9A). The timing of haddock egg hatching was not affected by exposure (Fig. S9B). Following exposure to water accommodated fractions (WAF) of oil Hansen et al (2018a) also observed no increase in acute mortality, but in contrast to the current work, WAF-exposed cod eggs displayed delayed hatching.

The larvae, sampled 2 days post hatch, displayed clear symptoms of reduced condition as evident through biometric analyses (Supporting Information SIH, Figure S10). Concentration-dependent reductions in length and body area were evident for both species. For cod, standard length was reduced compared to controls for the highest exposure, and body area was significantly smaller for medium ($p<0.05$) and high ($p<0.0001$) exposures. Similar results were obtained for haddock, where high exposures caused shorter larvae ($p<0.0001$) and reduced larvae body area ($p<0.05$). These results are consistent with previous studies on cod and haddock exposed to crude oil with a T-PAH exposure range similar to those used in the present experiment (Hansen et al., 2018a; Sørhus et al., 2015).

3.3. Indices of cardiotoxicity

Typical cardiotoxicity phenotypes in marine fish include bradycardia (reduced heart rate), pericardial edema, reduced stroke volume, arrhythmia, reduced contractility, poor looping, and failed ventricular cardiomyocyte proliferation (Incardona, 2017; Khursigara et al., 2017; Sørhus et al., 2017). Cod embryonic HR was higher (34.4 ± 1.4 bpm) than for haddock (23.9 ± 2.7 bpm), but opposite in larvae where HR was higher in haddock (60.0 ± 7.5 bpm) than in cod (50.6 ± 3.7 bpm). Significantly lower HR was observed in embryos exposed to the highest concentration compared to controls for both species ($p<0.0001$) (Fig. 2A). Compared to corresponding controls, cod displayed a larger drop in HR (22.4%) than haddock (17.6%). Lower HRs compared to controls were also observed after hatch (Fig. 2B) in larvae for both species (high treatment only) (Fig. 2B). Increase in pericardial edema (Fig. 2C) was observed for both species in a concentration-dependent manner, being significantly more severe than in controls for medium ($p<0.0001$) and high ($p<0.0001$) exposures (Fig. 2C). Although both species displayed comparable cardiotoxic effects of high treatment, cod also displayed significantly higher degree of deformation compared to controls at the medium concentration, whereas haddock did not, suggesting that cod may be more sensitive. Effects observed were consistent with exposures of haddock to dispersed crude oil within the same TPAH range (Sørhus et al., 2015; Sørhus et al., 2017). In haddock, bradycardia and pericardial edema was associated with a chemical blockage of calcium channels, disruption of ion channel biosynthesis and defects in cardiac cell differentiation (Sørhus et al., 2016). It is expected that these adverse outcome pathways are similar in cod, Studies using crude oil have, in contrast to our experiments with produced water, concluded that haddock are more susceptible to oil dispersions crude oil than cod. This has been explained by different chorion properties (haddock eggs are stickier than cod eggs) causing differences in kinetics and uptake routes between the two species. Thus, haddock may bind more oil droplets to chorion surface than cod (Hansen et al., 2018b; Sørensen et al., 2017). For produced water discharges, and specifically in the droplet-free exposures utilized in the present experiments, differences in chorion surface and their droplet-adhesion properties between the two species may be less of an issue than for acute oil spills.



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254 **Figure 2: Cardiotoxic responses in cod (red) and haddock (blue plotted as a function of exposure**
255 **concentration (in µg T-PAH/L). Responses given as mean ± SEM. A: Heart rate (HR), beats per min,**
256 **N=14-69) in embryos. B: Heart rate (HR, beats per min, N=11-16) in larvae. C: Pericardial edema**
257 **severity degrees in larvae (N=23-25). Significant differences (p<0.05) between groups within each**
258 **species is given with different letters (cod: A, B and C. haddock: A', B' and C'), i.e. identical letters**
259 **indicate no significant differences between groups (p<0.05). Note different scaling on the axes.**

260

261 **3.4. Craniofacial and jaw deformations**

262 The developing heart is considered a primary target for toxicity of crude oil compounds to early life
263 stages of fish, whereas most other aspects are likely secondary effects caused by loss of circulation
264 (Incardona, 2017; Incardona et al., 2004). One suggested secondary effect is reduced ability to inflate
265 and develop fin-folds. One-third of the cod larvae exposed to the high exposure displayed abnormal
266 marginal finfold where the anterior portion of the dorsal marginal finfold was collapsed or not present.
267 This was much more pronounced for haddock exposed to the high PW concentrations, for which 62.5%
268 of the larvae displayed collapsed dorsal marginal finfold. Normal or close to normal marginal finfold
269 was observed for the low and medium exposure concentration in both species.

270 Previously published studies on several fish species have associated cardiotoxicity with jaw and
271 craniofacial deformations (Incardona et al., 2004; Sørhus et al., 2015; Sørhus et al., 2016). Our PW
272 exposure to cod and haddock resulted in similar deformations to occur in a concentration-dependent
273 manner for both species (Fig. 3A-B). Compared to controls, significantly more jaw deformations,
274 analyzed by severity ranking (Fig. 3A) and jaw lengths (Fig. 3D), were found for cod at high treatment
275 ($p<0.0001$). Haddock displayed more severe deformations, with a near complete lack of upper and
276 lower jaw structures, than cod. Compared to controls, exposed haddock displayed significantly altered
277 jaw length for low ($p<0.05$), medium ($p<0.05$) and high ($p<0.0001$) exposure concentration and for
278 medium ($p<0.001$) and high ($p<0.0001$) treatment for jaw deformation. These results suggest that
279 haddock may be more sensitive to PW than cod. Importantly, however, at the highest treatment, no
280 individuals for any of the species displayed a normally developed jaw. We did not perform Alcian
281 staining for visualizing cartilage and bone structures on the larvae in our work. However, the
282 phenotype observed in 100% of the haddock larvae exposed to high PW exposure as embryos resemble
283 the most severely deformed larvae exposed to oil dispersions as reported by Sørhus et al (2016). These
284 larvae typically lack basocranium and have reduced or fused jaw cartilages (Sørhus et al., 2016).

Craniofacial deformations were analyzed for severity degree (Fig. 3B) as well as biometrical measurements of the distance between the eye and forehead (Fig. 3E) displaying almost identical relationships with exposure concentration as jaw deformation. All haddock larvae exposed to high PW concentrations displayed severe craniofacial defects with marked reductions in base structures of the skull. This was also estimated biometrically measuring the distance between the eye and forehead (Fig. 3E). For cod exposed to high PW extract concentrations, significantly shorter eye-to-forehead distance was found ($p<0.0001$) compared to controls. Haddock was more sensitive displaying significantly shorter eye-to forehead distance for all exposure concentrations (Low: $p<0.05$, Med: $p<0.01$, High: 0.0001). For cod exposed to high PW extract concentrations, significantly shorter eye-to-forehead distance ($p<0.0001$) and higher craniofacial deformity severity ($p<0.0001$) was observed. Haddock was more sensitive, displaying significantly higher craniofacial deformation severity for all PW extract treatments. Comparable deformation phenotypes have been observed in haddock exposed to dispersed oil with TPAH levels like our experiment (Sørhus et al., 2015; Sørhus et al., 2017). In addition to the craniofacial and jaw deformations, spinal curvatures (Supporting Information SII, Fig. S11) were observed in larvae for both species exposed to high concentrations ($p<0.0001$), and for cod for medium exposure ($p<0.001$) as well. Both species also displayed smaller eyes as a function of exposure concentration (SII, Fig. S11). This also appears to be a more sensitive endpoint in cod as small eye phenotype was significant for both medium ($p<0.01$) and high (0.0001) treatments, whereas for haddock significantly smaller eyes were only found in high treatment of haddock ($p<0.0001$).

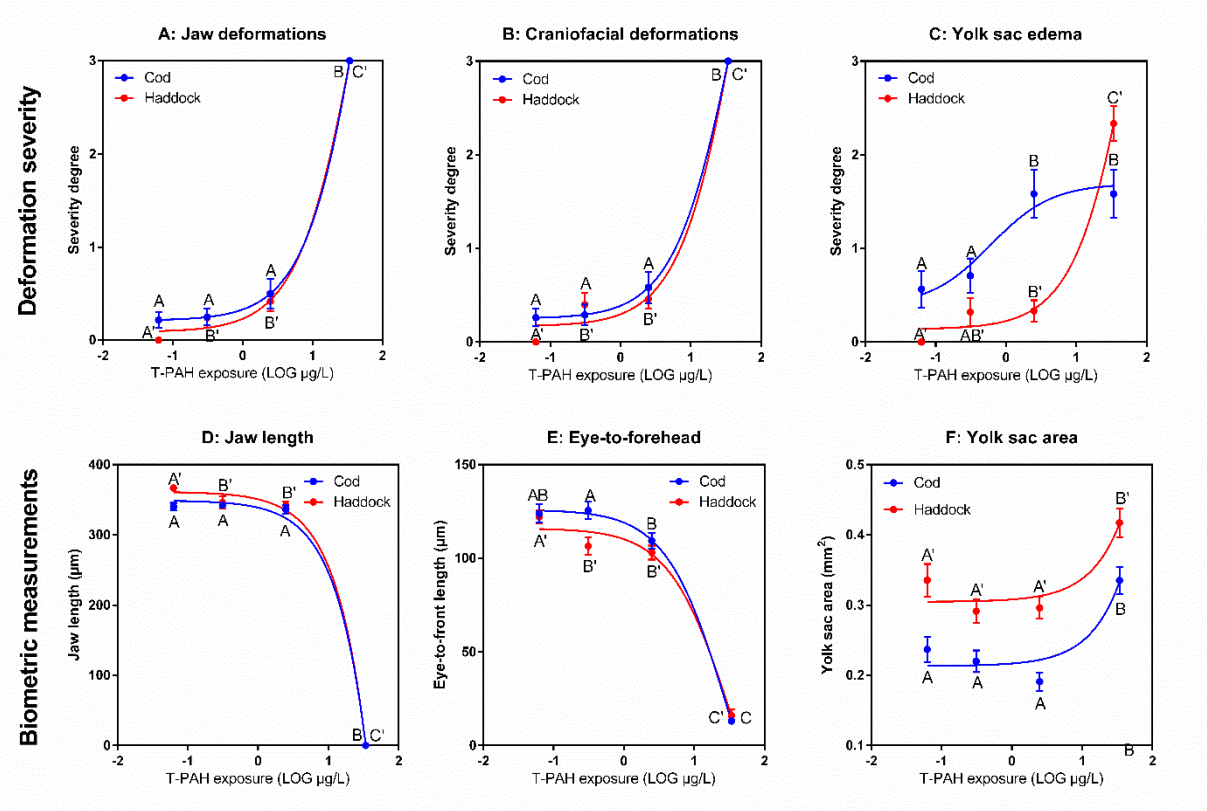

Figure 3: Biometric measurements in larvae exposed to produced water during embryogenesis plotted as a function of exposure concentration (in µg T-PAH/L); Deformation severities in jaw structure (A), craniofacial structures (B) and yolk sac (C), and biometric analyses data for jaw length (in µm) (D), eye-to-forehead distance (in µm) (E) and yolk sac area (in mm^2) (F) in cod (red) and haddock (blue). Data are displayed as mean ± SEM, N=23-25). Significant differences ($p<0.05$)

between groups within each species is given with different letters (cod: A, B and C. haddock: A', B' and C'), i.e. identical letters indicate no significant differences between groups.

3.5. Yolk sac consumption and edema

Before exogenous feeding is initiated 6-8 days after hatch for cod and haddock, the yolk sac is the only nutrition provider (Martell et al., 2005; Neilson et al., 1986). Two-dimensional yolk sac area was analyzed in lateral images. Although no significant concentration-dependent responses were observed in yolk sac area (Fig. 3F), there was a decrease for low and medium exposures for both species. This suggest that exposure to low and medium exposure concentrations come at an energetic cost, possibly through initiation of detoxification mechanisms. Comparable trends have been observed in yolk sac stages of the warm-water fish mahi-mahi (*Coryphaena hippurus*) after exposure to crude oil (Pasparakis et al., 2016). In mahi-mahi, reduced yolk sac area was observed at TPAH concentrations comparable to our highest exposure, however, the highest exposure in our studies resulted in larger yolk sac area compared to controls for both species ($p<0.05$). Increased yolk sac area has also been observed in haddock exposed to crude oil (Sørhus et al., 2017), and may be attributed to occurrence of narcosis and associated reduced metabolic rate and energetic demand. Yolk sac edema (Fig. 3C) was observed for both species being significantly more severe for cod exposed to medium ($p<0.05$) and high ($p<0.05$) exposure and for haddock exposed to high exposure ($p<0.0001$). As for the above-mentioned deformations, yolk sac edema has previously been shown for haddock exposed to dispersed oil with TPAH-concentrations in the same range as used in our produced water experiments (Sørhus et al., 2015).

4. Conclusions

The PW extract used to expose cod and haddock eggs caused no effect on egg survival, hatching success or larvae survival, although hatching was initiated earlier for cod exposed to the highest exposure concentration. Our studies, however, demonstrate that PW components can cause developmental effects in early life stages of fish. Cardiac toxicity and severe craniofacial and jaw deformation were observed for both species, with more larvae displaying higher severity in haddock compared to cod. Adverse effects were primarily associated with the highest PW exposure, designed to mimic a 50x dilution of the PW effluent, concentration levels which for regular discharges will typically only occur in the immediate vicinity of the discharge point. However, effects were also observed for the lower concentrations, e.g. mild craniofacial deformations were observed for haddock even at the lowest exposure concentration mimicking a 5000x dilution of the effluent. Thus, implementing a regulatory strategy to predict the risk of adverse embryotoxicity to occur following produced water discharges is clearly needed. To do so, it is important to identify which specific compounds and/or compound groups cause these effects, and to establish relationships between exposure, dose (preferably body residues) and effects. Current knowledge suggest that tricyclic PAHs is a good place to start, however, as produced water is a highly complex mixture, it is important to include the full range of produced water compounds.

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