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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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17

18   **Abstract**

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

34

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

36

37   **1. Introduction**

38

39   Regular discharges of produced water (PW) from the oil and gas industry represent the largest direct
40   discharge of effluent into the marine environment worldwide (Lee and Neff, 2011). Approximately 1.3
41   x 10^8 m^3 PW is released on the Norwegian continental shelf annually from offshore production
42   platforms (NOROG, 2017). PW contains an aqueous mix of formation water, oil and/or gas from the
43   reservoir, injected freshwater or brine water and added production chemicals. The chemical
44   composition of PW is therefore very complex and comprises a mixture of dissolved and particulate,
45   organic and inorganic compounds. Organic compound classes typically reported in PW include
46   saturated hydrocarbons, monoaromatic and polyaromatic hydrocarbons (MAHs, PAHs) as well as
47   oxygenated compounds, such as phenols, acids and ketones (Faksness et al., 2004; Lee and Neff, 2011).
48   Total 2016 PW releases from activities on the Norwegian continental shelf was estimated to include 1
49   600 tons of crude oil, 2221 tons BTEX, 576 tons phenols, 28 438 tons organic acids and 126 tons PAHs
50   (NOROG, 2017). This forms a cocktail of known and suspected toxicants, but limited knowledge is yet
51   available on the sub-lethal toxicity of produced water to marine cold-water species.

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

58   Developing fish embryos and yolk sac larvae are especially vulnerable to crude oil-derived pollutants
59   (Hodson, 2017; Incardona et al., 2004; Pasparakis et al., 2016; Sørhus et al., 2015). In these early life
60   stages of fish, cardiotoxicity has been identified as the most prominent effect of crude oil exposure,
61   typically in association with craniofacial and jaw malformation (Incardona et al., 2004). Cardiotoxicity,
62   manifested as pericardial edema, bradycardia, arrhythmia, reduced stroke volume, reduced
63   contractility, poor looping, and failed ventricular cardiomyocyte proliferation, has been shown
64   following low crude oil exposures (Incardona, 2017; Incardona and Scholz, 2016; Khursigara et al.,
65   2017; Sørhus et al., 2017; Sørhus et al., 2016). Cardiotoxicity has also been linked to other
66   developmental abnormalities in larvae including reduced swimming activity which ultimately may
67   affect predator avoidance behavior and long-term survival (Hicken et al., 2011). Limited knowledge
68   exists on the potential for produced water to cause cardiotoxic effects, particularly in cold water
69   species. Early life stages of Atlantic cod (*Gadus morhua*) exposed to diluted produced water effluents
70   (maximum 1%) displayed no effects on survival and hatching success, but displayed deformations and
71   a transient lack of pigmentation (Meier et al., 2010).

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

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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

121   solutions (200 ml) were transferred into 0.5 L-glass jars for exposure of fish embryos. Sub-samples of

122   the reconstituted PW solutions were analyzed as described above for exposure characterization.

123

124   **2.3. Fish exposure**

125   Fertilized Atlantic cod (*G. morhua*) and haddock (*M. aeglefinus*) eggs were collected from spawning

126   brood stocks kept in 7000 L tanks at Austevoll Research Station at the Institute of Marine Research

127   (IMR). Eggs (300 ml) were collected early in the morning from overnight spawning, transferred to sea

128   water in closed bottles which were insulated with bubble wrap, placed on ice in a styrofoam container

129   and sent to SINTEF Sealab in Trondheim using airfreight. At arrival, less than 12 hours after fertilization,

130   eggs were transferred to 50 L tanks with flow-through of filtered (1 µm) seawater (6 ± 1°C) delivering

131   one volume exchange of seawater per day. Natural sea water, collected from a depth of 80 m (below

132   thermocline) in a nonpolluted Norwegian fjord (Trondheimsfjord; 63°26' N, 10°23' E), was supplied by

133   a pipeline system from the source to our laboratories (salinity of 34 ‰, pH 7.6). Gentle air bubbling

134   kept embryos moving continuously in the tanks. Dead and unfertilized eggs were removed from the

135   tank daily. The embryos were acclimated for 10 days until being transferred to glass jars for exposure.

136   Three concentrations of PW extract were used, in addition to a negative control containing seawater

137   only. Approximately 200 fish eggs with embryos (11 dpf) were transferred to glass jars consisting of

138   200 mL exposure medium. Images of 11 dpf embryos of both species are given in Supporting

139   Information (SIB, Fig. S1). All treatments were run with four replicates (N=4), and eggs were exposed

140   for 4 days (11-15 dpf). During this time an extra 200 ml exposure solution was added to the glass jars

141   after 2 days to maintain the exposure concentration. After 4 days exposure, dead eggs were counted

142   and removed, and the surviving eggs were transferred to glass bowls (2 L) containing clean sea water

143   (1 L) and maintained at 6 ± 1°C until 2 days post hatch (2 dph). Survival and hatching were monitored

144   throughout the recovery period. Identical experiments were performed for cod and haddock eggs. A

145   complete time line of the exposure experiment is given in Supporting Information (SIB: Table S2).

146

147   **2.4. Microscopy, heart rate analyses and biometry**

148   Images and videos of 10-20 embryos (14 dpf) and individual larvae (2 dph) were taken through a

149   microscope (Eclipse 80i, Nikon Inc., Japan) equipped with Nikon PlanApo objectives (2x for egg videos

150   and whole larvae images and 10x for close-up larvae images and videos), a 0.5x videoadaptor and a

151   CMOS camera (MC170HD, Leica Microsystems, Germany). Videos were used as a basis for heart rate

152   (HR) analyses in individual embryos/larvae using automated video analyses. Briefly, this method

153   identifies the heart tissue region in the video through pixel intensity difference between frames. Then,

154   the time sequence of mean value of the intensity in that region is extracted. This signal tends to

155   oscillate in concert with heart contraction and expansion. After normalization and smoothing the

156   signal, the number of peaks is counted, which is interpreted as the number of heart beats, providing

157   an estimate of the heart rate. The method also performs an analysis of the video and signal quality,

158   which is used to indicate potential outliers (e.g. non-beating hearts, strong larval motion) (Nepstad et

159   al., 2017). Larvae images were used for biometric analyses using Image J (Schneider et al., 2012) and

160   blinded deformation ranking analysis adopted from Sørhus et al (2015). All larvae were analyzed for

161   standard length, yolk sac area, body area, eye diameter, jaw length and eye-to-forehead distance.

162   Representative images of larvae with highlighted traces of distances/areas are given in Supporting

163 Information (SID, Figures SI2-SI6). Morphological abnormalities (jaw deformations, craniofacial
164 deformations, pericardial edema and spine deformations) were determined for larvae (2 dph)
165 according to a severity degree scale (0-3 where 0 is normal, 1 is minor deformation, 2 is moderate
166 deformation and 3 is severe deformation) (Sørhus et al., 2015). Positioning of the marginal finfold was
167 also investigated, but not ranked in the same manner as the other deformations. Examples of control
168 and deformed larvae (2 dph) are given in Figure 1, where the main observed deformations are
169 indicated. Additional examples of larvae with different deformation ranking is provided in Supporting
170 Information (SIE, Fig. S7).
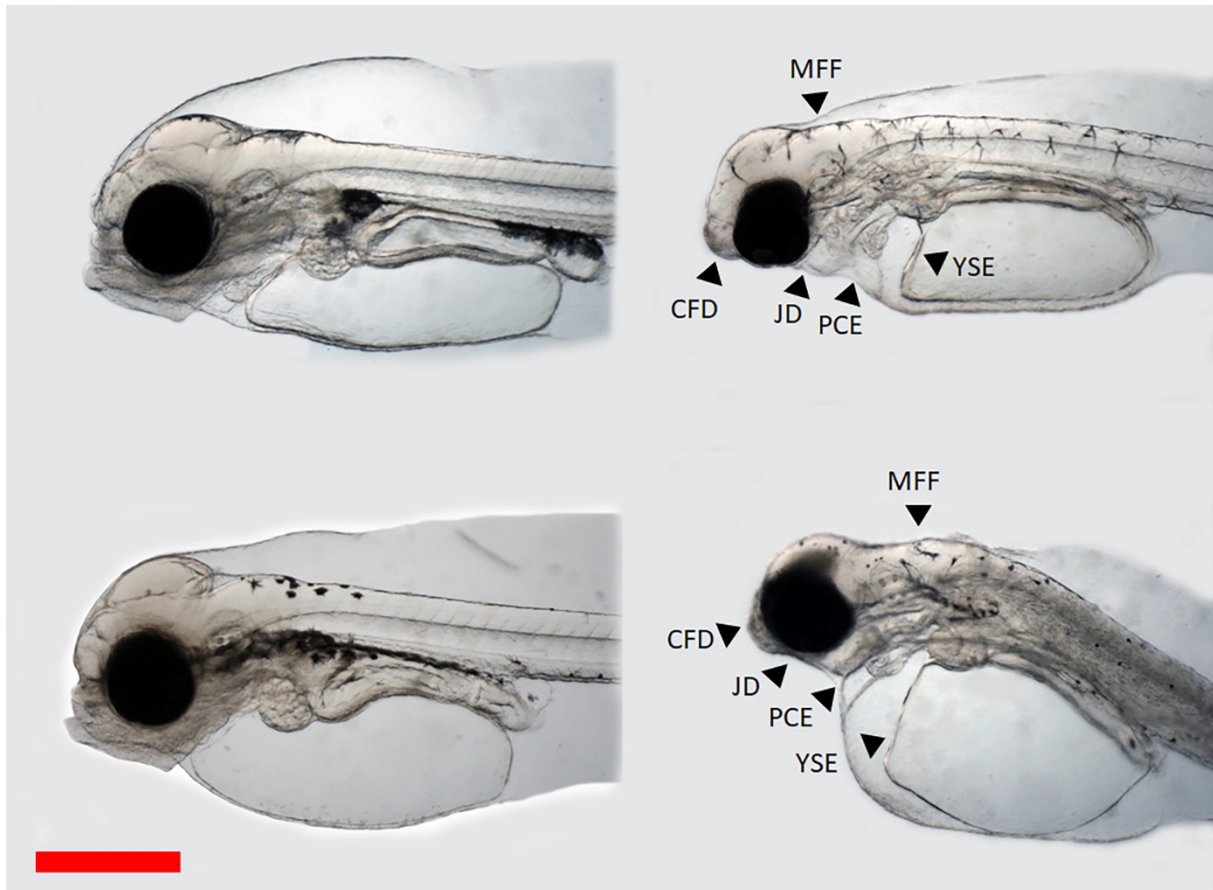
171

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

177

178 **2.5. Statistical analyses**

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

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

186

187 **3. Results and Discussion**

188

189 **3.1. Chemical characterization of produced water**

190 The total extractable material (TEM) of the whole effluent was 22 mg/L containing primarily PAH
191 (mostly naphthalenes) and phenols (Table 1). During reconstitution some loss of decalins and
192 naphthalenes was expected, but a good concentration series was obtained for all analyzed
193 components. Exposure solutions were prepared to be a dilution of the original produced water
194 starting at a concentration expected to be in a 50x dilution (high exposure) of whole effluent, and
195 then 10- and 100-fold dilutions for the medium and low exposures, respectively. There was an
196 apparent loss of C0-C1-naphthalenes and phenols during reconstitution, probably due to evaporation
197 during DCM removal. Toxicity was estimated based on T-PAH concentrations (45 PAHs and alkylated
198 homologues) in the individual treatments. Importantly, this does not mean that PAHs are the only
199 component group in produced water responsible for eliciting the studied toxic effects (Hansen et al.,
200 2018a), but provides a basis for comparison to other studies.

201

202 **Table 1: Chemical characterization of exposure solutions and the whole effluent used as a basis to**
203 **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

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

205

206 **3.2. Acute toxicity, hatching success and larvae condition**

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

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

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

226

227 **3.3. Indices of cardiotoxicity**

228 Typical cardiotoxicity phenotypes in marine fish include bradycardia (reduced heart rate), pericardial
229 edema, reduced stroke volume, arrhythmia, reduced contractility, poor looping, and failed ventricular
230 cardiomyocyte proliferation (Incardona, 2017; Khursigara et al., 2017; Sørhus et al., 2017). Cod
231 embryonic HR was higher (34.4 ± 1.4 bpm) than for haddock (23.9 ± 2.7 bpm), but opposite in larvae
232 where HR was higher in haddock (60.0 ± 7.5 bpm) than in cod (50.6 ± 3.7 bpm). Significantly lower HR
233 was observed in embryos exposed to the highest concentration compared to controls for both species
234 ($p<0.0001$) (Fig. 2A). Compared to corresponding controls, cod displayed a larger drop in HR (22.4%)
235 than haddock (17.6%). Lower HRs compared to controls were also observed after hatch (Fig. 2B) in
236 larvae for both species (high treatment only) (Fig. 2B). Increase in pericardial edema (Fig. 2C) was
237 observed for both species in a concentration-dependent manner, being significantly more severe than
238 in controls for medium ($p<0.0001$) and high ($p<0.0001$) exposures (Fig. 2C). Although both species
239 displayed comparable cardiotoxic effects of high treatment, cod also displayed significantly higher
240 degree of deformation compared to controls at the medium concentration, whereas haddock did not,
241 suggesting that cod may be more sensitive. Effects observed were consistent with exposures of
242 haddock to dispersed crude oil within the same TPAH range (Sørhus et al., 2015; Sørhus et al., 2017).
243 In haddock, bradycardia and pericardial edema was associated with a chemical blockage of calcium
244 channels, disruption of ion channel biosynthesis and defects in cardiac cell differentiation (Sørhus et
245 al., 2016). It is expected that these adverse outcome pathways are similar in cod, Studies using crude
246 oil have, in contrast to our experiments with produced water, concluded that haddock are more
247 susceptible to oil dispersions crude oil than cod. This has been explained by different chorion
248 properties (haddock eggs are stickier than cod eggs) causing differences in kinetics and uptake routes
249 between the two species. Thus, haddock may bind more oil droplets to chorion surface than cod
250 (Hansen et al., 2018b; Sørensen et al., 2017). For produced water discharges, and specifically in the
251 droplet-free exposures utilized in the present experiments, differences in chorion surface and their
252 droplet-adhesion properties between the two species may be less of an issue than for acute oil spills.
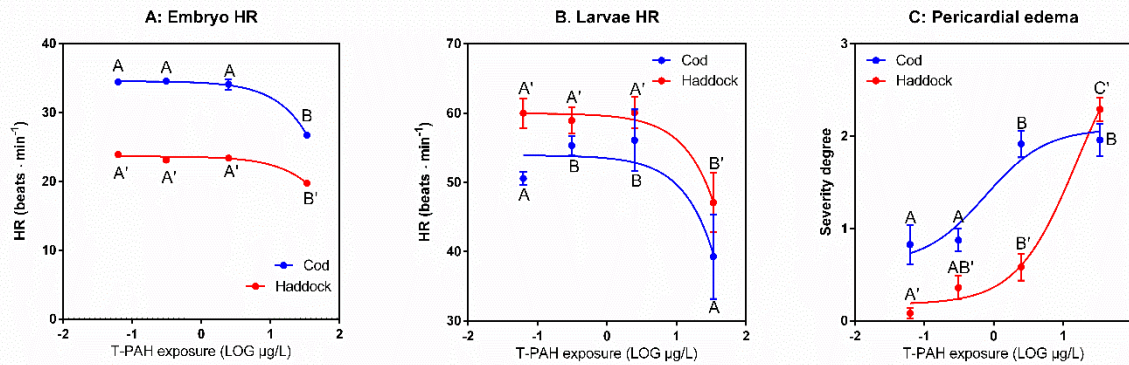
Figure 2: Cardiotoxic responses in cod (red) and haddock (blue plotted as a function of exposure concentration (in µg T-PAH/L). Responses given as mean ± SEM. A: Heart rate (HR), beats per min, N=14-69) in embryos. B: Heart rate (HR, beats per min, N=11-16) in larvae. C: Pericardial edema severity degrees in larvae (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 (p<0.05). Note different scaling on the axes.
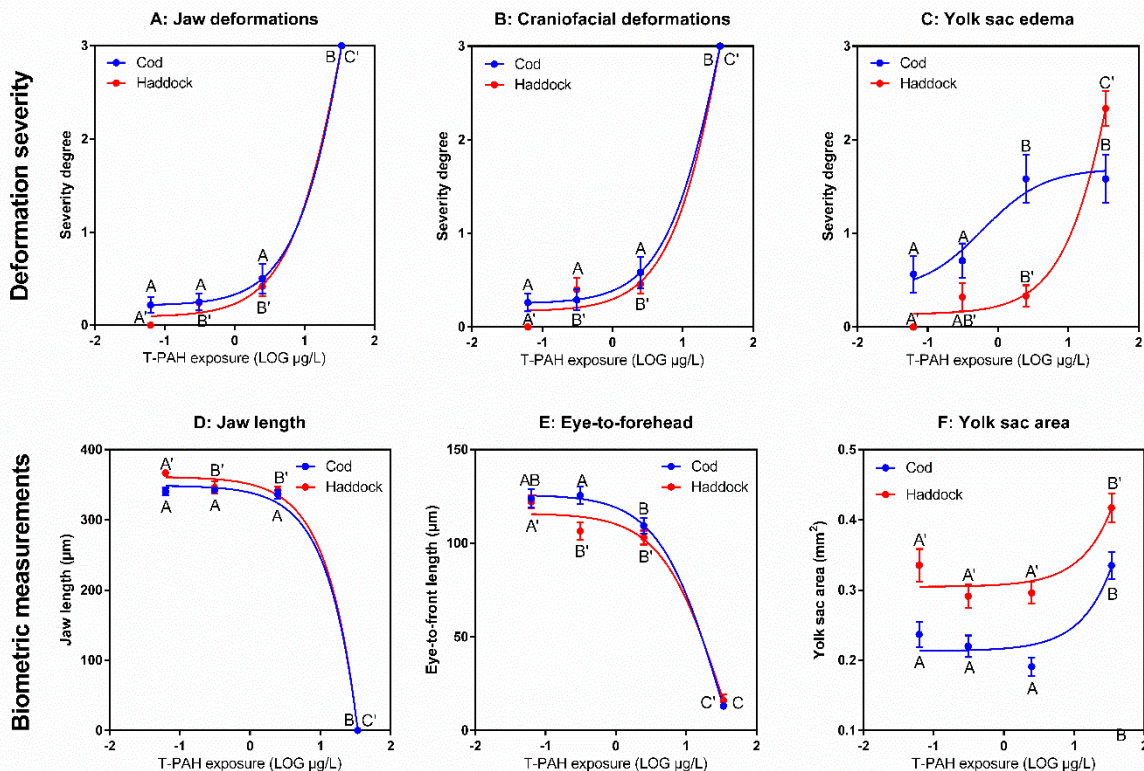
3.4. Craniofacial and jaw deformations

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

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

285 Craniofacial deformations were analyzed for severity degree (Fig. 3B) as well as biometrical
286 measurements of the distance between the eye and forehead (Fig. 3E) displaying almost identical
287 relationships with exposure concentration as jaw deformation. All haddock larvae exposed to high PW
288 concentrations displayed severe craniofacial defects with marked reductions in base structures of the
289 skull. This was also estimated biometrically measuring the distance between the eye and forehead (Fig.
290 3E). For cod exposed to high PW extract concentrations, significantly shorter eye-to-forehead distance
291 was found (p<0.0001) compared to controls. Haddock was more sensitive displaying significantly
292 shorter eye-to forehead distance for all exposure concentrations (Low: p<0.05, Med: p<0.01, High:
293 0.0001). For cod exposed to high PW extract concentrations, significantly shorter eye-to-forehead
294 distance (p<0.0001) and higher craniofacial deformity severity (p<0.0001) was observed. Haddock was
295 more sensitive, displaying significantly higher craniofacial deformation severity for all PW extract
296 treatments. Comparable deformation phenotypes have been observed in haddock exposed to
297 dispersed oil with TPAH levels like our experiment (Sørhus et al., 2015; Sørhus et al., 2017). In addition
298 to the craniofacial and jaw deformations, spinal curvatures (Supporting Information SII, Fig. S11) were
299 observed in larvae for both species exposed to high concentrations (p<0.0001), and for cod for medium
300 exposure (p<0.001) as well. Both species also displayed smaller eyes as a function of exposure
301 concentration (SII, Fig. S11). This also appears to be a more sensitive endpoint in cod as small eye
302 phenotype was significant for both medium (p<0.01) and high (0.0001) treatments, whereas for
303 haddock significantly smaller eyes were only found in high treatment of haddock (p<0.0001).

304

305 **Figure 3: Biometric measurements in larvae exposed to produced water during embryogenesis**
306 **plotted as a function of exposure concentration (in μg T-PAH/L); Deformation severities in jaw**
307 **structure (A), craniofacial structures (B) and yolk sac (C), and biometric analyses data for jaw length**
308 **(in μm) (D), eye-to-forehead distance (in μm) (E) and yolk sac area (in mm²) (F) in cod (red) and**
309 **haddock (blue). Data are displayed as mean ± SEM, N=23-25). Significant differences (p<0.05)**

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

312

313   **3.5. Yolk sac consumption and edema**

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

331

332   **4. Conclusions**

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

349

350

Acknowledgements

References

Faksness, L.-G., Grini, P.G., Daling, P.S., 2004. Partitioning of semi-soluble organic compounds between the water phase and oil droplets in produced water. Marine Pollution Bulletin 48, 731-742.

Hansen, B.H., Farkas, J., Nordtug, T., Altin, D., Brakstad, O.G., 2018a. Does Microbial Biodegradation of Water-Soluble Components of Oil Reduce the Toxicity to Early Life Stages of Fish? Environmental Science & Technology 52, 4358-4366.

Hansen, B.H., Sorensen, L., Carvalho, P.A., Meier, S., Booth, A.M., Altin, D., Farkas, J., Nordtug, T., 2018b. Adhesion of mechanically and chemically dispersed crude oil droplets to eggs of Atlantic cod (Gadus morhua) and haddock (Melanogrammus aeglefinus). The Science of the total environment 640-641, 138-143.

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proceedings of the National Academy of Sciences 108, 7086-7090.

Hodson, P.V., 2017. The Toxicity to Fish Embryos of PAH in Crude and Refined Oils. Archives of Environmental Contamination and Toxicology 73, 12-18.

Incardona, J.P., 2017. Molecular Mechanisms of Crude Oil Developmental Toxicity in Fish. Archives of Environmental Contamination and Toxicology 73, 19-32.

Incardona, J.P., Collier, T.K., Scholz, N.L., 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicology and Applied Pharmacology 196, 191-205.

Incardona, J.P., Scholz, N.L., 2016. The influence of heart developmental anatomy on cardiotoxicity-based adverse outcome pathways in fish. Aquatic Toxicology 177, 515-525.

Johnsen, S., Frost, T., Hjelsvold, M., Utvik, T.R., 2000. The Environmental Impact Factor-a proposed tool for produced water impact reduction, management and regulation, SPE International Conference on Health, Safety and Environment in Oil and Gas Exploration and Production. Society of Petroleum Engineers.

Khursigara, A.J., Perrichon, P., Bautista, N.M., Burggren, W.W., Esbaugh, A.J., 2017. Cardiac function and survival are affected by crude oil in larval red drum, Sciaenops ocellatus. Science of The Total Environment 579, 797-804.

Lee, K., Neff, J.M., 2011. Produced Water: Environmental Risks and Advances in Mitigation Technologies. Springer, New York.

Martell, D., Kieffer, J., Trippel, E., 2005. Effects of temperature during early life history on embryonic and larval development and growth in haddock. Journal of Fish Biology 66, 1558-1575.

Meier, S., Morton, H.C., Nyhammer, G., Grosvik, B.E., Makhotin, V., Geffen, A., Boitsov, S., Kvestad, K.A., Bohne-Kjersem, A., Goksoyr, A., Folkvord, A., Klungsoyr, J., Svardal, A., 2010. Development of Atlantic cod (Gadus morhua) exposed to produced water during early life stages Effects on embryos, larvae, and juvenile fish. Marine Environmental Research 70, 383-394.

Neff, J.M., Johnsen, S., Frost, T.K., Røe Utvik, T.I., Durell, G.S., 2006. Oil well produced water discharges to the North Sea. Part II: Comparison of deployed mussels (Mytilus edulis) and the DREAM model to predict ecological risk. Marine Environmental Research 62, 224-246.

398   Neilson, J.D., Perry, R.I., Valerio, P., Waiwood, K., 1986. Condition of Atlantic cod Gadus morhua
399   larvae after the transition to exogenous feeding: morphometrics, buoyancy and predator avoidance.
400   Mar. Ecol. Prog. Ser 32, 229-235.
401   Nepstad, R., Davies, E., Altin, D., Nordtug, T., Hansen, B.H., 2017. Automatic determination of heart
402   rates from microscopy videos of early life stages of fish. Journal of Toxicology and Environmental
403   Health, Part A 80, 932-940.
404   NOROG, 2017. 2017 Environmental Report: Environmental work by the oil and gas industry - facts
405   and development trends.
406   Pasparakis, C., Mager, E.M., Stieglitz, J.D., Benetti, D., Grosell, M., 2016. Effects of Deepwater Horizon
407   crude oil exposure, temperature and developmental stage on oxygen consumption of embryonic and
408   larval mahi-mahi (Coryphaena hippurus). Aquatic Toxicology 181, 113-123.
409   Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis.
410   Nature methods 9, 671-675.
411   Sørensen, L., Sørhus, E., Nordtug, T., Incardona, J.P., Linbo, T.L., Giovanetti, L., Karlsen, Ø., Meier, S.,
412   2017. Oil droplet fouling and differential toxicokinetics of polycyclic aromatic hydrocarbons in
413   embryos of Atlantic haddock and cod. Plos One 12, e0180048.
414   Sørhus, E., Edvardsen, R.B., Karlsen, Ø., Nordtug, T., Van Der Meeren, T., Thorsen, A., Harman, C.,
415   Jentoft, S., Meier, S., 2015. Unexpected interaction with dispersed crude oil droplets drives severe
416   toxicity in atlantic haddock embryos. Plos One 10.
417   Sørhus, E., Incardona, J.P., Furmanek, T., Goetz, G.W., Scholz, N.L., Meier, S., Edvardsen, R.B., Jentoft,
418   S., 2017. Novel adverse outcome pathways revealed by chemical genetics in a developing marine
419   fish. eLife 6, e20707.
420   Sørhus, E., Incardona, J.P., Karlsen, Ø., Linbo, T., Sørensen, L., Nordtug, T., van der Meeren, T.,
421   Thorsen, A., Thorbjørnsen, M., Jentoft, S., Edvardsen, R.B., Meier, S., 2016. Crude oil exposures reveal
422   roles for intracellular calcium cycling in haddock craniofacial and cardiac development. Scientific
423   Reports 6, 31058.
424   USEPA, 1996. Method 3510C: Separatory funnel liquid–liquid extraction.

425