Adhesion of mechanically and chemically dispersed crude oil droplets to eggs of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*)

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Abstract

Crude oil accidentally spilled into the marine environment undergoes natural weathering processes that result in oil components being dissolved into the water column or present in particulate form as dispersed oil droplets. Oil components dissolved in seawater are typically considered as more bioavailable to pelagic marine organisms and the main driver of crude oil toxicity, however, recent studies indicate that oil droplets may also contribute. The adhesion of crude oil droplets onto the eggs of pelagic fish species may cause enhanced transfer of oil components via the egg surface causing toxicity during the sensitive embryonic developmental stage. In the current study, we utilized an oil droplet dispersion generator to generate defined oil droplets sizes/concentrations and exposed Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) to investigate if the potential for dispersed oil droplets to adhere onto the surface of eggs was species-dependent. The influence of a commercial chemical dispersant on the adhesion process was also studied. A key finding was that the adhesion of oil droplets was significantly higher for haddock than cod, highlighting key differences and exposure risks between the two species. Scanning electron microscopy indicates that the differences in oil droplet adhesion may be driven by the surface morphology of the eggs. Another important finding was that the adhesion capacity of oil droplets to fish eggs is significantly reduced (cod 37.3%, haddock 41.7%) in the presence of the chemical dispersant.

Key words: Petroleum; fish embryo; adhesion; Arctic; dispersant

1. Introduction

Formation of mechanically dispersed oil droplets in the water column following an oil spill may be caused by many factors, including the nature of the spill (e.g. an underwater blowout) and turbulence caused by wave-action. This formation of oil droplets is often seen as beneficial in spill scenarios, as the higher oil-water surface area increases the rate of oil compound dissolution and subsequently biodegradation (Brakstad et al., 2015a; NRC, 2005). In some cases, dispersion of spilled oil is encouraged through intentional application of chemical dispersants to a slick or underwater plume (Brandvik et al., 2013). Mechanically dispersed oil droplets are typically < 100 µm (Muschenheim and Lee, 2002), while chemically dispersed droplets are typically smaller (Khelifa et al., 2008; Li et al., 2007). Produced water emissions also contains dispersed oil droplets, and the legislations on the Norwegian continental shelf is that produced water should not exceed 30 mg/L produced water.

It is generally considered that the dissolved fraction of crude oil is the most bioavailable to marine organisms, and therefore contributes most to bioaccumulation. However, oil droplets present in dispersions have the potential to significantly affect filter-feeders, which ingest oil droplets that match the size of their natural prey and coat feeding apparatus reducing feeding efficiency (Almeda et al., 2014; Hansen et al., 2012; Hansen et al., 2009). Importantly, in a crude oil dispersion in seawater, most of the oil component mass is present in the droplet phase. For oil dispersions in seawater with oil concentrations in the range 0,1 – 10 mg/L this also applies to the larger PAHs (MW>230 Da) and high logK_{OW} (>6) and their dissolved concentrations are generally very low. On the other hand, lighter components such as naphthalenes (e.g. naphthalene: logK_{OW}=3.17, MW=128,171 Da) are mostly found in the dissolved phase and only a small mass fraction is retained in the oil (Nordtug et al., 2011a) Previous studies have shown that oil droplets do not appear to contribute to the observed toxicity of oil dispersions to fish larvae (Carls et al., 2008; Nordtug et al., 2011b; Olsvik et al., 2011; Olsvik et al., 2010), or the uptake of PAHs by other marine species (Viaene et al., 2014). However, recent studies

have suggested that adhesion of oil droplets onto the chorion of fish eggs may be an important route of entry for oil components to the developing fish embryos (Sørhus et al., 2015; Sørhus et al., 2016).

It has been reported that cod and haddock eggs exposed to similar doses of mechanically dispersed crude oil were exhibiting significantly differences in PAH accumulation resulting in more severe toxicity (cardiotoxicity and larvae deformation) in the latter(Sørensen et al., 2017; Sørhus et al., 2015). The studies showed that dispersed oil droplets adhered to the chorion of haddock eggs, while the same phenomenon was not observed for cod eggs. The adhesion appeared to correlate with an increase in body residue of polycyclic aromatic hydrocarbons (PAHs) in the haddock eggs, as well as more severe malformations (Sørensen et al., 2017). At the embryo stage, the haddock and cod eggs are nearly identical in terms of size, colour and embryonic development (Fridgeirsson, 1978; Hall et al., 2004). Like most pelagic species, both cod and haddock eggs have a thin, homogenous, lamellated chorion (Lønning et al., 1988; Morrison et al., 1999). Therefore, the differences in oil droplet adhesion observed between eggs of the two species could be driven by variations in the chemistry and/or surface morphology of egg chorions facing the surrounding water.

Despite oil production and transport within spawning areas, both in Norwegian waters and globally, there is currently a lack of data on how dispersed crude oil droplets affect the early life stages of fish (Olsen et al., 2013). The areas around the Lofoten Islands of northern Norway, as well as the Barents Sea, and the Atlantic Arctic area, are considered especially vulnerable to oil spills since they are spawning and larval-drift areas for several commercially important species of marine fish, including Atlantic haddock (*Melanogrammus aeglefinus*) and cod (*Gadus morhua*) (Hauge et al., 2014; Misund and Olsen, 2013; Olsen et al., 2010). A more detailed understanding of the effects of dispersed crude oil on different fish species is therefore necessary to identify both species and regions that represent highest risks for oil spill impacts.

In the present study, the capacity for dispersed oil droplets to adhere to the chorion of cod and haddock eggs were estimated and compared. Furthermore, the potential differences in the adhesion properties of mechanically (MD) and chemically dispersed (CD) crude oil to the eggs was also assessed. Identical oil dispersions, in terms of concentrations and oil droplet sizes, of mechanically and chemically dispersed (added chemical dispersant) crude oil were prepared and eggs of both species were exposed for 24 hours. Body burden analyses of high logK_{OW} PAHs were used as a proxy for oil droplet adhesion estimation. Differences in cod and haddock egg surface morphology were investigated using scanning electron microscopy (SEM) imaging. The study should provide important information about potential species-differences in oil adhesion capacity and how chemical dispersants affect this process. For net environment benefit analyses and resource damage assessment processes in the event of an accidental oil spill, these results are key to decide on oil spill responses and to estimate exposure and toxicity to native fish populations.

2. Materials and Methods

2.1. Eggs

Fertilized eggs were collected from the stock fish facility at the Institute of Marine Research at Austevolden, Norway. The eggs were transported by air freight to Trondheim and kept at 5°C in incubator tanks until used for experiments and analyses.

2.2. Dispersion generation, egg exposure, sampling and analyses

Uniform oil dispersions were generated using an oil droplet generator (Nordtug et al., 2011a), where crude oil (Heidrun blend) was dispersed in filtered (0.22 µm cartridge filter) sea water through a series of nozzles yielding a constant flow of dispersion with a homogeneous droplet size. To generate the chemically dispersed oil (CD), the commercially available oil spill dispersant Dasic NS was premixed into the oil (4% w/w dispersant) prior to dispersion. Oil dispersions generated without the use of chemical dispersant are termed mechanically dispersed (MD). The two dispersions were generated

with identical set-up, but to achieve a similar oil droplet size distribution and concentration in the two treatments, the energy input (water flow and thus turbulence) for generating the dispersion with Dasic NS was reduced compared to the purely mechanically generated dispersion (Nordtug et al., 2011a). Both dispersions were generated at a nominal concentration of 1 mg oil/L. Droplet size distributions were verified by a Coulter Counter (Multisizer 3, with 100 µm aperture).

Freshly prepared dispersions were transferred to 2 L borosilicate bottles (N=4 for each treatment), 200 cod or haddock eggs (14 days post fertilization) were added, and the bottles capped with Teflon-lined caps (VWR International). The bottles were filled completely (no headspace) and mounted on a custom-built carousel incubation system, as previously described (Brakstad et al., 2015a; Brakstad et al., 2015b). Bottles filled only with filtered sea water and eggs (no oil) were used as negative controls (N=4). The carousel system maintained a constant clockwise rotation at a velocity of 0.75 rpm at 5°. After 24 h rotation, the bottles were taken off the carousel and sampled immediately.

The fish eggs were separated from the water phase by sieving the dispersions through a 100 µm size mesh. An aliquot of the water sample was also analysed for droplet size distribution and concentrations using Coulter Counter Multisizer. A sub-sample (40 mL) was removed for analysis of volatile organic compounds using purge and trap gas chromatography coupled to mass spectrometry (Faksness et al., 2015). The remaining volume of water was acidified (pH~2, HCl) and extracted by serial liquid-liquid extraction using dichloromethane (45-30-30 mL) for analysis of semi-volatile organic components (SVOC). Surrogate internal standards (*o*-terphenyl, naphthalene-*d*8, phenanthrene-*d*10, chrysene-*d*12 and phenol-*d*6) were added prior to extraction. The combined extracts were dried over sodium sulfate and concentrated to approximately 1 mL before addition of recovery internal standards (5α-androstane, fluorene-*d*10). The total extractable material (TEM) was quantified using GC coupled to a flame ionization detector (GC-FID), while decalins, PAHs, alkyl PAHs and alkyl phenols were quantified using GC coupled to mass spectrometry (GC-MS/MS).

Approximately 50 eggs were sampled for body residue analyses using the method described in Sørensen et al. (2016). After addition of surrogate standards (naphthalene-*d*8, biphenyl-*d*8, acenaphtylene-*d*8, anthracene-*d*10, pyrene-*d*10, perylene-*d*12 and indeno[1,2,3-*cd*]pyrene-*d*12), the samples were homogenized in *n*-hexane-DCM (1:1 v/v, 2 mL) followed by addition of anhydrous sodium sulphate (150 mg) to remove residual water, vortex extraction (30 s) and centrifugation (2000 rpm, 2 min). The supernatant was collected and the extraction step repeated twice. The combined organic extract was concentrated to ~1 mL prior to clean-up by solid phase extraction (SPE) using silica (Agilent Bond Elut SI, 500 mg, Agilent Technologies, USA). The extract was eluted with dichloromethane in *n*-hexane (1:9, v/v, 6 mL). Immediately prior to the analysis, the volume of the purified extract was reduced to 100 µL under a gentle stream of N_2. PAHs and alkyl PAHs were analysed by GC-MS/MS as described in Sørensen et al (2017).

2.3. Fluorescence microscopy

The remaining eggs were transferred to clean, filtered sea water and imaged both in bright field and fluorescence using microscopy. A microscope (Nikon eclipse 80i, Nikon Corp., Tokyo, Japan) equipped with a 10× S Fluor objective (Nikon Corp., Tokyo, Japan; NA 0.50) was used to visualize crude oil droplet fluorescence on egg surface, induced by illuminating the specimen with a 120-W mercury arc lamp (xcite 120, EXFO Corp., Quebec, Canada) passing through a B-2A filter cube (Nikon Corp., Tokyo, Japan). Images were captured with a Peltier cooled CCD camera (DS 5Mc, Nikon Corp., Tokyo, Japan) controlled from a computer running NIS Elements F (Nikon Corp., Tokyo, Japan; v. 4.30).

2.4. Estimation of oil mass associated with eggs

To estimate the total amount of oil mass adhering to the eggs, the measured concentrations of individual low-solubility oil components ($\log K_{OW} > 6.0$, N=13 for each sample) associated with the eggs and in the bulk oil (Oil profile given in Supporting Information Table S3) were determined. Under the

assumption that loss through dissolution is negligible during the experiment, the ratio of the two concentrations provides the required estimate. The concentrations are:

$$C_{KO} = \frac{m_K}{m_O}, C_{KE} = \frac{m_K}{m_E}$$

where C_{KO} is the measured concentration of oil component K in the bulk oil (ug/g) and C_{KE} is the measured concentration of oil component K associated with the egg (ug/g), m_K is the mass of component K in the parent oil, m_O is the mass of the oil and m_E is the mass of the eggs in the sample. An estimate of total oil in the egg C_{OE}^K (g/g) based on component K is thus:

$$C_{OE} = \frac{C_{KE}}{C_{KO}} = \frac{m_K}{m_E}\frac{m_O}{m_K} = \frac{m_O}{m_E}$$

There are variations in these estimates for different components, so the final estimate is based on a component average (*N* components) for each sample:

$$\overline{C_{OE}} = \frac{1}{N}\sum_{K=1}^{N} C_{OE}^K$$

and the corresponding standard deviation to quantify the spread in the estimate.

2.5. Egg surface morphology

To investigate physical differences in the surface morphology of the eggs from cod and haddock, a batch of eggs from each species were subjected to imaging by scanning electron microscopy (SEM). SEM was performed with a secondary electron signal using a NOVA NANOSEM 650 FEI instrument. Sample preparation involved fixation in 3% glutaraldehyde (pH 7.4), dehydration in ascending concentrations of ethanol and finally critical point drying. Prior to observation the samples were coated with carbon to enhance the contrast.

2.6. Statistical analyses

The software GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) was used for visualizing data and performing statistical analyses. Two-way analysis of variance (ANOVA) was used to assess

differences between treatments. Tukey's multiple-comparison post hoc test was used to compare the exposed groups against the control or for comparison between exposure groups. A significance level of $p < 0.05$ was used for all tests.

3. Results and Discussion

Pelagic fish eggs are at risk of being exposed to oil dispersions if an acute oil spill occurs in spawning areas. Dissolved oil components will be taken up through passive diffusion, but adhesion of oil droplets onto the chorion of fish eggs may also be a contributing exposure route for less water-soluble oil components resulting in increased toxicity during this early stage. This study focussed on adhesion of MD and CD oil droplets on the chorion of fish eggs. This was done by exposing eggs to comparable MD/CD dispersions for a short period of time and estimate oil droplet associated with eggs based on concentrations of high $\log K_{OW}$ (>6.0) oil components analysed in eggs and parent oil.

Droplet size distribution analysis confirmed that the exposure experiments were conducted using comparable droplet sizes in all exposure treatments (Table 1). The measured average oil droplet sizes were slightly larger in the MD treatment (11.4-12.2 µm) than in the CD treatment (9.2-9.9 µm) (Table 1). A smaller droplet will have a lower surfacing velocity than a larger droplet and this may affect the droplet concentration over time. However, in the current experiment droplets were constantly kept in suspension by turbulence created by rotating the exposure bottles, and the observed size differences did not affect exposure concentrations. Thus, the difference in the droplet size range between CD and MD treatment is not expected to affect the conclusions of the study. Exposure concentrations of droplets as measured by the Coulter Counter indicated good comparability across all exposure treatments (1.05-1.08 mm^3/L at 0h and 0.85-0.96 after 24 h). GC-MS analyses confirmed that the chemical composition of the exposure treatments was almost identical for MD and CD as well as between cod and haddock exposures (Table 2). A detailed chemical composition of each exposure treatment is given in the Supporting Information (Tables S1).

211

212 Fluorescence microscopy imaging was performed to visualize oil droplets attached to the surface of
213 eggs after 24 h exposure to MD and CD. Although relatively few, adhered oil droplets were clearly
214 visible on the chorion of eggs from both species as well as from MD and CD treatments (example of
215 cod after MD-treatment given in Figure 1). Unfortunately, it was not possible to estimate any
216 quantitative differences in droplet number based on the images, possibly due to the low depth of field,
217 but oil droplets did not appear to be located at any specific location on the chorion for either of the
218 two species. Previous use of this methodology on dispersion-exposed copepods has successfully
219 provided insights into the filtration and adhesion characteristics of MD and CD crude oil (Hansen et al.,
220 2009; Nordtug et al., 2015).

221

222 The body burden of total PAHs and individual PAH classes determined in cod and haddock eggs differed
223 between treatments, being most different for high logK_{OW} compounds (logK_{OW} > 6.0; Table 2 and
224 Supporting Information Table S2). These components are mainly associated with oil droplets because
225 of their low water solubility (Nordtug et al., 2011a). Therefore, if egg analyses indicate the presence of
226 high molecular weight compounds, the eggs must be adhering droplets. In the current study, this
227 behaviour of different oil components is observed for haddock and, to a somewhat lesser extent, for
228 cod. Estimation of the oil mass on cod and haddock eggs was conducted using GC-MS/MS analysis of
229 the fraction of high logK_{OW} compounds (>6.0) associated with crude oil and eggs. This approach
230 assumes that high logK_{OW} components are only associated with eggs through adhered oil droplets and
231 not accumulated as dissolved compounds through the water phase. Although components with logK_{OW}
232 values > 6.0 have a very low solubility, some uptake by the eggs of these components from the water
233 phase over time is possible. Furthermore, oil droplets may also act as a reservoir for PAHs, replenishing
234 them in the dissolved phase when they are removed by uptake into the eggs (Redman, 2015). However,
235 these processes are slow, and the short exposure time (24 h), and the static exposure design
236 implemented in the current study, should reduce any significant uptake of high logK_{OW} components

from the water phase. It is therefore assumed that any high logK_{OW} components found to be associated with eggs come from the adhered oil droplets.

A significantly ($p<0.05$) higher oil mass was associated with haddock eggs compared to cod eggs in the MD exposure (Figure 2). This is consistent with a previous study where higher numbers of oil droplet were observed on haddock eggs compared to cod eggs (Sørhus et al., 2015). This higher association of oil droplets to haddock eggs may explain differences observed between these species in terms of oil toxicokinetics and sensitivity (Sørensen et al., 2017). Increased adhesion of oil droplets was found to correlate with an increase in PAH body residue in haddock eggs and more severe malformations in embryos (Sørensen et al., 2017).

The underlying mechanism causing this difference in oil droplet adhesion to cod and haddock eggs has not been elucidated but may be related to variations in chemistry and/or morphology of the chorions of the two species. SEM images of the chorion surface of cod and haddock eggs revealed significant morphological differences, suggesting this may contribute to the observed variations in oil droplet adhesion (Figure 3). The cod chorion was characterised by a rough surface comprised of long, densely packed filaments (Figure 3A), while the haddock chorion appeared much smoother with low-density distribution of nodules or filaments (Figure 3B). The haddock egg surface has been previously described as a featureless thin surface coating (Morrison et al., 1999), consistent with the observations in the current study.

It is suggested that this lack of features or filaments on haddock eggs may facilitate the adhesion of oil droplets owing to an increased contact area between the droplet and the egg surface. The larger filaments present on cod eggs suggest a significantly reduced contact area is available for droplets to attach to the chorion surface than for haddock. Interestingly, pores were visible on both egg types, but they are partly covered in cod eggs by the long filaments (Figure 3B). This may serve to protect cod

eggs against uptake of components from oil droplets, as the filaments increase the distance between the droplet and the pore, while for haddock there is potential for direct contact between droplets and pores. Furthermore, haddock eggs have an outer chorion membrane that is absent in cod eggs (Fridgeirsson, 1978), and it is believed that this outer membrane changes or even disappears during development of the embryo. As the current study was performed using eggs close to hatching, the haddock may thus have a thinner chorion than cod. These changes in haddock chorion during development have been shown to change the adhesion potential of oil droplets, with adhesion being confined to in defined regions of the haddock chorion during late exposure (Sørensen et al., 2017; Sørhus et al., 2015). This also suggests that chemical changes or differences in the surfaces of the two egg types might also influence adhesion of oil droplets and requires further study to fully understand the underlying mechanisms controlling oil droplet adhesion to fish eggs.

Both cod and haddock also displayed significantly lower oil mass association with eggs exposed to CD compared to eggs exposed to MD (Figure 2). Previous work has confirmed that there is no difference in the water soluble fraction of oil when the oil is chemically or mechanically dispersed at the same droplet size and concentration (Sørensen et al., 2014). Therefore, any difference in body burden should be directly related to the adhesion of droplets. Results from the current study suggest that mechanically dispersed oil is more "sticky" than chemically dispersed oil, leading to increased interaction with the chorion of fish eggs. This difference has previously been reported for the interaction between mechanically and chemically dispersed oil droplets and inorganic particles (Sørensen et al., 2014). Furthermore, a previous study has shown that differences in MD and CD oil may influence toxicity of oil dispersions. First-feeding cod larvae were exposed to MD and CD oil at comparable oil concentrations and droplet size ranges, the MD treatment elicited more significant responses in acute toxicity, transcriptional responses and metabolic alterations relative to CD exposures (Hansen et al., 2016). The differences in adhesion properties between CD and MD oil droplets may also be explained by the surface properties of the droplets. For CD, the dispersant will

289 align on the surface of the droplet with a hydrophilic component interacting with the water and a
290 hydrophobic component interacting with the oil (NRC, 2005). This changes the interfacial tension and
291 possibly the electric charge of the droplets in the CD oil droplets compared to the MD droplets, and
292 this may cause differences in the interactions between oil droplets and chorion components (proteins,
293 glycoproteins, mucosaccharides and lipids). This topic is not covered in the present work and deserves
294 attention for future research.
295

296 **3. Conclusions**

297 Oil droplets adhere to the chorion of both haddock and cod eggs, indicating this may be a significant
298 exposure route for larger, apolar crude oil components ($\log K_{OW} > 6.0$) when released into the marine
299 environment. Adhesion of droplets is more significant for haddock eggs relative to cod eggs, and may
300 be facilitated by morphological differences of the surfaces between the two egg types. MD oil droplets
301 were observed to adhere more frequently to eggs of both species than CD oil droplets, suggesting the
302 use of chemical dispersants under oil spill scenarios may help to reduce adhesion and certain
303 toxicological effects. A knowledge of fish egg morphology may be useful when undertaking risk
304 assessments of oil production or transport in marine regions known to be spawning grounds for
305 specific commercial fish species. Future research should focus on understanding the underlying
306 physicochemical mechanisms (membrane chemistry and morphology) controlling the adhesion of oil
307 droplets to fish eggs, as well as detailed understanding of the surface chemistry of oil droplets
308 with/without dispersant. Finally, dechorionizing eggs followed by extraction and PAH analyses of the
309 embryos may provide evidence of droplet-enhanced uptake of heavy oil components.
310

311 **Acknowledgements**

312 This work was funded by the Research Council of Norway (grant numbers 267820 and 280511). The
313 authors also want to thank two anonymous reviewers for valuable comments.

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Figure legends

Figure 1: Example of egg imaged in bright field (A) and fluorescence (B) using microscopy. Oil droplets are barely visible in bright field, but they show yellow-green stain using fluorescence. The example shows oil droplets adhered to the chorion of a cod egg exposed to mechanically dispersed (MD) crude oil. Inside the egg, the head of the cod embryo is displayed.

Figure 2: Estimated mass of oil adhered onto cod (COD) and haddock (HAD) eggs (in mg oil/g egg) after 24 h exposure to mechanically dispersed (MD) and chemically dispersed (CD) oil. Letters display significant differences ($p<0.05$) among treatments (mean ± STDEV, N=4 and each replicate consists of 13 components each using the whole data set in Two-way ANOVA analysis).

Figure 3: Scanning Electron Microscopy (SEM) of haddock (A) and cod (B) chorion after Karnovsky fixation. The cod image was taken at 20 000x magnification, and the haddock was taken at 14 000x magnification. The white bars indicate 3 µm.