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Transcriptional evidence for low contribution of oil droplets to acute toxicity from dispersed oil in first feeding Atlantic cod (*Gadus morhua*) larvae

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ABSTRACT

We evaluated the potential contribution of oil droplets to the toxicity of dispersed oil to first feeding fish larvae. Atlantic cod larvae were exposed to five concentrations of either artificially weathered (200 °C residue) dispersed oil (D1–D5) containing oil droplets [medium size 11–13 μm based on volume] and water-soluble fraction [WSF] or the filtered dispersions containing only the corresponding equilibrium WSFs only (W1–W5). The larvae were exposed for 4 days and harvested for transcriptional analysis at 13 days post hatching. The most significant differently expressed genes were observed in cod larvae exposed to the highest concentration of the dispersed oil (containing 10.41 ± 0.46 μg $\sum$PAH/L), with CYP1A showing the strongest response. Functional analysis further showed that the top scored network as analyzed with Ingenuity Pathway Analysis was "Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism". Oil exposure also increased the expression of genes involved in bone resorption and decreased the expression of genes related to bone formation. In conclusion, oil exposure affects drug metabolism, endocrine regulation, cell differentiation and proliferation, apoptosis, fatty acid biosynthesis and tissue development in Atlantic cod larvae. The altered gene transcription was dominated by the WSF and the corresponding oil droplet fraction only had a moderate contribution to the observed changes.

1. Introduction

As oil exploration and production in the Atlantic Ocean move northwards towards more sensitive areas like the Lofoten/Vesterålen area offshore of Northern Norway and the Arctic regions, spawning grounds for large stocks of commercial important fish species may be exposed to accidental oil spills. Due to the high dilution rate in the marine environment, toxic compounds present in accidentally discharged crude oil are generally found at low concentrations. Acute oil spills near spawning grounds of Atlantic cod (*Gadus morhua*) and wintering areas of juvenile Atlantic herring (*Clupea harengus*) may however endanger local populations of these species. The long-term impacts of oil spills on marine fish are generally thought to be relatively low, with an exception for the particularly vulnerable early life stages (Marty et al., 1997; Bue et al., 1998; Carls et al., 1999; Heintz et al., 1999; 2000; Incardona et al., 2004; 2005; Elskus et al., 2005). For larvae of the Atlantic cod, little is known about their ability to metabolize oil components, as well as the levels to which oil compounds cause narcosis, growth effects and death. A better understanding of the impact of spilled oil on Atlantic cod larvae is therefore needed.

Oil is a complex mixture of a range of different components like aliphatic hydrocarbons, aromatic hydrocarbons, phenols, and a substantial amount of unknown compounds described in the literature as unresolved complex mixture (UCM; Melbye et al., 2009). Following an acute oil spill, waves, wind and sunlight will cause weathering of the oil in the marine environment, altering the oil appearance and composition dramatically and dynamically. Over time the actual exposure of organisms to oil components will vary. In order to provide any meaningful information regarding the fate and effects of a marine oil spill computerized modeling tools are needed.

The weathering process generates oil-in-water dispersions, consisting of oil droplets in the water phase that may persist or resurface. Micron-sized oil droplets will to a minimal degree resurface, and they will be a source of oil compounds through leakage/dissolution as chemical equilibrium for oil compounds between the water phase and the oil droplets continuously will vary. Limited knowledge exists on the fate and effects of oil droplets in the water column in terms of lifetime, adhesion to particles, dissolution rates and toxicity. The extensive use of oil dispersants during the Deepwater Horizon accident in the Gulf of Mexico in 2010 has also highlighted the need for more knowledge on the toxic effects of oil droplets on fish larvae. Exposure studies carried out with pink salmon (*Oncorhynchus gorbuscha*) and pacific herring (*Clupea pallasi*) embryos after the Exxon Valdez accident showed that dissolved PAHs alone (i.e. without oil droplets) are sufficient for toxic impacts (Marty et al., 1997; Carls

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et al., 1999; Heintz et al., 1999). Studying zebrafish (*Danio rerio*), Carls et al. (2008) exposed fish embryos in a physical barrier separating dissolved PAH from oil droplets, and showed identical biological responses to water containing either dissolved PAH alone, or dissolved PAH plus droplets. Although the 2008 Carls study settles the case for fish embryos, the situation could be different for feeding-stage larvae.

Some studies have been made in order to investigate effects of produced water and single oil compounds on cod adults and larvae, e.g. developmental effects (Meier et al., 2010), reproductive effects (Meier et al., 2007a), glycerophospholipids/cholesterol metabolism (Meier et al., 2007b), endocrine system (Boitsov et al., 2007), redox status (Hasselberg et al., 2004), transcriptional effects (Lie et al., 2009a; Holth et al., 2010) and proteome effects (Bohne-Kjersem et al., 2010). However, in this work we aim to understand explicitly what contribution the oil droplets have to the toxicity, and in order to do so two vital prerequisites are applied. The first is an experimental system that generates a continuous controlled dispersion in equilibrium with the surrounding water and a filtered dispersion, consisting of the water-soluble compounds generated from the dispersion through a filter. Secondly, to understand which effects that occur, high throughput analytical tools, like microarrays, are applied. In this study we are combining a complex experimental system for exposure studies with transcriptional analyses of exposed cod larvae. Thus, information regarding the effects of droplets either as additional effects to the water-soluble fraction or as "droplet only" effects may be extracted. The use of the microarray will also provide information regarding possible modes of oil droplet toxicity. Furthermore, if there is a no additional effect of the droplets per se, effects modeling of acute oil spills will be simpler, as effects are more easily assessed by using only the water-soluble fraction of the oil as input from models.

The aim of this work was to evaluate the contribution of oil droplets to the mechanistic effects or toxicity caused by exposure of cod larvae to oil dispersion. The difference in transcriptional responses in cod larvae exposed to oil dispersion or the corresponding filtered WSF may be deciphered as additional toxicity and/or potential modes of oil droplet toxicity. Transcriptional responses in the cod larvae were determined by microarray and RT-qPCR analyses. Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) were applied for functional and pathway analysis. In a follow-up genomic profiling study we will look at chemically induced oil droplets and whether they have the same effects as physically made oil droplets in Atlantic cod larvae.

2. Materials and methods

2.1. Materials and experimental set up

Fresh crude oil was artificially weathered by heating to 200 °C. The resulting 200 °C + residue was dispersed into filtered seawater (5 μm) through a series of nozzles yielding a constant flow of dispersion with a homogenous droplet size. The principle of the exposure system (Fig. 1) is based on generating two dilution series, one with dispersion and a second with the corresponding water-soluble fraction (WSF) isolated from the dispersions. The concentration gradient used was logarithmic with a spacing of 0.5 log-units based on the amount of dispersed oil added. To distinguish between toxic stress caused by the dispersion and its pure WSF, each step in the dispersion dilution series was filtered. The filter units consisted of fine glass wool on top of a Watman GFC glass filter. Retention of oil droplets was verified by chemical analysis and particle counting to isolate the WSF of the parent dispersion in a parallel exposure vessel.

The exposure containers consisted of 5 L borosilicate glass bottles with their bottoms removed. Exposure solution and clean seawater (controls) were added in the lower part of the exposure container through Teflon tubing (bore 1 mm). Water was drained from the surface through a 300 μm plankton mesh. The temperature was controlled by submerging the units into a water bath. The flow-through in all exposure units was kept constant at 17.5 mL/min. A peristaltic pump (Watson–Marlow), equipped with Marphrene® tubing, was used to drive the dispersion through the glass filters. Dispersions were added passively and flow was adjusted by the height of the inlet water column using the inlet Teflon tubes as resistance. The principle of the exposure systems is shown in Fig. 1. Three identical exposure systems were used in order to achieve three biological replicates for every exposure concentration. The principle of the exposure units is shown in Fig. 1. The full description of the experimental set-up with verifications has been previously reported by Nordtug et al. (submitted A), and the system has also previously been used for experiments with cod larvae (Olsvik et al., 2010; Nordtug et al., submitted B) and copepods (Hansen et al., 2009).

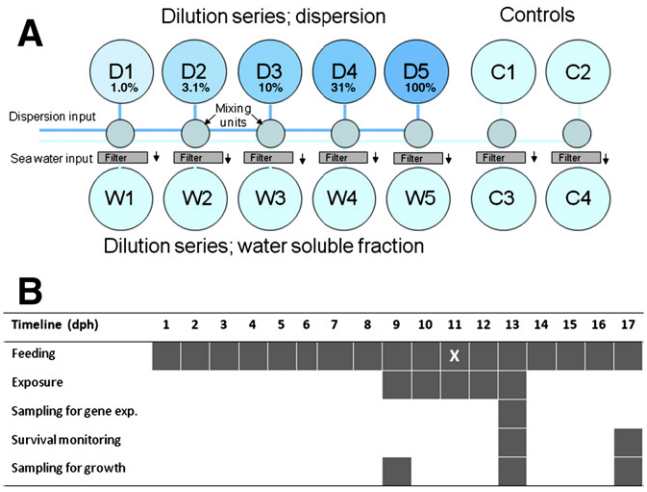

Fig. 1. A) Design of the experimental system. Three identical exposure systems were used in order to achieve biological replicates for every exposure concentration. Note that the dilution gradient is reversed relative to normal practice with the highest concentration furthest from the inlet. This is done in order to maintain a high flow rate of the dispersion through the inlet tube, thus minimizing settling of oil droplets within the tube. B) The figure shows an overview of the experiment (feeding, exposure period and when the cod larvae were sampled for gene expression analyses, growth measurement and survival was observed. X is the approximate time when the yolk sack was consumed. D1–D5 are dispersions with relative concentrations indicated, W1–W5 are the corresponding equilibrium WSFs. The maximum PAH concentration including naphthalenes of the dispersion (D5: 100%) was 28.09 μg/L (±SD = 1.5) (corresponding to approx. 0.5 mg/L total hydrocarbon).

Fertilized cod eggs (*Gadus morhua*) from Marine Harvest Cod (Norway) were transported to the laboratory, hatched and the larvae was maintained as previously described by Olsvik et al. (2010). Cod larvae were exposed to a gradient of different concentrations of dispersed oil or the corresponding water-soluble oil fraction 9 dph until 13 dph when larvae were sampled for gene expression analyses, and the experiment was ended at 17 dph after a recovery period in clean sea water. The effect of the exposure on the cod larvae was compared with identical control units containing non-exposed cod larvae. During the experiment the larvae were fed rotifers in green water (*Isochrysis galbana*). Larval survival and dry weights were also analyzed for with method and results described elsewhere (Nordtug et al., submitted B).

2.2. Larvae sampling and RNA extraction

At the end of the exposure period (on day 13), the whole cod larvae were immediately rinsed with distilled water and blotting paper and flash-frozen in liquid nitrogen, and stored at −80 °C before RNA isolation. To ensure enough RNA was available for downstream transcriptomic analysis, 10 larvae were pooled together from