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Biomarker candidate discovery in Atlantic cod (*Gadus morhua*) continuously exposed to North Sea produced water from egg to fry

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ABSTRACT

In this study Atlantic cod (*Gadus morhua*) were exposed to different levels of North Sea produced water (PW) and 17β-oestradiol (E_2), a natural oestrogen, from egg to fry stage (90 days). By comparing changes in protein expression following E_2 exposure to changes induced by PW treatment, we were able to compare the induced changes by PW to the mode of action of oestrogens. Changes in the proteome in response to exposure in whole cod fry (approximately 80 days post-hatching, dph) were detected by two-dimensional gel electrophoresis and image analysis and identified by MALDI-ToF-ToF mass spectrometry, using a newly developed cod EST database and the NCBI database. Many of the protein changes occurred at low levels (0.01% and 0.1% PW) of exposure, indicating putative biological responses at lower levels than previously detected. Using discriminant analysis, we identified a set of protein changes that may be useful as biomarker candidates of produced water (PW) and oestradiol exposure in Atlantic cod fry. The biomarker candidates discovered in this study may, following validation, prove effective as diagnostic tools in monitoring exposure and effects of discharges from the petroleum industry offshore, aiding future environmental risk analysis and risk management.

1. Introduction

Produced water is considered to be by far the most dominant discharge from offshore oil production, containing a variety of compounds, including oil products such as mono-, di- and poly-aromatic hydrocarbons (PAHs), alkyl phenols, and several heavy metals, which have previously been shown to cause adverse effects in fish (Neff, 2002). Some of these compounds are reported to be endocrine disrupting chemicals (Evanson and Van Der Kraak, 2001; Santodonato, 1997), that affect steroidogenesis (Evanson and Van Der Kraak, 2001; MacLatchy and Van Der Kraak, 1995), reproductive organs and reproduction (Meier et al., 2007a,b), in fish eggs, larvae, and fry (Hahn, 2001; Rolland, 2000). PAHs and metals present in produced water are also reported to cause oxidative stress (Hasselberg et al., 2004; Livingstone, 2001), possibly resulting in mutagenesis (Machala et al., 2001) and carcinogenesis (Santodonato, 1997).

Biological effects of exposure occurring at the early stages of development are of special interest, as these stages are found to be more sensitive to xenobiotic exposure (Bern, 1992; Colborn et al., 1993). In many teleost species, this period of enhanced vulnerability is from fertilization through the yolk sac embryo stage, which is prior to the larvae stage. However, studies have shown that food and water-borne exposure contributes significantly also to fry mortality (Rolland, 2000).

There has been an unprecedented decline in commercial marine fish species world-wide, resulting in a growing concern for the future viability of fishery resources and prompting a search for causes explaining this decline (Jackson et al., 2002). In many coastal ecosystems cod fish and other marine vertebrates are functionally or entirely extinct. Overfishing is believed to be the main reason for this (Worm et al., 2006), although pollution is considered an important contributing factor to reported declines (Lotze and Milewski, 2004). Atlantic cod was selected for this study as it is one of the key fish species, together with herring, for risk assessment studies in connection with oil and gas activities in Norwegian waters. Development of improved and more sensitive biomarkers may contribute with new knowledge that is important to risk management, as drilling and exploration of wells in new areas is progressing.

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At present, it is difficult to detect and monitor effects of exposure before they become a threat to health and reproduction and causing effects at the population level. Many field studies report late occurring adverse effects in various teleost species, often not visible in the exposed fish, but rather in its progeny (see reviews by Rolland, 2000; Schwaiger et al., 2002). Also several reports suggest that adverse effects are induced at lower levels of exposure than known biomarkers can detect (Denslow et al., 2001; Reid and MacFarlane, 2003). Hence, there is a need for more sensitive biomarkers that can act as early warning signals to detect and monitor the effects of oil and produced water on marine fish.

In this study, we have exposed Atlantic cod (*Gadus morhua*) at early life stages to different concentrations of produced water (Oseberg C, the North Sea) and 17β-oestradiol (E_2), a natural oestrogen, continuously for 90 days from egg to fry stage. Our aim was to investigate produced water-induced changes in cod fry, and, by comparing them to the responses induced by E_2, to see if any of these responses represent a similar mode of action (MOA).

2. Materials and methods

Acetic acid, Coomassie brilliant blue CBB G250, orthophosphoric acid, urea, thiourea, tris (hydroxymethyl)-ammonium methane, acetonitril, methanol and trifluoroacetic acid were all purchased from Merck (Damstadt, Germany). Ethanol was obtained from Arcus Kjemi (Oslo, Norway), and bovine serum albumin, BSA, 3-(3-cholamidopropyl)-dimetylammonio-1-propansulfonate, CHAPS, Triton X-100, DL-dithiothreitol, DTT, iodoacetamide, and alpha-cyano-4-hydroxycinnamic acid, CHCA, were purchased from Sigma–Aldrich (St. Louis, MO, US). Ampholine™ 3.5–10, DryStrip Cover fluid, 18 cm IPG strips pH 4.5–5.5 and IEF Electrode strips were purchased from GE Healthcare (Uppsala, Sweden). Ammonium dodecyl sulphate, SDS, agarose, 30% acrylamide/bis solution, 37.5:1, ammonium persulphate, TEMED, and Precision Plus Protein™ standard were purchased from Bio-Rad (Hercules, CA, US). Trypsin was obtained from Promega (Madison, WI, US), while Poros 20R2, Reverse Phase packing, was purchased from Applied Biosystems (Foster City, CA, US). Siliconised tubes were provided from Sorenson, BioScience, Inc. (Salt Lake City, UT, US). Peptide Calibration Standard and MTP384 Target plate polished steel TF were purchased from Bruker Daltonics (Leipzig, Germany).

2.1. Experimental design

The experimental set up was carried out in accordance with "The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments", and approved by The National Animal Research Authority in Norway. The experimental design was made to answer whether environmentally realistic doses of produced water (PW) gave effects on the developing cod egg, larvae and fry at chronic exposures. For this reason PW at 0.01%, 0.1% and 1% were selected as exposure concentrations. Modelling studies have calculated that PW may be diluted approximately 1:30 at 10 m, 1:100 at 100 m, and 1:1000, 1 km from the outlet pipe (Neff, 2002). Modelling calculations and in-field measurements in the North Sea have shown concentrations of dispersed oil around the largest oilfields to be approximately 1–3 ppb, which roughly corresponds to a dilution factor of 1:10,000 (Rye et al., 1998). Parallel treatments with 10 μg/l 17β-oestradiol were included in order to compare responses after PW with oestrogenic responses. All treatments were performed in triplicates, as well as the control (clean sea water). The exposure was carried out for 90 days, starting March 25, 2004, on fertilized cod eggs and ended at early fry stages June 22, 2004. Light and temperature regime were held as natural conditions in the Bergen area, Norway at this time of year. Cod larvae were fed natural zooplankton after end of the yolk sac stage until the end of the exposure period.

The eggs used in this study were obtained from wild cod caught in Tysfjorden in Lofoten, Norway. For spawning, one male and one female fish were placed in a spawning tank and the resultant eggs collected from a filter placed over the runoff outlet. To ensure a realistic level of biological variation in this study, we mixed eggs collected from five separate pairs of spawning cod. The average egg diameter (D) from each spawning pair was measured, and the number of eggs calculated according to the following formula: N (number of eggs per ml) = $1222 \times D^{-2.71}$ (Kjesbu, 1989). Using this formula, 60,000 eggs (12,000 from each of the five pairs) were added to 100 l of water in each exposure tank. Three identical 100 l tanks, each containing 60,000 fertilized cod eggs (1–2 days old), were subjected to one of the five different treatment regimes described above for 23 days (in March/April 2004), until day 3 post-hatch (dph). The larvae were counted and 6500 from each tank were transferred to a fresh 100 l tank and the treatment continued for 67 days (473-day-degrees), through the whole start-feeding phase. At the end of this 90-day period (June 22, 2004) the fish were approximately 2 cm in length and 10 mg in weight (dry weight).

The dilution factor in the tanks was controlled by gas chromatography/mass spectrometry analysis of alkyl phenols as described in Boitsov et al. (2004). The average result of two measurements of C_2 alkyl phenols taken throughout the experiments showed the dilution factors to range from 30% to 110% of the nominal doses.

2.2. Sample preparation

Whole individual cod fry (approximately 80 days post-hatching, dph) were homogenised in 6× (v/w) re-hydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% (v/v) Triton X-100, 0.5% (v/v) Ampholine 3–10, bromphenol blue) using a Potter Elvehjem homogeniser with a Teflon® pestle. The pestle was run up and down 6 times during homogenisation. The homogenised sample was subsequently centrifuged at 13,000 × g at room temperature for 20 min. The protein concentration was determined using a plate reader-modified Bradford's assay (Bradford, 1976).

2.3. Two-dimensional gel electrophoresis (2DE)

500 μg of sample was diluted in re-hydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% (v/v) Triton X-100, 0.5% (v/v) Ampholine 3-10, bromphenol blue) and added to IPG strips pH 4.5–5.5 (covering the majority of proteins in the sample, results not shown), 18 cm (Görg et al., 2000; O'Farrell, 1975). Four individuals from each treatment-group were analyzed by 2DE. Due to a limited amount of sample, less protein (135–250 μg) from samples exposed to 0.1% produced water was diluted in buffer and added to the IPG strips. Only three individuals from this group were analyzed by 2DE. The strips were re-hydrated for a minimum of 12 h and focused on a Multiphor II unit (GE Healthcare) according to the manufacturer's guidelines. The strips were equilibrated 15 min at room temperature in 0.25% DTT-containing SDS equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, bromphenol blue) and 15 min in a 4.5% iodoacetamide-containing SDS equilibration buffer (Görg et al., 2000; O'Farrell, 1975) prior to separation in the second dimension on 9% SDS-PAGE gels (Laemmli, 1970). Gels were run in an Ettan Dalt Twelve unit (GE Healthcare) at 1 W/gel, 20 °C, for approximately 17 h. The gels were then stained with colloidal Coomassie (Neuhoff et al., 1988). Coomassie-stained gels were scanned on GS-800 Calibrated Densitometer flatbed scanner (Bio-Rad) using PDQuest 7.2.0 software (Bio-Rad). The gels were scanned with high resolution: 127.0 μm × 127.0 μm.