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Differential gene expression and biomarkers in zebrafish (*Danio rerio*) following exposure to produced water components

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ABSTRACT

The main effluent from oil and gas production is produced water (PW), a waste that contains low to moderate concentrations of oil-derived substances such as polycyclic aromatic hydrocarbons (PAHs) and alkylphenols (APs). PW components may be present in seawater at low concentrations over large areas in the vicinity of oil and gas production facilities. In this study, zebrafish (*Danio rerio*) were exposed to control and three treatments (high-, pulsed-, low-dose) of a synthetic PW mixture for 1, 7 and 13 weeks. The aim was to investigate the development of transcriptome and biomarker responses as well as relationships between early responses and population-relevant effects. The synthetic PW contained a mixture of low-molecular-weight PAHs (<5 ring) and short-chain APs (C1–C4). The water-borne exposure levels (sum PAH) ranged from 0.54 ppb (low dose) to 5.4 ppb (high dose). Bile pyrene metabolites ranged from 17–133 ng g^{-1} bile in the control group to 23–1081 ng g^{-1} bile in the high exposure group. Similar levels have been observed in wild fish, confirming an environmentally relevant exposure. The expression of mRNAs of hepatic genes was investigated in the high exposure group using the Zebrafish OligoLibrary™ from Compugen. Functional clustering analysis revealed effects in the reproductive system, the nervous system, the respiratory system, the immune system, lipid metabolism, connective tissue and in a range of functional categories related to cell cycle and cancer. The majority of differentially expressed mRNAs of genes were down-regulated, suggesting reduction in gene transcription to be as relevant as up-regulation or induction when assessing biological responses to PW exposure. Biomarkers for effects of PAHs (cytochrome P450 1A) and environmental estrogens (vitellogenin) did not appear to be affected by the chronic exposure to low concentration of PW components. Effects at the population level included a reduction in condition factor in male fish from all exposed groups and spinal column deformations in the F1 generation of exposed groups. The different exposure regimes did not produce any significant differences in reproduction or recruitment. The results from this study demonstrate that environmentally relevant concentrations of PW affect gene expression and population-relevant endpoints in zebrafish, although links between the two were not obvious.

1. Introduction

Discharges of produced water (PW) from offshore oil and gas production have been a growing concern for the last decades due to the potential long-term effects on biota. The composition and volume of discharged PW is highly variable depending on geological characteristics and the age of a field. In recent years, as the oil fields in the North Sea age, the volume discharge of PW has increased. The volume of PW discharged in the Norwegian sector of the North Sea was 162 million m^3 in 2007 (OLF, 2008). Organic components in North Sea PW in 2006 were organic acids (94%), BTEX (benzene, toluene, ethylbenzene, xylene; 4.4%), alkylphenols (APs; 0.9%), phenols (0.5%) and polycyclic aromatic hydrocarbons (PAHs; 0.2%) (OLF, 2007), of which the environmental effects caused by PAHs and APs have been given the most attention.

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PAHs and APs are two diverse groups of chemicals of natural origin. PAHs are composed of two or more fused benzene rings, both homo- and heterocyclic, which may be alkylated. Depending on their size, structure and solubility, PAHs can be divided into carcinogenic and non-carcinogenic compounds. The most studied are the carcinogenic PAHs, e.g. benzo[a]pyrene which consists of five fused benzene rings. Studies on effects of benzo[a]pyrene are numerous and it is often used as a model compound for effects of PAH exposure on fish (e.g. Beyer et al., 1997). Alkylphenols are phenols with an attached carbon-chain of varying length and structure. The major concern with APs has been their estrogenic properties, as demonstrated by the potent endocrine disruptors octyl- and nonylphenol and their ethoxylates (Nimrod and Benson, 1996). The concentration of APs in PW generally increases with decreasing length of their alkyl-chain (Boitsov et al., 2004). In recent studies, more effort has been put into investigating effects of shorter chained APs and their estrogenic properties (Gimeno et al., 1998a; Seki et al., 2003; Barse et al., 2006; Tollefsen et al., 2008).

The use of early warning signals is based on a theoretical time-dependent relationship of responses from low to high levels of biological organization. Changes in gene expression will lead to changes in cellular processes, which may ultimately affect the organism and give rise to population level effects. Causal links between such early warning signals to chemical exposure and adverse organism or population level effects have not been widely documented, although a relationship in fish between PAH exposure and liver lesions have been indicated (Myers et al., 2003). The genomic tools available for North Sea fish species are not yet well developed (Douglas, 2006). There exists an expression array for flounder (Williams et al., 2003) as well as small cDNA libraries for cod (International Cod Genomic Consortium www.codgen.olsvik.info, Olsvik, P.A.; Atlantic Cod Genomics and Broodstock Development http://codgene.ca, Bowman, S. and Trippel, E.). In this study, in order to allow detection of genome wide responses, zebrafish (*Danio rerio*) was chosen as model species (Hill et al., 2005). The zebrafish genome has been widely studied (Teh et al., 2005) and more recently for changes in expression related to environmental stressors (Ton et al., 2003; van der Ven et al., 2005; Alestrom et al., 2006; Olsvik et al., 2007; Voelker et al., 2007). The Zebrafish OligoLibrary™ from Compugen (Alestrom zebrafish lab, http://microarray.no) was used to study differential mRNA expression of zebrafish genes.

The metabolism of xenobiotics in animals is conducted primarily through the phase I–III metabolising systems. The purpose of these systems is to increase the hydrophilicity of endogenous or exogenous substances through hydrolysis, reduction, oxidation (phase I) and/or conjugation (phase II), and finally the efficiency of transport and excretion (phase III). The regulation and activity of specific phase I or II metabolising system components have commonly been used to quantify effects of xenobiotics in aquatic species. The effects of PAHs in aquatic organisms have been assessed using phase I enzyme activity (cytochrome P450 1A; CYP1A) and subsequent adverse effects such as DNA adduct formation and histopathological lesions (Myers et al., 2003). In addition, the exposure load of PAHs in fish may be quantified by measuring PAH metabolites in bile (Aas et al., 2000). Adverse effects of PAHs may be mediated through the aryl hydrocarbon receptor (AhR) pathway, activated by dioxin and dioxin-like compounds such as the carcinogenic PAHs. The nature of interactions of 2- and 3-ring PAHs with the AhR is, however, less clear, with some evidence indicating effects mediated by an AhR independent mode of action (Incardona et al., 2005). Also, fluoranthene (a 4-ring PAH) has been shown to inhibit CYP1A activity in fish (Willett et al., 2001). Clearly, there is a need for more information about the effects of low-molecular-weight PAHs on fish as they are the most abundant and bioavailable PAHs in PW (Neff, 2002). The phase II metabolising system consists of conjugating enzymes such as sulfotransferases (SULT), glutathione *S*-transferases (GST) and UDP-glucuronosyltransferases (UDP-GT), increasing the excretion and transcellular transport of xenobiotics (Xu et al., 2005). However, phase II enzymes are generally less responsive than phase I systems.

The combination of ocean currents, fish movement and fluctuating discharge volumes causes any PW exposure to be low and variable. The biological effects of PW discharges from offshore activities have been monitored the last decade, and sub-lethal effects have been observed in cod (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus* L.) and saithe (*Pollachius virens* L.) (Hylland et al., 2006a). Furthermore, adaptation to low level contaminants has been observed in flounder (*Platichtys flesus* L.) caught in contaminated areas (Eggens et al., 1996). Exposure studies using PW or PW components includes observations on gene regulation (Olsvik et al., 2007), DNA adduct formation (Beyer et al., 2001; Skadsheim, 2004), effects on membrane lipid composition (Meier et al., 2007a), physiological stress (Stephens et al., 2000) and reproductive effects (Meier et al., 2007b). PW has also been shown to contain both estrogenic and anti-androgenic components in *in vitro* bioassays (Tollefsen et al., 2007). In the present study, hepatic gene expression, biomarker responses and population-relevant endpoints were analysed in zebrafish exposed to a mixture of PAHs and APs. The study aimed to investigate the development of biological responses during long-term PW exposure and to screen for early warning signals to any observed population-relevant effects.

2. Materials and methods

2.1. Experimental setup

Adult wild type zebrafish were purchased from a local fish dealer. The fish were sorted by sex then randomly distributed into 4 experimental groups in a total of 13 aquaria ($n = 1690$). The control group contained four replicate aquaria, while the exposure groups each contained three replicate aquaria. A total of 130 fish (65 males and 65 females) were held in each aquarium (25 l). The fish were acclimated to the exposure system for 1 week before start of the experiment. The flow of water was 75 l day^{-1} aquarium^{-1} and the aquaria were oxygenated by aeration. Water temperature was maintained at $26 \pm 2\,^{\circ}$C and pH at 7.1 ± 0.3 (average ± range). Photoperiod was kept at a light/dark cycle of 14/10 h. Fish were fed three times a day with live brine shrimps (once a day during weekends). Brine shrimp cultures were maintained in a separate laboratory to avoid chemical contamination of the shrimp cultures.

The fish were exposed for a total of 13 weeks to three different treatments of PAHs and APs combined in a cocktail, prepared according to median concentrations of the major classes of PAH and AP components in PW and estimated dilution factors (Table 1). The high dose had a nominal dilution factor 200 from PW, the low dose a nominal dilution factor 2000. Total nominal PAH and AP concentrations corresponded to 5.4 ppb PAH and 11.4 ppb AP in the high dose treatment and 0.54 ppb PAH and 1.14 ppb AP in the low dose treatment. To match the phenol content of PW, the treatments were also added 7 and 0.7 ppb phenol, respectively. A pulsed treatment was used to simulate the variation in a PW discharge. In the pulsed group, exposure alternated between high and control treatment with an interval of 1 week. The water was added salts according to ISO standard 7346-1 (1996), giving a concentration of 31.5 mg l^{-1} $NaHCO_3$ (Merck KGaA, Damstad, Germany), 147 mg l^{-1} CaCl (Mallinckrodt Baker, Phillipsburg, NJ, USA), 61.7 mg l^{-1} $MgSO_4$ (Mallinckrodt Baker) and 2.75 mg l^{-1} KCl (Merck KGaA). The solvent control group received acetone (5 μl l^{-1}; Merck KGaA). Chemi-