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Biomarker responses in polar cod (*Boreogadus saida*) exposed to the water soluble fraction of crude oil

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ABSTRACT

In order to mimic the biological effects of an oil spill in Arctic waters, we examined several types of biomarkers (genes, enzymes, metabolites, and DNA damage) in polar cod *Boreogadus saida* experimentally exposed to the water soluble fractions of crude oil. During 4 weeks of exposure, induction of the studied biomarkers exceeded baseline levels. The mRNA expression of the cytochrome P4501A1 (*cyp1a1*) gene was the most promising biomarker, with glutathione S-transferase (*gst*) as a suitable complement. The delayed ethoxyresorufin O-deethylase (EROD) and GST activities and their persistence following 2 weeks of depuration may allow detection of previous exposures in field samples. The composition of PAH metabolites in the bile indicated the bioavailability of different PAH size-classes. Although mRNA expressions of antioxidant defense genes were induced at start of the exposure, with the strongest responses from catalase and cytosolic superoxide dismutase, they were poor for oil monitoring purposes due to their very short response times. Significant DNA damage demonstrated genotoxicity even at low PAH concentrations (<15 $\mu g\,L^{-1}$) and was correlated with benzo(a)pyrene and pyrene metabolites in the bile.

1. Introduction

Some of the largest remaining oil and gas reserves are located in the Arctic (USGS, 2000). Therefore, the Barents Sea, as well as the Beaufort and Chukchi Seas, are becoming major areas of concern in terms of petroleum related activities. On the Norwegian side of the Barents Sea, a zero-discharge policy for the oil industry shifted the main environmental issue of concern in this region from operational discharges to accidental oil discharges. Oil spills and oil and gas drilling activities will potentially expose Arctic marine organisms to the water soluble fraction of crude oil. Hence, there is a need to develop polycyclic aromatic hydrocarbon (PAH) biomarkers for monitoring of these organisms in areas of future oil activities.

The most studied biomarker in temperate and boreal regions is the phase I cytochrome P450 1A (CYP1A) involved in the biotransformation of xenobiotics such as PAHs (van der Oost et al., 2003). The most common biomarker method used to measure CYP1A is the ethoxyresorufin O-deethylase (EROD) activity (Whyte et al., 2000). Gene expression studies of *cyp1a1* (isoform 1 of CYP1A) and other biomarkers have increased in the past two decades, but clear correlation between the responses of gene and enzyme biomarkers has not yet been demonstrated (George et al., 2004; Tom and Auslander, 2005). The phase II enzyme glutathione S-transferase (GST), commonly measured as GST activity, plays a role in the detoxification of oxidative stress products as well as in the conjugation of glutathione to xenobiotic metabolites to facilitate their excretion (Schlenk et al., 2008). Polycyclic aromatic hydrocarbons are well known to induce the production of reactive oxygen species (ROS), which cause cellular lesions such as lipid peroxidation or DNA damage. The most studied oxidative stress biomarkers beside GST are catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities (Akcha et al., 2000; Regoli et al., 2002). Additionally, DNA strand breaks are caused by the generation of toxic metabolites (Gravato and Santos, 2003) or excessive ROS production (Regoli et al., 2003) and can be detected with the comet assay – a biomarker of genotoxicity (Frenzilli et al., 2004). Finally, the measurement of PAH metabolites in the bile, through

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fluorescent spectrometry methods such as synchronous fluorescence scan (SFS) spectroscopy, is a relevant, fast and cost efficient way to evaluate the bioavailability of PAHs in marine organisms (Aas et al., 2000b; Meador et al., 2008). Responses of the above cited PAH biomarkers are well documented for temperate species (Goksøyr et al., 1994; Aas et al., 2000a) and commonly used in monitoring programmes (JAMP, 1998; Hylland et al., 2008). However, the response of these biomarkers in Arctic fishes is poorly studied.

To evaluate biomarker responses for oil monitoring in the Arctic, the polar cod *Boreogadus saida* was chosen as a relevant indicator species (Stange and Klungsøyr, 1997). Recent studies have investigated the seasonality of biomarkers in wild polar cod (Nahrgang et al., subm.) as well as the induction response to benzo(a)pyrene (B(a)P) in i.p. injected fish (Nahrgang et al., 2009). Laboratory and field studies on oil spill behavior in sea ice environments have shown transport of the water soluble fraction (WSF) of crude oil trapped in ice into the water (Faksness and Brandvik, 2008) with potential toxicity to sea ice organisms. Polar cod which lives in close association with the sea ice during part of its life cycle (Gradinger and Bluhm, 2004) may thus be exposed via the water and via the diet to the WSF of crude oil in both open water and in association with sea ice. The present study aims thus, to extend the acquired knowledge on the biomarkers responses by exposing polar cod chronically to the WSF of crude oil in an oil spill simulation. This study was carried out in parallel with a dietary exposure experiment (Nahrgang et al., in press).

2. Materials and methods

2.1. Fish sampling and rearing

Polar cod were caught with a Campelen Super 1800 bottom trawl in Rijpfjorden, Svalbard, Norway. A fish-lift prevented the fish from being injured during trawling (Holst and McDonald, 2000). Polar cod were kept on deck in a tank with running seawater during 24 h while being transferred to the Kings Bay Marine Laboratory in Ny-Ålesund (latitude ~79°N). Polar cod were acclimated to the laboratory conditions for 1 month in 700 L holding tanks with running and filtered seawater and were fed weekly till satiation with the pelagic amphipod *Themisto libellula*. The photoperiod was constantly dimmed light during periods of acclimation and the experiment (October–November 2007).

2.2. Experimental set-up

Polar cod were exposed to either the water soluble fraction of North Sea crude oil ($N = 3$ treatments × 24) using oiled rock columns (Carls et al., 1999) or clean seawater (control $N = 24$). This experimental set-up allowed mimicking an oil spill, with a decrease in aqueous PAH concentrations and a change in the relative abundance of PAH compounds over time. Gravel of approximately 1–8 mm in diameter (median = 3 mm) were washed, dried and mixed with crude oil at concentrations of 3, 6 and 12 g crude oil kg^{-1} gravel corresponding to low, medium and high oil treatments respectively, dried at room temperature 12–24 h and frozen pending column preparation. The oiled gravels were inserted in 1 m high PVC columns (12 kg per column). Particle filtered and UV treated seawater percolated upwards through the columns 960 ml min^{-1} to extract the water soluble fraction into the experimental tanks (160 L). The control tank received water percolated through a column containing clean gravel. To ensure the volatilization of BTEX compounds (benzene, toluene, ethylbenzene, and xylene) and an absence of particulate oil, the water was percolated through the columns 48 h before the transfer of polar cod into the experimental tanks. Fish were exposed continuously for 4 weeks. The columns were then removed, and tanks were provided with clean water for 2 weeks to study biomarker response during depuration. Six polar cod per treatment were sampled after their transfer to the experimental tanks, noted "week 0+", due to a delay of 7 h between the first and the last fish sampled at this time. Polar cod were again sampled after 2 and 4 weeks of exposure and following 2 weeks of depuration, noted as "week 6". Polar cod were sacrificed by sharp blow to the head. Fork length (±0.1 cm), weight (±0.1 g) and gender were recorded, and liver slices and bile were placed into separate cryovials, snap frozen in liquid nitrogen and stored at −80 °C prior to analysis. For comet assay analysis, blood samples were taken from the polar cod at week 4. In addition, blood samples were taken from 3 more polar cod from each oil exposed tanks and from all remaining controls (7 fish). To increase data accuracy for the controls, 14 additional unexposed fish from the acclimation tank were sampled for blood. Blood was drawn from the caudal vein with syringes (gauge 30; Omnican®) rinsed with heparin and thereafter prepared for DNA strand break determination as described in Section 2.3.5. Water samples were taken from each experimental tank (no replicates) on weeks 0+, 2, 4 and 6 and frozen at −20 °C prior to PAH analysis.

2.3. Analyses

2.3.1. Analysis of water for polycyclic aromatic hydrocarbons (PAHs)

The water samples were analyzed for the 16 Environmental Protection Agency (EPA) priority PAHs and naphthalenes, phenanthrenes and dibenzothiophenes (NPD) at the Norwegian Institute for Water Research. Briefly, an internal standard was added to the water samples that were extracted with dichloromethane. Samples were cleaned using Gel Permeation Chromatography (GPC), and extracts were analyzed by Gas Chromatograph/Mass Spectrometry (GC/MS) (Hewlett-Packard/Agilent technologies). The quantification of individual components was related to the internal standard.

2.3.2. Gene transcription analysis

The mRNA expression of cytochrome P450 1A1 isoform (*cyp1a1*), cytosolic pi-class homologue of glutathione S-transferase (*gst*), cytosolic superoxide dismutase (*sod*(*Cu*/*Zn*)) and mitochondrial (*sod*(*Mn*)), catalase (*cat*), phospholipid hydroperoxide glutathione peroxidase (*gpx*), and the housekeeping gene β-actin (*β-act*), were determined on six individual fish per treatment and sampling time (Ntotal = 96).

Total RNA was extracted from 40 mg of fresh liver tissue with the Stratagene "Absolutely RNA RT-PCR miniprep" kit, according to the manufacturer's instructions. Total RNA integrity (optical density 260/280 nm) and concentration were measured with a NanoDrop ND-1000 spectrophotometer. First-strand cDNA was synthesized from 5 μg of total RNA using the Stratagene "AffinityScript Multiple Temperature cDNA Synthesis" kit. Briefly, 5 μg of total RNA were mixed with 1 μl of oligo(dT), 0.8 μl of dNTP, 1 μl of random primers, and 2 μl of 10× buffer and adjusted to 19 μl with diethylpyrocarbonate treated water. The reaction was incubated at 65 °C for 5 min before adding 0.5 μl of RNase inhibitor and 1 μl of reverse transcriptase. The reaction was incubated for 1 h at 42 °C in an Eppendorf Mastercycler personal. The cDNA mixture was stored at −20 °C until use for real-time PCR reaction.

Primer pairs of *β-act*, *cyp1a1*, *gst*, *sod*(*Cu*/*Zn*), *sod*(*Mn*), *cat* and *gpx* were the same as applied by Nahrgang et al. (2009) and are presented with accession numbers in Table 1. Quantitative real-time Polymerase Chain Reaction (q-PCR) was performed in a LightCycler (Roche) following the manufacturer's instructions (1 cycle at 95 °C for 10 min, and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s). Each reaction contained 1 μl of cDNA template, 3.2 μl $MgCl_2$ (25 mM), 1 μl of master mix including the SyberGreen I fluorescent dye (Roche) and 2 μl of the gene-specific primer pair