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An investigation of the immunotoxicity of oil sands processed water and leachates in trout leukocytes

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ABSTRACT

Increased oil sands (OS) mining activity has raised concerns about impacts on aquatic organisms. This study sought to examine the effects of single representative compounds from OS (benzo(a)pyrene, naphthalene), a mixture of naphthenic acids (NAs), OS-processed water (OSPW) and OS leachate (OSL) extracts on rainbow trout leukocytes. Primary cultures of trout leukocytes were exposed to increasing concentrations of benzo(a)pyrene, naphthalene, NAs, OSPW and OSL for 48 h at 18 °C. Immunocompetence was followed by measuring changes in lymphocyte and macrophage viability and phagocytosis. Changes in the expression of 10 transcripts were also followed: interleukin 1, 2 and 6 (Il-1, Il-2 and Il-6), calreticulin (CRT), caspase 9 (Cas9), aryl hydrocarbon receptor (AhR), cyclooxygenase-2 (COX2), glutathione S-transferase (GST), catalase (CAT) and p53 tumor suppressor. The results revealed that exposure to OSPW extracts decreased the capacity of macrophages to engulf three beads or more, while the other compounds generally increased phagocytosis activity. Lymphocyte apoptosis was increased by all compounds and mixtures except naphthalene. Both OSPW and OSL induced apoptosis in macrophages. At the gene expression level, Cas9, CRT, Il-1 (inhibition) and Il-2 were specifically influenced by OSPW, while CAT, p53, COX2 and Il-1 (induction) transcripts were specifically expressed by OSL. Leukocyte exposure to OSPW produced characteristic changes in immunocompetence and genes involved in proinflammatory, apoptosis and protein damage (CRT) pathways which could not be explained by OSL, benzo(a) pyrene, naphthalene and NA mixture.

1. Introduction

The Athabasca is a large river that flows 1231 km north from the province of Alberta to the Mackenzie estuaries in the Northwest Territories of Canada. One particularity of the Athabasca watershed is that it includes oil sands (OS) deposits. With an estimated annual production of 25,000,000 to 30,000,000 m^3 of crude oil, it is considered the world's largest oil deposit (Gosselin et al., 2010). OS is composed of approximately 10% bitumen, 4% water and 86% clay and sand. The extraction process involves mixing OS with caustic hot water under aeration (Clark extraction method) to separate bitumen from the OS (Schramm and Smith, 1989). The resulting aqueous phase is referred to as OS processed water (OSPW). It is held in large ponds for weathering purposes, since OSPW is highly toxic to aquatic organisms. OSPW forms large tailing ponds that occupy volumes in the order of 900 million m^3. It has been suggested that tailing ponds should be reclaimed to eventually become viable systems, but their potential inadvertent leaching and drainage of OSPW into the Athabasca River's tributaries and groundwater could pose a risk to aquatic fauna. In addition, the aquatic environment already undergoes natural contamination from the leaching of natural deposits of OS in the Athabasca watershed and atmospheric contamination from mining activities (Kelly et al., 2010). Thus, the combined contamination caused by natural leaching of OS and potential release of OSPW complicates the understanding of the toxicity and hazard generated by OS extraction activities. It is therefore important to study the contamination and toxicity of OS water leachates in respect to OSPW. Compositional analysis of OSPW revealed a variety of compounds such as naphthenic acids (NAs), polycyclic aromatic hydrocarbons (PAHs), and metals such as cadmium, copper, nickel, silver, titanium and zinc (Lindeman et al., 2011; Pereira and Martin, 2015). OSPW are composed of NAs, which are weak aliphatic carboxylic acids following the $C_nH_{(2n-z)}O_2$ rule, and other organic compounds with N- and S-substituted ligands. NAs could reach levels as high as 70 mg/L in OPSW and as high as 50 mg/L in runoff and groundwater in the vicinity of OSPW ponds (Holowenko et al., 2002). Hence, this class of compounds is considered a major player in the manifestation of toxicity, although it cannot explain all the OSPW observed toxicity.

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The toxicity of OSPW and OS leachate (OSL) water has been studied in various aquatic organisms such as bacteria (Madill et al., 1999), algae (Debenest et al., 2012), chironomids (Anderson et al., 2012), daphnids (Puttaswamy et al., 2010), frogs (Hersikorn et al., 2010) and fish (Scarlett et al., 2013; Gagné et al., 2013). In fish, toxicity endpoints were mostly focused on reproduction and early life stage toxicity (Kavanagh et al., 2011; He et al., 2012). In addition to impacts of growth and reproduction, impacts on long-term survival could be assessed at the immune levels, since the immune system constitutes the main defence against invading microbial agents. The immune system in fish depends on both innate and acquired immunity. Innate immunity involves phagocytosis by macrophages and natural cytotoxic cells (Watts et al., 2001). Phagocytosis is the ingestion and destruction of foreign particles by highly reactive oxidizing agents such as nitric oxide and hydrogen peroxide in phagosomes (Hoeger et al., 2004). Acquired immunity results from the production of various interleukins and the production of antibodies by T and B lymphocytes (Brousseau et al., 2012). The effects of OS products on fish leukocytes have been investigated in a few studies. Rainbow trout were exposed to three types of artificial ponds (OSPW capped with 3 m of natural water; unextracted OS; and only Athabasca River water) and impacts to the immune system were examined (McNeill et al., 2012). As expected, the NA content was significantly higher in the pond containing OSPW, and high phenanthrene equivalents were detected in fish bile. Moreover, fish in ponds containing OSPW had significantly lower leukocytes, smaller spleens and increased incidence of opportunistic infection as determined by fin erosion. The fish showed decreased production of antibodies against *Aeromonas salmonicida* challenge, which collectively indicate immunosuppression. In a study of goldfish, 12 weeks of exposure to fresh OSPW decreased the production of reactive oxygen and nitrogen intermediates in macrophages. The expression of pro-inflammatory genes was enhanced by OSPW as determined by interleukin-1 (IL1-β1) with decreased levels in tumor necrosis factor (TNF-α2) and interferon-γ (IFN-γ) (Hagen et al., 2014). The enhanced proinflammatory response was further confirmed by the observation that fish exposed to OSPW controlled infection by parasite challenges better than non-exposed (control) fish. NAs could also contribute to reduced immunity, as shown by decreased B and T lymphocyte counts in spleens of rainbow trout exposed to NAs (MacDonald et al., 2013). Exposure of Walleye embryos to NA extracts revealed that the could lead to oxidative stress as suggested by induction of genes involved in oxidative stress such as catalase and superoxide dismutase (Marentette et al., 2017). Interestingly, the involvement of the AhR pathway was also involved suggesting that heavy PAH-type of compounds were also involved.

The purpose of this study was therefore to determine the direct effects of OS-related compounds (benzo(a)pyrene, naphthalene and a representative NA mixture) and OSPW/OSL (leachate) in primary cultures of rainbow trout leukocytes. *In vitro* exposure enabled us to determine the direct effects on the immune function of leukocytes, i.e., no influence from microorganisms which are found in natural OS-contaminated ponds. Immunocompetence was determined by measuring changes in leukocyte viability and phagocytosis activity in macrophages. In parallel, a suite of transcripts involved in immunity were also determined by qPCR methodology. An attempt was made to understand the contribution of BaP, naphthalene and NAs to the immunotoxigenomic properties of OSPW in rainbow trout leukocytes.

2. Materials and methods

2.1. Preparation of oil sands leachates and processed water in the laboratory

OSPW and OSL were prepared using shore oil sands from the Athabasca River in Fort McMurray (Alberta, Canada) in 2012. A simplified industrial protocol using a previous procedure (Debenest et al., 2012) was followed. Briefly, 500 mL of de-ionized water at pH 11 (NaOH 10 N) was heated at 75 °C in a glass beaker. Oil sands (200g) were then added under constant aeration (bubbling) for 3–4 h. Released bitumen from the oil sands was collected at the surface of the water during the extraction phase. Lastly, the mixture was allowed to decant overnight and centrifuged at 2700 × g for 20 min. The resulting supernatant is considered OSPW. For the OSL, 200g of oil sands were added to 500 mL of de-ionized water with no pH change (pH 7). The sand suspension was then mixed for 24 h at room temperature (30 rpm) to simulate natural leaching. As with OSPW, the mixture was decanted overnight and centrifuged at 2700 × g for 20 min. The supernatant was considered OSL. Both the OSPW and OSL were filtered on 0.4 µm pore filters (Millipore, USA) to remove any remaining suspended matter and microorganisms. A volume of 500 mL of OSPW and the same volume of OSL were passed through an activated reverse-phase C18 cartridge (360 mg) under 5 psi vacuum. The column was washed with 15 mL of double distilled water and eluted with 5 mL of analytical-grade ethanol to obtain the organic fraction. The ethanol fraction was kept in the dark at 4 °C until analysis.

2.2. PAHs and commercial naphthenic acid mixtures

Benzo(a)pyrene (BaP: CAS 50-32-8), naphthalene (Nap; CAS 91-20-3), and one commercial mixture of naphthenic acids (NAs) were examined (Merichem Chemicals, Alberta, Canada). For the NA mixture, a stock solution at 1 g/L was prepared in 0.1 M NaOH, and the pH was adjusted to pH 8 with concentrated HCl (2 M). BaP and Nap were prepared at 200 mg/mL and 4 mg/mL respectively in 100% DMSO. Serial dilutions were then prepared in sterile Leibovitz (L15) culture medium containing 20 mM Hepes-NaOH, pH 7.4, and 1xantibiotic /antimicotic (100 x preparation from Sigma-Aldrich) to reach 2.5, 5, 10, 40 µg/mL and 0.01, 0.1 and 2 µg/mL respectively. For OSPW and OSL, the cells were exposed to the 100 x ethanol fraction as described below at the following concentrations: 0.004%, 0.02% and 0.1%, corresponding to 0.4%, 2% and 10% concentrations of the undiluted sample. The final ethanol concentration for all exposure concentrations was set at 0.1%, and control cells received the appropriate amount of ethanol.

2.3. Fluorescence analysis of polycyclic aromatic hydrocarbons

The relative levels of PAHs in the NAs mix, OSPW and OSL ethanol extracts were determined using fixed wavelength analysis (Aas et al., 1995). NAs was prepared at 100 mg/L in ethanol, and the OSPW and OSL extracts were diluted to 0.1% in ethanol and transferred in dark microplates. For calibration, 100 µL of these solutions were added to 100 µL of standards of PAHs or ethanol blanks and analyzed by fluorescence using a dual monochromator-based microplate reader (Biotek Inc., USA). For light-weight PAHs (2- to 3-ringed PAHs), standard additions of naphthalene were used for calibration. Fluorescence readings for light PAHs were at 290 nm excitation and 340 nm emission. The data were expressed as µg naphthalene equivalents/L. For medium-weight PAHs (3- to 4-ringed PAHs), standard additions of pyrene were performed as described above. Fluorescence readings were taken at 325 nm excitation and 370 nm emission. The data were expressed as µg/L pyrene equivalents/L. For heavy-weight PAHs (4 rings or more), standard additions of BaP were used and fluorescence readings were taken at 385 nm excitation and 440 nm emission. The data were expressed as µg BaP equivalents/L. These wavelengths were selected to avoid spectral overlaps of the PAH size groups.

2.4. Primary cultures of trout leukocytes and immunocompetence evaluation

The anterior section of trout kidney was dissected out in fish previously anesthetized/euthanized in 0.1% tricaine (Boréal