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Molecular responses of Walleye (*Sander vitreus*) embryos to naphthenic acid fraction components extracted from fresh oil sands process-affected water

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ABSTRACT

Naphthenic acid fraction components (NAFCs) are constituents of oil sands process-affected water (OSPW), which is generated as a result of unconventional oil production *via* surface mining in the Athabasca oil sands region. NAFCs are often considered to be major drivers of OSPW toxicity to various taxa, including fishes. However, the molecular targets of these complex mixtures are not fully elucidated. Here we examined the effects in walleye (*Sander vitreus*) embryos after exposure to NAFCs extracted from fresh OSPW. Eleutheroembryos (exposed to 0, 4.2 or 8.3 mg/L NAFCs from 1 day post-fertilization to hatch) were subsampled, measured for growth and deformities, and molecular responses were assessed *via* real-time polymerase chain reaction (PCR). Fourteen genes were evaluated, with a focus on the aryl-hydrocarbon receptor (AhR) – cytochrome P450 pathway (*arnt*, *cyp1a1*), the oxidative stress axis (*cat, gst, sod, gpx1b*), apoptosis (*e.g. casp3*, *bax* and *p53*), growth factor signaling (*e.g.* insulin-like growth factors *igf1, igf1b,* and *igf1bp*), and tissue differentiation (*vim*). NAFC exposure was associated with an increase in the expression of *cyp1a1*, and a decrease in *gpx1b* and ribosomal protein *rps40*. These results indicate that NAFC effects on walleye early-life stages may be mediated through oxidative stress *via* pathways that include AhR.

1. Introduction

Surface mining of bituminous sands in the Athabasca oil sands region of northern Alberta, Canada, results in the generation of large volumes of oil sands process-affected water (OSPW) through the use of the Clark caustic water extraction method (FTFC, 1995a, 1995b). OSPW is stored in settling basins both to facilitate reuse, and adhere to the Government of Alberta's zero discharge policy (FTFC, 1995b; Giesy et al., 2010). Thus, an overarching aim of the remediation is storage of OSPW in end pit lakes with eventual release (CEMA, 2012). As of 2013, 976 million m^3 of OSPW has been generated, with a footprint of 220 km^2 (AESRD, 2015). The composition of OSPW is complex, including a wide range of acid-extractable organics (AEOs, also known as naphthenic acid fraction components or NAFCs), other organic compounds including polycyclic aromatic hydrocarbons (PAHs), inorganic salts, trace metals, and unextracted residual bitumen (Allen, 2008; Headley et al., 2013a; FTFC, 1995a,b). Once thought to be comparable to classical naphthenic acids ($C_nH_{2n+z}O_2$; Clemente and Fedorak, 2005), NAFCs are now understood to encompass a broad range of compounds including multiply oxygenated species, compounds containing nitrogen, sulfur or other heteroatoms, and aromatic or cage-like structures (Barrow et al., 2009; Bataineh et al., 2006; Headley et al., 2013a; Rowland et al., 2011a,b,c). Further characterization of NAFC constituents is ongoing (Headley et al., 2013b; Lengger et al., 2013; Noestheden et al., 2014; Pereira et al., 2013; West et al., 2013, 2014). NAFCs are often considered to be a primary driver of OSPW toxicity (Clemente and Fedorak, 2005; MacKinnon and Boerger, 1986), although empirical support for this role is incomplete (Giesy et al., 2014; Wiseman et al., 2013) and residual OSPW fraction toxicity remains after ozone-mediated degradation of NAFCs (Klamerth et al., 2015). A more complete understanding of OSPW composition and accurate analytical techniques is essential for developing environmental monitoring and remediation technologies for OSPW and its constituents (Brown and Ulrich, 2015; Frank et al., 2014, 2016).

Exposure to OSPW, or NAFCs extracted from OSPW, has been associated with a range of different effects in fishes. Acute toxicity,

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including mortality, cardiovascular abnormalities and spinal curvature, has been demonstrated in a wide range of species. These studies include embryos of fathead minnow (*Pimephales promelas*) exposed to OSPW (He et al., 2012a) or NAFCs extracted from fresh OSPW (Kavanagh et al., 2012; Marentette et al., 2015a,b), yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*) exposed to OSPW (Peters et al., 2007), and Japanese medaka exposed to NAFCs (Farwell et al., 2006) or diluted bitumen (Madison et al., 2015). Aromatic fractions of NAFCs extracted from fresh OSPW are reported to be more toxic to larval zebrafish (*Danio rerio*) than alicyclic fractions (Scarlett et al., 2013). Endocrine disruption has been demonstrated in fathead minnow exposed to either aged OSPW or NAFC extracts from fresh OSPW, resulting in changes to (*e.g.*, inhibition of) secondary sexual characteristics, plasma steroids, gonad size, and spawning (Kavanagh et al., 2011, 2012, 2013). Immunotoxic effects of OSPW and NAFCs have also been reported in goldfish (*Carassius auratus*; Hagen et al., 2012, 2014); however, OSPW effects on immune responses in rainbow trout (*Oncorhynchus mykiss*) do not appear to be attributed to NAFCs (Leclair et al., 2013; MacDonald et al., 2013).

Studies examining molecular responses to OSPW and, less frequently, to extracted NAFCs have pointed to multiple modes of action in fishes, including endocrine disruption. *In vitro* studies have demonstrated that fresh OSPW is both estrogenic and antiandrogenic (He et al., 2010, 2011), inducing expression of *vtg* (vitellogenin) and estrogen receptor in rainbow trout hepatocytes (Gagné et al., 2012, 2013) and mirroring sex-specific changes in estrogen-responsive transcripts in the brain, liver and gonad tissue of male and female fathead minnow (He et al., 2012b). Aromatic, but not alicyclic, fractions of NAFCs extracted from fresh OSPW also induced *vtg* expression in larval zebrafish (Reinardy et al., 2013). Some aromatic NAFCs have been identified as being structurally similar to estrogens (Rowland et al., 2011b) and some compounds have been predicted to interact with either estrogen or androgen receptors (Scarlett et al., 2012). Beyond endocrine disruption, *in vitro* studies have indicated a role for oxidative stress (*e.g.*, increased expression of superoxide dismutase, *sod*; glutathione-s-transferase, *gst;* catalase, *cat*; *etc.*; Gagné et al., 2012, 2013) in OSPW toxicity. Exposure to fresh OSPW induced expression of genes related to oxidative stress (*gst*, *sod,* caspase *casp9*), apoptosis (*casp9, ApopEn*) and the cytochrome P450 enzyme *cyp3a*, but not *cyp1a*, in embryo-larval fathead minnow (He et al., 2012a). Moreover, Wiseman et al. (2013) report that transcripts related to metabolism of xenobiotic compounds and oxidative stress (*gpx*, *gr*, *trx*, and others) were increased with untreated OSPW while transcripts associated with the complement immune system were decreased in relative abundance. When comparing RNA-seq data with real-time PCR, *cyp1a* was induced approximately 2-fold, along with glutathione enzymes and apoptosis-inducing factor 3, suggesting that these transcripts are responsive to OSPW exposure. In early life-stages of Japanese medaka, expression of *cyp1a* and incidences of pericardial edema were significantly greater in larvae co-exposed to retene and 5 × equivalent of dissolved organic compounds from Base-Mine Lake-OSPW compared to retene alone (Alharbi et al., 2016); changes in *cyp1a,* however, were not greater in larvae exposed to dissolved organic compounds from OSPWs alone. Taken together, there is evidence that developing fish activate xenobiotic metabolism and oxidative stress pathways following exposures to untreated and treated OSPWs.

Here, we examined the transcript responses resulting from exposure to NAFCs extracted from fresh OSPW in early life-stages of walleye (Percidae: *Sander vitreus,* formerly *Stizostedion vitreum*; Scott and Crossman, 1998). The walleye is a freshwater sportfish of major importance to recreational and (historically) commercial fisheries in Alberta, where populations that underwent past overexploitation and subsequent declines have begun to show signs of recovery (Sullivan, 2003) that complicate attempts to evaluate walleye health and condition in the lower Athabasca River (Schwalb et al., 2014). Potential effects of OSPW and NAFCs on walleye are of interest to consumers (Tolton et al., 2012) and walleye are a target fish species for oil sands end pit lake remediation (CEMA, 2012). As a percid, walleye also provide a point of comparison to existing molecular studies *in vivo* and *in vitro* that primarily target cyprinids (*e.g.*, fathead minnow, zebrafish) and salmonids (rainbow trout). We examined responses in walleye eleutheroembryos that were exposed from the blastula stage (1 day post-fertilization, or dpf) to hatch (12–14 dpf) in the laboratory. We also measured molecular responses for transcripts related to oxidative stress, aryl hydrocarbon receptor signaling, growth and development, cancer, and apoptosis, and compared these molecular responses to morphometric measurements (growth and deformities).

2. Methods

2.1. NAFC preparation

A 2000 L OSPW sample was collected in 2011 from an active settling basin at Industry A (now an end pit lake; OSTC and COSIA, 2012). NAFCs were extracted and purified as previously described (Frank et al., 2006; Marentette et al., 2015a,b), utilizing a method very specific to the AEO compounds. A stock solution was prepared in 0.05 M NaOH with a final nominal concentration of 1998 mg/L, determined *via* liquid chromatography/quadrupole mass spectrometry with time of flight detection (LC/QToF; Brunswick et al., 2015). Total aromatic (PAH) concentrations in this NAFC stock were very low, evaluated at 0.64 μg/L (evaluated with GC–MS; Marentette et al., 2015a). The extract was dominated by O_2 species (*i.e.*, $C_nH_{2n+Z}O_2$, 75.1% of all acids) which were predominately bi- and tricyclic compounds (Z = −4 and −6). Fewer compounds showed four to six rings, and monocyclic and acyclic acids were the least abundant of all (Marentette et al., 2015a).

Exposure solutions were prepared daily using municipal water that was carbon-filtered, dechlorinated and UV-sterilized (for water composition, see Supplementary information, Table S1). Both NAFC and solvent control (0.05 M NaOH) solutions were acidified to 8.3 ± 0.1 with 1.0 M HCl and all solutions were incubated to 15 ± 1 °C before use. Concentrations reported here are nominal although in similar beaker exposures with fathead minnows, these two nominal NAFC concentrations varied by less than 4% from mean measured NAFC concentrations (See Marentette et al. (2015b) for full details).

2.2. Walleye eleutheroembryo collection and early-life stage toxicity test

Eleutheroembryos (n = 38) were collected from daily static-renewal walleye early-life stage toxicity tests previously described (Marentette et al., 2015a). In brief, fertilized walleye eggs were obtained from White Lake Fish Hatchery (Ontario Ministry of Natural Resources, Sharbot Lake, ON), transported within 4 h of fertilization to the Aquatic Life Research Facility (Burlington, ON) and maintained overnight at 15 ± 1 °C, the optimal temperature for embryonic development (Koenst and Smith, 1976; McElman and Balon, 1979). By 1 dpf, viable embryos were readily identified at the blastula and early gastrula stages, pooled across four separate male-female spawnings and 30 randomly selected embryos were allocated to egg cups in aerated 1 L beakers filled to 600 mL. Hatching occurred between 9 and 16 dpf. As each group attained 50% hatch (12—14 dpf), up to five randomly selected eleutheroembryos at concentrations near and below the EC50 (*i.e.*, control, 4.2 mg/L and 8.3 mg/L) were subsampled from each of three beakers per