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Sensitivity of walleye (*Sander vitreus*) and fathead minnow (*Pimephales promelas*) early-life stages to naphthenic acid fraction components extracted from fresh oil sands process-affected waters

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ABSTRACT

Unconventional oil production in Alberta's oil sands generates oil sands process-affected water (OSPW), which contains toxic constituents such as naphthenic acid fraction components (NAFCs). There have been few studies examining effects of NAFC exposure over long periods of early-life stage development in fish. Here we examined the effects of NAFCs extracted from OSPW to embryo-larval fathead minnow, exposed for 21 days. We compared the sensitivity of fathead minnow to walleye reared to 7 days post-hatch (18–20 days total). EC50s for hatch success, including deformities, and total survival were lower for walleye (10–11 mg/L) than fathead minnow (22–25 mg/L), with little post-hatch mortality observed in either species. NAFC exposure affected larval growth at concentrations below the EC50 in fathead minnow (total mass IC10 14–17 mg/L). These data contribute to an understanding of the developmental stages targeted by oil sands NAFCs, as well as their toxicity in a greater range of relevant taxa.

1. Introduction

The Alberta oil sands contain 168 billion barrels of proven bituminous oil reserves, with total bitumen production in 2013 reaching 2.09 million barrels per day (ERCB, 2013; Alberta Energy, 2014). Bitumen extraction via the Clark hot water process generates 0.7–0.8 m^3 of oil sands process-affected water (OSPW) per tonne of oil sands; OSPW is stored in settling basins, or tailings ponds (FTFC, 1995a; FTFC, 1995b). OSPW contains a complex mixture of salts, metals, unextracted bitumen and organic constituents including a diverse range of acid-extractable organics, also known as naphthenic acid fraction components (NAFCs; Allen, 2008; Headley et al., 2013a, b). NAFCs are structurally more complex than classical naphthenic acids (NAs, $C_nH_{2n+z}O_2$; Clemente and Fedorak, 2005), and have been shown to be less toxic, with potentially different mechanisms of action (Nero et al., 2006; Peters et al., 2007; Marentette et al., 2015a). Characterization of individual NAFCs and their mixtures is an ongoing process (Headley et al., 2013a, 2013b; Pereira et al., 2013; Rowland et al., 2011a, 2011b; Wilde et al., 2015). Final storage options under consideration for OPSW include discharge into mined-out pits, forming end pit lakes with the capacity to eventually support fish populations (CEMA, 2012; AESRD, 2013). In support of these goals, and in light of the recently reported detection of NAFCs from OSPW in groundwater beyond containment systems, greater understanding of the toxicity of OSPW constituents such as NAFCs is needed (Giesy et al., 2010; He et al., 2012; Frank et al., 2014).

The toxicities of OSPW, reclaimed wetlands containing oil sands process materials, or NAFCs extracted from OSPW, to vertebrates have been examined in a range of mammals, birds and amphibians (Rogers et al., 2002; Gurney et al., 2005; Gentes et al., 2006; Harms et al., 2010; Hersikorn and Smits, 2011; Beck et al., 2014). However, the effects of exposure to OSPW, and NAFCs extracted from OSPW, have been explored most frequently in fish. Both OSPW and NAFCs are acutely toxic to the early-life stages (ELS) of fish, which may be the most sensitive period of an organism's life cycle (McKim, 1977). Dose-dependent mortality, rates of spinal, craniofacial and cardiovascular deformities, decreased yolk utilization and reduced hatch length have all been demonstrated variously in zebrafish larvae (*Danio rerio*, exposed for 96 h to NAFC from fresh OSPW; Scarlett et al., 2013), fathead minnow embryos (exposed to fresh OSPW for 7 d, He et al., 2012; exposed to NAFC from fresh OSPW for 9 d,

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Kavanagh et al., 2012), fathead minnow larvae (exposed to NAFC from fresh OSPW for 96 h, Kavanagh et al., 2012), Japanese medaka embryos (*Oryzias latipes*, exposed to OSPW or NAFCs until hatch, up to 18 d; Farwell et al., 2006; Peters et al., 2007) and yellow perch embryos (exposed to fresh OSPW until hatch; Peters et al., 2007).

Toxicity of extracted NAFCs over an extended period of fish ELS development has not yet been tested. Larvae (particularly the yolk-sac stage) are frequently more sensitive to toxicants than embryos, because they lack the protective barrier of the chorion (Von Westernhagen, 1988; Henn and Braunbeck, 2011). It is unclear if this is true of NAFC toxicity; for example, Kavanagh et al. (2012) reported that fathead minnow embryos exposed to NAFCs from fresh OSPW (from fertilization to 96 h post-hatch), were more sensitive than larvae; however, larvae were exposed after the transition to exogenous feeding (from 5 to 9 d post-hatch or dph).

Here, we aimed to expand on previous work examining the toxicity of NAFCs to fathead minnow ELS, by increasing the exposure period from <24-h post fertilization to include the larval period (terminating at 16 dph, just before the juvenile stage; 21 d total). The fathead minnow is a widely used model species in aquatic toxicology, is highly amenable to laboratory rearing (Ankley and Villeneuve, 2006), and is a forage fish native to freshwater systems across North America, including the Athabasca River and its tributaries (Scott and Crossman, 1998a). For a comparison species, we selected walleye (*Sander vitreus*, formerly *Stizostedion vitreum*), which may rank as the most important sportfish in Alberta (Sullivan, 2003), is native to the Athabasca River watershed (Scott and Crossman, 1998b), and is readily obtainable from hatcheries. The effects of OSPW and NAFC exposure on walleye are of interest (e.g., Tolton et al., 2012) but to date this fish has not previously been used in exposures to OSPW or NAFCs. We exposed walleye embryos from 1 d post-fertilization (dpf) to 7 dph (19–21 dpf) for a total test duration of 18–20 d. Our endpoints included time to hatch, eleutheroembryo size at hatch, and deformities at hatch in both species, as well as larval growth at test termination.

2. Materials and methods

2.1. NAFC preparation and measurement of NAFCs in exposure water

Two 2000 L OSPW samples, pumped from settling basins in active use at the time of collection, were collected in 2011 from Industry A (now an end pit lake; OSTC and COSIA, 2012) and B (hereafter, 2011 Industry A Fresh and 2011 Industry B Fresh; results of Orbitrap mass spectrometric characterization in Marentette et al., 2015a). NAFCs were extracted and purified using a previously defined method (Frank et al., 2006), creating stock solutions in 0.05 M NaOH with a final nominal concentration of 1998 and 1243 mg/L respectively, determined via liquid chromatography/quadrupole mass spectrometry with time of flight detection (LC/QToF; Brunswick et al., 2015). The LC/QToF analysis employed an Athabasca OSPW NAFC extract as reference for quantitative calibration, the reference material being generated in-house and previously characterized by electrospray ionization mass spectrometry (Headley et al., 2002). The method employed sample alkalination coupled with reverse phase gradient LC/MS-QToF in electrospray negative mode. NAFCs were identified by accurate mass employing time of flight detection and corresponding ions were extracted from the total ion scan. Calculation of total NAFC concentration employed pooled ion responses relative to those of the reference NAFC extract. An internal standard was included to confirm sample injection and compensate for any instrument drift.

A daily NAFC stock was prepared equivalent to the highest concentration used in the experiment, using carbon-filtered, dechlorinated and UV-sterilized municipal water (for water composition, see Supplementary information, Table S1). A solvent control was created with the equivalent quantity of NaOH to match the NAFC stock (NaOH control stock). Both stock solutions were acidified to pH 8.3 ± 0.1 with 1.0 M HCl. NAFC exposure solutions were diluted as needed with NaOH control, covered and placed in an incubator to reach 25 ± 2 °C overnight before use.

At three time points in each of the fathead minnow experiments, post-exposure water was collected from all three replicates, pooled by treatment group, filtered through a fine Nitex mesh to remove debris and uneaten food, and a 30-mL sample was preserved in amber glass at 4 °C to verify nominal exposure concentrations (LC/MS-QToF analysis completed within two to four months of sample collection; an in-house study is ongoing to confirm that NAFC concentrations are not affected by sample storage within that time period). For quantitation, the chromatographic peaks were extracted by accurate mass from a total ion scan and the results pooled to provide a total NAFC concentration relative to an Athabasca OSPW NAFC reference standard as described above. Measured NAFC concentrations in samples from fathead minnow exposures were very close to nominal concentrations (Table 1). See Disclaimer below for additional information.

2.2. Fathead minnow early-life stage toxicity test

Embryos <24 h post-fertilization (<1 d post-fertilization, dpf) from at least three clutches were collected daily from an external supplier (Aquatox Testing and Consulting, Inc., Aberfoyle, ON) and sorted according to criteria in Marentette et al. (2014). Eggs were primarily (>90%) in the blastula to early gastrula stages at the start of the test. Three replicates of six NAFC dilutions, plus six replicates of laboratory controls and three replicates of NaOH controls, were used in two 21-d early life-stage fathead minnow bioassays at 25 °C using 1 L beakers filled to 400 mL (0–4 dpf) or 800 mL (4–21 dpf), and aerated. These exposures in fathead minnows represented a 16 dph partial ELS test (cp. OECD Test Guideline 210, OECD, 1992). Larvae in these tests are typically in the late metalarval stage by 16 dph, just before the full transition to the juvenile stage typified by the complete resorption of all remaining medial finfold (Marentette et al., 2015b). As fathead minnow are sexually mature at 90 dph (Parrott, 2005), 21 d is >10% of the life cycle and thus a chronic toxicity test (Environment Canada, 2005). Twenty embryos per group were randomly selected and counted into an egg cup. Egg cups were constructed of a glass cylinder with fine nylon mesh at one end, fastened with silicone and elevated 2 cm above the bottom of the beaker (Colavecchia et al., 2004). Water was fully changed once per day (daily static-renewal exposures). Water quality (pH, dissolved oxygen (DO), total N, temperature and conductivity) was evaluated in each post-exposure solution at three time points over the course of the test (Supplementary information, Table S2).

Embryos and larvae were inspected each day for mortalities, which were recorded and removed. A mean *time to hatch* (TTH, days) was assigned to each group of 20 embryos, including data from all fully-hatched (viable and inviable) individuals. At 5 dpf, with hatch complete, all newly-hatched eleutheroembryos were assessed for viability. Non-viable eleutheroembryos were euthanized, and viable individuals were fed. *Hatch success* was defined as the percentage of eggs that produced viable eleutheroembryos without severe deformity (Birge et al., 1985). *Deformity rates* were the percentage of all hatched eleutheroembryos that exhibited either mild or severe deformities. These included cardiovascular abnormalities (pericardial and yolk-sac edemas, hemorrhages including hemostasis, and/or tubehearts), spinal curvature such as lordosis, kyphosis and scoliosis, and mobility problems. Such deformities often co-occurred with various craniofacial abnormalities,