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Toxicity of naphthenic acid fraction components extracted from fresh and aged oil sands process-affected waters, and commercial naphthenic acid mixtures, to fathead minnow (*Pimephales promelas*) embryos

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A B S T R A C T

Naphthenic acids (NAs) are constituents of oil sands process-affected water (OSPW). These compounds can be both toxic and persistent and thus are a primary concern for the ultimate remediation of tailings ponds in northern Alberta's oil sands regions. Recent research has focused on the toxicity of NAs to the highly vulnerable early life-stages of fish. Here we examined fathead minnow embryonic survival, growth and deformities after exposure to extracted NA fraction components (NAFCs), from fresh and aged oil sands process-affected water (OSPW), as well as commercially available NA mixtures. Commercial NA mixtures were dominated by acyclic O_2 species, while NAFCs from OSPW were dominated by bi- and tricyclic O_2 species. Fathead minnow embryos less than 24 h old were reared in tissue culture plates terminating at hatch. Both NAFC and commercial NA mixtures reduced hatch success, although NAFCs from OSPW were less toxic (EC50 = 5–12 mg/L, nominal concentrations) than commercial NAs (2 mg/L, nominal concentrations). The toxicities of NAFCs from aged and fresh OSPW were similar. Embryonic heart rates at 2 days post-fertilization (dpf) declined with increasing NAFC exposure, paralleling patterns of hatch success and rates of cardiovascular abnormalities (e.g., pericardial edemas) at hatch. Finfold deformities increased in exposures to commercial NA mixtures, not NAFCs. Thus, commercial NA mixtures are not appropriate surrogates for NAFC toxicity. Further work clarifying the mechanisms of action of NAFCs in OSPW, as well as comparisons with additional aged sources of OSPW, is merited.

1. Introduction

Naphthenic acids (NAs) are a complex group of naturally-occurring organic acids that have been classically defined by the formula $C_nH_{2n+Z}O_2$, where n is the number of carbons and *Z* indicates the number of hydrogens lost to ring formation (Clemente and Fedorak, 2005). NAs refined from petroleum are also commercially produced for use as textile and wood preservatives, surfactants, paint driers, and to promote adhesion during tire manufacture (Brient et al., 1995). Related compounds are also found in oil (bituminous) sands, such as the Athabasca region McMurray formation of Alberta, Canada. Surface mining of oil sands by means of the Clark hot water extraction process, and reuse of process water, leads to high concentrations of NAs in fluid tailings, which also contains other organic and inorganic compounds such as PAHs, salts, and trace metals (Allen, 2008; FTFC, 1995a; Giesy et al., 2010). In view of the toxicities of these mixtures, the importance of process water recycling, and the Government of Alberta's "zero discharge policy," process waters have been stored in settling basins, or tailings ponds (FTFC, 1995b; Giesy et al., 2010). The diverse family of carboxylic acids and other acid-extractable organic compounds (AEOs) from oil sands process-affected waters (OSPW) continues to be chemically characterized. The understanding of what comprises the acid-extractable fraction of these mixtures is evolving,

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due to the unveiling of new compound classes, including aromatic, adamantane or diamondoid structures (Rowland et al., 2011a,b,c) as well as sulphur- and nitrogen-containing compounds and multiple oxygenated acid species (Barrow et al., 2009; Bataineh et al., 2006; Headley et al., 2011, 2013a). The term 'naphthenic acid fraction components' (NAFCs) will be used here to capture this wide range of extracted components studied in this investigation.

NAFCs are considered a major driver of toxicity of OSPW (Clemente and Fedorak, 2005; MacKinnon and Boerger, 1986), although the toxicity of OSPW varies with age. Changes in NAFC composition over time in OSPW are thought to be associated with changes in toxicity; lower molecular weight NAs have been proposed to be more acutely toxic (Frank et al., 2008, 2009). These smaller acids are found at greater concentrations in freshly discharged versus aged OSPW, as they are more susceptible to microbial activity; compounds with increased alkyl branching in larger structures are more recalcitrant, persisting in aged waters (Bataineh et al., 2006; Johnson et al., 2010; Scott et al., 2005). Smaller, structurally simpler acids are similarly characteristic of commercially-produced NA mixtures (Grewer et al., 2010; Rowland et al., 2011c; Rudzinski et al., 2002; Scott et al., 2005) and simpler classical NAs as well as NAFCs are also readily degraded by microbes, with a resulting reduction of mixture toxicity (Clemente et al., 2004; Holowenko et al., 2002; Lai et al., 1996; MacKinnon and Boerger, 1986; Scott et al., 2005). As OSPW will eventually need to be incorporated into reclaimed landscapes such as end pit lakes, and NAFCs may be present in groundwater both near and far from oil sands development, understanding and mitigating NA toxicity is clearly important for regulatory decision-making (Frank et al., 2014; Giesy et al., 2010).

Commercial NAs and NAFCs exert multiple toxic effects on fish, potentially through several modes of action. For adult fish, endocrine disruption has been reported, possibly due to structural similarities of some NAFCs to the steroid hormones estradiol and estrone (Rowland et al., 2011b). Adult fathead minnow (*Pimephales promelas*) exposed for 21 days to aged OPSW, or 10 mg/L of a NAFC mixture extracted from fresh OSPW had reduced fecundity, and males had lower levels of plasma steroids (11-ketotestosterone and testosterone) and fewer tubercles (Kavanagh et al., 2011, 2012). Similarly, both male and female fathead minnow exposed for seven days to fresh OSPW showed altered transcription of estrogen-responsive genes in brain, liver and gonad tissue (He et al., 2012b). Aromatic NAFCs, and not alicyclic NAFCs, may be responsible for these changes (Reinardy et al., 2013), although OSPW alkalinity and pH may also play a role (Van den Heuvel et al., 2012). However, responses extend beyond endocrine disruption. Proliferative, inflammatory and structural changes to gill tissue, reducing surface area and impairing respiration, as well as liver histopathologies, were found after 21 days in yellow perch (*Perca flavescens*) exposed to commercial NA mixtures and NAFC from fresh OSPW (1–2 mg/L NAs), and yellow perch and goldfish (*Carassius auratus*) caged in OSPW (Nero et al., 2006a,b). Commercial NA mixtures affected immune system responses in goldfish; first increasing, then decreasing cytokine expression and host defense capacity against pathogens over 12 weeks (Hagen et al., 2012). NAFC extracted from aged OSPW stimulated blood cell proliferation but did not show specific toxic effects on immune cells (B- and T-lymphocytes) in rainbow trout (*Oncorhynchus mykiss*; MacDonald et al., 2013).

Early-life stage (ELS) assays indicate that both embryos and larvae of multiple species are vulnerable to NA exposure. Fresh OSPW as well as commercial NA mixtures caused a dose-dependent increase in craniofacial and spinal deformities, decreased yolk utilization and hatch length, hemorrhages, edemas and incomplete hatching in embryos of yellow perch and Japanese medaka (*Oryzias latipes*; Peters et al., 2007). Trends of reduced hatch length, delayed hatch and increased deformities were observed in medaka larvae exposed to NAFC concentrations below acute toxicity thresholds (Farwell et al., 2006). Fathead minnow embryos exposed to fresh OSPW showed increased rates of cardiovascular and spinal deformities, premature hatching and greater rates of spontaneous activity than control embryos (He et al., 2012a). These embryological effects may be due to increased oxidative stress and abnormally high rates of apoptosis, and not through cytochrome P4501A (CYP1A)-mediated toxicity (He et al., 2012a). Zebrafish (*Danio rerio*) larvae exposed for 96 h to fractions of NAFC from OSPW showed higher mortality in aromatic versus alicyclic fractions (Scarlett et al., 2013). In general, zebrafish larvae appeared to be more sensitive than fathead minnow larvae exposed to NAFC (Kavanagh et al., 2012). Toxicity of NAs and OSPW has been explored in early life stages of other vertebrates; metamorphosis of wood frog (*Lithobates sylvaticus*) tadpoles was delayed in newly-reclaimed but not aged reclaimed wetlands (Hersikorn and Smits, 2011). Commercial NA mixtures have also been shown to be acutely (at concentrations of 3-5 mg/L) and chronically toxic (at concentrations of 1–2 mg/L) to embryos and larvae of the wood frog, northern leopard frog (*Lithobates pipiens*) and western clawed frog (*Xenopus tropicalis*), affecting survival and metabolism, reducing growth and inducing deformities (Melvin and Trudeau, 2012a,b; Melvin et al., 2013).

Here we evaluated lethal and sublethal toxic effects of NAFC derived from both fresh (three samples) and aged OSPW, and three commercial NA mixtures, to early-life stages of fathead minnows. We hoped to expand on previous studies of NA toxicity to fish ELS (Farwell et al., 2006; Kavanagh et al., 2012; Peters et al., 2007) by testing a greater range of NAFC and NA mixtures, and incorporating additional endpoints, to determine whether exposures to commercial NA mixtures and NAFC extracts produced similar effects. Fathead minnow are widely used in aquatic toxicology (Ankley and Villeneuve, 2006) and, unlike zebrafish or medaka, are native to the Alberta oil sands region (Scott and Crossman, 1998). We reared embryos to hatch, evaluating time to hatch, deformities, growth and survival. We examined whether NAFC exposure affected embryonic heart rate early in development, which is an endpoint applicable for many toxicants (Carlsson and Norrgren, 2004; Incardona et al., 2014; Johnson et al., 2007) and a proxy for cardiovascular development, as a supplement to observations of deformities and growth.

2. Methods

2.1. Measurement of NA and NAFC concentrations in stock solutions

Three commercial NA mixtures (hereafter termed Merichem, Aldrich and Acros) were prepared at 400 mg/L in 0.05 M NaOH solutions (Merichem Company: Acid no. 181 mg KOH/gm, unsaponifiables 5.6%, water 0.13%; Sigma–Aldrich: Product no. 70340; Acros Organics: Cat no. 415281000). Large volumes of OSPW were collected in 2009 (∼3000 L collected from the discharge into the pond) and 2011 (∼2000 L pumped from within the pond) from Industry A's settling basin. In 2011, approximately 2000 L OSPW was also collected using a pump in the tailings pond of Industry B. At the time of OSPW collection, both settling basins were in active use, and are hereafter designated as 'fresh'. Also in 2011, approximately 2000 L of OSPW was collected from a test pond of Industry A, which had not received OSPW since its establishment in 1993 (Siwik et al., 2000), and hereafter is designated as 'aged'. NAFCs were precipitated with concentrated acid and then purified via diethylaminoethyl (DEAE) cellulose weak anion exchange and liquid–liquid extraction clean-up using dichloromethane, as described elsewhere (Frank et al., 2006; Kavanagh et al., 2012;