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Morphological and molecular effects of two diluted bitumens on developing fathead minnow (*Pimephales promelas*)

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ABSTRACT

Canada has experienced a significant increase in the transport of diluted bitumen (dilbit), a predominant oil sands product that combines bitumen with diluents derived from oil-gas condensates and other proprietary compounds. The toxicity of dilbit to fish embryos, which are immobile and thus at a high risk of exposure to oil in the event of a spill, remains largely unknown for most species. This study assessed the toxicity of water accommodated fractions (WAF) and chemically enhanced water accommodated fractions (CEWAF) of two winter dilbit blends, Access Western Blend (AWB) and Cold Lake Blend (CLB), to fathead minnow (*Pimephales promelas)* embryos. The TPH-F EC50s for malformations were 834 and 1058 µg/L for AWB WAF and CEWAF, respectively, and 500 and 715 µg/L for CLB WAF and CEWAF, respectively. Levels of *cyp1a* mRNA increased up to 46- and 69-fold, respectively, reflecting increasing exposure to polycyclic aromatic compounds (PACs) in AWB and CLB. Similarly, levels of *gst* mRNA were elevated up to 3.8-fold and 2.7-fold with increasing total concentrations of PACs in AWB and CLB, respectively. However, there were no significant changes in mRNA levels of *p53*, *sod, cat*, and *gsr*. These results suggest that the expression of *cyp1a* and *gst* may serve as biomarkers for dilbit exposure in fathead minnow, furthering our understanding of dilbit-responsive indicators of toxicity in fish species native to North America. This study is important as it utilizes the same exposure methodology to examine the toxicity of two commonly used Canadian dilbits, facilitating comparison of dilbit toxicity.

1. Introduction

Transport of diluted bitumen (dilbit) from Alberta's oil sands across Canada to refineries or marine ports depends on pipeline and railway shipment. In Canada, the two most commonly transported dilbits are Access Western Blend (AWB) and Cold Lake Blend (CLB). Dilbit is created by diluting extracted bitumen with diluents, such as oil-gas condensates, to decrease viscosity and facilitate its transportation, as raw bitumen is too viscous to flow through pipelines (Crosby et al., 2013). The precise chemical makeup of dilbit varies based on the extraction process, geographical source (Lee et al., 2015a), and seasonal variations in diluent concentration to adjust viscosity in response to temperature changes (Crosby et al., 2013).

When dilbit is spilled, the more volatile, lighter compounds in the diluent dissipate during the weathering process, which leaves behind the less volatile, heavier compounds, such as polycyclic aromatic compounds (PACs) (Yang et al., 2011). Because weathering occurs quickly following a spill, the chemical and physical properties and the environmental fate and behaviour of dilbit also change rapidly (Fingas, 2015; King et al., 2014). The loss of low molecular weight (LMW) alkenes and mono- and di-aromatic hydrocarbons associated with acute lethality are the main changes occurring in AWB and CLB during weathering (King et al., 2014). However, the evaporation of LMW compounds increases the viscosity of the residual oil and the concentration of 3–5-ringed alkyl-PACs, compounds associated with malformations and chronic toxicity in developing fish (Adams et al., 2014a; Bornstein et al., 2014). Thus, there is a growing concern about the potential effects of dilbit spills on aquatic ecosystems (reviewed in Alsaadi et al., 2018). While the toxicity of crude and refined oil to fish embryos is well established (Carls et al., 2000; Farwell et al., 2006; He et al., 2012; Martin et al., 2014; Schein et al., 2009), few studies have examined the effects of dilbit on aquatic species (Alderman et al.,

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2017a, 2017b; Barron et al., 2018; Madison et al., 2017, 2015; Philibert et al., 2016). Among others, Madison et al. (2015, 2017) found that the embryo toxicity of dilbit was related to the sum or total of all PAC (TPAC) concentrations in solutions prepared from mechanically-dispersed water accommodated fractions (WAF) and chemically enhanced water accommodated fractions (CEWAF).

PACs can lead to developmental toxicity by aryl hydrocarbon receptor (AhR) binding or by mechanisms independent of the AhR (Incardona, 2017). PACs that are strong AhR agonists bind to the AhR and form a PAC-AhR complex. The PAC-AhR complex forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (Arnt), which regulates gene transcription (Fujii-Kuriyama and Kawajiri, 2010). Regulated genes include those involved in phase I xenobiotic metabolism (cytochrome P450, *cyp1a*), phase II biotransformation enzymes (glutathione reductase, *gsr*; glutathione S-transferase, *gst*; and glutathione peroxidase, *gpx*), oxidative stress defence enzymes (superoxide dismutase, *sod*; catalase, *cat*) (He et al., 2012; Holth et al., 2014; Kim et al., 2013; Olsvik et al., 2012) and the tumorigenic response (tumour suppressing protein, *p53*) (Williams and Hubberstey, 2014). The regulation of genes involved in xenobiotic metabolism affects the potential of the organism to protect itself against exposure to xenobiotics (Altenburger et al., 2003).

The ability of PAC to bind to the teleost AhR predicts the potency of PAC to induce CYP1A activity (Billiard et al., 2002). As alkylated PACs interact more readily with the AhR, their accumulation by fish leads to increased CYP1A activity (Barron et al., 2004; Billiard et al., 1999; Hodson et al., 1996; Nebert et al., 2004, 2000; Whyte et al., 2000), increased production of reactive metabolites (Varanasi et al., 1989), and increased oxidative stress (Bauder et al., 2005). Together, these responses may contribute to adverse effects and mortality of early life stages of fish. Several studies have found that increased expression of *cyp1a* following exposure to toxic compounds preceded the occurrence of blue sac disease (BSD) (Bauder et al., 2005; Brinkworth et al., 2003; Cowey et al., 1985; Madison et al., 2017). BSD of fish embryos is a common response to exposure to compounds causing induction of cytochrome P450 enzymes. It is characterized by yolk sac edema which appears blue because the edematous fluid contains high concentrations of proteins that fluoresce. The edema may result from impaired cardiac function or membrane damage of yolk sac blood vessels (Billiard et al., 1999). Predicting the toxicity of dilbit is complicated by variations in its chemical composition. Thus, this study sought to identify the phenotypic and molecular effects of AWB and CLB exposure on developing fathead minnow (FHM) embryos to identify genes that may be used as biomarkers of dilbit toxicity following oil spills, and to examine how PACs, expressed in terms of TPAC content, are related to dilbit toxicity. We reasoned that because PAC concentrations are higher in CLB than AWB after weathering (King et al., 2014), embryos exposed to CLB will experience higher toxicity than those exposed to AWB. This study reports the chronic toxicity and gene expression changes in FHM embryos following exposure to previously unweathered AWB and CLB dilbit blends. However, the procedures for preparing test solutions included an initial 18 h open stirring step that would result in some loss of volatile constituents, so the observed baseline toxicity reflects simple weathering by evaporation. A systematic study of oils weathered by various mechanisms was not within the scope of this study.

2. Material and methods

2.1. Oils and chemicals

Unweathered AWB and CLB dilbit blends and Corexit® 9500A dispersant were supplied by the Centre for Offshore Oil, Gas and Energy Research, Fisheries and Oceans Canada (Dartmouth, NS, Canada). Mineral oil (Nujol) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental animals

FHM were selected as a test species because they are widespread in North American watersheds east of the Rocky Mountains (Scott and Crossman, 1973), including those where dilbit is produced and shipped. They prefer ponds, small lakes, and low-gradient streams and spawn in shallow water, under rocks, branches, or logs. Eggs were obtained from AquaTox Testing & Consulting Inc., Guelph, ON, Canada. They were collected from 10 breeding pairs of adults within 24 h of fertilization and transported in aquarium water at 25 ± 1 °C to Kingston, Ontario, where they were transferred to de-chlorinated Lake Ontario water from the City of Kingston's municipal freshwater supply at 25 ± 1 °C and pH 7.9 ± 0.2. Eggs came attached to a tile and were gently removed with a plastic scraper, mixed, placed in a plastic container, and immediately separated from mucous by sanitized tweezers. Healthy embryos (< 24 h post-fertilization) were carefully placed, egg by egg, into labeled jars using a pipette. At 20 h post-fertilization, the pericardial coelom enlarges, the heart anlage become visible, and the embryos exhibit first movements (USEPA, 1996). Approximately 30 h post-fertilization, the heartbeat is present and at 35.33 h post-fertilization, circulation begins (USEPA, 1996). Animal care protocols conformed to the Guidelines of the Canadian Council on Animal Care and the Queen's University Animal Care Committee.

2.3. Dilbit exposures

AWB and CLB dilbit experiments were run in sequence but are presented together to compare results. WAF and CEWAF of unweathered AWB and CLB were prepared following Madison et al. (2015, 2017). WAF was prepared by adding dilbit to water at a ratio of 1:9 according to density (AWB: 0.923 g/L; CLB: 0.922 g/L), stirring gently with a 19 mm Teflon-coated stir bar at 25% vortex for 18 h, and settling for 1 h. CEWAF was prepared by the same procedure with the exception that, after 18 h of stirring, Corexit® 9500A (oil dispersant; ECOLAB/NALCO, Illinois, USA) was added to the centre of the surface layer of dilbit at a dispersant-to-oil ratio of 1:20. The mixture was stirred for 1 h and settled for an additional hour. Fractions from the bottom aqueous layer were withdrawn with 60 mL and 40 mL plastic syringes (22 gauge 1.5″ needles with tips cut off) for WAF and CEWAF, respectively, and 10 mL syringes for controls. To minimize contact of the surface oil with the needle, a small amount of air was pushed out of the syringe to create a clear spot on the water surface where the needle was inserted. The filled syringes were placed horizontally in the fume hood to allow any large oil droplets suspended in the water to settle out and stick to the wall of the syringe before removing the aqueous phase. WAF stock solutions were transferred to 250 mL glass beakers before dilution to nominal concentrations of 0.32, 1.0, 3.2, 10, and 32% v/v. CEWAF stock solutions were transferred to 20 mL glass vials before dilution to nominal concentrations of 0.0001, 0.001, 0.01, 0.1, and 1% v/v. Stock solutions and dilutions were prepared daily for 24 h semi-static renewal exposures in clean exposure jars (< 2 mL of water left on embryos during changeover). Jars were cleaned with methanol-soaked wipes to remove residual oil, rinsed with de-chlorinated water, and re-used the following day.

2.4. Chronic toxicity test

Based on the number of healthy embryos received for each experiment, each treatment included duplicate jars of 30 embryos for AWB (total N = 60) and 40 embryos for CLB (total n = 80) in 200 mL of de-chlorinated water. Embryos were exposed to each treatment from under 24 h post-fertilization until hatch or to a maximum duration of 10 days if hatch was delayed. Each experiment included two controls, a negative (water) and a dispersant control (1% v/v of mineral oil dispersed with Corexit® 9500A). Jars were agitated on a shaker platform at 65 rpm to ensure water movement and continuous aeration. Within