Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*)

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**Abstract**

Due to a policy of no release, oil sands process-affected water (OSPW), produced by the surface-mining oil sands industry in North Eastern Alberta, Canada, is stored on-site in tailings ponds. Currently, ozonation is considered one possible method for remediation of OSPW by reducing the concentrations of dissolved organic compounds, including naphthenic acids (NAs), which are considered the primary toxic constituents. However, further work was needed to evaluate the effectiveness of ozonation in reducing the toxicity of OSPW and to ensure that ozonation does not increase the toxicity of OSPW. This study examined effects of untreated, ozone-treated, and activated charcoal-treated OSPW (OSPW, O3-OSPW, and AC-OSPW) on the early life stage (ELS) of fathead minnow (*Pimephales promelas*). Success of hatching of eggs, spontaneous movement, and incidences of hemorrhage, pericardial edema, and malformation of the spine of embryos were examined. To elucidate the mechanism of toxicity, concentrations of reactive oxygen species (ROS) were measured, and the abundances of transcripts of genes involved in biotransformation of xenobiotics, response to oxidative stress, and apoptosis were quantified by real-time PCR. Compared to the control group, which had an embryo survival rate of 97.9 ± 2.08%, survival was significantly less when exposed to OSPW (43.8 ± 7.12%). Eggs exposed to untreated OSPW exhibited a significantly greater rate of premature hatching, and embryos exhibited greater spontaneous movement. Incidences of hemorrhage (50.0 ± 3.46%), pericardial edema (56.3 ± 7.12%), and malformation of the spine (37.5 ± 5.38%) were significantly greater in embryos exposed to OSPW compared to controls. These effects are typical of exposure to dioxin-like compounds, however, abundance of transcripts of *cyp1a* was not significantly greater in embryos exposed to OSPW. Significantly greater concentrations of ROS, and greater abundances of transcripts *cyp3a*, *gst*, *sod*, *casp9*, and *apopen* compared to controls, indicated that exposure to OSPW caused
1. Introduction

There is increasing concern about the potential environmental effects of petroleum production from oil sands surface-mining in Alberta, Canada. One of the issues is that oil sands process-affected water (OSPW), which is generated by extraction of bitumen using the “Clark hot water extraction process”, is toxic to aquatic organisms. Due to a policy of no-release, more than 10^9 m^3 of OSPW are currently stored in on-site tailing ponds, with volumes continuously increasing (Del Rio et al., 2006). Remediation of this process water is a current priority of industries and government agencies (Government of Alberta, 2006; Del Rio et al., 2006).

The majority of the toxicity of OSPW has been attributed to the water soluble organic fraction, of which naphthenic acids (NAs) are one of the primary persistent constituents (Anderson et al., 2012a,b; Garcia et al., 2011a,b). NAs exist as a mixture, characterized as a group of cyclic and acyclic alkyl-substituted carboxylic acids with the general formula C\_nH\_m\_z O\_2, where n is the number of carbons and Z relates to the number of rings (Clemente and Fedorak, 2005; Frank et al., 2008; Headley and McMartin, 2004; Holowenko et al., 2002; Rowland et al., 2011a). Ozonation is a promising method for remediation of OSPW as it has been shown to significantly reduce the concentration of NAs (Scott et al., 2008; Martin et al., 2010; Gamal El-Din et al., 2011). However, further work was needed to fully evaluate the effectiveness of ozonation in reducing the toxicity of OSPW and to ensure that ozonation does not cause the formation of byproducts that might impart greater toxicity to OSPW.

OSPW is both acutely and chronically toxic to aquatic organisms (Clemente and Fedorak, 2005) and caused endocrine disrupting effects in vitro on sex steroid synthesis and receptor signaling (He et al., 2010, 2011). Exposure to OSPW decreased synthesis of and plasma concentrations of testosterone (T) and estradiol (E2) in yellow perch (Perca flavescens) and goldfish (Carassius auratus) (van de Heuvel et al., 1999; Lister et al., 2008). Abundances of transcripts of regulatory genes in all tissues of the hypothalamic-pituitary—gonad—liver axis were significantly different in fathead minnows (Pimephales promelas) exposed to OSPW (He et al., 2012). Exposure to OSPW also adversely affected reproductive capacity of fathead minnows. Fecundity was less, synthesis of sex steroids was altered, and less pronounced secondary sex characteristics were observed in male and female fathead minnows exposed to OSPW (Kavanagh et al., 2011, 2012). It is unknown which components of OSPW are responsible for these adverse effects; however, some NAs that are structurally similar to sex steroid hormones have been identified as potential candidate contaminants (Rowland et al., 2011b; Scarlett et al., 2012). Furthermore, a study using hepatocytes isolated from livers from rainbow trout revealed that exposure to OSPW resulted in greater expression of genes related to the biotransformation of xenobiotics, estrogenicity, and oxidative stress (Gagné et al., 2012).

OSPW is toxic toward early life stages of several species of fishes. When exposed to OSPW, oil sands sediment, or commercial NAs, a greater rate of premature hatching of eggs, lesser survival of embryos, greater incidence of deformities including hemorrhage, pericardial edema, and malformation of the spine were observed in fathead minnow, white sucker (Catostomus commersoni), yellow perch, or Japanese medaka (Oryzias latipes) (Colavecchia et al., 2004, 2006, 2007; Peters et al., 2007). In this study, potential toxicity of OSPW to embryos of fathead minnow was determined and the efficacy of ozonation to attenuate toxicity of OSPW was assessed. Assessment endpoints included rates of hatching of eggs, spontaneous movement of embryos, and morphological alterations such as malformations, especially of the spine, hemorrhage, and pericardial edema. Measurements of reactive oxygen species (ROS) and abundances of transcripts of genes related to biotransformation, responses to oxidative stress, and apoptosis were also measured to elucidate the mechanism(s) of toxicity of OSPW, and treated OSPW.

2. Materials and methods

2.1. Exposure waters

OSPW was collected from the West-In-Pit (WIP), an active settling basin on the Syncrude Canada Ltd. site at Fort McMurray, AB, Canada, in February 2010. The concentration of NAs in the OSPW was determined by use of ultra pressure liquid chromatography high resolution mass spectrometry (UPLC–HRMS) to be 19.7 mg/L (Wang, 2011). Ozonation of OSPW was conducted at the University of Alberta (Edmonton, AB, Canada) following standard procedures (Gamal El-Din and Smith, 2002; Wang, 2011). Ozone gas was generated from extra dry, high purity oxygen using an AGSO 30 Effizon ozone generator (WEDECO AG Water Technology, Herford, Germany). Prior to operation of the generator a 10 min stabilization period was utilized to obtain a stable ozone concentration in the feed-gas. The feed gas was sparged into the OSPW through a ceramic fine bubble gas diffuser located at the bottom of a PVC plastic reactor. During the ozonation process, concentrations of ozone in the feed- and off-gas lines were continuously monitored by two identical ozone...
monitors (model HC-500, PCI-WEDeco). The potassium iodide (KI) method was used to calibrate the ozone monitors periodically according to Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Treatment of the OSPW with ozone was continued until the total degradation of parent NAs reached approximately 90%, as determined by the remaining sum response of all UPLC-HRMS peak areas corresponding to NAs. Residual ozone and oxygen were stripped from the generator by purging for 10 min with purified nitrogen gas. Residual ozone in the reactor was determined by use of the Indigo method (APHA, 2005). Dose of ozone delivered by this system can be calculated (Equation (1)) (Gamal El-Din and Smith, 2002).

\[
\Delta O_3 = \int_0^t \frac{Q_{G,in} C_{G,in} - Q_{G,out} C_{G,out}}{V_L} dt - C_L 
\]

Where: \(\Delta O_3\) is the amount of utilized ozone (mg/L), \(C_{G,in}\) is the ozone concentration in the feed gas, which was calculated from reading the first ozone monitor (mg/L), \(C_{G,out}\) is the ozone concentration in the off gas, which was calculated from reading the first ozone monitor (mg/L), \(C_i\) is residue ozone concentration in the liquid phase (mg/L), \(V_i\) is effective reactor volume (L), \(Q_{G,in}\) is feed-gas flow rate (L/min), \(Q_{G,out}\) is off-gas flow rate (L/min), and \(t\) is ozone contact time (min).

Efficiency of removal of NAs by ozonation was greater than 90% with the total concentration of NAs having been decreased from 19.7 mg/L to 1.9 mg/L in the ozone-treated OSPW (O3-OSPW) as determined in a parallel study (Wang, 2011). The activated charcoal-treated OSPW (AC-OSPW) was prepared by mixing OSPW with 5% (w/v) activated charcoal according to Anderson et al. (2012a). Because organic compounds in OSPW bind to activated charcoal this water sample was a control to determine if toxic effects were due to organic compounds in OSPW.

2.2. Embryo exposure

The fathead minnow is a small fish species that is native to the oil sands region and is commonly used in aquatic toxicology testing in North America and whose life history is well known (OECD, 1992). Fathead minnows were cultured in 200 L tanks in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan. Three breeding tanks consisting of one sexually mature male and two sexually mature females were established in order to collect eggs for the exposure studies. Each breeding tank contained 20 L of dechlorinated tap water and half this volume was replaced daily. Tanks were maintained at 25 ± 1 °C with a 16/8 h day/night photoperiod. Fish were fed twice daily with frozen blood worms.

A 7-day assay to assess effects on development of embryos was designed based on OECD Guideline 210: Fish, Early Life-stage Toxicity Test (OECD, 1992). Fertilized embryos were collected within 1 h post fertilization (hpf) from the different breeding tanks and were pooled in a petri-dish containing control water. Eggs were rinsed 3 times in dechlorinated tap water and any unfertilized eggs were removed. Equal numbers of eggs were randomly placed into wells of a 6-well plate. Depending on the number of eggs available from each spawn the minimum number of eggs per well was 10 and the maximum number of eggs per well was 15 in an exposure replicate. Each well contained 2 mL of control water, which consisted of dechlorinated municipal tap water, OSPW, O3-OSPW, or AC-OSPW. The pH of the control water, OSPW, O3-OSPW, and AC-OSPW was 8.2, 8.7, 8.8 and 9.9, respectively. Fifty-percent of the volume (1 mL) was replaced daily with fresh test solutions. Exposures were performed at 25 ± 1 °C with a 16/8 h day/night photoperiod. Any dead eggs or larvae were removed daily. Exposure experiments were replicated 8 times and each experiment was performed with a separate batch of eggs.

Observations of embryos were made daily and values of measurement endpoints were recorded prior to the 50% water renewal. Daily measurements made included the number of live and dead embryos, the number of spontaneous embryo movements/minute (measured only at 26 hpf), rate of premature hatching, and prevalence of hemorrhage, pericardial edema, and spinal malformation. Exposures were terminated 168 hpf. At the end of exposure the cumulative percent occurrence of each endpoint was determined and used for statistical analyses. Percentage survival was calculated as the number of live larvae at the end of the experiment divided by the initial number of embryos.

2.3. Quantification of ROS

Concentration of ROS in embryos of fathead minnows were measured at 96 hpf by use of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) according to the manufacturer’s protocol (Invitrogen, Burlington, ON, Canada). Briefly, all live embryos from each exposure group were washed in dechlorinated tap water, homogenized in 120 μL of cold PBS, and centrifuged at 15,000 g at 4 °C for 20 min. A volume of 100 μL supernatant was recovered, diluted in pre-warmed PBS, and then added to a 96-well plate. CM-H2DCFDA was added to a final concentration of 1 μM and the plate was incubated at 37 °C for 30 min. Intensity of fluorescence was measured using a POLARStar OPTIMA microplate reader (BMG Labtech) with excitation and emission at 485 nm and 520 nm, respectively. Concentrations of ROS were normalized to protein content, which was determined by use of the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada). Exposure experiments were replicated 8 times and each experiment was performed with a separate batch of eggs.

2.4. Quantification of transcripts

Abundances of transcripts of ten genes related to biotransformation of xenobiotics, response to oxidative stress, and apoptosis were determined. All primers were designed using Primer 3 software and based on sequences obtained by Illumina RNA sequencing (unpublished data). Nucleotide sequences of primers and the biological functions of the target transcripts are given (Table 1).

Total RNA was extracted from embryos by use of the Qiagen RNeasy Plus Mini Kit according to the manufacturer’s protocol (Qiagen, Mississauga, ON, Canada) and stored at −80 °C until required. First-strand cDNA was synthesized from 1 μg of total RNA using an iScript™ cDNA Synthesis Kit.
Table 1 – Sequences of primers used for qPCR.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Gene</th>
<th>Primer pair (5′ – 3′)</th>
<th>Process</th>
<th>Annealing temp (°C)</th>
<th>Efficiency (%)</th>
</tr>
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<tbody>
<tr>
<td>18s</td>
<td>18S ribosomal RNA</td>
<td>F: GCCCTTGTAATTGGAATGACG</td>
<td>Reference gene</td>
<td>60</td>
<td>96.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCCCGAGATTCACATCGAGAAGGG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cyp1a</td>
<td>Cytochrome p450 1A</td>
<td>F: CCTGAGGAGAAGACTGAG</td>
<td>Phase I metabolism</td>
<td>60</td>
<td>85.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGAGCTACAGTGAGGGA</td>
<td>Phase I metabolism</td>
<td>60</td>
<td>89.2</td>
</tr>
<tr>
<td>Cyp3a</td>
<td>Cytochrome p450 3A</td>
<td>F: CGGAGAGACCTTCCCCAACAT</td>
<td>Phase II metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTTTTCTTGGAGACCGGTGTG</td>
<td>Oxidative stress</td>
<td>60</td>
<td>90.2</td>
</tr>
<tr>
<td>Gst</td>
<td>Glutathione-S-transferase</td>
<td>F: CCGGCAAGACGTTCACACAT</td>
<td>Phase II metabolism /</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGAATGCTGAAAATAGGG</td>
<td>Oxidative stress</td>
<td>60</td>
<td>91.5</td>
</tr>
<tr>
<td>Sod</td>
<td>Superoxide dismutase</td>
<td>F: CCAGACATGGCAGACCTTCTT</td>
<td>Oxidative stress</td>
<td>60</td>
<td>93.1</td>
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<tr>
<td>Cat</td>
<td>Catalase</td>
<td>F: TTATCGAGGAGTGCGTCTGTG</td>
<td>Apoptosis</td>
<td>60</td>
<td>95.4</td>
</tr>
<tr>
<td>Casp3</td>
<td>Caspase 3</td>
<td>F: GGCGGGGAGACACAGGAGAGAGAGAGG</td>
<td>Apoptosis</td>
<td>60</td>
<td>92.5</td>
</tr>
<tr>
<td>Casp9</td>
<td>Caspase 9</td>
<td>F: CCCGTTCCGCTCCACAGG</td>
<td>Apoptosis</td>
<td>60</td>
<td>97.8</td>
</tr>
<tr>
<td>ApopIn5</td>
<td>Apoptosis Inhibitor 5</td>
<td>F: GCCGACGAGAAGAGGAGAGAGAGAGG</td>
<td>Apoptosis</td>
<td>60</td>
<td>91.1</td>
</tr>
<tr>
<td>ApopEn</td>
<td>Apoptosis Enhancer</td>
<td>F: CCCGTTCCGCTCCACAGG</td>
<td>Apoptosis</td>
<td>60</td>
<td>96.3</td>
</tr>
<tr>
<td>Bax</td>
<td>Bax</td>
<td>F: AGCCATATACCGAGGAGGGAAGGAGG</td>
<td>Apoptosis</td>
<td>60</td>
<td>95.2</td>
</tr>
<tr>
<td>P53</td>
<td>P53</td>
<td>F: AGGGTGCAGGAGGAGGAGG</td>
<td>Apoptosis</td>
<td>60</td>
<td>95.2</td>
</tr>
</tbody>
</table>

Bio-Rad) according to the manufacturer’s instructions. The cDNA samples were stored at –20 °C until further analysis.

Quantitative real-time PCR (qPCR) was performed on an ABI 7300 Real-Time PCR System in 96-well PCR plates (Applied Biosystems, Foster City, CA, USA). A PCR reaction mixture for 1 reaction contained 10 µL of SYBR Green master mix (Applied Biosystems), 2 µL of sense/anti-sense gene-specific primers (Invitrogen, Carlsbad, CA, USA), and 8 µL of cDNA that was diluted in RNase free water (Qiagen). The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was: denaturing for 15 s at 95 °C and annealing and extension for 1 min at 60 °C for a total of 40 PCR cycles. Efficiency, uniformity, and linear dynamic range of each qPCR assay were assessed by construction of standard curves by use of serially diluted cDNA standards. Changes in abundances of transcripts of target genes were quantified by normalizing to 18s rRNA according to the method of Simon (2003). Exposure experiments were replicated 7 times and each experiment was performed with a separate batch of eggs.

2.5. Statistical analyses

Statistical analyses were conducted using SPSS 16.0 (SPSS, Chicago, IL, USA). All data are expressed as mean ± S.E.M. Normality of distributions of data was assessed by use of the Kolomogrov–Smirnov one-sample test and homogeneity of variance was determined by Levene’s test. When necessary, datasets were log2-transformed to meet assumptions of parametric tests. Non-transformed data are presented in figures. Statistical differences were evaluated by one-way ANOVA followed by post-hoc Tukey’s test. Differences were considered statistically significant at p < 0.05.

3. Results

3.1. Developmental toxicity of OSPWs

Exposure to OSPW resulted in a significantly greater incidence of premature hatching of eggs compared to the other treatments (Table 2). Cumulative rates of hatching of embryos exposed to OSPW were significantly greater than cumulative rates of hatching of embryos exposed to O3-OSPW or AC-OSPW at 48, 72, 96, 120, and 144 hpf. Embryos exposed to OSPW or O3-OSPW began hatching at 48 hpf and embryos exposed to AC-OSPW began to hatch at 72 hpf. No embryos hatched until 120 hpf in the control group. Exposure to OSPW significantly decreased the embryo survival rate (43.8 ± 7.12%) compared to that of control (97.9 ± 2.08%), O3-OSPW (93.8 ± 3.99%), and AC-OSPW (77.1 ± 7.12%) treated fish, respectively (Table 2).

At 26 hpf, embryos exposed to OSPW exhibited a significantly greater rate of spontaneous movement (48.6 ± 2.74 movements/min) compared to the control (25.3 ± 1.15 movements/min), O3-OSPW (29.25 ± 1.88 movements/min), and AC-OSPW (26.63 ± 1.23 movements/min) treatment groups.

Incidences of deformities were significantly greater in embryos of fathead minnows exposed to OSPW compared to the other treatments (Table 2 and Fig. 1). At the end of exposure (168 hpf) the total incidences of hemorrhage, pericardial edema, and malformation of the spine in embryos exposed to OSPW were 50.0 ± 3.49%, 56.3 ± 7.12%, and 37.5 ± 5.38%, respectively. Incidences of deformities of embryos exposed to O3-OSPW or AC-OSPW were significantly less than in embryos exposed to OSPW and were not significantly different from the controls, except for the significantly greater incidence of hemorrhage at 168 hpf in embryos exposed to O3-OSPW.
3.2. Abundances of transcripts

Exposure to OSPW affected abundances of transcripts of genes that encode cytochrome P450s compared to all other treatments (Fig. 2a). There were no significant differences in the abundance of transcripts of cyp1a among exposure groups. However, the abundance of transcripts of cyp3a was significantly greater by 2.35 ± 0.34-fold in embryos exposed to OSPW compared to the control group. The abundance of transcripts of cyp3a in embryos exposed to O3-OSPW or AC-OSPW were not significantly different from that of the control but were significantly less than in embryos exposed to OSPW (Fig. 2a).

Abundances of transcripts of genes involved in responses to oxidative stress were significantly affected by exposure to OSPW (Fig. 2b). Abundances of transcripts of gst and sod were significantly greater by factors of 2.15 ± 0.26 and 3.08 ± 0.74-fold, respectively, in embryos exposed to OSPW compared to the control group. Abundances of transcripts of gst and sod in embryos exposed to O3-OSPW or AC-OSPW were not significantly different from that of the control, but were significantly less than in embryos exposed to OSPW. Abundances of transcripts of cat were not significantly different among any of the exposure groups.

Abundances of transcripts of genes that regulate and mediate apoptosis were significantly affected in embryos exposed to OSPW (Fig. 2c). Abundances of transcripts of casp9 and apoœn were significantly greater by a factor of 3.26 ± 0.57 and 2.38 ± 0.25-fold, respectively, in the embryos exposed to OSPW compared to the control group. Abundances of transcripts of casp9 and apoœn were not significantly different in embryos exposed to O3-OSPW and AC-OSPW compared to the controls, but were significantly less than in embryos exposed to OSPW. Abundances of transcripts of casp3, p53, apopin5, and bax were not significantly different among any of the treatment groups.

3.3. Concentrations of ROS

Concentrations of ROS in embryos exposed to OSPW was significantly 1.68 ± 0.11-fold greater compared to that of the control embryos. Concentrations of ROS in embryos exposed to O3-OSPW or AC-OSPW were not significantly different from concentrations in control embryos but were, nonetheless, significantly less than the concentration in embryos exposed to OSPW (Fig. 3).

4. Discussion

Effects of OSPW on eggs and embryos of fathead minnows were consistent with the results of other studies. Several studies have reported reduced hatching success and survival of embryos exposed to oil sands sediment, PAHs, petroleum oil, and fractions of crude oil (Middaugh et al., 2002; Carls et al., 1999; Couillard, 2002; Colavecchia et al., 2004, 2006, 2007; Peters et al., 2007). These studies, together with the results of the present study, indicate that the organic compounds in OSPW are toxic to eggs and embryos, especially during organogenesis. Specifically, a greater incidence of premature hatching of embryos was a significant consequence of exposure to OSPW. Premature hatching has been attributed to rupturing of hatching glands due to stimulation of respiration or irritation by soluble hydrocarbons (Leung and Bulkley, 1979). The greater rate of spontaneous movement of embryos exposed to OSPW might be due to disruption in neurophysiological function (Drapeau et al., 2002). Greater spontaneous movement of fish embryos has been observed in embryos exposed to chemicals such as polybrominated diphenyl ethers (PBDEs) (Usenko et al., 2011).

Deformities observed during development of embryos exposed to OSPW were consistent with the results of other studies of oil sands sediments and OSPW. Those studies reported hemorrhage, pericardial edema, and malformation of the spine in fish embryos after exposure to oil sands sediment or OSPW (Colavecchia et al., 2004, 2006, 2007; Peters et al., 2007). These deformities are similar to symptoms of dioxin-induced “blue sac disease”, which is induced when PAHs and other dioxin-like compounds activate the aryl hydrocarbon receptor (AhR) (Fernandez-Salguero et al., 1996). The mechanism of toxicity due to activation of AhR signaling includes induction of CYP1A, oxidative stress, and damage to endothelial cells. TCDD-induced expression of CYP1A is correlated with oxidative damage to DNA, and is co-localized...
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with damage to tissues and programmed death of cells in both embryos and in visibly healthy post-hatch fry (Cantrell et al., 1996, 1998; Park et al., 1996). Although there is similarity of toxic effects caused by exposure to OSPW and dioxin-like compounds, since the abundance of transcripts of cyp1a was not greater in embryos exposed to OSPW compared to the control, the toxic effects observed during development during the present study seem to not be mediated by the AhR. Moreover, no significant AhR-mediated potency was detected when H4IIE-luc cells – an in vitro assay for the detection AhR agonists and antagonists (Hilscherova et al., 2000) – were exposed to OSPW (data not shown). These observations are supported by a recent study where the AhR binding potential of individual NAs from OSPW were modeled and it was determined that the compounds tested did not bind to the AhR (Scarlett et al., 2012). A role for sediment bound PAHs in the embryotoxic effects of oil sands sediments has been suggested (Colavecchia et al., 2004, 2006, 2007) but the current

Fig. 1 – Photographs of typical teratogenic responses of fathead minnow embryos: (a) hemorrhage, (b) pericardial edema, and (c) malformation of the spine. Images were taken at 20x magnification.

Fig. 2 – Fold-change in abundances of transcripts of genes related to (a) the metabolism of xenobiotics (b) oxidative stress, and (c) apoptosis. Bars represent the mean ± SEM of 7 independent replicate exposures and different letters indicate significant differences among treatment groups (one-way ANOVA with Tukey’s post-hoc test, \( p < 0.05 \)).
study suggests that organic compounds in OSPW that do not activate the AhR might cause these embryotoxic effects.

Organic compounds in OSPW might act as agonists of the pregnane-x-receptor (PXR). PXR is a nuclear receptor that is activated by f endogenous and exogenous chemicals that up-regulate expression of proteins involved in biotransformation of xenobiotics (Kliewer et al., 2002). One of the primary targets of PXR activation is induction of CYP3A, a phase I oxidative enzyme that is responsible for the metabolism of xenobiotics (Bertilsson et al., 1998). In addition, PXR can interact with factors binding to the antioxidant response element to elicit the pregnane induced response and up-regulates expression of phase II conjugating enzymes such as glutathione S-transferase (GST) (Falkner et al., 2001). In the present study, the abundance of transcripts of cyp3a was significantly greater in embryos exposed to OSPW compared to embryos exposed to control water, O3-OSPW, or AC-OSPW. This is consistent with a previous study where OSPW caused greater abundance of transcripts of cyp3a in hepatocytes isolated from rainbow trout (Gagné et al., 2012). As discussed below, the abundance of transcripts of gst was greater in embryos exposed to OSPW. This finding is evidence that compounds in OSPW are agonists of the PXR as this receptor can regulate expression of GST (Higgins and Hayes, 2011). The identities of agonists of the PXR in OSPW are not known. Aromatic acids have been identified in OSPW (Jones et al., 2012) so it will be important to determine whether these compounds activate signaling pathways regulated by the PXR.

Oxidative stress is a plausible explanation for the toxicity of OSPW to developing embryos. The malformations in embryos exposed to OSPW are consistent with those caused by oxidative stress (Deng et al., 2009; Mussi and Calcetta, 2010; Bui et al., 2012). Oxidative stress results when antioxidant defense mechanisms become saturated, and concentrations of ROS exceed the levels produced during normal functioning of cells. This exceedance of the capacity of cells to reduce ROS can then ultimately result in damage to tissues and cells (Zhang et al., 2012). Glutathione-S-transferase (GST), a phase II enzyme that facilitates detoxification of drugs, together with superoxide dismutase (SOD) and catalase (CAT), play key functions in clearance of ROS. Greater abundances of transcripts of these genes suggested that there was greater production of ROS in embryos exposed to OSPW. In a recent study that used hepatocytes from rainbow trout, exposure to extracts of OSPW and water accommodated with oil sands caused significantly greater abundance of transcripts of gst and sod (Gagné et al., 2012). This observation is consistent with the results of the present study. The source of the ROS is not known, but transformation of substrates by CYP1A and CYP3A results in production of reactive oxygen species (ROS) (Zangar et al., 2004) and generation of ROS in microsomes has been correlated with total P450 content and CYP3A activity (Shaik et al., 2010).

Exposure to OSPW might have caused apoptosis. Oxidative stress caused by ROS can induce apoptosis in developing embryos of zebrafish (Yamashita, 2003; Deng et al., 2009). These ROS can damage DNA and when damage to DNA is irreparable apoptosis is initiated by activation of the tumor suppressor protein p53. Once p53 is activated it induces up-regulation of pro-apoptotic proteins, including death receptors and their ligands (Langheinrich et al., 2002). B-cell lymphoma 2-associated X (BAX), which is a member of the BCL-2 family of genes, triggers a mitochondrial pro-apoptotic pathway by inducing mitochondrial outer-membrane permeabilization and promoting the release of cytochrome c, which, in turn, triggers activation of the caspase enzyme cascade (Bernardi et al., 2001; Gottlieb, 2001; Pyati et al., 2007). Caspase-9 (CASP9) is an initiator caspase, which has been linked to the mitochondrial apoptotic pathway. Activated CASP9 initiates cleavage of other inactive pro-caspases such as Caspase-3 (CASP3). CASP3 initiates apoptosis by cleaving cellular substrates, which results in shrinkage of cells and degradation of the contents of cells (Jaeschke et al., 2012). Apoptosis enhancing nuclease (APOOPEN) is an exonuclease that is induced by p53 following DNA damage and digests double-stranded DNA to form single-stranded DNA and amplifies DNA signals related to damage to DNA, which results in enhancement of apoptosis (Kawase et al., 2008). Apoptosis inhibitor 5 (APOPINS) is an inhibitor of apoptosis that prevents fragmentation of DNA after activation by CASP3 (Morris et al., 2006). The significantly greater abundances of transcripts of casp9 and apopen, and the lack of a change in the abundance of transcripts of apopin5 in embryos exposed to OSPW suggest that exposure to OSPW resulted in the activation of the oxidative stress-induced apoptotic pathway.

The source of oxidative stress that might have caused the toxic effects of OSPW toward embryos of fathead minnows is not known. Although some of the effects observed in embryos exposed to OSPW are similar to those caused by exposure to AhR agonists it was concluded that the primary mechanism of toxic action during development of embryos was independent of activation of the AhR. Rather, oxidative stress due to greater concentrations of ROS might be due to the metabolism of substrates by the CYP3A that is induced by binding of compounds to the PXR. Further studies are required to identify agonists of the PXR in OSPW.
Toxicity of OSPW to developing embryos of fathead minnow is caused by the organic fraction, possibly the NAAs. Reducing the concentration of the organic fraction of OSPW by either ozonation or activated charcoal significantly attenuated all of the adverse effects observed in embryos exposed to OSPW. Exposure to O3-OSPW or AC-OSPW did not cause any significant effects on embryo development except for limited incidences of premature hatching. With the exception of some hemorrhaging at 168 hpf in embryos exposed to O3-OSPW there were no cases of deformities in embryos exposed to O3-OSPW or AC-OSPW. Although oxidative stress might have been caused by osmotic stress from salts and metals in OSPW, considering the fact that both ozonation and activated charcoal treatment did not significantly reduce the amount of salts and metals (data not shown), the attenuation of effects were most likely attributed to the removal of organic content in OSPW. In addition, exposure to O3-OSPW and AC-OSPW did not cause any significant changes in generation of ROS or changes in abundances of transcripts of genes related to biotransformation, oxidative stress, and apoptosis. The results of the current study, together with those of previous studies (Anderson et al., 2012a, b; He et al., 2010, 2011; Garcia–Garcia et al., 2011c), suggest that ozonation is a promising method for remediation of OSPW.

In conclusion, the results of the present study demonstrated that exposure to OSPW caused adverse effects in developing fathead minnow embryos. Lesser survival, greater incidences of premature hatching, and greater incidences of deformities such as hemorrhage, pericardial edema, and malformation of the spine were caused by exposure to OSPW. The results suggest that caspase-activated apoptotic cell death, induced by oxidative stress resulting from metabolism of substrates by P450 enzymes that are not induced by activation of the AhR, was the primary mechanism of effects on embryos. Ozonation is a promising method for the remediation of OSPW because it significantly attenuated the developmental toxicity toward embryos of fathead minnow.

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