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Effects of chemical fractions from an oil sands end-pit lake on reproduction of fathead minnows

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HIGHLIGHTS

- Assessed endocrine-system effects of fractions of BML-OSPW collected in 2015.
- Exposure did not alter reproduction related endpoints of male or female minnows.
- Hepatosomatic index of male minnows exposed to 25% (v/v) BML-OSPW was greater.
- No effect on fertility, hatching or morphological indices of embryos were observed.

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ABSTRACT

Oil sands process-affected water (OSPW) is a byproduct of bitumen extraction in the surface-mining oil sands industry in Alberta, Canada. Organic compounds in OSPW can be acutely or chronically toxic to aquatic organisms, so part of a long-term strategy for remediation of OSPW is ageing of water in artificial lakes, termed end-pit lakes. BaseMine Lake (BML) is the first oil sands end-pit lake, commissioned in 2012. At the time of its establishment, an effects-directed analysis of BML-OSPW showed that naphthenic acids and polar organic chemical species containing sulfur or nitrogen contributed to its acute lethality. However, the chronic toxicity of these same chemical fractions has not yet been investigated. In this work, the short-term fathead minnow reproductive bioassay was used to assess endocrine-system effects of two fractions of BML-OSPW collected in 2015. One of the fractions (F1) contained predominantly naphthenic acids, while the other (F2) contained non-acidic polar organic chemical species. Exposure of minnows to F1 or F2 at concentrations equivalent to 25% (v/v) of the 2015 BML-OSPW sample (5–15% of the 2012 BML-OSPW sample) did not alter reproductive performance, fertilization success, or concentrations of sex steroids in female or male minnows. Additionally, there were no significant differences in fertility, hatching success, or incidence of morphological indices of embryos collected on day 7 or 14 from exposed breeding trios. However, exposure of male fathead minnow to 25% (v/v) intact 2015 BML-OSPW resulted in a significantly greater hepatosomatic index. Exposure of fathead minnow to refined fractions of dissolved organic chemicals in 2015 BML-OSPW, or a 25% (v/v) of the intact mixture did not affect fertility or fecundity as measured by use of the 21-day reproductive bioassay. These data will be useful in setting future threshold criteria for OSPW reclamation and treatment.

1. Introduction

As a complex mixture of salts, metals and dissolved organic

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compounds, oil sands process-affected water (OSPW) is a byproduct of bitumen extraction by the surface mining oil sands industry in Alberta, Canada (Hao et al., 2005). OSPW is toxic to aquatic organisms (Verbeek, 1994; Scarlett et al., 2013; Morandi et al., 2015; Kavanagh et al., 2011; Li et al., 2017) and is stored on-site in large tailings ponds where it is continuously recycled back into the extraction operations. OSPW toxicity may be actively treated, for example it has been demonstrated that ozonation or adsorption with activated charcoal can diminish the dissolved organic fraction which is responsible for most of the acute toxic potency (He et al., 2011; Anderson et al., 2012; He, 2012). Alternatively, end-pit lakes are one of the existing yet unproven strategies for passive, longer-term remediation of OSPW. Consisting of previously mined-out areas, which have been hydraulically disconnected from the surrounding environment and filled with OSPW or other oil sands related materials, end-pit lakes are designed to retain oil sands materials over a prolonged period of time to facilitate natural degradation of related chemicals of concern (Canadian Association of P, 2017). However, due to uncertainties related to the identity, toxicity and degradation potential of chemical species in OSPW, the effectiveness of the end-pit lake strategy remains uncertain (Weinhold, 2011). BaseMine lake (BML) was established in 2012 as the first commercial test of the oil sands end-pit lake strategy, thus representing a critical experiment for evaluation of this remediation strategy.

Naphthenic acids (NAs) are a complex mixture of organic carboxylates that are known to contribute to observed acute and chronic toxicity of OSPW (Scarlett et al., 2013; Morandi et al., 2015; Kavanagh et al., 2012; Frank et al., 2008; Hughes et al., 2017). In OSPW, NA species may contain up to 20 carbon atoms (typically less than 20 carbon atoms) and a wide range of double-bond equivalents (due to rings, double bonds or aromatic complexes), and for each species there are countless structural isomers that cannot yet be resolved using best chromatographic methods with negative ion electrospray (ESI^-) and ultrahigh resolution mass spectrometry (uHRMS) (Pereira et al., 2013; Pereira and Martin, 2015). Together, all NAs are termed 'O_2^- species', as each is composed only of carbon, hydrogen and two oxygen atoms, and are detected as negative ions. In addition to using ESI^-, it has been demonstrated that important classes of polar chemicals in OSPW can be detected in positive ion electrospray (ESI^+), and that many of these contain sulfur or nitrogen heteroatoms in their structures (Pereira et al., 2013). In particular, it has been shown that NO^+ species and SO^+ species have the potential to be bioaccumulated, and that these contribute to acute lethality and other sub-lethal effects of OSPW (Morandi et al., 2015; Alharbi et al., 2016; Peng et al., 2016; Zhang et al., 2015a). Significant advances have been made in elucidation of structures of some NAs and other sulfur-containing acids by use of two-dimensional gas chromatography interfaced to mass spectrometry, however these methods require derivitization of the original acids (West et al., 2014; Wilde and Rowland, 2018; Rowland et al., 2014).

Several studies have demonstrated potential for oil sands related materials to affect the endocrine system (He et al., 2011; He, 2012; Kavanagh et al., 2012; Wiseman et al., 2013a; Yue et al., 2015; Reinardy et al., 2013). Using the human breast cancer cell line T47-D or MDA cells, effects on receptor signaling in response to the sex steroids 17-β estradiol (E2) and testosterone (T) have been observed following exposure to OSPW (He et al., 2011). Similarly, exposure of goldfish (*Carassius auratus*) or fathead minnow (*Pimephales promelas*) to fresh OSPW, to OSPW that has been aged in a small scale demonstration pond for 20 years (i.e. Pond 9), or an extract of OSPW from the West In-pit settling basin results in changes to the concentration of circulating plasma sex steroids, as well as altered expression of key regulatory genes associated with the endocrine system of fathead minnow (Kavanagh et al., 2011, 2012, 2013; Lister et al., 2008; He et al., 2012). In addition, lesser fecundity and frequency of spawning, as well as less pronounced secondary sexual characteristics have been observed in fathead minnow exposed to OSPW, aged OSPW or OSPW extracts (Kavanagh et al., 2011; Hughes et al., 2017; Pereira et al., 2013). By use of the yeast estrogenic screening bioassay and an effects-directed analysis approach, Yue et al. (2015) demonstrated the contribution of a fraction containing O_2^-, O_3^- and O_4^- chemical classes, which includes NAs, to the estrogenic activity of OSPW. Furthermore, Reinhardy et al. observed greater mRNA abundance of genes coding for the lipo-phospho-protein, vitellogenin, in larval zebrafish (*Danio rerio*) following exposure to an esterifiable extract and aromatic NA extract of OSPW collected from the West In-pit settling basin, whereas no induction was observed following exposure to an extract containing predominantly alicyclic NAs (Reinardy et al., 2013).

Previously, using a fractionation and effects-directed analysis approach, it was demonstrated that NAs are responsible for most of the observed acute and chronic toxicity of dissolved organic fractions of OSPW, collected from BML in 2012 (BML-OSPW), which included effects on endocrine systems of exposed fishes (Morandi et al., 2015; He et al., 2011; Peng et al., 2016). In addition, it is now known that chemical species in BML-OSPW, containing sulfur and nitrogen, contribute to acute lethality and might be bioaccumulated into aquatic organisms (Morandi et al., 2015; Zhang et al., 2015b). However, there is currently limited information on contributions of these chemical species to chronic or sub-lethal toxicity endpoints. Therefore, the purpose of the present study was to gain a greater understanding of the potential for oil sands related chemicals to disrupt the reproductive performance and endocrine system of hatchery raised fathead minnow. BML-OSPW collected in 2015 and two fractions containing chemical species known to cause acute toxicity in BML-OSPW collected in 2012 were prepared and assessed by use of the 21-day fathead minnow reproductive bioassay.

2. Materials and methods

2.1. Chemicals and reagents

Acetic acid, methanol, dichloromethane, diethyl ether, and water (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous ethanol was purchased from Fischer Scientific (Edmonton, AB, Canada). Sulfuric acid 98% and sodium hydroxide were purchased from Osprey Scientific Inc. (Edmonton, AB, Canada).

2.2. OSPW sample collection

Approximately five hundred liters of surface water was collected from BML using a permanent sampling barge (Syncrude Canada Ltd) in August 2015, from which two hundred liters (200 L) was subsampled and stored in ten 20 L high-density polyethylene pails. The water was stored in the dark at 4 °C for two months prior to extraction.

2.3. Fractionation

For BML-OSPW collected in 2012, it was previously demonstrated that one fraction containing naphthenic acids, and another containing non-acidic polar organic species, were acutely toxic at their native concentrations to embryos of fathead minnow (Morandi et al., 2015). To obtain greater quantities of such fractions for longer-term exposures in the current work, the goal of the

fractionation method was to use a single technique, rather than three step-wise techniques used in our previous effects-directed analyses, to generate two fractions similar in chemical content to the two acutely toxic fractions in previous work, one fraction containing predominantly O_2^- species, and one containing predominantly O^+, O_2^+, SO^+, and NO^+ polar organic species (Morandi et al., 2015).

Suspended particulate matter was removed from 200 L of BML-OSPW collected in 2015 by use of vacuum filtration through Grade 4 glass fiber filters (1.2 μm nominal particle retention, Fisher Scientific, Nepean, ON, Canada). Solid phase extraction (SPE) of BML-OSPW was completed by using multiple Oasis HLB SPE columns (35 cc, 6 g sorbent per cartridge, 60 μm particle size, Waters Limited, Ontario, Canada). The SPE workflow is presented in Fig. S1. In detail, each SPE cartridge was conditioned with 20 mL of MeOH and equilibrated with 20 mL of HPLC grade water. The SPE procedures were carried out using a 20-position extraction manifold (Waters Limited, Ontario, Canada) with the exit valve connected to the vacuum pump with an inline flask as a liquid trap. Each cartridge was then slowly loaded with approximately 2.1 L of filtered BML-OSPW over 12 h. The cartridges were then washed in turn with 40 mL of MeOH:H_20 (50:50, v/v) and 20 mL of MeOH: 2% acetic acid (55:45, v/v). The corresponding eluents were termed Wash-1 and Wash-2 (Fig. S1) and were not used for any exposures because HPLC- Orbitrap MS analysis indicated no analytes of interest.

Another eluent was collected from the SPEs by adding 40 mL of MeOH: 5% NH_4OH (65:35, v/v) to each cartridge, and this fraction was termed Eluent-1 (Fig. S1). Total eluent from ten SPE cartridges were combined, methanol was evaporated by rotary evaporator and the remaining aqueous phase was adjusted to pH 12 with concentrated NaOH. This aqueous phase was extracted 3 times using 40 mL of DCM that was then evaporated to dryness, and this was termed Eluent 1-Basic (Fig. S1). The pH of the remaining aqueous sample was then adjusted to pH 2 with concentrated H_2SO_4 and extracted 3 more times using 30 mL of DCM, which was termed fraction 1(F1, Fig. S1).

Eluent-2 was then generated by using 25 mL of pure MeOH to elute any remaining analytes from each SPE cartridge, and this was combined with Eluent 1-Basic to produce fraction 2 (F2, Fig. S1). The F1 and F2 fractions were blown to dryness by use of a rotary evaporator and nitrogen gas. Dry weight of fractions were measured and are presented as mg of dried extract per L of the original filtered BML-OSPW. F1 and F2 were dissolved in methanol for Orbitrap MS analysis, and in ethanol for biological assays.

2.4. Characterization of fractions by HPLC-Orbitrap

Profiles of organic compounds in fractions were determined by use of high performance liquid chromatography coupled to an ultrahigh-resolution Orbitrap mass spectrometer (HPLC-Orbitrap). The HPLC (Transcend, Thermo Fisher Scientific) consisted of a degasser, a 1250 bar quaternary pump, an auto-sampler, and a column oven. Separation was performed on a Hypersil Gold C18 analytical column (50 × 2.1 mm, 1.9 μm particle size, Thermo Fisher Scientific) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 2 μL was used in all analyses. Mobile phases consisted of (A) 0.1% acetic acid in water, and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection. The Orbitrap MS was operated with an ESI source in either positive or negative mode, with separate injections of the same sample for each analysis. Ionization voltage was set at ± 4 kV, while the sheath, aux, and sweep gas flows were set to 40, 25 and 2 (arbitrary units), respectively. Vaporizer and capillary temperature were 325 °C and 300 °C, respectively. Acquisition was performed in full scan mode (*m/z* 100 to 500) at 1.2 Hz with resolving power set to a nominal value of 240,000 at full width half-maximum at *m/z* 400. For data presentation in the current work, each chemical species detected was binned according to its corresponding heteroatom class in either ESI^+ or ESI^-, and hetero-atomic class profiles are presented as relative abundance compared to unextracted BML-OSPW.

2.5. Fathead minnow 96-hr exposure

Studies with fathead minnows were conducted at the Aquatic Toxicology Research Facility in The Toxicology Centre, University of Saskatchewan. Expression of genes along the brain-gonad-liver axis that are key regulators of reproduction were quantified in male and female minnows exposed to OSPW and fractions of OSPW for 96-hr because previous work has demonstrated rapid changes in abundances of endocrine-related transcripts following exposure to endocrine disrupting chemicals (Ankley and Villeneuve, 2015). Two female and one male fathead minnow (8−14 months of age) were acclimatized in 10-litre aquaria by use of a static renewal system for three weeks prior to exposure (n = 4). A 50% water renewal was completed daily and each third day 75% of waters were replaced. Light: dark cycle was maintained at 16:8 and temperature of water was 26 ± 1 °C. Fish were fed bloodworms twice daily to satiety and detritus was removed from tanks approximately 1-h post-feeding. Upon test initiation, fathead minnows were exposed to city of Saskatoon municipal water, solvent control (0.002% ethanol, S. Control), 25% (v/v) OSPW, 25% (v/v) equivalent (equ.) F1, or 25% (v/v) equ. F2 diluted in city of Saskatoon municipal water. On the day of test termination, fish were anaesthetized by use of aquacalm and their brain stem was cut by use of dissecting scissors. Phenotypic sex determined by use of presence of secondary sexual characteristics, mass (grams; g), and length (centimeters; cm) were recorded for each fish. Liver, brain (including pituitary), and gonad from each fish were stored in pre-weighed vials, weighed, and immediately snap frozen by use of nitrogen and stored at −80 °C.

2.6. Fathead minnow 21-day exposure

Effects on reproduction were determined according to USEPA test method *Short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow* (*Pimephales promelas*) (Environmental Protec, 2001). To establish baseline fecundity and fertility data, two female and one male fathead minnow were acclimatized in 10-litre aquaria, as outlined above, with two breeding tiles, for a one month period prior to initiation of exposure. Following the pre-exposure period, tanks producing a minimum of 10 eggs/female/day were exposed for 21-days to one of; Saskatoon municipal water, S. Control (0.002% EtOH), 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, or 25% (v/v) equ. F2. Exposures were static renewal, and 50% of the exposure solution was replaced in each tank daily, except for each third day when 75% of the solution was renewed. Tanks were checked for eggs twice daily approximately 45 min subsequent to feeding. Eggs were removed from breeding tiles and placed into 25 ml petri dishes containing dechlorinated city of Saskatoon municipal tap water. The number of eggs and success of fertilization were recorded by use of a dissecting microscope. A subset of 10 eggs was collected from each tank on day 7 and 14 following the first spawning event. Embryos were placed in 25 mL petri dishes with 20 mL dechlorinated city of Saskatoon municipal water, maintained at 26 ± 1 °C and half of the water solution was replaced daily. Embryos were checked for survival daily and dead embryos were removed.

Following hatch, fathead minnow hatchlings were checked by use of a dissecting microscope to assess the presence/absence of deformities including; spinal curvature, hemorrhaging, pericardial and yolk sac edema, and craniofacial malformations. On the day of termination of the reproduction assay, fish were anaesthetized and blood was collected by use of heparinized tubes and caudal vein incision, and immediately stored on ice. Blood plasma was separated within 6 h of test termination as described in the next section. Phenotypic sex determined by use of presence of secondary sexual characteristics, mass (g), length (cm), and presence or absence of secondary sexual characteristics (e.g. tubercles) were recorded. Liver, brain, and gonad were weighed immediately in pre-weighed vials, snap frozen by use of liquid nitrogen, and stored at −80 °C. Condition factor (K), hepatosomatic (HSI), and gonadosomatic (GSI) indices were calculated (Environmental Protec, 2001).

2.7. Measurement of plasma 17-β estradiol and testosterone concentrations

The E2 and T enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI) were used to measure concentrations of E2 and T in plasma of female and male fish, respectively. Due to limited plasma volumes, concentrations of E2 were determined at the tank level by pooling blood plasma from all females (n = 2) in one tank. Blood plasma was collected by centrifugation of blood for 15 min @ 2600 rpm, and immediately stored at −80 °C. Samples were extracted three times by use of 0.5 mL DCM or diethyl ether for E2 and T, respectively. Extracts were combined, blown-down to near dryness by use of nitrogen gas, and immediately suspended in the assay media. Assays were performed as described by the manufacturer.

2.8. Quantification of gene expression

Abundances of transcripts of several genes (androgen receptor, *ar*; estrogen receptor alpha, *erα*; estrogen receptor beta, *erß*, vitellogenin 5, *vtg5*; aromatase, *cyp19a*) that are important for regulation of reproduction in fathead minnows were quantified. Total RNA was isolated from livers and gonads of male and female minnow exposed during the 96 h assay by use of the RNeasy Plus kit, according to the protocol provided by the manufacturer (Qiagen, Mississauga, ON, Canada). Concentration and purity of RNA was determined by use of a NanoDrop spectrophotometer (Fisher Scientific). Complementary DNA (cDNA) was synthesized using 1 μg of total RNA by use of the QuantiTect® reverse transcript kit, according to the protocol provided by the manufacturer (Qiagen). Quantitative real-time polymerase chain reaction (qPCR) was performed in 96-well plates by use of CFX96 Real-Time PCR System (BioRad, Mississauga, ON, Canada). A 35 μL reaction mixture of 2x concentrated SsoFast™ EvaGreen® Supermix, 2.5 μL of cDNA, 10 pmol gene-specific primers, and nuclease free water was prepared for each cDNA sample and primer combination. Each sample of cDNA was analyzed in duplicate with 10 μL reaction volumes per well. The reaction mixture for qPCR was denatured at 95 °C for 10 min followed by a thermal cycle profile consisting of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 cycles. Abundances of transcripts were quantified by normalizing to the housekeeping gene B-actin and the analysis was performed using CFX Manager™software. The analysis software corrects for differences in reaction efficiencies. Reaction efficiencies were determined by performing qPCR on serial dilution of a pool of cDNA that was created by combining equal volumes of cDNA from male and female fathead minnows exposed to freshwater, the S. Control, OSPW, and fractions of OSPW.

2.9. Statistical analysis

All statistical comparisons were made at the 5% significance level ($\rho < 0.05$). Data were tested for normality by use of the Kolmogorov-Smirnov test, while homogeneity of variance was tested by use of Levine's test. If data did not meet the assumptions of normality they were log-transformed. When data met the assumptions of normality and homogeneity, effects of OSPW and fractions of OSPW on mean eggs produced per female per day, percentage fertilization, percentage hatching, time to 50% hatch (TTH), morphometric indices and concentrations of sex steroids in blood plasma were compared to the S. control and were determined by use of one way analysis of variance (ANOVA) followed by Dunnett's test, with aquariums as experimental units of replication.

3. Results

3.1. Characterization of BML-OSPW and fractions

Similar to our previous study on the fractionation of BML-OSPW collected in 2012, the F1 fraction accounted for 86% of the original mass spectral intensity of O_2^- species detected in unfractionated BML-OSPW, demonstrating high recovery for naphthenic acids, and only minor amounts of the original $O_3^{+/-}$, O_4^- and SO_2^- species (Fig. 1). The F2 fraction accounted for 70% of the original O_2^+ class content, 95% of the SO^+ and NO^+ content, and 65% of the O^+ content based on mass spectral intensity. To provide context and comparison to our previous work, the organic content of BML-OSPW collected in 2015 (41.5 mg/L, measured by total organic carbon analysis of filtered BML water) was less than BML-OSPW collected in 2012 (150 mg/L, measured by total organic carbon analysis of filtered BML water) and used for our previous acute toxicity identification study (Morandi et al., 2015). Due to the different water and perhaps also due to differences in fractionation procedures, the organic mass contents of F1 and F2 in the current work (12.9 and 2.2 mg/L, respectively) were less than fractions from our previous work using 2012 BML-OSPW (20.0 and 14.0 mg/L). Compared to the original mass of organics in 2015 BML-OSPW, these two fractions accounted for 31% (F1) and 5% (F2) of the organic mass of the unfractionated sample.

3.2. Reproductive performance of fathead minnow exposed to OSPW and fractions

Exposure of fathead minnow breeding trios to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, and 25% (v/v) equ. F2 for 21-days did not affect cumulative fecundity when compared to control fish (Fig. 2 & Table S1). However, a delay in production of eggs by minnows exposed to fractions of BML-OSPW or intact BML-OSPW was observed during the initial days of exposure (Fig. 2) when compared to control fish. Similarly, morphometric analysis demonstrated no significant effects on length, mass, K, GSI, or concentrations of E2 or T in blood plasma of female and male fish, respectively. However, exposure of male fathead minnow to 25% (v/v) BML-OSPW resulted in significantly greater HSI compared to that of unexposed, control fish (Table 1 and Table S2).

3.3. Embryo-larval indices

Cumulative percent fertilization of embryos collected from fathead minnow breeding trios exposed over 21-days to BML-OSPW and fractions of BML-OSPW did not significantly differ between exposed and control exposed fish. Time to 50% hatch and incidence of malformations of embryos collected on day seven or 14 did not significantly differ between breeding trios of fathead

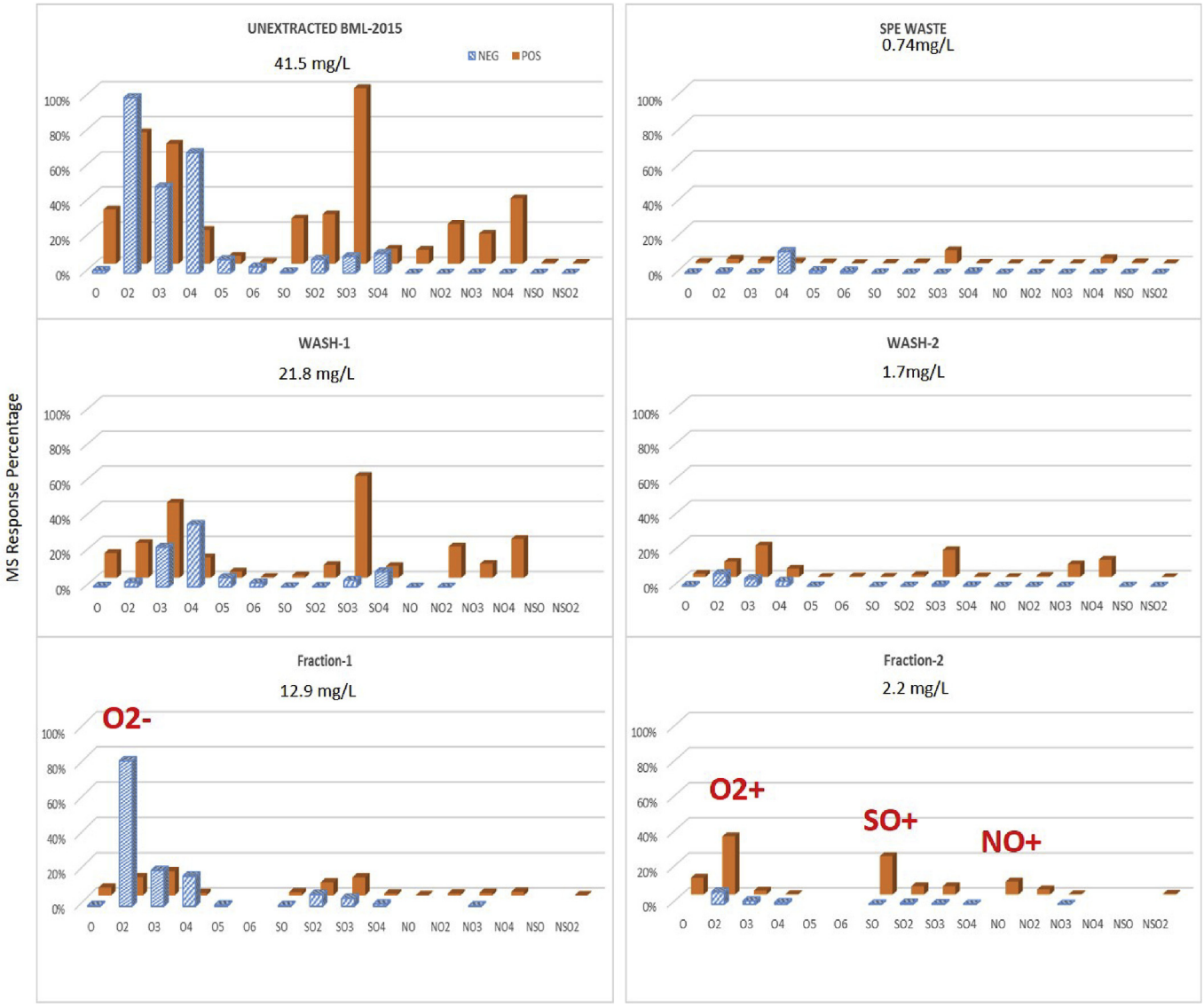

Fig. 1. Relative abundance of species by heteroatom class in positive (orange) and negative (blue) electrospray ionization modes of HPLC-Orbitrap MS, in unfractionated BML-OSPW collected in 2015, and in various fractions produced by SPE: waste, Wash-1, Wash-2, F1 and F2. The organic content (based on gravimetric weight for SPE fractions and total organic carbon analysis of filtered BML before SPE fractionation) is presented as the average mass per litre of filtered BML-OSPW. (*colour required).* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

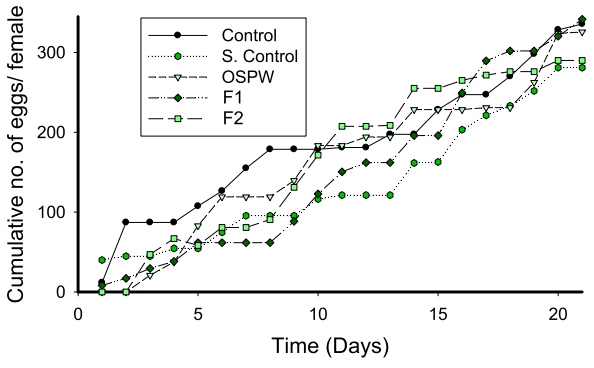

Fig. 2. Cumulative production of eggs by fathead minnows exposed to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, and 25% (v/v) equ. F2. Cumulative number shown as eggs/ female. (*colour required).* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

minnow exposed to BML-OSPW or fractions of BML-OSPW compared to unexposed, control fish (Table 2).

3.4. Effects on expressions of genes

Abundances of transcripts of *vtg5, ar, era, erb,* and *cyp19a* were not significantly different between male and female minnows exposed to BML-OSPW or fractions of BML-OSPW compared to unexposed, control fish.

4. Discussion

The fractionation method generated fractions with similar profiles of dissolved organic chemicals as those produced previously (Morandi et al., 2015). The F1 fraction contained the majority of NAs (i.e. O_2^- class) and the F2 fraction contained the majority of non-acidic species such as O^+, O_2^+, SO^+ , and NO^+ (Fig. 1). However, the organic content of BML-OSPW collected in 2015 and related

Table 1
Condition factor (K), hepatosomatic index (HSI), gonadosomatic index (GSI), and plasma concentrations of testosterone or estrogen for male and female fathead minnows respectively following 21-days of exposure to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1 or 25% (v/v) equ. F2. Data are shown as mean ± SEM. Asterisk denotes significant difference from control ($p < 0.05$, one-way ANOVA followed by Dunnett's multiple range test).

Exposure	Gender	K		HSI		GSI		Testosterone (ng/mL)	
		mean	sem	mean	sem	mean	sem	mean	sem
Control	Male	1.93	0.06	2.50	0.45	3.3	0.66	5.35	1.58
S. Control	Male	1.72	0.16	2.04	0.52	2.52	0.51	4.18	1.56
25% v/v OSPW	Male	2.24	0.20	4.20*	0.31	1.7	0.49	3.66	1.75
25% equ. F1	Male	1.93	0.25	2.50	0.31	2.5	0.46	5.42	1.16
25% equ. F2	Male	2.15	0.37	2.04	0.17	2.23	0.35	4.20	1.49
Exposure	Gender	K		HSI		GSI		Estradiol (ng/mL)	
		mean	sem	mean	sem	mean	sem	mean	sem
Control	Female	3.41	0.24	3.66	0.74	13.3	1.53	7.78	2.31
S. Control	Female	3.18	0.38	4.18	0.62	8.34	2.01	4.34	1.29
25% v/v OSPW	Female	3.23	0.14	2.84	0.40	9.11	1.36	4.55	1.65
25% equ. F1	Female	3.30	0.18	3.44	0.32	8.19	1.32	6.36	3.64
25% equ. F2	Female	3.17	0.26	2.87	0.24	13.6	0.93	3.40	0.86

Table 2
Mean percent fertilization of embryos collected throughout 21-day exposure, time to 50% hatch and percentage incidence of malformations of embryos ($n = 10$) collected on days 7 and 14 ($n = 4$) from fathead minnow breeding trios exposed to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, 25% (v/v) equ. F2, control or S. Control (0.002% EtOH).

Exposure	Cumulative fertilization		TTH (days) Day 7		TTH (days) Day 14		Incidence malformations	
	mean (%)	sem	mean (%)	sem	mean (%)	sem	mean (%)	sem
Control	97.5	0.86	5.00	0.5	5.25	0.29	7.00	0.70
S. Control (2E-03% EtOH)	97.8	1.68	4.50	0.35	4.33	0.29	6.00	1.20
25% (v/v) OSPW	95.2	0.55	5.33	0.57	4.75	0.25	5.00	0.50
25% (v/v) equ. F1	96.9	0.97	5.40	0.25	4.20	0.35	4.00	1.00
25% (v/v) equ. F2	95.7	1.59	5.25	0.29	4.8	0.63	7.00	1.10

fractions was less than BML-OSPW collected in 2012. Exposure of fathead minnow breeding trios to 25% (v/v) BML-OSPW or 25% (v/v) refined fractions of BML-OSPW did not result in any significant effect on reproductive capacity or the endocrine systems of males or females. Due to the large volumes of OSPW required for the single concentration used in this study, we were unable to test for responses over more concentrations, but the lack of significant effects contrasts with results of other studies, which have demonstrated that OSPW, aged OSPW, and NA extracts can affect the endocrine system (Kavanagh et al., 2011, 2012, 2013; He et al., 2011; He, 2012; Lister et al., 2008; Yue et al., 2014; Wiseman et al., 2013b-). However, the lack of observed effects in this study might be due to the dilution of BMLOSPW collected for this work (4-fold dilution of 100% OSPW), while previous studies have mainly used 100% OSPW or equivalents thereof (Kavanagh et al., 2011, 2012, 2013; He et al., 2011; He, 2012; Lister et al., 2008; Yue et al., 2014; Wiseman et al., 2013b). Furthermore, these results are interesting because exposure of fathead minnow to 100% OSPW collected from the former West In-pit, now BML, had previously been demonstrated to alter abundances of transcripts at all levels of the brain-gonad-liver axis in both male and female fathead minnows (He et al., 2012). Also these results were interesting because F1 contained predominantly acidic chemical species (i.e. $O_{2\text{-}4}^{-}$ classes) that are known to have estrogenic activity (Yue et al., 2015) and affect fecundity and sex steroid synthesis of fathead minnow (Kavanagh et al., 2012). The $O_{2\text{-}4}^{-}$ chemical classes accounted for approximately 90% of total mass spectral intensity of F1 under ESI^{-} (Fig. 1). Therefore, results of this study suggest that dissolved organic chemicals in BML-OSPW at 25% (v/v), which are known to have acute and chronic toxicity when exposed at higher concentrations (Morandi et al., 2015; Alharbi et al., 2016; Peng et al., 2016; Zhang et al., 2015a) had no effect on reproductive performance of fathead minnows at the concentration used here. Additionally, results of this study demonstrate significant dilution of BML-OSPW between 2012 and 2015 as the concentration of organics has decreased from >150 mg/L to <42 mg/L. Dilution of BML-OSPW since 2012 is likely due to active pumping of water from Beaver Creek Reservoir by Syncrude Canada Ltd to BML, but could also be due to surface run-off, or perhaps degradation of dissolved organic compounds (Alberta Energy Regulator., 2013).

Although no effects on the endocrine system were observed, exposure of male fathead minnow to 25% (v/v) intact BML-OSPW resulted in significantly greater HSI compared to control exposed fish. Previously, it has been demonstrated that exposure of fathead minnow for 21-days to OSPW that had been aged in an experimental reclamation pond (Pond 9) resulted in decreased spawning success and fecundity, but no effect on HSI (Kavanagh et al., 2011). Similarly, minnows exposed under laboratory conditions to a NA extract for 21-days exhibited decreased spawning, fecundity, and lesser concentrations of T in blood plasma, but had greater HSI (Kavanagh et al., 2012). In contrast to these results, laboratory fish exposed to OSPW collected from Syncrude's demonstration pond, demonstrated no effect on spawning, fecundity or HSI (Kavanagh et al., 2012), but wild minnows collected from the same pond had larger K, HSI, and GSI when compared to reference fish (Kavanagh et al., 2013). Although results are inconsistent among intact OSPW and fractions of dissolved organic species from OSPW, HSI appears to be a sensitive indicator of longer term exposure to OSPW and OSPW derived chemicals. However, the causative agent(s) of increased HSI remain unknown and might be attributed to chemicals in WASH-1 or -2, which included the chemical classes O_4^{-}, O_3^{+}, $SO_3^{+\cdot}$, NO_2^{+} and NO_4^{+}.

To the knowledge of the authors, this is the first assessment of reproductive performance of fathead minnow exposed to refined fractions of BML-OSPW, one containing predominantly acidic species including NAs (F1) and the other, predominantly non-acidic chemical species (F2). Furthermore, these results suggest that exposure of fathead minnow breeding trios to dissolved organic chemicals in BML-OSPW collected in 2015 at 25% (v/v) have no effect on reproductive performance. Although there has been dilution of BML-OSPW since our original study completed in 2012, as indicated by the lower concentrations of organics in BML-OSPW collected in 2015, the results of this study suggest that the intact BML-OSPW mixture is more toxic than refined fractions as identified by use of HSI. The identity of causative agents of larger HSI measured in male fathead minnow remains unknown however, HSI appears to be a sensitive indicator of exposure to intact OSPW.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126073.

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Effects of chemical fractions from an oil sands end-pit lake on reproduction of fathead minnows

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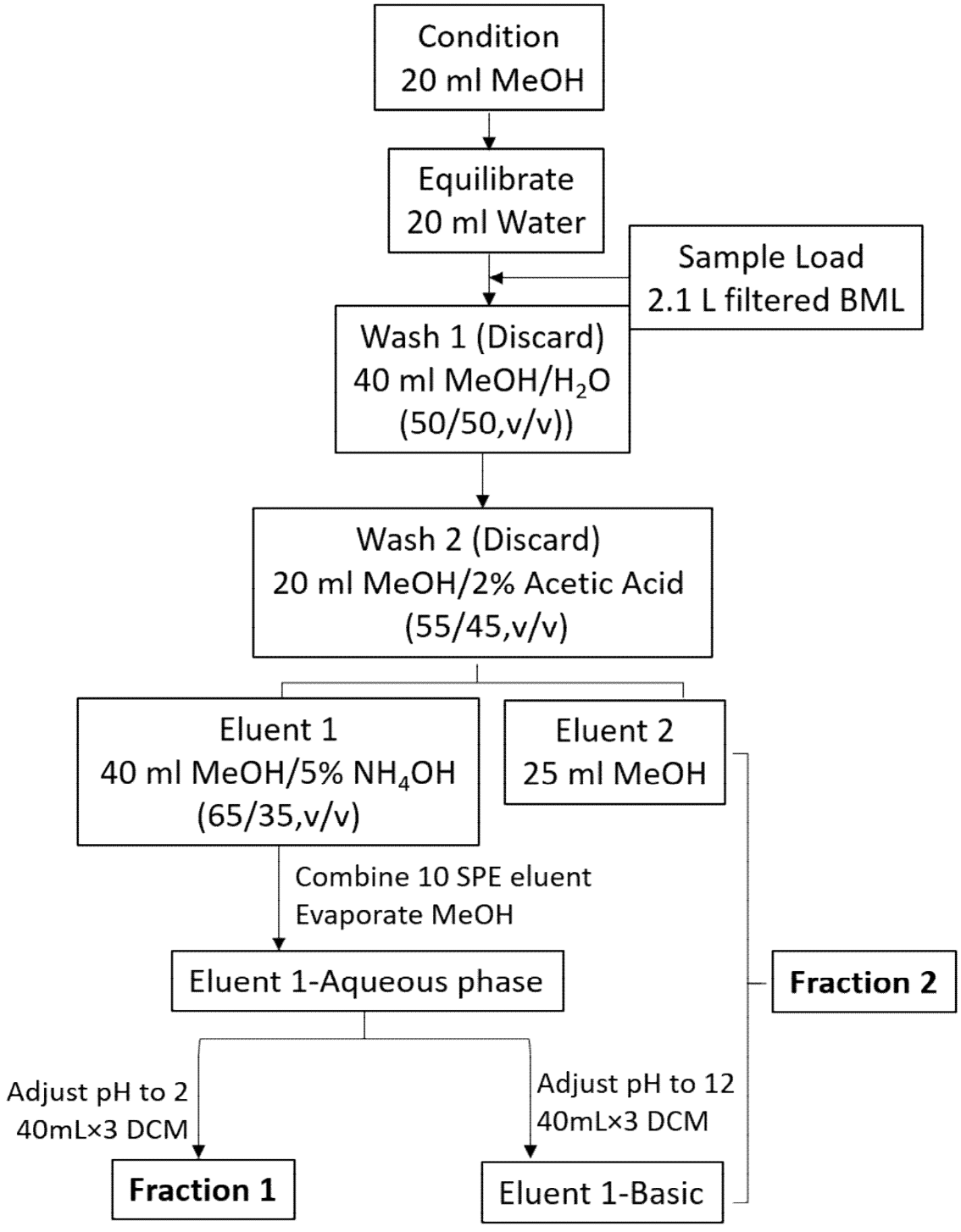

Figure S1. Fractionation schematic of BML-OSPW by use of SPE.

Table S1 Mean number of clutches and eggs per clutch of breeding trios of fathead minnow following 21-day exposure to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, 25% (v/v) equ. F2, control or S. Control (0.002% EtOH).

	Number of clutches		Eggs per clutch	
Exposure	mean	sem	mean	sem
Control	4.75	0.75	130	19
S. Control	4.5	0.5	123	17.45
25% v/v OSPW	4.25	0.48	160	38.6
25% equ. F1	5	0.95	132	12.2
25% equ. F2	4.8	0.58	118	9.75

Table S2 Mean length and weight of fathead minnow following 21-day exposure to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, 25% (v/v) equ. F2, control or S. Control (0.002% EtOH).

		Length (cm)		Weight (g)	
Exposure	Gender	mean	sem	mean	sem
Control	Male	6.33	0.62	3.31	0.54
S. Control	Male	6.43	0.49	3.85	0.88
25% v/v OSPW	Male	6.25	0.62	3.25	0.76
25% equ. F1	Male	6.46	1.03	3.67	1.4
25% equ. F2	Male	5.95	0.72	3.10	1.42
		Length (cm)		Weight (g)	
Exposure	Gender	mean	sem	mean	sem
Control	Female	4.93	0.43	1.49	0.33
S. Control	Female	5.1	0.44	1.76	0.59
25% v/v OSPW	Female	5.26	0.23	1.65	0.24
25% equ. F1	Female	4.97	0.41	1.56	0.41
25% equ. F2	Female	5.08	0.4	1.69	0.46