

# Effect of oil sands process-affected water on toxicity of retene to early life-stages of Japanese medaka (*Oryzias latipes*)



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## ABSTRACT

Toxicity of oil sands process-affected water (OSPW) to aquatic organisms has been studied, but effects of co-exposure to OSPW and polycyclic-aromatic hydrocarbons (PAHs), which are an important class of chemicals in tailings ponds used to store OSPW, has not been investigated. The goal of the current study was to determine if organic compounds extracted from the aqueous phase of relatively fresh OSPW from Base-Mine Lake (BML-OSPW) or aged OSPW from Pond 9 experimental reclamation pond (P9-OSPW) modulated toxic potency of the model alkyl-PAH, retene, to early life-stages of Japanese medaka (*Oryzias latipes*). Embryos were exposed to retene by use of a partition controlled delivery (PCD) system made of polydimethylsiloxane (PDMS) until day of hatch. Incidences of pericardial edema and expression of *CYP1A* were not significantly greater in larvae exposed only to dissolved organic compounds from either OSPW but were significantly greater in larvae exposed only to retene. Expression of *CYP1A* and incidences of pericardial edema were significantly greater in larvae co-exposed to retene and 5 × equivalent of dissolved organic compounds from BML-OSPW compared to retene alone. However, there was no effect of co-exposure to retene and either a 1 × equivalent of dissolved organic compounds from BML-OSPW or 5 × equivalent of dissolved organic compounds from P9-OSPW. While there was evidence that exposure to 5 × equivalent of dissolved organic compounds from BML-OSPW caused oxidative stress, there was no evidence of this effect in larvae exposed only to retene or co-exposed to retene and a 5 × equivalent of dissolved organic compounds from BML-OSPW. These results suggest that oxidative stress is not a mechanism of pericardial edema in early-life stages of Japanese medaka. Relatively fresh OSPW from Base Mine Lake might influence toxicity of alkylated-PAHs to early life stages of fishes but this effect would not be expected to occur at current concentrations of OSPW and is attenuated by aging of OSPW.

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## 1. Introduction

During surface mining of oil sands in Northern Alberta, Canada, liquid fine tailings generated from extraction of bitumen are held in tailings ponds. Over time, particulates, such as silt and clay, settle to form a layer of mature fine tailings (MFTs), leaving behind an aqueous layer of oil sands process affected water (OSPW) (Allen, 2008). Currently, contents of tailings ponds are not discharged to the ambient environment, but rather OSPW is recycled for use in

extraction of bitumen. As a result, salts, metals, and other inorganic and organic chemicals become concentrated in OSPW in tailings ponds. Currently, more than 1 billion m<sup>3</sup> of OSPW is held in tailings ponds (Giesy et al., 2010).

The composition of chemicals in the dissolved organic fraction of OSPW is complex, with more than 3000 elemental compositions having been detected by use of ultra-high resolution mass spectrometry (Pereira et al., 2013). Included in this mixture are a variety of acidic, basic, and neutral species containing oxygen (O<sub>x</sub>), sulfur (S), and nitrogen (N) (Barrow et al., 2010; Grewer et al., 2010; Pereira et al., 2013; Morandi et al., 2015; Alharbi et al., 2016). Dissolved organic compounds in OSPW cause a variety of adverse effects, including inhibition of growth of Chironomids (Anderson et al., 2011, 2012), oxidative stress (He et al., 2012a; Wiseman et al., 2013a,b) immunotoxicity (MacDonald et al., 2013; Wang et al.,

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2013), endocrine disruption (He et al., 2010, 2011, 2012b; Leclair et al., 2015) and impaired reproduction of fishes (Kavanagh et al., 2011, 2012), developmental malformations in early-life stages of fishes (He et al., 2012a), and acute lethality (Morandi et al., 2015).

In addition to the dissolved organic chemicals in OSPW, polycyclic aromatic hydrocarbons (PAHs) and their alkylated analogs occur in tailings ponds. However, it is thought that PAHs are associated with the sediment phase of tailings ponds and that concentrations in water are small (Colavecchia et al., 2004, 2006, 2007). For example, concentrations of individual lower molecular mass PAHs range from 10 to 330 ng/L in porewater of MFTs (Madill et al., 1999) and total concentrations of PAHs range from 1150 to 1600 ng/L in the upper clarified zone of tailings ponds (Galarneau et al., 2014). PAHs are natural components of bitumen and there is concern about adverse effects that these chemicals might have on aquatic environments in the oil sands mining region (Madill et al., 1999; Conly et al., 2002; Kelly et al., 2009; Colavecchia et al., 2006). PAHs and alkyl-PAHs cause adverse effects to early-life stages of fishes, including oxidative stress, and blue sac disease (BSD) that is characterized by induction of expression of CYP1A and a suite of developmental malformations, including pericardial edema (Hodson et al., 2007; Billiard et al., 1999; Bauder et al., 2005; Lin et al., 2015; Mu et al., 2014; Wolińska et al., 2013; Turcotte et al., 2011).

Effects of co-exposure to dissolved organic compounds from OSPW and PAHs has not been investigated. However, there is evidence that this co-exposure scenario might cause greater effects than exposure to PAHs alone. Dissolved organic chemicals in OSPW have properties similar to those of surfactants (Clemente and Fedorak, 2005; Frank et al., 2008; Schramm and Smith, 1985), and bioaccumulation of PAHs is greater when fish are co-exposed to PAHs and a surfactant (Tan et al., 2010). Greater bioaccumulation of PAHs has been attributed to inhibition of members of the ATP-binding cassette (ABC) superfamily of transporter proteins. For example, concentrations of metabolites of benzo[a]pyrene (BaP) in cells are greater when activities of ABC-transporter proteins are inhibited (Hessel et al., 2013; Ebert et al., 2005; Kranz et al., 2014). Recently it was demonstrated that dissolved organic compounds in OSPW can inhibit the function of ABC-transporter proteins in early-life stages of Japanese medaka (*Oryzias latipes*) (Alharbi et al., 2016).

The goals of the current study were to determine if dissolved organic compounds in OSPW modulate adverse effects of exposure to PAHs, and to compare effects of relatively fresh OSPW with OSPW that had been aged in an experimental reclamation pond. Early-life stages of Japanese medaka were exposed to the alkylated-PAH, 7-isopropyl-1-methylphenanthrene (retene), and the dissolved organic fraction of OSPW. Effects of retene, and the potential for dissolved organic chemicals in OSPW to modulate effects of retene, were determined by quantifying pericardial edema, and expression of genes important for metabolism of retene and the response to oxidative stress.

## 2. Materials and methods

### 2.1. Chemicals and OSPW

Retene (purity=95%) was purchased from Chem Service, Inc. (West Chester, PA, USA). Polydimethylsiloxane (PDMS) aquarium-grade sealant was purchased from Marineland (Blacksburg, VA, USA). Acetone, hexane, dichloromethane (DCM), and ethyl-acetate, each of HPLC grade, were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was obtained from GreenField Ethanol Inc (Brampton, ON, Canada). Two samples of OSPW were collected on the site of Syncrude Canada Ltd.

(Fort McMurray, AB, Canada). One sample was from Base Mine Lake (BML-OSPW), which is an end-pit-lake constructed from the West-In-Pit settling basin that received input of tailings from the main extraction facility until December 2012, after which all input of tailings from the extraction plant was ceased. The other sample was from an experimental reclamation pond called Pond 9 (P9-OSPW) that was constructed in 1993 and has not received input of OSPW since that time. Samples were collected in September of 2012, shipped to the University of Saskatchewan (Saskatoon, SK, Canada) and stored in the dark until extraction. Samples were inspected visually immediately upon arrival and were observed to be free of any residual bitumen or MFT.

### 2.2. Extraction of dissolved organics from OSPW

Basic and neutral compounds, but not acidic compounds, in the water soluble organic fraction of OSPW can modulate activity of ABC proteins in early-life stages of Japanese medaka (Alharbi et al., 2016). Therefore, a method to isolate basic and neutral compounds into one fraction that contains lesser amount of acidic compounds was used for extraction of OSPW (Vieno et al., 2006). Briefly, 1000 mL of each sample of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove any particulate matter and the pH of the OSPW adjusted to pH 9 by use of  $\text{NH}_4\text{OH}$ . Pre-concentration of samples was performed in one generic step by use of EVOLUTE<sup>®</sup> ABN sorbent (Biotage, Charlotte, NC, USA). Before addition of OSPW, cartridges were conditioned with 6 mL of methanol followed by 6 mL of ultrapure Milli-Q water (Millipore, Mississauga, Canada). Five cartridges were used for each sample of OSPW, and 200 mL of OSPW was extracted on each cartridge. OSPW was passed through cartridges under vacuum at a flow rate of 10–15 mL min<sup>-1</sup>. At a pH 9, acidic compounds will not bind the ABN sorbent. Subsequently, cartridges were washed with Milli-Q water and allowed to dry under vacuum for 30 min. Basic and neutral compounds were isolated in one step in 6 mL of methanol without use of vacuum. Samples were pooled, evaporated to dryness under a gentle stream of nitrogen, and reconstituted in 500  $\mu\text{L}$  of absolute ethanol. Therefore, the concentration of dissolved organic compounds in the final samples was 2000  $\times$  greater than in the original samples of OSPW.

### 2.3. Chemical profiling of OSPW

The profile of organic chemicals in extracts of BML-OSPW and P9-OSPW was determined by use of an Q Exactive mass spectrometer (OrbitrapElite, Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Dionex<sup>™</sup> UltiMate<sup>™</sup> 3000 UHPLC system (Thermo Fisher Scientific). Separation was achieved on a Betasil C18 column (5  $\mu\text{m}$ ; 2.1 mm  $\times$  100 mm; Thermo Fisher Scientific) with an injection volume of 5  $\mu\text{L}$ . The mobile phase consisted of ultrapure water (A) and methanol (B). Initially, 20% B was increased to 80% in 3 min, then increased to 100% at 8 min and held static for 19.5 min, followed by a decrease to initial conditions of 20% B and held for 2 min to allow for equilibration of the column. The flow rate was 0.25 mL min<sup>-1</sup>. Temperatures of the column and sample chamber were maintained at 30 and 10 °C, respectively. Data were acquired using full scan mode in both positive electrospray (ESI<sup>+</sup>) and negative (ESI<sup>-</sup>) ion mode. Briefly, MS scans (200–2000  $m/z$ ) were recorded at resolution  $R=70,000$  (at  $m/z$  200) with a maximum of  $3 \times 10^6$  ions collected within 200 ms, based on the predictive automated gain control. General mass spectrometry settings applied for positive and negative ion modes were as follows: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350 °C. Chemical species were grouped according to heteroatom empirical formula classes in ESI<sup>-</sup> or ESI<sup>+</sup> electrospray:

$O_x^{+/-}$  (where  $x=1-5$ ),  $N^{+/-}$ ,  $NO_x^{+/-}$  (where  $x=1-4$ ),  $S^{+/-}$ ,  $SO_x^{+/-}$  (where  $x=1-5$ ), or  $NO_xS^{+/-}$  (where  $x=1-4$ ). Only those chemicals in the total ion mass spectrum that had a peak threshold >600, a mass spectral signal-to-noise ratio (S/N) >3, were present at relative abundances of at least 2%, and that produced discernible extracted ion chromatographic peaks (i.e., S/N > 3) were reported, as described previously (Alharbi et al., 2016).

#### 2.4. Effects of co-exposure to retene and OSPW

Embryos of Japanese medaka were collected from a breeding culture that is maintained in the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, SK, Canada). Culturing of adult fish and rearing of embryos until they were required for exposures was conducted in dechlorinated City of Saskatoon municipal tap water at a temperature of 28 °C and a photoperiod of 16 h:8 h (light:dark). All protocols were approved by the University of Saskatchewan's Committee on Animal Care and Supply and adhered to the Canadian Council on Animal Care guidelines for humane animal use (UCACS Protocol #20090108).

Embryos were exposed to retene by use of a partition controlled delivery system with PDMS (Kiparissis et al., 2003; Turcotte et al., 2011; Lin et al., 2015). Briefly, retene was dissolved into a solution of DCM:hexane (15:85) at a ratio of 6 mg of retene per mL of solution. An appropriate volume of this mixture was added to PDMS to achieve a final concentration of retene of 15  $\mu\text{g mL}^{-1}$ . A negative control was prepared by use of the same procedure but without retene. Next, 2 mL was transferred into each 20 mL glass vial and vials were placed in a fume hood until all solvent was evaporated. Vials were wrapped in tin foil and all procedures were conducted in the dark to avoid any photo-modification of retene (Oris and Giesy, 1987).

Prior to exposure of embryos to retene, a study was conducted to assess partitioning of retene from PDMS to OSPW or the solvent control, which was City of Saskatoon municipal tap water containing 0.25% v/v of ethanol. Sampling of solutions was performed at 24, 96, 168 and 240 h in vials free of embryos. For exposures of embryos, 15 mL of the solvent control, 5  $\times$  or 1  $\times$  equivalent of the dissolved organic fraction of BML-OSPW, or 5  $\times$  equivalent of the dissolved organic fraction of P9-OSPW were added to vials containing PDMS with or without 15  $\mu\text{g mL}^{-1}$  of retene. These concentrations of the dissolved organic fraction of BML-OSPW and P9-OSPW did not affect survival of embryos (data not shown). Next, 12 embryos that were raised in freshwater until 4 dpf were added to each vial and placed on an orbital shaker (VWR International, Radnor, PA, USA) at 700–900 rpm and at 28 °C. Each day the number of embryos that were alive or dead or that had hatched was determined, and any dead embryos were removed. Exposures were terminated at 192 h of exposure. Samples of solutions were taken at 24 h and 192 h of exposure to quantify concentrations of retene. Solutions were not replaced during the exposure. Incidences of pericardial edema in larvae were evaluated by visualizing larvae with a light microscope at 10X magnification. Samples were stored at –80 °C for quantification of gene expression. All exposures were performed on 5 separate batches of embryos ( $n=5$ ).

#### 2.5. Quantification of retene by GC/MS

Concentrations of retene in the solvent control or OSPW were determined by use of liquid-liquid extraction according to a method described by El-Amrani et al. (2013). Briefly, 1 mL of solution was extracted twice with 500  $\mu\text{L}$  of a 1:1 mixture of hexane:ethyl acetate. Samples were centrifuged at 9000 g for 5 min, the organic phase was removed, and the remaining supernatant was concentrated under a gentle stream of nitrogen until samples were almost

dry. Next, samples were reconstituted in 95  $\mu\text{L}$  of nonane:hexane (15:85) and transferred to an auto-sampler vial fitted with a glass insert and 5  $\mu\text{L}$  of a solution containing 1  $\mu\text{g mL}^{-1}$  of fluorinated-polybrominated diphenyl ether 47 (F-BDE-47 in nonane) was added to samples as an internal standard for analysis by use of an Agilent (Santa Clara, CA, USA) 7890A gas chromatograph (GC) system coupled to an Agilent 5975C mass spectrometer (MS) operated in the electron impact ionization mode (EI). Samples at a volume of 1  $\mu\text{L}$  were injected at an injection port temperature of 280 °C in the splitless mode. Chromatographic separation was achieved by use of a DB-5MS (60 m  $\times$  0.250 mm  $\times$  0.1  $\mu\text{m}$ ) fused silica capillary GC column (Agilent). The carrier gas was helium at a constant flow of 1.5 mL min<sup>-1</sup>. The following GC oven temperature program was used: 100 °C for 1 min, 5 °C/min to 190 °C for 2 min, 20 °C/min to 220 °C for 2 min, and 40 °C/min to 300 °C for 4 min. The GC/MS transfer line was maintained at 28 °C. Selected ion monitoring was  $m/z$  234/219 for retene and 343/235 for F-BDE-47 (IS). Recovery of retene spiked into water at 10 or 350  $\mu\text{g L}^{-1}$  was 86.5  $\pm$  (RSD < 2%) and 104  $\pm$  (RSD < 5%), respectively. The limit of detection was 0.6  $\mu\text{g L}^{-1}$  and the limit of quantification was 2.4  $\mu\text{g L}^{-1}$ .

#### 2.6. Quantitative real-time PCR

Total RNA was extracted from each of the 5 pools of 6 larvae by use of the Qiagen RNeasy Protect Mini Kit according to the protocol provided by the manufacturer (Qiagen, Mississauga, ON, Canada). Quantities of total RNA were determined by use of a ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and integrity of RNA was determined by use of denaturing agarose gel electrophoresis. The cDNA was synthesized from 1  $\mu\text{g}$  of total RNA by use of the QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) according to the protocol provided by the manufacturer. Quantitative real-time quantitative PCR (qPCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 50  $\mu\text{L}$  reaction mixture of 2X concentrated QuantiFast SYBR Green Master Mix (Qiagen), an optimized concentration of cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water was prepared for each cDNA sample and primer combination. All reactions were conducted in duplicate with 20  $\mu\text{L}$  reaction volumes per well. The PCR reaction mixture was denatured at 95 °C for 10 min prior to the first PCR cycle. The thermal cycle profile consisted of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Amplification of a single PCR product was confirmed by melt curve analysis and abundances of target genes were quantified by normalizing to expression of the housekeeping gene *RPL-7* (Pfaffl, 2001). Primers to amplify cytochrome P450 1A (*cyp1a*), ATP-binding cassette subfamily c member 2 (*abcc2*) and subfamily G member 2 (*abcg2*), superoxide dismutase (*sod*), catalase (*cat*), and glutathione-s-transferase pi (*gstp*) were designed by use of Primer express 3.0 software (Applied Biosystems) and were purchased from Invitrogen (Mississauga, ON, Canada). Sequences of primers are given in Table 1. Prior to qPCR, the efficiency of each primer set was established by performing qPCR with cDNA that was generated by pooling equal volumes of each sample of cDNA and that was serially diluted by 5-fold from undiluted to 1:3125. Reactions without cDNA were performed to check for contamination of reagents and check for primer-dimers. All primer sets had a coefficient of determination ( $R^2$ ) of at least 0.99 and efficiencies of 84–106%.

#### 2.7. Statistical analyses

Effects of treatments on survival, rate of hatching, incidence of pericardial edema, gene expression, and concentrations of retene

**Table 1**  
Sequences of primers used to quantify expression of target genes in larvae of Japanese medaka.

Target transcript	Accession #	Sense primer (5'-3')	Antisense primer (5'-3')	Efficiency (%)
<i>cyp1a</i>	AY297923	GAGCACCTGGTCAAAGAGATAG	AGCACATGCCACAGATAACA	96
<i>gstp</i>	NA <sup>a</sup>	GAGCACCTGGTCAAAGAGATAG	AGCACATGCCACAGATAACA	96
<i>cat</i>	XM004069460	GAGGAGCAAGAGCAGCAATC	ATGAAGCACAGAGGCTGGAG	84
<i>sod</i>	XM004076213	AGAGAATCTCCGCGTGAAGTT	CCACGATTTGGTTGATGACCTC	96
<i>abcc2</i>	NA <sup>a</sup>	GGCGGTACATTAGGAGAGG	ACGTACACAGAACAGCAA	91
<i>abcg2</i>	NA <sup>a</sup>	AGGGTAAGCAGGGGATGACT	GAGAGCTCAACGATCAGGG	106
<i>rpl-7</i>	DQ118296	GTCGCCTCCCTCCACAAAG	AACTCAAGCTGCCAACAAAC	94

<sup>a</sup> Nucleotide sequence was from the Ensembl database (<http://uswest.ensembl.org/Oryzias.latiipes/Info/Index>).

**Table 2**  
Concentrations of retene ( $\mu\text{g L}^{-1}$ ) in aqueous phase in vials with and without larvae. The concentration of retene in PDMS was  $15 \mu\text{g mL}^{-1}$ .

Aqueous Media	Vials without fish		Vials with fish
	Time (h)	$[\mu\text{g L}^{-1}]$	$[\mu\text{g L}^{-1}]$
0.25% v/v ethanol	24	$27.8 \pm 0.6$	$65.9 \pm 7.0$
	96	$27.6 \pm 3.9$	NA
	168	$25.9 \pm 1.8$	NA
	192	$28.8 \pm 1.4$	$77.7 \pm 5.9$
1 × BML-OSPW	24	$30.6 \pm 1.9$	$90.7 \pm 4.0$
	96	$30.2 \pm 1.5$	NA
	168	$35.4 \pm 1.4$	NA
	192	$46.7 \pm 7.5$	$113.1 \pm 5.1$
5 × BML-OSPW	24	$180.5 \pm 5.27$	$108.9 \pm 4.5$
	96	$179.4 \pm 5.71$	NA
	168	$184.3 \pm 6.85$	NA
	192	$188.4 \pm 9.81$	$112.4 \pm 4.7$
5 × P9-OSPW	24	$71.8 \pm 4.2$	$97.9 \pm 0.7$
	96	$74.7 \pm 0.1$	NA
	168	$76.1 \pm 3.7$	NA
	192	$83.3 \pm 3.8$	$96.2 \pm 2.8$

NA = not analysed.

Values represent the mean  $\pm$  standard error of 3 independent replicates. In vials containing only the solvent control or OSPW the concentration of retene was less than the limit of detection.

were determined by use GraphPad Prism 5 software (San Diego, CA, USA). Normality of data was assessed by use of the Kolmogorov Smirnov one-sample test and homogeneity of variance were determined by use of Levene's test. If necessary, data were log transformed to ensure normality and homogeneity of variance. Significant differences among treatments were evaluated by use of a one-way ANOVA followed by Tukey post-hoc test. Differences were considered significant at  $p \leq 0.05$ .

### 3. Results

#### 3.1. Profiles of organic chemicals in BML and P9

Extraction of OSPW at pH 9 resulted in samples that were enriched in basic and neutral chemicals relative to acidic chemicals. Relative abundances of species detected by use of ESI<sup>-</sup>, which is used to detect organic acids, were lesser than abundances of species detected by use of ESI<sup>+</sup>, which is used to detect polar organic neutral and basic chemicals (Fig. 1). For example, relative abundance of naphthenic acids (NAs; O<sub>2</sub><sup>-</sup>) was less than relative abundance of O<sub>2</sub><sup>+</sup>, which are not NAs but had been proposed to be dihydroxy, diketo or ketohydroxy chemicals (Pereira et al., 2013). However, a small amount of acidic chemicals were present in the final extracts,

Classes of heteroatoms in extracts of BML-OSPW and P9-OSPW were similar, but differences in elemental compositions and relative intensities of species of heteroatoms were observed. In both extracts, intensity of species containing oxygen (O<sub>x</sub><sup>-</sup>) or sulfur (SO<sub>x</sub><sup>-</sup>) were greater than intensities of chemical species containing nitrogen (NO<sub>x</sub><sup>-</sup>) (Fig. 1A). When analysed by use of ESI<sup>-</sup>, intensity

of O<sub>2</sub><sup>-</sup> was 3.5-fold greater in BML-OSPW compared to P9-OSPW, and the same was observed for the intensity of O<sub>3</sub><sup>-</sup> and O<sub>4</sub><sup>-</sup> chemical species which were 2.5- and 3.0-fold greater, respectively, in BML-OSPW compared to P9-OSPW. Intensities of SO<sub>2</sub><sup>-</sup>, and SO<sub>3</sub><sup>-</sup> chemicals species were 1- and 5-fold greater, respectively, in BML-OSPW compared to P9-OSPW. When analysed by use of ESI<sup>+</sup> mode (Fig. 1B). Intensities of O<sup>+</sup>, O<sub>2</sub><sup>+</sup>, O<sub>3</sub><sup>+</sup> and O<sub>4</sub><sup>+</sup> chemical species were 1.5-, 1.6-, 2- and 3.1-fold greater in P9-OSPW than in BML-OSPW. Intensities of S<sup>+</sup>, SO<sup>+</sup>, SO<sub>2</sub><sup>+</sup> and SO<sub>3</sub><sup>+</sup> were 4.5-, 4.9-, 26- and 4.6-fold greater in BML-OSPW compared to P9-OSPW. Intensities of N, NO<sup>+</sup>, NO<sub>2</sub><sup>+</sup>, NO<sub>2</sub>S<sup>+</sup>, NO<sub>3</sub><sup>+</sup>, NO<sub>3</sub>S<sup>+</sup>, NO<sub>4</sub><sup>+</sup> and NO<sub>4</sub>S<sup>+</sup> were greater by 3-, 3-, 4-, 5-, 2.3-, 2.9-, 4- and 3.3 fold, respectively, in BML-OSPW compared to P9-OSPW.

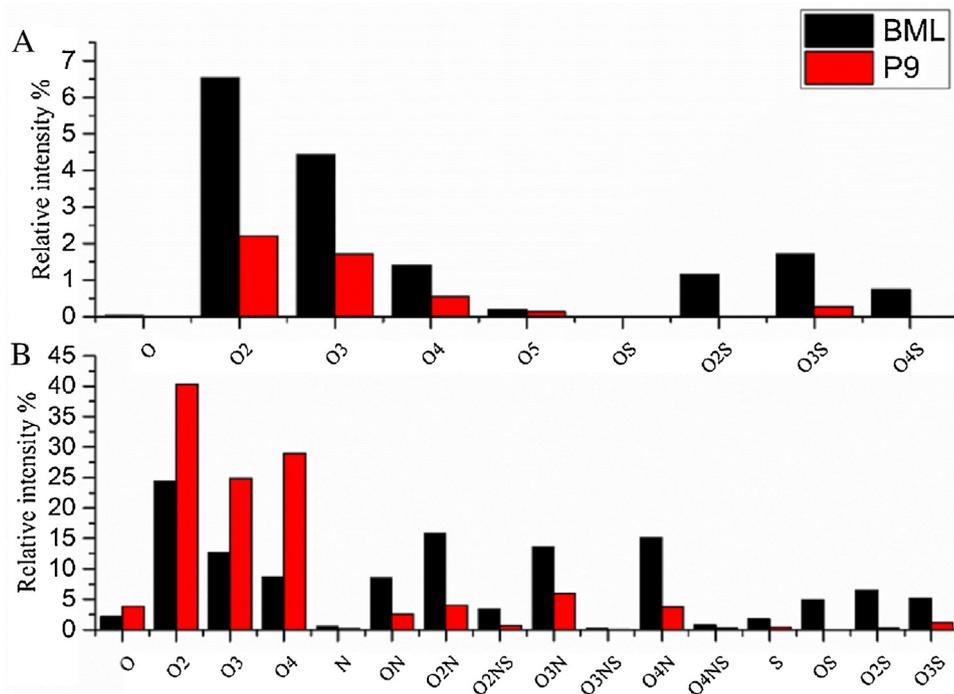
#### 3.2. Concentrations of retene in water and OSPW

Prior to initiation of exposures a preliminary study was conducted to confirm partitioning of retene from the PDMS into freshwater or OSPW (Table 2). Concentrations of retene in vials containing PDMS without retene were less than the limit of quantification. In vials containing PDMS with retene at  $15 \mu\text{g mL}^{-1}$  concentrations of retene ranged from 25.9 to  $28.8 \mu\text{g L}^{-1}$  when the aqueous phase was 0.25% v/v of ethanol. Concentrations of retene were greater when the aqueous phase was OSPW. In vials containing 1 × equivalent of BML-OSPW, concentrations of retene ranged from 30.2 to  $46.7 \mu\text{g L}^{-1}$  and in vials containing 5 × equivalent of BML-OSPW concentrations of retene ranged from 179.4 to  $188.4 \mu\text{g L}^{-1}$ , which is 7-fold greater compared to the concentration in 0.25% v/v of ethanol. The concentration of retene in vials containing 5 × equivalent of P9-OSPW ranged from 71.8 to  $83.3 \mu\text{g L}^{-1}$ , which is 2.8-fold greater compared to the concentration in 0.25% v/v of ethanol but 2-fold lesser compared to the concentration in 5 × equivalent of BML-OSPW.

In vials containing embryos, concentrations of retene in freshwater or OSPW were quantified at 24 h of exposure and upon termination of the exposure (Table 2). In vials containing PDMS without retene, concentrations of retene were less than the limit of quantification. In vials containing PDMS with retene at  $15 \mu\text{g mL}^{-1}$ , concentrations of retene was approximately equal at both time points (see Table 2). Concentrations of retene in vials containing retene and OSPW were greater than in vials containing 0.25% v/v of ethanol. However, the difference in concentrations of retene among vials containing different samples of OSPW and retene were small.

#### 3.3. Lethality, hatching and incidences of malformations

Mortality of embryos exposed to either treatment was less than 10% and there were no significant differences among treatments (data not shown). Percentage of eggs hatched by 192 h of exposure was greater than 90% for all exposures, and there were no significant differences among treatments. However, there were differences in the time to hatch (Table 3). Hatching of embryos exposed only to the solvent control began at 144 h of exposure. However, hatching of embryos exposed only to 5 × equivalent of



**Fig. 1.** Elemental composition and relative intensities of water-soluble species of chemicals by heteroatom class in BML-OSPW and P9-OSPW acquired in (A) ESI<sup>-</sup> and (B) ESI<sup>+</sup>.

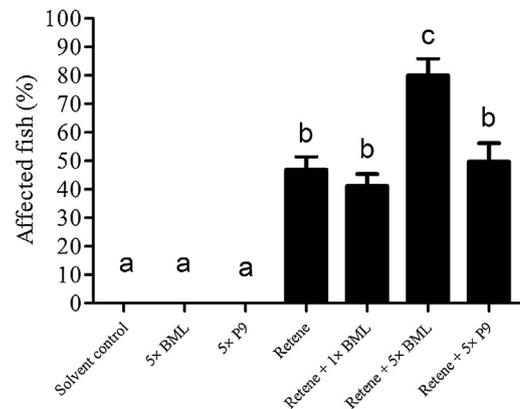
**Table 3**  
Effects of exposure to OSPW, retene or OSPW and retene on hatching of embryos.

Treatment	% of eggs hatched (cumulative)		
	144 h	168 h	192 h
Solvent control	27.0 ± 10.1 <sup>a</sup>	96.9 ± 9.6 <sup>a</sup>	96.9 ± 9.6 <sup>a</sup>
5 × BML-OSPW	0 <sup>b</sup>	36.0 ± 8 <sup>b</sup>	99.5 ± 9 <sup>b</sup>
1 × BML-OSPW	16.7 ± 12 <sup>c</sup>	35.0 ± 1 <sup>b</sup>	97.0 ± 15 <sup>b</sup>
5 × P9-OSPW	0 <sup>b</sup>	37.8 ± 7.2 <sup>b</sup>	96.2 ± 8 <sup>b</sup>
15 µg mL <sup>-1</sup> Retene	0 <sup>b</sup>	51.2 ± 23 <sup>b</sup>	93.4 ± 23 <sup>b</sup>
15 µg mL <sup>-1</sup> Retene + 5 × BML-OSPW	0 <sup>b</sup>	31.2 ± 3.4 <sup>b</sup>	97.9 ± 3.8 <sup>b</sup>
15 µg mL <sup>-1</sup> Retene + 1 × BML-OSPW	4.2 ± 4.2 <sup>c</sup>	33.4 ± 10.8 <sup>b</sup>	95.5 ± 15 <sup>b</sup>
15 µg mL <sup>-1</sup> Retene + 5 × P9-OSPW	0 <sup>b</sup>	36.5 ± 7.2 <sup>b</sup>	96.5 ± 7.2 <sup>b</sup>

Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in hatching were determined by use of one-way ANOVA followed by Tukey's post hoc test ( $n=5$ ,  $p \leq 0.05$ ) and different letters indicate significant effects.

either BML-OSPW or P9-OSPW did not begin until 168 h of exposure. This effect was attenuated by diluting BML-OSPW as hatching of embryos exposed to 1 × of BML-OSPW began at 144 h of exposure. Hatching of embryos exposed to 15 µg mL<sup>-1</sup> of retene began at 168 h of exposure and this effect was not modulated by exposure to either sample of OSPW.

Exposure to OSPW increased incidences of malformations caused by exposure to retene (Fig. 2). No incidences of malformations were observed in embryos in the solvent control or either sample of OSPW. Incidences of malformations of embryos exposed only to retene was 46.87%. Co-exposure to retene and either 1 × equivalent of BML-OSPW or 5 × of P9-OSPW did not modulate effects of retene as incidence of malformations were 41.14% and 49.74%, respectively. However, incidences of malformations of embryos exposed to retene and 5 × equivalent of BML-OSPW was 79.98%.

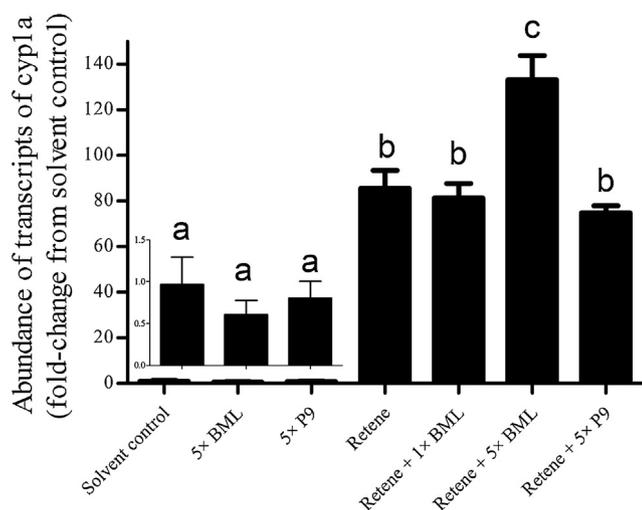


**Fig. 2.** Percentage of larvae of Japanese medaka with pericardial edema. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in incidences of pericardial edema were determined by use of one-way ANOVA followed by Tukey's post hoc test ( $n=5$ ,  $p \leq 0.05$ ) and are indicated by different letters.

### 3.4. Expression of genes

Expression of *cyp1a* was quantified as a biomarker of exposure to agonists of the aryl-hydrocarbon receptor (AhR) (Fig. 3). Compared to embryos exposed to the solvent control, abundance of transcripts of *cyp1a* was 85.6-fold greater in embryos exposed only to retene. Exposure to 1 × equivalent of BML-OSPW or 5 × of P9-OSPW did not significantly alter the response to retene as abundance of transcripts of *cyp1a* was 81.3- and 74.9-fold greater, respectively. However, in embryos co-exposed to retene and 5 × equivalent of BML-OSPW the abundance of transcripts of *cyp1a* was 133.2- fold greater compared to the solvent control.

Expression of genes encoding proteins that are important for the response to oxidative stress were quantified (Fig. 4). Compared to larvae exposed to the solvent control, abundances of transcripts of



**Fig. 3.** Abundances of transcripts of *cyp1a* in larvae of Japanese medaka. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in gene expression were determined by use of one-way ANOVA followed by Tukey's post hoc test ( $n=5$ ,  $p \leq 0.05$ ) and are indicated by different letters.

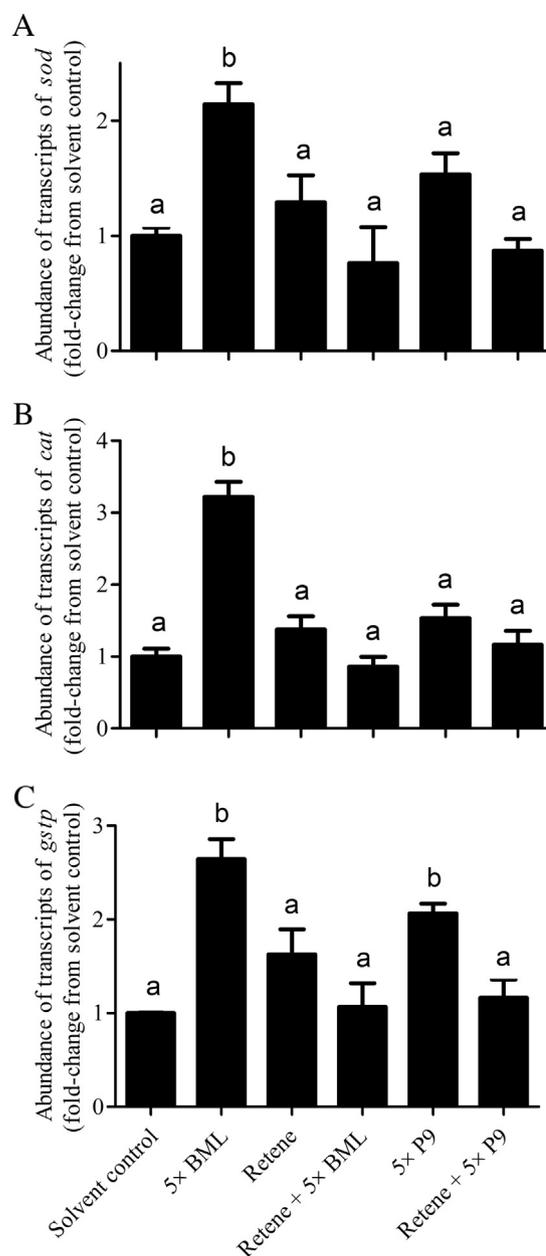
*sod*, *cat* and *gstp* were 2.1-, 3.2- and 2.6-fold greater, respectively, in larvae exposed to 5 × equivalent of BML-OSPW, and abundance of transcripts of *gstp* was 2.0-fold greater in larvae exposed to 5 × equivalent of P9-OSPW. However, abundances of transcripts of *sod*, *cat* and *gstp* were significantly lesser in larvae co-exposed to retene and 5 × equivalent of OSPW compared to larvae exposed only to 5 × equivalent of OSPW.

Expression of genes encoding proteins that are important for phase III metabolism of xenobiotics were quantified (Fig. 5). Compared to embryos exposed to the solvent control, abundances of transcripts of *abcc2* and *abcg2* were 12.4- and 5.3-fold greater, respectively, in larvae exposed to 5 × equivalent of BML-OSPW. Abundances of transcripts of *abcc2* and *abcg2* were not significantly greater in larvae co-exposed to 5 × equivalent of BML-OSPW and retene compared to controls, but were significantly less than in embryos exposed to 5 × equivalent of BML-OSPW. Exposure to 5 × of P9-OSPW did not affect abundances of transcripts of *abcc2* or *abcg2*.

#### 4. Discussion

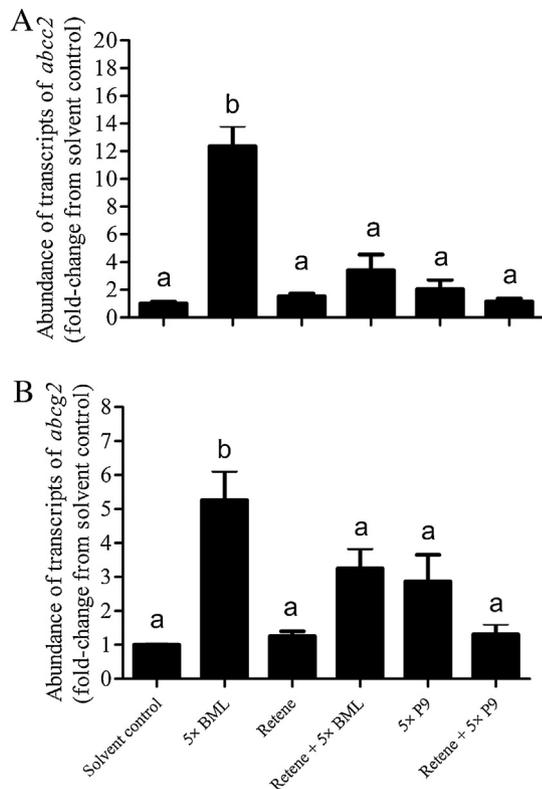
This study is the first to investigate whether dissolved organic compounds in OSPW affect toxicity of PAHs, which are an important component of tailings ponds used to store OSPW in the surface mining oil sands industry in northern Alberta, Canada. Exposure to retene, either sample of OSPW, or co-exposure to retene and OSPW did not cause acute lethality to embryos. The lack of acute lethality of embryos exposed to retene is consistent with the absence of mortality in marine medaka (*Oryzias melastigma*) exposed to concentrations of retene less than  $200 \mu\text{g L}^{-1}$  (Mu et al., 2014), and larvae of rainbow trout (*Oncorhynchus mykiss*) exposed to a nominal concentration of  $320 \mu\text{g L}^{-1}$  (Billiard et al., 1999; Bauder et al., 2005) or  $100 \mu\text{g L}^{-1}$  (Scott and Hodson, 2008).

Greater expression of CYP1A, and a greater prevalence of pericardial edema in larvae exposed to retene is consistent with results of previous studies that linked exposure to retene with blue sac disease (BSD) (Billiard et al., 1999; Brinkworth et al., 2003; Bauder et al., 2005; Scott and Hodson, 2008). Neither greater abundance of transcripts of *cyp1a* nor greater prevalence of pericardial edema was observed in larvae exposed only to OSPW. This is consistent with results of another study where abundances of



**Fig. 4.** Abundances of transcripts of (A) *sod*, (B) *cat* and (C) *gstp* in larvae of Japanese medaka. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in gene expression were determined by use of one-way ANOVA followed by Tukey's post hoc test ( $n=5$ ,  $p \leq 0.05$ ) and are indicated by different letters.

transcripts were not significantly greater in larvae of fathead minnow (*Pimephales promelas*) exposed to OSPW from the west-in-pit settling basin, which was commissioned as Base Mine Lake in 2013 (He et al., 2012b). However, in that study, embryos exposed to OSPW exhibited several manifestations that are characteristic of blue-sac disease, including pericardial edema. That effect was attenuated by treating OSPW with ozone or activated carbon, both of which reduce concentrations of dissolved organic compounds in OSPW, suggesting that the dissolved organic chemicals caused pericardial edema without activating the AhR (He et al., 2012b). It is not known why BML-OSPW in the current study did not cause pericardial edema, but at the time of the study by He et al. (2012b) the west-in-pit was receiving input of fresh tailings from a bitumen extraction facility, so there might have been differences in



**Fig. 5.** Abundances of transcripts of (A) *abcc2*, and (B) *abcg2*, in larvae of Japanese medaka. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in gene expression were determined by use of one-way ANOVA followed by Tukey's post hoc test ( $n = 5$ ,  $p \leq 0.05$ ) and are indicated by different letters.

composition of chemicals in OSPW. Also, because the extracts used in the current study were enriched in basic and neutral chemicals relative to acidic chemicals, the pericardial edema observed in the study by He et al. (2012b) might have been caused by acidic compounds. Compared to exposure only to retene, co-exposure to retene and 1  $\times$  of BML-OSPW or 5  $\times$  of P9-OSPW did not result in greater abundance of transcripts of *cyp1a* or greater incidences of pericardial edema. However, compared to fish exposed only to retene, or fish co-exposed to retene and 1  $\times$  equivalent of BML-OSPW or 5  $\times$  equivalent of P9-OSPW, abundance of transcripts of *cyp1a* and incidences of pericardial edema were greater in fish co-exposed to retene and 5  $\times$  equivalent of BML-OSPW.

The mechanism(s) of greater expression of CYP1A and incidences of pericardial edema in embryos co-exposed to retene and 5  $\times$  of BML-OSPW are not known. Because expression of CYP1A and induction of blue-sac disease in early-life stages of fishes in response to exposure to PAHs, including retene, is dependent on activation of the AhR (Billiard et al., 2006; Incardona et al., 2006; Scott and Hodson et al., 2008), these effects might be a result of the dissolved organic compounds in OSPW increasing activation of the AhR by retene. An example of this effect is greater activation of the AhR by TCDD in the presence of humic acids that do not activate the AhR (Bittner et al., 2009). In that study it was proposed that humic acids increased bioavailability of TCDD by enhancing solubility. Studies have shown that lower molecular weight organic acids increase solubilities of PAHs in water (Doring and Marschner, 1998; Ling et al., 2009). Chemicals in OSPW have properties of surfactants (Clemente and Fedorak, 2005; Frank et al., 2008; Schramm et al., 1984; Schramm and Smith, 1985), and surfactants enhance bioavailability of hydrophobic organic compounds

in water (Edwards et al., 1992; Jafvert et al., 1994; Volkering et al., 1995; Badr et al., 2004; Subramaniam et al., 2004). For example, synthetic non-ionic surfactants increased apparent solubilities and rates of dissolution of naphthalene and phenanthrene (Volkering et al., 1995). In the current study, regardless of the aqueous phase, retene desorbed from the PDMS to the aqueous phase, but, as indicated by results of the trial in the absence of embryos, the concentration of retene was greater when the aqueous phase was OSPW, and was greatest when the aqueous phase was 5  $\times$  of BML-OSPW. The lesser concentrations of retene observed in the presence of 1  $\times$  equivalent of BML-OSPW or 5  $\times$  equivalent of P9-OSPW likely was because the concentration of dissolved organics was smaller in these samples than in 5  $\times$  equivalent of BML-OSPW. Several studies have shown that concentrations of dissolved organic chemicals, including carboxylic acids such as NAs, are approximately 50% lesser in OSPW from Pond 9 than in OSPW from the west-in-pit (Han et al., 2009; Anderson et al., 2012).

In addition to greater exposure resulting from enhanced desorption of retene from PDMS caused by OSPW, greater abundance of transcripts of *cyp1a* and greater incidences of pericardial edema might be caused by greater uptake of retene in larvae co-exposed to 5  $\times$  equivalent of BML-OSPW compared to embryos exposed to retene alone or co-exposed to retene and 1  $\times$  equivalent of BML-OSPW or 5  $\times$  equivalent of P9-OSPW. Greater uptake of retene might result from alterations of cell membranes caused by dissolved organic chemicals in OSPW. Change in permeability of cell membranes was proposed as a mechanism by which activation of AhR by TCDD was greater in the presence of humic acids (Glover and Wood, 2005; Bittner et al., 2009). Studies have shown that surfactants increase permeabilities of cell membranes (Xia and Onyuksel, 2000). For example, bioaccumulation of BaP was greater in catfish exposed to the surfactant C-12 linear alkyl-benzene-sulfonate compared to freshwater (Tan et al., 2010). It is not known whether dissolved organic chemicals in OSPW alter cell membranes, but such an effect would enhance bioavailability of retene and therefore might result in greater toxicity.

It has been suggested that blue-sac disease in early-life stages of fishes exposed to retene might be caused by oxidative stress resulting from generation of reactive oxygen species (ROS) produced during metabolism of retene by CYP1A enzymes (Billiard et al., 1999; Bauder et al., 2005). Also, there is an abundance of evidence that dissolved organic chemicals in OSPW cause oxidative stress. Concentrations of ROS were greater in embryos of fathead minnows exposed to OSPW from the west-in-pit settling basin (He et al., 2012b). Concentrations of lipid hydroperoxides were greater in larvae of *Chironomus dilutus* exposed to OSPW from the west-in-pit settling basin and greater abundances of transcript of several genes important for the response to oxidative stress, including *gst*, *sod*, and *cat* were greater in fathead minnows and *Chironomus dilutus* exposed to OSPW from the west-in-pit settling basin (Wiseman et al., 2013a,b; He et al., 2012a). Therefore, it was hypothesised that co-exposure to OSPW and retene would cause a greater effect than exposure to retene alone. However, expressions of genes important for the response to oxidative stress do not support this mechanism of toxicity. Abundances of transcripts of *gst*, *sod* and *cat*, were not significantly greater in larvae exposed only to retene or co-exposed to retene and 5  $\times$  equivalent of BML-OSPW, both of which caused pericardial edema. However, abundance of transcripts of *cat* was greater in larvae exposed to 5  $\times$  equivalent of BML-OSPW, which did not cause pericardial edema. These results suggest that oxidative stress does not play a significant role in the incidence of pericardial edema observed in this study.

Inability to metabolise the greater amounts of retene that larvae co-exposed to retene and 5  $\times$  of BML-OSPW were exposed to might have contributed to the greater effects of retene on these fish. The ABC-transporter proteins, ABCB2 and ABCG2, eliminate

glutathione-, glucuronide-, and sulfate-conjugated metabolites of xenobiotics, including glutathione-conjugated metabolites of PAHs, from cells (Konig et al., 1999; Ebert et al., 2005; Hessel et al., 2013). Therefore, greater incidences of pericardial edema in larvae co-exposed to 5× of BML-OSPW and retene compared to larvae exposed only to retene might have been a result of inhibition of activity of ABC-transporter proteins, which has been reported previously (Alharbi et al., 2016). In addition to this potential mechanism of toxicity, greater toxicity might have been caused by effects on expression of genes important for metabolism of retene. Previously, it has been demonstrated that abundances of transcripts of *abcc2* and *abcg2* are greater in fathead minnows exposed to OSPW (Wiseman et al., 2013a). Results of the current study support this effect because abundances of transcripts of *abcc2* and *abcg2* was greater in larvae exposed to 5× of BML-OSPW. Also, the lack of any effect of exposure only to retene on expression of *abcc2* and *abcg2* is consistent with effects of retene on expression of these genes in embryos of rainbow trout (*Oncorhynchus mykiss*) (Vehniäinen et al., 2016). However, expression of *abcc2* and *abcg2* in larvae co-exposed to retene and 5× equivalent of BML-OSPW was significantly lesser compared to larvae exposed only to 5× of BML-OSPW. The mechanism of this response is not known, but the same response occurred for expression of *gst*, which is important for phase II metabolism of PAHs. If lesser abundances of transcripts of *abcc2*, *abcg2* and *gst* in larvae co-exposed to retene and 5× equivalent of BML-OSPW compared to larvae exposed only to 5× equivalent of BML-OSPW resulted in lesser abundances of these proteins it might have compromised the ability to detoxify the greater amounts of retene that these fish were exposed to and might have contributed to the toxic effects that were observed.

Results of the current study demonstrate that dissolved organic compounds in OSPW have potential to alter partitioning and effects of PAHs on aquatic organisms. While mechanisms by which these effects occur are not known at this time, it appears that dissolved organic compounds in OSPW might increase exposure and uptake of PAHs by aquatic organisms. However, it does not appear that acute effects related to activation of the AhR occur at environmentally relevant concentrations. Also, results of the study provide additional evidence that aging of OSPW in reclamation ponds is an effective method to attenuate adverse effects of OSPW. The study emphasises the importance of future research to investigate interactions among chemicals that co-exist in tailings ponds rather than focusing on one specific group of chemicals.

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