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Inhibition of ABC transport proteins by oil sands process affected water

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ABSTRACT

The ATP-binding cassette (ABC) superfamily of transporter proteins is important for detoxification of xenobiotics. For example, ABC transporters from the multidrug-resistance protein (MRP) subfamily are important for excretion of polycyclic aromatic hydrocarbons (PAHs) and their metabolites. Effects of chemicals in the water soluble organic fraction of relatively fresh oil sands process affected water (OSPW) from Base Mine Lake (BML-OSPW) and aged OSPW from Pond 9 (P9-OSPW) on the activity of MRP transporters were investigated in vivo by use of Japanese medaka at the fry stage of development. Activities of MRPs were monitored by use of the lipophilic dye calcein, which is transported from cells by ABC proteins, including MRPs. To begin to identify chemicals that might inhibit activity of MRPs, BML-OSPW and P9-OSPW were fractionated into acidic, basic, and neutral fractions by use of mixed-mode sorbents. Chemical compositions of fractions were determined by use of ultrahigh resolution orbitrap mass spectrometry in ESI^+ and ESI^- mode. Greater amounts of calcein were retained in fry exposed to BML-OSPW at concentration equivalents greater than 1× (i.e., full strength). The neutral and basic fractions of BML-OSPW, but not the acidic fraction, caused greater retention of calcein. Exposure to P9-OSPW did not affect the amount of calcein in fry. Neutral and basic fractions of BML-OSPW contained relatively greater amounts of several oxygen-, sulfur, and nitrogen-containing chemical species that might inhibit MRPs, such as O^+, SO^+, and NO^+ chemical species, although secondary fractionation will be required to conclusively identify the most potent inhibitors. Naphthenic acids (O_2^-), which were dominant in the acidic fraction, did not appear to be the cause of the inhibition. This is the first study to demonstrate that chemicals in the water soluble organic fraction of OSPW inhibit activity of this important class of proteins. However, aging of OSPW attenuates this effect and inhibition of the activity of MRPs by OSPW from Base Mine Lake does not occur at environmentally relevantconcentrations.

1. Introduction

In the surface mining oil sands industry extraction of bitumen from oil sands generates oil sands process affected water (OSPW) that is retained on-site in tailings ponds and settling basins that, as of 2009, covered an area of approximately 170 km^2 (Government of Alberta, 2011). Because oil sands mining companies do not discharge OSPW to the wider environment, the volume of OSPW

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stored in tailings ponds will increase as surface mining operations expand. Therefore, methods to remediate OSPW are needed. One strategy companies are exploring for remediation and reclamation of OSPW is use of end pit lakes (EPLs) constructed by filling mined-out pits with products of the extraction of bitumen, including OSPW (Gosselin et al., 2010). The expectation is that toxicity of OSPW in EPLs will decrease because of biodegradation of chemicals in the water soluble organic fraction of OSPW (Del Rio et al., 2006; Han et al., 2008, 2009), and that EPLs will eventually be capable of sustaining life.

The chemistry of tailings ponds is complex. Liquid tailings are a mixture of water, residual bitumen, sand, silt, and inorganic and organic compounds. Over time particulates (silt and clay fractions) settle to form a layer of mature fine tailings (MFTs), leaving behind an aqueous layer of OSPW. Two constituents of tailings ponds that have the potential to cause toxicity to aquatic organisms are polycyclic aromatic hydrocarbons (PAHs) and acid-extractable organic compounds, including naphthenic acids (NAs; $C_nH_{2n+z}O_2$), in the water soluble organic fraction of OSPW. 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 1150–1600 ng/L in the upper clarified zone of OSPW (Rogers et al., 2002; Galarneau et al., 2014). The water-soluble organic fraction of OSPW has been described as a "supercomplex mixture" (Jones et al., 2012). Much of the characterization of the water-soluble organic fraction has focused on NAs, but advances in ultra-high-resolution mass spectrometry have identified a variety of oxygen-, sulphur- and nitrogen-containing compounds in this mixture (Barrow et al., 2010; Pereira et al., 2013; Morandi et al., 2015).

Several mechanisms by which OSPW could cause toxicity have been identified. OSPW that is fresh causes acute lethality, and it has been proposed that the mechanism of this effect is narcosis (Frank et al., 2008; Scarlett et al., 2013; Morandi et al., 2015). Also, OSPW causes a variety of sub-lethal effects, including endocrine disruption (Lister et al., 2008; He et al., 2010, 2011, 2012a; Van den Heuvel et al., 2012; Kavanagh et al., 2011, 2012, 2013; Leclair et al., 2015), oxidative stress (He et al., 2012b; Wiseman et al., 2013a,b), and alterations to immune function (Garcia-Garcia et al., 2011; McNeill et al., 2012; MacDonald et al., 2013; Hagen et al., 2014). In many of these studies effects were caused by the water-soluble organic fraction of OSPW.

A variety of natural and synthetic chemicals can inhibit members of the ATP (energy-dependent efflux pumps)-binding cassette (ABC) superfamily of transporter proteins. These inhibitors might not be toxic themselves, but might cause toxicity of other chemicals by inhibition of these transporter proteins, a process known as chemosensitization (Smital and Kurelec, 1997; Kurelec et al., 2000; Ferreira et al., 2014; Kurth et al., 2015). ABC proteins are important for detoxification of xenobiotics because they actively transport a variety of structurally diverse chemicals, and their metabolites, from cells thereby protecting organisms from adverse effects (Leslie et al., 2005; Klaassen and Lauren, 2010; Hessel et al., 2013). In teleost fishes, PAHs and their metabolites are transported from cells by multidrug resistance-associated proteins (MRP) 1–6 (ABCC1–6) (Bard, 2000; Ferreira et al., 2014; Luckenbach et al., 2014). Although it is not known if constituents of OSPW inhibit ABC proteins, water-soluble fractions of crude oil inhibit ABC transporters in larvae of the marine invertebrate, the fat innkeeper (*Urechis caupo)* (Hamdoun et al., 2002). If constituents of OSPW inhibit activity of MRPs it could exacerbate accumulation and effects of PAHs or their bio-activated metabolites on aquatic organisms. Therefore, the objective of this study was to determine if the water soluble organic fraction of OSPW affects the activity of MRPs by use of a model species of teleost fish, the Japanese medaka (*Oryzias latipes*). Also, semi-quantification of chemicals in fractions by use of ultra-high resolution orbitrap mass spectrometry, were performed to identify classes of chemicals in OSPW that might cause effects on activity of MRPs.

2. Materials and methods

2.1. Chemical, reagents, and OSPW

MK-571, an inhibitor of MRPs (Fischer et al., 2013; Zaja et al., 2007), was purchased from Cayman Chemical Company (Anne Arbor, MI, USA) and calcein-AM was from AAT Bioquest (Sunnyvale, CA, USA). Dimethylsulfoxide (DMSO) and trypan blue were from the Sigma Chemical Company (St. Louis, MO, USA). All solvents used were of analytical grade. 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. 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. Both samples were collected in September of 2012, shipped to the University of Saskatchewan (Saskatoon, SK, Canada), and used for fractionation immediately upon arrival.

2.2. Fractionation of OSPW

Both samples of OSPW were fractionated into acidic, basic, and neutral fractions of polar organic compounds by use of mixed-mode sorbents (MMS). Prior to fractionation 500 ml of each sample of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove any particulate matter, then acidified to pH 2 by use of concentrated HCl (37%). Next, for isolation of basic fractions, pre-concentration was performed in one step by use of 500 mg of mixed-mode Strata®-X Polymeric-C solid-phase sorbent in plastic cartridges (Phenomenex, Milford, MA, USA). This matrix is a porous copolymer with a weak mixed-mode cation that provides dual modes for the retention and adsorption of lipophilic and hydrophilic compounds was well as ionic compounds. Before addition of OSPW cartridges were conditioned with 6 mL of methanol and 6 ml of acidified water. The 500 ml of filtered and acidified OSPW was passed through the cartridges under vacuum. Next, cartridges were washed with 2% (v/v) of formic acid and were allowed to dry under vacuum for 30 min. The first elution was performed with 100% of methanol and this extract contained acidic and neutral compounds. The second elution was performed with 5% (v/v) of NH_4OH in methanol and this fraction contained basic compounds. To separate acidic and neutral compounds a pre-concentration of samples was performed by use of Strata®-X-A 500 mg solid-phase matrix in plastic cartridges (Phenomenex). This polymeric sorbent is water wetable and provides dual modes of retention – anion exchange and reversed phase. Prior to use the cartridge was conditioned by washing with 100% methanol followed by 5% (v/v) of NH_4OH (aq). Next, elutant I from the Strata®-X Polymeric-C sorbent was evaporated to approximately 0.5 ml, adjusted to a pH of 10–11 with NaOH, and then passed through the cartridge without vacuum. Cartridges were washed with 5% (v/v) of NH_4OH (aq) and left to dry under vacuum for 30 min. Finally, the fraction containing neutral compounds was eluted with 100% methanol and a fraction containing acidic compounds was eluted with 2% (v/v) of formic acid in methanol. Fractions were dried, and reconstituted in ethanol for bioassays. A pooled sample representative of the organics fraction was generated by pooling equal volumes of the acidic, neutral, and basic fractions. Blank samples, which were city of Saskatoon municipal tap water, were extracted by use of this method.

2.3. Profiling of fractions

The profile of chemicals in fractions of BML-OSPW and P9-OSPW was analysed by use of reversed-phase liquid chromatography paired with a linear ion trap-orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA, USA) in both negative (ESI^-) or positive (ESI^+) electrospray according to the method described by Pereira et al. (2013). Chemical species detected in each fraction were grouped according to heteroatom empirical formula classes in ESI^- or ESI^+ 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 $x = 2$). Briefly, the HPLC instrument was an Accela System (Thermo Fisher Scientific, San Jose, CA), consisting of a degasser, a 600 bar quaternary pump, an autosampler, and a column oven. Chromatographic separation was performed on a Cosmosil C_{18} MS-II column (100 × 3.0 mm, 2.5 μm particle size) (Nacalai USA, San Diego, CA) at 40 °C. Flow rate was 0.5 ml/min and the injection volume was 3 μL. The mobile phases were 0.1% (v/v) acetic acid in water (Solvent A) and 100% methanol (Solvent B). Mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B at 14 min, and returning to 5% B in 1 min, followed by a 4 min hold prior to the next injection. In this study, mass values were restricted to singly charged ions with a molecular mass of 100–500 Da and signal to noise ratios greater than 3. Elemental compositions (i.e, $CcHhNnOoSs$) were calculated with the elemental composition tool within the Xcalibur software (Thermo Scientific, Bremen, Germany), and by use of the following restrictions: 0–40 ^{12}C, 0–2 ^{13}C, 0–100 ^{1}H, 0-2 ^{14}N, 0–8 ^{16}O, 0–2 ^{34}S, and 1 ^{32}S. Molecular formulas were also confirmed by the presence of ^{13}C isotopes. Determination of elemental composition was based on the accurate mass m/z by matching the theoretical mass with the observed mass of each ion to within 5 ppm (typically <2 ppm). Data was qualitatively analysed based on the distribution of heteroatom classes, type, and number of carbon atoms. 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.

2.4. Acute lethality

Effects of OSPW on survival of fry of Japanese medaka were quantified to determine sub-lethal concentrations that could be used to determine effects on activity of MRPs. Japanese medaka (*Oryzias latipes*) were cultured in the Aquatic Toxicology Research Facility at the University of Saskatchewan. Eggs were collected daily from the culture system and maintained in embryo rearing medium (ERM) (1 g/L NaCl, 0.030 g/L KCl, 0.040 g/L $CaC_{l2}{\cdot}H_2O$, 80 mg/L MgSO4, and 1 mg/L Methylene Blue in distilled water) until hatch. All culturing of adult fish and rearing of embryos was conducted at a water temperature of 28 °C with a photoperiod of 16 h:8 h (light:dark). Acute lethality caused by BML-OSPW and P9-OSPW were determined first at an enrichment equivalent to 5× the concentration in OSPW that was not fractionated. Next, if toxicity was observed, dose-response relationships were established by determining lethality at 0.5, 1, 2.5, and 5× equivalents. Lethality was determined after 6 h and 24 h of exposure. The concentration of ethanol in exposure solutions did not exceed 0.1% (v/v). Lethality was determined with 3 batches of fry, and each time exposures were conducted in triplicate with 10 fry per exposure. Experiments with fish was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (Protocol #20090108).

2.5. Effect of OSPW on accumulation of calcein by Japanese medaka

Japanese medaka at the fry stage of development (developmental stage 40, which extends from hatching until appearance of fin rays in the caudal and pectoral fins) were used for the assay. Accumulation of calcein in fry was quantified by modification of a method described by Fischer et al. (2013). Briefly, a single fry was transferred to each well of a 24 well plate with a clear bottom (VisiPlate-24 Black, PerkinElmer, Woodbridge, ON, Canada) and was exposed to either 5 μM of MK-571 (positive control), 5× equivalent of the pooled fraction of BML-OSPW or P9-OSPW, 5× equivalents of fractions of BML-OSPW, or 0.5, 1, 2.5 and 5× equivalents of the pooled organic fraction of whichever OSPW might have caused inhibition of activity of ABC transporters. Fry were exposed to DMSO or ethanol at 0.1% (v/v) to control for effects of solvents. After addition of chemicals, plates were incubated at 28 °C for 15 min, after which calcein-AM was added to a final concentration of 1 μM. Next, the plate was incubated for 60 min at 28 °C and then placed on ice to slow activity of esterase enzymes, and each well was washed three times with dechlorinated water warmed to 28 °C. Accumulation of calcein was determined by quantifying fluorescence inside fry by use of a Zeiss Axio ObserverZ1 inverted microscope (Zeiss, Toronto, Ontario, Canada) with an AxioCam ICc1 camera (excitation 470, emission 525 nm at 5× magnification). All fish were imaged from a dorsal viewpoint. Images were captured by use of AxioVision software V.4.8 (Zeiss, Toronto, Ontario, Canada) and analyzed by use of ImageJ software V.1.48 (NIH, Bathesda, Maryland, USA). Corrected total fluorescence (CTF), a measure which was corrected for size of the sample, was used as the measure of accumulation of calcein (Formula 1). CTF was a function of integrated density (an extensive quantity and product of area and mean gray value), area of sample (the size of the selected fish), and mean fluorescence of background (calculated by determining the mean of intensities of fluorescence of the background of the image). Two independent experiments were conducted and each exposure was replicated 4 times per experiment.

$$CTF = IntegratedDensity - (AreaofSelection \times MeanGreyValue) \quad \text{(Formula1)}$$

2.6. Analysis of data

Effects of OSPW on survival of embryos and accumulation of calcein in fry was 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 was determined by use of Levene's test. If necessary, data were $\log_{10}$ transformed to ensure normality and homogeneity of variance. Effects of treatments relative to controls were evaluated by use of one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at a ρ-value <0.05. Effects of samples of OSPW on survival of fry were compared to effects of the solvent. Effects of samples of OSPW on accumulation of calcein were compared to effects on fry exposed to calcein-AM without inhibitors. Fluorescence of fry exposed to calcein-AM dissolved in DMSO was not significantly different from fluorescence of fry exposed only to DMSO.

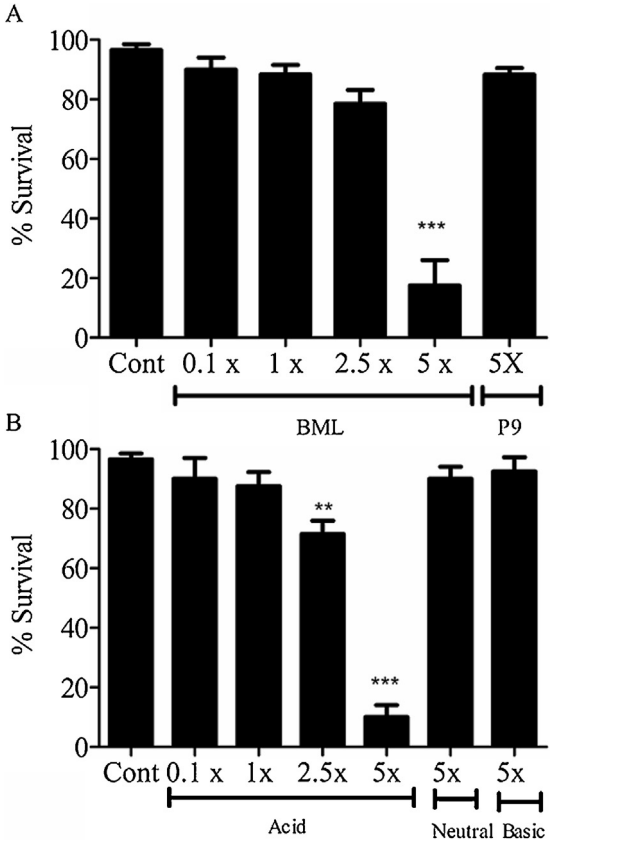

Fig. 1. Survival of fry of Japanese medaka exposed to (A) 0.1, 1, 2.5, or 5× equivalent of the organic fraction of BML-OSPW or a 5× concentration of the organic fraction of P9-OSPW, and (B) 0.1, 1, 2.5, or 5× equivalent of the acidic fraction of BML-OSPW or a 5× concentration of the neutral fraction or basic fraction of BML-OSPW. Survival of fry is expressed as mean ± standard deviation of three independent studies in which there were three replicate exposures with 10 fry per replicate. Significant differences in survival compared to control were determined by use of one-way ANOVA followed by Dunnett's post-hoc test and are designated by an asterisk. (* = $P \leq 0.05$, ** = $P \leq 0.01$, and *** = $P \leq 0.001$).

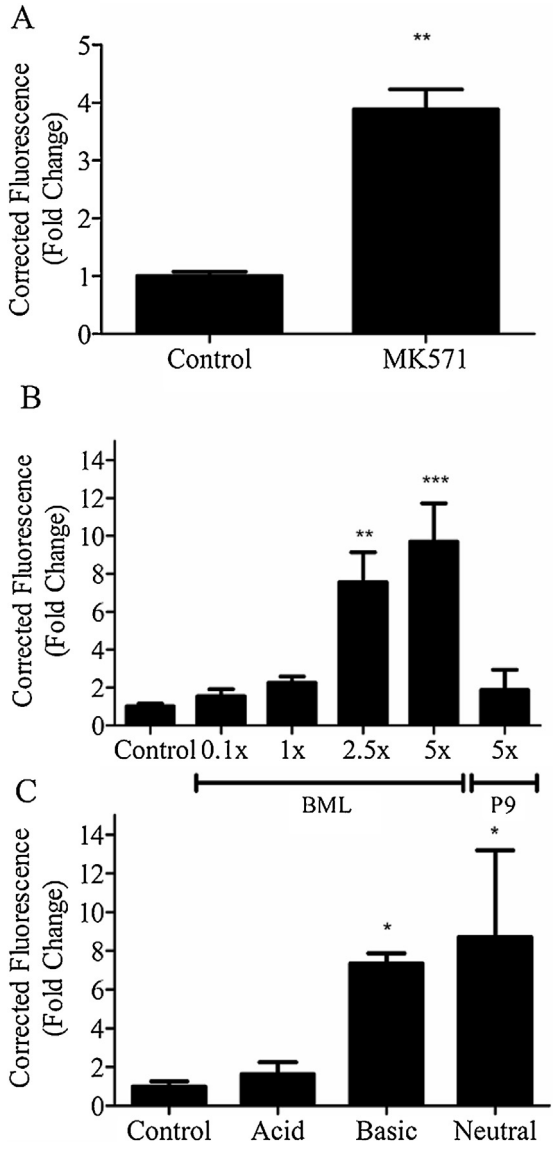

Fig. 2. Accumulation of calcein in fry of Japanese medaka exposed to (A) MK-571 (B) 5×, 2.5×, 1×, and 0.5× equivalents of the pooled organic fraction of BML-OSPW and 5× equivalent of the pooled organic fraction of P9-OSPW, and (C) 5× equivalents of acidic, basic and neutral fractions of BML-OSPW. Fry were exposed to (5 μM) MK-571 and OSPW for 15 min prior to co-exposure to calcein-AM for 1 h. Fry used as controls were exposed to embryo rearing medium containing 0.1% v/v DMSO. Accumulation of calcein was measured as fluorescence and data is expressed as mean ± standard deviation of two independent experiments in which the number of replicates per experiment was four. Differences that are statistically significant from the control are indicated by an asterisk. (* = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$).

3. Results

3.1. Acute lethality

Exposure to organic chemicals extracted from the water soluble organic phase of BML-OSPW affected survival of fry of Japanese medaka. No effects on survival were observed at 6 h of exposure to the pooled, acidic, neutral, or basic fraction of BML-OSPW (data not shown). Survival of fry exposed for 24 h to a 5× equivalent of the pooled fraction of BML-OSPW was significantly lesser (17.5 ± 8.5%) compared to the solvent control (96.55 ± 1.99%) (Fig. 1A). Neither 0.5, 1, nor 2.5× equivalent of the pooled organic fraction of BML-OSPW affected survival of fry. Survival of fry exposed to 2.5 or 5× equivalent of the acidic fraction of BML-OSPW was 71.44 ± 4.5% and 10 ± 4.2%, respectively (Fig. 1B). Neither the neutral nor basic fraction of BML-OSPW affected survival of fry at either of the equivalent concentrations tested. Survival of fry exposed to a 5× equivalent that of the pooled organic fraction of P9-OSPW was not different from the solvent control (Fig. 1A) at the equivalent concentrations tested.

3.2. Effects of OSPW on accumulation of calcein in fry

Exposure to organic compounds from the water soluble phase of BML-OSPW inhibited efflux of calcein from fry. The amount of calcein in fry exposed to MK-571 was 3.88 ± 0.34-fold greater compared to fry exposed to the solvent control (Fig. 2A). Amounts of calcein in fry exposed to 2.5 or 5× equivalent of the pooled fraction of BML-OSPW were 7.6 ± 1.6- and 9.7 ± 2.0-fold greater, respectively, compared to fry exposed to the solvent control (Fig. 2B), but amounts of calcein in fry exposed to 0.5 or 1× equivalent of the pooled fraction of BML-OSPW were not different from the solvent control. The amount of calcein was not greater in fry exposed to a 5× equivalent of the pooled fraction of P9-OSPW (Fig. 2B). Amounts of calcein in fry exposed to 5× equivalent of the neutral or basic fraction of BML-OSPW were 8.2 ± 4.4- and 7.5 ± 0.25-fold greater, respectively, compared to solvent controls (Fig. 2C). The amount of calcein in fry exposed to the acidic fraction of BML-OSPW was not different from the solvent control (Fig. 2C).

3.3. Comparison of chemicals in fractions of BML-OSPW

As a qualitative first step to identify chemicals in BML-OSPW that might have inhibited activity of MRPs, the profile and relative intensities of chemicals in neutral and basic fractions, which inhibited efflux of calcein from fry, were compared to relative intensities of chemicals in the acid fraction, which did not inhibit efflux of calcein. In ESI^- mode, which detects organic acids, chemical species containing oxygen and chemical species containing sulfur were more abundant in the acidic fraction than in the neutral or basic fraction, while chemical species containing nitrogen were not detected in either fraction (Fig. 3A). Intensity of NAs, which are O_2^- chemical species detected in the acidic fraction by use of ESI^- mode, was 3.0- and 8.9-fold greater in the acidic fraction compared to the neutral and basic fraction, respectively. Also, intensity of O_3^- and O_4^- chemical species was much greater in the acidic fraction than in the neutral or basic fraction, and O_5^- chemical species were detected only in the acidic fraction.

Polar organic neutral and basic compounds were detected by use of ESI^+ mode (Fig. 3B). Chemical species containing oxygen, sulfur, or nitrogen were detected in each fraction, but there were differences in intensities among fractions. Intensities of chemical species containing oxygen was greater in the acidic fraction and neutral fraction compared to the basic fraction. The most abundant chemical species in the neutral fraction was O_2^+, and intensity was 1.4- and 51-fold greater than in the acidic and basic fractions, respectively. These chemical species are not NAs, but might be dihydroxy, diketo, or ketohydroxy compounds (Pereira et al., 2013). The greatest intensity of any chemical species in either fraction was of SO_3^+ in the acidic fraction. S^+ and SO^+ chemical species were detected in the neutral and basic fractions but were not detected in the acidic fraction. The greatest number of chemical species containing nitrogen were detected in the basic fraction. Specifically, NO^+, NO_2^+, NO_3^+, NO_4^+, and NO_2S^+ were detected in the basic fraction. The NO^+ chemical species was detected in neutral and basic fractions, and intensity in the neutral fraction was approximately 9-fold greater in the basic fraction. The only chemical species containing nitrogen in the acidic fraction was NO_3^+, and intensity was greater than in either the neutral or basic fraction.

3.4. Comparison of chemicals in fractions of BML-OSPW and P9-OSPW

Whereas the amount of calcein in fry exposed to either the basic or neutral fraction of BML-OSPW was significantly greater than that in controls, neither the basic nor neutral fraction of P9-OSPW caused this effect. Therefore, the profile and intensities of chemicals in these basic and neutral fractions from BML-OSPW were compared to the basic and neutral fractions of P9-OSPW to qualitatively identify chemicals that might inhibit MRPs. Chemicals in the acidic fraction of BML-OSPW and P9-OSPW were not compared because calcein dye did not accumulate in fry exposed to the acidic fraction of BML-OSPW. The comparison was limited to chemical species detected by use of ESI^+ because the majority of chemical species detected by use of ESI^- were in the acidic fraction.

There were differences in intensity of chemical species containing oxygen between the neutral and basic fraction of BML-OSPW and P9-OSPW. The greatest difference was that intensity of O^+ species was approximately 8-fold greater in the neutral fraction of BML-OSPW compared to the neutral fraction of P9-OSPW. This species was detected in the basic fraction of P9-OSPW but not BML-OSPW. Intensity of O_2^+ species was greater in both fractions of BML-OSPW, but the difference was less than 2-fold. In contrast, intensity of O_3^+ was approximately 5-fold greater in the neutral and basic fraction of P9-OSPW compared to BML-OSPW.

Intensities of chemical species containing sulfur were greater in neutral and basic fractions of BML-OSPW compared to P9-OSPW (Fig. 4). The most abundant chemical species containing sulfur in either fraction were SO^+ and SO_2^+. Intensities of these species in the neutral fraction of BML-OSPW were 7.43- and 1.98-fold greater, respectively, than in the neutral fraction of P9-OSPW. Neither chemical species containing sulfur that was detected in the basic fraction of BML-OSPW was detected in the basic fraction of P9-OSPW (Fig. 4).

The most pronounced difference between the neutral and basic fractions of BML-OSPW and P9-OSPW was the difference in the number and intensities of species containing nitrogen (Fig. 4). For both samples of OSPW, a greater number of chemical species containing nitrogen were detected in the basic fraction compared to the neutral fraction. Most chemical species containing nitrogen that were detected in the basic fraction of BML-OSPW were detected in the basic fraction of P9-OSPW, but the intensity was much greater in BML-OSPW. The most abundant chemical species containing nitrogen in the basic fraction of BML-OSPW was NO^+, and the intensity was 5-fold greater than in P9-OSPW. The exception was that intensity of NO_2^+ was greater in the neutral fraction of P9-OSPW than BML-OSPW, and NO_3^+ was detected in the neutral fraction of P9-OSPW but not BML-OSPW. NO_2S^+ was detected only in the basic fraction of BML-OSPW (Fig. 4B).

4. Discussion

Results of this study suggest that activity of MRPs, which are members of the ATP-binding cassette (ABC) superfamily of membrane-transporter proteins, might be inhibited by organic compounds dissolved in OSPW. Previous studies have shown that OSPW affects expression of genes encoding members of the ABC superfamily of transporters (Wiseman et al., 2013b; Gagné et al., 2012). However, this is the first study to demonstrate that activity of a specific family of ABC transporters, the MRPs, is inhibited by organic compounds dissolved in OSPW.

Inhibition of MRPs by constituents of the water soluble organic fraction of OSPW could lead to accumulation of chemicals, and their metabolites, which could cause adverse effects on aquatic organisms. Numerous studies have shown that inhibition of the activity of ABC transporters might exacerbate bioaccumulation and toxicity of chemicals, a process known as chemosensitization (Smital and Kurelec, 1997; Kurelec et al., 2000; Ferreira et al., 2014; Kurth et al., 2015). As an example, cytotoxicity of the anticancer drug paclitaxel was greater in the mammalian cell line caco-2 exposed to long-chain polyunsaturated fatty acids that inhibit activity of *p*-glycoprotein (*P*-gp), which is a member of the ABC superfamily (Kuan et al., 2011). Also, inhibitors of *P*-gp increased the cytotoxicity of several drugs to embryos of the innkeeper worm (*U. caupo*) (Toomey and Epel, 1993). With respect to tailings ponds, inhibition of MRPs by chemicals in the water soluble organic fraction of OSPW could inhibit efflux of PAHs and their metabolites from cells, and this could result in greater toxicity. Bioaccumulation of benzo[a]pyrene (BaP) was greater in catfish exposed to the surfactant C-12 linear alkylbenzenesulfonate (LAS), and it was hypothesised that inhibition of *P*-gp by LAS might have been a mechanism of this effect (Tan et al., 2010). Mortality of embryos of zebrafish co-exposed to inhibitors of an ABCB4 protein and the PAH phenanthrene was greater compared to embryos exposed only to phenanthrene (Fischer et al., 2013). Also, DNA damage in MCF-7 cells co-exposed to BaP and inhibitors of ABC proteins was greater than in cells exposed only to BaP (Myllynen et al., 2007). PAHs, which exist primarily in their alkylated form in oil sands materials, can exert adverse effects on fishes (Lin et al., 2014). While the metabolism and clearance of alkylated PAHs has not been eluci-

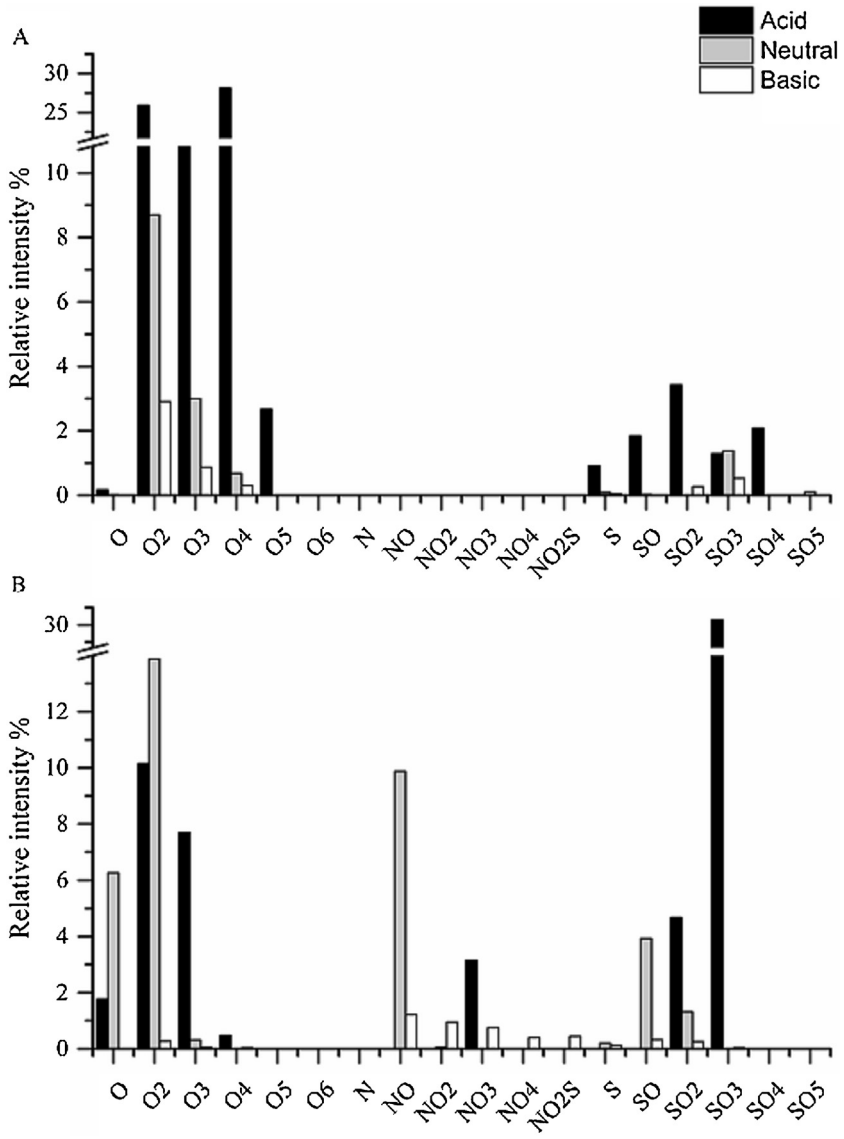

Fig. 3. Elemental composition and relative intensity of water-soluble chemicals in acidic, basic and neutral fractions of BML-OSPW acquired in (A) ESI^- and (B) ESI^+.

dated, glucuronic and glutathione conjugated metabolites of PAHs are excreted from cells by MRPs (Hessel et al., 2013; Srivastava et al., 2002). Thus, inhibition of MRPs might enhance toxicity of PAHs associated with tailings ponds and other oil sands materials, and future studies should be designed to investigate this potential mechanism of toxicity. These studies also should investigate the mechanism of chemosensitization by constituents of OSPW. Chemosensitizers can inhibit efflux of compounds either by competitive inhibition or by other mechanisms such as blocking the ATPase activity, disruption of phosphorylation of the transport protein, or alteration of membrane fluidity and permeability (Ferreira et al., 2014; Kurth et al., 2015).

Identities of specific chemicals in BML-OSPW that inhibit MRPs are not known. Activity of ABC transporters can be inhibited by structurally unrelated compounds, including non-ionic surfactants (Bogman et al., 2003), dietary fatty acids (Kuan et al., 2011), and biodegraded petroleum hydrocarbons (Hamdoun et al., 2002). Because the acidic fraction of BML-OSPW was composed primarily of chemicals containing oxygen or sulfur that were detected by use of ESI^-, which detects organic acids, results of the current study suggest that acids, including NAs (O_2^-), in BML-OSPW likely do not inhibit activity of MRPs. Several chemicals detected by use of ESI^+, which is used to detect polar organic neutral and basic compounds, were more abundant in the neutral and basic fractions of BML-OSPW compared to both the acidic fraction of BML-OSPW and the neutral and basic fraction of P9-OSPW, and therefore might cause inhibition of MRPs. Specifically, S^+, SO^+, NO^+, and NO_2^+ were detected in the neutral and basic fraction of BML-OSPW but not in the acidic fraction of BML-OSPW. Also, these chemicals were present in the neutral and basic fractions of P9-OSPW but at lesser relative intensities compared to BML-OSPW. In addition, O^+ in the neutral fraction of BML-OSPW, and NO_3^+, NO_4^+, NO_2S^+, SO_2^+ in the basic fraction of BML-OSPW were more abundant than in P9-OSPW. Properties of some of these chemicals are consistent with several properties of chemosensitizers, including positive charge at physiological pH, and the presence of a basic nitrogen atom (Ecker et al., 1999; Kurth et al., 2015). Also, based on partitioning to polydimethylsiloxane (PDMS) coated stir bars, it was determined that O^+, SO^+, and NO^+ are among the most hydrophobic (i.e., apparent K_{ow} up to 203,000 for SO+ species), and therefore have the greatest potential to bioaccumulate, of any chemicals in the aqueous phase of OSPW (Zhang et al., 2015). Moderate to high lipophilicity is a

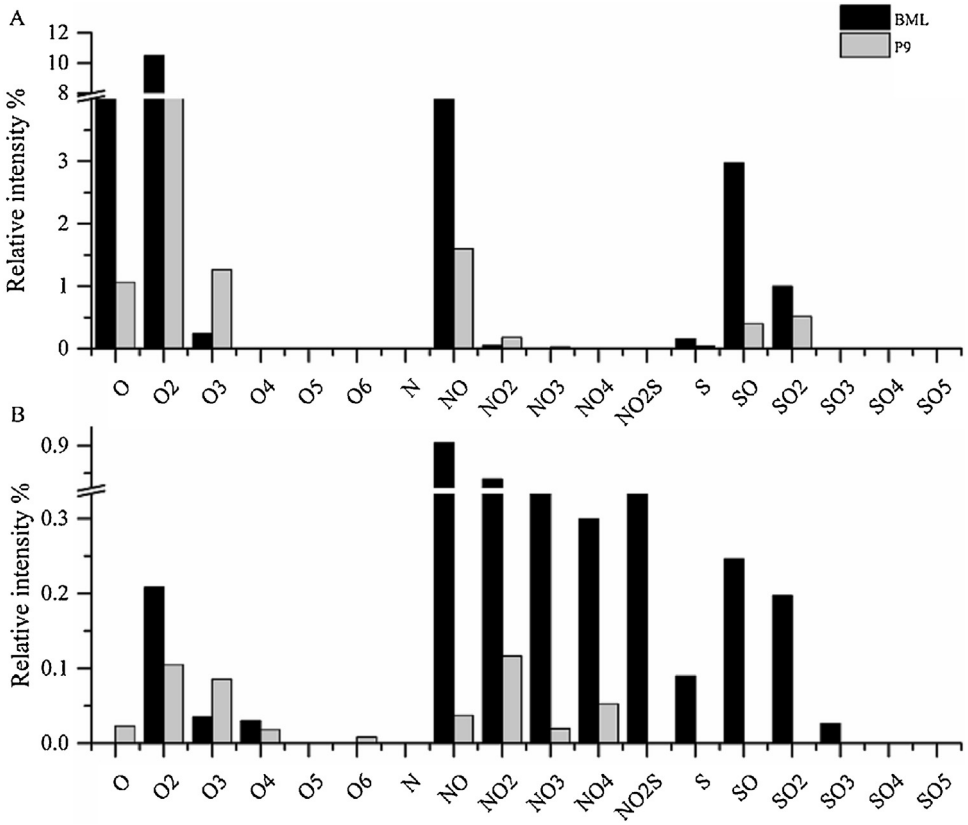

Fig. 4. Comparison of the elemental composition and relative intensity of species containing sulfur or nitrogen in (A) Basic and (B) Neutral fractions of BML-OSPW and P9-OSPW acquired in ESI^+ mode.

property of chemosensitizers (Ecker et al., 1999; Kurth et al., 2015). Additional fractionation steps are required to determine whether each, or a subset, of these compounds inhibits MRPs.

This study is the first to report inhibition of ABC transporters by organic compounds in OSPW. The data suggest that at concentrations currently occurring in BML or Pond 9, organics extracted from OSPW would not be expected to have adverse effects on fish in short-term exposures. However, indirect effects of these organics in the presence of other chemicals (ie. PAHs) from tailings ponds have not been investigated. Also, because some chemicals identified as potentially inhibiting MRPs have the greatest potential for bioaccumulation, long-term effects on organisms that might inhabit tailings ponds under future reclamation scenarios, such as end-pit lakes, should be investigated.

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