Research article

Journal of Applied Toxicology

Received: 24 June 2016, Revised: 6 September 2016, Accepted: 6 September 2016 Published online in Wiley Online Library: 24 October 2016

(wileyonlinelibrary.com) DOI 10.1002/jat.3397

Toxicokinetics and toxicodynamics of chlorpyrifos is altered in embryos of Japanese medaka exposed to oil sands process-affected water: evidence for inhibition of P-glycoprotein

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ABSTRACT: Oil sands process-affected water (OSPW) is generated during extraction of bitumen in the surface mining oil sands industry in Alberta, Canada. Studies were performed *in vitro* by use of Caco-2 cells, and *in vivo* with larvae of Japanese medaka (*Oryzias latipes*) to determine if organic compounds from the aqueous phase of OSPW inhibit ATP binding cassette protein ABCB1 (permeability-glycoprotein, P-gp). Neutral and basic fractions of OSPW inhibited activity of P-gp in Caco-2 cells by 1.9- and 2.0-fold, respectively, while the acidic fraction had the least effect. The organophosphate pesticides chlorpyrifos (a substrate of P-gp) and malathion (not a substrate of P-gp), were used as model chemicals to investigate inhibition of P-gp in larvae. Co-exposure to chlorpyrifos and an extract of OSPW containing basic and neutral compounds reduced survival of larvae to 26.5% compared to survival of larvae exposed only to chlorpyrifos, which was 93.7%. However, co-exposure to malathion and the extract of OSPW did not cause acute lethality compared to exposure only to malathion. Accumulation and bioconcentration of chlorpyrifos, but not malathion, was greater in larvae co-exposed with the extract of OSPW. The terminal elimination half-life of chlorpyrifos in larvae exposed to chlorpyrifos in freshwater was 5 days compared with 11.3 days in larvae exposed to chlorpyrifos in OSPW. Results suggest that in non-acute exposures, basic and neutral organic compounds in the water-soluble fraction of OSPW inhibit activity of P-gp, which suggests that OSPW has the potential to cause adverse effects by chemosensitization. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: Bitumen; naphthenic acid; chemosensitization; chlorpyrifos; tailings ponds; ABC protein

Introduction

Extraction of bitumen in the surface mining oil sands industry in northern Alberta, Canada, generates liquid fine tailings that are held in tailings ponds and recycled for use in the extraction of bitumen. As fine tailings settle a layer of mature fine tailings is formed at the base of tailings ponds, leaving behind an aqueous layer of oil sands process-affected water (OSPW) (Allen, 2008). Because companies do not discharge the contents of tailings ponds to the ambient environment there is more than 1 billion m^3 of OSPW currently being held in tailings ponds, and this volume will increase as surface mining continues (Government of Alberta, 2011). Chemical compositions in tailings ponds are complex. In addition to a variety of metals and salts, more than 3000 elemental compositions containing oxygen (O_x), sulfur (SO_x) and nitrogen (NO_x) have been detected in the water-soluble organic fraction of OSPW by use of ultrahigh resolution mass spectrometry (Barrow *et al.*, 2010; Grewer *et al.*, 2010; Pereira *et al.*, 2013). In addition, polycyclic aromatic hydrocarbons (PAHs) and dibenzothiophenes are constituents of tailings ponds and are associated primarily with fine tailings (Galarneau *et al.*, 2014; Madill *et al.*, 1999; Rogers *et al.*, 2002).

Exposure to OSPW causes a variety of toxicities. Disruption of sex steroid synthesis and signaling (He *et al.*, 2010, 2011, 2012a; Leclair *et al.*, 2015), impaired reproduction of fish (Kavanagh *et al.*, 2012), immunotoxicity (MacDonald *et al.*, 2013) and impairment of growth and development of invertebrates (Anderson *et al.*, 2012) and fish (He *et al.*, 2012b) have been reported. Recently, a novel mechanism by which OSPW might cause adverse effects was reported. Specifically, dissolved organic compounds in OSPW inhibit activity of the ATP-binding cassette (ABC) superfamily proteins (Alharbi *et al.*, 2016a). Members of the ABC superfamily of transporters are involved in a variety of

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processes, including transport of endogenous and exogenous chemicals across biological membranes (Epel *et al.*, 1998; 2008; Kurelec, 1992; Leslie *et al.*, 2005; Luckenbach *et al.*, 2014; Schinkel & Jonker, 2003). ABC transporters are important in the defense against xenobiotic compounds by mediating the active efflux of parent compounds or products of their phase II biotransformation (Luckenbach *et al.*, 2014). For example, multidrug resistance proteins (MRPs), which are members of the ABC superfamily of proteins, remove glutathione-conjugated metabolites of PAHs from cells (Hessel *et al.*, 2013; Kranz *et al.*, 2014). However, numerous chemicals that are structurally diverse can competitively or non-competitively inhibit activity of ABC transporters, causing greater bioaccumulation of substrates of ABC transporters and greater sensitivity to these substrates, a phenomenon known as chemosensitization (Kurelec, 1997; Kurth *et al.*, 2015; Luckenbach *et al.*, 2014). Therefore, there is the potential for compounds that might otherwise be considered as not posing a threat to the health of organisms to cause adverse effects, particularly when they occur as part of complex mixtures with toxic compounds that are substrates of ABC proteins (Epel *et al.*, 2008; Kurelec, 1997). Although numerous chemicals are chemosensitizers, whether chemosensitization is a mechanism of toxicity at environmentally relevant concentrations of chemicals is not known (reviewed in Kurth *et al.*, 2015).

The permeability-glycoprotein (P-gp or ABCB1) is the most studied ABC transporter and understanding of chemosensitization is based mostly on results of studies of P-gp (Luckenbach *et al.*, 2014). Efflux activity of P-gp protects cells from the effects of a variety of natural and anthropogenic xenobiotics that are diverse in their physiochemical properties (Leslie *et al.*, 2005). Inhibition of this transporter is of toxicological importance as any disruption of its functioning can enhance toxicities of compounds that are substrates of P-gp. For example, mortality of zebrafish (*Danio rerio*) embryos increased when they were co-exposed to inhibitors of P-gp and the environmentally relevant PAH, phenanthrene or the anticancer drug vinblastine (Fischer *et al.*, 2013). Similarly, the mortality of zebrafish embryos was greater when they were exposed to vinblastine and perfluorooctane sulfonate, which inhibits P-gp (Keiter *et al.*, 2016).

It has been proposed that chemicals in the water-soluble organic fraction of OSPW might act as chemosensitizers by inhibiting activity of ABC transporters but it was not known if these chemicals inhibit P-gp (Alharbi *et al.*, 2016a). Therefore, the goal of this study was to use *in vitro* and *in vivo* methods to determine if compounds in the water-soluble organic fraction of OSPW inhibit P-gp and cause chemosensitization to substrates of P-gp. First, the effects of organic compounds extracted from the aqueous phase of OSPW on accumulation of the fluorescent dye calcein-AM, which is a substrate of P-gp, was quantified in Caco-2 cells. Second, the effects of organic compounds extracted from the aqueous phase of OSPW on uptake, depuration and toxicity of the organophosphate pesticides, chlorpyrifos and malathion, to larvae of Japanese medaka was determined. Although chlorpyrifos or malathion are not of toxicological relevance in the context of oil sands mining or tailings ponds used to hold OSPW, these model chemicals were used to investigate P-gp inhibition and chemosensitization by OSPW. Cytochrome P450 enzymes convert chlorpyrifos to a toxic metabolite, chlorpyrifos oxon, which causes acute lethality by inhibition of acetylcholine esterase (Fukuto, 1990). P-gp affords protection from acute lethality by active efflux of chlorpyrifos (Zaja *et al.*, 2011) and chlorpyrifos oxon (Lanning, 1996). Therefore, it was hypothesized that inhibition of P-gp by OSPW would cause an increase in concentrations of chlorpyrifos and therefore an increase in chlorpyrifos oxon in cells, which would result in greater incidence of acute lethality. Malathion causes acute lethality by the same mechanism, but it is not a substrate of P-gp (Zaja *et al.*, 2011). Therefore, inhibition of P-gp by OSPW would not be expected to cause an increase in concentrations of malathion in cells and therefore would not cause an increase in acute lethality.

Materials and methods

Chemicals and oil sands process-affected water samples

Verapamil was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA) and calcein-AM was from AAT Bioquest (Sunnyvale, CA, USA). Dimethylsulfoxide (DMSO) and trypan blue were from the Sigma Chemical Company (Oakville, ON, Canada). Caco-2 cells (human colon adenocarcinoma cells) were purchased from the American Type Culture Collection (Rockville, MD, USA). Fetal bovine serum (product no. F6178) that was sterile filtered was purchased from the Sigma Chemical Company. Penicillin/streptomycin, 10 mM minimal essential medium non-essential amino acids, 100 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin streptomycin, phosphate-buffered saline, Hanks' balanced salt solution, trypsin-EDTA (0.25% trypsin with 1 mM EDTA.4Na) and Dulbecco's modified Eagle's medium with high glucose, L-glutamine, pyruvate and phenol red (product no. 11995-065) were purchased from ThermoFisher Scientific (Burlington, ON, Canada). Chlorpyrifos, chlorpyrifos oxon, malathion and malathion-d_{10} standards, each of which was >98% purity, were purchased from AccuaStandard (New Haven, CT, USA). Acetone, hexane, dichloromethane and ethyl-acetate, each of high-performance liquid chromatography grade, were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was obtained from GreenField Ethanol Inc. (Brampton, ON, Canada). OSPW was collected from Base Mine Lake, 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 new inflow of fresh OSPW from the extraction plant was ceased (Syncrude Canada, Ltd., Fort McMurray, AB, Canada). Samples were collected during September 2012 and shipped to the University of Saskatchewan (Saskatoon, SK, Canada), where, upon arrival, they were inspected visually and observed to be free of any residual bitumen or mature fine tailings. OSPW was stored in the dark until extraction to prevent any potential photomodification of dissolved organic compounds.

In vitro assays

Extraction of dissolved organic chemicals from oil sands process-affected water. Acidic, basic and neutral fractions of OSPW were generated by use of a method that has been described previously (Alharbi *et al.*, 2016a). Because the goal of the study was to investigate inhibition of P-gp by chemicals in the water-soluble organic fraction of OSPW, a 500 ml sample of OSPW was passed through a Whatman® glass microfiber filter (GF/D 0.47 mm; Fisher Scientific) to remove particulates. Next, the sample was acidified to pH 2 by use of concentrated HCl (37%). To isolate the basic fraction, cartridges containing 500 mg of mixed-mode Strata®-X Polymeric-C solid-phase sorbent (Phenomenex, Milford, MA, USA) were conditioned with 6 ml

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methanol and 6 ml acidified water and OSPW that had been acidified to pH 2 was passed through cartridges under vacuum. Cartridges then were washed with 2% of formic acid and dried under vacuum for 30 min. Acidic and neutral compounds were isolated in the first elution with methanol. The second elution was performed with 5% (*v*/v) of NH_4OH in methanol and this fraction contained basic compounds. Acidic and neutral compounds from the first elution were separated by use of 500 mg of Strata®-X-A solid-phase matrix in plastic cartridges (Phenomenex). Before use, cartridges were conditioned by washing with 100% methanol followed by 5% (v/v) of NH_4OH (aq.). Eluant I from the Strata®-X Polymeric-C sorbent (acidic and neutral compounds) was evaporated to approximately 0.5 ml, adjusted to pH 10–11 with NaOH, and then passed through the Strata®-X-A solid-phase matrix by use of gravity. The cartridge was washed with 5% (v/v) of NH_4OH (aq.) and left to dry under vacuum for 30 min. A fraction containing neutral compounds was eluted with 100% of methanol and a fraction containing acidic compounds was eluted with 2% (v/v) of formic acid in methanol. Each fraction was dried under a gentle stream of nitrogen and reconstituted in 500 μl of absolute ethanol to generate a sample in which the concentration of organics was enriched to an equivalent of 1000× greater than in the original sample of OSPW. A pooled sample representative of the organic fraction was generated by pooling equal volumes of the acidic, neutral and basic fractions. The profile of heteroatom classes in these fractions has been described previously (Alharbi *et al*., 2016a). These samples were used in the calcein-Am assay that was performed in Caco-2 cells.

Cytotoxicity. The human colon adenocarcinoma cell line (Caco-2) is the most common *in vitro* model used to investigate substrates and inhibitors of P-gp (Balimane & Chong, 2005; Elsby *et al*., 2008; Siissalo *et al*., 2007). Effects of OSPW on viability of Caco-2 cells were quantified to identify sublethal concentrations that could be used to determine inhibition of P-gp. Cell viability was determined by use of the CellTiter 96® AQueous One Cell Proliferation Assay (MTS) according to the protocol provided by the manufacturer (Promega Corporation, Madison, WI, USA). Caco-2 cells (5000 cells per well) were seeded into each well of a 96-well plate and placed in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h the medium, which was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, was replaced with medium containing 0.5, 1, 2.5 or 5× equivalent of the pooled sample of OSPW or 5× equivalent of the acidic, basic or neutral fraction. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 1 or 24 h. The concentration of ethanol in exposure solutions was 0.1% (*v*/v) and controls were performed to ensure this concentration was not cytotoxic. At the end of the exposure period, cells were washed with fresh media and incubated with the MTS solution in a humidified atmosphere of 5% CO_2 for 4 h at 37 °C. Viability of cells was quantified using a spectrophotometric Elx800TM microplate reader (BioTek Instruments, Winooski, VT, USA). Three independent experiments were conducted and there were eight replicates ($n = 8$) per experiment.

Effect of oil sands process-affected water on accumulation of calcein in Caco-2 cells to assess inhibition of the permeability-glycoprotein. An assay with the fluorescent dye, calcein-AM, was used to determine the effects of the acidic, basic and neutral fractions on activities of P-gp in Caco-2 cells, based on a method described elsewhere (Volpe, 2011). Caco-2 cells overexpress P-gp and other membrane transporters comparable to the human small intestine (Englund *et al*., 2006). Calcein-AM, which is the acetoxymethyl ester of calcein, enters cells by passive diffusion and is hydrolyzed enzymatically into the fluorescent dye calcein by esterases. Because calcein is transported from cells by ABC transporters, including inhibition of activity of P-gp, for example by verapamil, this results in accumulation of calcein that can be measured as an increase of intracellular fluorescence (Bansal *et al*., 2009; Eneroth *et al*., 2001; Glavinas *et al*., 2011). Briefly, 5000 cells were seeded in each well of a 96-well black plate with clear bottoms (Thermo Scientific). Cells were incubated for 48 h in a humidified atmosphere of 5% CO_2 at 37 °C to maintain 80–90% confluence. The medium was replaced after 24 and 48 h. Medium was removed and cells were washed with phosphate-buffered saline warmed to 37 °C, then cells were exposed for 15 min in a humidified atmosphere of 5% CO_2 at 37 °C to either 10 μM of verapamil dissolved in DMSO (positive control), 0.5, 1, 2.5 or 5× equivalent of the pooled organic fraction, or 5× equivalent of fractions of OSPW. Cells were exposed to either DMSO or ethanol at a final concentration of 0.1% to control for solvent effects. Next, cells were washed twice with media warmed to 37 °C, then 200 μl of media containing samples of OSPW or verapamil and calcein-AM was added and plates were incubated for 60 min in a humidified atmosphere of 5% CO_2 at 37 °C. Fluorescence of calcein (excitation 485 nm, emission 538 nm) was recorded immediately by use of a microtiter plate reader (Elx800TM microplate reader; BioTek Instruments). The accumulation of calcein in cells was calculated by subtracting the background fluorescence measured in a blank well and the fold-change in fluorescence caused by exposure to fractions was calculated compared to the solvent control.

In vivo assays

Isolation of basic and neutral compounds from oil sands process-affected water. The method used to prepare OSPW for *in vivo* assays was different from the method used to generate fractions of OSPW for use in Caco-2 cells. Because results of the *in vitro* assay indicated that basic and neutral compounds, but not acidic compounds, in the water-soluble organic fraction of OSPW inhibit P-gp, a method that isolates basic and neutral compounds into one fraction and contains smaller amounts of acidic compounds was used to prepare samples for assays with embryos (Vieno *et al*., 2006). Details of this method have been described previously (Alharbi *et al*., 2016b). Briefly, 1000 ml 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 NH_4OH. Pre-concentration of samples was performed in one generic step by use of EVOLUTE®ABN sorbent (Biotage, Charlotte, NC, USA). Before addition of OSPW, two cartridges were conditioned with 6 ml of methanol followed by 6 ml of ultrapure Milli-Q water (Millipore, Mississauga, Canada). Next, 500 ml of OSPW was passed through each cartridge under vacuum at a flow rate of 10–15 ml min^{-1}. At 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 the use of a vacuum. Samples were pooled, evaporated to dryness under a gentle stream of nitrogen and reconstituted in 500 μl of absolute ethanol. Therefore, the concentration of dissolved organic compounds in the final sample was 2000× greater than in the original sample of OSPW. The profile of heteroatom classes in these fractions has been described previously (Alharbi *et al*., 2016b).

Acute lethality. Embryos of Japanese medaka (orange–red strain) were collected from a culture 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). Protocols for culturing of fish were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (UCACS-AREB; no. 20090108).

Effects of OSPW on acute lethality of chlorpyrifos and malathion were quantified. Larvae at 8 ± 1 dpf were exposed for 24 or 48 h to 100 or 250 μg l^{-1} chlorpyrifos or 15 mg l^{-1} malathion, either in freshwater or in 1 or 2.5× equivalent of OSPW. Acute lethality of larvae exposed only to 2.5× equivalent of OSPW also was quantified. Exposures were performed in six-well tissue culture plates with 5 ml of solution in each well. Each exposure consisted of 10–12 larvae and all exposures were conducted five times ($n = 5$) each with a new batch of larvae.

Body burden of chlorpyrifos and malathion. Concentrations of chlorpyrifos, chlorpyrifos oxon and malathion were quantified in larvae to assess the effect of OSPW on body burden. Larvae at 8 ± 1 dpf were exposed to 100 μg l^{-1} chlorpyrifos or malathion either in freshwater or in 1× equivalent of OSPW because these exposure conditions did not cause acute lethality. Exposures of larvae to chemicals was performed in six-well tissue culture plates with 5 ml of solution per well. Each exposure consisted of 30 larvae and all exposures were conducted in triplicate ($n = 3$), each with a new batch of larvae. Ten larvae and 1 ml of solution were sampled at 24 h of exposure. Next, larvae exposed only to chlorpyrifos or malathion were transferred to a solution of freshwater and larvae exposed to chlorpyrifos or malathion in 1× equivalent of OSPW were transferred to a solution of 1× equivalent of OSPW for 24 h to assess depuration. Ten larvae and 1 ml of solution was sampled at the end of the depuration period. Samples were stored at −20 °C until required for analysis.

Kinetics of bioaccumulation and depuration of chlorpyrifos. Effects of OSPW on kinetics of uptake and depuration of chlorpyrifos were determined. Protocols used in this assay have been described previously (El-Amrani *et al.*, 2012). The concentration of chlorpyrifos was 10 μg l^{-1}, the same concentration used previously to quantify bioaccumulation of chlorpyrifos in larvae of zebrafish (El-Amrani *et al.*, 2012). This concentration is less than 1% of the LC_{50} of chlorpyrifos and therefore meets recommendations for assessing bioaccumulation in fish (OECD, 2012). Approximately 100 larvae at 8 ± 1 dpf were placed in glass petri dishes (100 × 15 mm) containing 50 ml of chlorpyrifos either in freshwater or 1× equivalent of OSPW. Larvae were collected at 0, 2, 6, 12, 21, 29, 45 and 48 h of exposure during the uptake phase. For the depuration phase, larvae were transferred to petri dishes containing 50 ml of freshwater or 1× equivalent of OSPW and larvae were sampled at 50, 54 and 72 h. Larvae were killed by use of hypothermic shock in ice water (Strykowski & Schech, 2015). Ten larvae and 1 ml of solution were collected at each time point and larvae were stored at −20 °C until required for analysis. No mortality was observed during the uptake or depuration phases of the study. Bioaccumulation and depuration of chlorpyrifos was calculated using a model that uses first-order kinetics to describe uptake and depuration (El-Amrani *et al.*, 2012; Gobas & Zhang, 1992; Mackay & Fraser, 2000; Sanz-Landaluze *et al.*, 2015; Tu *et al.*, 2014). Rate of uptake Eqn (1) and depuration Eqn (2) of chlorpyrifos were calculated.

$$\frac{dC_B}{dt} = k_1.C_w - k_2.C_B \quad (1)$$

$$\frac{dC_B}{dt} = -k_2.\ C_B \quad (2)$$

where C_B is concentration of chlorpyrifos in larvae (expressed in ng g^{-1} *w*/w), *t* is the exposure time (h), k_1 is the first-order uptake constant (l kg^{-1} wet mass h^{-1}), C_w is concentration in solution (expressed in ng ml^{-1}) and k_2 is the first-order elimination rate constant (per h).

The bioconcentration factor (BCF) of chlorpyrifos was calculated Eqn (3).

$$BCF_k = k_1/k_2 \quad (3)$$

The terminal elimination half-life ($t_{½}$) of chlorpyrifos in larvae was calculated Eqn (4).

$$t_{½} = 0.693/K_2 \quad (4)$$

Quantification of chlorpyrifos and malathion by liquid chromatography-tandem mass spectrometry. Concentrations of chlorpyrifos, chlorpyrifos axon and malathion in solutions and larvae were determined by use of a method described previously (El-Amrani *et al.*, 2012). One ml of each exposure solution was extracted twice with 500 μl of a 1: 1 mixture of hexane/ethyl acetate before analysis. Pools of larvae were rinsed several times with nano-pure water to remove any chemicals adsorbed to the body surface. Next, larvae were homogenized in 1 ml of a 1: 1 mixture of hexane/ethyl acetate, followed by ultrasonication for 1 min. After extraction, samples of larvae and exposure solutions were centrifuged at 9000 g for 5 min, and the organic phase was removed and concentrated under a gentle stream of nitrogen until dry. Next, samples were reconstituted in 195 μl of 100% of methanol and transferred to an autosampler vial fitted with a glass insert and 5 μl of a solution containing 1 μg ml^{-1} malathion-d_{10} was added to samples as an internal standard for analysis. After extraction, samples were filtered through a 13 mm × 0.2 μm nylon syringe filter (Whatman) and into a 1.5 ml amber LC vial. Separation of chlorpyrifos, chlorpyrifos oxon and malathion was performed by use of high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) on a Kinetex C_{18} 100 A column (Phenomenex, 100 mm × 4.6 mm, 5 μm particle size), using water (A) and methanol (B) as solvents at a flow rate of 0.250 ml min^{-1}. The solvent gradient was 10% B increasing to 85% B over 15 min, then increasing to 95% B at 25 min, before returning to 10% B and holding for 5 min. Mass spectra were collected by use of an ABI SCIEX (Milford, MA, USA) 3000 triple quadrupole tandem mass spectrometer fitted with an electrospray ionization source operated in positive ionization mode (multiple reaction monitoring), by using the following operation parameters: temperature 500 °C; capillary voltage, 5.5 kV; collision gas, nebulizer gas, curtain gas were 4, 8 and 10 respectively. Data were analyzed was by use of Analyst 1.4.1 software (Applied Biosciences, Foster City, CA, USA). Multiple reaction monitoring, recovery, limit of detection and limit of quantification are presented in Table 1.

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Table 1. MRM, recoveries, RSDs, LODs and LOQs of chlorpyrifos, chlorpyrifos oxon and malathion analyzed by use of liquid chromatography–tandem mass spectrometry

Chemical	LOD ($ng\,g^{-1}$)	LOQ ($ng\,g^{-1}$)	Concentration in exposure solutions		Concentration in larvae		MRM	
			Spiked ($ng\,ml^{-1}$)	Recovery ±RSD%	Spiked ($ng\,g^{-1}$)	Recovery ±RSD%	Quantification ion	Confirmation ion
Chlorpyrifos	0.19	0.73	50	98 ± 5.4%	500	97 ± 2.8%	349.9 > 197.9	349.9 > 97
Chlorpyrifos oxon	0.11	0.45	10	96.4 ± 3.7%	250	94 ± 3.3%	336 > 280	336 > 308
Malathion	1.67	6.2	50	95.7 ± 7.8%	500	97.4 ± 1.4%	331 > 127	331 > 99
Malathion d_{10} (IS)	ND	ND	ND	ND	ND	ND	343.3 > 132	343.3 > 100

IS, internal standard; LODs, limits of detections; LOQs, limits of quantifications; MRM, multiple reaction monitoring; ND, parameter not determined; RSDs, relative standard deviations.

Statistical analysis

Effects of treatments on cell viability, accumulation of calcein in Caco-2 cells, survival of larvae, and concentrations of chlorpyrifos, chlorpyrifos oxon and malathion in larvae or solutions was determined by use of GraphPad Prism 5 software (San Diego, CA, USA). Normality of data was assessed using the Kolmogorov–Smirnov one-sample test and homogeneity of variance was determined using 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 an unpaired *t*-test (for internal concentration of chlorpyrifos and malathion and accumulation of calcein-AM in caco-2 exposed to verapamil) or one-way ANOVA followed by Tukey's *post-hoc* test (for cell viability, accumulation of calcein-AM in Caco-2 cells exposed to OSPW and survival of larvae of Japanese medaka). Differences were considered significant at $P \leq 0.05$. Concentrations of chlorpyrifos in larvae and in solutions were used for calculations of BCF by use of Origin 9.1 software (OriginLab Corporation, Northampton, MA, USA).

Results

In vitro assays

Cytotoxicity of oil sands process-affected water. Organic chemicals extracted from the water-soluble phase of OSPW were acutely toxic to Caco-2 cells, but only at concentrations greater than 1× equivalent in the original sample of this OSPW, and only after 24 h of exposure. Cytotoxicity was not observed after 1 h of exposure (data not shown). Viability of Caco-2 cells exposed to a 5× equivalent of the pooled sample of OSPW was significantly less (79.6 ± 1.1%) than the solvent control (Fig. 1A). At equivalents of 0.5, 1 or 2.5× the pooled sample of OSPW was not cytotoxic. Neither the neutral nor the basic fraction of OSPW was cytotoxic at concentrations tested. Viability of cells exposed to 5× equivalent of the acidic fraction of OSPW was significantly lesser (74.3 ± 1.0%) compared to the solvent control (Fig. 1B).

Effects of oil sands process-affected water on accumulation of calcein in Caco-2 cells. Efflux of calcein from Caco-2 cells was inhibited by the water-soluble organic fraction of OSPW. Retention of calcein in cells exposed to verapamil, which is an inhibitor of P-gp, was 1.5 ± 0.1-fold greater compared to cells exposed to the solvent control (Fig. 2A). Exposure to 1, 2.5 or 5× equivalent of the pooled fraction of OSPW increased cellular retention of calcein by 1.2 ± 0.03-, 1.2 ± 0.04- and 1.5 ± 0.05-fold, respectively, compared to cells exposed to the solvent control (Fig. 2B). Retention of calcein in cells exposed to 5× equivalent of acidic, basic or neutral fractions of OSPW was significantly greater by 1.3 ± 0.02-, 1.9 ± 0.01- and 2.0 ± 0.01-fold, respectively, compared to cells exposed to the solvent control and the amount in cells exposed to neutral and basic fractions was greater than in cells exposed to the acidic fraction (Fig. 2C).

In vivo assays

Acute lethality. Co-exposure to chlorpyrifos and OSPW had a significant effect on the survival of larvae (Fig. 3A,B). After 24 h of exposure, survival of larvae exposed to the solvent control, 2.5× equivalent of OSPW, or 100 or 250 $\mu g\,l^{-1}$ of chlorpyrifos was 97.5

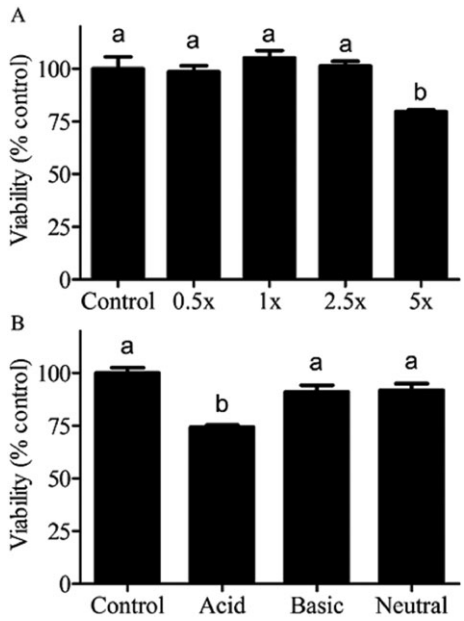

Figure 1. Viability of Caco-2 cells exposed to (A) four concentrations of pooled sample of oil sands process-affected water, and (B) a 5× equivalent of the acidic, basic or neutral fraction of oil sands process-affected water. Viability is expressed as a percentage of control. Cells were exposed for 24 h. Data are expressed as mean ± standard deviation of three independent experiments in which the number of replicates per experiment was eight. Significant differences in viability were determined by use of one-way ANOVA followed by a Tukey's *post-hoc* test ($n = 8$, $P \leq 0.05$) and are designated by different letters. Error bar represents the standard deviation.

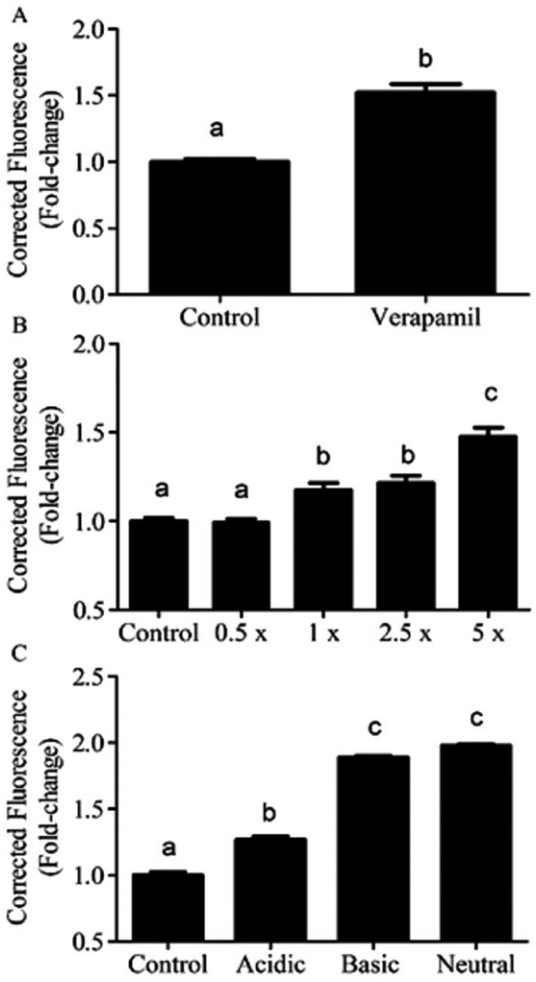

Figure 2. Accumulation of calcein in Caco-2 cells exposed to (A) 10 μM verapamil, (B) pooled sample of OSPW and (C) acidic, basic and neutral fractions of OSPW. Cells were exposed to calcein-AM for 15 min before co-exposure to either (B) 5×, 2.5×, 1× and 0.5× equivalents of the pooled organic fraction of OSPW for 1 h or (C) 5× equivalents of the acid, neutral and basic fraction of OSPW for 1 h. Control cells were exposed to cell culture medium containing 0.1% *v*/v DMSO. Accumulation of calcein was measured as fluorescence and data are expressed as mean ± standard deviation of three independent experiments in which the number of replicates per experiment was eight. Effect of verapamil was assessed by use of an unpaired *t*-test. Effects of OSPW were assessed by use of a one-way ANOVA followed by Tukey's *post-hoc* test. Significant differences among treatments are indicated by different letters ($n = 8$, $P \leq 0.05$). Error bar represents the standard deviation. OSPW, oil sands process-affected water.

± 2.0%, 91.6 ± 8.3%, 93.7 ± 6.3% and 88 ± 6.3%, respectively, and no differences in survival was observed among these groups. However, survival of larvae co-exposed to 2.5× equivalent of OSPW and 100 or 250 μg l^{-1} of chlorpyrifos was 26.5 ± 3.0%, and 15.8 ± 2.2%, respectively, which was significantly lesser compared to the effect of the solvent control, 2.5× equivalent of OSPW and chlorpyrifos alone. Survival of larvae co-exposed to 1× equivalent of OSPW, which is an environmentally relevant concentration, and 100 or 250 μg l^{-1} of chlorpyrifos was 94 ± 6.0% and 85 ± 9.0%, respectively, which is not significantly different from the effect of exposure to the solvent control or 100 or 250 μg l^{-1} of chlorpyrifos alone.

Co-exposure to OSPW and malathion did not affect survival of larvae (Fig. 3C). After 24 h of exposure, no differences in the survival of larvae exposed to the solvent control, 2.5× equivalent of OSPW, 15 mg l^{-1} of malathion, or co-exposed to 2.5× equivalent of OSPW and 15 mg l^{-1} of malathion were observed. Because there were no effects after 24 h, the exposure was extended to 48 h. After 48 h of exposure, survival of larvae exposed to 15 mg l^{-1} of malathion or co-exposed to 2.5× equivalent of OSPW and 15 mg l^{-1} malathion was 67.9 ± 6.5% and 69.3 ± 3.6%, respectively, which is not different from each other but significantly less than survival of larvae exposed to the solvent control or 2.5× equivalent of OSPW.

Effects of oil sands process-affected water on body burden of chlorpyrifos or malathion. Chlorpyrifos, chlorpyrifos oxon and malathion were quantified in larvae exposed to a nominal concentration of 100 μg l^{-1} of either chemical, either alone or with 1× equivalent of OSPW, because these exposures did not cause acute lethality (Table 2). Before the initiation of exposures, concentrations of chlorpyrifos and malathion in freshwater and 1× equivalent of OSPW were less than the limit of detection but concentrations in freshwater and 1× equivalent of OSPW that were spiked with 100 μg l^{-1} chlorpyrifos were 75.1 ± 2.9 and 76.2 ± 5.8 μg l^{-1}, respectively, which are not significantly different. Concentrations of chlorpyrifos in larvae exposed to these solutions for 24 and 48 h were significantly different. Concentrations of chlorpyrifos in samples of solutions taken at 24 h of exposure also were significantly different. After 24 h of depuration, concentrations of chlorpyrifos in freshwater were less than the limit of quantification but mean concentration in the 1× equivalent of OSPW was 16.9 ± 2.9 μg l^{-1}. In addition, concentrations of chlorpyrifos in larvae that had been exposed to chlorpyrifos in 1× equivalent of OSPW were significantly greater than concentrations in larvae exposed to chlorpyrifos in freshwater.

Concentrations of chlorpyrifos oxon in larvae exhibited a similar trend as the parent compound (Table 2). Before exposures, concentrations were less than the limit of detection in all solutions, regardless of whether the solution was spiked with chlorpyrifos. After 24 h of exposure, concentrations of chlorpyrifos oxon were less than the limit of quantification in the solution of chlorpyrifos in freshwater but were 0.6 ± 0.1 μg l^{-1} in the solution of chlorpyrifos in 1× equivalent of OSPW. In larvae sampled at 24 h of exposure, concentrations of chlorpyrifos oxon were significantly lesser in larvae exposed to chlorpyrifos in freshwater compared to chlorpyrifos in 1× equivalent of OSPW. Similar to this observation, after 24 h of depuration, concentrations of chlorpyrifos oxon in larvae that had been exposed to chlorpyrifos in freshwater were significantly greater than concentrations in larvae that had been exposed to chlorpyrifos in 1× equivalent of OSPW.

Exposure to OSPW did not affect concentrations of malathion in larvae (Table 2). Before initiation of exposures, concentrations of malathion in freshwater or 1× equivalent of OSPW were less than the limit of detection but concentrations in freshwater or 1× equivalent of OSPW that were spiked with 100 μg l^{-1} of malathion were 63.4 ± 3.6 and 66.8 ± 2.8 μg l^{-1}, respectively, which were not significantly different. After 24 h of exposure of larvae to these solutions, concentrations of malathion in freshwater or 1× equivalent of OSPW were not different. In addition, concentrations of malathion were not different in larvae exposed to malathion in freshwater or 1× equivalent of OSPW for 24 or 48 h. After 24 h of depuration the concentration of malathion in freshwater and in 1× equivalent of OSPW was less than the limit of quantification. There was no difference in concentrations of malathion in larvae exposed to malathion in freshwater or 1× equivalent of OSPW.

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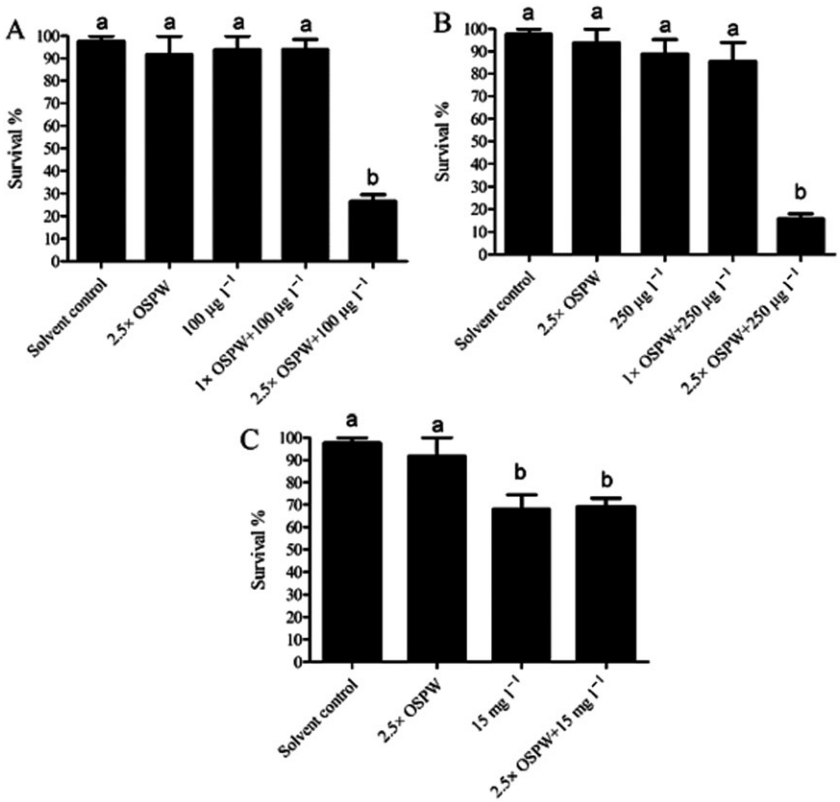

Figure 3. Survival of larvae of Japanese medaka exposed to (A) 100 $\mu g\,l^{-1}$ of chlorpyrifos, (B) 250 $\mu g\,l^{-1}$ of chlorpyrifos or (C) 15 $mg\,l^{-1}$ of malathion. Approximately 12 larvae at 8 ± 1 dpf were exposed to chlorpyrifos or malathion alone or co-exposed with 1× or 2.5× equivalent of OSPW. Effects of chlorpyrifos were determined after 24 h of exposure. Because malathion did not cause effects after 24 h of exposure the effects were evaluated after 48 h of exposure. Significant differences from control were determined by use of one-way ANOVA followed by Tukey's *post-hoc* test and different letters indicate significant differences ($n = 5$, $P \leq 0.05$). Error bar represents the standard deviation. OSPW, oil sands process-affected water.

Effects of oil sands process-affected water on the kinetics of uptake and depuration of chlorpyrifos. Differences in body burdens of chlorpyrifos after exposure and depuration in freshwater and OSPW suggested that OSPW affects bioaccumulation of chlorpyrifos. Therefore, effects of OSPW on kinetics of bioaccumulation and depuration of chlorpyrifos were assessed. In general, the profile of uptake and depuration of chlorpyrifos was similar in larvae exposed to chlorpyrifos in freshwater or the 1× equivalent of OSPW (Fig. 4). In both exposures, concentrations of chlorpyrifos in larvae increased rapidly during the first 2 h of exposure and were greatest at 24 h, but concentrations of chlorpyrifos did not reach steady state by 48 h in either exposure. However, concentrations of chlorpyrifos were greater by 2-fold in larvae after 24 h of co-exposure to chlorpyrifos in 1× equivalent of OSPW compared to freshwater. At the end of the depuration phase, concentrations of chlorpyrifos were 8-fold greater in larvae exposed in OSPW compared to freshwater.

The BCF of chlorpyrifos was different in larvae co-exposed with 1× equivalent of OSPW compared to freshwater. At the end of the uptake phase, which was 48 h, the mean concentration of chlorpyrifos in freshwater was 4.3 ± 0.5 $\mu g\,l^{-1}$ but was 1.8 ± 0.3 $\mu g\,l^{-1}$ in 1× equivalent of OSPW. The log BCF in larvae exposed to chlorpyrifos in freshwater was 3.43 but was 3.95 in larvae exposed to chlorpyrifos in 1× equivalent of OSPW. The rate of uptake of chlorpyrifos (k_1) was 374 $\mu g\,l^{-1}\,h^{-1}$ in larvae exposed to chlorpyrifos in freshwater but was 543.4 $\mu g\,l^{-1}\,h^{-1}$ in larvae exposed to chlorpyrifos in 1× equivalent of OSPW. The terminal elimination half-life of chlorpyrifos in larvae exposed to chlorpyrifos in freshwater was 5 days compared to 11.3 days in larvae exposed to chlorpyrifos in 1× equivalent of OSPW. Finally, the rate of depuration of chlorpyrifos (k_2) was 0.14 $\mu g\,l^{-1}\,h^{-1}$ in larvae exposed in freshwater but was 0.06 $\mu g\,l^{-1}\,h^{-1}$ in larvae exposed in 1× equivalent of OSPW. At the end of the depuration phase, the concentration of chlorpyrifos in solution was less than the limit of quantification in all exposures.

Discussion

Results of this study suggest that basic and neutral chemicals in the aqueous phase of OSPW inhibit P-gp, a member of the ABC superfamily of proteins. Previously, inhibition of MRP, which also is a member of the ABC superfamily of proteins, by basic and neutral chemicals in the aqueous phase of OSPW was demonstrated (Alharbi *et al.*, 2016a). Together, these studies suggest that chemosensitization might be a mechanism of toxicity of OSPW.

Greater mortality of larvae co-exposed to chlorpyrifos and the extract of basic and neutral chemicals from the aqueous phase of OSPW are evidence of a chemosensitizing effect caused by inhibition of P-gp. Chlorpyrifos (log K_{ow} 4.7) and malathion (log K_{ow} 2.7) are lipophilic compounds that enter cells by diffusion and are bioactivated via oxidative desulfuration to their oxon metabolite that causes acute lethality by inhibiting acetylcholinesterases (Fukuto, 1990; Ecobichon, 2001). However, transport of chlorpyrifos, but not malathion, from cells by P-gp protects against acute toxicity (Zaja *et al.*, 2011). Therefore, greater lethality caused by co-exposure to chlorpyrifos and the extract of neutral and basic chemicals from the aqueous phase of OSPW likely was a result of

Table 2. Concentrations of chlorpyrifos, chlorpyrifos oxon and malathion in solutions and in Japanese medaka larvae during assessment of body burden of these chemicals

Chemical measured	Exposure scenario	Exposure				Depuration	
		Solution (0 h)	Solution (24 h)	Solution (48 h)	Larvae (24 h)	Larvae (48 h)	Solution (24 h)	Larvae (24 h)
CPF	CPF	75.1 ± 2.9[a]	34.2 ± 0.9[a]	ND	5625 ± 534[a]	5747 ± 495[a]	<LOQ	2541 ± 206[a]
	CPF + OSPW	76.2 ± 5.8[a]	19.5 ± 1.7[b]	ND	8693 ± 774[b]	8811 ± 460[b]	16.9 ± 2.9	5119 ± 210[b]
CPF oxon	CPF	<LOQ	<LOQ	ND	25.4 ± 2.0[a]	ND	<LOQ	13.1 ± 0.8[a]
	CPF + OSPW	<LOQ	0.6 ± 0.1	ND	56.1 ± 5.0[b]	ND	0.74 ± 0.1	67.8 ± 8.0[b]
MA	MA	63.4 ± 3.6[a]	29.6 ± 0.7[a]	ND	3698.8 ± 141[a]	4063.5 ± 101[a]	<LOQ	85.2 ± 15[a]
	MA + OSPW	66.8 ± 2.8[a]	30.8 ± 1.8[a]	ND	3617.2 ± 150[a]	4016.5 ± 93[a]	<LOQ	83.7 ± 18[a]

CPF, chlorpyrifos; CPF oxon, chlorpyrifos oxon; <LOQ, concentration was less than the limit of quantification; MAL, malathion; ND, concentration was not determined.
Statistical analysis compared concentrations of chlorpyrifos, malathion or chlorpyrifos oxon at each time point and comparisons only were made for different exposures to the same chemical. Different letters indicate a statistical difference.

Figure 4. Effect of OSPW on kinetics of uptake and depuration of chlorpyrifos. Concentrations of chlorpyrifos in larvae of Japanese medaka were determined at during 48 h of exposure and 24 h of depuration. Approximately 100 larvae at 8 ± 1 dpf were exposed to 10 $\mu g\ l^{-1}$ of chlorpyrifos either alone or in co-exposure with 1× equivalent of OSPW. Depuration was performed in solutions without chlorpyrifos. Concentrations are presented as natural-log (Ln) of the arithmetic mean concentration. Three exposures ($n = 3$) were performed with separate batches of larvae. Error bar represents the standard deviation. FW, freshwater; OSPW, oil sands process-affected water. [Colour figure can be viewed at wileyonlinelibrary.com]

inhibition of P-gp resulting in accumulation of chlorpyrifos in larvae. Results of this study support this conclusion because at concentrations of chlorpyrifos that were sublethal, concentrations of chlorpyrifos and chlorpyrifos oxon were greater in larvae when they were co-exposed to basic and neutral compounds from OSPW, including a 1× equivalent of these chemicals, which is the environmentally relevant concentration. Greater concentrations of chlorpyrifos in larvae were matched by lesser concentrations of chlorpyrifos in the extract of OSPW compared to freshwater after 24 h of co-exposure. The greater concentration of chlorpyrifos in OSPW compared to freshwater after 24 h of depuration likely was because the greater amount of chlorpyrifos that accumulated during the co-exposure with OSPW was being depurated. Malathion is not transported from cells by P-gp (Zaja *et al.*, 2011), so inhibition of this protein by OSPW would not increase concentrations of malathion in larvae and therefore would not result in greater acute lethality. Results of the current study support this mechanism of toxicity because survival of larvae exposed to malathion was almost the same compared to larvae co-exposed to malathion and the extract of basic and neutral chemicals from OSPW. These results are consistent with those from other studies where concentrations of substrates of P-gp are greater in tissues exposed to inhibitors of P-pg. For example, inhibitors of P-gp increased accumulation of anticancer drugs within cells, and this resulted in greater cytotoxicity (Callaghan *et al.*, 2014). In addition, the sensitivity of zebrafish (*D. rerio*) embryos toward phenanthrene was greater when they were exposed to inhibitors of P-gp (Fischer *et al.*, 2013).

Kinetics of uptake and depuration of chlorpyrifos were altered in larvae co-exposed to the extract of OSPW, which is evidence that the basic and neutral chemicals from OSPW inhibit P-gp. The BCF and half-life of chlorpyrifos were greater in larvae that were co-exposed to 1× equivalent of basic and neutral chemicals from the aqueous phase of OSPW compared to the solvent control. Although BCFs can be predicted from the log K_{ow} by use of

quantitative structure–activity relationships (Van der Oost *et al*., 2003), factors affecting the toxicokinetics of compounds can affect BCFs. For chemicals that are eliminated from cells by ABC proteins, inhibitors of these proteins would increase internal concentrations of toxicants and thus toxicity (Choi *et al*., 2011; Fischer *et al*., 2013; Kasinathan *et al*., 2014). The rate of efflux of chlorpyrifos is expected to be slower when the activity of P-gp is inhibited. The greater half-life of chlorpyrifos in larvae that were co-exposed to 1× equivalent of basic and neutral chemicals from OSPW compared to freshwater supports this. In addition, concentrations of chlorpyrifos and chlorpyrifos oxon were greater in larvae during the depuration phase, which can be explained not only because concentrations of chlorpyrifos were greater in larvae during uptake phases, but also because efflux of chlorpyrifos by P-gp was inhibited by basic and neutral chemicals from OSPW. The implication of this result is that internal concentrations of the substrate of P-gp (chlorpyrifos) are maintained relative to a given external concentration in freshwater while in the presence of environmentally relevant concentrations of OSPW, the equilibrium is affected by inhibition of P-gp.

Results of this study suggest that basic and neutral chemicals in the water-soluble fraction of OSPW have properties similar to those of inhibitors of P-gp. However, identities of these chemicals currently are not known. There is evidence that inhibitors of P-gp have unique physiochemical properties such as presence of two planar aromatic domains, high lipophilicity at a physiological pH with a log $K_{ow} > 2.92$ and moderate to high molecular mass (Wang *et al*., 2003; Zamora *et al*., 1988). Another characteristic of inhibitors of P-gp is the presence of a cationic charge usually in a nitrogen-containing cyclic ring (Ecker *et al*., 1999; Wang *et al*., 2003; Zamora *et al*., 1988). Chemicals containing nitrogen have been detected in the aqueous phase OSPW (Barrow *et al*., 2010; Grewer *et al*., 2010; Pereira *et al*., 2013). Abundances of these chemicals are greatest in neutral and basic fractions of OSPW and are detected by use of Orbitrap mass spectrometry in positive electrospray ionization (Alharbi *et al*., 2016a). It has been suggested that chemicals containing nitrogen that are present in the basic and neutral fractions of OSPW from Base Mine Lake might inhibit activity of MRPs in early life stages of Japanese medaka (Alharbi *et al*., 2016a). Accumulation of calcein in Caco-2 cells and chlorpyrifos in larvae of Japanese medaka exposed to neutral and basic fractions of OSPW compared to the acidic fraction of OSPW supports this finding. In addition, activities of ABC proteins, including P-gp, is inhibited by surfactants (Rege *et al*., 2002). Anionic surfactants or acid extractable compounds are major constituents of OSPW (Clemente & Fedorak, 2005; Frank *et al*., 2008), but non-ionic and cationic surfactants are yet to be characterized. Although dissolved organic compounds in OSPW have values of log K_{ow} ranging from −2 to 3, most acidic compounds, including naphthenic acids (O_2 chemicals that are detected by use of negative electrospray ionization) have log K_{ow} values less than 0. However, polar basic and neutral compounds had values of log K_{ow} greater than 0 at physiologically relevant pH (Wang *et al*., 2003; Zamora *et al*., 1988; Zhang *et al*., 2015). These results support the conclusion that basic and neutral chemicals in OSPW might inhibit the activity of P-gp. Lesser effects of the acid fraction on accumulation of calcein-AM in Caco-2 cells are probably because chemicals in this fraction are acidic and lacking some of the important features of inhibitors of ABC transporters.

In conclusion, the current study provides evidence that dissolved organic compounds in the aqueous phase of OSPW inhibit the activity of P-gp and therefore have the potential to act as chemosensitizers. However, several uncertainties about the environmental relevance of these findings must be addressed in future studies. For example, because the composition of chemicals in OSPW and fine tailings has not been characterized fully, it is not known if substrates of P-gp exist in either of these media. Therefore, studies aimed at identifying substrates of P-gp in tailings ponds are warranted. In addition, the effects of polar basic and neutral compounds from the aqueous phase of OSPW that has been aged on activity of P-gp should be assessed to identify if this potential mechanism of toxicity is attenuated by aging of OSPW in reclamation ponds and end pit lakes.

Acknowledgments

This research was supported by a Collaborative Research and Development Grant from the Natural Science and Engineering Research Council of Canada and Syncrude Canada Ltd. to J.P.G. (project no. C4288) and, in part, by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (project no. 326415–07) to J.P.G. The authors acknowledge the support of an instrumentation grant from the Canada Foundation for Innovation. J.P.G. was supported by the Canada Research Chair program, and a Distinguished Visiting Professorship in the School of Biological Sciences at the University of Hong Kong, and the Einstein Professor Program of the Chinese Academy of Science. H.A. was supported by King Saud University. We acknowledge the support of the Aquatic Toxicology Research Facility at the Toxicology Centre, University of Saskatchewan, in providing space and equipment for the culturing and breeding of Japanese medaka. The authors thank Syncrude Canada Ltd. for supplying OSPW.

Conflict of interest

The authors did not report any conflict of interest.

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