# Rapid assessment of the toxicity of oil sands process-affected waters using fish cell lines

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Abstract Rapid and reliable toxicity assessment of oil sands process-affected waters (OSPW) is needed to support oil sands reclamation projects. Conventional toxicity tests using whole animals are relatively slow, costly, and often subjective, while at the same time requiring the sacrifice of test organisms as is the case with lethal dosage/concentration assays. A nonlethal alternative, using fish cell lines, has been developed for its potential use in supporting oil sands reclamation planning and to help predict the viability of aquatic reclamation models such as end-pit lakes. This study employed six fish cell lines (WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHML, FHMT) in 24 h viability assays for rapid fluorometric assessment of cellular integrity and functionality. Forty-nine test water samples collected from the surface of oil sands developments in the Athabasca Oil Sands deposit, north of Fort McMurray, Alberta, Canada, were evaluated in blind. Small subsample volumes (8 ml) were mixed with 2 ml of 5× concentrated exposure media and used for direct cell exposures. All cell line responses in

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L. E. J. Lee (⊠) Faculty of Science, University of the Fraser Valley, Abbotsford, BC, Canada V2S 7M8 e-mail: lucy.lee@ufv.ca terms of viability as measured by Alamar blue assay, correlated well with the naphthenic acids (NA) content in the samples ( $R^2$  between 0.4519 and 0.6171; p<0.0001) when data comparisons were performed after the bioassays. NA or total acid-extractable organics group has been shown to be responsible for most of the acute toxicity of OSPW and our results further corroborate this. The multifish cell line bioassay provides a strong degree of reproducibility among tested cell lines and good relative sensitivity of the cell line bioassay as compared to available in vivo data that could lead to cost effective, high-throughput screening assays.

Keywords Fish cell lines  $\cdot$  Toxicity  $\cdot$  Naphthenic acids  $\cdot$  Alamar blue  $\cdot$  Oil sands

## Introduction

Currently, the most commonly used animal test in regulatory ecotoxicology is the fish acute lethality assay (protocol 203, OECD 1992) often used to evaluate chemicals and industrial effluents. However, fish acute lethality tests (96-h lethal dose for 50% of test fish or LD<sub>50</sub> assays, or expressed as lethal concentration LC<sub>50</sub>) are costly and highly variable with subjective endpoints (Hrovat et al. 2009). They often represent an integrative endpoint, making it difficult to differentiate routes of toxic mechanisms (Dayeh et al. 2005a). Species such as rainbow trout (Oncorhynchus mykiss), fathead minnow (Pimephales promelas), and bluegill sunfish (Lepomis macrochirus) are among the most frequently used fish for aquatic toxicity testing (Hrovat et al. 2009). Although, analogous LD<sub>50</sub> assays for mammalian tests have been banned since 2002 (e.g., OECD testing protocol 401, OECD 2002), fish LD<sub>50</sub> assays are still in common use. A new "Fish Testing Framework" have recently been evaluated by OECD, and alternative bioassay tests, including in vitro assays, are now being advocated (OECD 2012).

The Athabasca Oil Sands (AOS) in northern Alberta, Canada, has the potential to produce close to 170 billion barrels of oil (Energy Resources Conservation Board 2012). The ecological footprint of the surface mining and in situ extraction methods has been increasingly the focus of environmental concerns and demands for acceptable reclamation strategies (Holroyd and Simieritsch 2009; Kelly et al. 2010; Lemphers et al. 2010). Surface oil sands mining lead to major disturbances and tailings storage re-shape the landscape. This type of development results in negative impacts to surface water and wetland habitats, and alters large volumes of waters (Dyer et al. 2008; Kelly et al. 2010). Oil sands operators have made a commitment to reclaim these disturbed areas with both terrestrial and aquatic approaches being examined. Aquatic reclamation will entail the construction and evaluation of both lake and wetland components, which must eventually be certified and left as viable aquatic components to the region. These reclaimed aquatic systems, e.g., end-pit lakes (Johnson and Miyanishi 2008), must be self-sustaining, with at least the equivalence of their predevelopment capability. Before reclamation of aquatic systems can be deemed successful, the impacts and effects on various trophic levels and their interactions must be understood. Tools for rapid and effective evaluation of toxicity of the components of these reclaimed systems are thus needed.

The water associated with the extraction process, broadly termed oil sands process-affected water (OSPW) reflect the release of solubilized constituents from the ore and its processing. Constituents of OSPW include dissolved inorganics (e.g., salts, trace metals) and organics (e.g., naphthenic acids (NAs), aliphatic and aromatic hydrocarbons (PAHs)) constituents (Leung et al. 2001). Of these, NAs have been attributed to be responsible for most of the toxicity associated with OSPW to both aquatic and wildlife (MacKinnon and Boerger 1986; Rogers et al. 2002; Nero et al. 2006; Peters et al. 2007; Frank et al. 2008; Kavanagh et al. 2011, 2012; Kannel and Gan 2012).

Bioassays based on fish cell lines may be useful in the study of aquatic reclamation strategies since they offer fast and easy protocols for assessing the response of test waters to an array of higher trophic organisms without the need for lethal endpoints (Castaño et al. 2003). The tests are robust with physiologically relevant storage and testing temperatures, ease of maintenance (Bols et al. 2005), and tolerance of simple culture media (Schirmer 2006). A variety of relatively quick and inexpensive in vitro tests do exist (Microtox<sup>®</sup>, Daphnia IQTM, Thamnotoxkit F) but are based on bacterial or invertebrate models and may not necessarily be environmentally relevant for vertebrates (Lee et al. 2008). Toxicity endpoints obtained with fish cell lines compare well with fish lethality bioassays in their relative sensitivity toward

toxicants (Schirmer 2006). However, cell monocultures employing a single cell line often show decreased absolute sensitivity when compared to in vivo studies (Saito et al. 1994; Magwood and George 1996; Segner 2004), most likely due in part to the invariable reduction in target sites compared to a whole organism (Schirmer 2006). Using several cell lines from varying species and tissue origins may ameliorate this limitation, rendering the target site diversity of the in vitro assay more akin to that of a whole organism. Six cell lines of varying tissue and species origin were chosen for this study (Table 1) based on one or more of the following criteria: economic relevance of origin species, sensitivity to direct OSPW exposure of origin tissue, tissue function, and indigeneity of origin species. These included two cell lines from rainbow trout, two from fathead minnow, a goldfish cell line, and a cell line originally thought to be derived from walleye but which under quality control evaluation in this study, turned out to be derived from bluegill sunfish.

Effective whole animal alternatives also require rapid and sensitive viability assays. This study used a previously reported fluorometric assay performed with three indicator dyes on the same set of cells as reported by Dayeh et al. (2005a), and used to deduce toxic mode of action and increase strength of data interpretation (Schirmer 2006). The evaluated parameters included: metabolic activity (reduction of Alamar Blue or resazurin by enzymes in cytoplasm and mitochondria of viable cells), membrane integrity (conversion of 5'-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) into 5-carboxyfluoroscein by membrane esterases), and lysosomal function (retention or accumulation of 3-amino-7-dimethylamino-2-methylphenazine hydrochloride or Neutral red in healthy lysosomes). The assays were performed in 96-well tissue culture plates, which conserve resources and reduce the amount of test agents and cell requirements. A fluorometric multiwell plate reader linked to a computer also makes measurement and analysis rapid and easy (O'Connor et al. 1991).

This study evaluated, in blind, the cytotoxicity of 49 OSPW and non-OSPW samples from the Syncrude Canada Ltd. lease site in northeastern Alberta. Water samples were taken from a diverse cross-section of water bodies that represented a range of oil sands impacts, including none (natural surface waters), fresh or active sources (settling basins and waters from the seepage control systems), and reclamation waters (experimental field sites containing aged OSPW and oil sands-processed materials (OSPM)). The results of toxicity evaluation of these samples using the fish cell lines were compared to the physicochemical composition of the tested waters. This study provides evidence that fish cell line bioassays could be biologically relevant tools in the initial assessment of OSPW toxicity and perhaps augment some of the traditional methods for assessing toxicity in a rapid and cost-effective manner.

Table 1. Reported  $LC_{50}$  and generated  $EC_{50}$  values for four fish species cell lines with tested reference chemicals.

Fish/cell line	96 h LC <sub>50</sub> (in vivo data	) or 24 h EC <sub>50</sub> (in vitro data) ( $\mu$	$(n) \pm SD(n)$	
	CuSO <sub>4</sub>	SDS <sup>a</sup>	CNA	Cr.NA
Bluegill <sup>b</sup>	$3.83 \pm 4.78 (28)^{c}$	4.5 (1) <sup>c</sup>	6.32±0.838 (4) <sup>c, d</sup>	64.9±14.5 (3) <sup>e</sup>
WF-2 cell line				
AB	8.13±1.5 (4)	31.35±5.37 (3)	42.91±4.2 (3)	66.64±9.08 (3)
CFDA-AM	8.32±0.9 (4)	141.3±58.5 (3)	$-^{f}(3)$	402.1±149.3 (3)
NR	g	18.1±5.09 (3)	13.44±2.1 (3)	120.9±19 (3)
Rainbow trout <sup>b</sup>	$0.20 \pm 0.27 \ (76)^{\rm c}$	$11.0\pm12.06~(9)^{\rm c}$	25.0 (1) <sup>c, h</sup>	64.9±14.5 (3) <sup>e</sup>
RTL-W1 cell line				
AB	4.04±0.143 (4)	11.51±0.45 (3)	15.48±3.6 (3)	45.26±2.44 (3)
CFDA-AM	3.92±1.1 (4)	34.09±7.73 (3)	$-^{f}(4)$	158.8±24.2 (3)
NR	_g	16.26±6.3 (3)	18.22±5.36 (3)	174.23±26.1 (3)
RTgill-W1 cell line				
AB	6.06±1.25 (3)	5.89±1.23 (3)	6.84±1.95 (3)	76.35±12.63 (3)
CFDA-AM	7.08±0.67 (3)	398.93±104.5 (3)	81.48±52.83 (3)	682.7±163.4 (3)
NR	27.0±7.87 (3)	4.11±0.16 (3)	4.23±0.73 (3)	126.07±72.4 (3)
Fathead minnow <sup>b</sup>	$0.95 \pm 1.82 (299)^{c}$	10.18±4.83 (4) <sup>c</sup>	32.93±36.69 (3) <sup>c, i</sup>	64.9±14.5 (3) <sup>e</sup>
FHMT cell line				
AB	3.47±0.84 (4)	21.75±0.67 (3)	24.7±14.22 (3)	146.3±46.61 (3)
CFDA-AM	38.7±3.49 (3)	2168±152 (3)	$-^{f}(4)$	536.7±128.2 (3)
NR	37.8±17.1 (3)	27.18±0.87 (3)	12.67±4.39 (3)	319.9±159.95 (3)
FHML cell line				
AB	7.83±3.6 (3)	14.0±1.43 (3)	14.15±8.36 (5)	74.49±15.27 (3)
CFDA-AM	4.9±2.6 (3)	122.7±61.56 (3)	$-^{f}(4)$	370.2±58.36 (3)
NR	67.69±19.2 (3)	26.2±3.8 (3)	13.98±10.51 (3)	103.67±51.7 (3)
Goldfish <sup>b</sup>	$0.41 \pm 0.49$ (6) <sup>c</sup>	$28.4(1)^{c}$	58.33±14.43 (3) <sup>c, j</sup>	$64.9 \pm 14.5 (3)^{e}$
GFSK-S1 cell line				
AB	5.09±0.29 (4)	9.08±0.47 (3)	24.31±2.65 (3)	101.6±8.2 (3)
CFDA-AM	3.5±1.6 (3)	180.14±36.8 (3)	$-^{f}(3)$	390.97±185.7 (3)
NR	g	20.09±2.67 (3)	26.44±5.13 (3)	163.8±28.6 (3)

Bold  $EC_{50}$  values within a cell line (*rows*) and for a single toxicant (column) were found not to be statistically different from one another (p>0.05) <sup>a</sup> Sodium dodecyl sulfate searched as sodium lauryl sulfate in http://www.pesticideinfo.org/

<sup>b</sup>96-h LC<sub>50</sub> mortality data reported in literature for whole fish species as gathered from http://www.pesticideinfo.org/

<sup>c</sup> Average of all available 96-h LC<sub>50</sub> mortality data from http://www.pesticideinfo.org/ for each species

<sup>d</sup> Average 96-h LC<sub>50</sub> mortality data from http://www.pesticideinfo.org/ for "naphthenic acid" for bluegill

<sup>e</sup> Refers to Microtox assay data done by Frank et al. (2006) and not the fish LC<sub>50</sub>, as data specific to these fish were not available

 ${}^{\rm f}{\rm EC}_{50}$  value not calculated because relative fluorescence units did not fall below 50%

<sup>g</sup> Neutral red assay not done for CuSO<sub>4</sub>

<sup>h</sup> Data retrieved from http://www.pesticideinfo.org/ for CNA (CAS #61790-13-4) specific to rainbow trout was not found, but 96-h LC<sub>50</sub> data for a close relative Chum salmon, *Oncorhynchus keta*, is presented

<sup>i</sup> Data retrieved from http://www.pesticideinfo.org/ for CNA (CAS #61790-13-4) specific to fathead minnow was not found, but 96-h LC<sub>50</sub> data for two small fish Zebrafish, *Danio rerio*, and common round gobi, *Neogobius melanostomus*, is presented as averaged data

<sup>j</sup> Data retrieved from http://www.pesticideinfo.org/ for CNA (CAS #61790-13-4) specific to goldfish (*Carassius auratus*) was not found, but 96h LC<sub>50</sub> data for two other cyprinid fish Kutum (*Rutilus frisii kutum*), and Caspian roach (*Rutilus rutulius caspicus*) is presented as averaged data

#### **Materials and Methods**

*Fish cell cultures and maintenance*. Six fish cell lines of varying tissue and species origin were chosen for this study. WF-2 (Wilensky and Bowser 2005) originally reported as being derived from Walleye (*Sander vitreus*) was a gift from Dr. Paul Bowser, Cornell University. Two rainbow trout (*O. mykiss*) cell lines derived from liver (RTL-W1; Lee et al. 1993) and gills (RTgill-W1, Bols et al. 1994; ATCC CRL-2523); two fathead minnow (*P. promelas*) cell lines derived from testis (FHMT; Vo et al. 2010) and liver (FHML; Lee et al. 2009); and GFSk-S1 cells (Lee et al. 1997), derived from goldfish skin (*Carassius auratus*), were obtained from the Lee Lab at Wilfrid Laurier University (now relocated to the University of the Fraser Valley).

Cells were routinely cultured in 75 cm<sup>2</sup> tissue culture flasks at room temperature (20±2°C) in Leibovitz's L-15 culture medium (Sigma, St Louis, MO) supplemented with fetal bovine serum (Sigma; 10% for WF-2, Rtgill-W1, GFSK-S1, FHMT and 5% for FHML, RTL-W1) and antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin; Gibco, BRL). Culture supplies and subcultivation procedures were as previously described (Bols and Lee 1994; Schirmer et al. 1994). Prior to toxicant exposures, 100 µl of cell suspension were plated in 96-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NY) at cell density ranging from  $2 \times 10^4$  to  $9 \times 10^4$  cells per 100 µl in L-15/ex and allowed to adhere for 24-h. L-15/ex is a simple exposure medium originally developed by Schirmer et al. (1997). This media has been shown to support cell viability but lacks supplements that may interact with toxicants during experiments avoiding potential errors in derived effective concentrations. L-15/ex is also quite inexpensive and can be used to assess whole-water samples such as produced water and effluents (Dayeh et al. 2002).

*Reference chemicals evaluation.* Preliminary testing of all cell lines was done with known toxicants of varying physicochemical characteristics using the fluorometric viability assays as described below in order to validate sufficient bioassay sensitivity. A stock solution of the model toxicant CuSO<sub>4</sub> (Sigma) in L-15/ex was filter sterilized and serial dilutions (0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100 µg/ml) made using L-15/ex for 24-h exposures.

As the chief toxicant in OSPW is thought to be NAs which display surfactant-like properties, sodium dodecyl (lauryl) sulfate (SDS, Sigma), an anionic surfactant, was tested at 0.1, 1, 10, 20, 25, 30, 40, 50, 100, and 1,000  $\mu$ g/ml. This chemical was chosen as a reference sample (positive control) for the viability assays as suggested by Schirmer et al. (2008).

A commercial (Acros Organics—AC41528-0025) naphthenic acid preparation (CNA—0.1, 1, 10, 20, 25, 30, 40, 50, 100, and 1,000  $\mu$ g/ml), and a crude naphthenic acid 55

extract (Cr.NA—0.1, 1, 7, 15, 30, 60, 120, 250, 500, and 1,000  $\mu$ g/ml; prepared as per Frank et al. (2006)) were also tested. The CNA stock solution was prepared by dissolving solid CNA in ethanol and diluted to the highest experimental concentration (1,000  $\mu$ g/ml), then filter sterilized (0.2  $\mu$ m filters) and serially diluted with L-15/ex. Appropriate vehicle controls (0.1% EtOH) were also included in the assays.

OSPW sample preparation. Water samples from a number of sources, both process-influenced and natural, at the Syncrude Canada's Mildred Lake site, located north of Fort McMurray, AB, were collected by Syncrude personnel and shipped to Waterloo, ON, Canada. Samples in 100-ml vials were kept refrigerated in the dark at 4°C. Since direct testing of water samples is not feasible with cell cultures, 8 ml of each OSPW sample were added to 2 ml of 5× concentrated L-15/ex solution. Each sample was then filter sterilized (0.2 µm syringe filters), resulting in 80% OSPW sample concentration in a buffered media that could be used for direct cell exposures. Evaluation of sample toxicity was performed in at least three independent experiments with a minimum of six replicates per sample (see below for exposure details). Chemical analysis of OSPW samples was performed at Syncrude Canada's Edmonton Research Centre using standard protocols, but identity and composition of the samples were not revealed until after cell viability assays were completed. NA analysis on each water sample was done at Syncrude using the Fourier transform infrared spectroscopy (FT-IR) method of Jivraj et al. (1995). With this method, the NA concentration is defined as the total acid-extractable organics (TAO-acid extraction from the water into methylene chloride at pH2-2.5). After taking the extract to dryness, it was reconstituted into methylene chloride, and adsorption at wavelengths of about 1,705 and 1,745 cm<sup>-1</sup> was obtained using a Nicolet model 8700 FT-IR spectrometer (Thermo Electron Corporation).

Osmolality and pH of raw and L-15/ex-containing samples were measured using a vapor pressure osmometer (Westcor 5001B) and a pH meter, respectively. Cell viability assays were done on the WF-2 cell line to verify that the measured fluctuations in pH (6.9–9.1) were not cytotoxic.

Chemical and OSPW sample exposures. Cells that had been plated in 96-well tissue culture plates in L-15/ex media were allowed to attach and spread for 24-h at room temperature after which the L-15/ex media was removed and the cells were exposed to 100  $\mu$ l/well of the filter-sterilized test chemicals or the 80% OSPW samples in replicates of six to eight wells for 24-h before viability assays were performed. Chemical and OSPW exposures were performed at least three times per cell line. It should be noted that previous experiments had confirmed fish cell lines to survive for at least 48-h in L-15/ex made up in both cell culture water (Schirmer et al. 1997) and industrial effluents (Dayeh et al. 2002). *Cell line viability assays with fluorometric indicator dyes.* Viability of cells was measured using Alamar Blue (AB; Biosource International DAL1100) and CFDA-AM (Sigma) or neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride; Gibco), as described by Dayeh et al. (2005a). Briefly, after the 24-h incubation period, OSPW samples and control chemicals were removed and cells were treated with the fluorometric dye solutions and incubated for 1 h at room temperature, after which fluorescence was quantified using a SpectraMax Gemini XS microplate reader (Molecular Devices 02518) with excitation and emission wavelengths of 530 and 595 nm for AB, 485 and 530 nm for CFDA-AM, and 530 and 645 nm for NR.

Data analysis. Mean relative fluorescence units (RFUs) for each OSPW sample or chemical concentration (performed in at least six replicates) were calculated and expressed as percent of control cells (L-15/ex control made up with tissue culture-grade water). The normalized data were compared for variances between experimental replicates which were performed in at least three separate occasions for each chemical or sample. Once the viability data was plotted, regression and correlation analyses were done with the revealed chemical composition of the OSPW samples using GraphPad InStat 3.06. EC<sub>50</sub> values were calculated using GraphPad Prism 5.02. Unpaired t test was used to compare two  $EC_{50}$  values and an analysis of variance (ANOVA) was used for comparing three or more. For OSPW samples, significant deviation of cell viability from control was measured by one-way analysis of variance, followed by Dunnett's test ( $\alpha$ =0.05). Regression analysis was done through the generation of a Pearson correlation matrix to identify simple correlations between cell viability and OSPW sample components. Multiple regression analysis was also done to look for multicollinearity within data for the individual components of the OSPW samples.

### Results

*Cell line identity validation.* Each of the tested cell lines was submitted for species of origin identification to the Guelph Barcode of Life project, as reported for other cell lines (Lee et al. 2011). PCR amplification and DNA sequencing of the mitochondrial cytochrome c oxydase I and 18S ribosomal RNA genes (as per Ivanova et al. 2007) allowed correct identification for five of the six cell lines as to the species of origin. WF-2 was the only cell line not identified as originating from walleye, *Sander vitreous*, as originally thought. Rather, WF-2 was identified as derived from bluegill sunfish, *L. macrochirus*. These fish are widely distributed in North American streams and lakes (Scott and Crossma 1973), and like walleye, bluegill belong to the Perciformes and are native in Alberta, as well as being used as model toxicology

organisms. Thus, findings using WF-2, as model cells from indigenous fish species, are considered relevant for this study.

Standard curve generation for cell viability assays. Standard curves were generated for the three fluorometric indicator dyes using all six cell lines. Increases in measured RFUs corresponded to the increased presence of viable cells. Figure 1 shows a representative standard curve for FHML cells assayed with the three fluorometric dyes. For all cell lines tested, strong linear correlation was found between increasing cell numbers and increasing Alamar blue fluorescence units ( $R^2$  values ranged from 0.811 to 0.992 indicating a strong correlation between increasing number of viable cells and increasing RFUs). This was also true for the other two fluorescence assays as previously reported by Dayeh et al. (2005a). Thus, in an experiment where cells are exposed to putative toxicants, cytotoxicity can be deduced from and quantified by decreases in measured RFUs.

Cell line responses to reference chemicals. Cell exposure to the four chemical compounds tested, CuSO<sub>4</sub>, SDS, CNA, and Cr.NA, caused an overall dose-dependent decline in cell viability as measured by at least two fluorometric indicator dyes. EC<sub>50</sub> values (effective concentrations causing a 50% decline in cell viability) calculated for individual cell lines (Table 1) for CuSO<sub>4</sub> using AB and CFDA-AM ranged from 3.5 to 8.3 µg/ml except for FHMT (38.7±3.49 µg/ml) and were not statistically different (p>0.05) from one another for either of the two viability tests except for FHMT. NR was not used to assay CuSO<sub>4</sub> toxicity as it had yielded confounding results in a previous study (Dayeh et al. 2005b). This finding was reproduced by exposing RTgill-W1, FHML, and FHMT cells to CuSO<sub>4</sub> ranging from 0.01



**Figure 1.** Fluorometric assays as a measure of viable cell numbers for FHML. Standard curves were generated to determine the correlation of increasing cell numbers and relative fluorescence units as measured by three indicator dyes. Cells at various dilutions were plated in a 96-well microplate and incubated for 24 h at room temperature. Cells were then exposed to AB, CFDA-AM, and NR. Six-well replicates (n=6) were used for each cell concentration. Data points are shown as mean RFUs of the six wells with *error bars* representing standard deviations.

to 100  $\mu$ g/ml for 24-h in L-15/ex and assaying with NR. Results showed a similar biphasic curve with decreases in viability observed until 10  $\mu$ g/ml at which point cell viability appeared to increase again (data not shown).

Viability data for SDS, CNA, and Cr.NA showed relatively similar dose-dependent decreases in cell viability as measured by both AB and NR. In most cases, the EC<sub>50</sub> values obtained for both fluorometric dyes were not statistically different from one another (p>0.05). Where values were significantly different, they were still relatively close and well within the same order of magnitude. The only exception was the data obtained for Cr.NA using the WF-2 cell line (p=0.0466). Viability data for SDS, CNA, and Cr.NA as measured by CFDA-AM was erratic compared to AB and NR values. In many instances, viability of cells did not drop below 50% of the control, and EC<sub>50</sub> values could not be calculated, thus this is indicated by a dash in Table 1. Where EC<sub>50</sub> values could be calculated for these chemicals, the values were highly variable ranging from 34±8 to 2,168±152 µg/ml.

Reported lethal dosages expressed as LC<sub>50</sub>-96-h (lethal concentration for 50% of the test organisms in vivo) for each of the test compounds determined for the comparable cell line derived species in vivo (summarized from available literature) are listed in Table 1. Where multiple values were found in the literature, the mean was calculated and standard deviation indicated. If literature LC50 values were not found for the comparable organisms, available literature data for related species were averaged as indicated in Table 1. Appearance of cells was also monitored during experiments via phase contrast microscopy. All cell lines showed similar morphological differences pre- and post-exposure. In all cases, conspicuous changes in morphology were present at the highest chemical concentrations as compared to preexposure morphology. Figure 2 is an example of a phase contrast micrograph taken before and after 24-h chemical exposure; in this case, showing the WF-2 cell line before and after exposure to CNA. Cell membrane integrity appears to have been compromised and the cells show no morphological similarity to their pre-exposure counterparts.

Fish cell line responses to OSPW samples. Fish cell lines were exposed to iso-osmotic OSPW samples prepared 4:1 in  $5 \times$  concentrated L-15/ex for a final 80% of original water sample in the buffered exposure media. Osmolality of the prepared samples ranged from 270-326 mOsm/kg which were within the acceptable physiological range of 260-320 mOsmol/kg (Waymouth 1970). Decreases in cell viability were detected using all three fluorometric indicator dyes, dropping below 50% of controls (4:1 tissue culture grade water in 5× L15/ex) for some OSPW samples. Cell line responses to OSPW samples as measured by AB were the most consistent both from trial to trial for each cell line and between cell lines (Fig. 3). One-way ANOVA followed by Dunnett's test ( $\alpha$ =0.05) identified a number of OSPW samples for which the mean RFUs deviated significantly from the control values (not identified in the graphs for lack of space). OSPW samples (Table 2)8, 12, 13, 16, 19, 42, and 43 resulted in a decrease in viability below 50% of the control for all cell lines. Samples 23, 35, 36, 44, 47, and 48 showed similar decreases below 50% of the control in all cell lines but one (most often GFSk-S1). Samples7, 17, 18, 20, 24, and 37 showed a similar decrease in three or more cell lines. All samples showing such decreases below 50% were found to be significantly different than the control (p < 0.01), with the exception of samples 47 and 48 using the GFSk-S1 cell line.

The chemical composition of the OSPW samples was unknown throughout the testing period so as to not bias reporting. When the composition of the samples was revealed (Table 2), data analysis was done to identify correlations between the viability data and the relative concentrations of the various OSPW sample components. A



**Figure 2.** Phase contrast micrographs of WF-2 cells before and after 24 h exposure to CNA. WF-2 cells were exposed to varying concentrations of CNA in L-15/ex media for 24 h. Phase contrast images were taken before (*A*) and after cell exposure to 50  $\mu$ g/ml CNA (*B*). WF-2

cells have totally deteriorated and appear as a monolayer of homogenous remains after exposure to CNA indicating severe membrane damage. Such micrographs were taken of all cells before and after exposure to each chemical.



**Figure 3.** Fish cell line responses to 24 h exposure to OSPW samples as measured by Alamar Blue viability assay. FHML, FHMT, RTgill-W1, RTL-W1, GFSk-S1, and WF-2 cells were exposed to iso-osmotic OSPW samples for 24 h at 18°C. Cell viability was then measured by

AB. Data points represent the mean of four separate experiments (each experiment consisted of six6-well replicates for each OSPW sample). Cells were plated at densities ranging from  $3.3 \times 10^4$ - $8.0 \times 10^4$  cells/well.

correlation was found between the concentration of NAs (TAOs) present in a given sample and the measured viability of cells exposed to that sample. Specifically, with increasing concentrations of NAs, decreases in cellular viability were noted. The highest correlation observed was noted with WF-2 cells as measured by AB ( $R^2$ =0.617, p<0.0001) and ranged from 0.452 to 0.566 for the remaining cell lines (p<0.0001). The concentration of NA or TAO in the OSPW samples in this study ranged from 0.30 to 82.30 µg/ml (Table 2).

Regression analysis was also done on the other components found in the OSPW samples revealing a high degree of correlation between the viability of cells and the concentration of OSPW constituents associated with the dissolved inorganics, specifically, Na, HCO<sub>3</sub>, and sample conductivity (data not shown). For this reason, multiple regression analyses were performed comparing concentrations of the individual OSPW components with each other, as well as with viability data. This analysis revealed a high degree of multicolinearity indicating concomitant increases between a number of the OSPW sample components (Table 2). The  $R^2$ values for multicolinearity were quite high (>0.9) for conductivity, sodium, chloride, and bicarbonate. A Pearson correlation matrix, comparing select OSPW sample components and RFUs (WF-2, AB; Table 3), shows significant correlations between rising levels of NA, HCO<sub>3</sub>, and between sample conductivity and major ions (Na, Cl, and K).

#### Discussion

Six fish cell lines of varying species and tissue origins were used in this study to evaluate their applicability for assessing toxicity of OSPW. Five of the six cell lines were confirmed to their originating fish species by DNA barcoding as per Ivanova et al. (2007). However, WF-2, although, originally reported to be of Walleye (*S. vitreus*) origin (Wolf and Mann 1980; Wilensky and Bowser 2005), were identified as originating from Bluegill, *L. macrochirus*. It is unclear how the cell line became misidentified, but contamination and/or misidentification has occurred with aquatic animal cell lines (Winton et al. 2010; Lee et al. 2011). Fortunately, *L. macrochirus* is indigenous to the Athabasca region (Scott and Crossman 1973) and bluegill is a common organism used for toxicity testing (US EPA 2002). Therefore, the WF-2 cell line may be a very useful tool for developing effective bioassays to assess the potential toxicity of oil sands reclamation scenarios.

Cell exposure to four reference chemicals caused an overall dose-dependent decline in cell viability in all cell lines as measured by at least two of three fluorometric indicator dyes (Fig. 1). Although all three fluorometric indicator dyes did not always yield EC<sub>50</sub> values similar to one another, two of the assays were usually not significantly different from one another and were within the same order of magnitude as reported LC<sub>50</sub> values for the same or similar compounds (Table 1). For each cell line, AB was the most consistent from trial to trial and compared well with reported effective concentrations in vivo, although overall in vivo values were slightly more sensitive than our derived data. However, the LC<sub>50</sub> data listed in Table 1 were derived from 96-h exposures which may account for some of the comparable insensitivity of the 24-h cell line bioassays in this study. Increasing exposure times have been noted to enhance cytoxicity of SDS for RTgill-W1 cells (Glawdel et al. 2009), thus it is expected that cells'  $EC_{50}$  with longer exposure periods would likely decrease, similar to that seen

Table 2	. Annotated chemical com	position of	f evaluated OSPW	V samples (in milligrams p	ber liter u	unless stat	ed otherwis	se; chemic	al data prc	vided by 3	Syncrude (	Canada Ltd)		
Site	Sample	Type <sup>a</sup>	Sample date	Conductivity (µS/cm)	Ηd	$NA^{b}$	$\mathrm{NH}_4$	Na	К	Mg	Са	CI	$\mathrm{SO}_4$	$CO_3 + HCO_3$
1	FE1	ю	Jul-07	704	7.2	1.4	0.29	LL	0.5	32.7	57.6	4.6	259	182
2	FE2	2	Jul-07	696	7.6	3.0	0.27	144	0.5	16.9	21.6	35.0	38	367
3	FE3	2	Jul-07	069	7.6	2.9	0.30	143	0.5	16.5	19.5	31.0	4	348
4	FE4	2	Jul-07	667	7.6	3.6	0.28	137	0.5	17.6	18.0	27.0	58	322
5	FES	2	Jul-07	2,340	7.9	11.2	2.1	614	8.0	39.0	20.0	140	LLL	481
9	FE6	2	Jul-07	1,260	7.7	2.5	0.21	273	0.5	30.8	19.7	34.0	308	385
7	TPWPond	2	Jul-07	2,040	8.1	21.6	0.18	528	6.2	10.1	9.0	240	122	664
8	STORPond	2	Jul-07	2,740	8.2	36.8	0.29	795	7.2	11.3	10.8	320	234	959
6	BPIT	2	Jul-07	1,530	8.0	8.3	0.35	379	5.1	18.0	14.2	110	164	575
10	SHALWL-Ditch	3	Jul-07	620	7.4	0.4	0.21	91	0.5	38.1	19.3	14.0	125	223
11	CT Pond	2	Jul-07	3,750	7.7	27.7	0.01	1,040	14.6	33.5	35.9	650	1,220	357
12	MLSP-OP	4	Jul-07	1,920	7.6	44.2	2.3	548	0.5	11.6	27.8	220	72	1,030
13	BCV-AS	4	Jul-07	2,490	8.0	24.1	0.01	628	0.5	21.6	45.9	480	89	821
14	MLAKE	1	Jul-07	340	7.4	0.3	0.16	23	0.5	10.1	35.9	12.0	30	160
15	BCV-B16	4	Jul-07	1,280	7.8	1.9	0.23	176	0.5	34.6	103.0	150	229	346
16	DD-B2506	4	Jul-07	2,310	7.5	65.5	2.7	702	9.0	14.2	18.1	240	295	1,050
17	MLSB	4	Jul-07	3,200	7.6	5.5	16.9	704	15.6	11.9	19.5	440	424	648
18	WIP	4	Jul-07	3,380	7.7	15.4	13.4	844	15.0	10.7	16.0	530	384	742
19	DDW	4	Jul-07	2,740	7.9	30.2	2.6	677	8.9	12.7	15.9	250	300	696
20	WIP	4	Jul-07	3,460	7.7	21.4	14.9	793	14.2	10.8	16.3	520	383	825
21	Golden Pond	б	Jul-07	1,680	8.8	3.4	<0.01	225	1.1	57.6	115	38.0	746	163
22	SUN_High SO4 WL	2	Aug-08	2,980	7.6	15.2	<0.01	437	15.9	118	200	4.4	1,590	239
23	SUN_4m CTWL	2	Aug-08	1,953	8.3	22.3	0.22	326	13.5	58.5	83.3	43.0	595	512
24	SUN_NatWL	2	Aug-08	1,242	9.1	44.1	0.56	292	11.9	14.1	19.4	17.0	204	504
25	CNRL	1	Jul-07	256	9.3	2.4	<0.01	22	0.6	8.7	23.0	4.7	22	120
26	South Beaver	1	Jul-07	345	7.6	3.2	<0.01	31	0.8	10.3	41.3	6.0	5	231
27	SCL_NWID Ditch	1	Jul-07	663	8.2	2.3	0.11	94	1.6	22.1	39.7	56.0	38	333
28	SUNCTWL_Waste Area	2	Aug-08	868	8.7	7.0	0.18	112	9.8	32.4	52.4	6.5	308	169
29	U-SHAPED Pond	2	Jul-07	342	8.9	4.6	0.17	37	1.0	8.0	29.1	25.0	79	80
30	FEI	б	Aug-08	729	7.7	1.3	0.12	78	1.0	30.0	53.9	5.6	249	173
31	FE2	2	Aug-08	688	8.4	3.2	0.70	148	1.0	15.4	14.9	33.0	52	322
32	FE3	2	Aug-08	674	8.5	2.4	0.14	147	1.0	15.0	13.5	29.0	55	308
33	FE5	2	Aug-08	2,680	9.0	10.6	0.23	630	8.7	37.6	15.1	140	784	403
34	FE6	2	Aug-08	1,252	9.0	2.5	0.34	268	1.0	29.3	12.4	37.0	341	259
35	TPWPond	2	Aug-08	2,080	9.2	20.0	0.28	519	1.0	8.7	5.7	230	119	553
36	STORPond	2	Aug-08	3,010	8.8	45.0	<0.01	780	7.6	11.0	9.7	310	275	896
37	BPIT	2	Aug-08	1,584	9.1	12.1	0.15	378	1.0	15.8	8.9	112	188	419

Table	<b>2.</b> (continued).														
Site	Sample	Type <sup>a</sup>	Sample	date Cond	luctivity (µS/cm)	Ηd	$NA^{b}$	$\rm NH_4$	Na	К	Mg	Са	CI	$\mathrm{SO}_4$	CO <sub>3</sub> +HCO <sub>3</sub>
38	DEEP WL	3	Aug-08	547		7.8	0.9	<0.01	72	1.0	23.9	30.9	12.0	79	258
39	SHALWL-Ditch	С	Aug-08	748		8.6	0.6	<0.01	113	1.0	37.1	19.9	15.0	174	218
40	CTPond	2	Aug-08	4,730		8.7	29.0	<0.01	1,080	15.0	32.3	31.2	069	1,260	298
41	CT ProtoPd	2	Aug-08	540		8.9	5.5	0.10	124	1.0	3.5	7.4	69	29	158
42	MSLB-OP	4	Aug-08	2,230		7.6	68.5	2.2	570	5.5	11.7	28.1	210	85	1,025
43	SCP1	4	Aug-08	2,270	-	8.0	46.6	0.5	557	5.6	17.6	40.0	250	124	914
44	BCV-AS	4	Aug-08	2,280		8.1	19.8	<0.01	519	1.0	18.2	44.8	340	91	756
45	BCV-B16	4	Aug-08	1,261		7.5	5.4	<0.01	159	1.0	32.0	94.9	130	185	351
46	ETB Pond	2	Aug-08	535		8.9	12.9	<0.01	123	1.0	3.0	7.5	55.0	29	184
47	DD-B2506	4	Aug-08	2,850		7.6	82.3	2.8	706	9.5	14.4	18.9	250	310	1,020
48	DD-2503	4	Aug-08	2,950		7.2	75.8	2.7	733	10.5	14.6	24.3	280	301	1,040
49	MLAKE	1	Aug-08	287		8.2	0.4	<0.01	18	1.0	9.8	33.7	8.0	31	138
	Hq	NA	Cond.	HCO <sub>3</sub>	Na	$SO_3$		$\mathrm{NH}_4$	К		Mg	Ca		CI	RFUs
Hq	1.0000														
NA	-0.1764	1.0000													
Cond.	-0.1214	0.5774	1.0000												
HCO <sub>3</sub>	-0.3067	0.8282	0.6339	1.0000											
Na	-0.1119	0.6500	0.9686	0.7206	1.0000										
$SO_3$	-0.0091	0.1058	0.6512	-0.0486	0.5215	1.00	00								
$\rm NH_4$	-0.2706	0.1175	0.4461	0.3653	0.4224	0.08	20	1.0000							
К	-0.1028	0.4536	0.7885	0.3907	0.7262	0.68	41	0.5314	1.0(	000					
Mg	-0.1034	-0.1754	0.1663	-0.2783	-0.0197	0.732	26	-0.1958	0.2(	695	1.0000				
Са	-0.2048	-0.1417	0.0477	-0.2431	-0.1513	0.50	61	-0.1671	0.13	304	0.8256	1.0	000		
CI	-0.1376	0.4514	0.8696	0.5687	0.8952	0.37	71	0.4981	0.59	923	-0.1721	-0-	.1733	1.0000	
RFUs	-0.0592	-0.7856	-0.6345	-0.8245	-0.6619	-0.0-	994	-0.1347	-0-	4042	0.0721	0.0	978	-0.4616	1.0000

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with in vivo data. Differences in sensitivity by primary cultures of rainbow trout hepatocytes to a range of alkylphenols and alkylated nonphenolics were noted by Tollefsen et al. (2008) when evaluated by AB and CFDA-AM. Their  $EC_{50}$  values derived using AB were lower than those using CFDA-AM for most chemicals tested. Additionally, Tollefsen et al. (2012) evaluated the toxicity of synthetic NAs using trout hepatocytes with both AB and CFDA-AM but with 96-h exposures and their findings also show higher EC<sub>50</sub> values with CFDA-AM. Their reported EC<sub>50</sub> values for the mixtures of NAs, which were also variable, were within the ranges of the data reported here. Thus, toxicity resulting in metabolic inhibition was observed at lower concentrations than loss of membrane integrity. These differences in measured viability between indicator dyes may point to an inherent strength of cell line bioassays allowing physiological mechanism of toxic action to be further investigated.

The OSPW samples tested in this study represent a range of sources, from natural non-OSPW surface waters in the area of Syncrude's Mildred Lake surface operation to OSPW taken from active tailings settling basins. As such, they show a range in both dissolved inorganic (conductivity from <300 to >4,700 uS/cm) and organic (NA from <1 to >80 ug/g) constituents (Table 2). The characteristics of the OSPW vary with source, role (process water associated with extraction or waters from experimental reclamation test sites), and age (waters representing freshly produced process streams to those aged under natural conditions for almost 20 yr). Therefore, the waters tested are likely representative of waters present at both an operational and reclamation stage of a surface oil sands development.

The implementation of successful wet landscape reclamation options, such as wetlands and end-pit lakes (EPLs), must involve the evaluation of potential negative biological impacts associated with both the salt and NA containing OSPW (Leung et al. 2003). Analytical chemistry techniques can be helpful in characterizing and quantifying the components of such whole-water mixtures and for examining trends and changes over time in various reclamation options. But at present, scientists have little ability to follow and predict accurately their potential toxicity on aquatic biological systems, such as fish. This latter requirement is being met by applying standard laboratory bioassays, with eventual incorporation of ecosystem testing as these reclamation systems develop. Easy, fast, and reliable nonlethal bioassays for small-scale and flux assessments of processes within these reclamation strategies are lacking. To this end, nonanimal toxicity models utilizing fish cell lines may be used in the initial assessment of toxicity of samples in a rapid, inexpensive, and ethical manner. It is worthy to note though, that a report with a mammalian cell line provided no direct evidence of cytotoxicity to NA (He et al. 2010), whereas Garcia-Garcia et al. (2011) noted differences in immune responses in isolated mice macrophages exposed to NAs and OSPW. Thus, a species and tissue sensitivity comparison should be carried out to elucidate this. It is also interesting to note the toxicity of CNA appeared greater than the Cr.NA extract, consistently yielding lower EC<sub>50</sub> values. Similar results were found by Nero et al. (2006) when exposing young-of-the-year yellow perch to a commercial and an extracted oil sands NA mixture resulting in LC<sub>100</sub> values of 3.6 and 6.8 mg/L, respectively. A relationship to toxicity and aging processes of the NA has shown that the more labile NAs are lost quickly through bioremediation and these seem to account for most of the acute toxicity of the NAs (Scott et al. 2005; Han et al. 2009). Degradation processes result in the hydroxylation of the parent NAs (Grewer et al. 2010). Relative toxicity of OSPW is likely to reflect NA composition and degree of degradation of the mixtures.

NAs are naturally found in waters in the AOS region. In most surface waters, concentrations are less than 1 µg/ml, while ground waters in contact with oil sands formations may exceed 10 µg/ml. These NA are possibly added by the continuous leaching and solubilization from the bitumen during erosion and contact with exposed oil sand. However, during the bitumen extraction process where pH is elevated, NAs are more readily liberated and dissolved into process waters, resulting in concentrations that may exceed 100 µg/ml (Leung et al. 2003). NAs have been shown to be toxic to a number of organisms including phytoplankton and plants (Wort and Patel 1970; Leung et al. 2001), fish, zooplankton, rats, and luminescent bacteria (Clemente and Fedorak 2005). Dokholyan and Magomedov (1983) studied acute NA toxicity by exposing various fish species to 12-100 mg/l (µg/ml) NA for 10 d, generating LC<sub>50</sub> values ranging from 25 to 75 mg/l. Dorn (1992) found fish to be even more sensitive to oil refinery effluents showing significant toxicity in effluents containing NA concentrations as little as  $2.5-5 \mu g/ml$ .

These toxic endpoints for fish are comparable to those reported for OSPW (MacKinnon and Boerger 1986; Schramm et al 2000). Toxicity to aquatic organisms is quite broad, but the toxic response does depend on the characteristic of NA mixtures (Tollefsen et al. 2012). The dramatic differences in EC<sub>50</sub>s reached in each study is likely because each used differing sources of NAs (e.g., commercial preparations, oil refinery effluents, or NAs isolated from fresh or aged OSPW). This is important because the complex nature of NAs makes estimates of effective concentrations variable, probably because NA samples of similar concentrations from different sources are likely to contain differing relative makeup of molecular weight, ring structures and degree of hydroxylation (Nero et al. 2006; Han et al. 2009), making sample-to-sample comparisons difficult (Headley and McMartin 2004; Clemente and Fedorak 2005; Kannel and Gan 2012). Nero et al. (2006) also found that the addition of 1 g/L of salt ( $Na_2SO_4$ ) reduced the NA toxicity by 40–50%. The addition of salt to the NA solution may change the surfactant properties of the NAs in the OSPW and reduce apparent toxicity. Therefore, salinity may be an important factor when measuring OSPW toxicity and could be a valuable area of research in the future.

When the cytotoxicity results were analyzed with the revealed chemical composition of the OSPW samples (Table 2), a strong correlation ( $R^2$ =0.6171 for WF-2 cells; p < 0.0001) between decreasing cell viability and increasing NA concentrations were noted (Fig. 4). This was consistent with all tested cell lines, and OSPW samples with high NA content could be readily identified. This particular bioassay could provide a valuable initial assessment of a broad range of OSPW samples thus enhancing the time- and costeffectiveness of traditional environmental toxicological assessment. Acute toxicity of OSPW has been shown to decrease with time (MacKinnon and Boerger 1986). This decrease in toxicity appeared to follow an increase in the proportion of NAs or TAOs that contain  $\geq 22$  carbons (Holowenko et al. 2002), which implies OSPW sample toxicity is influenced primarily by low molecular weight NAs. Frank et al. (2008) showed continual decreases in toxicity as measured by the Microtox assay with increases in the proportion of higher-molecular weight NAs. Furthermore, the recent development of higher resolution mass spectrometry has provided additional support for the shift in proportion of high molecular weight NAs as well as the degree of hydroxylation (Han et al. 2009; Grewer et al. 2010). When waters from experimental test sites undergoing natural bioremediation over extended periods were examined, the results supported the argument that microbial degradation



**Figure 4.** Representative graph showing correlation between cell viability and naphthenic acid concentration of evaluated OSPW samples. WF-2 cells were exposed to OSPW samples for 24 h after which cell viability was measured using AB, CFDA-AM, and NR. This was done for all six cell lines. This graph shows significant correlation between cell viability as measured by AB and concentration of NA present in 49 individual OSPW samples ( $R^2$ =0.6171; p<0.0001).

of low-molecular weight NAs is occurring over time and specific pathways of degradation seem to be underway.

In Table 2, the chemical composition and general type grouping of the samples taken from the Syncrude lease are given. In the current study, the actual history and age of the samples was not included. This is an important factor since the time of isolation from contact with fresh process tails will result in a shift in the concentration of the NAs, but more importantly will lead to different high-to-low molecular weight composition ratios and degree of hydroxylation which is another factor that will be reflected in toxicity. Naphthenates occur together with other compounds, such as hydrocarbons and salts (dominated by sodium, sulfate, and chloride). There is evidence for toxicological effects of salts derived from the extraction process (Leung et al. 2001). The complex nature of naphthenates, along with the presence of these additional process-affected substances makes the ecotoxicological evaluation of OSPW difficult. Studies have indicated that some of the OSPW toxicity is related to salinity, major ions content, and possibly additional factor, such as PAHs (van den Heuvel et al. 1999; Leung et al. 2003). In the examination of the current data using the fish cell line bioassay, multiple regression analysis was done to identify additional components of the OSPW samples that may contribute to the observed toxicity. Correlations with cell viability similar to those found with NA concentrations were found between concentrations of sodium and bicarbonate, as well as sample conductivity as measured by AB and with CFDA-AM for HCO<sub>3</sub>. Such increases in these OSPW components and concomitant decreases in cell viability imply they may also induce toxicity. However, a high degree of multicollinearity was found between the concentrations of NA, Na, HCO<sub>3</sub>, and sample conductivity (major ions). That is, with increases in NA, similar increases are seen in Na, Cl, HCO<sub>3</sub>, and sample conductivity.

The major ions in OSPW typically responsible for high conductivity include Na, Cl,  $HCO_3$ , and  $SO_4$ , which come primarily from the ore during the extraction process, added process chemical aids, or through byproducts of upgrading and utilities (Mikula et al 1996; Allen 2008). This means that these OSPW constituents will follow a similar path as the added NAs, resulting in them being strongly correlated. Thus, the concentration of NA and total ionic content in a given sample are likely to be coincidentally correlated with cell viability.

Conductivity is often used as a measure for the total ions dissolved in freshwater (Goodfellow et al. 2000). With regard to toxicity, conductivity can be used as a general screening tool. The conductivity of a freshwater effluent above 2,000  $\mu$ S/cm may indicate a concentration of dissolved solids high enough to induce toxicity in aquatic organisms. However, the correlation between increasing conductivity and toxicity may vary with ionic composition

of effluent samples and therefore may not be the best predictor of toxicity. That is, cations and anions are not present individually, but instead are associated with other ions making conductivity itself a poor predictor of toxicity. Twenty of the OSPW samples in this study are reported to have a conductivity measurement in excess of 2,000 µS/cm. Allen (2008) states that even if salinity concentrations in process water are insufficient to be acutely toxic, it may act as a stressor effectively increasing the toxicity of other compounds present in the effluent. Therefore, it is critical to compare ion concentrations in the effluent to literature or lab-derived toxic effect concentrations (Goodfellow et al. 2000). Reported 96-h LC<sub>50</sub> values for HCO<sub>3</sub> for rainbow trout and bluegill were 7,700 and 7,100 µg/ml (OECD 2002), respectively. OSPW sample 16 had the highest concentration of HCO<sub>3</sub> of the samples tested  $(1,050 \mu g/ml)$ making HCO<sub>3</sub> an unlikely source of toxicity on its own. Similarly, studies have shown that Na<sup>+</sup> is not generally a major contributor to freshwater aquatic toxicology; in fact, the absence of  $Na^+$  can be more toxic (Mount et al. 1997). Generally, toxicity with regard to Na<sup>++</sup> is concerned with the associated anion (Goodfellow et al. 2000).

Multispecies cell line testing is a value-added approach to assessing toxicity, especially the possibility of using "indigenous" species cell lines to better evaluate and/or provide relevance to the interpretation of bioassays. It rapidly and inexpensively compares possible species response differences and provides an alternative to ethically unacceptable lethality tests. As can be noted in Table 1 for the compiled in vivo data for the 96-h LC<sub>50</sub> values (from pesticideinfo.com), for the corresponding species response to the tested reference chemicals, there is great variability in the averaged data (with wide standard deviations) to these chemicals and between species, thus multispecies comparisons should also be carried out at the cellular level which may be difficult to do with primary cultures (Gagne et al. 2012; Tollefsen et al. 2012).

At present, in vitro models are not likely appropriate replacements for established in vivo methods. However, the ability of fish cell lines to consistently identify OSPW samples with high concentrations of NAs is a positive step and could lead to their implementation as convenient models for the initial screening and augmented assessment of OSPW samples leading to a prediction of fish success in oil sands reclamation scenarios, such as EPLs.

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