ALBERTA INNOVATES

Isolation and Characterization of Oil Sands Process-Affected Water (OSPW) Fractions and Assessment of Their Toxicity Using Mammalian Cells

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UNIVERSITY OF ALBERTA

1. PROJECT INFORMATION:

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3. PROJECT PARTNERS

We would like to acknowledge Suncor Energy for providing us with OSPW samples from their demonstration pit lake located at Lake Miwasin.

A. EXECUTIVE SUMMARY

Untreated oil sands process-affected waters (OSPW) are stored long-term in tailings ponds and to date, no standardized water treatment method exists that would help permit rapid and large-scale release of these contaminated waters into the environment. Efforts to remediate and/or detoxify OSPW must be carefully monitored for their efficacy and feasibility and therefore the main objectives of this project were to:

(1) To develop methods to isolate the OSPW-inorganic fraction (IF) and OSPW-organic fraction (OF)
(2) To investigate the immunotoxicological effects of acute OSPW-IF and OSPW-OF exposures alone or in combination *in vitro* using a mammalian macrophage immune cell line.
(3) To establish bioactive profiles for OSPW before and after treatment using solar-activated zinc oxide photocatalytic oxidation or electro-oxidation, and to perform water quality assessments on each sample (i.e., NA quantification and profiling).

Our results provide evidence that OSPW contains factors that can activate the antimicrobial and proinflammatory responses of immune cells (i.e., they are immunotoxic). Specifically, using the murine macrophage RAW 264.7 cell line, we establish the bioactivity (i.e., macrophage activating status) of two different OSPW samples and their isolated fractions. The waters examined were obtained from a pilot scale demonstration pit lake (DPL) created to assess Suncor's patented Permanent Aquatic Storage Structure (PASS) treatment technology. Here, we directly compared the bioactivity of two DPL water samples, which included expressed water from the PASS-treated tailings (termed the before water capping sample; BWC) as well as an after water capping (AWC) sample consisting of a mixture of expressed water, precipitation, upland runoff, coagulated OSPW and added freshwater. Significant inflammatory bioactivity was associated with the AWC sample and its organic fraction (OF), whereas the BWC sample had reduced bioactivity that was primarily associated with its inorganic fraction (IF). Furthermore, we show that by using two advanced oxidation processes (AOPs) to treat the samples that OSPW bioactivity is significantly reduced, which correlates with removal of naphthenic acids, a marker organic compound targeted by the solar-activated zinc oxide photocatalytic oxidation or electro-oxidation.

Overall, these results indicate that at non-toxic exposure doses, the murine macrophage RAW 264.7 cell line serves as a sensitive and reliable biosensor for the screening of bioactive/immunotoxic constituents within and among discrete OSPW samples. Bioactive-guided profiling of OSPW represents a potentially promising *in vitro* tool for the continued monitoring of various water samples undergoing both passive and active remediation treatment protocols.

B. INTRODUCTION

Sector Introduction:
Northern Alberta contains the largest oil sands (i.e., bitumen) reservoir worldwide, and the third largest known oil reserve globally (Xue *et al.*, 2018, Hewitt *et al.*, 2020). Bitumen (i.e., viscous black organic hydrocarbons found naturally, or refined during petroleum distillation) is extracted from sediment using the modified Clark hot water extraction process that involves mixing heated caustic water with mined ore, generating oil sands process affected water (OSPW) (Han *et al.*, 2009). OSPW is a complex, dynamic, and heterogeneous mixture of inorganic and organic components (Headley *et al.*, 2013). Of the known components in OSPW, the naphthenic acid fraction compounds (NAFC) and the polyaromatic hydrocarbons (PAHs) are generally perceived as major organic toxicants of significance that induce cell death or narcosis through nonspecific and detrimental plasma membrane effects (Li *et al.*, 2017, Cancelli & Gobas, 2020). However, there are thousands of species of known chemical compositions of naphthenic acid (NA) compounds in OSPW, and thus determining the toxicity of specific NAs has proven to be difficult (Bartlett *et al.*, 2017). In addition to organic toxicants, there are also several inorganic components in OSPW such as sodium, chloride, ammonia, and bicarbonate that exceed concentrations found in the Athabasca River by 8-200 fold (Allen 2008). In addition to PPE, volatile organic contaminants such as the benzene, toluene, ethylbenzene, and the xylene (BTEX) group are also present in OSPW (Allen, 2008).

Due to a zero discharge government mandate enacted in 1993 by the Alberta Environmental Protection and Enhancement Act (Giesy *et al.*, 2010, Hazewinkel & Westcott, 2015), these contaminated waters are stored on site in tailings ponds, and are reused during subsequent extraction events (Allen, 2008). The zero-discharge policy was initially created to maximize the rates of water reuse by requiring oil sands operators to recycle the tailings pond water in subsequent extractions. However, the bitumen extraction efficiency of OSPW decreases over time due to accumulated salts (Hazewinkel & Westcott, 2015, Martin, 2015), and thus additional freshwater is required to supplement OSPW during reuse. Of the ~2-4 barrels of water needed to extract a barrel of surface bitumen (Jordaan, 2012), ~0.5-2.5 barrels are sourced from freshwater supplementation (Quinlan & Tam, 2015), resulting in the continuous expansion of tailings ponds. As of 2015, 1.18 trillion litres of OSPW has been estimated to reside in tailings ponds, which presents a significant environmental concern (McNeill & Lothian, 2017).

Issues surrounding the fate of OSPW have been of substantial importance to multiple stakeholders. Governmental initiatives have pushed for oil companies to have tailings ponds in a "ready to reclaim state" within 10 years after mining is fully completed at a site (Martin, 2015), yet industrial estimates for reclamation range from 15 - 70 years (McNeill & Lothian, 2017). Reclamation is defined as the creation of a self-sustaining environmental ecosystem on disturbed land (Alberta Environment and Water, 2012). Acute toxicity of OSPW has been shown using a number of experimental models (MacDonald *et al.*, 2013, Sansom *et al.*, 2013, Scarlett *et al.*, 2013, Bauer *et al.*, 2019, Phillips *et al.*, 2020), and as a result, various OSPW remediation strategies have been explored over the past decade, incorporating both engineering (i.e., active) and biological (i.e., passive) applications (Toor *et al.*, 2013, Demeter *et al.*, 2014, Huang *et al.*, 2015, Xue *et al.*, 2018, Chegounian *et al.*, 2020). Currently, there is no consensus on how best to reclaim tailings ponds to self-sustaining ecosystems in an economically feasible way. Chemical compositions of OSPW have been analyzed through combinations of techniques including (but not restricted to) mass spectrometry, fractionation, and fluorescence spectroscopy (Frank *et al.*, 2008, Han *et al.*, 2009, Rowland *et al.*, 2012, Scarlett *et al.*, 2013, Frank *et al.*, 2014, Morandi *et al.*, 2015, Frank *et al.*, 2016, Bartlett *et al.*,

2017, Ahad *et al.*, 2020). These studies have shown that OSPW constituents vary based on multiple factors such as the operator source, effluent age, the treatment method performed, and its status (active tailings ponds versus settling basins, versus dyke seepage runoffs, etc.), necessitating further interrogation into the biological effects of these mixtures. As government policies require OSPW to be eventually remediated and incorporated into independently functioning ecosystems, contemporary monitoring of OSPW is needed to rapidly, sensitively, and cheaply track compositional changes that can further offer informed, directed, and effective treatment options.

Measuring the toxicological effects of OSPW has mostly been performed using aquatic species (MacDonald *et al.*, 2013, Toor *et al.*, 2013, McQueen *et al.*, 2017, Bauer *et al.*, 2019). While these whole organism models provide useful insights on toxicant effects at a macro level, the inherent complexity of whole animal systems lend themselves to inter-replicate variations, or unexpected variables that influence downstream results (Li *et al.*, 2017). Beyond these potentially confounding factors, are the more publicized critiques on animal ethics considerations, and the reliability of extrapolating results between different animal models (Kathy, 2018). For example, recent work done by Bauer et al. demonstrated that the acute toxicity endpoints of aged OSPW in eight different aquatic organisms are both species and fraction dependent (Bauer *et al.*, 2019). Thus, governmental regulations incorporating both acute and long-term toxicity endpoints using whole animals must take into consideration downstream flora and fauna community effects.

Alternatively, *in vitro*-based approaches can serve as surrogates to reduce the number of resources, time, and ethical limitations of assays that require the rearing and processing of live animals. *In vitro* assays can also be useful for measuring specific effects of a toxicant without needing to consider factors such as sex, fecundity, ecological networks, intra- and inter- specific interactions. Within the framework of *in vitro* assays are biosensors, which feature a biological component, such as enzymes, cells or tissues, to detect specific factors of interest (Wijesuriya & Rechnitz, 1993, Wang *et al.*, 2005). The substantial diversity of bioreceptors available allows broad applications including environmental monitoring, food safety, and clinical diagnoses through examining the bioactivity (functional response) of the biosensor system (Gupta *et al.*, 2019). For example, immune cell-based biosensor systems can detect an array of stimuli using an arsenal of innate receptors and associated genetic machinery designed to respond to various stimuli rapidly and specifically. In turn, this makes the construction and maintenance of immune cell-based biosensors conventionally cheaper and simpler to other counterparts, as they do not require complex cellular organization like tissue biosensors, nor do they need to be isolated and purified like molecular biosensors reliant on antibodies or enzymes.

During this project, we demonstrate the use of an immune cell-based bioassay as a new *in vitro* model for the detection of bioactive (i.e., immune cell activating) constituents within OSPW. Specifically, we use the mouse macrophage immune cell line, RAW 264.7 cells, as cellular biosensors to directly test and compare the bioactive profiles between various OSPW samples and their organic and inorganic fractions. Macrophages are an integral part of innate immunity in most species and are equipped to rapidly sense and destroy foreign debris, pathogens, and dead cells through a potent array of antimicrobial responses (Buchmann, 2014, Mills & Ley, 2014, Varol *et al.*, 2015). Strategically positioned at entry sites of the body (Ángeles Esteban, 2012), macrophages effectively survey for incoming threats that when detected, rapidly activate the cells from a basal resting form into a distinct pro-inflammatory state. This switch is associated with notable induction of secreted biomolecules that can be used to detect and monitor their bioactivity. Therefore, using bioassays that track macrophage activation can be incorporated into a bioactive-guided

profiling system to help track and ultimately detect constituents of potential concern (i.e., immunotoxicological factors) within these complex waters. Specifically, our results support the use of macrophages as a cell-based biosensor that can be employed to interrogate biological responses after their direct exposure to sub-lethal OSPW doses collected from a single demonstration pit lake at two different time periods. Then, by examining activation of various antimicrobial activity pathway and immunologically relevant gene transcriptional responses, we report on the establishment of bioactive-guided profiles for both whole OSPW and their subsequent inorganic and organic fractions in a sensitive and reliable manner. Furthermore, we tested the utility of our bioassay system to monitor two advanced oxidation processes that are used to treat OSPW for the removal of organic compounds such as naphthenic acids. Overall, by establishing and utilizing a macrophage biosensor system as means of surveillance of aquatic remediation efforts in the Albertan industry, we have provided a new method that can be incorporated in future sampling frameworks examining the complex dynamics of OSPW undergoing passive and active treatment regimes in support of end goal reclamation efforts.

Knowledge Gaps:

OSPW is a very complex mixture of suspended solids, salts, inorganic compounds, dissolved organic compounds, and trace metals. Organic compounds found in OSPW include NAs, benzene, humic and fulvic acids, phenols, PAHs, and toluene, among others. Despite the advances in analytical techniques, comprehensive characterization of all OSPWs is not possible. OSPW contains thousands of organic compounds that have not been identified so far because of the complexity of the OSPW mixture and the variety of different structures of NAs (in addition to other organics) present in OSPW. While the NAs in OSPW are generally identified as main drivers of toxicity in OSPW, there are other components such as inorganic ions that have been shown to modify the toxicity effect in exposure studies with model organisms (Puttaswamy and Liber, 2012). Liquid-liquid extraction (LLE) using dichloromethane (DCM) has been commonly used to extract NAs from OSPW. However, the NA recovery using LLE extraction is usually not high (<80%), and the DCM extraction typically requires pH adjustment to protonate NAs for better extraction. Compared to LLE, solid phase extraction method consumes less solvent and could be an alternative for the OSPW organic and inorganic separation.

There are concerns about the environmental and human health impacts because of any possible future release of treated OSPW into the environment. OSPW has been reported to cause both acute and chronic toxicity to a variety of organisms, including fish, amphibians, and mammals. Although several studies have reported the toxicity of NA species towards different organisms, little research has been carried out regarding the OSPW and NA toxicity in mammals. Our previous findings indicate that a concentration-dependent acute and sub-chronic OSPW immunotoxicity in fish and mammals can be significantly reduced by ozone treatment. However, the impacts of inorganic species and potential inorganic-organic complexes to the overall toxicity of OSPW remain unclear.

The key gaps in our knowledge regarding OSPW immunotoxicity that were addressed by this project are:

- Can the inorganic fraction of OSPW be efficiently isolated and used in immunotoxicological assays?
- Can immune cell-based bioassays be used as an *in vitro* tool for the detection and tracking of inflammatory components in different OSPW samples?
- Using an immune cell-based bioassay system, can we differentially assess the immunotoxicological effects of two distinct OSPW samples and their fractions?

- Can immune cell-based bioassays contribute to the temporal monitoring of Suncor's Lake Miwasin Demonstration Pit Lake?
- What specific effects do the OSPW-OF and OSPW-IF have on the viability and functions of mammalian immune cells (e.g., macrophages)?
- Are there any additive and/or synergistic effects that occur among the organic and inorganic constituents of OSPW that contribute to the overall immunotoxicity of the whole OPSW?
- What effect does solar-activated zinc oxide photocatalytic oxidation have on the organic components (i.e., NAs) of OSPW and how does this affect the bioactivity of the water?
- What effect does electro-oxidation have on the organic components (i.e., NAs) of OSPW and how does this affect the bioactivity of the water?

C. PROJECT DESCRIPTION

Project Objectives:

The specific project goals were: (1) To determine if the inorganic Fraction (IF) of OSPW can be efficiently isolated by developing a sequential multi-step extraction; (2) To determine if the inorganic and organic constituents of whole OSPW contribute to immunotoxicity by investigating the specific effects of acute OSPW-IF and OSPW-OF exposures alone *in vitro* using a mammalian cell; (3) To determine the possible additive and/or synergistic effects that occur among OSPW constituents by investigating the possible effects of combined (i.e., reconstituted) acute OSPW-IF and OSPW-OF exposures; and (4) To perform chemical analysis and immunotoxicology assessments of a raw Athabasca River water sample (i.e., reference sample).

Additional project goals were later added to employ our optimized bioactivity profiling system on two different OSPW treatment processes. Specifically, we generated bioactive signatures for the following six water samples: Untreated OSPW1 from Syncrude; OSPW1 after treatment using solar-activated zinc oxide photocatalytic oxidation and after electrooxidation; Untreated OSPW2 from Suncor, and OSPW2 after treatment using solar-activated zinc oxide photocatalytic oxidation and after electrooxidation. Each of these samples were examined using our immune cell-based water monitoring bioassays that use a selected series of reliable biomarkers. Comprehensive bio profiling of each water sample was also complimented with water quality analyses performed in the Gamal El-Din laboratory to detect and quantify various water parameters including but not restricted to naphthenic acids.

Our results contribute an important scientific foundation for the development of management strategies and regulations when considering safe release of treated process-affected waters to the environment. It also provides a new cell-based monitoring tool for the assessment of the progression of detoxification (i.e., reduction of inflammatory factors) of OSPW in end-pit lakes.

(1) To develop methods to isolate OSPW-IF and OSPW-OF fractions.

OSPW is a complex mixture of a variety of inorganic species, organic species, and suspended particles. The inorganic species are mainly anions and cations in addition to trace elements, while the organic species are primarily naphthenic acids (NAs). Although several studies have reported the toxicity of NA species

towards different organisms, the impacts of inorganic species and potential inorganic-organic complexes to the overall toxicity of OSPW remain unclear. To conduct further toxicological investigations of OSPW inorganic and organic species, it is important to develop a sample preparation method to effectively isolate inorganic and organic fractions of OSPW (OSPW-IF and OSPW-OF).

As previously highlighted, isolation of NAs has been commonly performed using liquid-liquid extraction (LLE), with dichloromethane (DCM) as a solvent. However, the efficiency of extraction of NAs using this method is usually <80%, and generally requires the addition of hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) to protonate OSPW NAs that allow for enhanced extraction. This adjustment of pH is expected to inevitably affect the chemical composition of OSPW samples. Solid phase extraction (SPE) is also widely used to extract and separate OSPW organic fractions. Compared to the LLE procedure, adding acid to decrease OSPW pH is not necessary for SPE methods using anion exchange cartridges. However, samples are acidified and then buffered afterwards prior to further testing This makes the SPE method a potential new method to obtain OSPW inorganic matrix. Compared to LLE, the SPE method consumes less solvent and could be an alternative for the OSPW organic and inorganic separation. In addition, carbonaceous material adsorption is also an efficient method to remove the organic matrix in OSPW and achieve the separation of OSPW-IF and OSPW-OF. For carbonaceous adsorption, pH adjustment is also not needed, which will preserve the inherent composition of inorganic fractions.

To achieve the separation of OSPW-IF and OSPW-OF, several SPE methods and the activated carbon adsorption method were investigated. SPE cartridges with different sorbents material such as alkyl-bonded silicas, copolymers and ion exchange resins were tested to investigate their ability to capture the organic constituents. The organic and inorganic matrix separation efficiency of the self-packed activated carbon cartridge was also tested.

(2) To investigate the effects of acute OSPW-OF and OSPW-IF exposures alone or in combination *in vitro* using a mammalian immune cell line.

Despite recent advances in analytical techniques, the comprehensive characterization of OSPW constituents is very difficult. We developed methods for efficient isolation and chemical characterization of the inorganic fraction (IF) of OSPW. We previously reported that ozonation of the organic fraction (OF) of OSPW significantly (80%) reduced the NAs content, which resulted in significant decrease in acute and subchronic toxicity in aquatic organisms and mice (Li et al., 2019, Hagen et al., 2012). The OSPW OF is generally focused on as it contains the NA constituents, however, our *in vitro* results also indicated that the whole OSPW was significantly more acutely toxic to cells compared to OSPW-OF. This strongly suggests that components present in the IF contribute to the overall toxicity of whole OSPW. It is also possible that interactions between the organic and inorganic constituents of OSPW may synergize to exert toxic effects on cells. Therefore, we employed the RAW 264.7 mouse macrophage cell line as an *in vitro* bioindicator system, and using our newly developed *in vitro* protocols, we assessed the relative contributions of OSPW-OF and the OSPW-IF to the overall acute toxicity of mammalian cells.

(3) To establish bioactive profiles for OSPW before and after treatment using solar-activated zinc oxide photocatalytic oxidation or electro-oxidation, and to perform water quality assessments on each sample (i.e., NA quantification and profiling).

The main goal of this part of the project was to examine the effects of two different AOPs on the immunotoxicologial activities of two representative OSPW samples using the RAW 264.7 mammalian

macrophage cell line and a series of *in vitro* bioassays designed to monitor cytokine gene and protein expression, as well as the ability of these cells to activate antimicrobial responses. Furthermore, we anticipate that our bioactive signatures will reveal new information regarding the absence/presence of various constituents during the two OSPW treatment processes. We hypothesized that each treatment process will remove specific OSPW components (i.e., organics such as NAs) and the removal or reduction of these components will be reflected by discrete changes in one or more of our selected biomarkers.

D. METHODOLOGY

OSPW samples

The OSPW samples examined in this study were collected from Lake Miwasin, a (DPL) constructed by Suncor in 2017, and officially completed in 2018. Oil sands companies construct DPLs to test long-term treatment and storage approaches of OSPW and tailings. The fluid tailings (which consists of OSPW, sand, clay, and residual bitumen) was first processed through a patented treatment system using Suncor's permanent aquatic storage structure (PASS) technology (Suncor Energy Inc., 2019). Briefly, inorganic coagulants are added to fluid tailings to facilitate the aggregation of ultra-fine minerals and bitumen particles. These compounds to precipitate and sink to the bottom of the reservoir to facilitate mineralization or sequestration of select metals (Revington et al., 2016). Afterwards, polyacrylamide was added to enable the dewatering of the fluid tailings which were deposited at the bottom of the DPL in October 2017. Subsequently, an initial water cap was first pumped out of Lake Miwasin in August 2018, which consisted of expressed water from the treated tailings after further settling, precipitation, and run off. The water capping process dilutes treated tailings and OSPW (COSIA, 2018). Freshwater was also added in August 2018, and OSPW treated with a coagulant was incorporated into Lake Miwasin in October 2018 to fill the lake up to its pre-designated elevation and mixture of approximately equivalent portions of treated OSPW and freshwater. The result is termed a pilot scale DPL that is being monitored extensively by multidisciplinary research groups to characterize the physiochemical, abiotic, and biotic changes that occur in Lake Miwasin over a 15-year period in a passive remediation approach (Suncor Energy Inc., 2019). In this study, we tested the expressed water from the PASS-treated tailings (i.e., BWC/OSPW2) as well as an AWC (OSPW1) sample, which consisted of a mixture of expressed water, precipitation, upland runoff, coagulated OSPW and added freshwater (with a total of roughly 57% of process water and 43% of freshwater). Furthermore, the isolated OF and IF from the BWC and AWC samples were also tested for bioactivity and compared to the original whole waters.

OSPW samples were collected on site from Lake Miwasin and shipped to the University of Alberta and stored at 4°C in a refrigerated cold room. Water samples were filtered using a syringe 0.45µm membrane filter (Millipore Sigma) before use in all immune cell-based bioassays. All bioassays were completed at sub-lethal concentrations using the methods to assess cytotoxicity described previously by our group (Qin et al., 2019).

Isolation of OSPW organic and inorganic fractions

Fractionation of BWC and AWC was performed as previously described (Qin et al., 2019). Briefly, to obtain the OF, concentrated hydrochloric acid was added to whole OSPW until a final pH of 2 was attained. The acidified OSPW was then filtered through a pre-conditioned solid phase extraction (SPE) cartridge connected to a vacuum manifold at a flow rate of 1ml/min to facilitate organic adsorption. Cartridges

were then rinsed with ultra-pure water to remove salts and dried before the elution of the organic matter from the cartridge, using acidified methanol. After eluent collection, the solvent was evaporated, and the precipitate was re-dissolved in $NaHCO_3$ buffer that was balanced to the pH of the starting whole OSPW sample (pH 8.7) to ensure full dissolution of organic material. To obtain the IF, granular activated carbon (GAC) was pre-treated with HCl and NaOH to remove impurities and then left to dry overnight. Whole OSPW filtered through the cleaned GAC was then loaded into a column, and the effluent containing the inorganic components was collected via gravity filtration. Finally, the IF was passed through a 0.45µm nylon membrane filter.

Cell culture conditions

The mouse macrophage cell line RAW 264.7 (ATCC® TIB-7™) was cultured in complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich) supplemented with 5% Fetal Bovine Serum (FBS, Sigma) and 100 U penicillin - 100µg/ml streptomycin (Gibco) (Phillips et al., 2020). Cells were incubated at 37°C with 5% CO_2 and passaged into new 75 cm^2 vented flasks (Biolite) every 2-3 days using a 0.25% Trypsin in 1mM Ethylenediaminetetraacetic acid (Trypsin-EDTA, Sigma) using the isolation procedure as described[4].

Effect of OSPW exposures on inducible nitric oxide synthase (iNOS) protein levels

Intracellular iNOS protein levels were examined as an initial macrophage bioactive marker using an iNOS-specific PE-conjugate monoclonal antibody (eBioscience/Invitrogen) as previously described (Phillips et al., 2020). Briefly, RAW 264.7 cells were seeded in 500µl of supplemented DMEM at a density of $3x10^5$ cells per well in 24-well plates and incubated for 24 hours at 37°C/5% CO_2 prior to exposures. The following day, cells were washed twice with 500µl of PBS and then exposed for 24 hours with indicated %v/v dilutions (i.e., 10-50% v/v) of each OSPW and their fractions. As a positive control for the qualitative increase of intracellular iNOS levels by stimulated macrophages, cells were incubated with 1.25µl of heat-killed DH5α *Escherichia coli* (OD_{600}: 0.6) after a 2-hour pre-treatment with PBS or OSPW samples. After a further 22 hours in the presence of OSPW (or matched PBS controls), cells were harvested with Trypsin-EDTA and then fixed with 4% formaldehyde at 4°C for 30 minutes. Cells were then permeabilized using a 1X-permeabilization buffer (eBioscience/Invitrogen) at room temperature for 30 minutes. Permeabilization buffer was then removed, and cells were stained with the iNOS-PE antibody (0.06µg/sample, Invitrogen) for 30 minutes at 4°C. Cells were then washed twice with 500µl of PBS and then resuspended in 200µl of PBS. Finally, cells were analyzed using an Attune NxT (Thermofisher) flow cytometer at the Flow Cytometry Facility (Faculty of Medicine and Dentistry, University of Alberta) to detect the proportion of iNOS positive macrophages in a population of 10,000 analyzed cells per sample. Experiments were performed in triplicate, with the average of three biological experiments for each treatment pooled to obtain the average between assays.

Effect of OSPW exposures on nitric oxide (NO) production

iNOS activity was determined by monitoring macrophage NO production using the colorimetric Griess. Briefly, RAW 264.7 cells were seeded and treated as described in section 2.4. After 24-hours, plates were centrifuged at 300 g for five minutes and supernatants were collected and transferred to a new 96-well plate. NO levels were detected via the addition of 50µl each of 1% sulphanilamide (Sigma) and 0.01% naphthyl ethylenediamine dihydrochloride (NED) (Sigma) in 2.5% phosphoric acid (Sigma). Plates were incubated for 7 min prior to analysis using a SpectraMax M_2 microplate reader (Molecular Devices, USA) set to an absorbance wavelength of 570nm. The concentration of NO produced was determined using a sodium nitrite standard curve. Data collected from each set of triplicates was pooled together to obtain

the average NO concentration for a given treatment during an individual experiment. Each experiment was performed in triplicate.

Macrophage surface receptor expression levels following OSPW exposures

Two distinct cell surface immune receptor proteins termed CD86 (B7-2 Antigen) and CD206 (mannose receptor) were also examined following exposure of the macrophages to whole OSPW and their fractions. Briefly, RAW 264.7 cells were plated into a 24-well plate at a cell density of $3x10^5$ per well and incubated overnight at 37°C/5% CO_2. The next day, cells were rinsed twice with 500µl of PBS and then incubated with 50% v/v of whole BWC or AWC and their fractions for 24 hours. Macrophages cultured in media alone and PBS 50% v/v treatments were used to assess baseline surface receptor expression levels. Cells were harvested after 24 hours and transferred to a 1.5ml centrifuge tube and centrifuged at 500 g for 3 minutes. Cells were then fixed using 4% formaldehyde and surface stained using CD86-GFP or CD206-GFP conjugated anti-mouse monoclonal antibody (Biolegend) for 30 min at 4°C using antibody concentrations of 1:200 and 1:100 v/v, respectively. After staining, cells were washed twice with 500µl of PBS and then re-suspended in 200µl of PBS. The proportion of cells staining positive for CD86 and CD206 were detected by flow cytometry using the Attune NxT flow cytometer at the Flow Cytometry Facility (Faculty of Medicine and Dentistry, University of Alberta). Each surface receptor staining experiment was performed in triplicate.

Effect of OSPW exposures on macrophage cytokine protein secretion activity

Untreated ($3x10^5$) RAW 264.7 cells were plated in wells of a 24-well culture plate and incubated overnight at 37°C/5% CO_2. The following day cells were washed twice with 500µl of PBS and then exposed to whole BWC, AWC, and their fractions separately at 50% v/v dilution for 24 hours. After the 24-hour exposure period cells were centrifuged at 500 g for three minutes and supernatants were collected and stored at -20°C. A 70µl aliquot of each exposure sample was then shipped on dry-ice to Eve Technologies Inc. (Calgary, Canada) for cytokine/chemokine detection using a Mouse Cytokine/Chemokine 31-plex Array. Data presented is representative of at least three independent experiments performed. Protein secretion was normalized to relative protein secretion activity to allow direct comparison between the different cytokines as follows: the percent relative secretion activity for each exposure was calculated across all individual experiments and their values then averaged together to give the mean % secretion activity for each marker. These mean secretion activity values were then used to compare differences in the relative secretion activity for each of the markers graphed (IP-10, MIP-2, MCP-1) for whole and fractionated waters for BWC and AWC and their respective controls to determine the highest relative secretion response.

Effect of OSPW exposures on the induction of macrophage antimicrobial and proinflammatory gene transcription

Quantitative (q)PCR experiments were performed as previously described to examine macrophage gene expression levels[4]. Briefly, RAW 264.7 macrophages were seeded at $3x10^5$ cells/well in a 24-well plate in DMEM, and left to adhere to the plate overnight at 37°C. The following day, culture media was aspirated, and the adherent cells were washed with 500µL of PBS before exposing them to 500µL of OSPW samples at 50% v/v. To examine the pro-inflammatory gene expression profiles of macrophages after exposure to OSPW, RNA was extracted after 2, 4, 6, 8, or 10 hours following a standard phenol-chloroform protocol to isolate total RNA. RNA concentrations and their relative quality were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher). cDNA was then synthesized using the Superscript III First-Strand Synthesis System kit (Invitrogen) according to manufacturer's recommended standard protocols.

The selected immune genes analysed in this study were *inos*, interferon inducible protein 10 (*ip-10*), monocyte chemoattractant protein 1 (*mcp-1*), and macrophage inflammatory protein 2 (*mip-2*). All primer sequences used were obtained from published literature (Table 1). In house validation of these primer sets were performed to confirm primer specificity and efficiency at optimized concentrations (Table 1). *gapdh* was used as the endogenous control gene based on its consistent expression in all treatments. 1ng of cDNA was used as qPCR template and all qPCR experiments were run on the Applied Systems' 7500 Fast Real-Time PCR system using the manufacturer's software. qPCR reactions were composed of 2µL of primer, 5µL of SYBR™ green master-mix (MBSU, University of Alberta), and 2µL of cDNA per well.

Water chemical analyses

The Soils, Water, Air, Manures, and Plants (SWAMP) Lab at the University of Alberta performed quantification of chemical elements using spectrometry methods. Water samples (i.e., unfiltered whole water samples and fractionated samples) were digested with nitric acid at high temperature and pressure to dissolve particulate matter, and analyzed on through ICP-MS (Javed et al., 2020). Additional analyses such as dissolved organic carbon and NA quantification were performed as previously described by the Gamal El-Din Lab (Qin et al., 2019).

Statistical analyses

95% confidence intervals (CI) were generated from the difference of the means between populations. If the lower and upper limits of the CI do not contain the value 0, then the two treatments initiate statistically significant different bioactivity responses from each other (Tan and Tan, 2010). Standard error of the mean (SEM) is plotted for error bars on all graphs, and all experiments are performed with a minimum of N=3 biological replicates.

Advanced Oxidation Processes for treatment of OSPW

Photocatalysis

The photocatalytic experiments were conducted under illumination from Newport Solar Simulator (Oriel Instruments, USA) with power supply maintained at 225 W. Irradiance was measured to be 277.92 W/m2, with the UVA+B+C content of the radiation recorded as 16.72 W/m2. All experiments were conducted in 250 mL cylindrical reactor capacity using 100 mL OSPW. The photocatalytic treatment of the OSPW was conducted with the catalyst nanoparticles applied in suspension. ZnO particles (BET specific surface area = 3.3 m2/g) were added to OSPW in a glass beaker to make the desired catalyst concentration and the contents were steadily mixed for 30 min to achieve adsorption equilibrium on the particles. The contents were subsequently exposed to steady collimated radiation from the simulated solar light for photocatalytic reactions to take place while samples were taken at predetermined time intervals. The reacting mixture was steady mixed at 550 rpm throughout each experiment with the contents maintained at room temperature. Samples were immediately filtered (filter size = 0.2 µm) to remove catalyst particles and stored at 4 °C for subsequent analyses. To account for the removal by adsorption and photolytic breakdown of the target compounds, dark and photolytic experiments were respectively conducted as controls in which catalyst particles contained OSPW were steadily stirred over time without illumination (dark) and OSPW illuminated over time in the absence of photocatalyst. For comparison, similar experiments were performed using TiO2 (BET specific surface area=46.8 m2/g) as the catalyst.

Electrooxidation

All electrolysis experiments were conducted in an open, undivided cylindrical glass cell of 7 cm of diameter and 600 mL of capacity, constantly stirred with PTFE magnetic bar to maintain mass transport of substance towards/from the electrodes surface. The anode was a 40 cm^2 double-faced coated BDD mesh (thin film deposited on Nb, NeoCoat, Switzerland), while the cathode was a stainless-steel mesh of similar surface area. The electrodes were placed parallel to each other at the center of the electrolytic cell at an electrode distance of approximately 2 cm. Except otherwise stated, all experiments were performed with 450 mL of real OSPW (without addition of any chemical) since its conductivity was high enough for good electrical conduction. The experiments were conducted at 10 mA/cm2.

E. PROJECT RESULTS

Tasks 1a and 3a: Development of methods to isolate OSPW-IF and OSPW-OF and to then fractionate two different OSPW samples for further examination during this project.

To achieve the separation of OSPW-IF and OSPW-OF, several SPE methods and the activated carbon adsorption method were investigated. SPE cartridges with different sorbents material such as alkyl-bonded silicas, copolymers and ion exchange resins were also tested to investigate their ability to capture the organic constituents. The organic and inorganic matrix separation efficiency of the self-packed activated carbon cartridge was then assessed, and the key results of this project task are summarized below.

- The highest organic fraction extraction was obtained using a hydrophilic modified, styrene-based solid phase extraction polymer with 95.4 ± 0.7% of dissolved organic carbon (DOC) and 90.0 ± 5.3% of naphthenic acid (NA) recovered from OSPW, which were higher than those obtained using the traditional dichloromethane liquid-liquid extraction (48.8 ± 0.2% of DOC and 81.0 ± 2.6% of NA recovery) or other SPE cartridge.

- We show the first isolation method for OSPW inorganic fraction by removing 96.1 ± 0.2% of DOC in OSPW using granular activated carbon.

- OSPW-OF showed significant cytotoxicity at 14 mg/L O_2-NA and affected the cellular metabolic activity at 10 mg/L of O_2-NAs.

- No significant cytotoxicity or effect on cellular metabolic activity was observed for whole OSPW, OSPW-IF and the reconstituted fractions.

- This work outlines a new process for the isolation of the major components of OSPW (i.e., organics and inorganics), which allows for subsequent examination of their relative immunotoxicological effects using a series of optimized immune cell-based bioassays that are the testing platform used throughout the remainder of this project.

Tasks 1b and 3b: Chemical Analysis of two different Suncor DPL Water Samples and a Reference Sample

The original water chemical analyses of Suncor DPL Water Samples (and their fractions) were performed by the Gamal El-Din lab in Fall 2019, which established baseline chemical component data for total organic carbon, NA species, cations, and anions of relevant constituents. These same waters were then further analyzed by the Dr. Shotyk lab (Fall 2020) using (inductively coupled plasma mass spectrometry) ICP-MS,

ion chromatography, and ICP-OES to quantify trace elements, major anions, and major cations, respectively. Water samples were analyzed as whole waters (WH) both unfiltered (UF) and filtered (FL) using a 0.45 µm membrane, in addition to their inorganic (IF) and organic (OF) fractions. The two OSPW waters used in our study were sampled from Suncor's DPL after being capped with freshwater (i.e., Suncor DPL Water Sample #2; aka AWC) and before the water cap was added (Suncor DPL Water Sample #1; aka BWC), thus their chemical constitutional comparisons allowed for an assessment of the temporal changes in physiochemical parameters. We also included a reference sample (i.e., Athabasca River Water) to the project for comparison. The key results from this project task are shown in **Tables 1 and 2** and are summarized as follows:

- In general, the fractionation of water samples increased the amount of most constituents examined. For example, 11/18 elemental ppb concentrations were higher in filtered Suncor DPL Water Sample #1 compared to the unfiltered water sample. In contrast, 7/32 absolute concentrations increased after filtering Suncor DPL Water Sample #2.

- Elements strontium (Sr) and lithium (Li), known OSPW markers, were detected in both water samples; Sr levels were 3.2-fold higher in Suncor DPL Water Sample #1 (UF), while Li was 1.6-fold higher in the UF Suncor DPL Water Sample #2. Trace elements associated with bitumen enrichment such as rhenium (Re), nickel (Ni), vanadium (V), and molybdenum (Mo) were also present in both samples, with Re (2.7x), Ni (2.2x), and Mo (2.2x) being higher after the water cap (OSPW1), and V higher (17.3x) before the water cap (OSPW2).

- Toxic and/or high priority pollutants such as arsenic (As), copper (Cu), lead (Pb), nickel (Ni), selenium (Se), and zinc (Zn) were detected in both water samples. Higher amounts of As were found in the IF of the Suncor DPL Water #1 (56.7 ppb) and Suncor DPL Water #2 (28.8 ppb). Ni was 1.8x higher in the IF of Suncor DPL Water #1 and Se was 2.4x higher in Suncor DPL Water #2 although at relatively low overall concentrations (5 ppb>). Zn was 1.2x higher in the Suncor DPL IF Sample #2 but was detected in the highest concentration in the Suncor DPL OF #1 (40.6 ppb). Like Zn, Cu was detected at a 6.1x higher concentration in UF Suncor DPL Water #2, however, it was measured at its highest concentration at 135 ppb in the OF. It should be noted that further water analytic analyses must be performed to confirm these findings as our trace element data revealed possible technical issues as in some cases the fractionated samples showed values that were orders of magnitudes higher than the whole water. Repeated experiments also require procedural blank data.

- Environmental lead (Pb) is usually present as an inorganic form. Our analysis indicates that the highest concentration of Pb was found in the Suncor DPL OF Sample #2, with a 5.2-fold difference between the two OF samples examined. As mentioned in the previous point, further water analyses need to be performed to determine the discrepancies between whole vs. fractionated water analytic data.

- The reference sample used for this project (Athabasca River Water) was generally lower in all selected ions when compared to whole OSPW1 and OSPW2 (**Table 2**), which is due to the brackish properties of OSPW. Naphthenic acid analyses were not performed on the reference sample.

- The OSPW2 sample displayed higher salinity than OSPW1. As the incorporation of a freshwater cap in approximately equal proportions to OSPW2 was completed to create OSPW1, this decrease in salinity is anticipated due to the dilution process.

Concentration (µg/L)	BWC (OSPW 2) Whole	BWC (OSPW 2) Organic Fraction	BWC (OSPW 2) Inorganic Fraction	AWC (OSPW 1) Whole	AWC (OSPW 1) Organic Fraction	AWC (OSPW 1) Inorganic Fraction
Aluminum	<LOQ	23.2	53.1	153	47.1	57.4
Antimony	1.13	0.04	2.96	1.17	2.74	0.04
Arsenic	5.7	0.54	56.7	3.56	28.8	0.52
Barium	34.7	29.2	222	42.8	134	29.2
Cadmium	0.1	0.06	0.33	0.08	<LOQ	0.04
Cesium	0.33	0.04	0.66	0.14	0.33	0.03
Chloride*	1 045 160	0	880 980	429 130	0	388 830
Chromium	<LOQ	0.54	<LOQ	<LOQ	<LOQ	0.77
Cobalt	2.97	0.15	0.45	0.65	0.44	0.12
Copper	1.45	29.5	36.6	8.78	13.3	135
Iron	<LOQ	4.66	<LOQ	4.56	<LOQ	11.1
Lead	0.008	0.175	0.134	0.03	0.127	0.91
Lithium	81.7	1.04	56.9	135.5	1.32	120
Manganese	0.14	1.06	4.77	0.29	4.72	2.95
Molybdenum	366	5.88	170	182	35.5	3.56
Napthenic Acid Species (O_2-O_6)*	52 660	40 520	560	31 920	25 550	500
Nickel	15.9	1.73	5.34	8.66	4.48	1.04
Nitrate*	2 850	0	0	2260	0	0
Nitrite*	50	0	0	50	0	50
Phosphate*	1 220	0	4 480	330	0	1960
Rhenium	0.7057	<LOQ	0.015	0.2844	<LOQ	<LOQ
Rubidium	34	0.26	24.6	14.4	12.3	0.26
Selenium	<LOQ	<LOQ	4.52	3.13	1.87	<LOQ
Silver	<LOQ	1.37	<LOQ	<LOQ	<LOQ	0.61
Sodium*	1 281 304	14 119	1 153 975	506 206	12 896	474 852
Strontium	1246	5.18	705	423	394	5.61
Sulphate*	1 067 030	0	986 820	295 440	0	318 860
Uranium	7.16	0.043	39.7	4.68	5.61	0.023
Vanadium	0.68	<LOQ	4.95	12.9	2.34	<LOQ
Zinc	<LOQ	40.6	16.4	13.6	21.1	29.2

Table 1. Composition of selected chemicals from oil sands process-affected water (OSPW) and Athabasca River water. Trace elements were quantified as per Shotyk et al. (2017), and chemical compounds were quantified as per Qin et al. (2019) in µg/L. Whole and fractionated water samples from before water capping (BWC, OSPW 2) and after water capping (AWC, OSPW 1) are detailed. <LOQ indicates below the limit of quantification. Asterisks beside chemicals indicate the water was filtered prior to quantification.

				Fold Change Differences (Absolute)	
	BWC (OSPW 2)	AWC (OSPW 1)	Athabasca River Water	BWC-ATH	AWC-ATH
Concentration (µg/L)	**Whole**	**Whole**	**Whole**	**Whole**	**Whole**
Aluminum	<LOQ	153	13.08		11.7
Antimony	1.13	1.17	0.088	12.8	13.3
Arsenic	5.7	3.56	0.6	9.5	5.9
Barium	34.7	42.8	51.8	1.5	1.2
Cadmium	0.1	0.08	0.01	10.0	8.0
Cesium	0.33	0.14	0.003	110.0	46.7
Chloride*	1 045 160	429 130			
Chromium	<LOQ	<LOQ	0.1		
Cobalt	2.97	0.65	0.03	99.0	21.7
Copper	1.45	8.78	6.4	4.4	1.4
Iron	<LOQ	4.56	58.7		12.9
Lead	0.008	0.03	0.08	10.0	2.7
Lithium	81.7	135.5	5.15	15.9	26.3
Manganese	0.14	0.29	0.5	3.6	1.7
Molybdenum	366	182	0.691	529.7	263.4
Napthenic Acid Species (O_2-O_6)*	52 660	31 920			
Nickel	15.9	8.66	1.61	9.9	5.4
Nitrate*	2 850	2260			
Nitrite*	50	50			
Phosphate*	1 220	330			
Rhenium	0.7057	0.2844	0.0032	220.5	88.9
Rubidium	34	14.4	0.76	44.7	18.9
Selenium	<LOQ	3.13	0.17		18.4
Silver	<LOQ	<LOQ	0		
Sodium*	1 281 304	506 206			
Strontium	1246	423	183.6	6.8	2.3
Sulphate*	1 067 030	295 440			
Uranium	7.16	4.68	0.48	14.9	9.8
Vanadium	0.68	12.9	0.25	2.7	51.6
Zinc	<LOQ	13.6	6.96		2.0

Table 2. Composition of selected chemicals from oil sands process-affected water (OSPW) and Athabasca River water. Trace elements were quantified as per Shotyk et al. (2017), and chemical compounds were quantified as per Qin et al. (2019) in µg/L. Whole water samples from before water capping (BWC, OSPW 2) and after water capping (AWC, OSPW 1) are detailed. <LOQ indicates below the limit of quantification. Hatched cells indicate data not available. Cells highlighted in red have at least a 5-fold difference (in absolute value) between the whole OSPW indicated and the reference sample (indicated as ATH). Asterisks beside chemicals indicate the water was filtered prior to quantification.

Tasks 2a and 4a: *In vitro* toxicity assessments of two Suncor DPL Waters their Fractions plus a Reference Water

The primary goal of Tasks 2a and 4a were to determine if OSPW contains inflammatory components and if a selected suite of immune cell-based bioassays can be used to detect and track these among the two test waters and their fractions. Overall, the ability of OSPW exposures to activate immune cell antimicrobial and proinflammatory activities was considered as immunotoxicity since the cells used in these assays were always in a non-activated/resting state prior to exposures. Then relative to control samples, OSPW-induced bioactivities were detected and quantified as described below revealing that OSPW contains inflammatory/immunotoxic components, which we can precisely detect and compare among the various samples tested.

Effects of OSPW exposures on immune cell viability

Prior to examining the effect of OSPW exposures on immune cell responses, it was first necessary to ensure that the exposure doses chosen did not cause any effects on cell health. Therefore, using an established cell culture viability assay, we performed OSPW dose responses at the maximal exposure period of 24 hrs and then examined cell health. Overall, whole OSPW and its organic and inorganic components are differentially cytotoxic to mammalian macrophages. In general, after 24 hr exposure to the cells, 50% v/v of OSPW-OF #1 is significantly cytotoxic, whereas >70% v/v of the whole OSPW #1 and its IF were required to induce cell death. A similar cytotoxic profile was observed for the second Suncor DPL water tested and overall, these data show that when the new extraction procedure is performed, the OSPW-OF is relatively more toxic to cells when compared with raw OSPW and OSPW-IF. Based on the cytotoxicity data, we chose sub-lethal OSPW exposure doses (i.e., <70% v/v) when performing all immune assays described in the subsequent sections.

Effects of OSPW exposure on the antimicrobial activity of macrophages

We examined the ability of immune cells (i.e., mouse macrophages) to produce nitric oxide (NO; a major antimicrobial molecule used for killing microbes) as a specific indicator of OSPW-mediated bioactivity. To do this we monitored inducible nitric oxide (*inos*) gene expression, iNOS protein levels, as well as the production of NO (the product of iNOS enzyme activity) following exposures. Since resting macrophages do not express the *inos* gene, have negligible amounts of iNOS protein nor produce NO, induction of these biomarkers following exposure is a direct indicator of the immunotoxic effects of the tested water samples. The key results obtained from these antimicrobial assays are described in detail below.

inos transcription was maximally induced following exposure of macrophages to OSPW1 (the AWC sample) and its OF

By using quantitative PCR (qPCR) to examine *inos* gene expression, we confirmed at a molecular level that Suncor DPL Waters activate macrophages (Fig. 1). Specifically, macrophages exposed to whole OSPW1, had a statistically significant 10-fold increase from the 2 to 4-hour time point in *inos* gene expression when compared to the control cells (Fig. 1A). This increase in expression was sustained for up to 10 hours post exposure. In comparison, macrophages exposed to 50% (v/v) of whole OSPW2 only showed a statistically significant increase in *inos* expression after 4 hours when compared to the PBS-exposed control cells. *inos* expression levels following exposure of the cells to the OSPW2-IF or OSPW2-OF were all statistically different from the control group at all timepoints but with no induction levels higher than 4-fold were observed (Fig. 1B). Comparatively, macrophages exposed to the OSPW1-OF showed an early, and relatively large, sustained induction of *inos* (Fig. 1C) that ranged between ~3-60-fold depending on the

exposure period, whereas cells treated with the OSPW1-IF had only relatively minor changes in their *inos* expression levels, although other than the 4-hour time point, were still significantly higher when compared to the control exposure (Fig. 1C). **These data show that OSPW-mediated activation of macrophages can be detected as early as 2 hours after exposure and suggests that the whole OSPW1 (the AWC sample) contains organic constituents with potent macrophage activating (i.e., inflammatory) properties**. In comparison, whole OSPW2 (the BWC sample) did induce detectable and significant *inos* expression (Fig. 1A at 4hrs), however this bioactivity was relatively diminished following fractionation, and it did not segregate to the OF (Fig. 1B) as was shown following OSPW1-OF exposures (Fig. 1C).

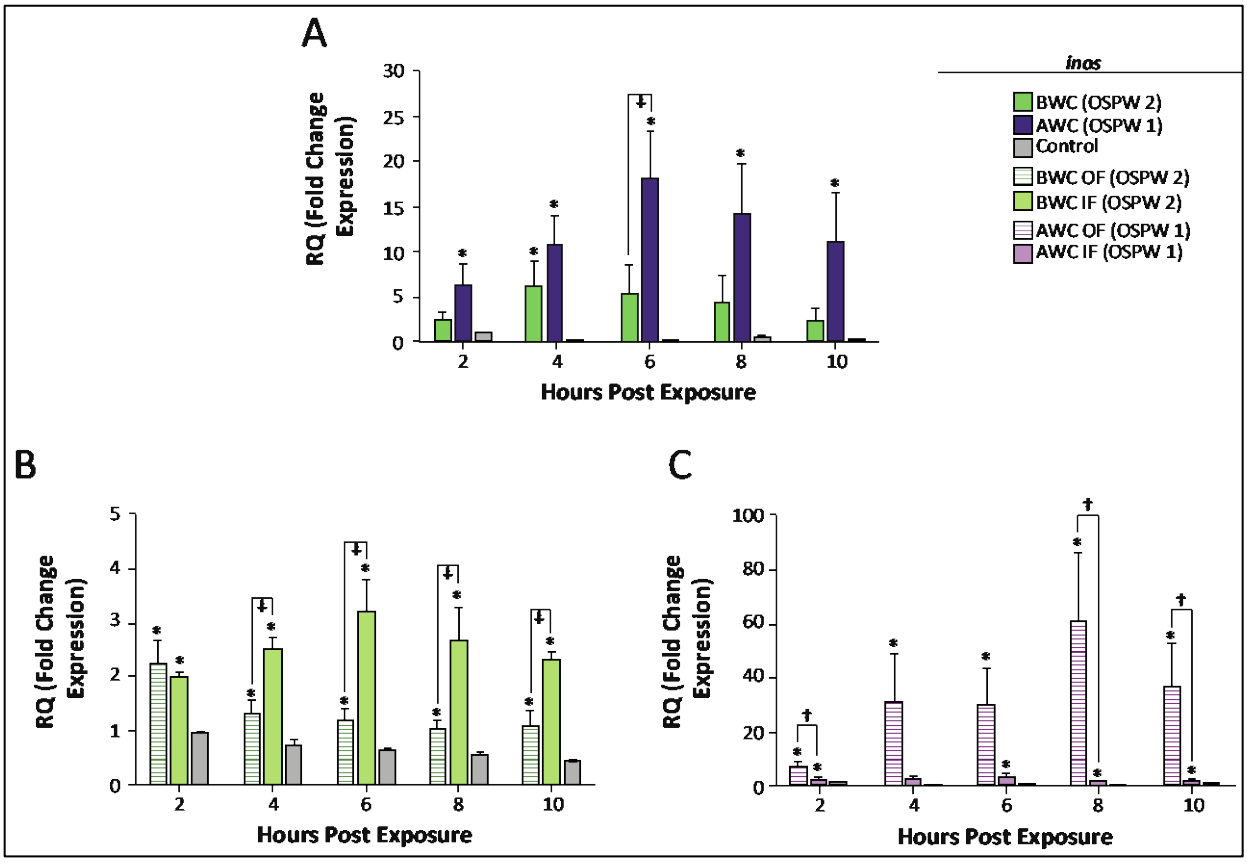

Figure 1. *inos* is differentially expressed based on source and fractionation of oil sands process-affected water (OSPW). 3 x10^5 RAW macrophage cells were exposed to a 50% v/v displacement of OSPW to cell culture media. Cells were harvested at 2-hour intervals (to a total exposure period of 10 hours), and the expression of the antimicrobial gene *inos* was examined in comparison to a baseline control of untreated (PBS exposed) cells using RT-qPCR. (A) Cells exposed to whole OSPW1 (the after water cap (AWC) sample) and whole OSPW2 (the before water cap (BWC) sample), (B) the OSPW2 OF and IF, and (C) the OSPW1 OF and IF were all examined for fold-change differences. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

Exposure of macrophages to whole OSPW1 and its OF induced intracellular iNOS enzyme expression and nitric oxide secretion activity

We detected the presence of intracellular iNOS protein in OSPW-exposed macrophages by flow cytometry as a second bioindicator supporting that OSPW-exposed macrophages activated the iNOS antimicrobial pathway. Furthermore, since *inos* expression levels were significantly increased following treatment of the cells with whole OSPW1 and the OSPW1-OF, we wanted to determine if this effect predictably extended to an increase in intracellular iNOS protein levels. As shown in Figure 2, whole OSPW1 (Fig. 2A) and OSPW1-OF (Fig. 2B) significantly increased the proportion of cells that stained positive for intracellular iNOS when compared to either the untreated PBS control group or whole OSPW2-exposed cells. Of interest, an inverse relationship between exposure doses and bioactivity were observed as the lowest %v/v of whole OSPW1 and OSPW1-OF (i.e., 10% v/v) induced the maximum relative proportions of iNOS staining compared to cells treated with 50% v/v (Fig. 2A and 2B). In comparison, no significant increases in iNOS staining were observed when the cells were treated with the OSPW1-IF at any of the doses tested (Fig. 2C). Furthermore, when the cells were exposed to whole OSPW2 and its fractions, the only statistically significant effect was associated with the 50% OSPW-2 IF exposure group (Fig. 2C), which only marginally caused an increase in the %iNOS-PE(+) cells at ~10%.

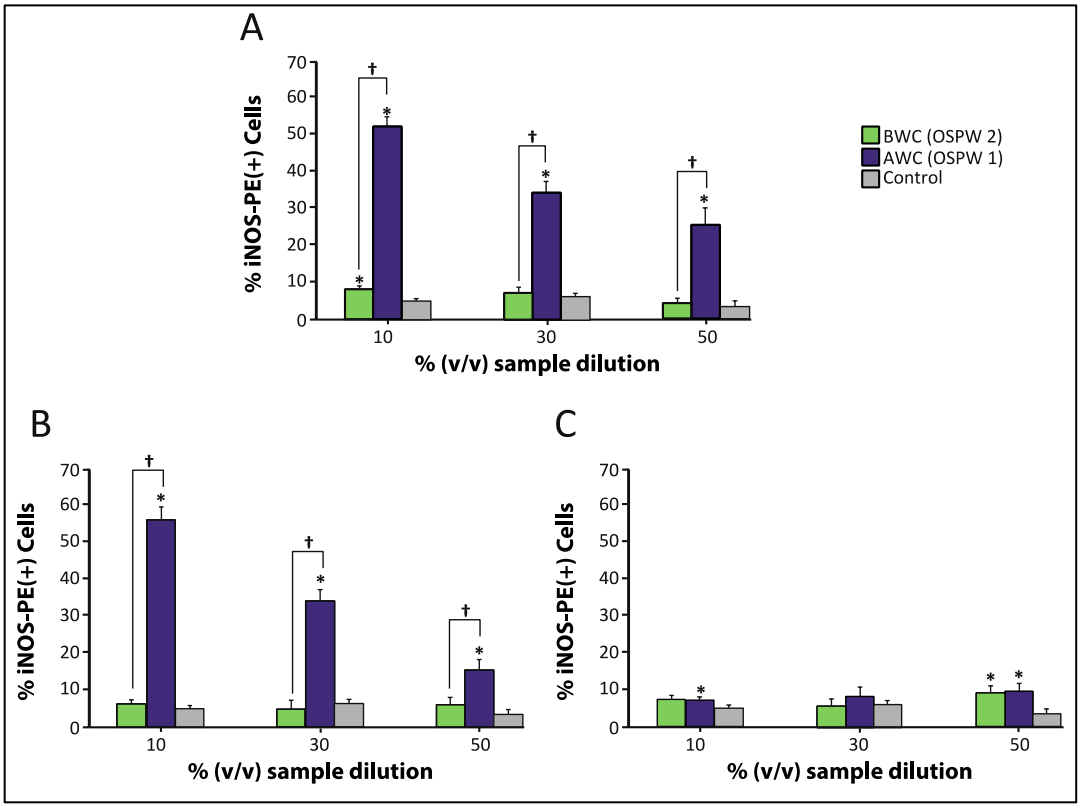

Figure 2. Increases in the proportion of cells that stained positive for intracellular iNOS following exposure to whole OSPW1 and its OF. 3 x10^5 RAW macrophage cells were exposed to a 10%, 30%, or 50% v/v displacement of OSPW to cell culture media. Cells were harvested after 24 hrs and the intracellular presence of the iNOS protein was examined using flow cytometry and compared to a baseline control of untreated (PBS exposed) cells. The relative proportion of iNOS staining cells was determined as a percentage from the total analyzed population of 10,000 cells per sample after staining with a monoclonal anti-iNOS-PE antibody stain. (A) Whole OSPW1 (the after water cap (AWC) sample) and OSPW2 (the before water cap (BWC) sample exposures. (B) OSPW1-OF and OSPW2-OF

exposures. (C) OSPW1-IF and OSPW2-IF exposures. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

Production of the antimicrobial compound NO was subsequently examined following OSPW1 or OSPW2 exposures as a correlative indicator of macrophage iNOS enzyme activity (Fig. 3). As expected, whole OSPW1 induced the secretion of nitrite (NO_2^-) and this bioactivity was also associated with the OSPW1-OF (Fig. 3) Overall, the NO_2^- secretion levels of macrophages following whole OSPW1 and OSPW1-OF exposures ranged between ~13-20 µM, which were all statistically higher than the levels produced by the PBS (control)-exposed cells as well as the levels observed following whole OSPW2 and OSPW2-OF exposures at all doses tested (Fig. 3A and 3B). Interestingly, as observed for iNOS protein levels, the highest levels of NO_2^- secretion occurred at the lower exposure doses of 10% and 30% v/v when compared to the 50% v/v exposures for whole OSPW1 and OSPW1-OF (Fig. 3A and 3B). Furthermore, the OSPW1-IF and OSPW2-IF exposures did not stimulate NO_2^- production when compared to the control groups at all concentrations tested (Fig. 3C). However, whole OSPW2 exposures at all doses tested and the 10% and 30% v/v OSPW2-OF exposures did significantly increase the NO_2^- production response of macrophages when compared to the PBS-exposed cells. However, these measured increases were all significantly lower than the whole OSPW1 and OSPW1-OF-induced bioactivity responses. **Overall, these results correlate with the *inos* expression and iNOS protein data and further support that whole OSPW1 contains organic factors that potently activate the macrophages.**

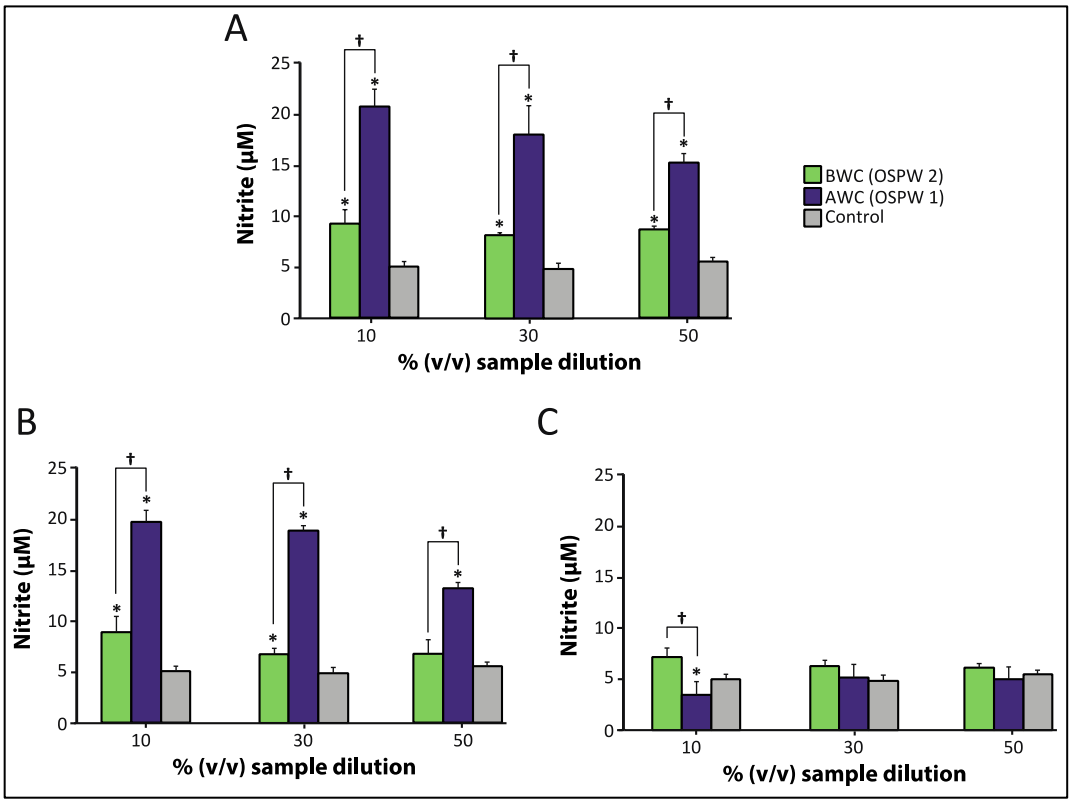

Figure 3. Nitric oxide (NO) secretion is significantly induced following exposure to whole OSPW1 and its OF. $3x10^5$ RAW macrophage cells were exposed to a 10%, 30%, or 50% v/v displacement of OSPW to cell culture media. Cells

were harvested after 24 hours and the extracellular presence of nitrite, a proxy for nitric oxide, was quantified in cell supernatants through the Griess reaction colorimetric assay in µM. (A) Cells exposed to whole OSPW1 (the after water cap (AWC) sample) and whole OSPW2 (the before water cap (BWC) sample). (B) OSPW1-OF and OSPW2-OF exposures. (C) OSPW1-IF and OSPW2-IF exposures. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

OSPW1 and OSPW2 exposures both significantly increase the proportion of cells expressing two selected surface immune markers

We examined the effects of OSPW exposures on the surface protein expression profile of macrophages as an additional bioindicator for the immunotoxic effects of OPSW. Activated macrophages display functional phenotypes that can be broadly categorized based on their functional properties as M1 (proinflammatory) and M2 (anti-inflammatory). Therefore, we selected a classical M1 macrophage marker (CD86) and the M2 marker CD206 for further analyses of macrophage activation following OSPW exposures. As shown in Fig. 4A, whole OSPW1, whole OSPW2, and their fractions all induced significant increases in the proportion of macrophages expressing CD86 when compared to the control cells. Specifically, macrophages exposed to PBS (control) had a relatively low (~2%) or basal level of populational CD86 expression. However, when the cells were exposed to whole OSPW1 (50% v/v) for 24 hours, ~38% of the treated cells stained positive for CD86 (Fig. 4A). A significant increase in CD86 positive cells was also observed following exposure to OSPW1-IF, but this increase (i.e., 13%) was significantly lower than the levels observed when the cells were exposed to whole OSPW1 or the OSPW1-OF, which both had similar overall effects (Fig. 4A). Exposure to the whole OSPW2, also induced a significant increase in the CD86 positive population (~20% CD86 positive cells), however, this effect and that following the OSPW2-IF exposure were both significantly lower when compared to the proportion of CD86 positive cells detected following OSPW2-OF exposures (~38%; Fig. 4A). The effects observed for whole OSPW2, and its fractions were statistically different from each other, while the whole OSPW1 and OSPW1-IF, as well as OSPW1-IF and OSPW1-OF were statistically different when compared to each other**. Overall, these data suggest that both whole OSPW1 and whole OSPW2 contain factors that increase the proportion of cells positive for the M1 macrophage marker CD86 with the OFs from each sample showing the greatest overall bioactivity effects.**

In general, when macrophages express M1 markers, they often suppress M2 markers during a process known as functional polarization. To determine if the induction of CD86 was associated with a concomitant decrease in an M2 marker, we also examined the expression of CD206 in parallel exposure studies. As shown for CD86, exposure to whole OSPW1, whole OSPW2, or their fractions induced significant increases in the proportion of macrophages expressing CD206 when compared to control cells (Fig. 4B). For example, cells exposed to PBS (control) had a relatively low (~6%) level of populational CD206 expression, however, when the macrophages were treated with either whole OSPW1 or OSPW2 and their fractions, significant increases in the CD206 positive staining population were observed, which ranged between 16%-28% (Fig. 4B). These increases were all significantly higher than the control, but unlike what we observed for CD86, no discernable pattern or segregation of bioactivity was consistently observed for the OF; although we noted that the OSPW1-OF exposure-induced increase in CD206 positive cells was statistically higher when compared to the whole OSPW1 and OSPW1-IF levels. Overall, regardless of the macrophage surface protein marker tested, OSPW exposures significantly increased the proportion of cells expressing CD86 and CD206. **This shows that in addition to intracellular iNOS protein levels,**

macrophage surface proteins can also be used as potential biomarkers to track OSPW-induced macrophage activation status.

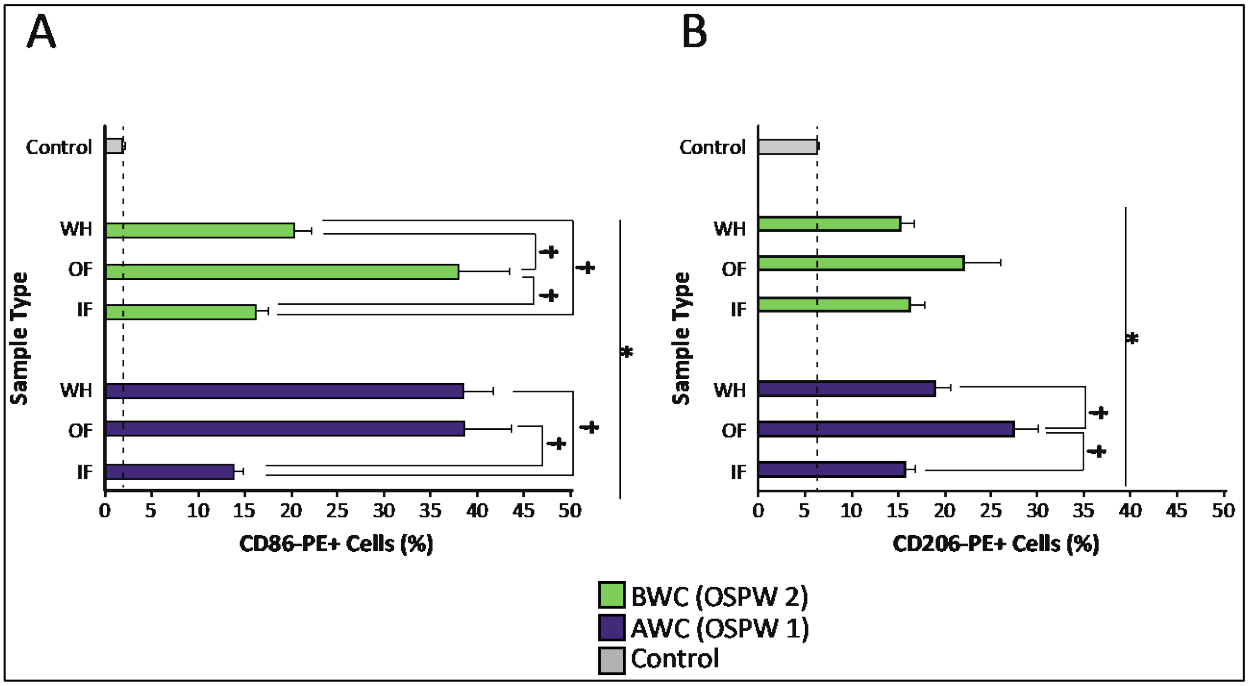

Figure 4. Expression of the macrophage surface receptors CD86 and CD206 are increased after OSPW exposures. $3x10^5$ RAW macrophage cells were exposed to a 50% v/v displacement of OSPW to cell culture media and were harvested after 24 hours. Cells were harvested after 24 hours and the population of cells with the surface receptor CD86 or CD206 expressed were examined through specific receptor staining (anti-CD86-FITC or anti-CD200-FITC monoclonal mouse antibodies) and subsequent flow cytometry analysis. Data is represented as a percent of cells positive for either receptor out of an analyzed 10 000 cells per sample. (A) CD86+ and (B) CD206+ cells were recorded for both waters and their fractions. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

Macrophage cytokine protein secretion activities following OSPW1 and OSPW2 exposures

We also examined cytokine secretion profiles following exposure of macrophages to Suncor DPL Waters using cytokine array technology. Generally, cytokines represent a diverse group of bioactive proteins produced by activated immune cells. When cells produce cytokine proteins, these factors are secreted into the supernatant, which can then be collected and analyzed using a microbead-based capture technology. Consequently, many different cytokine proteins can be specifically, simultaneously, and quantitatively monitored directly from small volumes of cell culture media. This is an extremely useful tool for determining if OSPW and its fractions have the potential to activate various cytokine secretion pathways. In addition, monitoring cytokine secretion as a bioactive indicator for OSPW constituents can also be used to generate distinct secretion profiles. These data then allow for direct comparisons between different water samples and/or whole vs. fractionated OSPW based on their cytokine secretion bioactivity.

The secretion of three selected pro-inflammatory cytokine proteins, interferon gamma-induced protein 10 kDa (IP-10), macrophage inflammatory protein-2 (MIP-2), and monocyte chemoattractant protein-1 (MCP-1) were quantified from the supernatants of macrophages exposed to the two different OSPW samples and their fractions (Fig. 5). After a 24-hour (50% v/v) exposure, both whole OSPW1 and OSPW2 induced statistically significant increased secretion levels of all three cytokines compared to their respective controls (Fig. 5A). Following whole OSPW2 exposure, cytokine secretion activity ranged from a 33-55% increase, while following whole OSPW1 exposure, the cytokine secretion activities ranged between 40-50% (Fig. 5A).

Notably, differential cytokine secretion activity was observed when the OSPW1 and OSPW2 isolated fractions were tested. Specifically, OSPW1-OF exposure induced cytokine secretion activities (34-42%) that were all significantly higher when compared to the controls, whereas the cytokine secretion activities induced following OSPW2-OF exposures (Fig. 5B) were only statistically significant from controls (≤6%) for the MIP-2 and MCP-1 chemokines (Fig. 5B), and these levels were markedly lower than the OSPW1-OF-induced activities (Fig. 5B). In comparison, when the OSPW2-IF exposed macrophage supernatants were tested, a significant 38-49% increase of cytokine secretion activity was observed (Fig. 5C). In contrast, the cytokine secretion activities following OSPW1-IF exposures were ≤17% for all three cytokines measured, of which only MIP-2 was statistically different than the control (Fig. 5C). **Overall, these results indicate that whole OSPW1 and OSPW2 exposures activate macrophage pro-inflammatory cytokine secretion and that when fractionated, these activities differentially segregated to the OSPW1-OF and the OSPW2-IF. These data also clearly show that both fractionation of OSPW and subsequent reconstitution of the fractions can be sensitively monitored based on discrete changes in bioactivity detected using cytokine secretion bioassays.**

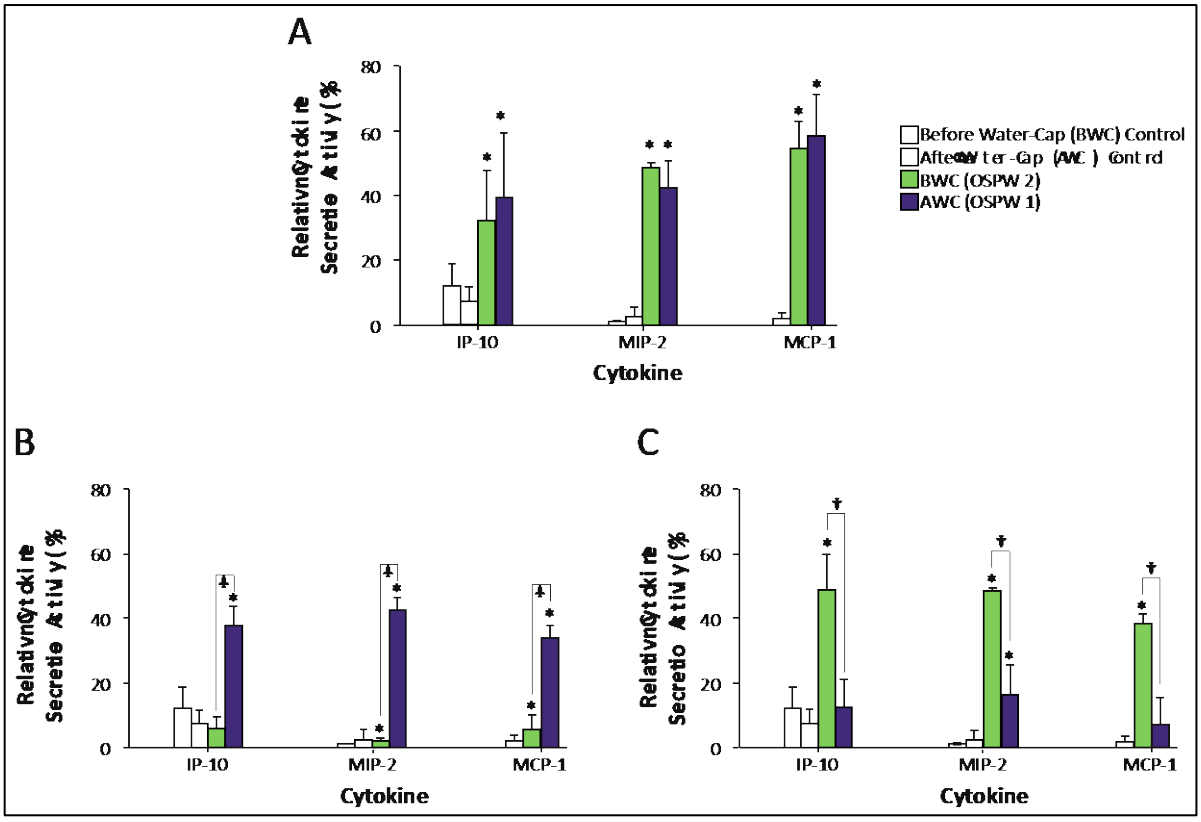

Figure 5. Macrophage cytokine protein secretion activities following OSPW1 and OSPW2 exposures $3x10^5$ RAW macrophage cells were exposed to a 50% v/v displacement of OSPW to cell culture media and after 24 hours the supernatants were collected and sent for IP-10, MIP-2, and MCP-1 cytokine quantification to Eve Technologies Corporation. Data is represented as the normalized secretion percentage per cytokine. (A) Whole OSPW1 (the after water cap (AWC) sample) and whole OSPW2 (the before water cap (BWC) sample) exposed cells. (B) OSPW1 and OSPW2 organic fraction (OF), and OSPW1 and OSPW2 inorganic fraction (IF)-exposed cells were examined for fold-change differences. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

Induction of proinflammatory cytokine gene transcription following OSPW exposures

We used qPCR to examine the expression of *ip-10*, *mcp-1*, and *mip-2* genes, providing a sensitive and much earlier temporal assessment of Suncor DPL water bioactivity. These experiments were also performed to validate the observations made using the cytokine secretion assays, which were performed after overnight (i.e., 24 hrs) exposures. The early temporal (i.e., 2-10 hour) gene expression levels of the proinflammatory cytokine genes *ip-10*, *mip-2*, and *mcp-1* were examined by qPCR after macrophages were exposed to the two different OSPW samples and their fractions (Fig. 6). The key results for each gene examined are detailed below and **in general our transcriptional cytokine gene profiling again confirms that OSPW contains potent bioactive components that can be detected as early as 2 hrs following exposure of the cells. Furthermore, as shown earlier, fractionation of the AWC Suncor DPL (OSPW1) increases its potency as an inducer of cytokine gene expression, whereas fractionation of BWC Suncor DPL (OSPW2) does not increase its potency as an activator of cytokine gene expression.**

Overall, each gene examined showed distinct expression patterns following exposure of the cells to the whole OSPW samples and their organic and inorganic fractions. Specifically, *mip-2* expression had the highest relative induction levels at the earliest time point examined (e.g., 2 hours = ~250-fold change) when cells were exposed to 50% v/v of the whole OSPW1 (Fig. 6A). Then, *mip-2* levels decreased over time down to a final RQ (relative quantification, equivalent to fold-change) of 23 after 10 hours. A similar trend was also observed for whole OSPW2, decreasing from a maximum 83 RQ at 2 hours down to 11 RQ at 10 hours (Fig. 6A). The 2- and 6-hour exposures for both whole waters, and the whole OSPW1 exposures at 4 and 8 hours were all statistically significant from the control cells treated with PBS. Furthermore, OSPW2-IF induced a 348 RQ expression of *mip-2* at 2 hours, an effect that also gradually reduced over the exposure period (Fig. 6B). OSPW2-OF exposures also induced *mip-2* expression but at a notably smaller magnitude when compared to the OSPW2-IF effects, decreasing from 40 RQ to 11 RQ at the initial and final exposure times; 2 hours and 10 hours, respectively. Although all exposure-induced expression levels were statistically different from the control, only the first two time points (i.e., 2 hours and 4 hours) showed significant differences in the *mip-2* RQ values between the OSPW2-OF and OSPW2-IF exposures. However, when the macrophages were exposed to the OSPW1-OF for 2 hours, a relatively large induction of *mip*-2 expression (RQ of 1216), which decreased down to 523 RQ at 10 hours, was observed (Fig. 6C). In comparison, OSPW1-IF exposures induced a relatively low RQ of 84 for *mip-2* at 2 hrs, and only a RQ of 10 after 10 hours.

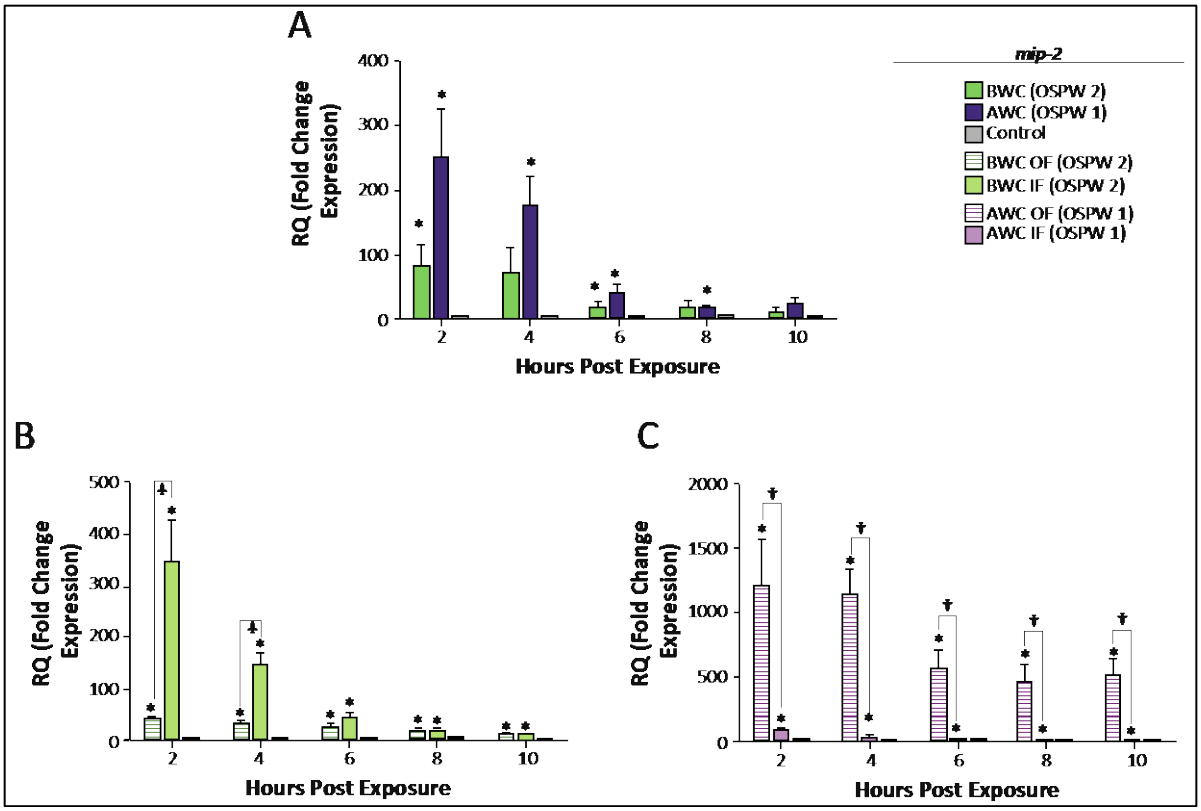

Figure 6. *mip-2* expression following exposure of macrophages to oil sands process-affected waters (OSPWs). 3 x10^5 RAW macrophage cells were exposed to a 50% v/v displacement of OSPW to cell culture media. Cells were harvested at 2-hour intervals (to a total exposure period of 10 hours), and the expression of the pro-inflammatory gene *mip-2* was examined in comparison to a baseline control of untreated (PBS exposed) cells using RT-qPCR. (A) Cells exposed to whole OSPW1 (the after water cap (AWC) sample) and whole OSPW2 (the before water cap (BWC) sample), (B) the OSPW2 OF and IF, and (C) the OSPW1 OF and IF were all examined for fold-change differences. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

mcp-1 transcription increased consistently over time when macrophages were treated with the whole OSPW samples (Fig. 7A). For example, after 2 hours, both waters induced relatively low expression levels of *mcp-1* (OSPW2 = ~7 RQ and OSPW1= 9 RQ), which then progressively increased over time, peaking at 10 hours (OSPW2 = 37 RQ, and OSPW1 = 41 RQ). Other than the whole OSPW2 exposures at 4 and 8 hours, all increases in *mcp-1* expression levels were statistically significant when compared to the control, but not between the two whole waters (Fig. 7A). When the macrophages were exposed to the OSPW2-IF, a similar trend of induced *mcp-1* transcription occurred over the exposure period (Fig. 7B). In comparison, *mcp-1* levels following OSPW2-OF exposure did not reach double RQ digits, instead only peaking to a 7-fold change at 2 hours, which decreased over time to a value of only 2 RQ at 10 hours. Despite these relatively small inductions levels, all OSPW2-OF exposures caused statistically significant inductions of *mcp-1* when compared to the controls. Alternatively, the OSPW2-IF exposure-induced increases in *mcp-1* where all significantly higher than those induced by the OF at each exposure period (Fig. 7B). Finally, in agreement with *mip-2* expression data (Fig. 6C), OSPW1-OF exposure induced higher *mcp-1* gene expression levels when compared to cells treated with the OSPW1-IF (Fig. 7C). However, unlike the trend

observed for *mip-2,* the *mcp-1* expression increased sharply between the 2- and 4-hour exposures (34 RQ to 122 RQ), peaked at 8 hours (201 RQ), and then decreased down to 137 RQ at 10 hours. Finally, the OSPW1-IF-induced *mcp-1* expression levels were relatively lower overall when compared to the OSPW1-OF effects, but all OSPW1-IF data points, excluding the 2-hour time point were statistically significant from the control sample (Fig. 7C).

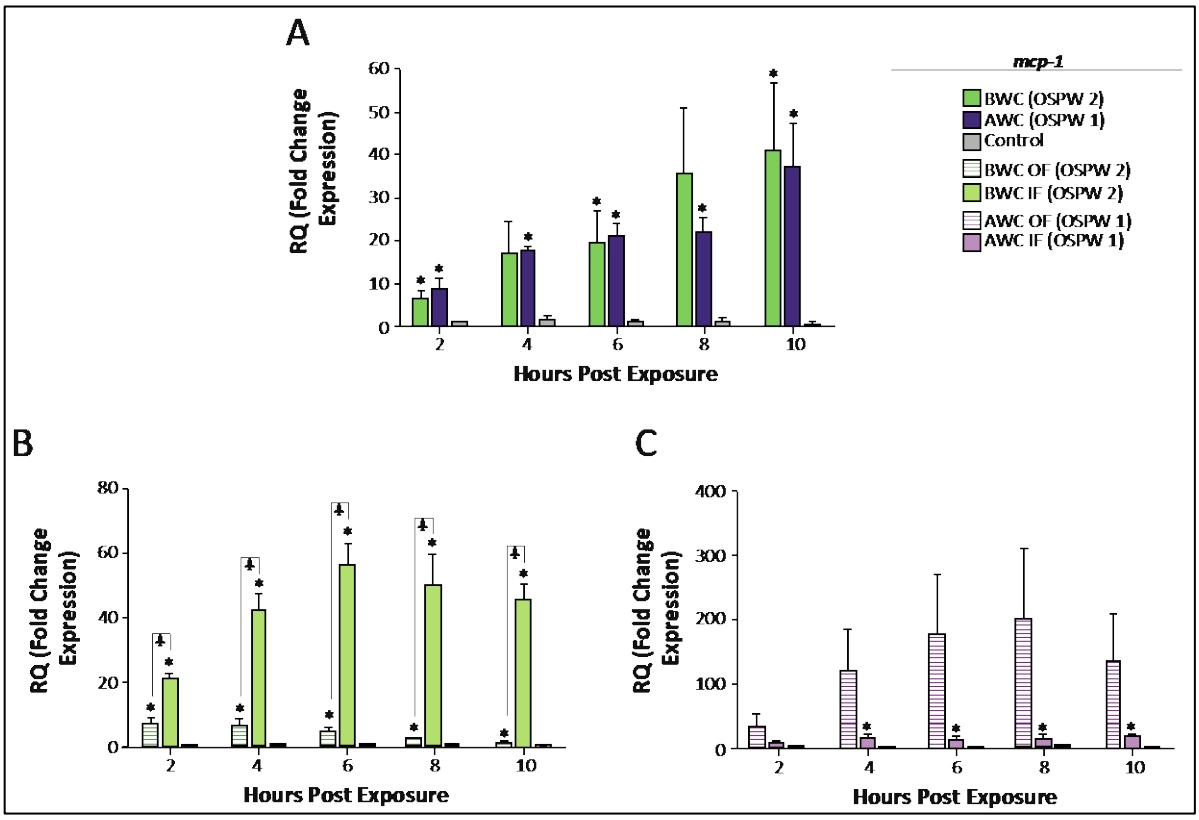

Figure 7. *mcp-1* following exposure of macrophages to oil sands process-affected waters (OSPWs). 3 x10^5 RAW macrophage cells were exposed to a 50% v/v displacement of OSPW to cell culture media. Cells were harvested at 2-hour intervals (to a total exposure period of 10 hours), and the expression of the pro-inflammatory gene *mcp-1* was examined in comparison to a baseline control of untreated (PBS exposed) cells through RT-qPCR. (A) Cells exposed to whole OSPW1 (the after water cap (AWC) sample) and whole OSPW2 (the before water cap (BWC) sample), (B) the OSPW2 OF and IF, and (C) the OSPW1 OF and IF were all examined for fold-change differences. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

ip-10 inductions showed a relative bell-shaped pattern following exposure of the cells to the different OSPWs and their fractions (Fig. 8). For example, an early expression peak at 4 hours for whole OSPW2 (70 RQ), and 6 hours for whole OSPW1 (149 RQ) gradually diminished over the exposure period until the final time of 10 hours (Fig. 8A). All whole OSPW1 exposure-induced effects were statistically significant when compared to the control, but only the 2-hour exposure after whole OSPW2 was statistically significant. As observed for both *mip-2* and *mcp-1* expression, the OSPW2-IF was the primary bioactive fraction, initiating its maximal response of ~3-fold change at 6 hours, with a gradual decrease to <1-fold at 10 hours (Fig. 8B). In comparison, macrophages treated with the OSPW2-OF had consistently low (<1-fold change) levels of

ip-10 expression at all time points measured (Fig. 8B) but all these treatment-induced expression levels were statistically significant from the control and between each fraction for the OSPW2 exposures (Fig. 8B). Finally, Macrophages exposed to OSPW1-OF had the highest *ip-10* induction levels with a relative temporal bell-shaped curve expression pattern observed, increasing from 131 RQ at 2 hours to a peak expression of 572 RQ at 6 hours, which then decreased to 141 RQ after 10 hours (Fig. 8C). Following OSPW1-IF exposures, the overall expression values were significantly lower, however all IF exposure-induced transcription levels being significantly different from the control and between fractions (Fig. 8C).

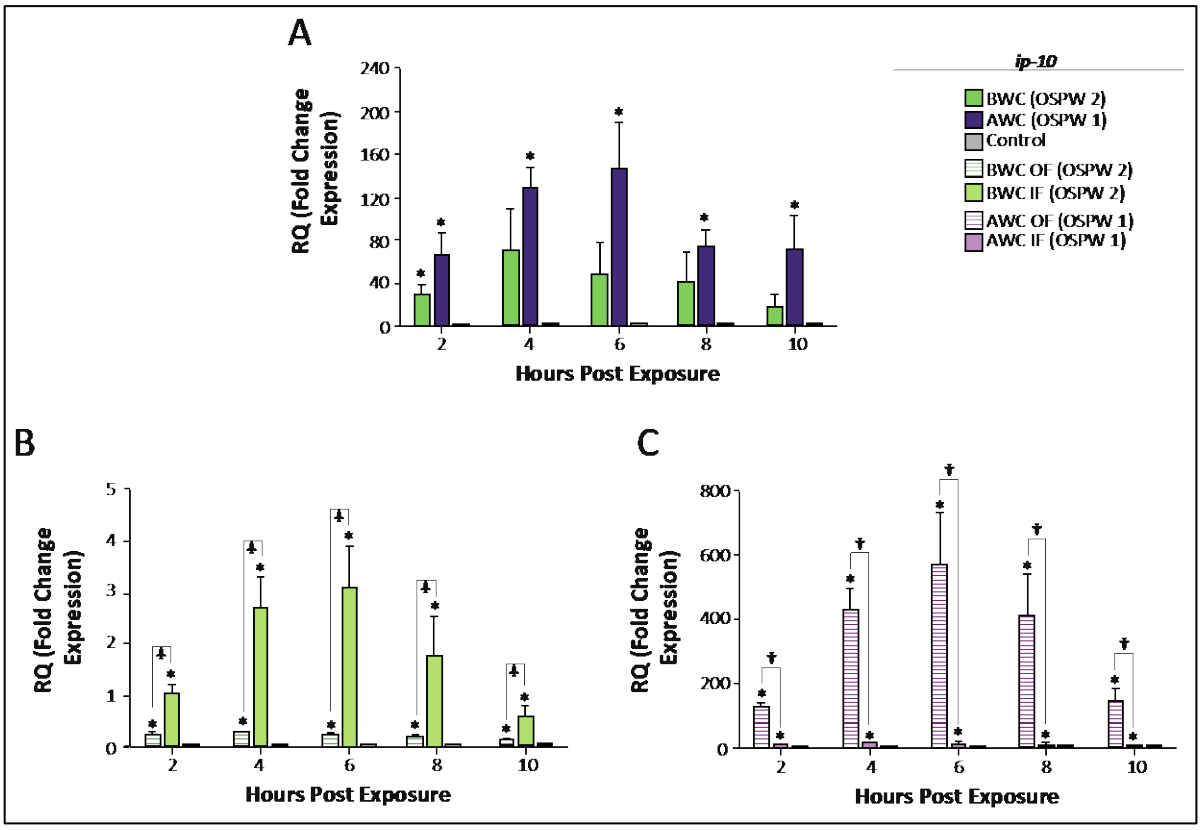

Figure 8. *ip-10* following exposure of macrophages to oil sands process-affected waters (OSPWs). 3 x10^5 RAW macrophage cells were exposed to a 50% v/v displacement of OSPW to cell culture media. Cells were harvested at 2-hour intervals (to a total exposure period of 10 hours), and the expression of the pro-inflammatory gene *ip-10* was examined in comparison to a baseline control of untreated (PBS exposed) cells through RT-qPCR. (A) Cells exposed to whole OSPW1 (the after water cap (AWC) sample) and whole OSPW2 (the before water cap (BWC) sample), (B) the OSPW2 OF and IF, and (C) the OSPW1 OF and IF were all examined for fold-change differences. Bars display the mean of biological experiments performed in triplicate, with standard error of the mean (SEM) error bars. Asterisks indicate statistical significance compared to the control, while daggers indicate significant differences between water sources or fractions interpreted from a 95% confidence interval.

OSPWs vs. Reference Sample Bioactivity Profiles

We compared the effects of OSPW1 and OSPW2 exposures on macrophage bioactivity relative to the effects of exposing the cells to Edmonton tap water or the reference sample obtained from the Athabasca River. Notably, all four water samples did not affect the health of the exposed cells since all had 0% Cytotox. activities (Fig. 9). OSPW had 75% activity for iNOS and 100% for NO (Fig. 9A), whereas OSPW2

(Fig. 9B) and the Athabasca River water (Fig. 9D) had 25% activity for these two markers while Edmonton tap water (Fig. 9C) had 0% activity. In fact, other than two cytokine proteins (TNF-alpha and VEGF), tap water (Fig. 9C) had no relative activity for any of the biomarkers tested. Interestingly, the overall bioactivity profile for Athabasca River water (Fig. 9D) was like that of OSPW2 (Fig. 9B) showing two active peaks for MIP-2 and TNF-alpha secretion. However, when compared with OSPW1, the Athabasca River water sample had lower relative activity for IL-6, KC, LIF, MCP-1, and VEGF. **Overall, these results show that municipal tap water is the least bioactive and that both OSPW2 and the representative Athabasca River Water sample show enhanced bioactivity for two out of the thirteen markers tested. In comparison, OSPW1, which is the after water cap sample of Suncor's DPL at Lake Miwasin is the most bioactive sample showing relative increases in nine out of the thirteen examined biomarkers tested. While this approach provides qualitative comparisons between different water samples using multiple inflammatory markers, a more comprehensive sampling and testing protocol is required to make conclusions about the overall bioactivity of Athabasca River samples in proximity to as well as distal to tailings ponds and DPLs in the Alberta oil sands region. Additional testing is also required to generate research-supported conclusions on bioactivity and its association with ecotoxiciology.**

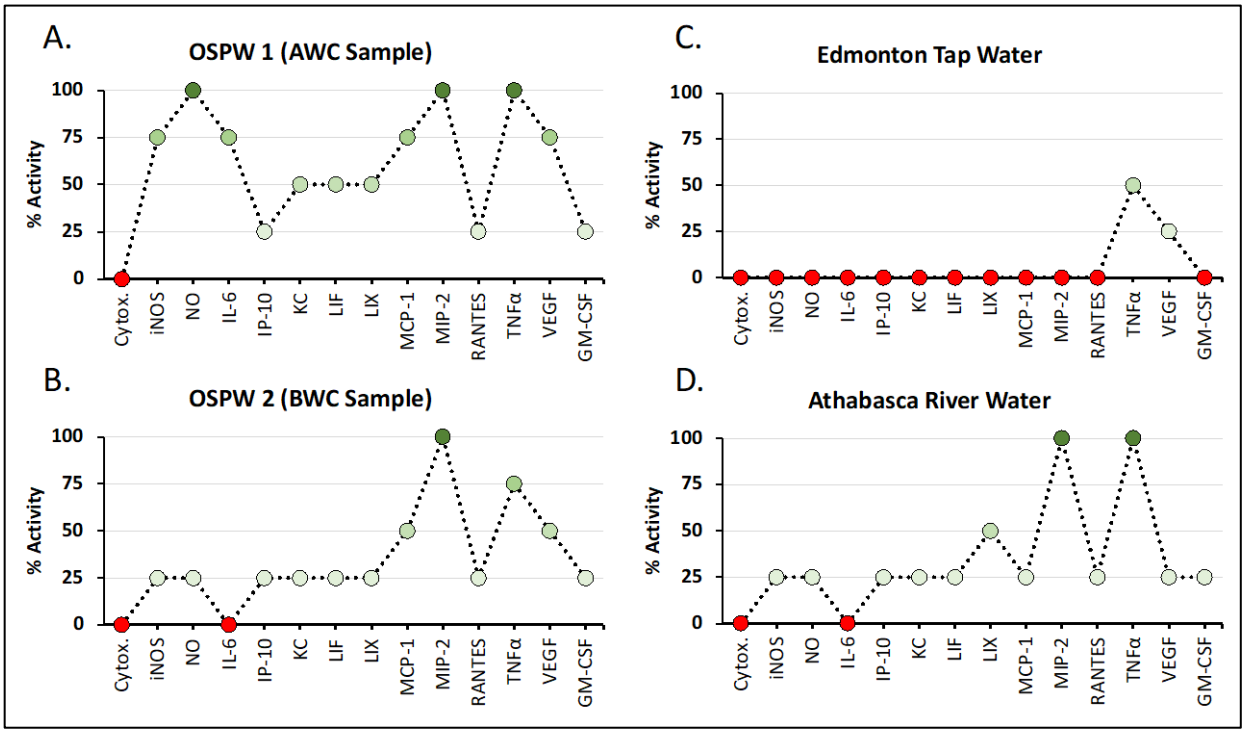

Figure 9. Bioactivity profile comparisons of two OSPW samples with Edmonton municipal tap water and a reference Athabasca River water sample. Each water sample was comprehensively tested using the series of bioassays listed on the x-axis by exposing the cells to 50% v/v of the samples for 24 hrs. To allow for cross-assay and cross-sample comparisons, data was normalized and presented as % relative activity. To calculate the % relative activity, samples with the highest bioassay response for each assay were set to 100% and the other samples were then plotted as equal to (e.g. 100%) or proportionately lower in activity using the set intervals of 75%, 50%, 25%, and 0% (if not activity was observed). Cytox. refers to cell viability data, iNOS and NO refer to levels of the iNOS protein and NO production, respectively, and the remaining biomarkers are all different proinflammatory cytokine proteins that were measured (IL-6, IP-10, KC, LIF, MCP-1, MIP-2, RANTES, TNF-alpha, VEGF, and GM-CSF).

Examination of the possible interactions between the OSPW-OF and OSPW-IF on macrophage functional responses

We also examined possible interactions between the OF and IF of OSPW1 using the immune cell-based bioassay focused on the NO antimicrobial pathway. Interestingly, these experiments showed that when the NO-inducing OSPW1-OF was mixed with varying concentrations of the OSPW1-IF (i.e., the non-active fraction), we observed that **the IF was uniquely capable of suppressing the bioactivity of the OF**. For example, when cells were treated with a constant 10% v/v dose of OF mixed with a range of IF concentrations (i.e., 10% v/v-70% v/v), a clear dose-dependent abrogation of the OF bioactivity was observed because of an IF-induced inhibition of this response (Fig. 10A). Specifically, compared with controls (i.e., mixing the OF with PBS instead of IF), a 32%, 54%, and 82% inhibition of OF-induced NO production occurred when 10% v/v of the OF was mixed with 20%, 50%, and 70% v/v of the IF, respectively. Furthermore, when we assessed iNOS protein levels in the exposed cells, a similar trend for the IF-induced inhibition of the OF bioactivity was observed (Fig. 10B). **This indicates the presence of inorganic constituents within Suncor OSPW1 that can suppress the bioactivity of the organic constituents. These data also suggest that fractionation of OSPW may potentiate its bioactivity by disrupting OF-IF complexes.** In comparison, since neither fraction of Suncor OSPW2 induced the NO antimicrobial pathway, we were unable to examine possible interactions between the OF and IF of this water sample using the NO antimicrobial pathway.

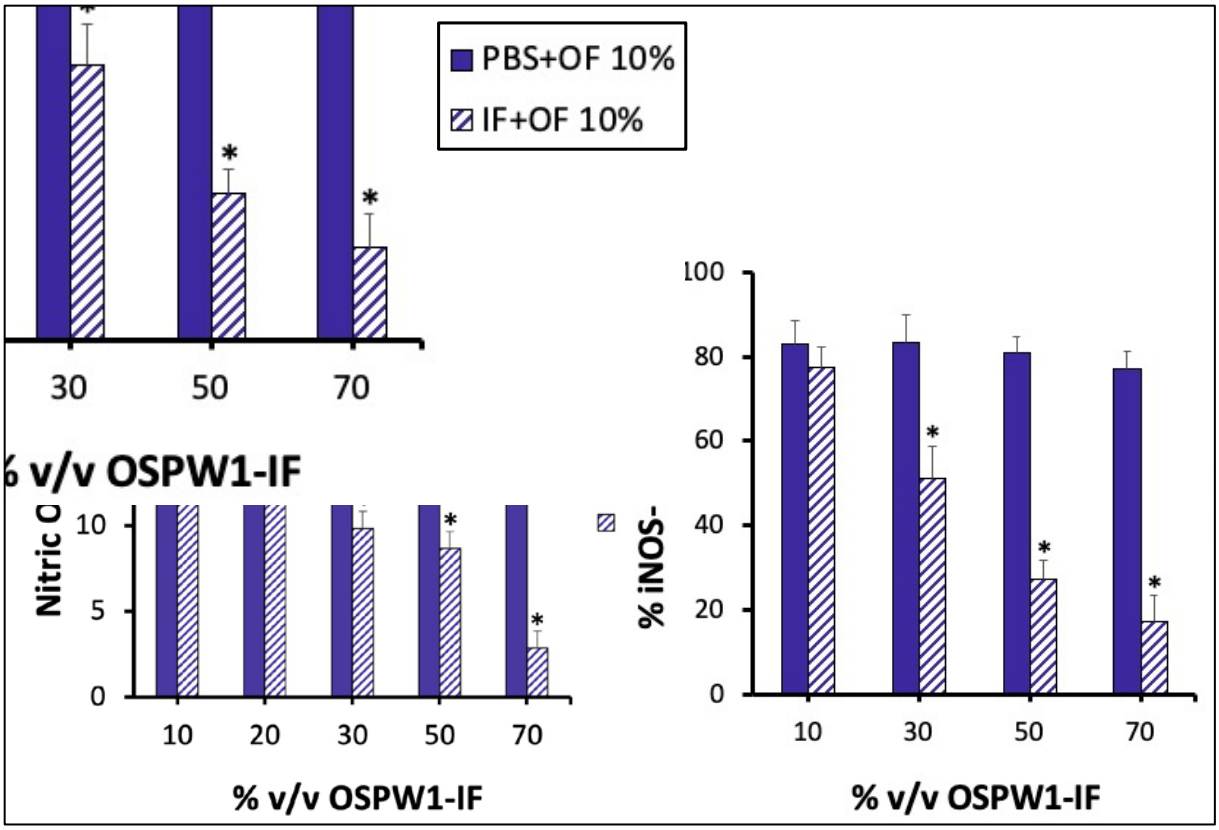

Figure 10. OSPW1-OF-mediated bioactivity is abrogated when mixed with various exposure doses of the OSPW1-IF component. RAW 264.7 cells were exposed to samples containing 10% v/v OSPW1-OF mixed with 10-70% v/v of either PBS or IF for 24 h. After the 24 h exposure, bioactivity of the OSPW1-OF were quantified by (A) the production of nitric oxide (NO) measured via Griess Reaction or by (B) using flowcytometry to measure changes in the percentage of cells positive for the intracellular protein iNOS (*) indicates significant differences in the responses of cells exposed to IF/OF mixtures relative to the responses measured for cells exposed to the PBS/OF mixtures at each indicated dilution (ie. 10% to 70% v/v). Data shown represent the mean ± SEM from four independent experiments and significance values were determined by one-way ANOVA analysis ($p < 0.05$) using GraphPad Prism 9.2.

We then examined possible interactions between the OF and IF of OSPW1 using the cytokine secretion bioassay. Here, we followed a similar protocol described above for assessing how OF and IF mixtures affected the NO-inducing activity. Again, since the OF was primarily responsible for cytokine-inducing bioactivity, we directly examined what effect(s) mixing it with the IF (i.e., the non-bioactive fraction) had on this response. To do this we mixed 10% v/v of the OF with varying concentrations of the IF or PBS as a control. For simplicity, and to summarize our major findings, only data for 10% OF v/v mixed with 50% v/v of the IF are described. Overall, mixing the OSPW1-OF with the bioactive OF, significantly potentiated the cytokine secretion bioactivity of the OF. This affect was also cytokine specific. For instance, the relative IP-10 secretion activity of the OF was enhanced by 73% (±7.2%) when this fraction was mixed with IF relative to when it was mixed with the same dose of PBS. To a lesser, but still at a significant level, mixing OF with IF enhanced the relative cytokine secretion activity of the OF by 46.5% (±7.7%) (RANTES) and 33.7% (±4.3%) (LIF). In comparison, no relative enhancement of the OF-induced cytokine secretion of G-CSF, TNF-alpha, MIP-2, or MCP-1 were detected when the OF was mixed with the IF. **Overall, these data**

clearly demonstrate a potent interactive effect for when the OF and IF of OSPW1 were reconstituted. However, this effect was only specific for some but not all cytokine markers examined, and the potentiation of the OF-induced cytokine secretion effect was contrary to the significant inhibitory effects that the IF had on the OF-induced NO secretion activity described earlier. Regardless, **these data support that interactions between organic and inorganic constituents of an OSPW sample profoundly affects their bioactivity profiles** (i.e., inhibition of the NO antimicrobial response vs. enhancement of proinflammatory cytokine secretion).

Finally, we also tested for possible interactions between the OF and IF of OSPW2 using the cytokine secretion bioassay. Since the IF of this water was uniquely responsible for cytokine-inducing bioactivity, we directly examined what effect(s) mixing it with the OF (i.e., the non-bioactive fraction) had on this response. To do this we mixed 10%-40% v/v of the IF with matching doses of the OF or PBS as a control, which as described below shows that interactions between organic and inorganic constituents of OSPW2 profoundly affects their bioactivity. For example, alteration of IF-mediated secretion of VEGF occurred when it was mixed with equal doses of the OF. Specifically, when 20% v/v each of OSPW2-IF and OF are recombined and then added to cells, the relative cytokine secretion activity of the macrophages was enhanced by 68% (±26%). However, when 40% v/v of each fraction were recombined, the relative cytokine secretion activity of the IF was augmented by 40% (±33%). The relative cytokine secretion activity of the IF for TNF-alpha, MIP-2, MCP-1, G-CSF, and IL-6 were also significantly enhanced by mixing it with equal volumes of the OF. Each of these cytokines showed variable responses specific for each set of reconstitution doses tested. Specifically, for MIP-2 secretion, the lowest mixing/reconstitution dose tested (10% v/v) caused a 41% (±2%) increase in secretion activity relative to a -9% (±8%) of cytokine secretion observed at a higher reconstitution dose (i.e., 40% v/v of IF mixed with 40% v/v OF) indicating this dose did not enhance MIP-2 secretion activity. Alternatively, for MCP-1, the relative cytokine secretion activity of macrophages was 262% (±16%) at 10% v/v mixtures but -9% (±17%) at 40% v/v mixtures. In contrast, when IP-10 secretion was examined, a notable inhibition of cytokine secretion activity was observed when the IF was reconstituted with the OF. This occurred at all doses examined resulting in overall suppression of IP-10 secretion activity ranging between 17-40%. Finally, RANTES secretion activity was inhibited 19% (±8%), 34% (±1%), 45% (±3%), and 74% (±1%) when the cells were treated with 10% v/v, 20% v/v, 30% v/v, and 40% v/v equal mixtures the OSPW2-IF reconstituted with OF, respectively. **Overall, these results demonstrate that by using a sensitive immune cell-based bioassay, we can detect specific and varying effects on immune cell bioactivity following reconstitution of the organic and inorganic fractions of OSPW2.**

Bioactive profiling of OSPW samples before and after treatment using AOP:

Main Tasks:

1. To establish bioactive profiles for OSPW before and after treatment using solar-activated zinc oxide photocatalytic oxidation or electro-oxidation
2. To perform water quality assessments on each sample (i.e., NA quantification and NA profiling).

Our original plan detailed in Amendment No.3 was to examine an Untreated and Solar-activated zinc oxide photocatalytic oxidation treated sample (OSPW1) as well as an Untreated and Electro-oxidation treated sample (OSPW2). However, this work was expanded further to include the examination of the following waters:

i) Untreated OSPW1 (Syncrude)
ii) Solar-activated zinc oxide photocatalytic oxidation treated OSPW1 (Syncrude)
iii) Electro-oxidation treated OSPW1 (Syncrude)
iv) Untreated OSPW2 (Suncor)
v) Solar-activated zinc oxide photocatalytic oxidation treated OSPW2 (Suncor)
vi) Electro-oxidation treated OSPW2 (Suncor)

Photocatalysis is a promising method for treating OSPW due its strong potential in degrading naphthenic acids, its requirement of less expensive and relatively safe chemicals, and relatively easy scale up for potential industrial applications. We investigated the performance of nanoparticle zinc oxide (ZnO) as a semiconductor catalyst activated by simulated solar radiation to treat real oil sands process water (OSPW). Zinc oxide nanoparticles were added to a glass beaker containing 200 mL of OSPW to achieve the desired catalyst concentration. The contents were mixed steadily for at least 30 minutes to achieve adsorption equilibrium on the particles. The mixture was then subsequently exposed to steady collimated radiation from a solar simulated system for photocatalytic reactions to take place under steady stirring at 500 rpm for 2 hr and 4 hr. Electrochemical advanced oxidation processes (EAOPs) are very efficient and effective treatment techniques for the degradation of persistence organic pollutants. These processes possess some distinguish advantages over other AOPs such as high versatility, eco-friendly, limited, or low chemical requirements and high amenability. We assessed the performance of electro-oxidation to treat real OSPW using a boron-doped diamond electrode. The experimental conditions included a current density of 10 mA cm^{-2} (400 mA/ 6.67 volts), and treatment times of 1 h (60 min) and 6 h (360 min). Filtered samples were taken at the different reaction times indicated and stored at 4^{o}C for subsequent analyses.

For water quality assessments, the Gamal El-Din lab conducted analyses to determine NA concentrations and species, DOC, Ammonia, Nitrate, Nitrite, Chloride, Sulphate, Phosphate, Bromide, and Fluoride. In addition, major analyte profiles will also be performed.

For the bioassays, samples were filtered using a 0.45 µm membrane filter and then added to the cell culture media at a 50% v/v dose. This is a standard dose used for all samples as 50% v/v is highest media displacement dose that can be performed without adversely affecting the cells. For bioactive profiling, no cytotoxicity can occur during the exposure period (i.e., 24 hr), so initially all samples were screened using a standard cell viability assay prior to examining bioactivity using our immune cell-based bioassays.

Key Results:
As expected, both untreated OSPW samples were potent activators of immune cells (i.e., macrophages) based on our antimicrobial assay (Fig. 11) as well as cytokine transcriptional activity assessment (Fig. 12 and cytokine secretion profiling (Fig. 13) experiments.

While both OSPW samples activated the iNOS antimicrobial pathway, the average NO production induced by untreated Syncrude (OSPW1) sample was higher than the activity induced following exposure of the cells to the Suncor OSPW2 sample (Fig.11). However, when the samples were treated using electrooxidation (1 hr and 6 hr treatments) or solar-activated zinc oxide photocatalytic oxidation (2 hr and 4 hr treatment), their ability to induce NO production were significantly reduced to baseline

levels.

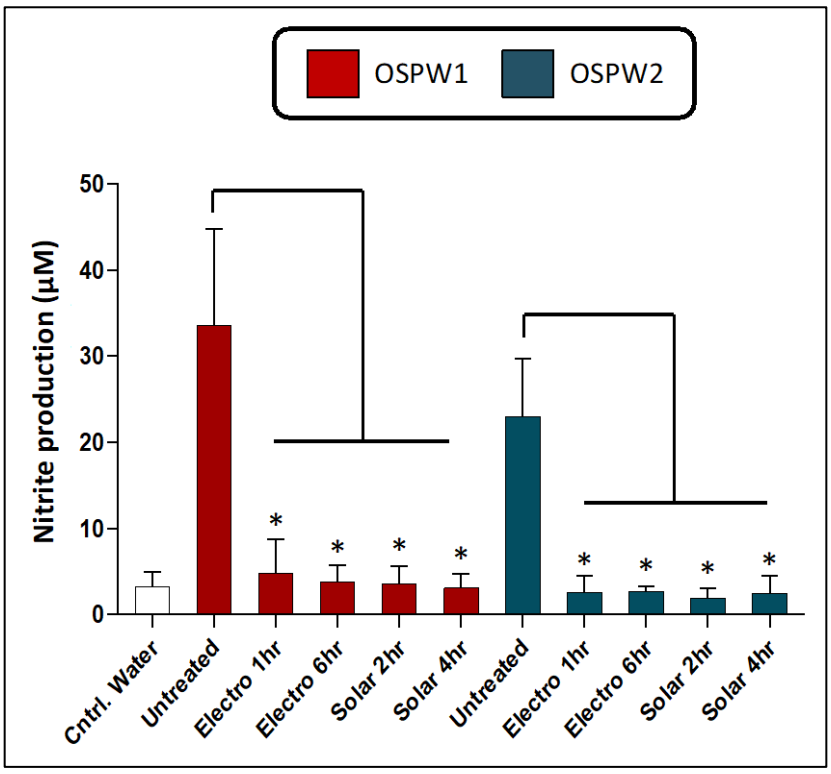

Figure 11. Effects of AOPs on OSPW-induced Nitric oxide (NO) production
$3x10^5$ RAW macrophage cells were exposed to a 50% v/v displacement of the various untreated and treated OSPW samples. Cells were harvested after 24 hours and the extracellular presence of nitrite, a proxy for nitric oxide, was quantified in cell supernatants using the Griess reaction colorimetric assay in µM. Data shown represent the mean ± SEM from four independent experiments and significance values were determined by one-way ANOVA analysis (* = $p < 0.05$) using GraphPad Prism 9.2. PBS = Phosphate Buffered Saline, Cntrl. Water = Control water (sterile).

Next, we examined the effects of the same OSPW treatments on the ability of OSPW samples to induce transcriptional activity of selected proinflammatory cytokine genes. As shown in Figure 12, both the untreated Syncrude (OSPW1) and the untreated Suncor (OSPW2) samples activated the expression of the proinflammatory cytokine genes *il-6*, *il-8*, *tnf-α*, and *il-1ß* after a 6 hr exposure of the cells. Then, as shown for NO production, gene expression levels were all significantly reduced after electrooxidation and solar photocatalysis. Unlike the broad inhibitory effects, we observed for NO production, varying levels of cytokine gene expression were observed, and, in some instances, there were observable trends in the inhibitory effects between the two OSPW samples. For example, *il-6* expression was effectively blocked when OSPW1 was treated, whereas although treated OSPW2 had significantly reduced *il-6* levels when compared to the untreated sample, there was a clear effect of extended treatment times (i.e., 6 hrs for electrooxidation and 4 hrs for solar photocatalysis) causing enhanced inhibition of cytokine gene expression (Fig. 12A). In general, this trend was consistent for the other cytokine genes examined and indicated differential susceptibility of the two OSPW samples to treatment-mediated abrogation of their inflammatory activities. It is important to note that all treatments had significant inhibitory effects on both Syncrude and Suncor OSPW-mediated activation of proinflammatory cytokine

gene expression levels.

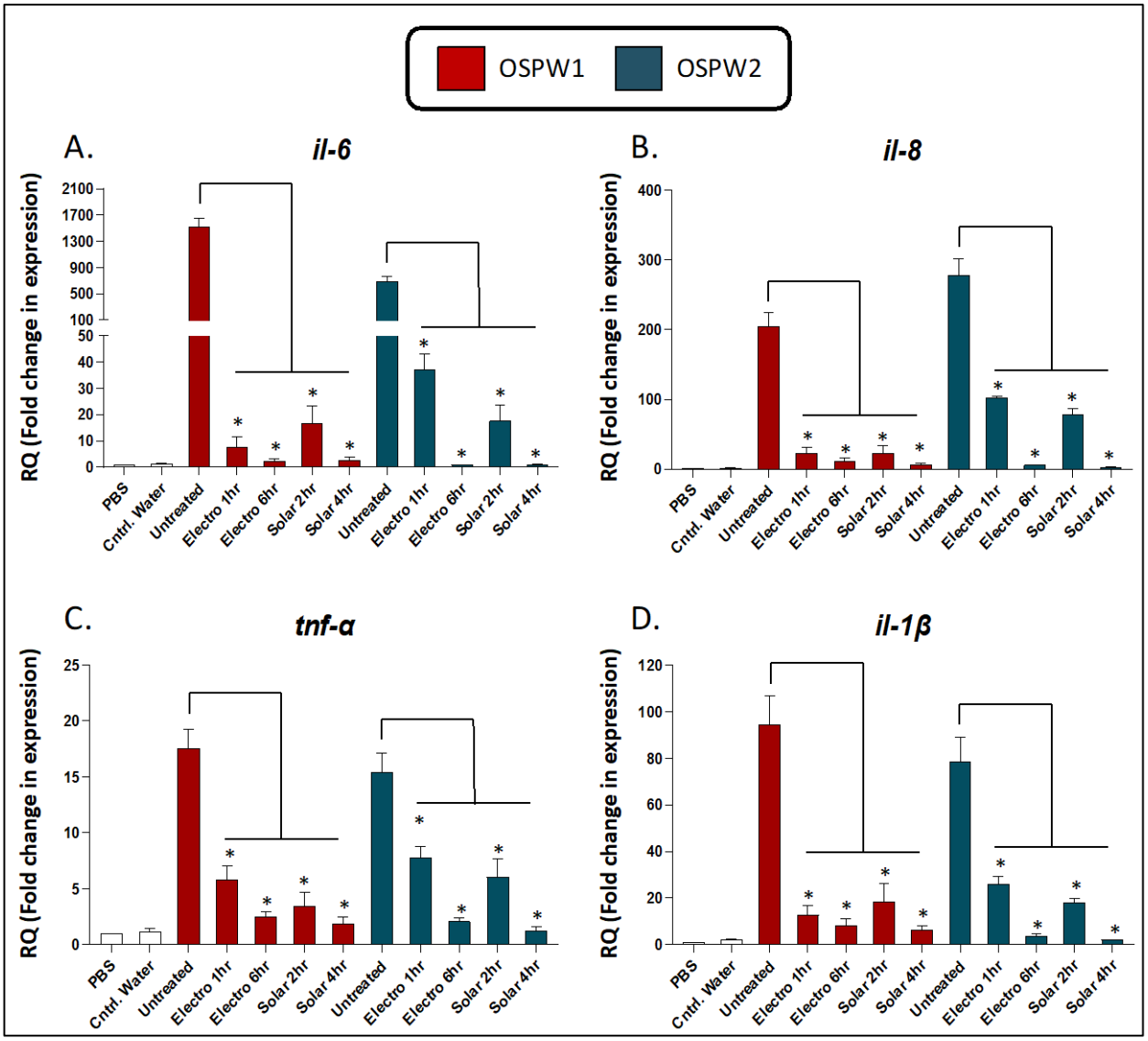

Figure 12. Effects of AOPs on OSPW-induced proinflammatory cytokine gene transcription levels. Quantitative (q)-PCR was performed following 6 hr exposures (25% v/v) of the cells to untreated and treated OSPW samples as indicated to determine the transcription of (A) *il-6*, (B) *il-8*, (C) *il-1ß* and (D) *tnf-α*. Data shown represent the mean ± SEM from three independent experiments and significance values were determined by one-way ANOVA analysis (* = p <0.05) using GraphPad Prism 9.2. PBS = Phosphate Buffered Saline, Cntrl. Water = Control water (sterile).

To further assess the effects of electrooxidation and solar photocatalysis on OSPW-induced immune cell activation we followed up the 6 hr cytokine gene expression experiments (Fig. 12) with cytokine secretion assays. For these experiments, cells were exposed to both untreated OSPW samples for 24 hrs after which their supernatants were collected and analyzed for cytokine protein levels. When IL-6 secretion was tested, it was evident that the Syncrude (OSPW1) sample induced much higher (i.e., ~1000 pg/mL) levels of IL-6 than the Suncor (OSPW2) sample, which peaked at only ~70 pg/mL (Fig. 13A). However, despite the differential ability of these untreated samples to activate IL-6 secretion, electrooxidation and solar photocatalysis treatment of both waters, at both treatment times, nearly abolished all detectable IL-6 protein secretion in the supernatants (Fig. 13A). For IL-8, both OSPW1 (~5000 pg/mL) and OSPW2 (~6000 pg/mL) were potent inducers of secretion (Fig. 13B). Then following treatment of the water, electrooxidation and solar photocatalysis significantly inhibited IL-6 secretion but the shorter 1 hr electrooxidation treatment was the least effective when compared to the 6 hr electrooxidation, whereas both short (2 hr) and long (4 hr) solar photocatalysis of the waters were very effective blockers of IL-8 secretion. Finally, examination of TNF-α and IL-1ß secretion levels again showed that the untreated samples had the highest overall secretion levels that after treatment, were significantly reduced. (Fig. 13C and 13D) For both TNF-α and IL-1ß, Syncrude (OSPW1) was a stronger inducer of their secretion than the Suncor (OSPW2) sample but OSPW1 was more sensitive to both electrooxidation and solar photocatalysis. As shown for IL-8, the Suncor water sample was relatively resistant to the 1hr electrooxidation treatment but overall sensitive to the effects of solar photocatalysis.

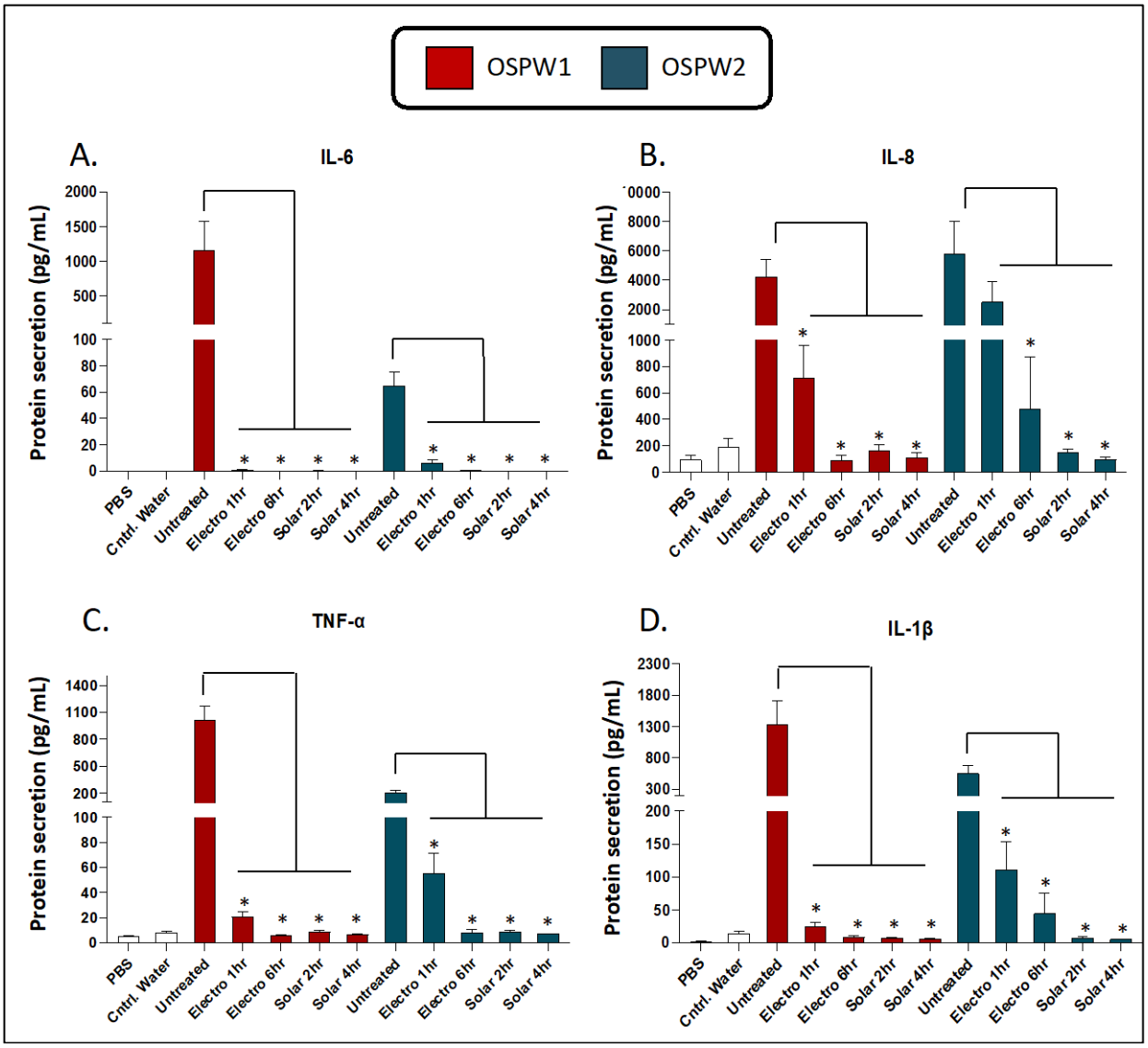

Figure 13. Effects of AOPs on OSPW-induced proinflammatory cytokine secretion activity

Cytokine secretion assays were performed following 24 hr exposures (25% v/v) of the cells to untreated and treated OSPW samples as indicated to determine the secretion levels of the proinflammatory cytokine proteins (A) IL-6, (B) IL-8, (C) IL-1ß and (D) TNF-α. Data shown represent the mean ± SEM from three independent experiments and significance values were determined by one-way ANOVA analysis (* = $p < 0.05$) using GraphPad Prism 9.2. PBS = Phosphate Buffered Saline, Cntrl. Water = Control water (sterile).

Parameter	OSPW 1					
	Raw	EO 1hr	EO 6hr	SPO 2hr	SPO 4hr	Raw
pH						8.85
Salinity						2780.00
Turbidity (NTU)						1.73
UV_{254} (cm^2)						0.96
Alkalinity ($CaCO_3$ mg/L)						
Conductivity (mS/cm)						4.80
Chloride	1161.04	890.30	3.21	1169.77	1197.64	
Sulfate	344.06	346.31	331.28	344.13	341.94	
Nitrate						
Carbonate						
O_2-NAs	16.63	0.41	0.30	6.88	2.89	36.50
O_3-NAs	7.30	0.63	0.00	7.01	5.55	15.90
O_4-NAs	6.85	0.68	0.00	5.46	4.17	10.20
O_5-NAs	1.11	0.07	0.00	1.61	1.62	2.30
O_6-NAs	0.24	0.01	0.00	0.55	0.64	0.64
Total Napthenic Acid Species (O_2-O_6)	32.13	1.80	0.30	21.51	14.87	65.63
Dissolved Organic Carbon (DOC)	68.48	36.16	3.33	68.30	67.62	78.20
DOC Removal Efficiency (%)		47.19	95.13	0.26	1.26	
Chemical Oxygen Demand (COD)	213.00	0.00	0.00	212.00	203.00	
COD Removal Efficiency (%)		100.00	100.00	0.47	4.69	
Total Dissolved Solids						2960.00

Parameter	OSPW 1						
	Raw	EO 1hr	EO 6hr	SPO 2hr	SPO 4hr	Raw	S
pH						8.85	
Salinity						2780.00	
Turbidity (NTU)						1.73	
UV_{254} (cm^2)						0.96	
Alkalinity ($CaCO_3$ mg/L)							
Conductivity (mS/cm)						4.80	
Chloride	1161.04	890.30	3.21	1169.77	1197.64		
Sulfate	344.06	346.31	331.28	344.13	341.94		
Nitrate							
Carbonate							
O_2-NAs	16.63	0.41	0.30	6.88	2.89	36.50	
O_3-NAs	7.30	0.63	0.00	7.01	5.55	15.90	
O_4-NAs	6.85	0.68	0.00	5.46	4.17	10.20	
O_5-NAs	1.11	0.07	0.00	1.61	1.62	2.30	
O_6-NAs	0.24	0.01	0.00	0.55	0.64	0.64	
Total Napthenic Acid Species (O_2-O_6)	32.13	1.80	0.30	21.51	14.87	65.63	
Dissolved Organic Carbon (DOC)	68.48	36.16	3.33	68.30	67.62	78.20	
DOC Removal Efficiency (%)		47.19	95.13	0.26	1.26		
Chemical Oxygen Demand (COD)	213.00	0.00	0.00	212.00	203.00		
COD Removal Efficiency (%)		100.00	100.00	0.47	4.69		
Total Dissolved Solids						2960.00	
Total Suspended Solids							

Table 3. Composition of selected chemicals from oil sands process-affected water (OSPW) pre and post-treatment. Raw OSPW samples were collected from different industries and subjected to solar photocatalytic (SPO) treatments for two or four hours, or electro-oxidation (EO) treatments for one or six hours. Trace elements and water physiochemical parameters were quantified. All chemical measurements are in mg/L unless specified. <LOQ indicates below the limit of quantification. Hatched cells indicate parameter not measured.

Overall, these data show that the two AOPs examined in this project significantly reduce the immunotoxic activities of OSPW based on our antimicrobial and proinflammatory cytokine gene and protein secretion assays. This suggests that the inflammatory components present in the two representative OSPW samples tested are sensitive to AOP, which implies they could be organic compounds and/or organic containing complexes. Furthermore, each OSPW tested displayed their own unique susceptibility to AOP-mediated reduction of bioactivity with an indication that solar photocatalysis was more effective than electrooxidation regarding the removal of the inflammatory activities of OSPW. While the data shown in Table 3 clearly shows that the total and classical Naphthenic acid species are effectively reduced in both OSPW1 and OSPW2 samples following electrooxidation and solar photocatalysis, this does not confirm they are the inflammatory compounds responsible for the activities observed. However, as a tracer for the effectiveness of each treatment, they reliably show removal of target organic species, which correlates with the loss of immunotoxic activities as measured in our bioassays. It is likely that NAs could play some role in

immune cell activation, but this requires substantially more research.

F. KEY LEARNINGS

Project Learnings:

i) Our water quality analyses showed some discrepancies between the sum of OSPW fractions and the whole water samples. This was likely caused by low concentrations and analytical artefacts in the extraction process, but the cause of these issues would require further experiments. However, not all constituents detected in the tested waters are present in high levels; however, assessments should be conducted on a case-by-case basis regarding toxicants. For example, there are compounds found at low concentrations such as selenium (Se), observed at 4.52 ppb in Suncor DPL Water IF #1, and silver (Ag; 1.37 ppb) in Suncor DPL OF #1, which represent the highest measurements for both elements in our dataset. While these concentrations seem minimal in an absolute quantification sense, both values exceed the freshwater long-term concentration standard (Se: 1 ppb, Ag: 0.25 ppb) according to the Water Quality Guidelines for the Protection of Aquatic Life mandated by the Canadian Council of Ministers of the Environment. This reinforces the need to ensure monitoring occurs at highly sensitive concentrations and that we consider individual components and their toxicity during analysis. More detailed examination of the presence, behavior, and fates of compounds detected in our study are also required.

ii) Overall, most constituents in the recent SWAMP lab dataset (Fall 2020) are lower than the baseline data we reported in Fall 2019. This is expected as OSPW components are known to be affected by the passage of time. Specifically, changes observed over the 12-month ageing period between analyses may be attributed to spontaneous chemical reactions, such as the possibility of organometallic complexes forming due to differences in polarity (i.e., negatively charged humic acids found naturally in OSPW binding to positively charged metal ions such as Ag^{+}, Na^{+}, or Al^{3+} to form stable complexes). Furthermore, physiochemical parameters of OSPW may change due to the extant (and currently unclassified) microbial communities that utilize various compounds in these samples for nutrients. Classification of the microbial taxa in Suncor DPL Water #1 and Suncor DPL Water #2 remains a future task that may better elucidate the metabolic potential and chemical component dynamics in these complex samples. In addition, ongoing chemical analysis of OSPW samples will be helpful to examine changes over time and may help us further understand our downstream bioactive-guided profiling data obtained using our *in vitro* immune cell-based bioassays.

iii) The bioactivity of different Suncor DPL Waters and their fractions can be rapidly detected and quantified using qPCR-based monitoring of antimicrobial and cytokine genes.

iv) Using *inos* expression as a marker of macrophage activation, Suncor DPL Water #1 but not #2 was confirmed as a potent inducer of this antimicrobial response. Furthermore, our data supports those organic constituents within Suncor DPL Water #1 activate *inos* expression.

v) We have identified using reconstitution experiments that the IF of Suncor DPL Water #1 contains factors that are uniquely capable of suppressing the OF-mediated activation of the NO antimicrobial pathway. These data also suggest that the fractionation methods used to separate the water samples may

potentiate its bioactivity, by possibly disrupting organic-inorganic (i.e., organometallic) complexes that exist in the whole water.

vi) Further to the cytokine secretion data, we have shown that the OF isolated from Suncor DPL Water #1 activates secretion of seven different proinflammatory cytokine proteins. No cytokine secretion activity was detected when the cells were exposed to the IF, which reinforces those unknown organic factors within this water can induce various cytokine secretion pathways

vii) qPCR-based monitoring of the selected cytokine genes *ip-10*, *mcp-1*, and *mip-2* confirmed for Suncor DPL Water #1 that it's OF but not IF is responsible for the observed bioactivity. Each cytokine gene displayed a unique temporal expression profile over the 2-10 hr periods examined when cells were treated either WH or the OF. Interestingly, a significant enhancement of each cytokine gene expression was observed when the cells were exposed to the OF vs. the whole Suncor DPL Water #1. This clearly indicates that fractionation of the water increases its potency as an activator of cytokine gene expression. As mentioned previously, this could be due to the release of bioactive organic components from inhibitory inorganic factors.

viii) Examination of interactions between the OF and IF of Suncor DPL Water #1 showed significant potentiation of OF-induced cytokine secretion for some but not all cytokines monitored. Overall, these data clearly demonstrate a potent interactive effect for when the OF and IF of Suncor DPL Water #1 are reconstituted. However, the enhancement of the OF-induced cytokine secretion effect was contrary to the significant inhibitory effects that IF had on the OF-induced NO secretion activity described in point vi). These data add further evidence supporting those interactions between organic and inorganic constituents of OSPW samples can profoundly affect their bioactivity.

ix) Further to the cytokine secretion data, our results also show that the IF of Suncor DPL Water #2 (but not the OF) is responsible for activating cytokine secretion. Interestingly, although this water did not activate the NO antimicrobial pathway, whole Suncor DPL Water #2 and its IF potently activated cytokine secretion. Notably it was the OF from Suncor DPL Water #1, not its IF, that induced cytokine secretion by exposed macrophages.

x) qPCR-based monitoring of *ip-10*, *mcp-1*, and *mip-2* confirmed for Suncor DPL Water #2 that its IF was responsible for the observed bioactivity. As previously described, each cytokine gene displayed a unique temporal expression profile over the 2-10 hr periods examined when cells were treated with either WH or the IF of Suncor DPL Water #2. For two of the cytokine genes examined (i.e., *ip-10* and *mip-2*), the overall fold-increases measured were ~50% lower than the levels observed after the cells were exposed to the first water. Also, our data shows that unlike DPL Water #1, fractionation of Suncor DPL Water #2 did not increase its potency as an activator of cytokine gene expression.

xi) Examination of interactions between the OF and IF of Suncor DPL Water #2 showed variable effects on the IF-induced cytokine secretion activity. These effects ranged from OF-mediated enhancement of the IF-induced cytokine secretion activity for TNF-alpha, MIP-2, MCP-1, G-CSF, and IL-6. Conversely, the effect of mixing OF with IF caused a significant inhibition of the IF-induced secretion of IP-10 and RANTES. These

data suggest that reconstitution experiments combined with sensitive immune cell-based bioassays show that interactions between OSPW IF and OF components have profound effects on macrophage responses.

xii) Both Electro and Solar oxidation-based treatment protocols dramatically reduced OSPW-induced macrophage activation. By monitoring the expression levels of key proinflammatory genes, secreted cytokine proteins, and antimicrobial factors we showed that treated OSPW significantly reduced the ability of OSPW to activate the inflammatory response of immune cells, which correlated with the removal of organic constituents (i.e., NA species). This indicates that at acute sub-lethal exposure doses, oxidation treatment also reduces immunotoxicity, which may be due to the degradation of NAs in OSPW.

xiii) In our recently published work, the effectiveness and kinetics of ZnO-based solar photocatalytic degradation of the NAs and PAHs in real OSPW were assessed and specific details from this publication related to this report are as follows (Suara et al., 2022). In addition to examining the relative degradation of the different classes of NAs under this photocatalytic treatment, the effect of the photocatalytic treatment on its overall effect on immunotoxicity (i.e., macrophage activation) was evaluated. The observed results indicated that solar activated ZnO photocatalysis is effective in degrading NAs and PAHs in real OSPW and that the toxicity of the OSPW was considerably reduced by the photocatalytic treatment. Solar activated ZnO was also observed to have outperformed solar activated TiO2 in OSPW, which could be attributed to the favorable physico-chemical conditions because of the high pH of OSPW and the isoelectric point of ZnO. Additionally, the NAs degradation was structurally dependent with DBE and carbon showing strong influence, while the reactions were observed to be dictated by hydroxyl (•OH) and superoxide (O2•−) radicals. The results of this study indicate the potential of solar-activated ZnO photocatalysis as an effective OSPW treatment method.

xiv) Further studies are required to the identify specific constituents within OSPW samples that are responsible for activating immune cells, and how targeted removal of these factors can be reliably and sensitively detected as reductions in proinflammatory biomarkers.

xv) Our immune cell-based bioassays take advantage of the versatile sensing system of macrophages to detect and monitor proinflammatory constituents in OSPW. This project also showcased that immune cell-based bioassays serve as an additional sensitive bioindicator for examining the effects of untreated and treated OSPW exposures on mammalian immune cells.

Discussion on broader impacts of the learnings:

Our results showed that macrophages differentially responded to two distinct OSPW samples associated with the same geographic location. Macrophage *inos* levels were up-regulated in response to the whole AWC OSPW2 and its OF, whereas BWC OSPW1 exposures triggered only a marginal fold-change whether whole or fractionated. The whole AWC and its OF also induced a high level of relative bioactivity when intracellular iNOS protein and NO secretion were examined. Activation of the iNOS pathway is a hallmark antimicrobial response of stimulated macrophages (MacMicking et al., 1997). This conserved pathway involves induced expression of *inos* followed by transcription of the intracellular enzyme iNOS, which then catalyzes the production of reactive nitrogen intermediates that are potently destructive to microbes. This

innate antimicrobial response is not activated in resting macrophages but is rapidly deployed by stimulated cells and thus serves as a sensitive bioactivity marker following short (i.e., 2-24 hr) exposures to OSPW samples.

Incorporating freshwater into the cap at Lake Miwasin's treated tailings introduces environmental constituents such as prokaryotic assemblages and different sources of organic carbon unlikely to be present in the original OSPW sample (Pernthaler, 2013). Tailing ponds microbial communities generally contain lower species diversity than environmental samples due to more restrictive carbon sources and concentrated toxins (Yergeau et al., 2012). This suggests that the influx of foreign microbiota (relative to the BWC microbiome) after water capping (wherein both OSPW and environmental water were used to form the water cap) may have contributed to the robust induction of the iNOS pathway. In contrast, whole BWC OSPW1, BWC OF, and BWC IF exposures induced very little iNOS bioactivity in comparison to the AWC sample. Previous studies have suggested that OSPW retains persistent inorganics that are toxic to invertebrates (Bauer et al., 2019), and also capable of inducing macrophage activation (Phillips et al., 2020). For example, ions such as those associated with salinity (Na^+, Cl^- and HCO_3^-), and metals such as nickel and boron have been cited as toxicants of concern in OSPW (McQueen et al., 2017; White and Liber, 2018), and these may also potentially contribute to the BWC IF-induced bioactivity observed in this study.

Our results also support our previous findings (Phillips et al., 2020), which showed macrophage activation following exposure to an OSPW sample collected from an active tailings pond from a different site. Furthermore, in this previous work, we showed that the OSPW IF induced transcription of the oxidative stress gene *hmox1*, and the DNA damage inducible gene *gadd45*, while suppressing the production of the iNOS protein. While our current project only monitored proinflammation effects of OSPW (i.e., not cell stress markers), we also noted a minimal increase of iNOS staining and NO production following exposure of the cells to either the BWC IF or AWC IF. The BWC sample tested in the current study are most comparable to the OSPW used in *Phillips et al*, where the bioactivity observed was also associated with the IF when proinflammatory gene expression was examined (Phillips et al., 2020). This may be due to the accumulation of inorganic constituents in OSPW before dilution. Additionally, as the BWC sample used in the present study was collected under the ice, it is possible that the concentration of solutes was increased due to the salt rejection effect (DeGrandpre et al., 2021). While both OSPW samples obtained from two different oil sands operators induced fraction-dependent responses in macrophages, it is important to note that recovery of the OF in these different studies were obtained using two separate approaches (dichloromethane, or DCM (Phillips *et al.,* 2020) versus solid phase extraction, or SPE, this study. As reported, using SPE has a higher recovery of the OF components such as NAs and dissolved organic carbon (Qin et al., 2019). With the consideration of both datasets, we show that different oil sands mines and operator-sourced samples have distinct bioactivities, and our ability to examine the iNOS pathway allows for fine scale quantitative resolution between water samples and their fractions.

Whole AWC (OSPW2) and the AWC OF exposures maximally increased the proportion of cells that expressed the surface receptor CD86, whereas only BWC OF exposure caused maximum increases of CD86. Overall, the proportions of CD86+ staining cells in these experiments organized into a pattern of OF ≥Whole>IF, like the proportion of CD206+ cells observed when macrophages were treated with AWC sample. However, although the BWC OF induced the highest proportion of CD206+ expressing macrophages, significant levels of CD206+ staining cells were also observed following treatments with both whole BWC OSPW1 and its IF. Although there is a larger portion of CD86+ macrophage cells

compared to CD206+ cells after OSPW exposures, it is evident that the tested OSPW samples can significantly activate the surface expression of these two different immune receptor proteins.

Macrophages that express increased levels of CD86 are typically in an activated state due to their responses to proinflammatory cytokines and factors secreted by other immune cells, as well as exogenous factors such as lipopolysaccharide (LPS), a component of Gram-negative bacteria (Chavez-Galan et al., 2015). In contrast, CD206 is generally present on macrophages challenged with allergens, parasites, or those that are involved in the wound resolution phase of inflammation (Chavez-Galan et al., 2015; Martinez et al., 2008). While the specific constituents of OSPW that induce immune receptor up regulation are unknown, it may be that the increased CD86+ cell staining is a direct response to bacterial components in the samples, such as LPS, which is heat-stable and bioactive at nanogram concentrations (Li and Boraschi, 2016; Mosser and Zhang, 2008). LPS has also been shown to be a primary agonist of pro-inflammatory responses using river water samples (Wichmann et al., 2004), and LPS in tandem with bacterial DNA has shown to synergistically increase NO production in macrophages (Gao et al., 1999). Additionally, macrophage receptor expression levels are known to be differentially regulated in response to viruses (Sang et al., 2015), plant phytochemicals (Saqib et al., 2018), the external surface shape of constituents (Rostam et al., 2015), and chemical and ionic concentrations (Fernandez et al., 2017; Gardner, 1984); all factors that potentially exist and contribute to the complexity in environmental and industrial water samples. Furthermore, CD86 and CD206 are co-expressed by cells that receive complex mixtures of stimuli (Smith et al., 2016)**,** which are likely occurring in OSPW as they possess constituents that are not clearly elucidated and likely possess both immune activating and repressing constituents. Overall, examination of these select surface receptors offers an additional approach to track the bioactivity of macrophages exposed to OSPW.

Three proinflammatory cytokine biomarkers were also used to monitor the immunotoxicity of OSPW using both gene expression and protein secretion activity assays. Importantly, as with the iNOS pathway, the three cytokines examined in this study (IP-10, MIP-2, and MCP-1) are not expressed or secreted by resting (i.e., untreated) macrophages. However, we showed that OSPW and their fractions are potent inducers of cytokine secretion, with high expression of all cytokines observed following exposure of the cells to whole AWC OSPW2 and whole BWC1, AWC OF, and the BWC IF. Furthermore, induction of cytokine gene expression occurred as early as 2 hours post-exposure, compared to the 24-hour period when cytokine secretion activities were examined. Among the cytokine genes tested, *ip-10* followed a trend like the *inos* gene expression pattern, peaking midway through the exposure period. The *mcp-1* and *mip-2* expression patterns conveyed opposite transcriptional responses, with *mcp-1* generally showing increasing fold-change values over time, while the RQ for *mip-2* was the highest at the mid exposure time points, which then gradually decreased by 10 hours after water exposures.

In agreement with the cytokine secretion activity data, bioactivity following exposures primarily associated with the AWC OF and BWC IF samples. As *mcp-1*, *mip-2*, and *ip-10* are all involved in immune cell mobilization during acute inflammation (Sokol and Luster, 2015), this may indicate that the AWC OF and BWC IF possess immune cell activating factors such as various microbial components as previously discussed, as well as ions, residual heavy metals, or high salinity. Specifically, for the IF-induced responses, high salinity has been shown to prime macrophages towards a proinflammatory state (Zhang et al., 2015), and oil sands process-affected materials, which includes both solids and water, is generally brackish due to the natural salts present in the sediment as well as from the Clark hot water extraction process (Li et al., 2020; Ripmeester and Sirianni, 1981). Using freshwater mussels, salinity was shown to have an additive

effect on toxicity when supplemented at concentrations that are relevant to OSPW alongside NAFCs (Bartlett et al., 2017). Trace inorganic elements such as arsenic, copper, nickel, or molybdenum are also enriched in OSPW (Small et al., 2015), and ions such as iron or nickel can both affect macrophage homeostasis (Ganz, 2012), and viability (Gardner, 1984). Inorganic components are persistent factors of toxicity in *in vivo* models of aged tailings pond effluent (Bauer et al., 2019), and they were also previously shown to induce pro-inflammation gene transcription in macrophages (Phillips et al., 2020).

Regarding organic components that may initiate the observed proinflammatory effects, eukaryotic and microbial communities including prokaryotic bacteria, and phages offer candidate biotic factors of interest that likely affect macrophage responses (Al-Shayeb et al., 2020; Hadwin et al., 2006; Richardson et al., 2020; Yergeau et al., 2012). Major microbial constituents in OSPW have been detailed in a recent review from our lab, where these members facilitate inter-species interactions such as predation or biofilm generation, the geochemical cycling of elements, and the degradation of toxicants (such as the NAFCs) of interest (Hussain and Stafford, 2023). These communities and their various components should be taken in consideration when examining the inflammatory activity of OSPW. Furthermore, the OSPW OF, which contains the abiotic NAFCs, is highly toxic to fish, invertebrates, and aquatic plants (McQueen et al., 2017). It also induced the stress response of *Escherichia coli* (Morandi et al., 2017), and has been implicated as a modulator of fish immune systems (Hagen et al., 2012). Overall, the relative impact that each component has with respect to the activation of macrophages are unknown, however, it is expected these abiotic and biotic factors alone or in variable combinations within complex OSPW mixtures have the potential to uniquely modulate macrophage responses that are detectable using various immune cell-based bioassays.

G. OUTCOMES AND IMPACTS

Project Outcomes and Impacts:
Using several standard immune cell biomarkers, we convincingly show that macrophages can acutely discriminate bioactivity in different waters and their fractions. Although the constituents responsible for inducing bioactivity have not been identified, tracking this activity in different samples may be useful for future monitoring approaches. At this time, we cannot assume that the bioactivity observed is a surrogate for toxicity, as this will also require further investigations. However, the unique ability to acutely discriminate bioactive samples using several robust assays, presents an important opportunity to integrate this *in vitro* cell-based approach into ongoing monitoring protocols. Moving forward, targeted OSPW treatment regimes, passive remediation technologies, and detailed constituent analyses, combined with our suite of bioactivity assays can be used to help identify and then track various OSPW constituents of interest that induce immune cell bioactive responses.

OSPWs are extremely heterogeneous mixtures, and the type and amounts of biotic and abiotic constituents found in these waters are influenced by multiple external factors. As there is no standardized protocol on how best to reclaim OSPW (Martin, 2015), all remediation efforts must be investigated to ensure safe environmental reintegration of tailings ponds. The biological effects of the Albertan oil sands and its by-products have been assessed in terrestrial and aquatic organisms (Bauer et al., 2019; Bechtel et al., 2009; Colavecchia et al., 2004; Gentes et al., 2007; MacDonald et al., 2013; Rogers et al., 2002; Scarlett et al., 2012), however rearing whole animals can be costly and require prolonged maintenance to observe changes. Our study demonstrates the utility of an *in vitro* immune cell-based series of sensitive

bioactivity assays as a rapid and economic biosensor system for examining OSPW-induced immune cell activation as a potential indicator of water quality.

The outcomes of the proposed research will be beneficial not only for the oil sands companies in terms of the availability of new metrics for assessing OSPW remediation efforts, but also for regulatory agencies that can use our data to help further inform guidelines for establishing water quality parameters. The results generated from this research may also provide decision makers with the engineering and scientific data/evidence necessary for making sound decisions on the sustainable development of Canada's oil sands resources.

Project Outputs:

Journal Articles:

Lillico DME., Hussain NAS., Choo-Yin Y., Qin R., How ZT., Gamal El-Din M., and Stafford JL. (2022). Using immune cell-based bioactivity assays to compare the inflammatory activities of oil sands process-affected waters from a pilot scale demonstration pit lake. *Journal of Environmental Sciences*. DOI: 10.1016/j.jes.2022.07.018

Hussain NAS., and Stafford JL. (2022). Abiotic and biotic constituents of oil sands process-affected waters. *Journal of Environmental Sciences*. 127, 169-186

Suara MA., Ganiyu SO., Paul S, Stafford JL., and Gamal El-Din M. (2021) Solar-activated zinc oxide photocatalytic treatment of real oil sands process water: Effect of treatment parameters on naphthenic acids, polyaromatic hydrocarbons and acute toxicity removal. *Science of The Total Environment*. 819, 153029.

Phillips NAI., Lillico DME., Chen R., McAllister M., Gamal El-Din M., Belosevic M., and Stafford JL. (2020). Inorganic fraction of oil sands process-affected water induces mammalian macrophage gene expression and acutely modulates immune cell functional markers at both the gene and protein levels. *Toxicology in Vitro. 66: 104875*.

Qing R., Lillico DME., Huang R., Belosevic M., Stafford JL., and Gamal El-Din M. (2019). Separation of oil sands process water organics and inorganics and examination of their acute toxicity using standard in vitro bioassays. *Science of The Total Environment*. 695, 133532

Presentations:

Stafford JL. *Using mammalian cell lines to detect and monitor bioactive constituents in untreated and treated OSPW samples*". Canada's Oil Sands Innovation Alliance (COSIA) Mine Water Management Workshop (Invited speaker). November 30-December 1, 2021. (Remote).

Hussain NAS., Lillico DME., Richardson E., Choo-Yin Y., Hanington PC., and Stafford JL. *An immune cell-based assay for detecting and monitoring bioactive constituents withing oil sands process-affected waters (OSPW).* Society of Environmental Toxicology and Chemistry (SETAC) North America 42nd Annual Meeting, Portland, OR, USA (Talk). November 17, 2021. (Remote).

Choo-Yin Y., Lillico DME., and Stafford JL. *Bioactivity profiling of oil sands process affected waters using an immune cell-based bioassay system.* 60th Annual Meeting of the Canadian Society of Zoologists (Talk) May 17-21, 2021. (Remote).

Platform Speaker (November 2020): "*Immune cell-based bioindicator system for tracking bioactive constituents within oil sands process-affected waters*"
Society of Environmental Toxicology and Chemistry (SETAC) North America 41st Annual Meeting, Fort Worth Texas, USA (online). Immunoecotoxicology: Immunoenhancing & Immunosuppressing Effects of Contaminants & their Relevance to Risk Assessment.

Choo-Yin Y., Lillico DME., and Stafford JL (2020). SETAC North America 41st Annual Meeting. 2020 SETAC In Focus: Environment Quality Through Innovative Science. Fort Worth, Texas, USA (online). Development and use of an immune gene-based bioindicator system for tracking bioactive constituents within oil sands process-affected waters on mouse macrophages (poster).

Platform Speaker (November 2019): "*Isolation and characterization of OSPW fractions and assessment of their toxicity using mammalian cells*"
Society of Environmental Toxicology and Chemistry (SETAC) North America 40th Annual Meeting, Toronto, Ontario, Canada. Addressing existing challenges in immune-ecotoxicology session: from tool development to risk assessment.

Invited Speaker (October 2019): "Developing a cell-based bioindicator system for identifying and tracking bioactive constituents within OSPW and its fractions."
Canada's Oil Sands Innovation Alliance (COSIA), Edmonton, Alberta, Canada. Mine Water Release Workshop

Theses:

MSc Thesis: "Effects of oil sands process-affected waters on mouse gene expression profiles"; defended April 2020. Department of Biological Sciences, University of Alberta.

H. BENEFITS

This project builds on an important scientific foundation for the development of management strategies and regulations when considering safe release of treated process-affected waters to the environment. It also provides new sensitive tools for the assessment of the progression of detoxification of OSPW in end-pit lakes as well as the monitoring of untreated and AOP-treated waters.

Oil sands development provides significant economic benefits to Canada, including employment, government revenue, and investment in research and development. The proposed research is built in alignment with support for the ongoing efforts for the economic development of Alberta oil sands resources and enhances the leadership and safety stewardship of Alberta scientists and government policy makers. As bitumen surface mining continues to supply oil as a relatively low-cost fuel source, it is imperative that strategies for remediating large volumes of contaminated tailings are developed and

instituted. Due to challenges associated with ever growing volumes of tailings waters in the environment, a rapid, sensitive, and inexpensive biological screening system is needed. Faced with the impractical, expensive, and unethical use of live animals in toxicity testing, cell-based bioassays offer a potential alternative. We have now identified the critical tests that will augment and perhaps eventually replace lethal end-point assays. To that end, our *in vitro* mammalian immune cell bioindicator system will further expand the ranges of waters that can be examined (this project) leading to the optimization and selection of critical tests for overall OSPW biological testing and monitoring. To assist industry in their role as environmental stewards, our cell-based assays will set the stage for the design of robust biosensors offering an alternative detection method that is relatively inexpensive, practical, and simple, yet specific and sensitive to OSPW-mediated effects on cells.

I. RECOMMENDATIONS AND NEXT STEPS

The PASS technique involves the deposition of treated tailings (through the process of coagulation and flocculation) into a pit, which is subsequently filled (i.e., capped) with both OSPW and environmental water forming the basis of reclamation endeavours at Lake Miwasin (COSIA, 2018; Suncor Energy Inc., 2019). The water testing scheme detailed in this study reflects the pre- and post-introduction of a water cap to treated tailings during the early construction of this pilot DPL. We detected temporal changes occurring in a single geographic location underpinned by anthropogenically-introduced treatments to OSPW. These responses will be compared to future samples taken from Lake Miwasin to examine how the naturally driven remediation progress affects the early inflammatory-inducing properties of the lake. Importantly, by broadening our analyses to include detailed chemical and microbial community profiles, it may also be possible to correlate OSPW exposure-induced macrophage activation with distinct changes in the former two constituents over a monitoring timeframe.

Macrophages have been extensively used as *in vitro* tools to study toxicity of fatty acids (Martins de Lima et al., 2006), ozone (Becker et al., 1991; Driscoll et al., 1987), ambient particulate matter (Sawyer et al., 2010; Zhou and Kobzik, 2007), and cigarette smoke (Gunella et al., 2006; Thomas et al., 1978). Immune cells, including macrophages, have also been directly used as indicators of water quality based on the detection of inflammatory agents in river waters as well as those surrounding treatment facilities (Adebayo et al., 2014; Jagals et al., 2003; Pool and Magcwebeba, 2008; Pool et al., 2000; Wichmann et al., 2004). Specifically, the RAW 264.7 mouse macrophage cell line have been used to examine bioactive compounds in plant extracts (Adebayo et al., 2017; Indriana et al., 2016; Karinchai et al., 2021; Saravanan et al., 2015; Shine et al., 2020; Wang et al., 2020), ship diesel fuel (Sapcariu et al., 2016), and as a bioindicator for immunotoxicity of treated sewage (Makene and Pool, 2015). In general, macrophages offer a sensitive, resource- and time-effective approach as a biomonitoring system to supplement whole-organism toxicity exposures. Additionally, the macrophage biosensor system serves to establish responses between treated tailings and early remediation stage OSPW that can be compared against future samples at Lake Miwasin. Further studies are required to help elucidate the abiotic and biotic constituents and mixtures in OSPW that contribute to the bioactivity observed in this study.

J. KNOWLEDGE DISSEMINATION

As reported in Section J., the knowledge gained from this project has been communicated through the publication of several journal articles, the presentation of the data at various conferences and meetings, through graduate student theses, and through yearly AI reporting. Currently in my lab there are two MSc students and a PhD student working in this area in collaboration with our Engineering partners led by the Gamal El-Din group. Their thesis projects will continue to advance from the findings reported during this project with a focus on implementing the cell-based bioassay methods for examining and monitoring various environmental waters including untreated and treated OSPW. Furthermore, their research will further validate the utility of immune cell-based bioassay as an additional water monitoring tool to be integrated into standard testing. These findings will be disseminated through additional publications, presentations, reports, and theses.

K. CONCLUSIONS

Overall, significant bioactivity was associated with the whole and OFs of both waters, with some activity corresponding to the IF of the BWC depending on the bioassay used. As the abiotic and biotic constituents of the treated tailings before and after the water cap are not known, further investigation into the processes underpinning the macrophage responses will be the focus of future experiments. A preliminary examination of the chemical constituents within the two whole OSPWs and their fractions have identified elements such as rhenium, nickel, vanadium, and molybdenum as enriched in the BWC sample. These elements are associated with bitumen deposits (CEC, 2020), and thus may contribute to the increased bioactivity observed regarding the BWC IF-induced cytokine secretion, as nickel, cadmium, manganese, and chromium have known immunotoxicological effects (Jalčová and Dvorožňáková, 2014; Theron et al., 2011; White et al., 1979). These elements may also have been concentrated at the time of sampling due to salt rejection during the formation of ice (DeGrandpre et al., 2021). NA species (O_2-O_6) were present as expected at highest concentrations in both whole OSPWs and their OFs and these organics may also contribute to the activation of macrophages. There have been multiple studies demonstrating that both commercial and oil sands derived NAs have biological effects on animals and cells, ranging from goldfish (Hagen et al., 2012), to macrophages (Garcia-Garcia et al., 2011), aspen (Kamaluddin and Zwiazek, 2002), or freshwater organisms such as fathead minnows, water fleas, crustaceans, insects, or cattails (Kinley et al., 2016). The disruption of membrane integrity, also termed narcosis, is likely the main mode of toxicity of NAs (Frank et al., 2008; Li et al., 2017). However, at sub-lethal doses, it is difficult to clearly identify how organic components, including NAs, may affect macrophage bioactivity. Studies examining aged OSPW have recorded lower concentrations of elements than what we detected in our test waters (Bauer et al., 2019), although the OSPW sample Bauer and colleagues have assessed was aged statically for 18 years before chemical quantification and toxicity testing proceeded (Suncor Energy Inc., 2019). The high salinity concentration normally found in OSPW and in our samples has also been shown to impact organism and cellular viability (Miles et al., 2019; Morandi et al., 2017; Sansom et al., 2013). Additionally, how components interact as separate entities isolated in each fraction, and together in raw OSPW is yet to be elucidated. Previous research in our lab using a different OSPW sample supports the observation that fractionation of OSPW induces responses in macrophages that are distinct from the whole water

exposures (Fu et al., 2017; Qin et al., 2019), and other studies have also showed this concept using *in vivo* exposure experiments (Bauer et al., 2019; Morandi et al., 2015). Further research into chemical kinetics may provide insight into how interactions between abiotic components impact biological responses.