Monitoring Macrophage Immune Gene Expression Profiles as an Early Indicator System for Examining the Bioactivity of Oil Sands Process Affected Waters

by

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Abstract

Oil sands process-affected waters (OSPW) are by-products of bitumen (i.e. oil) extraction from the oil sands located in northern Alberta. These large volumes of water are held in tailings ponds and cannot be released due to their potential toxic effects. Overall, tailings ponds and mining operations have changed the landscape immensely and industry is mandated to reclaim these sites to self-sustaining ecosystems. Consequently, OSPW must be remediated so that it can be safely released into the environment. Unfortunately, remediation efforts are hampered by the variability of constituents found in each OSPW source which restricts industry's ability to implement common, effective, and accepted treatment strategies. Furthermore, while individual culprits of toxicity have been identified, such as naphthenic acids, very little is known about the interactive and dynamic effects of different components that likely influence each tailings pond's toxicity potentials. These include, but are not limited to, inorganic constituents, microbial communities, and various environmental factors such as pond ageing and location. It is therefore necessary to examine and monitor the effects of OSPW with a focus on identifying the major toxic and/or bioactive impacts. To do this, my research aimed to further optimize an immune cell-based bioindicator system using a mouse macrophage cell line to rapidly and sensitively detect changes in inducible pro-inflammatory/antimicrobial genes following OSPW exposures. I focused on two OSPW sources: the first was collected from Lake Miwasin prior to the placement of a freshwater cap (termed OSPW #7). Lake Miwasin is a demonstration pit lake established Fall 2018 that contains large volumes of alum treated OSPW and is the focus of a large-scale monitoring program examining passive remediation efforts. After the addition of the freshwater cap, a second sample was collected from Lake Miwasin, which is termed OSPW #2 throughout the thesis. Following exposure of the cells to these

different whole OSPW samples, I observed a rapid and significant basal upregulation of pro-inflammatory gene expression. In resting cells, OSPW #2 exposure induced an overall greater magnitude of response than OSPW #7, however, when I examined bacterial-stimulated macrophage gene expression activity, only OSPW #7 caused significant abrogation of antimicrobial gene expression. Overall, this suggests that each OSPW sample contained bioactive constituents that can activate or suppress antimicrobial responses depending on the activation state of the macrophages. Furthermore, when macrophages were exposed to the inorganic (IF) and organic (OF) fractions of each OSPW, bioactivity (i.e. changes to gene expression) segregated to the OF of OSPW #2 and the IF of OSPW #7. These results showed that OSPW-mediated bioactivity can be traced to specific fractions, which is an asset for identifying bioactive constituents. When the OSPW #2 IF and OF were reconstituted to examine potential interactions between the fractions, no clear gene expression profile changes were observed. However, following treatment of the cells with an untreated and alum treated OSPW, discrete bioactive responses were documented. Overall, my data suggests that macrophages can serve as sensitive cell-based biomonitoring tools that show unique gene expression profiles following exposure to different samples. Accordingly, this assay can be used as a bioindicator for OSPW-mediated biological effects and for examining changes in bioactivity that may aide in OSPW characterizations to help guide ongoing remediation efforts.

Preface

This thesis is an original work by Yemayá Yue Choo-Yin. No part of this thesis has been previously published.

This thesis is dedicated to my greatest comforter and biggest distraction,

Salem “Bad Boi” Choo-Yin.

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List of Abbreviations

AEF	Acid extractable fraction
AEO	Acid extractable organics
AhR	Aryl-hydrocarbon receptor
AOP	Advanced oxidation process
APC	Antigen-presenting cell
BML	Base Mine Lake
CF	Coagulation/flocculation
COD	Chemical oxygen demand
DC	Dissolved component
DDT	Dichloro-diphenyl-trichloroethane
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDA	Effects-directed analysis
EPL	End-pit lake
ERK	Extracellular signal-regulated kinase
EROD	Ethoxyresorufin-O-deethylase
FBS	Fetal bovine serum
FPW	Flowback and produced water
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSI	Gonadosomatic index
IC50	50% inhibitory concentration
IF	Inorganic fraction
IFN-γ	Interferon-γ
IL-	Interleukin-(1, 1β, 1β1, 2, 4, 6, 8)
IMPROVER	Systems biology verification for Industrial Methodology for PROcess VErification in Research
iNOS	Inducible nitric oxide synthase
IP-10	Interferon-inducible protein 10

LC50	50% lethal concentration
LPS	Lipopolysaccharide
LSI	Liver somatic index
MAM	Microbial anti-inflammatory molecule
MCP-1	Monocyte chemoattractant protein 1
MFT	Mature fine tailings
MIP-2	Macrophage inflammatory protein 2
MS	Mass Spectrometry
NA	Naphthenic acid
NAFC	Naphthenic acid fraction
NFAT5	Nuclear factor of activated T cell 5
NF-κβ	Nuclear factor-κβ
NO	Nitric oxide
OF	Organic fraction
OSLW	Oil sands leaching water
OSPW	Oil sands process-affected water
PAC	Polycyclic aromatic compound
PACl	Polyaluminum chloride
PAH	Polycyclic aromatic hydrocarbon
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PICD	Phagocytosis-induced cell death
POP	Persistent organic pollutant
PPARγ	Peroxisome proliferator-activated receptor γ
PRR	Pathogen recognition receptor
qPCR	Quantitative PCR
RC-OF-IF	Recombined OF and IF
RF	Mildred Lake site Pond 9's recombined organic fractions
ROS	Reactive oxygen species
SCL	Syncrude Canada Ltd.

SEI	Suncor Energy Inc.
SPEAR	Species at risk
SPM	Suspended particulate matter
SSI	Spleen somatic index
TGF-β1	Transforming growth factor-β1
TIE	Toxicity identification evaluation
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VE	Volumetric equivalents

Chapter I

General Introduction

1.1 Introduction

The impacts of anthropogenic activities and contaminant release into the environment represent a complex problem associated with public concerns and thus investigating their effects is essential to designing guidelines that mediate their safe release and maintain ecosystem health. The Alberta oil sands are at the centre of this conversation as they generate large amounts of waste waters that visually affect the landscape via production of massive tailings ponds.

The Canadian oil sands are located in northern Alberta and comprise the Athabasca, Peace River, and Cold Lake regions (Canadian Association of Petroleum Producers (CAPP) 2021). This area covers ~142 000 km^2 of boreal forest and is one of the largest oil reserves in the world (Alberta Energy Regulator (AER) 2021a; CAPP 2021). It is estimated that it holds 165 billion barrels of bitumen, which is an important natural resource and contributor to the Canadian economy (AER 2021a). Alberta's oil sands industry is unique compared to other petroleum-based industries because it does not require drilling. Oil sands lay within 70 m of the land's surface and can be directly removed by surface mining. Bitumen, the target source in oil sands processing, exists as oil mixed with water, clay, and sand (CAPP 2021). To isolate bitumen, industry uses the Clark's hot water process, but this generates a massive amount of water waste as 2 to 4 units of water are required to isolate 1 unit of bitumen. This liquid waste by-product is called oil sands process-affected water (OSPW) and must be stored in tailings ponds that currently cover ~220 km^2 (reviewed by Giesy et al. 2010; Government of Alberta 2021). OSPW is defined as all water that has contacted the oilsands and it contains many known toxic

constituents, or components at toxic concentrations, and consequently this contaminated water cannot be directly released into the environment without remediation. As such, OSPW toxicity and its massive storage requirements are an impediment to the obligatory remediation of oil sands processing-impacted regions under the *Environmental Protection and Enhancement Act* (EPEA) and the *Conservation and Reclamation Regulations* (AER 2021b). Therefore, understanding how OSPW exerts toxicity is an essential step in the remediation process, and accordingly, methods for monitoring OSPW effects and identifying constituents of concern are required.

1.2 Research Objectives

Quantifying OPSW bioactivity is required to assess the efficacy of remediation strategies, observe changes in OSPW over time, and identify mediators of adverse effects. Bioactivity describes the ability of a substance, regardless of whether it is endogenous or xenobiotic, to induce a biological response. Bioactivity may overlap with toxicity, which describes adverse effects mediated by a toxicant, but this is not always the case. Specifically, in this thesis, bioactivity refers to OSPW-induced changes in immune gene expression profiles at non-toxic (i.e. sub-lethal) OSPW exposure doses.

Characterizing OSPW is a difficult task for industry as each OSPW source contains unique compositions. For example, individual OSPW sources are distinct based on their processing history as well as environmental factors, and this makes tailings pond remediation efforts a complex task (reviewed by Li et al. 2017). The overall objectives of this thesis were to further establish and optimize the use of a cell-based bioindicator system for examining OSPW bioactivity via alterations in selected pro-inflammatory gene expression. Specifically, I aimed; 1)

to examine the effects of whole OSPW and its organic fraction (OF) and inorganic fraction (IF) on immune cell gene expression levels; 2) to examine and confirm if OSPW OF and IF interactions affected inflammatory gene expression patterns, and; 3) to determine if immune gene expression profiling could serve as a sensitive bioassay for examining the effects of alum treatment on OSPW. To do this, I optimized a sensitive qPCR-based immune-gene expression system using RAW 264.7 mouse macrophages following exposure to whole OSPW, OSPW IF and OF alone or in combination, as well as an untreated and an alum-treated OSPW. Overall, my thesis work establishes a high throughput immune gene-expression based bioindicator system that generates outputs much earlier (2 hrs) than protein level analyses (24 hrs), with demonstrated sensitivity to the differences between complex OSPWs.

Chapter II

Literature Review

2.1 OSPW Toxicity

Large volumes of OSPW, which refers to all water that has contacted the oil sands, are generated following bitumen extraction then stored in tailings ponds (Miskimmin et al. 2010). OSPW characterization and toxicity assessments are hampered by processing location and methods, ore quality, and weather; which generate waste waters with unique attributes thus preventing conclusions that apply to all OSPW sources (Li et al. 2017). Nevertheless, all OSPWs are primarily composed of water, (70-80 wt%), but they also contain solids such as silt and clay (20-30 wt%), as well as the bituminous residue not isolated during the extraction procedure (as reviewed by Mahaffey and Dubé 2017). In general, the OSPW organic fraction (OF) contains naphthenic acids (NAs), polycyclic aromatic hydrocarbons (PAHs), and phenols, whereas the inorganic fraction (IF) is composed primarily of metals and ions. Generally, acute and chronic toxicity are attributed to the OF and specifically to NAs (Li et al. 2017). NAs are carboxylic acids derived from petroleum sources that act as surfactants (Mahaffey and Dubé 2017). Surfactants in the environment are cause for concern, specifically to aquatic organisms, since they can increase cell membrane permeability or contribute to cell death by interacting with bioactive membrane molecules (Ivanković and Hrenović 2010). In comparison, the roles of the IF constituents in OSPW toxicity are not yet clear, but they likely contribute to overall toxicity via interactions with organic constituents and thus require adequate consideration during the design and validation of OSPW remediation efforts (Qin, Lillico, et al. 2019). Currently, OSPW

studies have focused on potential OSPW-mediated effects. These assessments can be categorized as *in vitro* and *in vivo* and cover various taxa and endpoints.

2.1.1 In vitro studies

In vitro studies have contributed significantly to our understanding of OSPW toxicity and there are several benefits for using *in vitro* approaches. Generally, exposure times are shorter thus generating faster results, and fewer resources are required compared to whole organism-based studies. In this section, I will discuss some of the *in vitro* toxicity tests that have been routinely used to investigate the potential impacts of OSPW.

Vibrio fischeri is a marine bioluminescent bacterium. Changes to their bioluminescence intensity in response to xenobiotics, like OSPW, is a specific indicator of toxicity and the major principle of the commonly used Microtox Assay. For example, exposure of *V. fischeri* to both fresh and aged OSPW-derived NAs resulted in 50% inhibitory concentrations (IC50s) between 71.8 and 83.9 mg/L using the Microtox Assay (Bartlett et al. 2017). This suggests that the NAs from this OSPW source were toxic as they caused a dose-dependent inhibition of bacterial bioluminescence.

The Comet Assay, or single cell gel electrophoresis, is another *in vitro* assay but it measures contaminant-mediated genotoxicity. In studies, genotoxicity was observed after rainbow trout (*Oncorhynchus mykiss*) hepatocytes were exposed to synthetic NAs, PAHs, OSPW, and oil sands leaching water (OSLW). Diamondoid NAs, which are polycyclic saturated hydrocarbons with “diamond” carbon skeletons, showed greater toxicity than other NAs; and OSPW exposure resulted in greater genotoxicity than OSLW. Nevertheless, all four treatments were toxic at environmentally relevant concentrations (Lacaze et al. 2014). Similarly, NAs,

PAHs, OSPW, and OSLW impacted gene expression and functions related to the immune competency of rainbow trout leukocytes. Specifically, Gagné et al. (2017) found that OSPW exposure inhibited macrophage phagocytosis (the engulfment of large particles via the plasma membrane) compared to the increased phagocytic response observed when other compounds were tested. In addition, only OSPW and OSLW exposures induced macrophage apoptosis indicating that OSPW and OSLW have immunotoxic effects ranging from inhibition of antimicrobial responses to genotoxicity (Gagné et al. 2017).

In vitro studies have also shown immunotoxic effects in mammalian cell lines in response to OSPW and its fractions, and specific constituents. For example, OSPW exposures inhibited cell proliferation, induced cytotoxicity, caused changes in cell morphology, upregulated the expression of various stress genes, and inhibited the phagocytic activity of mouse macrophages. Additionally, gene expression of pro-inflammatory bioactive molecules called cytokines, including *interleukin-1β* (*il-1β)* and *interferon-inducible protein-10* (*ip-10)*, were upregulated and downregulated, respectively, after exposure of resting macrophages to OSPW (Fu et al. 2017). During this study, Fu et al. (2017) observed that many cellular perturbations were mediated by whole OSPW but not its isolated OF. This was unexpected as toxicity is commonly attributed to the OF. Therefore, Fu et al. (2017) suggested that the IF is a contributor to toxicity and may account for the differential effects observed between the OF and the whole OSPW in these experiments. In agreement that OSPW IF has toxic effects, Phillips et al. (2020) observed cytotoxicity of RAW 264.7 macrophages treated with OSPW or the IF at >14 mg/L NA at volumetric equivalents (VE). Again, this suggests that inorganic constituents contribute to toxicity and may be the main proponents of toxicity instead of organic constituents in these specific OSPW samples. Nevertheless, the expression of stress and antimicrobial genes were

differentially affected after exposure to OSPW as well as the OF, and IF at concentrations of 4 mg/L, 8 mg/L, 12 mg/L NA or VE (Phillips et al. 2020; Fu et al. 2017). Therefore, the endpoints selected to monitor OSPW are imperative, and a stimulus may produce unique results depending on the endpoint chosen.

Beyond macrophage studies, mouse embryonic stem cells exposed to NAs showed increased expression of cardiac marker genes. This supported the Mohseni et al. (2015) observation that 7 days after NA exposure, significantly more beating clusters were observed in treatment groups ranging from 0.025 mg/L to 2.5 mg/L NA, indicating accelerated cardiac development. Additionally, neuroectodermal markers were upregulated following exposure to 2.5 mg/L NA. Overall, this suggests that exposures to NA may impact mammalian embryonic development through cardiovascular and nervous system pathways (Mohseni et al. 2015). In human placental trophoblasts, NAs at concentrations ranging from 1.25 mg/L to 125 mg/L increased the quantity of intracellular reactive oxygen species (ROS), but only the highest concentration altered gene expression related to steroidogenesis, oxidative stress, and inflammation. Perturbation of all three pathways are associated with placental dysfunction in whole organisms (Raez-Villanueva et al. 2019). Consequently, NAs elicit cellular responses over a range of concentrations that effect several pathways, and these observations support previous observations that NAs are a major OSPW toxicant.

In summary, *in vitro* systems have provided a convenient and effective way to examine OSPW toxicity. Specifically, cell models have allowed researchers to identify pathways affected by OSPW and OSPW constituents, which are essential for understanding the modes of action of these complex waters.

2.1.2 In vivo studies

Various benefits are associated with conducting toxicological assessments *in vivo*. For example, *in vivo* studies provide biologically relevant results. Furthermore, when selecting a model organism relevant to the oil sands region, these results are not only biologically relevant, but may also provide applicable insight into potential OPSW-mediated effects on members of the surrounding ecosystems. As a result, a myriad of study organisms ranging from invertebrates to mammals have been used in OSPW assessments using *in vivo* approaches.

2.1.2.1 Invertebrates

Several invertebrate species have been used to document the toxicity of OSPW. These species are primarily aquatic, or have an aquatic life-stage, and include water fleas, midges, mayflies, snails, and mussels.

Daphniids are a primary focus in assessing the effects of OSPW *in vivo*. Water fleas (*Daphnia magna*) experienced increased lethality in response to OSPW exposures and an acute exposure feeding rate IC50 value of 5.34% OSPW (Lari et al. 2016). Chronic exposures also affected feeding, reproduction, activity levels, and development. After a 10 day exposure, 10% OSPW lowered reproduction by 39% and neonates raised in the same OSPW concentration were both shorter and lighter than unexposed neonates by 7% and 55%, respectively. Moreover, adults lost 35% of their mass during these 10 day exposures (Lari et al. 2017c). OSPW concentrations greater than 5% lowered their ability to detect food and lowered their overall activity levels and distance swam (Lari et al. 2016; Lari et al. 2017a). Again, 10% OSPW reduced activity level and total distance swam after 1 day and 10-day exposures. OSPW exposure at 1% only reduced their distance travelled after 10 days (Lari et al. 2017a). Examining the effects of OSPW on feeding

demonstrated that several aspects of nutrient acquisition were affected. By using OSPW samples separated into their dissolved component (DC), and its suspended particulate matter (SPM), Lari, et al. (2017d) showed that different OSPW components uniquely affected feeding activity. Specifically, water fleas exposed to OSPW and SPM did not beat their thoracic limbs with the same frequency as the controls, while the DC exposure lowered peristaltic movement and OSPW also inhibited trypsin activity, a protease found in the gut. Consequently, undigested algal cells were found in the hind gut and feces indicating lower digestion efficiency compared to unexposed daphniids (Lari et al. 2017d).

Exposure of midges (*Chiromonus dilitus*) to fresh OSPW sampled in 2009 and 2010 from Syncrude Canada Ltd.'s West In-Pit pond also showed toxic effects. For example, OSPW collected in 2009 lowered survival by 45% after 10 days while the 2010 sample had no effect. For the remaining endpoints including body mass, larval case integrity, larval activity, larval pupation, and adult emergence; both OSPW samples had reduced, or abnormal outcomes, compared to the controls (Anderson et al. 2012a; Anderson et al. 2012b; Wiseman et al. 2013). OSPW exposures also upregulated and downregulated genes associated with oxidative stress and endocrine signalling. Further, observed lipid peroxidation supports the implication of oxidative stress in midges whereby exposed larvae had 2227.5 nmol/mg of lipid hydroperoxides compared to 767.4 nmol/mg in control larvae (Wiseman et al. 2013).

Invertebrate exposures to NAs, which are major mediators of OSPW toxicity, have also been conducted. Exposure of saltwater mussels (*Mytilus galloprovincialis*) to diamondoid NAs induced DNA damage in both haemocytes and gill cells (Dissanayake et al. 2016). Water fleas (*Ceriodaphnia dubia*) exposed to commercial NAs had an LC50 value of 2.8 mg/L over a 7 day exposure period, while midges showed less sensitivity to NAs with an LC50 of 6.5 mg/L (Kinley

et al. 2016). Although commercial NA exposures are beneficial to understanding NA-mediated toxicity, they are not always representative of bitumen-derived NAs as a result of the extraction processes. Comparisons between commercial NAs and different samples of energy-derived NAs showed that freshwater mussel (*Lampsilis cardium*) larval sensitivity is dependent on the source. For example, bitumen processing-derived NAs from an active pond at Industry A reduced viability at 70 mg/L compared to the Industry B active pond NAs at 25 mg/L. Furthermore, commercial NAs lowered viability to <10% at 2.5 mg/L (Bartlett et al. 2017). Alternatively, Johnston et al. (2017) exposed pond snails (*Lymnaea stagnalis*) to lightweight NAs and energy-derived NAs and found that they shared the same concentration-dependent effects with an IC50 of 32 mg/L. Increasing concentrations decreased egg hatching with total inhibition reached at 50mg/L. Embryo growth was increasingly impaired with longer exposures, and later exposures in development, to concentrations less than the IC50 (Johnston et al. 2017). Therefore, commercial NAs can produce similar results to OSPW-mediated toxicity, however this is not universal and differences in magnitude of effects should be considered when drawing conclusions from studies that only focus on commercial NAs.

As mentioned earlier, OSPW inorganic constituents may also contribute to toxicity. Sodium, magnesium, potassium, and calcium concentrations were 55% higher downstream of a bitumen processing area than upstream that Pilote et al. (2018) suggested is from leaching OSPW. Bioaccumulation in 2012 of dissolved metals in “giant floater” freshwater mussels (*Pyganodon grandis*) located downstream of the oil sands in the Steepbank River included aluminum, vanadium, cobalt, and molybdenum; all were concentrated primarily in the gill tissue. The 90% reduced water flow of 2013 resulted in more particulate matter and greater accumulation of cadmium and lead in digestive glands, while nickel and strontium were the only

metals that had greater accumulation in the gills. This indicates that water flow impacts the dilution of metals, and particulate matter concentrations, that influence the uptake pathways in mussels. Furthermore, in 2012, metabolic consequences of metal bioaccumulation were observed: metallothionein in the gills and lipid peroxidation in digestive glands are indicators of oxidative stress and these biomarkers were not observed in 2013. This suggests that oxidative stress is triggered by gill accumulations and dissolved components as opposed to particulate matter and digestive gland sequestration (Pilote et al. 2018). Thus, metal leachates from bitumen processing, including OSPW, may be harmful to aquatic invertebrates with their toxicity being mediated by environmental factors including water flow rates and current.

Seasonality also impacts toxicity effects in water fleas exposed to OPSW collected during the winter; and OSPW summer samples also showed unique toxicity outcomes. For example, cold-treatment increased mortality, reduced size, and eliminated egg production. Daphniids in warm treatments had a reduced number of neonates while both season treatments resulted in lower mass (Lari et al. 2018). Consequently, researchers must consider environmental factors and their impacts on OSPW itself, as well as how they influence the dispersal of OSPW through the environment, when monitoring OSPW toxicity.

In addition to individual constituent toxicity, interactions between constituents are also of concern. Howland et al. (2019b) found that mayfly (*Hexagenia* spp.) survivorship decreased 48% after exposure to a PAH spiked sediment and aqueous NA mixture compared to NAs alone (Howland et al. 2019b). As a result, exposing invertebrates to multiple OSPW components may be a potential avenue for analyzing the additive and synergistic effects that are likely taking place in these complex waters.

Lastly, and most relevant, is the impact of OSPW on invertebrate communities. An outdoor mesocosm study exposing a field-collected invertebrate community to 10, 50, and 100% OSPW showed a decrease in sensitive taxa by 69% and species richness declined following 50% and 100% OSPW exposures. Exposure to 100% OSPW also reduced the richness of tolerant taxa by 63% (Howland et al. 2019a). Additionally, field monitoring of sites in the McMurray Formation area found that PAHs at concentrations lower than water flea acute sensitivity levels increased both the sensitivity and the generation time of invertebrate communities (Gerner et al. 2017). Overall, these community studies offer important insight into potential biodiversity changes mediated by OSPW exposures.

2.1.2.2 Fish

The effects of OSPW exposures have been extensively examined in fish due to their entirely aquatic lifecycle, versatility as study organisms, and their importance to aquatic ecosystems. Remediation aims to establish self-sustaining ecosystems from OSPW and thus the impacts on higher trophic populations, like those of fish, is imperative to understand.

One major point of interest is the effect of OSPW on the ability of fish to use social and environmental cues during exposures. It was found that OSPW at concentrations of 0.1% and 1% induced an olfactory response from rainbow trout causing OSPW avoidance (Lari and Pyle 2017b). However, as OSPW concentration and exposure time increased, OSPW impaired the ability of rainbow trout to detect important food and social cues (Lari et al. 2019). In short-term exposures, this effect was reversed once OSPW was removed. This effect also appeared to be influenced by the coating of the olfactory epithelium with OSPW suspended particulate matter (Lari and Pyle 2017b; Lari et al. 2019). Similarly, Reichert et al. (2017) found that olfactory

impairment after a 30 minute exposure was reversible, but the effects of 7 day exposures were persistent, which may be attributed to salinity as saline controls evoked similar results to 10% OSPW exposures (Reichert et al. 2017). Overall, this suggests that long term exposures to OSPW may negatively impact the ability of fish to feed and interact with others. Avoidance of OSPW components may affect migration but may also protect populations. By detecting point releases of OSPW, fish populations can effectively reduce or avoid exposures altogether resulting in the maintenance of healthy fish populations in OSPW impacted areas.

In addition to chronic lab exposures to OSPW, fish health has also been examined in fathead minnows (*Pimephales promelas*) inhabiting an OSPW-based pond. This chronic, and multigenerational exposure resulted in changes to reproductive characteristics and overall health indicators. For example, when compared to fish from reference sites, those living in OSPW had a higher condition factor based on their larger gonadosomatic index (GSI), liver somatic index (LSI), and spleen somatic index (SSI). The condition factor describes the relationship between weight and length of fish; a fish with a high condition factor is heavier compared to other fish of the same length. Thus, OSPW-exposed fathead minnows were heavier than reference fish. Furthermore, once sexual maturity was reached, fathead minnows darkened, but this effect was not observed in fish exposed to OSPW. In addition, operculum and gill deformities including epithelial cell proliferation, fusion of lamellae, and necrosis were observed when fish were exposed to OSPW (Kavanagh et al. 2013). Overall, OSPW exposures induced several adverse effects in a naturally exposed fish population, which indicates the potential impacts it may have if released on a larger scale prior to remediation.

The immunotoxic effects of OSPW have also been examined using the goldfish (*Carassius auratus*) model system. Acute exposure to fresh OSPW induced an antimicrobial response

including the increased expression of *interleukin-1β1* (*il-1β1*). Interestingly, following sub-chronic exposures, Hagen et al. (2014) observed the downregulation of pro-inflammatory genes like *interferon-γ* (*ifn-γ*) and *tumour necrosis factor-α* (*tnf-α*). During infection with the parasite *Trypanosoma carassii*, goldfish acutely exposed to OSPW experienced lower parasitemia compared to unexposed fish supporting the enhanced inflammatory effects observed at the gene level (Hagen et al. 2014). Although lower parasitemia is beneficial, alterations to the immune response, such as the maintenance of a pro-inflammatory state, can lead to adverse outcomes including immune-related tissue damage.

NA-mediated effects were also observed in fish. For example, developmental effects from exposures were documented in zebrafish (*Danio rerio*). Specifically, zebrafish exposed to OSPW-isolated NAs had several deformities including yolk sac edema and spinal malformation as well as the upregulation of genes encoding P450 aromatase, estrogen receptors, and vitellogenin suggesting endocrine disruption and estrogenic activity (Wang et al. 2015b). Walleye (*Sander vitreus*) embryos also experienced spinal deformities as well as cardiovascular and craniofacial abnormalities. The altered expression of genes, such as those in the aryl-hydrocarbon receptor (AhR) pathway, indicated that NAs likely induced oxidative stress (Marentette et al. 2017).

Likewise, the above-mentioned immunotoxicity data from goldfish following OSPW exposures may also be attributed to NAs as goldfish exposed to NAs experienced comparable effects. This includes the upregulation of inflammatory genes following acute exposures and the resulting greater immune response to parasite challenges, as well as the sub-chronic down regulation of inflammatory genes, which coincide with lower control of parasite challenges (Hagen et al. 2012). In contrast, OSPW and OSPW-derived NAs did not cause similar adverse

effects to rainbow trout immunity. For instance, in rainbow trout, OPSW reduced the number of leukocytes in circulation and altered their presence in immune organs, and lowered antibody production. However, these effects were not as prevalent after NA treatments suggesting that they are not the only contributor to the observed immunotoxic effects (Leclair et al. 2013).

2.1.2.3 Birds

The assessments of OSPW adverse effects have also been conducted in birds. Birds are a unique study organism as they do not necessarily have aquatic habitats that would be directly influenced by OSPW release. However, they often rely on aquatic landscapes, like wetlands, and have dietary components with aquatic life stages (e.g. invertebrates). This may result in the manifestation of OSPW effects through these indirect exposure routes via the food chain.

When comparing reclaimed oil sands mining sites and reference wetlands, Smits et al. (2000) observed no change in tree swallow (*Tachycineta bicolor*) reproductive success, growth, or immune response. However, an increase in ethoxyresorufin-O-deethylase (EROD), which is indicative of increased xenobiotic metabolism and detoxification, was observed and correlated with higher sediment concentrations of PAHs (Smits et al. 2000). Alternatively, tree swallows inhabiting oil sands effluent-fed wetland sites had smaller nestlings and they also had higher EROD activity. Furthermore, inclement weather including heavy rainfall and sudden decreases in temperature, although impactful at all nesting sites, comparatively increased nestling mortality in birds that fed at locations near mining effluents (Gentes et al. 2006). Fernie et al. (2018) observed that at sites impacted by mining, but not by OPSW, the rainfall levels of 2012 (compared to 2013) lowered reproductive successes, reducing nestling and fledgling mass. This was suspected to be influenced by the accumulation of polycyclic aromatic compounds (PACs)

from oil sands mining activities due to changes in available diet and food sources (Fernie et al. 2018). Interestingly, exposure of tree swallows to NAs 7 to 13 days post-hatch did not result in adverse effects on growth, blood chemistry, organ condition, or EROD activity, suggesting that other OSPW constituents, such as PACs, or other environmental factors are primarily responsible for the adverse health effects (Gentes et al. 2007a).

Conversely, short term exposures of both juvenile and adult domestic mallards (*Anas platyrhynchos domestica*) to OSPW did not affect their survival or adult body mass. Changes to potassium and bicarbonate levels in blood, and the levels of released thyroid hormones and corticosterone, changed compared to controls but not beyond documented healthy ranges for mallards and thus it was not deemed biologically significant. However, bioaccumulation of vanadium was documented at levels 244% higher than controls, which may suggest toxicity (Beck et al. 2014).

In summary, although birds may not be the most likely of study organisms when examining OSPW toxicity, they have provided insights into the indirect pathways through which OSPW exposure may occur or may cause negative effects. These include pathways via food and water consumption, and reduced resiliency to environmental stresses.

2.1.2.4 Mammals

Few *in vivo* studies have been performed examining the effects of OSPW and OSPW constituents in mammals. Like birds, most mammals do not have aquatic life histories, and this may be why OSPW studies have not focused on them. Nevertheless, they do rely on landscapes that are likely to be perturbed by OSPW during remediation or release, and thus it is important to understand OSPW effects in mammalian systems.

OSPW exposure studies have been conducted in mice with a focus on reproductive and developmental endpoints. Specifically, acute and sub-chronic exposures of female mice to doses of the OSPW OF matching environmentally relevant NA concentrations (1-10 mg/L) did not affect pregnancy, lactation, or hormone profiles. Moreover, gene expression associated with detoxification, oxidative stress, DNA repair, and cell proliferation, were not affected unless the NA concentration was increased to 55 mg/L during acute exposures (Li et al. 2019). Rats exposed to a high (60 mg/kg) concentration of NA during a sub-chronic exposure period (90 days) developed heavier livers, brains, and kidneys, and transient changes to their food and water consumption. The high-dosed rats drank more water throughout the exposure but ate less food between exposure day 1 and 11. This resulted in lower weights compared to control and low-dosed rats (0.6 mg/kg) (Rogers et al. 2002). Consequently, it appears that mammals require high doses of NAs to display adverse effects and may be unaffected by lower, environmentally relevant, concentrations.

2.2 Remediation Strategies

Due to the increasing volumes of OSPW and its known toxicity potential, remediating these waters is necessary to safely reclaim northern Alberta landscapes affected by oil sands mining operations. Remediation requires carefully converting the contaminated waters to acceptable non-toxic levels that can be released in to, and interact with, surrounding ecosystems without harm. In addition to release, remediation also requires transforming decommissioned mining sites into self-sustaining ecosystems. Possible strategies to accomplish this include ageing, advanced oxidation processes, coagulation/flocculation, and membrane filtration; all of which will be discussed in the following sections of this literature review.

2.2.1 Ageing

Currently, the most common and cost-effective method of OSPW remediation is the process of ageing. As a passive process, ageing allows oil sands waste products to detoxify over time with little to no human interference. This makes it an ideal remediation strategy compared to active processes, like advanced oxidation, coagulation/flocculation, and membrane filtration, which all require labour and resources.

To examine the efficacy of OSPW ageing, Syncrude Canada Ltd. (SCL) created shallow, aerated storage lagoons of OSPW which mirrored other industrial effluents' ageing strategies. Likewise, prior to its reclamation in 1999, Suncor Energy Inc. (SEI) monitored tailings pond 1A, which had been inactive from 1977 to 1984 to assess the ageing process (MacKinnon and Boerger 1986; Nix and Martin 1992).

Alternatively, ageing can be accomplished by constructing end-pit lakes (EPLs) where processing waste from mines is moved to, and held in, areas of mining pits that are no longer operational. The processing waste is then covered with a layer, or "cap", of freshwater or mixed freshwater and OSPW, which acts as a barrier to contain the tailings. This is known as water capping at which time the water cap's composition is primarily OSPW (Consortium Fine Tailings Fundamental 1995; Dompierre et al. 2016). This strategy was examined in the late 1980's and early 1990's by major oil sands companies, like SCL and SEI, at which time both companies began constructing bench scale and field prototypes that included capping mature fine tailings (MFT) with unprocessed water or with OSPW (Siwik et al. 2000; Nix and Martin 1992).

Early assessments of ageing demonstrated an increase in detoxification in aerated OSPW compared to anaerobic conditions. There was also a general trend towards lower toxicity

over time in aquatic invertebrates, like water fleas, and reduced acute toxicity to rainbow trout fingerlings (MacKinnon and Boerger 1986; Nix and Martin 1992). Regardless, water fleas exposed to OSPW still had lower fecundity than those in the reference lake and although rainbow trout hatching improved over time, there was still a high post-hatch mortality rate indicating that chronic toxicity was still persistent (Nix and Martin 1992).

Recently, several studies have investigated the effectiveness of ageing by comparing fresh and aged OSPW to each other. Midge larvae had lower mass after exposure to fresh OSPW compared to aged OSPW as well as changes in antioxidant gene expression, lipid peroxidation, and increased endocrine gene expression (Wiseman et al. 2013). 2014 surface water from Base Mine Lake (BML), an EPL established in 2012 to assess EPL efficacy, did not affect larval survival of midges or water fleas, but delayed key events in the midge lifecycle. In comparison, the reduced reproduction of water fleas due to BML 2014 was not observed in BML 2015 (White and Liber 2020). Overall, ageing does reduce OSPW toxicity, but this process will take time and toxicity will likely persist for many years.

Similarly, analyses of OSPW constituents after ageing have been performed as well. Assessment of the organic compounds within fresh OSPW and aged OSPW showed that Japanese medaka (*Oryzias latipes*) fry accumulated calcein, which suggested inhibition of membrane transport proteins, after fresh OSPW exposure but not after aged OSPW exposure (Alharbi et al. 2016). Conversely, examination of NA toxicity between fresh and aged OSPW found that NA fractions (NAFCs) from fresh and aged OSPW induced mortality and developmental abnormalities in fathead minnows that were primarily cardiovascular in nature, with no difference between the OSPW ages (Marentette et al. 2015). Following the Marentette et al. (2015) protocols, Bartlett et al. (2017) expanded the comparison of NAFCs to their effects on

an amphipod crustacean (*Hyalella azteca*) and freshwater mussels. They observed few differences between the fresh and aged NAFCs toxicity (Bartlett et al. 2017). Similarly, exposure of larval fathead minnows by Loughery et al. (2019) to fresh and aged NAFCs induced cardiovascular and spinal deformities. Surprisingly, they were more prevalent in aged NAFC. Both fresh and aged NAFCs induced expression of transcriptomes associated with metabolism, detoxification, and the immune system (Loughery et al. 2019). Overall, these studies further illustrate the difficulty of OSPW characterizations and that conclusions about detoxification efficacy are dependent on the analysed constituent, the study organism, and the endpoints assessed.

Although most studies focus on the OSPW OF regarding toxicity and detoxification, the IF also contains constituents of concern. White and Liber (2018) identified Na^+, Cl^-, and HCO_3^- at high-risk concentrations in the BML surface water over the course of 3 years. Over time Na^+ and Cl^- concentrations decreased, but HCO_3^- and metals such as boron and nickel remained elevated despite ageing and dilution from SCL water pumping. Interestingly, researchers observed water fleas (*Daphnia pulex*) in a sample from 2016 suggesting that BML may be able to eventually maintain an ecosystem of salt-tolerant organisms (White and Liber 2018).

Investigation into the efficacy of ageing at the SCL Mildred Lake mine site produced conflicting results across different model organisms. Siwik et al. (2000) conducted a study examining the effects of OSPW aged for 6 years on the growth of fathead minnows. They compared treatments of MFT capped with unprocessed water, MFT capped with OSPW, MFT capped with runoff water, and OSPW alone. It was shown that fish raised in MFT capped with either runoff or OSPW had lower survival rates. It was also shown that fish raised with MFT and/or OSPW grew larger in lab treatments, but after introduction into field mesocosms, had

reduced growth and weight gain compared to control fish suggesting that physical characteristics of aged OSPW, such as light and turbidity, affected development as opposed to chemical characteristics (Siwik et al. 2000). Alternatively, a recent investigation of the Mildred Lake Pond 9 site after 18 years of ageing compared whole OSPW, organic fractions that differed in extraction method (F1, F2, F3), and the fractions recombined (RF), and found differing levels of sensitivity across species. Fathead minnows and Japanese medaka were both sensitive to F3. Fathead minnows had reduced hatch success, hatch length, and increased larval abnormalities, whereas Japanese medaka had reduced hatch-length and time-to-hatch. Interestingly, these effects were not observed in whole OSPW. It was hypothesized that F3-induced toxicity was due to its increased polarity and higher degree of aromaticity, and its concentration of acid extractable organics (AEOs). These results suggest that interactions between constituents in aged whole OSPW decreased toxicity in fish. This outcome was not observed in invertebrates, such as freshwater amphipods and freshwater mussels that displayed reduced survival after exposure to whole OSPW. This result suggested that the IF and/or interactions between constituents contributed to persistent invertebrate toxicity despite 18 years of ageing, which contrasts the outcome observed in fathead minnows and Japanese medaka. In the mayfly (*Hexagenia* spp.) no affects on survival regardless of treatment were observed (Bauer et al. 2019). These studies on the Mildred Lake site again reinforce the hurdles in assessing toxicity and remediation efficiency.

Beyond comparisons between fresh and aged OSPW, mechanisms that mediate the ageing process have been identified. Bacterial and algal species were found to be mediators of OSPW detoxification through their ability to degrade constituents of concern including NAs, PAHs, and the AEO (Folwell et al. 2016a; Folwell et al. 2016b; Lengger et al. 2013; Yue et al.

2015; Ruffell et al. 2016). The efficiency of microbial biodegradation was dependent on environmental conditions such as an aerobic or anaerobic environment, and the age of the OSPW. Thus, biodegradation can often be improved by combining it with other remediation techniques. For instance, gamma irradiation exposure of fresh and aged OSPW stimulated different organisms with unique biodegradation potentials. For example, *Pseudomonas* spp. alone reduced OSPW toxicity but also effectively remediated it in combination with ozonation (VanMensel et al. 2017; Zhang et al. 2015). Secondly, photodegradation occurring naturally from sunlight also contributed to ageing mediated OSPW remediation. UV works directly by transforming chemical structures or indirectly through photosensitizers that generate reactive radicals (Liu et al. 2017). This resulted in the degradation of organic OSPW constituents that can be facilitated by inorganic photosensitizers such as nitrate (Challis et al. 2020; Shu et al. 2014; Qin, How, et al. 2019).

Ageing, and EPLs specifically, are considered an ideal solution. It has been proposed that remediating mining wastes through naturally occurring biological and geochemical processes with the addition of fresh-water inputs, such as precipitation or natural ground runoff, will contribute to improving water quality (Dompierre et al. 2016, 2017). From an economical standpoint, EPLs incur few initial costs and are easily incorporated into the broader goal of mine site reclamation put forward by the EPEA and the *Conservation and Reclamation Regulations* by filling decommissioned mining pits (Dompierre et al. 2017; White and Liber 2020; AER 2021b). However, due to concerns over EPLs' ability to effectively cap MFTs, maintain an ecosystem and protect the surrounding watershed, and sustain plasticity to hydrological changes; recent applications to approve EPLs have been denied, and further demonstrations of the effectiveness of EPLs are required (AER 2018, 2019).

2.2.2 Advanced oxidation processes

Advanced oxidation processes (AOPs) are oxidation processes that use radicals to degrade persistent organic, and sometimes inorganic, compounds (Deng and Zhao 2015). It has been employed in other waste-water treatments and is thought to be a potential avenue of remediation for OSPW.

As previously mentioned, photodegradation is a natural process occurring between sunlight and various OSPW constituents. However, to improve this natural process, several studies have examined the effectiveness of adding photocatalysts, like titanium dioxide, that enable the photodegradation reaction to occur more efficiently. These studies have found that photocatalysts are effective at targeting constituents with greater aromaticity, carbon number, and structural complexity; while eliminating nitrosonium (NO^+) and sulfur oxide (SO^+) classes, leading to lower acute toxicity in *V. fischeri* (de Oliveira Livera et al. 2018; Leshuk et al. 2018, 2016). Thus, it is proposed that photocatalyst-mediated photodegradation may be complimentary to, and work alongside, natural biodegradation in ageing OSPW.

Ozonation has also been investigated as a remediation strategy for OSPW that primarily targets NAs. For instance, after ozonation, Lyons et al. (2018) observed that NAs decreased 92% and He et al. (2012) found that fathead minnow exposed to ozonated OSPW did not experience the toxicity induced by untreated OSPW such as embryonic developmental defects and lower survival rates. Similarly, untreated OSPW inhibited nitric oxide (NO) production in bone marrow-derived macrophages, but this was not observed following exposure to ozone-treated OSPW. In contrast, despite a reduction in NAs, ozone-treated OSPW still induced toxicity in *V. fischeri* (Wang et al. 2013). Specifically, NAs were degraded 55% to 98% by ozonation yet

toxicity remained in *V. fischeri* and goldfish primary kidney macrophages suggesting that NA degradation by-products and other constituents in OSPW contribute to this persistent toxicity (Klamerth et al. 2015). Consequently, ozonation paired with biodegradation is demonstrated as a better remediation tactic. Environmental microbial communities are able to withstand ozonation with no adverse effects to their rate of biodegradation. In addition, specific conditions like ozonation dose and aerobic environment can select for microorganisms known to degrade aromatic constituents and help reduce toxicity (Brown et al. 2013; Dong et al. 2015; Vaiopoulou et al. 2015). For instance, the toxicity of OSPW to *V. fischeri* was eliminated after ozonation and biodegradation with Pseudomonads (Zhang et al. 2015).

As such, AOPs are effective at reducing the effects of certain toxic organic constituents, like NAs, but are not able to remediate toxicity entirely due to the complex and unique mixtures of organic and inorganic constituents in different OSPWs. As a result, recent studies have shown the benefits to administering AOPs in tandem with microbial biodegradation to remediate major toxic constituents, their by-products, and other components that contribute to OSPW toxicity.

2.2.3 Coagulation/flocculation

Coagulation/flocculation (CF) is the process of binding small particles together to form larger particles that eventually precipitate and can be isolated (Thomas et al. 1999). Due to OSPW complexity and turbidity, CF is thought to be a beneficial pre-treatment for removing fine solids to facilitate secondary remediation strategies and can contribute to remediation. For example, CF using alum and a cationic polymer decreased the concentration of NAs by 37%. The cationic polymer, however, induced toxicity in midges, thus it is not recommended as a flocculant. Wang et al. (2015a) compared a synthesized polyaluminum chloride (PACl) with

83.6% Al_{13} to PACl with 30.5% Al_{13} of the total aluminum ions and found that the higher Al_{13} percent resulted in reduced *V. fischeri* toxicity. Additionally, both PACl-types were effective at reducing turbidity and removing metal cations with low pK_a compared to those with high pK_a, but neither removed NAs (Wang et al. 2015a). Thus, CF can be an effective pre-treatment for OSPW remediation, but this is dependent on the CF agent. Moreover, the precipitation during CF generates waste that must be separated prior to continued OSPW treatments and that waste may also require further processing (Quinlan and Tam 2015).

2.2.4 Membrane filtration

Membrane filtration is a high-throughput technique of water treatment that involves filtering liquid through a solid material that excludes the passage of select solutes (Hendricks 2011). As such, membrane filtration is an option for OSPW remediation with the goal of removing toxic constituents and facilitating downstream remediation treatments.

However, effective membrane filtration is dependent on the filter used and influent characteristics. Comparison between polymeric membranes with different levels of hydrophilicity found that hydrophobic membranes adsorbed more of the acid extractable fraction (AEF) compared to hydrophilic membranes. Moreover, the OSPW pH impacted NA adsorption with OSPW at pH 5.3 adsorbing 26±2.4% of NAs compared to insignificant amounts adsorbed at pH 8.7 (Moustafa et al. 2014).

Unfortunately, a caveat to membrane filtration is fouling, or the build up of material and consequent blocking of filters. Alpatova et al. (2014) found that this can be remedied by pre-treating OSPW with CF before filtering it through a 1kDa ceramic ultrafiltration membrane. Based on this membrane pore size, they observed up to 38.6±2.7% removal of the chemical

oxygen demand (COD), but also observed low AEF and NA removal efficiency due to their size (<1kDa). Hence, a proposed follow-up of the OSPW treatment using nanofiltration and reverse osmosis to collect smaller constituents was suggested (Alpatova et al. 2014). Similarly, Dong et al. (2014) found that treating ceramic microfiltration membranes with SiO_2 reduced fouling and that filtration following CF removed 93% of suspended solids, 17% of total organic carbon, and 10% of COD. Total organic carbon and COD removal increased to 98% and 100%, respectively, if reverse osmosis treatment followed, which also removed a myriad of metals from OSPW (Alpatova et al. 2014).

Alternatively, membrane filtration can take advantage of the natural biodegradation processes of microbial communities by using biofilms. OSPW supplemented with growth medium had greater biofilm biomass compared to OSPW alone resulting in the degradation of COD, sulfate, and nitrogen after 6 months (Liu et al. 2015). Additionally, using granular activated carbon as support medium for biofilm growth enabled the development of a diverse microbial community, compared to OSPW, that enabled the degradation of COD, AEF, and classical NAs by 51%, 56%, and 96%, respectively. Many members of the biofilm community were carbon degraders resulting in a greater decrease in NAs with higher carbon content (Islam et al. 2014). This reduction in OSPW constituents can translate to reduced toxicity. Yue et al. (2016) observed that following a reduction in the AEF, including NAs, that toxicity to *V. fischeri* decreased 73% and estrogenic activity in yeast decreased 22%. However, like in other remediation strategies, persistent toxicity, likely due to degradation products, required further treatments (Yue et al. 2016).

2.3 Bioindication

Bioindication and biomonitoring refer to the use of a biological system to assess changes to the environment and these terms are often used interchangeably (as they will be in this thesis). More specifically, bioindication is a method of qualifying the effects of a stressor whereas biomonitoring quantifies these impacts on an ecosystem (reviewed by Parmar et al. 2016).

Bioindication has become an important tool in the fields of environmental toxicology and conservation/ecology. Some of the key benefits of bioindication are its cost-effectiveness compared to other sampling methods and the ability to assess the outcome of interactions between many factors. Nevertheless, the success of bioindication depends on the selection of effective indicators. Characteristics considered valuable in an indicator are:

1. The ability to display early signs of stress;
2. The ability to remain sensitive and produce varying degrees of response relative to the stressor intensity; and
3. The ability to measure not only the change itself, but the cause (reviewed by Carignan and Villard 2002).

Consequently, indicators vary and depend largely on the stimulus or ecosystem being monitored.

2.3.1 Aquatic biomonitoring

Assessing water quality is imperative and has become a key focus of biomonitoring. A variety of organisms have been employed in this venture ranging from microorganisms to whole

animals and this is dependent on the environmental stressors being monitored. For example, sulfur-oxidizing bacteria were sensitive monitors for stream pollutants as they were inhibited within 2 hrs of the stream being intentionally contaminated with swine wastewater or with nitrite and chromium ions (Hassan et al. 2019). Santos et al. (2019) found a correlation between seasonality and anthropogenic activities that led to nutrient fluctuations and changes to the density of high and low nucleic acid bacteria. This suggests that bacterial communities can serve as indicators for several factors simultaneously impacting an aquatic ecosystem. Similarly, Patang et al. (2018) found that macroinvertebrates tolerant to organic compounds dominated in polluted rivers compared to more sensitive species, and thus changes in biodiversity are indicative of pollution levels. Naturally, aquatic monitoring is largely dependent on fish indicators due to their presence and importance to water systems. Bioindication involving fish spans many contaminants and includes the monitoring of heavy metals, persistent organic pollutants (POPs), and microplastics within fresh and marine waters (Zrnčić et al. 2013; Panseri et al. 2019; Su et al. 2019).

Another factor to consider when choosing an aquatic bioindicator is their role in the ecosystem food web. Trophic level describes the position of a species in a food chain and determines the transfer of energy through an ecosystem. Organisms at higher trophic levels consume those at lower levels and this results in greater sequestration of substances, like contaminants, as trophic levels increase. Thus, trophic level is an important consideration in water quality assessments. For example, the levels and effects of dichloro-diphenyl-trichloroethane (DDT), a major pesticide of the mid 20th century and a POP, has been monitored successfully in aquatic apex predators like birds and marine mammals because of the increase in contaminant bioaccumulation in apex predators. This has been accomplished by measuring

eggshell thickness and DDT concentrations in lipid-rich organs where organics accumulate like in the blubber of aquatic mammals and the uropygial gland of birds (reviewed by Hellou et al. 2013). In comparison, the Reid et al. (2013) review of Eurasian otters as bioindicators in Ireland concluded that, because of their broad niche and their dietary plasticity, they do not effectively indicate changes in nutrification and eutrophication (increases in environmental nutrients from natural and anthropogenic processes, respectively) in their habitats. Thus, a variety of organisms may be used to effectively monitor water quality, but this efficacy is dependent on the organism's niche and the endpoints chosen.

2.3.2 OSPW biomonitoring

The premise of using an organism as an indicator for OSPW toxicity is the same as other environmental biomonitoring processes. However, it differs because rather than assessing the effects of a contaminant on an ecosystem, this is examining inherently contaminated sources that are contained to specific areas. Consequently, indicator selection often depends on their likelihood of becoming exposed based on their proximity to tailings ponds. This nuance results in more laboratory studies compared to field studies and a developing interest in cell-based toxicity tests.

2.3.2.1 Field studies

Field studies are an asset to OSPW biomonitoring as they generate data that is more relevant to the effects OSPW has on ecosystems. The study by Kavanagh et al. (2013) examined OSPW-mediated effects on the reproductive and overall health of fathead minnows collected from a population that was unintentionally introduced into a demonstration pond constructed by SCL. Although Kavanagh et al. (2013) did not propose them as bioindicators, this presented a

rare opportunity to monitor toxicity in a natural OSPW fish population. Likewise, SCL and SEI reclaimed wetlands and areas of active mining have been monitored using tree swallows that served as effective higher trophic level indicators due to their insectivorous diet that relies heavily on aquatic systems. Tree swallow endpoints measured included growth and mortality rates, detoxification, parasite loads, immune responses, and bioaccumulation levels (Smits et al. 2000; Gentes et al. 2006; Gentes et al. 2007b; Harms et al. 2010). Overall, these studies conducted on wild OSPW-affected populations are the most environmentally relevant, but they are not always feasible.

In instances when field studies are not tenable or whole field sites are not accessible, constructing mesocosms that mimic natural ecosystems are a viable option. Fathead minnows reared in mesocosms constructed within sites with various levels of OSPW wastewater contamination displayed unique growth patterns (Siwik et al. 2000). This study demonstrated the use of mesocosms to replicate the development of fish should they become exposed to these OSPW sources and, aside from mesocosm construction, little experimental manipulation was carried out by the researchers. However, mesocosm studies do offer the benefit of more experimental control. For example, Howland et al. (2019a) divided a macroinvertebrate community into a set of mesocosms with various OSPW treatment concentrations and observed shifts in community assemblages. Overall, they observed that, at higher OSPW concentrations, sensitive taxa had depleted richness and abundance compared to tolerant taxa and unexposed individuals. Therefore, mesocosm studies pertaining to OSPW offer the relevance of field studies, but with greater control of experimental variables and confounding factors.

It is critical to note, in addition to choosing effective indicators and establishing sound experimental designs, the importance of standardized methodologies when discussing OSPW

bioindication and bioindication in general. For instance, Gerner et al. (2017) assessed changes in invertebrate communities residing in the Athabasca river and other nearby waterways. They observed heightened sensitivity and decreased generation time over three years from bitumen-derived components. To quantify these results, they established a variant of the Species at Risk (SPEAR) approach specific to this scenario. In general, SPEAR identifies species characteristics that increase a species' vulnerability to a stressor. High SPEAR values indicate tolerance and low values indicate sensitivity. Previously, SPEAR has been optimized to measure pesticides, salinity, and organic pollutants with various endpoints in specific regions around the world (Schäfer and Liess 2013). Going forward, OSPW biomonitoring should utilize standardized tests like SPEAR as a means of effectively tracking species changes over time and facilitating comparisons between studies.

2.3.2.2 Laboratory studies

Most studies examining the effects of OSPW on organisms have been conducted in laboratory settings. Study organisms range from cells to whole mammals as covered in section 2.1. Providing the study organism had a sensitive response, most experimental designs could be considered for biomonitoring if they were repeated over time. Currently, *in vivo* assessments comprise the overwhelming majority of OSPW research (see section 2.1.2) although there have been several emerging *in vitro* studies (see section 2.1.1).

Of the *in vitro* studies, there is a broadening interest in OSPW perturbation to cell function. The Microtox Assay, for instance, has been useful in assessing changes in toxicity during experimental remediation processes and strategies (Leshuk et al. 2016; Wang et al. 2013; Zhang et al. 2015; Wang et al. 2015a; Yue et al. 2016). As previously mentioned, Fu et al.

(2017), Qin, Lillico, et al. (2019), and Phillips et al. (2020) have all examined the effects of OSPW and its fractions on RAW 264.7 mouse macrophages. They propose the use of macrophages as cell-based bioindicators due to their sensitivity and rapid response to changes in their environment. For example, inducible nitric oxide synthase (iNOS) protein levels were detected as early as 6 hrs after OSPW exposures and revealed differential results depending on the exposure water (Phillips et al. 2020). Consequently, macrophages (as representative innate immune cells) possess the sensitivity for not only a rapid response but also produce variable outputs specific to different stimuli.

2.3.3 The immune system and macrophages as bioindicators

The immune system encompasses the cells, molecules, and interactions that take place within an organism that have evolved to maintain homeostasis and recognize and respond to xenobiotic threats (reviewed by Thomas et al. 2013). In short, it functions as an individual's alarm system for internal (e.g. tissue injury) and external (e.g. microbes) threats. Accordingly, the immune system satisfies the core tenets of a bioindicator described by Carignan and Villard (2002) due to its sensitivity and rapid response. The immune system can recognize a physiological disturbance, generate a cellular response, and produce an output that can be measured and interpreted quickly (i.e. hrs). Perhaps the most important feature of the immune system in relation to biomonitoring is its sensitivity to a variety of stimuli, both endogenous and exogenous, making it a versatile indicator system (Thomas et al. 2013). As such, immuno-toxicological and immuno-bioindication studies span several classes of stimuli and indicator organisms.

Macrophages possess several potent antimicrobial functions and secrete bioactive

proteins called cytokines that are markers for macrophage functions and phenotypes. Consequently, macrophages provide several endpoints that can be monitored following exposure to a xenobiotic. As previously discussed, the RAW 264.7 mouse macrophage cell line has been demonstrated by our lab for use in bioassays designed for monitoring OSPW and may allow identification of constituents of potential concern. In addition, other studies have also employed macrophages as sensitive bioindicators.

For example, metal biomonitoring has been accomplished using various macrophage endpoints. In fish, the response of macrophage centres (the precursors to germinal centres responsible for B-cell maturation and antibody specification in higher vertebrates) was documented following exposure to mercury and arsenic (Meinelt et al. 1997; Datta et al. 2009). Arsenic downregulated the antimicrobial abilities of macrophages and thus prevented the clearance of a bacterial infection (Datta et al. 2009). In humans, Bai et al. (2019) established a link between urinary lead concentrations and carbon loaded airway macrophages, which have previously been used as bioindicators for traffic pollution, as a means to monitor an individuals' exposure to air pollutants.

Similarly, macrophages are often used as indicators of pollution. Several comparisons have been made between clean and contaminated aquatic environments, which primarily focus on changes in macrophage population size, localization, and pigmentation (Fishelson 2006; Bala et al. 2015; Pavloski et al. 2020). For example, in three different species of South African birds, Steyn and Maina (2015) found higher concentrations of macrophages in urban bird populations compared to rural populations and unique differences between species. Consequently, they propose the use of birds and their macrophages to monitor air quality in cities.

Lastly, molecules secreted by macrophages have also been used as biomarkers, which

are quantifiable indicators, as opposed to the examination of macrophages themselves. Cytokines that are secreted from macrophages following a specific stimulus act as cellular messengers and aide in coordinating the immune response. Complimentary to what Datta et al. (2009) observed, Nayak et al. (2007) showed the down regulation of two important antimicrobial cytokines, IL-1β and TNF-α, following exposure to low levels of arsenic in zebrafish. Also in zebrafish, aphantoxins are released from an aquatic cyanobacterium resulting in perturbations to cytokine secretion compared to unexposed fish (Zhang et al. 2019). In addition to cytokines, iNOS, an enzyme that catalyses the synthesis of NO which has many antimicrobial functions, can be monitored for environmental changes. For instance, the pesticide deltamethrin induced iNOS in several organs depending on exposure time and dose in carp (*Cyprinus carpio L.*) (Arslan et al. 2017). Thus, macrophages and their secreted molecules have served as successful bioindicators and biomarkers, respectively, for several contaminants and a variety of endpoints.

2.4 Summary

OSPW is the result of bitumen extraction from the oil sands and is contained in tailings ponds pending remediation. It is primarily a mixture of water, clay, and silt, but is also a complex mixture of several organic and inorganic constituents with demonstrated toxic effects in a range of *in vitro* and *in vivo* models. OSPW toxicity has largely been attributed to the OF, however constituents within the IF undoubtedly play a role independently or synergistically with the organics. To better understand OSPW toxicity and the effects its constituents have on the environment, many exposure studies, both *in vivo* and *in vitro*, have quantified the impacts through changes to endpoints like morphology, development, survival, function, gene expression, and death. Altogether, these studies highlight the breadth of impacts OSPW has, but also the variability in the magnitude of effects emphasizes the major challenge in its assessments.

OSPW is not only a complex mixture of constituents, it is also unique depending on the source. As a result, it is impossible to make sweeping statements about its overall harmfulness and this has hindered ongoing remediation efforts. Remediation is the process of removing or lowering OSPW toxicity to the point that it can be safely released into the environment. Several different strategies of remediation have been tested ranging from passive approaches like ageing and biodegradation via environmental factors, to active approaches like membrane filtration and coagulation/flocculation. These strategies primarily target organic constituents and despite many of the active processes successfully reducing toxicity, they would require further remediation through other approaches. Therefore, based on active labour and cost, passive processes are more desirable.

To ensure the efficacy of remediation and to monitor the effects of OSPW in general, bioindication strategies have been employed. Researchers have conducted field studies at OSPW impacted locations using higher trophic level organisms. Mesocosms are also a promising strategy to examine chronic OSPW effects, particularly in smaller organisms like invertebrates. Laboratory assessments have dominated OSPW research, and they have predominantly been *in vivo*, but recently, *in vitro* indicators are being proposed and established in this field. Cell indicators and their biomarkers, like macrophages and their cytokines, are a promising avenue of OSPW assessment due to their sensitivity and their shorter output timeframe. Therefore, I aimed to establish an immune cell-based bioindicator system that capitalizes on the benefits of *in vitro* systems and the previously shown qualities of macrophages as indicators. Specifically, I aimed to demonstrate that, following exposure to OSPW, OSPW fractions, and manipulated OSPW; macrophages recognize the nuances between samples and generate unique responses by differentially expressing inducible pro-inflammatory genes. By doing so, I demonstrate that

macrophages are a dynamic system that can serve as an effective bioindicator to assist OSPW characterization and facilitate industry remediation efforts by monitoring changes in bioactivity.

Chapter III

Materials and Methods

3.1 Cell Culture Maintenance

The RAW 264.7 mouse macrophage cell line (ATCC®TIB-71™) was cultured in 75 cm^2 vented culture flasks containing Dulbecco's Modified Eagle's medium-high glucose (DMEM; Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and 1% penicillin/streptomycin (Invitrogen). Cells were harvested and/or passaged once they reached confluency. Old media was aspirated, and the cells were washed with phosphate-buffered saline (PBS; Sigma Aldrich) before detaching them from the flask's surface with 2 mL of trypsin-EDTA 0.25% (Sigma Aldrich). The trypsin was neutralized with 3 mL of serum-containing media. One millilitre of harvested cells was passaged into 9 mL of media in a new flask and incubated at 37°C with 5% CO_2.

RAW 264.7 stocks were stored at -80°C in FBS supplemented with 10% dimethyl sulfoxide (DMSO; Fisher Scientific).

3.2 OSPW

Suncor Energy Inc. provided several water samples that were collected from Lake Miwasin. Lake Miwasin, formerly called Demonstration Pit Lake, is a reclaimed mine site. It consists of large volumes of OSPW that have been coagulated with alum to remove suspended particles as seen in Wang et al. (2015a). This treated OSPW was then covered with a freshwater cap to promote the formation of a sustainable aquatic ecosystem (SEI 2021).

More specifically, the original untreated OSPW (Suncor DPL OSPW #6) was treated, as described above, and used to fill Lake Miwasin. This treated OSPW is termed Suncor DPL OSPW #3. Both samples of OSPW #6 and OSPW #3 were collected from the sources that filled the lake in October 2018. Prior to the addition of the freshwater cap, surface water from Lake Miwasin was collected in March 2018 (Suncor DPL OSPW #7). After capping the lake, a final sample was collected in October 2018 (Suncor DPL OSPW #2).

In addition to testing whole (i.e. raw) water samples, OSPW #2 and #7 were both fractionated into their organic and inorganic components using a recently developed hydrophilic lipophilic solid phase extraction method and granular activated carbon protocol as described in Qin et al. (2019). The results of chemical analyses on OSPW #2, OSPW #7, and their OFs and IFs are found in Table 3.1.

All waters were stored in the dark at 4°C prior to, and during, the experiments described in this thesis.

Table 3.1 A subset of OSPW constituents.

Constituent (mg/L)	OSPW #2	OSPW #7	OSPW #2 OF	OSPW #7 OF	OSPW #2 IF	OSPW #7 IF
(O_2-O_3) NAs	31.92	52.66	25.55	40.52	0.5	0.56
Chloride	429.13 ±0.17	1045.16 ±11.89	0±0	0±0	388.83 ±0.07	880.98 ±16.25
Sulphate	295.44 ±1.36	1067.03 ±14.53	0±0	0±0	318.86 ±0.47	986.82 ±23.45
DOC*	49.05 ±0.59	70.5 ±1.87	44.76 ±0.04	53.54 ±0.30	1.21 ±0.04	1.68 ±0.02
Al	0.0825 ±0.0021	0.0479 ±0.0131	0.252 ±0.0231	0.1003 ±0.0146	0.1221 ±0.0014	0.0793 ±0.006
B	2.4689 ±0.0142	6.1082 ±0.0437	0.0994 ±0.0289	0.1229 ±0.0471	0.0276 ±0.0201	0.463 ±0.0158
Ca	28.8303 ±0.0244	19.3029 ±0.0223	0.6048 ±0.072	0.194 ±0.0753	16.0622 ±0.2694	28.6528 ±0.2405
Fe	-	-	0.0646 ±0.0133	0.045 ±0.0121	-	-
K	9.3222 ±0.024	21.061 ±0.0919	0.0529 ±0.0091	0.0459 ±0.0006	8.1576 ±0.0625	18.3441 ±0.0499
Li	0.1215 ±0.0028	0.0613 ±0.0015	0.0017 ±0.0005	0.0018 ±0.0004	0.1082 ±0.0013	0.0582 ±0.0008
Na	506.2061 ±2.8325	1281.3038 ±11.8124	12.8955 ±0.0469	14.1185 ±0.0971	474.8517 ±1.9397	1153.9753 ±17.4888
Sr	0.3723 ±0.0006	1.1723 ±0.0096	0.006 2±0.0008	0.001 ±0	0.3605 ±0.0001	0.9507 ±0.0014
Ti	0.0027 ±0.0003	0.0111 ±0.0004	0.003±0.00 02	0.0007±0.0 001	0.0022 ±0.0001	0.0092 ±0.0002

*Dissolved organic carbon

3.3 OSPW Exposures

To ensure that macrophages were only exposed to OSPW and not any microorganisms that may be present in the water samples, OSPW samples were filtered using 0.45 μm surfactant-free cellulose membrane bottle-top filters (Thermo Scientific) or 0.45 μm mixed cellulose esters membrane syringe filters (Sigma Aldrich) prior to exposing the cells. Filtered OSPW samples were then mixed with DMEM without phenol red and 5% FBS to achieve a range of OSPW exposure doses (Table 3.2). Dilutions of OSPW samples used to examine the interactions between OSPW #2 OF and IF are found in Table 3.3. In these experiments, PBS served as a

negative control (a substitute for OSPW) as it does not affect the immune response of macrophages but displaces the same amount of cell culture media.

Table 3.2 Dilutions for the examination of OSPW #2 and #7 and their fractions.

OSPW	Organic Fraction	Inorganic Fraction	PBS	DMEM
50%	-	-	-	50%
-	50%	-	-	50%
-	-	50%	-	50%
-	-	-	50%	50%

Table 3.3 Dilutions for OSPW #2's organic and inorganic fraction's recombination.

Organic Fraction	Inorganic Fraction	PBS	DMEM
10%	-	-	90%
-	10%	-	90%
10%	30%	-	60%
10%	-	30%	60%
10%	50%	-	40%
10%	-	50%	40%
10%	70%	-	20%
10%	-	70%	20%
-	70%	10%	20%

During exposures, RAW 264.7 macrophages were seeded at $3x10^5$ cells/well in a 24-well plate in 500 µL of media (100% DMEM). Cells were left to adhere to the plate overnight at 37°C. The following day, culture media was aspirated, and the adherent cells were washed with 500 µL of PBS before exposing them to 500 µL of the OSPW treatments outlined in Tables 3.1 and 3.2 for 2, 4, 6, 8, and 10 hrs.

Basal exposure experiments were performed to examine any possible OSPW-mediated effects directly on resting macrophages. Examination of OSPW effects on microbe-activated macrophages was performed by treating macrophages with 1.25 µL of heat-killed *Escherichia coli* (OD 600) following a 2-hr OSPW pre-treatment after which cells were incubated with OSPW and *E. coli* simultaneously for the duration of the time course.

Cells stimulated with *E. coli* for 6 hrs served as a positive control, demonstrating expected pro-inflammatory gene induction profiles. Under normal circumstances, resting macrophages will not express the pro-inflammatory genes examined in this thesis. However, after recognizing a stimulus like *E. coli*, macrophages are activated leading to the potent expression of pro-inflammatory genes. Thus, this display of bioactivity from a known source was compared to the bioactivity observed by OSPW-exposure alone (i.e. basal gene induction).

3.4 RNA Extraction

To examine the pro-inflammatory gene expression profile of macrophages after exposure to OSPW, RNA was extracted at 2, 4, 6, 8, or 10 hrs following a phenol-chloroform protocol. OSPW/cell media mixtures were aspirated from wells using an aspirator and 200 µL of TRIzol (Invitrogen) was then added to each well. Cells were homogenized in TRIzol and incubated at room temperature for 10 minutes before being transferred to 120 µL of chloroform (Fisher Scientific) and vortexed. The phenol-chloroform homogenate was incubated at room temperature for 10 minutes then centrifuged for 15 minutes at 12,000 rcf. Four hundred microlitres of the aqueous layer was added to 400 µL of isopropanol (Fisher Scientific) and inverted 5 times prior to a 10-minute room temperature incubation. The mixture was centrifuged for 10 minutes at 12,000 rcf. The supernatant was then aspirated, and the pellets were washed with 500 µL of 75% reagent grade ethanol (Sigma Aldrich) then centrifuged for 5 minutes at 12,000 rcf twice. The supernatant was aspirated after which RNA pellets air-dried and then dissolved in 20 µL nuclease-free water (Integrated DNA Technologies; IDT) overnight at 4°C.

RNA concentrations and relative quality were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher). Quality RNA samples had 260/280 and 260/230 ratios

greater than 1.8. The nucleotides of RNA absorb light at 260 nm, therefore smaller ratios indicate protein and ethanol contamination, respectively.

Ethanol precipitation was performed on samples with ratios lower than 1.8 by adding 1/10 the sample volume of sodium acetate pH 5.2 and 400 µL of 100% ethanol. Samples were incubated for at least 20 minutes in -80°C before being centrifuged for 20 minutes at 7600 rcf. The supernatant was removed, and the pellets were washed with 70% ethanol and centrifuged for 15 minutes at 12,000 rcf. The supernatant was removed before the RNA pellets were dried, dissolved, and analysed as stated above.

3.5 Quantitative PCR (qPCR)

A qPCR method was used to examine relative changes in the expression of pro-inflammatory genes compared to a reference sample. The reference sample is a sample that shows the baseline expression of the target genes to which experimental groups are compared. Thus, in relative qPCR, the expression of target genes from experimental samples are presented as fold changes relative to the reference sample's expression of the same target genes which are automatically set to a fold change of 1.

3.5.1 cDNA synthesis

cDNA was synthesized using the Superscript III First-Strand Synthesis System kit (Invitrogen) according to manufacturer's recommended standard protocols.

3.5.2 qPCR primer validation

The genes analysed in this study were inducible nitric oxide synthase (*inos*), interferon inducible protein 10 (*ip-10*), monocyte chemoattractant protein 1 (*mcp-1*), and macrophage

inflammatory protein 2 (*mip-2*). All are proinflammatory genes and important contributors to the antimicrobial functions of activated macrophages. For example, iNOS is an enzyme that catalyzes the synthesis of NO, which plays a key role in host defense and cell signalling (as reviewed by Bogdan 2001). IP-10, MCP-1, and MIP-2 are important cytokines and chemoattractants that coordinate the migration of immune cells to areas of inflammation thus promoting an effective inflammatory response (as reviewed by Deshmane et al. 2009; Liu et al. 2011; Qin et al. 2017). 18S rRNA (*18s rrna*) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) served as the endogenous controls for the qPCR assay.

Primer validation proceeded with a 1:4 serial dilution of cDNA to assess the efficiency of each primer pair and to determine the final cDNA and primer concentrations. Firstly, *18s rrna* and *gapdh* were selected as endogenous control genes due to their declining linear gene expression that correlated with greater cDNA dilutions. Endogenous genes must be ubiquitously expressed and remain unaffected by the experimental treatments. They are particularly important because they account for variability in gene expression between experimental replicates due to imperfections in technique and procedure (Kozera and Rapacz 2013). During a qPCR run, the endogenous control must be consistently expressed (±2 threshold cycle; C_t).

C_t refers to the number of cycles before SYBR™ green reaches the threshold of detection. SYBR™ green fluoresces upon binding to double-stranded DNA hence the importance of primer specificity. Accordingly, a greater C_t indicates a lower starting concentration of the gene of interest.

The efficiencies of primers for their respective target genes were determined by graphing the difference in C_t cycle between them and the endogenous control against the log of the cDNA dilutions. The slope of this graph must be between -0.1 and 0.1 indicating that the

target gene primers amplify with comparable efficiency to the endogenous control. Since these primers were selected from published articles, as listed in Table 3.4, further sequencing was not performed. The final cDNA dilution selected was a 1/20 dilution as it resulted in 1 ng of total RNA per reaction, which allowed for the efficient amplification of each target gene by each pair of primers. Primer sequences and their final concentrations are found in Table 3.4.

It should be noted that *18s rrna* was replaced by *gapdh* as the endogenous control after varied expression following certain OSPW treatments.

Table 3.4 qPCR primers used in this thesis. Primers were ordered from IDT. [a]Primer sequences were selected from Stephens et al. (2011) , [b]primers were selected from Girbl et al. (2018), [c]primers were selected from Taciak et al. (2018), [d]primers were selected from Bandow et al. (2012).

Gene	Forward Primer	Reverse Primer	Final Concentration
18s rrna[a]	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA	2.5 µM
gapdh[b]	TCGTGGATCTGACGTGCCGCCTG	CACCACCCTGTTGCTGTAGCC GTA	3 µM
inos[c]	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC	5 µM
ip-10[d]	GGATCCCTCTCGCAAGGA	ATCGTGGCAATGATCTCAACA	3 µM
mcp-1[d]	AGCACCAGCCAACTCTCACT	CGTTAACTGCATCTGGCTGA	3 µM
mip-2[d]	GAAGTCATAGCCACTCTCAAGG	TTCCGTTGAGGGACAGCA	3 µM

**inos* and *ip-10*'s primer concentrations when compared to *18s rRNA* were 3 µM and 1.5 µM, respectively.

3.5.3 qPCR parameters

qPCR was performed on Applied Systems' 7500 Fast Real-Time PCR system using the manufacturer's software. qPCR reactions were composed of 1 µL of primer, 2 µL of nuclease-free water, 5 µL of SYBR™ green master-mix (MBSU, University of Alberta), and 2 µL of cDNA per well, and each sample was run with 3 technical replicates.

The cycling parameters were as follows: holding stage at 95℃ for 2 minutes; cycling stage of 40 cycles that alternates between 95℃ for 15 seconds and 50℃ for 1 minute; melt curve stage of 95℃ for 15 seconds, 50℃ for 1 minute, 95℃ for 30 seconds, and 50℃ for 15 seconds.

3.6 Statistics

Statistics were completed on GraphPad Prism 9.0 software. Statistical significance of the fold change in gene expression was determined following a Shapiro-Wilk test for normality. If the conditions were met for a gaussian distribution ($\alpha=0.05$), an unpaired t-test was completed. If the distribution of data was not normal, a Mann-Whitney test was conducted. A p-value < 0.05 was considered significant.

Chapter IV

Effect of OSPW Exposures on Macrophage Pro-Inflammatory Gene Expression Profiles

4.1 Introduction

To assess complex mixtures like OSPW, model organisms and endpoints that are sensitive to nuances between samples are necessary. In general, cells can serve as effective biosensors since they receive, process, and integrate multiple complex stimuli that result in quantifiable responses. Specifically, changes to immune cell activities including effects on their gene expression, can serve as excellent indicators of OSPW's bioactivity because immune cells are sensitive to, and respond to, various homeostatic changes (reviewed by Taniguchi et al. 2009). In this thesis, I assessed the utility of macrophages, which are important effector cells of the immune system, as sensitive bioindicators to monitor OSPW-mediated effects on immune gene expression profiles following short term exposures.

The mammalian immune response is divided into two major branches: adaptive immunity, and innate immunity. Adaptive immunity is found in all jawed vertebrates. It is characterized by a slower response than that of the innate immune system but is specific to the identified threat and confers immunological memory. Immunological memory is long-lasting immunity to certain pathogens. Once established, it mediates the quick and efficient targeting of previously encountered threats (reviewed by Bartl et al. 2003; reviewed by McCullough and Summerfield 2005). In comparison, innate immunity is ancient and is therefore found throughout the animal kingdom (Bartl et al. 2003). It is characterized by an immediate and general response to threats that does not confer classical immune memory. For the purposes of this study, the

innate immune system, as opposed to the adaptive immune system, is the ideal option for monitoring the effects of OSPW as it mediates primary and rapid responses to homeostatic changes.

The innate immune system also consists of physical barriers, like skin and mucus, but is internally coordinated by a suite of immune cells that contribute to inflammation (McCullough and Summerfield 2005). Inflammation refers to the acute localized tissue response to injury and/or infections, which is primarily mediated via the activation of immune cells that produce a broad array or proinflammatory proteins referred to as cytokines and chemokines. Consequently, inflammation is often localised to the site of injury with the goal of eliminating the threat (e.g. microbes) and then rapidly resolving the inflammatory response (Nathan 2002; Medzhitov 2008).

Should a pathogen overcome the host's physical barriers, the first step of inflammation is pathogen recognition by innate immune cells. Pattern recognition receptors (PRRs) are expressed on the cell surface and they recognize pathogen-associated molecular patterns (PAMPs) that are unique molecular signatures of various pathogens. One major PRR family is the toll-like receptors (TLRs). TLRs are an ancient family of receptors and members can be grouped based on the recognition of similar PAMPs. For example, TLR1, TLR2, and TLR6 all bind to lipids. TLR4 is an exception, however, as it recognizes lipopolysaccharide (LPS) present on *E. coli*, heat shock proteins, and viral and plant molecules, which are structurally distinct. The recognition of PAMPs by PRRs triggers downstream signalling cascades leading to the release of cytokines (reviewed by Akira et al. 2006). Pro-inflammatory cytokines promote the inflammatory response. Specifically, chemokines, a subset of cytokines, mediate the movement

of other immune cells to areas of inflammation in a process known as chemotaxis (Zhang and An 2007). The cytokines examined in this thesis (IP-10, MCP-1, and MIP-2) fall into this category.

Upon recruitment, innate immune effector cells attempt pathogen clearance by mediating antimicrobial functions including phagocytosis and degranulation. Phagocytosis is the receptor-mediated physical engulfment of large insoluble particles by the plasma membrane, which forms an internal envelope called a phagosome. This structure then fuses with lysosomes to aid in the destruction of the internalized particle. The main phagocytic cells of the immune system are macrophages, neutrophils, eosinophils, osteoclasts, dendritic cells, and monocytes (reviewed by Gordon 2016). Another major antimicrobial function called degranulation is the exocytotic release of antimicrobial molecules into the extracellular environment. Within granulocytic cells (neutrophils, mast cells, basophils, and eosinophils), these molecules are contained within specialized secretory vesicles called granules (reviewed by Lacy 2006). Once the pathogen has been contained and/or cleared, inflammation is resolved and PRRs are no longer activated by PAMPs.

A major consequence of inflammation is the generation of apoptotic cells that require clearance. Apoptosis is a tightly regulated process resulting in cell death that is essential to maintaining homeostasis (reviewed by Hengartner 2000). During inflammation, apoptosis is triggered by the infection of cells but also by the phagocytosis of pathogens, which leads to phagocytosis-induced cell death (PICD) as a means of limiting pathogen spread within the host (Fox et al. 2010; Medzhitov 2008). Furthermore, apoptosis is the outcome for immune cells who are no longer required to promote inflammation. For example, neutrophils present at inflammation sites rapidly undergo apoptosis following environmental cues that alter pathways like the extracellular signal-regulated kinase (ERK) pathway. Interestingly, this programmed

immune cell death also contributes to the attenuation of the pro-inflammatory response during the resolution phase. Annexin A1 is secreted by apoptotic neutrophils and prevents further recruitment towards the inflammation site in conjunction with changes to chemokine structures that prevent immune cell migration cues (reviewed by Ortega-Gómez et al. 2013). Cell clearance is mediated by signals to phagocytes which remove apoptosed cells in a process called efferocytosis (reviewed by Doran et al. 2020). Efferocytosis triggers the secretion of anti-inflammatory proteins like transforming growth factor-β1 (TGF-β1), which downregulates pro-inflammatory cytokine synthesis and NO production (Campisi et al. 2014; Ortega-Gómez et al. 2013; Zhang and An 2007). This results in a shift from the secretion of pro-inflammatory molecules to the secretion of anti-inflammatory wound-healing molecules. This change in immune cell phenotype and function is imperative to preventing chronic inflammation and further tissue damage.

If pathogen clearance is not achieved by the innate immune system, some innate immune cells can present antigens on their cell surface to lymphocytes of the adaptive immune system. Broadly, antigens are molecules that can be bound by immune cell receptors or antibodies and illicit an immune response (reviewed by Eter et al. 2000). They are often present on the surface of pathogens and are expressed by antigen-presenting cells (APCs) following phagocytosis. This facilitates immune memory development by the adaptive immune system (reviewed by McCullough and Summerfield 2005).

Macrophages represent an innate immune cell population considered vital for the survival of all animals. These cells are also known to broadly exist in two functional states: as resting/basal or activated/stimulated macrophages. Resting or quiescent macrophages are found in all tissues of the body where they perform various homeostatic roles in development,

metabolism, and tissue maintenance such as clearing cellular debris, and maintaining iron and heme levels (Mosser et al. 2020; Wynn et al. 2013; Soares and Hamza 2016). Upon activation, macrophages undergo a phenotypic change which is dependent on the type of stimulation received; this phenomenon is generally known as polarization (reviewed by Mosser and Edwards 2008; reviewed by Yang and Ming 2014). Classical macrophage activation is the result of pro-inflammatory cytokine recognition, such as TNF-α or IFN-γ, and/or the recognition of a bacterial, fungal, or viral pathogen (via their distinct PAMP signatures). Classically activated macrophages display a pro-inflammatory phenotype and possess microbicidal functions including cytokine and NO secretion, both markers of antimicrobial activity. By contrast, alternatively activated macrophages are polarized towards a wound-healing or tissue repair phenotype and display anti-inflammatory functions in response to cytokines like interleukin 4 (IL-4) as well as certain helminth (parasitic worm) infections (Mosser and Edwards 2008). The dichotomous states and various phenotypes of macrophages are what make them suitable for my thesis work. Specifically, their sensitive and predictable responses serve as effective on/off indicators for OSPW bioactivity, which has previously been reported by our lab using intracellular iNOS protein detection and cytokine secretion assays.

For example, in resting macrophages, Lillico et al. (unpublished data) found that exposure to whole OSPW #2 and its OF, but not whole OSPW #7, increased the number of iNOS positive cells. However, in stimulated macrophages, both whole OSPW #2 and #7 and their fractions significantly decreased the number of iNOS positive cells. Similarly, both OSPW #2 and #7 upregulated MCP-1, MIP-2, and IP-10 basal secretion levels, but following microbial stimulation, only the secretion of IP-10 was inhibited, whereas MCP-1 and MIP-2 secretion levels were unaffected. These observations suggested that OSPW contains bioactive constituents

that can both stimulate resting macrophages and inhibit activated macrophages depending on the pro-inflammatory marker examined.

In this thesis chapter, I examined OSPW-mediated effects on basal and stimulated pro-inflammatory gene expression profiles over time. Specifically, I aimed to establish macrophages as sensitive bioindicators for OSPW exposures at the gene level and to examine the pro-inflammatory gene expression profiles following OSPW exposures. The specific aims of this chapter were: 1) To examine the effects of OSPW #2 and OSPW #7 and their fractions on the basal expression of *inos*, *ip-10*, *mcp-1*, and *mip-2*, and 2) To examine the effects of OSPW #2 and #7 on the stimulated expression of *inos*, *ip-10*, *mcp-1*, and *mip-2*. My data shows that both OSPW #2 and #7 contain bioactive constituents that significantly upregulated basal immune gene expression levels and that this bioactivity can be traced to specific OSPW fractions. Interestingly, stimulated gene expression was primarily inhibited by OSPW #7 except in the case of *mcp-1* where OSPW #2 and #7 resulted in upregulation. Ultimately, I show that macrophages are sensitive bioindicators and, in the case of *mip-2*, display gene upregulation as early as 2 hrs after initial OSPW exposure.

4.2 Results

4.2.1 Basal immune gene expression profiles of mammalian macrophages following OSPW exposures

RAW 264.7 cells were exposed to 50% v/v of whole OSPW #2 and #7 as well as their fractions, or PBS (negative control), for 2, 4, 6, 8, and 10 hrs. All samples were normalized to the PBS control at 2 hrs, and *18s rrna* or *gapdh* were used as endogenous controls. A p-value < 0.05 was used to designate statistical significance between the various experimental groups.

Following OSPW #2 exposures, *inos* was upregulated compared to the control after 4, 6, 8, 10 hrs, with peak expression observed at 6 hrs (19.4-fold change; Fig. 4.1A). OSPW #2 IF exposures induced the expression of *inos* at 2, 4, 6, and 10 hrs but to no greater than a 3-fold change, while exposure to OSPW #2 OF resulted in significant upregulation at 2, 4, 6, and 8 hrs, with the highest fold change of 61.6 observed after 8 hrs (Fig. 4.1B). When compared to each other, OSPW #2 OF exposure caused significantly greater upregulation of *inos* than when the macrophages were exposed to OSPW #2 IF at 2, 4, and 6 hrs; with the differences in fold change ranging from 4.6 at 2 hrs to 28.5 at 4 hrs. *inos* only reached a significant 2.5-fold change at 10 hrs compared to the control after exposure to OSPW #7 and OSPW #7 OF did not significantly affect *inos* levels. Compared to the control, OSPW #7 IF exposure caused low (no greater than a 3.2-foldchange), yet significant *inos* induction at 2, 4, 6, and 10 hrs and significantly greater expression compared to OSPW #7 OF at all time points except 2 hrs, with the greatest difference being 2-fold at 6 hrs (Fig. 4.1C).

Compared to the control, *ip-10* levels were significantly upregulated following exposure to OSPW #2 at 2, 4, 6, and 8 hrs with fold changes of 67.2, 129, 148.6, 73.9, respectively (Fig. 4.2A). Both OSPW #2 IF and OF exposures induced significant upregulation at all time points compared to the control. *ip-10* had significantly greater expression levels following OSPW #2 OF exposures, which varied between a 131 and a 572.3-fold change, compared to OSPW #2 IF, which only ranged between a 6.4 and 18.8-fold change (Fig. 4.2B). The greatest difference between OSPW #2 OF and IF exposure-induced changes in *ip-10* was 556.5-fold at 6 hrs (Fig. 4.2B). *ip-10* levels showed significant and sustained upregulation following OSPW #7 exposure across all time points examined, peaking at a 69.6-fold change after 4 hrs (Fig. 4.2A). Relative to the control, OSPW #7 IF and OF exposures induced *ip-10* expression after 2, 4, 6, and 10 hrs,

however the upregulated expression levels following OPSW #7 IF exposure was significantly greater than OSPW #7 OF at 2, 4, and 6 hrs with fold differences of 16.1, 48.2, 57.1, respectively (Fig. 4.2C).

Following exposure to OSPW #2, *mcp-1* was significantly upregulated where it peaked after 10 hrs with a 37-fold increase (Fig. 4.3A). This observed upregulation was also occurred after exposure to both the IF and OF of OSPW #2, however the IF exposure did not induce a fold change greater than 20.6 after 10 hrs while the OF reached a 201-fold change after 8 hrs (Fig. 4.3B). OSPW #7 exposure also upregulated *mcp-1* expression, which reached a maximum fold change of 40.9 at 10 hrs (Fig. 4.3A). OSPW #7 OF exposure significantly upregulated the expression of *mcp-1* across all time points with the greatest upregulation occurring after 2 hrs with a 7.4 RQ and the lowest after 10 hrs with an RQ of 1.8 (Fig. 4.3C). *mcp-1* was upregulated post OSPW #7 IF exposure at 2, 4, 6, and 10 hrs with expression ranging from 21.5 at 2 hrs to 56.4 at 6 hrs at which time its expression was also significantly greater than its expression after OSPW #7 OF exposure (Fig. 4.3C). The greatest disparity between OSPW #7 OF and IF occurred at 6 hrs with an RQ difference of 51.5 (Fig. 4.3C).

Following exposure of the cells to OSPW #2, *mip-2* was significantly upregulated at all times examined and peaked at 2 hrs before drastically reducing its expression over time to 23.5 RQ at 10 hrs (Fig. 4.4A). OSPW #2 IF and OF exposures also induced the upregulation of *mip-2*. Specifically, OSPW #2 IF exposure upregulated *mip-2* by 84.5 RQ at 2 hrs before it progressively decreased over time (Fig. 4.4B). OSPW #2 OF exposure induced a 1217-fold change after 2 hrs before the observed expression also decreasing to 523.2 RQ at 10 hrs (Fig. 4.4B). *mip-2* expression post OSPW #2 OF exposure was significantly greater than after OSPW #2 IF exposure, with the greatest difference observed at 2 hrs with a fold difference of 1132.5.

Mip-2 was significantly upregulated after treatment with OSPW #7, except at 10 hrs, where expression peaked at 2 hrs with an 83.1-fold change (Fig. 4.4A). OSPW #7 IF and OF exposures significantly upregulated *mip-2* expression, and at 2, 4, and 6 hrs the IF exposure showed greater expression than following exposure to the OF. Finally, the IF-induced *mip-2* expression peaked at 348.3 RQ after 2 hrs where it was 307.5 RQ greater than *mip-2* expression following the OF exposure (Fig. 4.4C).

4.2.2 Stimulated immune gene expression profiles of mammalian macrophages following OSPW exposures

To examine the effects of OSPW exposures on stimulated macrophage immune gene expression, cells were exposed to 50% v/v of OSPW (or PBS) for 2 hrs before they were stimulated with heat-killed *E. coli* and then continuously incubated with OSPW (or PBS) and bacteria for the duration of the experiment. PBS (50% v/v) treatment represented the control group and changes in gene expression were relative to the levels observed after the 2 hr pre-exposure to PBS. All time points described below refer to the time after the initial OSPW exposure (i.e. 2 hrs before bacterial stimulation) and a p-value < 0.05 was used to designate statistical significance between the various experimental groups.

As observed previously, exposure to either whole OSPW #2 or #7 significantly upregulated *ip-10*, *mcp-1*, and *mip-2* expression levels at 2 hrs compared to the control. However, in contrast to Figure 4.1A, *inos* expression was upregulated after 2hrs during these exposures (Fig. 4.5A).

After 6, 8, and 10 hrs, the control gene expression was upregulated due to bacterial stimulation of macrophages that resulted in pro-inflammatory gene expression. Comparatively, OSPW #7 exposure significantly downregulated bacterial-stimulated *inos* expression levels by

37.8, 111.1, and 85.2 RQ, respectively (Fig. 4.5A). Similarly, *ip-10* expression was significantly inhibited after exposure to whole OSPW #7 at 6 and 8 hrs by a 251 and 95.1-fold, respectively, when compared with the control (Fig. 4.5B). At 10 hrs, OSPW #2 significantly downregulated *mip-2* expression by a 2138.7-fold change while OSPW #7 downregulated *mip-2* at 8 and 10 hrs by 1302.6 and 1771 RQ, respectively (Figure 4.5D). Conversely, stimulated expression of *mcp-1* gene expression was not inhibited following exposure to either OSPW #2 or OSPW #7. In fact, whole OSPW #2 and #7 exposures significantly upregulated the expression of *mcp-1* after 6 and 8 hrs compared to the control, with the greatest upregulation for both observed at 6 hrs with increases of 462 and 227 RQ, respectively (Fig. 4.5C).

4.3 Discussion

In this chapter, I further investigated the use of macrophages as sensitive bioindicators of OSPW bioactivity using gene expression analysis at early time points. This was performed by examining changes in the resting and activated states of macrophages following exposure to different OSPW samples. Overall, pro-inflammatory gene expression endpoints served as potential early biomarkers following OSPW exposures. Using gene expression quantification, I have demonstrated that macrophages generate unique responses to various OSPW samples at early time points and I propose their potential use as an OSPW biomonitoring tool.

Resting macrophages are primarily responsible for clearing cellular debris and they do not express antimicrobial or pro-inflammatory markers (Mosser and Edwards 2008). However, like the previously observed protein-level results by Lillico et al. (unpublished data), I observed OSPW-mediated stimulation of macrophages. My results showed that certain pro-inflammatory genes were upregulated in resting macrophages following OSPW exposure suggesting that whole

OSPW #2 and OSPW #7 contain bioactive constituents that trigger macrophage activation. The level of bioactivity observed was dependent not only on the whole water and fraction tested, but also on the gene examined. For instance, the expression of *inos* was upregulated by whole OSPW #2 and this bioactivity, although statistically significant in both fractions, largely segregated to the OF, suggesting that organic constituents are more bioactive than the inorganic components within this water. Interestingly, whole OSPW #7 did not significantly upregulate *inos,* however its IF displayed small, yet significant, bioactivity. By comparison, both whole waters displayed bioactivity by upregulating *ip-10*, *mcp-1*, and *mip-2*. Like *inos*, *ip-10* and *mip-2* upregulation suggests that OSPW #2-mediated bioactivity is largely attributed to the OF, while OSPW #7-mediated bioactivity is attributed to its IF. The induction of *mcp-1* gene expression after OSPW #2 OF exposure trended to be larger than what I observed following OSPW #2 IF exposure, but this was not statistically significant. OSPW #7 IF exposure induced greater expression of *mcp-1* compared to OSPW #7 OF. Overall, OSPW #2 and OSPW #7 contain bioactive constituents that activated resting macrophages and these constituents segregated to the organic and inorganic fractions of these waters, respectively.

By contrast, the bacterial-stimulated expression of *inos*, *ip-10*, and *mip-2* was significantly downregulated following exposure to whole OSPW #7. The downregulation of *mip-2* contradicts earlier observations at the functional level that demonstrated that neither OSPW #2 nor #7 influenced protein levels compared to untreated cells when macrophages were stimulated. Likewise, OSPW #2 lacks the inhibitory effects observed at the functional level when compared to the expression of *inos* and *ip-10*, but this difference is likely remedied by the extensive regulation of protein synthesis in eukaryotic cells (Rhoads 1993). Nevertheless, my results do suggest that whole OSPW #7 has significant inhibitory effects on the *inos*, *ip-10*, and *mip-2*

pathways at the level of transcription. Interestingly, *mcp-1* was an exception as its bacterial-stimulated expression was upregulated following OSPW #2 and OSPW #7 exposures. Ultimately, OPSW-mediates perturbations to normal pro-inflammatory gene expression and this is depending on the macrophage activation state and the gene examined.

4.3.1 Bioactivity

OSPW-mediated activation of resting macrophages and the observed inhibition of stimulated gene expression suggests that OSPW bioactivity may be due to agonistic and antagonistic interactions of certain constituents with macrophage receptors that cause perturbations to normal pro-inflammatory gene expression. Effectors that mediate the expression of genes examined herein include TLRs, nuclear factor-κβ (NF-κβ), and TNF receptors, among others, that are possible candidates responsible for triggering OSPW-mediated bioactivity (reviewed by Ley et al. 2016).

Discrete changes in pro-inflammatory gene expression in resting macrophages following acute exposures to OSPW suggests that constituents within these waters can activate specific antimicrobial pathways. Others have also reported similar bioactivity in unstimulated cells. For example, human trophoblast cells expressed *il-1β* following exposure to a NA mixture, and changes to the expression of the inflammatory genes *il-1*, *il-2* and *il-6* in OSPW-exposed trout leukocytes was documented by Gagné et al. (2017). Alteration of the expression of the AhR (binds to planar aromatic hydrocarbons), which has a role in regulating immunity, was also documented by Gagné et al. (2017), suggesting that it may be an OSPW target causing alterations of immune cell responses (reviewed by Gutiérrez-Vázquez and Quintana 2018). In general, ligand mimicry may be considered as a potential method of action by OSPW. Feng et al.

(2019) demonstrated this potential mode of action via the creation of a TLR2 agonist mimic that replicates the pro-inflammatory action of its classical ligands: lipoproteins common to bacterial membranes (reviewed by Oliveira-Nascimento et al. 2012).

Alternatively, the stimulated inhibition of *inos*, *ip-10*, and *mip-2* may be due to competitive and/or non-competitive inhibition mechanisms. Fu et al. (2017) also observed the downregulation of *ip-10* in stimulated RAW 264.7 cells, however this inhibitory outcome was not replicated by Phillips et al. (2020) in a study which primarily observed the stimulated upregulation of pro-inflammatory genes. This discrepancy may be attributed to two main factors: 1) the differences in the immune gene profiles examined, and 2) the unique composition of the different OSPW samples tested. Accordingly, we see an overall lack of investigation into the mechanistic action of OSPW because these waters are unique and complex, and standardized endpoints have yet to be established in the field of environmental toxicology for the examination of OSPW, specifically.

As mentioned previously, relationships between OSPW constituents and receptors are not well studied, especially as they pertain to innate immunity, however research in other biological systems has yielded interesting results. For example, using murine preadipocyte fibroblasts, OSPW exposure caused agonistic behaviour by activating the peroxisome proliferator-activated receptor γ (PPARγ), which resulted in adipogenesis (Peng et al. 2016). Adipogenesis is the differentiation of progenitor cells into triglyceride-containing adipocytes that store energy and are colloquially referred to as fat cells (reviewed by Ghaben and Scherer 2019). Through a series of pull-down assays and mass spectrometry (MS), OSPW ligands for PPARγ were identified as hydroxylated/polyoxygenated carboxylic acids and hydroxylated/native

sulfates (Peng et al. 2016). Furthermore, NAs were identified as estrogen receptor agonists while PAHs showed antagonistic activity towards androgen receptors in yeast (Thomas et al. 2009).

It should also be noted that despite OSPW samples being filtered prior to exposing the cells (to eliminate the presence of whole microorganisms that may be present in the waters), this level of filtering does not eliminate smaller/soluble microbial products or components. For example, ligands from bacteria such as LPS, peptidoglycan, and flagellin act as agonists by activating macrophage receptors, which result in pro-inflammatory responses (O'riordan et al. 2002). Conversely, bacterial components can mediate an anti-inflammatory response, which was observed by Quévrain et al. (2016) whereby *Faecalibacterium prausnitzii*'s microbial anti-inflammatory molecule (MAM) inhibited NF-κβ dose dependently in epithelial cells. Thus, the potential agonists and antagonists found in OSPW may not exclusively be by-products of bitumen extraction but instead could also be derived from microbial communities. An additional level of complexity are the relatively unknown interactions that may occur between inorganic and organic substance present at variable levels in different OSPW samples.

4.3.2 Bioactivity of OSPW organic and inorganic fractions

OSPW #2 fractions were both bioactive and statistically different from the control, however OSPW #2-mediated bioactivity significantly segregated to the OF for all the genes except *mcp-1*. Bioactivity from the OF is expected as OSPW effects have primarily been attributed to organic constituents (Li et al. 2017). For example, whole OSPW, NAs, and PAHs were found to perturb the immune response of leukocytes, and in particular, immune gene expression (Gagné et al. 2017). Similarly, commercial NAs increased the expression of inflammation markers like IL-1β (Raez-Villanueva et al. 2019). Although both Gagné et al.

(2017) and Raez-Villanueva et al. (2019) recorded changes in gene expression due to organic constituents of OSPW, namely NAs, OSPW is a complex mixture that is unique to its collection site based on several factors like age, weathering, and processing method; as such, commercial mixtures may not always depict accurate OSPW effects.

In comparison to the observed induction of basal gene expression from OSPW #2 fractions, OSPW #7 IF had significantly greater bioactivity than OSPW #7 OF at most time points examined. The organic components of OSPW are thought to be the major contributors to toxicity and as a result, few studies have examined the potential role of the IF. Nevertheless, both Fu et al. (2017) and Phillips et al. (2020) conclude that the IF contributes to OSPW toxicity based on interactions with the OF constituents and that these interactions may also mediate the polarization of RAW 264.7 macrophages towards a healing (i.e. non-inflammatory) phenotype.

Alterations of ion-driven mechanisms by inorganic OSPW constituents may contribute to the activation of, or the inhibition of, macrophage functions observed in this study. For instance, high concentrations of sodium in the extracellular environment can stimulate macrophages towards their pro-inflammatory phenotype. (reviewed by Kirabo 2017; Müller et al. 2019). Specifically, macrophages exposed to sodium had greater expression of iNOS through nuclear factor of activated T cell 5 (NFAT5) activation. In agreement, Jantsch et al. (2015) observed that mice with high salt diets accumulated a higher concentration of cutaneous sodium which activated macrophages and resulted in better control of *Leishmania major* infections. In contrast, Di et al. (2018) showed that inhibition of the potassium efflux channel, TWIK2, prevented activation of macrophages. Therefore, increases in ions in the extracellular environment do have the potential to activate and/or inhibit macrophages and, if not ions themselves, changes to ionic mechanisms could lead to these differential effects.

Various metals have been identified in OSPW including titanium, iron, lithium, aluminum, boron, and strontium (Mahaffey and Dubé 2017; Harkness et al. 2018). These metals have varying, and often conflicting, effects on inflammation and macrophages, but they may contribute to the observed effects of the IF reported in this thesis. For example, titanium nanoparticles upregulate pro-inflammatory markers including iNOS, TNF-α, and extracellular ROS in murine macrophages (Scherbart et al. 2011). Similarly, chronic ingestion of these nanoparticles by mice reduced the number of immune effector cells, including macrophages, which was associated with chronic inflammation (Mu et al. 2019). Likewise, iron oxide nanoparticles were shown to mediate the polarization of macrophages to their pro-inflammatory phenotype (Zanganeh et al. 2016). Agoro et al. (2018) also observed that iron influenced the polarization of macrophages but as an antagonist to the pro-inflammatory phenotype. They demonstrated that iron-loaded basal macrophages displayed fewer pro-inflammatory co-stimulatory molecules and stimulated macrophages had a reduced pro-inflammatory response. In rats, lithium increased free radicals like NO, which in turn upregulated the expression of IL-6, but this was not replicated *in vitro* where Makola et al. (2020) observed the attenuation of the pro-inflammatory response by lithium in activated RAW 264.7 cells (Mezni et al. 2017; Makola et al. 2020). Likewise, strontium also downregulated pro-inflammatory cytokine expression in RAW 264.7 macrophages and this was hypothesized to occur through the NF-κβ pathway (Zhu et al. 2016). When treated with LPS, increasing doses of aluminum and boron increased the expression of IL-8 and IL-1β in mouse cell line epithelial cells, and iNOS and pro-inflammatory cytokines in mouse peritoneal macrophages, respectively (De Chambrun et al. 2014; Routray and Ali 2016). Consequently, the role of metals in OSPW-mediated bioactivity is not easily inferred and unsurprisingly, examination of their effects on basal versus stimulated cells does generate

conflicting results akin to what I observed between the whole water exposed basal and stimulated macrophages (i.e. the basal upregulation, and the stimulated inhibition of the immune genes examined in this study).

Although analyses have identified several metals common to OSPW, their roles within these complex mixtures are not well-studied and this presents a sizeable gap in our understanding of OSPW toxicity. Extensive examination of the effects of OSPW-mediated organic constituents has skewed the discourse surrounding the main proponents of bioactivity in these waters and neglects the roles of inorganic factors. Going forward, more emphasis should be placed on examining the effects of, and interactions between, inorganic constituents within the context of OSPW at environmentally relevant concentrations.

4.3.3 Bioindication

Regardless of the mode of action behind the observed bioactivity, this chapter of my thesis established that macrophages are sensitive bioindicators in response to acute and sub-lethal OSPW exposures. Previous research from our lab also used macrophages as functional bioindicators but used samples collected 24 hrs after OSPW exposures. Thus, shifting towards a gene-based bioassay was beneficial as exposure times were significantly shortened (i.e. 2-10 hrs), however the sensitivity of macrophages to OSPW at shorter time intervals was unknown. Moreover, it was unknown whether OSPW exposure caused effects at the gene level and if they did, would a qPCR assay be sensitive enough to detect these changes. Fortunately, I observed changes in gene expression as early as 2 hrs using a qPCR-based assay. Therefore, using OSPW-exposed RAW 264.7 macrophages and a qPCR-based assessment of their immune gene profiles

verifies that macrophages do indeed provide an *in vitro* system that can generate unique water profiles for monitoring the effects of OSPW on pro-inflammatory gene expression.

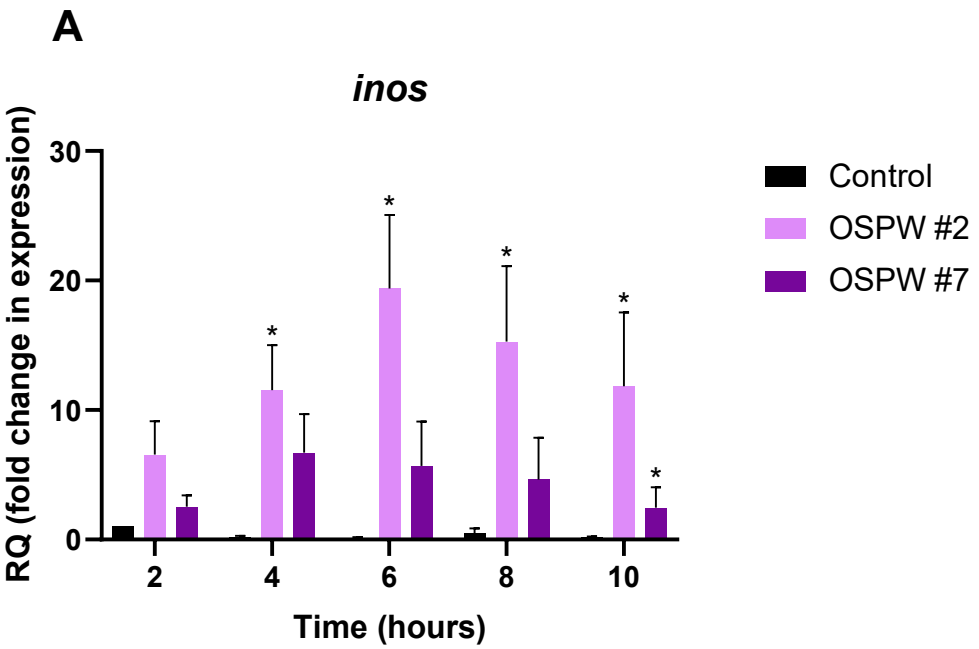
It is important to note, however, that the development and use of this *in vitro* bioassay does not translate to biological significance. Although this system can be used to effectively monitor OSPW samples, these results should not be extrapolated to whole organism nor environmental levels. Not only does *in vitro* cell-line work neglect the dynamic interactions between cells and whole biological systems, but alterations to gene expression does not directly translate to a consequential functional response. Regardless, immune gene profiling does allow for direct comparisons between various OPSW samples and their fractions following sub-lethal and acute OPSW exposures.

4.3.4 Future directions

This immune cell-based bioassay should be expanded to encompass a larger suite of pro-inflammatory genes. This is imperative as I observed differential changes in expression depending on the immune gene examined. Observing different results between different endpoints suggests that only monitoring a few genes for bioactivity provides a narrow view of these xenobiotic effects, which may lead to uninformed conclusions. Therefore, when examining OSPW-mediated effects on the innate immune response, a more accurate assessment of bioactivity can be determined from a broader scope of immune genes profiled. Such genes could include other pro-inflammatory markers like TNF-α, IL-1β, or IL-6. Additionally, examining additional OSPW sources and pairing their bioactivity profiles with constituent analyses may aide in identifying constituents of concern beyond just bioactive fractions.

Another avenue of research based on the results of this thesis chapter would be further examination of the effects of OSPW on stimulated macrophages. I only examined the effects of whole OSPW. Examination of the bioactivity of the OF and the IF would confirm whether inhibitory effects of OSPW also segregate to specific fractions and if this corresponds with the segregation of bioactivity in resting macrophages.

Lastly, and deviating from the goal of biomonitoring, identifying pathways through which OSPW-mediated bioactivity is acting may also be an avenue to parse out constituents of concern. One strategy would be to examine the expression of pro-inflammatory receptor genes. By identifying activated receptors, potential agonists or antagonists could be determined based on similar ligand binding structures or through a pull-down assay methodology as described by Peng et al. (2016). Likewise, in the case of OF-mediated bioactivity, ascertaining the role of microbial components would aide in developing better targeted remediation strategies that would not only aim to reduce bitumen processing-derived constituent toxicity, but the toxicity of microbial products.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30
20
10
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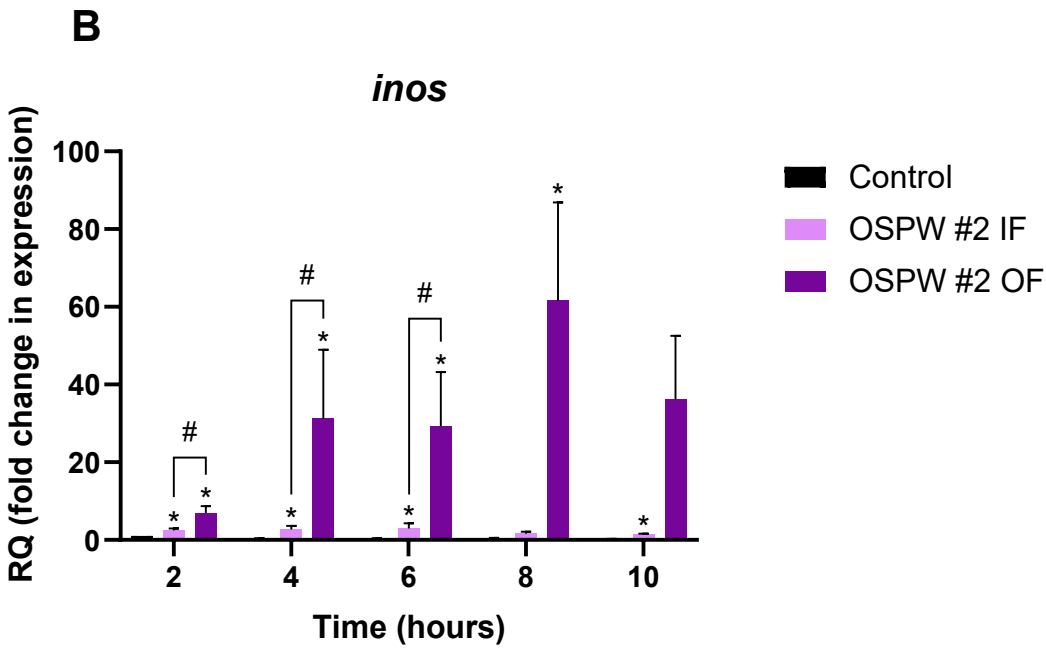
2
4
6
8
10
Time (hours)
Control
OSPW #2
OSPW #7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OSPW #2 OF

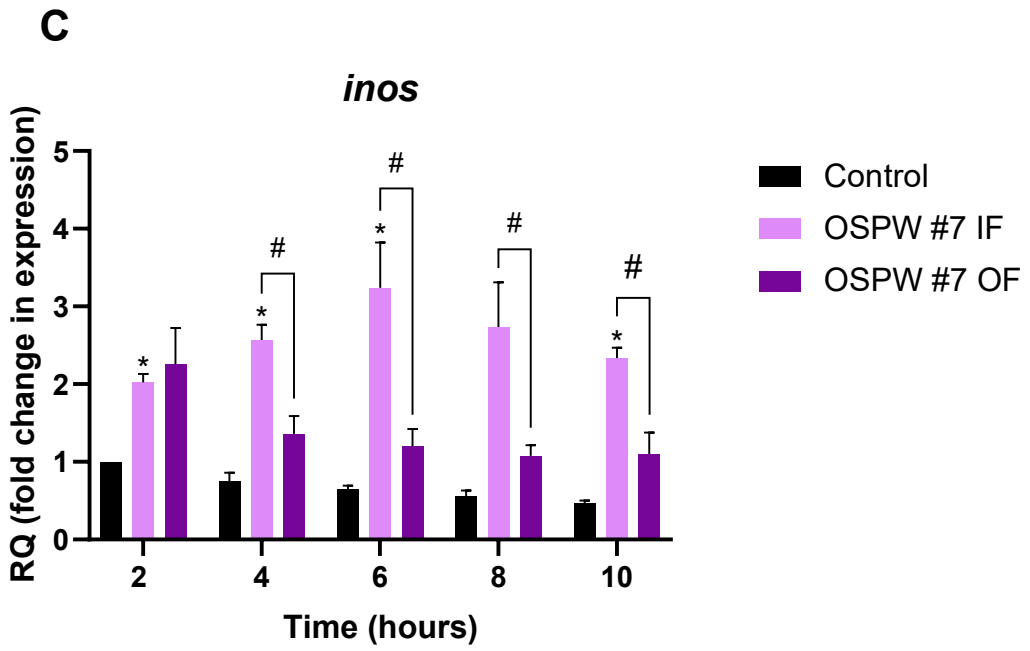
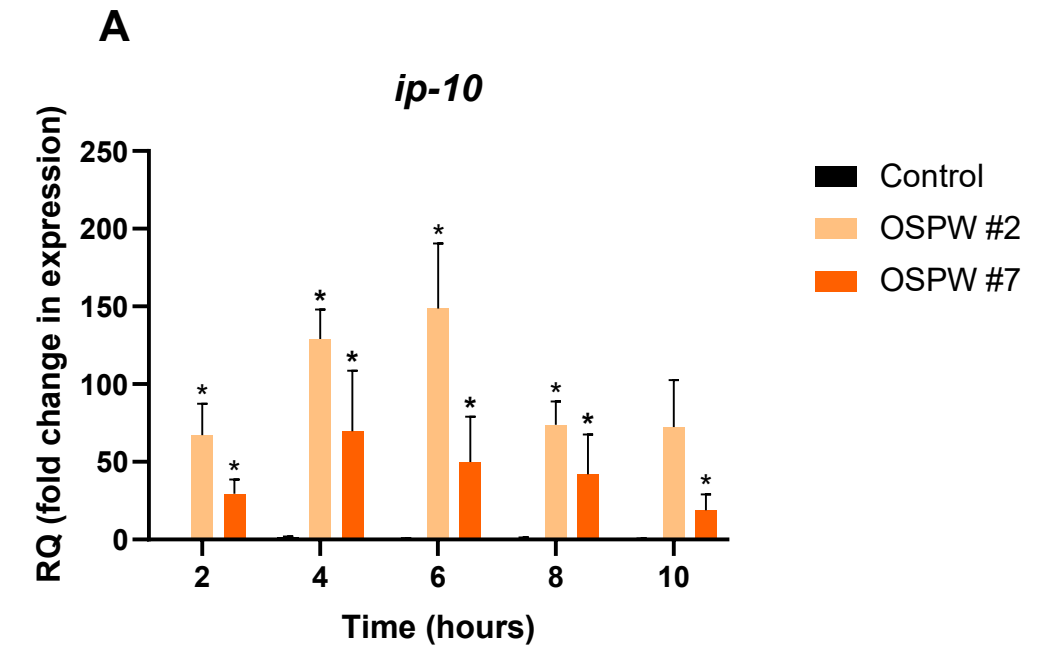


Figure 4.1 RAW 264.7 *inos* expression following OSPW exposure. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500µL of 50% v/v OSPW #2 or #7 (A; n=4), OSPW #2's IF or OF (B; n=4), and OSPW #7's IF or OF (C; n=3) then incubated at 37°C. RNA extraction occurred after 2, 4-, 6-, 8-, and 10-hrs following exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qPCR. Results are expressed as a fold change in gene expression compared to the endogenous controls, *18s rrna* A), or *gapdh* (B, C), and normalized to Control (PBS 50% v/v) at 2 hrs. Bars represent mean + SEM (n=4). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * represent significance ($p < 0.05$) of a treatment compared to Control at a given time point. # represent significance ($p < 0.05$) between treatments at a given timepoint.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ip-10
RQ (fold change in expression)
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200
150
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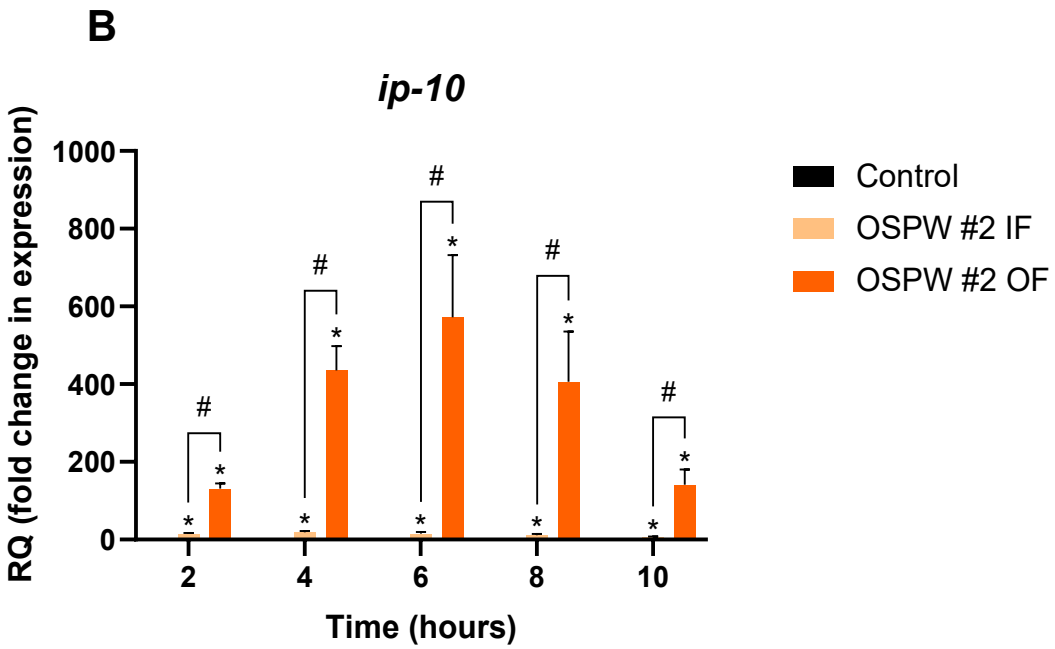
2
4
6
8
10
Time (hours)
Control
OSPW #2
OSPW #7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2
4
6
8
10
Time (hours)
Control
OSPW #2 IF
OSPW #2 OF

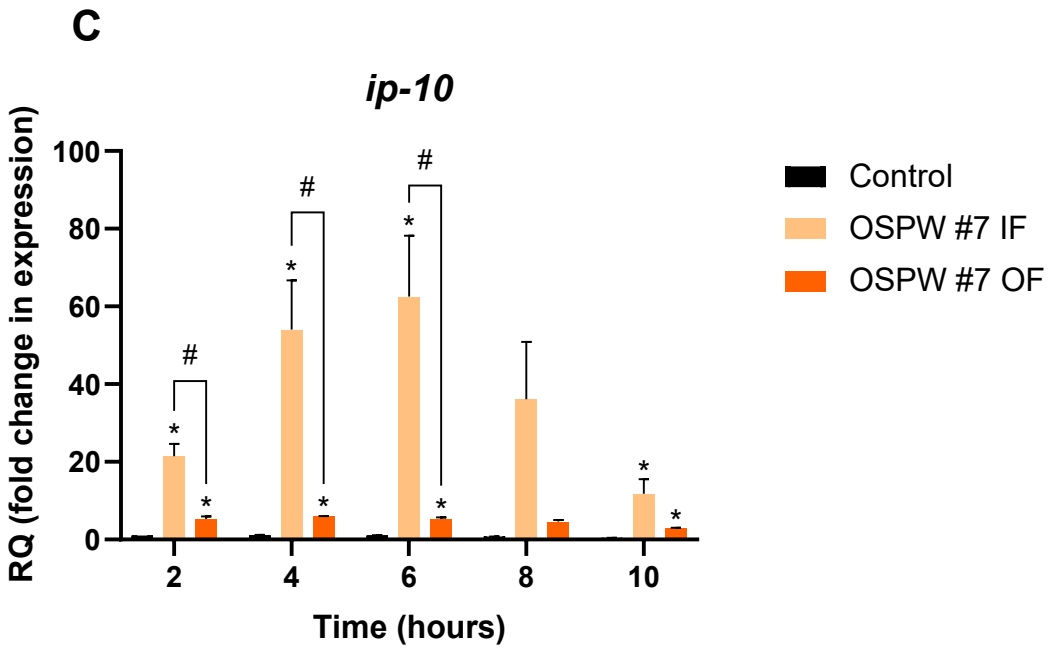
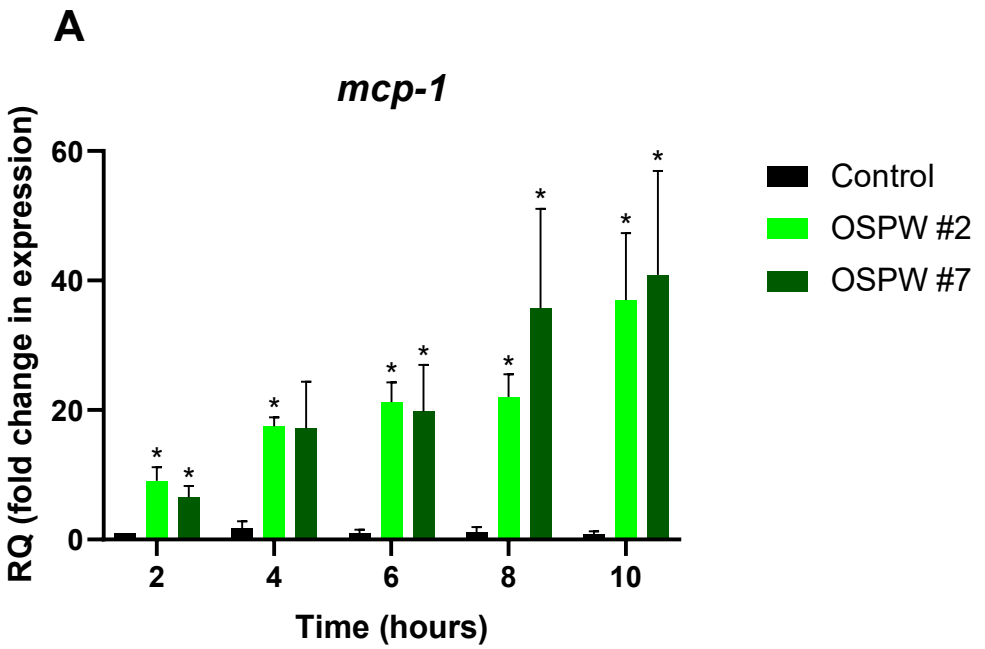


Figure 4.2 RAW 264.7 *ip-10* expression following OSPW exposure. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500µL of 50% v/v OSPW #2 or #7 (A; n=4), OSPW #2's IF or OF (B; n=4), and OSPW #7's IF or OF (C; n=3) then incubated at 37°C. RNA extraction occurred after 2, 4-, 6-, 8-, and 10-hrs following exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qpCR. Results are expressed as a fold change in gene expression compared to the endogenous controls, *18s rrna* (A) or *gapdh* (B, C), and normalized to Control (PBS 50% v/v) at 2 hrs. Bars represent mean + SEM (n=4). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * represent significance ($p < 0.05$) of a treatment compared to Control at a given time point. # represent significance ($p < 0.05$) between treatments at a given timepoint.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mcp-1
RQ (fold change in expression)
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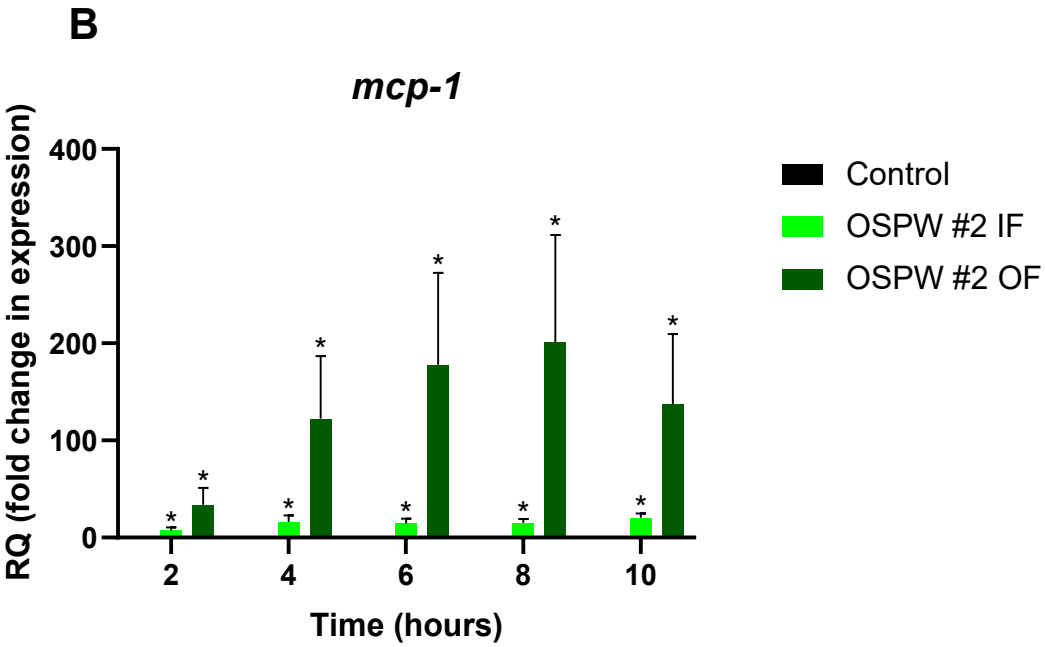
40
20
0
2
4
6
8
10
Time (hours)
Control
OSPW #2
OSPW #7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OSPW #2 OF

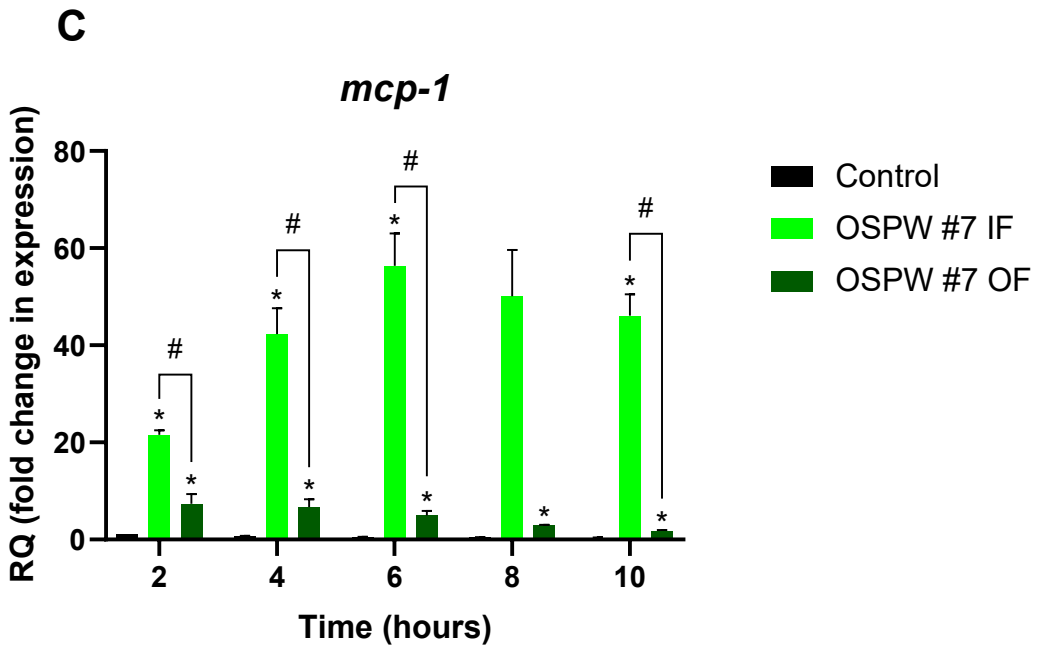
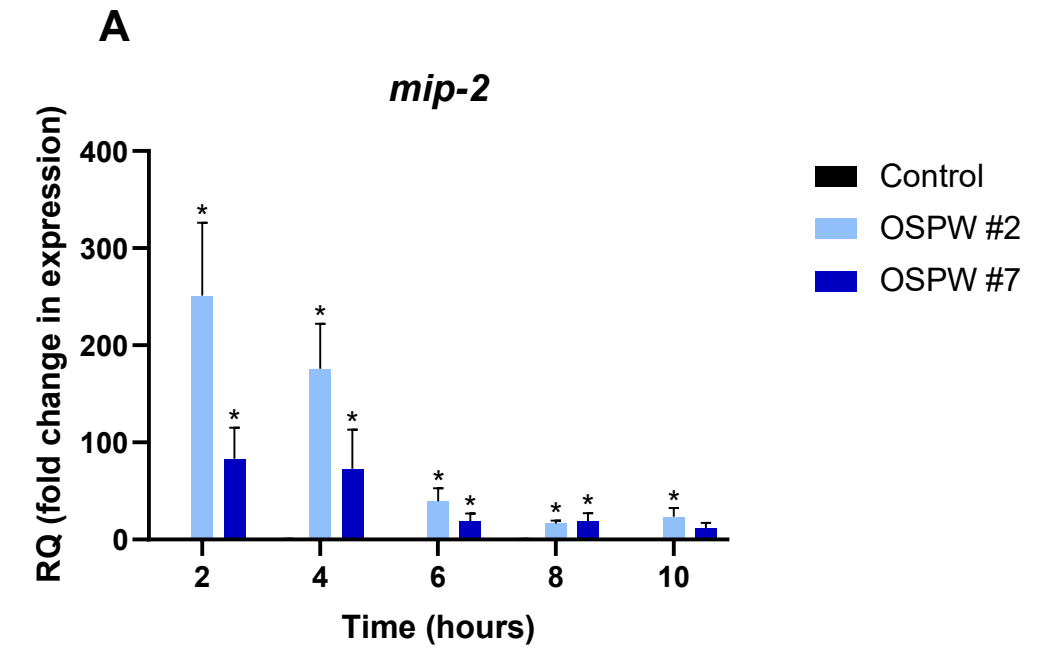


Figure 4.3 RAW 264.7 *mcp-1* expression following OSPW exposure. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500µL of 50% v/v OSPW #2 or #7 (A; n=4), OSPW #2's IF or OF (B; n=4), and OSPW #7's IF or OF (C; n=3) then incubated at 37°C. RNA extraction occurred after 2, 4-, 6-, 8-, and 10-hrs following exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qpCR. Results are expressed as a fold change in gene expression compared to the endogenous controls, *18s rrna* (A) or *gapdh* (B, C), and normalized to Control (PBS 50% v/v) at 2 hrs. Bars represent mean + SEM (n=4). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * represent significance ($p < 0.05$) of a treatment compared to Control at a given time point. # represent significance ($p < 0.05$) between treatments at a given timepoint.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mip-2
RQ (fold change in expression)
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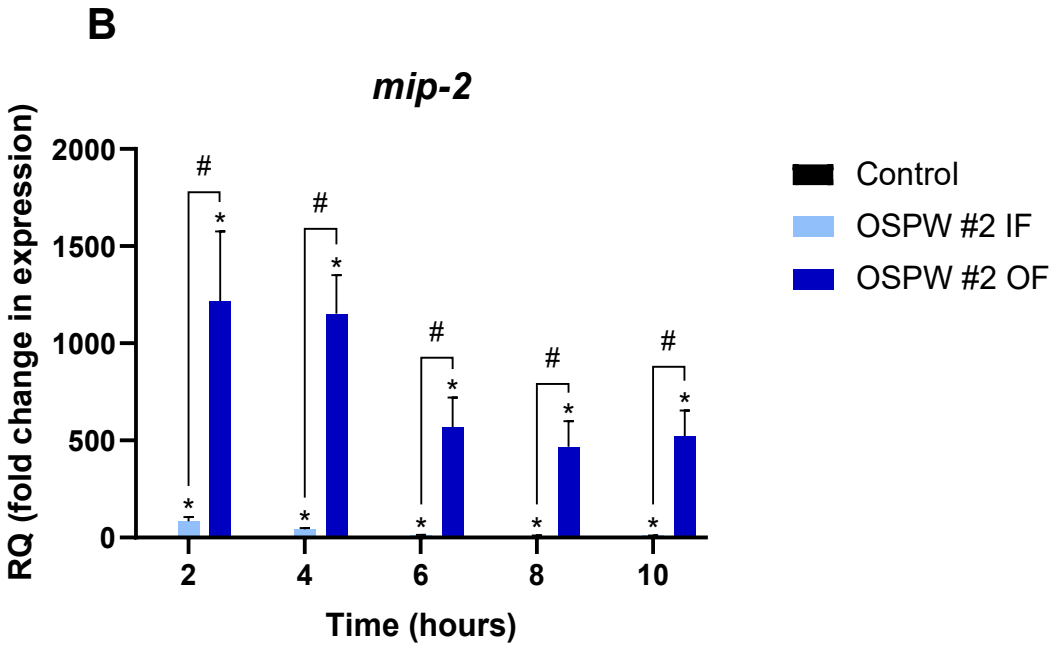
Time (hours)
Control
OSPW #2
OSPW #7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RQ (fold change in expression)
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4
6
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10
Time (hours)
Control
OSPW #2 IF
OSPW #2 OF

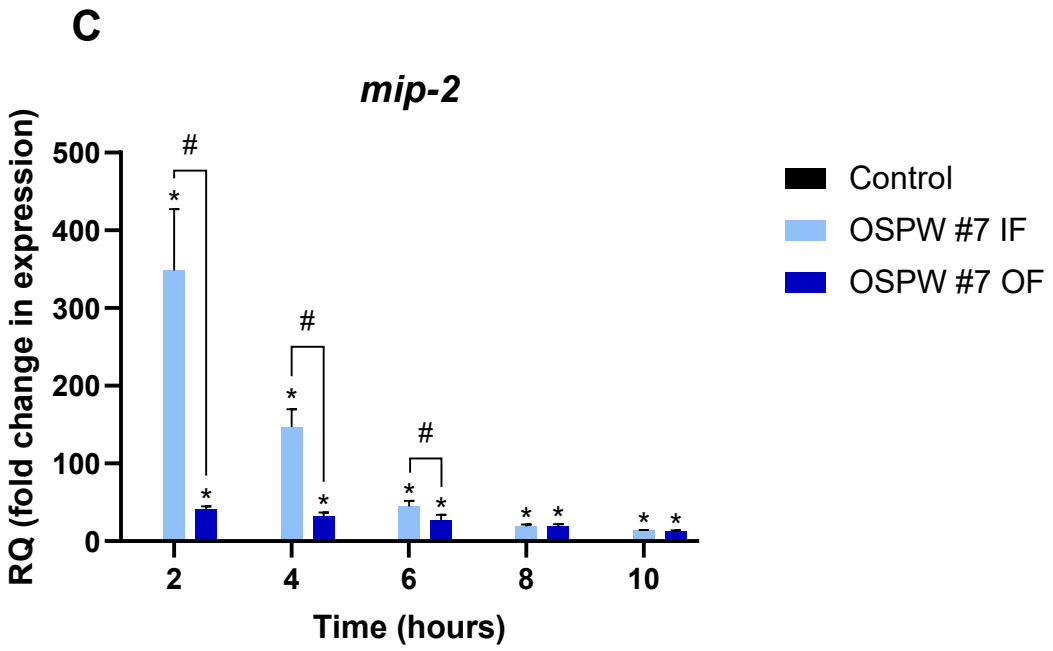
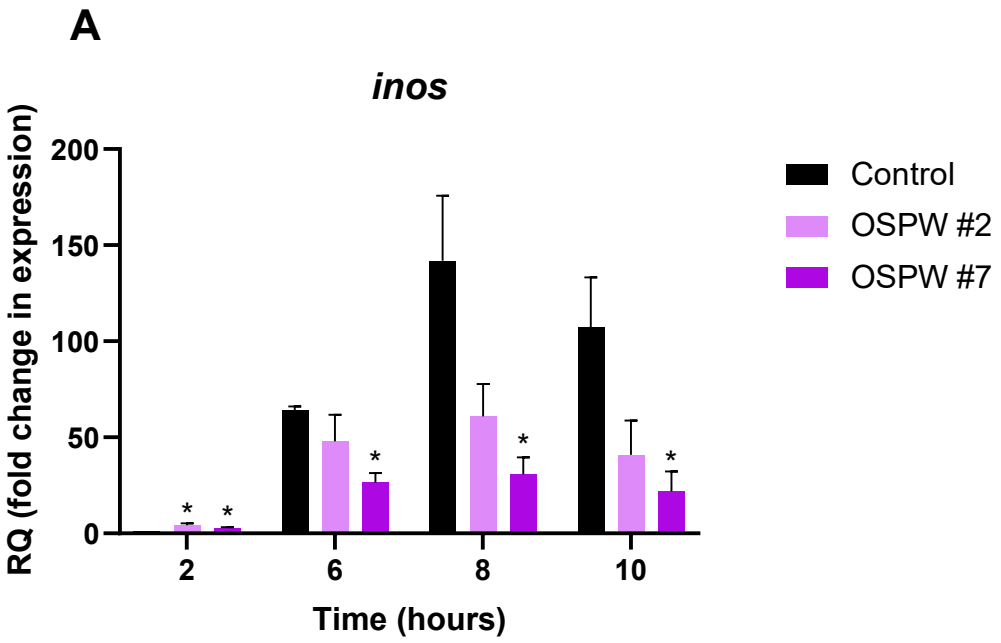


Figure 4.4 RAW 264.7 *mip-2* expression following OSPW exposure. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500µL of 50% v/v OSPW #2 or #7 (A; n=4), OSPW #2's IF or OF (B; n=4), and OSPW #7's IF or OF (C; n=3) then incubated at 37°C. RNA extraction occurred after 2, 4-, 6-, 8-, and 10-hrs following exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qpCR. Results are expressed as a fold change in gene expression compared to the endogenous controls, *18s rrna* (A) or *gapdh* (B, C), and normalized to Control (PBS 50% v/v) at 2 hrs. Bars represent mean + SEM (n=4). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * represent significance ($p < 0.05$) of a treatment compared to Control at a given time point. # represent significance ($p < 0.05$) between treatments at a given timepoint.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RQ (fold change in expression)
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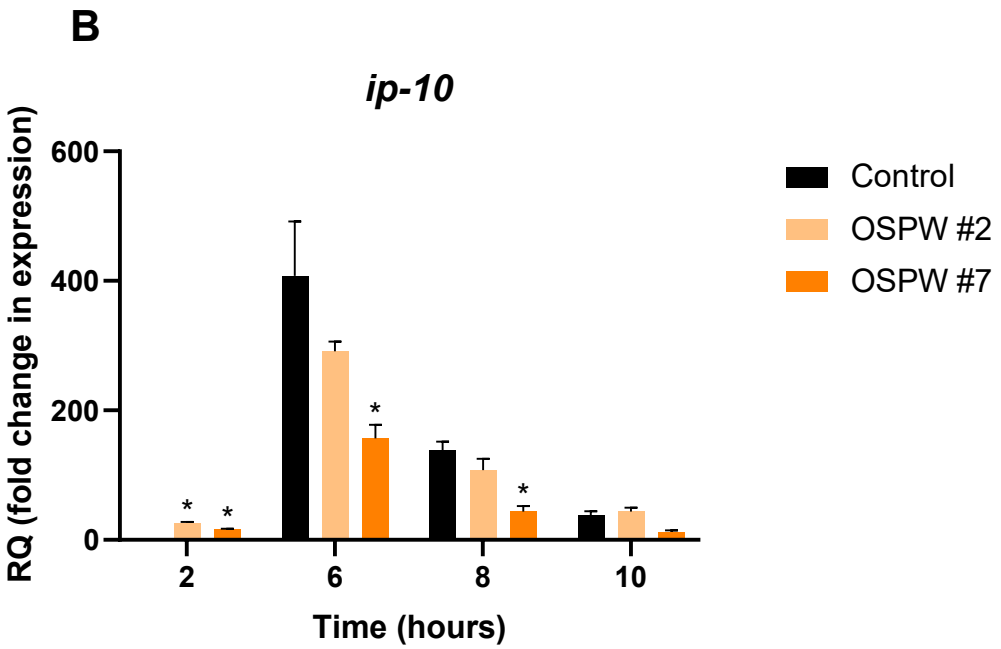
Time (hours)
Control
OSPW #2
OSPW #7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RQ (fold change in expression)
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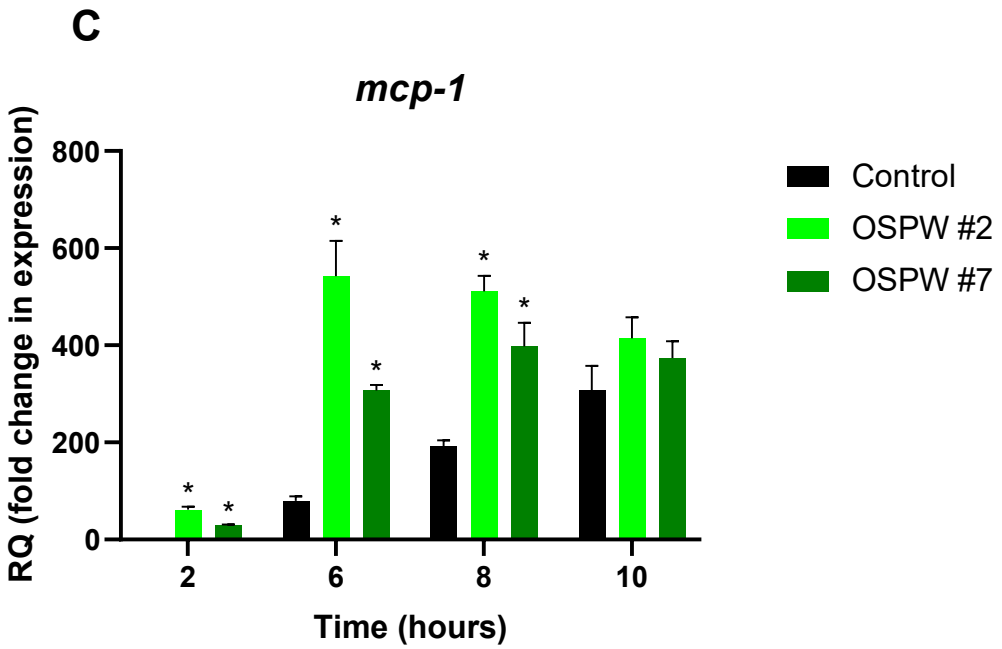
Time (hours)
Control
OSPW #2
OSPW #7


C
mcp-1
RQ (fold change in expression)
800
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Time (hours)
Control
OSPW #2
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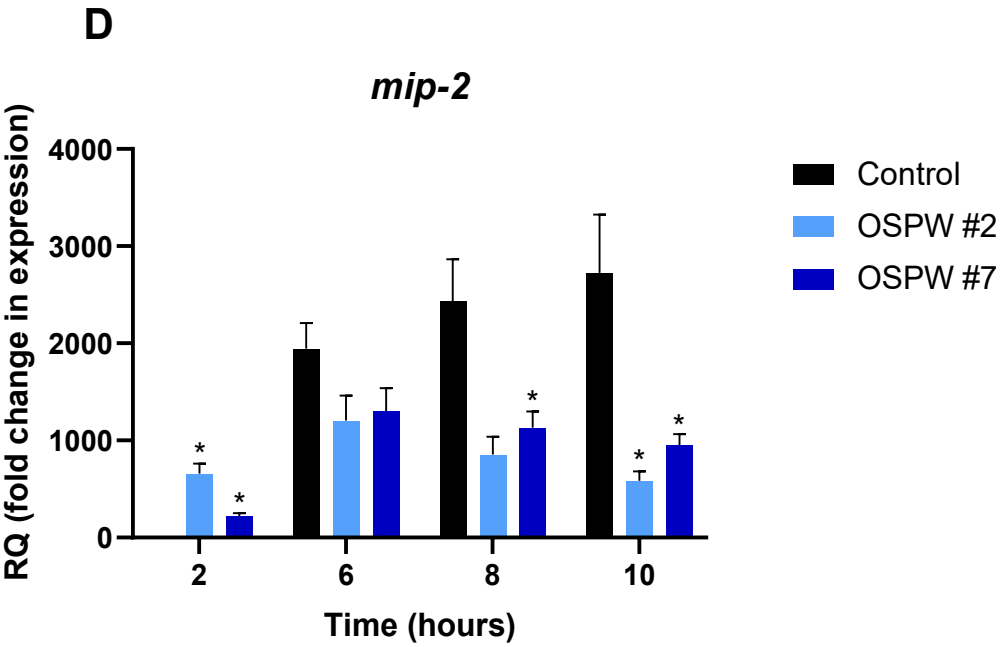
D
mip-2
RQ (fold change in expression)
4000
3000
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Time (hours)
Control
OSPW #2
OSPW #7

Figure 4.5 Pro-inflammatory gene expression of stimulated macrophages following OSPW exposure. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500µL of 50% v/v OSPW and incubated at 37°C. Macrophages were given 1.25 µL of heat killed *E. coli* after 2 hrs of OSPW exposure. RNA extraction occurred after 2-, 6-, 8-, and 10-hrs following exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qpCR. Gene expression of iNOS (A), IP-10 (B), MCP-1 (C), and MIP-2 (D) is expressed as a fold change in expression compared to the endogenous control, *gapdh*, and normalized to Control (PBS 50% v/v) at 2 hrs. Bars represent mean + SEM (n=3). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * denotes significance ($p<0.05$) compared to the control at a given time point.

Chapter V

Examination of Macrophage Pro-Inflammatory Gene Expression Profiles as a Tool for Monitoring OSPW Fraction Recombination and OSPW Treatment Protocols

5.1 Introduction

In the previous chapter, I established macrophages as sensitive *in vitro* bioindicators of OSPW-mediated bioactivity using a qPCR assay. I observed that of the two waters examined, OSPW #2 and OSPW #7, both induced basal pro-inflammatory gene expression in macrophages and also inhibited their stimulated pro-inflammatory gene expression. When the OF and IF of each water was analysed separately, bioactivity largely segregated to one fraction or the other. Specifically, OSPW #2 bioactivity segregated to the OF while the IF from OSPW #7 was primarily responsible for changes in gene expression. Consequently, we can infer that the OSPW constituents that induced the documented changes in gene expression are present in both fractions (i.e. organic and inorganic constituents) although the identity of these bioactive factors remains unknown.

When I analyzed changes in gene expression due to OSPW #2 and OSPW #2 OF exposures, I observed that the induced gene expression was greater when macrophages were exposed to the OF alone compared to the whole water at the same dose. This may suggest that, in the whole water, interactions between the IF and the bioactive OF may attenuate the activity of the latter. For example, OSPW #2 exposure upregulated *mip-2* by 251 RQ after 2 hrs. This is much lower than the induction of *mip-2* following OSPW #2 OF exposure at the same time point, which was 1216.9 RQ (Fig. 4.4A/B). Thus, I hypothesize that interactions between the OF

and the IF can influence the bioactivity of whole OSPW #2, while also having their own unique bioactivity when fractionated.

Interactions between organic and inorganic constituents have been documented in several studies focusing on industrial processes and regions. For instance, inorganic sedimentary constituents contribute to the formation of petroleum basins as seen with the oxidization of hydrocarbons by minerals containing iron. Moreover, water is a facilitator of these organic transformations as it is a source of hydrogen and oxygen (reviewed by Seewald 2003). Similarly, Yuan et al. (2019) observed that water facilitates interactions between oil and feldspar, a silicate mineral, that caused oil degradation and mineral alterations in sediment basins. Examination of aluminum mobilization and speciation at a mine dump showed that pH and ion concentrations were major contributors to aluminum interactions. For example, at a site with high pH, aluminum formed more complexes with organics, whereas sites with high concentrations of sulphate decreased interactions between aluminum and organics (Rivas-Pérez et al. 2016). Thus, organic-inorganic interactions are present in areas influenced by anthropogenic activities and are dependent on several abiotic factors including pH, ionic concentration, and access to water.

The consequences of the interactions between organic and inorganic components has also been examined during bioaccumulation. Specifically, California blackworms (*Lumbriculus variegatus*) were exposed to flowback and produced water (FPW) from hydraulic fracturing that was either untreated, or treated with activated carbon, to measure the role of organic constituents on the bioaccumulation of inorganic elements. Results showed that the presence of organics in FPW increased bioaccumulation compared to treated FPW. For example, FPW-exposed California blackworms were less efficient at eliminating inorganic elements compared to those exposed to treated FPW and the untreated control worms, which had similar levels of elimination

after 7 days (Mehler et al. 2021). Consequently, interactions between phases contribute to the adverse effects observed in study organisms and should not be overlooked when assessing complex mixtures.

Likewise, Lillico et al. (unpublished data) observed interactions between OSPW #2 OF and IF using immune cell-based bioassays that quantified NO production and pro-inflammatory cytokine secretion in macrophages. The OF-induced enhancement of a macrophage antimicrobial response (i.e. NO production) was markedly reduced following the addition of the non-bioactive IF. Specifically, when 10% v/v OSPW #2 OF was combined with increasing concentrations of IF (30%, 50%, and 70% v/v), the IF reconstitutions (compared to PBS controls) caused a significant dose dependent inhibitory effect on the OF-induced NO response. Following the same dosage scheme, IF and OF interactions were assessed by measuring the secretion of various pro-inflammatory cytokine proteins. Contrary to the inhibitory effects on NO production observed during OF+IF reconstitutions, the IF surprisingly enhanced OF-mediated cytokine secretions in a selective manner. For example, relative IP-10 secretion was increased by 73% when the cells were treated with OF reconstituted with the IF (Lillico et al. unpublished data). Overall, this demonstrated that synergistic and antagonistic interactions exist between OSPW fractions when they are reconstituted, and these interactions are likely important contributors to OSPW bioactivity profiles. Furthermore, these observations also suggest that the process of fractioning OSPW into the OF and IF may disrupt stabilized chemical interactions and/or complexes present in the whole OSPW.

If OSPW toxicity is attributed, at least in part, to interactions between constituents, then targeted remediation processes that aim to disrupt or remove constituents that facilitate such chemical interactions are viable targets for reducing OSPW adverse effects. Alum treatments are

an example of one such remediation technique that can target certain organic constituents within OSPW. In general, alum is often incorporated into coagulation/flocculation water treatment schemes and involves the addition of aluminum sulfate to contaminated waters. Once in the water, the alum hydrolyses, resulting in positively charged species that bind to, and neutralize negatively charged particles, leading to their aggregation and facilitates removal (Yukselen and Gregory 2004; Jiang 2015). Pourrezaei et al. (2011) observed that following an alum coagulant treatment, NAs were reduced 37% and oxidized NAs were reduced 86%. Accordingly, large reductions in the concentrations of certain OSPW constituents can alter OSPW bioactivity, as discussed in section 2.2, and this may be due to changes in the interactions between OSPW constituents that were inferred by Lillico et al. (unpublished data).

In this chapter, I used the RAW 264.7 bioindicator system to examine the potential synergistic effects between OSPW #2 OF and IF. I quantified these interactions by measuring changes in pro-inflammatory gene expression in resting macrophages. To do this, I repeated the same exposure concentrations as Lillico et al. (unpublished data) by exposing cells to 10% OF alone or the OF reconstituted with increasing (i.e. 30%, 50%, or 70% v/v) of the IF. Additionally, I assessed the ability of macrophages to detect changes in OSPW bioactivity after water treatment with an alum by comparing pro-inflammatory gene expression in macrophages exposed to an alum-treated and untreated OSPW. Overall, the aims of this chapter were: 1) To examine potential interactions between OSPW #2 IF and OF by monitoring the basal expression of *inos*, *ip-10*, and *mip-2*, and 2) To examine the efficacy of alum treatment by comparing changes in basal *inos*, *ip-10*, *mcp-1*, and *mip-2* expression following exposure to a treated and untreated OSPW. The results of this chapter showed that, at the gene level, macrophages could not provide conclusive evidence that the IF and OF interacted with each other regardless of time

point or gene examined. In contrast, treated OSPW trended towards being less bioactive than untreated OSPW across all genes examined and across most time points. This data suggests that macrophages are sensitive indicators that can monitor remediation efforts via gene expression levels, however they are unable to show the nuanced interactions between fractions that were observed at the functional level.

5.2 Results

5.2.1 Examining OF-IF interactions following OSPW #2 fraction reconstitution

RAW 264.7 macrophages were exposed to OSPW #2 OF alone or in combination with increasing concentrations of the IF. OSPW #2 IF at 10% was used as a negative control because it previously displayed low bioactivity at the gene level. At 2 hrs, it was also the qPCR reference sample to which all other samples were normalized. OSPW #2 OF at 10% v/v displayed unencumbered OF bioactivity. *gapdh* was the endogenous control.

As shown in Figure 5.1, macrophages treated with 10% v/v of the OF upregulated *inos*, *ip-10*, and *mip-2* expression after 6 and 10 hrs, which was expected based on my previous demonstration of bioactivity (Fig. 5.1A/B/C).

After 6 hrs, there was no significant difference between the reconstituted IF+OF treatments and the OF+PBS controls, regardless of the concentration of IF, on the expression of *inos* (Fig. 5.1A). However, the OF+IF (30% v/v) induced *inos* expression that was significantly lower than OF+PBS (30%) at 10hrs by 22.4 RQ (Fig. 5.1A). OF+IF (50% v/v) and OF+IF (70% v/v) did not cause significant changes in *inos* expression when compared to their controls (Fig. 5.1A).

Exposure to OF+IF did not induce significantly different magnitudes of *ip-10*

expression compared to OF+PBS after 6 hrs (Fig. 5.1B). Likewise, OF+IF (30% v/v) was not different than OF+PBS (30% v/v) after 10hrs. In contrast, *ip-10* expression was enhanced following exposure to OF+IF (50% v/v) and OF+IF (70% v/v) compared to their PBS controls (Fig. 5.1B). Specifically, OF+IF (50% v/v) induced *ip-10* expression was 37 RQ greater than *ip-10* expression following OF+PBS (50% v/v) treatment. OF+IF (70% v/v) enhanced *ip-10* expression by 64.8 RQ compared to OF+PBS (70% v/v) as seen in Figure 5.1B.

mip-2 expression was enhanced after 6 hrs following exposure to OF+IF (70% v/v) compared to OF+PBS (70% v/v) by 125.8 RQ (Fig. 5.1C). Lower concentrations of IF in the OF+IF (30% v/v) and OF+IF (50% v/v) treatments did not induce *mip-2* expression that was significantly different than the OF+PBS controls. Similarly, after 10 hrs OF+IF reconstitutions did not result in *mip-2* induction that differed from the OF+PBS controls except when IF was 70% v/v. OF+IF (70% v/v) significantly upregulated *mip-2* expression by a fold-change of 57.7 compared to OF+PBS (70% v/v) (Fig. 5.1C).

5.2.2 Effects of alum treatment on OSPW bioactivity

Macrophages were exposed to 50% v/v OSPW #2, OSPW #3, and OSPW #6 for 2, 4, 6, 8, and 10 hrs. OSPW #2 was sampled from Lake Miwasin post alum treatment and after the placement of a water cap. OSPW #3 and OSPW #6 were identical water sources, however OSPW #3 was alum treated to remove suspended particles while OSPW #6 was left untreated. PBS was the negative control and PBS at 2 hrs was the reference sample that was set to 1.0 and to which all other treatments were normalized. *gapdh* was the endogenous control and statistical significance was determined from $p<0.05$.

As expected, OSPW #2 exposure significantly upregulated *inos* at 2 and 4 hrs. OSPW

#3 induced *inos* expression was only significant after 8 hrs, however this fold change was lower than 2-fold. OPSW #6 induced gene expression after 2 and 8 hrs by 3.8 and 14.9 RQ, respectively, at which time it was significantly greater than OSPW #3 (Fig. 5.2A).

OSPW #2 exposure significantly upregulated *ip-10* expression at 2, 4, and 6 hrs. Expression at 6 hrs was the highest with a fold change of 252.7. OSPW #3 exposure caused significant gene induction at all time points examined with the highest change in expression at 4 hrs (e.g. 10.7 RQ). OSPW #6 exposure induced significant upregulation compared to the control after 2, 6, and 10 hrs. OSPW #6 exposure also induced gene expression that was significantly greater than OSPW #3 at 2 and 6 hrs by 34.7 and 154.8 RQ, respectively (Fig. 5.2B).

All treatments significantly induced the expression of *mcp-1* at all time points compared to the control. OSPW #2 caused the greatest gene induction at 10 hrs with a fold change of 253.6. OSPW #3 induced stable upregulated gene expression but these levels were significantly lower than those observed after OSPW #6 exposure at all time points. The greatest difference between OSPW #3 and OSPW #6 induced gene expression occurred at 10 hrs with when OSPW #6 induced *mcp-1* expression that was 182.8 RQ greater than OSPW #3 (Fig. 5.2C). Similar to OSPW #2 effects, OSPW #6 induced increasing gene expression over time, which peaked at 10 hrs with an RQ of 199.1 (Fig. 5.2C).

OSPW #2 exposure at 2 hrs caused significant upregulation of *mip-2* with an RQ of 1159. OSPW #2 also caused significantly greater gene expression at 6 and 10 hrs where *mip-2* expression was 259.2 and 141.6 RQ, respectively. OSPW #3 only significantly induced gene expression at 2 hrs with an 87-fold change, and after 10 hrs with a 66.9-fold change. OSPW #6 exposures caused significant gene upregulation at every time point except 4 hrs. OSPW #6

induced gene expression peaked at 2 hrs with 753.9 RQ, where it was also significantly greater than the OSPW #3 induced expression by 666.9 RQ (Fig. 5.2D). Finally, OSPW #6 exposure had the lowest RQ value at 10 hrs with 75.5 RQ (Fig. 5.2D).

5.3 Discussion

In this chapter, I used the macrophage bioindicator system established in Chapter IV to examine if interactions between OSPW #2 OF and IF could be detected at the gene level. I also assessed the efficacy of macrophages as bioindicators based on their ability to generate discrete responses following exposure to a treated OSPW sample. These experiments served to broaden our knowledge of macrophage sensitivity following OPSW exposures and to determine if macrophages could produce discrete responses to manipulated OSPW samples.

In the previous chapter, I showed that macrophages responded to 50% v/v OSPW #2 exposure by upregulating the expression of pro-inflammatory genes and that this bioactivity segregated to the OF. However, OF exposures resulted in greater magnitudes of gene induction compared to whole water, which suggested that the process of fractioning the OF and the IF concentrated the OF components or possibly, that in the whole water, interactions between bioactive organic components are attenuated by binding to certain organics. Based on basic NA analyses of the OSPW#2 whole water and the OF, it appears that concentration of the OF components did not occur following fractionation (Table 3.1). In this chapter, I assessed interactions between the fractions by reconstituting a baseline concentration of 10% v/v OF with various concentrations of the IF (30% v/v, 50% v/v, and 70% v/v).

OF-IF interactions were previously observed at the functional level but were not replicated at the gene level despite following the same treatment scheme. Lillico et al.

(unpublished data) observed that as the concentration of IF increased, OF bioactivity was inhibited which resulted in reduced NO production. At the antimicrobial gene expression level, this effect was only observed after 10 hrs when the expected 10% v/v OF-mediated *inos* induction was significantly abrogated when the bioactive OF was reconstituted with 30% v/v of the IF. Throughout my examinations of *inos* expression during OF-IF reconstitutions, this was the only condition that agreed with the data shown at the protein secretion level and the only display of IF-mediated inhibition of the OF-induced *inos* expression.

Moreover, Lillico et al. (unpublished data) showed that, opposite to the effects on NO synthesis, greater concentrations of IF enhanced the secretion of pro-inflammatory cytokines. This was not conclusively observed when quantifying gene expression. Dose dependent effects of the IF were not shown when measuring either *ip-10* or *mip-2* expression. I only observed similar results pertaining to *ip-10* expression after 10 hrs. Specifically, at 10 hrs, reconstitutions of 10% v/v OF with 30% or 50% v/v IF induced greater expression of *ip-10* than the matched PBS reconstitution controls. Likewise, reconstitution of 10% v/v OF with 70% IF upregulated *mip-2* significantly more the PBS reconstitution controls after 6 and 10 hrs. Therefore, the dose dependent upregulation of cytokine secretions following increased reconstitution doses of IF was not observed using gene expression analysis. These results indicate that, at the gene level, macrophages are not suitable for detecting possible interactive effects between different components.

Alternatively, macrophages did have discrete responses following exposure to treated and untreated OSPW samples and the reliability of these responses was dependent on the endpoint examined. For example, OSPW #3, an alum treated sample, induced lower gene expression levels of *inos*, *ip-10*, and *mip-2* at certain time points when compared to macrophages

exposed to the untreated OSPW #6. However, *mcp-1* expression was significantly lower after OSPW #3 exposures compared to OSPW #6 across all time points examined. The greatest difference in magnitude of gene expression between these waters was observed after quantifying *mip-2* expression at 2 hrs. Thus, macrophages can detect specific changes in treated OSPW using bioactive markers, but consistent and reliable results depend on the careful selection of responsive endpoints.

5.3.1 Potential synergistic and antagonistic effects between the IF and OF

Studies examining the impacts of OSPW have suggested that the IF contributes to toxicity (Fu et al. 2017; Phillips et al. 2020). Furthermore, these interactions were observed when measuring NO and the secretion of pro-inflammatory cytokines in a dose dependent manner as IF concentration increased and OF concentration stayed the same (Lillico et al. unpublished data). However, this was not seen at the gene expression level as there was not a dose-dependent pattern of IF effects on OF bioactivity regardless of the gene examined. Potential reasons for these results are: 1) there was not enough time for interactions or complexes between OF and IF constituents to form; 2) the environment required for interactions to occur was not provided, and; 3) the biological effects of IF-OF interactions occur downstream of gene expression.

Aside from the functional observations by Lillico et al. (unpublished data), other studies have observed organic and inorganic constituent interactions. An example of complexes that may be mediating OSPW bioactivity are organometallics. Organometallics are organic-metal complexes that are defined as having a covalent bond between a carbon and a metal. The formation of these bonds are often man-made, however they can also occur naturally or due to interactions mediated by microorganisms (Chau and Wong 1980). Organometallics can be found

in a variety of environments and Crompton (1988) lists the major routes of release as anthropogenic activities or natural processes including land erosion in waterways. Organometallics have not yet been identified in OSPW, but are likely present as they have been observed in other industrial and mining effluents (Fish et al. 1983; Wong et al. 1997; Rivas-Pérez et al. 2016). Pertaining to this study, organometallics are potential mediators of OSPW effects due to their bioactivity that has been observed in other studies.

Organometallics have documented bioactivity, and this has been studied as it pertains to inflammation and cancer treatments. Specifically, organometallics appear to have strong anti-inflammatory properties. Iqbal et al. (2015) observed that a silver(I) *N*-heterocyclic carbene complex inhibited the synthesis of TNF-α and IL-1 in human macrophages and also activated caspases. These observations may contribute to the anti-proliferation of human colorectal cancer cells (Iqbal et al. 2015). Pro-apoptotic effects were also observed in gold(I) *N*-heterocyclic carbene that inhibited selenoenzyme thioredoxin reductase, which contributes to apoptosis (Bertrand et al. 2014). Similarly, gold(I) complexes supressed TNF-α and IL-1β but not anti-inflammatory cytokines in stimulated macrophages (Trávníček et al. 2012). Therefore, the above organometallics possess the anti-inflammatory characteristics I expected to see following recombination of the OF with the IF on *inos* expression levels.

In contrast, certain organometallics display bioactivity which might result in pro-inflammatory outcomes. Iron(III)-salophene-exposed neuroblastoma cells had upregulated ROS production and certain pathways were activated, such as Jun N-terminal kinase and p38 MAPK, which can respond to inflammatory signals. Thus, organometallics can also mediate pro-inflammatory effects, which may account for the enhanced up-regulation of pro-inflammatory cytokines observed by Lillico et al. (unpublished data). However, neither the anti-inflammatory

nor the pro-inflammatory effects of organometallics were observed in my qPCR-based experiments.

As previously mentioned, microorganisms can also mediate metal biotransformation and this may account for my inconclusive reconstitution results (Chau and Wong 1980). Methylation is one process of transformation used by microorganisms that involves the addition of a methyl group to a metal. This causes formation of an organometallic that is more likely to bioaccumulate due to greater lipophilicity (reviewed by Roane et al. 2015). Although OSPW samples were filtered to remove large particulate matter and microorganisms prior to exposure, it is possible that small organisms were able to bypass the filter and remain present in the exposure samples. Accordingly, if they are present in the organic fraction, methylation of metals upon OF-IF recombination may not have adequate time to take place resulting in an insignificant concentration of organometallics that are unable to produce the discrete results that were observed when we monitored cytokine secretion activity after 24 hrs (vs. 2-10 hrs in my study).

The second potential explanation for my results posits that experimental conditions did not favour interactions between the IF and the OF. Although it seems less plausible because I replicated the conditions performed for the cytokine secretion assays, the exposure periods in my experiments were shorter and this would result in a higher pH and greater nutrient availability in the extracellular environment at the time of sampling compared to the samples collected for protein analysis. Consequently, the earlier timepoints when I analyzed gene expression responses may not have favoured OF-IF interactions. Rivas-Pérez et al. (2016) observed that pH and ion concentrations were key factors that influenced the interactions between aluminum and organics and thus they may have also influenced interactions in this experiment. Lastly, there is a possibility that OF-IF interactions take place downstream of gene expression, such as during

protein synthesis or protein transport and secretion and would simply not be observed in a gene-monitoring experiment as reported here.

5.3.2 Macrophage sensitivity to an OSPW treatment protocol

As discussed in section 2.2, alum treatments are an efficient method of removing constituents of concern, particularly organics from OSPW. The addition of an alum neutralizes negatively charged constituents following its hydrolyzation into positive species in water. Neutralization allows for the aggregation of particles, which can then be filtered out (Yukselen and Gregory 2004; Jiang 2015). Given the sensitivity macrophages displayed when treated with various OSPW samples in Chapter IV, it was not surprising that they produced unique outputs in response to a treated and untreated water. However, I was expecting to observe sustained differences between OSPW #3 and OSPW #6 where OSPW #3 was predicted to show significantly lower gene expression responses across all time points and for all the genes examined herein.

Of the genes examined, *mcp-1* and *mip-2* displayed the most consistent results. Upregulation of *mcp-1* in response to OSPW #6 increased over time but was always significantly greater than the upregulation induced by OSPW #3. Although OSPW #3 did induce *mcp-1* expression compared to the control, it never rose above 17 RQ compared to OSPW #6 RQ values that ranged from 43.2 to 199.1. Therefore, *mcp-1* offers reliable results and is suitable for monitoring samples over time. In contrast, *mip-2* did not have sustained significant differences in expression when macrophages were exposed to treated vs. untreated OSPW. However, *mip-2* expression levels produced the greatest magnitude of difference at only 2 hrs. In comparison, a significant difference between OSPW #3- and OSPW #6-induced *inos* and *ip-10* expression was

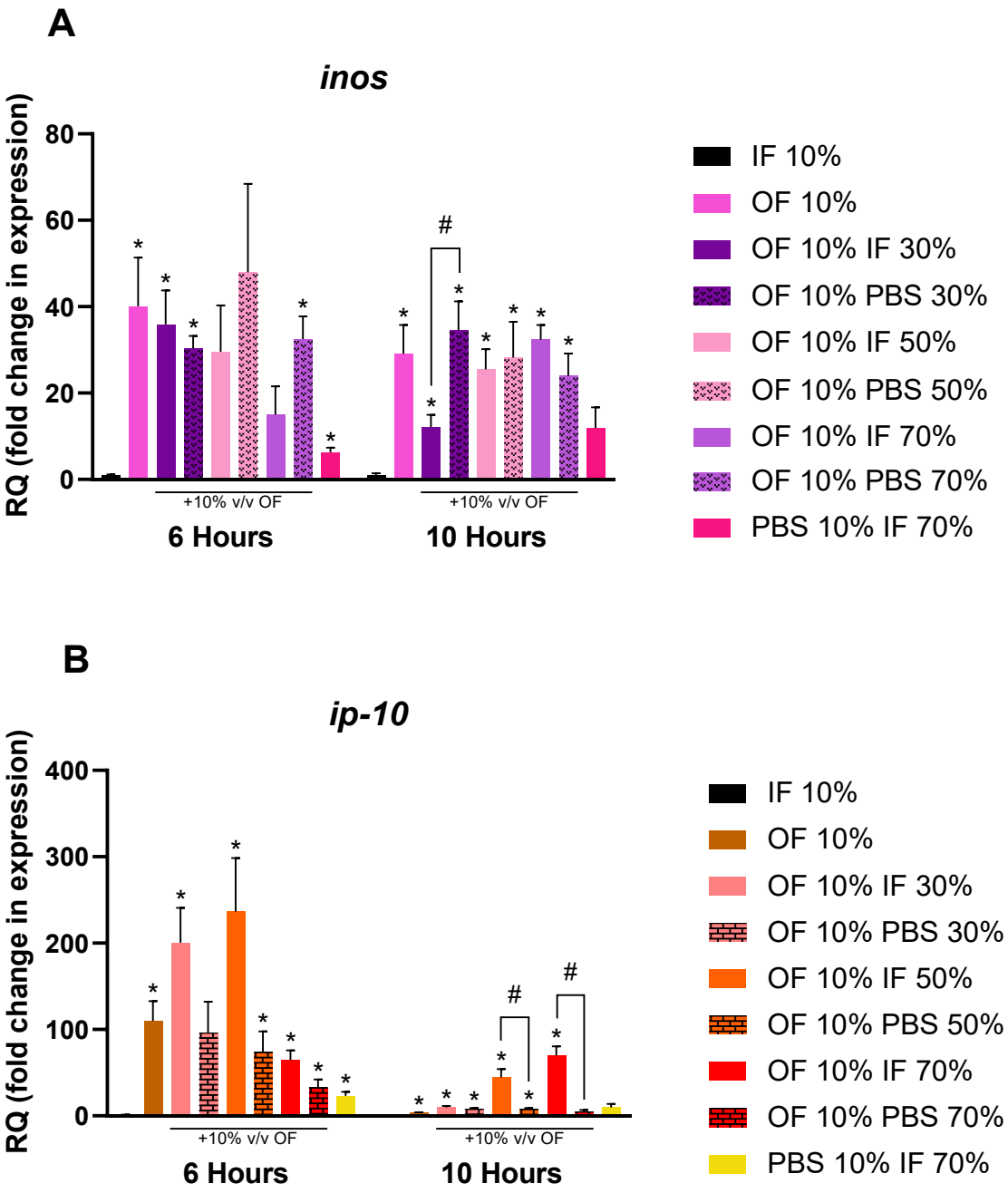
not maintained over time like *mcp-1* expression was. Likewise, I did not observe the large difference in expression magnitude between OSPW #3- and OSPW #6-induced gene expression when examining *inos* and *ip-10* induction that was documented for *mip-2* after 2 hrs. Although both genes showed a trend for treated OSPW having lower bioactivity than untreated OSPW, this was not statistically significant for most time points. Consequently, *mcp-1* and *mip-2* expression are better biomarkers for assessing bioactivity in manipulated OSPW samples. *mcp-1* induction in response to OSPW exposure offers sustained differences between a treated OSPW and an untreated OSPW, which would be an asset for biomonitoring the effects of remediation strategies over short periods of time. Alternatively, *mip-2* expression levels in response to these waters generated a discrete output with a large magnitude of difference for each water after 2 hrs. Overall, this is beneficial and results in a rapid bioindicator test when monitoring alum-treated OSPW.

5.3.3 Future directions

The presence and role of organometallics in OSPW should be substantiated. Wong et al. (1997) proposed using the sensitive responses of algae as an indicator system to identify these complexes within various waters. Wastewaters from two gold mines and industrial effluents from a chemical plant and a pulp and paper mill underwent chemical analyses to confirm whether they contained organometallic complexes prior to exposing them to two algal species (*Chlorella* and *Cladophora*). The overall goal was to compare the effects generated by the collected water samples on algal species to the effects observed following their exposure to the organometallics, alone. This would show whether algae have discrete responses to the presence of organometallics via X-ray microanalysis. Mine waters did procure fine structural damage to the endoplasmic reticula and mitochondria of *Chlorella*, which was supported by X-ray spectrum

identification of organometallics (Wong et al. 1997). A similar methodology could be used to identify these constituents within OSPW using the macrophage bioassay system. This would be similar to the creation of unique water profiles for OSPW samples based on the differential expression of pro-inflammatory genes and would broaden our understanding of bioactivity within OSPW.

The efficacy of remediation and the ability of macrophages to detect and respond to treated OSPW samples should be expanded further. Firstly, macrophages should be exposed to more OSPW samples that have been remediated or samples that have undergone different remediation strategies. This would increase our understanding of macrophage sensitivity and their ability to detect changes to constituent compositions. Secondly, expanding the number of pro-inflammatory genes examined beyond *mcp-1* and *mip-2* may produce a suite of reliable biomarkers for monitoring OSPW remediation efforts and offer more robust data from which to further substantiate the utility of immune cell-based biomonitoring.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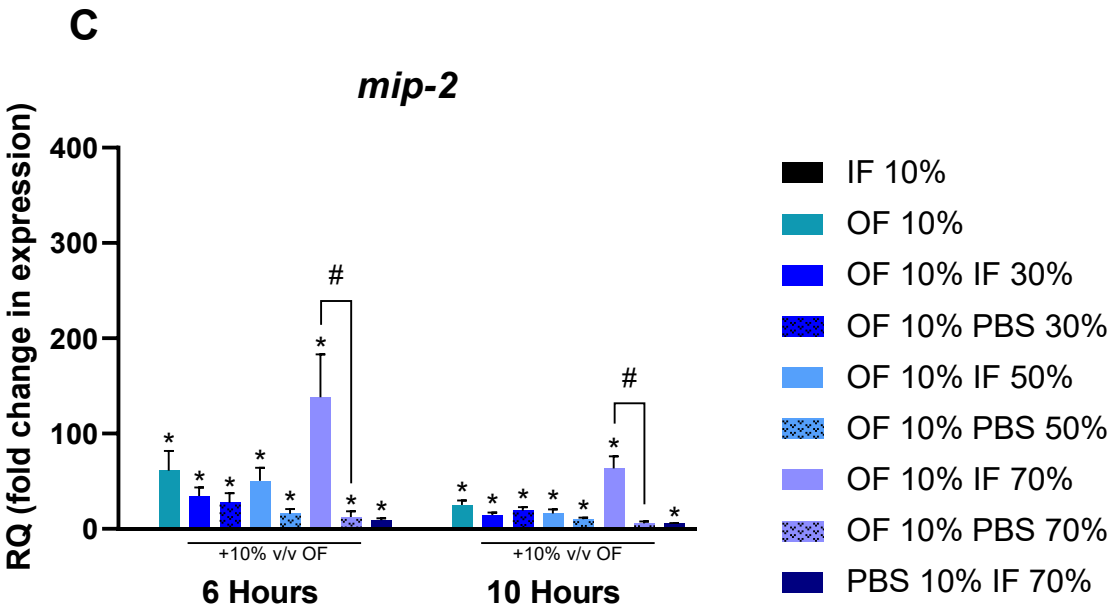
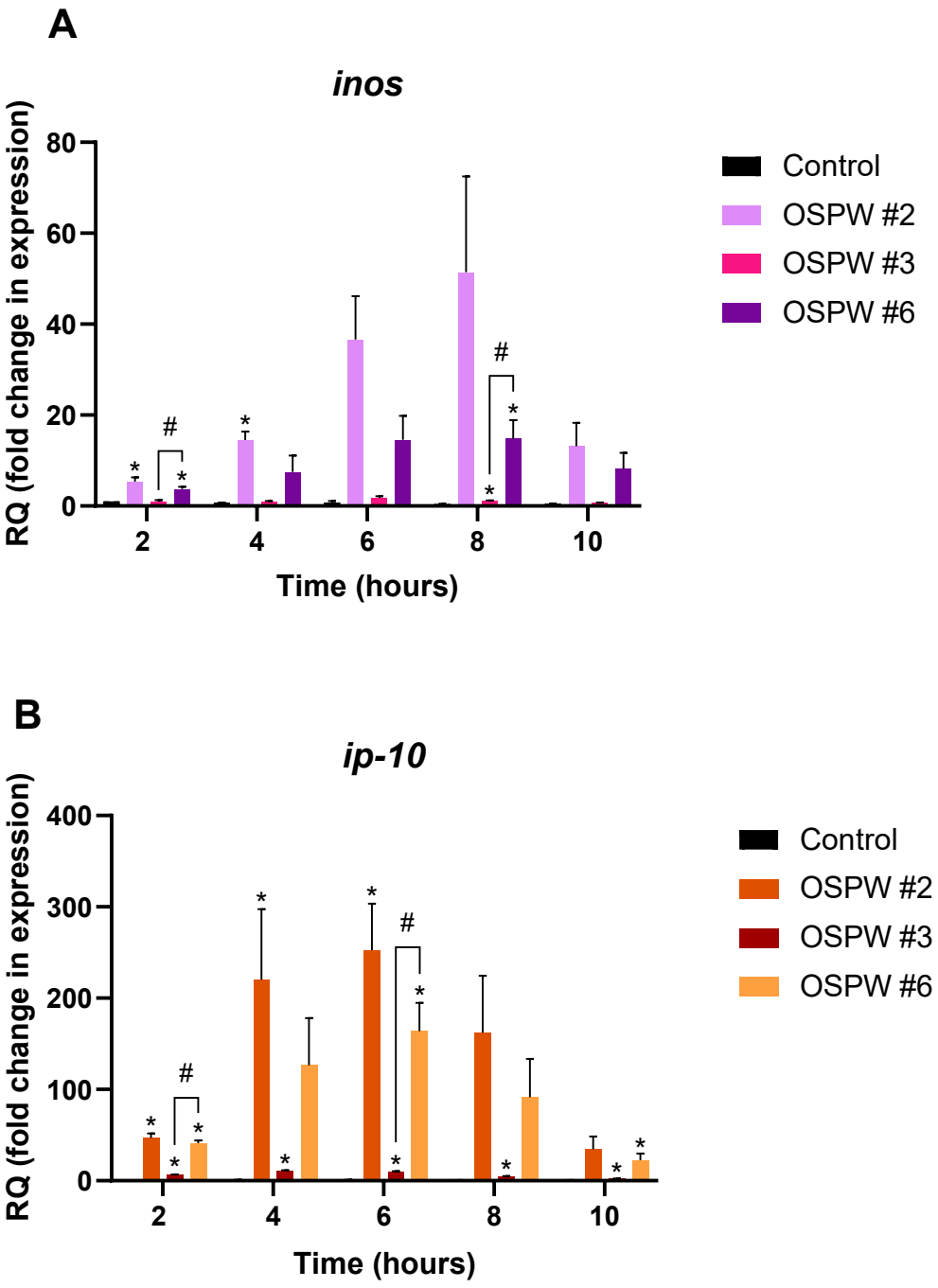


Figure 5.1 Pro-inflammatory gene expression of resting macrophages following exposure to OSPW #2 organic fraction and inorganic fraction recombined. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500 µL of recombined OSPW #2 fractions and incubated at 37°C. IF 10% contained 10% v/v IF and was a negative control; OF 10% contained 10% v/v OF. Mixing consisted of a baseline OF concentration (10% v/v) and increasing IF concentrations (30% v/v, 50% v/v, 70% v/v). PBS served as a negative control for IF with corresponding concentrations of 30% v/v, 50% v/v, and 70% v/v. PBS 10% IF 70% contained 10% v/v PBS and 70% v/v IF and measured the effects of 70% v/v IF alone. RNA extraction occurred after 6 and 10 hrs of exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qPCR. Gene expression of iNOS (A), IP-10 (B), and MIP-2 (C) is expressed as a fold change in expression compared to the endogenous control, *gapdh*, and normalized to IF 10% at 2 hrs. Bars represent mean + SEM (n=3). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * denotes significance ($p<0.05$) compared to the control at a given time point. # denotes significance ($p<0.05$) between treatments.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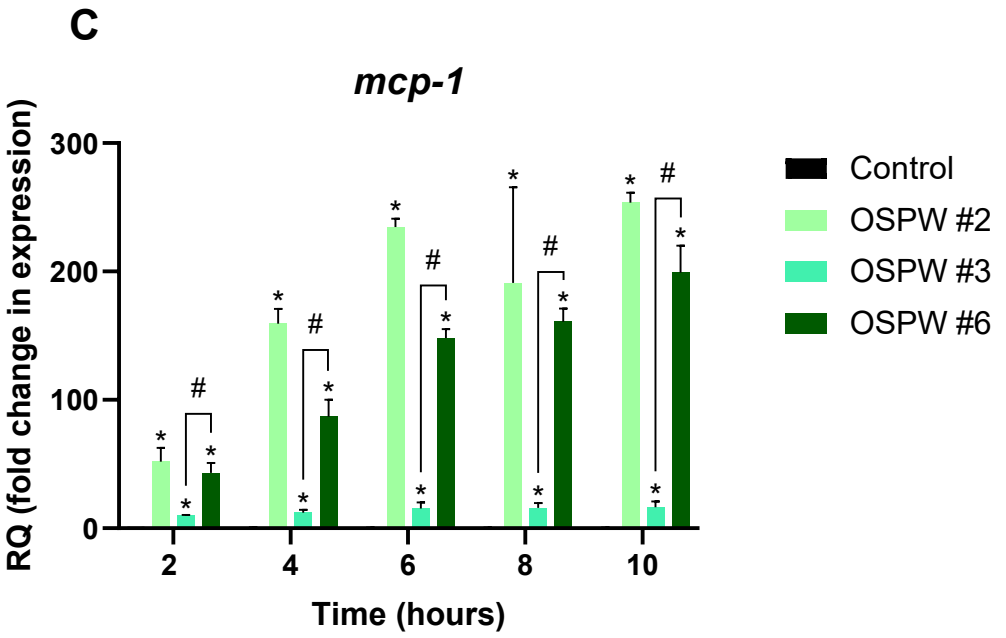
OSPW #3
OSPW #6


C
mcp-1
RQ (fold change in expression)
300
200
100
0
2
4
6
8
10
Time (hours)
Control
OSPW #2
OSPW #3
OSPW #6

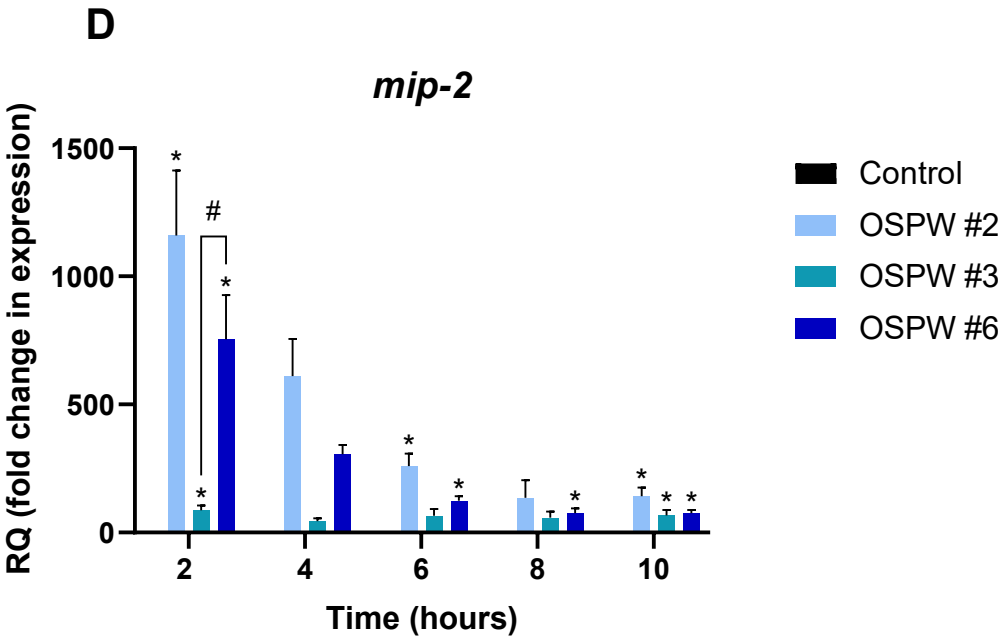

D
mip-2
RQ (fold change in expression)
1500
1000
500
0
2
4
6
8
10
Time (hours)
Control
OSPW #2
OSPW #3
OSPW #6

Figure 5.2 Pro-inflammatory gene expression of macrophages following exposure to treated and untreated OSPW. RAW 264.7 macrophages seeded at $3x10^5$ cells/well were exposed to 500 µL of 50% v/v of OSPW #2 (treated), OPSW #3 (treated), or OSPW #6 (untreated). OSPW #3 and #6 are from the same OSPW source. Macrophages were incubated at 37℃ and RNA extraction occurred after 2, 4, 6, 8, and 10 hrs following exposure using TRIzol. 1 µg of RNA was used to synthesize 10 µL of cDNA that was analyzed via qPCR. Gene expression of iNOS (A), IP-10 (B), MCP-1 (C), and MIP-2 (D) is expressed as a fold change in expression compared to the endogenous control, *gapdh*, and normalized to Control (PBS 50% v/v) at 2 hrs. Bars represent mean + SEM (n=3). Statistical significance was determined following a Shapiro-Wilk test of normality and either an unpaired t-test or a Mann-Whitney test depending on distribution. * denotes significance ($p<0.05$) compared to the control at a given time point. # denotes significance ($p<0.05$) between treatments

Chapter VI

General Discussion

6.1 Summary of Thesis Findings

Alberta's oil sands are in the Athabasca, Cold Lake, and Peace River regions of northern Alberta, which spans ~142 000 km^2 (CAPP 2021). These oil deposits contain an estimated 165 billion barrels of bitumen and roughly 20% of this oil deposit can be surface mined due to its accessibility (AER 2021a; CAPP 2021). The oil sands contain bitumen, clay/silt, and water (AER 2021a). To isolate the bitumen, industry uses a caustic water process that generates large quantities of wastewater that is held long-term in tailings ponds. This water is known as OSPW and due to its potential harmful effects, it must be remediated prior to its release (Giesy et al. 2010). A major setback for OSPW remediation efforts is the fact that each tailing pond contains OSPW with unique compositions. For example, individual OSPW sources are distinct based on their processing history as well as environmental factors, and this makes tailing pond remediation efforts a complex task (Li et al. 2017).

The overall objectives of this thesis were to establish and evaluate the use of a cell-based bioindicator system for examining OSPW bioactivity via alterations in selected pro-inflammatory gene expression. Specifically, I aimed 1) to examine the effects of whole OSPW and its OF and IF on immune cell gene expression; 2) to examine and confirm if OSPW OF and IF interactions affected inflammatory gene expression patterns, and; 3) to determine if immune gene expression profiling could serve as a sensitive bioassay for examining an alum treated OSPW. To do this, I optimized a sensitive qPCR-based immune-gene expression system using

RAW 264.7 mouse macrophages following exposure to whole OSPW, OSPW IF and OF alone or in combination, as well as an untreated and an alum-treated OSPW.

6.2 The Potential Roles of Environmental Microbes and OSPW-Detected Bioactivity

Bioactivity is the ability of a substance to elicit a biological response irrespective of whether this response is beneficial or harmful. OSPW bioactivity and/or toxicity has been examined in a plethora of model organisms following various exposure schemes and analytical methods. Adverse effects following exposure is primarily attributed to OSPW organic constituents like NA species. However, little attention has been given to the role of microorganisms in mediating bioactivity.

Microbial communities have been documented in OSPW, but analyses have focused on their role in remediation efforts. For example, Brown et al. (2013) monitored microbial density in ozone treated OSPW as a means of evaluating community changes and did not observe a difference in density despite increased biodegradability of organic carbon. This suggests that microbial communities were altered resulting in greater organic carbon degradation, however the analyses were not robust enough to elaborate on these population changes. By contrast, VanMensel et al. (2017) monitored changes in fluid fine tailings microbial communities after gamma irradiation and found that many of the genera present after treatment were associated with constituent degradation including hydrocarbons. In anaerobic conditions, the most abundant phylum was Proteobacteria and specifically the genera of *Enterobacter*, *Pseudomonas*, *Smithella*, and *Syntrophus*. In aerobic conditions, *Geobacter*, *Diaphorobacter*, and *Ferruginibacter* were increased in abundance post gamma irradiation. This method of microbial monitoring was more effective than that reported by Brown et al. (2013) since changes in

specific populations could be assessed. Unsurprisingly, it was also observed that bacterial communities changed over time when comparing fresh to aged tailings (VanMensel et al. 2017). Thus, microbial communities are present in these waters, but most analyses have focused on their role in constituent remediation at varying levels of population analyses. The role of microbial communities as mediators of adverse effects has yet to be explored in the context of OSPW.

As discussed, immune cells recognize and respond to microbial PAMPs via PRRs, which trigger intracellular cascades leading to the release of pro-inflammatory molecules (Akira et al. 2006). Thus, it is very likely that water samples containing microorganisms would lead to bioactivity as observed in this thesis. In this study, prior to OSPW exposures, all waters were filtered using 0.45 μm filters to eliminate potential large particulates present in these samples. However, when Wang et al. (2007) filtered samples of freshwater through 0.45 μm sterile filters, they observed that as little as 5.56%, and as great as 87.07%, of bacteria were able to pass through depending on the water sample. Similarly, Proteobacteria were shown to pass through 0.22 μm filters and comprised 24-67% of the bacteria in the filtered water (Maejima et al. 2018). Therefore, it is possible that bacteria, or bacterial components, remain in filtered OSPW samples.

Another potential mediator of OSPW bioactivity is not the microbial community directly, but microbial components or molecules from their secretome. The secretome refers to all molecules secreted by a cell that mediate biological processes such as inter-cell communication and virulence (reviewed by Hathout 2007; reviewed by Zubair et al. 2020). Consequently, proteins secreted by the microbial population of OSPW may be inducing the bioactivity observed in this study. One of the most well studied microbial secretomes is that of the gut microbiome. Changes to the microbiome and the resultant secretome produce immune responses, especially those related to inflammation. For instance, obesity alters the gut

microbiome leading to the secretion of deoxycholic acid, which stimulates the secretion of pro-inflammatory cytokines from hepatic stellate cells (Yoshimoto et al. 2013). Similarly, secretions from two non-pathogenic *E. coli* strains stimulated the NF-κB pathway and pro-inflammatory cytokine upregulation in intestinal epithelial cells (Zargar et al. 2015). Intestinal inflammation associated with Chron's disease can be attributed, at least in part, to the gut microbiome secretome, which influences inflammation in mice (Günther et al. 2020). Thus, OSPW bioactivity may be mediated by the microbial community's secretome, which is often a direct response to their environment (Hathout 2007).

Microbial responses to OPSW are not as well researched as those in more complex organisms. Studies that have examined the impacts of OSPW exposures on microorganisms have focused on *V. fischeri* which is a marine species of bacteria with bioluminescence. Changes in levels of bioluminescence are quantified following exposure to xenobiotics and have been used as a standard toxicity indicator. Consequently, this bacterial-based Microtox assay has been an asset for biomonitoring OSPW and assessing the efficacy of remediation strategies (Leshuk et al. 2016; Wang et al. 2013; Zhang et al. 2015; Wang et al. 2015a; Yue et al. 2016). Endogenous or native microbial communities in OSPW are affected by external influences on their environments as was shown by VanMensel et al. (2017), and alluded to in Brown et al. (2013). However, Folwell et al. (2016) observed effects on microorganism populations as a response to OSPW constituents, specifically. For example, addition of PAHs altered bacterial and fungal community composition as high molecular weight PAHs increased the abundance of *Pseudomonas*, *Bacillus*, and *Microbacterium* genera (Folwell, McGenity, and Whitby 2016). Thus, OSPW microbial communities do recognize and respond to OSPW constituents and these

responses should be evaluated as they pertain to the secretome and their potential pro-inflammatory effects.

Other biological factors in OSPW that are potential mediators of the bioactivity I observed are phages. Phages are viruses that infect bacteria and are found in freshwater sources. Interactions between phages and bacteria in aquatic environments are not well understood and therefore recent studies have been focused on characterizing the phage population through metagenomics. Sequencing of two freshwater samples generated >2000 complete phage genomes. Between two water sources, there was only one nearly identical phage (Kavagutti et al. 2019). Thus, the phage populations between sources are unique, which presents a massive hurdle in understanding their ecosystem impacts. Chen et al. (2020) also completed a metagenomic analysis and identified phages that are likely to infect methanotrophic bacteria. These phages encode an important subunit of methane monooxygenase which catalyses methane oxidation and is similar to the subunit found in methanotrophs. Regions of phage-subunit abundance correlated with methanotroph habitats suggesting an evolutionary relationship between these bacteria and phages. Likewise, because phages express this subunit, they may contribute to methane oxidation (Chen et al. 2020). Therefore, although phage-host interactions are unclear in ecosystems and have not been assessed in OSPW, they are very likely to be present and may generate responses in bacterial populations that will induce changes to their secretomes, which could be detected by the immune cells used in my bioassays.

Furthermore, it is unclear whether the potential interactions between immune cells and OSPW phages would induce a pro-inflammatory immune response. Generally, phages have been shown to induce anti-inflammatory properties (Jónczyk-Matysiak et al. 2017). When gp12, a phage tail adhesin that promotes phage binding to *E. coli*, was examined in mice, Miernikiewicz

et al. (2016) observed inhibition of the expected LPS-stimulated pro-inflammatory response and suggested that gp-12 binds to LPS. Consequently, this binding of gp-12 to LPS prevented LPS recognition by immune cells and subsequent inflammation. However, phages were shown to induce pro- and anti-inflammatory gene expression profiles in blood monocytes independent of bacterial endotoxins (Van Belleghem et al. 2017). Thus, phage-immune cell interactions are possible, as are phage-host interactions, that influence immune responses. It is therefore impossible to predict how phages might mediate OSPW bioactivity, but they should be considered in OSPW examinations.

6.3 Bioassay Utility

In this thesis, I used macrophages as sensitive biosensors following OSPW exposures. Compared to previous studies in our lab that focused on macrophage antimicrobial responses and cytokine secretion (i.e. 'functional experiments') after 24 hr OSPW exposures, I have demonstrated that macrophages are not only able to generate discrete gene expression profiles following exposure to different OSPW samples, but that they are also display these expression responses as early as 2 hrs after exposure. Consequently, macrophages serve as rapid and sensitive indicators for assessment of the bioactivity of these complex waters.

Firstly, I exposed resting (basal) and bacterial-stimulated macrophages to OSPW to examine potential OSPW effects on the resting and induced expression of select pro-inflammatory genes. In resting macrophages, OSPW #2 and OSPW #7 were considered bioactive as they both significantly upregulated gene expression, however OSPW #2 generally induced a greater magnitude of responses. When individual fractions were examined, bioactivity segregated to OSPW #2 OF and surprisingly to OSPW #7 IF. This suggests that bioactivity from

two independent whole OSPW samples are variably attributed to their organic and inorganic fractions. This doesn't mean that all bioactive components are exclusively present in one fraction or the other, however my assays showed a predominant difference in the OF and IF-induced bioactivity of these two OSPWs. In stimulated macrophages, OSPW #7 inhibited the induced expression of all genes tested except for *mcp-1*. Overall, both waters were bioactive and perturbed pro-inflammatory gene expression but the adverse effect, whether it be upregulation or downregulation, was dependent on the fractions examined or the state of macrophage activation.

I also tested the utility of this bioassay by exposing macrophages to manipulated samples of OSPW. Macrophages generated discrete responses to waters pre- and post-alum treatment, particularly when quantifying *mcp-1* and *mip-2* expression. However, reconstituting the OF and the IF did not generate conclusive results that demonstrated interactions between the fractions. I hypothesized that increasing concentrations of IF would influence OF bioactivity. Specifically, a baseline concentration of OF with increasing concentrations of IF was expected to attenuate the OF induction of *inos* and promote induction of pro-inflammatory cytokines based on data collected at the protein level after 24 hrs of exposure. However, in my studies, no dose dependent interactions between the IF and the OF were observed at the gene expression level.

Ultimately, this macrophage bioassay generates rapid and sensitive responses to unique and complex water samples. This can serve two important functions: 1) generating OSPW pro-inflammatory gene expression profiles and, 2) monitoring changes in OSPW bioactivity. Profiling OSPW is an important first step towards understanding these complex and dynamic waters. It provides a baseline knowledge of OSPW bioactivity and allows for direct comparisons between different OSPWs, their fractions, and even samples undergoing treatment. Following the establishment of unique OSPW bioactive profiles, biomonitoring can then take place as a tool for

assessing changes in a water overtime or during treatment-specific remediation efforts. Combined with other assessments (i.e. water quality analysis and microbial community profiling), immune cell-based bioactive monitoring can assist in assessment of the efficacy of remediation strategies as well as the natural ageing and weathering process. Understanding the changes in these waters over time is important as we progress towards establishing metrics that will inform ongoing reclamation efforts and the end goal of OSPW release into the natural environment.

Overall, this immune cell bioindicator system offers several technical advantages compared to other biomonitoring strategies. For one, *in vitro* systems are more economical than maintaining whole organisms *in vivo* models and do not require adherence to ethical or animal care standards (Archibald 2018). The labour and time required for conducting *in vitro* assays is also generally less than more complex whole animal model systems. These attributes are important for large scale assessments such as those required for OSPW examinations, and a cell-based bioassay is easily modified to the needs of the assessment project. For example, scaling-up a cell line bioassay is easier than other model systems. Cell lines provide a virtually unlimited ability to increase the repertoire of OSPW samples examined or the number of experimental replicates conducted. Therefore, cell-based bioindicator assays are a cost-effective, labour and time efficient, high throughput method of gathering information.

As discussed earlier in this thesis, cells offer many advantages as a model system from a biological perspective. All cells respond to internal and external stimuli which triggers internal responses and leads to an output that can be quantified (Taniguchi et al. 2009). My thesis focused on optimizing an immune cell bioassay that takes advantage of macrophage activation and the resulting phenotypic dichotomy as they are sensitive to homeostatic changes and are likely to

interact with xenobiotics like OSPW (Mosser and Edwards 2008). In general, all cell types have the capacity to be a bioindicator and can be used to examine OSPW bioactivity. Increasing the suite of cells used to assess OSPW would benefit biomonitoring efforts and improve the scope of profiling by facilitating the analysis of other cell-specific endpoints. Since different cell types have different functions, increasing the number of cell types used in biomonitoring would enable different OSPW-receptor interactions that may provide new insights regarding water quality analyses when combined with other metrics such as constituent analyses.

Another important facet of this bioassay was the optimization of qPCR to quantify pro-inflammatory gene expression following OSPW exposure. Using changes in gene expression is an asset to this indicator system as exposure times are shorter compared to the length of exposure time required to collect functional data (i.e. 2 hrs vs 24 hrs). This results in faster collection of data and an overall more rapid indicator assay. Furthermore, gene expression assessments allow for the monitoring of several biomarker genes simultaneously from the same treatment and sample collection, which is a benefit of isolating total RNA from exposed cells. Consequently, the most simplistic approach to bolstering this system would be to examine a larger collection of genes. I previously suggested including other pro-inflammatory genes like *tnf-α* and *il-6* however biomarkers of stress, such as heme oxygenase 1 (*hmox1*) and growth arrest and DNA damage 45 (*gadd45*) as seen in Phillips et al. (2020), or members of the cytochrome P450 family that are involved in xenobiotic metabolism, would also be informative and should be considered (Marentette et al. 2017; Lyons et al. 2018). Therefore, beyond my demonstration that select immune genes are sensitive and rapid markers for OSPW bioactivity, endpoints are not limited to immune genes and the effects of OSPW-mediated bioactivity can be expanded to assess other cellular response networks.

One major limitation of this bioindicator assay, from an ecological standpoint, is the lack of overall biological relevance. Although I observed upregulated pro-inflammatory gene expression following OSPW exposures, this does not translate to a pro-inflammatory response in a whole organism. In general, gene expression cannot be extrapolated to functional outputs. Using a cell line negates interactions between cells and systems within an organism. Therefore, the conclusions drawn in this thesis cannot be applied to the realities of OPSW exposures on tissues, organs, or animals. Consequently, this bioassay is limited to bioindication by quantifying changes in gene expression via qPCR and at this stage is not a surrogate for toxicity assessments or whole animal functional changes, including inflammatory reactions.

6.4 Complementary Analyses to Improve the Significance of Bioassay Outputs

As mentioned, this bioassay lacks biological significance on its own and is restricted to measuring changes in gene expression at the cell level following OSPW exposures. However, there are types of analyses that can be conducted in tandem with this bioassay that can provide relevant data to support the observations produced by this system.

6.4.1 Chemical analyses for identifying bioactive constituents

Chemical analyses of these complex waters can be paired with my bioindication data. Comparing the constituents present in water samples and correlating them to bioactive samples could aide in identifying constituents of interest. For instance, characterizing the chemicals within OSPW #7 IF would begin the process of singling out inorganic constituents of concern that are likely mediating bioactivity.

Several methods of constituent identification and characterization exist. Variations of MS have thus far been successful techniques in chemical analysis of OSPW and are most often

used, especially for organic constituents. MS quantifies the mass:charge ratio of molecules and their intensity, which can identify specific constituents within complex samples as well as their abundance. Similar to the goal of creating bioactive profiles for OSPW sources using the macrophage bioassay, Headley et al. (2011) analysed the polar organic constituents within several Athabasca regional water samples and OSPW. The presence of distinct populations of sulfur-containing species suggests that they could be used to distinguish OPSW sources. Huang et al. (2018) also used MS to characterize the NAs in various OSPWs and oil sands-impacted water samples and found distinct populations of NAs and aromatic NAs that could be used as water identifiers. Inorganic constituents have also been profiled from these waters. For example, boron, strontium, and lithium isotopes were identified in OSPW using MS by Harkness et al. (2018), where they were also proposed as source identifiers based on their unique distributions and as indicators to monitor OSPW migration through ecosystems.

Spectroscopy and chromatography approaches are used as chemical analyzers for OSPW samples and oftentimes accompany MS. Spectroscopy measures the interaction of light with a sample and chromatography is the physical separation of components in a mixture. Cations, like Na^+ and K^+, have been analyzed using spectrophotometry and anions, such as Cl^- and NO_2^-, have been analyzed using chromatography (Harkness et al. 2018; White and Liber 2018). Kavanagh et al. (2009) used fluorescent spectrophotometry to compare OSPW to commercial NAs with a known concentration and proposes this method as a means of identifying and monitoring OSPW constituents. Thus, several approaches to measuring constituents within these complex waters are available.

Pairing the data generated by the macrophage bioassay with chemical analyses is referred to as an effects-directed analysis (EDA) or a toxicity identification evaluation (TIE). The

general methodology of an EDA/TIE is as follows: *in vitro* or *in vivo* models are exposed to a complex sample to observe potential biological responses. If changes to endpoints are observed, the sample is fractionated, and these fractions are again examined via the bioassay for effects on the same endpoints. Fractions that induce effects are chemically analyzed to identify the bioactive constituents (reviewed by Hong et al. 2016). This strategy has previously been used to assess OSPW. Morandi et al. (2015) combined the data from Microtox assays and fathead minnow lethality experiments after exposure to fractionated OSPW and found that a fraction containing predominantly NAs caused the greatest effects. Likewise, an MS analysis of fractions that induced rainbow trout fry lethality revealed that NAs with ≥17 carbons were the most toxic (Hughes et al. 2017). Exposure of Japanese medaka to organic fractions of increasing polarity induced nuclear factor erythroid 2-related factor 2 that regulates cellular oxidative stress resistance. Using MS analysis, SO_3^+ and O_3^+ were shown to be the mediators of oxidative stress (Sun et al. 2017). Consequently, EDA using the established macrophage bioassay is a promising avenue for determining the identity of various mediators of OSPW bioactivity observed in this thesis.

6.4.2 Whole organism studies

The simplest way of associating the bioactivity observed in this study to biologically significant outputs would be to replicate exposures in whole organisms. Although it seems redundant to use this bioindicator system alongside an *in vivo* model, it can be of benefit. Using *in vivo* models generally results in longer exposure times and sample collection requires greater processing compared to a cell line. Therefore, screening for OSPWs with the greatest bioactivity, as observed in the cell-based bioassay, and only exposing the whole organism model to those samples could streamline *in vivo* exposures. De Brabanter et al. (2013) compared metabolism of

a cannabinoid in human liver microsomes to chimeric mice with humanized livers to determine the relevance of the *in vitro* indicator system. They found that the *in vivo* model was able to confirm metabolites observed in the *in vitro* model. This reinforces the results of the cell bioassay and supports its use as a bioindicator whose biological relevance can be supported with *in vivo* experiments.

One important consideration for this strategy is the relationship between the *in vitro* and *in vivo* models. As mentioned previously, macrophages are a cell type found in all vertebrates and invertebrates and are even considered relatives of aquatic amoeba. This suggests that, regardless of the RAW 264.7 cell line being from mice, results obtained using these mammalian immune cells can be compared with other organisms that possess macrophage-like cells. However, the translatability between *in vitro* models and *in vivo* models, and between species, is unclear. As a result, the systems biology verification for Industrial Methodology for PROcess VErification in Research (IMPROVER) has attempted to establish how well data was analysed for rat and human experiments that exposed epithelial cells to 52 types of stimuli. Specifically, IMPROVER examined how similar the analyses were and found variability between the statistical significances calculated by 49 different research groups. This suggests that, not only will experimental variation impact the translation of biological significance between models, but so will methods of analyses (Rhrissorrakrai et al. 2015). Overall, this would be a challenge for confirming biological relevance but could be mitigated by analyses being conducted by the same project operators as opposed to collaborating between researchers.

6.5 Future Directions

The introduction of environmental baseline water samples is an important addition to the repertoire of OSPWs that have been examined in my thesis and previously in our lab. Currently, pro-inflammatory gene expression profiles generated using this bioassay have only been performed on process waters (i.e. OSPW). This disregards the natural bioactivity that regional water samples might possess. As a result, the effects mediated by the OSPW samples examined in this study may be misattributed to OSPW constituents from the bitumen extraction process as opposed to innate bioactivity present in the oil sands region water sources. Potential baseline waters that could be analysed include Athabasca river water collected upstream of oil sands mining areas or the freshwater sources that are being used as freshwater caps for EPLs. Many of these environmental samples have already been examined using the 24 hr functional assays described in Lillico et al. (unpublished data) but their analysis using this gene-based bioassay would be informative. By expanding the range of waters tested to include natural sources, an effective baseline of expression could be established, which would aide in assessing OPSW bioactivity and remediation targets.

Likewise, more attention should be paid to the influence the microbial communities have on OSPW bioactivity. One of the objectives for using a cell-based bioassay was to develop a tool to identify potential constituents of concern within OPSW. Thus, to mitigate the contributions of microbes to pro-inflammatory gene upregulation, better filtration of samples should be implemented. More broadly, generating microbial profiles for water samples and examining their interactions within OSPW would be a benefit to this assay by providing a more holistic view of potential OSPW bioactivity mediators.

In terms of evaluating OSPW bioactivity, the suite of pro-inflammatory genes being analysed should be expanded. Bioactivity was differentially upregulated based on the gene examined. By increasing the number of genes assessed, a clearer understanding of OSPW effects will be generated. This was emphasized following macrophage exposure to alum treated and untreated OSPW as I observed unique results and differences in statistical significance depending on the gene. For instance, *mcp-1* expression was significantly lower after OSPW #3 exposure compared to OSPW #6 exposure across the entire time course. Conversely, *inos* expression was not consistently different between OSPW #3 and OSPW #6. Therefore, OSPW does mediate bioactivity, but the bioactive effects observed are dependent on the endpoint that is quantified. By only examining a small repertoire of genes, observing nuances in bioactivity between OSPW is diminished. Other genes that could be monitored in addition to those in this thesis include other pro-inflammatory genes (*tnf-α*), stress genes (*gadd45*), immune cell receptors (TLRs), or genes involved in metabolism and biotransformation like the cytochrome P450 family.

Lastly, this bioassay should complement other analytical approaches for assessing OSPW. I have discussed the pairing of my data with microbial and chemical analyses however there are several other avenues of research that would provide insight into OSPW effects. For example, sediment analyses can also identify and characterize constituents. This can determine which OSPW components precipitate and settle during the ageing process compared to those that remain in the aqueous environment. Moreover, it can evaluate the movement of constituents between phases which may affect bioactivity over time (Fawcett et al. 2015). Soil analysis and accompanying plant analysis provide insights into the movement of OSPW constituents through an ecosystem (El Azhari et al. 2017). Moreover, examining the uptake of pollutants in plants can

determine which constituents are likely to bioaccumulate in higher trophic levels (Naikoo et al. 2019). Therefore, assessing OSPW bioactivity using this assay in tandem with other examination strategies will aide in our understanding of OSPW impacts on northern Alberta's landscapes.

6.6 Final Conclusion

In this thesis, I developed a cell-based bioindicator assay that quantifies changes in pro-inflammatory gene expression via qPCR following macrophage exposure to OPSW. OSPW is a complex mixture of constituents that must be remediated prior to its release into surrounding ecosystems. Consequently, it is imperative that methods are established that enable OSPW monitoring and characterization as a means of assessing its potential impacts on the environment. Macrophages were selected as a bioindicator system due to their rapid response to homeostatic changes. I monitored changes in the expression of *inos*, *ip-10*, *mcp-1*, and *mip-2*, that also serve as functional biomarkers. Using this bioassay, I observed unique basal upregulation of these genes in response to OSPW #2 and OSPW #7. When I examined the IF and the OF, I observed that bioactivity segregated to the OF from OPSW #2, and the IF from OSPW #7. In stimulated macrophages, however, OSPW #7 treatments selectively abrogated gene expression. This suggests that agonistic and antagonistic interactions occur between OSPW constituents and macrophages and that these depend on the macrophage state of activation and the gene examined.

In my second chapter, I assessed the sensitivity of this bioassay by exposing cells to OSPW #2 IF-OF reconstituted mixtures and alum treated and untreated OSPW. The results from the reconstitution experiments were inconclusive despite strong evidence at the protein level showing that interactions between OSPW #2 OF with increasing concentrations of IF reduced

the presence of *inos* and enhanced the secretion of cytokines in resting macrophages. I hypothesize that interactions between fractions were not facilitated in my exposure environment, which led to inconclusive data. Finally, discrete differences in gene expression were induced following exposure to treated OSPW #3 and untreated OSPW #6, where treated water caused lower pro-inflammatory gene upregulation compared to untreated water. *mcp-1* and *mip-2* were the best indicators of the effects of alum treatment based on a consistent expression pattern and a large magnitude of expression level differences, respectively.

Overall, my thesis work has established a high throughput immune gene-expression based bioindicator system that generates outputs much earlier (2 hrs) than protein level analyses (24 hrs) and is sensitive to the differences between complex OSPWs. In the future, this bioassay should be complimented by other types of analyses that will contribute to a more thorough understanding of OSPW for monitoring, and perhaps remediation, purposes.

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