Toxic effects induced in mammalian immune cells after *in vitro* exposure to oil sands process-affected water and its fractions

by

Nicole Anne Irene Phillips

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology, Cell and Developmental Biology

Department of Biological Sciences

University of Alberta

Abstract

Oil sands process-affected water (OSPW) is produced by the surface mining industry in Alberta as a byproduct of the Clark hot water process, the currently used extraction method of crude oil from bitumen/tar sands. Under a provincial zero release policy all produced OSPW must be stored on site. The footprint and volume of stored OSPW represents a difficult environmental challenge. The characterization of OSPW toxicity is critically important for remediation efforts, as well the protection of ecosystem and public health. Chemical analysis of OSPW indicates a highly complex mixture of over 400 constituents of both organic and inorganic origin. OSPW can be physically separated into the organic fraction (OSPW-OF) and inorganic matrix (OSPW-IM). Exposure of test organisms to the organic fraction of OSPW impairs immune function at a cellular and organismal level. These effects include both stimulatory and suppressive deviations from homeostasis which may lead to increased susceptibility to infection and chronic inflammation. It is well established that the naphthenic acid containing OSPW-OF induces dose-dependent toxicity in aquatic and terrestrial organisms. In contrast, the potential toxic effects of the OSPW-IM remain to be comprehensively examined. The overall objective of my thesis was to evaluate the toxic effects in mammalian immune cells exposed to OSPW-IM.

The effects induced by the whole (crude) OSPW, OSPW-OF, and OSPW-IM were compared *in vitro* using the RAW 264.7 macrophage-like cell line and a number of different bioassays. The results showed that acute exposure of cells to OSPW-IM significantly affected cellular viability, metabolism, gene expression, protein synthesis and function. The magnitude of toxic effects induced by exposure of cells to the OSPW-IM was similar to those

induced by whole OSPW and not OSPW-OF. The exposure to either the whole OSPW or OSPW-IM caused functional changes in macrophages that may profoundly influence the ability of the host to maintain homeostasis and/or control exposure to pathogens. The gene expression analyses in conjunction with enzyme abundance and function revealed that whole OSPW and OSPW-IM possessed immunomodulatory properties causing changes in macrophage polarization status.

The results of my thesis research represent the first comprehensive analysis of the toxic effects of OSPW-IM and its contributions to the overall toxicity of whole OSPW, using multiparametric analysis of toxicity caused by complex xenobiotic mixtures.

Preface

My thesis research is the first comprehensive evaluation of the toxic effects of the inorganic matrix of OSPW. I designed and performed majority of the experiments presented in this thesis under the supervision of Dr. Miodrag (Mike) Belosevic and Dr. James L. Stafford, Department of Biological Sciences at the University of Alberta in Edmonton, Alberta, Canada.

Select data in Chapter 3 of this thesis has been published as: Fu, L., Li, C., Lillico, D., Phillips, N., Gamal El-Din, M., Belosevic, M., and Stafford, J., 2017. Comparison of the Acute Immunotoxicity of Nonfractionated and Fractionated Oil Sands Process-Affected Water Using Mammalian Macrophages. Environmental Sciences & Technology, 51 (15): 8624-8634. In this paper, I performed cell culture and set up, OSPW exposures and specifically generated results shown in Figures 1, S1, and S2.

The results presented in Chapters 4 and 5 of this thesis were generated by myself with the exception of flow cytometry assays, which were done in collaboration with Dr. D. Lillico.

Acknowledgements

The opportunity to embark on this research journey would not have been possible without the support, encouragement, and patience of my supervisor, Dr. Belosevic. My sincerest and deepest appreciation go to him and the members of my committee: Dr. Stafford and Dr. Tierney, who guided me to the completion of this thesis. Dr. Stafford, I am especially grateful for the technical guidance, company to conferences, and advocacy you have provided for me. Dr. Tierney, thank you for seeding my interest in research and leading me to the Belosevic lab.

Mike, I am so proud to have been a member of your lab and to join the legacy you have created in our scientific community. Thank you for teaching me resolve, how to think critically and of course- to learn from and value my failures.

To all of the previous Belosevic students: thank you for leaving behind a culture that has driven me to do my best. Dr. Dan Barreda, Dr. Dustin Lillico, Dr. Jordan Hodgkinson, Dr. Jiasong Xie, and Dawn Kieller; working with you all has taught me so much about the scientific process. Danyel Evseev, you have been a great friend and go-to for trouble-shooting, a special thank you for your infinite optimism. Mark McAllister, you are one of my dearest friends. Our midnights in the lab, laughable failures, pep-talks, and picking each other back up are some of my favourite and most cherished memories from our time on the third floor.

Thank you to the staff at MBSU, particularly Troy Locke, for being an invaluable resource in learning and completing molecular experiments. Similarly, thanks to the staff in Bio-Stores and the administrative staff of the fourth floor. I would also like to thank members of the Gamal El-Din lab in the Faculty of Engineering for providing me with more than enough water for my experiments.

This thesis would not have been possible without the help and support from my parents, Wayne and Patti, and my brother, Rocky. Mom and Dad, thank you for encouraging me to follow my own path, and persevere, even when it was bumpy.

Lastly, I would like to acknowledge financial support provided by Natural Sciences and Engineering Research Council in the form of an operating grant to Dr. Belosevic, The Department of Biological Sciences (Teaching Assistantship), the Faculty of Graduate Studies and Research (Queen Elizabeth II Scholarship), and the Alberta Innovates Technology Futures Scholarship.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AEO	Acid extractable organics
AhR	Aryl hydrocarbon receptor
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BMDM	Bone marrow derived macrophage
C-NAs	Commercial naphthenic acids
CCME	Canadian Council of Ministers of the Environment
CFS	Coagulation/floccation/sedimentation
CytoD	Cytochalasin D
DCM	Dichloromethane
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
EROD	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HCl	Hydrochloric acid
HMDS	Hexamethyldisiliazene
IC50	Inhibitory concentration 50%; the concentration that induces an inhibitory effect of 50%
IDRs	Innate defence regulators
LC50	Lethal concentration 50%; the median lethal concentration
LOEC	Lowest observable effects concentration
LOEL	Lowest observable effects level
LPS	Lipopolysaccharide
MATC	Maximum acceptable toxicant concentrations
MC	Media/medium control

mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide
MW	Molecular weight
NaOH	Sodium hydroxide
NAs	Naphthenic acids
NO	Nitric oxide
NOEC	No observable effects concentration
NOEL	No observable effects level
NSD	No significant difference
OSPW	Oil sands process-affected water
OSPW-IM	Oil sands process-affected water inorganic matrix
OSPW-OF	Oil sands process-affected water organic fraction
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PKM	Primary kidney derived macrophages
qPCR	Quantitative real time polymerase chain reaction
rgEPO	Recombinant goldfish erythropoietin
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RXN	Reaction
ScEM	Scanning electron microscopy
SEM	Standard error of the mean
Std	Standard
TBST	Tris-buffered saline + Tween 20
TDS	Total dissolved solids
TOC	Total organic carbon
UPLC/HRMS	Ultra-performance liquid chromatography/high resolution mass spectrometry
V/E	Volume equivalent
V/V	Volume per volume concentration
W/W	Weight per weight concentration

Chapter 1: General Introduction

1.1 Background

Surface mining from the Alberta oil sands produces large volumes of oil sands process-affected water (OSPW) as a by-product of the Clark hot water extraction process; a method that separates bitumen from contaminants including clay, sand, organic compounds and metals under hot (79-93 ^{0}C) water and caustic soda (1). OSPW is currently stored in tailings ponds at site under a zero-release policy (Alberta Environmental Protection and Enhancement Act, 1993) which is surrounded by significant controversy and concern over environmental impacts as well as ecosystem health (2). Characterization of OSPW is challenging because of its complexity, containing well over 400 constituents that vary between different companies and source sites. Most broadly, OSPW is separated into the organic fraction (OSPW-OF) and inorganic matrix (OSPW-IM). OSPW has been reported to cause both acute and sub-chronic toxicity to a variety of organisms, including microorganisms, aquatic invertebrates, fish, amphibians, birds, and mammals (3–8). The principal toxic component(s) of OSPW that affect test organisms are yet to be fully identified. The toxicity of OSPW has largely been suggested to be due to naphthenic acids (NAs) that comprise about 40 to 45 % of the organic fraction. Comparatively, little is known about the contribution of the inorganic matrix to the toxicity of OSPW.

Exposure of fish and mammals to the organic fraction of OSPW impairs immune function at a cellular and organismal level. These effects include both suppressive and stimulatory deviations from homeostasis leading to an inability to clear, and/or increased susceptibility to infection, and chronic inflammation. The organic fraction has been shown

to induce dose dependent toxicity in aquatic and terrestrial test systems and has been the primary focus of OSPW research to date, while toxicity of the inorganic matrix has largely been undetermined. Preliminary experiments analyzing toxicity of unfractionated (whole) OSPW have shown similar toxic effects as the organic fraction but at lower NA concentrations suggesting a key role of the inorganic constituents in direct, or additive/synergistic toxicity when present in whole OSPW.

The organic fraction of OSPW contains remnant bitumen, naphthenic acids (NAs), phenols, asphaltenes, cresols, polyaromatic cyclic hydrocarbons (PAHs), humic and fluvic acids, phthalates and BTEX (benzene, toluene, ethylbenzene and xylenes) (9,10). The toxicity of the organic fraction is currently attributed to NAs, a complex mixture of aliphatic and (poly) alicyclic carboxylic acids, but NAs constitute less than half of the organic fraction by percent, suggesting that toxicity is due to compounds in addition to NAs (10,11). Several studies have shown that exposure of fish and mammalian cells *in vitro* and aquatic and terrestrial organisms *in vivo* to the organic fraction of OSPW alters viability, development, endocrine regulation and immune responses (7,12–17) . Commercial NAs (C-NAs) have been used in comparison with the OSPW-OF where both have been attributed to decreases in respiratory burst, phagocytosis, and antimicrobial response of murine macrophages (11). Alternatively, macrophages from goldfish exposed acutely to C-NAs showed increased pro-inflammatory cytokine expression, nitric oxide production and respiratory burst, however these weakened following sub-chronic exposures (4). In the case of murine macrophages, the magnitude of the suppressed responses were insufficient to indicate that NAs are the only toxic component of the organic fraction of OSPW (7).

In comparison to the OSPW-OF, there has been limited investigation into the toxic effects of the OSPW-IM on test systems. Composed of mainly metals and salts, most insight to OSPW-IM toxicity on organisms has stemmed from extrapolating the toxicity of individual components (10,19). Unfortunately, this approach does not account for possible additive/synergistic effects of the multiple contaminants within OSPW. Several known toxicants within the OSPW-IM have been shown to have a significant role in immunosuppression, neurotoxicity, and developmental impairments (20–24). Heavy metals such as lead, cadmium, and mercury have been associated with increased susceptibility of mammalian hosts to bacterial and viral infections (25). Specifically, animals exposed to lead or mercury have reduced antibody titers following pathogen challenge or immunization, while cadmium was shown to be directly toxic to macrophages (25). In addition to heavy metals; aluminum, arsenic, cobalt, copper, iron, molybdenum, vanadium and nickel are present in amounts exceeding exposure recommendations by the Canadian Council of Ministers of the Environment (CCME) (10). This is of importance since resistance to infectious agents by experimental animals was shown to be reduced when exposed to arsenic compounds, cobalt sulphate, and nickel (26–28).

The focus of my thesis research was to compare toxic effects of whole OSPW, OSPW-OF and OSPW-IM in RAW264.7 mammalian macrophage-like cell line. I proposed to generate a dataset that would contribute to the development of effective and consistent toxicity screening tools for risk assessments and regulations regarding OSPW management for the protection of aquatic, wildlife, environmental, and public health.

1.2 Research objectives

As an established *in vitro* bio-indicator system, the RAW 264.7 mammalian macrophage-like cell line was a suitable candidate for the first evaluation of inorganic constituents of OSPW. The primary objective of my thesis research was to assess the toxic effects and contributions of inorganic components of oil sands process-affected water to whole effluent toxicity in comparison to the organic fraction. My specific objectives were to:

1. Evaluate the acute toxicity of the OSPW-IM, and its contributions to whole OSPW toxicity.
2. Assess the immunotoxic potential of whole OSPW and its fractions.
3. Determine a mechanism of immunomodulatory effects of whole OSPW and OSPW-IM

Chapter 2: Literature Review

2.1 Immunotoxicology

Immunotoxicology is defined as the study of toxic effects on the immune system following exposure to xenobiotics (29). Assessing the immune system is achieved through careful analysis of immunologic events at multiple levels; from regulation to function, to pathogen challenge itself. The collection of xenobiotics capable of altering immunologic events is expanding and includes known pharmaceutical drugs as well as environmental pollutants.

Important information can be gathered when analyzing the immunotoxic potential of environmentally sourced xenobiotics; where these contaminants induce a threshold of effect capable of increasing disease sensitivity or neoplastic potential, there is an obvious requirement for monitoring of animal, human, and environmental health (30). On the other hand, the potential to harvest the sensitive and tightly regulated responses of the immune system as a bio-indicator tool for the assessment of toxic potential of xenobiotics may play an important role in protection of affected populations through the development of guidelines and policy to limit exposure in a native setting.

2.1.1 Advantages of immune-based assays:

Immune based assays present many advantages in the multidisciplinary field of toxicology. For example, in clinical toxicology there has been an expansion of available assays that are highly specific for an array of different metabolites and/or drugs that induce toxic effects. The time to identification of suspect drugs or poisons is key in clinical

toxicology; making several advantages of immune-based methods important in selecting their use in a clinical setting. Some of these advantages are: rapid delivery of results in a near-patient setting, robust results with small sample volume, sensitivity, and low-cost (31,32). Examples of immune-based techniques used in clinical toxicology include: Cloned Enzyme Donor ImmunoAssay (CEDIA ®), Enzyme Linked ImmunoSorbent Assay (ELISA), Enzyme Multiplied ImmunoAssay (EMIT ®), Fluorescence Polarization ImmunoAssay (FPIA), and RadioImmunoAssay (RIA) (31). Currently, the use of rapid antibody-based urine screening tools [Enzymatic ImmunoAssays (EIAs)] are the standard for drug screening at most points of care and patient management (33). The main benefit of using immunological techniques is the growing availability of the number of tests that are highly sensitive and specific. The quick application of EIAs to rule out negative specimens such that only positives are subjected to more rigorous analysis for identification has increased the efficiency of substance identification and decreased the costs (31,33).

Immune-based test methods have also become important in environmental toxicology and provide more sensitive measures of toxic effects as compared to lethal dose (LD_{50}), or lethal concentration (LC_{50}) values. The basis of toxicology using an "assembly line" system, where potential toxicants are assessed *in vivo* for their LD/LC_{50} values, MATC (maximum acceptable toxicant concentration), LOEL (lowest observable effects level), LOEC (lowest observable effects concentration), NOEL (no observable effects level), and NOEC (no observable effects concentration) to set limits of safe exposure in guidelines have documented limitations when being extrapolated to human, and eco-system protection; driving a shift toward high-throughput *in vitro* systems (34,35). Immune-based methods for

the evaluation of environmental pollutants can be done using immune cells in an *in vitro* setting or by assessing immune parameters *in vivo*. Analysis *in vivo* may take advantage of measuring multiple immune readouts, both conserved functions like phagocytosis, or organism specific, such as hemocyte counts in invertebrates, or adaptive immunity in mammals (30,36). Considering the commercial availability of kits and reagents for immunological assays, utilizing immune cells for *in vitro* studies can drastically expand the endpoints available when compared to tissue cell-lines.

Using an immunotoxicological approach in marine mammals, a robust assessment of environmental pollutants was compiled *in vivo* and compared to the *in vitro* assays, showing little variation in their results suggesting, and supporting that *in vitro* assays may predict the outcome of *in vivo* toxic effects (37). Similarly, the assessment of toxic potential of nanomaterial using common indicators *in vivo* have been identified. They include hemolysis, complement activation, thrombogenicity, pyrogenicity, cytokine induction, and phagocytosis– all of which are outputs that can be evaluated using *in vitro* systems (38). Whereas *in vivo* studies are invariably resource heavy, expensive, and ethically restrictive, the use of *in vitro* assessments can either replace or, in most instances provide preliminary information regarding the potential toxicity of test xenobiotics. In comparison to toxicological assessments of survival, which have significantly different results between *in vivo* and *in vitro* studies, the potential to reduce the footprint of whole-animal use is another advantage to using immunotoxicological approaches for assessment of toxic effects of xenobiotics.

2.2 Immunotoxicology and environmental pollutants

Considering the difficulty of transforming toxicological data into risk assessments, identifying systems that produce meaningful results in the context of a whole organism, animal population or eco-system is critical (34). For example, generating an immunotoxicological index score for marine mussels showed a correlation with pollution intensity (39). This index then enabled sites to be classified and monitored by assessing the mussel populations for their immune status using a variety of tests such as total hemocyte counts, lysosomal stability, and phagocytosis (39). The index score reflected differences in the xenobiotic composition of contaminated sites, indicating the potential sensitivity of immunological readouts, an important factor for application in risk-assessment (34,39).

Environmental pollutants of anthropogenic origins such as heavy metals, pesticides, and insecticides have been shown to have immunotoxic effects in test organisms. Wood mice (*Apodemus sylvaticus* L.) are a commonly used test organism for monitoring heavy metal pollution as an endemic population in Europe. These mice are susceptible to several parasites including nematodes and ectoparasites and when challenged with these pathogens, their ability or inability to control the infections was directly related to their immunological host defense prowess (40,41). Mice caught closer to a non-ferrous heavy metal smelter had increased rates of infection with the nematode *Heligmosmoides polygyrus*, increased spleen mass, and increased numbers of apoptotic cells; all correlated with increasing concentrations of organ-bound heavy metals (40). In rapidly urbanizing areas, wetlands have become sources of heavy metal and other xenobiotic accumulation (42). In the native frog species *Euphlyctis hexadactylus*, the weight of lymphoid organs, number of peripheral blood leukocytes, and immune functions such as phagocytosis were negatively affected, and the

results paralleled the *in vitro* assay findings, providing complimentary evidence of immunosuppression (43).

Pesticides and insecticides are purposely used to produce selective-toxicity on target species, unfortunately these often result in secondary effects which includes modulation of immune responses in invertebrates, fish, and higher vertebrates. Organophosphorus pesticides (OP) have been shown to induce histopathological changes in immune tissues and organs, lymphocyte maturation, and function (44). Other pesticide types, such as carbamates have been implicated in altering the immune response leading to autoimmune diseases, hypersensitivity reactions, and cancers, by acting through multiple mechanisms at enzymatic and signal transduction levels (45). Pesticides and insecticides present a unique toxicological dilemma, where they are released according to guidelines that have been determined that they do not present an acute-toxicity risk to non-target organisms, but their ability to modify immune responses may cause other toxic effects and be associated with elevated morbidities and mortalities.

2.3 Immunotoxicology and industrial wastewaters

Outside of environmental pollutants, industry-produced wastes also present a unique toxicological risk. Wastewaters generated from many industrial processes may be potentially toxic. For example, wastewaters produced by industries such as textile dyeing or pulp and paper have successfully implemented remediation techniques within guideline parameters for environmental release (46,47). Although these guidelines may be based on the more typical and traditional toxicological assessments such as LD/$LC_{50\%}$, NOEL, NOEC, LOEL, and LOEC values, it is currently accepted as remediated water. While all industries producing

potentially contaminated wastewaters should continue monitoring for, and assessing additional toxic effects, such as immunotoxicity, the wastewater produced by surface mining of tar-sands, oil sands process-affected water (OSPW) is not currently eligible for release under any guidelines due to its uncharacterized toxicity and complex composition.

2.3.1 OSPW

The Alberta oil sands are considered the third largest oil reserve in the world, with estimates that over 170 billion barrels of recoverable oil are available using current method of extraction (1). Unfortunately, recovery of unconventional crude oil sources such as the oil sands contributes to drastic changes in the landscape and is associated with controversy surrounding exploitation of such resources. Oil sands process-affected water (OSPW) is a by-product of the Clark hot water extraction process; a method that separates bitumen from contaminants including clay, sand, organic compounds, and metals under hot (79-93 °C) water and caustic soda (1,48). OSPW is defined by Natural Resources Canada as any water which has contacted oil sands, therefore, the surface mining industry in Alberta is constantly producing large volumes of OSPW that must be stored on-site due to a zero-release policy (Alberta Environmental Protection and Enhancement Act, 1993) (19). The footprint and volume of stored OSPW, estimated to cover over 180 square kilometers in 2017 and growing, has gained international attention and represents a unique environmental challenge in Alberta due to unknowns in characterizing its toxicity for remediation efforts.

OSPW contains a variety of both inorganic and organic toxicants that have demonstrated significant perturbation to test organism homeostasis, which has contributed to concern surrounding accidental release, seepage, precipitation contamination in the

hydrologic cycle, and bioaccumulation. Therefore, it is important to develop effective and consistent toxicity screening tools for risk assessments and regulations regarding OSPW management for the protection of aquatic, wildlife, environmental, and public health.

Characterization of OSPW and its associated toxicity is challenging due to its complex nature and differences in content when generated by different companies and/or mining sites. Most broadly, OSPW components can be physically separated into organic and inorganic components. Naphthenic acids (NAs), phenols, and polycyclic aromatic hydrocarbons are major components of the organic fraction (OSPW-OF), while heavy metals such as lead, arsenic, mercury, and salts are contained within the inorganic matrix (OSPW-IM). OSPW has been reported to cause both acute and sub-chronic toxicity to a variety of organisms, including aquatic invertebrates, fish, amphibians, birds, and mammals (1-5,7,49, 50). The principal toxic component(s) of OSPW that affect test organisms have not been fully identified due to the complexity and number of different constituents in OSPW.

2.4 Toxicity of OSPW

2.4.1 In vitro studies

Acute toxicity of OSPW has been studied using multiple *in vitro* systems including primary cells, cell lines, and bacteria to assess endocrine disruption, stress, functional impairments, and cell viability (18,51–53). Development of *in vitro* models for OSPW toxicity screening is a useful tool because of the repeatability, high capacity, and reduced cost relative to *in vivo* systems. *In vitro* results are typically presented as inhibitory, or lethal doses causing 50% reductions in a function (IC_{50}) or viability (LC_{50}), respectively. This allows for the results of different toxicant exposures and studies to be easily compared by

similar metrics. Perhaps the most advantageous is the speed that *in vitro* analysis can be completed for preliminary identification of toxicity and water remediation effectiveness.

The Microtox assay using *Vibrio fischeri* has been used to directly measure narcosis following acute exposure to NAs. Relatively lower molecular weight NAs demonstrated increased toxicity to *V. fischeri*, contradicting the increased bioaccumulation capacity of high molecular weight NAs, and therefore increased narcotic potential (54).

Studies using human cell lines exposed to OSPW have shown anti-androgenic and estrogenic effects, likely mediated by modulation of different receptor pathways. The anti-androgenicity was observed following exposure to ozonated, as well as non-ozonated OSPW (which degrades up to 90% of NAs) indicating that components other than NAs are contributing to the endocrine disruptive effects (51).

Work evaluating the immunotoxic potential of OSPW showed that exposure of macrophages to the OSPW organic fraction (OSPW-OF) containing high NAs concentration impaired nitric oxide and respiratory burst production and phagocytic potential of murine macrophages (18). More recently the OSPW-OF was shown to not have significant effects on the function of murine macrophages at lower NA concentrations, but whole OSPW at the equivalent NA concentrations as OSPW-OF did (13). Additionally, it was recently found that ozonation of whole OSPW did not change the observed inhibitory effects with respect to nitric oxide response, and cytokine production, however previous work indicated that

ozonation of OSPW eliminated the suppressive effects of OSPW-OF on bone marrow derived macrophages of mice (55,56).

2.4.2 In vivo studies:

OSPW has been reported to cause toxic effects in a variety of organisms. Endocrine disruption, developmental impairment, oxidative stress, and immune dysregulation have been documented following exposure to OSPW. Understanding the disruptive effects of OSPW is key to permitting accurate monitoring of nearby regions through toxicological tests and bioassays.

2.4.2.1 Invertebrates:

Multiple studies using different OSPW sources have documented physiological impacts of OSPW exposure to both adults, and developing invertebrates. Effects on the freshwater crustacean, *Daphnia magna,* have been assessed and it was reported that after 96 hours of exposure the LC_{50} was 16-27% OSPW (V/V) (57). Interestingly, more recent studies have reported values of LC_{50} over 100% (V/V) for *D. magna* (53,58). This demonstrates the complexity and unpredictability of OSPW coming from different sources, and of different ages. Sub-lethal concentrations of OSPW have been shown to impair feeding, reproduction, and growth of *D. magna* which could further limit high energy processes required for a successful life history (58).

In addition to the freshwater crustaceans, that spend their entire lifecycle in an aquatic environment the midge, *Chironomus dilutus,* has also been found to have physiological changes upon exposure to non-lethal concentrations of OSPW. Considering the role of *C.*

dilutus as a major biomass contributor in Alberta wetlands, a reduction in mass and successful development to adults may present challenges for the entire food chain (3,59). Additionally, the expressions of genes involved in oxidative stress and endocrine signalling were found to change following exposure of both fresh and aged OSPW indicating possible effects of these pathways (59). Although the measured indicators of toxicity were associated with NA concentration, the toxic effect on gene expression persisted in aged OSPW, where NAs and organic compounds should be degrading, suggesting that non-organic components may have a role in induction of these effects.

2.4.2.2 Amphibians:

Surface waters are the primary location for amphibian growth and development making them highly sensitive to water contaminants, especially in their larval stages. At concentrations of NAs as low as 6 mg/L, 100% of larval and embryonic mortality has been observed in frogs (60). Considering ground water NA concentrations have been recorded as high as 51 mg/L, and concentrations in the Athabasca River are up to 0.9 mg/L the potential for animal contact with NAs close to or above the threshold levels for physiological effects is not irrelevant (61). Frogs exposed to doses below the lethal concentration have experienced significant decreases in growth and development, including when exposed in a wetland with seepage water (60,62). It is well established that fitness and survival in later life stages is affected by tadpole size. Therefore, reduction in their growth following exposure to NA concentrations found in natural OSPW and surrounding environmental waters could extend into the lifetime of frogs and other amphibians.

2.4.2.3 Birds:

Wetlands containing OSPW constituents form as a by-product of storage in dykes through seepage, as well as are intentionally built as part of reclamation strategies. Those that form incidentally by seepage through the deep dykes are home to water that is up to 80% original tailings water (50). These wetlands are highly attractive to several bird species as breeding grounds (50). Ducklings raised on dyke seepage wetlands, or consolidated tailings wetlands were smaller in body size than those on a reference wetland free of OSPW contamination; because body morphology (including size) is related to survival and breeding success of birds, effects could contribute to relevant ecological losses. Other research has reported that ducklings exposed to OSPW did not have any significant differences in mass or survival compared to reference water, indicating that frequent, short term exposure may not induce toxic effects (63).

Regardless of inconsistencies in the literature regarding acute toxicity, it was found that ducklings in dyke seepage wetlands had significantly increased amounts of polycyclic aromatic hydrocarbon metabolites than the reference and EROD activity in tree swallows exposed to consolidated tailings was 2 times higher than control and 1.9 times higher for those in a wetland receiving dyke seepage water. This indicates that birds can retain at least some of the contaminants we know of in OSPW.

2.4.2.4 Fish:

Adult rainbow trout and fathead minnows have been subject to multiple $LC_{50\%}$ studies using fresh OSPW. The recovered LC_{50} concentrations have ranged from <4% to 35% (V/V) for rainbow trout and from 6-8.5% (V/V) for minnows in 96 hour exposures (53,57,64).

Other work in younger life cycle stages of fish, including fingerlings and early life stages (days post fertilization to hatch) of rainbow trout, fathead minnows, and walleye indicate they are more sensitive to OSPW with respect to lethality (65,66). For example, fathead minnow embryo survival rates decreased by approximately 55% upon exposure to OSPW (67). In addition to direct effects on survival, embryos of zebrafish experienced increased heart rates and rates of muscle deformities when exposed to crude oil (68).

Reproduction impairment has been documented in several fish species following exposure to OSPW or OSPW-NA extracts. Since NAs have been, and are, the currently accepted predominant toxicant of OSPW, there has been focus on using only NA extracts for animal exposures to determine their specific role. Fathead minnows exposed to NA extracts had reduced plasma concentrations of testosterone and 11-ketotestosterone, limiting spermatogenesis and reproductive behaviour in these fish (66,69). Yellow perch, and goldfish have also been shown to have decreased testosterone and estradiol concentrations following OSPW exposure (16,70). Additionally, some NAs have structural similarity to steroid sex hormones, meaning they could directly feedback through receptors such as the estrogen receptor (67).

Challenging exposed fish with pathogens illustrated the difference between acute and sub-chronic effects of OSPW. Acute exposure enhanced ability of fish to control infection, likely because of an induced pro-inflammatory state observed by elevated pro-inflammatory cytokine transcripts (15). The gene expression became significantly down regulated

following longer (sub-chronic) exposure to OSPW, which could later negatively impact the ability to control infections (15).

2.4.2.5 Mammals:

Toxicology studies in mammals exposed to OSPW are less common than other *in vivo* systems. Rats and mice have been the primary subjects, showing organ damage, reproductive impairment, and immunotoxicity (7,18,55,71). Organ damage, specifically heart, liver, and brain in rats was observed using extremely high NA doses compared to what would be observed in a natural interaction between OSPW and an animal (71). Following exposure to more environmentally relevant doses, macrophage function as well as immune gene expressions were affected in mice, but the expression changes did not corroborate with cytokine protein levels (7,72). Additionally, a comprehensive analysis of only OSPW-OF exposure to pregnant mice demonstrated an overall insignificant effect on many parameters including: pregnancy rate, resorption, fetal and placental weight, as well as hormone levels and histology (56,72). There have not been any studies reported using OSPW-IM specifically in mammals *in vivo* to date, leaving the possibility of non-organic compound mediated toxicity still an open question.

2.5 OSPW and its fractions

2.5.1 Whole (crude) OSPW

Spectrophotometric analysis has indicated that OSPW is composed of over 400 chemical constituents, of both organic and inorganic origin. Further complicating assessments of OSPW is the variability in its composition between different production sites and companies which generate it. The composition can be so variable between sources that OSPW can be used as a "chemical fingerprint" to track its creator (73). The potential

chemical interactions, synergistic or counteractive, that may occur depending on constituents and relative volumes or amounts is vast further contributing to the difficulty in predicting crude OSPW toxicity and designing consistent assessment systems or tools.

2.5.2 OSPW organic fraction (OSPW-OF):

The organic fraction of OSPW contains remnant bitumen, naphthenic acids (NAs), phenol, asphaltenes, cresols, polyaromatic cyclic hydrocarbons (PAHs), humic and fluvic acids, phthalates and BTEX (benzene, toluene, ethylbenzene and xylenes) (9). The toxicity of the organic fraction is currently attributed to NAs, a complex mixture of aliphatic and (poly) alicyclic carboxylic acids, but NAs constitute less than half of the organic fraction, approximately 40 to 45%, suggesting that toxicity is due to compounds in addition to NAs (10,11,74). Several studies have shown that exposure of fish and mammalian cells *in vitro* and aquatic and terrestrial organisms *in vivo* to the organic fraction of OSPW alters immune responses (4,7,48).

The structure of NAs includes a hydrophilic tail and hydrophobic head, they are typically low molecular weight and therefore able to insert into the lipid bilayer of cells and mimic surfactant activity (71,75). This membrane-disrupting ability makes NAs directly cytotoxic *in vitro* at high doses, however in whole-organism scenarios the toxicity of NAs is observed by assessing fish survival and mammalian and fish immune responses. Fish species and life stages display variable sensitivities to acute toxicity following NA exposure; ranging from the extremely sensitive larval zebrafish where 13.1 mg/L was acutely toxic to sturgeon which are able to withstand 50 mg/L (76,77).

Immune responses of mammals and fish have shown impairment following exposure to NAs and/or OSPW. Commercial NAs (C-NAs) have been used in comparison with the OSPW organic fraction with both demonstrating decreases in respiratory burst, phagocytosis, and antimicrobial response of murine macrophages (7). Alternatively, macrophages from goldfish exposed acutely to C-NAs showed increased pro-inflammatory cytokine expression, nitric oxide production and respiratory burst, however these weakened as exposure time was extended to sub-chronic (4,7).

PAHs are known to have immunotoxic, carcinogenic, and endocrine disrupting properties, these effects are related to their structure as planar aromatic compounds with an anthracene backbone and variable aliphatic additions (78,79). Specifically, PAHs have repeatedly been shown to alter the immune responses of fish and mammals in both the innate and adaptive arms of the immune system (78,80,81). Phagocytes exposed to PAHs have been shown to have impaired phagocytic capacity, chemotaxis, and respiratory burst, while lymphocytes showed reduced proliferation (78). This is further supported by evidence that PAH exposure reduces resistance of fish to bacteria and parasites (82,83). Interference with the fish immune response by PAHs has been suggested to occur by direct interaction with immune cells (78). Mice exposed *in vivo* to high doses of PAHs exhibited lymphoid tissue atrophy and abnormal morphologies in their thymus, spleen, and bone marrow as well as lymphocyte apoptosis (78,84). Immunosuppression in mammals was induced by lower doses than those documented to be directly cytotoxic and was assessed by demonstrating reduced receptor expression, inhibition of cytokine secretion and reduced T-cell dependent B-cell responses *in vivo* and *in vitro* (78). Similar to the direct effects on immune cells, PAHs have

been shown to induce neoplasia by direct PAH-DNA binding, as well as activity as tumor promoters in mammals (79). PAHs are produced by combustion reactions, and are naturally found in oil deposits, however, they are a broad class of molecules which can induce different physiological effects. Currently, their specific role in OSPW toxicity remains to be elucidated.

For both BTEX (benzene, toluene, ethylbenzene, and xylenes) and phenols in OSPW, sub-lethal exposure impairs the immune system of fish, however, in comparison to studies that evaluated NA and PAH toxic effects there is a paucity of data specifically addressing immunotoxicity. BTEX exposures have been shown to cause cytotoxicity and endocrine disruption. Cellular narcosis was observed in fish exposed to BTEX, as well as *in vitro* in HeLa cells (85,86). The roles of fluvic and humic acids appear to be more related to their ability to alter metal-ion chemistry by forming complexes that reduce toxicity to organisms (87). Fluvic acids may also increase the removal of metal ions during water treatment, complicated in OSPW by the separation of metal ions and fluvic acids into the inorganic matrix and organic fraction respectively.

2.5.3 OSPW inorganic matrix (OSPW-IM):

In comparison to NAs or the OSPW-OF, investigations of inorganic constituent toxicity have been largely ignored. Being composed of mainly metal and salts, most insight to the toxicity of OSPW inorganic components has stemmed from extrapolating the toxicity of individual components (10,19). However, this mechanism of study fails to account for potential synergistic effects of multiple contaminants within OSPW.

Heavy metal toxicity has demonstrated immune system suppression in addition to other effects such as neurotoxicity and developmental impairments. Heavy metal contained within the OSPW-IM include arsenic, lead, cadmium, mercury, selenium, vanadium and zinc. Arsenic is known to be carcinogenic, and mediated its carcinogenicity by inhibiting DNA repair and initiating DNA structural changes; observed in rodents and humans *in vitro* (88,89). Altered expression and potential mutation of p53, an important cell cycle regulator, is also documented as a potential mechanism of arsenic mediated tumor formation (89). Lead, cadmium, and mercury exposure have been associated with increased susceptibility of mammalian hosts to bacterial and viral infections (25). Specifically, animals exposed to lead or mercury have reduced antibody titers following pathogen challenge or immunization, while cadmium is directly toxic to macrophages (90–92). Alternatively, selenium is stimulatory toward immune responses and has been shown to recover antibody titer, colony formation, Th1 immune responses (93,94). Opposing effects on aspects of the immune response, such as antibody titer, highlights the potential interplay and complexity of metals found in the OSPW-IM and the ineffectiveness of analyzing toxicity from individual components only.

In addition to heavy metals; aluminum, cobalt, copper, iron, molybdenum, and nickel are all present in OSPW in amounts exceeding recommendation by the Canadian Council of Ministers of the Environment. Resistance to infectious agents by experimental animals was reduced when exposed to cobalt sulphate, and nickel. Nickel specifically weakens macrophage phagocytosis (28).

Salt concentrations in the OSPW-IM present a handful of problems related to direct effects on organism osmotic balance as well as interactions with the metal contaminants. Salt toxicity is species specific, as well as ion specific and has caused reduced survival, growth, and reproduction of freshwater organisms (10). Furthermore salt-mediated impairment of transport systems within fish may limit the uptake of other OSPW compounds, such as NAs, which provides a protective effect to the fish relative to toxicant entry but limits gas exchange ability (95). Salt anions further complicate the overall toxicity of OSPW-IM by interaction with metals to produce compounds that reduce metal-specific uptake to organisms.

2.6 Remediation:

Currently biodegradation and aging are the leading remediation strategies used by the oil industry. OSPW is stored in tailings ponds and left for native microbial populations to degrade the organic compounds over years of time. Total NA amounts have been measured to decrease over time, however toxicity persists (96).

Remediation techniques to date have also focused on degrading organic components of OSPW; ozonation, advanced oxidation processes, and biodegradation have generated promising results for the degradation of NAs. Removal of suspended solids, colloidal particles and high molecular weight NAs can be accomplished using CFS (coagulation, floccation, sedimentation). Organics can also be removed by the use of simple activated carbon/charcoal filtration systems. The end product of all these processes is water still containing the inorganic constituents, for which the toxicity profile remains unknown.

2.7 Summary:

OSPW currently stored on-site must eventually be remediated for release back into the ecosystem. At present, aged OSPW is considered applicable for release where there has been sufficient time to degrade organic constituents, including NAs. However, the predominant barriers to successful large-scale release remain; including the gaps in knowledge surrounding OSPW toxicity and access to remediation techniques that are effective and applicable on an industrial scale. Toxicity studies have been disproportionally focused on the organic components of OSPW, specifically NAs. There has not been sufficient research or efforts put into the analysis of inorganic constituents to declare that they are insignificant in whole OSPW observed effects.

The complex and variable composition of OSPW makes understanding the potential environmental, animal, and aquatic health effects challenging. There is a particular need for further research into the role of inorganic constituents alone, or in whole effluent toxicity. Use of whole animals, mammals or aquatic organisms, cannot be replaced by *in vitro* models, however, there is a desire to reduce the use of animals in research. Cell lines bring many benefits to the table of high throughput toxicity research which may be used by the industry as a tool for assessing and monitoring industrial wastewaters, including OSPW.

Chapter 3

Exposure to oil sands process-affected water (OSPW) and its fractions exert differential acute toxicity in RAW 264.7 cells[1]

3.1 Introduction

Oil sands process-affected water (OSPW) is produced by oil sands surface mining in Alberta as a result of their extraction method, the Clark hot water extraction process. The volume of water produced is increasing with estimates there will be more than 1 billion m^3 of OSPW, which must be stored on site under a provincial zero-release policy, by 2025 (97). Characterization of OSPW toxicity is a formidable challenge due to its complex and variable nature. The naphthenic acid (NA) containing organic fraction (OSPW-OF) has traditionally been the focus of the vast majority of toxicological studies. In comparison, there has been very little research done on the evaluation of the OSPW inorganic matrix (OSPW-IM) toxicity (2-5).

OSPW has been the subject of many toxicological investigations, demonstrating that exposure induces adverse toxic effects on several physiological systems. Whole OSPW has been implicated in changes to immune, reproductive, and endocrine systems of aquatic and terrestrial organisms *in vivo* (15,16,18,65,71). To date, research done using exposures to relatively high concentrations of NAs, or OSPW-OF *in vitro* has invariably shown that OSPW-OF (and/or NAs) exerted toxic effects. In *Vibrio fischeri*, it was shown that as NA concentrations in wetlands increased, the $IC_{50\%}$ decreased, indicating that OSPW-contaminated wetlands became more toxic with additional NAs (99). In a rat cell line OSPW

[1] Part of this chapter has been published in *Environmental Science and Technology* (2017), 51(15), 8624-8634

organics were shown to be directly cytotoxic at a NA concentration of 50 mg/L (12). While NAs, and other organic components such as PAHs, or BTEX have been shown to induce toxic effects *in vitro* in bacteria, fish, and mammalian cells, the NA doses tested were much higher than the concentration of NAs in a typical tailings pond (which is under 20 mg/L). Our previous experiments using the mammalian macrophage-like cell line, RAW 264.7, found that whole OSPW was significantly more toxic at NA concentrations from 10-18 mg/L, compared to the equivalent NA doses of OSPW-OF. This suggested that at NA concentration of less than 20 mg/L, the inorganic constituent(s) and/or potential interactions between the OSPW-IM and OSPW-OF may significantly contribute to whole OSPW toxicity (13). Currently, OSPW-IM contributions to whole effluent toxicity remain to be fully elucidated.

In this chapter I report on the differential effects of OSPW, OSPW-OF, and OSPW-IM to acute toxicity in mammalian macrophage-like cells. The purpose of these studies was to use a developed *in vitro* immunological system to investigate and compare properties of OSPW and its fractions (organic and inorganic) to the whole effluent toxicity. Acute toxicity was assessed by measuring effects on cellular viability and cytotoxicity, metabolic activity, and changes in the surface membrane morphology of cells.

3.2 Materials and methods

3.2.1 Cell culture

The RAW 264.7 cell line (ATCC® TIB-71 ™) was cultured in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher) at 37°C + 5% CO_2, supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; ThermoFisher).

3.2.2 Oil sands process-affected water (OSPW): whole (crude), organic fraction (OSPW-OF), and inorganic matrix (OSPW-IM)

Whole OSPW was provided and collected by Syncrude Inc. personnel from the Aurora tailings pond in Fort McMurray, Alberta. Whole OSPW was stored in barrels at 4°C until required for further processing or use in biological assays.

The OSPW-OF, containing both neutral and acid organic components, was isolated using a dichloromethane (DCM) liquid-liquid extraction method previously developed in our laboratories, using a ratio of 100 mL DCM to 1 L OSPW (7,13,56). To completely dissolve weak organic acids, whole OSPW was pH-adjusted using NaOH to pH=10.5. Three rounds of liquid-liquid extraction with DCM were performed. The remaining aqueous phase was subject to three additional rounds of DCM extraction after adjustment of the pH using HCl to pH=2. All collected organic phases were pooled prior to removal of DCM using a rotary evaporator, resulting in the final OSPW-OF. Finally, the OSPW-OF was dissolved in pH=10 distilled water, and chemically analyzed using UPLC/HRMS to measure the NA content which was 52,000 mg/L, compared to 18.38 mg/L for whole OSPW. A schematic of this procedure is shown in Figure 3.1.

The OSPW-IM was generated by first filtering whole OSPW using a 0.22 μm nylon membrane filter and then passing the filtrate through an activated carbon packed column at a rate of 3 mL/minute. The flow through containing the purified OSPW-IM was collected and stored at 4°C until use. The total organic carbon of the OSPW-IM was 0.59 mg/L, compared to 49.86 mg/L for whole OSPW. A schematic of this procedure is shown in Figure 3.2.

Whole OSPW and OSPW-IM have basic pHs. The native pH of whole OSPW was 8.5 and the native pH of OSPW-IM was 8.9. To accommodate for potential pH effects, rather than constituent effects, a sub-sample of both waters was collected and pH-adjusted using 12M HCl. The pH adjustments required less than 1 mL of HCl per 100 mL of water, limiting the risk of significant effects on the osmolarity of OSPW or OSPW-IM. The results of the analyses of different test waters used in my thesis (whole, OSPW-OF, and OSPW-IM) are shown in Tables 3.1 and 3.2.

3.2.3 Exposure of RAW 264.7 cells to OSPW, OSPW-OF, and/or OSPW-IM

Whole OSPW doses were based on NA concentrations, with the stock OSPW having a concentration of 18.4 mg/L, and OSPW-OF a concentration of 52,000 mg/L, while OSPW-IM doses were generated using volumetric equivalents (V/E) to the whole. OSPW dilutions were done in colourless DMEM (ThermoFisher Scientific) with FBS held constant at 5% per well to achieve final NA concentrations of: 1, 2, 4, 6, 8, 10, 12, 14, 16, 17 mg/L (or V/E). FBS was reduced to 5% to accommodate the highest selected dose of whole OSPW, 17 mg/L. Media displacement for exposure doses >10 mg/L (or V/E) was over 50% by volume, therefore a dilution control of phosphate buffered saline (PBS) was introduced and used in volume equivalents to whole OSPW (also diluted using colourless DMEM with 5% FBS).

Relative contributions, and potential additive or synergistic effects of OSPW-OF and OSPW-IM constituents were investigated using two different mixing matrices. The first mixing procedure used was to simply reconstitute the volume and NA concentration of whole OSPW by combination of OSPW-OF, in the correct NA concentration, and addition of OSPW-IM to achieve an equivalent final volume. This was done to assess the potential

effects of the separating procedures on OSPW constituents and/or toxicity. Final NA concentrations were 1, 2, 4, 6, 8, 10, 12, 14, 16, 17 mg/L, the specific volumes used for this mixing scheme are shown in Table 3.3.

To assess potential synergistic and/or additive effects of OSPW-OF and OSPW-IM constituents, a diluted OSPW-OF stock of 18 mg/L with OSPW-IF was mixed in a volumetric series of sample-mixtures. For example, mixture 2 was composed of 90% OSPW-OF (18 mg/L) and 10% OSPW-IF; mixture 3 was composed of 80% OSPW-OF (18 mg/L) and 20% OSPW-IM; and so on. The mixtures were done with OSPW-IM + OSPW-OF, as well as OSPW-IM + PBS, and PBS + OSPW-OF to account for any potential permissiveness of displacement effects. The exhaustive list of volumes used for this mixing scheme are shown in Table 3.4.

3.2.4 LIVE/DEAD cell counts

RAW 264.7 cells were seeded at a density of $2.5x10^5$ cells in 1 mL of complete medium per well, in 24-well plates and incubated at 37°C for 24 hours before exposure to different OSPW test waters. Exposures were completed as outlined in previous section (section 3.2.3), 18 hours post-exposure media was aspirated and cells detached using 1 mM EDTA + 0.25% trypsin (Sigma) for collection. Collected cells were washed then stained for 30 minutes with 0.1% *LIVE/DEAD* stain in DMSO (Invitrogen). Cells were again washed, followed by fixation in 1% paraformaldehyde for 20 minutes at 4°C prior to examination of fluorescence intensity using an ImageStream Mark II (Amnis Corporation) flow cytometer. A total of 2,500 cells were counted per sample and evaluated as live or dead based on the relative fluorescence, where dead cells displaced an increased relative fluorescence by

approximately 50-fold. Three independent experiments (n=3) were performed and results are presented as the mean ± SEM.

3.2.5 The 3-(4,5- dimethylthiazolyl- 2)- 2,5 diphenyltetrazolium bromide (MTT) assay

RAW 264.7 cells were seeded at a density of $1x10^5$ cells in 100µL of complete medium per well, in 96-well plates and incubated at 37°C overnight before exposure to OSPW test waters as outlined above (section 3.2.3). A sample of OSPW only (no cells) was included as a blank control, and to prevent interpretation of artificial effects on the readout of MTT activity by constituent interference with the assay itself. Eighteen hours post-exposure supernatants containing OSPW test waters (select doses of OSPW, OSPW-OF, OSPW-IM, mixtures, or PBS) were aspirated and a 5 mg/mL MTT (Invitrogen) solution in colorless DMEM (ThermoFisher) was added to the cultures. After 4 hours of incubation at 37°C, DMSO was added to solubilize products and the plates were incubated for 30 minutes at 37°C in 5% CO_2. The colorimetric assessment was done using a SpectraMax M_2 plate reader (Molecular Devices) at the absorbance of 540nm. The MTT assay assesses the metabolic activity of cells by taking advantage of MTT, a tetrazolium dye, that produces a purple formazan product when reduced by functional cellular oxidoreductase enzymes. MTT results are used to calculate percent of MTT activity (relative to untreated controls) following exposure to OSPW. This was calculated relative to an untreated control according to the following formula:

$$\%\ \text{MTT Activity} = \frac{(\text{mean A540 experimental})}{(\text{mean A540 control})} \times\ 100\%$$

Three independent experiments (n=3) were performed, results presented as the mean ± SEM.

3.2.6 Scanning electron microscopy (ScEM) of RAW 264.7 cells

Scanning electron microscopy (ScEM) was used to generate images of RAW 264.7 cells following exposure to OSPW, OSPW-OF, OSPW-IM, and PBS. RAW 264.7 cells were seeded onto glass coverslips in 2 mL of complete medium at a density of 5.0 x10^5 cells per well in 12-well plates at 37°C in 5% CO_2. The following day cells were exposed to 4, 8, or 12 mg/L (V/E) OSPW test waters as previously described (Section 3.2.3). After 18 hours of exposure, medium was aspirated and cells were immediately fixed at room temperature, using ScEM fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer). The samples were processed for ScEM 24 hours after fixing using the HMDS (hexamethyldisiliazane) procedure at the University of Alberta, Department of Biological Sciences Microscopy Unit (100). The processed coverslips were mounted on ScEM stubs, carbon coated and imaged *in lens* at 1,000X, 5,000X, and 10,000X using a Zeiss EVO LS15 EP-SEM operated by the University of Alberta, Department of Earth and Atmospheric Sciences Scanning Electron Microscopy Laboratory. Images were collected from two independent exposure experiments, and the results shown are representative of the majority of the cell population in each of the experimental groups.

3.2.7 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis of data was performed using a two-way ANOVA, followed by the Tukey's post hoc test, using Prism 6.0 (Graphpad software, CA, USA). Differences between treatment groups were considered statistically significant when $p < 0.05$.

3.3 Results

3.3.1 Viability of RAW 264.7 cells

Cell survival and cell death following exposure to OSPW, OSPW-OF, or OSPW-IM were assessed using the *LIVE/DEAD* Fixable Yellow Stain (Invitrogen). This stain was specific for free amines which are disproportionately available when membrane integrity is lost, causing an increase in the relative fluorescence by about 50% in cells compared to those with intact membranes. Exposure to whole OSPW or OSPW-IM significantly affected cell viability at 16 mg/L (V/E) (Figure 3.3). The PBS dilution control did not significantly affect the number of dead cells at any exposure dose, causing a 7% increase is dead cells compared to non-exposed controls at 17 mg/L (V/E) (Figure 3.3). Comparatively, at 17 mg/L exposure to whole OSPW there was an approximately 17 times greater number of dead cells compared to non-exposed controls and an approximately 6 times greater number of dead cells compared to the PBS dilution control (Figure 3.3). Cells exposed to OSPW-OF at doses tested did not have significantly affected cellular viability compared to non-exposed controls, however at 17 mg/L (V/E) there was a significant difference in the number of live and dead cells between OSPW-OF and whole OSPW or OSPW-IM (Figure 3.3).

3.3.2 Metabolic activity of RAW 264.7 cells

The MTT assay assesses the metabolic activity of cells by taking advantage of MTT, a tetrazolium dye, that produces a purple formazan product when reduced by functional cellular oxidoreductase enzymes. MTT activity of non-exposed (control cells) was set at 100%, while the values of MTT activity for any of the exposed cells, including the PBS dilution controls were expressed as a percentage of the specific activity compared to non-exposed cells.

3.3.2.1 Exposure to OSPW, OSPW-OF, or OSPW-IM

There was no significant reduction in MTT activity for cells exposed to the PBS dilution control, compared to non-exposed controls (Figure 3.4A). Exposure to whole OSPW or OSPW-IM reduced cellular metabolic activity in dose-dependent manner, where both test waters caused significant reductions in MTT activity compared to the PBS dilution control at 12 mg/L or higher (V/E) (Figure 3.4A). Comparatively, a 12.5 times higher concentration of OSPW-OF was required to significantly affect MTT activity (Figure 3.4B). Exposure to whole OSPW, OSPW-IM, and OSPW-OF reduced MTT activity to less than 15% but at different doses: 17 mg/L for whole, 16 mg/L (V/E) for OSPW-IM, and 300 mg/L for OSPW-OF (Figure 3.4). Cells exposed to 14 mg/L (V/E) of whole OSPW or OSPW-IM caused differential inhibition of metabolic activity: OSPW caused approximately a 60% reduction, while OSPW-IM caused approximately an 85% reduction in activity (Figure 3.4A).

OSPW, and the OSPW-IM are alkaline waters with the native pH of whole OSPW being ~8.5, and OSPW-IM slightly higher at ~8.9. An aliquot of each water was pH-adjusted with HCl to achieve a physiological pH near 7.2. Cells exposed to whole OSPW or OSPW-IM of both the native pH, or adjusted pH showed similar dose-dependent decreases in metabolic activity (Figure 3.5). At both pHs (native, and adjusted), whole OSPW significantly reduced MTT activity at NAs concentration of 10 mg/L; there were no significant differences between the native pH and adjusted-pH treatments at any dose tested (Figure 3.5A). Exposure to the native or adjusted pH OSPW-IM also resulted in a significant reduction of MTT cellular metabolic activity at 10 mg/L V/E, however, there was a significant difference in the degree of reduction between the native pH, which caused,

approximately, a 20% reduction, and the adjusted-pH which caused an approximately 60% reduction but this difference was not sustained at higher doses (Figure 3.5B).

3.3.2.2 Exposure to mixtures of OSPW-OF and OSPW-IM

There was no significant difference in MTT activity of cells exposed to whole OSPW compared to OSPW-IM + OSPW-OF (Figure 3.6). The mixture of OSPW-IM + OSPW-OF exhibited similar dose-dependent decrease in MTT activity as whole OSPW, with both causing a significant reduction in metabolic activity at NA concentration of 10 mg/L or higher (Figure 3.6) compared to the PBS dilution control. The PBS dilution control did not significantly affect metabolic activity at any dose in comparison to non-exposed controls (Figure 3.6).

MTT activity in cells exposed to mixtures of OSPW-IM and OSPW-OF where the NA concentration was inversely related to percent volume of OSPW-IM in each mixture was assessed. As cells were exposed to increasing amounts (V/V) of OSPW-IM, metabolic activity decreased in a dose-dependent manner, with a significant reduction at 60% (V/V) OSPW-IM (7.2 mg/L NAs) (Figure 3.7A). This directly contrasted the trends observed in the PBS-only dilution controls, as well as where PBS replaced the volume equivalent of OSPW-IM which had no significant reduction in MTT activity (Figure 3.7B).

3.3.3 Membrane morphology of RAW 264.7 cells

At all of the doses tested (4, 8, 12 mg/L) there were no morphological changes in the integrity of cell membranes of cells exposed to OSPW-OF (Figure 3.8A-E). The PBS dilution control at 4 mg/L V/E, and 8 mg/L V/E looked marginally different from the non-exposed

media control cells at 1,000X and 5,000X magnification, however, for all doses examined there were very obvious differences in cell membrane morphology when cells were exposed to whole OSPW compared to the PBS dilution control. Whole OSPW exposed cells displayed stringy webs of membrane projections while the PBS exposed cells remained, round and smooth (Figure 3.8B-E). Cells exposed to OSPW-IM displayed some of these projections at 4 and 8 mg/L, but it was not as severe as those treated with whole OSPW (Figure 3.8 B, C). At 12 mg/L (V/E) the surfaces of cells exposed to OSPW-IM appeared to be morphologically equivalent to the whole OSPW experimental group cells, which was also evident at the highest magnification tested (10,000X) (Figure 3.8 DE).

3.4 Discussion

Organic components of OSPW including NAs, phenols, PAHs, and BTEX have been individually evaluated extensively for their toxicity in a variety of test systems. Several studies have shown that exposure of aquatic and terrestrial organisms, as well as fish and mammalian cells *in vitro* to OSPW-OF has significant endocrine disrupting, carcinogenic, developmental and immunotoxic effects (3,5,7,49,50). Conversely, the possible toxic effects of inorganic components such as salts, dissolved ions, as well as heavy and other metals within OSPW have not been widely performed although many of the known inorganic contaminants in whole OSPW are in amounts above CCME guidelines and are documented toxicants (10,101). In this chapter, I presented the results of the acute toxic effects following exposure of RAW 264.7 cells to whole OSPW, OSPW-OF, and/or OSPW-IM. Toxicity was quantified by assessing cell viability, cytotoxicity, cellular metabolic activity and morphological changes in the cell membrane after exposure to different OSPW test waters.

The combined use of the MTT assay for metabolic activity provided a more sensitive readout of intermediate toxic effects in these cells compared to the *LIVE/DEAD* cell counts. The MTT assay is frequently employed to calculate percent viability directly, however, my results of the *LIVE/DEAD* counts in conjunction with the MTT assay suggest that cells with fractional losses in their metabolic activity remain viable, while exhibiting up to ~90% reductions in their metabolic activity. It has been suggested that use of the MTT assay for interpretation of cellular viability should be restricted to where metabolic processes and metabolic rates are expected to remain constant (102). MTT is reduced by active cells using NAD(P)H-dependent cellular oxidoreductase enzymes, this reaction takes place within the mitochondria and is sensitive to mitochondrial stability as well as the availability of NAD(PH) (103). Mitochondrial function and regulation is sensitive to xenobiotic exposure *in vivo* and *in vitro* causing a range of disruptions in cellular metabolism (104).

Viability of cells was significantly reduced when exposed to OSPW, while exposure to OSPW-OF had no impact (at equivalent NA concentrations), up to 17 mg/L. These findings are supported by the metabolic assessments, where OSPW-OF had no effect until the dose was increased by 12.5 times. In addition, my results support the findings of a previous study where mouse bone marrow-derived macrophages were found to tolerate exposure to OSPW-OF containing 50 mg/L NAs (18). Interestingly, the exposure of RAW 264.7 cells to OSPW-IM caused similar toxic effects when compared to whole OSPW. This suggests that inorganic constituents, at doses of less than 20 mg/L (V/E), are significant contributors to the overall whole OSPW effluent toxicity. When cells were exposed to mixtures of OSPW-IM and OSPW-OF, where the NA concentration is inversely related to

percent volume of OSPW-IM, mitochondrial activity continued to decrease in a dose dependant manner suggesting a dominant toxic contribution of OSPW-IM constituents. Specifically, the toxic effects were similar when cells were exposed to the OSPW-IM + OSPW-OF mixture to those exposed to OSPW-IM + PBS, where PBS was added in volume equivalents to OSPW-OF. This indicated that the addition of organic components, including NAs, had no supplementary effect on the metabolic activity of RAW 264.7 cells. These results are supported by a previous study where ozonation of OSPW, which effectively reduces the amount of NAs by ~90%, did not significantly ameliorate toxicity in RAW 264.7 cells (56). The implication of a dominant inorganic matrix-mediated toxicity is particularly interesting because there has been a traditional focus in the literature on the effects induced by the OSPW organic constituents, primarily NAs. My study is the first comprehensive evaluation of the potential toxicity of the OSPW-IM.

Both whole OSPW and OSPW-IM are brackish waters with native pHs outside of a physiological range for mammalian cells, suggesting that the observed increase in relative toxicity compared to OSPW-OF could, at least in part, be due to pH effects. Mammalian cells exposed to pHs outside of the normal physiological range (~7.0-7.4) are known to become more sensitive to secondary effects such as temperature and osmolarity (105–107). Therefore, I decided to examine the effects of pH by reducing OSPW and OSPW-IM pH to within the physiological range and then examining their toxicity. In general, lowering the pH to the physiological range did not change the toxic effects observed at native pH for both whole OSPW and OSPW-IM (there was only one instance of a difference between the adjusted pH, and native pH treatments, for a number of different exposure scenarios

performed in these experiments). At 10 mg/L V/E the pH-adjusted OSPW-IM reduced metabolic activity by ~20% while the native pH was ~50%, however this difference was not sustained at other exposure doses tested. Mammalian cells express a multitude of pH-sensitive protein classes such as specific ion transporters, ion channels and pumps to respond to the changes in the pH (108). Tumour cells specifically up-regulate four classes of pH regulators: proton pumps, sodium-proton exchangers, the bicarbonate transporter family, and monocarboxylate transporter family because of the essential role pH plays in enhancing cellular proliferation (27-28). RAW 264.7 cells are leukaemia-virus transformed macrophage-like cells, which may have an enhanced ability to tolerate the increased pH of OSPW and OSPW-IM. Immortal cell lines allow for rapid, inexpensive, and consistent screening of xenobiotics, but the results of such screens should keep in mind the characteristic physiological differences of transformed cells. More specifically, conclusions should be interpreted with this in mind and effects not directly extrapolated to a whole organism without substantial evidence *in vivo*.

The membrane morphology of cells exposed to whole OSPW showed dramatic changes when compared to the PBS dilution control or medium control. Exposure to whole OSPW (4 to 12 mg/L NAs), caused stringy membrane extensions and the exposure to OSPW-IM also caused similar cell membrane changes that were most evident at the highest dose examined (12 mg/L). In contrast, the exposure to OSPW-OF at the same doses (4 -12 mg/L NAs content) did not cause any major changes in cell membrane morphology and the cell membrane was similar to that of non-exposed (control) cells. Previously, this membrane morphology of RAW 264.7 cells exposed to 10 mg/L or higher doses of whole OSPW was

associated with an increase in oxidative and genotoxic stress gene expression (13). Given that some changes in cell membrane morphology were evident after exposure to only 4 mg/L (V/E) of whole OSPW or OSPW-IM, a dose that did not cause significant changes in cell viability or cellular metabolism, suggests that other, more subtle, toxic effects of whole OSPW or OSPW-IM may occur at relatively low doses. The importance of these subtle effects caused by exposure of cells to low doses of whole OSPW or OSPW-IM remains to be elucidated. It is possible that the changes in the observed cell membrane morphological changes could affect ion channel function, membrane polarity of cells or cell to cell communication.

Table 3.1 Measured parameters of OSPW and OSPW-IM

Parameter	Whole OSPW	OSPW-IM
pH	8.41	8.80
Total dissolved solids (TDS) (mg/L)	2050.00	–
Total alkalinity ($CaCO_3$ mg/L)	644.15	–
Hardness ($CaCO_3$ mg/L)	182.50	–
Chloride (Cl) (mg/L)	457.25	–
Sulfate (SO_4-S) (mg/L)	131.80	–
Biochemical oxygen demand (BOD) (mg/L)	13.61	–
Chemical oxygen demand (COD) (mg/L)	224.79	–
Total organic carbon (TOC) (mg C/L)	49.86	0.59
Naphthenic acids (NAs) (mg/L)	18.38	–

Table 3.2 Analysis of NAs from OSPW and OSPW-OF

Parameter (mg/L)[a]	OSPW	OSPW-OF
NAs	18.38	1099.38
O-NAs	8.75	237.98
O_2-NAs	9.57	304.4
O_3-NAs	3.98	65.69
O_4-NAs	0.62	17.00

[a] NAs: $C_nH_{2n+z}O_2$; O-NAs: $C_nH_{2n+z}O_3$; O_2-NAs: $C_nH_{2n+z}O_4$; O_3-NAs: $C_nH_{2n+z}O_5$; O_4-NAs: $C_nH_{2n+z}O_6$;

Table 3.3 Reconstitution of whole OSPW based on NA concentration and total volume

NAs (mg/L)	Whole (uL)[a]	OSPW-OF (µL)[b]	OSPW-IM (µL)[c]
Media Control	0	0	0
1	54.4	0.19	54.21
2	108.8	0.38	108.42
4	217.6	0.76	216.84
6	326.4	1.14	352.26
8	435.2	1.52	433.68
10	544.1	1.9	542.20
12	652.9	2.28	650.62
14	761.7	2.66	759.04
16	870.5	3.04	867.46
17.5	952.1	3.33	948.77

[a] Whole (µL): Volume of whole required for respective NA concentration in 1.0 mL total volume
[b] OSPW-OF (µL): Volume of 1/10 dilution, in pH=10 PBS, of OSPW-OF stock (52 000 mg/L)
[c] OSPW-IM (µL): Calculated by subtracting OSPW-OF (µL) from whole OSPW (µL)

Table 3.4 Serial dilution of OSPW-OF by percent volume in OSPW-IM

Sample Number	% OSPW-OF[a]	NAs (mg/L)	%OSPW-IM
Sample 1	100	18	0
Sample 2	90	16.2	10
Sample 3	80	14.4	20
Sample 4	70	12.6	30
Sample 5	60	10.8	40
Sample 6	50	9	50
Sample 7	40	7.2	60
Sample 8	30	5.4	70
Sample 9	20	3.6	80
Sample 10	10	1.8	90
Sample 11	0	0	100
Media Control	0	0	0

[a] OSPW-OF: 18mg/L dilution in pH=10 PBS, OSPW-OF stock concentration is 52 000 mg/L

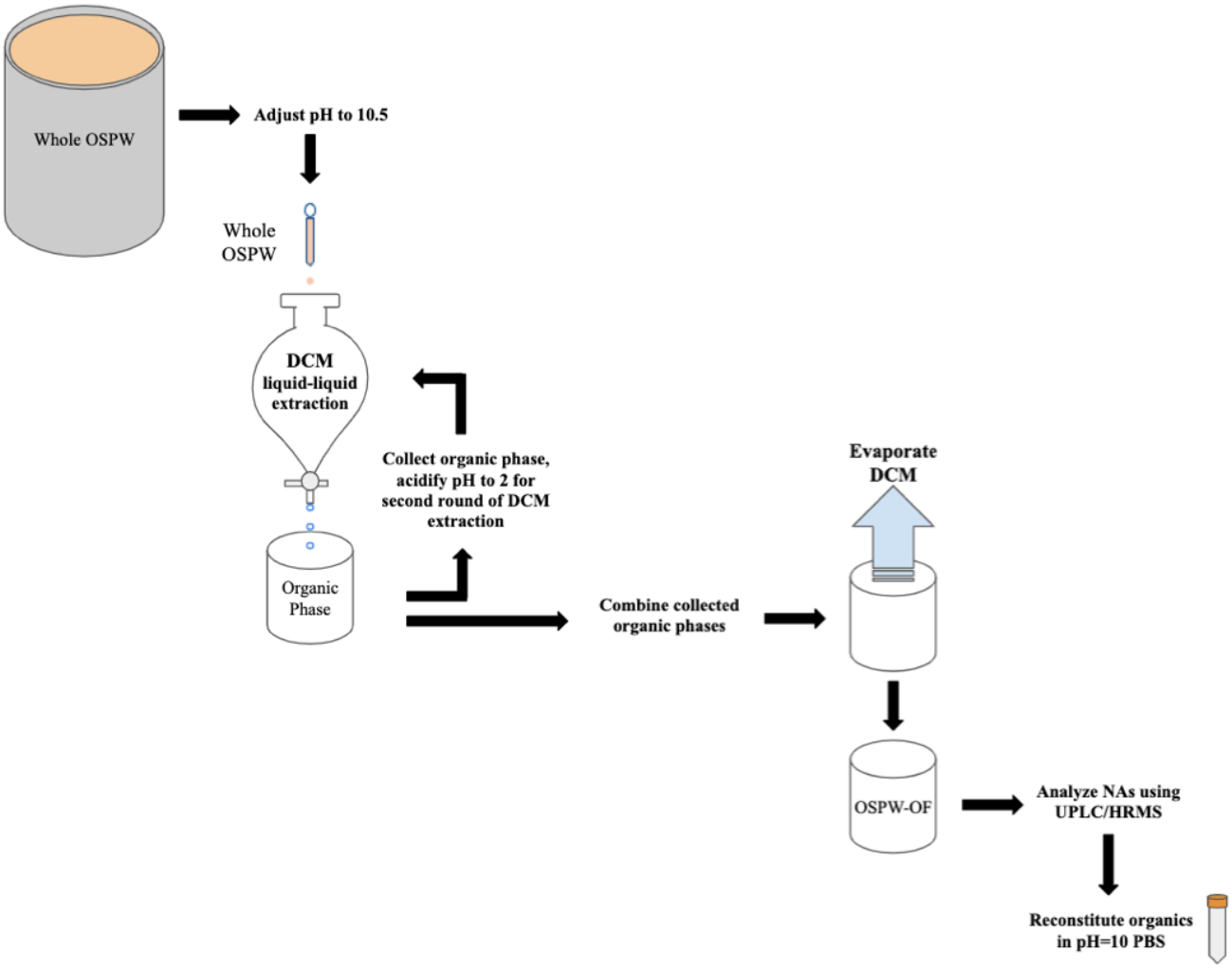


Figure 3.1 Procedure for the extraction of OSPW organic fraction (OSPW-OF) from whole OSPW.

[a]Modified from (56). UPLC is ultra-performance liquid chromatography, HRMS is high-resolution mass spectrometry, used for analysis of naphthenic acid (NA) populations in whole OSPW and OSPW-OF.

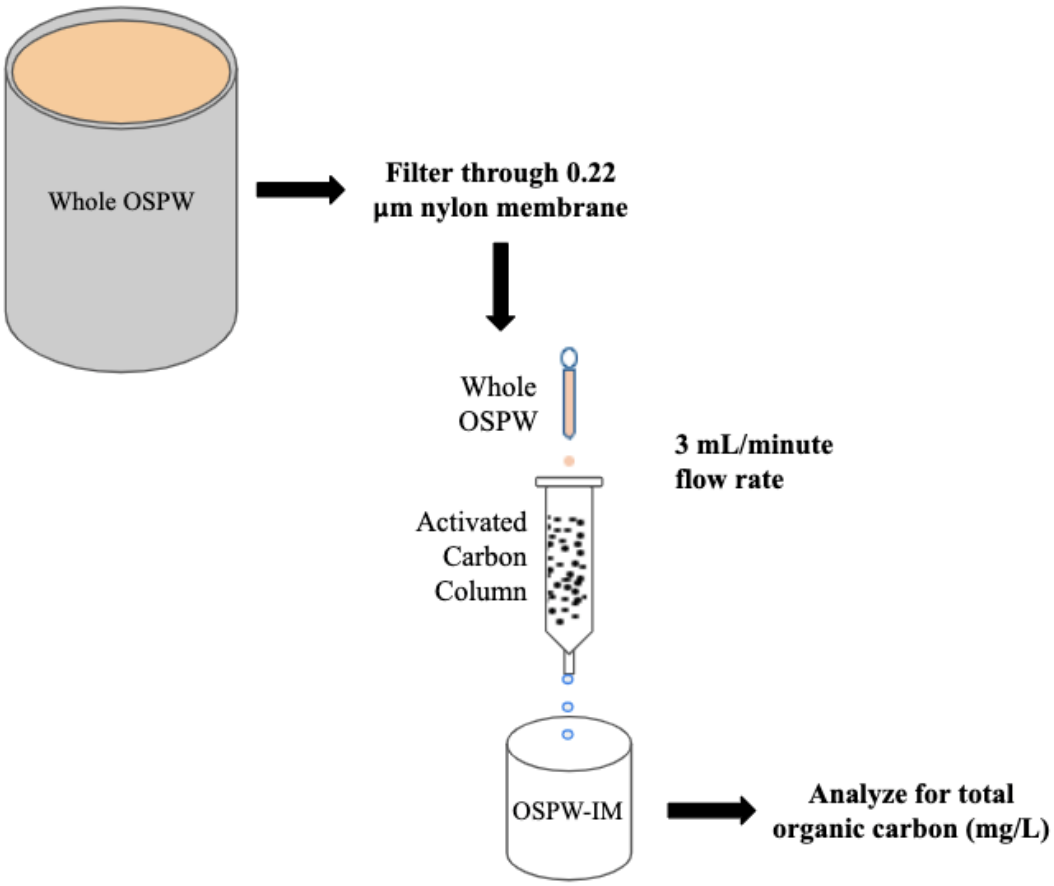


Figure 3.2 Procedure for the collection of OSPW inorganic matrix (OSPW-IM) from whole OSPW.

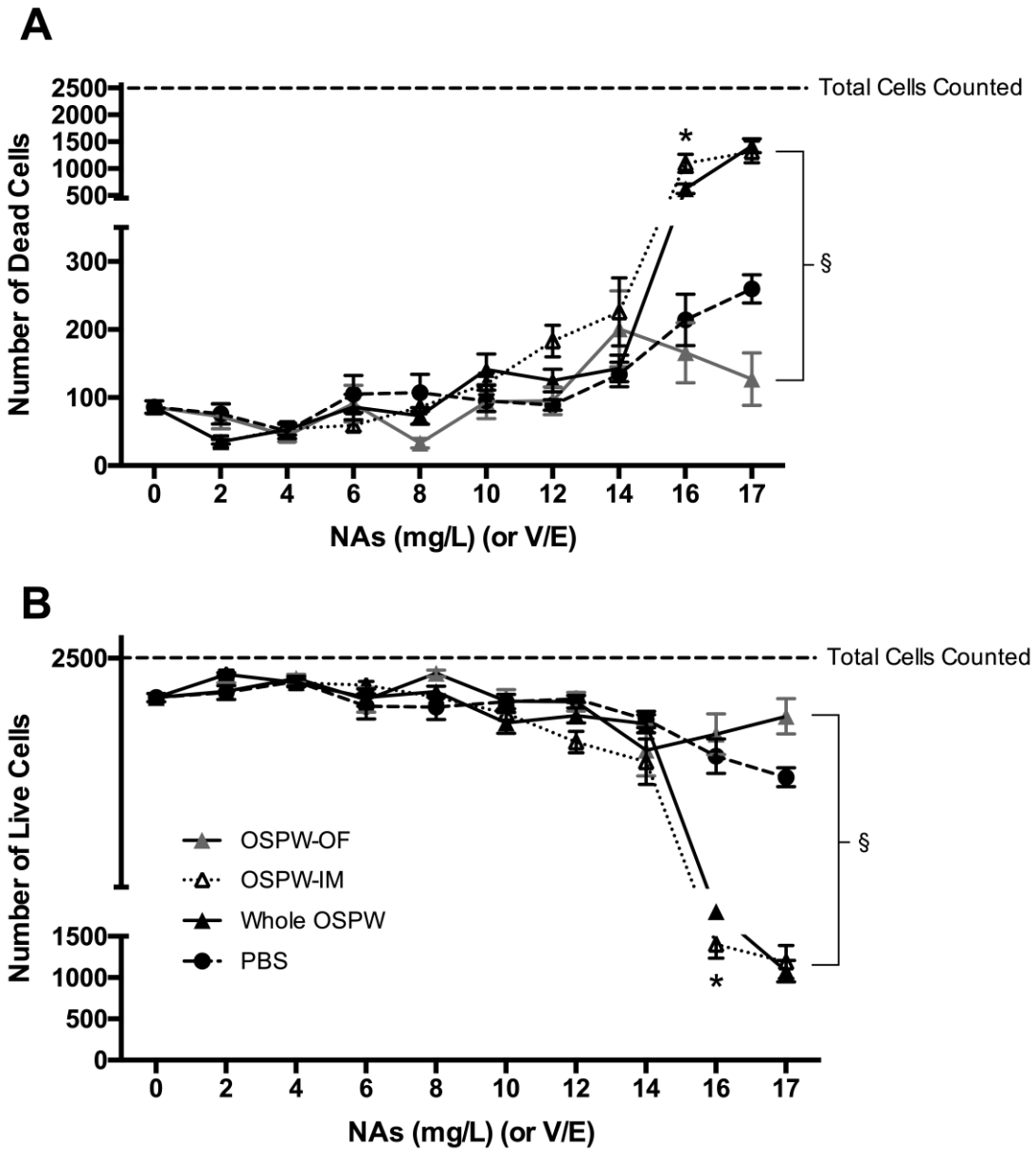


Figure 3.3 Viability of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. PBS is the dilution control, 0 mg/L NAs indicates the non-treated, medium controls. After 18 hours of exposure, cells were stained with LIVE/DEAD Fixable Yellow Stain (Invitrogen), and 2,500 cells were counted as dead (A) or live (B) using an ImageStream flow cytometer. All plotted values are the mean ± SEM from 3 independent experiments. Statistics performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to dilution control, § indicates $p < 0.05$ between the indicated experimental group dose points.

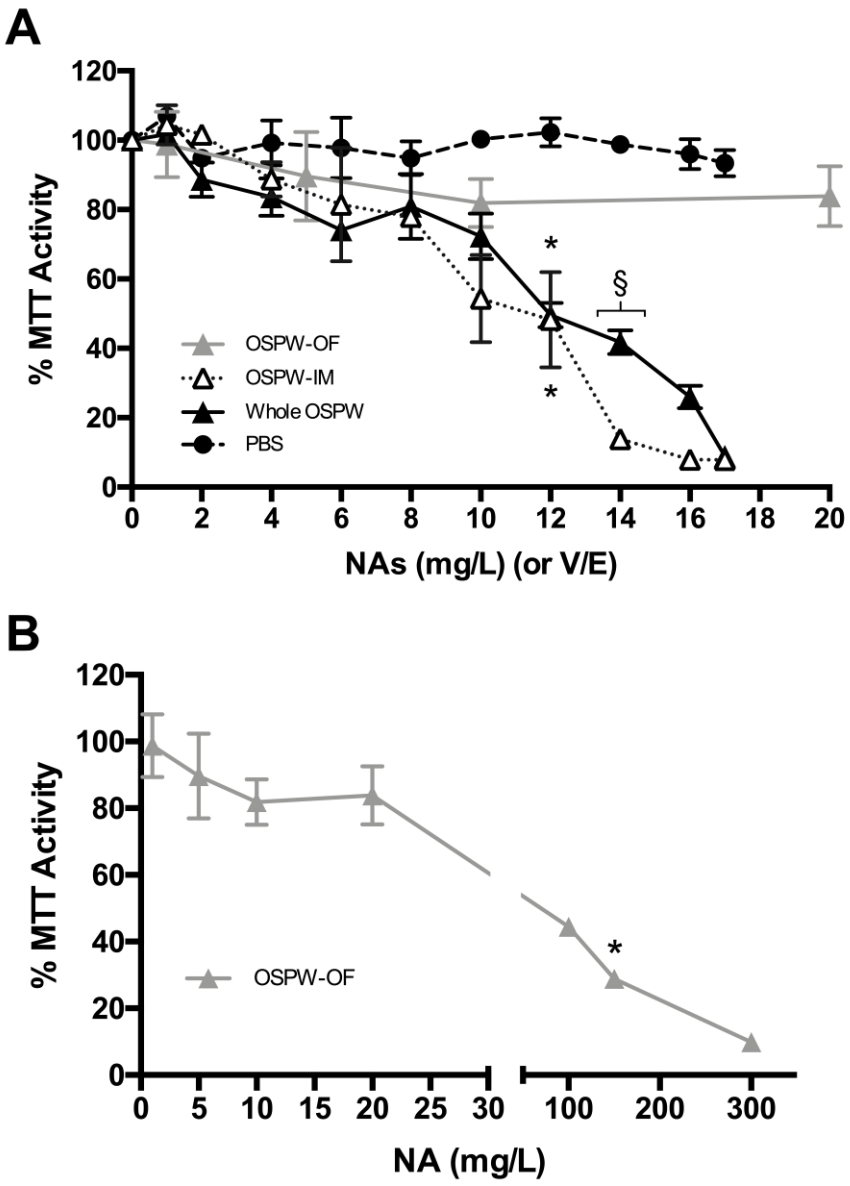


Figure 3.4 Metabolic activity of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. PBS is the dilution control. After 18 hours of exposure, cellular metabolism was determined using the MTT assay. The data are represented as the percentage of MTT (metabolic) activity relative to non-exposed controls (0 mg/L NAs). All plotted values are the mean ± SEM from 3 independent experiments. Statistics in (A) were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to dilution control, § indicates $p < 0.05$ between the indicated experimental groups. Statistics in (B) were performed with a t-test, * indicates $p < 0.05$ compared to non-exposed controls.

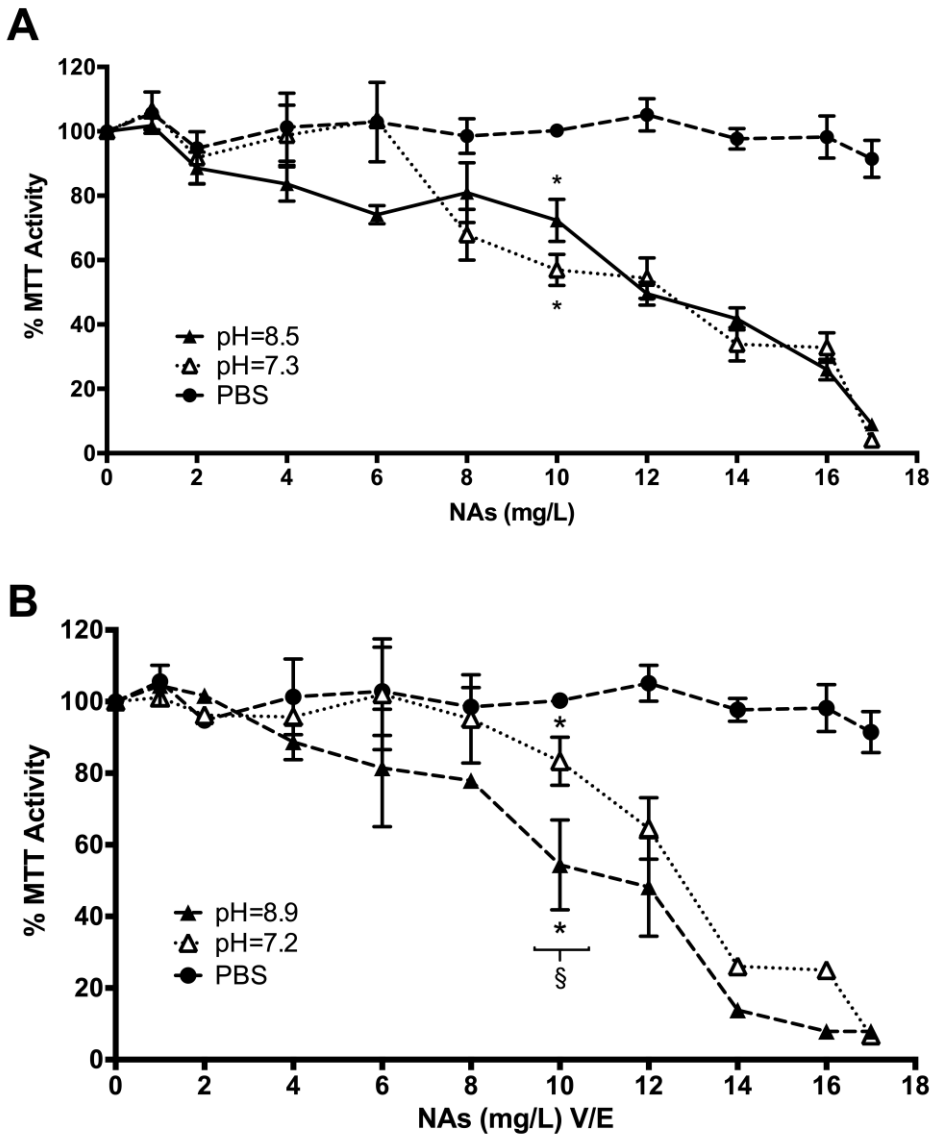


Figure 3.5 Effects of pH on metabolic activity of RAW 264.7 cells.
Cells were exposed to the native, basic pH or pH-adjusted (~7.2) whole OSPW (A) or OSPW-IM (B). PBS is the dilution control. After 18 hours of exposure, cellular metabolism was determined using the MTT assay. The data are represented as the percentage of MTT (metabolic) activity relative to non-exposed controls (0 mg/L NAs). All plotted values are the mean ±SEM from 3 independent experiments. Statistics performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to dilution control, § indicates $p < 0.05$ between the native pH and pH-adjusted groups.

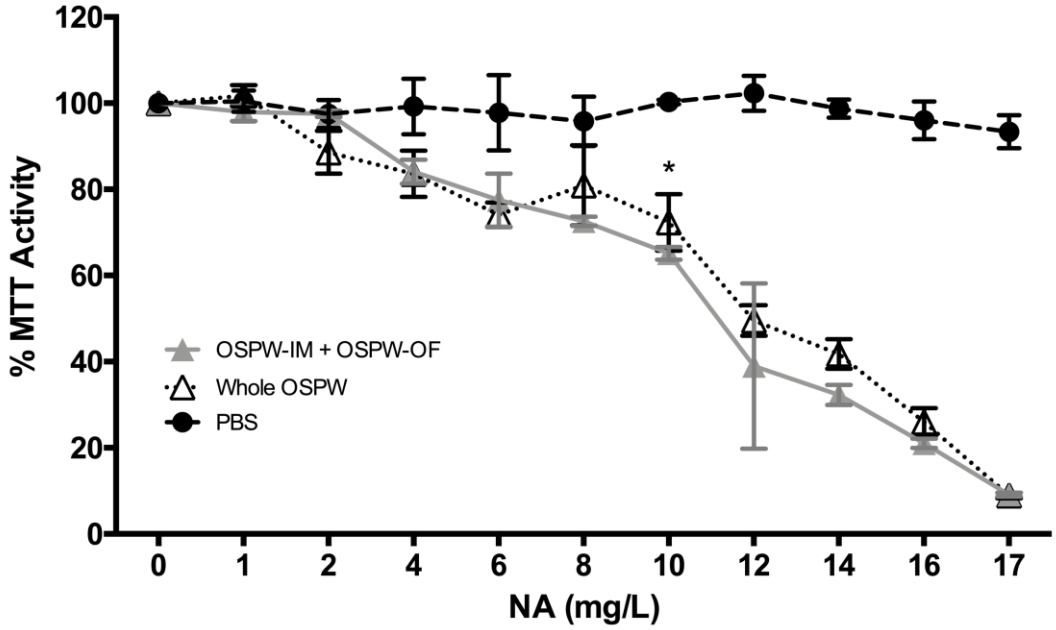


Figure 3.6 Effects of organic/inorganic separation processes on the metabolic activity of RAW 264.7 cells.

Cells were exposed to a mixture of OSPW-IM to achieve the same final volume, and OSPW-OF to achieve the same NA concentration as whole OSPW at those doses. PBS is the dilution control. After 18 hours of exposure, cellular metabolism was determined using the MTT assay. The data are represented as the percentage of MTT (metabolic) activity relative to non-exposed controls (0 mg/L NAs). All plotted values are the mean ±SEM from 3 independent experiments. Statistics in (A) were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to dilution control.

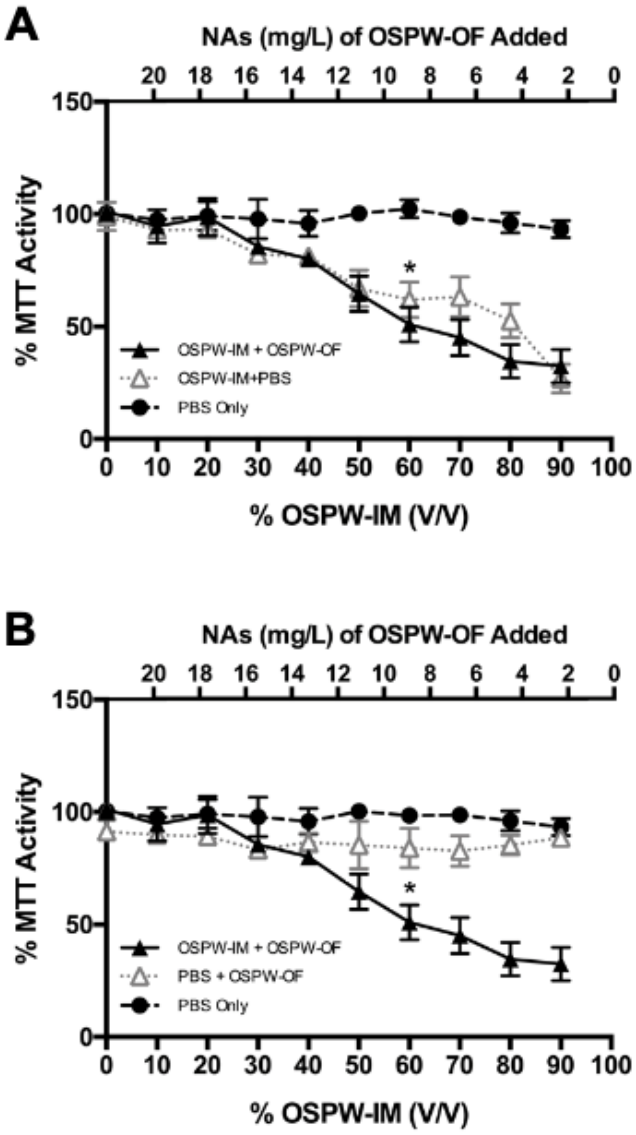


Figure 3.7 Relative contributions od OSPW-IM and OSPW-OF to effects on metabolic activity of RAW 264.7 cells.

Cells were exposed to a volumetric series of OSPW-IM + OSPW-OF, OSPW-IM + PBS (A), or PBS + OSPW-OF (B) by percent volume, with PBS replacing the respective component as a displacement control. After 18 hours of exposure, cellular metabolism was determined using the MTT assay. The data are represented as the percentage of MTT (metabolic) activity relative to non-exposed controls (0 mg/L NAs). All plotted values are the mean ±SEM from 3 independent experiments. Statistics in (A) were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to dilution control.

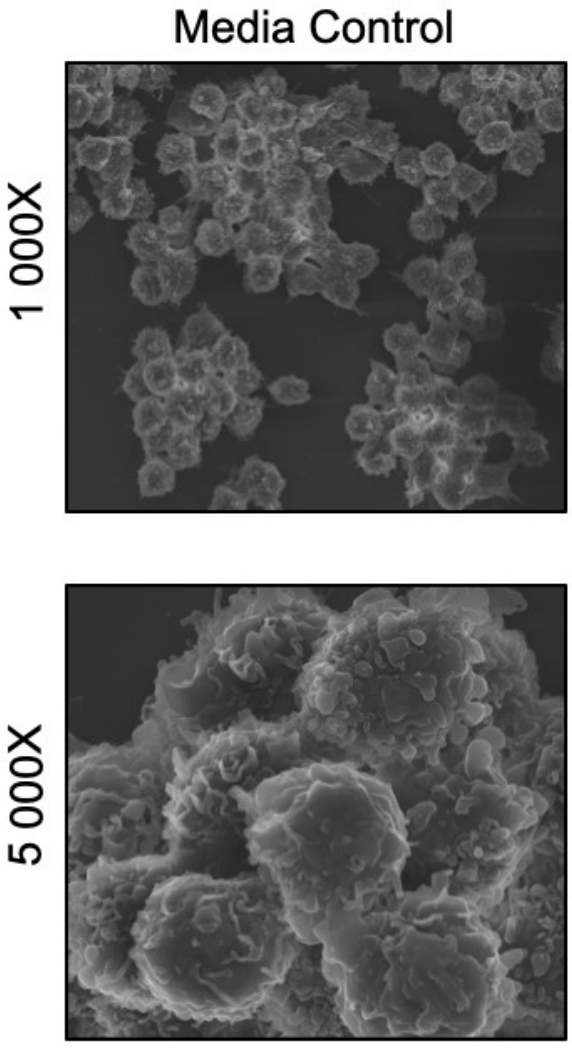
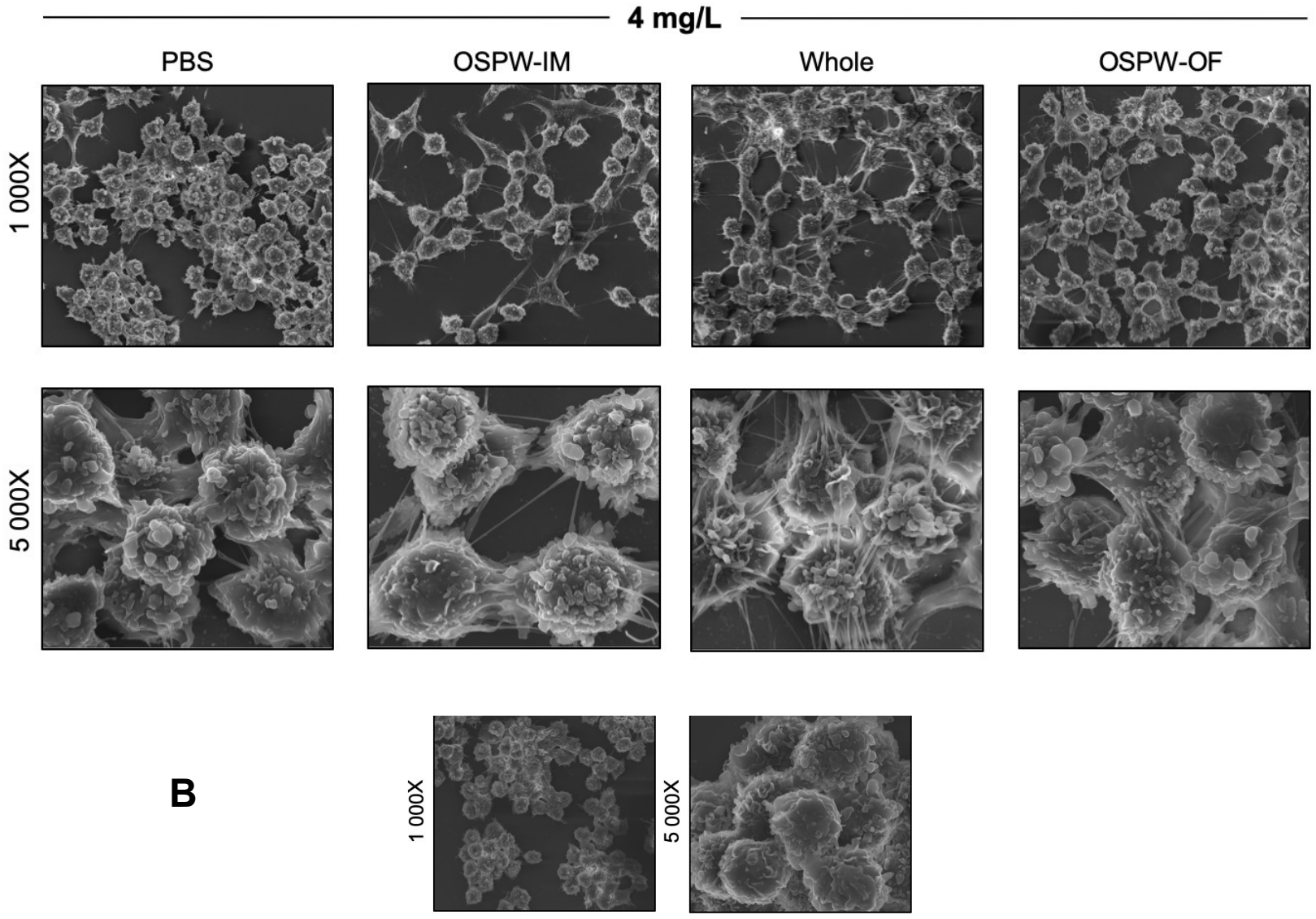
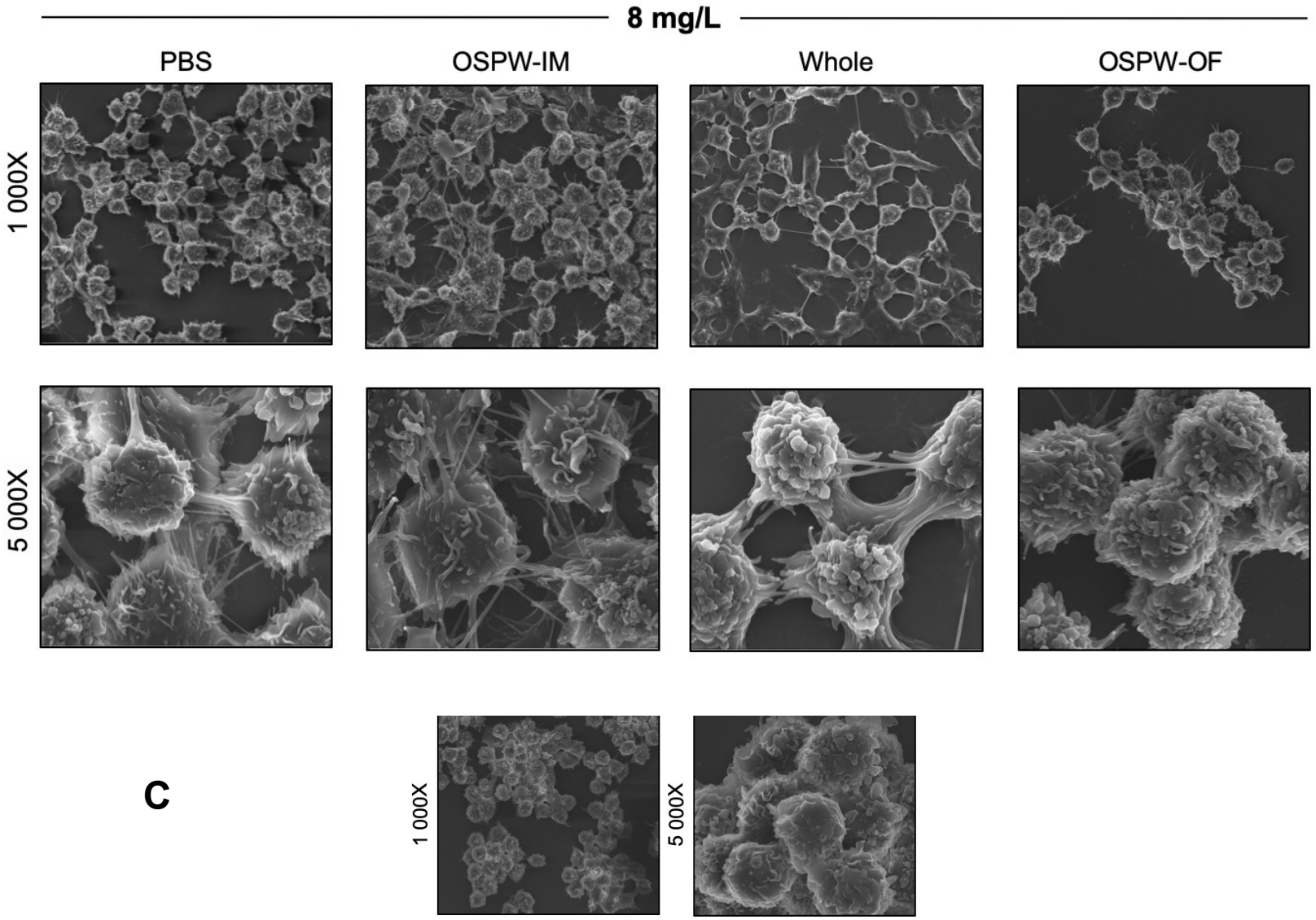
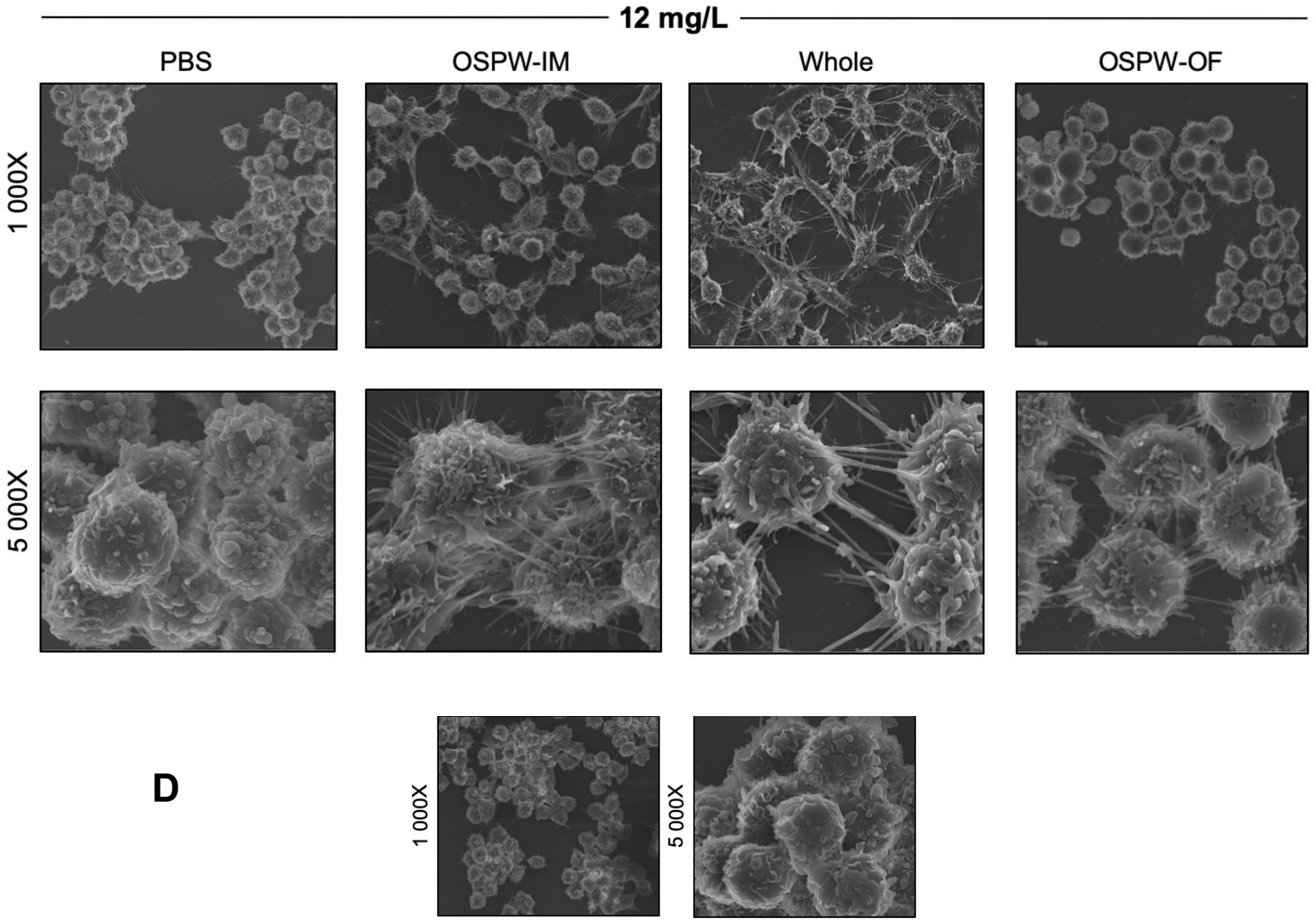
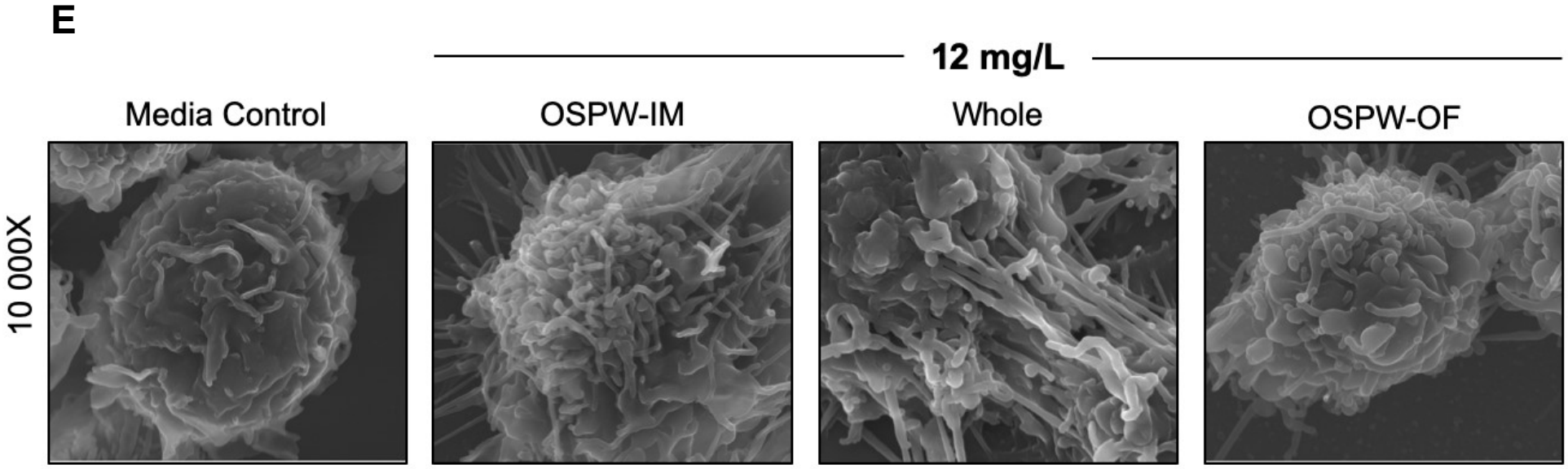


Figure 3.8 (A-E) Membrane morphology of RAW 264.7 cells.
ScEM was used to assess the surface morphology, cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. (A) media control, panel is shown with all doses for simplicity, (B) 4 mg/L, (C) 8 mg/L, (D) 12 mg/L, (E) 10,000X magnification. PBS is the dilution control. After 18 hours exposed cells were fixed with 2.5% glutaraldehyde + 2% PFA, then processed for imaging using the HMDS procedure. Processed samples were coated with carbon and imaged *in lens* at 1,000X, 5,000X, and 10,000X using a Zeiss EVO LS15 EP-SEM. Cells from two independent exposure experiments were imaged, shown are representative images for each treatment.


4 mg/L
PBS
OSPW-IM
Whole
OSPW-OF
1 000X
5 000X
B
1 000X
5 000X


8 mg/L
PBS
OSPW-IM
Whole
OSPW-OF
1 000X
5 000X
C
1 000X
5 000X


12 mg/L
PBS
OSPW-IM
Whole
OSPW-OF
1 000X
5 000X
D
1 000X
5 000X


E
12 mg/L
Media Control
OSPW-IM
Whole
OSPW-OF
10 000X

Chapter 4

Exposure to oil sands process-affected water (OSPW) and fractions affects gene expression of RAW 264.7 cells

4.1 Introduction

Acute toxicity of OSPW has been studied using multiple *in vitro* systems including primary cells, cell lines, and bacteria to assess endocrine disruption, stress, functional impairments and effects on viability (13–16) . These readouts are often coupled with gene expression of select target genes that may have a role in the anticipated mechanism(s) of toxicity. In murine embryonic stem cells exposed to OSPW, changes in the expression of cardiac and neural development genes was proposed as a mechanism for causing developmental abnormalities in the heart and nervous system (111). Common targets of toxicologic gene expression analysis include genes that are involved in metabolism, stress-response, and DNA damage. However, the selected target genes may also be specific to an effect of interest such as enzymes involved in endocrine/hormone biosynthesis and regulation, or protein effectors and enzymes required for immune responses.

The expression of genes involved in mammalian cell response to various stressors has been well studied. It has been shown that environmental or physiological causes of stress are complex, and that numerous molecular pathways are involved (112). The importance of protecting cellular genomic DNA can be interpreted by just how many arms of response exist to protect daughter cells from gaining potentially harmful mutations; cells experiencing genotoxic stress may activate programmed cell death pathways or cell cycle arrest programs (113). The Gadd45 family of proteins are rapid responders to genotoxic stress and act to

arrest the cell cycle, participate in DNA repair, and potentially modulate the induction of apoptosis (112,114). In addition to causing damage to cellular DNA, oxidative stress can also result in injury to lipids and proteins and therefore must be appropriately controlled (115). Upregulation of *hmox1* (heme oxygenase one) has been associated with an enhanced resistance to oxidative stress (116,117). Heme oxygenase quickly responds *in vivo* to induced oxidative stress, within 3 hours at the mRNA level, and within 6 for protein activity (118).

Immune gene expression is induced in response to specific situations requiring a host return to homeostasis during, or following tissue damage, infection, or tumour development. Pro-inflammatory genes are upregulated to initiate a cytotoxic response, while anti-inflammatory gene products promote wound-healing and tissue-repair. Macrophages have a specific set of mediators they generate for autocrine or paracrine signalling, as well as for their function as phagocytes and antigen presenting cells. For signalling in an acute phase response, macrophages produce pro-inflammatory cytokines including tumour necrosis factor alpha (TNFα) and interleukin-one beta (IL-1β) (119). TNF-α promotes chemotaxis, phagocytosis, and production of antimicrobial molecules by macrophages (120). IL-1β is a granulocyte chemoattractant, and contributes to the induction of systemic immune responses (121). In their alternative role of fostering a wound-healing environment, macrophages produce anti-inflammatory cytokines. Transforming growth factor-beta (TGFβ) is an anti-inflammatory cytokine that effectively suppress an active immune response promoting tissue repair and eventual return to homeostasis (122,123).

The signalling, response, and function of macrophages in both active responses, as well as in shutting down these responses, makes them central regulators of host immunity. To accomplish these opposing outcomes, macrophages exist in two activation states; corresponding to different phenotypes. The first is the classically activated M1 macrophage phenotype which is pro-inflammatory and is also known as the "killer" phenotype. M1 macrophages possess antimicrobial functions and are responsible for control of different pathogens. The alternative activation state is that of the M2 macrophage phenotype, where these cells control anti-inflammatory host response and are responsible for shifting the response from inflammation to wound/tissue-repair. Classically and alternatively activated macrophages compete for the same substrate, L-arginine, to accomplish their activity. M1 macrophages express the inducible nitric oxide synthase (iNOS) enzyme that catalyzes the conversion of L-arginine to citrulline and eventual production of reactive nitrogen intermediates (RNI) that are cytotoxic to variety of pathogens (124). In contrast, M2 macrophages express the arginase enzyme to promote cell proliferation and collagen deposition. These pathways are mutually exclusive and maintained as such by several control mechanisms including arginase mediated uncoupling of iNOS, translational repression of iNOS, and destabilization of the iNOS protein (45,46). Markers of the M1 phenotype are expression of iNOS, and pro-inflammatory cytokines; TNFα, IL-1β, IL-12 (45). M2 phenotype markers include expression of arginase, and anti-inflammatory cytokines; TGFβ, and IL-10 (125,126).

In this chapter I report the effects of OSPW, OSPW-OF, and OSPW-IM on expression of the genes associated with cellular stress and immune function discussed above in

mammalian macrophage-like cells. The purpose of these studies was to further investigate toxic effects of OSPW and its fractions using our developed *in vitro* bioindicator system.

4.2 Materials and methods

4.2.1 Stimulation of RAW 264.7 cells

RAW 264.7 cells were cultured as previously described in Chapter 3 (section 3.2.1) then seeded in 2 mL of complete media at a density of $1.8x10^6$ cells per well in 6-well plates and incubated at 37°C for 24 hours prior to exposure to different test waters. Cells in duplicate wells were exposed to 4, 6, 8, 10, or 12 mg/L of OSPW (or V/E) using the procedure outlined in Chapter 3 (section 3.2.3), an additional high dose of OSPW-OF (50 mg/L) was also tested.

After two hours of incubation, exposed to only OSPW, one well of each exposure dose was stimulated with LPS (1 ug/mL per well) and the other had an equivalent volume of PBS (diluent) added. The stimulated cells, with OSPW, were then left to incubate for an additional 16 hours (18 hours total) to induce a pro-inflammatory profile.

To assess the induction, and potential effects of OSPW on induction of immune genes, RAW 264.7 cells were exposed to 4, 8, or 12 mg/L and stimulated as above for 0.5, 3, 6, or 22 hours (2.5, 5, 8, and 24 hours, respectively).

4.2.2 Gene expression

Gene expression of several target genes associated with stress and immune response/function was assessed using qPCR. The expression of the following genes was determined: tumour necrosis factor alpha (*tnf*α), interleukin one beta (*il-1*β), inducible nitric

oxide synthase (*inos*), arginase (*arg*), transforming growth factor beta (*tgfβ),* heme oxygenase one (*hmox1*), and growth arrest and DNA damage-inducible protein (*gadd45*).

Total RNA from both samples of stimulated and non-stimulated RAW 264.7 cells was extracted using a phenol-chloroform extraction method. Medium from each culture well (± OSPW and ± LPS) was aspirated then 1 mL of TRIZOL was immediately added to the well and incubated for 5 minutes at room temperature to homogenize and dissociate all nucleoprotein complexes. The homogenized solution was then mixed with chloroform and incubated for 3 minutes at room temperature, prior to centrifugation at 12,000 x *g* for 15 minutes to separate phases. The total RNA in the aqueous phase was precipitated by addition of isopropyl alcohol. RNA was pelleted and washed 3 times by centrifugation at 12,000 x *g* for 10 minutes, and the remaining pellet was left to dissolve overnight at 4°C in DEPC-treated water.

The Superscript III cDNA Synthesis kit (ThermoFisher) was used to reverse transcribe 4 μg of RNA to cDNA with a final concentration of 0.2 μg/μL. The cDNA in qPCR was ran diluted 1/20 dilution in nuclease free water at a 2:8 ratio with 2X SYBR Green Master Mix (MBSU, University of Alberta). For cells stimulated 22 hours, the qPCR reactions were performed and analyzed using a 7500 Fast Real-Time PCR System (Applied Biosystems). For samples stimulated for 0.5, 1, 3, 6, or 22 hours were analyzed using a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The cycling parameters were: 95°C, 2 minutes; 95°C 15 seconds; 60°C, 1 minute; 95°C 15 seconds; 60°C 1 minute; 95°C, 30 seconds; 60°C, 15 seconds (melting curve); for 40 cycles. Gene expression was

analyzed using the ddCT (Delta-Delta-CT) method, fold difference (RQ) was calculated according to the Applied Biosystems software manual relative to the endogenous control gene, hypoxanthine-guanine phosphoribosyl-transferase (*hprt1*). Relative quantitation (RQ) values were normalized for whole OSPW and OSPW-IM to the PBS dilution control, and OSPW-OF to the non-exposed (medium) control because of the respective medium displacements.

Primers were validated using a standard curve and considered acceptable where R^2 >0.98, all products were then subject to agarose gel electrophoresis, cloned using the TOPO-TA Cloning kit (Invitrogen) and sequenced to confirm specificity. A list of primers and their sequences is shown in Table 4.1.

4.2.3 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis of experimental data was performed using a two-way ANOVA, followed by the Tukey's *post hoc* test (Prism 6.0, Graphpad software, CA, USA). Statistical analysis of the various qPCR controls, PBS dilution control or non-exposed (medium) controls, were analysed using a two-way ANOVA, followed by a Dunnett's *post hoc* test or t-test, using Prism 6.0 software. Differences between treatment groups were considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1 Expression of stress response genes

Following exposure to whole OSPW, OSPW-IM, or OSPW-OF at a range of doses from 4-12 mg/L (V/E) for 18 hours, RAW 264.7 cells were assessed for the expression of oxidative stress response gene, *hmox1*, and cell growth arrest and DNA damage inducible

gene, *gadd45*. Cells exposed to whole OSPW or OSPW-IM showed dose-dependent changes in the expression of *hmox1*, this was statistically significant compared to controls after exposure to doses greater than 8 mg/L (V/E) (Figure 4.2). The expression of *hmox1* was not significantly increased following exposure to OSPW-OF at doses less than or equal to 12 mg/L (Figure 4.2A). Following exposure to high dose (50 mg/L NA content) of OSPW-OF there was a significant upregulation in *hmox1*, however the magnitude of increase was still approximately 5 times lower compared to the increase in expression when cells were exposed to 12 mg/L (NA content) of whole OSPW (Figure 4.2A). In contrast, the expression of *gadd45* significantly increased after exposure to 12 mg/L and 50 mg/L of OSPW-OF but was not affected after exposure to whole OSPW or OSPW-IM for 18 hours (Figure 4.2B). The exposures to whole OSPW and OSPW-IM did cause a significant increase in *gadd45* expression but at the early timepoints for all doses tested, but this was not sustained after exposure for 8 hours (Figure 4.3D-F). Similar to the induction profile of *gadd45* over time, there was a significant increase in *hmox1* expression following treatment with OSPW-OF at 12 mg/L, a 6-fold change in expression at 2.5 hours, but this was not persistent (Figure 4.3C).

4.3.2 Expression of immune genes

Following exposure to whole OSPW, OSPW-IM, or OSPW-OF at a range of doses from 4-12 mg/L (V/E) for 18 hours, RAW 264.7 cells were assessed for the expression of pro-inflammatory cytokine, anti-inflammatory cytokine, and enzyme genes. The expression of at least one of the pro-inflammatory cytokines assessed (*il-1β* and *tnfα*) was significantly changed after exposure to whole OSPW or OSPW-IM at majority of the doses tested (Figure 4.4). Alternatively, exposure to OSPW-OF affected only expression of *il-1β* at the high dose, 50 mg/L, and there were no changes in *tnfα* mRNA levels (Figure 4.4A). Whole OSPW and

OSPW-IM induced significant increases in the mRNA levels of *il-1β* at 4 mg/L (V/E), the relative increase was sustained at 8 mg/L (V/E) by OSPW-IM only (Figure 4.4A). Exposure to whole OSPW or OSPW-IM caused inverse trends in the mRNA levels of *il-1β* which was significantly increased at the lowest dose compared to *tnfα,* where there was no effect at 4 mg/L but a significant reduction at 3 times the exposure dose (Figure 4.4).

Tracking the expression of *il-1β* and *tnfα* at earlier time points showed a consistent effect of whole OSPW and OSPW-IM on early expression of the pro-inflammatory genes. Specifically, after exposure for 2.5 hours *il-1β* mRNA levels increased at all doses tested (Figure 4.5A-C). This trend of increased expression of *il-1β* after treatment of cells with whole OSPW or OSPW-IM was short-lived and the mRNA levels of returned to baseline levels by 8 hours of exposure. The expression pattern of *tnfα* was not as predictable and showed variable up- and down-regulations between different exposure doses and times (Figure 4.5D-F). Generally, OSPW-OF exposures were associated with significant changes in *tnfα* mRNA levels at exposure times of less than or equal to 8 hours (Figure 4.5D-F). Whole OSPW or OSPW-IM treated cells showed changes in their *tnfα* expression after incubation for 2.5 hours. By 24 hours there were no significant changes in *tnfα* expression (Figure 4.5D-F).

Cells exposed to whole OSPW, OSPW-IM or OSPW-OF for 18 hours showed no significant changes in expression of the anti-inflammatory cytokine *tgfβ* at any of the doses tested (Figure 4.6). Similarly, there were no changes to the expression with any treatment at 24 hours of exposure (Figure 4.7). Exposure to whole OSPW or OSPW-IM showed

significant increases in *tgfβ* from 5 to 8 hours of incubation in the 12 mg/L treatment (Figure 4.7C). OSPW-OF did not affect expression at any time or dose tested (Figure 4.7).

After exposure to whole OSPW or OSPW-IM at a range of doses from 4-12 mg/L (V/E) for 18 hours, RAW 264.7 cells showed a global down-regulation of *inos* mRNA (Figure 4.8A). In comparison, expression of the enzyme *arg* (arginase) showed a dose-dependent increase when cells were exposed to whole OSPW (4 mg/L-12 mg/L, NAs content; Figure 4B). Macrophages express two different but functionally related arginase enzymes and both *arg1* and *arg2* expression were similarly increased following OSPW exposures. Subsequent reference to arg expression throughout this thesis refers to *arg2.* In cells exposed to OSPW-IM 12 mg/L (V/E), *arg* mRNA levels were significantly higher (Figure 4.8B). Exposure of RAW 264.7 cells to OSPW-OF did not affect either enzyme at any dose.

Examination of the expression of *inos* and *arg* over time showed a general trend of down-regulation in the expression of genes involved in the pro-inflammatory pathway (*inos*) with a corresponding upregulation of the expression of gene involved in in the anti-inflammatory pathway (*arg*) when the RAW 264.7 cells were exposed to whole OSPW or OSPW-IM (Figure 4.9). This trend was most obvious in the 12 mg/L (V/E) treatment where there was a time-dependent transition from a significant increase in *inos* expression to a significant decrease over a period of 21.5 hours (Figure 4.9C). Exposure to whole OSPW significantly increased the mRNA levels of *arg* at all doses by 24 hours (Figure 4.9D-F). Alternatively, OSPW-IM only caused a statistically relevant increase in *arg* at the highest

dose, 12 mg/L (V/E), however there is an observable dose-dependent increase in the mRNA levels as time progressed at all treatments (Figure 4.9D-F). Exposure to OSPW-OF at any dose, or time did not cause deviations from baseline expression of either pro-inflammatory or anti-inflammatory enzymes (Figure 4.9).

4.4 Discussion

The mRNA levels of stress response genes suggest that whole OSPW and its fractions induce cellular stress. Expression of *hmox1* appeared to be dose-dependent in response to whole OSPW or OSPW-IM. There were no instances where whole OSPW significantly affected stress gene expression and OSPW-IM did not. Interestingly, there were instances in *hmox1* expression where the magnitude of effect was statistically different between whole OSPW and OSPW-IM which may suggest a role of organic constituents in increasing the oxidative stress experienced by treated cells. Low doses of a volatile organic mixture that included benzene, toluene, and xylene was shown to significantly increase DNA and oxidative damage by reducing levels of a peroxidase enzyme, effectively limiting the ability to mount an antioxidant response (127). This was not supported by my findings since expression of *hmox1* in cells treated with OSPW-OF did not exhibit changes in *hmox1* at lower doses.

Based on composition alone, it was expected that treatment with whole OSPW or OSPW-IM would cause increased oxidative effects compared to OSPW-OF, due to the ionic and metal constituents (128,129). Metal ion oxidative-toxicity is affected by solubility, absorbability, and intra-cellular reactivity, which may be further complicated by the addition of organic compounds. Previous reports in the literature have indicated that the presence of

dissolved organic compounds affected the bioavailability of metals- typically resulting in reduced toxicity; however these were not complex mixtures (130–132). Alternatively, the observed relative fold differences between whole OSPW and OSPW-IM could be due to slight changes in the water chemistry, other than removal of organics, caused by the separation process. This would be difficult to ascertain because of the extremely complex composition of whole OSPW.

The expression of *gadd45*, an indicator of genotoxic stress, was induced at early timepoints for all doses of OSPW and OSPW-IM tested and returned to baseline by 24 hours. At 18 hours, OSPW-OF caused a significant increase in the mRNA levels at 12 mg/L while whole OSPW did not. The induction of *gadd45* appeared to be both time and dose dependent following treatments with either whole OSPW or OSPW-IM. The observation that cells exposed to whole OSPW, OSPW-IM, or high doses of OSPW-OF experienced oxidative stress suggests a potential mechanism of concurrent DNA damage. While there is a significant induction of the cellular antioxidant response, indicated by *hmox1* upregulation, this response can become over-burdened by accumulation of oxidative stressors. Oxidative damage affects DNA, in addition to other molecules such as lipids and proteins, which in turn would encourage cells to elicit a genotoxic stress response (113,127,133). This stress gene expression analysis following exposure to whole OSPW, where there is an early, short-lived upregulation in *gadd45,* as well as the observed sustained increase in *hmox1* mRNA levels, is in agreement with our previous study where we proposed that inorganic components, or interactions between inorganic and organic constituents, may significantly contribute to whole OSPW toxicity in mammalian macrophages (13).

Pro-inflammatory cytokines are important in host response to infectious agents, and establishment of a robust and effective systemic immune response. TNF-α deficient animals are known to be significantly more susceptible to mortality and morbidity during infections with bacteria or parasites (134,135). There were inconsistent, and unpredictable trends of *tnfα* expression in cells treated with whole OSPW, OSPW-IM, or OSPW-OF which may suggest a more general dysregulation rather than a specific immunostimulatory or immunosuppressive effects. NAs are known to act as surfactants, which may be inducing membrane damage sufficient to increase *tnfα* expression, in response to the release of intracellular material following exposure (136–139).

Levels of *il-1β* mRNA were significantly increased at 2.5 hours in all doses, similar to the expression pattern observed of *gadd45*. Cellular responses to DNA damage trigger changes to the surrounding environment through a variety of mechanisms that can result in abnormal cytokine or free-radical production (140). Examining the changes in the *il-1β* and *tnfα* expression, there were instances where the relative increases observed after exposure of cells to either whole OSPW and OSPW-IM were significantly different. This was observed at early time points (<5 hours), at the low and intermediate dose tested, 4 or 8 mg/L (V/E) respectively. Again, these difference in the expression may indicate a minor contributory role of organic constituents in whole OSPW.

Considering the cells were stimulated with the pro-inflammatory stimulus LPS, expression of the anti-inflammatory cytokine gene, *tgfβ,* was expected to remain at the same levels as controls. This was generally true after exposure to OSPW-OF. On the other hand,

at time points <18 hours there were significant upregulations in the mRNA levels of *tgfβ* after exposure of cells to either whole OSPW or OSPW-IM. Between 5- and 8-hours post-exposure the expression of *tgfβ* increased significantly. Interestingly, the observed increase at this time point was directly contrasted by a return to baseline expression of the pro-inflammatory cytokine *il-1β*.

Macrophages possess the ability to alter their activation states in order to promote a resolution phase following inflammation (141). Stimulation with type 2 cytokines, notably IL-4 and IL-13, can cause macrophages to become alternatively activated. This pathway induces a functional shift from inflammation (M1) to tissue repair (M2). The classically and alternatively activated pathways are mutually exclusive and are defined by the dichotomous expression and activity of the enzymes iNOS and arginase which utilize the same substrate (L-arginine) for their activity (142,143). Under normal circumstances, a macrophage faced with a pathogen challenge, or mimic such as LPS, would generate a pro-inflammatory response to eliminate the threat (144). Remarkably, following exposure to doses as low as 4 mg/L (V/E) of whole OSPW or OSPW-IM RAW 264.7 cells had a significant decrease in the mRNA levels of *inos*, coinciding with a dose-dependent increase in arginase (*arg*) expression. This suggests that exposure to whole OSPW or OSPW-IM may be altering the functional phenotype of these cells. Interestingly, even at the highest tested dose (12 mg/L (V/E)) *inos* expression was significantly increased at early timepoints while *arg* expression remained at control levels; indicating that the inability to appropriately upregulate *inos* is not because of a general inhibition in these cells. Toxicology literature generally reports that deficiencies in a typical response using a well-characterized pathway are caused by generic

inhibitory, or “toxic” effects of the xenobiotic; my results provide evidence that environmental contaminants may function by altering the functional cellular phenotypes of immune cells such as macrophages.

Table 4.1 Primer sequences of genes in qPCR analysis.

Gene		Primer Sequence (5'→ 3')
hprt1	**Hypoxanthine-Guanine Phosphoribosyltransferase** Endogenous Control	**F:** GTTAAGCAGTACAGCCCCAAAATG **R:** AAATCCAACAAAGTCTGGCCTGTA
tnfα	**Tumour Necrosis Factor-Alpha** *Pro-inflammatory Cytokine*- Chemotaxis, phagocytosis and NO production enhancement of macrophages	**F:** CACAAGATGCTGGGACAGTGA **R:** TCCTTGATGGTGGTGCATGA
il-1β	**Interleukin One-Beta** *Pro-inflammatory Cytokine*- Granulocyte chemoattractant, induction of systemic response, fever	**F:** GCACTACAGGCTCCGAGATGAAC **R:** TTGTCGTTGCTTGGTCT CCTTGT
inos	**Inducible Nitric Oxide Synthase** *Antimicrobial Response*- marker of M1 macrophage phenotype	**F:** GTTCTCAGCCCAACAATACAAGA **R:** GTGGACGGGTCGATGTCAC
arg	**Arginase** *Wound Repair*- marker of M2 phenotype	**F:** TCCTCCACGGGCAAATTC **R:** GCTGGACCATATTCCACTCCTA
tgfβ	**Transforming Growth Factor-Beta** *Anti-inflammatory Cytokine*- Reduces pro-inflammatory cytokine production by other cells.	**F:** CTCCCGTGGCTTCTAGTG **R:** GCCTTAGTTTGGACAGGATCTG
hmox1	**Heme Oxygenase One** *Stress Response*- Oxidative stress	**F:** CCTGGTGCAAGATACTGCCC **R:** GAAGCTGAGAGTGAGGACCCA
gadd45	**Growth Arrest and DNA Damage-Inducible Protein** *Stress Response*- DNA damage inducible	**F:** AGTCAGCGCACCATTACGGT **R:** GGATGAGGGTGAAATGGATCTG

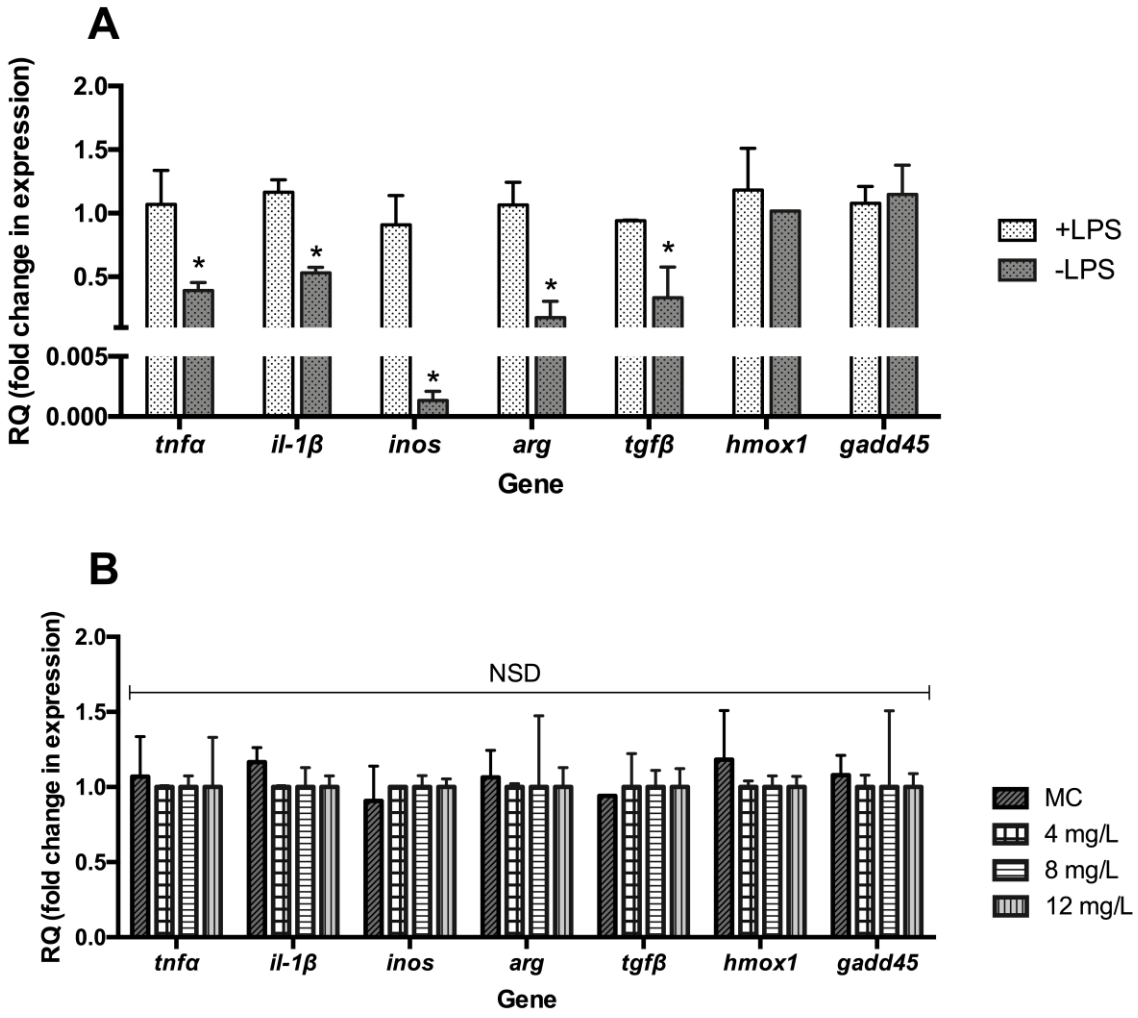


Figure 4.1 Gene expression of RAW 264.7 cells following control treatments.
(A) Cells were cultured in complete medium and treated with LPS (1 μg/mL) or V/E of diluent for 18 hours prior to extraction of total RNA. (B) Cells were cultured in complete medium (MC), or V/E of PBS for the respective doses listed and treated with LPS as described prior to extraction of total RNA. RNA was reverse transcribed to cDNA. mRNA expression levels are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. Values are the mean ±SEM of 4 independent experiments. Statistics performed with a t-test (A), or a two-way ANOVA with a Dunnett's *post hoc* test (B), * indicates $p < 0.05$ between groups, NSD is no statistical difference.

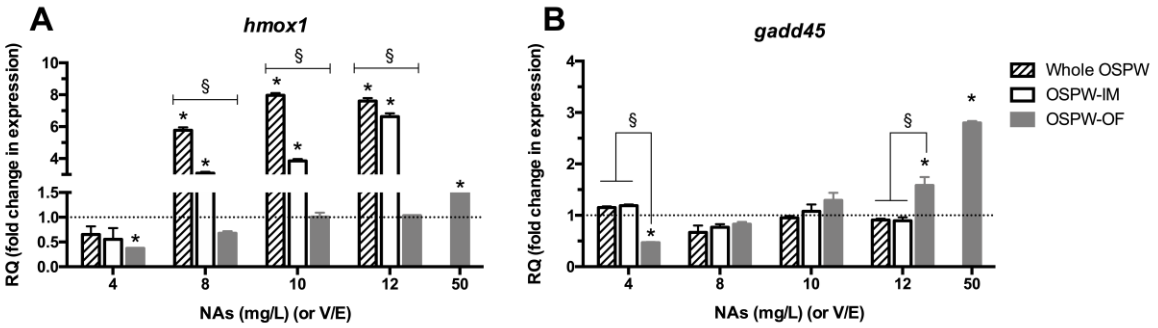


Figure 4.2 Stress gene expression of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 16 hours (18 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of stress response gene *hmox1* (A) and DNA-damage inducible gene *gadd45* (B) are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

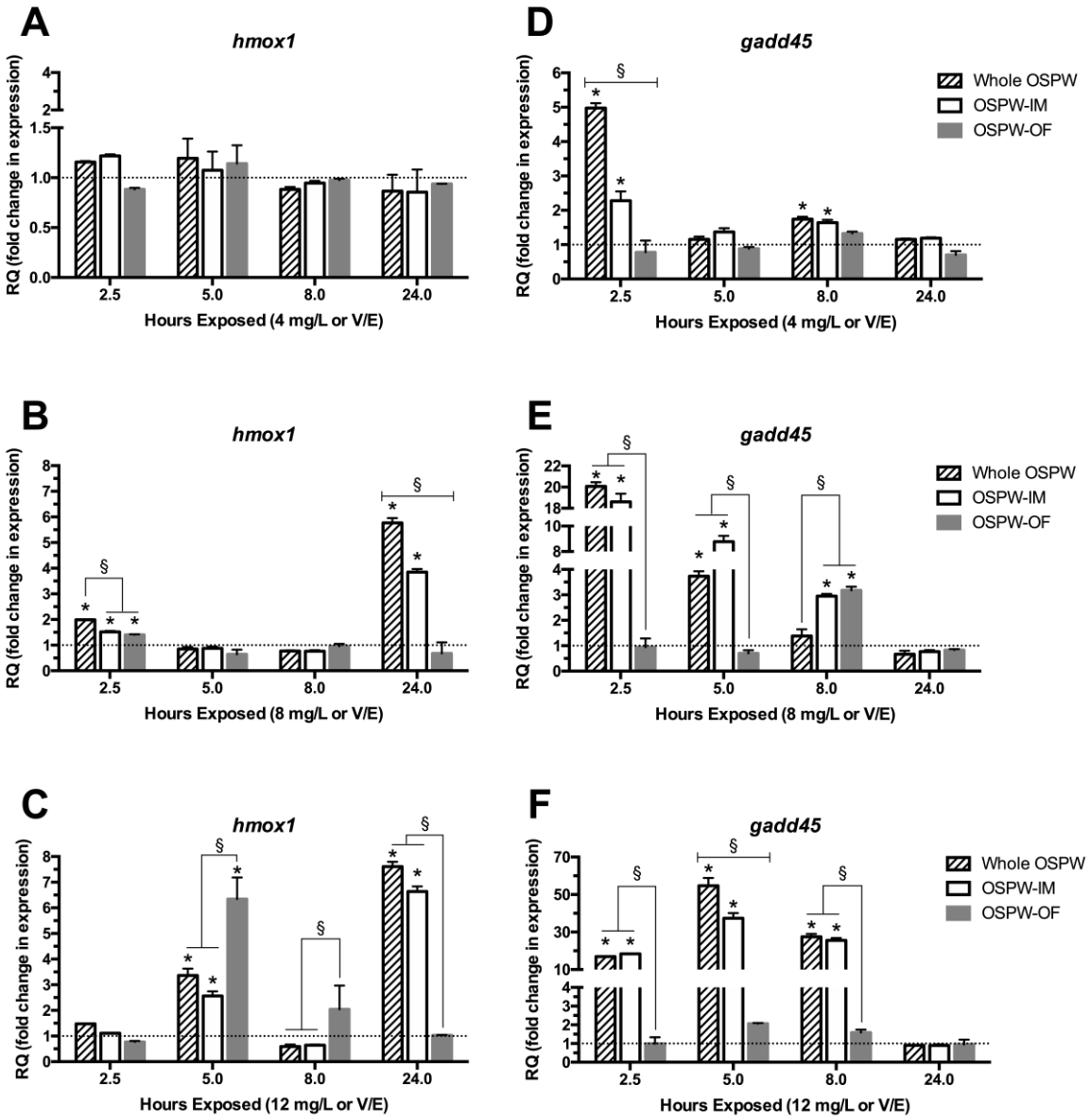


Figure 4.3 Effects of exposure time on stress gene expression of RAW 264.7 cells. Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 0.5, 3, 6, or 22 hours (2.5, 5, 8, or 24 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of stress response gene *hmox1* (A-C) and DNA-damage inducible gene *gadd45* (D-F) are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

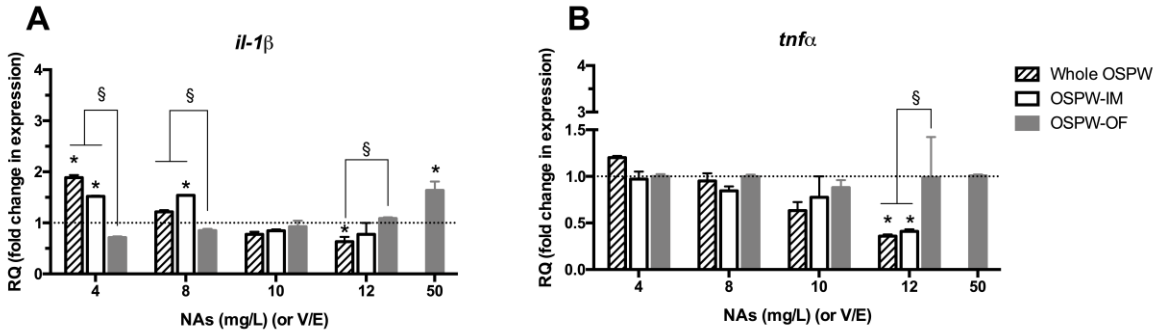


Figure 4.4 Pro-inflammatory cytokine gene expression of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 16 hours (18 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of pro-inflammatory immune genes *il-1β* (A) and *tnfα* (B) are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

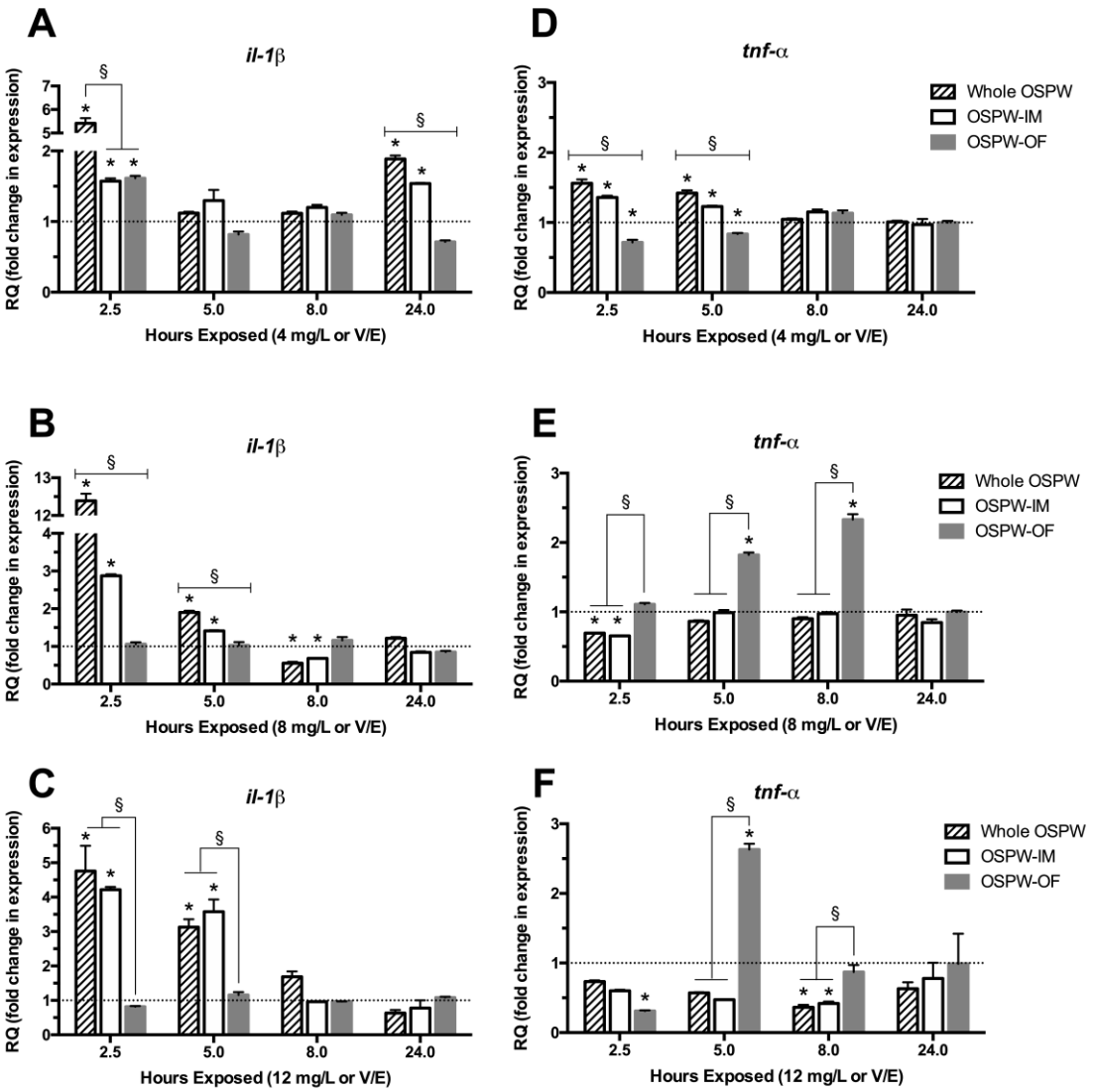


Figure 4.5 Effects of exposure time on pro-inflammatory cytokine gene expression in RAW 264.7 cells.

Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 0.5, 3, 6, or 22 hours (2.5, 5, 8, or 24 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of pro-inflammatory immune genes *il-1β* (A-C) and *tnfα* (D-F) are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

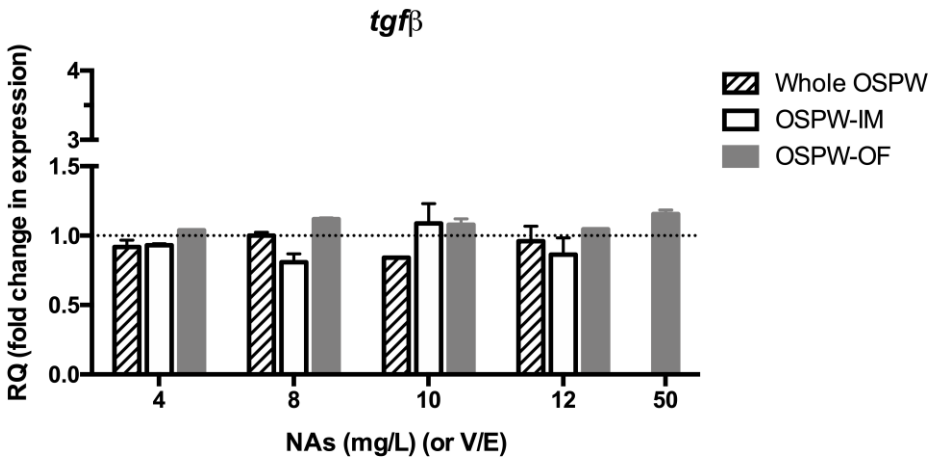


Figure 4.6 Anti-inflammatory cytokine gene expression of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 16 hours (18 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of the anti-inflammatory immune gene *tgfβ* are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

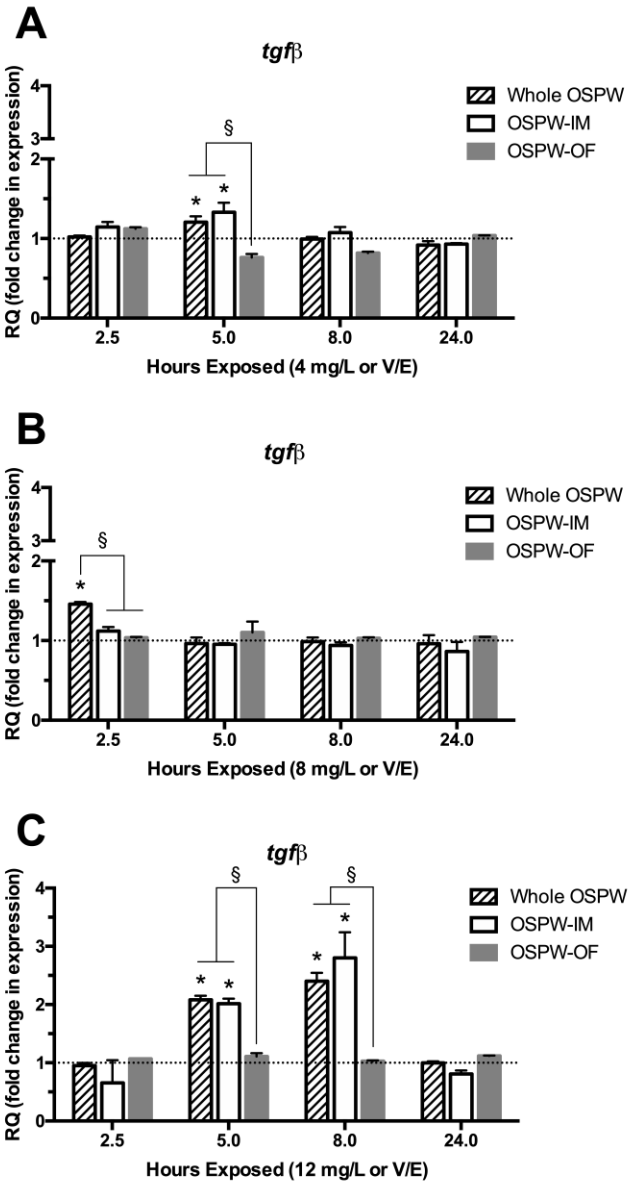


Figure 4.7 Effects of exposure time on anti-inflammatory cytokine gene expression in RAW 264.7 cells.

Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 0.5, 3, 6, or 22 hours (2.5, 5, 8, or 24 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of the anti-inflammatory immune gene *tgfβ* are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

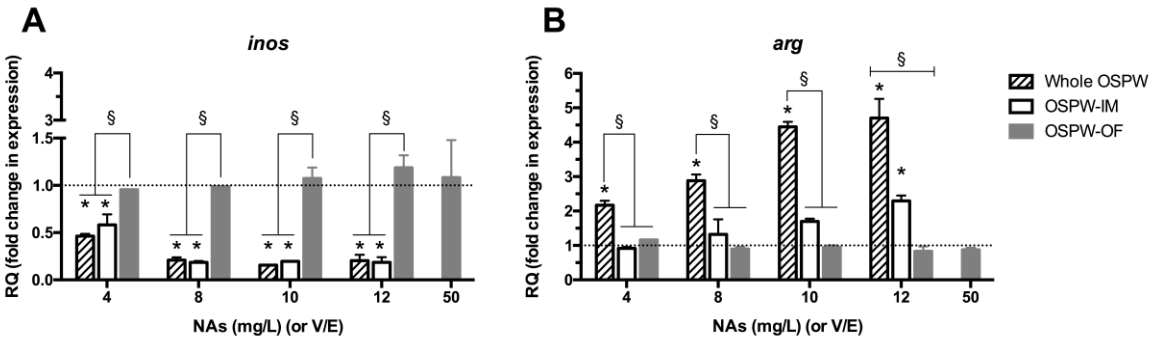


Figure 4.8 Gene expression of enzymes involved in M1 and M2 macrophage phenotype polarization in RAW 264.7 cells.

Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional16 hours (18 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of pro-inflammatory immune gene (*inos*)(A) and anti-inflammatory gene, arginase (*arg*) (B) are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

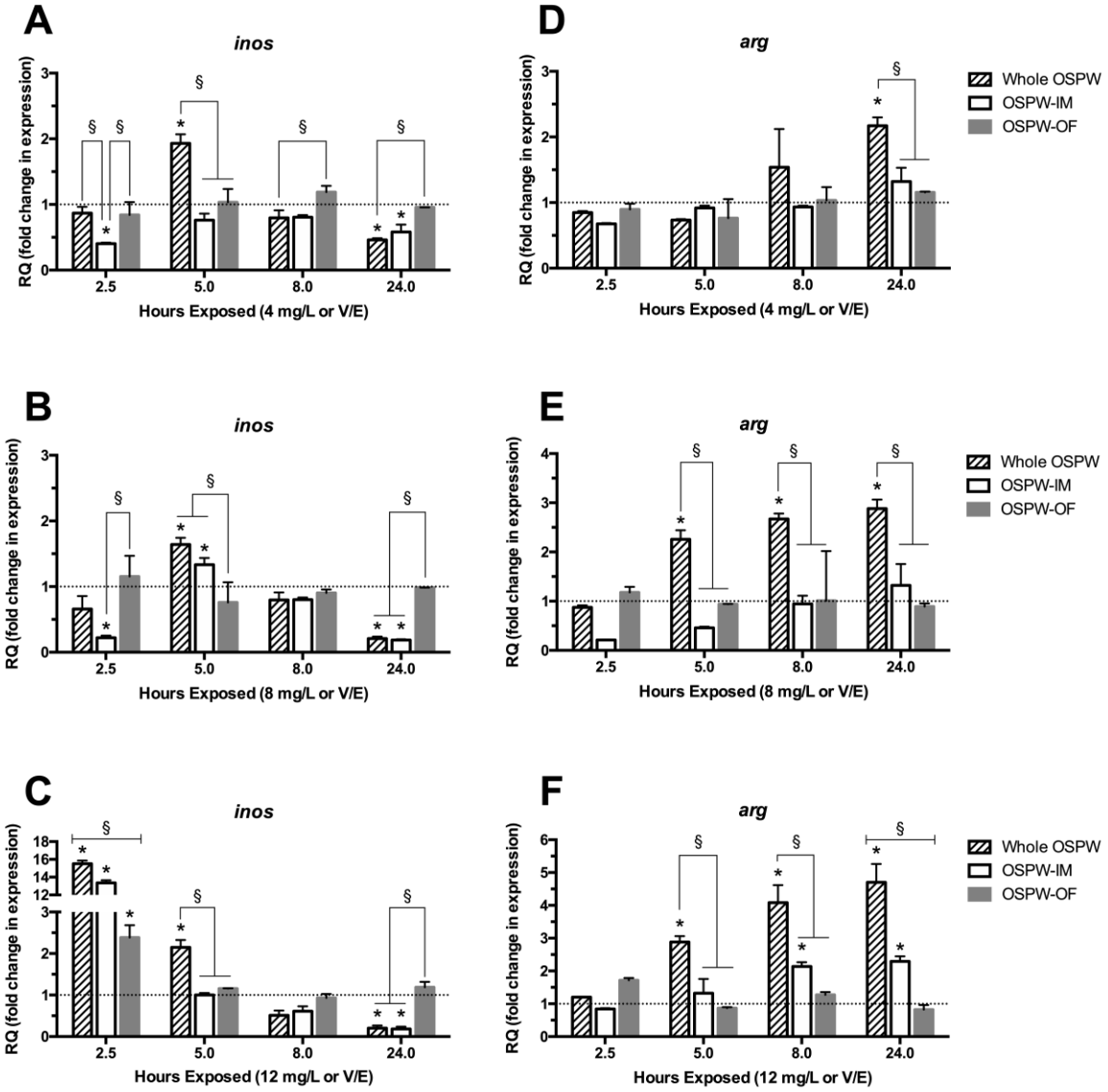


Figure 4.9 Gene expression of enzymes involved in M1 and M2 macrophage polarization over time in RAW 264.7 cells

Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure cells were stimulated with LPS (1 μg/mL) for an additional 0.5, 3, 6, or 22 hours (2.5, 5, 8, or 24 hours total). Total RNA was then extracted and reverse transcribed to cDNA for qPCR analysis using the ddCT method. Results of the mRNA expression levels of pro-inflammatory, M1 immune gene (*inos*) (A) and anti-inflammatory, M2 gene *arg* (B) are presented as RQ, representing their fold differences relative to the endogenous control gene *hprt1*. RQ values of whole OSPW, OSPW-IM were normalized to the PBS dilution control and OSPW-OF values were normalized to non-exposed (media) controls, represented by the dashed line at RQ=1. All plotted values are the mean ± SEM from 4 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to control, § indicates $p < 0.05$ between the indicated experimental groups.

Chapter 5

Differential toxic effects on the immune functions of RAW 264.7 cells exposed *in vitro* to whole OSPW and its fractions

5.1 Introduction

Exposure of fish and mammals to the organic fraction of OSPW impairs immune function at a cellular and organismal level (145). These effects include both suppressive and stimulatory deviations from homeostasis leading to an inability to clear and/or increased susceptibility to infections and chronic inflammation (146–148). The organic fraction (OSPW-OF) containing high concentrations of naphthenic acids (NAs) has been shown to induce dose dependent toxicity in aquatic and terrestrial organisms and has been the primary focus of toxicity assessments to date, while toxicity of the inorganic matrix (OSPW-IM) has largely been ignored. Recently, it has been shown that the toxicity of unfractionated (whole) OSPW has similar toxic effects in RAW 264.7 cells as the OSPW-OF, but at much lower NAs concentrations suggesting that other components present in OSPW, such as OSPW inorganic matrix (OSPW-IM), may also contribute to toxicity in either direct, additive or synergistic manner (13).

The immune system is tightly-regulated to balance protection from, and elimination of pathogens without inducing excessive damage caused by chronic inflammation. Therefore, an appropriate response requires specific activation, generation of the protective response, clearance of the pathogens, and a return to homeostasis. Previous immunotoxicity studies have examined key genes in induction of an immune response (18,51,52). Immune gene expressions are induced by specific activation events, initiating the appropriate signaling

cascades in immune cells, such as macrophages, which in turn generate the required mediators and effector molecules to eventually return the host to homeostasis.

Changes in the gene expression profile and activity of macrophages demonstrates their response plasticity. The generation of macrophage phenotypes was reported to occur through complicated interplays of environmental signals as well as the hardwired differentiation programs (124). Both macrophage phenotypes (M1 and M2) are sensitive to this process. These phenotypes are mutually exclusive and maintained as such by several regulatory mechanisms; including those facilitated by their central effector-enzymes, iNOS and arginase, respectively. Characterization of macrophage phenotypes requires a comprehensive approach; examining the gene expression patterns, protein expression patterns, as well as the resulting cellular functions. Traditionally, these evaluations are centered around the dichotomous relationship between iNOS and arginase, which utilize the same substrate, L-arginine (149,150).

In the previous chapter, I reported the effects of whole OSPW and OSPW-IM on expression of M1 and M2 macrophage phenotype associated genes, most importantly iNOS and arginase. The results indicated that exposure to either OSPW or OSPW-IM induced a shift in mRNA levels of the genes encoding these key enzymes despite stimulation designed to induce an M1 macrophage phenotype. In this chapter I report the effects of OSPW, and OSPW-IM exposures on immune function of RAW 264.7 cells.

5.2 Materials and methods

5.2.1 Phagocytosis assay

RAW 264.7 cells were cultured as previously described in Chapter 3 (Section 3.2.1) then seeded in 1 mL of complete medium at a density of $3x10^5$ cells per well in 24-well plates and cultured for 24 hours at 37°C in 5% CO_2 atmosphere before exposure to OSPW. Cells were exposed to 4, 8, or 12 mg/L OSPW, OSPW-OF (or V/E for OSPW-IM) using the procedure outlined in Chapter 3 (Section 3.2.3) for either 2 or 24 hours, then washed twice with PBS prior to addition of 5 µL of 2 mg/mL pHrodo *E. coli* targets (ThermoFisher). Phagocytosis was left to proceed for 1 hour at 37°C. The cells were then washed twice with PBS, and detached using 1 mM EDTA + 0.25% trypsin (Sigma) for collection. RAW 264.7 cells were fixed with ice-cold 4% paraformaldehyde (PFA) and moved to 96 well plates for analysis by flow cytometry using the ImageStream Mark II (Amnis Corporation) flow cytometer. The percent (%) phagocytosis was determined, i.e. the number of cells positive for pHrodo *E. coli* based on fluorescence intensity. To emit fluorescent signal, the pHrodo *E. coli* must be exposed to an acidic pH which occurs only after successful internalization. One set of medium-only cells (controls) were treated with 10 µM of the cytoskeleton inhibitor cytochalasin D (Millipore Corporation, USA), which blocked phagocytosis.

5.2.2 Nitric oxide assay

Nitric oxide production was assessed after bacterial lipopolysaccharide (LPS) stimulation of RAW 264.7 cells using the Griess reaction. A density of $1x10^5$ RAW 264.7 cells per well were seeded in 100 µL of complete medium into 96-well plates and cultured for 24 hours at 37°C in 5% CO_2 atmosphere for 24 hours, before exposure to OSPW as previously described in Chapter 3 (Sections 3.2.1 and 3.2.3). The following day exposures to

1, 4, 6, 8, 10, or 12 mg/L OSPW (or V/E for OSPW-IM) were performed in two sets of triplicates for each. After 2 hours incubation exposed to only OSPW, one triplicate was stimulated with LPS (1 µg/mL per well) and the other triplicate had an equivalent volume of PBS (diluent) added as outlined in Chapter 4 (Section 4.2.1). A sample of OSPW only (no cells) was included as a blank control, and to prevent interpretation of artificial effects on nitric oxide production by constituent interference with the assay itself. After exposure for an additional 0.5, 1, 3, 6, 12 or 22 hours (2.5, 3, 5, 8, 14, and 24 hours of total times of exposures, respectively), 75 µL of supernatant was collected and reactive nitrogen intermediate production (nitrite concentration) determined using the Griess reaction. Supernatants were mixed with 50 µL of 0.1% N-[1-naphthyl]-ethylenediamine in 2.5% phosphoric acid, then 50 µL of 1% sulphanilamide in 2.5% phosphoric acid. Nitrite concentrations were determined by measuring absorbance at 540nm on a SpectraMax M_2 plate reader (Molecular Devices) and a nitrite standard curve generated for each experiment. A sample nitrite standard curve is shown in Figure 5.1.

5.2.3 Arginase activity assay

Enzymatic activity of arginase was assessed in response to bacterial LPS stimulation of non-exposed cells and cells exposed to OSPW, OSPW-OF or OSPW-IM. RAW 264.7 cells were cultured as previously described in Chapter 3 (Section 3.2.1) then seeded in 2 mL of complete media at a density of $1.8x10^6$ RAW 264.7 cells per well in 6-well plates and incubated at 37°C for 24 hours before exposure to OSPW. The following day exposures to 4, 8, or 12 mg/L of OSPW (or V/E for OSPW-IM) were performed using the procedure outlined in Chapter 3 (Section 3.2.3), and cells were incubated for two hours. After two hours of exposure to either OSPW or OSPW-IM, a subset of the cells was stimulated with LPS (1

μg/mL per well) as previously described in Chapter 4 (Section 4.2.1) and incubated for an additional 22 hours (24 hours total) at which time the cells were harvested using 1 mM EDTA + 0.25% trypsin (Sigma). Cells were enumerated using a hemocytometer, and $1.0x10^6$ cells were collected for determination of arginase activity.

In triplicate, $1.0x10^6$ cells were lysed at room temperature for 10 minutes using 100 μL of 10 mM Tris-HCl, pH=7.4 with 1 μM pepstatin A (Sigma), 1 μM leupeptin (Sigma), and 0.4% Triton X-100. Insoluble material was removed by centrifugation, and 40 μL of supernatant was kept and mixed with 10 μL of deionized endotoxin-free water. The arginase reaction was used to determine the rate of urea formation using the Arginase Activity Assay Kit (Sigma). The enzymatic reactions were incubated for 180 minutes at 37°C. The urea production was determined by measuring absorbance at 430 nm on a SpectraMax M_2 plate reader (Molecular Devices) using a urea standard curve generated for each experiment. The arginase activity was used to calculate arginase levels with the following equation, where one unit of arginase is the amount that will convert 1.0 μmol of L-arginine to ornithine and urea per minute at 37°C, a sample calculation is shown in Figure 5.2:

$$\text{Arginase}\left({}^{\text{Units}}/_{\text{L}}\right) = \frac{\text{A430}_{sample} - \text{A430}_{blank}}{\text{A430}_{standard} - \text{A430}_{water}} \times \frac{1\text{mM} \times 50\mu\text{L} \times 10^3}{\text{V} \times \text{T}}$$

V= Sample Volume (μL), T= Rxn Time (mins), 1mM= Urea Std Concentration, 50 μL= Rxn Volume (μL)

5.2.4 Expression of iNOS and arginase proteins

5.2.4.1 Western blotting

Production of arginase and iNOS protein was assessed using Western blotting. RAW 264.7 cells were cultured as previously described in Chapter 3 (Section 3.2.1) then seeded in 2 mL of complete medium at a density of $1.8x10^6$ cells per well in 6-well plates and cultured for 24 hours at 37°C in 5% CO_2 atmosphere prior to OSPW, OSPW-OF or OSPW-IM exposures. Cells were exposed to 4 or 12 mg/L OSPW or V/E of OSPW-IM using the procedure outlined in Chapter 3 (Section 3.2.3). After exposure for 2 hours OSPW, OSPW-OF or OSPW-IM, a subset of the cells was stimulated with LPS (1 μg/mL per well) according to the procedure described in Chapter 4 (Section 4.2.1). Cells were lysed at 22 hours post-stimulation using 1% Triton-X 100 in PBS containing a 1% Proteinase Inhibitor Cocktail (Calbiochem). Whole cell lysate was then mixed in 4X Lamelli buffer with 5% β-mercaptoethanol and boiled for 10 minutes at 95°C prior to being run on an 8% SDS-PAGE gel.

Proteins were transferred from the SDS-PAGE gel to nitrocellulose membranes using the BioRad Trans-Blot system at 125V for one hour. Following transfer, membranes were incubated overnight at 4°C in 5% skim milk solution. After blocking, membranes were washed in TBST three times for 10 minutes prior to incubation with the primary antibody (1:5000) for arginase (ThermoFisher) or iNOS (ThermoFisher) at 4°C for one hour. Membranes were again washed 3X for 10 minutes with TBST between incubations using the primary and secondary (1:10 000 dilution) of rabbit-anti-mouse HRP conjugated antibody (ThermoFisher) for one hour at 4°C. After a final wash, membranes were developed using

SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher) and imaged using a BioRad ChemiDoc Imager (Molecular Biology Service Unit, University of Alberta).

5.2.4.2 Flow cytometry assessment of iNOS

Production of iNOS protein was assessed quantitatively using the iNOS-PE monoclonal antibody (eBioscience/Invitrogen) and flow cytometry. RAW 264.7 cells were cultured as previously described in Chapter 3 (Section 3.2.1) then seeded in 2 mL of complete medium at a density of $3x10^5$ cells per well in 24-well plates and incubated for 24 hours at 37°C in 5% CO_2 atmosphere for 24 hours prior to OSPW exposures. The following day the cells were exposed to 4 or 12 mg/L OSPW, OSPW-OF or V/E for OSPW-IM using the procedure described in Chapter 3 (Section 3.2.3), and cells were incubated for 2 hours before stimulation with LPS (1 μg/mL per well) as outlined in Chapter 4 (Section 4.2.1). Cells were harvested with 1 mM EDTA + 0.25% Trypsin (Sigma) at 22, 10, or 4 hours post-stimulation and fixed by adding 250 μL of 4% paraformaldehyde for 20 minutes at 4°C to each well. Following fixation cells were permeabilized using permeabilization buffer (eBioscience/Invtitrogen) at 4°C for 30 minutes, the permeabilization buffer was then removed and cells were stained with iNOS-PE antibody (0.06 μg/sample) for 30 minutes at 4°C. The stained cell cultures were then analyzed using an ImageStream Mark II (Amnis Corporation) flow cytometer.

5.2.5 Statistical analysis

Data are presented as Mean ± Standard error of the mean (SEM). Statistical analysis of experimental data was performed with a two-way ANOVA, followed by the Tukey's *post hoc* test, using Prism 6.0 (Graphpad software, CA, USA). Statistical analysis of the various

functional controls, PBS dilution control or non-exposed (medium) controls, were performed using a two-way ANOVA, followed by a Dunnett's *post hoc* test or t-test, using Prism 6.0. Differences between treatment groups were considered statistically significant when $p < 0.05$.

5.3 Results

5.3.1 Phagocytosis

Exposure to whole OSPW, OSPW-IM, or OSPW-OF had no effect on the phagocytic ability of RAW 264.7 cells (Figure 5.3 & Figure 5.4). Increasing the incubation time with the phagocytic target pHrodo *E. coli* also did not change the average percent of phagocytosis with all treatment groups exhibiting 30-40% phagocytosis after exposure to test waters for 2- and 24-hours exposure, respectively (Figure 5.3, Figure 5.4).

5.3.2 M1 and M2 macrophage phenotype activity

5.3.2.1 Exposure to OSPW, OSPW-OF, or OSPW-IM

Following exposure to whole OSPW, OSPW-OF or OSPW-IM (V/E) at a range of doses from 4-12 mg/L for 24 hours, there was a significant reduction in the ability to produce RNI at 4 mg/L of OSPW-IM (V/E), which was sustained to the highest tested dose, 12 mg/L OSPW-IM (V/E) following stimulation with LPS (Figure 5.6A). OSPW-OF did not affect production of RNI at any of the tested doses (Figure 5.6A). At 6 mg/L (V/E) of whole OSPW or OSPW-IM the stimulated cells produced equivalent amounts of nitric oxide as the non-stimulated, negative control group ($p > 0.3$, Table 5.1, Figure 5.6A). This effect was sustained at all subsequent doses of whole OSPW or OSPW-IM. Alternatively, there was no significant effect of exposure to whole OSPW, OSPW-IM, or OSPW-OF on nitric oxide production in non-stimulated cells which remained at the same amount as non-stimulated controls (Figure 5.6B). As expected nitric oxide production of RAW 264.7 cells stimulated with LPS and

equivalent volume of PBS showed consistent nitrite production which was absent in cells not stimulated with LPS (Figure 5.5).

Activity of the M2 enzyme, arginase, was significantly increased after exposure to 4 mg/L (V/E) of whole OSPW or OSPW-IM compared to the M1 induced- control (dotted line; Figure 5.7). Following exposure to 12 mg/L (V/E), arginase activity further increased; becoming significantly higher than the non-stimulated medium control, and 4 mg/L (V/E) treatment (Figure 5.7). Whole OSPW treated cells showed the largest increase in their arginase activity, more than doubling when the dose was increased from 4 mg/L to 12 mg/L (Figure 5.7). OSPW-IM also showed a dose-dependent increase, however at 12 mg/L it was significantly less than whole OSPW (Figure 5.7). The M1 induced control cells had less than 50% of the arginase activity retained by baseline (untreated) RAW 264.7 cells (Figure 5.6).

5.3.2.2 Exposure to mixtures of OSPW-OF and OSPW-IM

Production of RNI in cells exposed to mixtures of OSPW-IM and OSPW-OF where the NA concentration was inversely related to percent volume of OSPW-IM in each mixture was assessed. As cells were exposed to increasing amounts (V/V) of OSPW-IM, the amount of nitric oxide produced was decreased in a dose-dependent manner, with a significant reduction at 10% V/V OSPW-IM (16.2 mg/L NAs) (Figure 5.8A). This directly contrasted the trends observed where PBS replaced the volume equivalent of OSPW-IM (PBS + OSPW-OF) which had no significant effect on production of RNI (Figure 5.8B).

5.3.3 Proteins

The production of M1 and M2 enzymes, iNOS and arginase respectively, was assessed using Western blot. Following exposure to 4 mg/L (V/E) the observed banding of arginase, at the expected size of ~35 kDa, was similar to the non-stimulated media control (Figure 5.9A). The arginase signal intensity increased from the non-stimulated baseline, and 4 mg/L (V/E) doses, in cell lysates harvested from cells treated with 12 mg/L (V/E) of whole OSPW or OSPW-IM (Figure 5.9A). The LPS-stimulated media control had a very faint band in comparison to all other groups (5.9A). In contrast, the LPS-stimulated media control group showed a strong band at the predicted iNOS size of ~135 kDa (Figure 5.9B). The Western blot of iNOS after exposure to 4 mg/L (V/E) was similar to the stimulated non-exposed cells (Figure 5.9B). Banding was visible for iNOS in all groups, but was markedly weaker in those from a 12 mg/L (V/E) whole OSPW or OSPW-IM exposures, with these being most comparable in intensity to the non-stimulated media control (Figure 5.9B). Taken together, the banding profiles following treatment with 12 mg/L whole OSPW or OSPW-IM of the M2 associated enzyme arginase (Figure 5.9A) showed a contrasting trend of signal intensity when compared to that of the M1 associated enzyme, iNOS (Figure 5.9B).

Quantitative evaluation of iNOS protein was done using flow cytometry of cells exposed to whole OSPW, OSPW-IM or OSPW-OF with LPS stimulation. These results agree with the Western blot findings, where at 4 mg/L the number of cells positive for iNOS was not significantly different in any treatment group from the non-exposed control (Figure 5.10). This absence of effect was consistent for all time points tested; 6, 12, and 24 hours (Figure 5.10). Following exposure to 12 mg/L (V/E) of whole OSPW or OSPW-IM there was a significant reduction in the number of iNOS positive cells, and this reduction appeared to be

time dependent where at the early time there was no significant effect, moving to a significant decrease at 12 hours that was sustained at 24 hours (Figure 5.10). There was no effect of OSPW-OF on the number of iNOS positive cells in any time or dose examined (Figure 5.10).

5.4 Discussion

The ability to induce a robust and effective response to pathogens is key to host survival. However, equally important is the alternative activation state of macrophages (M2) which promotes a resolution phase following inflammation (141). The signalling, response, and function of both macrophage phenotypes makes them complex regulators of the entire immune response. Under normal circumstances a macrophage activated with a potent pro-inflammatory stimulus, such as LPS, would normally generate an M1 type response (killer macrophage phenotype) that includes production of antimicrobial and cytotoxic products such as reactive nitrogen intermediate (RNI), reactive oxygen intermediates (ROI) and antimicrobial peptides (AMPs). The activity of iNOS would be regulated and modulated by engagement of several LPS specific cellular receptors including CD 14 and TLR 4 inducing downstream signalling cascades that eventually cause the activation NFκB and its translocation to the nucleus (151). Following exposure to whole OSPW or OSPW-IM the ability to produce RNI was shown to be significantly impaired, likely due to the observed decrease of iNOS protein that catalyzes breakdown of L-arginine to citrulline and eventual production of RNI. The reduction in iNOS protein levels, and the reduction in RNI produced coincides with dose-dependent increases in arginase activity and protein levels. This suggests that exposure to whole OSPW or OSPW-IM altered the functional phenotype of RAW 264.7 macrophage-like cells.

When cells were exposed to mixtures of OSPW-IM and OSPW-OF, where the NA concentration was inversely related to percent volume of OSPW-IM, the ability to produce nitric oxide continued to decrease in a dose-dependent manner, similar to that observed for whole OSPW only. This suggests a dominant immunomodulatory effect caused by OSPW-IM constituents. Specifically, the immunotoxic effects were similar when cells were exposed to the OSPW-IM + OSPW-OF mixture to those exposed to OSPW-IM + PBS, where PBS was added in volume equivalents to OSPW-OF, indicating that the addition of organic components, including NAs, had no supplementary effect on reduced RNI production. These results are supported by a previous study where ozonation of OSPW, which effectively reduced the amount of NAs by ~90%, did not improve the production of RNI by cells exposed to whole OSPW from 1-10 mg/L NAs (56). The implication of a dominant inorganic matrix-mediated effect is of particular interest since the major focus in the literature is on the toxicity induced by the OSPW organic constituents, primarily NAs (152–155).

The results of gene expression studies shown in the previous chapter, indicated that early exposure of cells to test waters caused significantly overexpressed *inos*. The results presented in this chapter that this overexpression of *inos* does not correspond to an increase in the protein production, and furthermore, there was also the inability of the cells to produce RNI following exposure to as low as 4 mg/L (V/E) of whole OSPW or OSPW-IM. In contrast, the results indicate that increasing mRNA levels of arginase coincided with increased protein signal intensity and function. Expression of arginase is known to reduce the availability of extracellular arginine, which suppresses the iNOS pathway and ability of cells to generate nitric oxide response (126,156–158). Arginase maximal activity is >1000

fold higher than iNOS, resulting in rapid consumption of the shared substrate, L-arginine (159). Furthermore, M2 macrophages may use exhaustion of extracellular arginine to enhance their anti-inflammatory role as they do not require employment of an arginine recycling pathway compared to M1 macrophages (126).

The number of iNOS positive cells, decreased after exposure to OSPW 12 mg/L (NAs content). At early exposure time point (6 hours) there were fewer positive cells including in the controls, which was likely due to the limited rate at which proteins can be translated (160). For example, at the 12-hour time point, ~50% of cells in the control group were iNOS positive, compared to ~16% at 6 hours. Interestingly, the percent of iNOS positive cells in the whole or OSPW-IM treated groups was reduced to <10% at 12 hours, from ~20% at 6 hours. One would expect that if protein synthesis as a whole was experiencing translational blockage or inhibition, the amount of iNOS produced would remain constant over time. Instead there was a substantial decrease in the amount of iNOS positive cells; suggesting an active mechanism for the loss of iNOS occurred in the exposed cells. Arginase has been implicated in mediating destabilization and uncoupling of the iNOS protein in addition to translational repression (126,161). The Western blot results showed a dose-dependent increase in arginase, suggesting that RAW 264.7 cells produced additional arginase protein after exposure to whole OSPW or OSPW-IM, instead of synthesizing iNOS. This observation was also supported by measurements of arginase activity, that increased significantly compared to that observed for M1 induced control and from the non-stimulated, baseline control. Together these results indicate that exposure to whole OSPW or OSPW-IM constituents causes a shift in macrophage phenotypes.

Following exposure to whole OSPW or its fractions there were no changes in phagocytic ability of the RAW 264.7 cells. This was unexpected with the results of the previous gene expression and other functional assays which strongly indicate a phenotypic shift toward an alternatively activated state. M2 macrophages retain partial phagocytic activity but preferentially use endocytosis instead (162). While M2 cells maintain the structural ability to phagocytose, their targets are mainly apoptotic cells, or self-cellular debris, and therefore they become less competent in their internalization of pro-inflammatory targets due to the changes in their receptor repertoire (150,162). Given the complexity of OSPW, it is possible that an M2-enhanced phenotype has been induced, but has maintained some ability to perform classically activated functions, such as phagocytosis of bacteria. Synthetic innate defense regulators (IDRs) are peptides with designed immunomodulatory effects to steer the innate immune response (163–165). Treatment with a specific cationic IDR produced an intermediate macrophage phenotype that displayed select characteristics of both M1 and M2 phenotypes, suggesting that immunomodulators may induce non-defined phenotypes within the continuum of M1 to M2 defined status (165).

Conventionally macrophage polarization is thought to occur by environmentally-specific cues including cytokines, growth factors, and pathogen associated molecular patterns (124). The common factor between these cues is that they are of biological origin. My results showed that anthropogenic xenobiotics such as OSPW and its fractions can also alter macrophage polarization at the gene, protein and functional levels which may have profound influence on the ability of exposed hosts to control infectious diseases.

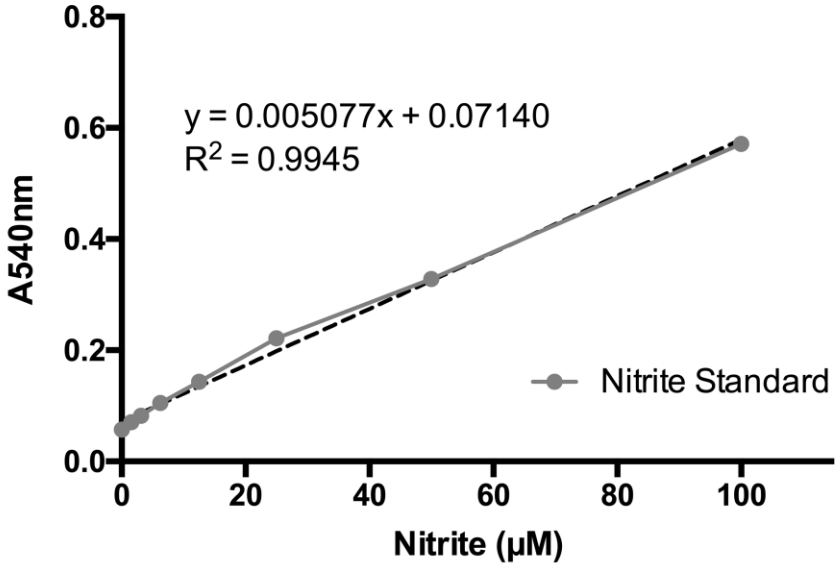


Figure 5.1 Sample nitrite standard curve.

$$\text{Activity } \left({}^{\text{Units}}/_{\text{L}}\right) = \frac{\text{A430}_{sample} - \text{A430}_{blank}}{\text{A430}_{standard} - \text{A430}_{water}} \times \frac{1\text{mM} \times 50\mu\text{L} \times 10^3}{\text{V} \times \text{T}}$$

V= Sample Volume (µL), T= Rxn Time (mins), 1mM= Urea Std Concentration, 50 µL= Rxn Volume (µL)

$$\text{Activity } \left({}^{\text{Units}}/_{\text{L}}\right) = \frac{\text{A430}_{sample} - \text{A430}_{blank}}{\text{A430}_{standard} - \text{A430}_{water}} \times \frac{1\text{mM} \times 50\mu\text{L} \times 10^3}{40\mu\text{L} \times 180\text{mins}}$$

$$\text{Activity } \left({}^{\text{Units}}/_{\text{L}}\right) = \frac{0.75 - 0.07}{0.54 - 0.04} \times \frac{1\text{mM} \times 50\mu\text{L} \times 10^3}{40\mu\text{L} \times 180\text{mins}}$$

$$\text{Activity } \left({}^{\text{Units}}/_{\text{L}}\right) = 1.36 \times 6.94\, {}^{\text{Units}}/_{\text{L}} = \mathbf{9.44}\ {}^{\mathbf{Units}}/_{\mathbf{L}}$$

Figure 5.2 Sample arginase activity calculation.

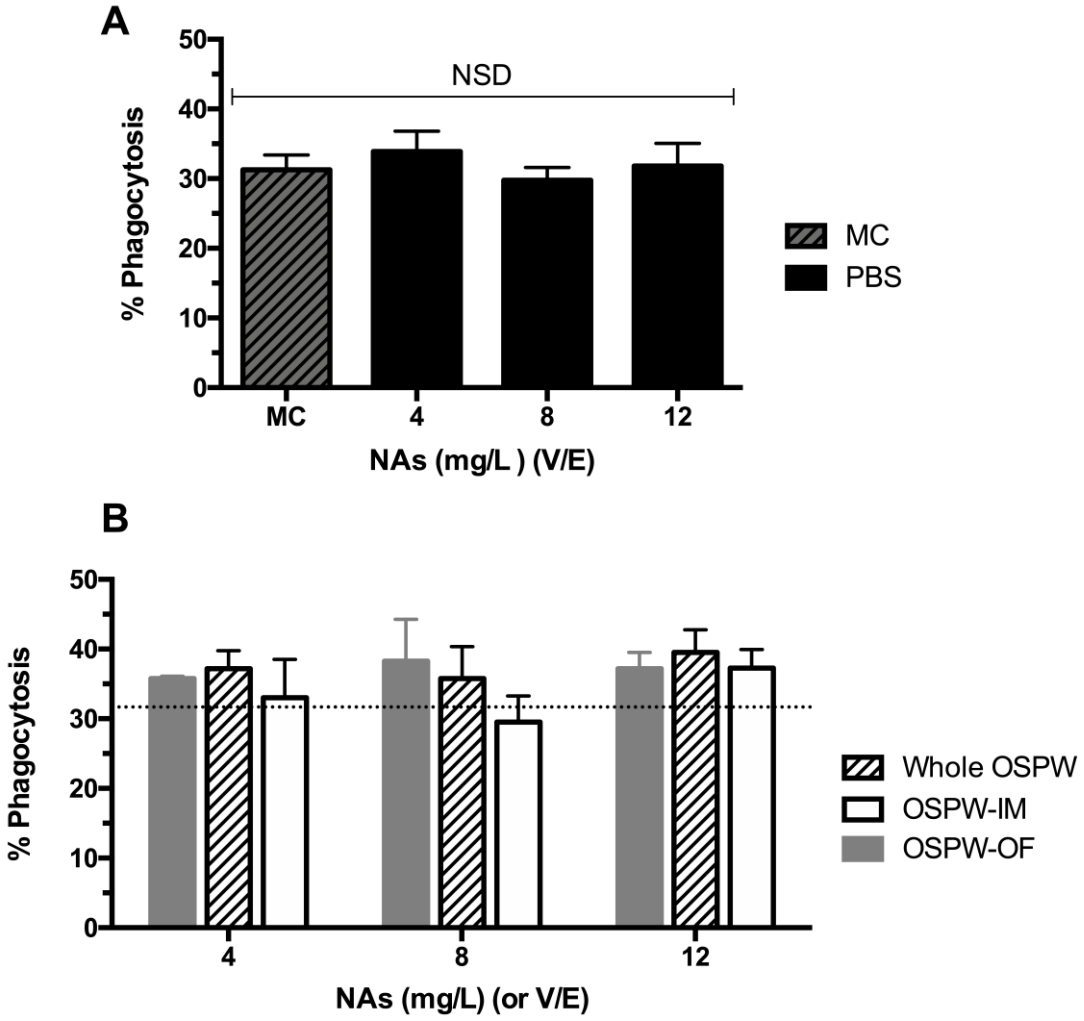


Figure 5.3 Phagocytosis of RAW 264.7 cells treated with OSPW for 2 hours.
(A) PBS is the dilution control, MC is the non-treated, medium control (B) Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. After 2 hours of exposure, cells were given pHrodo (green fluorescent) *E. coli* for 1 hour, then harvested and measured for the number of pHrodo *E. coli* positive cells (percent phagocytosis) using an ImageStream flow cytometer. All plotted values are the mean ± SEM from 3 independent experiments. Statistics performed using a two-way ANOVA with a (A) Dunnett's or (B) Tukey's *post hoc* test, NSD indicates no significant difference between designated groups, * indicates $p < 0.05$ compared to average control which is represented by the dashed line in (B).

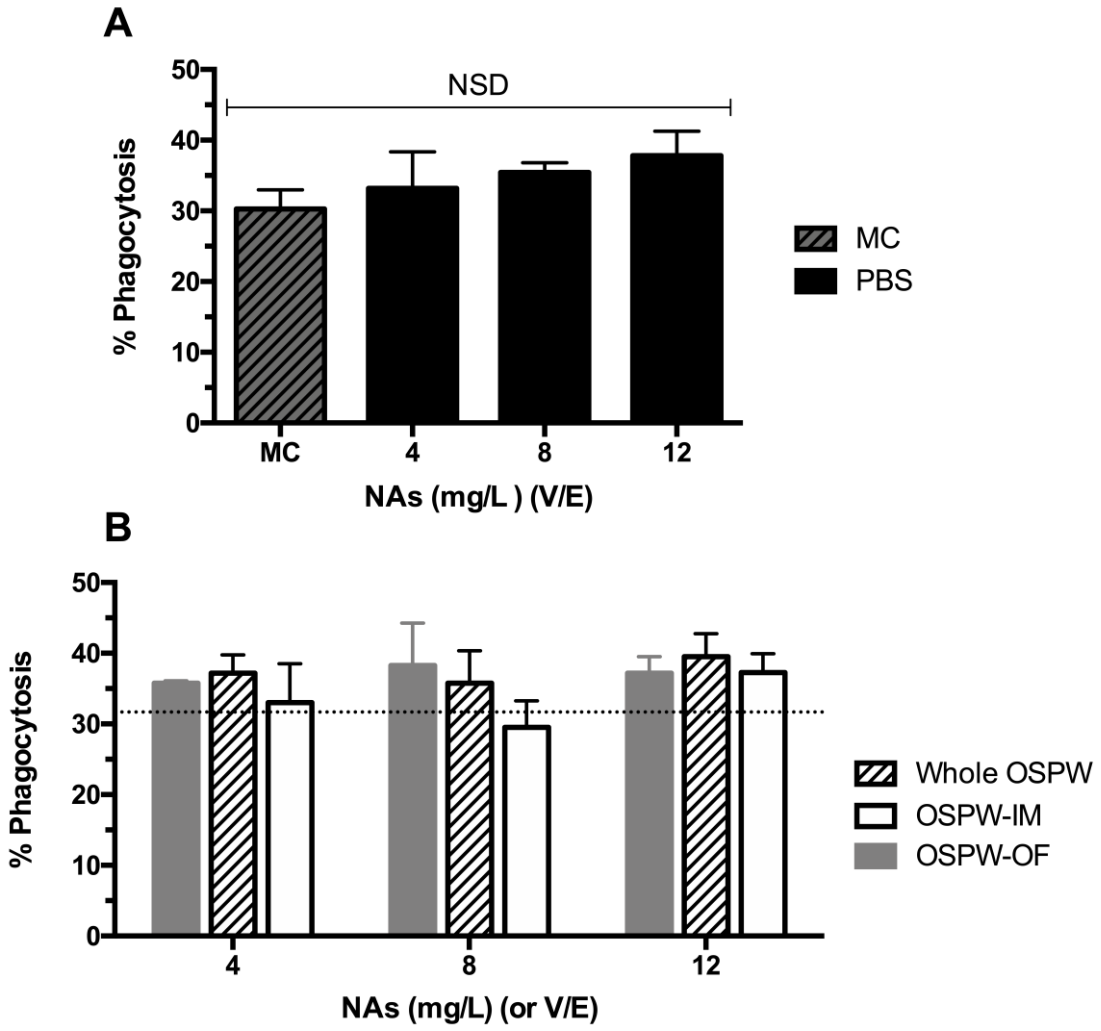


Figure 5.4 Phagocytosis of RAW 264.7 cells treated with OSPW for 24 hours.
Cells were exposed to whole OSPW, OSPW-IM, or OSPW-OF as indicated. (A) PBS is the dilution control, MC is the non-treated, medium control. (B) After 2 hours of exposure, cells were given pHrodo (green fluorescent) *E. coli* for 1 hour, then harvested and measured for the number of pHrodo *E. coli* positive cells (percent phagocytosis) using an ImageStream flow cytometer. All plotted values are the mean ± SEM from 3 independent experiments. Statistics performed using a two-way ANOVA with a (A) Dunnett's or (B) Tukey's *post hoc* test, NSD indicates no significant difference between designated groups, * indicates $p < 0.05$ compared to average control which is represented by the dashed line in (B).

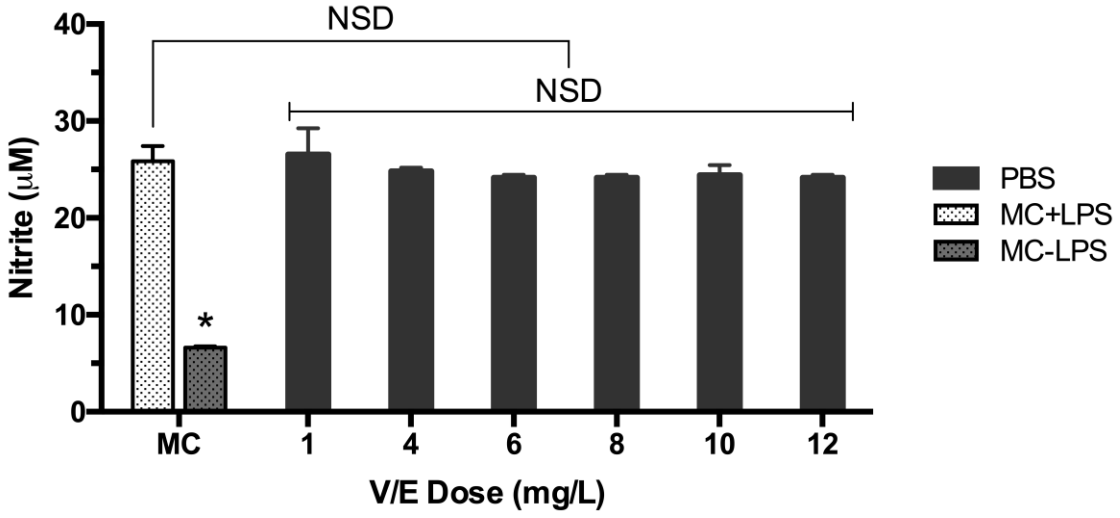


Figure 5.5. Nitric oxide production of RAW 264.7 cells following control treatments. Cells were cultured in complete medium (MC), or V/E of PBS for the respective doses listed and treated with LPS (1 μg/mL) or V/E of diluent for 24 hours. Nitric oxide response was analyzed using the Griess reaction, results are presented as the amount of produced nitrite. Values are the mean ±SEM of 3 independent experiments. The data were analyzed using two-way ANOVA followed by Dunnett's *post hoc* test, * indicates $p < 0.05$ from MC, NSD is no statistical difference.

Table 5.1 Results of Tukey's multiple comparisons test for production of nitric oxide by LPS stimulated RAW 264.7 cells and non-stimulated controls.[1]

Comparison	Adjusted *p* value	Summary
1 mg/L (V/E)		
WH vs OSPW-IM	0.9629	NSD
OSPW-IM vs Non-stimulated	<0.0001	****
WH vs Non-stimulated	<0.0001	****
4 mg/L (V/E)		
WH vs OSPW-IM	0.9951	NSD
OSPW-IM vs Non-stimulated	0.0125	*
WH vs Non-stimulated	0.0098	**
6 mg/L (V/E)		
WH vs OSPW-IM	0.9979	NSD
OSPW-IM vs Non-stimulated	0.3937	NSD
WH vs Non-stimulated	0.4303	NSD
8 mg/L (V/E)		
WH vs OSPW-IM	0.9946	NSD
OSPW-IM vs Non-stimulated	0.8771	NSD
WH vs Non-stimulated	0.8227	NSD
10 mg/L (V/E)		
WH vs OSPW-IM	0.9964	NSD
OSPW-IM vs Non-stimulated	0.9352	NSD
WH vs Non-stimulated	0.9632	NSD
12 mg/L (V/E)		
WH vs OSPW-IM	0.9384	NSD
OSPW-IM vs Non-stimulated	>0.9999	NSD
WH vs Non-stimulated	0.9261	NSD

[1] WH = OSPW, non-stimulated is the average nitrite produced by unexposed cells that were not treated with LPS.

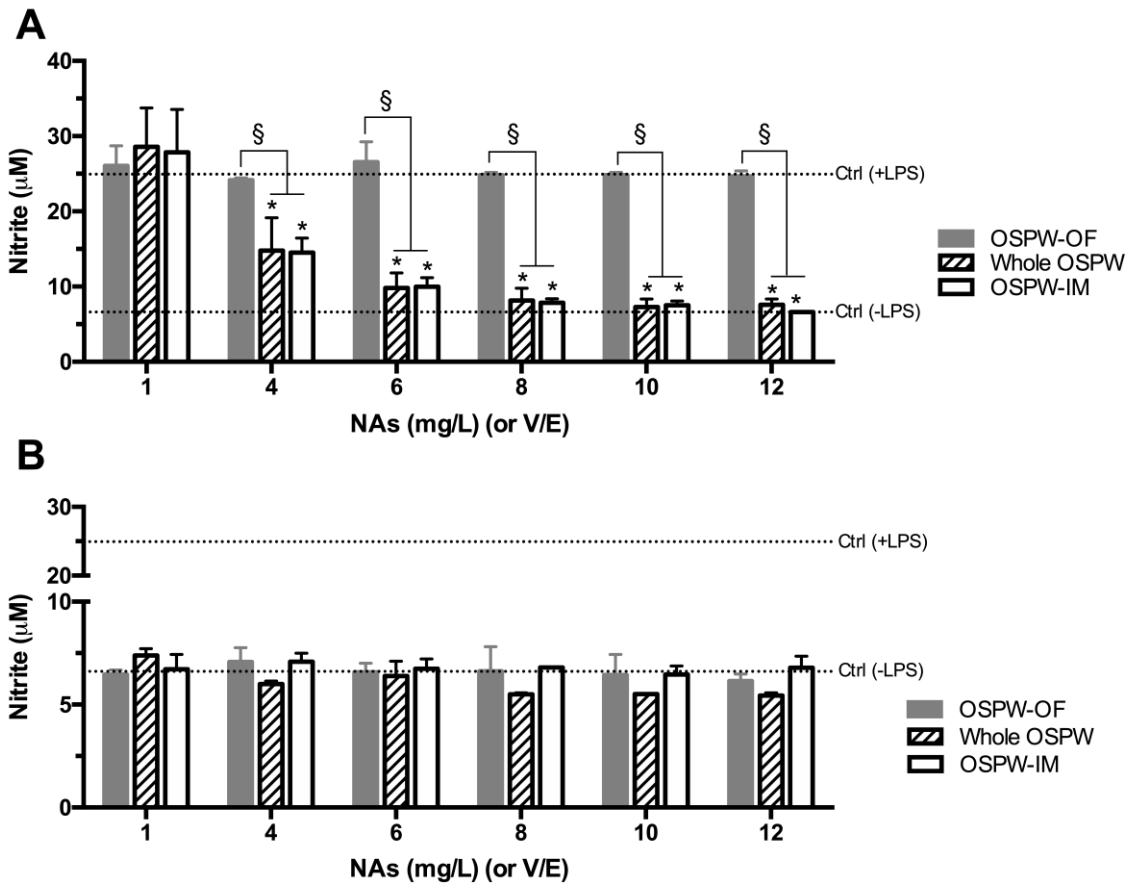


Figure 5.6 Nitric oxide production of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM or OSPW-OF as indicated. After 2 hours of exposure, cells were (A) stimulated with LPS (1 μg/mL) or (B) the V/E of diluent for an additional 22 hours (24 hours total). Nitric oxide response was analyzed using the Griess reaction, and the results are presented as the amount of produced nitrite. Ctrl (+LPS) is the averaged positive control (non-exposed), Ctrl (-LPS) is the averaged non-stimulated, negative control. All plotted values are the mean ±SEM of 3 independent experiments. Statistics performed with a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ from (A) the positive control (Ctrl (+LPS)) or (B) the negative control (Ctrl (-LPS)), § indicates $p < 0.05$ between the indicated experimental groups.

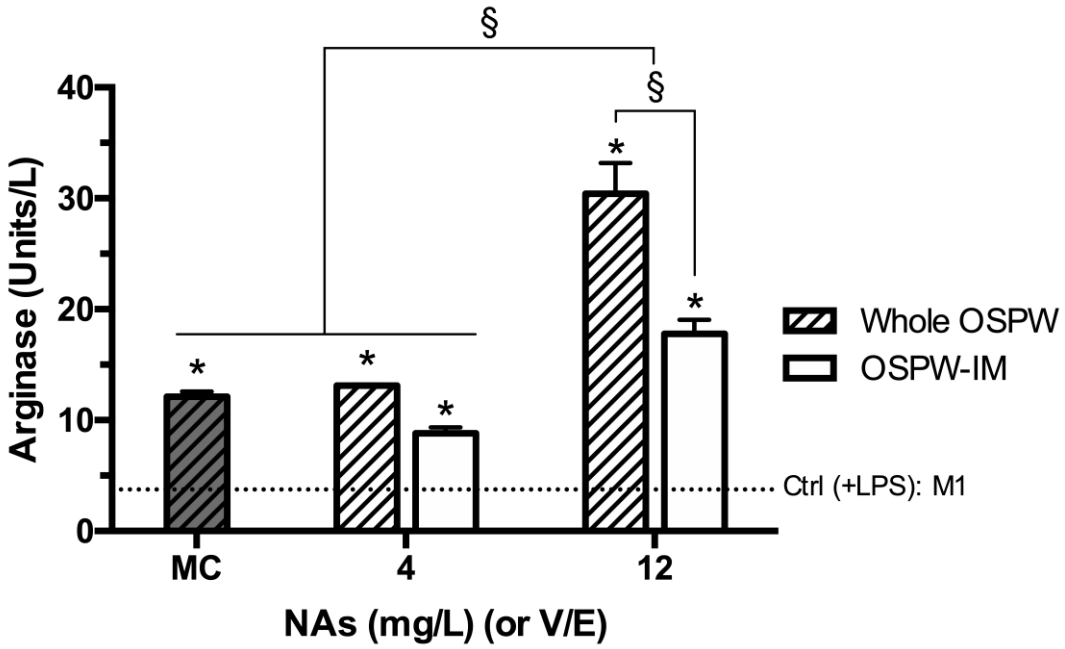


Figure 5.7 Arginase activity of RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM or OSPW-OF as indicated. After 2 hours of exposure, cells were stimulated with LPS (1 μg/mL), except for MC (media control) which received the V/E of diluent, for an additional 22 hours (24 hours total). Arginase activity was measured using the Arginase Activity Assay Kit (Millipore), one unit of arginase is the amount that will convert 1.0 μmol of L-arginine to ornithine and urea per minute at 37°C. Ctrl (+LPS) is the averaged negative control (non-exposed MC stimulated with LPS). All plotted values are the mean ±SEM of 3 independent experiments. Statistics performed with a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ from Ctrl (+LPS), § indicates $p < 0.05$ between the indicated experimental groups.

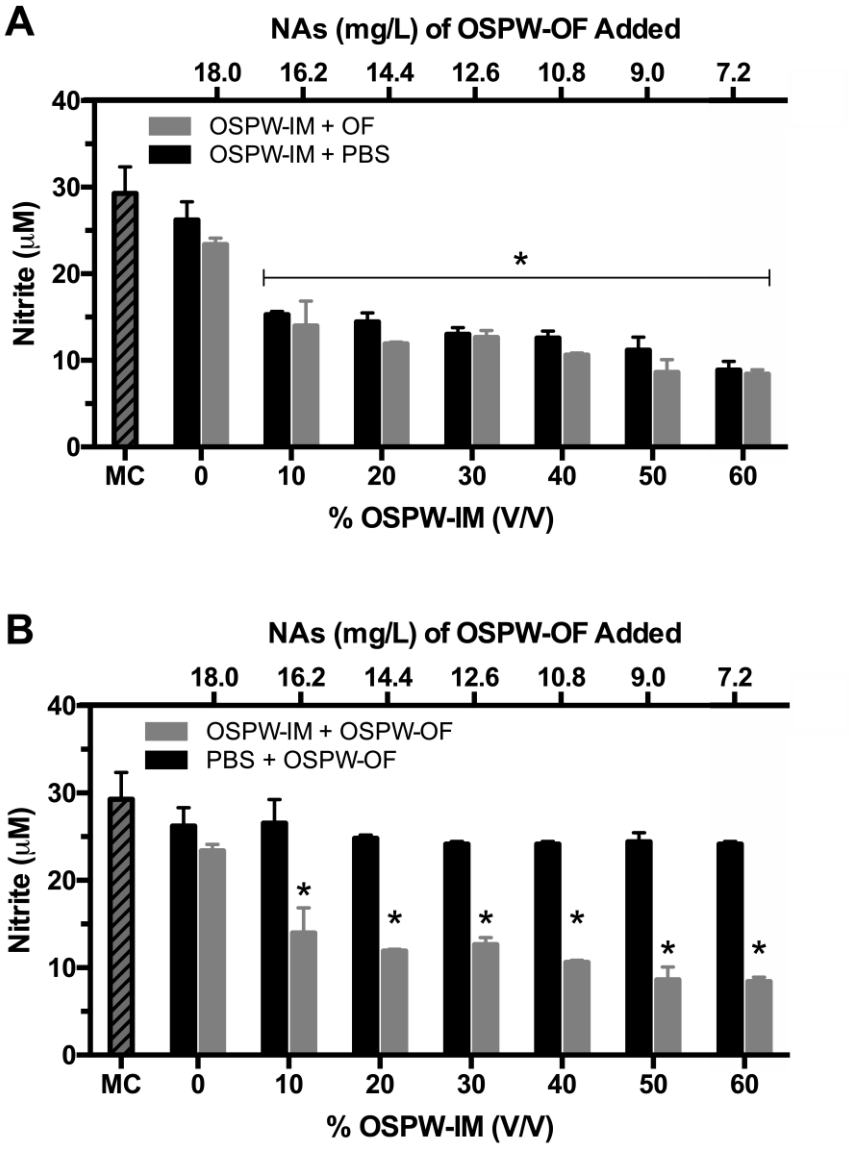


Figure 5.8 Relative contributions of OSPW-IM and OSPW-OF to effects on nitric oxide production by RAW 264.7 cells.

Cells were exposed to a volumetric series of OSPW-IM + OSPW-OF, OSPW-IM + PBS (A), or PBS + OSPW-OF (B) by percent volume, with PBS replacing the respective component as a displacement control. After 2 hours of exposure, cells were stimulated with LPS (1 μg/mL) for an additional 22 hours (24 hours total). Nitric oxide response was analyzed using the Griess reaction, and the results are presented as the amount of produced nitrite. All plotted values are the mean ±SEM from 3 independent experiments. Statistics were performed using a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ compared to dilution control.

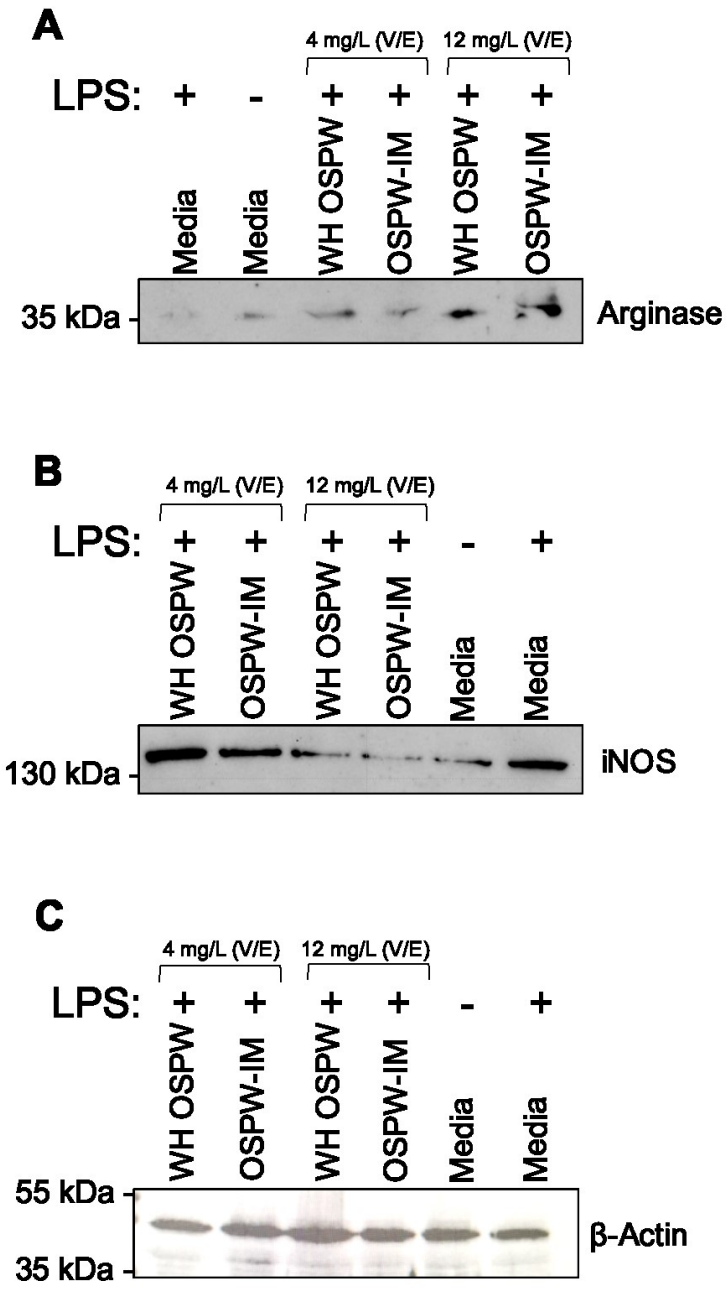


Figure 5.9 Western blots of enzymes involved in M1 and M2 macrophage phenotype polarization in RAW 264.7 cells.

Cells were exposed to whole OSPW or OSPW-IM as indicated. After 2 hours of exposure, cells were stimulated with LPS (1 μg/mL) for an additional 22 hours (24 hours total). Whole cell lysate was ran on an 8% SDS-PAGE gel, then transferred to nitrocellulose membranes for Western blotting to assess (A) arginase 1 and (B) iNOS proteins, (C) is the β-actin loading control. Western blots were ran using commercial monoclonal primary antibodies to the target (1:5 000 dilution) and rabbit-anti-mouse HRP conjugate as the secondary antibody (1:10 000 dilution).

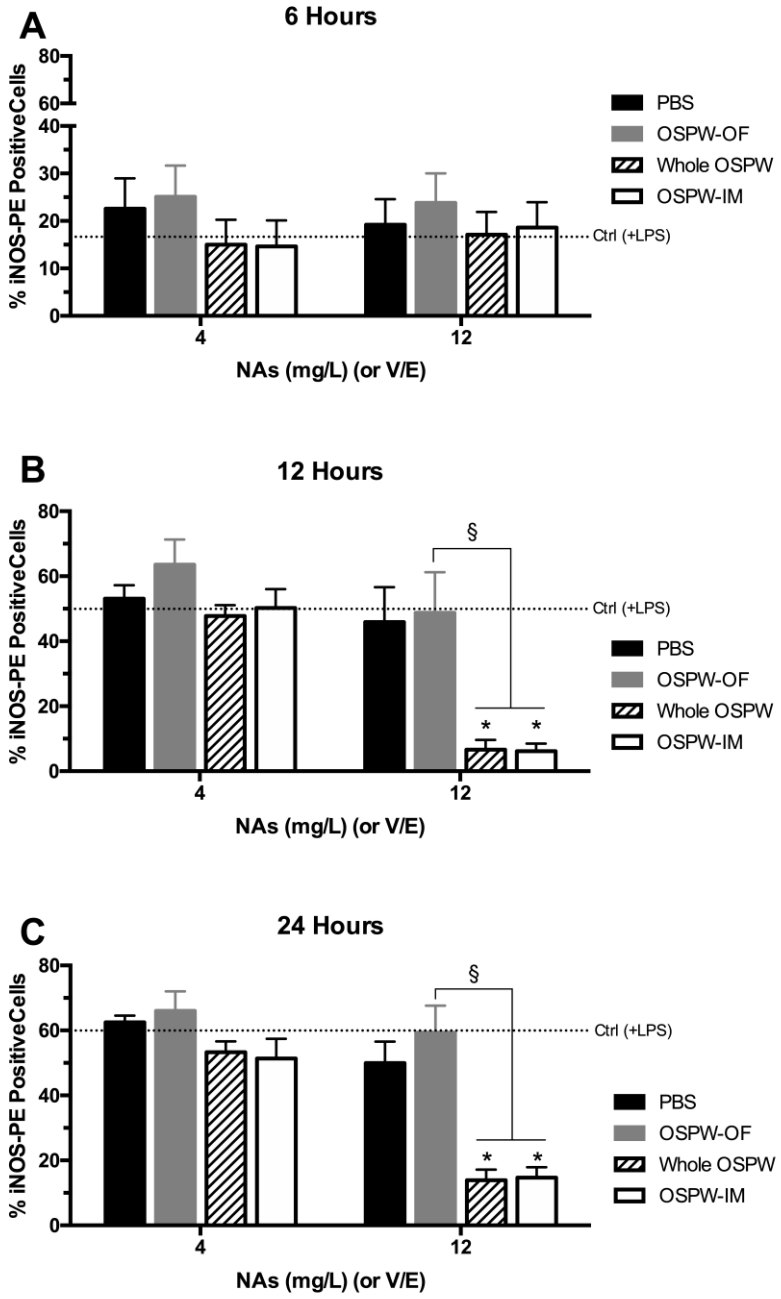


Figure 5.10 Levels of iNOS protein in RAW 264.7 cells.
Cells were exposed to whole OSPW, OSPW-IM or OSPW-OF as indicated. After 2 hours of exposure, cells were stimulated with LPS (1 μg/mL) for an additional (A) 4 hours, (B) 10 hours, or (C) 22 hours. Production of iNOS protein was assessed using the iNOS-PE monoclonal antibody (Invitrogen). The results are presented as the percent of cells positive for staining with the antibody, counted with an ImageStream flow cytometer. Ctrl (+LPS) is the averaged positive control (non-exposed). All plotted values are the mean ±SEM of 3 independent experiments. Statistics performed with a two-way ANOVA with a Tukey's *post hoc* test, * indicates $p < 0.05$ from the positive control (Ctrl (+LPS)), § indicates $p < 0.05$ between the indicated experimental groups.

Chapter 6: General Discussion

6.1 Introduction

The main objectives of this thesis research were to: (1) evaluate the acute toxicity of OSPW-IM, and its contribution to whole effluent toxicity; (2) assess the immunotoxic potential of whole OSPW and its fractions; and (3) identify a mechanism of immunomodulatory effects of whole OSPW and OSPW-IM. The acute- and immuno-toxicity of OSPW and its fractions were assessed using the RAW 264.7 mammalian macrophage-like cell line. RAW 264.7 cells were selected because they have previously been established as an *in vitro* bio-indicator system for OSPW.

6.2 OSPW-IM contributions to whole OSPW toxicity

Characterization of the primary toxicants in complex mixtures is very difficult due to the infinite additive/synergistic, and antagonistic interactions that can occur among the constituents, and their relative amounts in the mixture. OSPW is extremely complex, being composed of more than 400 chemical constituents. Using physical separation processes one can isolate the organic or inorganic components of OSPW allowing for independent assessment of the toxic effects induced by OSPW fractions and comparisons to those induced by the whole effluent. While the literature has primarily been focusing on the OSPW-OF constituents, namely NAs, as the primary contributor to toxicity, our lab recently reported that whole OSPW was significantly more acutely toxic than the OSPW-OF containing the equivalent concentrations of NAs (13). This observation suggested that inorganic components and/or potential interaction between the OSPW-IM and OSPW-OF may significantly contribute to whole OSPW toxicity. Previously, there was little known

regarding the role of OSPW-IM in whole effluent toxicity and my thesis research provides evidence that OSPW-IM contributes significantly to the overall OSPW toxicity.

The toxic effects induced in RAW 264.7 macrophage cells after exposure to whole OSPW, OSPW-IM, and OSPW-OF were comprehensively evaluated, and the main conclusions reached based on the results obtained were that OSPW-IM was significantly more toxic to cells when compared to OSPW-OF. In general, OSPW-IM induced toxicity was similar to that induced by the whole OSPW at equivalent doses and exposure times. It is important to note that the exposure of RAW 264.7 cells to OSPW-OF containing <20 mg/L NAs did not induce significant toxic effects in contrast to exposures of cells to the equivalent doses of whole OSPW or OSPW-IM (V/E). At the gene expression level there were occasional instances of significant relative differences between whole OSPW and OSPW-IM, which could suggest possible interactions between the inorganic matrix and organics found in whole OSPW. These occasional gene expression differences between whole OSPW and OSPW-IM more likely indicate changes to the water chemistry as a by-product of the organic removal process that we have not been able to ascertain simply because of the complexity and number of constituents. Gene expression, and qPCR specifically, are extremely sensitive measures of cellular regulation that allows us to pick up on minor transcriptional changes that may not correspond to biologically relevant/different responses (166,167).

When RAW 264.7 cells were exposed to mixtures of OSPW-IM + OSPW-OF the toxicity of the mixtures was confirmed by decreases in both cellular metabolic activity and

the production of reactive nitrogen intermediates of exposed cells. These experiments were done using mixtures of OSPW-IM and OSPW-OF where the NA content was inversely related to percent volume of OSPW-IM. Mitochondrial activity, as determined by the MTT assay, continued to decrease in a dose dependant manner when exposed to a mixture containing OSPW-IM. Thus, regardless of the addition of organic components, including NAs, the observed suppressive effects on metabolic activity were associated with a dominant OSPW-IM toxicity. For both the metabolic and immune function experiments, the OSPW-IM mixtures induced toxicity similar to that induced by whole OSPW, and importantly, there were no further enhancements of toxic effects on the metabolic activity or immune function of RAW 264.7 cells with the addition of OSPW-OF. This indicates that inorganic constituents, at doses of less than 20 mg/L (V/E), are significant contributors to the whole OSPW immunotoxicity. These results are supported by previous research in our lab where ozonation of OSPW, which effectively reduces the amount of NAs by ~90%, did not significantly ameliorate toxicity in RAW 264.7 cells (56).

The results of my experiments on OSPW-IM, either independently or in mixtures of OSPW fractions, indicate that inorganics found within whole OSPW are significant mediators of cellular effects at transcriptional, protein, functional, and metabolic levels that impact cellular health and plasticity. This directly contradicts the majority of OSPW research to date which has identified OSPW organics, specifically NAs, as the principal toxic component in OSPW (9–11,61,74,152,154,155). Targeted evaluations of NA toxicity have been performed using OSPW NA extracts (NAEs), rather than the entire OSPW-OF. Exposure to NAEs in mammalian and aquatic species have shown effects on reproduction,

development, immune response and organ histology (7,18,66,71,95). When analyzed *in vitro,* the NAEs have been associated with molecular weight- dependent cytotoxicity, and time-dependent AhR agonist activity (aryl hydrocarbon receptor, described as a molecular sensor for toxicant presence in the environment) (12,168).

Some observed effects of OSPW exposure can logically be attributed to the NA components, such as endocrine disruptive properties, since many NAs share structural similarity to steroid sex hormones (51). For example, aromatase (*cyp19a*) which is responsible for transformation of androgens to estrogens has become a target gene for investigations of endocrine disrupting effects. Following exposure to OSPW there was a time-dependent, and dose-dependent increase in the expression of aromatase, up to 3-fold when treated with undilute OSPW in a human cell line (51). However, both the *in vitro* and *in vivo* studies published to date that examined the NA-induced toxicity have employed NAs doses that are in excess of what would be biologically and environmentally relevant. The range of NA concentration in OSPW storage ponds is typically between 5-40 mg/L in reclamation ponds or wetlands, and from 20-80 mg/L in fresh settling basins (169). In the Athabasca river NA concentrations are <1 mg/L (1). For example, exposure to OSPW acid extractable organics demonstrated significant genotoxic and mutagenic activity *in vitro,* but this was observed only after exposure to extremely high doses, between 7 and 10 times higher than the environmental sample (175). In contrast, I observed a significant increase in the expression of DNA damage-associated (genotoxic stress-induced) protein, *gadd45,* at environmentally relevant concentrations (<20 mg/L V/E), after exposure to whole OSPW or OSPW-IM. Exposure of mouse bone marrow derived macrophages to 50 mg/L OSPW-OF

or NAEs produced suppression of antimicrobial response markers, including production of RNI and expression of *inos* mRNA (7,18). Again, I observed similar effects in the iNOS pathway following exposure to whole OSPW or OSPW-IM but at low concentrations (<10 mg/L). This suggests that at environmentally appropriate doses the inorganic components of are major contributors to the overall OSPW toxicity.

Even ignoring the multiple detoxification pathways available to organisms that would affect tissue-specific concentrations, using experimental doses above these ranges, or doses exceeding an expected natural exposure may provide valuable mechanistic data. However, this should not be extrapolated to propose or define target pollutants in OSPW in the context of environmental protection. Acute exposure to high doses of NAs (300 mg/kg W/W) *in vivo* using rats was associated with histopathological effects, inflammation, and anorexia (176). Toxicity persisted, including reduced body weight, reduction in liver mass, and negative effects on breeding success (176,177). Although significant toxicity was reported to occur using 60 to 300 mg/kg W/W, this dose range was as much as 50 times higher than a "worst-case- daily water intake" by a natural interaction of animals in the environment containing OSPW (56). Our lab further explored the potential effects of orally administered, environmentally relevant NA concentrations, up to 55 mg/L, on pregnancy and reproductive success in mice and reported no significant effects (72). In aquatic test systems, muscle deformity and increased heart rate have been observed in developing embryos exposed to OSPW, associated with changes to transcription factor mRNA levels (68,111). Similarly, effects on the transcriptome of minnows were attributed to the organic fraction because non-ozonated OSPW changed the abundance of 195 transcripts, compared to 132 after ozonation (178). However, in both examples these exposures were performed in non-diluted OSPW

with NA concentrations >15mg/L, which is unlikely to represent characteristics of an accidental release to natural water systems, or exposure to native fish populations (68,178). Furthermore, it was demonstrated that fish are able to sense OSPW contaminants, present at as low as 0.1%, using their olfactory system and in a natural setting will swim away from these sites effectively limiting their risk of exposure to high contaminant concentrations (179,180).

6.3 Immunomodulation of mammalian macrophages after exposure to whole OSPW and OSPW-IM and/or OSPW-OF fractions

Previous studies in our lab indicated that OSPW induced immunomodulatory effects, both *in vitro* and *in vivo*, with specific reference to macrophage function (4,7,13,18). Macrophages are ubiquitously distributed throughout the body acting as key orchestrators of immune responses, recovery, and disease. Receptor-mediated recognition and endocytosis of pathogens encountered during normal surveillance can lead to classical activation of macrophages. Additionally, these cells can be activated in a paracrine fashion by other cells secreting pro-inflammatory cytokines such as IFNγ (142,150). These classically activated M1 macrophages (also known as the "killer" macrophages) promote destruction of pathogens, and induce a systemic pro-inflammatory immune response by production of antimicrobial products, pro-inflammatory cytokines, and an eventual induction of adaptive immunity as required. On the other hand, alternatively-activated macrophages become induced by type two cytokines such as IL-4 and IL-13 which increase in response to progressive host damage during infection or injury (142,181). Due to the negative effects of chronic inflammation, a repair and immunosuppressive phase is required following successful clearance and control of infectious pathogens. This phase is mediated by M2

macrophages, which play a critical role in promotion of adaptive immunity and coordination of post-injury return to homeostasis.

Based on the above, I decided to examine the immunomodulatory effects caused by exposure of RAW 264.7 cells to whole OSPW, OSPW-IM, and/or OSPW-OF. The immunomodulatory effects of whole OSPW or OSPW-IM promoting a shift to an M2 phenotype was both time and dose dependent, but also inevitable, since after 24 hours of exposure there was a significant reduction in iNOS mRNA and production of RNI regardless of dose. Focussing on the highest dose, 12 mg/L (V/E), I observed significantly increased *inos* expression at early timepoints while arginase remained at control levels. This indicates that whole OSPW or OSPW-IM does not affect the transcription of iNOS mRNA directly. Therefore, these treatments also do not interfere with the upstream LPS induced signalling pathway progressing from receptor binding, to adaptor recruitment/phosphorylation, and finally transcription factor activity within the first 5 hours (182). After 6 hours, the cells were capable of producing iNOS protein at the same level as the LPS stimulated, non-exposed controls. Together, this suggests that the overexpression of *inos* mRNA at early timepoints enables short-term baseline translation of the protein. Conversely, after 24 hours of exposure, the intensity of arginase bands on Western blots following exposure to 12 mg/L OSPW-IM (V/E) increased compared to both the non-stimulated controls, and 4 mg/L OSPW-IM (V/E) treatments. The increase in Western blot band intensity was associated with a time-dependent increase in *arginase* mRNA expression. The exposure to 12 mg/L OSPW-IM (V/E) induced a significant upregulation of *inos* and *arginase* mRNA. However, these pathways are by definition mutually exclusive, suggesting that 5 hours of exposure may be the window of

time in which cells "switch" and begin transitioning from a primarily M1 to an M2 phenotype. There was also a significant increase in the mRNA levels of *tgfβ* which is known to be produced by alternatively activated macrophage, supporting the observation of M1 to M2 phenotype switch (122). This was further supported by the significant reduction in the mRNA levels of pro-inflammatory genes (*tnfα,* and *il-1β*).

The observed immunomodulatory effects occurred at the transcriptional, translational, and functional levels, suggesting that there are likely multiple points of action that can alter polarization status in response to OSPW exposure. Arginase directly destabilizes iNOS proteins and may be present in sufficient amounts to do so following exposure to low doses of OSPW (126). This could explain why we see a normal population of iNOS positive cells at 24 hours exposed to 4 mg/L, but still observe a significant decrease in the production of RNI. Overexpression of arginase can rapidly consume intracellular arginine which has effects on substrate availability for iNOS, and additionally induces negative regulation of a translation initiation factor, reducing iNOS protein synthesis (157).

After 24 hours of exposure to 12 mg/L (V/E) of whole OSPW or OSPW-IM there was a universal reduction of M1 phenotype defining characteristics. The mRNA levels of pro-inflammatory cytokines IL-1β, TNF-α, and iNOS; the percent of iNOS positive cells; as well as Western blot band intensity decreased. This is directly opposed by trends of M2 properties: increase in arginase and Il-1β mRNA and higher arginase activity, suggesting that OSPW/OSPW-IM directly promotes the activation of the arginase pathway, since most observed changes in M1 are known to be mediated by arginase directly (124,142,183).

Previous immunotoxicity assessments of OSPW *in vitro* and *in vivo* using fish and mammals have shown that exposure was associated with decreased pro-inflammatory cytokine production, reduced control of infections, and suppression of pro-inflammatory cellular functions (4,13,52,184). These studies independently concluded that OSPW had immunomodulatory effects. My results provide evidence for a potential mechanism by which OSPW/OSPW-IM immunomodulate macrophage functions.

Disease outcomes are strongly influenced by the polarization of macrophages in the appropriate direction. Improper regulation of M1/M2 induction is associated with inflammatory or immunosuppressive conditions for the host (149). The importance of polarization in the control of infections can be deduced by the multitude of pathogen-mediated mechanisms that target it as an immune evasion strategy (185). *Mycobacterium tuberculosis* infamously makes use of M2-like polarization for its persistence in the host; macrophages infected with *M. tuberculosis* have increased expression of M2 markers and reduced antimicrobial function (185,186). Exploitation of this regulation is utilized by bacteria and parasites, demonstrated by arginase 1 knockout macrophages which effectively controlled challenge with *M. tuberculosis* or *Toxoplasma gondii* and increased host survival (187). In *T. gondii* specifically, it was speculated that knocking out arginase relieved induced inhibition of the nitric oxide response, permitting pro-inflammatory anti-parasitic functions. Beyond pathogen control scenarios, persistent overexpression of arginase is found in psoriatic skin environments where pathophysiology of psoriasis is associated with relieved nitric oxide-mediated inhibition of keratinocyte proliferation (188). In some instances, host-directed induction of M2 macrophages is important in protection from an excessive immune

response or immune-mediated pathology. This is most commonly observed in host-parasite interactions. For example, *Schistosoma mansoni* infection in mice deficient for the IL-4 receptor experienced 100% mortality, indicating that IL-4 signalling, which encourages M2 polarization, in macrophages was essential for protection from organ damage (185). Together these examples demonstrate that in a physiological setting, macrophages display functional and phenotypic plasticity on a case-by-case basis; the proper induction of the appropriate phenotype of either M1 or M2 is critical in protecting from site and source specific disease pathophysiology.

The observed potent induction of M2 macrophages following exposure to low doses of OSPW/OSPW-IM *in vitro* could indicate a mode of action during *in vivo* exposures where the immunomodulatory effects observed appear to be dominated by inorganic components of OSPW. Whole organisms may be able to detoxify, or attenuate chemical compounds by mechanisms not available to *in vitro* systems, however detoxification of metals is highly unsuccessful without clinical intervention due to their ability to escape physiological control mechanisms such as transport compartmentalization or specific constituent binding (189). Following *in vivo* exposures of rainbow trout and yellow perch to OSPW, a reduced ability to control bacterial and viral infections was documented (190,191). My findings suggest that this may be due, at least in part, to an increase in the population of M2 macrophages, which as first responders to infection would not be able to orchestrate an appropriate, systemic pro-inflammatory response. Mice with severe tissue damage through thermal burns showed a systemic promotion of M2 macrophages, and upon subsequent oral administration of

pathogen challenge the mice were described as “LPS tolerant” as they did not mount a pro-inflammatory response to control bacterial load and dissemination (192).

In addition to being associated with the promotion of tissue repair, and dampening of reactive type-one mediators, M2 macrophages are also coupled to Th2-polarized immune responses. Characteristics of a Th2 type response include production of type-two cytokines such as IL-4 and IL-13, B-cell isotype switching, and an antibody-oriented effector response (185). Antibody production is strongly favoured in response to extracellular pathogens, and is associated with effective control of the pathogens by the host. Pathogens, including *Trypanosoma* spp., specifically target this polarization induction as a dominant immune evasion strategy. A number of extracellular *Trypanosoma* spp. products induce a Th1 response which is characteristically ineffective in clearance of extracellular pathogens (193,194,194). Our lab previously used an established parasitic infection model to look at outcomes following exposure to OSPW *in vivo.* Goldfish were exposed to OSPW, and challenged with the extracellular parasite, *Trypanosoma carassii* (15). The results of these experiments showed that OSPW-exposed fish had significant reductions in the number of parasites during the course of the infection compared to the non-exposed control (15,171). It was proposed that this was due to OSPW-induced overexpression of pro-inflammatory cytokines, observed at the mRNA level, in this time frame which enhanced an effective pro-inflammatory response for pathogen clearance. However, the cytokines analysed and shown to be overexpressed were type 1 cytokines during the acute phase of the infection: TNF-α, IFNγ, and IL-1β, which typically do not promote successful extracellular pathogen control (15,171,195). It would have been interesting to examine the Th2 cytokine gene expression

during the elimination of *T.carassii* infection (subchronic exposure period), in order to ascertain whether an M1 to M2 switch occurred in animals exposed to OSPW.

It is well established that the anti-inflammatory hormone, EPO, can induce M1 to M2 phenotype switching of macrophages, which may be host-protective in this host-parasite association (196,197). This was supported by recent evidence from our lab that has specifically implicated inflammation in the disease outcomes of typical *T. carassii* infections. Injection of goldfish with recombinant erythropoietin (rgEPO) caused a decrease in parasitaemia similar to what we observed in OSPW-exposed fish (15,171,198). Extracellular pathogens are most effectively cleared by Th2 type responses, which are associated with M2 macrophages, and furthermore balance with anti-inflammatory signals protects hosts from excessive self-injury (194,199). Therefore, the observed positive disease outcomes in goldfish exposed acutely to fresh OSPW may actually be mediated by changes to a macrophage polarization bias associated with OSPW factors that preferentially induce an M2 phenotype, despite pro-inflammatory stimuli and/or parasite factors that would normally favour an M1 polarization.

As the exposure time of goldfish to OSPW *in vivo* was increased from acute to sub-chronic (12 weeks), there was further reduction of the pro-inflammatory profile. Specifically, primary kidney-derived macrophages from OSPW-exposed, *T. carassii*-infected fish had decreased M1 functional capacity with respect to respiratory burst and production of nitric oxide (15). My analysis *in vitro* corroborated these observations; where low dose OSPW exposure for extended time resulted in decreased antimicrobial function. Furthermore, in this

sub-chronic period, OSPW-exposed fish had a global decrease in the expression of pro-inflammatory cytokines after a significant overexpression following short-term exposure (4,15). This is a similar temporal profile to my *in vitro* gene expression analysis where pro-inflammatory cytokines were significantly upregulated early, and transitioned to downregulation at later time points. However, I also assessed anti-inflammatory parameters which showed time- and dose- dependent increases at the transcriptional, translational, and functional level suggesting that where I observed a decrease in the expression of pro-inflammatory markers, there was a concurrent increase in the anti-inflammatory capacity of the system. In the context of the parasitic infection, goldfish exposed sub-chronically and challenged with *T. carassii* showed increased susceptibility and mortality, supporting a time-dependent effect of OSPW mediated immune modulation; where the induced anti-inflammatory state has limited the ability to mount an effective response, rather than in the acute exposure where a slight bias toward M2 phenotypic responses and effects was beneficial and protective within a robust *in vivo* immune response (4,193). Ultimately, an effective *in vivo* immune response must be carefully balanced between type one and two mediators, and functions to protect the host from excessive inflammatory damage, while also harnessing inflammation to control the pathogens (200). Short-term exposure to OSPW may be alleviating the type 1 bias favoured by an extracellular pathogen such as *T. carassii* but extended induction of a large-scale immunosuppressive state could mediate non-specific tolerance to infectious agents.

Conventionally macrophage polarization occurs by environmentally-specific cues including cytokines, growth factors, and pathogen associated molecular patterns (124). The

common factor between these cues is their biological origin, unlike anthropogenic xenobiotics such as OSPW. The concentration of NAs found in fresh tailings is typically <20 mg/L, the average reported in wetlands are 7.95 mg/L, but high OSPW character wetlands report concentrations >46.7 mg/L, meaning that the immunomodulatory effects of whole OSPW, and its associated inorganic components, could pose a direct threat to native organisms in contact with OSPW storage sites (201). Furthermore, *in vivo* experiments have indicated that sub-chronic exposure to OSPW has immunosuppressive effects, and labelled OSPW as directly immunotoxic (4,7,15,18). My thesis research provided evidence that in addition to direct immunotoxicity, the exposure to OSPW and its inorganic matrix induces changes to immune function/status of the host, which undoubtedly may influence the ability of the host to control infectious diseases.

6.4 Future directions

Future research on OSPW toxicity in RAW 264.7 cells should include the assessment of additional macrophage phenotype markers and products. Expanding on the biomarkers of polarization may further elucidate the specific targeted mechanism of M2 phenotype induction. Given the complexity of immune pathways as a whole, through redundant and conserved signalling systems, a targeted or whole transcriptome analysis using next generation RNA sequencing after treatment with whole OSPW or OSPW-IM would significantly expand our working knowledge of OSPW toxicity at the molecular level. Identification of precise molecular regulators could contribute to the development of high-throughput toxicity screening of OSPW and other complex industrial wastes.

Given the complexity of OSPW as a contaminant, and its variable nature by source and site, profiles of additional OSPWs should be generated and compared for effects on regulation of the M1/M2 pathway which may present alternative results depending on variations in composition, specifically inorganics. While exploitation of this pathway and bio-assay system *in vitro* presents utility as a tool for profiling, monitoring, and screening OSPW in an industrial/environmental protection sense or for identification of target xenobiotic constituents, the indicated mode of action presents a significant concern for immune health of native species present at industrial sites. Analysis of select phenotype markers and activity should be assessed *in vivo* using test organisms exposed to OSPW. Additionally, repeat pathogen challenge experiments that expand the analyses of M2 associated parameters in addition to the usual pro- and anti-inflammatory factors could indicate a similar effect *in vivo*, further supporting the mechanism of immunomodulation suggested here using *in vitro* studies.

6.5 Conclusion

This thesis research analysed the immunotoxic effects of whole OPSW, OSPW-OF, and OSPW-IM *in vitro* using the RAW 264.7 mammalian macrophage-like cell line. The results indicate that at <20 mg/L (V/E) inorganic constituents significantly contribute to reductions in cellular viability, metabolism, gene expression, protein synthesis and function. Effects on cellular viability and metabolism demonstrated dose dependent decreases following exposure to whole OSPW or OSPW-IM, but not OSPW-OF at environmentally relevant concentrations. Gene expression of stress-induced, and pro/anti-inflammatory cytokines was affected in a dose and time dependent manner following treatment with low dose (<10 mg/L) whole OSPW or OSPW-IM. Additionally, the temporal shift from

overexpression of pro- to anti-inflammatory genes over 24 hours corroborated with enzyme abundance and function suggested that inorganic compounds present in whole OSPW have specific immunomodulatory effects on macrophage polarization status in response to bacterial stimuli. Together, these results highlight the consequences of analysing only expected immune pathways in traditional immunotoxicology and the importance of using multiparametric constituent analysis in studies of complex xenobiotic mixtures similar to OSPW.

The implication of a dominant inorganic matrix-mediated toxicity, demonstrated in my thesis research, is of importance because there has been a traditional focus in the literature on the effects induced by the OSPW organic constituents, primarily NAs. My study is the first comprehensive evaluation of the potential toxicity of the OSPW-IM. These results could have significant impact on current remediation tactics and OSPW management.

References

1. Giesy JP, Anderson JC, Wiseman SB. Alberta oil sands development. Proc Natl Acad Sci U S A. 2010 Jan 19;107(3):951–2.

2. Del Rio LF, Hadwin AKM, Pinto LJ, MacKinnon MD, Moore MM. Degradation of naphthenic acids by sediment micro-organisms. J Appl Microbiol. 2006;101(5):1049–61.

3. Anderson J, Wiseman SB, Moustafa A, Gamal El-Din M, Liber K, Giesy JP. Effects of exposure to oil sands process-affected water from experimental reclamation ponds on Chironomus dilutus. Water Res. 2012 Apr 15;46(6):1662–72.

4. Hagen MO, Garcia-Garcia E, Oladiran A, Karpman M, Mitchell S, El-Din MG, et al. The acute and sub-chronic exposures of goldfish to naphthenic acids induce different host defense responses. Aquat Toxicol. 2012 Mar 1;109(Supplement C):143–9.

5. Melvin SD, Trudeau VL. Toxicity of naphthenic acids to wood frog tadpoles (Lithobates sylvaticus). J Toxicol Environ Health A. 2012;75(3):170–3.

6. Beck EM, Smits JEG, Clair S, Cassady C. Evidence of low toxicity of oil sands process-affected water to birds invites re-evaluation of avian protection strategies. Conserv Physiol. 2015 Jan 1;3(1).

7. Garcia-Garcia E, Pun J, Hodgkinson J, Perez-Estrada LA, El-Din MG, Smith DW, et al. Commercial naphthenic acids and the organic fraction of oil sands process water induce different effects on pro-inflammatory gene expression and macrophage phagocytosis in mice. J Appl Toxicol JAT. 2012 Dec;32(12):968–79.

8. Bartlett AJ, Frank RA, Gillis PL, Parrott JL, Marentette JR, Brown LR, et al. Toxicity of naphthenic acids to invertebrates: Extracts from oil sands process-affected water versus commercial mixtures. Environ Pollut. 2017 Aug 1;227:271–9.

9. Allen EW. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. J Environ Eng Sci. 2008 Feb 27;7(2):123–38.

10. Li C, Singh A, McPhedran K, Chelme-Ayala P, Belosevic M, Gamal El-Din M. Synthesis of Toxicological Behavior of Oil Sands Process-Affected Water Constituents. 2014.

11. Swigert JP, Lee C, Wong DCL, White R, Scarlett AG, West CE, et al. Aquatic hazard assessment of a commercial sample of naphthenic acids. Chemosphere. 2015 Apr 1;124(Supplement C):1–9.

12. Leclair LA, Pohler L, Wiseman SB, He Y, Arens CJ, Giesy JP, et al. In vitro assessment of endocrine disrupting potential of naphthenic Acid fractions derived from oil sands-influenced water. Environ Sci Technol. 2015 May 5;49(9):5743–52.

13. Fu L, Li C, Lillico DME, Phillips NAI, Gamal El-Din M, Belosevic M, et al. Comparison of the Acute Immunotoxicity of Nonfractionated and Fractionated Oil Sands Process-Affected Water Using Mammalian Macrophages. Environ Sci Technol. 2017 Aug 1;51(15):8624–34.

14. Peters LE, MacKinnon M, Van Meer T, van den Heuvel MR, Dixon DG. Effects of oil sands process-affected waters and naphthenic acids on yellow perch (Perca flavescens) and Japanese medaka (Orizias latipes) embryonic development. Chemosphere. 2007 May 1;67(11):2177–83.

15. Hagen MO, Katzenback BA, Islam MDS, Gamal El-Din M, Belosevic M. The Analysis of Goldfish (Carassius auratus L.) Innate Immune Responses After Acute and Subchronic Exposures to Oil Sands Process-Affected Water. Toxicol Sci. 2014 Mar 1;138(1):59–68.

16. Lister A, Nero V, Farwell A, Dixon DG, Van Der Kraak G. Reproductive and stress hormone levels in goldfish (Carassius auratus) exposed to oil sands process-affected water. Aquat Toxicol. 2008 May 1;87(3):170–7.

17. Collier TK, Anulacion BF, Arkoosh MR, Dietrich JP, Incardona JP, Johnson LL, et al. Effects on Fish of Polycyclic Aromatic HydrocarbonS (PAHS) and Naphthenic Acid Exposures. Fish Physiol. 2013 Jan 1;33:195–255.

18. Garcia-Garcia E, Pun J, Perez-Estrada LA, Din MG-E, Smith DW, Martin JW, et al. Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. Toxicol Lett. 2011 May 30;203(1):62–73.

19. Gosselin P, Hrudey S, Naeth M, Plourde A, Therrien R, Vand der Kraak G, et al. Environmental and health impacts of Canada's oil sands industry. Royal Society of Canada, Ottawa. 2010;

20. Snoeijs T, Dauwe T, Pinxten R, Vandesande F, Eens M. Heavy Metal Exposure Affects the Humoral Immune Response in a Free-LivingSmall Songbird, the Great Tit (Parus major). Arch Environ Contam Toxicol. 2004 Apr 1;46(3):399–404.

21. Dunier M. Water pollution and immunosuppression of freshwater fish. Ital J Zool. 1996 Jan 1;63(4):303–9.

22. Belyaeva EA, Sokolova TV, Emelyanova LV, Zakharova IO. Mitochondrial Electron Transport Chain in Heavy Metal-Induced Neurotoxicity: Effects of Cadmium, Mercury, and Copper. Sci World J. 2012:1–14.

23. Garza A, Vega R, Soto E. Cellular mechanisms of lead neurotoxicity. Med Sci Monit. 2006 Mar 1;12(3):RA57–65.

24. Jezierska B, Ugowska K, Witeska M. The effects of heavy metals on embryonic development of fish (a review). Fish Physiol Biochem Dordr. 2009 Nov;35(4):625–40.

25. Koller LD. Immunotoxicology of heavy metals. Int J Immunopharmacol. 1980 Jan 1;2(4):269–79.

26. Dangleben NL, Skibola CF, Smith MT. Arsenic immunotoxicity: a review. Environ Health. 2013 Sep 2;12(1):73.

27. Harkin A, Hynes MJ, Masterson E, Kelly JP, O'Donnell JM, Connor TJ. A Toxicokinetic Study of Nickel-Induced Immunosuppression in Rats. Immunopharmacol Immunotoxicol. 2003;25(4).

28. Sunderman FW, Hopfer SM, Lin S-M, Plowman MC, Stojanovic T, Wong SH-Y, et al. Toxicity to alveolar macrophages in rats following parenteral injection of nickel chloride. Toxicol Appl Pharmacol. 1989 Aug 1;100(1):107–18.

29. Luster MI, Simeonova PP, Germolec DR. Immunotoxicology. In: Delves PJ, editor. Encyclopedia of Immunology (Second Edition) [Internet]. Oxford: Elsevier; 1998. p. 1365–9.

30. Galloway TS, Depledge MH. Immunotoxicity in Invertebrates: Measurement and Ecotoxicological Relevance. :19.

31. George S. Position of immunological techniques in screening in clinical toxicology. Clin Chem Lab Med. 2004;42(11):1288–309.

32. Cox MN, Baum CR. Toxicology reviews: immunoassay in detecting drugs of abuse. Pediatr Emerg Care. 1998 Oct;14(5):372–5.

33. Zachary J. Nelson, Samuel J. Stellpflug, Kristin M. Engebretsen. What can a urine drug screening immunoassay really tell us? J Pharm Pract. 2016;29(5):516–26.

34. Eason C, O'Halloran K. Biomarkers in toxicology versus ecological risk assessment. Toxicology. 2002 Dec 27;181–182:517–21.

35. Shukla SJ, Huang R, Austin CP, Xia M. The Future of Toxicity Testing: A Focus on In Vitro Methods Using a Quantitative High Throughput Screening Platform. Drug Discov Today. 2010 Dec;15(23–24):997–1007.

36. Luster M I, Dean J H, Boorman G A. Cell-mediated immunity and its application in toxicology. Environ Health Perspect. 1982 Feb 1;43:31–6.

37. Desforges JPW, Sonne C, Levin M, Siebert U, Dietz R. Immunotoxic effects of environmental pollutants in marine mammals. Environ Int. 2016;86:126–39.

38. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Controlled Release. 2013;172(2):456–66.

39. Auffret M, Rousseau S, Boutet I, Tanguy A, Baron J, Moraga D, et al. A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranea. Ecotoxicol Environ Saf. 2006 Mar 1;63(3):393–405.

40. Tersago K, De Coen W, Scheirs J, Vermeulen K, Blust R, Van Bockstaele D, et al. Immunotoxicology in wood mice along a heavy metal pollution gradient. Environ Pollut. 2004 Dec 1;132(3):385–94.

41. Topashka-Ancheva M, Metcheva R, Teodorova S. A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria II: chromosomal aberrations and blood pathology. Ecotoxicol Environ Saf. 2003 Feb;54(2):188–93.

42. Rai PK. Heavy metals in water, sediments and wetland plants in an aquatic ecosystem of tropical industrial region, India. Environ Monit Assess. 2009 Nov;158(1–4):433–57.

43. Priyadarshani S, Madhushani WAN, Jayawardena UA, Wickramasinghe DD, Udgama PV. Heavy metal mediated immunomodulation of the Indian green frog, Euphlyctis hexadactylus (Anura_Ranidae) in urban wetlands. Ecotoxicol Environ Saf. 2015;116:40–9.

44. Galloway T, Handy R. Immunotoxicity of Organophosphorous Pesticides. Ecotoxicology. 2003 Feb 1;12(1):345–63.

45. Dhouib I, Jallouli M, Annabi A, Marzouki S, Gharbi N, Elfazaa S, et al. From immunotoxicity to carcinogenicity: the effects of carbamate pesticides on the immune system. Environ Sci Pollut Res. 2016 May 1;23(10):9448–58.

46. Tüfekci N, Sivri N, Toroz İ. Pollutants of Textile Industry Wastewater and Assessment of its Discharge Limits by Water Quality Standards. Turk J Fish Aquat Sci. 2007 Feb 1;7(2):97–103.

47. Pokhrel D, Viraraghavan T. Treatment of pulp and paper mill wastewater—a review. Sci Total Environ. 2004 Oct 15;333(1):37–58.

48. Masliyah J, Zhou ZJ, Xu Z, Czarnecki J, Hamza H. Understanding Water-Based Bitumen Extraction from Athabasca Oil Sands. Can J Chem Eng. 2004 Aug 1;82(4):628–54.

49. Heuvel MR van den, Hogan NS, Roloson SD, Kraak GJVD. Reproductive development of yellow perch (Perca flavescens) exposed to oil sands-affected waters. Environ Toxicol Chem. 2012 Mar;31(3):654–62.

50. Gurney KE, Williams TD, Smits JE, Wayland M, Trudeau S, Bendell-Young LI. Impact of oil-sands based wetlands on the growth of mallard (Anas platyrhynchos) ducklings. Environ Toxicol Chem. 2005 Feb 1;24(2):457–63.

51. He Y, Wiseman SB, Hecker M, Zhang X, Wang N, Perez LA, et al. Effect of Ozonation on the Estrogenicity and Androgenicity of Oil Sands Process-Affected Water. Environ Sci Technol. 2011 Aug 1;45(15):6268–74.

52. Wang N, Chelme-Ayala P, Perez-Estrada L, Garcia-Garcia E, Pun J, Martin JW, et al. Impact of Ozonation on Naphthenic Acids Speciation and Toxicity of Oil Sands Process-Affected Water to Vibrio fischeri and Mammalian Immune System. Environ Sci Technol. 2013 Jun 18;47(12):6518–26.

53. Zubot W, MacKinnon MD, Chelme-Ayala P, Smith DW, Gamal El-Din M. Petroleum coke adsorption as a water management option for oil sands process-affected water. Sci Total Environ. 2012 Jun 15;427–428(Supplement C):364–72.

54. Frank RA, Sanderson H, Kavanagh R, Burnison BK, Headley JV, Solomon KR. Use of a (Quantitative) Structure–Activity Relationship [(Q)Sar] Model to Predict the Toxicity of Naphthenic Acids. J Toxicol Environ Health A. 2009 Dec 31;73(4):319–29.

55. Garcia-Garcia E, Ge JQ, Oladiran A, Montgomery B, El-Din MG, Perez-Estrada LC, et al. Ozone treatment ameliorates oil sands process water toxicity to the mammalian immune system. Water Res. 2011 Nov 15;45(18):5849–57.

56. Li C. The evaluation of toxic effects induced by exposure of mammals to oil sands process-affected water and its organic fraction. 2018.

57. MacKinnon MD, Retallack JT. Preliminary characterization and detoxification of tailings pond water at the Syncrude Canada Ltd. oil sands plant [Energy production]. 1982 [cited 2018 Jan 8]; Available from: http://agris.fao.org/agris-search/search.do?recordID=US8207556

58. Lari E, Wiseman S, Mohaddes E, Morandi G, Alharbi H, Pyle GG. Determining the effect of oil sands process-affected water on grazing behaviour of Daphnia magna, long-term consequences, and mechanism. Chemosphere. 2016 Mar 1;146(Supplement C):362–70.

59. Wiseman SB, Anderson JC, Liber K, Giesy JP. Endocrine disruption and oxidative stress in larvae of Chironomus dilutus following short-term exposure to fresh or aged oil sands process-affected water. Aquat Toxicol. 2013 Oct 15;142–143(Supplement C):414–21.

60. Melvin SD, Trudeau VL. Growth, development and incidence of deformities in amphibian larvae exposed as embryos to naphthenic acid concentrations detected in the Canadian oil sands region. Environ Pollut. 2012 Aug 1;167(Supplement C):178–83.

61. Headley JV, McMartin DW. A Review of the Occurrence and Fate of Naphthenic Acids in Aquatic Environments. J Environ Sci Health Part A. 2004 Dec 27;39(8):1989–2010.

62. Pollet I, Bendell-Young LI. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. Environ Toxicol Chem. 2000 Oct 1;19(10):2589–97.

63. Beck EM, Smits JEG, St. Clair CC. Health of Domestic Mallards (Anas platyrhynchos domestica) Following Exposure to Oil Sands Process-Affected Water. Environ Sci Technol. 2014 Aug 5;48(15):8847–54.

64. M D MacKinnon. A study of the chemical and physical properties of Syncrude's tailings pond, Mildred Lake. Environ Res Rep 1981-1 Syncrude Can Ltd Edmonton, AB. 1981;

65. Kavanagh RJ, Frank RA, Oakes KD, Servos MR, Young RF, Fedorak PM, et al. Fathead minnow (Pimephales promelas) reproduction is impaired in aged oil sands process-affected waters. Aquat Toxicol Amst Neth. 2011 Jan 17;101(1):214–20.

66. Kavanagh RJ, Frank RA, Burnison BK, Young RF, Fedorak PM, Solomon KR, et al. Fathead minnow (Pimephales promelas) reproduction is impaired when exposed to a naphthenic acid extract. Aquat Toxicol Amst Neth. 2012 Jul 15;116–117:34–42.

67. He Y, Wiseman SB, Wang N, Perez-Estrada LA, El-Din MG, Martin JW, et al. Transcriptional Responses of the Brain–Gonad–Liver Axis of Fathead Minnows Exposed to Untreated and Ozone-Treated Oil Sands Process-Affected Water. Environ Sci Technol. 2012 Sep 4;46(17):9701–8.

68. Philibert DA, Lyons D, Philibert C, Tierney KB. Field-collected crude oil, weathered oil and dispersants differentially affect the early life stages of freshwater and saltwater fishes. Sci Total Environ. 2019 Jan 10;647:1148–57.

69. Borg B. Androgens in teleost fishes. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 1994 Nov 1;109(3):219–45.

70. van den Heuvel MR, Power M, MacKinnon MD, Dixon DG. Effects of oil sands related aquatic reclamation on yellow perch (Perca flavescens). II. Chemical and biochemical indicators of exposure to oil sands related waters. Can J Fish Aquat Sci. 1999 Jul 1;56(7):1226–33.

71. Rogers VV, Wickstrom M, Liber K, MacKinnon MD. Acute and Subchronic Mammalian Toxicity of Naphthenic Acids from Oil Sands Tailings. Toxicol Sci. 2002 Apr 1;66(2):347–55.

72. Li C, Fu L, Lillico DME, Belosevic M, Stafford JL, Gamal El-Din M. Exposure to Organic Fraction Extracted from Oil Sands Process-Affected Water Has Negligible Impact on Pregnancy and Lactation of Mice. Environ Sci Technol. 2019 Jun 18;53(12):7083–94.

73. Headley JV, Peru KM, Mohamed MH, Frank RA, Martin JW, Hazewinkel RRO, et al. Chemical fingerprinting of naphthenic acids and oil sands process waters—A review of analytical methods for environmental samples. J Environ Sci Health Part A. 2013 Aug 24;48(10):1145–63.

74. Brient JA, Wessner PJ, Doly MN. Naphthenic acids. Encyclopedia of Chemical Technology. 1995

75. Ivanković T, Hrenović J. Surfactants in the environment. Arh Hig Rada Toksikol. 2010 Mar;61(1):95–110.

76. Scarlett AG, Reinardy HC, Henry TB, West CE, Frank RA, Hewitt LM, et al. Acute toxicity of aromatic and non-aromatic fractions of naphthenic acids extracted from oil sands process-affected water to larval zebrafish. Chemosphere. 2013 Sep;93(2):415–20.

77. Dokholyan BK, Magomedov AK. Effect of sodium naphthenate on survival and some physiological-biochemical parameters of some fishes. Voprosy Ikhtiologii. 1983.

78. Davila DR, Davis DP, Campbell K, Cambier JC, Zigmond LA, Burchiel SW. Role of alterations in ca $^{2+}$ -associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. J Toxicol Environ Health. 1995 Jun;45(2):101–26.

79. Baird WM, Hooven LA, Mahadevan B. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ Mol Mutagen. 2005;45(2–3):106–14.

80. Jacobson KC, Arkoosh MR, Kagley AN, Clemons ER, Collier TK, Casillas E. Cumulative Effects of Natural and Anthropogenic Stress on Immune Function and Disease Resistance in Juvenile Chinook Salmon. J Aquat Anim Health. 2003;15(1):1–12.

81. Simalowicz RJ, DeVito MJ, Riddle MM, Williams WC, Birnbaum LS. Opposite Effects of 2,2′,4,4′,5,5′-Hexachlorobiphenyl and 2,3,7,8-Tetrachlorodibenzo-p-dioxin on the Antibody Response to Sheep Erythrocytes in Mice. Toxicol Sci. 1997 Jun 1;37(2):141–9.

82. Arkoosh MR, Clemons E, Huffman P, Kagley AN, Casillas E, Adams N, et al. Increased Susceptibility of Juvenile Chinook Salmon to Vibriosis after Exposure to Chlorinated and Aromatic Compounds Found in Contaminated Urban Estuaries. J Aquat Anim Health. 2001;13(3):257–68.

83. Khan RA. Health of Flatfish from Localities in Placentia Bay, Newfoundland, Contaminated with Petroleum and PCBs. Arch Environ Contam Toxicol. 2003 May 1;44(4):0485–92.

84. Reynaud S, Deschaux P. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. Vol. 77. 2006. 229 p.

85. Di Marzio W, Galassi S, Todeschini R, Consolaro F. Traditional versus WHIM molecular descriptors in QSAR approaches applied to fish toxicity studies. Chemosphere. 2001 Jul;44(3):401–6.

86. Shen Y. In Vitro Cytotoxicity of BTEX Metabolites in Hela Cells. Arch Environ Contam Toxicol. 1998 Apr 1;34(3):229–34.

87. Saar RA, Weber JH. Fulvic acid: modifier of metal-ion chemistry. Environ Sci Technol. 1982 Sep 1;16(9):510A-517A.

88. Pershagen G. The carcinogenicity of arsenic. Environ Health Perspect. 1981 Aug;40:93–100.

89. Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. Heavy Metal Toxicity and the Environment. In: Luch A, editor. Molecular, Clinical and Environmental Toxicology: Volume 3: Environmental Toxicology. Basel: Springer Basel; 2012. p. 133–64.

90. Hawley DM, Hallinger KK, Cristol DA. Compromised immune competence in free-living tree swallows exposed to mercury. Ecotoxicology. 2009 Jul 1;18(5):499–503.

91. Shenker BJ, Rooney C, Vitale L, Shapiro IM. Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. I. Suppression of T-cell activation. Immunopharmacol Immunotoxicol. 1992;14(3):539–53.

92. Mustafa MG, Cross CE, Tyler WS. Interference of Cadmium Ion With Oxidative Metabolism of Alveolar Macrophages. Arch Intern Med. 1971 Jun 1;127(6):1050–8.

93. Avery JC, Hoffmann PR. Selenium, Selenoproteins, and Immunity. Nutrients. 2018 Sep 1;10(9).

94. Strom S, Johnson RL, Uyeki EM. Mercury toxicity to hemopoietic and tumor colony-forming cells and its reversal by selenium in vitro. Toxicol Appl Pharmacol. 1979 Jul 1;49(3):431–6.

95. Nero V, Farwell A, Lee LEJ, Van Meer T, MacKinnon MD, Dixon DG. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicol Environ Saf. 2006 Oct 1;65(2):252–64.

96. Leung SS, MacKinnon MD, Smith REH. The ecological effects of naphthenic acids and salts on phytoplankton from the Athabasca oil sands region. Aquat Toxicol Amst Neth. 2003 Jan 10;62(1):11–26.

97. Al jibouri AKH, Wu J. Model development for naphthenic acids ozonation process. Environ Sci Pollut Res. 2015 Feb 1;22(4):2558–65.

98. Grewer DM, Young RF, Whittal RM, Fedorak PM. Naphthenic acids and other acid-extractables in water samples from Alberta: What is being measured? Sci Total Environ. 2010 Nov;408(23):5997–6010.

99. Holowenko FM, MacKinnon MD, Fedorak PM. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Res. 2002 Jun 1;36(11):2843–55.

100. Bray DF, Bagu J, Koegler P. Comparison of hexamethyldisilazane (HMDS), Peldri II, and critical-point drying methods for scanning electron microscopy of biological specimens. Microsc Res Tech. 1993 Dec 15;26(6):489–95.

101. Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol. 2008 May 22;82(8):493.

102. Kupcsik L. Estimation of Cell Number Based on Metabolic Activity: The MTT Reduction Assay. In: Stoddart MJ, editor. Mammalian Cell Viability: Methods and Protocols. Totowa, NJ: Humana Press; 2011. p. 13–9. (Methods in Molecular Biology).

103. Fotakis G, Timbrell JA. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett. 2006 Jan 5;160(2):171–7.

104. Burgos-Aceves MA, Cohen A, Paolella G, Lepretti M, Smith Y, Faggio C, et al. Modulation of mitochondrial functions by xenobiotic-induced microRNA: From environmental sentinel organisms to mammals. Sci Total Environ. 2018 Dec 15;645:79–88.

105. Michl J, Park KC, Swietach P. Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. Commun Biol. 2019 Apr 26;2.

106. Gerweck L, Rottinger E. Enhancement of Mammalian Cell Sensitivity to Hyperthermia by pH Alteration. Radiat Res. 1976 Sep 1;67(3):508–11.

107. Brusick D. Genotoxic effects in cultured mammalian cells produced by low pH treatment conditions and increased ion concentrations. Environ Mol Mutagen. 1986;8(6):879–86.

108. Ruffin VA, Salameh AI, Boron WF, Parker MD. Intracellular pH regulation by acid-base transporters in mammalian neurons. Front Physiol. 2014 Feb 13;5.

109. Izumi H, Torigoe T, Ishiguchi H, Uramoto H, Yoshida Y, Tanabe M, et al. Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. Cancer Treat Rev. 2003 Dec 1;29(6):541–9.

110. Raghunand N, Gillies RJ. pH and drug resistance in tumors. Drug Resist Updat. 2000 Feb 1;3(1):39–47.

111. Mohseni P, Hahn NA, Frank RA, Hewitt LM, Hajibabaei M, Van Der Kraak G. Naphthenic Acid Mixtures from Oil Sands Process-Affected Water Enhance

Differentiation of Mouse Embryonic Stem Cells and Affect Development of the Heart. Environ Sci Technol. 2015 Aug 18;49(16):10165–72.

112. Liebermann DA, Hoffman B. Gadd45 in stress signaling. J Mol Signal. 2008 Sep 12;3(1):15.

113. Yang J, Yu Y, Duerksen-Hughes PJ. Protein kinases and their involvement in the cellular responses to genotoxic stress. Mutat Res Mutat Res. 2003 Jan 1;543(1):31–58.

114. Vairapandi M, Balliet AG, Hoffman B, Liebermann DA. GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. J Cell Physiol. 2002 Sep;192(3):327–38.

115. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: Signaling for suicide and survival. J Cell Physiol. 2002;192(1):1–15.

116. Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci. 1997 Sep 30;94(20):10925–30.

117. Kelly KA, Havrilla CM, Brady TC, Abramo KH, Levin ED. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Perspect. 1998 Jul;106(7):375–84.

118. Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, et al. Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. J Clin Invest. 1992 Jul;90(1):267–70.

119. Baumann H, Gauldie J. The acute phase response. Immunol Today. 1994 Feb 1;15(2):74–80.

120. Riches DW, Chan ED, Winston BW. TNF-alpha-induced regulation and signalling in macrophages. Immunobiology. 1996 Oct;195(4–5):477–90.

121. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood. 1996 Mar 15;87(6):2095–147.

122. Liu Z, Kuang W, Zhou Q, Zhang Y. TGF-β1 secreted by M2 phenotype macrophages enhances the stemness and migration of glioma cells via the SMAD2/3 signalling pathway. Int J Mol Med. 2018 Dec;42(6):3395–403.

123. Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. J Exp Med. 1993 Aug 1;178(2):605–13.

124. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol. 2011 Nov;11(11):750–61.

125. Lu H-L, Huang X-Y, Luo Y-F, Tan W-P, Chen P-F, Guo Y-B. Activation of M1 macrophages plays a critical role in the initiation of acute lung injury. Biosci Rep. 2018 Apr 27;38(2).

126. Rath M, Müller I, Kropf P, Closs EI, Munder M. Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. Front Immunol. 2014 Oct 27;5.

127. Wang F, Li C, Liu W, Jin Y. Oxidative damage and genotoxic effect in mice caused by sub-chronic exposure to low-dose volatile organic compounds. Inhal Toxicol. 2013 Apr 1;25(5):235–42.

128. Gallego SM, Benavídes MP, Tomaro ML. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Sci. 1996 Dec 20;121(2):151–9.

129. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med. 1995 Feb 1;18(2):321–36.

130. Fisher NS, Frood D. Heavy metals and marine diatoms: Influence of dissolved organic compounds on toxicity and selection for metal tolerance among four species. Mar Biol. 1980 Nov 1;59(2):85–93.

131. Gadd GM, Griffiths AJ. Microorganisms and heavy metal toxicity. Microb Ecol. 1977 Dec 1;4(4):303–17.

132. Flemming CA, Trevors JT. Copper toxicity and chemistry in the environment: a review. Water Air Soil Pollut. 1989 Mar 1;44(1):143–58.

133. Cadet J, Douki T, Gasparutto D, Ravanat J-L. Oxidative damage to DNA: formation, measurement and biochemical features. Mutat Res Mol Mech Mutagen. 2003 Oct 29;531(1):5–23.

134. Zganiacz A, Santosuosso M, Wang J, Yang T, Chen L, Anzulovic M, et al. TNF-α is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. J Clin Invest. 2004 Feb 1;113(3):401–13.

135. Oladiran A, Beauparlant D, Belosevic M. The expression analysis of inflammatory and antimicrobial genes in the goldfish (Carassius auratus L.) infected with Trypanosoma carassii. Fish Shellfish Immunol. 2011 Oct;31(4):606–13.

136. Wang J, Cao X, Sun J, Chai L, Huang Y, Tang X. Transcriptional responses of earthworm (Eisenia fetida) exposed to naphthenic acids in soil. Environ Pollut. 2015 Sep 1;204:264–70.

137. Chen C, Zhou Q, Liu S, Xiu Z. Acute toxicity, biochemical and gene expression responses of the earthworm Eisenia fetida exposed to polycyclic musks. Chemosphere. 2011 May 1;83(8):1147–54.

138. Roubalová R, Dvořák J, Procházková P, Elhottová D, Rossmann P, Škanta F, et al. The effect of dibenzo-p-dioxin- and dibenzofuran-contaminated soil on the earthworm Eisenia andrei. Environ Pollut. 2014 Oct 1;193:22–8.

139. Ayala A, Perrin MM, Meldrum DR, Ertel W, Chaudry IH. Hemorrhage induces an increase in serum TNF which is not associated with elevated levels of endotoxin. Cytokine. 1990 May 1;2(3):170–4.

140. Coates PJ, Lorimore SA, Wright EG. Cell and tissue responses to genotoxic stress. J Pathol. 2005;205(2):221–35.

141. Porcheray F, Viaud S, Rimaniol A-C, Léone C, Samah B, Dereuddre-Bosquet N, et al. Macrophage activation switching: an asset for the resolution of inflammation. Clin Exp Immunol. 2005;142(3):481–9.

142. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci J Virtual Libr. 2008;13:453–61.

143. Zhang M, Hutter G, Kahn SA, Azad TD, Gholamin S, Xu CY, et al. Anti-CD47 Treatment Stimulates Phagocytosis of Glioblastoma by M1 and M2 Polarized Macrophages and Promotes M1 Polarized Macrophages In Vivo. PLOS ONE. 2016 Apr 19;11(4):e0153550.

144. Rossol M, Heine H, Meusch U, Quandt D, Klein C, Sweet MJ, et al. LPS-induced Cytokine Production in Human Monocytes and Macrophages. Crit Rev Immunol. 2011;31(5).

145. Li C, Fu L, Stafford J, Belosevic M, Gamal El-Din M. The toxicity of oil sands process-affected water (OSPW): A critical review. Sci Total Environ. 2017 Dec 1;601–602:1785–802.

146. Hagen MO, Garcia-Garcia E, Oladiran A, Karpman M, Mitchell S, El-Din MG, et al. The acute and sub-chronic exposures of goldfish to naphthenic acids induce different host defense responses. Aquat Toxicol. 2012 Mar;109:143–9.

147. Garcia-Garcia E, Pun J, Hodgkinson J, Perez-Estrada LA, El-Din MG, Smith DW, et al. Commercial naphthenic acids and the organic fraction of oil sands process water induce different effects on pro-inflammatory gene expression and macrophage phagocytosis in mice. J Appl Toxicol. 2012 Dec 1;32(12):968–79.

148. Garcia-Garcia E, Pun J, Perez-Estrada LA, Din MG-E, Smith DW, Martin JW, et al. Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. Toxicol Lett. 2011 May 30;203(1):62–73.

149. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol. 2014;5:614.

150. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014 Mar 3;6.

151. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sci. 2004 Jun 25;75(6):639–53.

152. Madill REA, Orzechowski MT, Chen G, Brownlee BG, Bunce NJ. Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: Bioassays with mature fine tailings pore water. Environ Toxicol. 2001;16(3):197–208.

153. Li C, Fu L, Stafford J, Belosevic M, Gamal El-Din M. The toxicity of oil sands process-affected water (OSPW): A critical review. Sci Total Environ. 2017 Dec 1;601–602:1785–802.

154. MacKinnon MD, Boerger H. Description of Two Treatment Methods for Detoxifying Oil Sands Tailings Pond Water. Water Qual Res J. 1986 Nov 1;21(4):496–512.

155. Clemente JS, Fedorak PM. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere. 2005 Jul 1;60(5):585–600.

156. El-Gayar S, Thüring-Nahler H, Pfeilschifter J, Röllinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. J Immunol Baltim Md 1950. 2003 Nov 1;171(9):4561–8.

157. Lee J, Ryu H, Ferrante RJ, Morris SM, Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc Natl Acad Sci U S A. 2003 Apr 15;100(8):4843–8.

158. Choi B-S, Martinez-Falero IC, Corset C, Munder M, Modolell M, Müller I, et al. Differential impact of l-arginine deprivation on the activation and effector functions of T cells and macrophages. J Leukoc Biol. 2009;85(2):268–77.

159. Durante W, Johnson FK, Johnson RA. Arginase: A Critical Regulator of Nitric Oxide Synthesis and Vascular Function. Clin Exp Pharmacol Physiol. 2007;34(9):906–11.

160. Nathan C, Xie Q. Regulation of Biosynthesis of Nitric Oxide. J Biol Chem. 1994;269(19):13725–8.

161. Shu B, Feng Y, Gui Y, Lu Q, Wei W, Xue X, et al. Blockade of CD38 diminishes lipopolysaccharide-induced macrophage classical activation and acute kidney injury involving NF-κB signaling suppression. Cell Signal. 2018;42:249–58.

162. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, Functional, and Plasticity Features of Classical and Alternatively Activated Human Macrophages. Am J Respir Cell Mol Biol. 2015 Apr 14;53(5):676–88.

163. Nijnik A, Madera L, Ma S, Waldbrook M, Elliott MR, Easton DM, et al. Synthetic Cationic Peptide IDR-1002 Provides Protection against Bacterial Infections through

Chemokine Induction and Enhanced Leukocyte Recruitment. J Immunol. 2010 Mar 1;184(5):2539–50.

164. Rivas-Santiago B, Castañeda-Delgado JE, Santiago CER, Waldbrook M, González-Curiel I, León–Contreras JC, et al. Ability of Innate Defence Regulator Peptides IDR-1002, IDR-HH2 and IDR-1018 to Protect against Mycobacterium tuberculosis Infections in Animal Models. PLOS ONE. 2013 Mar 21;8(3):e59119.

165. Pena OM, Afacan N, Pistolic J, Chen C, Madera L, Falsafi R, et al. Synthetic Cationic Peptide IDR-1018 Modulates Human Macrophage Differentiation. PLOS ONE. 2013 Jan 7;8(1):e52449.

166. Mestdagh P, Feys T, Bernard N, Guenther S, Chen C, Speleman F, et al. High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. Nucleic Acids Res. 2008 Dec 1;36(21):e143–e143.

167. Waters MD, Fostel JM. Toxicogenomics and systems toxicology: aims and prospects. Nat Rev Genet. 2004 Dec;5(12):936.

168. Frank RA, Kavanagh R, Kent Burnison B, Arsenault G, Headley JV, Peru KM, et al. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere. 2008 Jul 1;72(9):1309–14.

169. Li C, Fu L, Stafford J, Belosevic M, Gamal El-Din M. The toxicity of oil sands process-affected water (OSPW): A critical review. Sci Total Environ. 2017 Jun 11;601–602:1785–802.

170. Anderson J, Wiseman SB, Moustafa A, Gamal El-Din M, Liber K, Giesy JP. Effects of exposure to oil sands process-affected water from experimental reclamation ponds on Chironomus dilutus. Water Res. 2012 Apr 15;46(6):1662–72.

171. Hagen MO, Katzenback BA, Islam MDS, El-Din MG, Belosevic M. The Analysis of Goldfish (Carassius auratus L.) Innate Immune Responses After Acute and Subchronic Exposures to Oil Sands Process-Affected Water. Toxicol Sci. 2013 Nov 27;kft272.

172. Hersikorn BD, Ciborowski JJC, Smits JEG. The effects of oil sands wetlands on wood frogs (Rana sylvatica). Toxicol Environ Chem. 2010 Sep 1;92(8):1513–27.

173. Kavanagh RJ, Frank RA, Solomon KR, Van Der Kraak G. Reproductive and health assessment of fathead minnows (Pimephales promelas) inhabiting a pond containing oil sands process-affected water. Aquat Toxicol. 2013 Apr 15;130–131:201–9.

174. Kavanagh RJ, Frank RA, Oakes KD, Servos MR, Young RF, Fedorak PM, et al. Fathead minnow (Pimephales promelas) reproduction is impaired in aged oil sands process-affected waters. Aquat Toxicol. 2011 Jan 17;101(1):214–20.

175. Zetouni NC, Siraki AG, Weinfeld M, Pereira ADS, Martin JW. Screening of genotoxicity and mutagenicity in extractable organics from oil sands process-affected water. Environ Toxicol Chem. 2017;36(5):1397–404.

176. Rogers VV, Wickstrom M, Liber K, MacKinnon MD. Acute and Subchronic Mammalian Toxicity of Naphthenic Acids from Oil Sands Tailings. Toxicol Sci. 2002 Apr 1;66(2):347–55.

177. Rogers VV. Mammalian toxicity of naphthenic acids derived from the Athabasca oil sands. Ottawa: National Library of Canada = Bibliothèque nationale du Canada; 2004.

178. Wiseman SB, He Y, Gamal-El Din M, Martin JW, Jones PD, Hecker M, et al. Transcriptional responses of male fathead minnows exposed to oil sands process-affected water. Comp Biochem Physiol Part C Toxicol Pharmacol. 2013 Mar 1;157(2):227–35.

179. Lari E, Pyle GG. Rainbow trout (Oncorhynchus mykiss) detection, avoidance, and chemosensory effects of oil sands process-affected water. Environ Pollut. 2017 Jun 1;225:40–6.

180. Reichert M, Blunt B, Gabruch T, Zerulla T, Ralph A, Gamal El-Din M, et al. Sensory and Behavioral Responses of a Model Fish to Oil Sands Process-Affected Water with and without Treatment. Environ Sci Technol. 2017 Jun 20;51(12):7128–37.

181. Van Dyken SJ, Locksley RM. Interleukin-4- and Interleukin-13-Mediated Alternatively Activated Macrophages: Roles in Homeostasis and Disease. Annu Rev Immunol. 2013;31(1):317–43.

182. Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther. 2003 Nov 1;100(2):171–94.

183. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. Immunity. 2014 Jul 17;41(1):14–20.

184. Gagné F, Bruneau A, Turcotte P, Gagnon C, Lacaze E. An investigation of the immunotoxicity of oil sands processed water and leachates in trout leukocytes. Ecotoxicol Environ Saf. 2017 Jul;141:43–51.

185. Muraille E, Leo O, Moser M. Th1/Th2 Paradigm Extended: Macrophage Polarization as an Unappreciated Pathogen-Driven Escape Mechanism? Front Immunol. 2014;5.

186. Refai A, Gritli S, Barbouche M-R, Essafi M. Mycobacterium tuberculosis Virulent Factor ESAT-6 Drives Macrophage Differentiation Toward the Pro-inflammatory M1 Phenotype and Subsequently Switches It to the Anti-inflammatory M2 Phenotype. Front Cell Infect Microbiol. 2018;8:327.

187. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol. 2008 Dec;9(12):1399–406.

188. Bruch-Gerharz D, Schnorr O, Suschek C, Beck K-F, Pfeilschifter J, Ruzicka T, et al. Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation. Am J Pathol. 2003 Jan;162(1):203–11.

189. Jan AT, Azam M, Siddiqui K, Ali A, Choi I, Haq QMohdR. Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants. Int J Mol Sci. 2015 Dec 10;16(12):29592–630.

190. Leclair LA, MacDonald GZ, Phalen LJ, Köllner B, Hogan NS, van den Heuvel MR. The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (Oncorhynchus mykiss). Aquat Toxicol. 2013 Oct 15;142–143:185–94.

191. McNeill SA, Arens CJ, Hogan NS, Köllner B, van den Heuvel MR. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. Ecotoxicol Environ Saf. 2012 Oct 1;84:254–61.

192. Shigematsu K, Asai A, Kobayashi M, Herndon DN, Suzuki F. Enterococcus faecalis translocation in mice with severe burn injury: a pathogenic role of CCL2 and alternatively activated macrophages (M2aMphi and M2cMphi). J Leukoc Biol. 2009 Oct;86(4):999–1005.

193. Oladiran A, Belosevic M. Immune Evasion Strategies of Trypanosomes: A Review. J Parasitol. 2011 Oct 18;98(2):284–92.

194. McGuirk P, Mills KHG. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol. 2002 Sep 1;23(9):450–5.

195. Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev. 1996 Oct;9(4):532–62.

196. Nairz M, Sonnweber T, Schroll A, Theurl I, Weiss G. The pleiotropic effects of erythropoietin in infection and inflammation. Microbes Infect. 2012 Mar 1;14(3):238–46.

197. Broxmeyer HE. Erythropoietin Surprises: An Immune Saga. Immunity. 2011 Jan 28;34(1):6–7.

198. McAllister M, Phillips N, Belosevic M. Trypanosoma carassii infection in goldfish (Carassius auratus L.): changes in the expression of erythropoiesis and anemia regulatory genes. Parasitol Res. 2019 Apr 1;118(4):1147–58.

199. Raes G, Beschin A, Ghassabeh GH, De Baetselier P. Alternatively activated macrophages in protozoan infections. Curr Opin Immunol. 2007 Aug 1;19(4):454–9.

200. Infante-Duarte C, Kamradt T. Th1/Th2 balance in infection. Springer Semin Immunopathol. 1999 Dec 1;21(3):317–38.

201. Neville LA, McCarthy FMG, MacKinnon MD, Swindles GT, Marlowe P. Thecamoebians (Testate amoebas) as proxies of ecosystem health and reclamation success in constructed wetlands in the oil sands of Alberta, Canada. J Foraminifer Res. 2011;41(3):230–47.