SUB-LETHAL EFFECTS OF OIL SANDS PROCESS-AFFECTED WATER (OSPW) IN TWO AQUATIC ORGANISMS: *DAPHNIA MAGNA* AND RAINBOW TROUT

EBRAHIM LARI
Bachelor of Science, Zabol University, Iran, 2006
Master of Science, Shahid Beheshti University, Iran, 2009

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Dr. G. Pyle Supervisor	Professor	Ph.D.
Dr. J. Rasmussen Thesis Examination Committee Member	Professor	Ph.D.
Dr. A. Gruber Thesis Examination Committee Member	Associate Professor	Ph.D.
Dr. K. Liber External Examiner University of Saskatchewan	Professor	Ph.D.
Dr. S. Rood Internal External Examiner	Professor	Ph.D.
Dr. R. Laird Chair, Thesis Examination Committee	Associate Professor	Ph.D.

Dedication

I dedicate this dissertation to my beloved wife and best friend, Effat Mohaddes, who has offered unwavering support and encouragement during my doctoral journey. Thanks Effat for your support and counsel.

I also want to offer thanks to my mother, Shahla Taghikhani, who always supported me, whatever path I took.

Abstract

My thesis contributes to a better understanding of the impacts of OSPW on aquatic organisms by investigating its sub-lethal effects on an invertebrate (*Daphnia magna*) and a vertebrate (rainbow trout) model species. Studies conducted on *D. magna* demonstrated that OSPW impairs feeding, reduces hemoglobin content, increases oxygen consumption, and reduces growth, reproduction and macronutrient reserves of exposed animals at 1 to 10% concentrations. I also investigated the interaction of OSPW with the chemosensory system of rainbow trout (*Oncorhynchus mykiss*). I demonstrated that rainbow trout are able to detect and behaviourally avoid low concentrations (0.1%) of OSPW. However, even a short-term (< 5 min) interaction with OSPW reduced olfactory acuity. The toxic effect of OSPW on the fish olfactory system gradually increased with increasing exposure time. Overall, the results of my thesis suggest that untreated OSPW, even at low concentrations (1% OSPW), can affect different aspects of aquatic animals.

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CHAPTER 1: Introduction

Covering over 140,000 km^2 in the Peace River (Bluesky-Gething deposit), Cold Lake (Clearwater deposit) and Athabasca (Wabiskaw-McMurray deposit) regions, the oil sands regions of Northern Alberta, Canada, encompass the largest reserve of bitumen in the world (Allen, 2008; ERCB, 2010). With an estimated 178 billion barrels of recoverable oil, reserves in Alberta's oil sands contain the world's third-largest oil reserves after Saudi Arabia and Venezuela (Holden et al., 2011; Environment Canada, 2013). The oil sands consist of a thick mixture of bitumen, sand, silt, clay, trapped gases, pore water, trace metals and minerals (Lo et al., 2006). Production of crude oil from bitumen has grown from 1.2 million barrels per day in 2008 to approximately 2.4 million barrels per day in 2015 and is predicted to grow to 3.95 million barrels per day by 2030 (CAPP, 2015). This rapid growth in oil sands extraction activities in this area has increased concern about the potential environmental impacts on the surrounding ecosystems related to air emissions, water use, wastewater production, potential groundwater contamination, land and habitat disturbances (Dowdeswell et al., 2011).

Currently, the main method used for bitumen recovery is open-pit mining in which oil sands are scraped from the surface of the deposit and trucked to refineries (CAPP, 2015). Extraction of bitumen from oil sands requires a large volume of fresh water. In the "Clark hot water extraction process," oil-containing sand is mixed with hot (79-93°C) water to separate bitumen from other constituents including clay and residual sand (Giesy et al., 2010). In this process, some untargeted compounds (inorganic and organic) are also separated from bitumen. For each 1 m^3 of synthetic crude produced by surface mining, 11 t

of oil sand is mined, 2.5 m^3 of hot water is used (80% recycled from tailings pond), and 3.3 m^3 raw oil sands tailings are added to the tailings ponds, which over time become 2 m^3 mature fine tailings (RSC, 2010). The resultant tailings contain solids, including sand, silt and clay, unrecovered hydrocarbons, and oil sands process-affected water (OSPW) (Han et al., 2009). For storage and remediation purposes, additives such as gypsum (a by-product of flue gas desulfurization) and anionic polyacrylamide might be added to the tailings waste (Haveroen et al., 2005; Allen, 2008; Ramos Padrón et al., 2011).

Because industrial operators are not currently permitted to discharge OSPW back into the Athabasca River, OSPW is currently being stored in on-site tailing ponds that cover an area of about 130 km^2 (Iqbal et al., 2013). It is estimated that these tailing ponds contain approximately one billion m^3 of tailings (altogether), which are 1–3% bitumen, 20–30% sand-silt-clay and 70–80% water (Del Rio et al., 2006; Collins et al., 2016). The massive quantity of stored OSPW raised public concern which led to proposing targets for a reduction of liquid tailings by the Energy Resources Conservation Board of Alberta (ERCB, 2009). In its latest released regulation via the Alberta Energy Regulator (AER), the Government of Alberta required oil sands operators to minimize the volume of liquid tailings stored in tailings ponds and leave the operation sites at a ready-to-reclaim state within ten years from the end of the mine's life (Government of Alberta, 2015). Consequently, companies are eventually responsible for remediating and releasing OSPW to the natural environment or turning tailings ponds into active ecosystems through the development of end-pit lakes. This mandate presents a major challenge for the industrial and academic communities to develop effective reclamation processes. Such a release would need to minimize the environmental impact on downstream ecosystems. Releasing

OSPW in a manner that minimizes downstream impacts or reclaiming tailings ponds to active ecosystems require a good knowledge of OSPW's chemical composition and characteristics, toxicity to resident biota, and its behaviour in aquatic ecosystems, along with good knowledge of the ecosystem into which it is being released.

Oil sands process-affected water is a complex mixture of a wide range of components. The most dominant and the primary persistent components in OSPW are organics such as naphthenic acids (NAs), oxidized NAs and related organic acids containing sulfur or nitrogen, as well as alkylated polycyclic aromatic hydrocarbons (aPAHs) (Clemente and Fedorak, 2005; Holden et al., 2011; Yang et al., 2011; Islam et al., 2014). However, given their small molecular masses, some PAHs volatilize rapidly from solution. For example, the concentration of selected PAHs in an OSPW sample from an active settling pond was below detection limit (0.2 ng/L) (Morandi et al., 2015). Additionally, OSPW contains different ionic forms of elements such as nickel (Ni), vanadium (V), aluminium (Al), iron (Fe), and chromium (Cr) and salts (e.g. Na^+, Cl^-, SO_4^{2-}, and HCO_3^-) (Melita and Gumrah, 2010; Debenest et al., 2012). Since activated carbon adsorption or ozonation drastically attenuates the toxic effects of OSPW, it is likely that the dissolved organic components are the primary toxic components of OSPW to aquatic organisms (He et al., 2011; He et al., 2012b; Niasar et al., 2016).

Oil sands process-affected water is both acutely and chronically toxic to a variety of aquatic organisms (e.g. Hersikorn et al., 2010; Kavanagh et al., 2011; Scarlett et al., 2013). Studies on the toxicity of OSPW to invertebrates have mainly investigated survival (Puttaswamy et al., 2010; Wiseman et al., 2013a), growth (Anderson et al., 2012a; Goff et al., 2013), and biochemical disruption (Wiseman et al., 2013a). Studies on early life stages

of *Chironomus dilutus* demonstrated that OSPW impairs growth, pupation, and emergence of larvae and interrupts endocrine balance of exposed animals; however, several years of natural degradation of OSPW components reduces OSPW effects on the larvae of *C. dilutus* (Anderson et al., 2012a; Wiseman et al., 2013a). On the other hand, a few days of ozonation attenuates the same effects more effectively than passive aging (Anderson et al., 2012b).

Several studies have investigated the toxicity of OSPW to fish. The lethal toxicity (96-h LC50) of OSPW to rainbow trout from an active tailing pond was calculated to be as low as 67% OSPW (Toor et al., 2013). In a study on two fish species, the eggs of yellow perch (*Perca flavescens)* and Japanese medaka (*Oryzias latipes*) were exposed to several dilutions of OSPW; both species demonstrated a reduction in hatch rate and deformity in larvae (Peters et al., 2007). In another reproduction study, male yellow perch raised in an OSPW-contaminated lake showed a significant reduction in testicular development and in circulating testosterone and 11-ketotestosterone, while no changes were observed in ovarian size or circulating steroid levels in female perch (van den Heuvel et al., 2012). Rainbow trout (*Oncorhynchus mykiss*) exposed to OSPW showed an increase in stress hormones and a decrease in immune system function (McNeill et al., 2012). In molecular studies, genotoxic effects (oxidative DNA damage, changes in RNA transcription, etc.) of OSPW on fish were also characterised (Gagné et al., 2011; Gagné et al., 2012; Wiseman et al., 2013b; Lacaze et al., 2014). Early life stages of fish are more vulnerable to the toxic effects of OSPW. Oil sands process-affected water and its components cause mortality, deformation, cardiovascular abnormalities and inhibition of growth in the embryo of several fish species (Gagné et al., 2012; He et al., 2012a; Morandi et al., 2015; Marentette et al., 2017).

The vast majority of OSPW toxicology studies focus on individual toxic components of OSPW, mainly NAs (e.g. Frank et al., 2008; Young et al., 2008; Young et al., 2011; Kavanagh et al., 2012) and PAHs (e.g. Short et al., 2008; Hogan et al., 2010). Considering the complex composition of OSPW, it is less likely that knowledge of the toxicity of its individual constituents can be generalized to the toxicity of OSPW. However, these studies contribute to identifying the most toxic components of OSPW so they can be targeted in remediation studies. There is also a need for the identification of biomarkers and endpoints to OSPW exposure and effects, such that the effects of accidental spills can be diagnosed. Of relevance for ecological risk assessment is the need to develop biomarkers and ecotoxicological endpoints that relate to the survival and ecological importance using sensitive organisms that are likely to be exposed to OSPW released to natural receiving environments. These biomarkers and sensitive endpoints may also be used as references for testing the success of the remediation methods.

In order to assess potential effects of an accidental contaminant release to an aquatic environment, all potentially important confounding variables must be considered. Fed from glacier headwaters, in temperate latitudes, water quality variables (e.g. temperature, hardness, and alkalinity) in water bodies associated with the Athabasca River watershed change throughout the year (Hebben, 2009; Environment Canada, 2011). For instance, water quality assessment of the Athabasca River at two sampling sites (M5 and M6) adjacent to and downstream from oil sands operations during 2015-2016 showed that the average water hardness and alkalinity varied from 105 to 178 mg/L (as $CaCO_3$) and 92 to 157 mg/L (as $CaCO_3$) throughout the year, respectively (RAMP, 2016). Changes in water quality parameters can alter the toxicity of many environmental contaminants (Pynnönen,

1995; Park et al., 2009). For instance, several metals, including nickel, cadmium, vanadium and zinc show different levels of toxicity in waters that vary in water hardness (Markich et al., 2006). Unfortunately, studies on the effect of water quality on the toxicity of OSPW or its major components are scarce. Thus, it is necessary to provide data on the effect of changing water quality of receiving ecosystem on the toxicity of OSPW before considering any regulation that allows for raw or treated OSPW to be released to natural aquatic environments. Athabasca River water quality has been monitored for decades. Regardless of minor variances due to yearly weather differences, it seems that water quality variables tend to follow annual trends from year to year (Figure 1.1) (Hebben, 2009). These annual patterns follow the general northern hemisphere rivers that originate from mountains. Water quality variables (e.g. pH, hardness, alkalinity) vary together on an annual cycle.

Although, there is merit in studying the effect of each water quality variable on the toxicity of OSPW, investigating the effects of waters that represent seasonal characteristics would be more ecologically relevant than investigating each variable's effect separately.

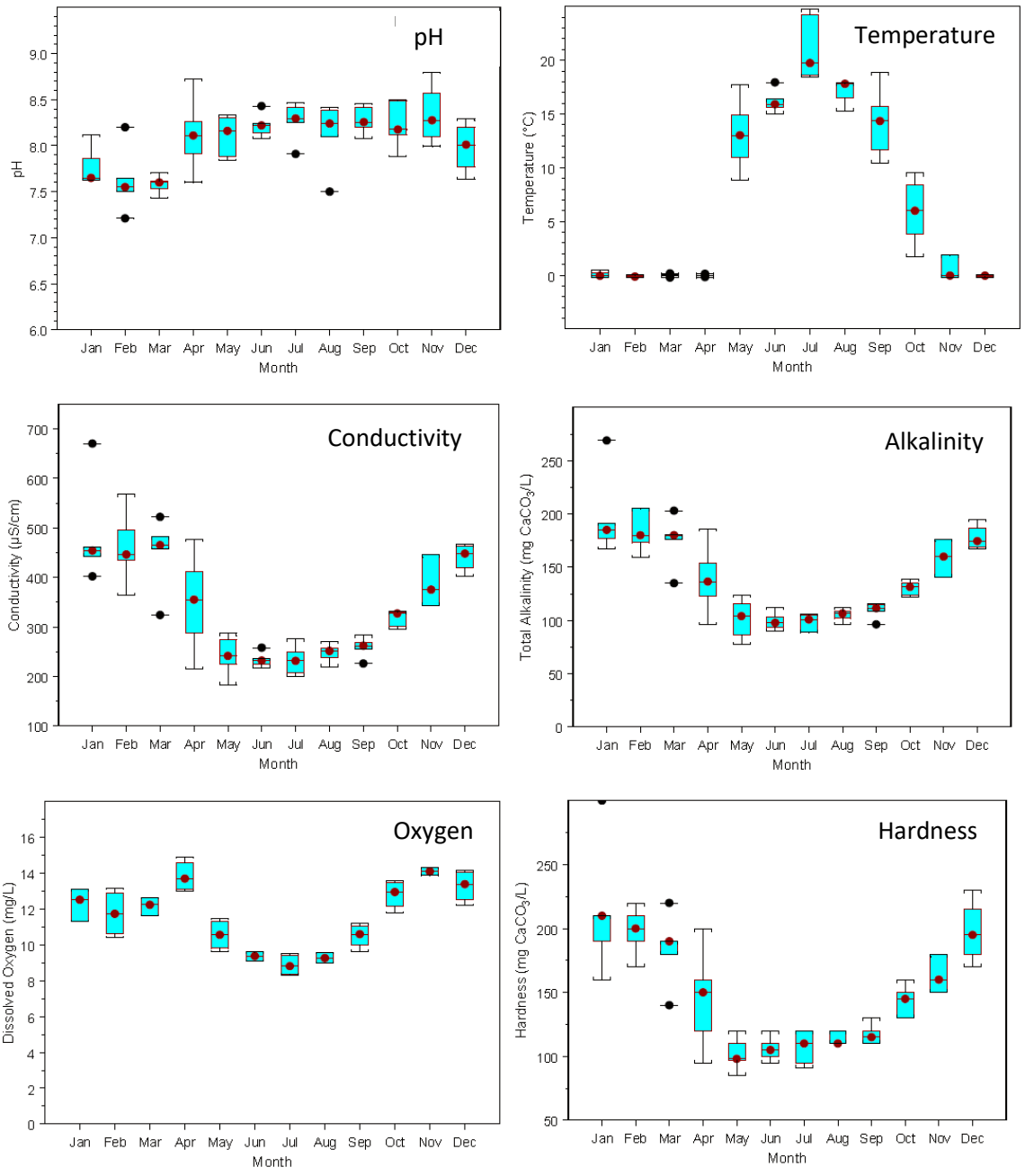


Figure 1.1. Annual water characteristics of Athabasca River varies with season in Fort McMurray from 1960 to 2007. Box plots display minimum and maximum values (upper and lower whiskers), first and third quartiles (top and bottom of boxes), the median values (red dots), and outliers (black dots) (Hebben 2009).

A great deal of research has been conducted to understand the effects of toxicants on the physiology and survival of many animals. As such, acute regulatory guidelines for aquatic pollutants in natural ecosystems have been traditionally based on acute lethality tests such as the 96 h LC50 (e.g. EPA, 2001; CCME, 2014); although in chronic guidelines impacts on development, growth, and, reproduction have also been considered (Scott and Sloman, 2004). Since behaviour serves as a link between physiological processes and ecological manifestations, there has been increasing interest, from the scientific community, in the use of animal behaviour models for evaluating the environmental impact of pollutants (Faucher et al., 2008). Thus, behavioural studies can be complementary to traditional regulatory guidelines in which physiological death is considered, while behavioural endpoints monitor "ecological death" in response to contaminants (Scott and Sloman, 2004). Ecological death refers to changes in an animal's behaviour in way that impairs their ability to function in an ecological context. For instance, it is shown that in their seasonal migration to tributaries the Pacific Ocean, salmon avoid hydrocarbon-contaminated waters (Weber et al., 1981). These salmon may not experience hydrocarbon toxity owing to their ability to avoid contaminated areas; however, their inability to reach spawning habitat may have negative consequences on their ability to reproduce. The same behavioural response might, to some extent, account for the severely reduced population of migratory fish in oil sands mining areas (Schwalb et al., 2015). Several studies show that various contaminants impair chemosensory-mediated food search and predator avoidance behaviour in fish (e.g. Dew et al., 2014; Lari, et al., 2015, Sakamoto et al., 2016). Impairment of such behaviours could reduce the chance of finding food and increase the chance of being preyed upon in contaminant in contaminated environments. Some drugs interfere with the stress response of fish, which increases the chance of being preyed upon

(Birceanu et al., 2015). Fish are an excellent model in this regard since many ecologically relevant fish behaviours are easily observed and quantified in a controlled environment. Furthermore, following the adverse outcome pathway (AOP) approach, the physiological, cellular, and molecular mechanisms by which a contaminant altered a normal behaviour should be investigated (Ankley et al., 2010). An AOP is a conceptual construct that aims to link the initial molecular interaction (initiating event) of a contaminant with the adverse outcome at the organism level. Thus, investigating the effects of a contaminant on relevant endpoints at different biological levels is required to develop accurate AOPs.

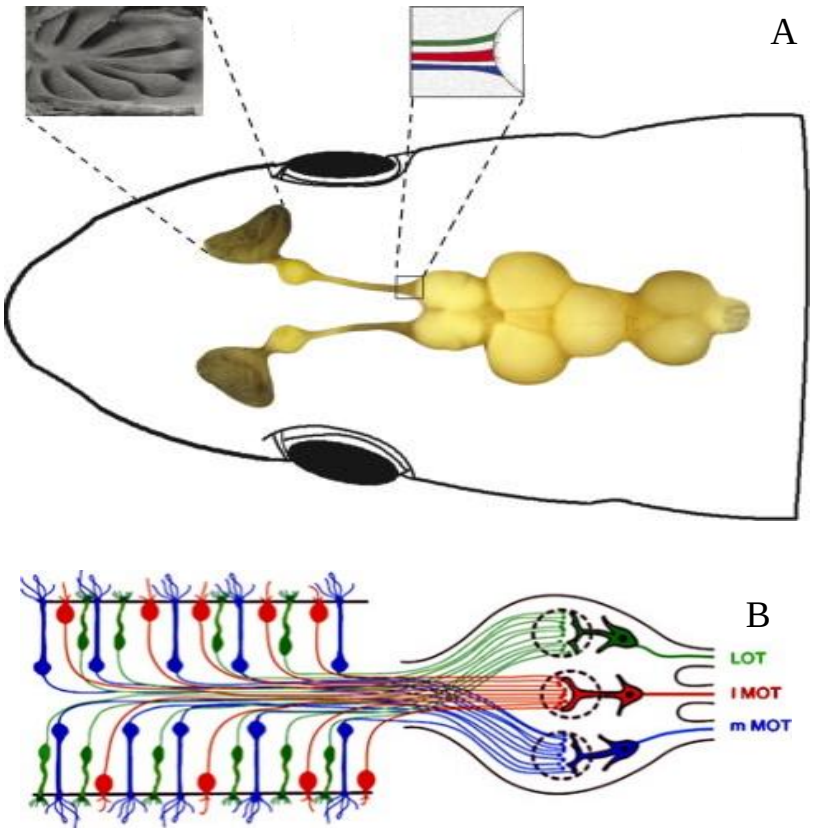
Many components of OSPW, including NAs, PAHs and metals, induce oxidative stress in target tissues of a variety of aquatic organisms from plankton (Wang et al., 2009; Yi et al., 2010) to fish (Gagné et al., 2012; Palanikumar et al., 2012; Lacaze et al., 2014). Additionally, since reactive oxygen species (ROS) cause cytotoxicity mainly via lipid peroxidation and enzymatic disruption (Gauthier et al., 2014), oxidative stress caused by elevated ROS production should be investigated as a potential mechanism of OSPW toxicity. Another well-known metabolic pathway related to hydrocarbons and metals is cytochrome P450 (CYP) (Ariyoshi et al., 1990; Gauthier et al., 2014, 2015). These enzymes are responsible for the modification and detoxification of hydrocarbons (Nebert and Dalton, 2006; Mu et al., 2012). Exposing fish to hydrocarbon or metal contaminants amplifies gene expression transcripts of the CYP enzymes (Ceyhun et al., 2011; Das and Mukherjee, 2013) and their activities (Baird et al., 2005). Therefore, monitoring the cytochrome P450 enzymes such as CYP1A1 and CYP3A4 for changes in response to hydrocarbon exposure would serve as biomarkers of the interaction of hydrocarbon components of OSPW in targeted tissues. Investigating enzymatic pathways that are indicators of interaction with

OSPW components along with tracing the contaminants' (OSPW) peculiar components such as NAs (Young et al., 2008) and some elements (like nickel and vanadium), Agusa et al. (2004) provide information on which tissues were the main targets of OSPW components and the tissues' responses to OSPW contamination. These studies are particularly important as they illustrate the interaction of the contaminants with the targeted tissue at the molecular level, which is the first step in developing an AOP.

A major fraction of OSPW (i.e. NAs) has surfactant properties (Headley and McMartin, 2004). Chemicals with surfactant properties are well known for penetrating into and disrupting the structure of biological membranes (Kannel and Gan, 2012). Naphthenic acids cause narcosis and receptors malfunction by disrupting cell membrane fluidity, surface tension, and permeability (Gunatilleka and Poole, 1999; Frank et al., 2008). Histopathological studies show that all major components of OSPW (i.e. organics and metals) provoke structural responses in different tissues of exposed animals (e.g. Nero et al., 2006; Corbett et al., 2014; Martins et al., 2016). Thus, histopathological and physiological investigation of exposed organisms could contribute towards an understanding of the mechanisms by which OSPW induces toxicity to aquatic animals.

Despite the significant number of studies on OSPW toxicity, many aspects related to the effects of OSPW on aquatic organisms are still unknown. For instance, many sublethal effects of OSPW and the mechanism(s) of effects to sensitive indicator species are not well understood. A fully intact chemosensory system is vital for fish survival as it plays a fundamental role in regulating important behaviours (Kasumyan, 1999; Tierney et al., 2010). Except for a few specially adapted species like flounders, the olfactory organs occur as a pair of chambers on the dorsal surface of the head, in front of the eyes (Figure 1.2A)

(Cox, 2008). Utilizing three classes of olfactory sensory neurons (OSN), microvillus, ciliated, and crypt cells (Figure 1.2B), the fish olfactory system plays a key role in foraging, mating, and detecting predators in their immediate surroundings (Døving, 1991; Hamdani and Døving, 2007; Dew and Pyle, 2014). Olfactory sensory neurons are bipolar neurons that interact with odorants at the apical surface of the olfactory epithelium. Sensory cilia or microvilli blanket the epithelial surface. Odour molecules bind to olfactory receptors on the OSNs, which produces an action potential that travels along the axon and propagates to the central nervous system via the olfactory bulb (Buck and Axel, 1991; Hamdani and Døving, 2007). Odorant molecules bind to a class of G protein-coupled receptors (GPCRs), known as olfactory receptors (ORs), at the apical surface of the OSN. This binding initiates a molecular signal transduction cascade that results in the production of second messengers cyclic AMP or inositol 1,4,5-phosphate (Jones and Reed, 1989). The net result of this signal transduction cascade is the depolarization of the sensory neuron leading to the production of an action potential (Jones and Reed, 1989). In the olfactory bulb, axons from each OSN sub-class (i.e. microvillus, ciliated, and crypt cells) sort on the basis of cell type and synaps to corresponding secondary neurons, making three nerve bundles—each carrying different information—that enter and terminate in different regions of the brain (Hamdani and Døving, 2007, Figure 1.2).



[1] Figure 1.2. Olfactory system of fish: A) Overview of the structure and the position of fish olfactory system and olfactory tract to the brain. Left inset picture is a scanning electron microscopy image of the olfactory rosette. B) Drawing of the three olfactory sensory neurons (OSN) and their distinct parallel pathways from the olfactory rosette, via the olfactory bulb to the olfactory tract in the fish olfactory system. Crypt (red), microvillous (green), and ciliated (blue) cells synaps with the secondary neurons that make lateral olfactory tract (LOT), lateral part of medial olfactory tract (mMOT), and medial part of medial olfactory tract (lMOT), respectively.

[1] Reprinted from *Progress in Neurobiology*, 82, E.H., Hamdani, K.B. Døving, The functional organization of the fish olfactory system, 80-86, 2007, with permission from Elsevier.

Fish can perceive minute concentrations of odorants in the surrounding water, as the olfactory rosettes are exposed directly to the environment. Therefore, the OSN might easily get impaired or incapacitated by contaminants (Azizishirazi et al., 2013; Dew and Pyle, 2014; Sakamoto et al., 2016). If a fish cannot detect and appropriately respond to a potential toxicant in its environment, serious damage can occur to the fish olfactory system that may lead to partial or complete loss of olfactory function (Baldwin and Scholz, 2003; Tierney et al., 2010). This loss of olfactory function may have important consequences for the fish population given its reduced ability to find food (and grow), avoid predators (and survive), or reproduce. Thus, because the olfactory system and olfactory-driven behaviours have important implications for fish, and because the olfactory system is directly exposed to OSPW when a fish enters a contaminated water, chemosensory-mediated behaviours are ideal ecologically relevant endpoints.

In addition to the impacts of OSPW on olfactory acuity and function, investigating the effects of OSPW on olfactory signaling (i.e., chemical communication) is important for predicting the ecological risk of OSPW-contaminated waters to fish. The ability of the olfactory system to detect a substance and whether it is perceived as attractive or repulsive odorant in the central nervous system, mediates fish behavioural responses to that substance. A fish's ability to detect and respond to chemicals, including environmental contaminants, varies based on several factors, including the concentration of the chemical, which needs to be above sensory threshold concentrations, and the presence of appropriate olfactory epithelial receptors (Tierney, 2016). Normally, fish detect and avoid harmful chemicals; but in some cases, they either cannot detect the presence of a potential toxicant, or they can detect it but fail to avoid it. Sometimes, fish are even attracted to potentially

harmful compounds, which is an obviously maladaptive response (reviewed in Tierney, 2016). For instance, Saglio et al. (2001) demonstrated that goldfish are attracted to the herbicides nicosulfuron and bentazone, and the fungicide prochloraz. Sometimes, a fish's response to a chemical is concentration dependent. For instance, the behavioural response of rainbow trout to copper changes from avoidance at 6.4 µg/L to attraction at 330 µg/L (Giattina et al., 1982). Thus, if fish are indifferent or attracted to a contaminant, such as OSPW, they might be exposed to potentially harmful concentrations.

Although several studies have investigated the toxic effects of contaminants such as metals and pesticides (Tierney et al., 2010; Tierney, 2016), the effects of crude oil (a product of bitumen) and bitumen and their derivatives on the olfactory system of fish are not well studied. A behavioural study by Lari at al. (2015) demonstrated that 24 h exposure to the water-soluble fraction (WSF) of crude oil impaired the feeding behaviour of Caspian roach (*Rutilus caspicus*). The same study also demonstrated that Caspian roach could detect WSF of crude oil at 1 mg/L (Lari et al., 2015).

Rainbow trout (*Oncorhynchus mykiss*) is a highly sensitive species to environmental stressors and toxicants (Miller and Hontela, 2011). Moreover, its abundance in freshwater environments, worldwide distribution, and frequent use in ecotoxicology studies makes rainbow trout a suitable environmental bioindicator species (Ondarza et al., 2012). Rainbow trout is also globally recognised as one of the most important commercial and sport-fishing species. Although not native to the most parts of Alberta, rainbow trout is endemic to the Athabasca River, and live around oil sands mining areas of Northern Alberta (Allen et al., 2016), making it ideal for OSPW studies.

One of the freshwater invertebrate animals most used and recommended as a standard model species for a variety of ecological studies is the cladoceran, *Daphnia magna*. *Daphnia* spp. are among the most predominant lentic, primary consumers (Smirnov, 2013). Being grazers, these metazooplankters occupy an important trophic position by linking primary producers to secondary consumers (Allen et al., 1995). Thus, any disturbance in *Daphnia* health and population dynamics can affect the ecosystem. *Daphnia* spp. are easy to culture in the laboratory, have a short life cycle, and can be maintained at high population densities in relatively small volumes of medium (Martins et al., 2007). Among ecotoxicologists, *D. magna* is well known to be sensitive to a wide variety of chemical and physical contaminants that are commonly found in the aquatic environment (Villarroel et al., 2003; Lampert, 2006; Harris et al., 2012). They react to changes in their environment with a variety of physiological, anatomical, and behavioural responses (Michels et al., 2000; Nakari and Huhtala, 2008), and mortality in the case of severe toxicity (Taylor et al., 1998; Lari et al., 2017a). Because they are commonly used in ecotoxicology studies, a great deal of information is available on the toxicity of a broad range of contaminants on *Daphnia magna*. This abundance of knowledge on the toxicity of contaminants on *D. magna* provides an opportunity to compare the toxicity of OSPW with other major contaminants and more importantly compare OSPW with its own components such as PAHs (Sponza and Oztekin, 2011) and metals (Le et al., 2013; Lari et al., 2017a). Therefore, *D. magna* can effectively serve as a model for planktonic invertebrates for investigating the toxicity of OSPW.

Thesis Project

This thesis investigates the sub-lethal effects of OSPW on some sensitive and environmentally relevant biological endpoints in an invertebrate (i.e. *D. magna*) and a vertebrate (i.e. rainbow trout) indicator species. Conducting this study in a laboratory provides greater control than in the field owing to potential external influences, including variable temperature, sunlight, water quality, and animal life stages.

The thesis is broken into two sections: studies on *D. magna* and studies on rainbow trout. The objective of the first section was to first, investigate the effects of OSPW on *D. magna* feeding, respiration, and circulation; and second, to illustrate the manifestation of the toxic effects of OSPW on these systems on *D. magna* performance and fitness. The differences in the toxicity of OSPWs from various sources will also be investigated alongside the first objective. Thus, in the first part of this project the following questions will be answered.

1) Do acute and chronic exposure to OSPW affect food consumption of *D. magna*?

2) Can a technique be developed to measure the activity of feeding appendages, gut activity, and heart rate in *D. magna*?

3) What are the mechanisms by which OSPW affects feeding behaviour in *D. magna*?

4) What are the effects of OSPW on the cardiovascular system of *D. magna*, especially on respiration and circulation?

5) What are the effects of OSPW on growth, macronutrient reserves, and reproduction of *D. magna*?

The final objective of this section is to understand how some annual water quality characteristics alter the toxicity of OSPW to *D. magna*. To address this question, I will study and compare the effects of OSPW on growth and reproduction of *D. magna* under a cold- and a warm-season scenario.

During the first section of this project, techniques will be developed to measure the targeted endpoints. Specifically, a novel apparatus will be developed for measuring thoracic limb (feeding and respiratory appendages) activity, mandible (feeding appendages) rolling, heart beat rate, and peristaltic activity in *D. magna* in response to OSPW exposure. A behavioural maze for investigating chemosensory in *Daphnia* spp. will be developed for the first time. An optical respirometry method will be adopted to measure oxygen consumption. Several techniques will be used to study the biochemical and enzymatic changes in *D. magna* in response to exposure to OSPW.

The objective of the second section of this thesis—investigations on the effects of OSPW on rainbow trout—is to first, investigate the interaction of the olfactory system with OSPW and the olfactory-mediated behavioural manifestations of this interaction upon the first encounter. To that end, the ability of fish to detect and behaviourally respond to OSPW contamination will be investigated using electro-olfactography (EOG) and a choice maze. The instantaneous effect of OSPW on olfactory acuity and olfactory-mediated behaviours will also be studied using the same methods. The purpose of these experiments is to determine how a highly mobile organism, with a potential to flee from contaminated waters, respond to OSPW and estimate their chance to successfully avoid exposure to OSPW.

A second objective of this work was to find out the toxic effects of OSPW on olfactory functionality and to elucidate the mechanism of those effects. For mechanistic studies histology, biochemical and genetic techniques will be applied. Following AOP, I expect to illustrate the molecular interaction and the biological pathways that leads to OSPW toxicity on the olfactory system and olfactory-mediated behaviours.

Anticipated Significance

Generally, toxicity studies are usually restricted to one or two levels of biological organization, but usually at levels below the population level. Understanding these effects at the population level is important for understanding how these effects might translate to the ecosystem level. Ultimately, linking mechanistic studies to altered survivorship will help predict the effects of contaminant exposure on organisms' populations. This study focused on toxicological effects four levels of biological organization—molecular cellular, physiological, and behavioural—in an attempt to draw a causative pathway(s) between two far ends of OSPW toxicity (i.e. initiating event and the adverse outcome at the organism level). This approach of tracing contaminant toxicity from the molecular initiating event to its adverse behavioural outcome is limited not only for OSPW toxicity but also other contaminants' toxicity.

The toxicants associated with oil sands mining has been shown to have adverse biological effects on aquatic organisms, as mentioned above. Although acute lethality tests are useful for creating water quality guidelines to protect aquatic organisms against physiological death (i.e. mortality), 'ecological death' that may occur at much lower toxicant concentrations is largely ignored. Indeed, environmental contamination measured

in natural ecosystems often occurs at concentrations well below those causing significant mortality. Since behaviour serves as the link between physiological and ecological processes, it is ideal for studying environmental pollutant effects.

I anticipate that this study will introduce some sensitive endpoints to be used in future ecotoxicology studies for evaluating OSPW toxicity, remediation methods, and tools for monitoring aquatic ecosystems affected by industrial oil sands mining. While olfaction plays an essential role in some of the most important aspects of aquatic organism survival (feeding, predator avoidance, mating), the impacts of oil sands mining-related toxicants on olfactory function and associated behaviours are relatively unknown. Impairment of chemosensory-mediated behaviours may influence the survival of the individuals and ultimately, the population. Considering that the olfactory system is constantly exposed to the external environment, it is at risk of exposure to contaminants. Consequently, we expect that the results from this project will provide ecologically relevant endpoints for studying the effects of OSPW on aquatic organisms.

A considerable number of studies have focused on the effect of environmental variables (hardness, pH, temperature, etc.) on the toxicity of contaminants. These studies investigated the effect of each element separately and showed that water quality variables affected the toxicity of many contaminants. In this study, I aim to expand on previous studies that demonstrate that water quality variables change the toxicity of contaminants, but from a more ecologically relevant perspective. Therefore, in our study we consider several water quality variables as they co-vary seasonally, in order to mimic water quality changes found in natural environment. Using this novel approach, we will be able to

determine how the toxicity of OSPW on aquatic organisms in the Athabasca River fluctuates throughout a year.

CHAPTER 2: Determining the effect of oil sands process-affected water on grazing behaviour of *Daphnia magna*, long-term consequences, and mechanism[2]

Abstract

Oil sands process-affected water (OSPW) is a byproduct of the extraction of bitumen in the open pit-mining oil sands industry and is currently stored in on-site tailings ponds. OSPW from three oil sands companies were studied to capture some of the variability associated with OSPW characteristics. To investigate the effect and mechanism(s) of the effect of OSPW on feeding behaviour, *D. magna* were exposed to low OSPW concentrations for 24 h and monitored for their feeding rate, olfactory response and swimming activity. The Al and Si content, which are indicators of suspended particulate matter in *D. magna* exposed to OSPW were investigated using energy-dispersive X-ray (EDX) spectroscopy. In long-term experiments, effects of exposure to OSPW for 21 days on feeding behaviour, growth, and reproduction of *D. magna* were evaluated. Feeding rates were similar among the three exposure populations, yielding a 24-h IC50 of 5.3% OSPW. Results of behavioural assays suggest that OSPW impairs the chemosensory function and reduces the total activity of *D. magna.* In EDX spectroscopy, Al and Si were detected in the body of the exposed *D. magna,* suggesting that *D. magna* filter clay particles from the OSPW solution. Results of the long-term exposure showed that OSPW significantly inhibits feeding behaviour, suppresses growth, and reduces reproductive output of *D.*

[2] A version of this chapter is published in *Chemosphere*, 146, Lari, E., Wiseman, S., Mohaddes, E., Morandi, G., Alharbi, H., Pyle, G.G., Determining the effect of oil sands process-affected water on grazing behaviour of *Daphnia magna*, long-term consequences, and mechanism, 362-370, 2016, with permission from Elsevier.

magna. There were no differences in the toxicity of the three samples of OSPW, which was in agreement with the fact that there were no differences in the species of dissolved organic compounds in the OSPW samples.

2.1. Introduction

The oil sands region of northern Alberta, Canada, encompasses a large deposit of bitumen in the Peace River, Athabasca, and Cold Lake areas (ERCB, 2010). It has been estimated that under a scenario of moderate growth the production of crude oil from raw bitumen will grow to about 3.95 million barrels per day in 2030 (CAPP, 2015). Rapid growth in oil sands extraction activities in this area has increased concern about the potential environmental impacts related to air quality, water use, wastewater production, groundwater contamination, and habitat disturbances in the surrounding ecosystems (Dowdeswell et al., 2011; He et al., 2012a; He et al., 2012b).

In the surface mining industry, extraction of bitumen from oil sands requires a large volume of fresh water. In the "Clark extraction process" oil sand is mixed with hot water and sodium hydroxide to separate bitumen from other constituents including clay and sand (Kavanagh et al., 2009). For each 1 m^3 of synthetic crude produced by surface mining, 11 t of oil sands are mined, 2.5 m^3 of water is used (80% recycled from tailings pond), and 3.3 m^3 of liquid fine tailings (LFT) that contains solids residual sand and clay, unrecoverable hydrocarbons, and oil sands process-affected water (OSPW) discharged to tailings ponds. Over time, the LFT dewater resulting in 2 m^3 mature fine tailings (MFT) and an aqueous layer of OSPW (Han et al., 2009; RSC, 2010).

Industrial operators are not permitted to discharge water affected by bitumen processing back into the Athabasca River (Giesy et al., 2010). Consequently, all OSPW is stored in on-site tailings ponds that cover an area of about 130 km^2, containing approximately 720 million m^3 of tailings that are 1–3% bitumen, 20–30% sand-silt-clay, and 70–80% water (Del Rio et al., 2006; RSC, 2010). The massive quantity of OSPW being stored on-site has raised public concern, which has led the Alberta Energy Regulator to propose targets for a reduction in the volume of liquid tailings stored in tailings ponds (AER, 2015). Consequently, companies will eventually be responsible for the remediation and release of OSPW to the natural environment. This mandate presents a major challenge for the industrial and academic communities because such a release would need to minimize the impact to the receiving environment. Thus, the release of OSPW requires knowledge of its chemical composition and characteristics, toxicity to resident biota, and behaviour in aquatic ecosystems, along with good knowledge of the characteristics of the ecosystem into which it is being released.

Oil sands process-affected water is a complex mixture of organic and inorganic chemicals (Grewer et al., 2010; Lengger et al., 2013). The dissolved organic fraction of OSPW is recognized as the main driver of acute toxicity, as aging, or treatment with activated charcoal or ozonation significantly attenuates or removes toxic effects (Anderson et al., 2012b; He et al., 2012b; Klamerth et al., 2015). However, the specific compounds responsible for the acute aquatic toxicity remain largely unknown (Frank et al., 2008; Klamerth et al., 2015). Of particular interest have been naphthenic acids (NA), a group of compounds defined as cyclic and alkyl-substituted carboxylic acids fitting the general formula $C_nH_{2n+Z}O_2$, where n is the number of carbons and Z is zero or an even negative

integer representing the hydrogen deficiency due to rings or double bonds, (Headley and McMartin, 2004) due to their elevated concentrations and aquatic toxicity (Frank et al., 2008; Scarlett et al., 2013). However, with the application of ultrahigh resolution mass spectrometry (uHRMS) it has been demonstrated that in addition to NA, the organic fraction of OSPW contains a wide variety of nitrogen and sulphur containing compounds as well as alkylated PAHs (alkyl-PAHs), terpanes, steranes, bicyclic sesquiterpenes, and diamondoids (Yang et al., 2011; Headley et al., 2013; Pereira et al., 2013a). Additionally, OSPW is enriched in elements such as nickel (Ni), vanadium (V), aluminum (Al), iron (Fe), and chromium (Cr) (Melita and Gumrah, 2010; Debenest et al., 2012).

It has been shown that OSPW is both acutely and chronically toxic to a variety of aquatic organisms (e.g. Hersikorn et al., 2010; He et al., 2012b; Scarlett et al., 2013). Studies on the toxicity of OSPW to invertebrates mainly investigate survival (Puttaswamy et al., 2010; Wiseman et al., 2013a), growth (Anderson et al., 2012a; Goff et al., 2013), and biochemical markers (Wiseman et al., 2013a). However, many aspects of effects of OSPW on aquatic organisms are still unknown. Consequently, there is a need for the establishment of biomarkers and endpoints indicative of exposure to OSPW and its adverse effects, such that the effects of accidental spills and monitoring detoxification of OSPW in end pit lakes can be diagnosed using species native to the Athabasca region.

The cladoceran, *Daphnia magna (D. magna)*, is a freshwater species commonly used as a standard bioindicator in a variety of ecological studies. This zooplankton grazer is easy to culture in the laboratory, has a short life cycle, and can be maintained at high population densities in relatively small volumes of media (Martins et al., 2007). The species *D. magna* is well known to be sensitive to many contaminants and physical threats that are commonly

found in the aquatic environment, and they can respond to these substances with a variety of physiological, anatomical and behavioural responses (Michels et al., 2000; Nakari and Huhtala, 2008). Based on their versatility in ecotoxicology studies, a great deal of information on the toxicity of a broad range of contaminants to *D. magna* has been generated, and these characteristics make them an attractive model for OSPW studies.

Chemosensory systems play an important role in the survival of aquatic animals (Tierney et al., 2010; Lari et al., 2015). *Daphnia* rely on their chemosensory system to locate food and avoid predators (Hunter and Pyle, 2004; Lovern et al., 2007). Consequently, impairment of chemosensory function and related behaviours of *Daphnia* threatens their chances of survival. Several studies have shown that different contaminants affect feeding (e.g. Villarroel et al., 1999; Ferreira et al., 2008) and predator avoidance (e.g. (Hunter and Pyle, 2004; Lovern et al., 2007; Mirza and Pyle, 2009) behaviours of *Daphnia*.

The aim of the present study was to characterize toxicity thresholds of OSPW on survival and feeding behaviour (olfactory related behaviour) of *D. magna* in order to define the concentrations of OSPW that can potentially impair chemosensory function, as well as the population (growth and reproduction) consequences of such an impairment. In order to address these questions, lethality and sub-lethal effects on feeding behaviour were investigated. Short-term experiments were followed by a chronic experiment in which the effect of OSPW on feeding, reproduction, and growth of *D. magna* was studied. In order to illustrate the mechanism of reduced feeding behaviour, three potential causes (impairment of olfactory function, reduction in total activity, and filtering of clay particles in OSPW solution) were investigated. An olfactory choice maze was used to investigate the effect of

olfactory impairment and changes in total activity on feeding behaviour. Aluminum (Al) and silica (Si) contents of *D. magna* body were tracked as indicators of consumed clay.

2.2. Materials and methods

2.2.1. Test-chemicals

Three samples of OSPW were used for this study. Samples were provided by three major oil sands companies in the region and will be referred as A-OSPW, B-OSPW, and C-OSPW, respectively. All samples were stored in 20 L plastic buckets at 4 °C. Approximately 100 mL of each sample of OSPW was sent to SGS Canada Inc. (Lakefield, Ontario, Canada) to measure the total recoverable concentrations of vanadium (V), nickel (Ni), copper (Cu), cadmium (Cd), and zinc (Zn) by use of inductively coupled plasma mass spectrometry (ICP-MS). Briefly, the samples were digested with HNO_3 and analyzed by ICP-MS. The samples were analyzed against 2% HNO_3 standardization materials.

2.2.2. Hydrocarbon analysis

A liquid-liquid extraction protocol was used to isolate the water-soluble organic fraction from one liter (1 L) of each OSPW. First, samples were filtered through a 0.7 µm glass microfiber filter to remove any suspended particulates (Fischer Scientific, Ottawa, ON, Canada). Then, the pH of the samples was adjusted to a pH of 2 by use of concentrated sulphuric acid (Fisher Scientific, Fair Lawn, NJ, USA), and extracted three times with 100 mL of dichloromethane. The organic phases were combined and evaporated to approximately five mL, transferred to a pre-weighed glass vial and blown to dryness using nitrogen gas. The dry extract was re-suspended in methanol.

The profile of chemicals in samples was analyzed by use of a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) as described previously (Pereira et al., 2013a), interfaced to a Dionex UltiMate 3000 ultrahigh-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific). Chromatographic separation was performed on a Cosmosil C18 MS-II column (100 × 3.0 mm, 2.5 μm particle size; Nacalai USA, San Diego, CA) at 40 °C. The flow rate was 0.5 mL/min, and the injection volume was 3 μL. The mobile phases were 0.1% (v/v) acetic acid in water (Solvent A) and 100% methanol (Solvent B). The mobile phase composition was 5% solvent B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min, followed by a 4 min hold prior to the next injection. Mass values were restricted to singly charged ions with a molecular mass of 100-500 Da and signal to charge ratios greater than 3. Elemental composition was based on the accurate mass *m*/*z* by matching the expected/theoretical mass with the observed mass of an ion within 5 ppm and the mass defect of the constituent atoms. Species detected in each fraction were binned according to heteroatom empirical formula classes in negative (-) or positive (+) electrospray modes: $O_x^{+/-}$ (where x = 1-6), $NO_x^{+/-}$ (where x = 1-4), $SO_x^{+/-}$ (where x = 1-4), or $NO_xS^{+/-}$ (where x = 1-2). Relative abundance of each chemical class (i.e. O_x, SO_x, NO_x) in samples was summed over all masses detected.

2.2.3. Test animals

All experiments were carried out with *D. magna* cultured under laboratory conditions. Cultures were maintained in 1 L glass beakers with 1 L of moderate hard water (100 mg/L as $CaCO_3$) that was renewed once a week, and fed daily with *Raphidocelis subcapitata* and also with a combination of yeast, CerophyllTM, and trout chow (YCT). About ten

daphniids per beaker were kept in a 16:8 h light:dark cycle regime and at 20±1°C. Neonates from *D. magna* that were three to five weeks of age were used for bioassays or to produce the next generation. A reference LC50 test with sodium chloride (NaCl) was performed every month to test the sensitivity of the culture. Daphniids that were less than 24 h of age were separated from the main cultures to other culture beakers and maintained under the same conditions until 6 days of age (referred to subsequently as one-week-old). The water that was used for culturing of *D. magna* was used for diluting the OSPW and as the control water in all experiments.

2.2.4. Acute lethality assay

In this experiment 10 daphniids (one-week-old) were exposed to 200 mL of different concentrations of OSPW (25, 50, 75, and 100%) and culture water as a control in 250 mL glass beakers, for 48 h. The number of dead individuals was recorded at the end of the 48-h period, and median lethal concentration (LC50) of each OSPW was calculated. Each treatment (different concentrations and OSPW samples) was replicated three times (n = 3).

2.2.5. Short-term feeding assay

In each trial, 6 groups of 5 daphniids (one-week-old) were exposed to a geometric dilution series of five concentrations of each sample of OSPW (1.25, 2.5, 5, 10, and 20%) and culture water as a control for 24 h. At the beginning of this period, they were fed a fixed ration of *R. subcapitata* (5×10^5 cell/L). To prevent algae from growing and reproducing during the test, all test samples were kept in complete darkness throughout the trial. At the end of the exposure, daphniids were removed, and the remaining water in each replicate was vigorously shaken to re-suspend any settled *R. subcapitata* cells. To

determine the amount of food consumed by *D. magna* the density of the remaining algae cells was determined three times by counting under a light microscope using a Neubauer chamber. The concentration of OSPW required to inhibit food consumption by 50% in 24 h (i.e., 24-h IC50) was estimated. Each trial was replicated 5 times. The effect of all three OSPW samples on feeding behaviour of *D. magna* was tested using this method.

2.2.6. Long-term exposure

To study the long-term effect of OSPW on *D. magna* (i.e. feeding rate, growth, and reproduction rate) 3 groups of 5 neonates *of D. magna* were exposed for 21 d to two dilutions (1 and 10%) of each OSPW (A-OSPW, B-OSPW, and C-OSPW) and culture water as a control. Daphniids were exposed to solutions in 250 mL beakers containing 200 mL of solution. The whole solution in every beaker was renewed with the same solution and the dead daphniids were counted and removed, on a daily basis. In order to investigate the effect of test solutions on the reproduction of *D. magna*, the number of neonates in every test beaker was counted and removed every day. The short-term (24 h) feeding test, as described in section 2.2.3, was also carried out on all groups at 7, 14, and 21 d of exposure (the feeding test was carried out in exposure beakers). After 21 days, all daphniids were euthanized by freezing. Dead daphniids were dried in an oven at 60°C for 48 h, and dry mass was determined as an indicator of produced biomass.

2.2.7. Choice maze

The preference of *Daphnia* to the food cue (filtered YCT) was investigated using a flow-through Y-maze. Arms of the maze were 75×12×25 mm and the discharge area was 75×25×25 mm in dimension. In order to align the water flow in the arms, a mesh net and a

perforated polystyrene plate were placed 7 and 15 mm, respectively, from the solution input at the distal end of each arm (Figure 2.1). The solution delivery system contained two tanks with capacities of 1 L that were placed 1 m above the tank and each was attached to one of the arms via a plastic tube. An adjustable clamp was used to control the flow in the corresponding arm.

In order to discharge solutions from the maze, two nested polystyrene pipes were used. The outer pipe had a 16-mm diameter with a 25-mm length and the inner pipe had an 8-mm diameter with a 20-mm length. The outer pipe had 5 semicircular grooves to pass the solution into the pipe and drain through the inner pipe. This system was applied to prevent whirl pooling at the discharge end. To prevent the test animal from entering the discharge area, its route was blocked by a plankton mesh. A food dye test showed that a chemosensory stimulus applied to the distal end of a maze arm could reach the drainage area within 10 s and mixing was minimal at the junction. Based on this test, the maze space was considered as two areas, A and B (Figure 2.1).

The 10 mm of the mid area of the maze closest to the mesh net that separated the discharge area was considered as the light trap section (i.e. start area; Figure 2.1). In order to keep the test animals in the start area, the whole maze was covered with a dark paperboard box except for the light trap area. The light trap area was lit by a white light placed 10 cm above the maze. For each test one *Daphnia* was gently placed in the start area and was given 10 min to acclimate. Immediately after the acclimation period, water was allowed to flow in both arms for 3 min. The water flow in one arm (randomly chosen) contained the food cue. Behavioural avoidance or preference to the chemosensory cue was estimated by the amount of time spent in each area (A or B) of the maze.

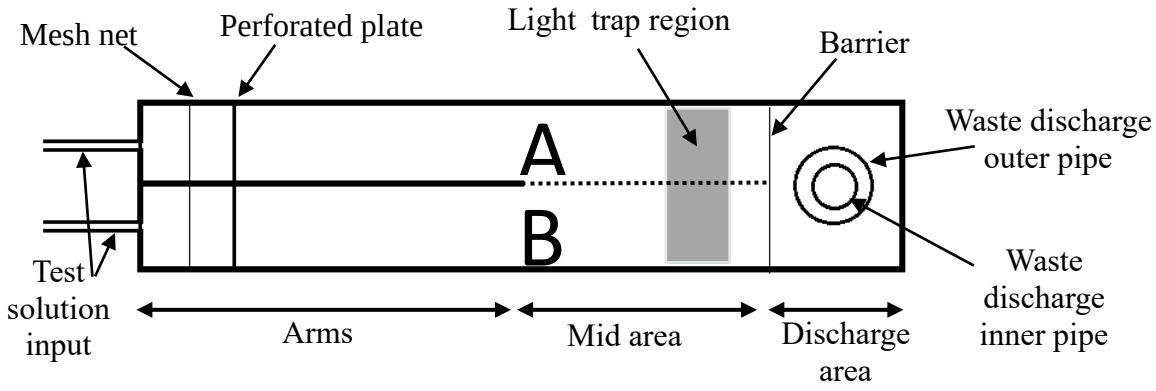


Figure 2.1. Choice maze apparatus. Test solutions are delivered through each of two input ports, where one input is a control and the other is a chemosensory stimulus. Each stream (A and B) is separated by a chemically impermeable barrier, in order to maintain a separation between the two chemosensory streams. Laminar flow is achieved by the flow alignment system. Dye trials demonstrate that laminar flow continues beyond the central impermeable barrier through to the waste discharge system. During a trial, animals are held within a light trap to acclimate to maze conditions. The test is initiated after chemosensory cues are injected into each arm, after which the entire apparatus is exposed to light allowing the test subjects to make a choice.

To estimate the avoidance/preference response, the following equation was used to convert the data to positive (attraction) and/or negative (avoidance) percentage (Lari et al., 2015):

$$\boldsymbol{Rv} = ((\mathbf{T_c} - \mathbf{T_W})/ (\mathbf{T_c} + \mathbf{T_W})) \times 100$$

Where ***Rv*** represents reaction value and $\mathbf{T_c}$ and $\mathbf{T_W}$ represent the time spent in arm channels supplied with the test olfactory cue and culture water, respectively. A positive ***Rv*** indicated a preference response, while negative ***Rv*** indicated an avoidance response. Active swimming of specimens during the test was timed and considered as an indicator of total activity. Each individual was used in the experiment only once. At the end of each set of trials, the whole system was washed thoroughly with water.

2.2.8. Clay consumption test

In each trial, six daphniids (one-week-old) were exposed to a geometric dilution series of five OSPW concentrations (1.25, 2.5, 5, 10, and 20%) and culture water and filtered OSPW (20% concentration) as controls for 24 hours. A mixture of equal volumes of the three OSPWs was used for this experiment. At the end of the exposure, *Daphnia* were removed and rinsed with distilled, de-ionized water (ddH_2O). The carapace and antennae were removed, and the remaining soft parts were pressed onto a scanning electron microscope (SEM) mount in order to expose the contents of the *Daphnia*'s gut. In order to prevent the interference of metal mounts in the reading from the samples, 18 mm carbon mounts (TED PELLA, INC., USA) were used. The presence of Al and Si in the samples was determined by energy-dispersive X-ray spectroscopy (EDX) using a tabletop SEM (TM-1000, Hitachi, Japan). Using a vacuum filtration system, 20 mL of the mixture OSPW

was filtered through a 0.45 µm cellulose nitrate membrane (Whatman, Germany). The membrane was dried in an oven (Isotemp, Fisher, USA) at 60 °C for 24 h. Afterward, a round piece (18 mm) of the membrane was cut and mounted on an 18 mm carbon mount. The EDX reading of the residuum of filtering OSPW was used as a reference for the clay particle.

2.2.9. Data analysis

The probit method was used to estimate LC50 and IC50 values. An analysis of covariance was performed in order to compare the toxic effect of the three different OSPW samples on the feeding rates of *D. magna*. In order to find the lowest observed effect level (LOEL), a one-way analysis of variance followed by a Dunnett's post-hoc test was applied. The long-term effect of each OSPW sample on feeding behaviour was analyzed using a repeated measures general linear model. The long-term effect of OSPW on reproduction and growth was determined by one-way analysis of variance. The results of the choice maze test (chemosensory and total activity) were also analyzed using a one-way analysis of variance. The differences between the groups were tested by Dunnett's post-hoc test. The LC50 and IC50 data were analyzed using the dose response curve (drc) package in R, version 3.2.1 (R Core Team, 2015) and the rest of the statistical analyses were done using IBM SPSS 22 software (IBM, USA).

2.3. Results

2.3.1. Chemical characteristics of the OSPWs

Concentrations of Ni, V, Zn, Cd, and Cu in each sample of OSPW are given in Table 2.1. The overall ionic composition of the three OSPW samples was similar. Amongst the targeted elements, V had the highest and Cd had the lowest concentrations of the analyzed metals. Distributions of chemical classes of dissolved organic compounds in the samples of OSPW are shown in Figure 2.2. In general, the profiles of chemical classes were similar. When detected by use of negative electrospray ionisation (ESI^-), oxygen-containing species (O_x^-) were present at the greatest relative concentrations compared to sulphur (SO_x^-) and nitrogen (NO_x^-) containing species across all samples. In samples from C-OSPW and A-OSPW the relative abundance of di-oxygenated (O_2^-) species (naphthenic acids, NAs) was greater than the tri- (O_3^-) and tetra- (O_4^-) oxygenated species. Conversely, in the sample from B-OSPW the relative abundance of tetra-oxygenated (O_4^-) species was greater than di- (O_2^-) and tri- (O_3^-) oxygenated species. Mono-oxygenated species (O^-) were detected in the A-OSPW at a low abundance but were less than the detection limit in samples of B-OSPW and C-OSPW. Sulphur containing species were detected in all samples, and SO_2^- were the most abundant species. Negligible amounts of nitrogen-containing species were detected in each sample by use of ESI^-.

Table 2.1. Mean trace metal concentrations of the OSPW samples (n = 3).

	Metals (µg/L) ± SD				
	Ni	V	Zn	Cu	Cd
A-OSPW	21.1 ± 0.1	26.9 ± 0.1	14.0 ± 0.1	5.29 ± 0.10	0.16 ± 0.01
B-OSPW	21.7 ± 0.1	27.6 ± 0.2	26.0 ± 0.2	11.70 ± 0.15	0.15 ± 0.01
C-OSPW	12.0 ± 0.1	16.7 ± 0.2	17.0 ± 0.1	4.09 ± 0.06	0.09 ± 0.01
OSPW mix	17.1 ± 0.3	20.2 ± 0.2	19.0 ± 0.2	7.3 ± 0.1	0.11 ± 0.02

When scanned by use of positive electrospray ionisation (ESI^+), the relative abundance of oxygen-containing species (O_x^+) was greater than sulphur (SO_x^-) or nitrogen (NO_x^-) containing species in each sample. Di-oxygenated species (O_2^+), which are not NAs but might be dihydroxy, diketo, or ketohydroxy compounds (Pereira et al., 2013) were most concentrated in the samples from B-OSPW and C-OSPW, while tri-oxygenated species (O_3^+) were greatest in the sample from A-OSPW. The relative abundance of sulphur containing species was greater than nitrogen-containing species in the samples from A-OSPW and B-OSPW. However, in the sample from C-OSPW the opposite was true as the relative abundance of nitrogen containing species was greater than sulphur containing species.

2.3.2. Acute toxicity test (LC50)

Values of LC50 could not be determined for the samples of OSPW. Acute lethality of *D. magna* exposed to 100% concentrations of A-OSPW, B-OSPW, and C-OSPW was 23.3%, 36.7% and 33.3%, respectively (Appendix 2.1). These results demonstrate that the LC50 of the OSPW samples was greater than 100% concentration of the OSPWs.

2.3.3. Short-term feeding test

The results of the acute feeding bioassays are presented in Figure 2.3. There were no differences among samples in effects on feeding [$F(2, 37) = 0.33$, $p = 0.72$], and the interaction between concentration and sample of OSPW was not significant [$F(2, 54) = 0.48$, $p = 0.62$; Figure 2.3]. As a result, the IC50 of all three OSPW samples was calculated at 5.34% (SE ± 0.07%; Appendix 2.2).

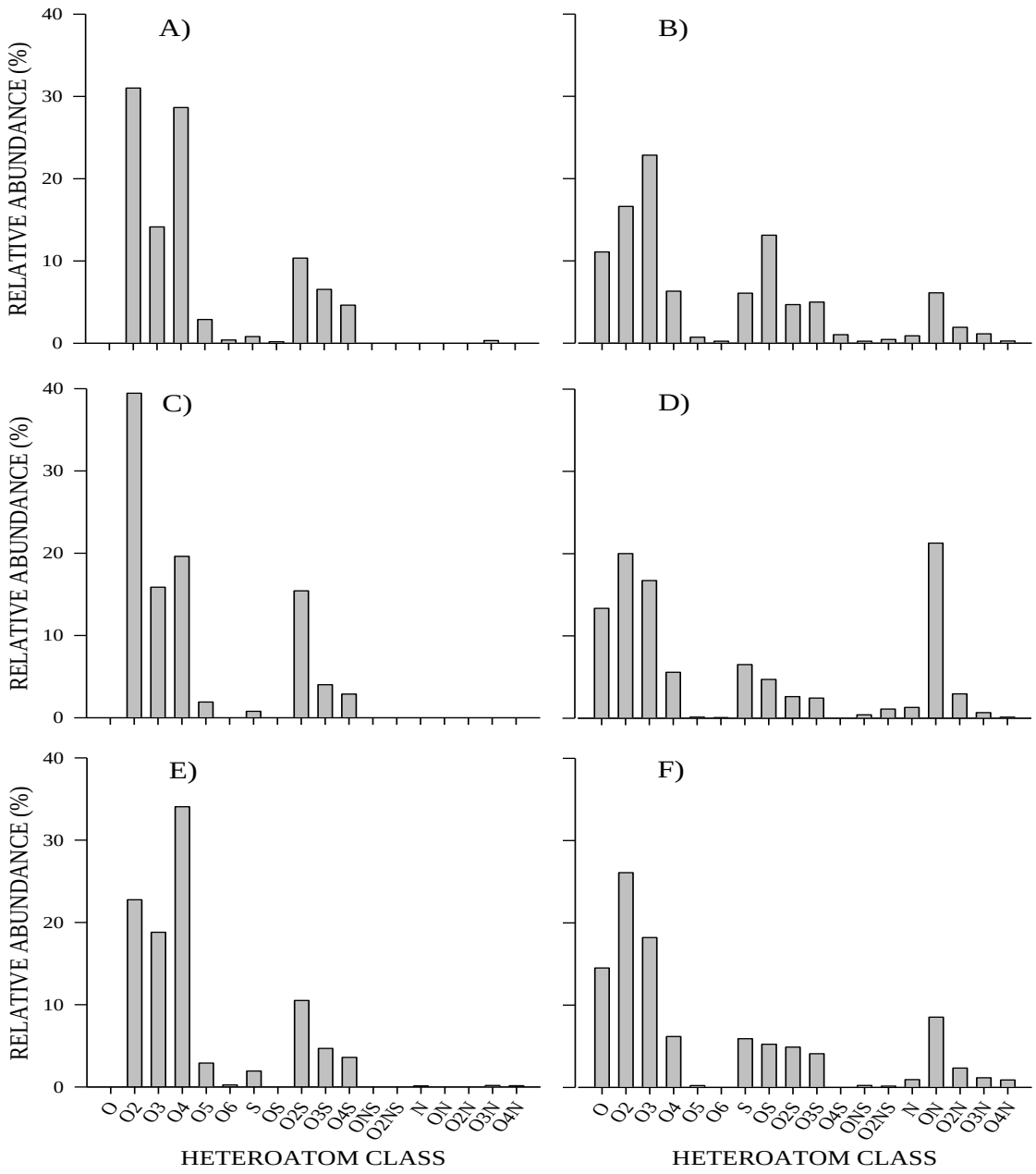


Figure 2.2. Relative abundance of dissolved organic species by heteroatom class in the acid extractable fraction of OSPW samples. A) A-OSPW in ESI-, B) A-OSPW in ESI+, C) C-OSPW in ESI-, D) C-OSPW in ESI+, E) B-OSPW in ESI-, F) B-OSPW in ESI+.

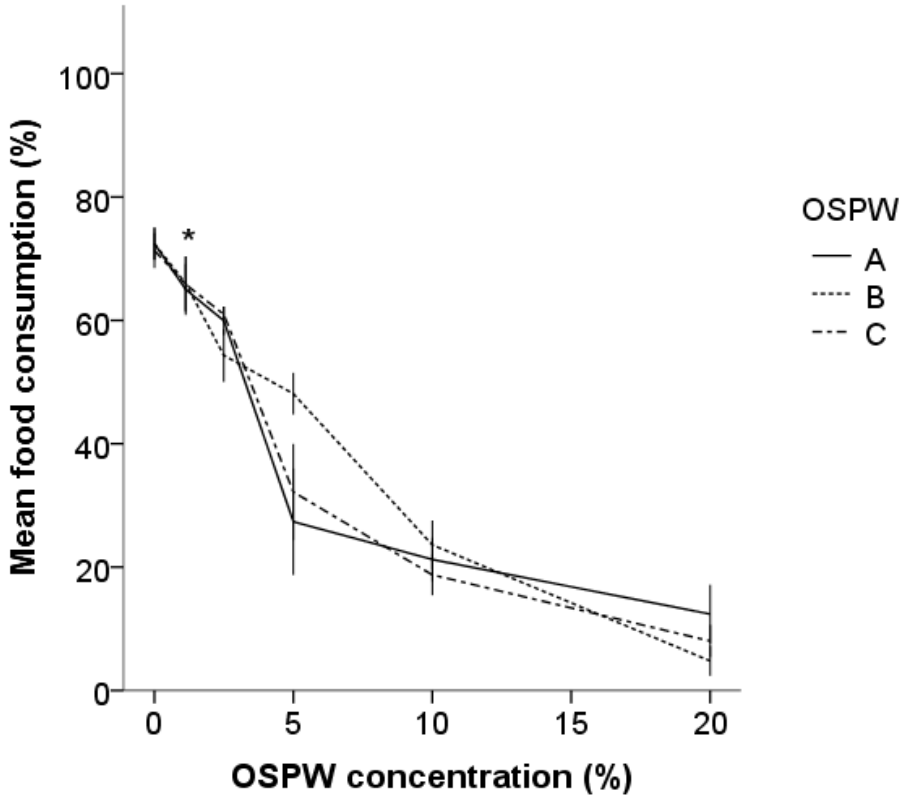


Figure 2.3. Effect of OSPWs on grazing behaviour of *Daphnia magna*. Asterisks (*) shows lowest observed effect level (LOEL). n = 5, Error bars: ± 2 SE.

2.3.4. Long-term exposure

The results of the chronic exposure showed that all three OSPW samples at both concentrations (1 and 10%) reduced the feeding behaviour of *D. magna*: A-OSPW [F (2, 33) = 99.74, $p < 0.001$], B-OSPW [F (2, 33) = 39.07, $p < 0.001$], and C-OSPW [F (2, 33) = 42.41, $p < 0.001$; Appendix 2.3]. The feeding rate of *D. magna* exposed to 10% of either OSPW was more significantly reduced than the feeding rate of individuals exposed to 1% OSPW.

Reproductive capacity of *D. magna* exposed to 1 and 10% concentrations of either sample of OSPW were significantly reduced: A-OSPW [F (2, 9) = 27.87, $p < 0.001$; Figure 2.4], B-OSPW [F (2, 9) = 25.33, $p < 0.001$; Figure 2.4], and C-OSPW [F (2, 9) = 54.71, $p < 0.001$; Figure 2.4; Appendix 2.4]. The growth of *D. magna*, exposed to either 1 or 10% concentrations of either sample of OSPW were significantly reduced: A-OSPW [F (2, 9) = 22.45, $p < 0.001$; Figure 2.5], B-OSPW [F (2, 9) = 13.87, $p < 0.001$; Figure 2.5], and C-OSPW [F (2, 9) = 28.82, $p < 0.001$; Figure 2.5] over the 21-d period (Appendix 2.4).

2.3.5. Choice maze

The results of the choice maze bioassay showed that exposure to concentrations of 5% and greater of the mixture of OSPW significantly impaired the food cue detection by *D. magna* [F (6, 133) = 4.47, p < 0.001; Figure 2.6; Appendix 2.5]. The total activity of *D. magna* was significantly reduced after exposure to 10 and 20% concentration of the mixture of the samples of OSPW [F (6, 133) = 7.22, p < 0.001; Figure 2.7; Appendix 2.5].

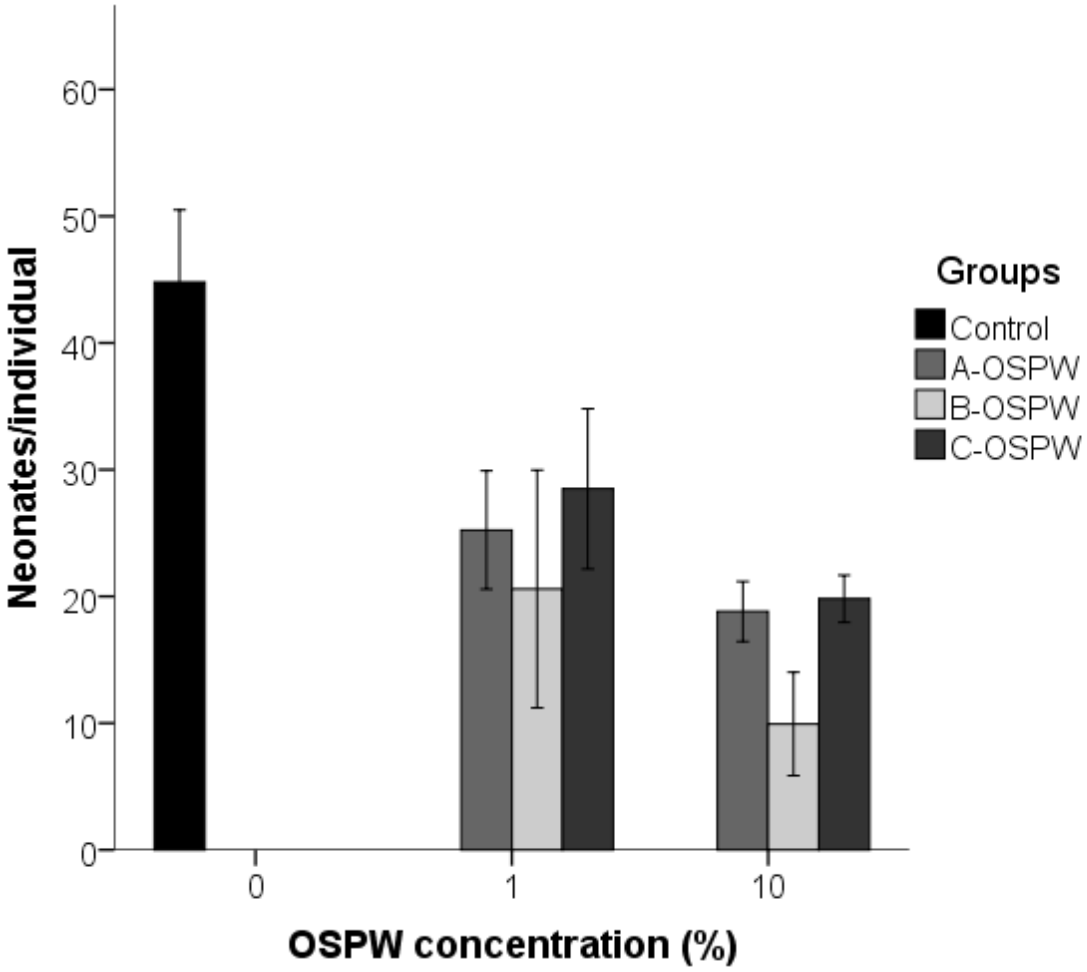


Figure 2.4. Long-term (21 d) effect of OSPWs on reproduction rate of *Daphnia magna.* A) A-OSPW, B) B-OSPW, C) C-OSPW. Asterisks (*) show significant difference with the control group. n = 4, error bars: ± 2 SE

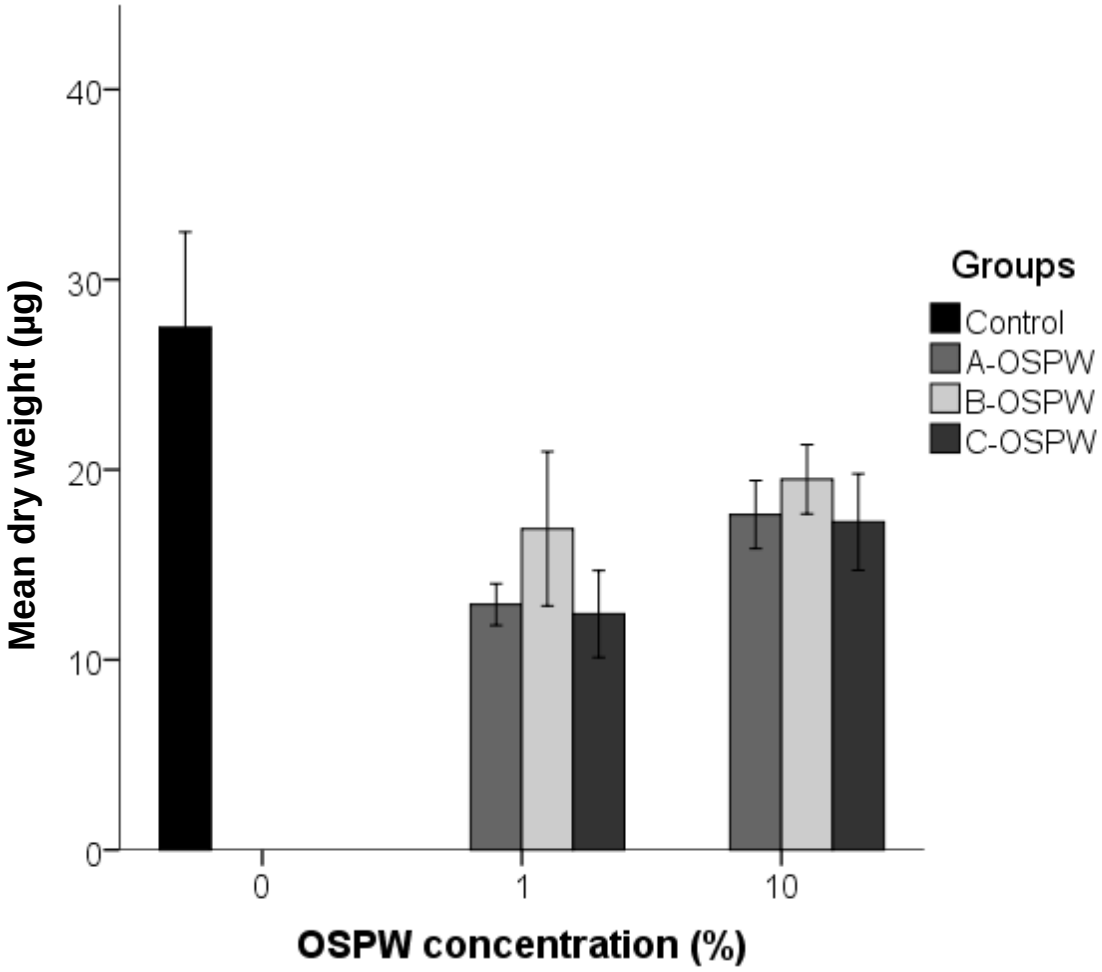


Figure 2.5. A) Long-term (21 d) effect of OSPWs on the growth rate of *Daphnia magna*. A) A-OSPW, B) B-OSPW, C) C-OSPW. Asterisks (*) show significant difference with the control group. n = 4, error bars: ± 2 SE

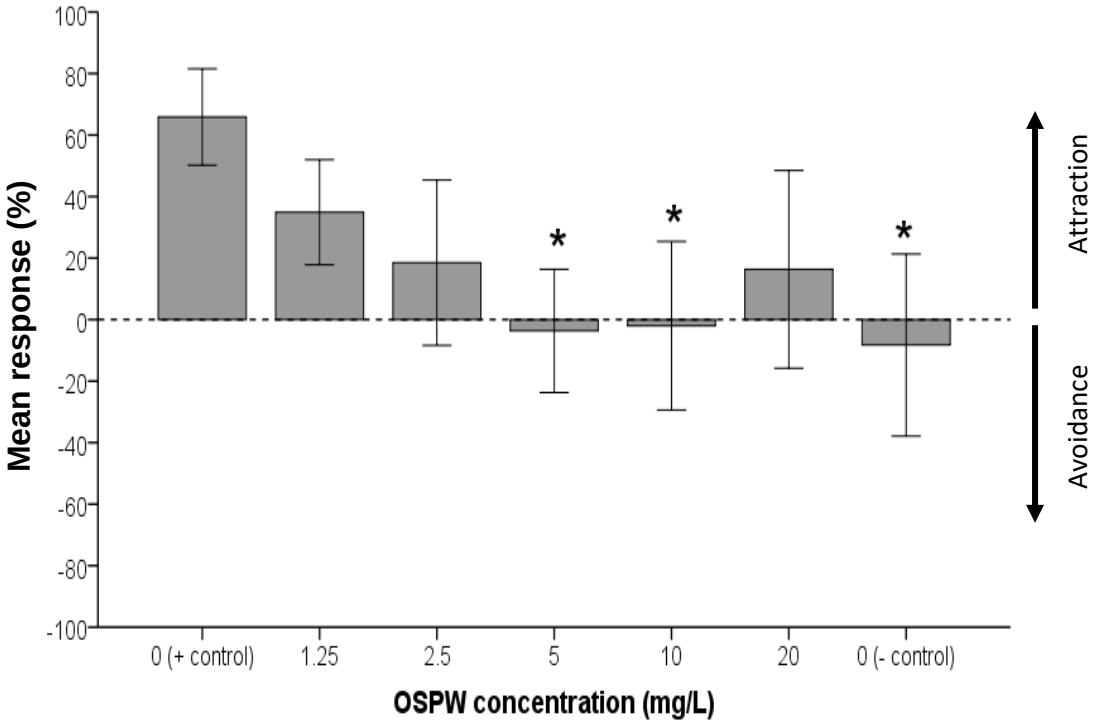


Figure 2.6. Short term (24 h) effect of an OSPW mixture on the response of *Daphnia magna* to food cue. Asterisks (*) show significant difference from the control group. n = 20, error bars: ± 2 SE.

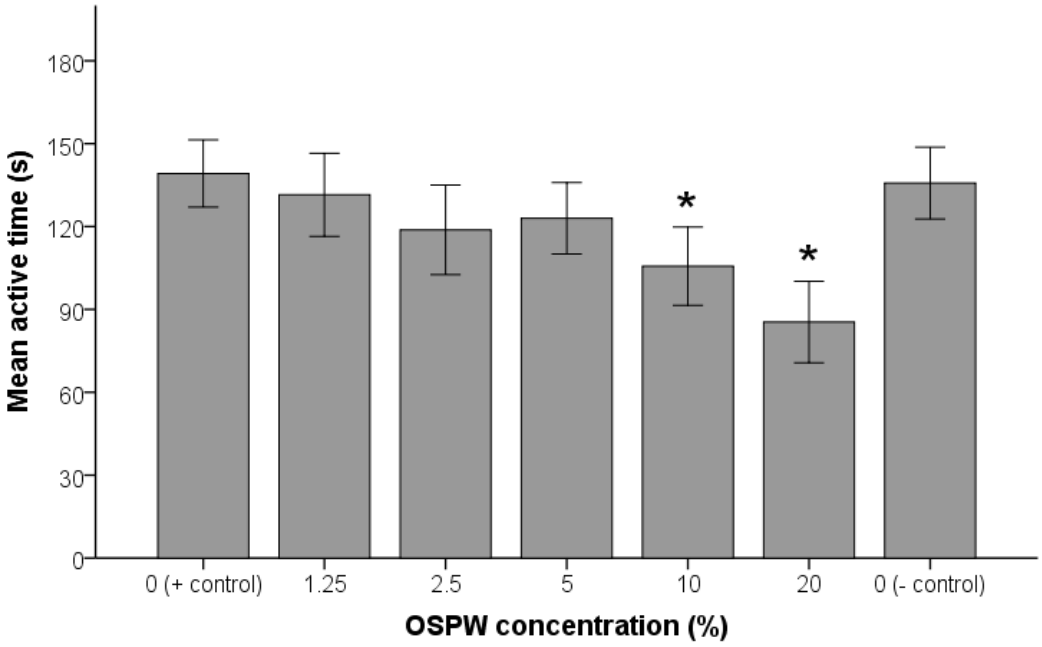


Figure 2.7. Short-term (24 h) effect of an OSPW mixture on the total activity of *Daphnia magna* to food cue. Asterisks (*) show significant difference with the control group. n = 20, error bars: ± 2 SE.

2.3.6. Clay consumption

The EDX assay detected Al and Si in all samples of *D. magna* exposed to 5, 10, or 20% OSPW. In the samples exposed to 2.5% OSPW only Si was detected and Al was less than the limit of detection. In samples from 1.25% OSPW, filtered OSPW, and control groups both Al and Si were below detection limits. High reading peaks for both Al and Si were observed from the residuum of filtering OSPWs. Samples of EDX readings of all treatment groups are presented in Appendix 2.6.

2.4. Discussion

One goal of the present study was to determine lethal concentrations of the OSPWs to *D. magna* by performing an acute lethality assay. The 48 h LC50s of all three OSPWs were greater than 100%. However, these results concur reasonably with the LC50 of 98% v/v OSPW on *D. magna* (Mackay and Verbeek, 1993). Comparing the reported LC50s of OSPW with LC50s of the studied oil-derived contaminants such as the water-soluble fraction (WSF) of crude oil (e.g. Ullrich Jr. and Millemann, 1983; Martínez-Jerónimo et al., 2005) and single hydrocarbons (e.g. Passino-Reader et al., 1997; Feldmannová et al., 2006), suggests that OSPW does not have a great potency for causing acute lethality.

The purpose of the short-term grazing test was to determine the effects of OSPW on the grazing behaviour of *Daphnia*. The results of the 24 h grazing tests of the studied samples were similar and the effect of the three OSPWs on the feeding rate of *D. magna* was not significantly different. The similarities in the toxicity and chemical properties of the investigated OSPWs in the present study suggest that despite the slight chemical

variances in the bitumen source and different processing methods, the chemical, and consequently, toxicological properties of the resultant OSPWs may be similar.

Although the effect of many contaminants on the feeding behaviour of *Daphnia* has been studied (e.g. Barata et al., 2008; Ferreira et al., 2008; Lopes et al., 2014), to the best of the author's knowledge, there are no published data on the effect of OSPW on the feeding behaviour of *Daphnia*. As is commonly accepted, feeding rate is the most important determinant of an animal's ability to obtain energy, which in turn affects energy metabolism. Therefore, a significant reduction in feeding behaviour of *D. magna* exposed to OSPW suggests that OSPW may also affect those physiological processes that have high energy requirements.

Growth and reproduction are among the most energy-consuming activities of animals and are of special importance at the population level. It is well known that toxicants affect reproduction (e.g. Villarroel et al., 2003; Muyssen et al., 2006; Lopes et al., 2014) and growth rate (e.g. Villarroel et al., 2003; Muyssen et al., 2006) of *Daphnia*. In the case of some toxicants (e.g. zinc and nano-zinc), it has also been shown that there is a correlation between the reduction of food consumption, growth rate, and reproductive capacity (Villarroel et al., 2003; Muyssen et al., 2006). The results of the long-term test showed that all three OSPW samples, at 1 or 10% concentrations, significantly impaired the feeding, reproduction and growth rate of *D. magna*, but showed no differences among the three OSPW samples. These results may be a consequence of the reduction in feeding rate, as was observed in the short-term test. Other metabolic and physiological mechanisms related to the toxicity of OSPW may also contribute to the observed reduction of *D. magna's* reproduction and growth rate, but the reduction in feeding rate seems to contribute to reduced reproduction and growth.

A goal of the current study was to investigate the potential causes of the reduction of feeding behaviour of *D. magna* exposed to OSPW. The results of the choice maze experiment illustrated that OSPW impairs the ability of *D. magna* to detect food cue odorants. In the feeding assay the food cells were homogenized in the test solution, so the test animals did not need to search for food. Consequently, it can be inferred that impairment of the chemosensory system did not play an important role in the observed reduction in feeding rate in feeding assay. However, in a natural environment where the food distribution is unequal, impairment of the ability to locate food may have an appreciable effect on the fitness of *D. magna.* Changes in the activity of filtering appendages and reduction in the activity of digestive enzymes may also play a role in the reduction of feeding rate, but these potential factors need further investigation. Since OSPW-induced impairment of *D. magna's* chemosensory system has not been reported before, investigations of the mechanism(s) and consequences of this phenomenon, especially in the population dynamics and ecological context, is warranted.

Results of the choice maze bioassay suggest that the reduction in feeding rate of *D. magna* exposed to OSPW might be caused by a reduction of total swimming activity. Several other studies have demonstrated a contaminant-induced reduction in total activity of *Daphnia* (e.g. Untersteiner et al., 2003; Zein et al., 2015). There are also some activity-related behaviours such as the rate of movement of the appendages that is thought to have an effect on the feeding rate of *Daphnia* (Hartmann and Kunkel, 1991). Studying the effect of OSPW on these behaviours may illustrate in more detail the effect of OSPW on *D. magna* feeding behaviour.

Binding of toxicants to suspended particulate matter reduces the bioavailability of contaminant, thereby attenuating any toxicity to aquatic organisms (Ma et al., 2002; Wölz

et al., 2008). However, studies show that suspended particulate matter may be filtered by *Daphnia* and consequently reduce their food consumption (Kirk, 1991). Results of the EDX experiment demonstrate that *D. magna* exposed to OSPW ingest a considerable amount of suspended particulate matter. Therefore, it can be concluded that filtering suspended particulate may play an important role in the reduction of feeding rate by filling the digestive system of *D. magna* with the non-digestible inorganic material (e.g., clay) and consequently reducing its capacity for consuming food. It is also worthwhile investigating the effect of the toxic chemicals bound to suspended particles on the feeding capacity of *D. magna*.

This study illustrates that exposure to sub-lethal concentrations of OSPW (1%) impairs feeding, growth, and reproduction of *D. magna*. Impairment of such ecologically relevant functions has been termed 'ecological death' (Scott and Sloman, 2004). Although *D. magna* may not be overtly harmed at the aforementioned concentrations of OSPW, their population may decrease as a result of a reduced reproduction rate among individuals. *Daphnia* are an important zooplankton in freshwater environments and changes in their population may result in shifts in food web structure. Additionally, the results of this study suggest that impairing the chemosensory function, reducing the total activity, and consumption of clay particles in OSPW play a role in the reduction of feeding rate in *D. magna*. The toxicity results, which are in agreement with the analysis of hydrocarbon and metallic elements content of the three OSPWs, showed that the three OSPW samples were similar.

CHAPTER 3: A novel apparatus for evaluating contaminant effects on feeding activity and heart rate in *Daphnia* spp.[3]

Abstract

Cladocerans are animals of significant importance to freshwater bodies such that changes in their populations may result in drastic shifts in food webs. Numerous studies have investigated the effects of toxicants, and the effects of chemical and physical habitat changes to these animals. Most of these studies investigated more general endpoints such as mortality, reproduction, growth, and food consumption over time, and less frequently examined molecular endpoints such as enzyme activity or gene expression. However, behavioral and physiological endpoints that link the organism and molecular level endpoints are scarce. In this study, we designed an apparatus that allows for the simultaneous investigation of three essential behavioral and physiological endpoints in *Daphnia*, including ventilation, food uptake rate, and heart rate. Using our apparatus, we studied the effect of cadmium (Cd), suspended particles, and food on the beating rate of thoracic limbs and the frequency of mandible rolling in *Daphnia magna*. We also studied the effect of temperature on the heart and thoracic limb beat frequency. The results show that both Cd and suspended particles reduce the activity of mandibles and thoracic limbs. Thoracic limb movements and heart rate increased gradually with temperature. Our

[3] A version of this chapter is published in *Ecotoxicology and Environmental Safety*, 135, Lari, E., Steinkey, D., Pyle, G.G., A novel apparatus for evaluating contaminant effects on feeding activity and heart rate in *Daphnia* spp., 381-386, 2017, with permission from Elsevier.

toxicity tests show that changes in feeding, ventilation, and heart rates are easily detected using this method.

3.1. Introduction

Cladoceran zooplankton of the genus *Daphnia* Muller, 1785 is an important component of freshwater food webs (Allen et al., 1995), and are sensitive to a wide range of environmental contaminants (Villarroel et al., 2003). *Daphnia* spp. are widely used as model organisms to investigate the effects of toxicants as well as chemical and physical habitat changes to aquatic environments (Komjarova and Blust, 2009; Lari et al., 2016b). A significant number of these studies focus on energy acquisition endpoints such as food uptake and digestion (e.g. Houde et al., 2013; Rocha et al., 2014), and energy-consumption endpoints such as respiration, energy storage, and metabolic rate (e.g. Weider and Lampert, 1985; De Coen and Janssen, 2003; Filho et al., 2011).

Physiological endpoints can link organismal survival and behavioral responses to cellular and molecular endpoints. Connecting biological levels is of particular importance both in understanding the mechanism of biological features of animals in adverse outcome pathways (AOP) in ecotoxicological studies (Ankley et al., 2010). For instance, the mechanism of the inhibitory effect of a contaminant on feeding rate of *Daphnia* can be surveyed by investigating a chain of endpoints from behavioral (e.g. thoracic limbs and mandibles activity) to physiological (e.g. gut peristaltic activity), and then molecular (e.g. digestive enzymes activity, gene expression) levels.

The beating rate of the thoracic limbs is a behavioral endpoint that is an indicator of both feeding and respiration in *Daphnia* (Smirnov, 2013). Constant beating of these limbs

propels food particles toward *Daphnia*'s food groove while at the same time provides propulsion for gas exchange over their body surface (Pirow et al., 1999a, b). Mandible rolling is another direct indicator of food uptake rate. Rotational motion of these two asymmetrical masticatory appendages crushes food particles and directs them toward the gut (Smirnov, 2013).

The myogenic (myocytes initiate contractions) heart of *Daphnia* is the main means of circulating hemolymph in cladocerans (Smirnov, 2013). The heart rate of *Daphnia* is easily altered by physical and chemical disturbance (Paul et al., 1997; Lovern et al., 2007) and is a physiologic indicator of metabolic rate in *Daphnia*.

Thoracic limb beating, mandible rolling, and heart rate have been applied, individually or together, to investigate several scenarios of habitat changes (*e.g.* water quality and temperature) and pollutants on *Daphnia*. Several studies have been dedicated to quantifying these three endpoints using various methods. However, these methods seemed to need modification to decrease the stress level to the specimens and increase time efficiency. The aim of the present study was to develop an efficient method for measuring all three endpoints simultaneously on one individual and increase the reliability of the measurement.

3.2. Materials and Methods

3.2.1. Test animals

All experiments were carried out with *D. magna* cultured under laboratory conditions for two years. For culturing, about ten daphniids were housed in 1 L beakers filled with

moderately hard reconstituted water (90 mg/L as $CaCO_3$). Water quality parameters are presented in Table 3.1.

The solution in each beaker was renewed once a week, and the daphniids were fed daily with *Raphidocelis subcapitata* and a combination of yeast, Cerophyll™, and trout chow (YCT). A 16:8 h light:dark cycle regime and the temperature at 20±1°C were maintained throughout. Each daphniid was maintained only for 5 weeks. Test neonates (i.e. less than 24 h of age) were separated from mothers of three to five weeks of age and maintained at a density of 80 individuals per 1 L beaker until 6-7 days of age. For tests that required a 24 h exposure time (i.e. to study the effects of physical and chemical contaminants and temperature; see sections 2.5 and 2.6), 6 day-old daphniids were used, while tests not requiring an exposure period (i.e. to study the acclimation time and the effects of food concentration; see sections 2.3 and 2.4) utilised 7 day-old daphniids. Daphniids at this age were used because they were a suitable size for handling and had not yet reached a reproductive stage.

Table 3.1. Water quality parameters as measured in the culture water during the study.

Parameter	Mean measured values ± SD (n = 3)
Hardness	91.9 ± 5.5 mg/L as $CaCO_3$
Alkalinity	166 ± 1.8 mg/L as $CaCO_3$
pH	8.1 (8.0 – 8.2)*
Temperature	22 ± 0.7 ºC
Cd**	40.3 ± 0.57 µg/L

* Median (range)

** In the effect of cadmium (Cd) experiment

3.2.2. Test setup

The test setup consisted of four main components: a daphniid test chamber, a micromanipulator system, a cue delivery system, and a recording system (Figure 3.1). The test chamber consisted of two 3.5 mL Acrylic Macro Cuvettes labeled cuvA and cuvB (BrandTech, Inc., USA) that were glued together lengthwise from the side. A 5-mm hole located 10 mm from the bottom of the cuvettes linked the interiors of cuvA and cuvB such that water could flow between them. A 2-mm drainage hole was located 5 mm from the top of cuvB's outer wall. Two 1 mm holes were pierced on the lid of cuvA, which provided openings for two plastic tubes into the chamber. One of the tubes was used for cue delivery, and the other acted as a vent for trapped air contained in the chamber. An insulin syringe needle was also inserted through the lid into cuvA 10 mm deep. The lid of cuvA was removable, which allowed the syringe head to be dipped in petroleum jelly (Vaseline, USA), in order to coat the first few millimeters of the needle tip. The dorsal carapace of the daphniid was gently affixed to the side of the needle coated with petroleum jelly. The other substance that may be used for attaching test daphniids to the needle is cyanoacrylate glue. One of the benefits of using petroleum jelly is that immediately after attaching the daphniid to the needle the animal can be inserted into the test chamber. Petroleum jelly is also neutral to the chemosensory system (Døving et al., 2006; Lari et al., 2015) and does not interfere with chemosensory-mediated behaviours such as feeding (Steinkey et al. in prep.). On the other hand, when fixed to the needle with cyanoacrylate glue, the daphniid should be left out of water for at least one minute to allow the glue to dry. Additionally, this glue has a scent that may affect chemosensory-mediated behaviours; however, the influence of cyanoacrylate glue has not yet been tested for its influence on chemosensory-mediated

behaviours. The downside of using petroleum jelly is that some test substances may be absorbed by it, which is not a concern for cyanoacrylate glue. However, considering the small amount of petroleum jelly that is used in this setup and the constant flow of the test solution, the absorption of organic test substances is negligible. Moreover, attaching the daphniid to the mounting post and inserting it into the test chamber in the least amount of time was considered to be an advantage of using petroleum jelly over the cyanoacrylate glue.

This setup allowed cues to be delivered into the top of cuvA, pass over the daphniid affixed to the needle, leave cuvA via the bottom opening into cuvB, and flow out of the entire system through the drain hole in cuvB. This two-chamber system allows for a flow through the apparatus without losing all the solution due to gravity. Two pieces (15×30 mm) of 1 mm thick strip magnets were glued to the back of the chambers and to the side of the micromanipulator. The chamber could then be easily attached to and detached, in order to maintain between tests, from the micromanipulator using these magnets.

The micromanipulator system allowed for precise positioning of the daphniid in three dimensions. The cue delivery system consisted of the cylinders of Luer-Loc tips 60 mL syringes (BD, Franklin Lakes, USA) that were connected to the cue delivery tube. Cue delivery was controlled by an electronic valve controller (V-6, Warner Instrument Corporation, USA). The recording system was a digital camera (FDK 23UP1300, Imaging Source, Germany) with a macro zoom lens (0.3~1X 1:45; MLM3X-MP, Computar, Japan) and was placed at a distance of 15 cm from the chamber. The chamber was lit using a microscope fibre-optic light source at 1000 lux (Fisher Scientific, USA).

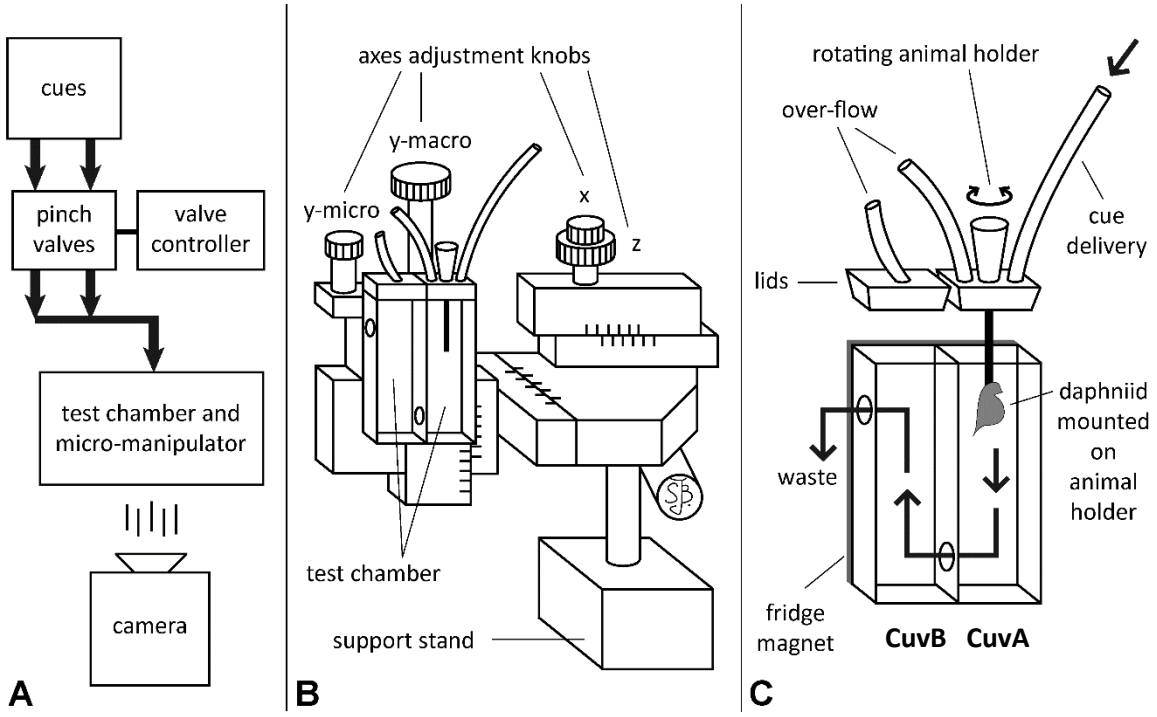


Figure 3.1. Apparatus setup. A) Setup overview. Cue delivery system (cue containers, pinch valves, valve controller) delivers cues to the test chamber which is attached to a micromanipulator. The test daphniid is glued to the animal holder inside the test chamber. The camera is placed in front of the chamber recording the activity of the test daphniid. B) The test chamber is attached to the micromanipulator via magnetic pieces. The position of the chamber can be adjusted by X, Z Y-macro, and Y-micro nubs. C) Test chamber; CuvA and CuvB are the two arenas in the chamber. A daphniid is glued to the tip of the animal holder and the cues are delivered via the cue delivery tube. The position of the specimen toward the camera is adjusted via the micro-manipulators.

To run tests, daphniids were placed in a test chamber and were left to acclimate for 10 min in the presence of the test cue. Afterward, the daphniid was recorded for 30 seconds at 90 frames per second (FPS). To measure the heart rate, the beating rate of the thoracic limbs, and mandibles rolling, the footages were reviewed in slow motion at 30% of normal speed. The movements were counted using a hand-held mechanical counter. The recording in the acclimation time test started at the beginning of the test. The recording in the other experiments started after a 10-min acclimation period.

3.2.3. Acclimation time

In order to decide on the acclimation time for the tests and measuring the changes in the stress level of daphniids in the chamber by passing time, ten daphniids were exposed to the test situation with 5×10^5 cells/mL of food algae delivered constantly throughout the test as a neutral cue. Daphniids were filmed immediately after being exposed and 5, 10, 15, and 20 min later. Heart rate and the beating rate of the thoracic limbs were monitored.

3.2.4. Effects of food concentration

Daphniids were tested for their response to increasing food concentrations on the endpoints listed above. To test this response, three groups of 10 daphniids were tested using two concentrations (5×10^5 and 5×10^6 cells/mL) of their food algae, *R. subcapitata,* and culture water as a control. The concentrations of food algae were chosen based on the concentrations that are commonly used in *D. magna* feeding tests (e.g. Rocha et al. 2014; Lopes et al. 2014). Food algae were constantly delivered to the chamber throughout the acclimation and test period. Using the method in section 2.2, mandible rolling frequency and beat rate of the thoracic limbs were measured.

3.2.5. Effects of physical and chemical contaminants

In order to test the effect of physical and chemical contamination on target endpoints, daphniids were exposed to clay (a prevalent physical challenge for filter feeder animals) and cadmium (a well-known metal toxicant), respectively. For physical contamination, two groups of 10 daphniids were exposed to 40 mg/L bentonite (clay) or culture water as a control. For chemical contamination, two groups of 10 daphniids were exposed to 45 µg/L of Cd or culture water as a control. Preliminary experiments show that the above-mentioned concentrations of clay and Cd reduce the food consumption of *D. magna* by 50% (Lari et al., 2017d). All four groups were fed 5×10^6 cell/L of *R. subcapitata*. After 24 h of exposure, mandibles grinding and the beating rate of the thoracic limbs of daphniids in all treatments were measured, using the method in section 2.2. The same solution as the exposure solution was delivered to the chamber throughout the acclimation and test period. Water samples were collected during exposures in Cd experiment in order to measure the actual Cd concentrations. Water samples were filtered through a 25-mm syringe filter with 0.45 µm cellulose acetate membrane (VWR, USA) into a 50-mL polyethylene conical tube (Falcon, Mexico) and acidified (0.2% final concentration) with trace metal grade HNO_3 (Fisher Scientific, Canada). Actual Cd concentrations were measured using Spectro Ciros Inductively-Coupled Plasma Optical Emission Spectrometer with an axial view plasma (Spectro Analytical Instruments, Kleve Germany) at Lethbridge Research and Development Centre, Lethbridge, AB, Canada (Table 3.1).

3.2.6. Effects of temperature

To determine the effect of temperature on heart rate and thoracic limb beating rate, four groups of 10 daphniids were exposed to an arithmetic progression of temperatures (5, 10, 15, 20 °C) in an agricultural growth chamber, for 24 h. At the end of the exposure, heart rate and thoracic limb, the beat frequency were measured at the same temperature as the treatments, using the method in section 3.2.2. Culture water with the same temperature as the treatment was delivered to the chamber throughout the acclimation and test period.

3.2.7. Statistical analysis

Shapiro-Wilk's test was used to test experimental data for the parametric assumption of normality. Homogeneity of variance was determined by a Bartlett's test in all two-sample comparisons. We analyzed the effect of the treatments (i.e. cadmium, clay, food) on the targeted endpoints (i.e. mandible rolling, heart and thoracic limb beat frequency) with multivariate analyses of variance (MANOVAs). In the acclimation time and food concentration tests, the significant MANOVAs were followed with a univariate analysis of variance (ANOVAs). Significant MANOVAs in the effect of Cd and clay were followed by Student's t-test. A linear regression analysis was used to determine if the temperature had a significant effect on heart rate and the beating rate of the thoracic limbs. All the statistical analyses were done using IBM SPSS 22 software (IBM, USA).

3.3. Results

3.3.1. Acclimation time

The results of the acclimation time showed that passing time had an effect on the measured endpoints [i.e. heart and thoracic limb beat frequency; Wilks' Lambda = 0.18, $F(8,88) = 15.30$, $p < 0.001$]. Both the movement of the thoracic limbs [$F(4, 45) = 45.32$; $P < 0.001$] and heart beat [$F(4, 45) = 45.89$; $P < 0.001$] reduced over the first ten minutes of acclimation and then stabilized for the remaining ten minutes (Figure 3.2), suggesting that the test daphniids were acclimated after 10 min exposure to the test situation (Appendix 3.1).

3.3.2. Effects of food concentration

Food concentration had an effect on *D. magna* [Wilks' Lambda = 0.16, $F(4,52) = 52.00$, $p < 0.001$]. Regular feeding behavior in *D. magna* could be changed by the presence of food in the water flowing through the test chamber. Changes were seen in both mandible grinding [$F(2, 27) = 4.191$, $p = 0.026$] and the beating rate of the thoracic limbs [$F(2, 27) = 71.61$; $p < 0.001$]. Increasing the concentration of *R. subcapitata* 10x had no apparent effect on the movement rate of the mandibles or the thoracic limbs. However, removal of the food cue increased mean thoracic limbs beating from 450 beats per minute to 684, but decreased mean mandibles rolling from 126 to 104 rolling movements per minute (Figure 3.3; Appendix 3.2).

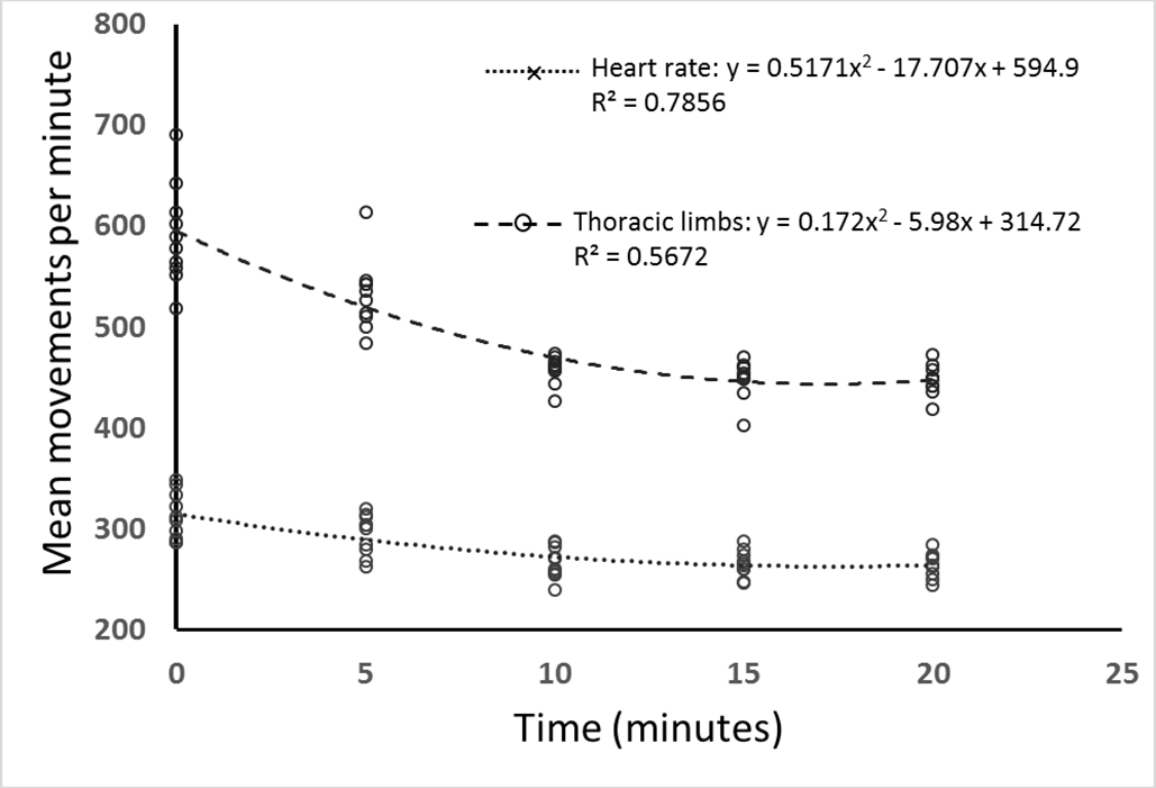


Figure 3.2. Shows the effect of acclimation time on both thoracic limbs and heartbeat of *Daphnia magna* in the absence of an algae food cue. Error bars represent +/- 1 SE; n = 10.

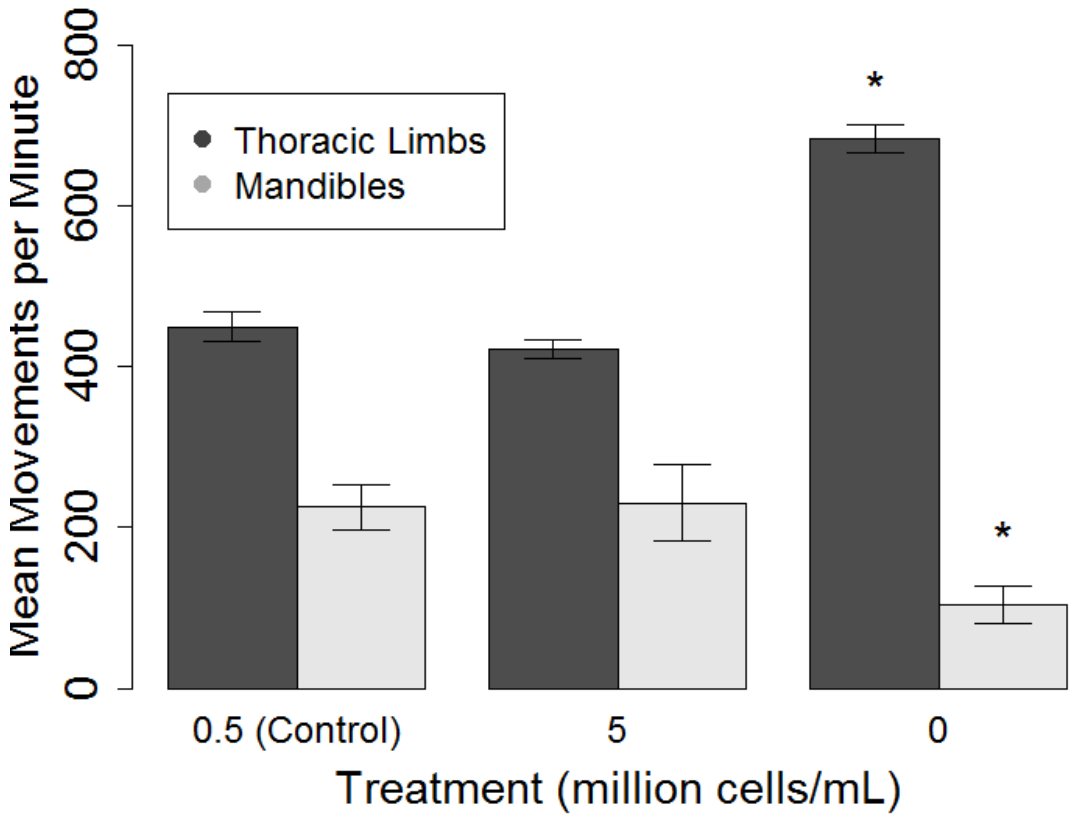


Figure 3.3. Changes in mandible and thoracic limb movements in *Daphnia magna* with changes in *R. subcapitata* density. Asterisks (*) show a significant difference from the control group. Error bars represent +/- 1 SE; n = 10.

3.3.2. Effects of particulate matter

The presence of clay in the water, affected the feeding apparatus of *D. magna* [Wilks' Lambda = 0.32, $F(2,22) = 23.11$, $p < 0.001$]. The addition of clay did not change the rate of mandibles grinding, with both the control and treatment daphniids averaging approximately 100 rolling movements per minute [$t(23) = 0.47$, $p = 0.65$]. The addition of clay did, however, reduce the mean number of thoracic limbs beating rate from 425 per minute to 209 [$t(30) = 46.51$, $p < 0.001$; Figure 3.4].

3.3.3. Effects of cadmium

The results of this test showed that the feeding apparatus of *D. magna* was affected by Cd [Wilks' Lambda = 0.42, $F(2,17) = 11.47$, $p = 0.001$]. Cadmium significantly inhibited both the beating rate of the thoracic limbs and the number of mandible rolling movements (Figure 3.5). Mean mandible rolling movements per minute were reduced from 188 down to 54 [$t(10) = 3.77$, $p = 0.004$] and mean thoracic limb movements per minute decreased from 420 to 314 [$t(12) = 3.44$, $p = 0.005$].

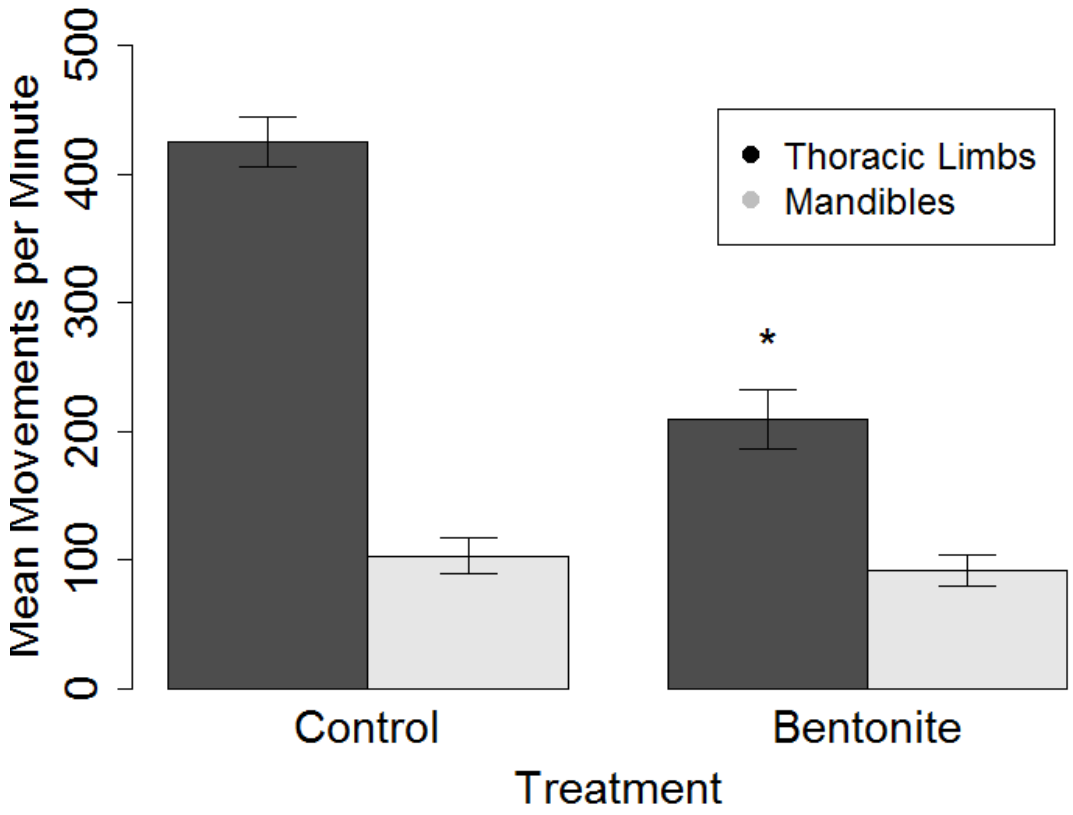


Figure 3.4. The frequency of mandible and thoracic limb movements *Daphnia magna* following 24 h exposure to 40 mg/L of bentonite (clay). Asterisks (*) show significant difference with the control group. Error bars represent +/- 1 SE; n = 10.

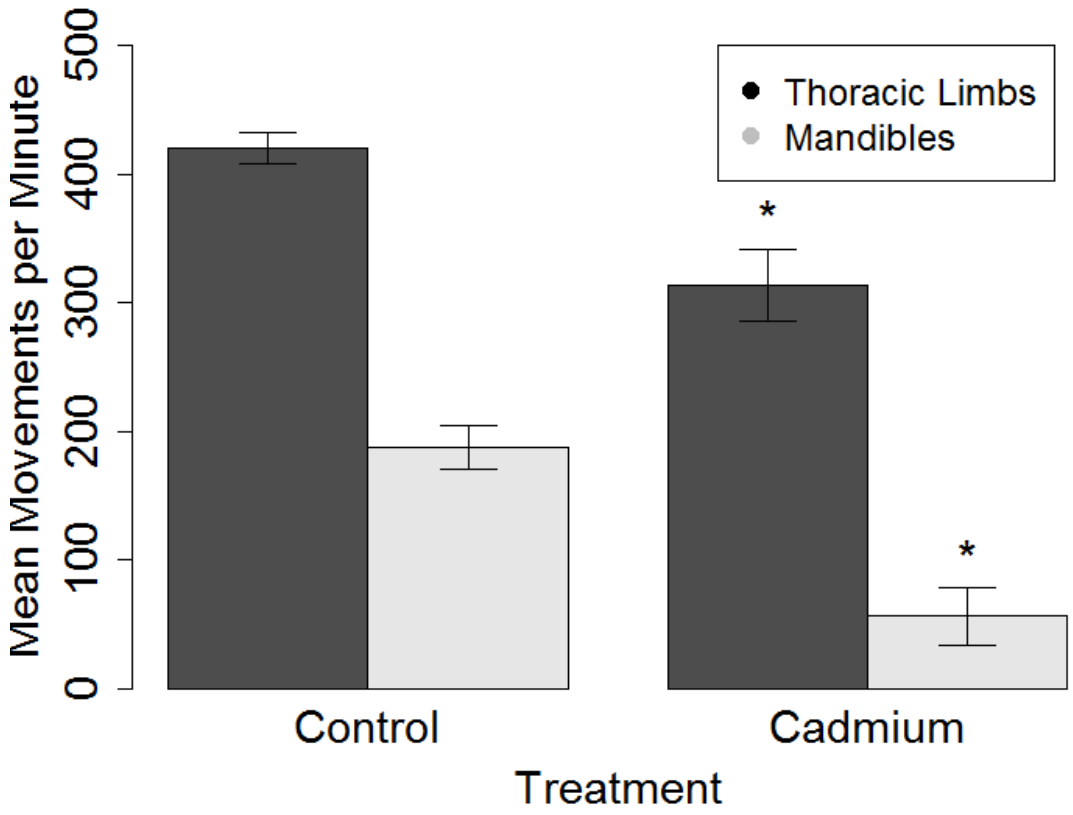


Figure 3.5. Mandible and thoracic limb movements in *Daphnia magna* following 24 h exposure to 45 µg/L of cadmium. Asterisks (*) show a significant difference from the control group. Error bars represent +/- 1 SE; n = 10.

3.3.4. Effects of temperature

The results showed that changes in the temperature of the surrounding environment, change the studied endpoints [i.e. heart and thoracic limb beat frequency; Wilks' Lambda = 0.12, $F(6,66) = 21.40$, $p < 0.001$]. Over a temperature range of 5 to 20 °C both heart beat [$F(3,34) = 63.14$, $p < 0.001$] and thoracic limbs movements [$F(3,34) = 31.04$, $p < 0.001$] increased consistently over the entire range (Figure 3.6). Qualitatively, over the temperature range tested, the strength of contractions and overall quality of the heart appeared to be vastly different between treatments; as it can be seen in the supplemental videos A and B. The heart in the 20 ºC looked full and round, in the cold water treatments it was slow and more transparent.

3.4. Discussion

The current study demonstrated a novel apparatus for studying daphniid behavioral and physiological responses to contaminants and habitat changes, including heart rate, thoracic limb beat frequency, and mandible rolling frequency. The method was tested for its ability to detect effects associated with contamination (i.e. cadmium and clay) and environment changes (i.e. food content and temperature). The results showed that thoracic limb beat frequency, mandible rolling, and heart rate are sensitive endpoints and are easily quantified by using this method.

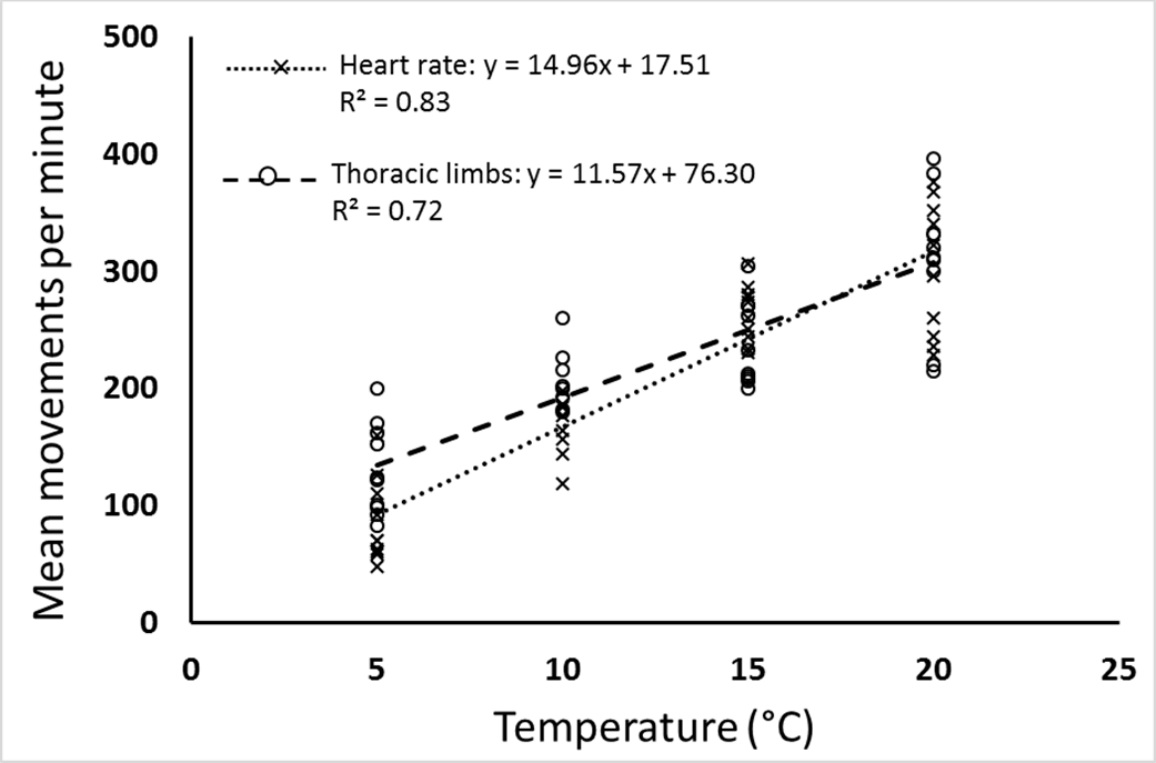


Figure 3.6. Heart rate and beat frequency of thoracic limbs in *Daphnia magna* following 24 h exposure to a series of temperatures (5, 10, 15, 20 °C). Error bars represent +/- 1 SE; n = 10.

The food concentration assay demonstrated that food scarcity increases the beating rate of thoracic limbs which increases the water current toward the daphniid's filtering fans. This behavioural change might be an attempt to bring in additional food to their feeding apparatus. Since there is no food available, mandible rolling (i.e. mastication) appears to be unnecessary and subsequently slows down, but never to zero. In contrast, in the presence of food (i.e. 5×10^5 and 5×10^6 cell/ml), the thoracic limb beat frequency declines while the mandible rolling frequency increases. The increased mandible rolling frequency would likely increase the rate of transfer of filtered food to the gut. These results corroborate the results of previous studies that suggest that the presence of food increases the mandible rolling frequency (Murtaugh, 1985) while decreasing the thoracic limb beat frequency (Peñalva Arana et al., 2007; Smirnov, 2013). Increasing the concentration of available food neither changed the mandible rolling frequency nor the thoracic limb beat frequency, probably because the beating rate of the thoracic limbs is also regulated by the demand for gas exchange (Smirnov, 2013). Kersting (1978) showed that food intake in *D. magna* increases by increasing the food particle concentration up to a critical concentration (2.5×10^6 cell/mL in two-weeks-old daphniids), after which the food intake rate remained constant. The food intake in the present study was not measured. However, the mandible rolling and the thoracic limb beat frequency did not change from 5×10^5 and 5×10^6 cell/ml algae.

The present assay was also sensitive for detecting the effects of environmental contamination on mandible beat frequency and thoracic limb movements. Increasing suspended clay particles reduced the beating rate of the thoracic limbs, which corroborates the results of a study by Kirk (1991) on the effects of suspended clay particles (50 mg/L)

on feeding activity of *Daphnia ambigua*. On the other hand, the present study found that the mandible-rolling rate remained constant in the presence of suspended clay (~ 100 beats per minute), while Kirk (1991) demonstrated a reduction in mandible-rolling frequency from 83 to 34 per minute. Because filtration in *Daphnia* is non-selective for any type of particle entering the filter, clay particles are actively taken up (Lari *et al.*, 2016). Because of this lack of selectivity, it is likely not the clay particles that reduce the mandible-rolling rate.

Domal-Kwiatkowska et al. (1994) showed that exposure to 150 µg/L of dissolved Cd reduced food consumption of *D. magna.* Lari et al. (2017a) reported half maximal inhibitory concentration (IC50) of Cd on food consumption of *D. magna* neonates at 3.04 µg/L. The results of the Cd toxicity tests in the present study showed a significant reduction in both thoracic limb beating and mandible rolling rate. These results suggest that one of the mechanisms involved in the inhibitory effect of Cd on food consumption of *D. magna* is impairing the activity of food collecting organs.

Both heart rate and the thoracic limb beat frequency increased with increasing temperature. It is well demonstrated that increasing temperature corresponds to a concomitant increase in metabolism and oxygen consumption. Increasing temperature increases the demand for gas exchange (Yurista, 1999), which stimulate the heart and thoracic limb beat rate. Heart rate has been suggested as an indicator of metabolic rate (Paul et al., 1998), and has been applied as an endpoint to investigate the effects of changing habitats (e.g. Paul et al., 1998; Lamkemeyer et al., 2003) and contaminants (e. g. (Lovern et al., 2007; Ona and Medina, 2015).

The methods described in the present study resulted from modifying several published methods. In some studies, daphniids were trapped in small droplets of water on a microscope slide (Villegas Navarro et al., 2003; Campbell et al., 2004). In other studies, daphniids were immobilized by being glued to a thin wire or hair and stuck on a slide or petri dish (Kirk, 1991) or ensnared between two plates (Paul et al., 1997; Paul et al., 1998). In these published methods daphniids are not or hardly able to move their antennae, and are forced to stay on their side. This situation may create excess stress, which threatens to confound the results of the experiments. Lamkemeyer et al. (2003) fixed *D. magna* in the bottom of their test chamber by gluing its head to a bristle in a way that its second antennae were still movable, which reduce the stress. Daphniids in those situations perform an extensive number of post-abdominal rejections and release unprocessed gut contents continuously (personal observation), which appears to be an indication of stress. However, biochemical studies (i.e. changes in stress hormone) are required to clarify whether the observed behaviour is due to increase in stress level or not. To reduce stress in the present study, daphniids were placed upright in the middle of the test chamber in a way that allowed them to move their appendages freely.

The results of the acclimation test showed that both heart rate and the rate of the thoracic limbs stabilize after 10 min of acclimation in the test chamber while acclimation time in other studies was 30 min or more (e.g. Lovern et al., 2007; Peñalva Arana et al., 2007). The heart rate of daphniids in this study stabilizes at the base rate faster than the previous studies, which suggests that the daphniids are less stressed as heart rate is a direct indicator of stress level in *Daphnia* (Smirnov, 2013).

The results of the experiments strongly suggest that both thoracic limb beating and mandible rolling rate are direct indicators of feeding behavior in *Daphnia*, and their changes can easily be quantified using the method demonstrated in the present study. These two endpoints can be used to investigate the mechanistic pathways leading to inhibitory effects of environmental stressors on food consumption in *Daphnia*. As feeding is the only source of energy in animals, whatever affects feeding inevitably affects many vital aspects of the animal's life (e.g. growth and reproduction) through altered metabolic pathways.

A direct indicator of the metabolic rate of animals is their heart rate, which can easily be monitored in *Daphnia* using the method demonstrated in the present study. Using the current method, heart rate may be studied as an indicator of the effect of stressors on the metabolic rate of *Daphnia* and possibly a link between the feeding impairment and metabolic status of *Daphnia*. The method presented in this study provides a reliable tool to quantify the effect of stressors on three endpoints (i.e. heart rate, beating rate of the thoracic limbs, and mandible rolling) that are directly related to feeding and metabolic rate and indirectly to growth and reproduction and consequently the fitness of *Daphnia*.

A useful feature of this study's chamber is that the angle of the test daphniid toward the recording system can easily be adjusted any time during the experiment, which makes it efficient for getting clear footage of the target organs. Different kinds of chemical (e.g. food and alarm) and physical (e.g. food and particles) cues, as well as contaminants, may be delivered in a consistent manner to the specimens via cue delivery system. The delivering cue can be switched to a different cue immediately during the test, enabling the investigators to observe the immediate reaction of *Daphnia* to the new cue. These features

provide a great deal of flexibility to this method making it suitable to several study designs and exposure scenarios.

To sum up, this method is an efficient tool for studying heart rate, thoracic limbs movement, and mandibles rolling with the low-stress level of the test animals, easy handling and manipulating the test daphniid's position and reduced time required to run the test. All of the endpoints mentioned above may be used to study the effect of stressors on the feeding and metabolic rate of *Daphnia*, which are the main sources of collecting energy and spending energy in animals.

CHAPTER 4: Oil Sands Process-Affected Water Impairs Feeding by *Daphnia magna*[4]

Abstract

Growth in the extraction of bitumen from oil sands has raised concerns about influences of this industry on surrounding environments. Water clearance rate (a surrogate of feeding rate by *Daphnia magna*) in water containing *D. magna* exposed to oil sands process-affected water (OSPW) and its principal components, dissolved component (DC) and suspended particulate matter (SPM), was reduced to 72, 29, and 59% of controls, respectively. This study also examined several possible mechanisms for the observed changes in algal cell density (i.e., feeding rate). There was no change in the digestive enzymes trypsin or amylase when *D. magna* were exposed to DC or SPM; however, exposure to total OSPW reduced trypsin activity. Mandible rolling or post-abdominal rejections, which are indicators of feeding and palatability of food, were not affected by any exposures to OSPW. The beating of thoracic limbs, which provides water flow toward the feeding groove, was reduced by exposure to SPM or total OSPW. Peristaltic activity was reduced by exposure to DC, which then might result in reduced digestion time in *D. magna* exposed to DC, SPM or whole OSPW. All treatments caused an increase in numbers of intact algae cells in the hindgut and excreted material. These results suggest that both DC and SPM affect the feeding of *D. magna* by impairing actions of the digestive system, but most probably not by reducing rates of ingestion.

[4] A version of this chapter is published in *Chemosphere*, 175, Lari, E., Steinkey, D., Morandi, G., Rasmussen, J.B., Giesy, J.P., Pyle, G.G., Oil sands process-affected water impairs feeding by *Daphnia magna*, 465-472, 2017, with permission from Elsevier.

4.1. Introduction

The oil sands mining industry in northern Alberta, Canada, is operating on the largest known deposit of bitumen in the world (ERCB, 2009). Both the current volume and rapid growth in the extraction of bitumen, producing an estimated 3.95 million barrels per day by 2030 (CAPP, 2015), has increased concern about potential effects on the surrounding ecosystems (Dowdeswell et al., 2011; He et al., 2012a). Oil sands process-affected water (OSPW), which is a byproduct of extraction of bitumen using the "Clark extraction process," is of main concern (Kavanagh et al., 2013).

Because industrial operators do not discharge OSPW back into the Athabasca River (Giesy et al., 2010), a large volume of OSPW produced throughout the years of mining, is currently stored on-site in tailings ponds (RSC, 2010). Recently, the Alberta Energy Regulator-mandated minimizing the volume of liquid tailings stored in tailings ponds and requires that companies leave operation sites at a ready-to-reclaim state within ten years of the end of the mine's life (Government of Alberta, 2015). Fulfilling mandates and tailings reclamation require knowledge of the chemical and physical characteristics of tailings, along with their effects on living organisms and the surrounding environment.

Oil sands process-affected water is a complex mixture of thousands of dissolved chemicals (organic and inorganic) and suspended particulate matter (SPM) (Del Rio et al., 2006; Debenest et al., 2012; Lengger et al., 2013). Depending on characteristics of the source bitumen, extraction method, and age, chemical and physical compositions of specific OSPWs might be different. However, chemical characteristics and toxicity of three OSPW samples collected from three different tailing ponds of different companies were similar (Lari et al., 2016b). Although, specific compounds responsible for toxicity of

OSPW are not well known, the dissolved organic fraction of OSPW, mainly napthenic acids (NA) and polyaromatic hydrocarbons (PAH), are the main drivers of its toxicity (Anderson et al., 2012a; Anderson et al., 2012b; Klamerth et al., 2015; Morandi et al., 2015).

Several studies have investigated toxic effects of OSPW and its major components on a variety of aquatic invertebrates (Morandi et al., 2016). NAs of lesser molecular mass, isolated from OSPW were more potent at reducing survival of *Daphnia magna* Straus, 1820 than were NAs of greater molecular mass (Frank et al., 2009). In contrast, unfractionated, whole OSPW is not lethal to planktonic animals such as *D. magna* or *Chironomus dilutus* Shobanov, 1999 larvae at equivalent concentrations observed in holding ponds (Wiseman et al., 2013a; Lari et al., 2016b). OSPW is also less potent at causing lethality than the water-soluble fraction of crude oil (Lari et al., 2016a). During longer-term exposures to sub-lethal effects of OSPW or isolated NAs exhibited various potencies for reduction of rates of growth of various planktonic organisms (Goff et al., 2013; Wiseman et al., 2013a; Lari et al., 2016b). Exposure of *D. magna* to small concentrations (IC50 = 5.34%) of OSPW for 24 h inhibited feeding (Lari et al., 2016b). That study also demonstrated that *D. magna* ingested SPM from suspensions of OSPW and suggested that SPM in OSPW reduced the rate of feeding by *D. magna* by filling their gut and reducing their capacity to ingest particles of more nutritious foods (Lari et al., 2016b). However, the exact mechanism for effects of OSPW and contributions of its constituents, dissolved components (DC) vs. SPM, were not fully elucidated. For instance it was not determined whether filling the gut is the only mechanism by which SPM reduces the feeding rate or if other mechanisms are also involved.

The present study follows up on results of a previous (Lari et al., 2016b), and aims to: 1) determine mechanisms by which OSPW reduced water clearance rate by *D. magna*, as a model species, and 2) investigate roles of DC and SPM. Potential causes of reductions in water clearance rate were investigated at three biological levels: behavioural (i.e. beating rate of the thoracic limbs, mandible rolling frequency, and rate of food rejection by the post-abdominal appendage), physiological (i.e. peristaltic activity and digestion efficiency) or biochemical (digestive enzyme activity).

4.2. Materials and methods

4.2.1. Test chemicals

For this study, three major oil sands companies in the Athabasca region of northern Alberta, Canada, provided samples of OSPW. In studies of lethality and sub-lethal effects on feeding, growth, and reproduction, the three OSPW exhibited the same potency (Lari et al., 2016b). Profiles of relative abundances of dissolved organic species in the three OSPWs were also similar. Therefore, in the present study a mixture of all three OSPWs in equal proportions (v/v) was used. The OSPW mixture was stored in 20 L plastic buckets at 4 °C. The DC of OSPW was prepared by filtering the mixture through a 0.45 µm cellulose nitrate membrane filter (Whatman, Germany). The filtrate was dried at 60 °C for 48 h and weighed to determine the SPM content of OSPW mixture (0.686 g/L). Because filtration changes the mean size of filtered particles and small particles are trapped in filter pores, filtration residuum was not used for making SPM solution. Suspended particulate matter solution and reconstituted whole OSPW was made by adding sediment (< 45 µm; Plainsman Clay

Limited, Canada), at the same rate as the measured residue filtered from OSPW (0.686 g/L) to culture water and filtered OSPW, respectively.

4.2.2. Chemical characterization

Samples were extracted as described previously (Lari et al., 2016b). Briefly, 1 L of OSPW was filtered by use of a 0.7 µm glass fiber filter (Fischer Scientific, Ottawa, ON, Canada). The sample was acidified to pH 2 by use of concentrated H_2SO_4 (Fischer Scientific, Fair Lawn, NJ, USA). The acidified solution was extracted twice with 200 mL of dichloromethane (DCM) in a 2 L separatory funnel. The two extracts were combined and blown-down by use of nitrogen gas to approximately 5 mL, transferred to a pre-weighed vial, blown to near dryness and weighed. The extract was re-suspended in methanol for chemical profiling. Profiling of extracts by use of HPLC-ultrahigh resolution MS (Orbitrap), operated in positive and negative ion mode, was completed as described previously (Lari et al., 2016b). Approximately 100 mL of OSPW was sent to SGS Canada Inc. (Lakefield, Ontario, Canada) to measure total recoverable concentrations of vanadium (V), nickel (Ni), copper (Cu), cadmium (Cd), and zinc (Zn) by use of inductively coupled plasma mass spectrometry (ICP-MS). Above mentioned elements are the most relevant trace metals in OSPW.

4.2.3. Test animals

All *D. magna* used in the present study were from a culture held under controlled laboratory conditions as described previously (Lari et al., 2016b). Culture water was reconstituted from double deionized water by adding 0.096 mg/L $NaHCO_3$, 0.06 mg/L $CaSO_4.H_2O$, 0.06 mg/L $MgSO_4$, 0.012 mg/L KCl, 2.4 µg/L Na_2SeO_4 and 3.2 µg/L vitamin

B12. The resultant culture water was moderately hard: hardness, 90 mg/L as $CaCO_3$; alkalinity: 165 mg/L as $CaCO_3$; pH, 8.3. To monitor sensitivity of the culture, lethality caused by the reference toxicant, sodium chloride (NaCl) was performed every month (Environment Canada, 1996). Neonates (i.e. ≤24 h old) from 3 to 5-week-old *D. magna* mothers were collected and raised at a density of approximately 80 individuals per liter of culture water, for 6 days (5^{th} instar). Each group of 80 *D. magna* was fed *Raphidocelis subcapitata* daily at a density of 10^5 cell/L. All experiments described in the present study were conducted with these 6-day-old *D. magna*. Water in which *D. magna* were cultured, was also used for diluting OSPW and as the control water in all experiments.

4.2.4. Exposures of *D. magna*

Experiments were conducted on *D. magna* that were exposed to 10% equivalent (1/10X) concentrations of DC, SPM or unfractionated, whole OSPW. This equivalent concentration of OSPW was chosen based on the inhibition curve for effects of the whole OSPW on the feeding by *D. magna* in an earlier study (Lari et al., 2016; IC75 = 10% OSPW). A fourth group was exposed to *D. magna* culture water in the absence of OSPW as a control. All groups were exposed to test solutions at the density of 5 individuals per 100 mL, with a fixed ratio of *R. subcapitata* (5×10^5 cell/mL) as food. In order to prevent growth and reproduction of *R. subcapitata,* test beakers were kept in complete darkness during 24-h exposures.

4.2.5. Inhibition of feeding

Studies of feeding were accomplished by use of five replicates for each treatment. Treatment solutions were sampled prior to the addition of *D. magna*, and held in the same

condition as the exposures without adding *D. magna*. After 24 h of exposure in complete darkness, *D. magna* were removed, the test solution was vigorously shaken, and density of remaining algae cells was enumerated by use of a Neubauer chamber (Marienfeld, Germany) and light microscope (Eclipse 80i, Nikon, Japan). In order to calculate consumption of food, the density of the remaining algae was compared to the density of algae in the sample without animals.

4.2.6. Activities of thoracic limb, mandible, and peristalsis and frequency of rejection of food

Rates of beating of thoracic limbs, mandible rolling frequency, the peristaltic activity of the gut, and frequency of rejection were measured by use of previously described methods (Lari et al., 2017e). Briefly, after a 24 h exposure (as described in section 2.4) individual *D. magna* were mounted inside a test chamber by gently adhering the back of its carapace with petroleum jelly (Vaseline, USA) to a holder inside the chamber. A 1 mL/min flow of test solution, identical to the exposure solution, was maintained throughout the test. Individual *D. magna* were given 10 min to acclimate to test conditions. Afterward, responses of individuals were recorded for 30 seconds at 60 frames per second (FPS) for measuring the beating rate of the thoracic limbs (reciprocating beats of the first pair), mandible rolling (semicircular reciprocating movement), and frequency of reverse peristaltic contractions (upward moving contractions) of the gut and 2 mins at 30 FPS for measuring the rejection frequency. The recording system consisted of a digital camera (FDK 23UP1300, Imaging Source, Germany) with a macro zoom lens (0.3~1X 1:45; MLM3X-MP, Computar, Japan) and was placed at a distance of 15 cm from the test chamber. The videos were reviewed in slow motion at 30% of normal speed to measure the

beating rate of the thoracic limbs and mandible rolling and reviewed at a normal rate to measure the peristalsis activity and rejection frequency using VLC media player. Fifty individuals (20 for thoracic limb and mandible activity, 20 for peristaltic activity, and 10 for post-abdominal rejection tests) were exposed to each dilution of OSPW. In some video recordings mandibles were not properly visible such that mandible rolling of those individuals could not be measured.

4.2.7. Activities of digestive enzymes

Digestive activity was assessed by use of kits for measuring activities of amylase (ab102532, Abcam, USA) and trypsin (ab102531, Abcam, USA). Following a 24 h exposure (as described in section 2.4), approximately 10 mg (10-15 individuals) of *D. magna* (24 h exposed as described in section 2.4) per replicate were rinsed in phosphate buffer solution (PBS), dried, crushed and diluted with reaction buffer. Samples were then centrifuged at 15,000 g for 15 min and the supernatant was collected to be used in each treatment and split between three wells of a 96 well plate analyzed by a microplate reader (Varioskan Flash, Thermo Scientific, Finland).

4.2.8. Gut contents

Contents of guts of *D. magna* (after 24 h of exposure as described in section 2.4) were studied to investigate the presence of SPM in the gut as well as the efficiency of digestion of ingested algal cells. To determine the presence of SPM in the gut, five individuals from each group were rinsed with distilled, de-ionized water (ddH_2O), their carapace and antennae were removed by dissection and the remaining soft parts were pressed onto a scanning electron microscope (SEM) 18 mm carbon mount (TED PELLA, INC., USA). A

qualitative feature, energy-dispersive X-ray spectroscopy (EDX), on a tabletop SEM (TM-1000, Hitachi, Japan) was used to detect the presence of aluminum (Al) and silica (Si) as indicators of SPM in guts of *D. magna* (Lari et al., 2016b).

In order to investigate the efficiency of the digestive system for processing ingested algae, the content of hindguts of each of 10 individuals were combined then diluted in 20 µL culture water, and the number of intact algae cells was counted using a Neubauer chamber. Feces of individuals from all groups were also examined for intact algal cells. Feces were collected from individual *D. magna* by placing them on concave microscope slides (VWR, USA), then examining them under a light microscope (Eclipse 80i, Nikon, Japan). Using a standard glass capillary tube (World Precision Instruments, USA), the first fecal output produced was collected. Because the amount of feces was not measurable and only portions of feces were collected immediately, numbers of algal cells were not quantified. Only presence or absence of intact algal cells was determined.

4.2.9. Data analyses

Analyses of feeding behaviour and digestive enzymes were done in R 3.2.1 (R Core Team, 2015). Assumptions of parametric statistical tests were tested. The Shapiro-Wilk test was used to test for normality of frequency distributions while Bartlett's test was used to assess homogeneity of variances. For each endpoint, differences among main effects (treatments) were determined by use of ANOVA with a Dunnett's *post-hoc* multiple range test, except for inhibition of feeding which was examined by use of Tukey's *post-hoc* analysis.

4.3. Results

4.3.1. Chemical characterization of OSPW

Concentrations of Cd and Cu were greater and less than Canadian Council of Ministers of the Environment (CCME) criteria for protection of aquatic life, respectively. No CCME criterion was available for V, Ni, or Zn (Table 2.1). Characteristics of organic compounds in OSPW are represented graphically (Figure 4.1). When detected by use of positive ion mode, oxygen- and nitrogen-containing compounds predominated, while sulfur-containing chemical species contributed little to total mass spectral response. Multi-oxygen containing chemical species ($O_{5\text{-}6}^+$) were the most abundant chemical classes, each representing >20% of total intensity, respectively. Conversely, mono-, di- and tri-oxygenated species contributed minor signals, representing less than 5% of total mass spectral response. Nitrogen-containing compounds accounted for approximately 50% of total mass spectral response and were enriched in N^+, ON^+ and O_2N^+ containing chemical classes. When characterized by use of negative ion mode, a similar distribution of classes of heteroatoms was observed as oxygen and nitrogen containing compounds predominated, representing greater than 30 and 67% of total mass spectral response, respectively. However, in contrast to positive ion mode, $O_{2\text{-}4}^-$ containing species accounted for greater than 28% of total intensity. Interestingly, the O_2^- chemical class, traditionally classified as naphthenic acids, contributed less than 8% of relative intensity. Comparison of nitrogen containing compounds revealed a similar profile to positive ion mode as the sample was enriched in N^-, ON^- and O_2N^- containing chemical species. Because the greater filter size used in chemical characterization might alter the profile of chemical classes compared to the biological assays the characterization is necessarily operationally defined.

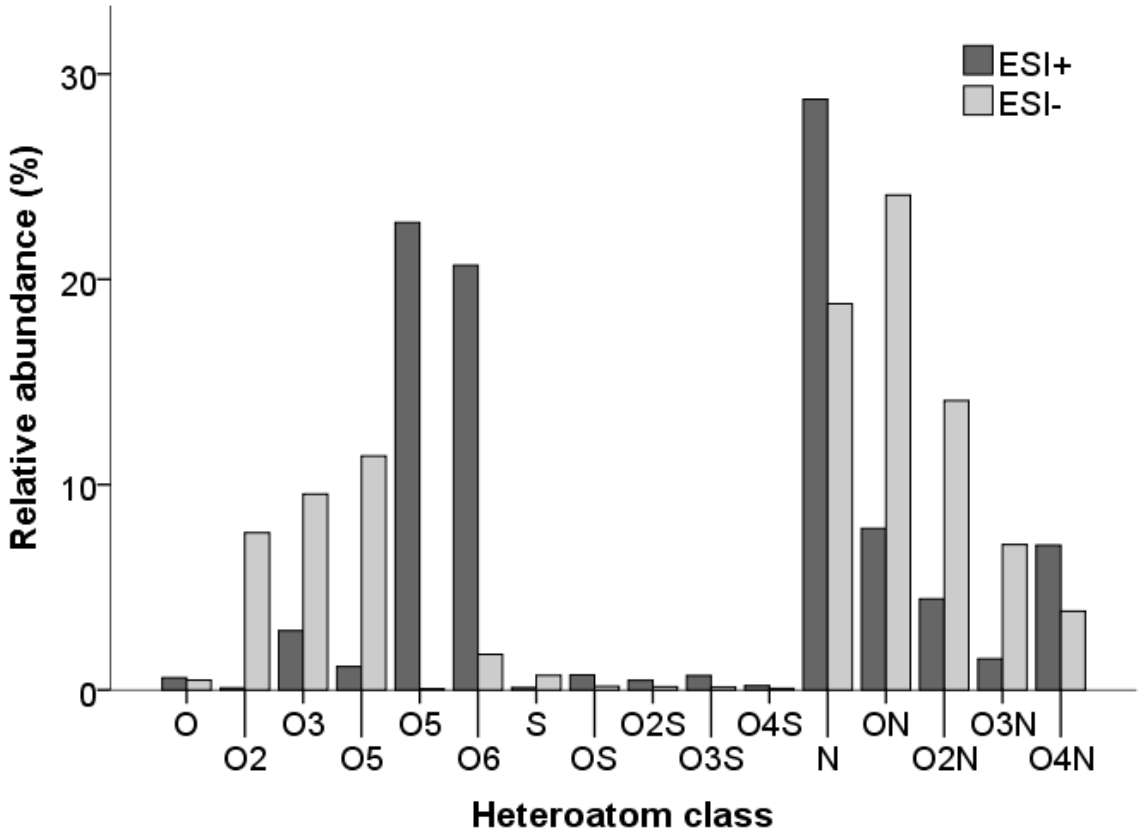


Figure 4.1. Distribution of classes of heteroatoms detected mode in the sample of oil sands process-affected water by use of HPLC ultrahigh resolution mass spectrometry (Orbitrap; HPLC-UHRMS) run in positive (ESI+) and negative (ESI-).

4.3.2. Inhibition of feeding

Compared to the control group, all three treatments reduced water clearing rate of *D. magna* [$F(3, 16) = 106.26$, $p < 0.001$; Figure 4.2]. The order of inhibition was: whole OSPW (72%) > SPM (59%) > DC (29%). Inhibition of feeding by SPM was significantly greater than that of DC ($p < 0.001$), which suggests that SPM is more effective at reducing feeding rate than DC (Appendix 4.1).

4.3.3. Movement of thoracic limb, mandible, peristalsis and frequency of rejection

None of the treatments had significant effects on frequency of post-abdominal rejection [$F(3, 28) = 1.10$, $p = 0.36$; Figure 4.3] or rate of mandible rolling [$F(3, 47) = 0.48$, $p = 0.70$; Figure 4.4] of *D. magna,* relative to that of the control. Treatments changed rate of beating of the thoracic limbs [$F(3, 36) = 21.76$, $p < 0.001$; Figure 4.4] and peristaltic activity [$F(3, 74) = 7.81$, $p < 0.001$; Figure 4.5]. Compared to the control group, exposure to DC did not alter rate of beating of thoracic limbs, while both SPM and whole OSPW reduced it. Peristaltic activity of the gut was reduced in individuals exposed to DC, but not in those exposed to SPM or whole OSPW (Appendix 4.2).

4.3.4. Digestive enzymes activity

There were no differences in activities of amylase among exposures [$F(3, 26) = 2.198$, $p = 0.11$; Figure 4.6]. However, trypsin activity was significantly [$F(3, 60) = 7.18$, $p < 0.001$; Figure 4.6] less in individuals exposed to whole OSPW exposure as compared to those exposed to either the control or DC (Appendix 4.3).

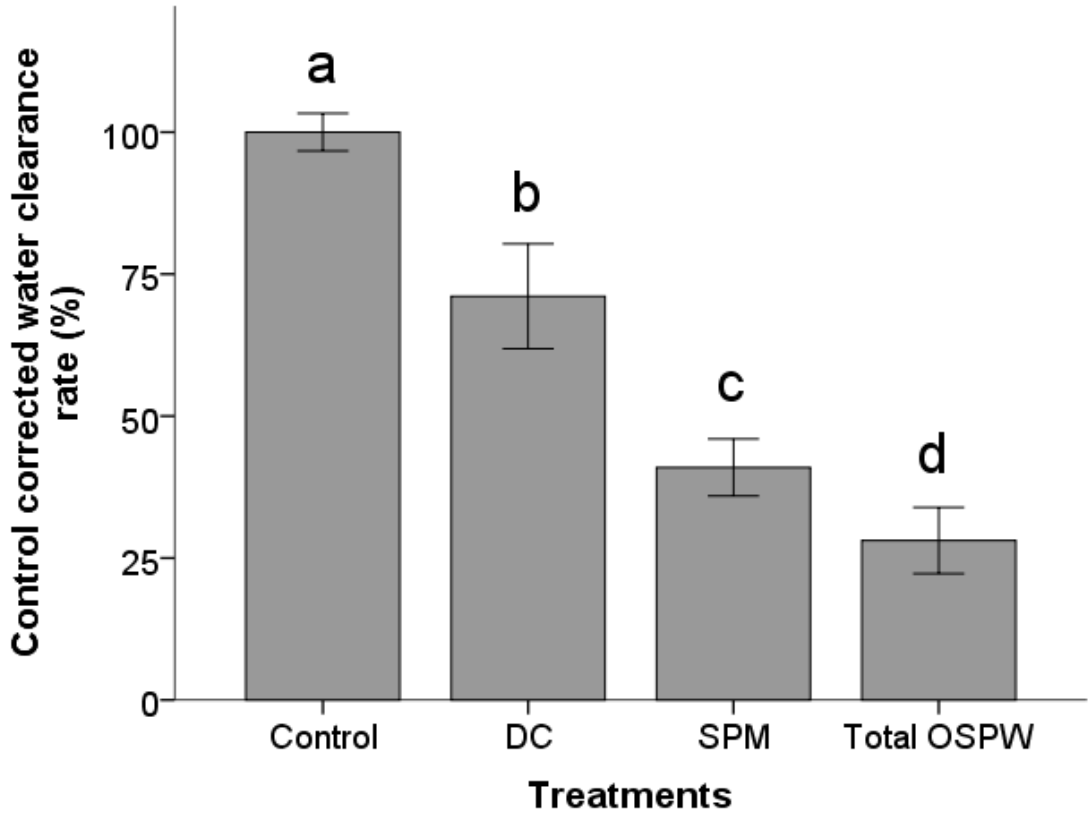


Figure 4.2. Effects of treatments on grazing by *Daphna magna*: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: suspended particulate matter in OSPW. n = 5, confidence limits: ± 2 SE.

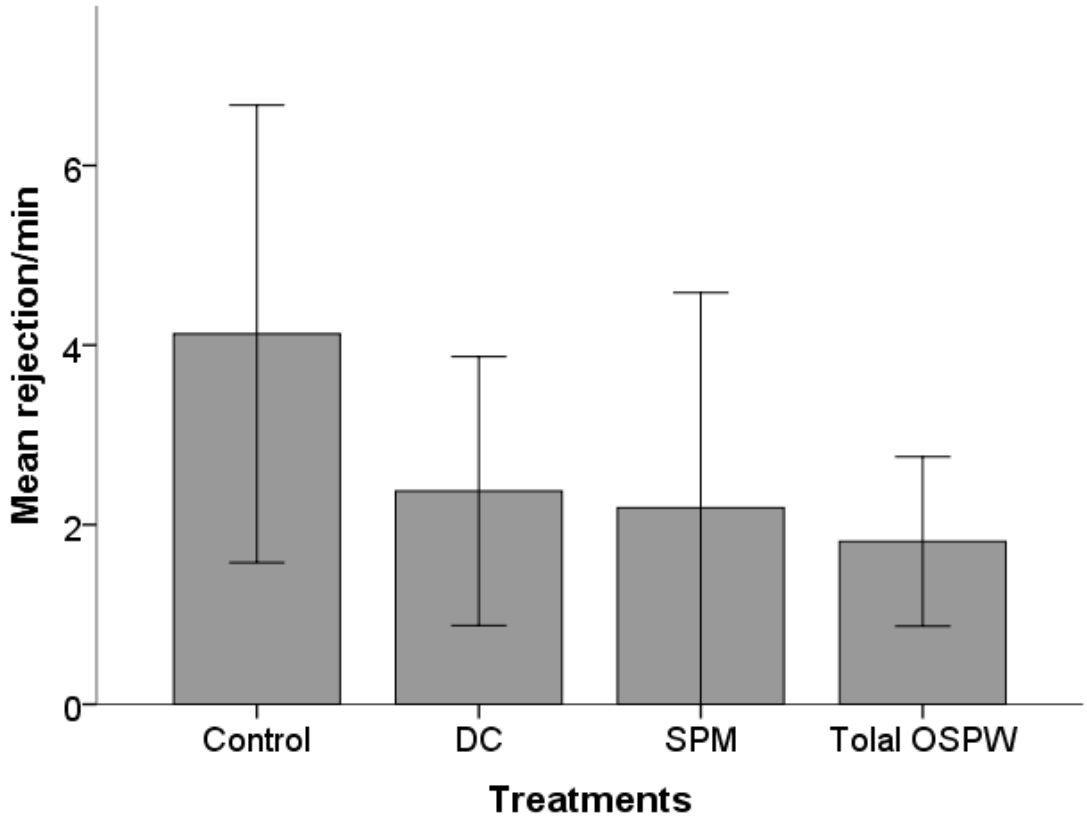


Figure 4.3. Effect of treatments on frequencies of post-abdominal rejection by *Daphnia magna*: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: suspended particulate matter in OSPW. n = 10, confidence limits: ± 2 SE.

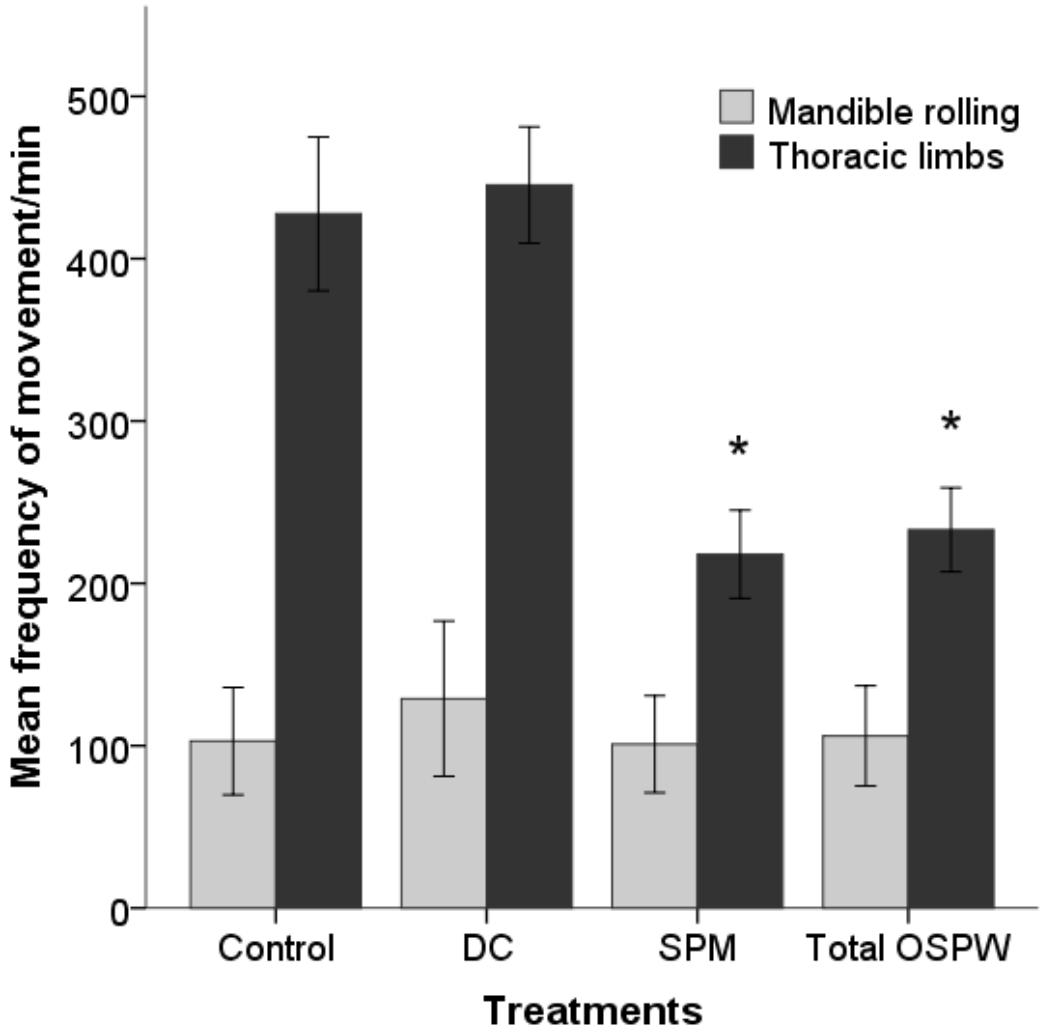


Figure 4.4. Effects of treatments on rates of the beating of thoracic limbs (n = 20) and mandible rolling (n = 13) of *Daphnia magna*. OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: Suspended particulate matter in OSPW. Asterisks (*) show a significant difference from the control group. Confidence limits: ± 2 SE.

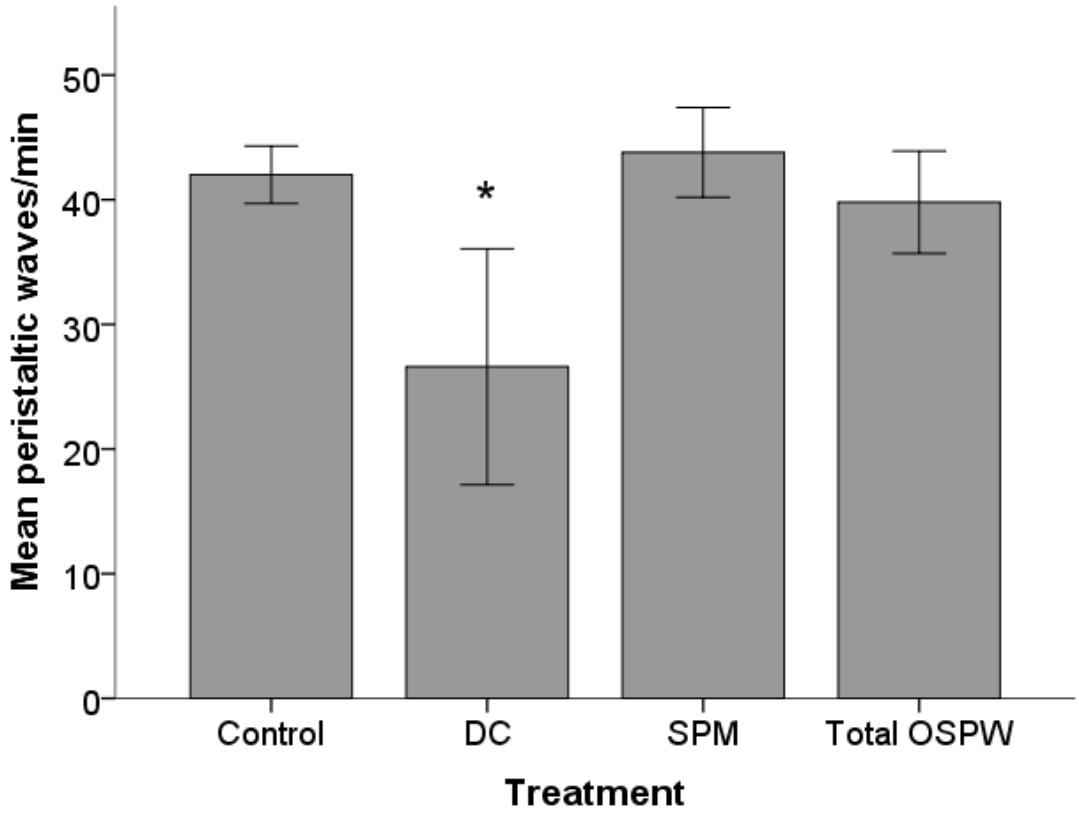


Figure 4.5. Effects of treatments on the peristaltic activity of *Daphnia magna*: OSPW: oil sands process-affected water; DC: dissolved component; SPM: Suspended particulate matter. Asterisks (*) show a significant difference with the control group. n = 20, confidence limits: ± 2 SE.

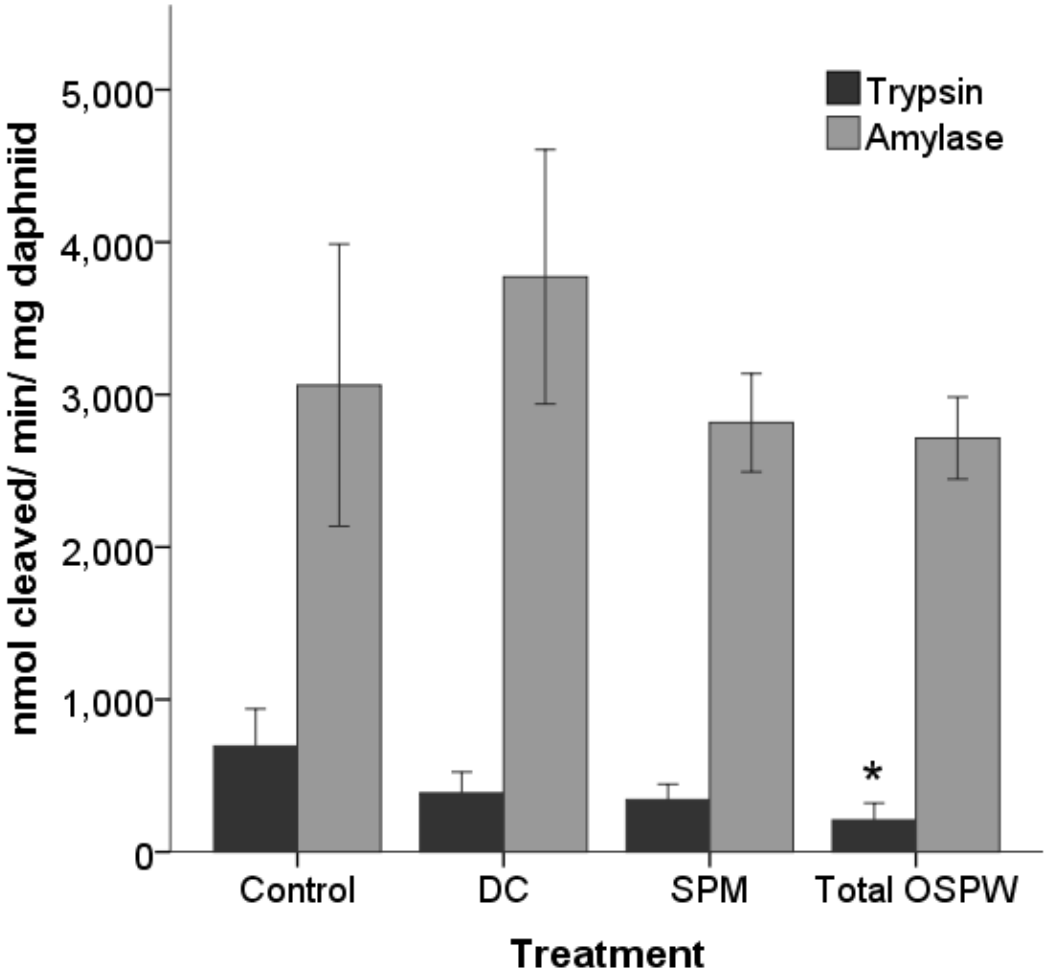


Figure 4.6. Effects of treatments on activities of digestive enzymes in the gut of *Daphnia magna*: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: suspended particulate matter in OSPW. Asterisk (*) shows a significant difference with the control group. Trypsin (n = 16), amylase (n = 8), confidence limits: ± 2 SE.

4.3.5. Contents of gut

The EDX measurements of *D. magna* exposed to SPM or whole OSPW showed clear peaks for both Al and Si, while neither Al nor Si was detected in *D. magna* exposed to DC or culture water (Appendix 4.4). These results indicate that during these exposures *D. magna* took up SPM from their environment. The color of *D. magna*'s gut exposed to SPM and total OSPW changed from green to brown (Appendix 4.5) over the course of the exposure. Numbers of intact algal cells in hindguts of *D. magna* exposed to all three types of fractions of OSPW were significantly greater than those of individuals in the control group [$F(3, 36) = 14.17$, $p < 0.001$; Figure 4.7; Appendix 4.6]. Mean numbers of intact algae cells in *D. magna* exposed to DC, SPM and total OSPW treatments were 11.5-, 13.5- and 23-fold greater than that in those of the control group, respectively, suggesting that all three treatments prevented *D. magna* to from digesting a significant number of ingested algal cells. Examining feces of *D. magna*, demonstrated that intact algal cells in feces of all ten specimens exposed to each of the three treatments, while on only one occasion were intact algal cells observed in feces of *D. magna* in the control group. The feces of *D. magna* in treatments was not in the form of fecal pellets and the excreted material, including the algal cells, was suspended in the water.

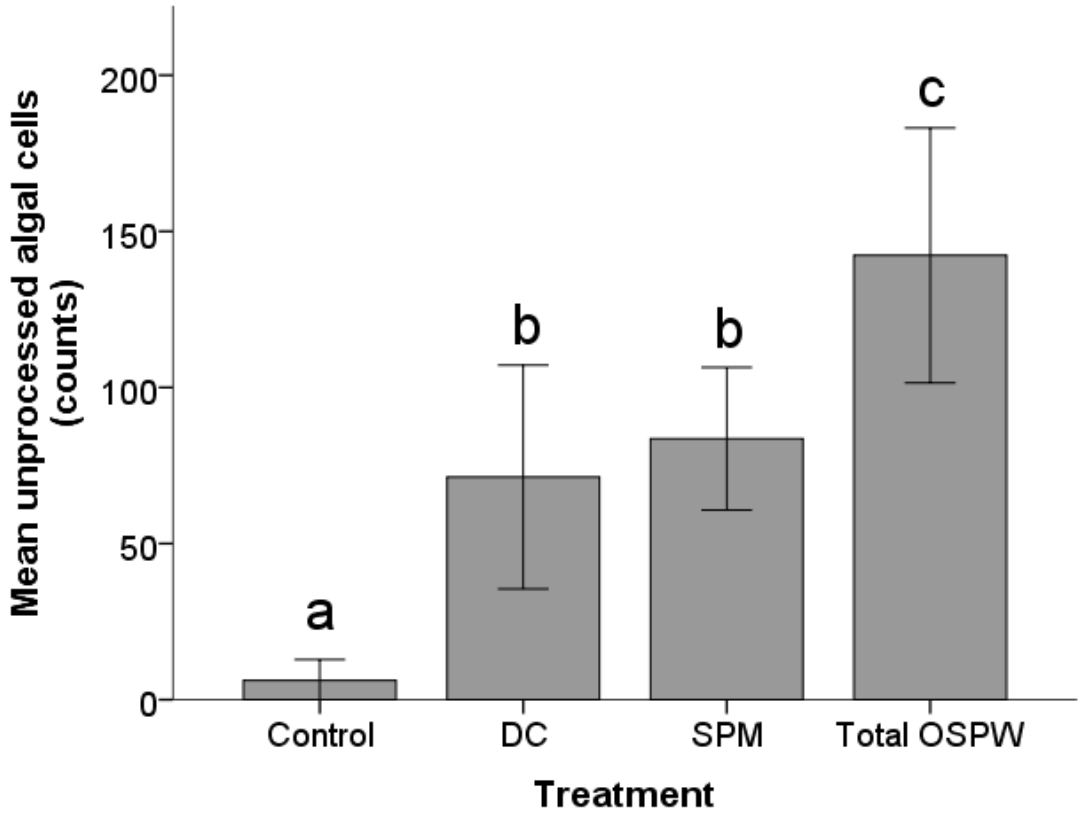


Figure 4.7. Effects of treatments on numbers of unprocessed algal cells in hindguts of *Daphnia magna*: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: Suspended particulate matter in OSPW. n = 10, confidence limits: ± 2 SE.

4.4. Discussion

Oil sands process-affected water has been shown to reduce rates of feeding of *D. magna* (Lari et al. 2016b). In the same study, the authors reported ingestion of SPM from OSPW by *D. magna*. The present study was designed to expand on those results in order to determine the mechanism by which OSPW modulates consumption of food by *D. magna* and to determine relative potencies of each component of OSPW (i.e. DC and SPM) on the feeding of *D. magna*. The DC sample impaired rate of feeding of *D. magna*, while the effect of SPM was significantly greater, which suggests that SPM more effectively reduces feeding of *D. magna* than did DC. Identifying chemical classes contributing to the observed toxicity was difficult due to the complexity of the mixture. The DC sample had greater abundances of nitrogen-containing chemical species and lesser abundances of NAs. However, it is important to note that the larger filter size used for chemical characterization might have affected the response of various chemical classes as it is known that ion suppression and chemical interactions can affect characterization (Bataineh et al., 2006). In *Daphnia*, movement of thoracic limbs directs water toward the feeding fans and oral groove, where food particles are trapped and collected (Smirnov, 2013). Particles collected in the food groove are then directed toward the mouth by the mandibles (Smirnov, 2013). Rates of the beating of thoracic limbs were reduced in individuals exposed to SPM or whole OSPW, but not in those exposed to DC. In contrast to dissolved constituents of OSPW, contaminants such as dissolved cadmium (45 µg Cd/L) can reduce rates of the beating of thoracic limbs and consequently rates of feeding of *D. magna* (Lari et al., 2017e). Exposure of *D. magna* to either of the insecticides cypermethrin (0.1 µg/L) or azoxystrobin (0.5 mg/L) caused lower beating rates of thoracic limbs (Friberg Jensen et al., 2010). Reduction

in thoracic limb beating rates of *Daphnia* in response to exposure to SPM or whole OSPW, which has been observed in several studies, suggests that increase in the concentration of particulate matter including food (Peñalva Arana et al., 2007; Lari et al., 2017e) and suspended minerals (Kirk, 1991; Lari et al., 2017e) reduce the beating rates of thoracic limbs. Thus, reduction of rates of the beating of thoracic limbs might not be due to toxicity, but, rather a response to the amount of particulate matter. Thus, this confounds the results and interpretation of effects of the whole OSPW on rates of feeding of *D. magna*.

Rates of feeding by *Daphnia* are directly correlated with rates of rolling of the mandible (Murtaugh, 1985). Contaminants such as metals (Lari et al., 2017e), pesticides (Gliwicz and Sieniawska, 1986; Friberg Jensen et al., 2010), crude oil (Wong et al., 1983), and biotoxins (Ghadouani et al., 2004; Rohrlack et al., 2005) can reduce rates of mandible rolling. Exposure to none of the fractions of OSPW used in the present study (i.e. DC, SPM, and total OSPW) altered rates of rolling of mandibles of *D. magna*. Results of the study reported here are in contrast with those reported previously (Kirk, 1991), where a reduction in the rate of rolling of the mandible in response to exposure to 50 mg/L particles was observed. However, the results are consistent with results of previous studies (Lari et al., 2017e) that suggested that particulate matter in the surrounding environment is less likely to affect rates of rolling of mandibles. Intake of particles by *Daphnia* spp. and consequently mandible rolling is directly proportional to concentrations of particles, unless a maximum rate of intake is reached (Smirnov, 2013). For instance, adding algal cells to water increased mandible rolling in *D. magna*; on the other hand, increasing the concentration of algae from 0.5 to 5 million cells/L did not change mandible rolling (Lari et al., 2017e).

Daphnia spp. select particles based on size (Burns, 1968; Geller and Müller, 1981) and probably electric charge (Gerritsen and Porter, 1982). *Daphnia magna* even ingest silver nanoparticles, which exert their toxicity immediately (Asghari et al., 2012). In response to a larger size (Webster and Peters, 1978) or abundance (Porter et al., 1982) of particles, *Daphnia* increase frequency of post-abdominal rejection. Frequency of post-abdominal rejection did not vary by experimental treatment, suggesting that particles in treatments were within the size and abundance limits of *D. magna* for ingesting particles. However, the presence of particles in the medium increases the frequency of post-abdominal rejection in *Daphnia pulex* (see Kirk, 1991). EDX measurements of contents of guts found Al and Si (main elements of soil) in the gut of *D. magna* exposed to SPM or total OSPW, which suggests that *D. magna* ingested SPM. Results of EDX and post-abdominal rejection tests along with the studies mentioned above that suggest low selectivity of *D. magna*, corroborate the result of the mandible rolling test that showed no reduction in response to the particulate matter.

In *Daphnia* spp., peristalsis of the gut controls transportation of food (Smirnov, 2013). A strong reverse peristalsis was first observed in 1925 in *D. magna* that were fed (Rankin, 1925). These reverse peristaltic waves originate as weak waves at the rectal end, and their intensity increases as the waves travel through, and vanish in the middle of the gut (personal observation). Peristaltic activity mixes contents of the gut with digestive enzymes and keeps ingested substances in the gut for a sufficient period to allow for digestion and nutrient uptake to take place. Results of studies of peristalsis showed that the presence of DC inhibits peristaltic activity while SPM and total OSPW did not. The reason that DC and total OSPW act differently toward suppressing peristalsis is not clear. A possible

explanation might be binding of toxicants in DC with SPM; however, further investigation is required. Reduction of peristaltic wave frequency reduces the efficiency of the gut in mixing its contents and decreases the clearance time, which is already short (31-35 min) (Esipova, 1971 reviewed in Smirnow, 2013) in *D. magna*.

Several contaminants can suppress activities of digestive enzymes of *Daphnia* (De Coen and Janssen, 1997; Zellmer et al., 2006; Houde et al., 2013). The two enzymes investigated in the present study (i.e. amylase and trypsin) are major digestive enzymes in guts of *Daphnia* (Agrawal et al., 2005; Houde et al., 2013). Activity of trypsin was reduced in *D. magna* exposed to OSPW, however, neither amylase nor trypsin was changed in response to exposure of *D. magna* to DC or SPM, which suggests that these two components of OSPW were not toxic to digestive enzyme activity in *D. magna* at concentrations in OSPW studied.

Concentrations of intact, undigested, algal cells in hindguts of *D. magna* from treatments were 11.5- to 23-fold greater than that of the control group. Intact algal cells were observed in feces of *D. magna* from all three experimental treatments, but were rare to non-existent in the controls. Since the feces was not pelletized, the excreted intact algal cells were suspended in the water again. Results of the two studies of algae in the hindgut and feces of *D. magna* clearly illustrate that both particulate and dissolved components of OSPW diminish the efficiency of the digestive system of *D. magna*. Particulate matter in OSPW does not paralyze the digestive system of *D. magna*. However, SPM becomes the dominant type of particle in the water and consequently dominant type of particle that *D. magna* ingested. It also increased total concentration of particulate matter, including food and mineral particulate matter in water. In this situation, SPM is the main type of particle

that *D. magna* ingested which filled the lumen of the gut, reducing the available space for food. Consequently, food algae made up a small portion of the material that *D. magna* ingested, which, in turn, resulted in faster clearance time because of reduced peristaltic activity. When *Daphnia* spp. are exposed to high concentrations of particles, they display luxury feeding behaviour. In this situation, they ingest a high quantity of particles, but less of the food gets digested. Moreover, reduced peristaltic activity and faster clearance time reduce time of contact of undigested food with digestive enzymes, thereby reducing digestive efficiency.

Although both SPM and DC of OSPW reduced consumption of food by *D. magna,* the inhibitory effect of the SPM was significantly greater than that of DC, suggesting that filtering SPM from OSPW may considerably reduce inhibitory effects of OSPW on the feeding of *D. magna* and perhaps other filter-feeding zooplanktons. The results of the mechanistic survey illustrate that neither SPM nor DC impair the food intake but impair the digestion of the ingested food. The mechanisms by which they impair the effective digestion of food in *D. magna* are different but both result in excreting undigested food.

CHAPTER 5: Effects of oil sands process-affected water on the respiratory and circulatory system of *Daphnia magna* Straus, 1820[5]

Abstract

Millions of cubic meters of oil sands process-affected water (OSPW), the major by-product of oil sand surface mining, is currently stored in tailings ponds. The present study investigated the effects of OSPW on the respiratory and circulatory system of Daphnia magna Straus 1820. The effect of OSPW on the activity (i.e. total movement and active time) of D. magna was also studied, as it has been shown to interact with the respiratory and circulatory system. Daphniids were exposed to both 1 and 10% OSPW for acute (1-day) and chronic (10-day) exposure periods. At the end of the exposures, daphniid oxygen (O2) consumption, heart rate, hemoglobin (Hb) content and activity were investigated. In response to chronic exposure to 10% OSPW, O2 consumption of D. magna increased, while the hemoglobin content and activity were reduced in both 1 and 10% OSPW. None of the OSPW treatments changed the heart rate of the test organisms. The results of the present study suggest that in response to increasing metabolic rate caused by OSPW exposure, D. magna conserve their energy by reducing their activity and probably by recycling macromolecules (i.e. hemoglobin).

[5] A version of this chapter is published in *Science of the Total Environment*, 605-606, Lari, E., Mohaddes, E., Pyle, G.G., Effects of oil sands process-affected water on the respiratory and circulatory system of *Daphnia magna* Straus, 1820, 824–829, 2017, with permission from Elsevier.

5.1. Introduction

Currently, 2.29 million barrels of crude oil per day are produced from the oil sands operations in Northern Alberta, Canada. It is estimated that this number will reach 3.95 million barrels per day by 2030 (CAPP, 2015). The Clark hot water extraction process is used during strip mining, which extracts crude oil from the sand and clay matrix using hot water (Kavanagh et al., 2009). Oil sands process-affected water (OSPW) is a major by-product of the Clark method, which is then stored in massive tailings ponds on-site until it can be recycled for other industrial processes (RSC, 2010).

In response to increasing concerns about potential environmental impacts of oil sand extraction, the Alberta Energy Regulator (AER) requires oil sands operators to minimise the volume of their fluid tailings within the range of the "end of mine life target." The AER also mandates that the operators make all the tailing areas ready to reclaim within ten years of the end of the mine's life (Government of Alberta, 2015). To fulfil these mandates, the operating companies in collaboration with academia are working towards reducing the amount of water used in extraction processes, understanding the toxic effects of OSPW, and its remediation.

The complex composition of OSPW makes it difficult to characterise all of its components fully. However, it is well known that hydrocarbons, mainly naphthenic acids (NA) and oxidized NAs and related organic acids with nitrogen and sulfur and metals are the major chemical components of OSPW (Lengger et al., 2013; Rowland et al., 2014). A significant amount of suspended particulate matter (SPM) also exists in OSPW (Liang et al. 2014). The dissolved organic fraction of OSPW is commonly recognized as the primary

driver of toxicity (Klamerth et al., 2015); however, other components such as SPM might also contribute significantly towards OSPW toxicity to some species (Lari et al., 2017d).

Several studies have investigated the effects of OSPW and its components – NAs in particular – on aquatic organisms. Although OSPW is not highly toxic to the survival of both planktonic animals (Wiseman et al., 2013a; Lari et al., 2016b), it may cause sub-lethal toxicity to aquatic animals. For example, OSPW causes oxidative stress (Wiseman et al., 2013a), hormone disruption (He et al., 2011; He et al., 2012a), feeding impairment (Lari et al., 2016b), growth reduction and reproduction impairment (Anderson et al. 2012; Lari et al., 2016b).

The sub-lethal effect of OSPW on some aspects of aquatic animals' lives is not well understood. For instance, the effect of OSPW on the development of the embryonic fish heart has been described (He et al., 2012a; Marentette et al., 2015). However, OSPW effects on cardiovascular systems and circulatory systems of non-embryonic fish and other aquatic animals remains unknown. Data on the effect of OSPW on the respiratory system of aquatic animals is also scarce. Oxygen (O_2) plays an essential role in providing energy for metabolic pathways and therefore is a direct indicator of the metabolic status of animals (De Coen and Janssen, 2003). Deviation from normal metabolic rate may increase the cost of living for individuals and eventually translate to changes at the population level. Respiratory and circulatory systems are the main agents for providing and transporting the O_2 required for maintaining cellular homoeostasis.

Like other animals, impairment of circulation and respiration might reduce survival in *Daphnia* spp. by reducing their ability to maintain cellular homeostasis and by increasing energy demand. The aim of the present study was: (1) To investigate the shift in metabolic

rate in response to OSPW exposure by measuring O_2 consumption. (2) To investigate the activity of principle systems – the circulatory and respiratory systems – involved in transporting gasses and chemicals in *D. magna* in order to illustrate the physiological changes by which *D. magna* adapt to changes in their metabolic rate.

5.2. Materials and methods

5.2.1. Test animals and chemicals

Six-day-old *D. magna* were used for the present study, with animals being cultured for over two years under laboratory conditions, as described in Lari et al. (2016). The culture was originally purchased from Carolina Biological Supply (Burlington, USA). A reference mortality test with sodium chloride (NaCl), as required by the Canadian Environmental Protection Service biological test method (Environment Canada, 1996), was performed every month to monitor the health and sensitivity of the culture. The culture showed normal response to NaCl during the course of the present study. An equal proportion (v/v) mixture of three OSPWs, provided by three major oil sands companies in the Athabasca region of northern Alberta, Canada, was used for the present study. These three OSPW samples were neither different in their toxicity to *D. magna*, nor was there a clear difference in their chemical properties (Lari et al., 2016b). The chemical properties (i.e. hydrocarbons and metals) of the three OSPW mixture was analysed and reported in Lari et al. (2017d) (Table 2.1; Figure 4.1). The OSPW mixture stock was stored in 20 L high-density polyethylene (HDPE) buckets at 4 °C, prior to use.

5.2.2. Exposures

For all toxicological endpoints tested, three groups of daphniids were exposed to each of 1 and 10% OSPW plus a culture water control, for one or ten days. The OSPW concentrations were chosen based on the results of Lari et al. (2016b), Because the median lethal concentration (LC50) was beyond 100% OSPW, the results of a sub-lethal endpoint (feeding behaviour) were used to determine OSPW exposure concentrations for the present study. A high and a low toxic effect concentration, 10% and 1% of OSPW were chosen for the present study. The former concentration inhibited the feeding behaviour of *D. magna* by 75% (IC75) and the latter was the lowest concentration that caused a significant inhibition in feeding behaviour (Lari et al., 2016b). All exposures were conducted in 250 mL glass tall-form beakers filled with 200 mL of the test solution and covered with transparent plastic throughout the duration of the exposure. Five daphniids were placed in each exposure replicates and fed with a fixed ration (5×10^5 cells/mL) of *Raphidocelis subcapitata* food. During the 10-day exposures, 80% of the exposure solution of the test beakers was renewed, and the neonates (i.e. newborn daphniids) were removed daily.

5.2.3. Oxygen consumption assay

Oxygen consumption of *D. magna* was measured in 10 mL glass chambers with tightly sealed lids. An O_2 sensor spot (PreSens, Germany) was placed in the chambers. In order to measure the O_2 consumption of *D. magna,* the chamber was filled with the same solution to which daphniids were exposed. Five or ten daphniids from chronic or acute exposures, respectively, were placed in the chamber and the lid of the chamber was sealed. Fewer *D. magna* were used in the chronic test to keep the volume/mass proportion equal between the

two exposure time points. Using a polymer optical fiber (PreSens, Germany) connected to an optical O_2 meter (Fibox 3, PreSens, Germany), the O_2 content of the solution was measured immediately after sealing the lid of the chamber and again 30 minutes later. Oxygen consumption was determined by subtracting the final reading from the initial one. The oxygen consumption in each replicate was corrected to the wet mass of *D. magna* in the chamber. To account for non-daphniid (i.e. microbial and organic material) oxygen consumption in each test solution, oxygen consumption was measured in exposure solution samples (1 and 10% OSPW and culture water) without daphniids as described above. To avoid the variance from empty or filled brood pouch, the few individuals with empty brood chamber were eliminated.

5.2.4. Heart rate assay

The heart rate of *D. magna* was measured using a bioassay described in Lari et al. (2017e). Briefly, a daphniid was mounted inside a test chamber filled with the exposure solution. To keep the O_2 content of the chamber constant throughout the test, a continuous flow of 1 mL/min of the exposure solution was maintained throughout the test. The daphniid was conditioned in the test chamber for 10 min. Afterward, using a digital camera (FDK 23UP1300, Imaging Source, Germany) with a macro zoom lens (0.3~1X 1:45; MLM3X-MP, Computar, Japan), the daphniid was recorded for 30 seconds at 60 frames per second (FPS). The heart rate was measured by manually reviewing the recorded videos in slow motion at 30% of normal speed with VLC media player (VideoLAN organization, France).

5.2.5. Hemoglobin assay

The hemoglobin (Hb) content of *D. magna* was measured by adapting a method described previously by Weider and Lampert (1985). In each trial, 20 or 10 daphniids from 1- or 10-day exposures, respectively, were placed in a 1.5 mL plastic microcentrifuge tube (Fisher Scientific, Canada) containing 50 µL of distilled deionized water (ddH_2O). Using a tissue grinder (mortar and pestle) daphniids were macerated and homogenised with 200 µL of ddH_2O and centrifuged at 4 °C for five minutes at 12,000 rpm in a refrigerated centrifuge (5804 R, Eppendorf, Germany). A 40 µL sample of the supernatant was placed in a cell of a 96-well plate (Thermo Fisher Scientific, USA) and 160 µL of 0.005% KCN (Sigma-Aldrich, USA) was added to the cell. The plate was placed in a microplate reader (Varioskan Flash, Thermo Scientific, Finland) and gently shaken for 5 min. Afterwards, Hb absorption was read at 415 nm (Weider and Lampert, 1985; (Muyssen et al., 2010). An absorption curve from a dilution series of 0.01 to 10% bovine Hb (Sigma-Aldrich, USA) was used to standardize the absorption values of the samples. Hemoglobin values of each sample were weight corrected and converted to mg Hb/g *D. magna*.

5.2.6. Motion activity assay

The behavioural setup that was used for measuring the motion activity of *D. magna* consisted of a light table, a testing arena, and a digital camera. An opaque polystyrene weighing dish (4.5 × 4.5 cm; Fisher Scientific, USA) was used as the testing arena. To prevent the water surface glare interrupting the video recording, the test arena was placed on a light table (15 × 15 cm), and the room lighting was minimal (zero lux). The test arena was filled (1 cm height) with the exposure solution, and a daphniid was gently placed in

the test arena. The daphniid was conditioned in the test chamber for 10 min. Afterwards, the daphniid was digitally recorded for 1 min. To quantify the time spent moving and distance moved, videos were analyzed using LoliTrack 4 video tracking software (Loligo Systems, Denmark).

5.2.7. Data analysis

Using Shapiro-Wilk's test, experimental data were tested for the parametric assumption of normality. Homogeneity of variance was determined by a Levene's test. To test for the significance of the effect of OSPW and exposure time along with their interaction on the studied endpoints (oxygen consumption, heart rate, hemoglobin content, time spent moving, and distance moved) a two-way ANOVA test was performed. In all cases, the ANOVA test was followed by a Dunnett's post hoc analysis. All statistics were calculated using IBM SPSS 22 software.

5.3. Results

5.3.1. Oxygen consumption

For oxygen consumption, the interaction between the exposure time and OSPW treatments was significant [$F(2, 54) = 3.74$, $p = 0.03$; Figure 5.1; Appendix 5.1], which showed that increasing the exposure time elevates the effect of OSPW on *D. magna* oxygen consumption. A 1-day exposure to either of the OSPW treatments (1 and 10% OSPW) did not affect the O_2 consumption of *D. magna*. However, in the 10-day exposure, O_2 consumption of daphniids exposed to 10% OSPW increased by 44% as compared to the

control group. However, despite the 19% increase, the O_2 consumption of the daphniids in 1% OSPW treatment was not statistically different from the control group.

5.3.2. Heart rate

The heart rate of *D. magna* varied among the exposure groups [i.e. 1 and 10% OSPW and control in 1- and 10-day exposures; $F (5, 54) = 6.26$, $p < 0.001$; Appendix 5.2]. However, the observed difference was not due to the OSPW exposures, because the average heart rate in all exposures were similar [$F (2, 54) = 0.65$, $p = 0.53$; Figure 5.2; Appendix 5.2]. The observed difference in heart rate was driven by daphniid ageing during 10-day exposure [$F (1, 54) = 27.16$, $p < 0.001$; Appendix 5.2], because daphniids from all three groups (1 and 10% OSPW and control) at 10-day exposure showed a significant reduction in heart rate (average: 24%) as compared to 1-day exposure. There was no interaction between the effect of exposure time and OSPW treatments [$F (2, 54) = 1.44$, $p = 0.25$; Appendix 5.2].

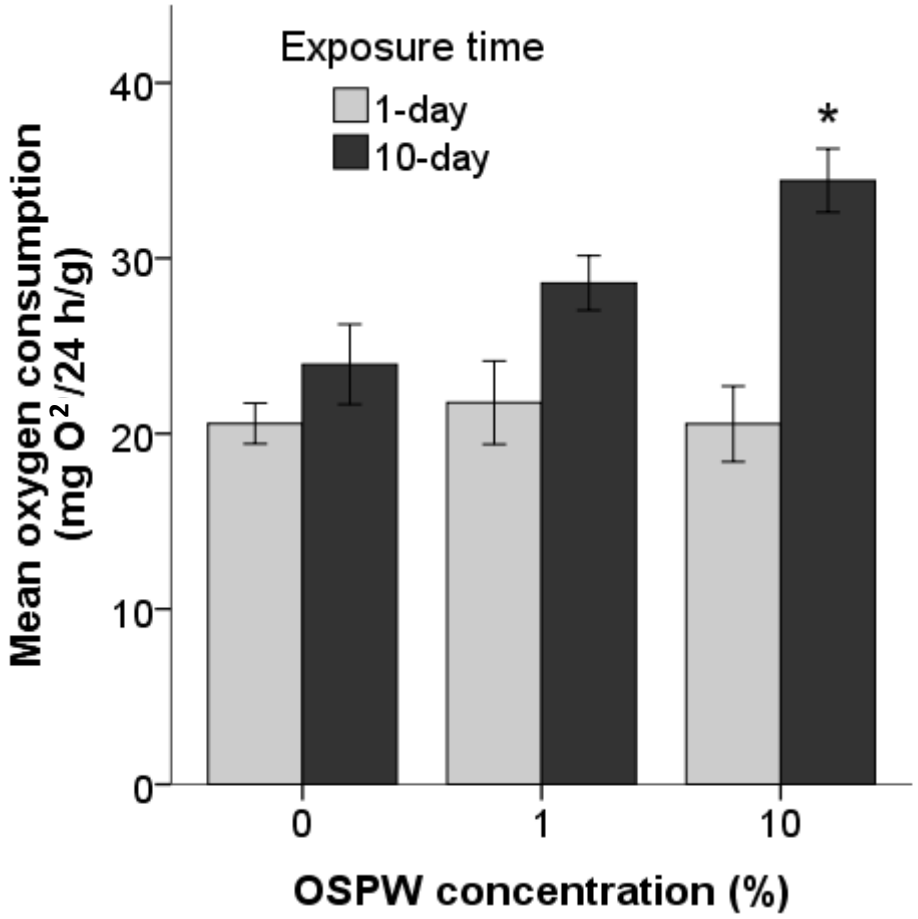


Figure 5.1. Effect of oil sands process-affected water (OSPW) on oxygen consumption of *Daphnia magna*. The asterisk (*) show a significant difference ($p \leq 0.05$) from the control group; n = 10, error bars: ± 1 SE.

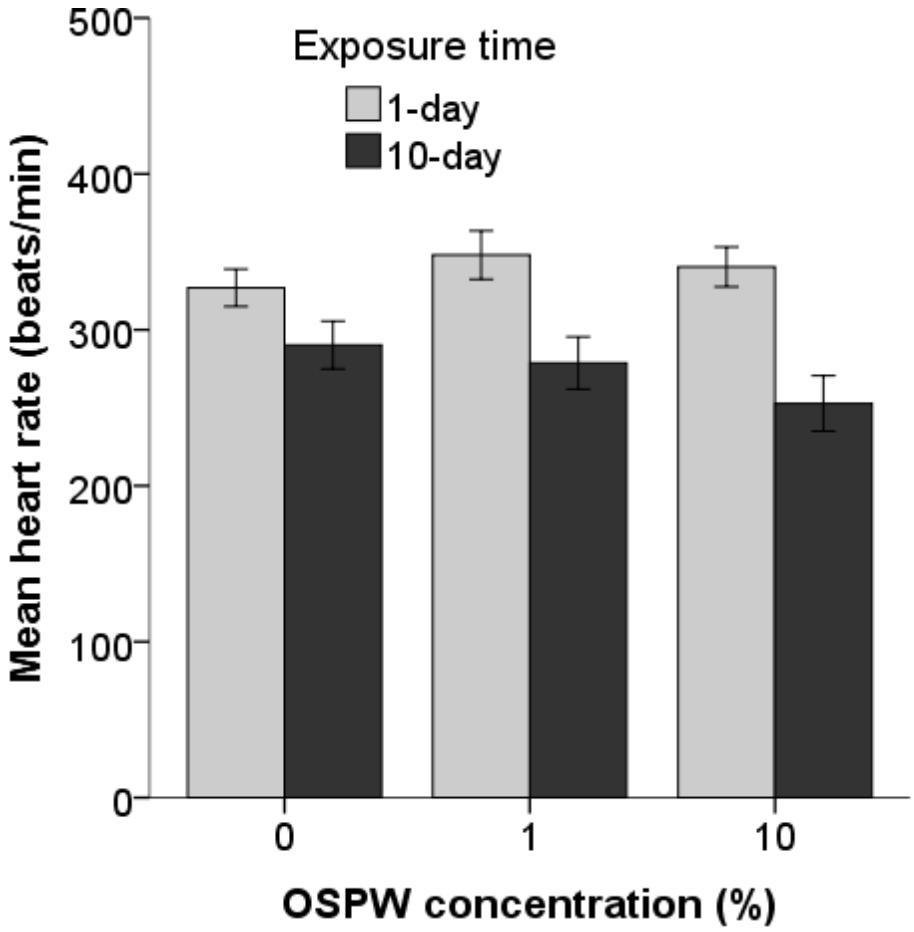


Figure 5.2. Effect of oil sands process-affected water (OSPW) on the heart rate of *Daphnia magna*; n = 10, error bars: ± 1 SE.

5.3.3. Hemoglobin

The Hb content of *D. magna* was affected by OSPW exposures (1 and 10%) as compared to the control [F (2, 24) = 4.28, p = 0.03; Figure 5.3; Appendix 5.3]. No difference was observed between the Hb content of *D. magna* exposed to 1 or 10% OSPW and the control group after 1-day exposure (Figure 5.3). On the other hand, the Hb content of *D. magna* exposed to both 1 and 10% OSPW was 18 and 26% lower, respectively, than the control in 10-day exposures (Figure 5.3). Although the difference between the control and OSPW exposures was only observed during the 10-day exposure, not the 1-day exposure, the interaction between the effect of exposure time and OSPW treatments was not significant [F (2, 24) = 2.71, p = 0.09; Appendix 5.3]. It appeared that only a chronic exposure to OSPW affected the hemoglobin content of *D. magna*.

5.3.4. Motion activity

The time spent moving in *D. magna* exposed to OSPW declined in both the 1- and 10-day exposures [F (2, 114) = 28.41, p < 0.001; Figure 5.4; Appendix 5.4]. The 1% OSPW treatment did not alter the time spent moving of *D. magna*, while 10% OSPW reduced activity in both 1-day and 10-day exposures by 24% and 49%, respectively (Figure 5.4). No interaction between the effect of OSPW and exposure time on the time spent moving was observed [F (2, 114) = 2.60, p = 0.08; Appendix 5.4].

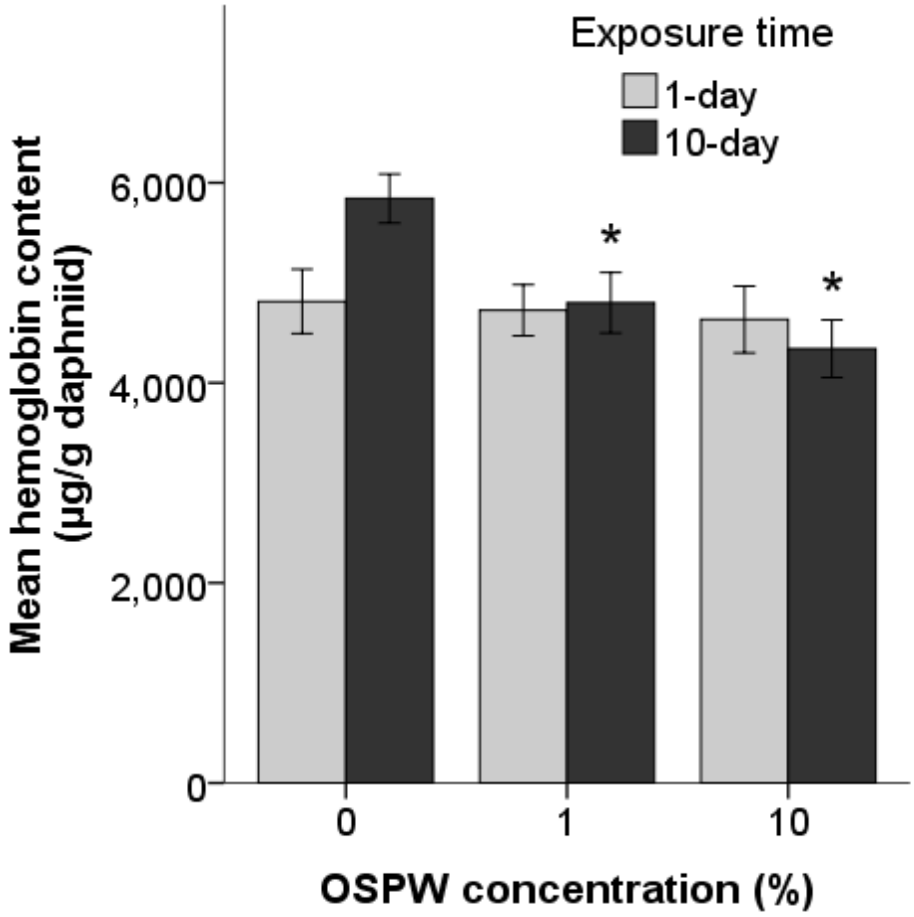


Figure 5.3. Effect of oil sands process-affected water (OSPW) on hemoglobin content of *Daphnia magna*. Asterisks (*) show a significant difference ($p \leq 0.05$) from the control group. n = 5, error bars: ± 1 SE

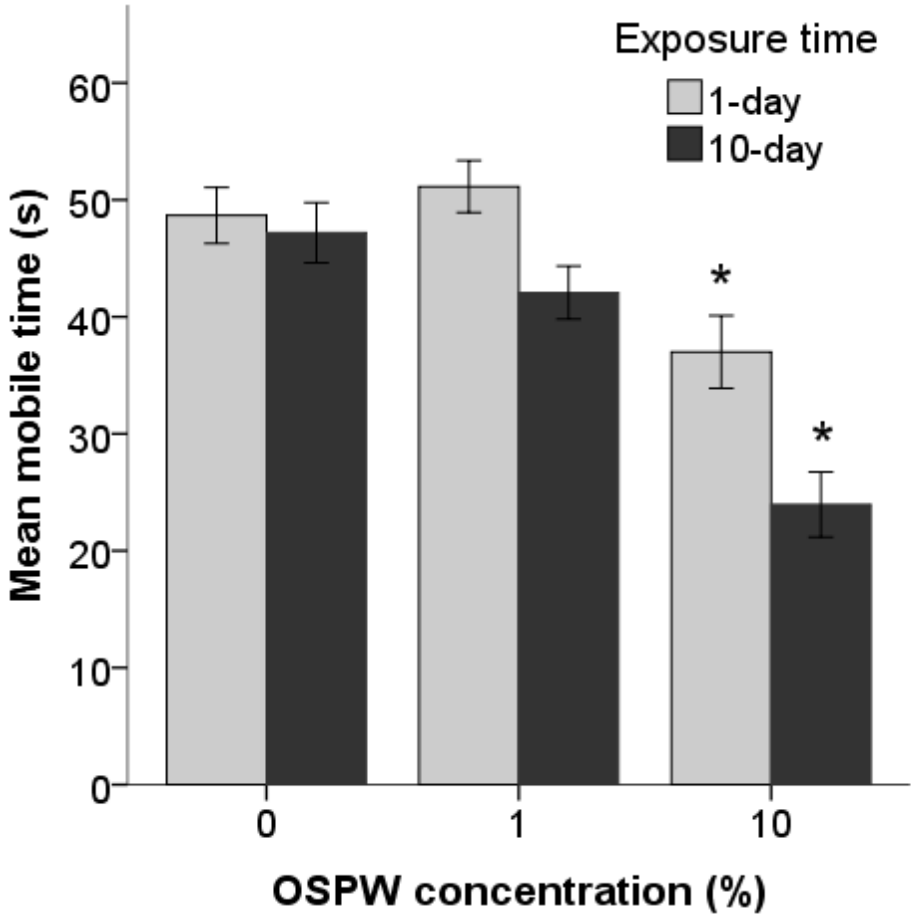


Figure 5.4. Effect of OSPW on the time spent moving of *Daphnia magna*. Asterisks (*) show a significant difference ($p \leq 0.05$) from the control group; n=20, error bars: ± 1 SE.

Both the 1% and 10% OSPW treatments reduced the distance moved by *D. magna* compared to the control [$F(2, 114) = 15.22$, $p < 0.001$; Figure 5.5; Appendix 5.4]. In the 1% OSPW treatment, a reduction of the distance moved by *D. magna* was observed only after ten days of exposure, whereas in the 10% OSPW treatment, a reduction of the distance moved was observed at both time points (Figure 5.5). No interaction between the effect of OSPW and exposure time on the distance moved was observed [$F(2, 114) = 1.57$, $p = 0.21$; Appendix 5.4].

5.4. Discussion

Oxygen consumption is an important endpoint in physiological and toxicological studies because it reflects the metabolic status of animals. In *Daphnia* spp. oxygen consumption has frequently been used to investigate the effect of changes in ecological factors such as food and O_2 concentration, and temperature (Armitage and Lei, 1979; Kobayashi, 1981; Bohrer and Lampert, 1988; Lamkemeyer et al., 2003). Oxygen consumption has also been used to investigate sub-lethal effects of contaminants (Muyssen et al., 2006). In most of these previous studies, O_2 consumption was directly measured (Barber et al., 1990, 1994; Lamkemeyer et al., 2003), while electron transport chain activity was determined as an indirect measurement of respiration in other studies (De Coen and Janssen, 1997, 2003).

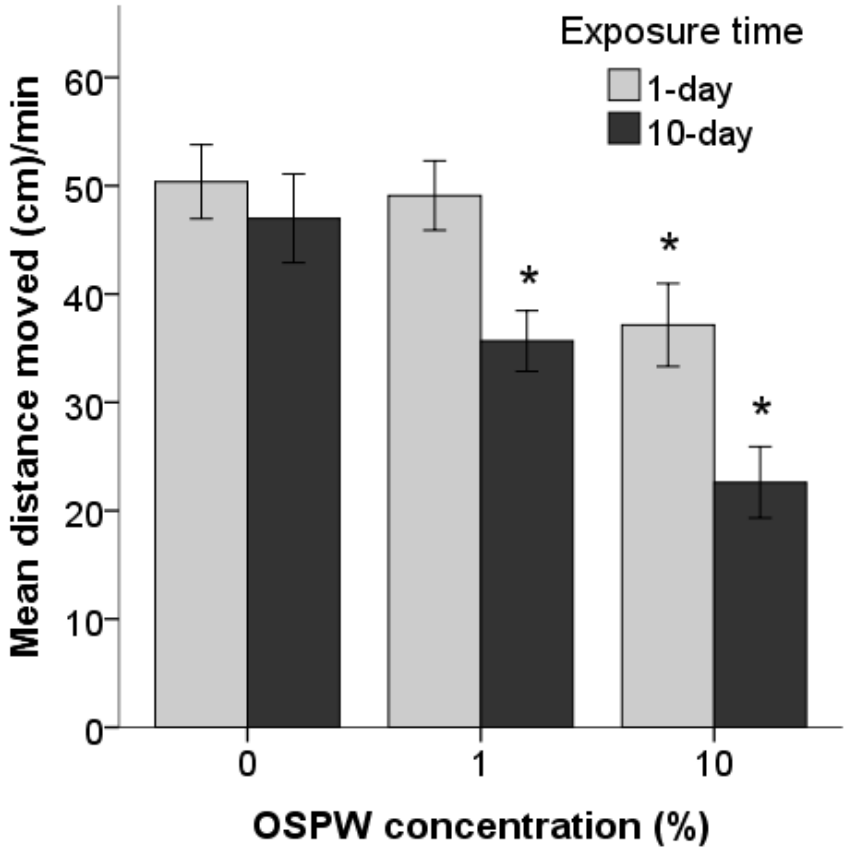


Figure 5.5. Effect of OSPW on the total activity of *Daphnia magna*. Asterisks (*) show a significant difference ($p \leq 0.05$) from the control group; n=20, error bars: ± 1 SE.

The results of the present study showed that in the acute exposure to OSPW, the concentrations of OSPW applied did not change the O_2 consumption in *D. magna*, but chronic exposure to higher OSPW concentrations (10% OSPW) increases the O_2 consumption in *D. magna*. In a study by Geiger and Buikema-Jr. (1981), 12 h exposure to water-soluble fractions of four petroleum derivatives (naphthalene, phenanthrene, No. 2 fuel oil, and coal-tar creosote) did not change the O_2 consumption of *Daphnia pulex* Leydig. It appears that in response to short-term exposure to oil derivatives, *Daphnia* spp. can maintain their metabolic rate by compensating for the energy spent for detoxification. In this regard, OSPW acts similarly to some other contaminants such as cadmium (Barber et al., 1990). However, by measuring metabolic rate using measures of electron transport chain activity, De Coen and Janson (2003) showed that a 96 h exposure to cadmium (Cd) increased the O_2 consumption at 8 µg/L Cd, but reduced the O_2 consumption in *D. magna* at higher concentrations (> 28 µg/L Cd). Results from the present study and those of previous studies suggest that in response to acute exposure to low concentrations of contaminants, *Daphnia* manage to maintain a normal metabolic rate by downregulating some of their less crucial activities such as mobility. However, when exposure continues, these physiological adjustments cannot cope with the increased energy demand.

The activity of daphniids exposed to 10% OSPW declined as early as 24 h into exposure. This result concurs with the results of a recent study with the same OSPW concentration and clone of *D. magna* that reported a reduction of activity in individuals exposed to 10% and higher concentrations of OSPW (Lari et al., 2016b). Food consumption in *D. magna* was demonstrated to be reduced at 50 and 75% in 5.3 and 10% OSPW, respectively (Lari et al., 2016b), and consequently reduces their energy supply. At the same

time, the stress caused by OSPW will increase the demand for energy. Considering the reduction in energy supply and increase in demand, it appears that the reduction in activity is an adaptive response to compensate for the increasing demand for energy. Reduced mobility might be one of the mechanisms by which *D. magna* managed to maintain their normal oxygen consumption after 24 h exposure to OSPW. In the long term, however, the energy compensating strategies such as reducing mobility cannot sustain the normal metabolic rate.

Although the demand for O_2 increased, the 10-day exposure to both 1 and 10% OSPW reduced the Hb content of *D. magna*. According to Moenickes et al. (2010), at the O_2 concentrations measured in the test solutions during the exposures to OSPW in the present study (~8 mg/L) and much lower concentrations (~5.5 mg/L), Hb plays no role in O_2 transportation in *D. magna*. At these concentrations – because of the small size of organizms and the fact that the pO_2 in daphniid's body is much lower than the surrounding water (Waterman, 1960) – the first two main mechanisms of O_2 transport (diffusive and convective) are able to efficiently deliver the required O_2 to the tissues (Moenickes et al., 2010). Only under extreme O_2 hypoxic conditions will the Hb content in *Daphnia* increase (Kobayashi, 1981; Smirnov, 2013). Hence, when physiological detoxification mechanisms increase the energetic demand and sufficient DO allows for efficient respiration without the need for elevated Hb. Additionally, it may be that Hb is not only produced as a respiratory protein but also as a form of protein storage (Schwerin et al. 2010b). We know that exposure to OSPW impairs food consumption in *D. magna* (Lari et al. 2016b), and the lack of food inhibits the production of Hb (Schwerin et al. 2010b). Therefore, downregulating the production of Hb in response to reduced energetic input and increased

energy demand might be a mechanism that reduces the Hb content of *D. magna* exposed to OSPW. The other mechanism by which the Hb of daphniids exposed to OSPW was reduced might be from the breakdown of stored Hb to provide amino acids for protein synthesis. However, further investigation is required to investigate these hypotheses concerning the reduction of Hb content of *D. magna* in response to OSPW exposure. In a recent study Lari et al. (2017c) demonstrated that the energy reserves of *D. magna* declined following chronic (10-day) exposure to 10% OSPW. Although protein content per mg dry weight was not reduced, the OSPW exposed animals produced less protein, as their average mass was significantly lower (35%) than the control.

Life stage factors such as age, maturity, and the presence and number of eggs in the brood pouch affect the Hb content of *D. magna* (Kobayashi and Nezu, 1986). If a contaminant affects any of the above, it might indirectly influence the Hb content of *D. magna*. Therefore, reduced clutch size in response to chronic (10 and 21-day) exposure to OSPW (Lari et al., 2016b; Lari et al., 2017c) may have contributed to the observed reduction of Hb. may have contributed to the observed reduction of Hb. However, since other factors (i.e. age, maturity, the number of broods) were similar to the control (Lari et al., 2017c), the effect of life stage factors should not be high.

There is an overwhelming number of studies on the effect of various contaminants on the heart rate of *Daphnia* (Villegas-Navarro et al., 2003; Dzialowski et al., 2006; Jeong et al., 2015; earlier studies are reviewed in Smirnov 2013). However, data on the effect of a common family of pollutants (i.e. petroleum derivatives) on heart rate are limited. Despite the significant increase in the demand for O_2 in 10% OSPW exposure, OSPW treatments in the present study did not change the heart rate of *D. magna* regardless of concentration

or exposure period. Increasing the O_2 transport via a diffusive pathway or increasing the movement of the limbs may partly compensate for not increasing the heart rate. Under normoxic conditions and normal O_2 demand, 50-60% of O_2 transport is through the non-circulatory (diffusive) pathway (Moenickes et al., 2010). Moreover, the movement of hemolymph is powered by both the heart and appendages (reviewed in Smirnov 2013). When food is limited in supply, *Daphnia* reduces its heart rate and increase the beating frequency of its limbs to regulate food and O_2 uptake (Pirow et al., 1999a; Pirow and Buchen, 2004). Paul et al. (1998) showed that even in anoxia, *D. magna* is able to maintain O_2 supply to its tissues. It appears that *D. magna* is equipped with different mechanisms that allow it to regulate its O_2 transfer without putting extra pressure on its heart, especially in the case of an energy shortage.

In summary, the results of the present study suggest that OSPW exposure imposes a metabolic burden on *D. magna*, which is manifested as an increase in O_2 consumption. This extra burden along with a shortage in energy uptake, due to feeding impairment (Lari et al., 2016b), obviates the need for establishing energy saving strategies, such as reducing mobility and recycling surplus macromolecules. It is very likely that these effects on energy intake and demand are the main causes of the observed reduction in energy reserves, fecundity and growth of *D. magna* in response to chronic exposure to OSPW (Lari et al., 2017c), which itself might translate to a shift in *D. magna* population. These effects might induce a *Daphnia* population and ecosystem disruption in OSPW containing waters. Conducting multigeneration and microcosm studies will illustrate the ecological consequences of the observed effects.

CHAPTER 6: Investigating the chronic effects of oil sands process-affected water on growth and fitness of *Daphnia magna* Straus 1820[6]

Abstract

The increasing amount of stored oil sands process-affected water (OSPW), a primary by-product of oil sands mining, is an environmental concern. In the present study, we investigated the chronic effects of OSPW on growth, reproduction, and macronutrient content in *Daphnia magna*. To do so, we exposed *D. magna* to 1 and 10% OSPW (a mixture of three OSPW samples provided by major oil sands mining operators in northern Alberta) for ten days. We measured the number of the neonates produced daily in each group throughout the exposure. At the end of the exposure, we measured the mass and length of the exposed daphniids and neonates. We also measured the carbohydrate, lipid, and protein content of exposed daphniids. In the 10% OSPW group, we observed a significant reduction in all of the measured endpoints except for body length and carbohydrate and protein content of exposed daphniids. In the 1% OSPW group, on the other hand, we found a reduction only in lipid content of exposed daphniids as compared to the control group. The results of the present study demonstrated that chronic exposure to 10% OSPW affects growth and fitness of *D. magna*, probably due to a reduction in energy intake that causes daphniids to deplete their energy reserves.

[6] A version of this chapter is published in *Science of the Total Environment*, 595, Lari, E., Steinkey, D., Mohaddes, E., Pyle, G.G., Investigating the chronic effects of oil sands process-affected water on growth and fitness of *Daphnia magna* Straus 1820, 594-600, 2017, with permission from Elsevier.

6.1. Introduction

Operating on the largest known bitumen deposit in the world containing 26.4 billion cubic meters of crude bitumen (AER, 2015), oil sands companies in northern Alberta, Canada, plan to increase the current production of crude oil from 2.29 to 3.95 million barrels per day by 2030 (CAPP, 2015). The predominant method for producing crude oil in this region is open-pit mining of bitumen and extracting the oil from sand and clay using the Clark extraction process. The by-products of this method are called "tailings," which consist of immense amounts of sand, clay, silt, oil sands process-affected water (OSPW), and bitumen residue (AER, 2015). Oil sands operators currently store these tailings on site in massive tailings ponds (RSC, 2010). In 2013, according to the mine operators, the tailings ponds contained a total of approximately 976 million cubic meters of fluid tailings (Alberta Energy, 2016; Pramanik, 2016).

The current volume of stored OSPW and the expected increase in volume that will result from future production has raised concerns about the environment and human health in the region. The most recent regulatory document released by the Government of Alberta mandates oil sands operators "minimize" the production of liquid tailings during operation and treat the tailings so they can be reclaimed within ten years from mine closure (AER, 2015). Treating liquid tailings with the intention of reclamation requires a sound understanding of the toxic effects of OSPW on the organisms that may come into contact with it.

Oil sands process-affected water is a complex mixture of organic and inorganic components. Due to its complex composition, it is currently not feasible to fully characterize the components, especially the organic portion (Rowland et al., 2011; Rowland

et al., 2014). Nevertheless, based on similarities in chemical structure, the organic components can be grouped into quantifiable classes (Peng et al., 2016; Zhang et al., 2016). Naphthenic acids (NAs) and cyclic and acyclic alkyl-substituted carboxylic acids with the general formula of $C_nH_{2n+z}O_2$, are the predominant constituents of OSPW (Peng et al., 2016). Naphthenic acids were previously considered to be the primary contributor to the toxic effects of OSPW, but a recent study showed that other classes of organic compounds also contribute to its toxicity (Morandi et al., 2015).

Owing to the effort devoted to studying the effects of OSPW and its components on aquatic animals in the last decade, knowledge in this field is expanding. Current literature on the sub-lethal effects of OSPW on aquatic animals covers a variety of endpoints on a diverse group of organisms. Researchers have demonstrated that OSPW in aquatic animals disrupts hormone secretion (He et al., 2012a; Wiseman et al., 2013a) and gene transcription (Wiseman et al., 2013b; Peng et al., 2016), causes oxidative stress (Wiseman et al., 2013a), and impairs feeding, growth, and reproduction (Anderson et al., 2012a; Goff et al., 2013; Lari et al., 2016b) to name but a few of the numerous known effects.

Daphnia spp. are some of the most dominant metazooplanktons (metazoan planktonic organisms) in freshwater ecosystems (Jürgens and Jeppesen, 2000), and are recommended test organisms for both acute and chronic toxicological studies (EPA, 2002b, a). Researchers have used *Daphnia* spp. as a model species to investigate the toxicity of OSPW and NAs, primarily by documenting acute endpoints (Frank et al., 2009; Marentette et al., 2015; Swigert et al., 2015). As previously demonstrated, the lethal toxicity of OSPW on aquatic organisms usually does not occur at ecologically relevant concentrations (Wiseman

et al., 2013a; Lari et al., 2016b). Therefore, we decided to investigate the sub-lethal effects of OSPW on *Daphnia magna* in the present study.

Previously, we studied the effects of OSPW on feeding behaviour, reproduction, growth (Lari et al., 2016b), respiration, and circulation (Lari et al., in review) of *Daphnia magna*. We also investigated the mechanisms by which OSPW affected the feeding behaviour of *D. magna* (Lari et al., 2017d). These results suggested that OSPW affected both the energy intake and demand in *D. magna*. In the present study, we investigated the endpoints that are influenced by the availability of energy . Thus, we investigated the effects of chronic exposure to OSPW on reproduction and physical condition (length and mass) of exposed daphniids and their offspring. We also measured energy reserves, in the form of protein, carbohydrate, and lipid content of the test animals. Finally, we calculated the total biomass that daphniids produced during the exposure.

6.2. Materials and Methods

6.2.1. Test chemicals and animals

The OSPW samples were obtained from three major oil sands operations in the Athabasca region of northern Alberta. We previously observed that the three OSPW samples showed the same toxicity at both lethal and sub-lethal (affecting feeding, growth, and reproduction) levels in *D. magna*, as well as the same relative abundance of dissolved organic species (Lari et al., 2016b). In the present study, a mixture of all three OSPW samples in equal proportions (v/v) was used. The hydrocarbon and major trace metal content of the OSPW sample was measured and reported in (Lari et al., 2017d), presented in Table 2.1 and Figure 4.1. The OSPW mixture was stored in at 4 ºC.

The daphniids used in the present study came from a culture held successfully under lab conditions for more than two years as described in (Lari et al., 2016b). Using a recipe from Environment Canada (1996), the quality of the reconstituted water used for the culture and experiments was maintained consistently at the following levels: hardness, 90-100 mg/L as $CaCO_3$; alkalinity, 165-170 mg/L as $CaCO_3$; pH, 8.4-8.6; and conductivity, 480-500 µS/cm. Six-day-old (5th instar) daphniids were used for the experiments throughout the study. The microalga, *Raphidocelis subcapitata*, was fed to daphniids in the main culture and throughout the experiments.

6.2.2. Exposures

A total of 30 replicates of five daphniids were exposed to each of the following three treatments for ten days: 1 and 10% OSPW and culture water as a control. To do so, daphniids were randomly assigned to 250 mL glass beakers filled with 200 mL of the test solution, spiked with 5×10^5 cells/mL *R. subcapitata* and kept at room temperature (20 ± 1 °C) under full spectrum fluorescent light (750 lux; 16:8 h light:dark). Each day, 80% of the exposure solutions was replaced with freshly prepared exposure solution and the neonates were counted and removed. Upon exposure initiation, five replicates of ten daphniids were randomly collected from the same batch of daphniids that were used for the exposures and were measured for dry mass, as described in section 2.3; the measurement represented initial mass.

6.2.3. Growth and reproduction

To investigate the effect of OSPW on *D. magna* reproduction, we kept a record of the number of neonates produced in five replicates from each treatment (i.e. 1 and 10% OSPW,

and control). To assess the effect of OSPW on the physical condition of neonates, the length and mass of the previous day's neonates was measured, as follows. A picture of three randomly collected neonates from each test beaker was taken, using a digital camera (FDK 23UP1300, Imaging Source, Germany) with a macro zoom lens (0.3~1X 1:45; MLM3X-MP, Computar, Japan), and their length was measured with the image analysis program ImageJ (freeware: http://rsb.info.nih.gov/ij/). All the neonates (including the ones that were used for length measurement) of the previous day in each beaker were collected in a 1.5 mL, pre-weighed microcentrifuge tube (Eppendorf, USA). After rinsing with culture water, the samples were dried in an oven (Isotemp, Fisher, USA) at 60 °C for 48 h. The average dry mass of single neonates was calculated by dividing the final mass of the samples by the number of neonates in the tube.

In order to investigate the effect of OSPW on the growth of exposed *D. magna*, the length and dried mass of the adult daphniids from the same replicates were measured using the same method as described above in this section. The biomass produced by an individual daphniid was calculated by applying the following equation:

Biomass produced (mg/individual) = final dry mass (mg) - initial dry mass (mg) + (number of neonates × neonate mass (mg))

6.2.4. Energy reserves

The effect of OSPW on energy reserves of *D. magna* was evaluated by measuring the carbohydrate, protein, and lipid content of the exposed daphniids. The enthalpy of combustion ratios presented in (De Coen and Janssen, 2003) were used to convert the mass of macromolecules to total energy available from proteins (24 J/mg), lipids (39.5 J/mg),

and carbohydrates (17.5 J/mg). Carbohydrate content was measured using a Total Carbohydrate Assay Kit (catalog no. MAK104, Sigma, USA). The sample preparation procedure consisted of homogenizing 10 mg of *D. magna* in 200 µL of assay buffer, centrifuging (Eppendorf Germany, 5804 R) the sample at 5000 g for 10 min, and collecting the supernatant and adding it to a 96-well microplate in replicates of 30 µL. To these replicates, 150 µL of 18 M sulfuric acid (EMD USA, 97%) was added and then incubated at 90 ºC for 15 min. After incubation, 30 µL of the developer solution was added to each well and incubation continued at room temperature for 5 min. Absorbance was measured at 490 nm using a microplate reader (Varioskan Flash, Thermo, USA) and read against a glucose standard.

Protein content of exposed daphniids was analysed using a Pierce™ BCA Protein Assay Kit (catalog no. 23225, Thermo, USA). The sample preparation process involved homogenizing a 10 mg sample of daphniids in 50 µL of distilled water, centrifuging the samples at 5000 g for 10 min, and adding the supernatant to a 96-well microplate. We then added 200 µL of the provided working reagent to each well and incubated the samples at 37 ºC for 30 min. Absorbance was measured in a microplate reader at 562 nm against a bovine serum albumin standard.

Lipid content was analysed using a total lipid assay procedure (Bennett et al., 2007). Sample preparation for this technique required homogenizing 20 mg of daphniids in 50 µL of 0.2 M sodium-citrate (VWR USA, 7810, pH corrected to 5.0 with HCl), centrifuging the samples at 5000 g for 10 min, and adding them to a 96-well microplate. Each sample received 180 µL of free glycerol reagent (Sigma, USA) and 45 µL of triglyceride reagent

(Sigma, USA), and then incubated at 37 ºC for 5 min. Absorbance was measured at 540 nm against a glycerol standard.

6.2.5. Data analysis

Parametric assumptions were tested using Shapiro-Wilk and Levene tests. The effect of OSPW on mass and length of both neonates and exposed daphniids was analysed using multivariate analyses of variance (MANOVA), followed by serial univariate analyses (ANOVA) to investigate the effect of OSPW on each endpoint (i.e. length and mass). We analysed the data from the other endpoints (i.e. number of neonates, total biomass, and carbohydrate, protein and lipid content) using an ANOVA, followed by a Dunnett's post-hoc test. The parametric assumption of homogeneity of variance was violated in the total biomass data, necessitating the use of a non-parametric Welch's ANOVA. Statistical significance was set a priori at $\alpha = 0.05$. All analyses were conducted using IBM SPSS 22 software (IBM, USA).

6.3. Results

6.3.1. Reproduction

Results showed that chronic exposure to OSPW reduced the reproduction rate in *D. magna* [$F(2,14) = 5.14$, $p = 0.02$; Figure 6.1; Appendix 6.1]. Reproduction in daphniids exposed to 1% OSPW was not different from the control; nonetheless, daphniids in the 10% OSPW exposure showed a 39% reduction in their reproduction rate.

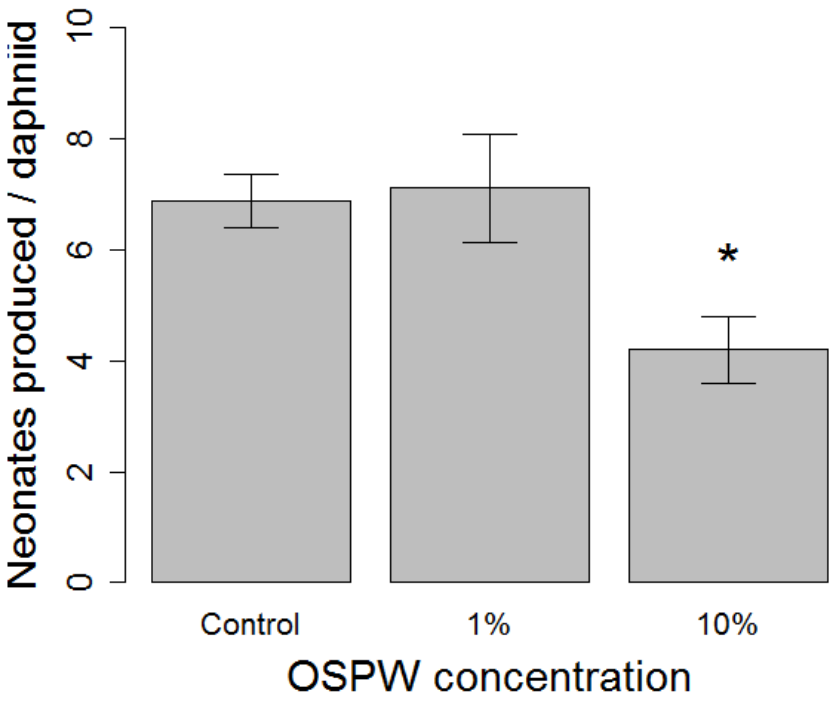


Figure 6.1. Reproduction (neonates produced/daphniid ± standard error) of *Daphnia magna* over 10 days of exposure to oil sands process-affected water (OSPW). Asterisks (*) denote significant differences between OSPW and control groups (n = 5).

Neonate condition (length and dry mass) was also affected by the OSPW exposures [Wilks' $\Lambda = 0.21$, $F(4,22) = 6.52$, $p < 0.01$, Appendix 6.1]. The neonates of the daphniids exposed to OSPW were shorter [$F(2,12) = 7.40$, $p < 0.01$; Figure 6.2] and weighed less [$F(2,12) = 13.0$, $p < 0.01$; Figure 6.2] than the control. However, the reduction in both length and dry mass was only significant in the 10% OSPW treatment, by 7 and 55% respectively.

6.3.2. Growth

Chronic exposure to OSPW affected the physical condition of adult *D. magna* [Wilks' $\Lambda = 0.40$, $F(4,22) = 3.17$, $p = 0.03$; Appendix 6.2]. The length of the daphniids in the OSPW treatments (1 and 10%) was not different from the control [$F(2,12) = 0.41$, $p = 0.67$; Figure 6.3; Appendix 6.2]. On the other hand, the dry mass of the daphniids in OSPW treatments was less than the control [$F(2,12) = 8.33$, $p < 0.01$; Figure 6.3; Appendix 6.2]. The reduction in the dry mass was only reduced significantly in the 10% OSPW exposure (35% reduction), but not in the 1% OSPW treatment.

The average dry mass of daphniids was 34 ± 4.2 µg at the beginning of the exposure. The differences among the groups' mass production was significant [$F(2,7.23) = 298.8$, $p < 0.001$; Figure 6.4; Appendix 6.2]. The mass produced in the 1% OSPW group was not significantly lower than the control. Nevertheless, the mass production in the 10% OSPW treatment declined by 58% relative to the control group (Figure 6.4).

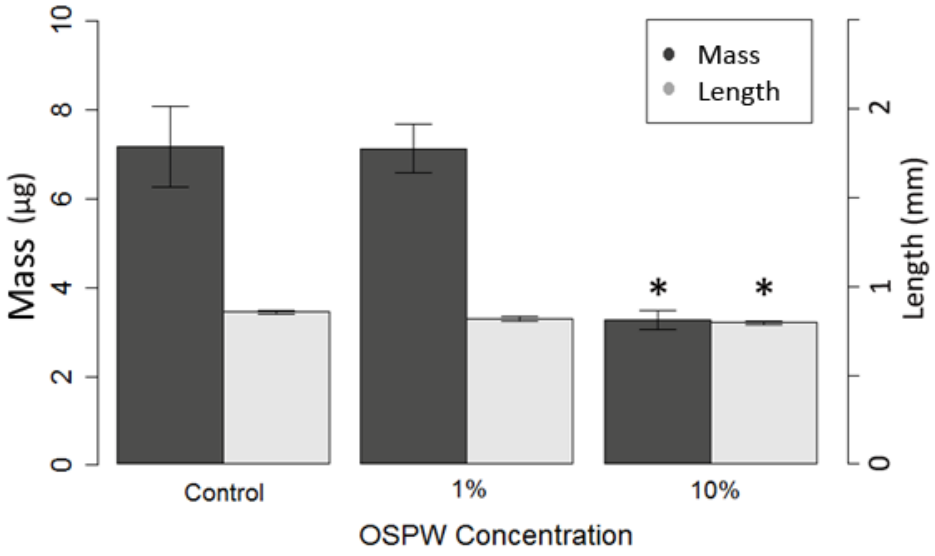


Figure 6.2. The size of offspring in both mass (µg ± standard error; n = 5) and length (mm ± standard error; n = 15) from *Daphnia magna* exposed to oil sands process-affected water (OSPW) for 10 days. Asterisks (*) denote significant differences between OSPW and control groups.

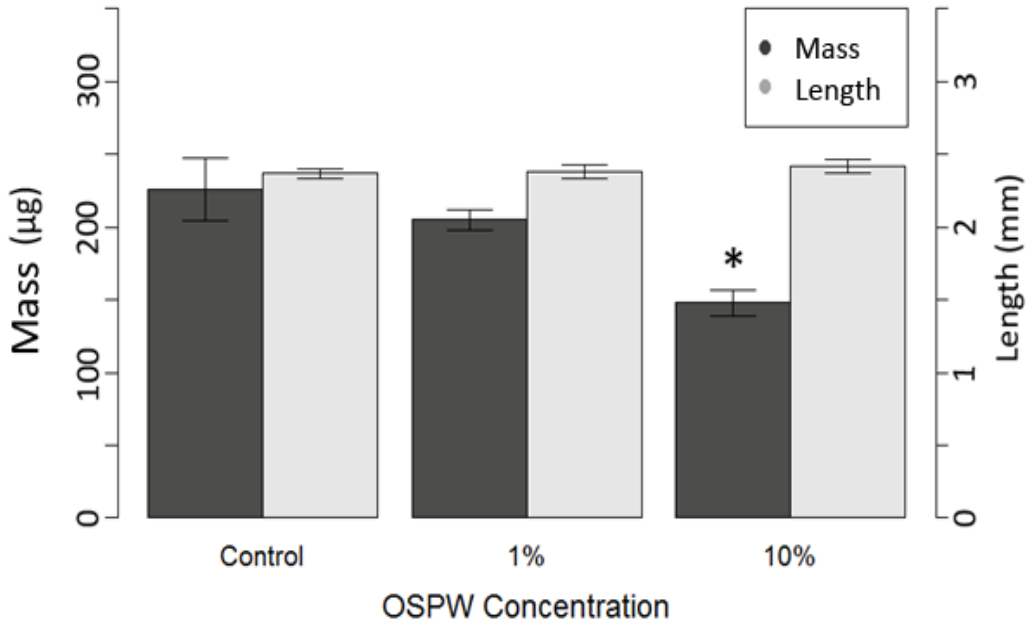


Figure 6.3. The size of adult *Daphnia magna* in mass (µg ± standard error; n = 5) and length (mm ± standard error; n = 15) after exposure to oil sands process-affected water (OSPW) for 10 days. Asterisks (*) denote significant differences between OSPW and control groups.

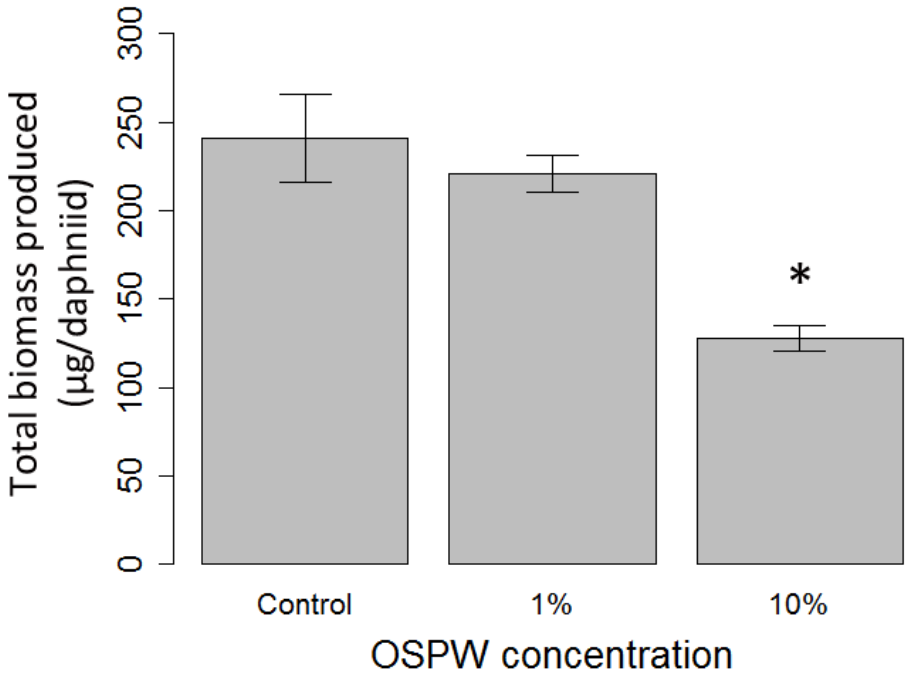


Figure 6.4. The total biomass (µg/daphniid ± standard error) produced from adult *Daphnia magna* during a 10-day exposure to oil sands process-affected water (OSPW). Biomass produced was calculated by subtracting initial mass from the final mass of exposed daphniids, and adding that to the number of neonates produced multiplied by the average neonate mass. Asterisks (*) denote significant differences between OSPW and control groups. n = 5.

6.3.3. Energy reserves

The quantity of carbohydrates and protein per mg of *D. magna* wet mass was not different between the control and 1 and 10% OSPW treatments [$F(2,27) = 0.92$, $p = 0.41$; Figure 6.5a; $F(2,15) = 0.24$, $p = 0.79$; Figure 6.5b, respectively; Appendix 6.3]. In contrast, the quantity of lipid per mg wet mass of *D. magna* was different amongst groups [$F(2,15) = 73.06$, $p < 0.01$; Figure 6.5c; Appendix 6.3]. The lipid content of the daphniids in both 1 and 10% OSPW treatment was lower than the control, by 29 and 41%, respectively.

6.4. Discussion

Food deprivation reduces growth and reproduction in *Daphnia* (Smirnov, 2013). Food deprivation might result from a paucity of nutritious particles in the surrounding environment (Bundschuh et al., 2016), impairment of the feeding behaviour by toxic chemicals (Domal-Kwiatkowska et al., 1994; Rocha et al., 2014), or interference of non-nutritious suspended particles on digestion efficiency (Kirk, 1991). Short term (24 h) exposure to 5.3 and 10% OSPW reduces the feeding rate of *D. magna* at 50 and 75%, respectively (Lari et al., 2016b). It seems that two of these mechanisms, toxic chemicals and suspended particles, can account for the reduction of food uptake in *D. magna* exposed to OSPW (Lari et al., 2017d).

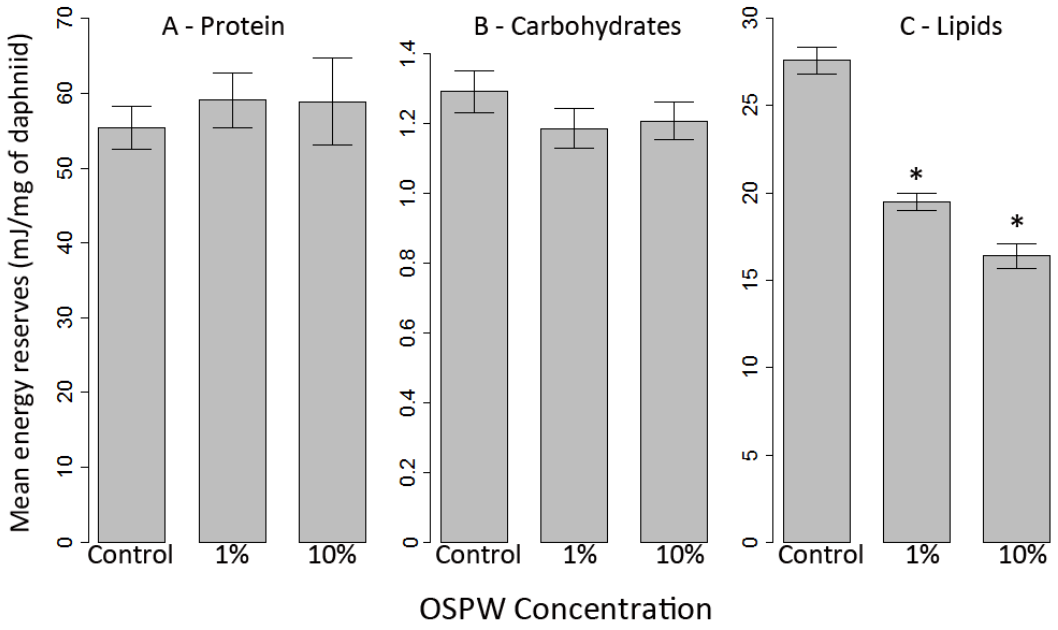


Figure 6.5. The energy stores (mJ/mg of daphniid ± standard error) in adult *Daphnia magna* after being exposed to oil sands process-affected water (OSPW) for 10 days. Energy as mJ/mg was calculated using the using the enthalpy combustion ratios presented in De coen and Janssen (2003). Asterisks (*) denote significant differences between OSPW and control groups.

In response to starvation or low quality food, *Daphnia* produce fewer but larger eggs than normal (Gliwicz and Guisande, 1992; Gliwicz and Boavida, 1996; Ravet and Brett, 2006). In contrast, *Daphnia* exposed to environmental contaminants show lower fecundity and produce smaller neonates than unexposed animals (Smutná et al., 2014; Sancho et al., 2016). We hypothesized that the reduction of fecundity in *D. magna* exposed to OSPW (Lari et al., 2016b) is mainly a result of reduced capacity to convert food to energy and increased energy demand, such that less energy would be available for reproduction. The present study showed that in response to chronic exposure to OSPW, *D. magna* decreased both the number and the size of their offspring. These results suggest that the effects of OSPW on the reproductive performance of *D. magna* is rooted in the toxicity of its chemical components (Lari et al. in review) and food deprivation (Lari et al., 2017d). Exclusive of our earlier study (Lari et al., 2016b), the literature on the effect of OSPW or its components on *Daphnia* reproduction is scarce. There are studies, however, that show that other petroleum derivatives reduce the reproduction rate in *Daphnia* (Geiger and Buikem, 1982; Calfee et al., 1999; Martínez-Jerónimo et al., 2005). For instance, Martínez-Jerónimo et al. (2005) studied the effect of water-soluble fractions (WSF) of seven different crude oils on the fecundity of *D. magna*, and observed that all seven WSFs impaired reproduction. Although they did not measure the condition of neonates, the results of the previous studies on the toxicity of petroleum derivatives on reproduction corroborate the results of the present study.

Daphnia produce small clutches of large eggs as a strategy to increase the chance of survival and fitness of their offspring when food is scarce. Small neonates tend to grow less and produce fewer, low-quality offspring compared to larger neonates (Brett, 1993;

Lampert, 1993). Since OSPW reduces both the number and quality of neonates produced, the magnitude of its effects on reproduction of *D. magna* may increase in the first generation (F1), which are smaller than normal. A study on *D. magna* by (Hammers-Wirtz and Ratte, 2000) demonstrated that the F1 of the parents (F0) exposed to the dispersant, Dispersogen A, displayed reduced fitness. The F1 of *D. magna* exposed to the pesticide, tetradifon, also showed the same trend (Villarroel et al., 2000). Another explanation for the reduced fecundity in exposed *D. magna* might be that OSPW interfered with normal endocrine function. It has been previously shown that OSPW can affect the production of hormones in the H295R cell line (Leclair et al., 2015), fish (He et al., 2012b) and *Chironomus dilutus* (Wiseman et al., 2013a). However, the effects of OSPW on the endocrine system of *D. magna* needs to be investigated.

Growth rate is an important and well-studied characteristic of *Daphnia* life history that has a direct impact on predator avoidance (Declerck and Weber, 2003; Manca et al., 2008) and fitness (Brett, 1993; Hammers-Wirtz and Ratte, 2000). The present study showed that *D. magna* chronically exposed to OSPW produced less biomass than the control, but did not differ in length. In *Daphnia*, reduced food intake (especially lipids) is the primary factor that constrains growth (Brett and Muller-Navarra, 1997). We have shown that exposure to 10% OSPW reduces the feeding rate of *D. magna* by 75% (Lari et al., 2016b), so based on the previous results the daphniids in the present study were starved during the exposure because of feeding impairment. Additionally, when exposed to OSPW, factors such as contaminant detoxification increase the metabolism and energy demand in *D. magna* (Lari et al. in review). Overall, it appears that reduced energy intake from impaired food intake

together with increased energy demands for detoxification combine to account for the reduction of growth that we observed in the OSPW exposed daphniids.

In our previous study (Lari et al., 2016b), we observed that *D. magna* exposed for 21 days to three OSPW samples (10%) from different sources exhibited a 38% to 55% reduction in growth as compared to the control group. Anderson et al. (2012a) demonstrated that chronic exposure to OSPW reduced the growth of *Chironomus dilutus* larvae by 64-77%. In a study on the effects of different crude oil derivatives on *Daphnia pulex*, chronic exposure to phenanthrene, No. 2 fuel oil, and naphthalene reduced growth (Geiger and Buikem, 1982). Petroleum derivatives have the potential to reduce the growth rate of *Daphnia* at low concentrations.

Comparing the forms of biomass produced in terms of growth and neonate production, we found that the amount of biomass associated with neonate production (e.g. 48.4 ± 11.3 µg in control group) is considerable as compared to the mass that they gained through growth during the same period (e.g. 192.7 ± 48.3 µg in control group). Consequently, 20% of the total biomass produced in the control group during the exposure period was in the form of neonate production. Therefore, the total biomass produced is a better measure of energy conversion to biomass among experimental treatments than growth alone.

The energetic test showed that the protein and carbohydrate content per unit mass (mg) were not different between the OSPW-exposed and control daphniids. It must be noted that this result does not suggest that the protein and carbohydrate content of an individual from OSPW groups was not reduced. Daphniids exposed to OSPW produced less biomass than the control. On the other hand, the lipid content per unit mass was reduced in both OSPW

exposure groups (i.e. 1 and 10% OSPW). These results suggest that the macronutrient content in daphniids exposed to OSPW was affected by the exposure. It is commonly accepted that energy is primarily stored in lipids in *Daphnia* (Smirnov, 2013). Consequently, energy is drawn from lipid stores when food intake is not sufficient to meet energetic demands (Becker and Boersma, 2005). Therefore, the reduction in the lipid content of *D. magna* in response to OSPW was predictable based on the results of our previous studies where OSPW and suspended particulate matter impaired the feeding behaviour of *D. magna* (Lari et al., 2016b; Lari et al., 2017e). Several studies measured the changes in carbohydrate, lipid, and protein content of *Daphnia* in response to different contaminants (De Coen and Janssen, 2003; Muyssen et al., 2006; Filho et al., 2011). However, they calculated and compared the macronutrient content per individual between treatments instead of per unit mass.

Although the lipid content of *D. magna* in both 1 and 10% OSPW was less than in the control, the total mass of the 1% group was not significantly lower despite the fact that they were 9.5% lighter than the control. As the primary means of energy storage (Becker and Boersma, 2005), it is expected that lipids are the most sensitive macronutrient to changes in energy budget. It appears that in the 1% OSPW group the exposure increased the demand for using stored lipid. However, because 1% OSPW did not change the amount of the other major macronutrients (i.e. carbohydrates and proteins) the total mass was not significantly less than the control.

In summary, the present study revealed that chronic exposure to OSPW at high enough concentrations reduces the growth and fitness of *D. magna*. The primary mechanisms by which OSPW affects these two traits of *D. magna* appears to be impairment of food intake,

increased energy demand and dirupted endocrine balance. In response to low energy income and increased energy demand, starved daphniids downregulate one of their most energy consuming activities (reproduction), exhaust their energy reserves (mainly lipids), and reduce their growth.

CHAPTER 7: Effects of seasonal changes on the toxic impacts of oil sands process-affected water on *Daphnia magna*

Abstract

Oil sands process-affected water (OSPW) is a major by-product of bitumen mining in northern Alberta, which can be potentially toxic to aquatic biota. The effects of environmental factors on the toxicity of OSPW are understudied. In the present study, the impacts of seasonal changes in water quality on the toxic effects of OSPW (1 and 10%) on *Daphnia magna* was examined. Animals were chronically exposed to OSPW under conditions that represented water quality of a cold or warm seasonal condition. At each seasonal scenario survival, growth (length and mass), and reproduction of exposed *D. magna* were investigated. Survival and length of *D. magna* were only affected by OSPW in the cold-season treatment. Exposure to OSPW reduced the mass of *D. magna* in both cold and warm season scenarios. *Daphnia magna* in the cold-season treatment did not reproduce or produce eggs during the course of the experiment. The results of the present study suggest that seasonal changes in water quality may alter the toxicity of OSPW on *D. magna*.

7.1. Introduction

The oil sands region of Northern Alberta, Canada, with an estimated 178 billion barrels of recoverable oil, encompasses the largest reserve of bitumen in the world (Holden et al., 2011; Environment Canada, 2013). Current production of crude oil from bitumen at 2.29 million barrels per day, is obtained primarily through open-pit mining and extracting the

oil from sand and clay using the Clark hot water extraction process (CAPP, 2015). Oil sands process-affected water (OSPW) is one of the main byproducts of this process, which is currently being stored on-site in massive tailings ponds (RSC 2010). To fully comply with the Government of Alberta's most recent regulations and mandates, oil sands mining operators are obligated to "minimise" accumulation of fluid tailings during operation and to treat the tailings so they are ready to reclaim within ten years of the end of mine life (AER, 2015). Reclaiming OSPW requires a sound understanding of its chemical composition and characteristics, toxicity to resident biota, and interaction of tailings with the aquatic environment.

Significant research efforts are focused on the effects of OSPW on aquatic animals, including both invertebrates and vertebrates (Lari et al., 2016b). Several studies have explored the effectiveness and practicality of remediation methods such as ozone (He et al., 2012a; Pereira et al., 2013b), UV (Shu et al., 2014) and chemical (Iqbal et al., 2013) treatments. On the other hand, the effects of environmental factors on the toxicity of OSPW are understudied, while several studies have demonstrated that they (i.e. temperature, hardness, alkalinity, pH, and dissolved organic matter) affect toxicity of other contaminants (Spry and Wiener, 1991; Heugens et al., 2003; De Schamphelaere and Janssen, 2004). Seasonal water quality changes may alter the toxic effects of OSPW and the responses of aquatic organisms to OSPW.

Fed from glacier headwaters, in temperate latitudes, water quality parameters of water bodies in Athabasca region change throughout the year (Environment Canada, 2011). For instance, the average water hardness and alkalinity of the Athabasca River near oil sands operations (sampling sites M5 and M6) during 2015-2016 varied from 105 to 178 mg/L (as

$CaCO_3$) and 92 to 157 mg/L (as $CaCO_3$) throughout the year, respectively (Table 7.1; Environment Canada, 2011; RAMP, 2016). It is important to note that water quality parameters are intertwined and change together over annual cycles. Therefore, while studying the effect of individual water quality variables illustrates the influence of each variable on the toxicity of contaminants, investigating the effect of bulk water quality changes reflects the potential seasonal fluctuation of the toxicity in the target environment.

Previously, we demonstrated that exposure to OSPW alters three major biological features (i.e. feeding, reproduction, growth) of *D. magna* (Lari et al., 2016b; Lari et al., 2017c). The aim of the present study was to investigate the effects of seasonal changes on the toxicity of OSPW. Thus, we examined the effects of OSPW on survival, reproduction, and physical condition (length and mass) of exposed daphniids and their offspring, in a cold and a warm season scenario.

7.2. Materials and Methods

7.2.1. Test chemicals and animals

Three OSPW samples were provided by three major oil sands operators in the Athabasca region of northern Alberta. The three OSPW samples showed the same toxic effects in *D. magna* at both lethal and sub-lethal (affecting feeding, growth, and reproduction) concentrations (Lari et al., 2016b). The relative abundance of dissolved organic species and trace metal content of the OSPW samples were also similar (Lari et al., 2016b). For these reasons, in the present study a mixture of all three OSPW samples, in equal proportions (v/v), was used. The relative abundance of dissolved organic species and

the trace metal content of the OSPW mixture were measured and reported in (Lari et al., 2017d), presented in Figure 4.1 and Table 2.1.

The daphniids used in the present study came from a *D. magna* clone, cultured successfully under lab conditions for approximately three years as described in Lari et al. (2016b). The quality of the reconstituted water used for the culture was maintained as reported in Table 7.1. Daphniids were fed the microalgae, *Raphidocelis subcapitata*, in the main culture and throughout the experiments. Neonates from 3-5 weeks-old daphniids were used for the experiments throughout the study.

7.2.2. Exposures

Two reconstituted waters were made — based on the seasonal water quality in Athabasca River downstream from oil sands mining activity (Table 7.1; RAMP, 2016) — as representatives of cold and warm seasons (Table 7.1). Half of the collected neonates from the culture were randomly assigned to the cold and the other half to the warm-season treatment, resulting in stocking densities of 80 daphniids/L. The cold-season neonates were transferred and held at a temperature of 10 ºC and the warm-season daphniids at 20 ºC for seven days before exposure to OSPW. The reared daphniids (both cold and warm-season treatments) were exposed to 1 or 10% OSPW (diluted with reconstituted water) or clean water as a control for ten days. For each replicate (n = 10), five daphniids were randomly assigned to a glass beaker filled with 200 mL of the test solution, spiked with 5×10^5 cells/mL *R. subcapitata* and kept at room temperature (20 ±1 °C) under fluorescent light (750 lux; 16:8 h light: dark). The exposure solutions were replaced, and the neonates and dead daphniids were counted and removed, daily.

7.2.3. Growth and reproduction

To investigate the effect of seasonal water chemistry changes on the toxicity of OSPW on *D. magna* reproduction, a cumulative count of the number of neonates produced in all replicates from each treatment (i.e. 1 and 10% OSPW, and control, in both cold and warm-season treatments) were recorded over the course of the 10-day exposure. The physical condition of the daphniids was also investigated as described in Lari et al. (2017c). Briefly, at day 10, a photograph of each *D. magna* was taken using a digital camera (FDK 23UP1300, Imaging Source, Germany) with a macro zoom lens (0.3~1X 1:45; MLM3X-MP, Computar, Japan) and used for measuring the total length (from the top of head to the base of apical spine) with the image analysis program ImageJ (freeware: http://rsb.info.nih.gov/ij/). Then, *D. magna* from each replicate were collected in a 1.5 mL microcentrifuge tube, dried at 60°C for 48 h, and weighed for the final dry mass.

7.2.4. Data analysis

Parametric assumptions were tested using Shapiro-Wilk and Levene tests for normality and homogeneity of variance, respectively. Survival and fecundity of the daphniids were analysed using a one-way ANOVA. The effect of OSPW on mass and length of the exposed daphniids was analyzed using a MANOVA, followed by univariate ANOVAs as posthoc analyses once significance was determined in the original MANOVA. All ANOVA analyses were followed by a Dunnett's post-hoc test. Statistical significance was set a priori at $\alpha = 0.05$. All analyses were conducted using IBM SPSS 24 software (IBM, USA).

Table 7.1. Water quality parameters of reconstituted waters used in the experiments (n = 3) and Athabasca River (regions M5 & M6; RAMP, 2016).

	Cold season (mean ± SD)		Warm season (mean ± SD)	
	Reconstituted	Athabasca	Reconstituted	Athabasca
Hardness (as mg/L $CaCO_3$)	176 ± 0.3	178 ± 7.5	102 ± 2.6	105 ± 5.7
Alkalinity (as mg/L $CaCO_3$)	147 ± 4.3	157 ± 5.2	91 ± 15.9	92 ± 1.2
Conductivity (µS/cm)	550.3 ± 19.7	466 ± 11.4	375.3 ± 21.0	250 ± 0.0
Dissolved oxygen (%)	107.3 ± 6.7	NA	91.1 ± 1.6	NA
pH	7.7 (7.6-7.8)*	7.9 (7.9-7.9)*	7.5 (7.4-7.6)*	8.1 (8.1-8.2)*

*Median (range)

7.3. Results

7.3.1. Survival

The results of the seasonal water chemistry experiment showed that OSPW did not affect the survival of *D. magna* in the warm-season treatment [F (2,27) = 2.80, p = 0.08; Figure 7.1; Appendix 7.1], but increased the mortality in the cold-season treatment [F (2,27) = 16.62, p = 0.001; Figure 7.1; Appendix 7.1]. The mortality of daphniids exposed to 10% OSPW in cold water was significantly higher than control at 43%. None of the other treatments affected the survival of *D. magna*.

7.3.2. Growth

In the warm season treatment, OSPW had no effect on the length of *D. magna* [F (2,126) = 2.07, P = 0.13; Figure 7.2; Appendix 7.2]. In contrast, length of *D. magna* in the cold-season treatment was different among groups [F (2,68) = 21.19, p < 0.001; Figure 7.2; Appendix 7.2]. Daphniids in both OSPW treatments (i.e. 1 and 10%) were significantly smaller (6 and 12%, respectively) than those in the control treatment.

Exposure to OSPW significantly affected the final mass of *D. magna* in both cold [F (2,12) = 8.01, p < 0.01; Figure 7.3; Appendix 7.2] and warm [F (2,12) = 4.78, p = 0.03; Figure 7.3; Appendix 7.2] season treatments as compared to the control. Mass of daphniids exposed to 10% OSPW was less than the control in both seasons, while 1% OSPW had no effect on the final mass of animals. In general, *D. magna* in the cold-season treatment (7.7 µg dry mass in control group) grew less than the ones in the warm-season treatment (278 µg dry mass in control group) at about 36 times.

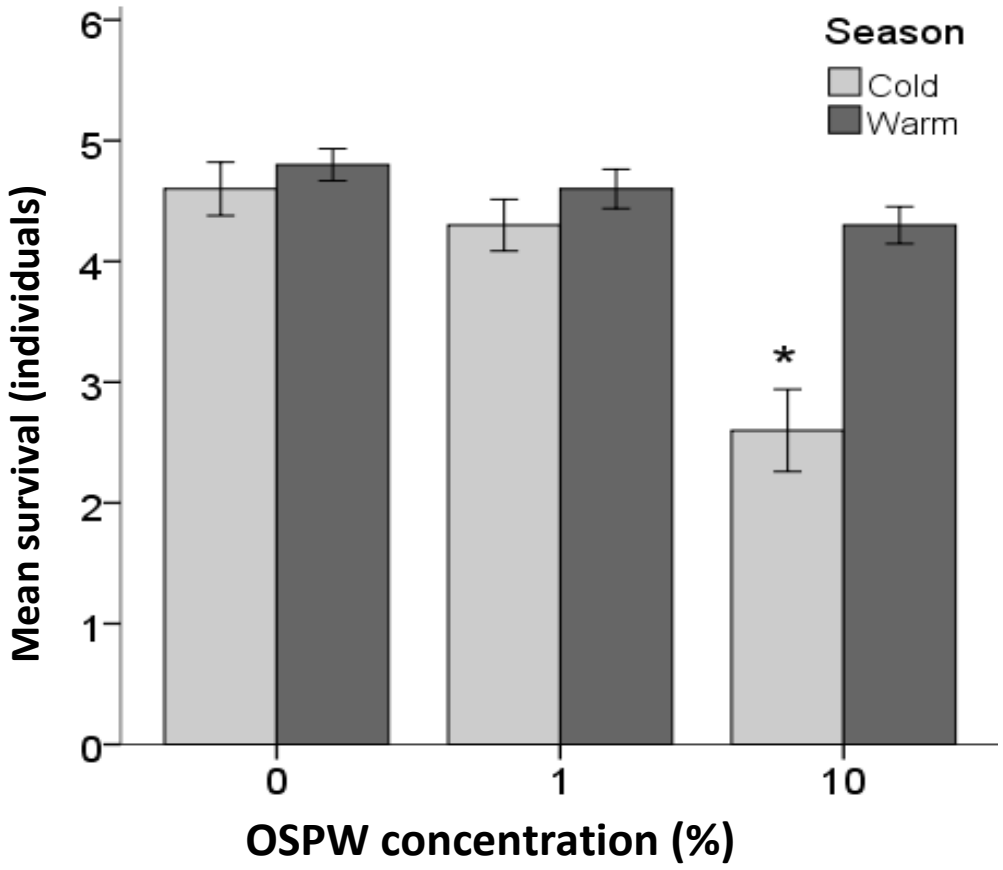


Figure 7.1. Effect of chronic exposure (10 days) to oil sands process-affected water (OSPW) on the survival of *Daphnia magna* under the cold or the warm-season treatment. An asterisk (*) shows a significant difference with the control group ($p < 0.05$). Bars represent means ± SE (n = 10).

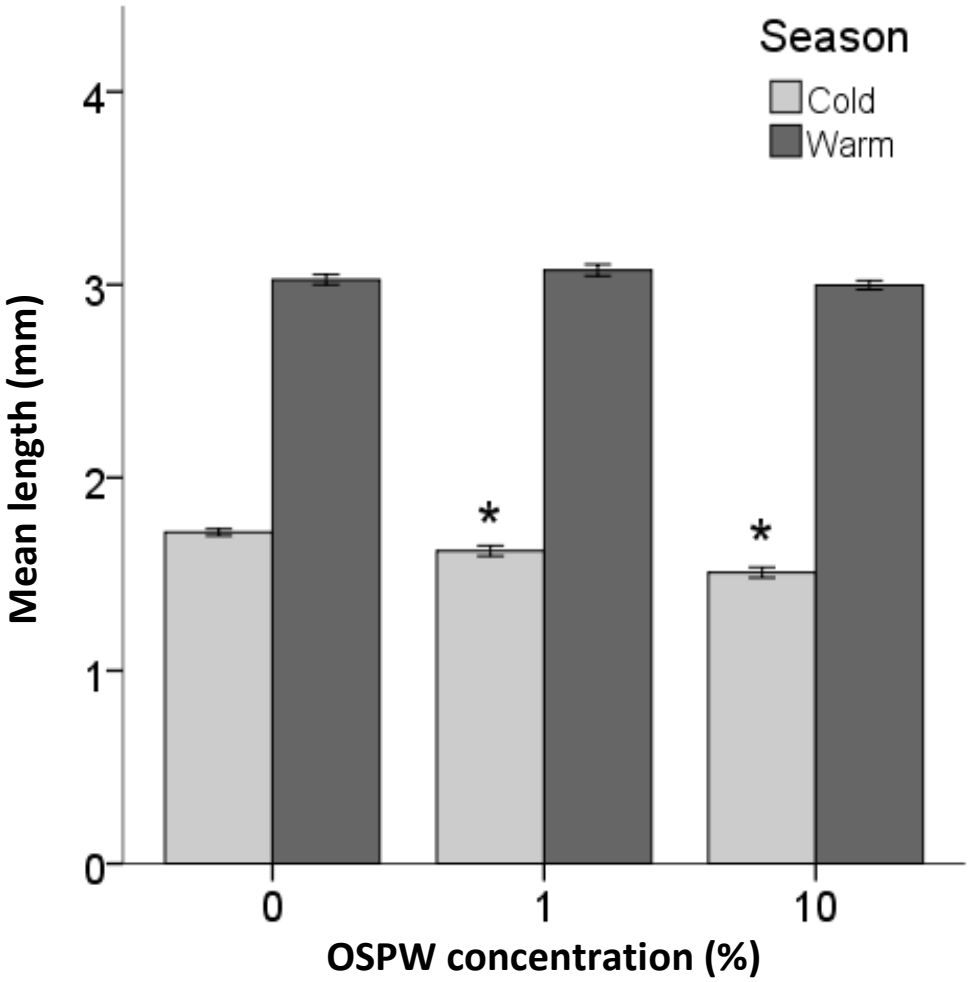


Figure 7.2. Effect of chronic exposure (10 days) to oil sands process-affected water (OSPW) on the length of *Daphnia magna* under the cold or the warm-season treatment. Asterisks (*) show a significant difference with the control group ($p < 0.05$). Bars represent means ± SE ($n = 10$).

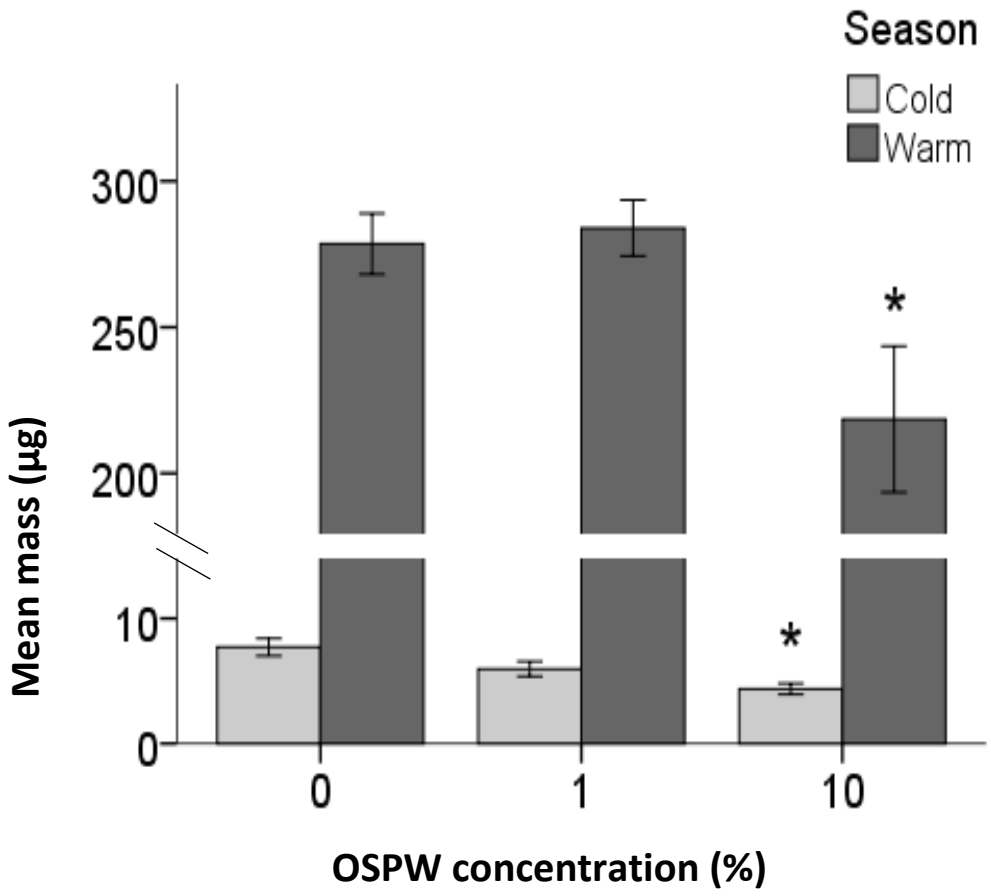


Figure 7.3. Effect of chronic exposure (10 days) to oil sands process-affected water (OSPW) on the mass of individual *Daphnia magna* under the cold or the warm-season treatment. Asterisks (*) show a significant difference with the control group ($p < 0.05$). Bars represent means ± SE ($n = 10$).

7.3.3. Reproduction

Exposure to OSPW affected the reproduction rate of *D. magna* in the warm-season treatment [$F(2,27) = 6.99$, $p < 0.01$; Figure 7.4; Appendix 7.1]. Daphniids in 10% OSPW produced fewer neonates than those in the control group, while 1% OSPW did not change the reproduction of *D. magna*. No individual exposed to the cold-season treatment produced eggs during the course of the exposure (Appendix 7.1).

7.4. Discussion

Environmental conditions affect the toxicity of contaminants. We previously demonstrated that OSPW impairs growth and reproduction of *D. magna* under standard laboratory conditions. In the present study, we investigated the consequences of environmental conditions changes on the toxic effects of OSPW by emulating the water quality variation in the Athabasca River downstream from oil sands mining activities (regions M5 and M6; Table 7.1). Of the measured water quality parameters, only the pH and conductivity of the reconstituted water used in this study were slightly lower than Athabasca River, which might be because of the presence of trace ions in the river water (Table 7.1).

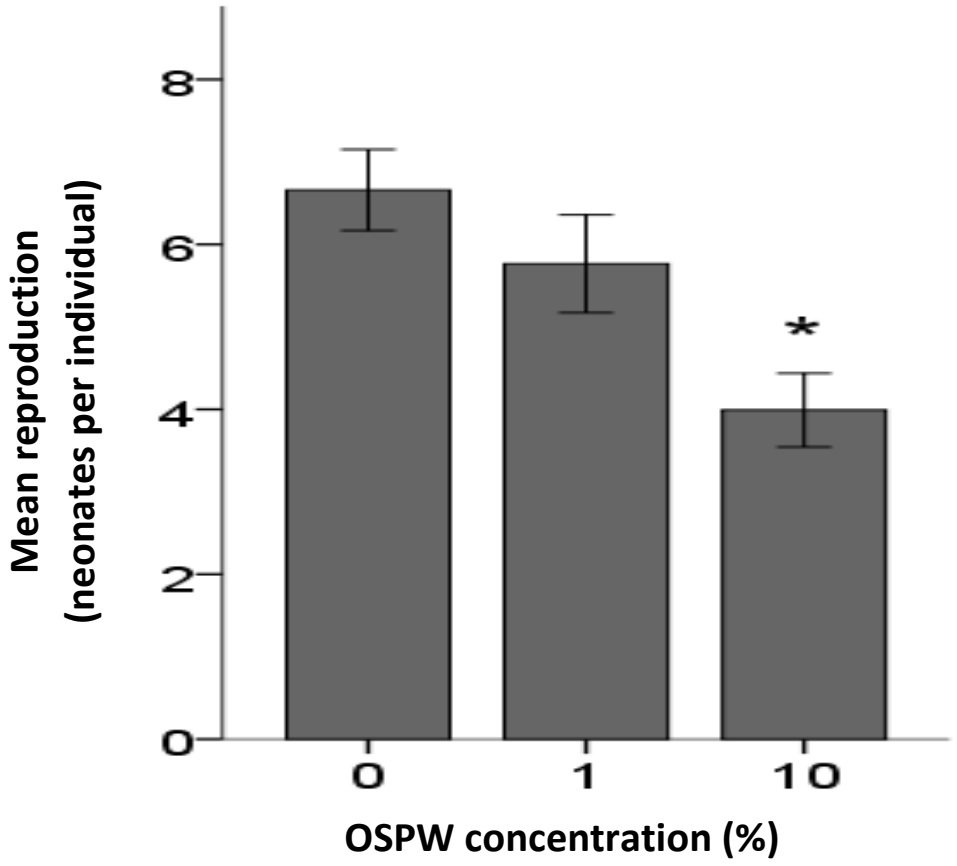


Figure 7.4. Effect of chronic exposure (10 days) to oil sands process-affected water (OSPW) on the reproduction of Daphnia magna under the warm-season treatment (no reproduction occurred under the cold-season treatment). An asterisk (*) shows a significant difference with the control group ($p < 0.05$). Bars represent means ± SE ($n = 10$).

Except for the 10% OSPW group in the cold-season treatment, the mortality data corroborated our previous results showing no significant increase in mortality of *D. magna* chronically exposed to 1 and 10% OSPW (Lari et al., 2016b). Water hardness is usually protective (Park et al., 2009) or has no effect (Verge et al., 2001) on the toxicity of contaminants that have been studied. Since hardness was greater in the cold-season treatment water than the warm season, it is unlikely that water hardness contributed to increased mortality in the the cold-season treatment. On the other hand, owing to its inefficiency in adapting phospholipid composition of the cell membrane, *D. magna* appears not to be well equipped for handling cold temperatures (Smirnov, 2013). In colder waters, *Daphnia* show compensatory control of physiological processes and enzyme activity (Schwerin et al., 2010a; Wojewodzic et al., 2011), increased minimum dissolved oxygen requirement (Koh et al., 1997), and reduced heart and thoracic limb rate (Lari et al., 2017e). High mortality in *D. magna* exposed to 10% OSPW in the cold-season treatment might be a combined outcome of OSPW toxicity and the stress from low temperature. Contrary to OSPW, however, low temperature is normally protective against lethality of contaminants such as metals (Heugens et al., 2003) and pesticides (Seeland et al., 2012).

Like survival, OSPW only affected the length of *D. magna* in the cold-season treatment. Our previous results (Lari et al., 2017c) showing chronic exposure to OSPW (up to 10% concentration) does not affect the length of *D. magna* were corroborated in the current study. Reduced temperatures increase the duration of each instar stage (Smirnov 2013). In eight species of Cladocera, Bottrel (1975) observed that a temperature drop from 20 to 10 ºC approximately triples the duration of instars at both juvenile and adult life stages. He also showed that the length of instars is shorter in early life stages. Given that

OSPW affected the length of *D. magna* in the cold-season treatment might be due to lower temperature and did not go through several molting during the experiment.

In accordance with previous studies (Hall and Burns, 2002; Sperfeld and Wacker, 2009), *D. magna* in all the warm-season treatments grew 36 times (in control groups) more than those in the in the cold-season treatment. However, the effect of OSPW on the mass of *D. magna* in the cold and the warm-season treatment was similar to each other and our previous findings in Lari et al. (2017c). The effect of OSPW on the growth of *D. magna* is the result of food deprivation and increased metabolic rate (Lari et al., 2017d; Lari et al. in review). It appears that seasonal changes do not alter the effects of OSPW on the growth of *D. magna*.

Daphniids in the cold-season treatment did not produce any eggs. Reduction or complete inhibition of reproduction in the cold-season treatment was expected, as several studies have demonstrated that reproduction of *Daphnia* spp. is highly temperature sensitive (Seeland et al., 2012; Scherer et al., 2013). On the other hand, individuals in the warm-season treatment produced 1-2 clutches. The impairing effect of OSPW on reproduction was also similar to our previous findings (Lari et al., 2016b; Lari et al., 2017c).

Known to be unable to overwinter in an active state (Farkas et al., 1984), *D. magna* perform poorly when temperatures fall below 15 ºC. Daphniids in the cold-season treatment grew much less than in the warm-season treatment and stopped reproducing entirely. However, investigating individual water quality variables (e.g. temperature, hardness, alkalinity) would provide an insight into which water quality variables play a role in increased toxicity of OSPW on *D. magna* under colder exposure conditions, as would be expected during winter. Further investigation of the influence of seasonal variation of

environmental characteristics on the adverse effects of OSPW on more model species will improve our knowledge on the behaviour of this contaminant in the environment.

CHAPTER 8: Rainbow trout (*Oncorhynchus mykiss*) detection, avoidance, and chemosensory effects of oil sands process-affected water[7]

Abstract

Oil sands process-affected water (OSPW) — a byproduct of the oil sands industry in Northern Alberta, Canada – is currently stored in on-site tailings ponds. The goal of the present study was to investigate the interaction of OSPW with the olfactory system and olfactory-mediated behaviours of fish upon their first encounter with OSPW. The response of rainbow trout (*Oncorhynchus mykiss*) to different concentrations (0.1, 1, and 10%) of OSPW was studied using a choice maze and electro-olfactography (EOG). The results of the present study showed that rainbow trout are capable of detecting and avoiding OSPW at a concentration as low as 0.1%. Exposure to 1% OSPW impaired (i.e. reduced sensitivity) the olfactory response of rainbow trout to alarm and food cues within 5 min or less. The results of the present study demonstrated that fish could detect and avoid minute concentrations of OSPW. However, if fish were exposed to OSPW-contaminated water and unable to escape, their olfaction would be impaired.

8.1. Introduction

The large volume of stored oil sands process-affected water (OSPW) – a major byproduct of surface mining bitumen (very heavy oil) – is an environmental concern in northern Alberta, Canada. With the intention of establishing an effective remediation and

[7] A version of this chapter is published in *Environmental Pollution*, 225, Lari, E., Pyle, G.G., Rainbow trout (*Oncorhynchus mykiss*) detection, avoidance, and chemosensory effects of oil sands process-affected water, 40-46, 2017, with permission from Elsevier.

reclamation program, as Alberta Energy Regulator mandates (AER, 2015), oil sands operators in collaboration with academia are investigating the effects of OSPW on organisms that may be found in aquatic habitats where potential exposure could occur.

Oil sands process-affected water is composed of water, organics, major ions, and suspended particulate matter (SPM). The detailed composition of OSPW varies with the composition of the ore being extracted, extraction process, and age (Allen, 2008). The dominant inorganics in OSPW (sodium, bicarbonate, chloride, and sulphate) are not normaly considered toxic but increase the salinity of OSPW. The concentrations of trace metals, which are more toxic than other inorganics, are very low in OSPW (Table 7.1). The dissolved organic component of OSPW is a complex mixture of acidic, basic, and neutral species containing oxygen, nitrogen and sulfur groups (Pereira et al., 2013a; Morandi et al., 2015; Figure 2.2). Because treating OSPW by activated carbon adsorption or ozonation dramatically attenuates the toxic effects of OSPW, it is generally accepted that the dissolved organic components are responsible for most toxicity to aquatic organisms (He et al., 2011; He et al., 2012b; Islam et al., 2015; Niasar et al., 2016).

Fish are both ecologically and environmentally important, which is why most environmental research has focused on them. Several studies used fish to investigate the toxic effects of OSPW (e.g. Peters et al., 2007; Zhang et al., 2016) and how remediation processes change the toxicity of OSPW (e.g. He et al., 2012a; Hagen et al., 2013). Fish have commonly been used to examine the effects of OSPW and naphthenic acids (NAs) (e.g. Knag et al., 2013; Marentette et al., 2015). Both OSPW and NAs are known to cause lethality (Toor et al., 2013), endocrine disruption (Knag et al., 2013; Leclair et al., 2015), immunotoxicity (Hagen et al., 2013; MacDonald et al., 2013), genotoxicity (Lacaze et al.,

2014), reproduction suppression (Kavanagh et al., 2012; Kavanagh et al., 2013), and embryo developmental malformation and mortality (Peters et al., 2007; He et al., 2012a; Scarlett et al., 2013) in fish. Despite the immense number of studies on the effects of OSPW on fish, literature on the interaction of OSPW on the olfactory system and olfactory-mediated behaviours are scarce.

In fish, the chemosensory system perceives chemical information from the surrounding environment that is vitally important to the survival of the animal (Tierney et al., 2010). Olfaction is one of the primary senses of fish, and they rely on their olfactory system to find food, avoid predators, and find mates. Thus, many substantial behaviours such as feeding, mating, social interactions, and predator avoidance are directly influenced by chemosensory signals (Bilberg et al., 2011). Olfaction is the most predominant chemosensory system, because it detects minute concentrations of chemicals and over long distances to inform the animal (Tierney et al., 2010). Olfactory sensory neurons (OSN) are grouped into three sub-classes: microvillus, ciliated, and crypt cells that sense food, social, and mating cues, respectively (Hamdani and Døving, 2007). To detect these cues, OSNs are in direct contact with the surrounding environment which makes them sensitive to minute concentrations of chemicals (Hara, 1992) and vulnerable to environmental contamination at the same time. Thus, olfaction is one of the most important endpoints that can be used to investigate the toxic effects of chemicals on fish.

The aim of the present study was to investigate rainbow trout's first chemosensory and behavioural response upon encountering OSPW. The first study tested whether or not the fish olfactory system can detect OSPW contamination, and determined its detection threshold concentration. Behavioural studies at OSPW threshold concentrations established

whether or not fish were attracted or repelled. The second study was designed to investigate how the presence of OSPW affected the ability of the olfactory system to perceive natural stimuli, such as food or chemical alarm cues.

8.2. Materials and Methods

8.2.1. Test Animals and Chemicals

Fingerling rainbow trout (8–12 cm total length; total of 128) were obtained from Allison Creek Brood Trout Hatchery Station, in Alberta, Canada. Fish were infected with *Gyrodactylus salmonis* (1.3 ± 0.8 parasites per cm^2 body surface) upon arrival. Since *G. salmonis* infection impairs the olfactory function of rainbow trout (Lari and Pyle, 2017a), fish were treated with a parasiticide (Parazipro, Hikari, USA) as described by Lari et al. (2017a). Fish were given two weeks to recover from treatment, as suggested in Lari et al. (2017b). After treatment, fish were kept in 400 L holding tanks, and the density was maintained at stocking densities lower than 5 g/L. The water quality in the holding tanks was monitored and maintained with the following parameters (mean ± SD; n = 5): temperature, 10 ± 0.2 ºC; dissolved oxygen, 8.4 ± 0.5 mg/L; hardness, 178 ± 4 mg/L as $CaCO_3$; alkalinity: 137 ± 3.6 mg/L as $CaCO_3$; pH, 8.23 (8.06-8.41); ammonia, 0.02 ± 0.01 mg/L; nitrate: 2.1 ± 0.9 mg/L; and nitrite, 0.017 ± 0.004 mg/L. Photoperiod was held constant on a 16:8 hr light:dark cycle. The University of Lethbridge Animal Welfare Committee approved all procedures associated with the present study (permit #1411).

The OSPW sample used in the present study was provided by a major oil sands company operating in the Athabasca region, Alberta, Canada. The OSPW sample was collected from a four-year-old, active tailing pond. The hydrocarbon (Figure 2.2) and major

trace metals content (Table 2.1) of the OSPW sample (labelled as OSPW-B) were analysed and reported in Lari et al. (2016b). The detailed methods for analysing the OSPW sample are presented in Lari et al. (2016b), Section 2.2. In general, oxygen-containing species (O_x^- and O_x^+) were more prevalent compared to sulfur (SO_x^-) and nitrogen (NO_x^-) containing species when detected by use of negative electrospray ionisation (ESI^-) and positive electrospray ionisation (ESI^+), respectively. The OSPW sample was stored at 4 °C in 22.7 L HDPE buckets with tight sealing lids (Pro-western Plastics LTD., Canada). To remove SPM from the OSPW, sample was centrifuged (5000 rpm, 10 min) before using in experiments.

8.2.2. Electro-olfactography Assay

To assess the olfactory system response to different chemosensory cues, an electro-olfactography (EOG) technique was used in accordance with the following procedure. Fish were anaesthetized by immersion in an MS-222 (tricaine methanesulfonate) solution, 120 mg/L TMS (AquaLife, Canada) and 360 mg/L $NaHCO_3$ (Fisher Scientific, USA) to buffer to pH 7.4. In order to facilitate access to the olfactory rosette, the septum that divides the anterior and posterior nares was removed. Gelatin- and saline-filled glass microelectrodes (40 g/L gelatine, EMD Chemicals, Germany; 9 g/L NaCl, Fisher brand, USA) with 4.16 ± 0.11 mΩ electric resistance were used for recording the EOG signals. A reference microelectrode was placed between the opening of the olfactory chamber and eye. The recording probe was positioned on the second largest lamella, at approximately 1/3 of the lamella length from the rosette's medial raphe. The gills were perfused with an aerated MS-222 solution, with a concentration equal to half of that in the anaesthetic solution, through a perfusion line inserted into the fish's mouth. A constant flow of culture water perfused

the olfactory chamber throughout the test. All the stimuli used in EOG experiments were also tested on dead fish that had their olfactory chambers treated with 3 M KCl for 10 min. The KCl treatment kills the tissue and denatures the cell membrane. The test on the dead olfactory rosette was carried out to investigate whether the EOG readings were the response of the olfactory system to the stimuli or the electric charge of the chemosensory cue delivery solutions.

In the present study 10^{-5} mol/L L-alanine (Fisher Scientific) was used to represent a food cue to examine the olfactory acuity of microvillous OSNs; and 10^{-5} mol/L taurocholic acid (TCA; Sigma-Aldrich, USA) was applied to represent a social cue to test the performance of ciliated OSNs. In the experiment in which the olfactory perception threshold of OSPW was investigated, 1 and 10% OSPW solutions were used as olfactory stimuli. Culture water was used as a blank stimulus in all experiments. Stimuli were delivered to the olfactory chamber in 3-second pulses by switching the olfactory perfusion water with the stimulus by means of an eight-channel valve controller (Warners Instrument Co., USA). To prevent olfactory attenuation, the chamber was given a 90 s rest time between stimulus deliveries, and the stimuli were delivered in a randomised manner. Each stimulus was delivered three times per fish. Signals received by the probes (glass microelectrode) were amplified by a differential amplifier (DP-311, Warner Instruments, USA), adjusted at high-pass 0.3 Hz, gain, 10. The amplified readings, then, were digitalized and recorded by a data acquisition system (PowerLab 4SP, ADInstruments, USA). The EOG response to a stimulus was determined by subtracting the minimum from the maximum point of the recorded peak. The average of the reading from the respective blank

was subtracted from the mean reading of each stimulus to calculate the final blank-corrected EOG response.

To study the olfactory perception threshold of OSPW, the response of fish to five stimuli was measured, 1 and 10% OSPW along with L-alanine and TCA as positive controls and culture water as blank. In this experiment, the readings from the blank were not subtracted from the readings from the stimuli but were used as a comparative control.

To investigate the immediate effect of exposure to OSPW on olfactory acuity, the response of each test animal to L-alanine and TCA was recorded in three phases respectively. In the first phase, the solutions used as gill and olfactory perfusion water and stimuli (i.e. L-alanine, TCA, and culture water) were made with clean water. For the second phase, immediately following from the first, 1% OSPW was added to the olfactory chamber perfusion water as well as the chemosensory stimulus delivery vehicle solutions (i.e. L-alanine, TCA, and culture water). Finally, OSPW was removed from the test system and the EOG responses to L-alanine and TCA were recorded again. In all three phases the EOG response of fish to chemical stimuli recorded as described earlier in the current section. In the control group, all three recording phases were done with all solutions made with clean water.

8.2.3. Behavioural Assay

Behavioural response of fish to OSPW was investigated using a flow-through choice maze. The chamber was made of an 80×40×20 cm (length, width, and height, respectively) of acrylic glass. The bottom of the chamber was transparent, and the walls were white. The first 50 cm of the chamber was divided into two identical (20 cm width) arms (A and B;

Figure 8.1). The last 10 cm was divided from the rest of the chamber by a perforated acrylic glass sheet (drainage area). Two drainages pipes were attached to two 2 cm diameter holes in the bottom of the maze in the drainage area; the centre of each hole was 5 cm from the front wall, and 10 cm from the nearby side wall (Figure 8.1). The remaining 20×40 cm (length by width) was considered as the acclimation of fish area. At the far end of each arm (away from the acclimation area) were placed four 0.5 cm diameter tubes, 4 cm apart from each other and 10 cm above the bottom of the chamber. All four tubes from one arm were connected to a peristaltic pump (Fisher Scientific, Canada) that pumped solutions to the corresponding arm at the rate of 2 L/min. Immediately below the opening of the tubes to the arm was placed a 10 cm airstone, mixing the delivered solution with the content of the maze. A perforated acrylic glass sheet and a 2 cm wide, plastic honeycomb were placed at 3 and 6 cm from the far end of each arm, respectively, to further mix the solution and create a laminar flow. The rear end of both arms was separated from the acclimation area by a perforated acrylic sheet (gates). Each gate was attached to a cable that passed through a pully with the opposite end accessible at the operator's desk, allowing for remote operation of the gates.

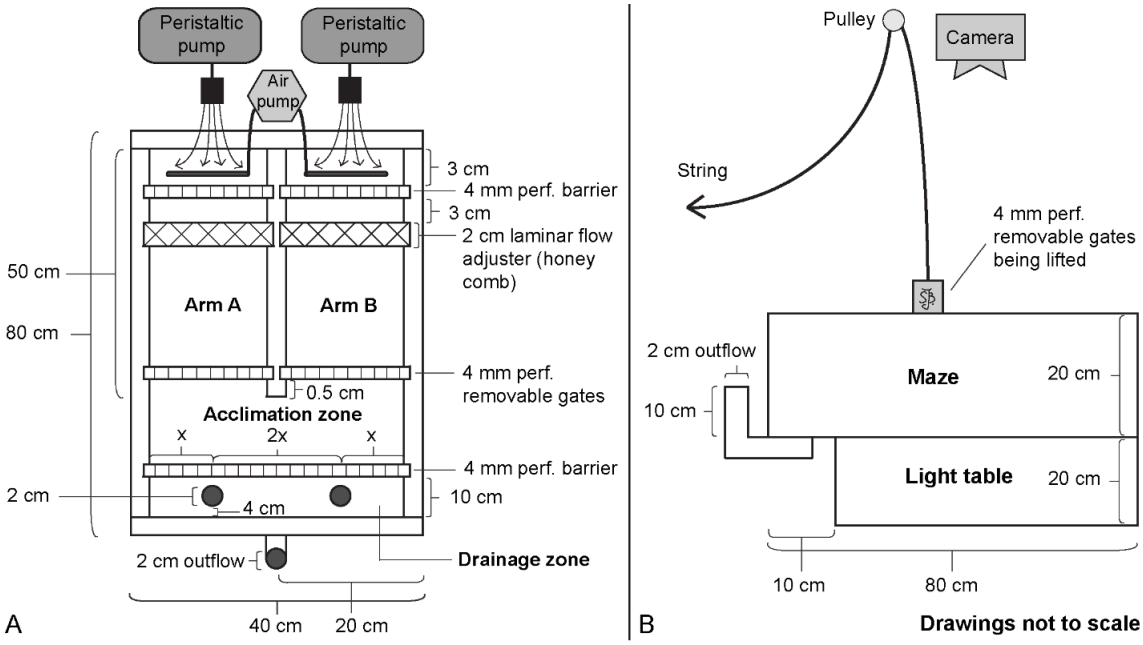


Figure 8.1. Flow-through choice maze. A) top view; B) side view. Each peristaltic pump forces solution to one arm of the maze through four tubes. An air pump, perforated (perf.) barrier, and honeycomb lattice allowed for laminar flow through each arm. The perforated barriers prevent fish from entering the arms during the acclimation period. Another perforated barrier devides the acclimation from drainage zone. Water drains out of the maze via two outlets in the drainage zone. A light table, possitioned under the maze lit the test area, and a camera placed over the maze records the movement of the fish.

For each trial, the maze was filled up to 10 cm height, the flow-through was started, and one fish was added to the acclimation area. The fish was conditioned to the chamber for 15 min. Then, the flow in one arm (randomly chosen) was switched to the test stimulus. A dye test with food colouring showed that the solution delivered at the far end of the arms reach the rear end in 40 s. Therefore, the gates were opened 40 s from the start of the stimulus delivery and the fish was video recorded for 2 min. The video footage was then analysed using video tracking software (Lolitrack 4, Loligo Systems, Denmark). The response of the fish to the stimulus (i.e. avoidance or attraction) was determined by the time spent in each arm using the equation described in (Lari et al., 2015).

In order to investigate the OSPW detection threshold, clean water was delivered to the maze during the acclimation period, after which 0.1 or 1% OSPW or clean water (control) was delivered to a randomly chosen arm as the stimulus. To determine the effect of OSPW on food search behaviour, the maze was filled with 1% OSPW or clean water for the control group. The same solution as the maze content was delivered during the acclimation period. Afterwards, 10^{-5} M L-alanine in 1% OSPW or clean water (control) was delivered to one randomly selected arm as stimulus.

To determine whether the attraction or avoidance response observed in the behaviour experiment was olfactory-driven, or if other chemosensory systems played a role, the reaction of anosmic fish to OSPW was also investigated. To create temporary anosmia in fish, the olfactory chamber was blocked with surgical glue (3M Vetbond, USA).

8.2.4. Statistical Analysis

All experimental data were tested for the parametric assumptions of normality and homogeneity of variance using a Shapiro-Wilk's and a Levene's test, respectively. Because the assumption of homogeneity of variance was violated in the EOG data for the detection of OSPW, a Welch's ANOVA test was used to analyse that data set. A repeated measures ANOVA was used to evaluate the data from the EOG test on the effect of OSPW on the olfactory acuity of rainbow trout. The behavioural experiment on OSPW detection was analysed using a one-way ANOVA, followed by a Tukey's post-hoc test to compare the response of rainbow trout to clean water, and 0.1 and 1% OSPW. To analyse the effect of OSPW on food searching behaviour of rainbow trout the response of fish to L-alanine in presence and absence of OSPW were compared using a Student's T-test. Statistical significance was set a priori at $\alpha = 0.05$. All data were analysed using an IBM SPSS 22 software (IBM, USA).

8.3. Results and Discussion

8.3.1. OSPW Detection Threshold

None of the stimuli used in the present study produced a significant EOG peak in dead fish [$F(5,12) = 0.92$, $p = 0.51$, Appendix 8.1], which ensured that the EOG recordings from live fish were an indicator of the OSNs' response to OSPW. In contrast, the stimuli provoked a significant response from the olfactory system of rainbow trout, as compared to the blank [$F(5,12.9) = 74.42$, $p < 0.001$, Figure 8.2; Appendix 8.1]. All stimuli except for 0.1% OSPW significantly excited the OSNs. The EOG response to OSPW at 1% concentration was not different from L-alanine but significantly lower than TCA. The

olfactory response to 10% OSPW was not significantly different from TCA and higher than L-alanine.

In the behaviour test, rainbow trout displayed an avoidance response to the presence of OSPW [$F(2,54) = 24.45$, $p < 0.001$; Figure 8.3; Appendix 8.2]. Fish avoided both 0.1 and 1% OSPW as compared to the control group. The avoidance of 1% OSPW was significantly stronger than 0.1%.

Numerous studies have investigated behavioural responses (i.e. attraction or avoidance) of fish to a large variety of chemicals such as food (e.g. Kasumyan and Marusov, 2005; Shamushaki et al., 2011; Marusov and Kasumyan, 2016) and alarm (e.g. Brown et al., 1995; Speedie and Gerlai, 2008; Júnior et al., 2012) cues, as well as environmental contaminants (reviewed in Tierney et al. 2010). The overwhelming majority of these studies claim that the observed responses are chemosensory-driven behaviours. In the case of several food and alarm cues, physiological data (i.e. EOG) support the idea that the olfactory system can detect these chemicals (e.g. (Harvey et al., 2010; Meredith et al., 2012; Valdés et al., 2015). On the other hand, to the best of our knowledge, literature in which the ability of the olfactory system to detect contaminants is investigated is scarce. Dew and Pyle (2014) demonstrated that calcium induces EOG responses and provokes avoidance behaviour in fathead minnows. Although, calcium is not considered a toxic substance to fish, the fact that it produces an olfactory response suggests that the olfactory system of fish may also be able to perceive trace metal contaminants (Moreira-Santos et al., 2008; Lari et al. in review), particularly the ones that mimic calcium such as cadmium (Wood, 2012).

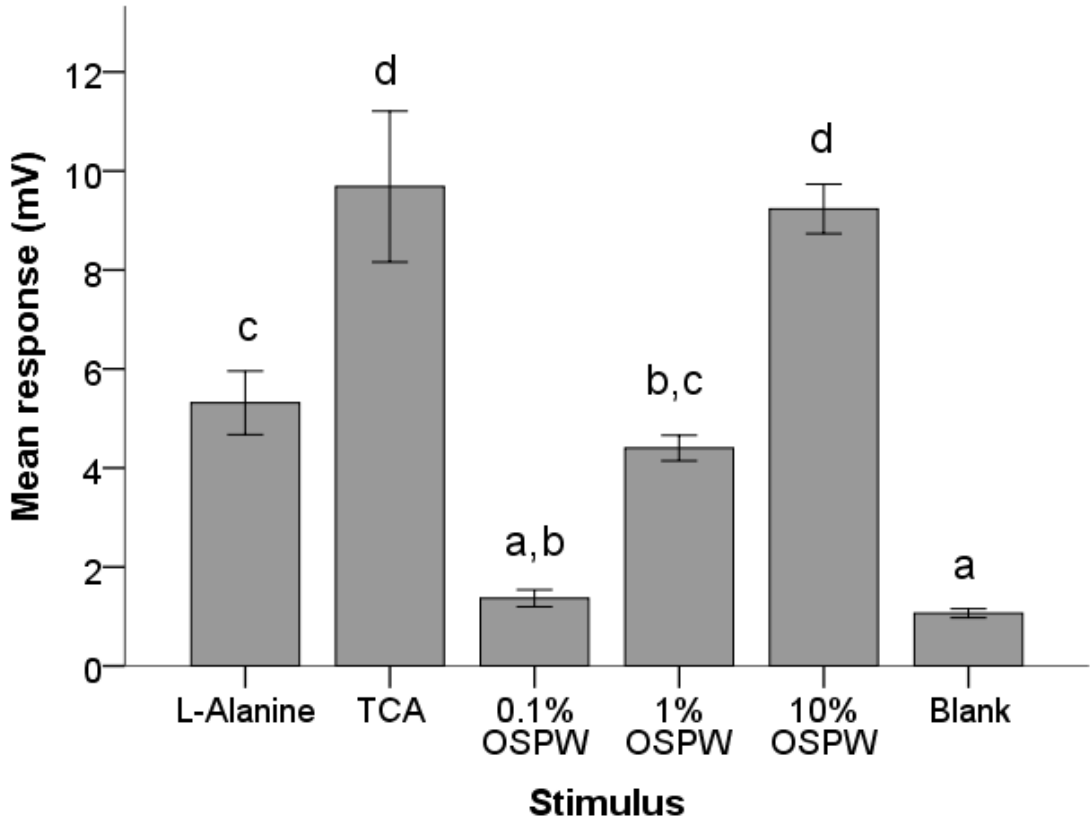


Figure 8.2. Electro-olfactography response (mean ± SE) of rainbow trout to L-alanine, taurocholic acid (TCA) and different concentrations of oil sands process-affected water (OSPW). Lower-case letters denote significant differences ($p \leq 0.05$; $n = 6$).

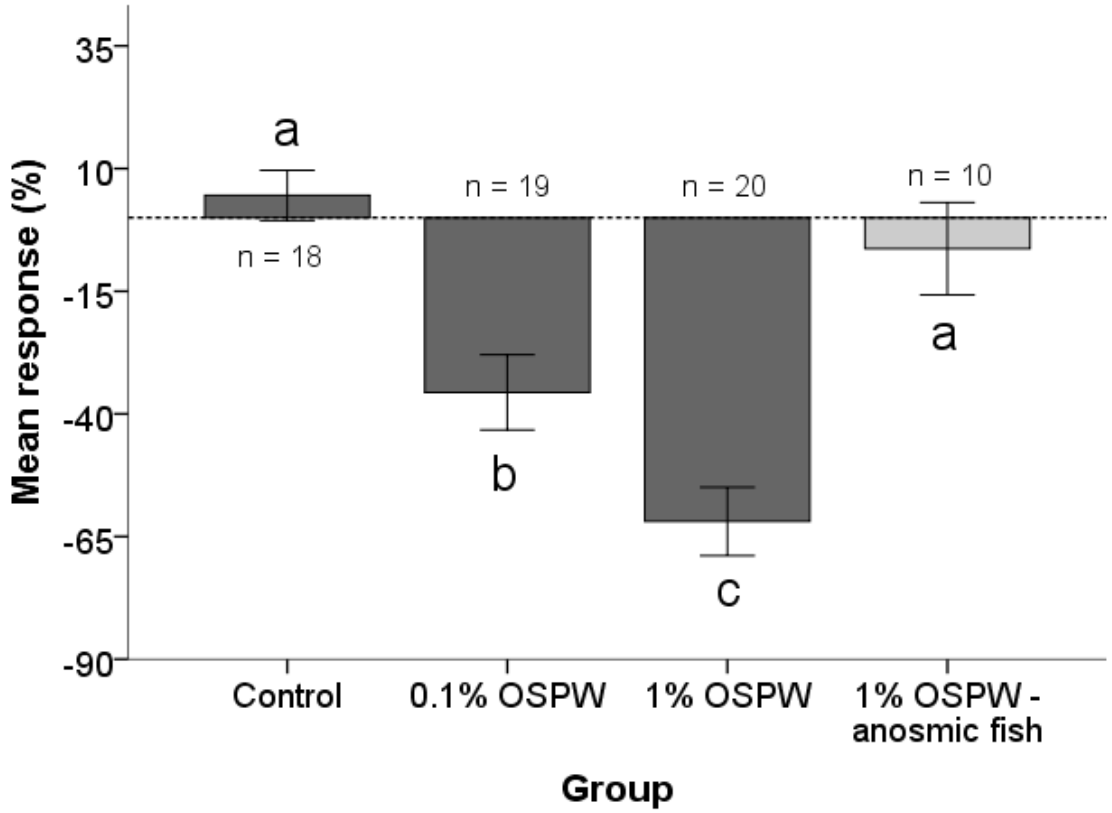


Figure 8.3. Behavioural responses (mean ± SE) of rainbow trout to different concentrations of oil sands process-affected water (OSPW). Negative responses indicate avoidance and positive responses show attraction to the chemosensory stimulus. Lower-case letters denote significant differences ($p \leq 0.05$).

In the present study, rainbow trout expressed an avoidance behaviour, accompanied by an olfactory response to OSPW, demonstrating that the observed avoidance was an olfactory-mediated behaviour. Notably, rainbow trout avoided OSPW at a concentration (i.e. 0.1%) one order of magnitude lower than the EOG threshold. Since the anosmic fish did not show a significant response to OSPW, the differences may be due to the limitation in the sensitivity of the EOG technique (Scott and Scott-Johnson, 2002; Baldwin and Scholz, 2005). For instance, with the EOG setup used in the present study, a significant response to L-alanine can be recorded from rainbow trout at a concentration as low as 10^{-5} M, while it is shown that rainbow trout manifest a feeding behaviour when exposed to 3×10^{-6} M L-alanine (Valentincic and Caprio, 1997). Furthermore, Hara (1973) using electroencephalogram (EEG) on the olfactory bulb demonstrated that rainbow trout are able to perceive free amino acids at concentrations as low as 10^{-7} to 10^{-8} M.

The literature on the behavioural response of fish when encountering OSPW is scarce. Nonetheless, several studies investigated the behavioural response of fish to petroleum hydrocarbons. Laboratory studies showed that fish avoid complex mixtures of hydrocarbons such as water soluble fraction (WSF) of crude oil (Lari et al., 2015) and coal liquids (Dauble et al., 1985; Gray, 1990), as well as single hydrocarbons, including mono- (Weber et al., 1981)and poly-cyclic aromatic hydrocarbons (Hinkle Conn et al., 1998). It is also reported that during their upstream migration, Pacific salmon (*Oncorhynchus* spp.) avoid entering waters contaminated by hydrocarbons (Weber et al., 1981). In general, the current literature implies that fish are capable of detecting and avoiding hydrocarbon-polluted waters.

8.3.2. Effect of OSPW on Olfaction

The OSPW treatment changed the response of fish to both L-alanine [treatment: Wilks' Lambda = 0.2, $F(2,9) = 11.82$, $p < 0.01$; treatment×group: Wilks' Lambda = 0.11, $F(2,9) = 36.67$, $p < 0.001$; Figure 8.4B; Appendix 8.3], and TCA [Wilks' Lambda = 0.06 treatment: $F(2,9) = 80.30$, $p < 0.001$; treatment×group: Wilks' Lambda = 0.05, $F(2,9) = 94.73$, $p < 0.001$; Figure 8.4A; Appendix 8.3]. The presence of OSPW immediately reduced the response of the fish olfactory system to natural cues, L-alanine and TCA, by 60% and 29% as compared to before exposure, respectively. However, the EOG response to the same chemical cues completely recovered shortly (> 2 min) after switching back to clean water.

In the behavioural test, the presence of OSPW changed the response of rainbow trout to L-alanine [$t(36) = 4.23$, $p < 0.001$; Figure 8.5; Appendix 8.4]. The existence of OSPW in the maze reduced fish attraction to L-alanine by 68%, as compared to the control group.

It was previously demonstrated that hydrocarbons impair the feeding behaviour of fish (Lari et al., 2015). However, the physiological data to support or refute the role of contaminant-induced olfactory impairment was missing. The behavioural and EOG data in the present study corroborate one another, suggesting that OSPW impairs the food search behaviour of rainbow trout by interrupting the perception of food cues.

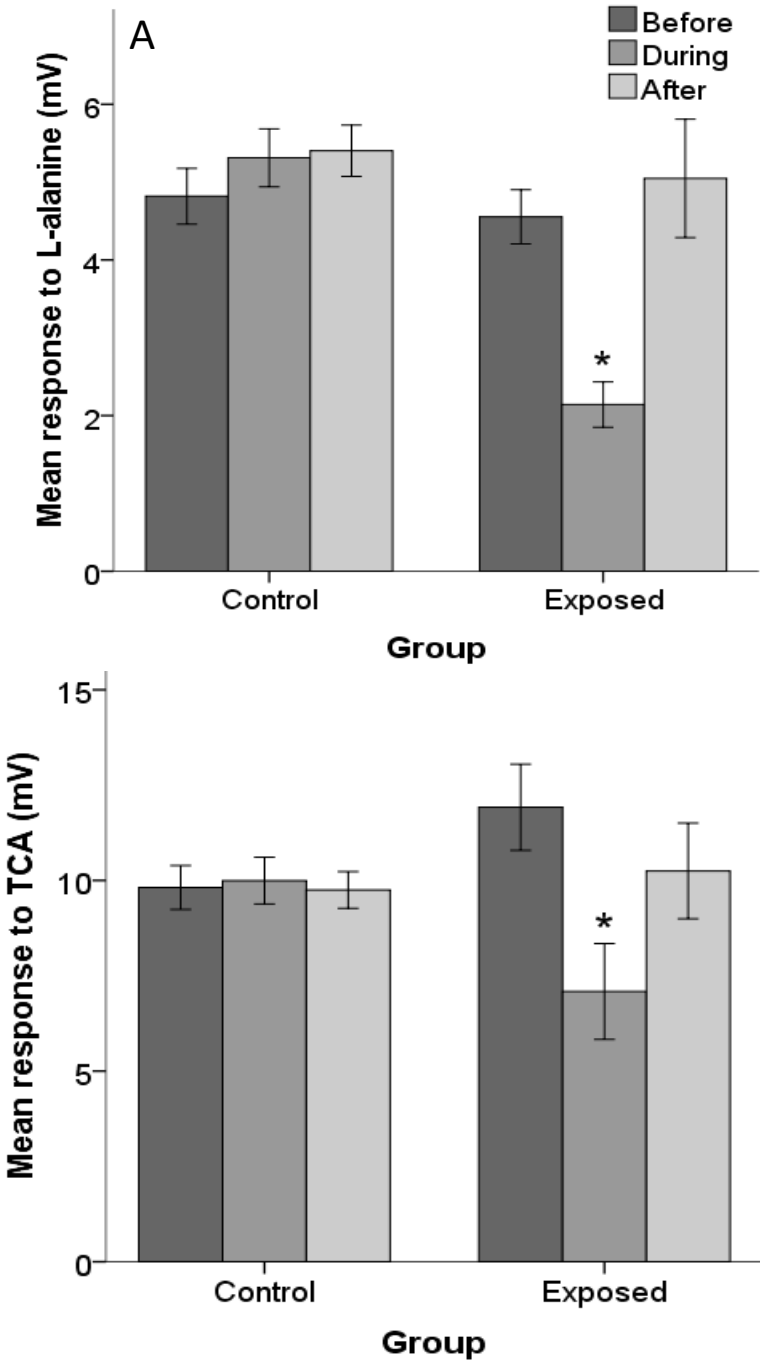


Figure 8.4. Electro-olfactography response (mean ± SE; n = 6) of rainbow trout to L-alanine (A), and taurocholic acid (TCA; B) before, during and after exposure to 1% oil sands process-affected water (OSPW).

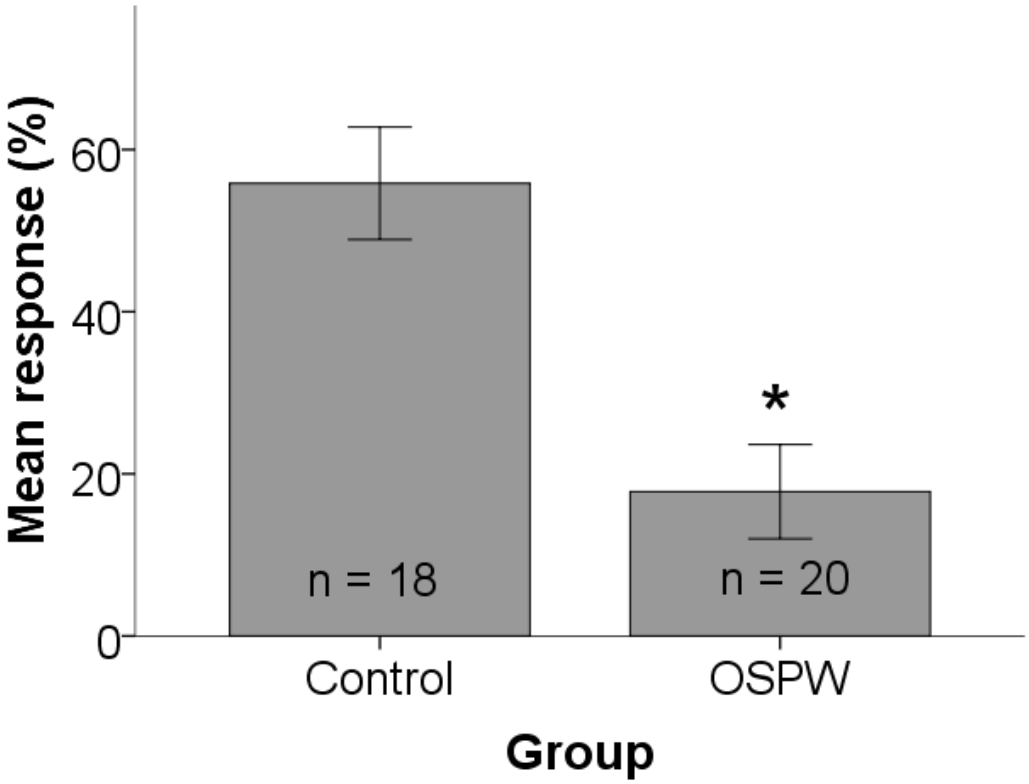


Figure 8.5. Behavioural response (mean ± SE) of rainbow trout to L-alanine in the presence and absence (control) of 1% oil sands process-affected water (OSPW). Asterisks (*) indicate significant differences ($p \leq 0.05$) between OSPW and control groups.

Immediate olfactory recovery from a short-term exposure of OSPW in the EOG experiment indicated that the impairment in perceiving the odorant molecules was not due to damage to the olfactory system. Considering that OSPW itself produces an olfactory response, it is plausible that the observed effect was either due to olfactory fatigue or blockage of the odorant receptors by OSPW components. However, because of the chemically complex nature of OSPW, testing this hypothesis is impractical. Exposure to individual contaminants have yielded transient effects on chemosensory function reported here for the complex mixture of OSPW, whereas others have reported more permanent effects. For instance Green et al. (2010) demonstrated that exposure to low concentrations of copper (80 and 160 nM) immediately suppressed the olfactory function in fathead minnow (*Pimephales promelas*), which recovered completely in a matter of min after removing the copper from the olfactory chamber. On the other hand, exposure to 35 µg/L silver and 45 µg/L nanosilver instantly reduced the EOG response of European perch (*Perca fluviatilis*), which did not recover when switched back to clean water (Bilberg et al., 2011).

An important point about the EOG results on the effect of OSPW on the olfactory acuity of rainbow trout was that exposure to OSPW impaired responses of both ciliated and microvillous OSNs. Consequently, exposure to OSPW induces a general impairment. Some contaminants such as cadmium are also known to cause general olfactory dysfunction (Dew et al., 2016), while others target specific sub-classes of OSNs. For instance, nickel and copper target specifically microvillous and ciliated OSNs, respectively (Dew et al., 2014). With its complex nature, containing thousands of organic and inorganic compounds, it is not surprising that OSPW acts as a general toxicant.

8.4. Summary

The results of the OSPW detection experiments (both behaviour and EOG) demonstrated that fish detect and avoid OSPW at a concentration as low as 0.1%. Studies have shown that fish sometimes fail to avoid (e.g. Hartwell et al., 1989; Tierney et al., 2007) or are even attracted to (e.g. Saglio et al., 2001; Tierney et al., 2011) certain toxic substances. The ability to avoid harmful concentrations of contamination is beneficial to animals in general. Nonetheless, despite its benefits, avoiding contaminated waters may interfere with seasonal migration and consequently reproduction of adult fish. Additionally, due to their lack of mobility, early life stages of fish (i.e. eggs and fry) are not able to avoid contact with contaminants. The behavioural reaction of different fish species to a substance in the same situation may be different (Stober et al., 1980; Gray, 1990). For instance, under controlled laboratory conditions, the avoidance threshold to total residual oxidant (TRO) for coho salmon occurred at 2 μg/L; however, not only did TRO not repel shiner perch (*Cymatogaster aggregata*) at concentrations lower than 175 μg/L, but it also induced preference behaviour at concentrations less than 100 μg/L (Stober et al., 1980). Investigating avoidance/attraction responses of other fish species to OSPW is therefore warranted.

Fathead minnows inhabiting areas contaminated with natural bitumen and areas affected by bitumen minig showed impaired olfactory response to standard chemosensory cues (Chow et al., in prep.). We showed that the fish olfactory system is sensitive to OSPW. Thus, olfactory related endpoints (i.e. EOG and behaviour) can be used as sensitive indicators of the toxic effects of bitumen related contamination. Because of their sensitivity and importance, olfactory related endpoints might also be used for comparing the toxicity

of raw and treated OSPW to determine the effectivenes of the potential remediation treatments.

In a brief exposure, OSPW suppressed olfaction in rainbow trout. However, the fish were able to behaviourally avoid it at a concentration that was an order of magnitude lower than that required to impair an EOG response. Therefore, in the case of an accidental release of OSPW fish may be able to detected and avoid the contamination if they encounter low concentrations of OSPW; but at higher concentrations (1% and over) their olfaction would likely be impaired, precluding their ability to avoid OSPW. The impairment was probably not a result of damage to the olfactory system, because olfaction recovered immediately after removing OSPW from the olfactory chamber. As OSPW contains several chemicals that stimulate an olfactory response in fish, the observed reduction in the sensitivity of the olfactory system in response to a breif exposure to OSPW might be a result of olfactory fatigue. Further studies, with longer exposure times, would improve our understanding of how OSPW interacts with the olfactory system and its effects on olfactory-mediated behaviours in situations where fish cannot or choose not to avoid OSPW.

CHAPTER 9: Oil sands process-affected water impairs the olfactory system of rainbow trout (*Oncorhynchus mykiss*)

Abstract

Oil sands process-affected water (OSPW), a byproduct of the extraction of bitumen in the surface mining of oil sands, is currently stored in massive on-site tailings ponds. Determining the potential effects of OSPW on aquatic ecosystems is of main concern to oil sands companies and legislators concerned about the reclamation of mining sites. In the present study, the interaction of OSPW with the chemosensory system of rainbow trout was studied. Using an electro-olfactography (EOG) technique, a 24 h inhibition curve was established to investigate the toxic effect of OSPW on the olfactory acuity and its response to chemical cues. Concentrations of OSPW that inhibit the olfactory system by 20 and 80% (IC20 and IC80) were estimated at 3 and 22% OSPW concentrations, respectively. To study the interaction of exposure time and OSPW exposure along with the mechanism of the toxic effect of OSPW, rainbow trout were exposed to 3 and 22% OSPW for 2, 24, and 96 h. An EOG investigation of the acuity of the olfactory system demonstrated a positive interaction between exposure time and concentration of OSPW, as an increase in either or both elevated the inhibitory effect of OSPW. To investigate whether or not structural damage of the olfactory epithelium could account for the observed chemosensory inhibitory effects of OSPW, the ultrastructure of the olfactory epithelium of exposed fish was investigated using scanning electron microscopy (SEM) and light microscopy (LM). The SEM micrographs showed no changes in the structure of the olfactory epithelium. The light micrographs revealed both an increase in the number of mucous cells generally, as well as frequency of

mucous cell in 22% OSPW, regardless of exposure duration. The results of the present study demonstrated that exposure to OSPW impairs the olfactory system of rainbow trout and its effects increase gradually with increasing exposure time. The present study demonstrated that structural epithelial damage contributed to the inhibitory effects of OSPW on the olfactory system.

9.1. Introduction

Extracting crude oil from massive oil sands deposits, located in Northern Alberta, is a leading industry in Canada. Open-pit mining followed by the Clark hot water extraction process is the primary method practised by operating companies for extracting bitumen from ore (Giesy et al., 2010). The water used in this process – oil sands process-affected water (OSPW) – is currently stored in massive on-site tailing ponds (Del Rio et al., 2006). The volume (approximately one billion m^3) (Collins et al., 2016) and toxicity of OSPW is an environmental concern to the public and a challenge for oil sands operators with regards to meeting the reclamation requirements at the end of the mine life (AER, 2015).

In the extraction process, bituminous materials contaminate the water with a wide range of organic compounds such as naphthenic acids (NAs) oxidized NAs and related organic acids containing sulfur or nitrogen, and poly-cyclic aromatic hydrocarbons (PAHs), trace metals, salts, and suspended particulate matter (SPM) (Giesy et al., 2010; Headley et al., 2013). However, the detailed composition of OSPW varies based on the chemical properties of the ore, extraction process, age and active or inactive profile of the tailing pond (Allen, 2008). Of the known components of OSPW, organic components are thought to be the main agents of toxicity (Morandi et al., 2016). Due to the complex composition

of the organic chemicals (Pereira et al., 2013a), pinpointing the chemical components responsible for the toxic effects is arduous.

Fish are of great importance, both environmentally and commercially. Therefore, the clear majority of ecotoxicology studies on the adverse effects of OSPW on aquatic organisms are focused on fish. Previous studies show that OSPW causes mortality in fish and their embryos (He et al., 2012a; Toor et al., 2013), immunotoxicity (MacDonald et al., 2013; Sun et al., 2014), genotoxicity (Lacaze et al., 2014), endocrine disruption (Knag et al., 2013; Leclair et al., 2015), reproduction suppression (Kavanagh et al., 2012; Kavanagh et al., 2013), and embryo developmental malformation (Peters et al., 2007; He et al., 2012a; Scarlett et al., 2013).

Chemosensation is vital for fish survival, because fish rely on chemosensation for perceiving and responding to food, evaluating the threat of predation, and identifying an appropriate mate (Tierney et al., 2010). Located on a rosette inside each olfactory chamber, olfactory sensory neurons (OSNs) detect minute concentrations of chemicals in the immediate environment (Hara, 1994). Three classes of OSNs, ciliated, microvillus, and crypt cells respond to social, food, and reproductive cues, respectively (Hamdani and Døving, 2007). To detect these chemical cues, OSNs are in direct contact with the surrounding water, which make them sensitive to low concentrations of toxicants and vulnerable to environmental contamination (Tierney et al., 2010). Thus, the olfactory system and olfactory-driven behaviours are ideal targets for investigating the toxic ecological effects of contaminants on fish.

Despite its importance and sensitivity, studies on the effects of OSPW on the fish olfactory system is limited. In a recent study (Lari and Pyle, 2017b), the interaction of

OSPW with the olfactory system and olfactory-mediated behaviours of rainbow trout (*Oncorhynchus mykiss*) when OSPW was first encountered in clean water was investigated. The aim of the present study was to investigate the interactive effects of exposure time and concentration on the toxicity of OSPW on rainbow trout olfaction. Following the adverse outcome pathway (Ankley et al., 2010), the mechanism of the effects of OSPW on the olfactory system was also studied.

9.2. Materials and methods

9.2.1. Test animals and chemicals

Juvenile rainbow trout (8–12 cm total length) were provided by Allison Creek Brood Trout Hatchery Station, in Alberta, Canada. A *Gyrodactylus salmonis* – an ectoparasite that invades external surfaces, including the olfactory chamber – infection (1.3 ± 0.8 parasites per cm^2 body surface) was diagnosed on fish upon arrival. Because *G. salmonis* impairs fish olfactory function (Lari and Pyle, 2017a), fish were treated with a parasiticide (Parazipro, Hikari, USA) as described in Lari et al. (2017b) and were given two weeks to recover from the treatment. Fish were kept in a 700 L holding tank, and the density was maintained at less than 5 g/L with a light:dark cycle held constant at 16:8 hr, respectively. The water quality in the holding tank was monitored and maintained with the following parameters (mean ± SD; n = 5): temperature, 10 ± 0.1 ºC; dissolved oxygen, 9.0 ± 0.4 mg/L; hardness, 178 ± 4 mg/L as $CaCO_3$; alkalinity: 137 ± 3.6 mg/L as $CaCO_3$; ammonia, 0.02 ± 0.01 mg/L; nitrate: 2.1 ± 0.9 mg/L; and nitrite, 0.017 ± 0.004 mg/L. Median pH of the water in the holding tanks was 8.23 (range: 8.06-8.41). All procedures associated with the present

study were approved by the University of Lethbridge Animal Welfare Committee (permit #1411).

The OSPW sample used in the present study was collected from a four-year-old, active tailings pond by a major oil sands company operating in the Athabasca region, Alberta, Canada. The organics and major trace metal composition of the OSPW sample were analysed and reported in Lari et al. (2016) (Figure 2.2 and Table 2.1; labelled as OSPW-B). The detailed methods for analysing the OSPW sample and its chemical properties are presented and discussed in Lari et al. (2016). The OSPW sample was stored at 4 °C in 22.7 L HDPE buckets with tight sealing lids (Pro-western Plastics LTD., Canada) before using in experiments.

9.2.2. Electro-olfactography assay

To investigate the effect of OSPW on the acuity of the olfactory system, physiological response of fish to standard chemosensory stimuli was studied using electro-olfactography (EOG) as described in Lari and Pyle (2017b). Briefly, fish were anaesthetized by immersion in MS-222 (120 mg/L tricaine methanesulfonate and 360 mg/L $NaHCO_3$). The septum that divides the anterior and posterior nares was removed and a gelatin- and saline-filled glass microelectrode was positioned on the second largest lamella of the olfactory rosette as the recording probe. The reference microelectrode was placed on the skin between the opening of the olfactory chamber and eye. A 10^{-5} mol/L solution of L-alanine (Fisher Scientific) or taurocholic acid (TCA; Sigma-Aldrich, USA) was used to stimulate activity in microvillous or ciliated OSNs, respectively. Fish culture water was used as a blank stimulus. Stimuli were delivered randomly to the olfactory chamber in 3-second pulses with 90 s rest time

between stimulus deliveries. A constant flow of culture water, matching that of the stimulus flow, perfused the olfactory chamber during the rest period to wash out the stimuli from the chamber. The EOG response to a stimulus was determined by measuring the amplitude of the corresponding recorded peak.

9.2.3. Inhibition curve

A 24 h inhibition assay was performed to estimate the concentrations of OSPW that could reduce the olfactory response of fish to chemical cues at 20 and 80% – IC20 and IC80 – respectively. In this test, six groups of six fish were exposed to a geometric dilution series of five concentrations of OSPW (2, 4, 8, 16, and 32%) and culture water as a control for 24 h. At the end of the exposure, their olfactory acuity was investigated using EOG, as described in section 9.2.2. The IC20 and IC80 concentrations – calculated in this test – were used as the exposure concentrations in the subsequent experiments.

9.2.4. Time and OSPW interaction

To investigate the effect of time on the toxicity of OSPW, fish were exposed to OSPW for 3, 24, and 96 h. During each exposure period, three groups of 6 fish were exposed to IC20 and IC80 OSPW concentrations. At the end of each period, fish olfactory acuity was assessed using EOG, as described in section 9.2.2. Since exposure to OSPW reduced the fish EOG response to TCA and L-alanine in a similar manner, only the response of fish to TCA was tested in this experiment.

9.2.5. Histopathology assays

To investigate the effect of OSPW on the olfactory tissue, both left, and right olfactory rosettes of the fish used in section 9.2.3 were sub-sampled to be studied, via a light microscopy (LM) and scanning electron microscopy (SEM). Left rosettes were preserved in Karnovsky's fixative (2% paraformaldehyde; 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4) for SEM. After fixation samples were washed with 0.1 M phosphate buffer, dehydrated in ascending concentrations of ethanol (70-100%), and critically dried in hexamethyldisilazane (Ted Pella, Inc., USA). Finally, samples were mounted on aluminium stubs, sputter-coated with platinum, and examined under a tabletop SEM (TM-1000, Hitachi).

9.2.6. Concentration of cAMP

Cyclic AMP was measured using a cAMP complete in vitro ELISA kit (Abcam, ab133051), in which a goat anti-rabbit immunoglobulin G binds with a cAMP antibody. Cyclic AMP then binds to the antibody in competition with a labelled colorimetric conjugate which was measured at 405 nm using a microplate reader (Varioskan Flash). Standards of known cAMP concentrations were used to compare to samples. Before the assay, frozen tissue samples were homogenized in 0.1 M HCl (0.1 g per mL). The homogenate was pelleted, and the supernatant was used for the assay. Two blanks were included, one with substrate only, and the other received all additions except a sample. Control wells were also monitored for non-specific binding and colorimetric maximums.

9.2.7. Statistical Analysis

All experimental data were tested for the parametric assumptions of normality and homogeneity of variance using a Shapiro-Wilk's and a Levene's test, respectively. A two-way ANOVA was used to evaluate the data from the EOG and cAMP test on the interactive effect of time and concentration of OSPW on the olfactory acuity of rainbow trout and cAMP concentration. A Tukey's post-hoc test was used to compare the treatments if the two-way ANOVA test showed a significant difference between treatments. Statistical significance was set a priori at $\alpha = 0.05$. All data were analysed using an IBM SPSS 22 software (IBM, USA).

9.3. Results

9.3.1. Inhibition curve

The olfactory response of rainbow trout to both L-alanine and TCA following the 24 h exposure at various concentrations was reduced in a concentration-dependent manner that was adequately fit with a log-logistic model (Figure 9.1 A&B; Appendix 9.1). The inhibitory effect of OSPW on microvillus (which respond to L-alanine) and ciliated (which respond to TCA) OSNs was similar. The concentration of OSPW required to inhibit the olfactory response to L-alanine and TCA by 20% (i.e. IC20) was determined to be 3.0% and 2.8%, and IC80 was calculated to be 22.0 % and 20.1%, respectively. Because the inhibitory effect of OSPW on the ciliated and microvillus OSNs was similar, only fish response to L-alanine was measured in the time-course experiment.

9.3.2. Time-course of OSPW toxicity

The results of the effect of OSPW on rainbow trout EOG responses to L-alanine over different exposure durations revealed a positive interaction between exposure time and OSPW concentration [$F(4,45) = 2.66$, $p = 0.04$; Figure 9.2; Appendix 9.2]. The inhibitory effect of both 3 and 22% OSPW was exacerbated by increasing exposure time. At 3% OSPW, the impairment of the olfactory system grew from 3.5% after 3 h of exposure to 17.7% after 24 h, and 35.3% after 96 h. At 22% OSPW concentration the inhibitory effect ranged from 59.4% after 3 h to 77.5% after 24 h and 86.1% after 96 h exposure period.

9.3.3. Histopathology

Using SEM, the surface structure of the olfactory epithelium of the OSPW exposed and the control fish was studied. The olfactory epithelium of all groups was completely intact, and no loss of cilia was observed (Figure 9.3). However, the surface of the olfactory epithelium in OSPW-exposed groups, especially in the 22% treatments, was covered with particles (Figure 9.3). Light microscopy of the olfactory epithelium ultrastructure showed no changes in the prevalence of the mucous or rodlet cells, or the integrity of the basal membrane in any of the 3% OSPW treatments (Figure 9.4 B). Fish exposed to 22% OSPW for 24 and 96 h showed local aggregations of mucous cells that formed large mucous cysts in the olfactory epithelium (Figure 9.4 C&D). Only in the 96 h, 22% OSPW treatment was an increase in the number of mucous cells observed.

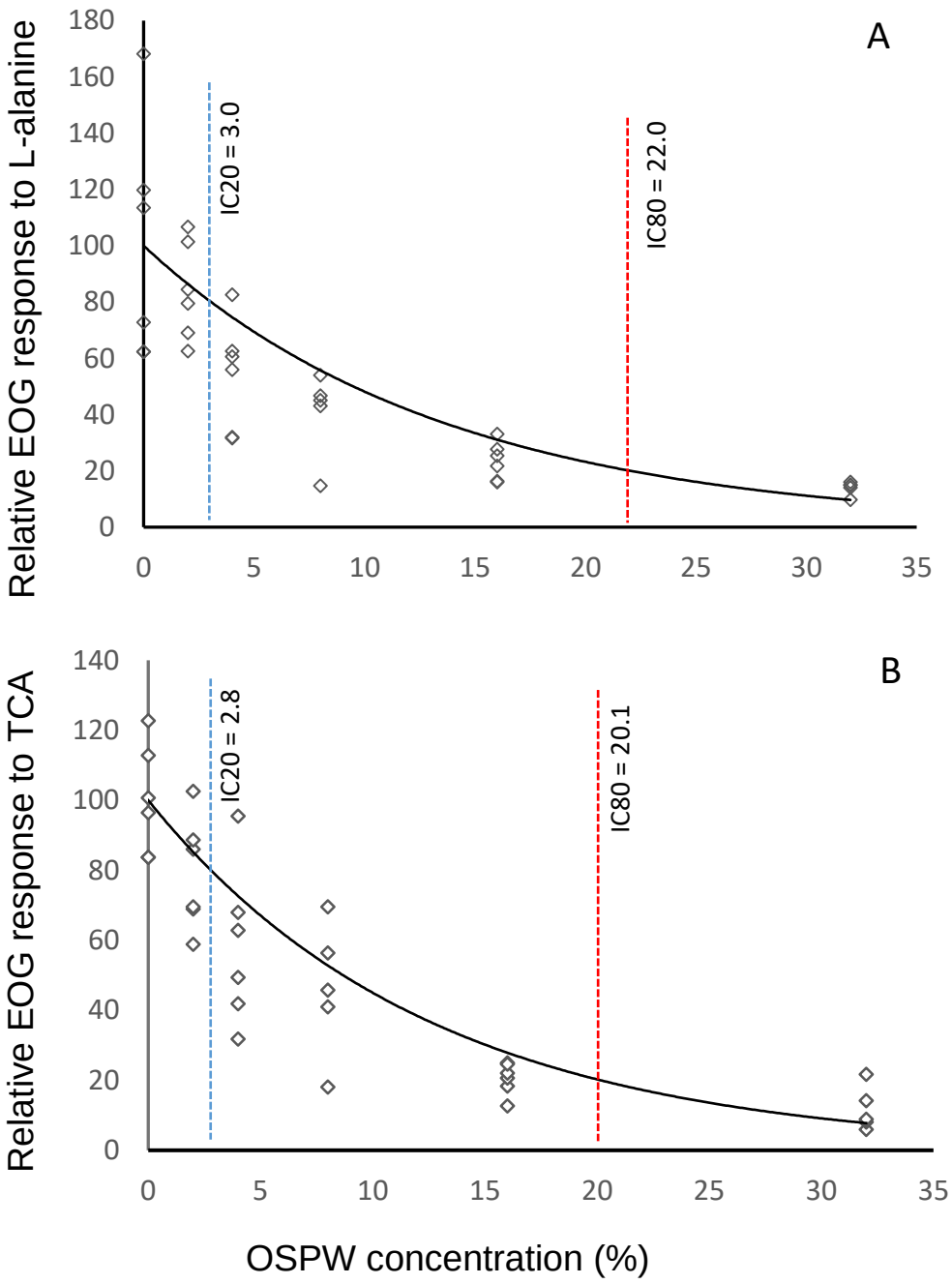


Figure 9.1. Inhibitory effect of OSPW on olfactory response of rainbow trout to: A) L-alanine, B) taurocholic acid (TCA); n = 6.

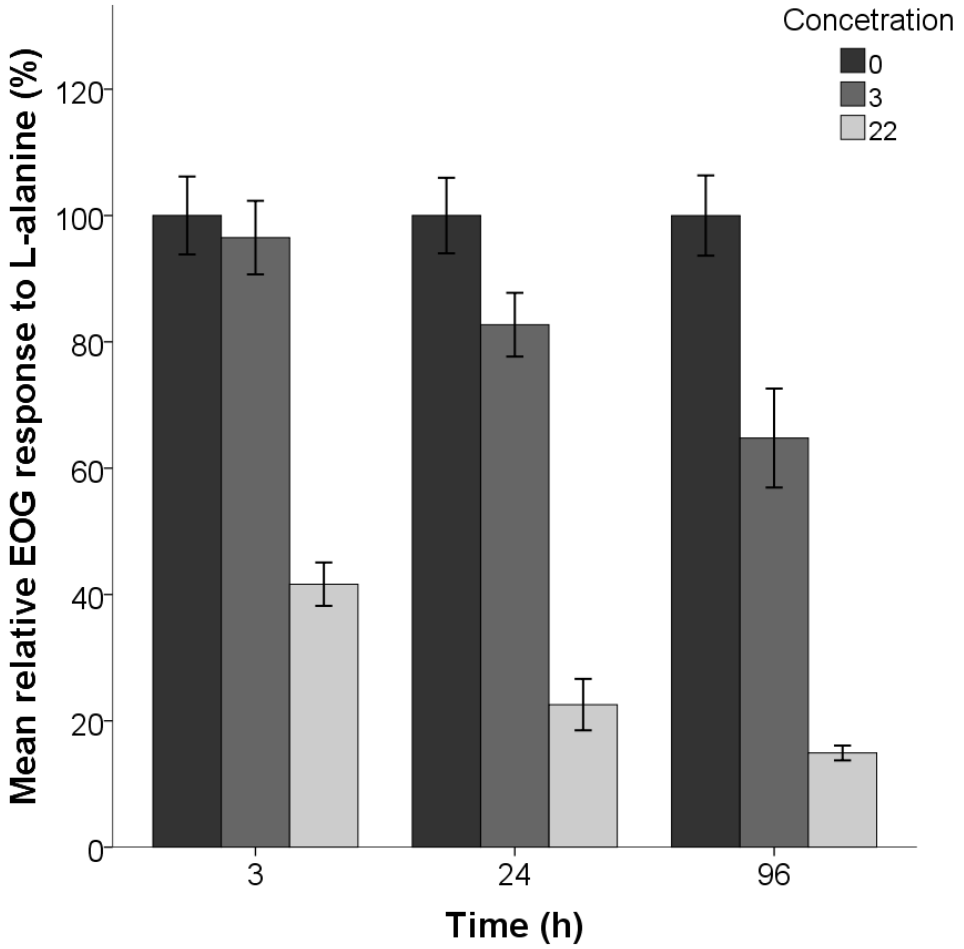


Figure 9.2. Inhibitory effect of oil sands process-affected water (OSPW) on olfactory response of rainbow trout to L-alanine, following 3, 24, and 96 h exposure. n = 6, error bars: ± SE.

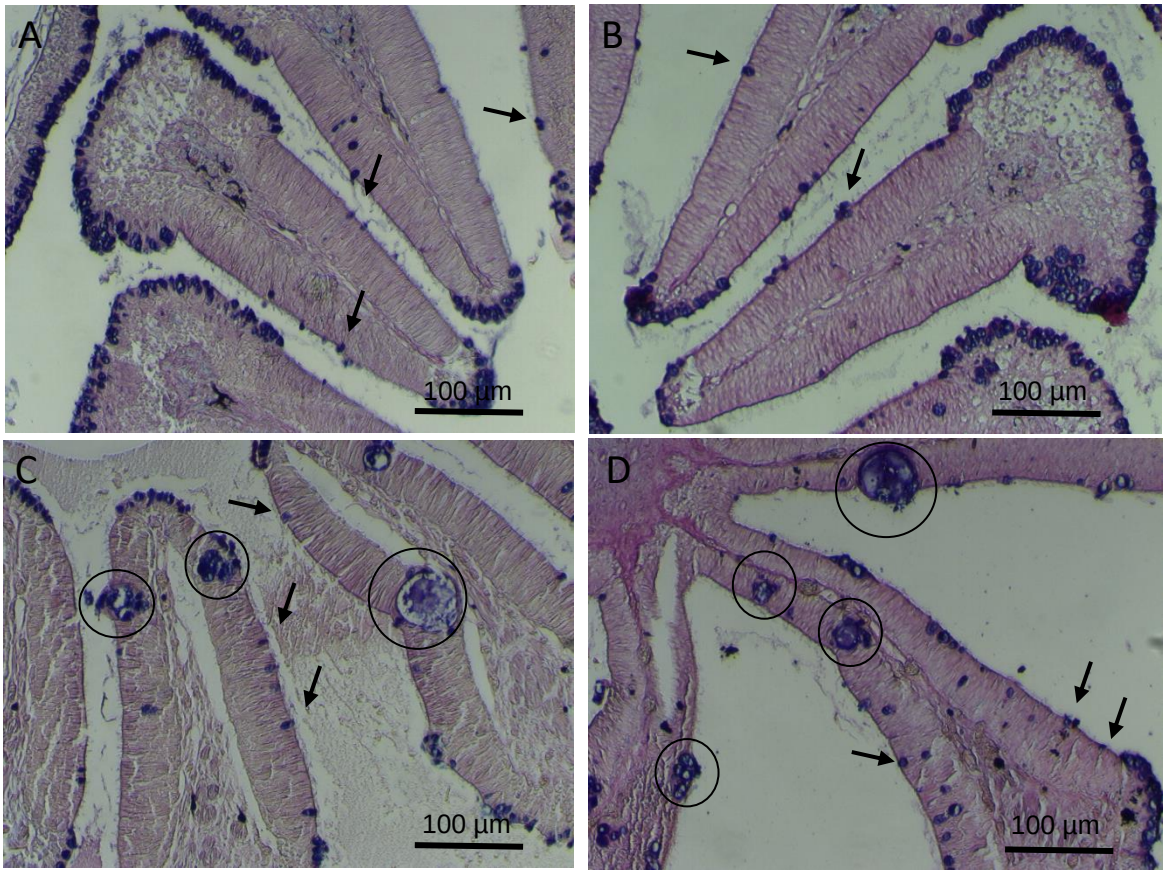


Figure 9.3. Olfactory epithelium of rainbow trout as observed with light microscopy: A) Control, B) Exposed 3% OSPW for 96 h, C) Exposed to 22% OSPW for 24 h, D) Exposed to 22% OSPW for 96 h. Arrows indicate mucous cells and circles indicate local aggregation of mucous cells.

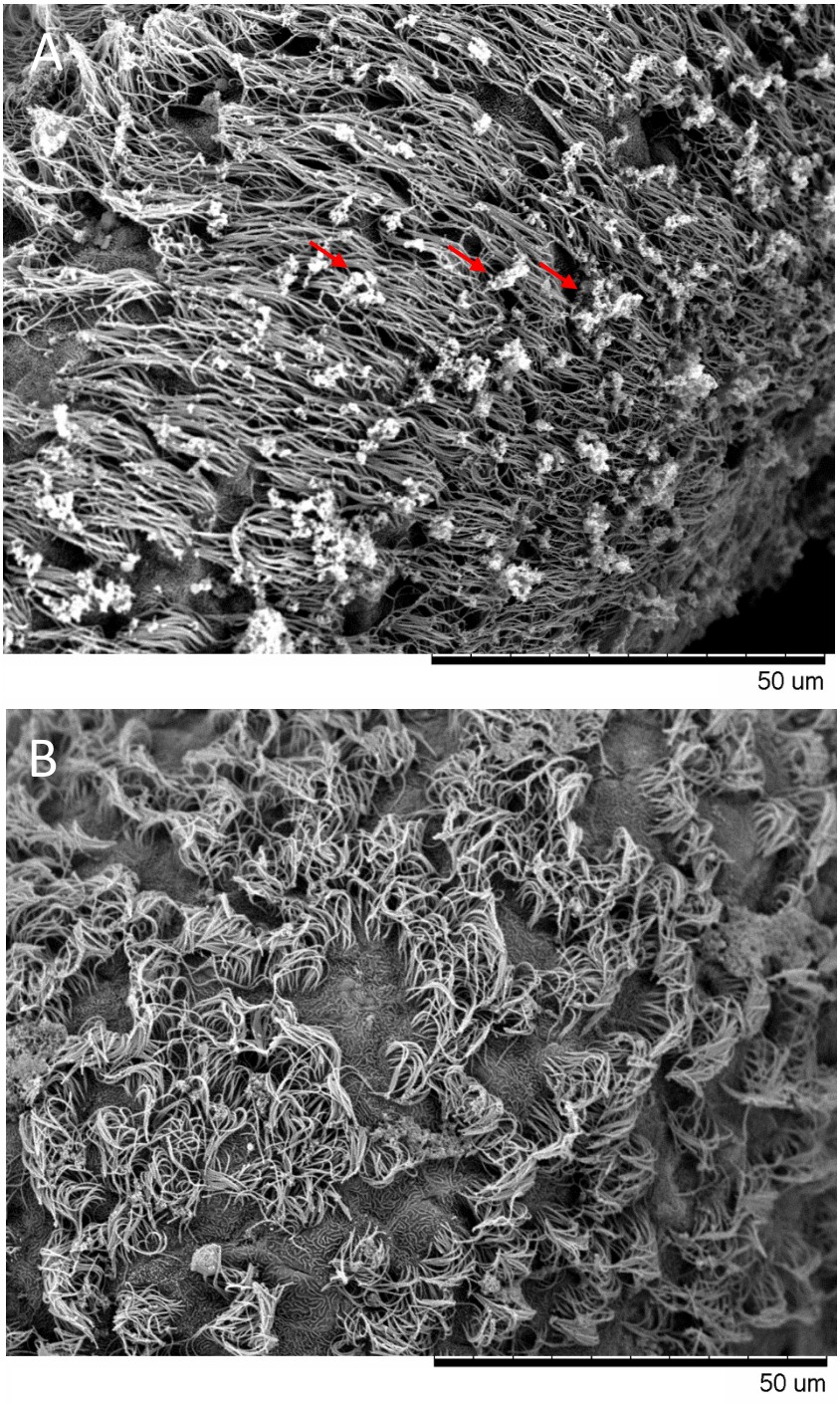


Figure 9.4. Scanning electron microscopy images of olfactory lamella of rainbow trout exposed to 22% oil sands process-affected water (A) and clean water (B). Arrows indicate particulate matter.

9.3.4. Concentration of cAMP

Two out of the four replicates of all groups (control, 3%, and 22% OSPW) from the 96 h exposure were lost during the analytical process. Therefore, the 96 h exposures were excluded from statistical analysis; however, the median values from these treatments were included in Figure 9.5. There was no interaction between exposure time and concentration of OSPW [$F(2,17) = 0.93$, $p = 0.41$; Figure 9.5; Appendix 9.3], and none of the OSPW treatments altered the concentration of cAMP in the olfactory tissue [$F(2,17) = 2.34$, $p = 0.13$; Figure 9.5; Appendix 9.3].

9.4. Discussion

The effect of OSPW on the olfactory system of rainbow trout best fits a reverse exponential curve, as the slope of the inhibition curve is constantly reduced as a function of increasing exposure concentration. The inhibitory effect of OSPW on both of the OSN classes (i.e. microvillus and ciliated) was similar. The IC20 was estimated to be 2.8% and 3% OSPW and the IC80 was estimated to be 20.1% and 22% for ciliated and microvillus OSNs, respectively. Some contaminants such as nickel (Ni) and copper (Cu) display OSN-specific toxicity. For instance, 25 µg/L Ni impairs the function of microvillous cells while a concentration as high as 500 µg/L Ni does not impair ciliated OSNs (Dew et al., 2014). On the other hand, some contaminants can induce a general olfactory toxicity, targeting both ciliated and microvillus OSNs equally. For instance, in a study on rainbow trout, 96 h exposure to cadmium (Cd) impaired ciliated and microvillus cells at the same rate (Dew et al., 2016).

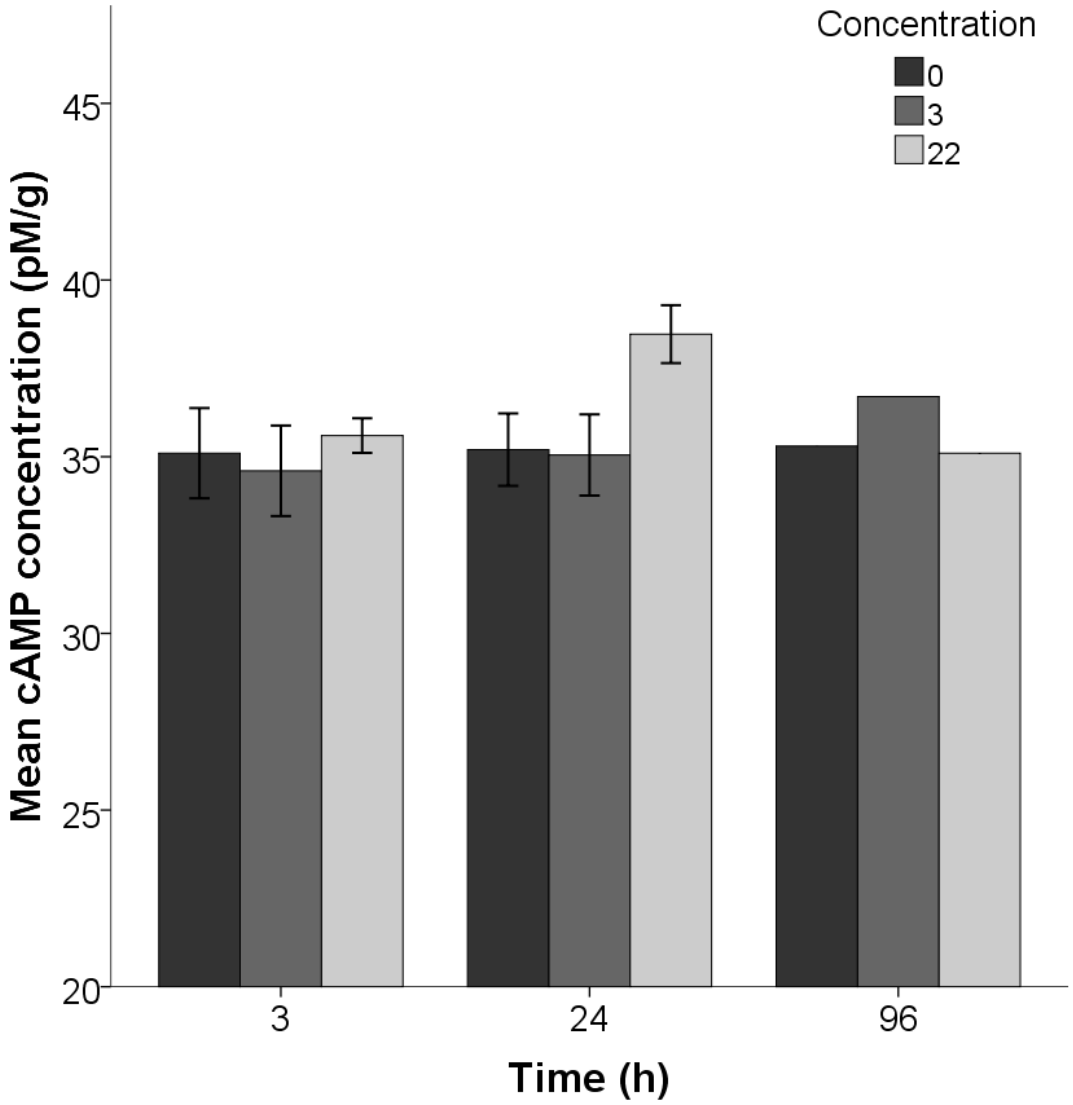


Figure 9.5. Effect of oil sands process-affected water (OSPW) on cAMP concentration in the olfactory epithelium of rainbow trout to L-alanine, following 3, 24, and 96 h exposure. n = 4, error bars: ± SE. Bars for 96 h treatments represent the median concentration of two replicates.

The results of the inhibition curve experiment suggest that OSPW is a general olfactory toxicant. Oil sands process-affected water is a complex mixture of thousands of components including organics, trace metals, and salts (Giesy et al., 2010; Headley et al., 2013). Since each component has different properties, showing a general olfactory toxicant behaviour was not unexpected from OSPW.

It was noteworthy that in the time and concentration experiment, the olfactory function of the fish exposed to 3 and 22% OSPW (calculated IC20 and IC80) was impaired by 18 and 78%, respectively. This result suggests that the fish response to OSPW exposure was proportional to exposure concentration. The inhibitory effect of OSPW on the olfactory system increased with exposure time. A 3 h exposure to 3% OSPW caused a minimal, insignificant reduction in the fish response to L-alanine by 3.5%, while the same concentration induced 35% impairment after 96 h exposure. *In vivo* and *in vitro* studies demonstrated that OSPW induces expression of both Phase I and Phase II metabolic proteins such as cytochrome P450 enzymes (CYPs), glutathione S-transferase (GST), and catalase (CAT), in fish (Gagné et al., 2013; Gagné et al., 2017; Marentette et al., 2017). Escalation of toxicity by increasing the exposure time implies that the metabolic mechanisms in the olfactory tissue were not efficiently detoxifying OSPW constituents, as reflected in the progressive toxicity associated with exposure time. In contrast, these mechanisms efficiently reduce the inhibitory effects of trace metals such as Cd and Cu, as demonstrated in a study on fathead minnows where olfactory recovery occurred during 24 h to 5-days of continuous exposure (Steinkey et al. in prep.).

A common response of the olfactory epithelium to some dissolved contaminants and particulate matter is to elevate mucus secretion, and in intense scenarios, to increase the

size and proliferation of mucous cells (Goldes et al., 1988; Beyers and Farmer, 2001; Ghosh and Mandal, 2014). Oil sands process-affected water contains compounds that are irritating to the rainbow trout olfactory system, and fish try to avoid them (Lari and Pyle, 2017b). Additionally, SEM micrographs showed that the particulate matter in OSPW stuck to the surface of the olfactory epithelium. Despite all interactions mentioned above, the number and size of the mucous cells in the olfactory tissue did not change in most of the OSPW treatments, except for the 96 h, 22% OSPW exposure. Olfactory epithelium of the 24 and 96 h exposure to 22% OSPW demonstrated aggregatiom or fusion of mucous cells. This histological effect might be a local response to irrritation caused by OSPW. The fact that the population of mucous cells in low concentration (i.e. 3% OSPW) treatments did not show mucous cell aggregations might suggest that the irritation was only sufficient to increase mucus production, but not to change the distribution or size of the mucous cells. An investigation of the mucus content of olfactory tissue will illuminate the regulation of mucus secretion in response to OSPW exposure.

At the ultrastructure level, contaminants might induce apoptosis (Saucier et al., 1991; Julliard et al., 1996; Wang et al., 2013) and necrosis (Brown et al., 1982) in OSNs. Considering the severely impaired olfactory acuity down to 14% of the normal response to L-alanine in 22% OSPW treatment, it is likely that OSN cell death might be one of the causes of olfactory impairment induced by OSPW. Further investigation of OSN apoptosis using microscopy (Julliard et al., 1996; Wang et al., 2013) and gene expression quantifying techniques (He et al., 2012a) will elucidate some of the remaining unknown effects of OSPW on the ultrastructure of the olfactory epithelium.

A very likely mechanism of the toxic effect of OSPW on the olfactory system might be through alteration in the structure and integrity of the cell membrane, leading to a disruption in the membrane functionality. Olfactory sensory neurons are bipolar neurons that interact with odorants at their sensory knob, on the surface of the olfactory epithelium, produce an action potential that travels along the axon and propagates to the central nervous system via the olfactory bulb (Buck and Axel, 1991; Hamdani and Døving, 2007). At the sensory site, odorant molecule binding to a class of G protein-coupled receptors (GPCRs), known as olfactory receptors (ORs), creates a cascade of cyclic AMP-dependent events leading to depolarization of the sensory neuron (Jones and Reed, 1989). Changes in the structure of the cell membrane interrupt this process. Naphthenic acids which are a major constituent of OSPW, display surfactant properties (Holowenko et al., 2002). Surfactants are well known for causing damage, morphology change, and rupture in cell membrane (Groot and Rabone, 2001; Rege et al., 2002). In an *in vitro* study, NAs extracted from OSPW changed the structure of cell membranes (Dr. Steve Wiseman, personal communication, 18 April 2017).

In summary, exposure to OSPW impairs the olfactory system of fish. Increasing exposure time and OSPW concentration increases the inhibitory effect of OSPW. Since fish rely on their sense of smell for finding food and avoiding predators, impairment of the olfactory system might reduce their chance of survival. Our attempt to elucidate the mechanism of OSPW effects on the olfactory system did not reveal many of the causes of toxicity, as an effect was only observed in 24 and 96 h exposures to 22% OSPW, while in most of the treatments an inhibition of the olfactory system was observed. Future studies might further illuminate the mechanism of OSPW toxicity by investigating OSNs

apoptosis, cell membrane integrity, and expression of contaminant metabolism and membrane proteins.

CHAPTER 10: Conclusions

The present study addressed a variety of research questions regarding the effects of oil sands process-affected water (OSPW) on two organisms, representing invertebrate (i.e. *Daphnia magna*) and vertebrate (i.e. rainbow trout) aquatic animals. The first part of the study was designed to investigate the effects of OSPW on the major biological functions (i.e. feeding, respiration, and circulation) of *D. magna* and how observed effects translate to organism- and population-level effects. I was also interested in understanding the effects of the chemical dissimilarities of OSPWs from various sources and seasonal changes in water quality variables (e.g. temperature, hardness, and alkalinity) on the toxicity of OSPW.

Three OSPW samples from different sources revealed that OSPW impaired feeding behaviour, reproduction, and growth of *D. magna*. However, the effects of these OSPW samples on the targeted endpoints, as well as their chemical characteristics, such as the trace metal concentration and the distribution of various hydrocarbons, was similar to each other (Chapter 2). This result allowed for the use of a mixture of all three OSPW samples for the rest of the first part of the present study.

A novel observation apparatus was developed to allow for the investigation of OSPW effects and their associated mechanisms on feeding behaviour (i.e. thoracic limbs, mandibles, post abdominal rejection, and peristaltic activity; Chapter 3) and cardiovascular function (i.e. heart rate; Chapter 3) in *D. magna*. An investigation of the mechanism of the effect on feeding behaviour demonstrated that suspended particulate matter (SPM) of OSPW impairs food consumption by reducing the efficiency of the food digestion process mainly via reducing the capacity of the gut for taking up food, reducing food contact with digestive enzymes, and reducing digestion time; while dissolved components (DC) reduce

the digestion time by impairing peristaltic activity. Impairment of digestive enzyme activity had little or no effect in the reduction of food consumption (Chapter 4).

Effects on the respiratory and circulatory system of *D. magna* showed an increase in oxygen consumption and a decrease in hemoglobin content in OSPW-exposed animals (Chapter 5). These results indicated that exposure to OSPW increased the metabolic rate, and consequently energy demand, of *D. magna*, most probably due to ongoing detoxification processes. The results of Chapters 2-5 suggested that due to increased energy demand and reduced nutrient supply, chronic exposure to OSPW might reduce energetic reserves, suppress growth, impair reproduction, and reduce the quality of offspring in *D. magna*. A full set of experiments with the above-mentioned endpoints confirmed this hypothesis (Chapter 6). Chronic (10-day) exposure to OSPW reduced reproduction, the size of the offspring, growth, and energy reserves in *D. magna*.

To address the final objective of the first part of the thesis – effect of seasonal changes in water quality parameters on the toxicity of OSPW – *D. magna* were chronically exposed to OSPW under a cold or a warm season water quality condition. The results demonstrated that during cold seasonal conditions, OSPW is more toxic to *D. magna* than it is during warm seasonal conditions (Chapter 7). The difference in OSPW toxicity became evident in survival and growth, and *D. magna* held under cold seasonal conditions did not reproduce at all over the course of the experiment. It appears that the stress from cold water helped to increase the adverse effects of OSPW on *D. magna*, as they are not well adapted to cold waters.

The results of the first part of the present project demonstrated that OSPW interferes with normal operation of several major functions in *D. magna* (i.e. feeding, respiration, and

circulation) and increases their energetic requirements due to an elevation of metabolic rate. The results of these interactions then externalise in the form of suppressed growth and reproduction, which themselves might induce a population and (or) ecosystem perturbation. Multigeneration and microcosm studies would illustrate the ecological consequences of the observed effects of OSPW on *D. magna* at the ecosystem level.

The objectives of the second part of the present study were first, to investigate the interaction of OSPW with the olfactory system and the resultant olfactory-mediated behavioural responses of rainbow trout during the first few minutes of an encounter with OSPW. The second objective was to study the interaction of the exposure time and OSPW concentration in inducing an inhibitory effect on the olfactory system and to elucidate the mechanisms by which OSPW induces adverse effects.

An electro-olfactograpy (EOG) technique was used to study the interaction of OSPW with the olfactory system at the physiological level. To study the ability of the olfactory system to detect OSPW, the EOG technique was modified to increase its sensitivity by a hundred-fold (unpublished data). The EOG results showed that rainbow trout could detect OSPW at a 1% concentration using olfaction. However, rainbow trout actively avoided OSPW at a concentration as low as 0.1% (Chapter 8). The observed difference between the behavioural and EOG results might be because of the lower sensitivity of EOG relative to an intact olfactory system that mediates the behavioural response. Further modifications are required to increase the sensitivity of the EOG analysis to better reflect the olfactory sensitivity required to mediate these behavioural responses. The results of the first encounter study also revealed that a brief exposure (less than 5 min) to 1% OSPW significantly impaired rainbow trout olfaction, although recovery occurred soon after

returning to clean water (Chapter 8). The fact that fish can detect and avoid OSPW at a concentration below the toxic concentration is promising. However, in many cases fish might not be abele to avoid OSPW contamination due to physical restrictions. Additionally, avoiding OSPW contaminated waters may interfere with their seasonal migration. Rapid (< 5 min) recovery from the toxic effect of OSPW suggests that the interaction of OSPW with the olfactory system occurs most probably on the surface of the olfactory epithelium, where components of OSPW might bind to odorant receptors (OR) of the olfactory sensory neurons (OSNs). Binding of those components with ORs would either produce a repetative signal discharge, which could lead to olfactory fatigue, or change the structure of the ORs such that they are rendered unable to perceive odour stimuli.

Rainbow trout displayed a concentration-dependent reduction of olfactory acuity following a 24 h exposure to 3 and 22% OSPW, concentrations that represent the IC20 and IC80 concentraitons. These two concentrations were used as reference concentrations for the following experiments. An EOG investigation of the interaction of OSPW concentration (3 and 22%) and exposure time (3, 24, and 96 h) showed a positive interaction between these two factors. Histopatological analysis and scanning electron microscopy showed no changes in the ultrastructure of the olfactory epithelium in any of the time/concentration treatments. The same result was observed in the study of cyclic adenosine monophosphate (cAMP). The fact that the impairment of the olfactory system increases with longer exposure time suggests that some other mechnism in adition to olfactory fatigue is involved in reducing olfactory acuity. Since no alteration in the ultrastructure of the tissue was observed, the toxic effects of OSPW on the olfactory systems might be a result of the interactions of OSPW with intracellular structures or cell

membrane of OSNs. Studying the gene expression and activity of known detoxification pathways (e.g. glutathione s-transferase, catalase, cytochrome P450s) and cell membrane (e.g. g-coupled protein receptors, pumps, and ligand-gated ion channels) proteins might provide insight into the molecular interactions of OSPW compounds with OSNs. As the cell membrane is the incipient site of interaction with odorants and contaminants, an investigation of the alteration in cell membrane structure, integrity and functionality will also contribute to understanding the mechanism by which exposure to OSPW inhibits olfactory acuity.

Data from the present study (both part 1 and 2) demonstrated that sublethal toxicity of OSPW occurs at much lower concentrations as compared to lethal concentrations. Considering the importance of the studied endpoints for the survival of aquatic animals – olfaction and feeding behaviour in rainbow trout and feeding, reproduction, and growth in *D. magna* – OSPW may affect the survival of individuals, which could lead to population-level effects at very low concentrations. Unfortunately, however, most acute water quality guidelines for protecting aquatic life are more lethality oriented. Thus, if sub-lethal endpoints were not taken into account, the toxicity of OSPW might be underestimated, and consequently, regulations might not be protective. For instance, Dew at al. (2016) showed that the current CCME guideline for cadmium is not protective of olfaction in fish.

Several endpoints that were used in the present study, in addition to their importance for *D. magna* and rainbow trout, appear to be sensitive to OSPW contamination. For instance, feeding behaviour in both species and olfaction in rainbow trout were impaired in response to short-term exposure to 1% OSPW. Such endpoints should be applied in the

investigation of the toxicity of contaminants such as OSPW, in order to develop protective and ecologically relevant regulations.

Except for olfactory acuity, which requires an EOG rig, the equipment required to measure most of the other endpoints examined in this thesis is simple and does not require specialized training or expertise. Therefore, these endpoints can be standardised for regular contamination monitoring. Feeding behaviour tests are ideal for monitoring acute exposures (for both rainbow trout and *D. magna*) and reproduction and growth for chronic exposures. These endpoints could also be used in investigating the efficacy of various remediation approaches.

The results of the present study and Lari et al. (2017a) demonstrated that food consumption of *D. magna* is sensitive to contamination. I also showed that impaired feeding has significant consequences on the well being and fitness of *D. magna*. A *Daphnia* feeding test is easy to conduct and can be standardised. Thus, feeding of *Daphnia* spp. has the potential to become a standard sub-lethal short term test for monitoring the toxicity of contaminants such as OSPW and investigating the efficiency of remediation processes. This endpoint also can be used in conducting acute water quality criteria for protecting aquatic life, especially for contaminants such as OSPW that display little lethality but show sub-lethal toxicity at low concentrations.

The fact that OSPW was toxic for both rainbow trout and *D. magna* at 1% suggest that both mining companies and regulators should establish regulations that prevent exposure of aquatic animals to raw OSPW at concentrations of 1% or higher. However, lower concentrations of OSPW might also have an ecological impact. Because fish avoid 0.1% OSPW, this concentration may interfere with the seasonal migration of fish. The avoidance

behaviour that fish display in response to OSPW contaminated waters might be one of the causes of the drastic reduction in the population of migratory fish (such as northern pike, walleye, and suckers) in tributaries around oil sands mining operation areas, as reported in Schwalb et al. (2015). Although the concentration of organic compounds reported in these waters is low, it might be enough to provoke avoidance behaviour in fish. Thus, a usually underrated behavioural response (i.e. avoidance/attraction), might play an important role in one of the most important ecological shifts that has been reported in oil sands mining areas. However, this hypothsis requires further investigation.

The results of the present study once again demonstrated that the olfactory system and olfactory-mediated behaviours of fish are sensitive to contamination. Several studies have demonstrated the importance of these endpoins in different aspects of fish life. Yet, olfaction and olfactory-mediated behaviours are not considered in either environmental risk assessment or in regulations intended to protect vulnerable freshwater populations. Since electrophysiology methods such as EOG and EEG provide a solid measurment of the acuity of the olfactory system, this endpoint can be standardised and adopted in regulatory framework. Althogh olfactory mediated behaviours are not easy to standardise, their importance for fish and the potential ecological consequences of their alteration calls for furrther efforts for adoptingthese endpoints in regulation making precesses.

Another considerable finding of the present study was that particulate matter plays an important role in the toxic effect of OSPW on feeding rate of filter feeder animals. Therefore, effective removal of particulate matter reduces the toxicity of OSPW to filter feeding animals. Since *D. magna* ingest SPM and excrete feces in the pellet form that sink to the bottom this species has the potential to be used for clearing SPM from OSPW

contaminated waters. Adding a concentration of algal cells to the water that provides enough energy for survival and reproduction of *D. magna* is the main challenge in for developing ans sustaining *D. magna* populations sizes capable of substantially clearing particulate matter in a remediation context.

Despite the effort in characterizing the chemical composition of OSPW and investigating its toxicity, studying the effects of seasonal changes in water quality on the toxicity of OSPW has been neglected. The results of the present study demonstrated that these seasonal changes affect the toxicity of OSPW on *D. magna*, indicating that a release of OSPW might have various effects on the receiving ecosystem in different times of the year. Although, *D. magna* is not well adapted to cold waters, the resuls of the seasonal changes studies in the present study shows a need for further investigation of the effects of water quality parameters of the receiving ecosystem on the toxicity of OSPW.

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APPENDICES

Appendix 2.1: Raw data of the lethal effect of single OSPWs (OSPW-A, OSPW-B, and OSPW-C) on *Daphnia magna*.

	Mortality (%)		
Concentration (%)	OSPW-A	OSPW-B	OSPW-C
0	0	0	0
0	0	0	0
0	0	0	0
25	0	0	0
25	0	0	0
25	0	0	0
50	0	0	0
50	0	10	0
50	0	0	0
75	10	0	20
75	0	0	0
75	0	20	10
100	30	30	40
100	10	40	20
100	30	40	40

Appendix 2.2: Raw data of the effect of acute (24 h) exposure to single OSPWs (OSPW-A, OSPW-B, and OSPW-C) on grazing rate of *Daphnia magna*.

	Feeding rate (%)		
Concentration (%)	OSPW-A	OSPW-B	OSPW-C
0	73.5	73.5	66.3
0	67.3	67.3	72.8
0	74.4	74.4	72. 2
0	74.1	74.1	70.6
0	72.8	72.8	74.8
1.25	61.4	60.6	61.7
1.25	61.1	60.3	59.7
1.25	70.7	65.5	66.6
1.25	71.4	71.0	71.4
1.25	65.3	67.8	69.8
2.5	55.6	56.0	60.8
2.5	46.6	61.8	58.9
2.5	59.7	62.5	61.4
2.5	55.65	60.5	62.8
2.5	54.0	58.9	60.8
5	42.4	18.4	26.6
5	47.3	19.7	22.0
5	52.7	39.5	39.5
5	49.8	35.9	42.8
5	48.2	23.4	29.9
10	28.9	27.0	21.8
10	18.7	16.3	17.8
10	20.0	19.9	15.5
10	23.3	22.5	23.4
10	27.2	20.5	15.2
20	3.3	7.6	6.8
20	5.9	7.6	12.4
20	4.9	20.5	4.6
20	1.3	13.2	9.1
20	8.5	12.9	7.2

Appendix 2.3: Raw data of the effect of chronic (21-day) exposure to single OSPWs (OSPW-A, OSPW-B, and OSPW-C) on grazing rate of *Daphnia magna*.

		Water clearance rate (%)		
	Concentration (%)	1 week	2 weeks	3 weeks
Control	0	38.4	47.6	57.1
	0	36.2	46.8	49.5
	0	40.1	44.0	52.5
	0	34.6	45.0	56.9
OSPW-A	1	16.6	16.6	19.1
	1	20.6	23.2	29.2
	1	17.2	29.1	32.7
	1	16.6	23.7	24.9
	10	10.8	6.9	9.4
	10	11.9	13.8	12.4
	10	9.2	18.7	16.0
	10	9.5	16.7	18.3
OSPW-B	1	20.9	30.7	31.8
	1	20.7	32.2	34.1
	1	16.9	32.5	37.7
	1	27.0	28.1	30.5
	10	16.7	21.6	20.7
	10	18.8	33.4	26.1
	10	13.8	24.1	28.4
	10	14.7	26.9	27.6
OSPW-C	1	19.7	35.3	30.3
	1	21.0	31.7	36.9
	1	17.8	35.0	39.9
	1	25.0	30.2	30.0
	10	18.2	19.3	18.1
	10	20.3	33.9	25.3
	10	15.3	21.9	23.3
	10	17.7	23.4	14.7

Appendix 2.4: Raw data of the effect of chronic (21-day) exposure to single OSPWs (OSPW-A, OSPW-B, and OSPW-C) on *Daphnia magna* reproduction and growth.

	Concentration (%)	Reproduction (neonate per individual)	Mass (µg)
Control	0	46.3	34.7
	0	41.8	23.0
	0	52.1	26.3
	0	39.0	26.0
OSPW-A	1	30.3	13.3
	1	16.2	14.2
	1	26.3	11.7
	1	9.7	12.5
	10	5.0	17.5
	10	9.8	16.3
	10	15.0	20.2
	10	10.0	16.6
OSPW-B	1	22.2	11.0
	1	34.7	15.8
	1	33.2	17.4
	1	24.0	16.4
	10	21.5	20.4
	10	21.3	18.4
	10	17.9	17.2
	10	18.6	19.0
OSPW-C	1	29.0	12.3
	1	26.3	11.2
	1	27.3	15.7
	1	18.5	10.5
	10	21.5	17.4
	10	18.3	20.4
	10	15.0	14.2
	10	19.6	17.0

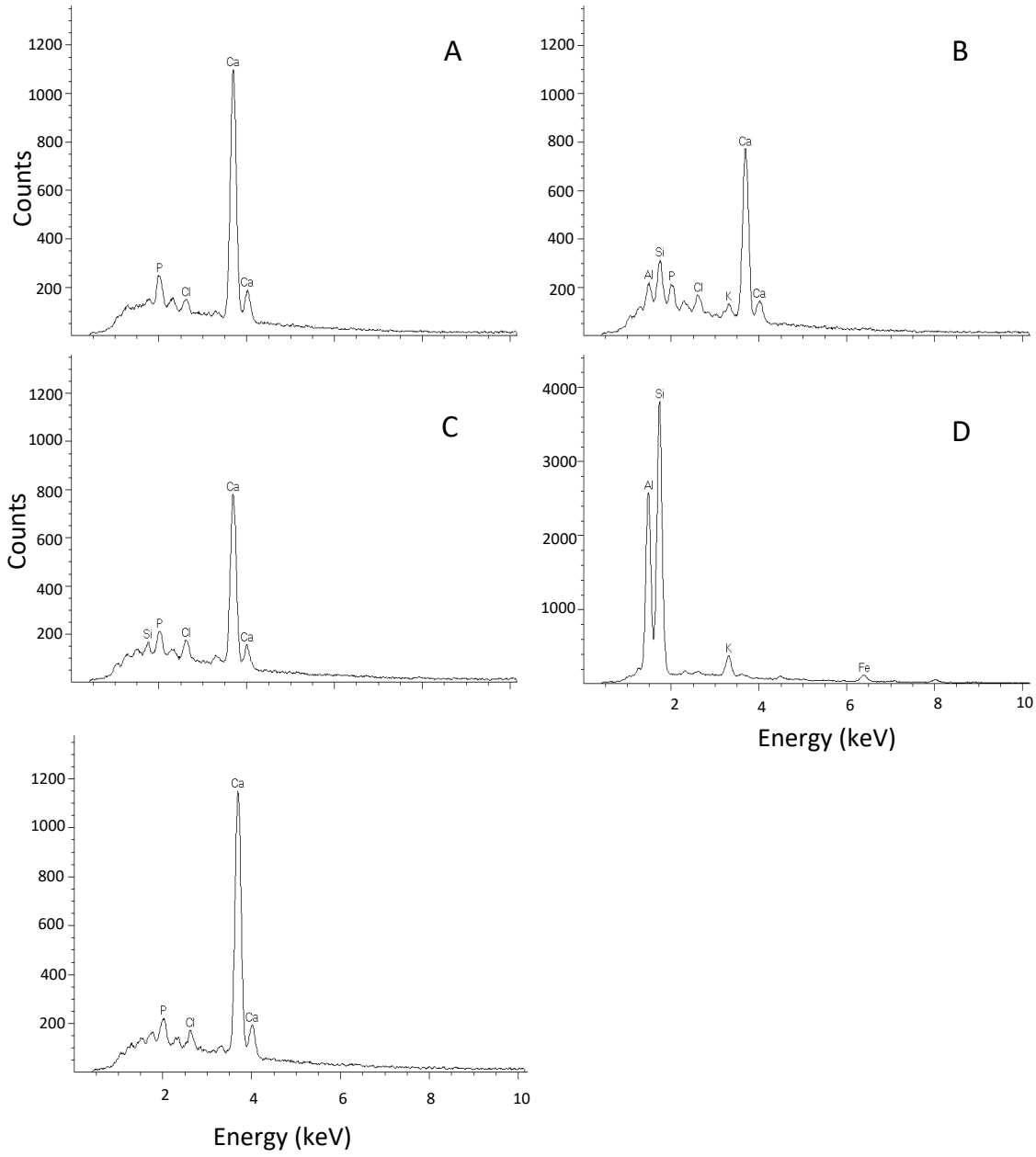
Appendix 2.5: Raw data of the behavioural response of *Daphnia magna* to 24 h exposure to a mixture of all three OSPWs (OSPW-A, OSPW-B, and OSPW-C).

Concentration (%)	target arm	Response (%)	Active time (s)
0	R	100.0	180
0	L	83.3	154
0	L	53.3	121
0	R	78.9	103
0	R	84.4	127
0	R	0.0	78
0	R	100.0	146
0	L	28.9	137
0	L	85.6	129
0	R	70.0	175
0	R	83.3	134
0	R	90.0	156
0	L	33.3	163
0	L	71.1	136
0	R	-12.2	127
0	L	8.9	93
0	L	100.0	166
0	R	96.7	132
0	R	85.6	173
0	R	76.7	154
1.25	L	-27.8	146
1.25	L	12.2	117
1.25	L	48.9	107
1.25	L	34.4	109
1.25	R	84.4	167
1.25	R	0.0	97
1.25	L	14.4	124
1.25	R	-10.0	156
1.25	R	61.1	109
1.25	L	26.7	177
1.25	R	90.0	174
1.25	R	-50.0	158
1.25	L	48.9	162
1.25	R	67.8	86
1.25	R	50.0	138
1.25	L	2.2	143
1.25	L	78.9	139
1.25	L	50.0	172
1.25	R	48.9	60

1.25	L	66.7	89
2.5	R	-18.9	166
2.5	R	71.1	121
2.5	L	-62.2	124
2.5	R	-86.7	45
2.5	L	87.8	145
2.5	R	-61.1	102
2.5	L	86.7	126
2.5	R	25.6	147
2.5	L	-32.2	153
2.5	R	60.0	90
2.5	L	92.2	106
2.5	L	-46.7	123
2.5	R	48.9	144
2.5	L	74.4	79
2.5	L	51.1	112
2.5	R	2.2	69
2.5	R	7.8	172
2.5	R	50.0	174
2.5	L	-62.2	111
2.5	R	82.2	67
5	L	-16.7	84
5	R	-2.2	172
5	L	-12.2	143
5	R	-42.2	138
5	L	18.9	89
5	L	-63.3	123
5	R	96.7	134
5	L	35.6	143
5	L	73.3	107
5	L	-25.6	65
5	L	-21.1	139
5	L	-6.7	165
5	R	45.6	113
5	R	33.3	79
5	L	-73.3	97
5	R	5.6	143
5	L	11.1	112
5	R	-57.8	129
5	L	-40.0	146
5	R	-32.2	139
10	R	7.8	124
10	L	90.0	115

10	R	11.1	156
10	L	-94.4	81
10	R	-56.7	97
10	L	-36.7	69
10	L	72.2	108
10	R	3.3	143
10	R	-51.1	130
10	L	21.1	126
10	R	-83.3	102
10	L	91.1	116
10	R	47.8	52
10	L	-28.9	141
10	R	-67.8	39
10	R	17.8	94
10	L	60.0	85
10	L	68.9	77
10	L	-96.7	145
10	R	-15.6	113
20	L	70.0	52
20	R	-4.4	67
20	L	-62.2	138
20	L	52.2	105
20	R	92.2	123
20	R	86.7	30
20	L	-64.4	89
20	L	61.1	93
20	R	-85.6	107
20	R	98.9	47
20	R	50.0	98
20	L	-51.1	45
20	L	86.7	137
20	R	80.0	104
20	L	86.7	82
20	R	25.6	73
20	L	-81.1	84
20	R	-67.8	128
20	L	52.2	34
20	R	-98.9	73
0/no cue	R	-100.0	160
0/no cue	R	90.0	122
0/no cue	R	94.4	174
0/no cue	L	-3.3	134
0/no cue	L	-22.2	126

0/no cue	R	-88.9	79
0/no cue	L	38.9	136
0/no cue	R	-97.8	137
0/no cue	L	7.8	123
0/no cue	R	-77.8	175
0/no cue	L	-40.0	134
0/no cue	L	90.0	136
0/no cue	L	-95.6	127
0/no cue	R	5.6	67
0/no cue	L	-75.6	156
0/no cue	R	38.9	163
0/no cue	L	10.0	178
0/no cue	R	75.6	154
0/no cue	L	-3.3	121
0/no cue	R	-12.2	113



Appendix 2.6: Samples of EDX readings: A) Control group with no Al or Si detected in their gut, B) 20% OSPW group, C) The lowest OSPW concentration (2.5%) group with only detectable amount of Si, D) Residuum of OSPW filtering, E) Filtered OSPW group with no Al or Si detected in their gut.

Appendix 3.1: Raw data of the effect of acclimation time on both thoracic limbs and heart rate of *Daphnia magna* in the absence of an algae food cue.

Time (min)	Heart rate (beat/min)	Thoracic limbs (beat/min)
0	322	642
0	298	552
0	286	518
0	312	614
0	286	690
0	308	578
0	344	590
0	290	558
0	334	602
0	348	564
5	312	614
5	304	514
5	268	510
5	314	526
5	280	542
5	284	500
5	320	542
5	262	484
5	300	536
5	304	546
10	270	474
10	288	458
10	240	426
10	282	456
10	260	462
10	254	466
10	272	462
10	258	444
10	256	470
10	286	460
15	280	470
15	266	462
15	248	452
15	288	454
15	264	434
15	260	402
15	268	450
15	260	448

15	246	460
15	274	460
20	270	442
20	272	448
20	244	472
20	274	450
20	256	458
20	264	442
20	264	450
20	262	462
20	250	436
20	284	418

Appendix 3.2: Raw data of changes in mandible and thoracic limb movements in *Daphnia magna* with changes in *Raphidocelis subcapitata* density.

Food Concentration (million cell/mL)	Thoracic limbs (beat/min)	Mandible (rolling/min)
0	382	80
0	386	80
0	344	92
0	352	84
0	328	58
0	332	4
0	356	0
0	292	64
0	310	62
0	340	0
5	210	208
5	218	80
5	208	122
5	200	26
5	208	138
5	188	144
5	258	2
5	206	40
5	228	182
5	182	214
0.5	276	18
0.5	248	102
0.5	200	124
0.5	186	154
0.5	216	80
0.5	232	156
0.5	234	138
0.5	198	84
0.5	258	160
0.5	200	108

Appendix 3.3: Raw data of changes in frequency of mandible and thoracic limb movements *Daphnia magna* following 24 h exposure to 40 mg/L of bentonite (clay).

Clay concentration (mg/L)	Thoracic limbs (beat/min)	Mandible (rolling/min)
0	361	197
0	502	112
0	529	B
0	380	B
0	344	B
0	465	170
0	384	180
0	388	B
0	340	B
0	531	B
0	458	71
0	463	110
0	479	96
0	164	30
0	437	180
0	385	43
0	417	99
0	499	36
0	499	3
0	474	113
40	284	B
40	300	0
40	330	B
40	370	B
40	0	B
40	238	B
40	0	B
40	146	184
40	279	B
40	202	B
40	294	88
40	242	68
40	199	79
40	238	90
40	156	103
40	217	190
40	230	76
40	234	80

40	223	52
40	0	B

B = Mandible view was blocked

Appendix 3.4: Raw data of changes in mandible and thoracic limb movements in *Daphnia magna* following 24 h exposure to 45 µg/L of cadmium.

Clay concentration (µg/L)	Mandible (rolling/min)	Thoracic limbs (beat/min)
0	460	212
0	408	188
0	330	260
0	390	146
0	428	216
0	416	242
0	462	136
0	446	98
0	438	232
0	424	148
45	150	16
45	200	14
45	452	104
45	362	64
45	318	8
45	372	148
45	292	0
45	394	208
45	296	4
45	304	0

Appendix 3.5: Raw data of changes in heart rate and beat frequency of thoracic limbs in *Daphnia magna* following 24 h exposure to a series of temperatures (5, 10, 15, 20 °C).

Temperature (°C)	Heart rate (beat/min)	Thoracic limbs (beat/min)
5	110	122
5	60	124
5	92	152
5	70	100
5	126	170
5	58	98
5	62	162
5	48	82
5	70	92
5	160	200
10	198	216
10	156	180
10	164	202
10	186	260
10	144	192
10	176	226
10	118	182
10	184	200
15	250	212
15	244	206
15	230	262
15	234	210
15	260	232
15	274	270
15	286	304
15	278	270
15	280	208
15	306	200
20	260	384
20	296	220
20	368	312
20	340	332
20	322	310
20	244	214
20	228	332
20	236	300
20	352	320
20	376	396

Appendix 4.1: Raw data of changes in grazing of *Daphna magna* exposed (24 h) to: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: suspended particulate matter in OSPW.

Treatment	Feeding rate (%)
SPM	30.5
SPM	25.1
SPM	27.4
SPM	22.0
SPM	30.9
DC	46.3
DC	51.9
DC	55.6
DC	44.4
DC	37.8
Total OSPW	22.4
Total OSPW	16.0
Total OSPW	14.0
Total OSPW	16.8
Total OSPW	24.0
Control	66.4
Control	69.0
Control	62.4
Control	67.3
Control	66.8

Appendix 4.2: Raw data of changes in mandible and thoracic limb movements, peristaltic activity, and post-abdominal rejection by *Daphna magna* exposed (24 h) to: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: suspended particulate matter in OSPW.

Treatment	Mandible (rolling/min)	Thoracic limbs (beat/min)	Peristalsis (waves/min)	Rejection/min
Control	197	361	38	1
Control	112	502	48	4.5
Control	B	529	40	0.5
Control	B	380	40	1.5
Control	B	344	46	7
Control	170	465	40	9
Control	180	384	44	1
Control	B	388	40	8.5
Control	B	340	38	-
Control	B	531	46	-
Control	71	458	-	-
Control	110	463	-	-
Control	96	479	-	-
Control	30	164	-	-
Control	180	437	-	-
Control	43	385	-	-
Control	99	417	-	-
Control	36	499	-	-
Control	3	499	-	-
Control	113	474	-	-
DC	B	0	36	2.5
DC	B	488	42	0.5
DC	114	449	28	5.5
DC	B	528	42	3.5
DC	207	462	44	5
DC	158	482	14	1.5
DC	B	405	4	0
DC	77	457	30	0.5
DC	89	377	6	-
DC	B	0	20	-
DC	81	434	-	-
DC	11	402	-	-
DC	220	432	-	-
DC	182	403	-	-
DC	77	507	-	-
DC	96	459	-	-
DC	B	428	-	-
DC	140	436	-	-
DC	B	453	-	-

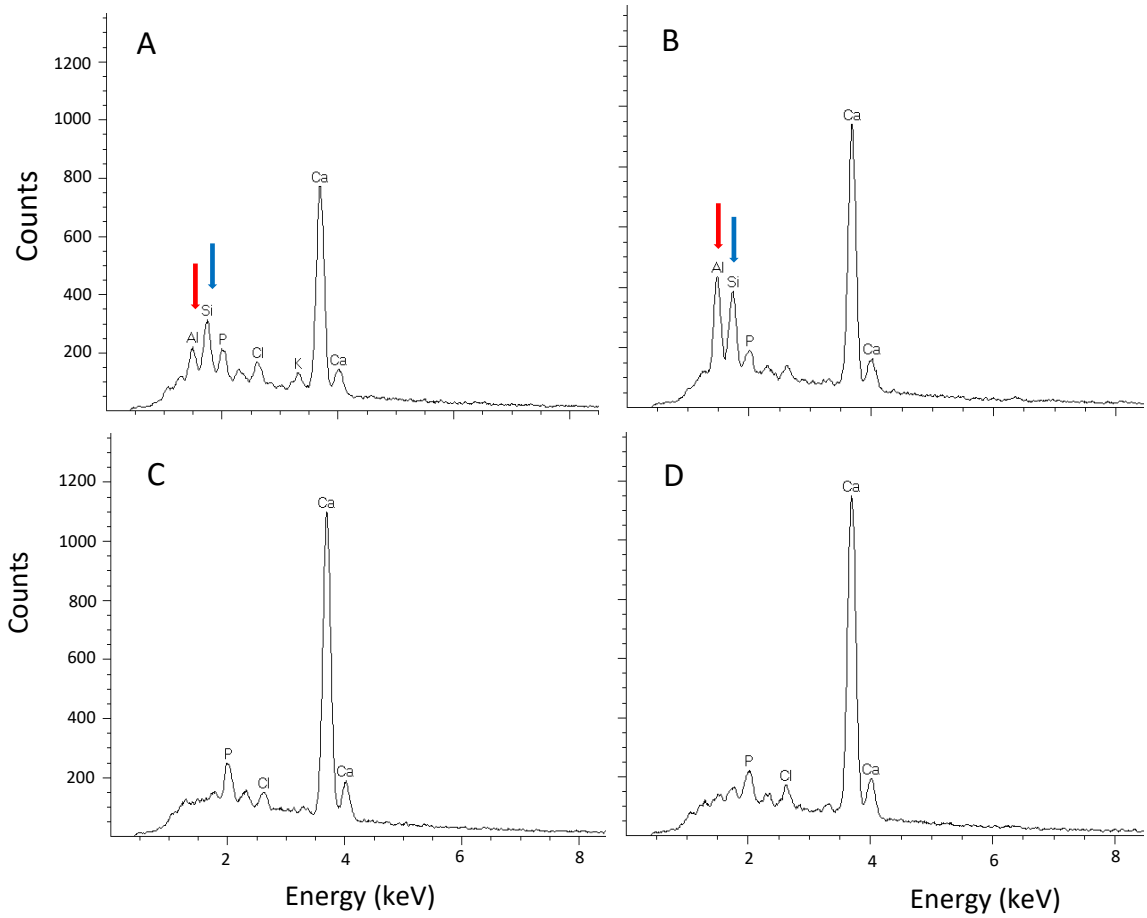
SPM	B	284	44	2.5
SPM	0	300	42	0
SPM	B	330	48	7.5
SPM	B	370	52	7.5
SPM	B	0	44	0
SPM	B	238	30	0
SPM	B	0	42	0
SPM	184	146	46	0
SPM	B	279	46	-
SPM	B	202	44	-
SPM	88	294	-	-
SPM	68	242	-	-
SPM	79	199	-	-
SPM	90	238	-	-
SPM	103	156	-	-
SPM	190	217	-	-
SPM	76	230	-	-
SPM	80	234	-	-
SPM	52	223	-	-
Total OSPW	B	0	26	3
Total OSPW	B	366	36	0
Total OSPW	66	255	48	3.5
Total OSPW	B	0	40	2.5
Total OSPW	190	243	44	2.5
Total OSPW	B	0	36	1
Total OSPW	21	272	40	2
Total OSPW	218	117	48	0
Total OSPW	B	323	38	-
Total OSPW	B	0	42	-
Total OSPW	115	255	-	-
Total OSPW	61	220	-	-
Total OSPW	53	283	-	-
Total OSPW	132	224	-	-
Total OSPW	113	242	-	-
Total OSPW	43	236	-	-
Total OSPW	144	288	-	-
Total OSPW	108	271	-	-
Total OSPW	67	200	-	-
Total OSPW	154	156	-	-

B = Mandible view was blocked

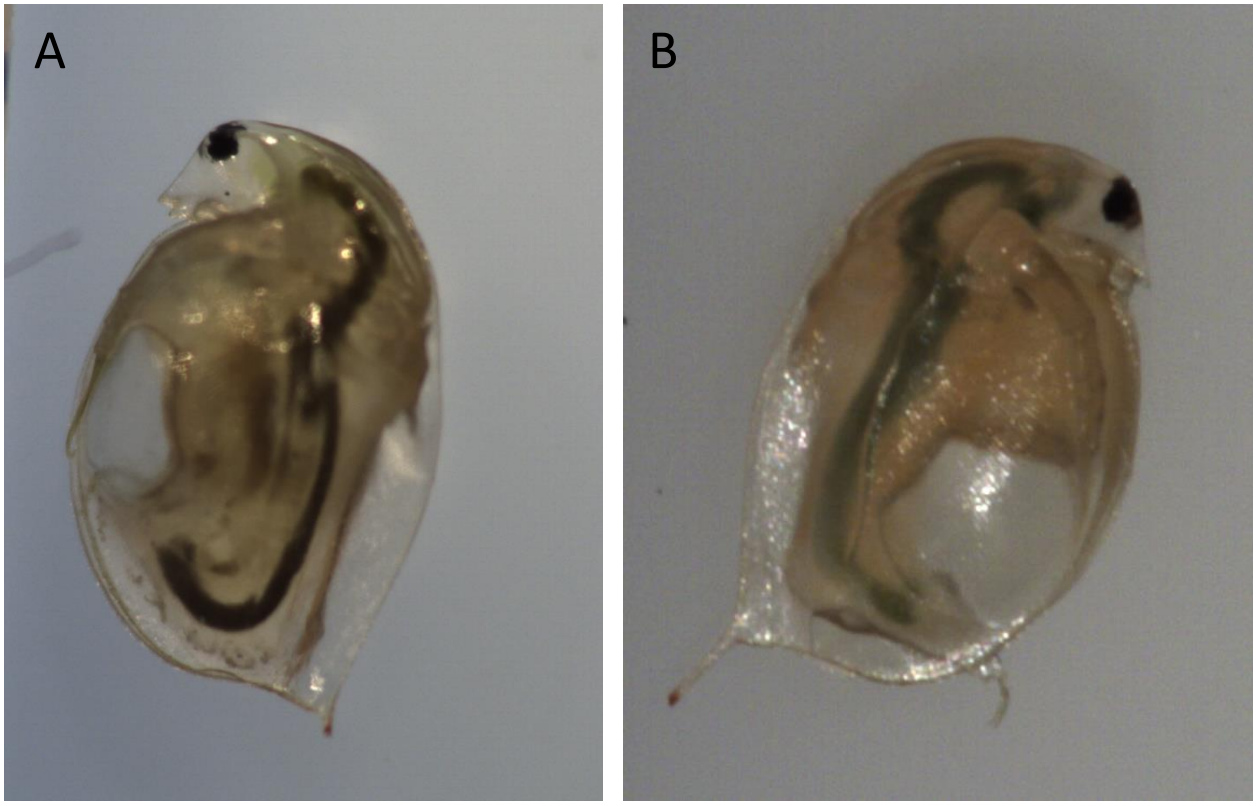
Appendix 4.3: Raw data of changes in activities of digestive enzymes in gut of *Daphnia magna* exposed (24 h) to: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: suspended particulate matter in OSPW.

Treatment	Trypsin activity (nM cleaved/min/mg daphniid)	Amylase activity
Control	845	2427
Control	481	2341
Control	1220	2867
Control	395	1899
Control	312	2290
Control	678	4822
Control	923	4787
Control	461	-
Control	486	-
Control	815	-
Control	704	-
Control	672	-
Control	816	-
Control	815	-
Control	854	-
Control	867	-
DC	682	2279
DC	369	2743
DC	602	2838
DC	450	2900
DC	62	4545
DC	295	4899
DC	364	5027
DC	265	4950
DC	707	-
DC	662	-
DC	713	-
DC	695	-
DC	725	-
DC	816	-
DC	837	-
DC	930	-
SPM	172	2163
SPM	511	2493
SPM	502	2878
SPM	390	2732
SPM	305	2391

SPM	464	3487
SPM	169	3207
SPM	222	3177
SPM	453	-
SPM	650	-
SPM	638	-
SPM	672	-
SPM	724	-
SPM	681	-
SPM	841	-
SPM	819	-
Total OSPW	126	2092
Total OSPW	402	2398
Total OSPW	185	2922
Total OSPW	127	2774
Total OSPW	97	3016
Total OSPW	82	3080
Total OSPW	437	2721
Total OSPW	236	-
Total OSPW	486	-
Total OSPW	401	-
Total OSPW	361	-
Total OSPW	681	-
Total OSPW	724	-
Total OSPW	268	-
Total OSPW	546	-
Total OSPW	371	-



Appendix 4.4: Samples of EDX readings from *Daphnia magna* exposed to: A) suspended particulate matter, B) total oil sands process-affected water (OSPW), C) dissolved components of OSPW, D) *D. magna* culture water (control). Red and blue arrows point out peaks for aluminum and silica, respectively.



Appendix 4.5: Samples of the gut color of *Daphnia magna* exposed to: A) total oil sands process-affected water (OSPW), B) *D. magna* culture water (control).

Appendix 4.6: Raw data of changes in numbers of unprocessed algal cells in hindguts of *Daphnia magna* exposed (24 h) to: OSPW: oil sands process-affected water; DC: dissolved component of OSPW; SPM: Suspended particulate matter in OSPW.

Treatment	Number of cells in hindgut
Control	200
Control	1360
Control	240
Control	0
Control	0
Control	40
Control	0
Control	480
Control	0
Control	160
DC	1160
DC	1640
DC	1640
DC	8240
DC	760
DC	1360
DC	3480
DC	2560
DC	2760
DC	4920
SPM	1000
SPM	2600
SPM	3160
SPM	5920
SPM	5000
SPM	1960
SPM	3760
SPM	2520
SPM	3440
SPM	4080
Total OSPW	3040
Total OSPW	7200
Total OSPW	10000
Total OSPW	5200
Total OSPW	3560
Total OSPW	2720
Total OSPW	4840

Total OSPW	7360
Total OSPW	3880
Total OSPW	9120

Appendix 5.1: Raw data of the effect of oil sands process-affected water (OSPW) on oxygen consumption of *Daphnia magna* over 1 and 10 days of exposure.

	Oxygen consumption (mgO2/24h/g)	
Concentration (%)	1-day	10-day
0	22.84	13.55
0	21.04	28.31
0	18.80	29.43
0	22.53	26.92
0	21.27	19.15
0	13.17	12.80
0	22.69	30.72
0	22.30	32.34
1	29.28	18.74
1	25.89	27.54
1	18.64	33.61
1	26.00	24.66
1	20.24	20.31
1	27.95	28.39
1	9.62	35.14
1	16.57	34.90
10	18.83	27.83
10	18.52	24.78
10	16.11	25.66
10	15.93	30.60
10	16.54	27.56
10	33.24	36.59
10	19.03	38.38
10	26.27	40.21

Appendix 5.2: Raw data of the effect of oil sands process-affected water (OSPW) on the heart rate of *Daphnia magna* over 1 and 10 days of exposure.

	Heart rate (beat/min)	
Concentration (%)	1-day	10-day
0	344	350
0	334	234
0	330	270
0	354	240
0	332	372
0	392	242
0	280	288
0	306	300
0	258	334
0	340	272
1	338	326
1	400	270
1	304	292
1	282	324
1	298	350
1	404	292
1	408	306
1	302	204
1	382	202
1	362	222
10	376	268
10	394	308
10	336	156
10	382	240
10	328	244
10	318	332
10	276	232
10	284	322
10	342	236
10	368	190

Appendix 5.3: Raw data of the effect of oil sands process-affected water (OSPW) on hemoglobin content of *Daphnia magna* over 1 and 10 days of exposure.

	Hemoglobin content (µg/g)	
Concentration (%)	1-day	10-day
0	4188.48	5431.75
0	4562.04	5612.83
0	5702.65	6220.66
0	4166.67	5341.61
0	5449.19	6604.87
1	4878.05	3771.55
1	5400.00	4556.07
1	4626.33	5337.08
1	3833.33	4888.89
1	4895.10	5449.19
10	4125.74	4719.39
10	4089.22	5223.88
10	5088.50	4257.91
10	5733.56	3609.63
10	4126.98	3891.71

Appendix 5.4: Raw data of the effect of oil sands process-affected water (OSPW) on the activity of *Daphnia magna* over 1 and 10 days of exposure.

	1-day		10-day	
Concentration (%)	Distance moved (cm)	Active time (s)	Distance moved (cm)	Active time (s)
0	40.20	42.43	13.24	37.22
0	38.97	46.65	35.17	29.78
0	65.04	56.78	43.68	45.26
0	43.86	42.48	48.75	44.96
0	58.90	60.96	27.22	28.17
0	47.88	48.09	68.48	59.78
0	57.97	58.48	58.09	52.57
0	52.57	45.48	62.84	60.13
0	74.04	67.7	40.89	54.00
0	25.18	28.74	71.70	53.96
0	24.24	37.24	45.88	48.13
0	35.53	39.43	38.97	46.65
0	43.68	45.26	77.97	58.48
0	48.42	44.35	38.20	42.43
0	27.26	28.17	24.18	28.74
0	68.46	59.79	32.57	45.48
0	58.28	52.05	74.04	67.70
0	62.06	60.95	65.04	56.78
0	70.23	54.48	43.86	52.48
0	64.70	53.95	28.90	30.96
1	31.90	29.70	54.82	43.12
1	55.92	58.35	19.33	29.04
1	77.33	69.61	36.40	40.91
1	54.62	54.96	32.49	42.39
1	47.23	46.61	27.74	32.39
1	68.38	57.83	22.13	36.91
1	32.02	47.78	26.74	35.70
1	49.62	50.78	42.02	41.00
1	63.46	60.61	47.85	44.09
1	50.41	48.70	31.89	40.09
1	49.62	50.78	25.92	38.35
1	46.74	55.70	32.02	47.78
1	31.90	39.70	37.33	39.61
1	25.92	38.35	64.38	67.83
1	68.38	67.83	46.74	55.70
1	32.02	47.78	30.41	48.70
1	63.46	60.61	49.62	50.78

1	50.41	48.70	31.90	39.70
1	37.33	39.61	40.18	48.74
1	45.18	48.74	13.46	18.61
10	10.67	13.52	10.67	13.52
10	41.03	42.22	19.25	19.04
10	14.85	19.35	48.93	52.57
10	20.14	20.39	15.46	18.87
10	18.36	20.22	19.18	27.52
10	53.06	56.43	16.53	18.22
10	25.03	32.39	8.64	11.04
10	54.03	43.74	2.23	5.48
10	49.33	34.04	4.02	7.74
10	67.13	69.83	29.55	22.30
10	20.14	30.39	10.67	13.52
10	25.01	32.35	20.14	30.39
10	42.14	48.39	8.01	12.35
10	54.02	43.12	22.14	28.39
10	19.33	29.04	54.02	43.12
10	38.02	41.54	19.33	29.04
10	65.18	48.74	38.02	41.54
10	41.03	34.22	28.36	20.22
10	36.18	29.74	41.03	34.22
10	48.36	50.22	36.18	29.74

Appendix 6.1: Raw data of changes in reproduction (neonates produced/daphniid) and the size of offspring in both mass and length of *Daphnia magna* over 10 days of exposure to oil sands process-affected water (OSPW).

Concentration (%)	Neonates per individual	Mean neonate mass (µg)*	Mean neonate length (mm)**
0	7.8	5.6	0.85
0	6.2	5.2	0.84
0	5.8	10.3	0.89
0	8.2	7.1	0.84
0	6.4	7.5	0.85
1	5.8	9.0	0.81
1	4.6	7.4	0.86
1	10.4	6.0	0.84
1	7.2	6.1	0.78
1	7.6	7.1	0.81
10	2.4	3.3	0.84
10	5.8	3.8	0.79
10	4.4	2.7	0.77
10	3.4	3.5	0.79
10	5.0	2.8	0.78

*Each number represents the average mass of the total neonates in the replicate.

**Each number represents the average length of three neonates.

Appendix 6.2: Raw data of changes in the size in both mass and length and total mass produced by *Daphnia magna* over 10 days of exposure to oil sands process-affected water (OSPW).

Concentration (%)	Mean mass (µg)*	Mean length (mm)**	Mean mass produced (µg/10-days)
0	243.3	2.40	253.0
0	303.3	2.43	329.0
0	183.3	2.44	181.5
0	196.7	2.27	220.9
0	206.7	2.32	220.7
1	186.7	2.35	204.9
1	196.7	2.38	196.7
1	220.0	2.24	248.4
1	200.0	2.50	209.9
1	223.3	2.44	243.7
10	156.7	2.47	130.6
10	116.7	2.55	104.7
10	143.3	2.28	121.2
10	170.0	2.41	147.9
10	153.3	2.39	133.3

*Each number represents the average mass of the total neonates in the replicate.

**Each number represents the average length of three neonates.

Appendix 6.3: Raw data of changes in energy stores in adult *Daphnia magna* after being exposed to oil sands process-affected water (OSPW) for 10 days.

Concentration (%)	Protein (mJ/mg)	Carbohydrate (mJ/mg)	Lipids (mJ/mg)
0	44.60	1.42	28.42
0	66.27	1.35	25.99
0	53.14	1.47	30.05
0	58.18	1.32	25.17
0	54.30	1.29	29.23
0	55.85	1.42	26.80
0	-	1.28	-
0	-	0.84	-
0	-	1.44	-
0	-	1.08	-
1	63.42	1.16	20.30
1	64.52	0.98	17.87
1	68.14	1.20	21.11
1	63.29	0.95	18.68
1	47.96	1.14	18.68
1	47.51	1.21	20.30
1	-	1.29	-
1	-	1.25	-
1	-	1.60	-
1	-	1.08	-
10	73.51	1.07	17.05
10	74.94	1.12	14.62
10	61.87	1.32	17.87
10	58.38	1.16	13.81
10	42.92	1.19	17.87
10	41.88	1.62	17.05
10	-	1.00	-
10	-	1.25	-
10	-	1.17	-
10	-	1.18	-

Appendix 7.1: Raw data of changes in survival rate and reproduction in adult *Daphnia magna* after being exposed to oil sands process-affected water (OSPW) for 10 days under cold and warm season condition.

	Warm season		Cold season	
Concentration (%)	Reproduction (neonate per individual)	Survival (%)	Reproduction (neonate per individual)	Survival (%)
0	7.0	80	0	80
0	4.4	100	0	80
0	8.4	100	0	60
0	8.0	100	0	100
0	4.2	100	0	100
0	6.6	100	-	-
0	8.4	100	0	100
0	5.6	80	0	100
0	7.8	100	0	100
0	6.2	100	0	100
1	6.5	80	0	80
1	4.0	100	0	100
1	10.0	80	0	80
1	4.8	100	0	60
1	4.2	100	0	100
1	5.8	100	0	80
1	7.7	80	0	80
1	4.7	80	0	100
1	4.6	100	0	100
1	5.2	100	0	80
10	6.5	80	0	40
10	2.2	100	0	60
10	4.0	80	0	60
10	5.2	80		0
10	4.7	80	0	60
10	4.5	80	0	60
10	3.0	80	0	60
10	4.7	80	0	60
10	2.6	100	0	80
10	2.4	100	0	40

Appendix 7.2: Raw data of changes in the size in both mass and length and total mass produced by *Daphnia magna* over 10 days of exposure to oil sands process-affected water (OSPW) under cold and warm season condition.

	Warm season		Cold season	
Concentration (%)	Mean mass (µg)*	Mean length (mm)**	Mean mass (µg)*	Mean length (mm)**
0	34	3.01	296	1.84
0		3.08		1.72
0	18	2.97	265	1.72
0		2.95		1.60
0	30	3.03	259	1.67
0		2.96		-
0	20	3.16	262	1.71
0		3.19		1.84
0	28	3.04	311	1.78
0		2.83		1.68
1	5	3.00	266	1.53
1		3.07		1.61
1	5	3.02	312	1.51
1		3.03		1.76
1	6	2.92	263	1.60
1		3.03		1.52
1	6	2.90	300	1.65
1		3.06		1.66
1	8	2.88	279	1.78
1		2.97		1.52
10	4	3.14	263	1.27
10		2.93		1.55
10	4	3.09	209	1.41
10		3.16		1.47
10	3	3.10	233	1.47
10		2.93		1.46
10	5	3.16	261	1.61
10		3.20		1.45
10	6	3.04	127	1.55
10		3.04		1.63

*Each number represents the average mass of the total neonates in two replicates.

**Each number represents the average length of the neonates in one replicate.

Appendix 8.1: Raw data of electro-olfactography (EOG) response of rainbow trout to L-alanine, taurocholic acid (TCA) and different concentrations (0.1, 1, and 10%) of oil sands process-affected water (OSPW).

EOG response (mV)

				OSPW		
	Water	L-alanine	TCA	0.1%	1%	10%
Live fish	1.3	6.7	8.9	1.6	4.6	11.0
	0.9	4.6	11.4	0.8	4.3	9.4
	0.9	7.7	16.4	1.5	5.5	9.2
	1.2	3.6	6.1	1.1	3.6	10.0
	1.3	4.2	7.6	2.0	4.1	8.2
	0.8	5.1	7.7	1.2	4.3	7.6
Dead fish	0.5	0.3	0.7	0.7	0.6	0.8
	0.2	0.2	0.4	0.2	0.2	0.3
	0.2	0.1	0.1	0.4	0.1	0.6

Appendix 8.2: Raw data of behavioural response of rainbow trout to two concentrations (1 and 10%) of oil sands process-affected water (OSPW).

	Concentration (%)	Cue arm	Response (%)
Normal fish	0	R	5.0
	0	L	21.7
	0	R	-13.3
	0	L	30.0
	0	R	26.7
	0	L	16.7
	0	L	-13.3
	0	R	30.0
	0	L	-41.7
	0	R	8.3
	0	L	-
	0	R	-15.0
	0	R	5.0
	0	L	30.0
	0	L	-13.3
	0	R	3.3
	0	R	31.7
	0	L	-5.0
	0	L	-
	0	L	-25.0
	0.1	R	-40.0
	0.1	L	-
	0.1	R	-60.0
	0.1	R	-45.0
	0.1	L	-51.7
	0.1	L	-38.3
	0.1	R	-75.0
	0.1	L	-48.3
	0.1	R	-40.0
	0.1	R	-40.0
	0.1	L	-50.0
	0.1	L	-38.3
	0.1	R	28.3
	0.1	R	-30.0
	0.1	R	-85.0
	0.1	R	-36.7
	0.1	L	38.3
	0.1	L	-46.7
	0.1	R	33.3

	0.1	R	-51.7
	1	L	-48.3
	1	R	-50.0
	1	L	-40.0
	1	L	-83.3
	1	R	55.0
	1	L	-81.7
	1	L	-66.7
	1	R	-70.0
	1	L	-66.7
	1	R	-50.0
	1	L	-63.3
	1	L	-83.3
	1	L	-71.7
	1	R	-70.0
	1	L	-100.0
	1	R	-53.3
	1	L	-68.3
	1	L	-83.3
	1	L	-73.3
	1	L	-70.0
Anosmic fish	0	R	10.0
	0	R	-28.3
	0	L	-6.7
	0	R	33.3
	0	L	-8.3
	0	L	-35.0
	0	R	31.7
	0	L	-43.3
	0	R	23.3
	0	L	-40.0

Appendix 8.3: Raw data of electro-olfactography response (EOG) of rainbow trout to L-alanine, taurocholic acid (TCA) before, during and after exposure to 1% oil sands process-affected water (OSPW).

	Response to L-alanine			Response to L-alanine		
Concentration (%)	Before	During	After	Before	During	After
0	3.8	5.2	5.9	11.0	10.9	10.3
0	5.0	5.7	5.2	8.6	8.7	8.6
0	4.1	4.2	4.6	8.6	8.9	8.9
0	4.6	4.9	4.7	11.3	12.3	11.0
0	6.2	6.9	6.7	11.0	10.6	11.0
0	5.3	5.1	5.3	8.4	8.6	8.6
1	3.8	1.8	3.1	10.3	5.1	6.7
1	4.1	1.9	3.8	9.2	5.0	8.3
1	5.4	2.4	4.2	10.3	4.4	9.1
1	5.2	3.3	5.8	13.5	8.2	10.2
1	5.4	2.3	8.3	16.7	12.6	15.4
1	3.5	1.2	5.0	11.6	7.2	11.8

Appendix 8.4: Raw data of behavioural response of rainbow trout to L-alanine in the presence and absence (control) of 1% oil sands process-affected water (OSPW).

Concentration (%)	Cue arm	Response (%)
0	R	58.3
0	L	43.3
0	R	83.3
0	R	-46.7
0	-	-
0	L	88.3
0	R	48.3
0	L	43.3
0	R	38.3
0	L	71.7
0	R	65.0
0	L	50.0
0	R	51.7
0	R	63.3
0	L	53.3
0	-	-
0	L	73.3
0	R	71.7
0	R	78.3
0	L	70.0
0.1	R	10.0
0.1	R	23.3
0.1	L	-2.2
0.1	L	26.7
0.1	R	-31.7
0.1	L	-41.7
0.1	L	15.0
0.1	R	41.7
0.1	L	16.7
0.1	R	26.7
0.1	L	28.3
0.1	R	51.7
0.1	L	-26.7
0.1	L	31.7
0.1	R	15.0
0.1	L	48.3
0.1	R	46.7
0.1	L	20.0

0.1	L	18.3
0.1	R	38.3

Appendix 9.1: Raw data of inhibitory effect of OSPW on olfactory response of rainbow trout to: A) L-alanine, B) taurocholic acid (TCA).

	EOG response (mV)	
Concentration (%)	L-alanine	TCA
0	87.2	49.2
0	32.3	40.9
0	37.7	47.1
0	32.3	40.9
0	58.9	60.0
0	62.1	55.2
2	35.8	33.7
2	32.5	28.8
2	43.8	34.0
2	41.2	42.0
2	52.6	43.3
2	55.3	50.1
4	16.6	15.5
4	16.4	20.5
4	29.0	30.7
4	31.4	24.1
4	32.4	33.2
4	42.8	46.7
8	7.6	8.8
8	22.3	22.3
8	23.4	34.0
8	24.2	20.0
8	28.0	27.6
16	8.5	9.0
16	8.3	6.2
16	13.2	10.1
16	17.1	10.8
16	11.3	12.2
16	14.4	12.0
32	7.8	10.6
32	7.3	2.9
32	8.3	4.0
32	5.0	4.3
32	7.8	6.9

Appendix 9.2: Raw data of inhibitory effect of oil sands process-affected water (OSPW) on the olfactory response of rainbow trout to L-alanine, following 3, 24, and 96 h exposure.

	EOG response to L-alanine (mV)		
Concentration (%)	3 h	24 h	96 h
0	44.3	38.0	45.1
0	46.3	46.3	53.7
0	54.4	53.0	62.3
0	57.8	50.6	49.9
0	37.6	57.4	51.8
0	51.0	57.3	68.4
3	37.5	38.2	21.9
3	56.9	33.0	43.1
3	49.7	48.4	35.8
3	47.6	37.8	23.8
3	40.6	47.6	44.6
3	48.9	45.2	45.4
22	17.3	13.3	8.9
22	17.0	11.6	5.0
22	15.7	19.6	9.2
22	25.8	11.0	8.3
22	22.6	4.7	9.0
22	22.9	8.2	8.9

Appendix 9.3: Raw data of effect of oil sands process-affected water (OSPW) on cAMP concentration in the olfactory e rainbow trout to L-alanine, following 3, 24, and 96 h exposure.

	cAMP concentration (pM/g)		
Concentration (%)	3 h	24 h	96 h
0	35.8	37.0	36.4
0	32.8	32.4	34.2
0	33.4	36.4	-
0	38.4	35.0	-
3	34.6	35.4	35.4
3	31.2	31.8	38.0
3	35.2	37.2	-
3	37.4	35.8	-
22	35.6	40.0	36.4
22	34.4	37.2	33.8
22	35.6	38.2	-
22	36.8	37.0	-