# ACUTE AND CHRONIC TOXICITY OF UNTREATED, AGED, AND OZONATED OIL SANDS PROCESS-AFFECTED WATER IN *CHIRONOMUS DILUTUS* LARVAE

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## ABSTRACT

One of the main issues associated with bitumen extraction in the Alberta oil sands is the production of oil sands process-affected water (OSPW). The OSPW is saline, alkaline, and containing high concentrations of inorganic and organic constituents, such as naphthenic acids (NAs). In accordance with environmental legislation, OSPW must be retained in on-site settling basins as the water has demonstrated toxicity towards a number of different aquatic and terrestrial organisms. Due to the large volumes of OSPW currently in containment, treatment methods are required to eliminate the toxicity and prepare the water for release in future reclamation scenarios. Benthic invertebrates, such as chironomids, represent an important component of aquatic food webs and ecosystems in the oil sands region, but the toxicity of OSPW towards these organisms had not been fully characterized. Additionally, the effects on toxicity of treating OSPW by aging or ozonation were unknown and needed to be assessed in preparation for a potential future release scenario.

To assess the toxicity of untreated, aged, and ozonated OSPW, 10-day and chronic exposures of *Chironomus dilutus* to OSPW were conducted; endpoints of interest included survival, growth, development, and behavior. For the studies described in this thesis, relatively fresh OSPW was sampled in two batches (designated 'WIP-OSPW-A' and 'WIP-OSPW-B') from the Syncrude Canada Ltd. West In-Pit (WIP) settling pond and from three experimental reclamation ponds – Big Pit, FE5, and TPW. Larvae were exposed to each of the treatment waters for both a 10-d and a chronic (until adult emergence) exposure period. Real-time PCR was used to assess gene expression of hemoglobins, endocrine-related receptors, and ribosomal protein following 1-, 4-, or 7- d

exposure to fresh or aged OSPW (WIP-OSPW or FE5) in order to investigate the underlying mechanisms of toxicity. The greatest concentrations of NAs were measured in the fresh WIP-OSPW (between 70 and 72 mg/L) and the total concentrations of NAs in the aged waters were between 13 and 35 mg/L.

Exposure to untreated OSPW resulted in both acute and chronic toxicity to *C. dilutus* larvae. Masses of larvae that were exposed to WIP-OSPW were 64% to 79% less than that of the respective control larvae ($p<0.001$). Cases built by larvae exposed to both fresh and aged OSPW were smaller and more fragile than those built by larvae exposed to freshwater. In terms of gene expression, the abundance profiles of transcripts of hemoglobin genes were significantly different in FE5-exposed larvae relative to the freshwater control. Exposure to both WIP-OSPW and FE5 resulted in differential expression of estrogen-related receptor, ultraspiracle protein, and ribosomal protein L15 in *C. dilutus* compared to the control. Similarly, chronic exposure to untreated OSPW resulted in significantly less pupation than in the controls, with 31% and 71% less pupation of larvae exposed to WIP-OSPW-A or WIP-OSPW-B, respectively ($p<0.05$). Rates of emergence were also significantly less for larvae exposed to WIP-OSPW, with only 13% and 8% of larvae emerging as adults when exposed to WIP-OSPW-A or WIP-OSPW-B, respectively, compared to 81% in the control ($p<0.0001$). However, larvae exposed to water from Big Pit, FE5 and TPW did not have significantly lesser masses than the controls ($p>0.05$). Aging of OSPW in reclamation ponds did not attenuate all chronic toxicity since exposure to TPW resulted in significantly less emergence and delayed emergence relative to the control. The OSPW aged in reclamation ponds retained

toxicity and, therefore, more aggressive, targeted treatment, such as ozonation, of OSPW is required.

To evaluate the effectiveness of ozonation in eliminating toxicity of OSPW, WIP-OSPW-A and WIP-OSPW-B were treated with 30 and 80 mg/L of ozone ($O_3$), respectively. There were no differences in survival of larvae exposed to ozonated-OSPW relative to the freshwater control ($p>0.05$). Ozonation also attenuated adverse effects on growth, pupation, and emergence seen in both batches of untreated OSPW, suggesting that ozonation may be an effective treatment for targeting the organic fraction of OSPW and reducing or possibly eliminating toxicity of OSPW to *C. dilutus.*

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*"The highest reward for a person's toil is not what they get for it, but what they become by it" – John Ruskin*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| ERR | Estrogen-related receptor |
| ESR | Ecdysteroid receptor |
| FTIR | Fourier transform infrared spectroscopy |
| GC | Gas chromatography |
| Hb | Hemoglobin |
| IC | Ion chromatography |
| ICP-MS | Inductively coupled plasma mass spectrometry |
| MS | Mass spectrometry |
| MW | Molecular weight |
| NA | Naphthenic acid |
| OSPW | Oil sands process-affected water |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RT-PCR | Real-time PCR |
| SD | Standard deviation |
| SEM | Standard error of mean |
| USP | Ultraspiracle protein |
| WIP | West In-Pit |

## PREFACE

Chapter 1 is a general introduction and Chapter 5 contains a general discussion and conclusions. Chapters 2, 3, and 4 of this thesis are organized as manuscripts for publication in scientific journals. Therefore, there is some repetition of introductions, materials, and methods throughout each data chapter. Chapter 2 was accepted to Water Research, Chapter 3 was submitted to Aquatic Toxicology and Chapter 4 was accepted to Environmental Science and Technology.

The content of Chapter 2 was reprinted (adapted) from Water Research, (DOI:10.1016/j.watres.2011.12.007). J. Anderson *et al., "*Effects of exposure to oil sands process-affected water from experimental reclamation ponds on *Chironomus dilutus,"* Copyright (2011), with permission from Elsevier.

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# CHAPTER 1:
# General introduction

## 1.1 Alberta oil sands

Current global energy demand is met primarily by fossil fuels, with these comprising more than 85% of total global energy supplies (Government of Alberta, 2008). Output of crude oil from conventional reserves, however, is declining approximately 3% per annum (National Energy Board of Canada, 2010). With energy demands expected to increase by 50% in the next two decades, mostly within non-OECD countries, emphasis is shifting from conventional oil production to the further development of alternative oil sources, such as oil sands (National Energy Board of Canada, 2010). Canada is home to the world's second largest proven oil reserves (the largest being those in Saudi Arabia), with the majority contained within the oil sands deposits of Alberta (Government of Alberta, 2006; Veil *et al.,* 2009).

The Alberta oil sands are divided into three primary deposit areas, the largest of which is the Athabasca area located near Fort McMurray (Figure 1.1). Alberta has over 170 billion barrels of proven oil in its reserves, most of which are located within oil sands in the form of bitumen (crude petroleum) (Government of Alberta, 2011a). Daily oil sands production in 2008 was 1.31 million barrels of bitumen per day (Government of Alberta, 2008) and that increased to 1.49 million barrels per day in 2009 (Government of Alberta, 2011a). In 2007, oil produced from the Alberta oil sands comprised 43% of all crude oil produced in Canada (Government of Alberta, 2008) and, due to increased production from the Athabasca deposit in particular, over 50% of Canada's oil supply originated in the Alberta oil sands in 2010 (CAPP, 2011; Frank *et al.*, 2009).

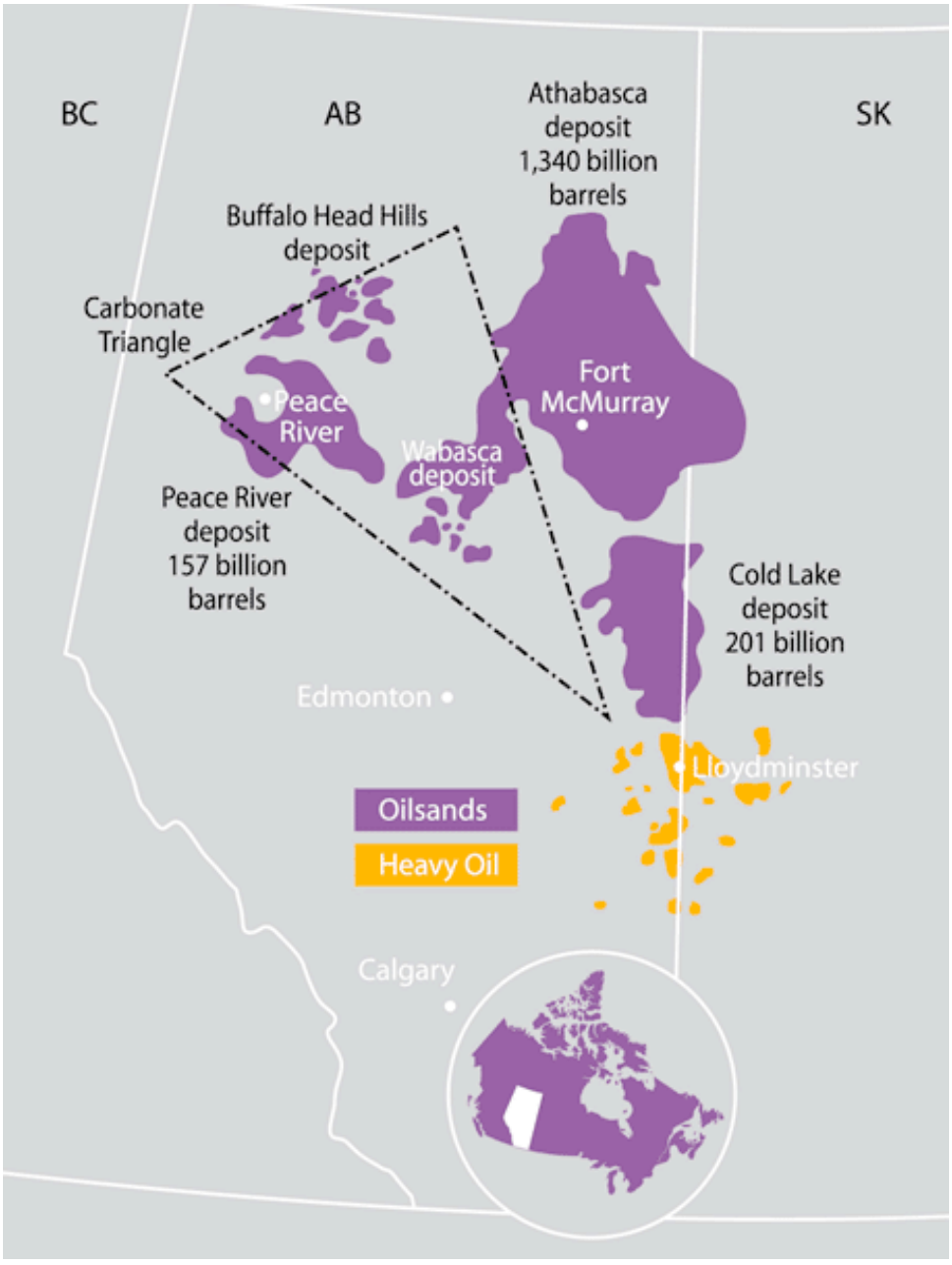


**Figure 1.1:** Deposits within the Athabasca oil sands region of Alberta. (Adapted from Oil Sands Developers Group, 2009)

*1.1.1 Extraction process*

The majority of bitumen extracted from surface-mined oil sands in Alberta is obtained using the "Clark hot water extraction process". This involves mixing oil sands with 79-93 °C water and caustic soda to separate bitumen from other constituents including clay, residual sand, inorganic compounds, and organic compounds, such as naphthenic acids (NAs). Extraction conditions can be optimized by use of 85 °C water at pH 8.5, resulting in over 90% efficient extraction of bitumen (Chalaturnyk *et al.*, 2002). The resultant process water (termed "oil sands process-affected water," OSPW) is moved to on-site active settling basins and stored (Hao *et al.,* 2005; Rogers *et al.,* 2002).

The extraction process, in addition to other operational procedures, requires considerable volumes of water and ultimately generates process water that becomes part of the management responsibilities and costs for oil sands companies (Veil *et al.,* 2009). For each cubic metre of oil sands processed, up to 4 cubic metres of tailings water are produced, even after the majority is recycled back into the extraction process (Holowenko *et al.,* 2002; Mikula *et al.,* 2008). Average freshwater use for extraction by Shell Canada from 2004 to 2008 was reported at 1.7 barrels of water per barrel of bitumen, with a representative 'normal' year having a ratio of closer to 2.3 barrels of freshwater per barrel of bitumen (Shell Canada, 2009). While recycling of OSPW can help reduce the demands on local freshwater resources and has been improving over time, the process concentrates NAs and other constituents within recycled water. Water recycling has implications for water quality and downstream treatment and reclamation, as well as for efficiency of bitumen extraction (Allen, 2008a). Oil sands companies are currently held to a zero-discharge to freshwater policy by the Alberta Environmental

Protection and Enhancement Act (1993), so all OSPW produced must be held on-site (Government of Alberta, 2008; Madill *et al*., 2001). This has resulted in greater than one billion cubic metres of tailings water in containment systems spread over more than 170 square kilometres in the Fort McMurray region of Alberta (Del Rio *et al.,* 2006; Government of Alberta, 2011b*)*, and this volume will continue to grow as the industry expands and production increases. Ultimately, the companies are responsible for reclaiming this water and finding ways to release it back into the local environment, which presents a major challenge for the industrial, government, and academic parties involved.

### 1.2 Naphthenic acids

One of the issues associated with the treatment of OSPW is reducing the concentration and toxicity of NAs in the process water. The term 'oil sands extractable organics' has been adopted recently as a result of more sophisticated methods for fractionating and identifying organic constituents of OSPW. This organic fraction of OSPW includes NAs and a complex array of other organic compounds (Headley *et al.,* 2011), but the research described in this thesis focused on classical NAs. The NAs are important organic constituents of OSPW and can account for up to 4% (by weight) of raw petroleum. These organic acids are naturally occurring in bitumen and become solubilized and concentrated in process water due to recycling of extraction water and the alkaline conditions created by the added sodium hydroxide, which promote retention of NAs in the water column (Headley and McMartin, 2004; Rogers *et al.,* 2002). Ambient levels of NAs vary depending upon the geological characteristics of the underlying area,

but background concentrations for waterways in the Athabasca region have typically been measured at less than 1 mg/L for total NAs (Headley and McMartin, 2004). In comparison, the total NAs concentrations in OSPW have been found to be as great as 110 mg/L (Headley and McMartin, 2004) and it is believed that these organic acids are responsible for the majority of the toxicity of OSPW to aquatic organisms (Herman *et al.,* 1994; Rogers *et al.,* 2002). For this reason, NAs are seen as primary targets for treatment efforts and are important indicators of potential downstream impacts post-release.

### *1.2.1 Structure and properties of naphthenic acids*

Naphthenic acids constitute a complex mixture of alkyl-substituted cycloaliphatic and acyclic carboxylic acids (Figure 1.2). These acids share the general formula $C_nH_{2n+z}O_2$, where n denotes the number of carbons and z specifies a homologous series and is an even integer between 0 and -12 (Clemente and Fedorak, 2005; Holowenko *et al.,* 2002; Lai *et al.,* 1996). In OSPW, NAs tend to have molecular weights less than 500 Da and are present as ionized naphthenate salts following extraction (Armstrong, 2008; Herman *et al.,* 1994). Characterization of this very complex mixture presents a significant analytical challenge (Rowland *et al.,* 2011a), and complete characterization of all NAs is unlikely to be achieved in the timeline required for addressing OSPW reclamation needs.

Since NAs contain one or more hydrophobic alkyl groups and hydrophilic carboxyl groups, NAs are generally considered surfactant-like compounds (Armstrong, 2008; Frank *et al.,* 2009). Their surfactant properties allow NAs to penetrate cell walls (Quagraine *et al.,* 2005), but at pH values greater than 6, as in the alkaline conditions of OSPW, NAs exist as ionized salts. These are considered to be less toxic because their

polar nature reduces their passage through biological membranes (Armstrong, 2008). It has been suggested that the probable mode of toxic action is narcosis, whereby the NAs are sufficiently hydrophobic to enter the lipid bi-layer of the cellular membrane and cause cellular disruption (Frank *et al.,* 2009). However, our understanding of the mechanism(s) of toxicity of NAs remains incomplete, so further research is required.

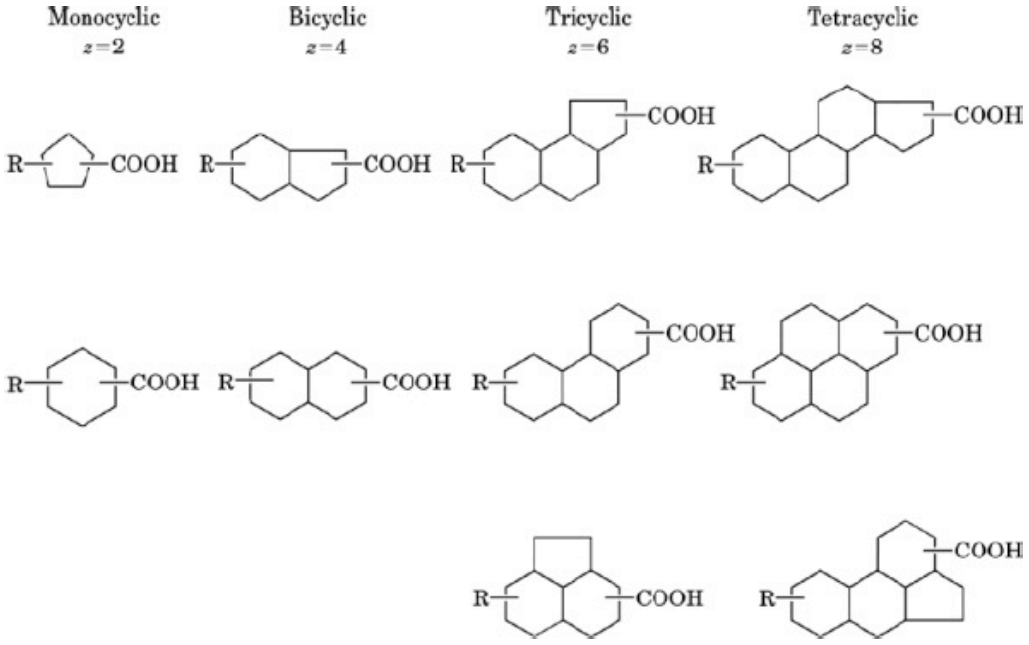


**Figure 1.2:** Typical structures of naphthenic acids, where R represents an alkyl group. In the case of z=0, the structure is acyclic and is simply R-COOH (Adapted from Brient *et al.,* 2000).

*1.2.2 Toxicity*

Acute and chronic toxicity of OSPW and NAs have been reported in both aquatic and terrestrial organisms, and can vary slightly depending on temperature and dissolved oxygen conditions (Siwik *et al.,* 2000). Under neutral or slightly alkaline conditions, such as those in tailings ponds, NAs are more water-soluble and, therefore, more available to aquatic organisms (Clemente and Fedorak, 2005). The toxicity of a specific NAs mixture can vary depending upon the structures and relative proportions of different individual acids. Generally, NAs with greater molecular weights (MW) (C>22) tend to be more persistent in the environment (Holowenko *et al.,* 2002). Additionally, a greater proportion of multi-ring structures tends to yield a less toxic mixture (Lo *et al.,* 2006), as does an increased number of carboxylic groups on greater MW NAs (Frank *et al.,* 2009). This lesser toxicity is likely due to the fact that more rings and a greater degree of carboxylation reduce hydrophobicity and make NAs less able to cross cell membranes (Frank *et al.,* 2009).

The toxicity of NAs towards different organisms has been studied in attempts to better understand the potential impacts of OSPW release on ecosystems downstream of the oil sands management areas. As would be expected, much of this work has focused on the effects of exposure to OSPW on growth, survival and reproduction of aquatic organisms. Phytoplankton populations exposed to OSPW were monitored in several studies and it was found that there was no significant change in total biomass, but there was a distinct shift in phytoplankton community composition (Leung *et al.,* 2003, Leung *et al.,* 2001).

Studies of toxicity have been conducted with a variety of fish species, including fathead minnow (*Pimephales promelas*), yellow perch (*Perca flavescens*), goldfish (*Carassius auratus*) and other small forage fish. Early life stages of fathead minnows exposed to sediments from tailings ponds manifested more malformations and greater mortality, as well as reduced body size and lesser hatching success than controls (Colavecchia *et al.*, 2004). Gills of fish tend to be a sensitive target organ as they represent a direct route of NAs uptake. Proliferations of mucous, epithelial, and chloride cells were observed in gills of yellow perch that had been exposed to OSPW (Nero *et al.,* 2006a). As well, significant histological changes in gill and liver were observed in perch and goldfish exposed for a 3 week period to either commercially available NAs or NAs extracted from OSPW (Nero *et al*., 2006b).

The endocrine system represents another important target for disruption following exposure to NAs or OSPW. After a 19-day exposure period, goldfish exhibited significantly lesser concentrations of testosterone and 17β-estradiol (E2) in blood plasma of both males and females compared to controls (Lister *et al*., 2008). Modulation of the endocrine system by OSPW has also been observed *in vitro*. Significantly lesser concentrations of testosterone and metabolism of E2 were observed *in vitro* in H295R cells exposed to untreated OSPW than in unexposed controls (He *et al.,* 2010). Similarly, OSPW exhibited both anti-androgenic and estrogenic responses through receptor-mediated pathways in MDA-kb2 and T47d Kbluc cells, respectively (He *et al.,* 2011). Based on these results, OSPW seems to have the potential to interfere with growth and reproduction of exposed organisms via several tissue systems and biochemical pathways. This potential for disruption was observed in a recent study where fathead minnows

exposed to aged OSPW had lesser concentrations of sex steroids in their blood and significantly fewer spawning events (Kavanagh *et al.,* 2011).

In addition to aquatic organisms, effects of OSPW exposure have been studied in terrestrial species. As in fish, the liver was a primary target organ in mammals. Wistar rats exposed to OSPW acutely or sub-chronically were adversely affected. There were both lethal and non-lethal effects, including: greater water consumption, anorexia, less blood glucose, fewer circulating leukocytes, greater muscle glycogen, and changes in amylase activity, total concentrations of protein, and cholesterol levels (Rogers *et al.,* 2002). Subsequent studies in rats that had been orally dosed with NAs demonstrated significantly impaired reproduction, probably due to failed embryonic implantation. The author suggested that these impairments were likely due to changes in cholesterol availability and a resultant decrease in progesterone synthesis (Rogers, 2003). A more recent study using macrophages derived from mouse bone marrow showed the potential for immunotoxic effects following exposure to commercial NAs and the organic fraction extracted from OSPW. Eight-week oral exposures resulted in impaired antimicrobial responses and expression of pro-inflammatory cytokines, which could have implications for the ability of the host animals to resist other immune challenges (Garcia-Garcia *et al.,* 2011a). Additional studies have shown dose- and time-dependent changes in expression of mRNA of pro-inflammatory genes in liver, spleen, and lymph nodes after the exposure regime as described in the previous study, with different expression profiles observed in animals exposed to commercial NAs compared to those exposed to the organic fraction isolated from OSPW (Garcia-Garcia *et al.,* 2011b). These studies suggest that the immune system may represent another target for organic acids from OSPW and that

commercial NAs are not necessarily an appropriate surrogate for the NAs and other organic constituents found in OSPW (Garcia-Garcia *et al.,* 2011b).

Other studies have examined effects of OSPW on birds inhabiting reclaimed mine sites, with tree swallows (*Tachycineta bicolor*) serving as a sentinel species. One study found greater production of thyroid hormones in nestlings living in wetlands containing mine tailings. The researchers suggested that this could have potential implications for behaviour, metabolism and survival in later life stages (Gentes *et al.,* 2007). Another study of tree swallows found a greater likelihood of dietary xenobiotic exposure in birds living in areas reclaimed after oil sands were removed. However, there were no significant changes in immune function, reproductive success, or growth rate in birds within reclaimed areas compared to those in reference wetlands (Smits *et al.,* 2000). The effects of exposure to OSPW and oil sands-derived materials depends on the specific species, the route and duration of exposure, and whether whole OSPW is used or extracted fractions, but the results of studies conducted to date suggest that a variety of organisms and physiological systems are sensitive to NAs and the organic fraction of OSPW. While it is may be feasible to limit risks for terrestrial organisms, there are concerns for aquatic organisms exposed to OSPW in the case of a spill scenario or reclamation using in-pit lakes.

### *1.2.3 Identification and quantification of NAs*

One of the greatest challenges of working with NAs and determining their toxicity is separating individual acids within mixtures in order to characterize their reactivity, structures and degradation profiles (Hao *et al*., 2005; Headley and McMartin, 2004). The

difficulty in quantifying individual constituents of NAs mixtures arises from the fact that the acids have similar structures and physico-chemical properties. They exhibit a limited range of volatility, molecular weight, and polarity (Holowenko, *et al.,* 2002). Since boiling point is directly proportional to carbon number, degree of cyclization, and molecular weight, methylated NAs can be distilled into fractions based upon their boiling points (Frank *et al.,* 2009). However, there is no single standard method used for analysis of NAs because there is a trade-off between high sample throughput and time for results and data to be returned (Headley *et al*., 2008).

Over the past two decades, a number of different approaches have been explored for use in NAs analysis and characterization. One analytical method that has been used historically to quantify NAs is Fourier transform infrared (FTIR) spectroscopy. In this method, which was developed by Syncrude Canada Ltd., aqueous OSPW samples are acidified and then NAs are extracted quantitatively into dichloromethane. The extracted samples are analyzed using FTIR to measure absorbencies of mono- and dimeric acids at 1743 and 1706 $cm^{-1}$, respectively. Most of the recently developed methods for quantification of NAs in OSPW involve mass spectrometry (MS), which has yielded the best information on compositions and molecular structures of NAs in mixtures, particularly when used in combination with other methods (Clemente and Fedorak, 2005). In the late 1980s, fluoride ion chemical ionization MS was used to separate NAs mixtures into groupings by their C and Z values (Dzidic *et al.*, 1988). Several years later, fast atom bombardment MS was used for mixture separation and found to be effective for quantification of components of the mixture that were polar, non-volatile and/or high in molecular weight, such as NAs (Fan, 1991). Two-dimensional gas chromatography (GC)

has been used in combination with time-of-flight mass spectrometry (ToF-MS) as a tool for comparing patterns of acyclic and monocylic NAs in different mixtures (Hao *et al.,* 2005). There are several additional mass spectrometric approaches that have been used, including different versions of chemical ionization, atmospheric pressure ionization, electroionization and electrospray ionization (reviewed by Headley *et al.,* 2008). These have been applied with varying degrees of success, but are generally more effective than conventional GC or HPLC methods.

Some of the most promising analytical approaches recently have involved a combination of GC or HPLC with mass spectrometry (Headley *et al.,* 2008), such as the use of HPLC and high resolution MS by Han *et al.* (2009). The majority of analytical approaches that have been used to date divide NAs mixtures into subgroups based upon carbon and ring numbers, but they have not been able to identify or separate individual acids. However, recently GCxGC-ToF-MS has been used to resolve the structures of individual NA isomers (Rowland *et al.,* 2011a). This approach may serve as a useful tool to assist in assessing the change in NAs profile by different treatment approaches, to further inform structure-activity relationships, and to identify the most toxic NA congeners. The technology for the quantification of NAs has been continually improving and new or modified analytical techniques will continue to emerge to help better understand the chemistry and toxicity of OSPW.

### 1.3 Reclamation and treatment strategies for OSPW

In accordance with environmental legislation, OSPW is currently stored in active settling basins and may not be released due to its potential to have adverse environmental

impacts (Government of Alberta, 2008; Madill *et al*., 2001). As the volume of OSPW continues to increase, there is interest in developing treatment strategies to reduce the toxicity of OSPW, primarily by developing methods to reduce the toxicity of NAs, and possibly allow the treated water to be re-introduced into the surrounding environment. A number of approaches have been proposed and research is currently underway to optimize some of the most promising treatment methods, including biodegradation, ozonation, and reclamation using wet or dry landscapes.

### *1.3.1 Biodegradation*

Biodegradation (also known as microbial degradation or "aging") of NAs has been an area of great interest and research over the past decade. There are microbial populations indigenous to the oil sands region that are capable of degrading NAs thereby reducing the acute toxicity OSPW, but the process is slow and incomplete (Herman *et al*., 1994; Holowenko *et al.,* 2002). In general, NAs with lesser MW and fewer rings tend to be most susceptible to biodegradation (Clemente and Fedorak, 2005; Frank *et al.,* 2008; Lai *et al.,* 1996). The least cyclic NAs are most rapidly degraded in settling basins and experimental reclamation ponds (Del Rio *et al.,* 2006*;* Han *et al.,* 2009). However, linear NAs with 5-12 carbons were found to be resistant to degradation (Del Rio *et al*., 2006), so some uncertainty exists as to the mechanisms involved in this degradation pathway and what factors are most important in determining susceptibility of individual NAs to biodegradation.

Since degradation of NAs occurs to some extent naturally, there is interest in optimizing environmental conditions to encourage bioremediation. *In situ* bioremediation

is limited by temperature, sunlight, nutrient availability and microbial biodiversity (McMartin, 2003). There are three approaches that could be used to address these limiting factors: 1) biostimulation, 2) bioaugmentation, 3) intrinsic treatment (Quagraine *et al.,* 2005). The biostimulation approach involves optimizing physical and chemical parameters for degradation by indigenous microbes that have the capability to degrade NAs (Quagaine *et al.,* 2005). Thus far, increasing dissolved oxygen and temperature and adding phosphate have been shown to significantly boost degradation rates of specific NA substrates (Lai *et al.,* 1996; Wong, 1998). However, these parameters can be difficult to optimize in the field, especially with the temperature variations that occur in northern Alberta (Allen, 2008b). The second approach for optimizing biodegradation is to inoculate OSPW with non-indigenous microbial populations that are capable of effectively degrading NAs. The effectiveness of this approach is contingent on degrading populations not being already present naturally. However, there are risks associated with introducing non-native organisms onto the landscape, lest they have detrimental impacts on the local flora and fauna (Quagraine *et al.,* 2005). Lastly, intrinsic treatment involves allowing an existing microbial population to proceed with biodegradation without additional anthropogenic interference. The demonstration reclamation ponds and wetlands currently in place are essentially using an intrinsic approach (Quagraine *et al.,* 2005). Since natural aging has been shown to be a very slow process, it is likely that some sort of optimization will need to be applied if biodegradation were used in large-scale reclamation efforts.

Early work with commercial NAs showed great potential for biodegradation, but it was found that these mixtures contained a greater proportion of lesser MW NAs and

less recalcitrant fractions than the mixtures found in OSPW (Scott *et al.,* 2005). As a result, commercial mixtures are not good surrogates for the NAs in OSPW, particularly in degradation studies, and true microbial degradation rates may be lesser than indicated by initial investigations (Rowland *et al.,* 2011b; Scott *et al.,* 2005). It was also previously thought that natural degradation would be one of the most cost-effective methods for treatment, but more recent observations of degradation in settling ponds have indicated that NAs in OSPW are poorly degraded on-site (Scott *et al.,* 2008). Additionally, a recent study showed evidence for persistent toxicity of aged OSPW, whereby reproductive capacity of fathead minnows was impaired by exposure to water aged in experimental reclamation ponds for close to 20 years (Kavanagh *et al.,* 2011). Biodegradation approaches to OSPW remediation are slow, especially at the environmental conditions naturally present in the Alberta oil sands region, and OSPW may still retain residual toxicity after several years of natural aging. While aging may be an appropriate treatment for OSPW until other technologies can be developed and scaled-up, it is not effective for the volumes of OSPW requiring treatment.

### *1.3.2 Ozonation*

To address the needs for OSPW reclamation, a more rapid and effective treatment method is required, and ozonation is one method of interest. Ozone ($O_3$) is a strong oxidizing agent that can break chemical bonds and form more polar compounds via either direct or indirect chemical reactions (Camel and Bermond, 1998; Martin *et al.,* 2010). Due to its great oxidation potential, $O_3$ is a popular method of choice for pre-oxidation, intermediate oxidation and/or final disinfection during municipal water

treatment (Camel and Bermond, 1998). Ozonation is used by some municipalities and private drinking water providers to treat surface or ground water to meet potable water standards. The purpose of including chemical oxidation as part of the treatment process is to: a) remove inorganic species (especially metal ions), b) encourage coagulation and flocculation, c) oxidize natural organic matter and organic micropollutants, and d) disinfect (Camel and Bermond, 1998). During such treatment, the cell membranes of bacteria are oxidized, which results in elimination of potentially pathogenic bacteria (Camel and Bermond, 1998).

In the context of the oil sands, application of $O_3$ has been suggested during pre-treatment or intermediate stages of treatment in order to reduce the toxicity of OSPW. Ozonation of OSPW was observed to reduce total concentrations of NAs in OSPW by 70% (to 20 mg/L) after a 50 min treatment and by 95% after a 130 min treatment. The resultant OSPW displayed an elimination of acute toxicity, as measured by the Microtox® bacterial luminescence assay (Scott *et al.,* 2008). The NAs were oxidized to other organic compounds in solution, as opposed to being mineralized to carbon dioxide as occurs during microbial degradation. It is believed that the detoxification mechanism involved in ozonation is different from that of aging. This conclusion is based on the observation that there was a shift towards low MW NAs post-ozonation, while microbial degradation results in a shift towards greater MW acid fractions remaining in the mixture (Scott *et al.,* 2008). Lesser toxicity towards microbes has also been observed following ozonation of both commercial NAs and OSPW, and ozonation also enhanced microbial degradation of the residual NAs, presumably by targeting some of the most persistent fractions and converting them to more bioaccessible forms (Martin *et al.,* 2010). A recent

study of structure-reactivity of NAs during the ozonation process found that those NAs with more rings and carbons were degraded most rapidly, leaving the less recalcitrant fractions and making ozonation a complementary process to microbial degradation (Perez-Estrada *et al.,* 2011).

Downstream effects of ozonation on organisms that could be exposed to ozonated OSPW are still somewhat unknown, but ozonation does not worsen endocrine effects (He *et al.,* 2010). Specifically, ozonation of OSPW was observed to attenuate effects on hormone production in H295R cells, to attenuate anti-androgenic effects in MDA-kb2 cells, and to result in no change in estrogenicity in T47d Kbluc cells (He *et al.,* 2011). The use of ozonation for treating OSPW has shown promise, but additional information about its mechanisms and effects is still required before it can be applied to reclamation on a large scale (He *et al.,* 2010; He *et al.,* 2011). This technology deserves additional consideration and was, therefore, selected as one of the experimental treatments for the research described in this thesis.

### *1.3.3 Wet and dry landscapes*

Reclamation of both wet and dry landscapes involves use of mature fine tailings (MFTs) themselves or the associated water and these landscapes represent strategies for creating new habitats in the Alberta oil sands region. Mature fine tailings (which are sometimes referred to as fine fluid tailings- FFTs) are sludges that have been allowed to settle to the point of containing 30-35 wt% solids, with the solids comprised of sand, silt, clay, bitumen and other residual particles suspended in process water after bitumen removal (Allen *et al.,* 2008a; Han *et al.,* 2009). The dry landscape option involves

combining de-watered MFTs from settling ponds with other materials, such as sand and soil, to create a terrestrial habitat where the tailings will eventually be incorporated into soil (Allen, 2008a; Han *et al*., 2009). Similarly, reclamation using wet landscapes would see the creation of pit lakes filled with tailings water and capped with freshwater (Allen, 2008a; Quagraine *et al.,* 2005). Wetlands can also be considered part of the wet landscape approach (Allen, 2008a), with the ultimate goal being the production of viable, self-sustaining aquatic ecosystems with functionality equivalent to other water bodies in the local area (Han *et al.,* 2009; Leung *et al,* 2003). In the 1980's, two of the major oil sands companies, Syncrude Canada Ltd. and Suncor Energy Inc., began to explore construction of wetlands and have continued to use test wetlands as trials for more widespread application (Allen, 2008a). Research on engineered wetlands has focused on optimization of primary mechanisms such as nitrification, de-nitrification, biodegradation and plant-uptake. There has been some concern raised regarding use of wetlands as a primary reclamation strategy since productivity and biodegradation will always be limited by seasonal variation in temperature due to the geographical location of the oil sands. However, the potential for minimal impacts on the environment, combined with the economic feasibility of wet landscapes, warrants serious consideration of this option (Allen, 2008b). There is limited information about tailings degradation *in situ,* since most work has been conducted under controlled laboratory conditions (Han *et al.,* 2009) so further research in the field would be beneficial and informative.

*1.3.4 Other potential reclamation methods*

There are a variety of other options that have been considered for the treatment of OSPW and some of these are currently under investigation to determine the logistical and economic feasibility at the scale required by the oil sands industry. There is some interest in phytoremediation, using terrestrial plants, macrophytes, or algae to take up and metabolize NAs. With terrestrial phytoremediation, research has focused on the rhizosphere and identifying plants with microbial associates that are able to degrade target compounds in OSPW (Armstrong, 2008). Aquatic remediation would use algal species, since it has been observed that some algae are able to survive in OSPW and biotransform specific NAs (Headley and McMartin, 2004). One unicellular alga, *Dunaliella tertiolecta,* was identified by Quesnel *et al.* (2011) as a species of interest since it was capable of degrading model NAs and some NAs within tailings water. However, the recalcitrant fraction (identified by the authors as the fraction predominately composed of structures with 10-15 carbons and Z designation of -4 to -6) of OSPW has proved to be the major hurdle because algae are not able to degrade these compounds (Quesnel *et al.,* 2011). Therefore, additional complementary strategies, such as ozonation or others, may be required to effectively implement a wider-scale phytoremediation approach.

Photolysis is another approach being considered for use in treatment of OSPW. Natural photolysis of NAs is negligible and affects neither their concentrations in nor toxicity of OSPW. This is due, in part, to the shallow penetration of the UV portion of the solar spectrum of wavelengths with sufficient energy to cause photolysis of NAs (300-400 nm). However, larger MW NAs are susceptible to photodegradation, so

photolysis could potentially be used to increase bioavailability of NAs and other target compounds for subsequent biodegradation (McMartin, 2003). Some of the major factors limiting advanced oxidation treatment via photolysis are the relatively high cost of application and the potential interference by the high concentrations of salts present in OSPW (Allen, 2008b).

Other traditional drinking water treatment options that have been explored for treatment of OSPW include the use of adsorbents, membranes, and reverse osmosis. Membranes could potentially be used for micro-, ultra-, or nano-filtration. With membrane techniques (including reverse osmosis), one limitation is bio-fouling, so prevention of bio-fouling is currently an active area of research (Allen, 2008b). The use of coagulants and flocculants as pre-treatments for OSPW can effectively remove organic carbon. While NAs concentrations were reduced with coagulant-flocculant treatment, the compounds themselves exerted toxic effects towards *C. dilutus* larvae and daphnids, so other treatments would be required to optimize this treatment method for OSPW (Pourrezaei *et al.,* 2011). The toxicity was likely due to the presence of aluminum ions from alum that was used as a flocculant. In all likelihood, there will be a continued trend towards developing an integrated approach to treating OSPW that will involve several methods working in parallel or sequentially to reduce NAs concentrations and toxicity (Headley and McMartin, 2004).

### 1.4 Biomarkers and monitoring

Biomarkers are generally defined as sublethal changes that occur in organisms as a result of exposure to toxicants and that can serve as early indicators of potential longer-

term effects (Cajaraville *et al.,* 2000; Lagadic *et al.,* 1994). Biomarkers can indicate exposure (i.e., biomarkers of exposure), toxic effect (i.e., biomarkers of effect), or susceptibility to stressors or toxicants and should be specific, quantitative, and easily measurable (Timbrell, 1998). Biomarkers are generally considered within individual organisms and focus particularly on molecular and biochemical changes (Au, 2004). Shifts at the population or community level typically indicate changing environmental quality and are referred to as "bioindicators" (Lagadic *et al.,* 1994). Markers at lower levels of organization are more specific and easier to reproduce, but it is often difficult to correlate the observed changes with ecologically relevant changes at higher levels of organization, such as population or community or ecosystem (Au, 2004; Lagadic *et al.,* 1994). In contrast, bioindicators are more ecologically relevant, but these typically take longer to become detectable and cannot serve as effective early warning systems (Au, 2004). Therefore, it is necessary to strike a balance between selecting reproducible biomarkers that only poorly predict true ecosystem changes and variable biomarkers that are relevant but less consistent and useful for monitoring programs. Operationally, biomarkers have been incorporated into monitoring systems in both Europe and North America (Cajaraville *et al.,* 2000) and are typically used in the first stage of risk assessments as a quick, inexpensive suite of tests to provide an initial indication of ecosystem function (Wedderburn *et al.,* 2000).

In order for specific molecular endpoints or biomarkers to serve as effective tools for prediction of future impacts, they are ideally very toxicant-specific and not induced by a large range of environmental factors (Au, 2004). It is also beneficial to select a marker that is not susceptible to physiological changes induced within the organism itself

(e.g. cortisol induction in response to handling stress can alter activities of other stress response enzymes or hormones, so these would not be ideal biomarkers of toxicant effects). Biomarkers can be used to indicate susceptibility of an individual or population to harm from a particular toxicant (Hong and Yang, 1997). However, since there is a wide range of genetic heterogeneity present within communities, it is necessary to be mindful that lab-reared test organisms might be more or less susceptible than those found within a particular ecosystem (Eisenhauer *et al.,* 1999). Also, it is necessary to have detailed information on base level conditions and normal seasonal variation in the species of interest in order to ensure that changes observed are truly a result of toxicant exposure. Even more importantly, biomarkers of exposure would ideally correlate with biomarkers of stress or health status of an organism, so that a change at the biochemical or molecular level can be known to manifest a response in the health or functioning of whole organisms (Cajaraville *et al.,* 2000). Identifying molecular biomarkers that are predictive of toxicity at the level of the whole organism is the ultimate goal in biomarker research and allows theses biomarkers to truly serve as an early warning system in monitoring contaminants in the environment, particularly if the change at the organism level can be observed directly or tested using a simple, inexpensive method.

A number of different bioindicators and biomarkers of stress and/or exposure to different toxicants have been developed for use in aquatic species. In mussels, potential biomarkers associated with industrial effluents include heart rate activity, tissue histopathology and lysosomal integrity (Wedderburn *et al.,* 2000). Au (2004) identified several markers in fish for general pollution monitoring applications, such as kidney and liver histopathology, epidermal hyperplasia, peroxisome proliferation, skeletal

malformation, and macrophage aggregates. For invertebrates, such as chironomids, acetylcholinesterase (AChE) has been used as a biomarker of toxicant exposure (Crane *et al.,* 2002), as have other detoxification enzymes (MFOs, etc.), changes in energetics (e.g. carbohydrate metabolism), and changes in behavior, development or reproductive activity (Lagadic *et al.,* 1994). Heat shock proteins (*hsps*) are commonly used to assess molecular responses to toxicant exposure in aquatic organisms (including *Chironomus* spp*.)* because they represent a family of highly conserved proteins within eukaryotes and are controlled by both inducible and constitutively-expressed genes (Lee *et al.,* 2006; Planello *et al.,* 2010). However, given their sensitivity to a number of toxicants, *hsps* are not sufficiently specific to be an appropriate biomarker of exposure or effect for one particular stressor.

A biomarker that is more commonly used in *Chironomus* is hemoglobin (*Hb*), since species of this genus exhibit instar-specific and tissue-specific synthesis of globin throughout the duration of the larval stage (Ha and Choi, 2008; Lee *et al.,* 2006). Specific to *Chironomus dilutus* is the expression of Balbiani ring genes. The Balbiani rings are giant puffs on chromosomes within larval salivary gland cells that represent areas of great RNA production and control production of components required for larval case building, including four silk proteins (Eeken, 2001). While the development of molecular biomarkers is a relatively new field within ecotoxicology, there are a number of molecular endpoints that have been used in aquatic organisms, specifically in *C. dilutus*, in order to assess the effects of exposure to toxicants and other environmental stressors.

## 1.5 Genomic applications in toxicology

Genomics has been defined as "the study of how an individual's entire genetic make-up translates into biological functions" (Snape *et al.,* 2004). Toxicogenomics involves examining the expression pattern of genes involved in adaptive responses following toxicant exposure (Snape *et al.,* 2004). In addition, toxicogenomics attempts to correlate changes in gene expression and cellular regulatory mechanisms with physiological responses observed following exposure to compounds of interest. However, it can be very difficult to establish these types of correlations (Neumann and Galvez, 2002). Gene expression can be dramatically different among individuals as a function of gender, age, nutritional status, life stage, metabolism, environmental conditions, and other factors, so these must be accounted for and controlled as much as possible (Miracle and Ankley, 2005; Neumann and Galvez, 2002). It is also important to remember that analysis of gene expression is temporally and spatially restricted, providing only a snapshot of gene transcription processes.

Until quite recently, there was only limited use of genomics in environmental toxicology, mainly due to a lack of DNA sequence reference libraries and tools for model environmental species (Denslow *et al*., 2007; Miracle and Ankley, 2005; Snape *et al.,* 2004). Gene technologies are currently less-developed for aquatic applications than mammalian, but genomes have been sequenced in *Daphnia pulex,* medaka (*Oryzias latipes)*, zebrafish (*Danio rerio*), Japanese pufferfish (*Takifugu rubripes*), and green spotted puffer (*Tetraodon nigroviridis)*, with commercial microarrays now available for a number of fish species including, but not limited to, zebrafish, medaka, and fathead minnow (Denslow *et al*., 2007; McTaggart *et al.,* 2009; Miracle and Ankley, 2005).

Unfortunately, *C. dilutus*, *C. riparius* and other popular ecotoxicological model benthic invertebrate species have not yet been fully sequenced to serve as genomic models (Snape *et al*., 2004), although some initial studies using polymerase chain reaction (PCR) and real-time PCR (RT-PCR) have been conducted with *C. riparius* (Ha and Choi, 2008; Lee *et al*., 2006).

High-throughput genomics tecnhologies can serve as rapid screening tools to minimize the effort involved in screening multiple variables controlling gene expression (Miracle *et al.,* 2003; Neumann and Galvez, 2002). These technologies can also be used to identify novel modes of action for contaminants and stressor-specific biomarkers of exposure (David *et al.,* 2010; Neumann and Galvez, 2002). By characterizing stress-induced gene expression patterns and correlating these with specific stressors (for those stressors that produce unique gene expression profiles), these patterns can be used to assess ecosystems and identify relevant ecosystem-specific drivers of stress or toxicity (Snell *et al.,* 2003). Genomics can be used to identify disruptions in developmental pathways (Maher *et al.,* 2009; Wang *et al.,* 2010), which may improve the ability to establish a direct correlation between early transcriptional events and physiological changes manifesting at higher levels of organization (Miracle *et al.,* 2003).

Open format transcriptome sequencing approaches, such as Illumina RNA-Seq (Illumina Inc.) and Roche 454 (Roche Applied Science), also hold potential for more extensive application in environmental toxicology than is currently offered by cDNA microarrays. These sequencing tools can be used to examine gene expression and, unlike arrays, are not limited to a specific set of genes, but are able to detect changes across the entire transcriptome (Morozova *et al.,* 2009). As well, the resultant sequences have been

found to be very reproducible and gene expression data agree more closely with the outcome from real-time PCR assays than do microarrays (Marioni *et al*., 2008). Though limited sequencing has been completed on the genome of *C. dilutus* or related species, no previous sequence information is required for Illumina, so this technology was used in this research thesis to gain an overall view of changes in gene expression in order to identify potential target genes for PCR array development.

### 1.6 *Chironomus dilutus* as a test organism

*Chironomus* spp. are commonly used for acute and chronic toxicity evaluations for a wide spectrum of toxicants, including metals, agrochemicals, endocrine disrupting chemicals, and industrial effluents (Muscatello and Liber, 2009; Soin and Smagghe, 2007; Taenzler *et al.,* 2007). The non-biting midge *C. dilutus* (formerly *C. tentans*) is ubiquitous throughout North American freshwater systems (Armitage *et al.,* 1995) and comprises, along with other Chironomidae, a large proportion of the invertebrate biomass within the Athabasca region of northern Alberta (Bendell-Young *et al.,* 2000). As a test organism, *C. dilutus* has a number of attractive characteristics including relative ease of culturing, a short life cycle (approximately 25-30 days at 23 °C), sensitivity to a variety of contaminants, and a number of developmental and reproductive endpoints which can be readily assessed (Benoit *et al.,* 1997). The life cycle of *C. dilutus* begins with an egg stage, which is followed by four larval instars (4-7 days each), a pupal stage (1-2 days), and finally an adult stage (Liber *et al.,* 1996; Taenzler *et al.,* 2007). Male adults typically emerge a few days earlier than females and adults survive for only a short time, perhaps up to 7 days (Taenzler *et al.,* 2007). The majority of the life cycle is completed under

aquatic conditions, making chironomid larvae appropriate subjects for water and sediment toxicity testing (Environment Canada, 1997; Taenzler *et al.,* 2007; US EPA, 2000).

### 1.7 Project rationale

Reclamation of OSPW represents a significant challenge for the oil sands industry and is of major interest for both private companies and the governmental agencies responsible for overseeing adherence to environmental regulations. As a result of the recent boom in oil sands extraction and processing, volumes of stored OSPW have reached a level requiring timely attention and effective treatment. The toxicities of OSPW and associated NAs have not yet been fully characterized, particularly in benthic invertebrates. There is also a need to understand the effectiveness and limitations at eliminating toxicity of different water treatment methods, including ozonation and biodegradation, so that appropriate and feasible options may be applied at the Athabasca oil sands. It is important to have information about both the chemical interactions of these processes and their potential for contributing to the toxicity towards aquatic organisms. Since chironomids are sensitive to OSPW and represent an important component of aquatic ecosystems in the oil sands region, *C. dilutus* was selected as a model invertebrate for studying the effects of untreated, aged, and ozonated OSPW.

### 1.8 Research goal and objectives

The overall goal of this thesis was to characterize the toxicity of OSPW to a model benthic invertebrate, *Chironomus dilutus* (formerly *C. tentans),* and to evaluate the

effectiveness of biodegradation and ozonation as treatments for reclaiming OSPW. In addition, this research sought to identify molecular biomarkers of exposure and effect for use in future monitoring programs. The specific objectives were:

1. To identify sensitive toxic endpoints in *C. dilutus* following acute and chronic exposure to OSPW.

$H_O$: There are no differences in survival, mass, pupation, or emergence between larvae exposed to untreated OSPW or freshwater.

2. To use molecular tools to characterize toxicity at the level of the whole organism and to identify potential biomarker(s) and high throughput screening methods for assessment of OSPW/NA exposure in *C. dilutus.*

$H_O$: There are no differences in gene transcription between larvae exposed to untreated OSPW or freshwater and therefore, no biomarkers of exposure to OSPW exist.

3. To determine whether natural biodegradation in reclamation ponds and/or ozonation treatment could effectively eliminate or reduce toxicity of OSPW to *C. dilutus*.

$H_O$: There are no differences in survival, mass, pupation, or emergence among larvae exposed to ozonated OSPW, aged OSPW, untreated OSPW, and freshwater.

# CHAPTER 2:
# Effects of exposure to oil sands process-affected water from experimental reclamation ponds on *Chironomus dilutus*

## 2.1 Introduction

Canada is home to the second largest proven oil reserves, with the majority located within the oil sands deposits of Alberta (Government of Alberta, 2006; Veil *et al.,* 2009). The oil sands are divided into three primary deposits and together these account for more than 40% of the oil produced in Canada (CAPP, 2011). The largest deposit is located at the Athabasca site located near Fort McMurray, AB and is estimated to contain 400 billion cubic meters of crude bitumen (National Energy Board, 2000). Extraction of bitumen from surface-mined oil sands involves use of the "Clark hot water extraction process" whereby oil sands are mixed with 79-93 $^{o}$C water and caustic soda to separate bitumen from other constituents including clay, residual sand, inorganic compounds, and organic compounds, such as naphthenic acids (NAs). The resultant tailing slurries are comprised of solids, including sand and clay, unrecoverable hydrocarbons, and oil sands process-affected water (OSPW) (Han *et al.,* 2009). OSPW is stored in active settling basins on-site (Hao *et al.,* 2005; He *et al.,* 2010; Rogers *et al.,* 2002) where the fine silt and clay fractions slowly densify to form mature fine tailings (MFTs) and release additional OSPW (Han *et al.,* 2009). Companies are prevented from discharging OSPW into surrounding freshwater systems, so all OSPW becomes part of the management responsibilities and costs for oil sands companies (Veil *et al.,* 2009).

The major impediment to the treatment and release of OSPW is its inherent toxicity which has been linked to organic compounds, including NAs, in the water (Rowland *et al.,* 2011a). NAs are broadly defined as a complex mixture of dissolved carboxylic acids

with the formula $C_nH_{2n+z}O_2$, where n indicates the number of carbons and z relates to the number of rings (Clemente and Fedorak, 2005; Holowenko *et al.,* 2002; Lai *et al.,* 1996). However, these classical structures represent only a portion of the acid-extractable fraction of OSPW (Grewer *et al.,* 2010) and in the present study, "NAs" refer to those extractable compounds measured by Fourier transform infrared spectroscopy (FTIR), which extend beyond the classical definition of naphthenic acids. The NAs are naturally occurring in bitumen and become solubilized and concentrated in tailings water as a result of the recycling of extraction water and the alkaline pH created by sodium hydroxide, which promotes retention of NAs in the water column (Headley and McMartin, 2004; Rogers *et al.,* 2002). The NAs and other acid-extractable organic compounds within OSPW represent an analytical challenge due to the complexity of the mixture and the variety of different individual structures present (Rowland *et al.,* 2011b). The toxicity of a NAs mixture is a function of the structures and relative proportions of different individual acids. Lesser molecular weight (MW) NAs are thought to be more acutely toxic than those with greater MW (Holowenko *et al.,* 2002). In OSPW, NAs with more rings also tend to be more persistent due to greater resistance to biodegradation (Holowenko *et al.,* 2002; Martin *et al.*, 2010). Biodegradation of NAs in OSPW settling ponds results in increasing proportions of mono-, di-, and tri-oxidized NAs (Han *et al.,* 2009). Acute toxicity of OSPW has been observed to decline over time, likely due to this shift in the NAs profile and the change in proportion of different carbon and ring numbers (Holowenko *et al.*, 2002).

Wet landscape reclamation has been proposed as an option for handling the volumes OSPW that are currently contained within active settling basins. This would represent a

passive treatment method whereby OSPW is transferred to hydrogeologically-secure pits under the assumption that toxicity and NAs concentrations will eventually diminish (Kavanagh *et al.,* 2011). After 24 months of natural aging and biodegradation, tailings pond water from the Syncrude Canada Ltd. lease site was found to have $LC_{50}$ (half maximal lethal concentration) values >100% for both 21-day rainbow trout (*Oncorhynchus mykiss*) survival tests and 21-day *Daphnia* life cycle tests (MacKinnon and Boerger, 1986). Similarly, Holowenko *et al.* (2002) found that OSPW from ponds aged for 7 and 11 years had $IC_{50}$ (half maximal inhibitory concentration) values >100% for acute toxicity and that the longest aged OSPW had the least acute toxicity and the greatest proportion of higher molecular weight (C>22) NAs. These trends suggest that biodegradation may be a feasible reclamation strategy, despite the long timelines required under natural conditions.

While the chronic effects of reclamation pond waters on fish have been studied (Kavanagh *et al.,* 2011), less was known about the effects of exposure to fresh or reclaimed OSPW on benthic invertebrates. For this reason, a model benthic invertebrate, *Chironomus dilutus* (formerly *C. tentans*), was chosen for this study. We hypothesized that effects of exposure to relatively fresh OSPW would be more severe or significant than effects of exposure to aged water from experimental reclamation ponds. The toxicity of each water sample was evaluated in terms of effects on the ecologically relevant endpoints of survival, growth, pupation, and emergence.

## 2.2 Materials and Methods

### *2.2.1 Test organisms*

Larvae for both the 10-day and chronic exposure studies were obtained by breeding adult *C. dilutus* midges from a laboratory culture housed in the Toxicology Centre at the University of Saskatchewan. Egg masses were raised to test age in several 15 L aquaria filled with control freshwater in an environmental chamber maintained at 23 ± 1 °C with a 16:8 hour light:dark regime. The water used for control freshwater in both culturing *C. dilutus* and during the tests was Saskatoon, SK municipal water that had been carbon filtered, bio-filtered, and aerated for 24 hr prior to placement into aquaria or test vessels.

### *2.2.2 Exposure waters*

*Chironomus dilutus* larvae were exposed to one of seven treatment waters: 1) control freshwater, 2) control saltwater (10 day acute exposure only), 3) untreated OSPW sampled from the West In-Pit in summer 2009 (designated 'WIP-OSPW-A'), 4) untreated OSPW sampled from the West In-Pit in winter 2010 (designated 'WIP-OSPW-B'), 5) Big Pit water, 6) FE5 water, or 7) TPW water. Samples from the three reclamation ponds were collected in summer 2009. The control freshwater had the following characteristics (mean ± SD): conductivity 436 ± 12 µS/cm, pH 8.19 ± 0.04, alkalinity 101 ± 3 mg/L as $CaCO_3$, and hardness 125 ± 8 mg/L as $CaCO_3$. Both WIP-OSPW-A and WIP-OSPW-B were collected from West In-Pit (WIP), an active settling basin located on the Syncrude Canada Ltd. lease site (near Fort McMurray, AB). WIP-OSPW represents relatively fresh, untreated process water that is regularly fed from the main extraction plant (as

described by Han *et al.,* 2009). Big Pit, FE5 and TPW are three on-site experimental reclamation ponds that have been aged by use of different approaches. Big Pit water has been aging since 1993 and is comprised of fine fluid tailings (FFTs) capped with freshwater. The FE5 pond was created in 1989 by capping FFTs with OSPW. Finally, TPW is OSPW that has been aging since 1993. A more detailed description of these OSPW samples is provided by Han *et al*. (2009).

Typically, OSPW has a total concentration of dissolved solids (TDS) in the range of 2,000 to 2,500 mg/L and the dominant ions include sulfate, bicarbonate, sodium, and chloride (Allen, 2008; Gamel El-Din *et al.*, 2011). A manually prepared saltwater control comprised of 938 mg/L NaCl, 506 mg/L $NaSO_4$ and 910 mg/L $CaSO_4$ was used in the 10-day acute exposure to simulate these components of OSPW in order to assess the contributions of salinity to toxicity of OSPW. For the chronic exposure study, two different inorganic controls were made by removing the majority of the organic fraction from untreated WIP-OSPW-A and FE5 (as described in Anderson *et al.,* 2011a). Each was mixed with 5% (w/v) 8-20 mesh particle size activated charcoal (Sigma-Aldrich, St. Louis, MO) and gently stirring for 4 hr at room temperature. Following the contact period, the larger charcoal particles were removed by sieving and the treated water was then vacuum-filtered through a 0.22 μm filter (Millipore Corporation, Billerica, MA) to remove all remaining charcoal particles. The resultant waters were designated AC-WIP-A and AC-FE5 (AC – "activated charcoal-treated").

Concentrations of NAs were measured by FTIR at the University of Alberta (Edmonton, AB). This method quantifies NAs based upon absorbance peaks of carboxylic acids at 1743 $cm^{-1}$ (monomer) and 1706 $cm^{-1}$ (dimer) (described in Holowenko

*et al.,* 2001), so it is possible that other organic compounds may be quantified by FTIR and some non-NAs could be missed. In WIP-OSPW-A, the concentration of NAs was 71.7 mg/L and in WIP-OSPW-B, the concentration of NAs in the water was 70.2 mg/L. Big Pit, FE5, and TPW had NAs concentrations of 23.2, 13.0, and 35.0 mg/L, respectively. The concentrations of NAs in AC-FE5 and AC-WIP-A were 0.92 mg/L and 6.4 mg/L, respectively, which represented 93% and 91% removal of total NAs. Ion chromatography (IC) and inductively coupled plasma mass spectrometry (ICP-MS) water chemistry analysis was conducted at the Water Quality Laboratory (University of Saskatchewan, Saskatoon, SK) for all waters. Methods for determination of metal concentrations are described in van de Wiel (2004). Samples for analysis were collected from individual beakers at the beginning, middle, and end of the exposure period for each treatment water and represented water chemistry one day after a water change.

### *2.2.3 Acute exposure*

#### *2.2.3.1 Effects on survival and growth*

The experiment was conducted under the environmental conditions described in Section 2.2.1 and by Anderson *et al.* (2011a). A modified 10-day static-renewal assay was used to assess effects of acute exposure to the treatment waters. Measured endpoints included survival, growth, and behavior of *C. dilutus* larvae. Ten 8-9 day post-hatch larvae were randomly assigned to each of four replicate 300 mL tall-form beakers per treatment group. Larvae were added without their cases in order to ensure direct exposure to the treatment waters and to investigate the effects of OSPW exposure on case building since pilot studies indicated that this was an endpoint to investigate. Approximately equal

sized larvae were placed into each replicate and 30 g of silica sand (particle size 200-400 μm) formed the substrate in each beaker. The mean initial wet mass was 0.633 ± 0.25 mg per individual, based on a representative subsample (3 x 10 animals).

Over the course of the study period, larvae were fed 0.67 mg dry weight TetraFin® fish food (Tetra Company, Blacksburg, VA) per individual per day and 50% of the water volume was replaced every 2 days. Prior to test initiation, oil sands-derived waters were aerated as needed until concentrations of ammonia were less than 1.0 mg/L (as per Liber *et al.,* 1996). Beakers were continually aerated during the test to maintain concentrations of dissolved oxygen (DO) greater than 7 mg/L, with mean water temperature of 23 ± 1.5 ° C. A different subgroup of beakers, including at least one representative beaker from each treatment, was sampled each day to measure oxygen and temperature using an Orion 3-Star RDO Portable Meter and Probe (Thermo Fisher Scientific Inc., Nepean, ON). Samples of water for analyzing conductivity, pH, total hardness, alkalinity, and total ammonia were collected on Days 0, 5, and 10.

The exposure was terminated on Day 10 and survival and wet mass of remaining larvae were reported on a per beaker basis. Wet mass was reported instead of dry mass so that tissues could be preserved for subsequent gene expression analysis. As well as the larvae themselves, larval cases that had been constructed over the course of the study were gently collected from the sand in each beaker and stored in 100% ethanol.

*2.2.3.2 Behavior*

Behavior of larvae was assessed over the course of the 10-day study period by making observations of case occupation behavior three times per day. The measured

endpoint was level of activity, as determined by frequency of observation outside of cases and body position relative to cases. A visual observation of all individual beakers was conducted at 11:00, 13:00, and 15:00 and a scoring system was used to indicate how many larvae were visible on the sand surface and the extent to which individual larvae were outside their cases. A score of 0, 1, 2, or 3 was assigned for each animal in each beaker. A score of 0 was assigned when the larva was completely encased and not visible at all on the sand surface. For a score of 1, less than half of the body length was exposed; a score of 2 was assigned when at least half of the body length was visible; and a score of 3 indicated that the entire larva was exposed and freely moving upon the surface of the sand. A total activity score was calculated for each beaker at each observation time point by multiplying the score value (0, 1, 2, or 3) by the number of larvae assigned that particular score and summing the totals.

*2.2.4 Chronic Exposure*

The effects of chronic exposure to the treatment waters were assessed by randomly assigning 10 approximately equal-sized larvae (8-9 days post-hatch) to each of 4 replicate 1 L, tall form beakers per treatment group. To each beaker, 500 mL of water and 30 g of silica sand were also added. The larger beaker size deviated from standard test protocols, but was used for the chronic exposure study in order to maintain a tolerable degree of animal density since significant growth occurred over the course of the exposure. Larvae were not removed from their cases during placement into test beakers, as opposed to during test initiation of the acute study. Beakers were maintained until individuals pupated and then emerged as adult midges. Emerging adults were

collected and their gender determined on a daily basis. Other endpoints assessed included survival, pupation success, and sex ratio. Test beakers were maintained until all individuals within the replicate had emerged or had died at whatever life stage they were able to achieve (larva, pupa, or adult). Feeding and daily water chemistry monitoring and maintenance and feeding regime followed the procedure described for the acute experiment.

*2.2.5 Data Analysis*

All statistical analyses were conducted using SYSTAT (version 12.0, Systat Software, Inc.). The experimental unit was the test beaker. Normality was assessed by the Shapiro-Wilk test and homogeneity was assessed by Levene's test. Statistical differences were determined by one-way ANOVA followed by Tukey's post-hoc test where either raw or log-transformed data met the assumptions of normality and homogeneity of variance. Data with unequal variances were analysed using Games-Howell post-hoc multiple comparisons. Non-normal behavioral data were assessed by Kruskal-Wallis one-way ANOVA. All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Differences were considered significant at $p < 0.05$.

Correlation and regression analyses to establish relationships between water chemistry parameters and biological endpoints were conducted using SigmaStat (version 3.5, Systat Software Inc.). Pearson correlations were tested between each of the individual endpoints: survival, mass, pupation, and emergence and each of the water chemistry parameters (cations, anions, and elemental concentrations).

## 2.3 Results

### *2.3.1 Acute exposure*

#### *2.3.1.1. Effects on survival and growth*

Survival was not significantly affected by exposure to OSPW from reclamation ponds, but tended to be less in WIP-OSPW (Figure 2.1A). In the freshwater and saltwater control treatments, the survival rates of 85% and 83% were sufficient to meet the 70% requirement for study validity (US EPA, 2000). There was a non-significant trend towards less survival in WIP-OSPW-A ($p= 0.06$) compared to the freshwater controls. There was significantly greater survival in Big Pit and FE5, representing aged OSPW, relative to the fresh WIP-OSPW-A ($p<0.03$).

Change in fresh mass was a more sensitive endpoint than survival as an indicator of exposure to OSPW. While there were no differences in growth between the freshwater control, saltwater control, and FE5 ($p>0.05$), masses of larvae in all other treatments were less than that in the controls (Figure 2.1B). Larvae exposed to water from Big Pit had 19% less mean mass than controls ($p<0.05$) and those exposed to water from TPW had 23% less mass ($p<0.01$). In the two WIP-OSPWs, mean larval mass was significantly less than that of the controls and the reclamation waters. *Chironomus dilutus* exposed to WIP-OSPW-A and WIP-OSPW-B were 64% and 79% smaller, respectively, than the freshwater controls following the 10-day exposure ($p<0.001$).

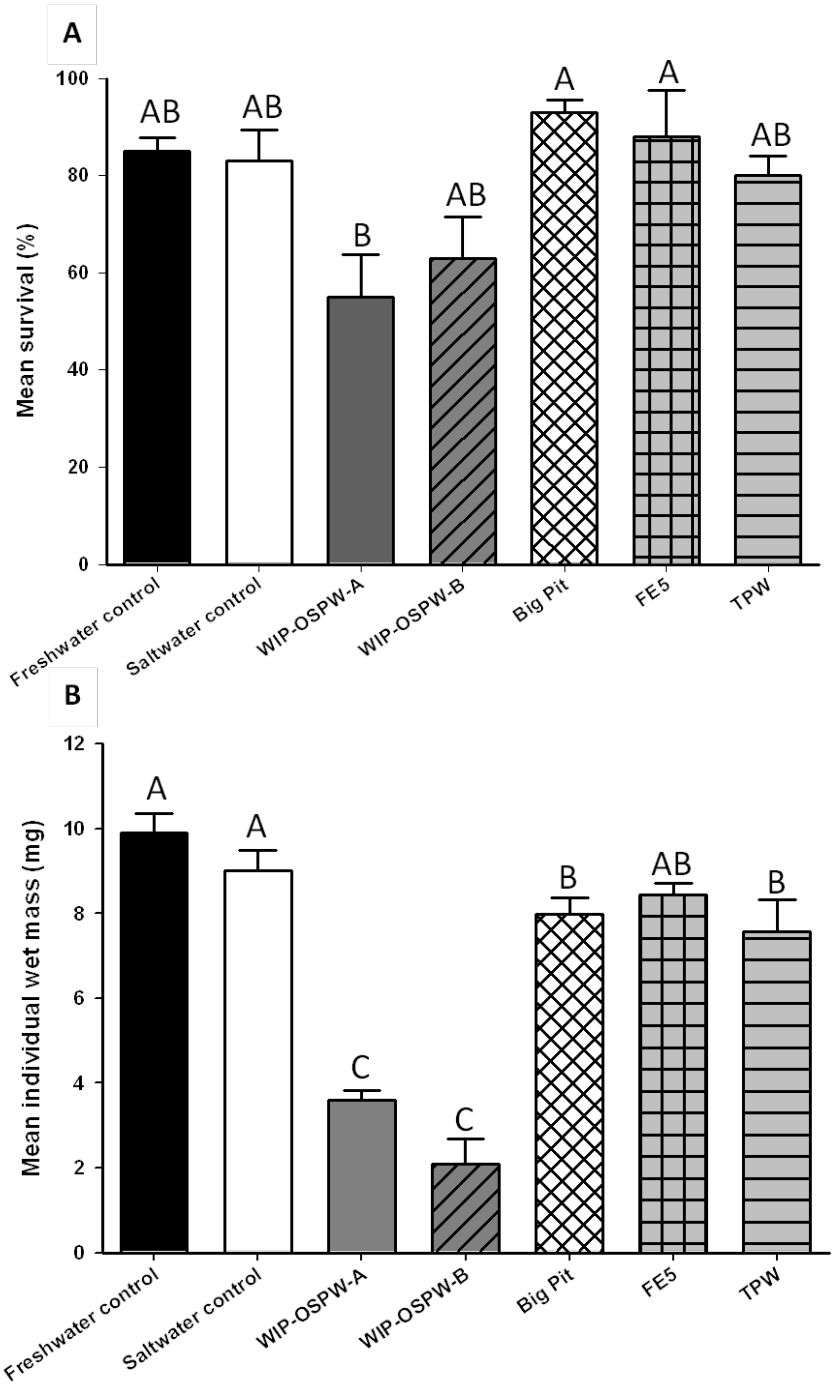


**Figure 2.1:** Mean survival (A) and mean individual wet mass (B) of *Chironomus dilutus* larvae following a 10-day exposure to untreated or reclaimed OSPW. Significant differences among treatments were determined using a one-way ANOVA followed by Tukey's HSD post-hoc test ($n$=4, $\alpha$ =0.05) and designated by different letters.

*2.3.1.2 Effects of OSPW on case building and occupation behavior*

The size, shape, and structural integrity of larval cases varied among treatments. Larvae exposed to freshwater produced cases that were tubular in shape and easily handled without causing damage. Cases produced by larvae exposed to water from the Big Pit and TPW reclamation ponds were slightly smaller than those of the controls, but relatively intact, while the cases produced by larvae exposed to water from the FE5 reclamation pond were even smaller and less solid than those in Big Pit. Cases produced by larvae exposed to WIP-OSPW-A and WIP-OSPW-B were the smallest of any treatment and were poorly constructed and very fragile.

Case occupation activity was assessed throughout the duration of the exposure. Behavioral data were pooled within observation times and assessed by day, due to significant differences between days ($p<0.05$). The trends in activity levels for each treatment group are shown in Figure 2.2. The saltwater controls did not behave significantly differently than the freshwater controls on any day during the exposure period. During the first six days of the exposure, animals exposed to WIP-OSPW (A and B) behaved similarly to those in the control treatments, except on Day 3, where larvae exposed to WIP-OSPW-B were significantly more active than the freshwater controls. On Day 6, behavior in WIP-OSPW-A and WIP-OSPW-B was significantly different from FE5 and Big Pit ($p<0.05$). From Day 7 to Day 9, larvae exposed to WIP-OSPW-A and WIP-OSPW-B were significantly less active outside of their cases than in any other treatment group ($p<0.05$), except they were not different from the freshwater controls on Day 8 and the saltwater controls on Day 9. Larvae exposed to waters from the experimental reclamation ponds were generally more active than larvae in freshwater

controls throughout the entire exposure period, with a greater number of individuals consistently observed fully or partially outside their cases. However, this result was not statistically significant. There was an overall trend towards increased activity as the exposure duration increased and larvae matured into later instars and progressed towards pupation.

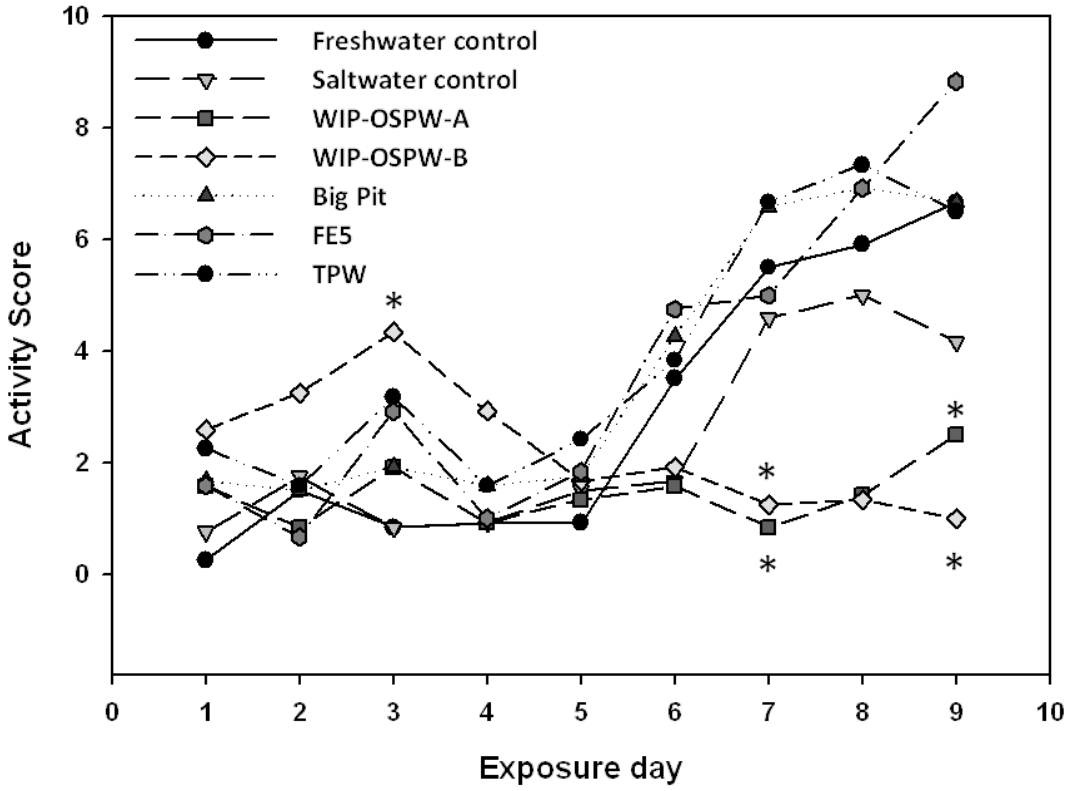


**Figure 2.2:** Trends in daily activity levels of *Chironomus dilutus* larvae over the course of a 10-day exposure to untreated or reclaimed OSPW. Mean scores were determined based on 3 daily observations at 11:00, 13:00, and 15:00. Significant differences from the freshwater control were determined by Kruskal-Wallis one-way ANOVA ($\alpha$ =0.05) and designated with an asterisk. Measures of variance were omitted from the figure for clarity.

*2.3.2 Chronic exposure*

*2.3.2.1 Effects on pupation and emergence*

Pupation and emergence of *C. dilutus* were observed in all treatments (Figure 2.3). Pupation was not significantly different between the freshwater and the activated charcoal-treated waters, with a rate of 96% observed in the freshwater treatments and 90% and 93% observed in the AC-WIP-A and AC-FE5, respectively. Compared to the freshwater controls, significantly less pupation occurred in WIP-OSPW-A and WIP-OSPW-B, where only 65% and 29%, respectively, of the larvae pupated, and in TPW, where 55% of larvae pupated ($p<0.01$). There was no difference in pupation between the remaining treatments and the controls ($p>0.05$).

Emergence was a more sensitive endpoint than pupation during exposure to oil sands-derived waters. Cumulative emergence is presented in Figure 2.4. There was no difference in emergence between *C. dilutus* exposed to the freshwater control and the activated charcoal-treated waters ($p>0.05$). Adults emerged in the freshwater controls at a rate of 81% of the initial larvae. Emergence rates of *C. dilutus* exposed to WIP-OSPW-A and B were significantly less than those in the freshwater controls, with only 13% and 8% of larvae reaching adulthood, respectively ($p<0.001$). Similarly, exposure to TPW significantly affected emergence, with only 23% of larvae reaching adulthood ($p<0.001$). The cumulative emergences in Big Pit and FE5 were not significantly different from that of the controls. However, there was a near-significant trend towards less emergence in FE5 ($p=0.09$).

Delays in emergence were observed in several of the treatments relative to the freshwater controls. Emergence of males and females was significantly delayed in TPW,

with adults emerging, on average, 9.0 days (males) and 8.6 days (females) later than males and females from the freshwater controls. Similarly, exposure to both AC-WIP-A and AC-FE5 resulted in delayed male emergence, by 4.7 and 8.4 days, respectively ($p<0.05$), but there were no differences in time to emergence of females. Emergence of males was also delayed in both WIP-OSPW-A and WIP-OSPW-B, but this was not significant, likely due to the small number of emerging adults. There were no differences in time to emergence between WIP-OSPW-A and WIP-OSPW-B and the freshwater controls, but again, the small number of emerging females in the WIP-OSPW treatments limited the ability to detect differences. There were no significant differences in the time to emergence of males or females exposed to Big Pit or FE5 compared to the freshwater controls ($p>0.05$).

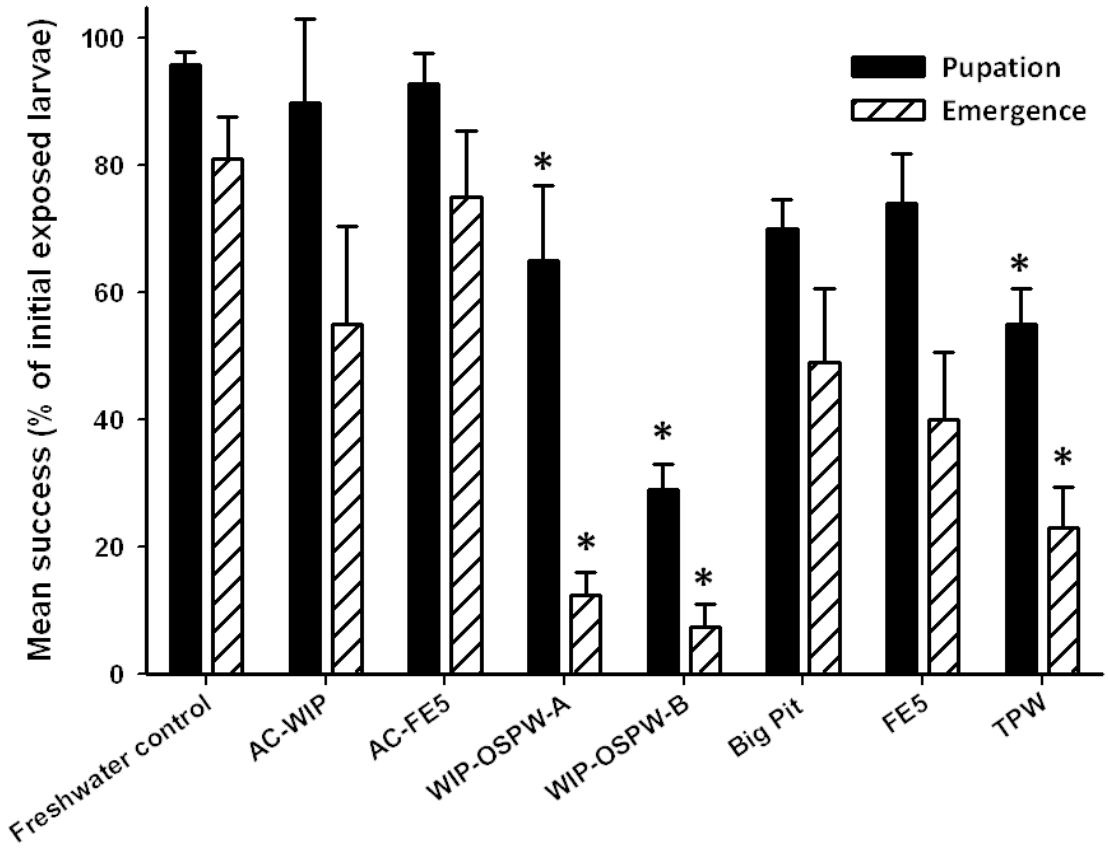


**Figure 2.3:** Mean pupation and adult emergence rates for *Chironomus dilutus* after chronic exposure to untreated or reclaimed OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD post-hoc test ($n$= 4 or 8, $\alpha$ =0.05) and designated with an asterisk.

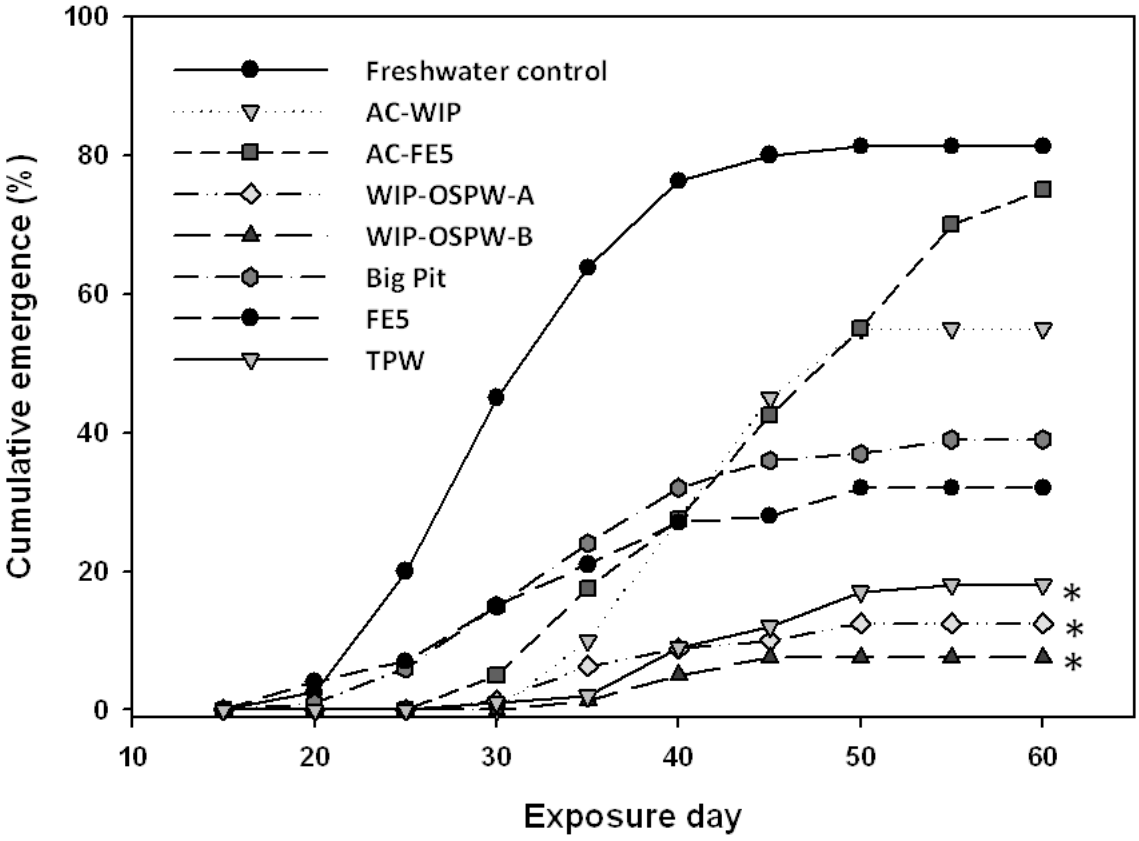


**Figure 2.4:** Cumulative emergence of adult *Chironomus dilutus* following exposure to untreated or reclaimed OSPW. Significant differences from freshwater controls determined by one-way ANOVA followed by Tukey's HSD post-hoc test (n= 4 or 8, α =0.05) and designated with an asterisk. Measures of variance were omitted from the figure for clarity.

*2.3.2.2 Water chemistry*

In general, all OSPWs had greater concentrations of metals, cations, and anions, as well as higher pH, than the municipal freshwater used in the control exposures (Table 2.1). The concentrations of metals were compared to applicable water quality guidelines

for environmental protection and the concentrations of a number of metals in the OSPW samples exceeded those guidelines, including Al, Mo, Se, and V (BC MOE, 2001; CCME, 2011, Ontario MOE, 1994; US EPA, 2002). The greatest numbers of parameters that had values that exceeded guideline levels were in the WIP-OSPW-A and AC-WIP-A, but all treatment waters surpassed guidelines for at least one parameter, with freshwater exceeding the recommended threshold for Cd.

The results of the correlation analysis are shown in Table 2.2. Total concentrations of NAs were significantly correlated with all four toxicological endpoints: survival, mass, pupation, and emergence ($p<0.01$). Concentrations of Ni, U, and Mn were also significantly negatively correlated with the measured endpoints ($p<0.05$) and other elements, including Cd, Sr, Ag, and Co, were negatively correlated to one or more endpoints.

**Table 2.1:** Measured water chemistry parameters for untreated and reclaimed OSPW (mean ± SD). Water quality guidelines are indicated in parentheses, and an asterisk designates cases where guidelines are exceeded.

| | | **Water chemistry parameter** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Anions (mg/L)** | | | | | **Cations (mg/L)** | | | |
| **Treatment water** | **NAs** | **$F^-$** | **$Cl^-$** | **$NO_2^-$** | **$SO_4^{2-}$** | **$NO_3^-$ (13)[a]** | **$Na^+$** | **$K^+$** | **$Mg^{2+}$** | **$Ca^{2+}$** |
| Freshwater Control | 1.2 | 0.17 ± 0.004 | 12.69 ± 0.95 | 1.41 ± 0.0 | 105.4 ± 4.25 | 2.20 ± 1.1 | 34.36 ± 1.19 | 3.57 ± 0.93 | 21.0 ± 1.4 | 15.7 ± 2.0 |
| AC-WIP-A | 10 ± 0.4 | 2.4 ± 0.3 | 572.5 ± 11 | 1.59 ± 1.4 | 616.7 ± 11.8 | 54.8* ± 7.6 | 949.4 ± 17.0 | 59.8 ± 1.5 | 28.9 ± 6.2 | 14.0 ± 2.2 |
| AC-FE5 | 6.6 ± 0.3 | 1.2 ± 0.03 | 161.4 ± 2.6 | 2.62 ± 2.4 | 892.2 ± 14.9 | n/a | 715.0 ± 14.4 | 55.3 ± 1.0 | 57.0 ± 2.6 | 15.7 ± 2.0 |
| WIP-OSPW-A | 72 ± 5 | 3.9 ± 0.2 | 616.3 ± 33 | n/a | 582.8 ± 30.4 | 94.4* ± 2.8 | 1024 ± 55.7 | 20.3 ± 1.1 | 15.0 ± 0.49 | 14.5 ± 1.4 |
| WIP-OSPW-B | 70 ± 0.5 | 3.7 ± 0.2 | 653.4 ± 25 | n/a | 582.8 ± 20.3 | 60.6* ± 1.9 | 1082 ± 40.2 | 19.0 ± 0.63 | 15.3 ± 0.22 | 15.5 ± 3.0 |
| Big Pit | 23 ± 0.3 | 1.7 ± 0.07 | 116.1 ± 0.84 | 1.08 ± 1.8 | 210.9 ± 2.17 | 4.59 ± 1.1 | 447.0 ± 3.41 | 4.62 ± 0.13 | 20.0 ± 0.38 | 11.6 ± 1.4 |
| FE5 | 13 ± 3 | 1.8 ± 0.1 | 147.2 ± 0.64 | 2.00 ± 0.86 | 793.1 ± 4.10 | 3.92 ± 0.0 | 671.6 ± 2.80 | 9.39 ± 0.072 | 44.1 ± 0.22 | 20.7 ± 1.4 |
| TPW | 35 ± 8 | 1.1 1.2 ± 0.2 | 271.3 ± 23 | n/a | 134.7 ± 38.5 | 1.83 ± 2.7 | 645.3 ± 25.2 | 5.94 ± 0.80 | 15.8 ± 6.7 | 7.85 ± 1.1 |

| | Water chemistry parameter | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Elements (µg/L) | | | | | | | | | | | | |
| **Treatment water** | **Al** (50) [b] | **As** (5) [a] | **Cd** (0.027) [a] | **Co** | **Hg** (0.77) [c] | **Mn** | **Mo** (73) [a] | **Ni** (80.67) [a] | **Pb** (2.39) [a] | **Se** (1) [a] | **Sr** | **U** (15)[a] | **V** (6) [d] |
| Freshwater control | 29 ±8 | 1.7 ±0.1 | 0.1* ±0.1 | 0.7 ±0.1 | 0.01 ±0.01 | 0.1 ±0.03 | 2 ±0.2 | 2.8 ±0.5 | 0.01 ±0.01 | 0.9 ±0.1 | 480 ±42 | 2.1 ±0.2 | 0.3 ±0.03 |
| AC-WIP-A | 879* ±140 | 7.2* ±1.4 | 0.3* ±0.1 | 0.5 ±0.2 | 0.8* ±0.05 | 0.2 ±0.1 | 290* ±20 | 1.9 ±1.0 | 0.07 ±0.05 | 6.3 ±1.1* | 1417 ±214 | 11.7 ±3.4 | 118 ±15* |
| AC-FE5 | 94* ±31 | 3.7 ±0.6 | 0.4* ±0.2 | 0.3 ±0.1 | 0.7 ±0.08 | 0.3 ±0.2 | 28 ±3 | 0.7 ±0.2 | 0.05 ±0.02 | 5.5* ±0.4 | 1048 ±52 | 1.6 ±0.2 | 99* ±3 |
| WIP-OSPW-A | 70* ±4 | 7.7* ±1.6 | 0.5* ±0.1 | 2.6 ±0.1 | 0.43 ±0.05 | 0.7 ±0.4 | 274* ±8 | 8.5 ±0.3 | 0.06 ±0.02 | 3.8* ±0.1 | 2710 ±53 | 28.7* ±1.9 | 12* ±0.2 |
| WIP-OSPW-B | 14 ±4 | 5.9* ±0.3 | 1.1* ±0.1 | 1.9 ±0.1 | 0.41 ±0.09 | 1.0 ±0.9 | 336 ±18 | 9.6 ±2.3 | 0.03 ±0.02 | 3.8* ±0.3 | 2715 ±128 | 34.9* ±3.7 | 9* ±0.4 |
| Big Pit | 43 ±8 | 8. 7* ±0.5 | 0.4* ±0.1 | 0.4 ±0.1 | 0.03 ±0.01 | 0.4 ±0.1 | 4 ±0.4 | 2.6 ±0.2 | 0.05 ±0.01 | 1.3* ±0.2 | 627 ±45 | 6.2 ±0.8 | 6 ±1 |
| FE5 | 37 ±4 | 4.9 ±0.2 | 0.3* ±0.1 | 0.4 ±0.1 | 0.05 ±0.02 | 0.2 ±0.04 | 3 ±0.4 | 2.0 ±0.4 | 0.04 ±0.03 | 1.2* ±0.2 | 1132 ±42 | 3.1 ±0.2 | 2 ±0.2 |
| TPW | 61* ±19 | 6.9* ±2.1 | 0.3* ±0.1 | 0.5 ±0.1 | 0.12 ±0.05 | 0.3 ±0.2 | 6 ±0.5 | 2.2 ±0.2 | 0.05 ±0.01 | 1.4* ±0.1 | 283 ±18 | 9.3 ±3.5 | 4 ±0.2 |

[a] CCME Water Quality Guidelines for the Protection of Aquatic Life (Freshwater) (hardness assumed to be 80 mg/L)
[b] British Columbia Ministry of Environment Water Quality Criteria for Aluminum
[c] US EPA National Recommended Water Quality Criteria

[d] Ontario Provincial Water Quality Objectives

**Table 2.2:** Coefficients for Pearson correlation relationships between survival, larval mass, pupation, and emergence of *Chironomus dilutus* larvae and water quality parameters of untreated and reclaimed OSPW. All correlation coefficients presented were significant at $\alpha = 0.05$.

| | **Water chemistry parameter** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Endpoint** | **NAs[a]** | **Ag** | **Ba** | **Cd** | **Co** | **Mn** | **Mo** | **Ni** | **Sb** | **Sr** | **U** |
| Survival[b] | -0.883 | n/s | -0.923 | n/s | -0.948 | -0.820 | -0.757 | -0.885 | -0.733 | -0.870 | -0.901 |
| Mass[c] | -0.974 | -0.712 | -0.910 | -0.877 | -0.867 | -0.980 | -0.707 | -0.951 | n/s | -0.855 | -0.961 |
| Pupation[b] | -0.817 | n/s | n/s | -0.862 | n/s | -0.825 | n/s | -0.734 | n/s | n/s | -0.820 |
| Emergence[b] | -0.907 | n/s | n/s | n/s | -n/s | -0.777 | n/s | -0.747 | n/s | n/s | -0.814 |



n/s = non-significant correlation relationship

[a] Total NAs concentration measured as mg/L, all other elements measured as µg/L
[b] Expressed as a percentage
[c] Measured in mg of wet mass

## 2.4 Discussion

Survival was not an especially sensitive indicator of exposure to OSPW, but exposure to WIP-OSPW did result in lesser survival. Survival was inversely related to concentrations of NAs, so aging, and the associated reduction in concentrations of NAs, appears to attenuate effects of OSPW on survival. Greater survival was also observed in a previous study using the same WIP-OSPW, whereby larvae exposed to ozone treated OSPW with lesser NA concentrations had greater survival than those in untreated OSPW (Anderson *et al.,* 2011a). These results offer further evidence for the role of NAs in toxicity of OSPW.

Growth is used extensively in toxicological assays as a measured endpoint because it represents a sensitive measure that requires integration of multiple physiological and biochemical processes to proceed normally (Sibley *et al.,* 1997). The significantly lesser masses of *C. dilutus* exposed to WIP-OSPW in the current study is consistent with the results of previous studies of the acute toxicity of WIP-OSPW towards *C. dilutus* (Anderson *et al.,* 2011a; Pourrezaei *et al.,* 2011). These results are also consistent with those observed in both field and lab-reared *C. dilutus* exposed to OSPW during larval development (Whelly, 1999). In the current study, larvae exposed to OSPW that had been aged for 18 years in the Big Pit and TPW reclamation ponds were significantly larger than those larvae exposed to WIP-OSPW. This result is consistent with the results of previous studies that have suggested that aging of OSPW attenuates acute toxicity (Holowenko *et al.*, 2002). Larvae exposed to FE5 water were not significantly smaller than the controls. Since concentrations of NAs in FE5 were less than the concentrations in the other reclamation ponds in this study (13.0 mg/L NAs in

FE5 vs. 23.2 and 35.0 mg/L in Big Pit and TPW, respectively), this result offers evidence that NAs are among the main drivers of the toxicity of OSPW. Evidence for NAs being the primary toxicant in OSPW was also observed in a previous study where greater growth was observed following treatment of WIP-OSPW using ozonation to deplete total concentrations of NAs (Anderson *et al.,* 2011a). Exposure to the saltwater control did not significantly affect growth, which suggests that the saline conditions present in OSPW do not inhibit the growth *C. dilutus.* While NAs seemed to be associated with the adverse effects, a role for as yet unidentified organic compounds, concentrations of which might be correlated with NAs, cannot be eliminated (Rowland *et al.,* 2011a). However, it can be concluded that the observed toxicity is likely associated with the acid-extractable organic fraction of OSPW.

Less growth of individual chironomids could have implications for the food web of the oil sands region as these organisms comprise a large proportion of the biomass within reclamation wetlands in the oil sands management area and in nearby water bodies (Bendell-Young *et al.,* 2000). While Chironomidae tends to be one of the dominant taxa of wetlands with OSPW inputs (Bendell-Young *et al.,* 2000), there has been generally low abundance of invertebrates in reclamation wetlands (Baker, 2007; Leonhardt, 2003). Acute sensitivity of *C. dilutus* as a model benthic invertebrate suggests that reclamation ponds constructed with fresh or less-aged OSPW might not support diverse, viable invertebrate communities until a certain degree of biodegradation of NAs has occurred.

Larval cases are important for the survival and development of *C. dilutus.* Cases provide protection from predators and possibly contaminants, are involved in respiratory functions, and provide a food source for developing larvae (Chaloner and Wotton, 1996;

Halpern *et al.,* 2002). Significant increases in tube desertion have been observed in *Chironomus luridus* as a response to chemical stressors, such as large doses of chloramines (Halpern *et al.,* 2002). Stress associated with exposure to OSPW could be responsible for the greater time spent unprotected on the substrate in the reclamation pond waters. Chironomid larvae represent a common prey item within aquatic food webs, but individuals regularly inhabiting cases are less likely to suffer predation than those who rarely or never occupy cases. Further, time spent without the protection of a larval case can serve as a significant predictor of rate of predation (Hershey, 1987). Based on the abnormal patterns of behavior observed over the course of the acute exposure, rates of predation of chironomids in reclamation ponds constructed with fresh or aged OSPW could be shifted and provide challenges for sustaining both the benthic invertebrate community and other aquatic organisms in these wetlands.

The frequency of observation outside of cases varied among the fresh OSPW and reclamation pond waters depending upon the exposure day, which might suggest different stress responses and sensitivities as a result of differences between the constituents of the waters. As the exposure period progressed, freshwater animals were observed more and more frequently outside their cases, with more of their body length visible. Larvae exposed to WIP-OSPW, especially WIP-OSPW-B, tended to be exceptionally active on the surface of the sand during the first several days of exposure, while larvae exposed to the reclamation pond waters were most active and remained outside their cases during the final three or four days of the study. Larvae exposed to Big Pit, FE5, or TPW behaved similarly to one another. The greatest number of days with greater activity outside cases than the freshwater controls was observed in TPW, which also had the greatest

concentration of NAs of the three reclamation ponds. Since the activity of *C. dilutus* exposed to the saltwater control was not significantly different from the freshwater controls, it is unlikely that salinity was responsible for the observed changes in behavior. Results of a previous study demonstrated that depletion of NAs by ozonation of WIP-OSPW resulted in normalization of *C. dilutus* measures of behavior related to occupation of their cases (Anderson *et al.,* 2011a). These results suggest that the acid-extractable organic fraction of OSPW is responsible for the observed changes in activity outside larval cases. In addition, the significant time and extent of case desertion during exposure to each of the reclamation pond waters suggests that OSPW retains its potential to disrupt behavior despite lesser concentrations of NAs, so disruption could be due in part to persistent NA congeners.

Exposure to OSPW significantly affected pupation and emergence of *C. dilutus*. Less cumulative emergence has been observed for *C. riparius* and *C. dilutus* exposed to several toxicants, including thiacloprid (Langer-Jaesrich *et al.,* 2010), 17α-ethinylestradiol (EE2) (Dussault *et al*., 2008), or mercury (Azevedo-Pereira and Soares, 2010). The observed differences in emergence of OSPW-exposed animals could be predicted from the results of the 10-day exposure based on relationships established for growth of *C. dilutus.* In previous studies, 90% growth inhibition in chironomid larvae led to greater fatalities while reductions in growth of 64% or greater were associated with delays in and less emergence, and changes to male:female ratios (Liber *et al.,* 1996). In addition, a minimum larval dry mass of 0.5-0.6 mg was required for successful emergence (Sibley *et al.,* 1997). Based upon a relationship between fresh and dry mass in third instar *C. dilutus* larvae established in pilot studies in our lab, this translates to a minimum wet mass of 5.5 mg in order to achieve successful emergence. This mass was

not achieved in either WIP-OSPW-A or WIP-OSPW-B, which had mean fresh masses of 3.6 mg and 2.1 mg, respectively (Anderson *et al.,* 2011a*).* The greater deficiency in fresh mass in larvae exposed to WIP-OSPW-B may be the reason for the greater impairment of both pupation and emergence, while larvae exposed to WIP-OSPW-A had sufficient metabolic capacity to pupate but not to emerge. Although WIP-OSPW-A and WIP-OSPW-B were sampled from the same settling pond, the profiles of NAs had subtle differences in the relative proportion of different structural groups (as described in Anderson *et al.* (2011a)). These differences might account for the differences in toxicity towards *C. dilutus* between the two batches of WIP-OSPW. While the mechanistic basis of the differences in emergence between WIP-OSPW and freshwater controls is not known, the lack of effects on emergence observed for animals exposed to the FE5 and Big Pit water, which have lesser concentrations of NAs, suggests that NAs, or other unknown organic compounds detected by FTIR, could be responsible for lesser emergence rates. Similarly, there is a trend within the reclamation waters whereby *C. dilutus* exposed to the reclamation pond with the greatest concentrations of NAs (TPW) pupated and emerged least, in addition to least growth. While there were slight decreases in pupation and emergence success in the activated charcoal-treated waters relative to the freshwater control, these were not significant and there were residual acid-extractable organic compounds remaining in AC-FE5 and AC-WIP. Therefore, based upon the results of the present study, it is unlikely that the observed effects in the oil sands-derived waters can be attributed to the inorganic fraction.

Exposure to aged oil sands water has been shown to cause developmental and reproductive effects in exposed organisms. Total cessation of spawning, with significantly smaller ovaries in exposed females and an overall reduction in fecundity,

has been reported in fathead minnows (*Pimephales promelas*) exposed to TPW (Kavanagh *et al.,* 2011). Concentrations of sex steroids were generally less in minnows exposed to OSPW and the greatest degree of reproductive impairment was observed in reclamation waters with the greatest concentrations of NAs. There was no impairment in FE5, which had greater conductivity than TPW, and the authors concluded that osmoregulatory stress was not the main driver for declines in reproductive output in TPW (Kavanagh *et al.,* 2011). Similarly, untreated WIP-OSPW disrupted sex steroid production *in vitro* and had both estrogenic and anti-androgenic properties *in vitro* (He *et al*., 2010; He *et al.,* 2011). It has been shown that structures of some NAs resemble estrogenic compounds and these may be responsible for the estrogenic activity of OSPW (Rowland *et al.,* 2011c). In the present study, there were delays in male emergence observed in both AC-WIP-A and AC-FE5, but it is not clear whether this was a result of residual NAs or other water chemistry or environmental parameters. Further study is required to determine whether the basis for impaired pupation and emergence in chironomids is primarily within metabolic or endocrine pathways.

Relationships between and among water chemistry parameters suggest that NAs and several metals are major drivers of toxicity of OSPW. The correlations were not intended to be predictive of toxic outcomes, but they can provide insight into the drivers of toxicity for oil sands water. Toxicity of OSPW NAs has been suggested in a number of organisms, including fathead minnows, yellow perch (*Perca flavescens*), *C. dilutus* and phytoplankton (Anderson *et al.,* 2011a; Colavecchia *et al.*, 2004; Leung *et al.,* 2003; Nero *et al*., 2006; Pourrezaei *et al.,* 2011) and NAs correlated strongly with lesser mass, survival, pupation, and emergence of *C. dilutus* in this study. The correlation analyses

used the total concentration of NAs, but as toxicity varies among profiles of NAs and structure (Holowenko *et al.,* 2002), sub-dividing the profile based upon ring series and carbon number might further refine the relationship. In addition to NAs, the models suggest that some portion of the toxicity observed could be associated with elements such as Ni, Mn, and U. The presence of a suite of metals could have contributed to the observed acute and chronic toxicity of OSPW in *C. dilutus.* However, the correlation was based on preliminary metals data and the concentrations do not necessarily reflect the bioavailability of those metals, so the relationship would benefit from further investigation. Exposure to metals can result in fewer emergences of adults and significantly delay in time-to-emergence (Wentsel *et al.,* 1978). Elevated uranium concentrations have been observed to cause less survival, greater time between moult phases, and less overall growth of *C. dilutus* (Dias *et al.,* 2008).

The only instance where a water guideline was surpassed in WIP-OSPW-A and not in AC-WIP-A was in the case of uranium, where treatment with activated charcoal reduced the concentration from 28.7 mg/L to 11.7 mg/L. Similarly, for FE5 and AC-FE5, there were no metals where FE5 exceeded the guideline concentrations and AC-FE5 did not. Clearly, the activated charcoal treatment had only minor effects on concentrations of the various water chemistry parameters measured. Additionally, ozonation has been observed to attenuate the toxicity of OSPW (Anderson *et al.,* 2011a; Scott *et al.,* 2008), and this treatment does not significantly change the concentrations of any of the metals of interest. These results support the hypothesis that the toxicity of OSPW can be attributed to organic compounds, including, but not limited to NAs (Anderson *et al*., 2011a).

In summary, both short-term and long-term exposure to untreated OSPW resulted in toxicity towards the benthic invertebrate, *C. dilutus*. Toxicity manifested predominately as lesser fresh masses in larvae, which then resulted in significantly impaired emergence. While survival and growth were unaffected by exposure to reclamation pond OSPW, less cumulative pupation and emergence was observed in all three ponds, especially TPW. A sufficient impairment of adult emergence and reproductive output, paired with increased susceptibility to predation due to behavioral changes, could have population level implications for chironomids in the oil sands region in a large-scale release scenario. Based on these results, in addition to the lengthy timelines involved in natural aging, use of biodegradation in reclamation ponds as a treatment approach for OSPW is not sufficient and does not completely eliminate toxicity of OSPW. Consequently, more aggressive methods of OSPW reclamation are required.

# CHAPTER 3:
# Expression of hemoglobins, endocrine receptors, and ribosomal protein L15 in *Chironomus dilutus* larvae following short-term exposure to oil sands process-affected water

## 3.1 Introduction

Global energy demands are expected to increase by 50% over the next two decades, mostly within non-OECD countries (National Energy Board of Canada, 2010). While the majority of current energy needs are met by fossil fuels from conventional production, emphasis is shifting towards alternative oil sources, such as oil sands (Government of Alberta, 2008). One of the largest reserves of petroleum hydrocarbons in the world is found in Canada within three primary bitumen deposit areas in the Athabasca region of Alberta (Government of Alberta, 2006; Veil *et al.,* 2009). Bitumen is extracted from surface-mined oil sands by use of the "Clark hot water extraction process". This results in saline, alkaline process water known as oil sands process-affected water (OSPW) (Hao *et al.,* 2005; Rogers *et al.,* 2002). Additionally, OSPW contains a complex mixture of organic compounds, including naphthenic acids (NAs), which have been implicated as the major drivers of acute and chronic toxicity of OSPW (Holowenko *et al.,* 2002; Rowland *et al.,* 2011a). Since companies are currently held to a policy of zero-discharge to surrounding freshwater environments, OSPW is held in active settling basins where fine silts and clays slowly densify to form mature fine tailings. Over time, concentrations of NAs are depleted to some extent by biodegradation, resulting in lesser acute toxicity of aged OSPW (Del Rio *et al.,* 2006; Han *et al.,* 2009; Holowenko *et al.,* 2002). The volumes of OSPW held in on-site settling basins continue to increase and represent a considerable management responsibility for the oil sands industry and expense for future reclamation (Del Rio *et al.,* 2006; Veil *et al.,* 2009).

Midges of the genus *Chironomus* (Insecta) comprise a large proportion of the biomass found within aquatic ecosystems of the Athabasca oil sands region (Bendell-Young *et al.,* 2000) and this, in addition to the ease with which they can be reared in the

laboratory, makes them desirable test organisms (Armitage *et al.,* 1995). Larvae of *Chironomus dilutus* are sensitive to a variety of toxicants and stressors, including metals, pesticides, and food limitation (Muscatello and Liber, 2009; Soin and Smagghe, 2006; Taenzler *et al.,* 2007). A number of studies have investigated effects of contaminant exposure on molecular responses of species within the genus *Chironomus*, including transcription of acetylcholinesterase (*AChE*), glutathione S-transferase (*GST*), heat shock proteins (*hsps*), and Balbiani ring proteins (Crane *et al.,* 2002; Ha and Choi, 2008; Nair *et al.,* 2011). In addition, hemoglobin genes (*Hbs)* have been presented as potential biomarkers of exposure to environmental contaminants for *Chironomus* spp. The presence of hemoglobin is relatively unique to these organisms since very few invertebrates possess *Hb* (Ha and Choi, 2008). As in vertebrates, *Hb* plays an important role in oxygen storage, transport, and diffusion. Additionally, in invertebrates, *Hb* facilitates a number of other biological functions, including maintenance of acid-base balance, detoxification reactions, and regulation of buoyancy (Weber and Vinogradov, 2001). Hemoglobin exists in both inducible and constitutively expressed isoforms, each with a different responsiveness to stressors. It has been suggested that this differential responsiveness allows *Chironomus* spp. to tolerate and thrive under a wide range of environmental conditions and to tolerate pollution (Ha and Choi, 2008; Lee *et al.,* 2006).

The endocrine system plays a critical role in *Chironomus* spp. by regulating developmental and reproductive processes, including metamorphosis, chitin synthesis, and molting (Nair *et al.,* 2011; Taenzler *et al.,* 2007). The estrogen-related receptor (*ERR)* is an important regulator of the development of *Drosophila* because it coordinates carbohydrate metabolism and cell proliferation during mid-embryogenesis (Tennessen *et*

*al.,* 2011). Ultraspiracle protein (*USP)* and ecdysteroid receptor (*ESR)* are ligand-dependent transcription factors that are members of the nuclear receptor superfamily (Henrich *et al.,* 2000). Together, *USP* and *ESR* form a heterodimer that mediates the transcriptional responses to ecdysteroids throughout different stages of development (Henrich *et al.,* 2000; Henrich *et al.,* 2009). In *Drosophila, USP* has both an activating and repressive function, so it plays a role in fine-tuning gene transcription and itself requires feedback mechanisms for regulation (Henrich *et al.,* 2000).

Exposure to environmental toxicants might alter gene expression, which could be linked to toxicological effects at the level of the whole organism, depending upon the duration and severity of exposure (Lee *et al.,* 2006). Changes in expression of genes can be difficult to correlate to changes in individual fitness due to the very sensitive and often transient nature of changes in gene expression (Au, 2004; Lagadic *et al.,* 1994). Exposure to OSPW impairs growth and development of *C. dilutus* (Anderson *et al.,* 2011a; Anderson *et al.,* 2011b). However, the mechanistic basis of these effects is unknown. Because these processes require energy and rely upon a coordinated system of endocrine responses, the goal of the current study was to investigate the effects of exposure to OSPW on the abundances of transcripts of genes involved in energy metabolism, endocrine pathways and protein synthesis. To this end, abundances of transcripts of hemoglobins (*HbB, HbD, HbE)*, endocrine-related receptors (*ERR, ESR, USP*), and ribosomal protein L15 (*RPL15)* were quantified in *C. dilutus* exposed to OSPW.

### 3.2 Materials and Methods

*3.2.1 Test organisms*

To obtain larvae for the exposure study, adult *C. dilutus* midges from an in-house laboratory culture (Toxicology Centre, University of Saskatchewan) were collected and bred. Resulting egg masses were placed into 15 L aquaria maintained at 23 ± 1 °C and raised to test age in an environmental chamber with a 16:8 hour light:dark photoperiod. During rearing, larvae were kept in control freshwater, which was Saskatoon, SK, municipal water that was aerated for 24 hours, bio-filtered, and carbon filtered prior to placement into aquaria or test vessels. The control freshwater had the following characteristics (mean ± SD): conductivity 422 ± 41 µS/cm, pH 7.88 ± 0.24, alkalinity 129 ± 7 mg/L as $CaCO_3$, and hardness 153 ± 15 mg/L as $CaCO_3$.

*3.2.2 Exposure waters*

*Chironomus dilutus* larvae were exposed to one of five treatment waters: 1) freshwater control, 2) untreated OSPW (designated 'WIP-OSPW'), 3) activated charcoal-treated WIP-OSPW (designated 'AC-WIP'), 4) FE5 water, or 5) activated charcoal-treated FE5 water (designated 'AC-FE5'). The freshwater for the control exposure was the same as that used for culturing *C. dilutus*. WIP-OSPW was collected in summer 2009 from West In-Pit (WIP), an active settling basin located on the Syncrude Canada Ltd. lease site (near Fort McMurray, AB). WIP-OSPW is regularly fed from the main extraction plant and represents relatively fresh, untreated process water (as described by Han *et al.,* 2009). FE5 is an experimental reclamation pond comprised of mature fine tailings capped with OSPW in 1989 and represents OSPW that has been subjected to some degree of natural biodegradation (Han *et al.,* 2009). The WIP-OSPW and FE5

water were stored in the dark from the time after collection until they were used in the exposure studies.

To create AC-WIP and AC-FE5, WIP-OSPW and FE5 water were each mixed with 5% (w/v) 8-20 mesh particle-size activated charcoal (Sigma-Aldrich, St. Louis, MO) and stirred gently for 4 hr at room temperature. Following the contact period, the largest charcoal particles were removed by sieving and the water was vacuum-filtered through a 0.22 μm Millipore Express Plus® filter (Millipore Corporation, Billerica, MA) to remove all remaining charcoal particles. This treatment removed much of the organic fraction of the parent waters in order to evaluate the relative contributions of the inorganic fraction (including salinity) and the organic fraction to the toxicity of OSPW. Fourier transform infrared spectroscopy (FTIR) was used to quantify the total concentrations of NAs and confirm removal of organic compounds. In WIP-OSPW, the total concentration of NAs was initially 71.7 mg/L and was reduced to 10 mg/L following treatment with activated charcoal. The initial concentration of NAs in FE5-OSPW was 13.0 mg/L and was reduced to 0.92 mg/L following activated charcoal treatment.

*3.2.3 Experimental design*

The study was conducted under the environmental conditions described in Section 3.2.1. A modified 7-day static-renewal assay was used to assess effects of acute exposure to the treatment waters on gene expression. Ten 9-10 day post-hatch larvae were randomly assigned to each of six replicate 300 mL, tall-form beakers per treatment group per time-point (1, 4, and 7 d.). Approximately equal-sized larvae were randomly placed into each replicate beaker and the substratum in each beaker was comprised of 30 g of

silica sand (particle size 200-400 μm). The initial mean wet mass was 1.17 ± 0.29 mg per individual, based on a representative subsample of 30 larvae taken on Day 0. Test larvae were sampled after 1, 4 and 7 days of exposure, frozen using liquid nitrogen, and stored at -80 °C until RNA extraction. Other measured endpoints included survival and mean wet mass (as an indicator of growth), which were reported on a per beaker basis. Over the course of the study period, larvae were fed 0.67 mg dry weight TetraFin® fish food (Tetra Company, Blacksburg, VA) per individual per day and 50% of the water volume was changed every 2 days.

Prior to initiation of a test, oil sands-derived waters were aerated as needed until concentrations of ammonia were less than 1.0 mg/L. Beakers were continually aerated during the test to maintain concentrations of dissolved oxygen (DO) near 7 mg/L. Different subgroups of beakers, including at least one representative beaker from each treatment, were sampled each day to measure oxygen and temperature using an Orion 3-Star RDO Portable Meter and Probe (Thermo Fisher Scientific Inc., Nepean, ON). Water samples for analysis of conductivity, pH, total hardness, alkalinity, and total ammonia were collected on Days 0, 1, 4, and 7.

### *3.2.4 Real-time PCR*

Quantitative real-time polymerase chain reaction (RT-PCR) was used to assess differences in the abundances of transcripts of target genes among treatment groups and sampling times. The sequences of the primers used for the quantification of hemoglobin

B, D, and E (*Hb B, D, E*), estrogen-related receptor (*ERR*), ultraspiracle protein (*USP)*, ribosomal protein L15 (*RPL15)*, and ecdysteroid receptor (*ESR)* are given (Table 3.1). Abundances of transcripts of target genes were normalized to the abundance of transcripts of β-actin according to the method of Simon (2003). The abundances of transcripts of genes of interest were then normalized to abundances of transcripts in freshwater controls on Day 1.

Larvae were sampled from each treatment group at each time-point (1, 4, and 7 days) and total RNA was isolated from pooled larvae collected from each beaker (approximately 10 animals per sample) using the TRIzol reagent (Invitrogen, Burlington, ON, Canada) following the manufacturer's instructions. Concentrations of total RNA were determined at A260 by use of a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The first strand cDNA was synthesized from 1 µg of total RNA using an iScript cDNA Synthesis kit (BioRad, Mississauga, ON, Canada) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the ABI 7300 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Separate 45 µl PCR reaction mixtures consisting of 2x Power SYBR® Green master mix (Applied Biosystems), cDNA, gene-specific primers, and nuclease free water (Qiagen, Mississauga, ON, Canada) were prepared for each cDNA sample and primer pair. A final reaction volume of 20 µl was transferred to each well and reactions performed in duplicate. The thermal cycle profile employed was as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing with extension for 1 min at 60 °C; and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for

15 s. Melting curve analyses were performed during the 60 °C stage of the final cycle to differentiate between desired PCR products and primer–dimers or DNA contaminants.

*3.2.5 Data analysis*

All statistical analyses were conducted using SYSTAT (version 12.0, Systat Software, Inc.). The experimental unit was the test beaker. The normality of each data set was assessed using the Kolomogrov–Smirnov one-sample test and homogeneity of variance was determined using the Levene's test. Significant differences were evaluated by one-way ANOVA with Tukey's post-hoc test where either raw or log-transformed data met the assumptions of normality and homogeneity of variance. Data with unequal variances were assessed by Games-Howell post-hoc test. Abundances of transcripts were assessed by Kruskal-Wallis one-way ANOVA followed by the Wilcoxon Signed Ranks test to determine differences from the freshwater control within each day and differences from Day 1. All data are presented as mean ± standard deviation (SD) unless otherwise stated. Differences were considered significant at $p < 0.05$.

**Table 3.1:** Primer sequences used for RT-PCR to assess gene expression in *Chironomus dilutus* larvae exposed to aged, activated charcoal-treated, or untreated OSPW.

| Target | Accession # | Sequence (5' – 3') | Annealing Temp |
|---|---|---|---|

| | | | |
|---|---|---|---|
| *β-actin* | AB070370 | F: TGATGAAGATCCTCACCGAACG<br>R: GGTTCATTACCGATTTGATG | 60 |
| *HbB* | X56272 | F: TTGAGCTTTTGAGATTCCAC<br>R: TTCGCTGGAAAGGATGTTG | 60 |
| *HbD* | X56272 | F: TTGAGATTCCACGGTTGTGA<br>R: AAGTTGACATCCTTGCTGCC | 60 |
| *HbE* | X56272 | F: TATGAGACGAGTGAGGCACG<br>R: ATTCGCTGGAAAGGATGTTG | 60 |
| *ERR* | GU070740 | F: TACCGACCTCCCACTTAACG<br>R: CAGCCAGAAATCTGATGCAA | 60 |
| *ESR* | S60739 | F: GGCCAATCAAGTAGCCGTTA<br>R: TTCTTCCTCCAGTTCGGTTG | 60 |
| *USP* | AF045891 | F: TCACCCGTACCAGTTCTTGG<br>R: ATGATGCGTTTGAGGAGAC | 60 |
| *RPL15* | HM622068 | F: TGGGTCGCTCAAGATGCTGCC<br>R: ACGACGCCATGCAGCACGA | 60 |

## 3.3 Results

### *3.3.1 Exposure conditions*

Concentrations of dissolved oxygen were maintained, on average, at greater than 5 mg/L for all treatments and at greater than 7 mg/L in AC-WIP, FE5-OSPW, and WIP-OSPW beakers, with a mean water temperature of 22.9 ± 0.2 °C. A summary of the measured water chemistry parameters is presented in Table 3.2. The mean pH values ranged from 7.88 to 9.32, where measured pH was generally least in the freshwater controls and greatest in the activated charcoal-treated waters. Similarly, conductivity and alkalinity were much greater in oil sands-derived waters than in the freshwater. The least hard water was WIP-OSPW and the hardest water was AC-FE5, with mean measured values in the study ranging from 67 to 225 mg/L as $CaCO_3$.

*3.3.2 Survival and growth*

Survival was unaffected by any of the exposures and did not differ significantly among treatment groups, with nearly 100% survival overall. However, differences in wet mass of *C. dilutus* larvae exposed to different treatments were observed (Figure 3.1). There were no significant differences in mean wet mass among treatments on Day 1. On Day 4, the larvae exposed to either AC-WIP or WIP-OSPW had significantly less fresh mass than the freshwater controls ($p<0.05$) (Figure 3.1). Additionally, larvae exposed to WIP-OSPW had significantly less mass than those exposed to FE5 ($p<0.05$). On Day 7, the mean wet mass of larvae exposed to WIP-OSPW, AC-WIP and AC-FE5 was significantly less than the mean wet mass of larvae exposed to freshwater ($p<0.01$). Larvae exposed to OSPW-WIP for 7 days had significantly less wet mass than those exposed to any other treatment water ($p<0.05$). The mean wet mass of larvae exposed to

AC-FE5 was significantly less than the mean wet mass of larvae exposed to FE5 ($p<0.05$).

**Table 3.2:** Measured water chemistry parameters for untreated and reclaimed OSPW over the duration of the exposure period. Values are presented as mean ± SD (n=8-12, except NAs, where n=2).

| | Water chemistry parameter | | | | | |
|---|---|---|---|---|---|---|
| **Treatment water** | **NAs (mg/L)** | **pH** | **Conductivity (µS/cm)** | **DO (mg/L)** | **Hardness (mg/L as $CaCO_3$)** | **Alkalinity (mg/L as $CaCO_3$)** |
| Freshwater control | 1.2 [a] | 7.88 ± 0.24 | 422 ± 41 | 6.7 ± 1.7 | 154 ± 15 | 129 ± 7 |
| AC-FE5 | 6.6 ± 0.3 | 9.30 ± 0.31 | 2958 ± 138 | 5.9 ± 1.8 | 225 ± 5 | 525 ± 12 |
| AC-WIP | 10.0 ± 0.4 | 9.32 ± 0.32 | 3830 ± 201 | 7.4 ± 1.1 | 107 ± 5 | 541 ± 15 |
| FE5 | 13.0 ± 3.0 | 8.93 ± 0.07 | 2790 ± 111 | 7.6 ± 1.0 | 182 ± 5 | 454 ± 37 |
| WIP-OSPW | 72.0 ± 0.5 | 8.85 ± 0.25 | 3702 ± 232 | 7.2 ± 1.4 | 67 ± 6 | 467 ± 13 |

[a] No SD value available due to analytical error
NAs = Total concentration of naphthenic acids (as measured by FTIR)
DO = Dissolved oxygen

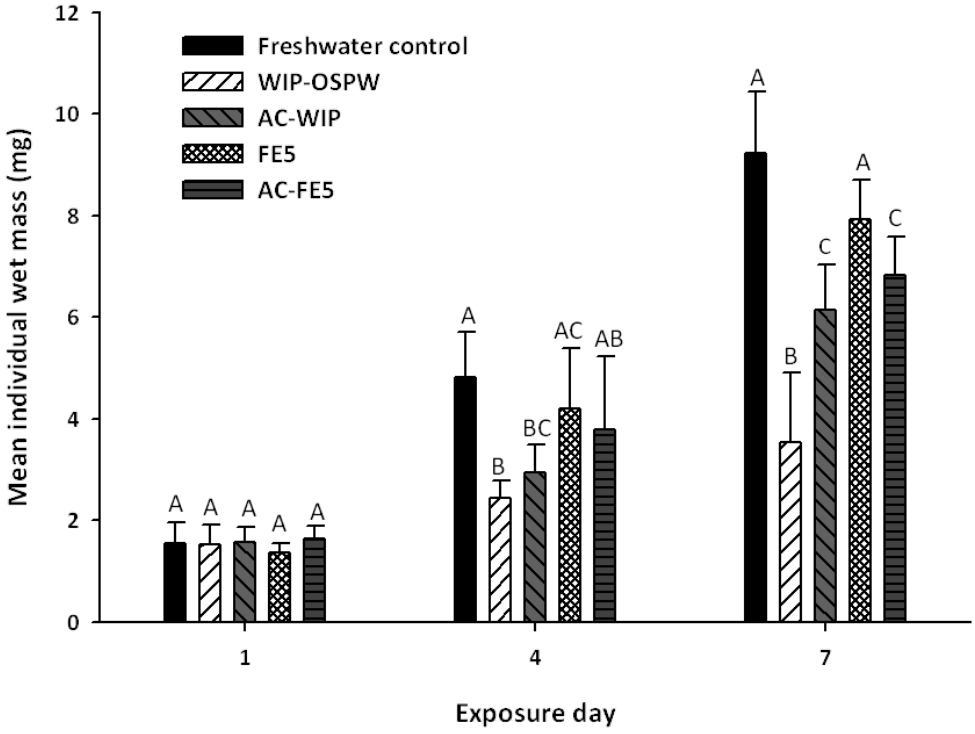


**Figure 3.1:** Mean (± SD) wet mass of *Chironomus dilutus* larvae following exposure to activated charcoal-treated, aged, or untreated OSPW for 1, 4, or 7 days. Significant differences were determined by one-way ANOVA followed by Tukey's HSD post-hoc test (n=4, $\alpha$ =0.05) and are designated by different letters.

*3.3.3 Transcript abundances of target genes*

Abundances of transcripts of target genes varied among treatments and changed as a function of duration of exposure. Abundances of transcripts of the different hemoglobin genes were generally fluctuating for the different sampling times (Figure 3.2). On Day 1, abundances of transcripts of *HbB, HbD and HbE* were significantly less in *C. dilutus* exposed to FE5 compared to the freshwater control and AC-FE5 ($p<0.05$). On Day 4, abundances of transcripts of *HbB* were significantly greater in FE5 than in WIP-OSPW ($p<0.05$) (Figure 3.2A). Compared to abundances of transcripts in the different treatment groups on Day 1, there was significantly less expression of *HbB* in AC-FE5, AC-WIP and the freshwater control treatment on Day 4 and in all treatment groups except FE5 on Day 7 ($p<0.05$). Abundances of transcripts of *HbD* were significantly greater in the freshwater control and WIP-OSPW compared to abundances of transcripts in FE5 on Day 7 ($p<0.05$) (Figure 3.2B). Over the duration of the exposure, expression of *HbD* was least on Day 4 in all treatments besides FE5. Abundances of transcripts of *HbE* were not significantly different among treatments on Day 4. However, on Day 7 the abundance of transcripts of *HbE* was significantly greater in WIP-OSPW and AC-FE5 than FE5 ($p<0.05$) (Figure 3.2C). Overall, with the exception of the abundance of transcripts of *HbE* in FE5, the abundance of transcripts of *HbE* was significantly greater on Day 1 than Day 4 or Day 7 in all treatments.

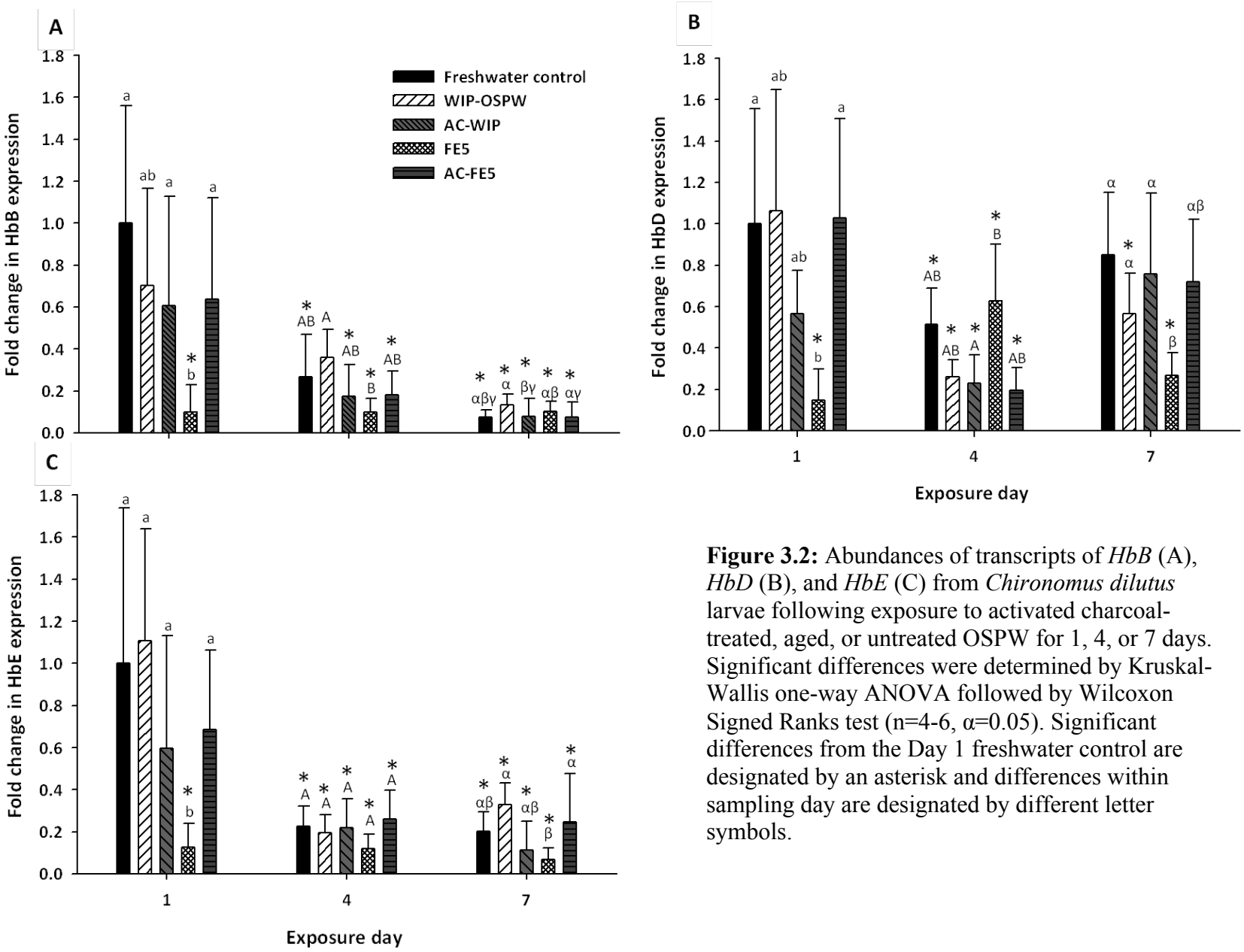


**Figure 3.2:** Abundances of transcripts of *HbB* (A), *HbD* (B), and *HbE* (C) from *Chironomus dilutus* larvae following exposure to activated charcoal-treated, aged, or untreated OSPW for 1, 4, or 7 days. Significant differences were determined by Kruskal-Wallis one-way ANOVA followed by Wilcoxon Signed Ranks test (n=4-6, α=0.05). Significant differences from the Day 1 freshwater control are designated by an asterisk and differences within sampling day are designated by different letter symbols.

The abundance of transcripts of *ESR* was subtly affected by exposure to the various treatments (Figure 3.3A). Neither WIP-OSPW nor FE5 affected the abundance of transcripts of *ESR* in exposed larvae. Similarly, abundances of transcripts of *ESR* were not affected by removal of organic compounds from WIP-OSPW or FE5, by use of activated charcoal. Abundance of transcripts of *ESR* was significantly less in FE5 than AC-WIP on Day 1 ($p<0.05$), but was not significantly different from any other treatment. On Day 4 and Day 7, there were no significant differences in abundances of *ESR* transcripts among any of the treatments ($p>0.05$). Overall expression of *ESR* was significantly less on Day 4 than Day 1 or Day 7 for all treatment groups.

The abundance of transcripts of *USP* varied as a function of time and with treatments (Figure 3.3B). The abundance of transcripts of *USP* in larvae exposed to AC-FE5 was significantly greater on Day 1 relative to the abundance in larvae exposed to FE5 or AC-WIP ($p<0.05$). On Day 4, the abundance of transcripts of *USP* in larvae exposed to WIP-OSPW was significantly less than the abundance in larvae from the freshwater control ($p<0.05$), but not from those in the other treatment groups. Larvae exposed to WIP-OSPW had significantly greater abundance of transcripts of *USP* than those exposed to FE5 or AC-WIP on Day 7 ($p<0.05$). Abundances of transcript of *USP* were significantly less on Day 4 than Day 1 for all treatments, and on Day 7 in freshwater, AC-WIP, AC-FE5, and FE5-exposed larvae.

Abundances of transcripts of the estrogen-related receptor gene (*ERR)* were transient over the course of the exposure and were affected by exposure to OSPW (Figure 3.3C). On Day 1, there were no significant differences in abundances of transcripts of *ERR* between the freshwater control and any of the other treatment groups ($p>0.05$).

However, the abundance of transcripts of *ERR* was significantly greater in larvae exposed to AC-FE5 than in those exposed to FE5 ($p<0.05$). On Day 4 of the exposure period, the larvae exposed to WIP-OSPW or AC-FE5 expressed significantly less abundance of transcripts of *ERR* than did the freshwater control larvae ($p<0.05$). However, the abundance of transcripts in WIP-OSPW or AC-FE5 was not significantly different from those in AC-WIP or FE5, respectively. There were no significant differences in abundances of transcripts of *ERR* among treatments on Day 7. Abundances of transcripts of *ERR* in all treatments were significantly less on Day 4 than on Day 1 or Day 7.

The abundance of transcripts of *RPL15* was transient throughout the exposure period and was affected by exposure to different treatment waters (Figure 3.4). There was significantly greater abundance of transcripts of *RPL15* in larvae exposed to AC-FE5 or AC-WIP than in those exposed to FE5 on Day 1 ($p<0.05$). On Day 4, abundance of transcripts of *RPL15* was greater in larvae exposed to AC-FE5 than those exposed to AC-WIP or WIP-OSPW ($p<0.05$). On Day 7, abundance of transcripts of *RPL15* was greater in larvae exposed to WIP-OSPW than in those exposed to FE5 ($p<0.05$). On Day 4 of the exposure the abundance of transcripts of *RPL15* in larvae exposed to WIP-OSPW were significantly less than the abundance on day 1. However, on Day 7, the abundance of transcripts of *RPL15* in WIP-OSPW was significantly greater than they were on Day 1 and Day 4 of the exposure. There were no other differences in expression of *RPL15* within treatments over the different sampling time-points.

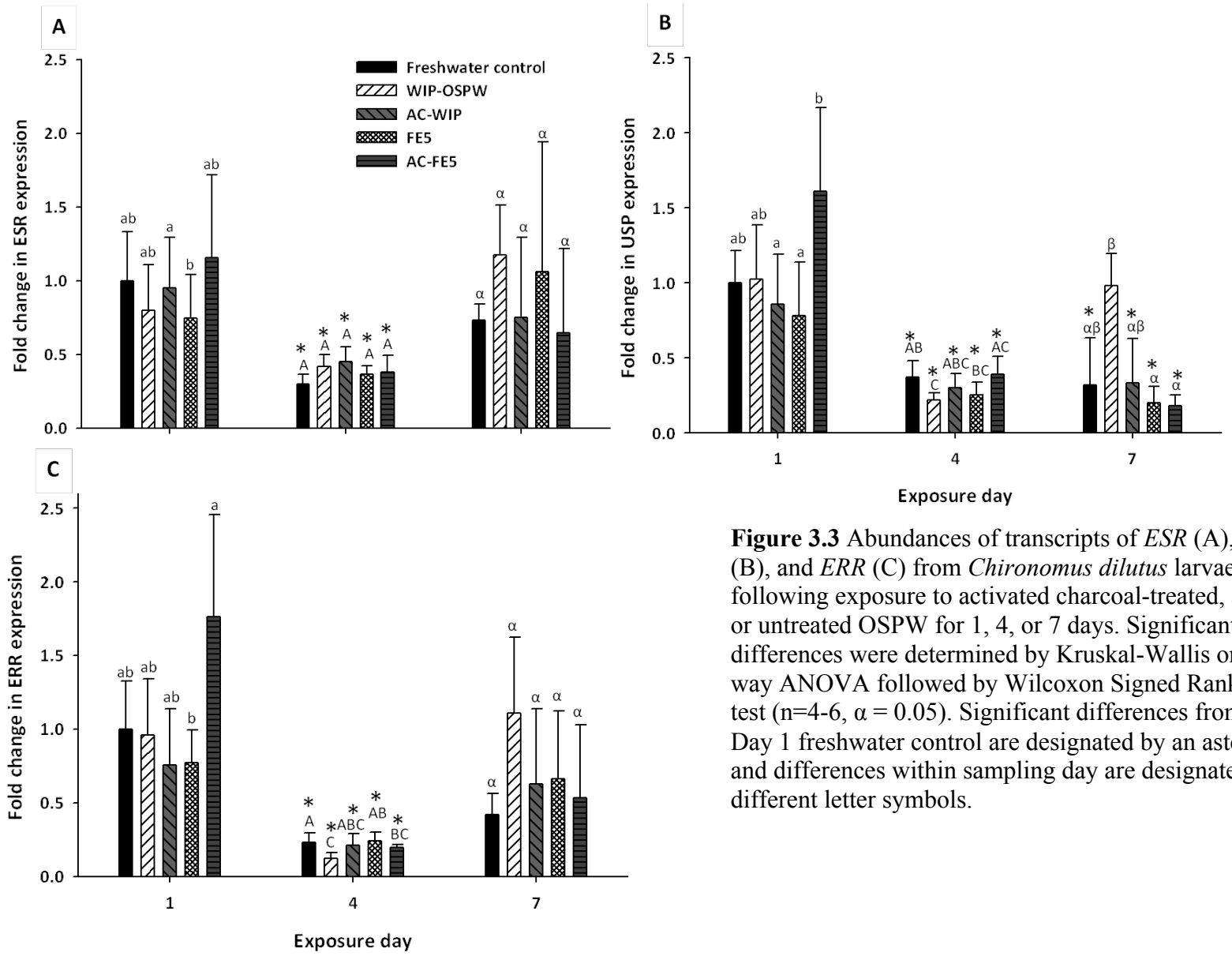


**Figure 3.3** Abundances of transcripts of *ESR* (A), *USP* (B), and *ERR* (C) from *Chironomus dilutus* larvae following exposure to activated charcoal-treated, aged, or untreated OSPW for 1, 4, or 7 days. Significant differences were determined by Kruskal-Wallis one-way ANOVA followed by Wilcoxon Signed Ranks test (n=4-6, $\alpha = 0.05$). Significant differences from the Day 1 freshwater control are designated by an asterisk and differences within sampling day are designated by different letter symbols.

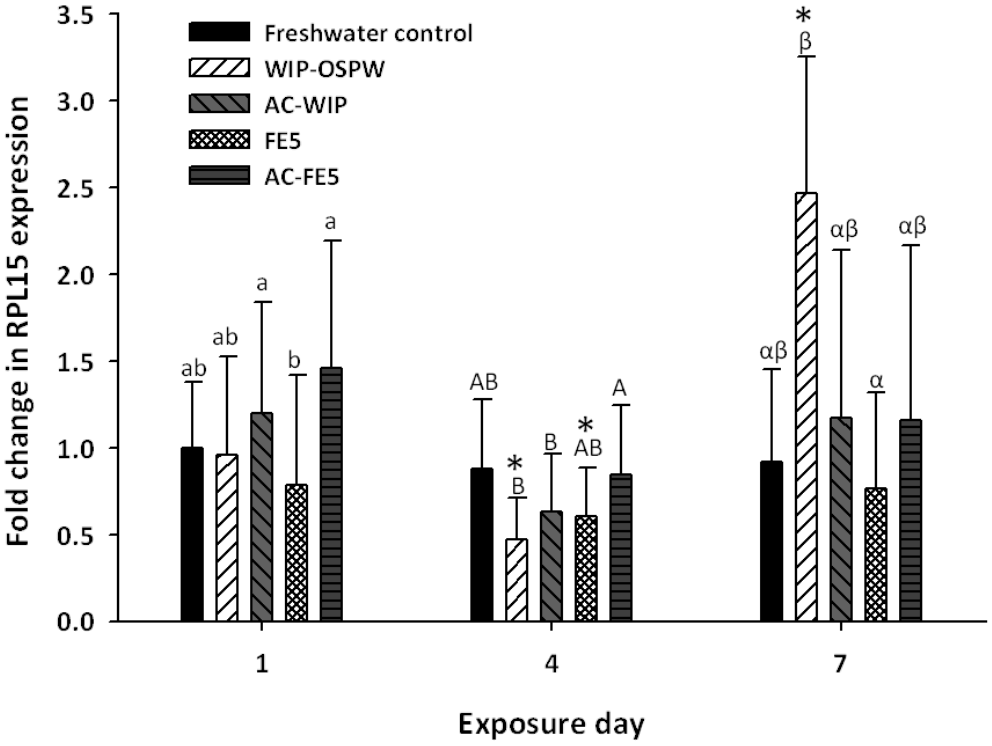


**Figure 3.4:** Abundances of transcripts of *RPL15* from *Chironomus dilutus* larvae following exposure to activated charcoal-treated, aged, or untreated OSPW for 1, 4, or 7 days. Significant differences were determined by Kruskal-Wallis one-way ANOVA follow by Wilcoxon Signed Ranks test (n=4-6, $\alpha$ =0.05). Significant differences from the Day 1 freshwater control are designated by an asterisk and differences within sampling day are designated by different letters symbols.

## 3.4. Discussion

Inhibition of growth was consistent with the results of previous studies that have investigated effects of OSPW on *C. dilutus.* In addition, the same pattern of inhibition of growth of *Chironomus* spp. has been observed following exposure to a number of other environmental contaminants and stressors, including reduced food availability (Liber *et al.,* 1996), uranium exposure (Muscatello and Liber, 2009; Dias *et al.,* 2008), and mercury exposure (Azevedo-Pereira and Soares, 2010; Chibunda, 2009). Previous studies have reported significant inhibition of *C. dilutus* larval growth as a result of a 10-day exposure to untreated OSPW (Anderson *et al.,* 2011a; Whelly, 1999). However, the current study represents the first time that inhibition of growth has been reported prior to the end of a 10-day exposure to OSPW. By Day 4 of the exposure period described here, *C. dilutus* exposed to WIP-OSPW had significantly less wet mass than larvae exposed to freshwater, and this difference was greater by Day 7. However, at no point during the study was the wet mass of *C. dilutus* exposed to the FE5 significantly less than that of the *C. dilutus* exposed to freshwater. This difference might be attributed to differences in the relative proportions of specific NAs or other organic constituents present in these OSPWs since biodegradation results in a proportional shift toward greater molecular weight and many-ringed NAs (Holowenko *et al.,* 2002; Martin *et al.,* 2010). A greater number of carboxyl groups and a greater proportion of multi-ring NA structures tend to result in a less-toxic mixture, probably due to lesser hydrophobicity and thus less ability to cross cell membranes (Frank *et al.,* 2009; Lo *et al.,* 2006). That explanation might apply to the aged OSPW used in this study. Although a reduction in of the organic fraction attenuates toxicity of OSPW (Anderson *et al.,* 2011a), inhibition of growth by WIP-OSPW was not attenuated after 4 days of exposure to AC-WIP. However, after 7 days of exposure, *C.*

*dilutus* exposed to AC-WIP had a significantly greater mean wet mass than *C. dilutus* exposed to WIP-OSPW, but were significantly smaller than *C. dilutus* exposed to the freshwater. This suggests that either the total concentration of NAs or the specific NAs remaining after the treatment with activated carbon was sufficient to inhibit the growth of *C. dilutus*.

Abundances of transcripts of seven genes were compared among treatment waters in order to investigate the underlying molecular basis for the effects of OSPW exposure on growth and development of *C. dilutus* (Anderson *et al.,* 2011a; Anderson *et al.,* 2011b). Three of the genes coded for isomers of hemoglobin (*Hb*). *Hb* is present within chironomid larval stages and the pattern of *Hb* protein synthesis is stage-specific and tissue-specific (Choi and Roche, 2004; Ha and Choi, 2008; Lee *et al.,* 2006). Larvae were obtained from egg masses laid on the same day in order to synchronize ages of larvae used in this study and to minimize differences in abundances of transcripts that could be attributed to age. Chemical-specific changes in expression of the *Hb* gene by chironomids have been observed in other studies in response to a variety of toxicants (Ha and Choi, 2008; Lee *et al.,* 2006), but patterns of expression and sensitivities vary among isoforms and among chemicals or stressors. For example, chironomid larvae exposed to the organophosphate insecticide, fenitrothion, expressed significantly less *Hb*, which correlated with less fresh body weight (Lee *et al.,* 2006). Another study found that exposure to atrazine resulted in down-regulation of 2 *Hb* genes and greater oxygen consumption (Anderson *et al.,* 2007). While larvae exposed to FE5 demonstrated significant down-regulation of hemoglobin genes in the current study, especially on Day 1, the slightly lesser masses were not significantly different from the masses of the

freshwater controls. Furthermore, WIP-OSPW caused very severe growth inhibition and FE5 caused altered *Hb* expression without affecting growth. These results suggest that FE5 and WIP-OSPW might have different mechanisms of toxicity, possibly due to different constituents, or proportions of constituents, within their respective organic fractions. Lesser abundances of transcripts of *HbB, HbD* and *HbE* in *C. dilutus* larvae exposed to FE5 for 1 day, and lesser abundance of transcripts of *HbD* on Day 7 of the exposure period were attenuated in *C. dilutus* exposed to AC-FE5. Evidence for the contribution of organic compounds to toxicity of OSPW is consistent with previous studies, which demonstrated that reduction of the organic fraction from OSPW attenuates toxicity of OSPW (Anderson *et al.,* 2011a; Pourrezaei *et al.,* 2011). This result is also consistent with the results of studies where ozonation of OSPW, which decreases concentrations of NAs, attenuated the toxicity of OSPW (Anderson *et al.,* 2011a; He *et al.,* 2010, 2011; Scott *et al.,* 2008).

A transient decrease in the abundance of transcripts of both *ESR* and *USP* was observed over the course of the study described here. Following the initial change in expression of *USP* between Day 1 and Day 4, abundance of transcripts of *USP* was relatively stable over the remainder of the experiment. A similar trend was reported following 12 h and 24 h exposure of *Chirionomus riparius* to cadmium, where constitutively expressed *USP* remained unchanged while the abundance of transcripts of *ESR* was significantly increased (Planello *et al.,* 2010). While expression of *ESR* did not increase significantly in the current study following exposure to oil sands-derived waters, the durations of exposure were longer than those used by Planello *et al.* (2010) and due to the time-sensitive and often transient nature of changes in expression of genes, the

sampling regime applied in this study might have captured different results. In a separate study, Sun *et al.* (2003) found that exposure to ecdysone agonists significantly increased expression of *USP* in codling moths. The significantly greater expression of *USP* in WIP-OSPW on Day 7 of the present study further suggests the potential of OSPW to modulate endocrine function. Previous studies have reported both estrogenic and androgenic effects of exposure to OSPW *in vitro* (He *et al.,* 2011), so it is likely that similar endocrine modulation could be observed *in vivo*.

Up-regulation of all three endocrine receptor genes was detected in Day 7 samples. This could coincide with the beginning of a new instar phase or developmental progression towards pupation, and might offer insight into the reasons for the significant impairment of pupation and emergence that was observed in previous studies (Anderson *et al.,* 2011a; Anderson *et al.,* 2011b). The most significant changes in abundance of transcripts from endocrine receptor genes were observed following 7 days of exposure to WIP-OSPW, the treatment which had the greatest total concentration of NAs, while the aged FE5, with lesser concentrations of NAs, did not produce significant changes in expression of these genes (13 mg/L NAs in FE5 vs. 72 mg/L NAs in WIP-OSPW). These results suggest that biodegradation might reduce or eliminate the organic fraction responsible for the observed changes in endocrine receptor gene expression in chironomid larvae.

In a recent study, *Chironomus riparius* had greater transcript abundance of *ERR* expression following exposure to the endocrine-disrupting chemicals bisphenol A (BPA) and 4-nonylphenol (NP) (Park and Kwak, 2010). Exposure to endocrine-disrupting chemicals has also been shown to alter the abundance of *ERR* transcript and result in

delayed pupation and larval development and lesser emergence in chironomids (Lee and Choi, 2007; Park and Kwak, 2010). Similarly, up-regulation of *ERR* gene expression occurred in larvae in the present study from the AC-FE5 treatment on Day 1 and WIP-OSPW on Day 7, with down-regulation in FE5 larvae on Day 1 and WIP-OSPW larvae on Day 4. Other studies have reported that OSPW and NAs might have endocrine modulating effects (He *et al.,* 2011) and that there are structural similarities between some NAs and estrogens (Rowland *et al.,* 2011c), so it is possible that the changes in expression of *ERR* can be attributed to effects of the NAs found in WIP-OSPW. Previous studies using the same WIP-OSPW and FE5 waters, as well as other aged oil sands waters, found that pupation and emergence of *C. dilutus* were significantly impaired and delayed following exposure to OSPW (Anderson *et al.,* 2011a; Anderson *et al.,* 2011b), and it is possible that these effects could be attributed to the observed changes in *ERR*-regulated metabolism. As a result, alterations to this system might have effects within an individual, as well as on populations if reproduction is sufficiently impaired.

Ribosomal proteins, such as *RPL15*, function primarily in protein synthesis, but can also serve a secondary role in regulation of development (Nair *et al.,* 2011; Wool, 1996). Following 24 hr exposure to silver nanoparticles, *C. riparius* larvae had reduced expression of ribosomal protein L15, which was suggested to have potential disruptive effects on protein production (Nair *et al.,* 2011). Similar down-regulation of *RPL15* was observed following 1-day and 7-day exposure to FE5, although the exposed *C. dilutus* larvae were no different in mean wet mass from the controls. In contrast, on Day 7 there was significantly up-regulated expression of *RPL15* in WIP-OSPW-exposed larvae relative to freshwater controls and these larvae had significantly less mass than the

controls. These results suggest that exposure to fresh and aged OSPW might have disruptive effects on ribosomal proteins and protein production, which can in turn have implications for larval development, but the mechanisms might differ in organisms exposed to different waters. As a result of aging, FE5 had a lesser concentration of total NAs than WIP-OSPW, and the relative proportions of different NAs in aged waters have been shown to vary from younger waters (Holowenko *et al.,* 2002; Martin *et al.,* 2010), so a possible explanation for the differences in mechanisms could be related to the specific components within the organic fraction of each water.

In general, abundances of transcripts for the genes investigated in this study were not very different in larvae exposed to OSPW that had been treated with activated charcoal compared to larvae from the freshwater control. The differences between these treatments were seen in the endocrine receptors, but not in hemoglobins or ribosomal protein, and this also corresponded with a significant degree of growth inhibition relative to the control. There were only two instances where AC-FE5 larvae had a significantly greater abundance of gene transcript than AC-WIP larvae (Day 1 *USP* and Day 4 *RPL15).* As AC-FE5-exposed larvae were slightly larger than AC-WIP-exposed larvae, these results suggest that the differences may have arisen from changes in transcriptional responses that are mediated by *USP* and/or from changes in ribosomal protein function. Significant differences in profiles of relative abundances of gene transcripts between larvae exposed to FE5 or AC-FE5 were more common than between larvae exposed to WIP-OSPW or AC-WIP, while wet masses were significantly different between both pairings, but to a greater extent in WIP-OSPW/AC-WIP. FE5 and WIP-OSPW-exposed larvae had very different profiles of abundances of hemoglobins transcripts, but

abundances of endocrine receptors and ribosomal protein were similar between the two treatments. This corresponded with significant growth inhibition in WIP-OSPW and no significant effects on growth in FE5, relative to the freshwater control.

Overall, this study showed that exposure to untreated OSPW can have significant consequences for proper growth and development of *C. dilutus* larvae. Larvae exposed to both aged and fresh OSPW displayed patterns of expression of genes that were significantly different from those of larvae in freshwater that served as controls, with changes in hemoglobins, estrogen-related receptor, ultraspiracle protein and ribosomal protein gene expression over the course of a 7-day exposure period. Additional use of OSPW fractionation and analytical identification techniques to compare the NAs profiles of these waters might assist in identifying the compounds or structural properties of compounds most closely associated with changes in gene expression and toxicity. Further analysis of transcriptional responses of other developmental and metabolic genes, along with quantification of hormone levels in exposed larvae, could help to more fully explain the organism-level effects that were observed in this study and in previous studies, and to offer potential biomarkers of OSPW exposure in chironomid larvae.

# CHAPTER 4:
# Effectiveness of ozonation treatment in eliminating toxicity of oil sands process-affected water to *Chironomus dilutus*

## 4.1 Introduction

Global energy demands are expected to increase by as much as 50% over the next two decades, driving a shift from conventional oil sources to further exploration and exploitation of alternative sources of fossil fuels, such as oil sands (Government of Alberta, 2006; Veil *et al.,* 2009). Water use is an issue associated with development of oil sands in the Athabasca region of Alberta, Canada. The "Clark hot water process" is used in surface mining operations to extract bitumen, the petroleum precursor, from oil sands. This involves addition of hot water and caustic soda to separate bitumen from residual sands, silts, clays, and other inorganic and organic compounds, and results in oil sands process-affected water (OSPW) that is stored in active settling basins (Hao *et al.,* 2005; Rogers *et al.,* 2002). OSPW is alkaline, saline, and produced at a rate of up to 4 $m^3$ per cubic metre of oil sands processed (Holowenko *et al.,* 2002), despite recycling OSPW to reduce the amount of freshwater used in the extraction process. Companies are held to a policy of zero-discharge to surface waters, and today over a billion cubic metres of OSPW are held in active settling basins (Del Rio *et al.,* 2006) and this volume will only increase as oil sands production continues.

In addition to salinity and alkalinity, OSPW contains a complex mixture of dissolved organic acids, referred to as naphthenic acids (NAs) (Headley and Martin, 2004). These are characterized as a group of carboxylic acids with the general formula $C_nH_{2n+z}O_2$, where n indicates the number of carbons and z relates to the number of rings (Clemente and Fedorak, 2005; Holowenko *et al.,* 2002; Lai *et al.,* 1996). NAs with more

rings tend to be more persistent in the environment (Clemente and Fedorak, 2005; Frank *et al.,* 2008; Lai *et al.,* 1996; Martin *et al.,* 2010). Also, NAs have been implicated as the primary cause of acute and chronic toxicity observed among taxa exposed to OSPW, although the exact mechanism of toxicity remains unproven (Clemente and Fedorak, 2005; Herman *et al.,* 1994; Rogers *et al.,* 2002).

There is extensive research underway to develop methods for reclaiming OSPW, such as constructed ponds, end-pit lakes and/or wetlands. Bioremediation is one possible option for reducing the toxicity of OSPW, but this can take several decades and might never result in sufficiently small concentrations of NAs to allow uninhibited development of biota. Thus, this mechanism of *in situ* remediation through natural degradation alone does not seem sufficient to treat the volumes of OSPW currently in containment (Herman *et al.,* 1994; Holowenko *et al.,* 2002). In order to effectively reduce or eliminate toxicity of OSPW, a treatment approach that can directly target NAs is required. Ozonation has been identified as a potentially effective treatment method since previous studies have demonstrated that ozone targets NAs with greater molecular weights and ring numbers, which tend to be more resistant to biodegradation (Martin *et al.,* 2010). Ozonation, which reduces the total concentration of NAs and changes the relative proportions of the different fractions of NAs in OSPW (Martin *et al.,* 2010), also reduces toxicity as measured by the Microtox® assay (Scott *et al.,* 2008) and attenuates endocrine disrupting effects on eukaryotic cells *in vitro (*He *et al.,* 2010; He *et al.,* 2011).

Toxicity of NAs and OSPW towards benthic invertebrates has not been extensively characterized, but sensitivities vary among taxa and between laboratory and field conditions (Whelly, 1999). Midges represent a group of ubiquitous, ecologically

significant freshwater organisms (Armitage *et al.,* 1995) and have been found to comprise a large proportion of the biomass within reclamation wetlands in the oil sands management area and in nearby water bodies (Bendell-Young *et al.,* 2000), which makes them appropriate test organisms. The benthic invertebrate *Chironomus dilutus* (formerly *C. tentans*) is commonly used in toxicological bioassays and has a number of attractive qualities as a test organism, including relative ease of rearing, short life cycle, and sensitivity to many contaminants (Giesy and Hoke, 1989; Sibley *et al.,* 1997). Therefore, in this study, effects of fresh OSPW and ozonated OSPW on *C. dilutus* were studied. It was hypothesized that exposure to untreated OSPW would have effects on survival of larvae, development, and behavior, and that ozonation would attenuate the toxicity of OSPW.

## 4.2 Materials and Methods

### *4.2.1 Test organisms*

Adult *C. dilutus* midges from an in-house laboratory culture (Toxicology Centre, University of Saskatchewan) were bred to obtain larvae for both the 10-day and chronic exposure assays. Egg masses were placed into several 15 L aquaria and raised to test age in an environmental chamber maintained at 23 ± 1 °C with a 16:8 hour light:dark regime. Prior to test initiation, larvae were kept in control freshwater, which was Saskatoon, SK, municipal water that was carbon filtered, bio-filtered, and aerated for 24 hr prior to placement into aquaria or test vessels.

*4.2.2 Exposure waters*

*Chironomus dilutus* larvae were exposed to one of six treatment waters: 1) freshwater control, 2) saltwater control (10-day acute exposure only), 3) WIP-1, 4) WIP-2, 5) WIP-1-30, or 6) WIP-2-80. The control freshwater had the following characteristics (mean ± SD): dissolved oxygen 8.13 ± 0.3 mg/L, conductivity 462 ± 33 µS/cm, pH 8.40 ± 0.20, alkalinity 100 ± 8 mg/L as $CaCO_3$, and hardness 142 ± 25 mg/L as $CaCO_3$. The control freshwater used was the same as that used for culturing *C. dilutus*.

The total concentration of dissolved solids (TDS) in OSPW is typically between 2,000 and 2,500 mg/L, with sodium, bicarbonate, chloride, and sulphate the dominant ions (Allen, 2008; Gamal El-Din *et al.,* 2011). Synthetic saltwater was therefore used as a second control to mimic these components of OSPW and was comprised of 938 mg/L NaCl, 506 mg/L $NaSO_4$ and 910 mg/L $CaSO_4$. During the chronic exposure, a different control for the inorganic fraction of OSPW was made by removing the majority of the organic fraction from untreated WIP-OSPW. This was done by mixing WIP-1 with 5% (w/v) 8-20 mesh particle size activated charcoal (Sigma-Aldrich, St. Louis, MO) and gently stirring for 4 hr at room temperature. After the contact period, the water was sieved to remove the larger charcoal particles, and then vacuum-filtered through a 0.22 µm filter (Millipore Corporation, Billerica, MA) to remove all charcoal particles. Fourier transform infrared spectroscopy (FTIR) was used to measure the total concentrations of NAs and confirm removal of the organic fraction; NAs were reduced from 23.6 mg/L to 6.4 mg/L in the AC-treated WIP-1. The estimated total concentrations of NAs in each of the untreated and ozonated OSPW samples was determined by ultra pressure liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) as previously

described (Martin *et al.,* 2010). In WIP-1, the concentration of NAs was 23.6 mg/L, which was reduced to approximately 12.1 mg/L following ozonation with 30 mg $O_3$/L. In WIP-2, the concentration of NAs in the untreated water was 19.7 mg/L, which was reduced to 1.9 mg NA/L by ozonation with 80 mg $O_3$/L (Wang, 2011). Treatment of WIP-1 with activated charcoal had no measurable effects on the concentrations of inorganic compounds (data not shown).

*4.2.3 Ozonation of OSPW*

Ozonation of WIP-OSPW was conducted at the University of Alberta (Edmonton, AB). Briefly, an ozone generator (WEDECO, GSO-40, Herford, Germany) was used to produce ozone gas from extra dry, high purity oxygen. To obtain a stable ozone concentration in feed-gas, the ozone generator was allowed to stabilize for 10 minutes. The feed gas, containing 6.2 to 7.7% w/w ozone (i.e., 83.7 to 96.1 mg/L), was sparged into the liquid phase though gas diffusers.

The ozone residual in the reactor was measured using the Indigo method (APHA, 2005). The gas flow rate was measured by flow meter (4-20L/min). The used ozone dose for this system can be calculated from Equation 1:

$$\Delta O_3 = \int_0^t \frac{(Q_{G,in}C_{G,in} - Q_{G,out}C_{G,out})}{V_L} dt - C_L$$

$$\int_0^t \frac{(Q_{G,in}C_{G,in} - Q_{G,out}C_{G,out})}{V_L} dt - C_L \qquad (1)$$

Where: $\Delta O_3$ is the amount of utilized ozone (mg/L), $C_{G,in}$ is the concentration of ozone in the feed gas (mg/L), $C_{G,out}$ is the concentration of ozone in the off gas (mg/L), $C_L$ is the

residue concentration of ozone in the liquid phase (mg/ L), $V_L$ is the effective reactor volume (L), $Q_{G,in}$ is the feed-gas flow rate (L/min.), $Q_{G,out}$ is the off-gasing flow rate (L/min.), and t is the ozone contact time (min.).

After treatment with ozone, the OSPW was purged for 10 min by a stream of purified nitrogen gas to strip the ozone residual and oxygen off the reactor. WIP-1 was treated with an ozone dose of 30 mg/L (resulting in WIP-1-30) and WIP-2 was treated with 80 mg/L (resulting in WIP-2-80), representing a lesser and greater degree of ozonation, respectively.

### *4.2.4 Acute exposure*

#### *4.2.4.1 Effects on survival and growth*

The experiment was conducted under the same environmental conditions as described in Section 4.2.1. Effects of acute exposure to the treatments were assessed by use of a modified 10-day static-renewal assay with endpoints of survival, growth, and behavior of *C. dilutus* larvae. Ten 8-9 day post-hatch larvae were randomly assigned to each of four replicate 300 mL, tall-form beakers per treatment group. Each beaker contained 30 g of silica sand (particle size 200-400 μm) and approximately equal sized larvae were placed into each replicate. Larvae were initially removed from their cases in order to expose them directly to the treatments without a protective case and to explore the effects of OSPW upon case building since preliminary studies suggested that case building might be disrupted by exposure to OSPW. Based on a representative subsample (3 x 10 animals), the mean initial wet mass was 0.63 ± 0.25 mg per individual.

During the exposure period, larvae were fed 0.67 mg dry weight TetraFin® fish food (Tetra Company, Blacksburg, VA) per individual daily and 50% of the water volume was replaced every 2 d. Concentrations of ammonia were monitored in all oil sands-derived waters prior to initiating tests and waters were aerated until ammonia concentrations were less than 1.0 mg/L before exposures commenced. Beakers were continually aerated during the test and concentrations of dissolved oxygen (DO) maintained at 7 mg/L or greater, with mean water temperature of 23 ± 1.5 ° C. Oxygen and temperature were measured each day from a subsample of beakers, including at least one representative beaker from each treatment, by use of an Orion 3-Star RDO Portable Meter and Probe (Thermo Fisher Scientific Inc., Nepean, ON). Samples of water from each treatment group were collected on Days 0, 5, and 10 and analyzed for conductivity, pH, total hardness, alkalinity, and total ammonia.

The assay was terminated on Day 10 and survival rate and wet mass of surviving midges determined and reported on a per beaker basis. Wet mass was measured in order to preserve animals for future analysis of gene expression. In addition to the larvae themselves, constructed cases were gently collected from the sediment in each beaker and stored in 100% ethanol. Preliminary surveys of the effects of WIP-OSPW on *C. dilutus* performed in our lab indicated that case building might be affected by exposure to WIP-OSPW, so this endpoint was added. Representative photographs showing the gross morphology of these cases were taken at 18 to 22 x magnification using an Olympus SZ61 zoom stereo microscope equipped with a Q-Color 5 Olympus digital camera (Olympus America Inc., Pennsylvania, PA).

*4.2.4.2 Effects on behavior*

Behavior of larvae was assessed over the course of the 10-day exposure period. To do so, observations of behavior were made three times daily. The measurement endpoint was amount of activity in terms of frequency of observation outside the case and body position relative to its case. A 15-minute visual observation of all individual beakers was also conducted at 11:00, 13:00, and 15:00. A scoring system was used to indicate the number of larvae visible on the sand surface and the extent to which larvae were outside their cases. A score of 0, 1, 2, or 3 was assigned for each animal in each beaker. A score of 0 was assigned when the larva was completely encased and not visible on the sediment surface. For a score of 1, less than half of the body length was exposed; a score of 2 indicated that at least half of the body length was visible; and a score of 3 was assigned if the entire larva was exposed and freely moving on the surface of the sand. An overall activity score was calculated for each beaker at each observation time point by multiplying the score value (0, 1, 2, or 3) by the number of larvae assigned that particular score and summing the totals.

*4.2.5 Chronic exposure*

To assess effects of chronic exposure to these same treatments, ten larvae (8-9 days post-hatch) were randomly assigned to each of four replicate 1L, tall-form beakers per treatment group. Each beaker contained 500 mL of water, with the larger beaker size used to reduce the density of larvae per unit volume, which deviated from standard procedure, but minimized potential stress due to crowding. Larvae were allowed to remain in their cases during placement into test beakers, as opposed to the acute exposure

where they were removed from their cases prior to exposure to treatments. Individuals were allowed to pupate and then emerge as adult midges. Adults were counted and collected on a daily basis. Endpoints assessed included survival, pupation rate, adult emergence, and sex ratio. The test beakers were maintained until all individuals within the replicate had emerged, or alternatively, until all individuals had died at whatever life stage they were able to achieve (larva, pupa, or adult).

Feeding and daily water quality monitoring and maintenance followed the procedure described in Section 4.2.4. Concentrations of ammonia were less than 1.0 mg/L prior to test initiation and concentrations of DO were greater than 7 mg/L. Water temperatures in all beakers were 23 ± 1.5° C. Water from each treatment group was collected every five days and analyzed for conductivity, pH, total hardness, alkalinity, and total concentrations of ammonia.

*4.2.6 Statistical analysis*

All statistical analyses were conducted using SYSTAT (version 12.0, Systat Software, Inc.). The experimental unit was the test beaker. Statistical differences were assessed by one-way ANOVA followed by Tukey's post-hoc pair-wise comparisons where either raw or log-transformed data met the assumptions of normality and homogeneity of variance. Normality was determined by the Shapiro-Wilk test and equality of variance tested by Levene's test. Non-normal data were assessed by ANOVA on Ranks followed by a Holm-Sidak multiple comparison post-hoc test. Data with unequal variances were assessed by Games-Howell post-hoc pair-wise comparisons. All

data are presented as mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$.

## 4.3. Results

### *4.3.1 Acute exposure*

#### *4.3.1.1 Water characteristics*

General water chemistry measured on Days 0, 5, and 10 of the exposure period did not differ significantly over time or among replicates, so water chemistry data for each parameter were pooled within treatment groups. Mean measures for conductivity, pH, hardness, alkalinity, and ammonia are presented in Table 4.1. Values for all parameters except hardness were greater in both ozonated and untreated OSPW than those in the freshwater control. Mean DO concentrations were greater than 7.0 mg/L in all treatments over the course of the experiment and there was no difference in DO among any of the treatment groups. Concentrations of ammonia were between 0.1 and 1.2 mg/L for all treatments over the course of the study. Total concentrations of NAs in the treatment waters were: 23.6 mg/L in WIP-1, 12.1 mg/L in WIP-1-30, 19.7 mg/L in WIP-2, 1.9 mg/L in WIP-2-80, and 6.4 mg/L in AC-WIP-1.

#### *4.3.1.2 Effects on survival and growth*

An overall survival of 85% in the freshwater control group and 83% in the saltwater control group met the 70% requirement for study validity (US EPA, 2000). In WIP-1, the observed survival of 55% was significantly less than the freshwater control ($p<0.05$), but there were no significant differences in survival observed among any of the

other treatment waters or with the controls (Figure 4.1). Survival of 95% in WIP-1-30 was significantly greater than in untreated WIP-1 ($p<0.01$). Survival in WIP-2-80 was not significantly different from that in untreated WIP-2.

Inhibition of growth was observed following exposure to untreated OSPW (Figure 4.2). *Chironomus dilutus* exposed to WIP-1 and WIP-2 had mean body masses that were 64% and 79% less, respectively, than the freshwater control following the 10-day exposure period ($p<0.001$). The masses of larvae from the ozonated-OSPW treatments were also significantly less than larvae exposed to freshwater or saltwater ($p<0.05$). *Chironomus dilutus* exposed to WIP-1-30 or WIP-2-80 had mean body masses that were by 22% and 32% less, respectively, relative to the freshwater control. However, these larvae had a mean mass that was significantly greater than individuals exposed to untreated OSPW ($p<0.01$).

**Table 4.1:** Water chemistry characteristics measured for ozonated and untreated OSPW and controls over the course of a 10-day exposure period (mean ± SEM). Samples were collected in triplicate and analyzed on Days 0, 5, and 10.

| | Treatment Water | | | | | |
|---|---|---|---|---|---|---|
| **Water quality parameter** | **Freshwater control** | **Saltwater control** | **WIP-1-30** | **WIP-1** | **WIP-2-80** | **OSPW- 80** |
| [NAs] (mg/L) | 0 | 0 | 12.1 | 23.6 | 1.9 | 19.7 |
| Conductivity (µS/cm) | 462 ± 33 | 3857± 269 | 3981 ± 171 | 3929 ± 180 | 4313 ± 157 | 4243 ± 124 |
| pH | 8.40 ± 0.2 | 8.98 ± 0.2 | 8.78 ± 0.3 | 8.72 ± 0.4 | 8.85 ± 0.3 | 8.86 ± 0.3 |
| Hardness (mg/L CaCO3) | 142 ± 25 | 98 ± 5 | 87± 7 | 86 ± 5 | 104 ± 3 | 85 ± 11 |
| Alkalinity (mg/L CaCO3) | 100 ± 8 | 603 ± 54 | 492 ± 16 | 497 ± 29 | 582 ± 18 | 549 ± 50 |
| Ammonia (mg/L) | 0-0.8 | 0.2-0.8 | 0-0.7 | 0-0.8 | 0.1-0.7 | 0.1-1.2 |

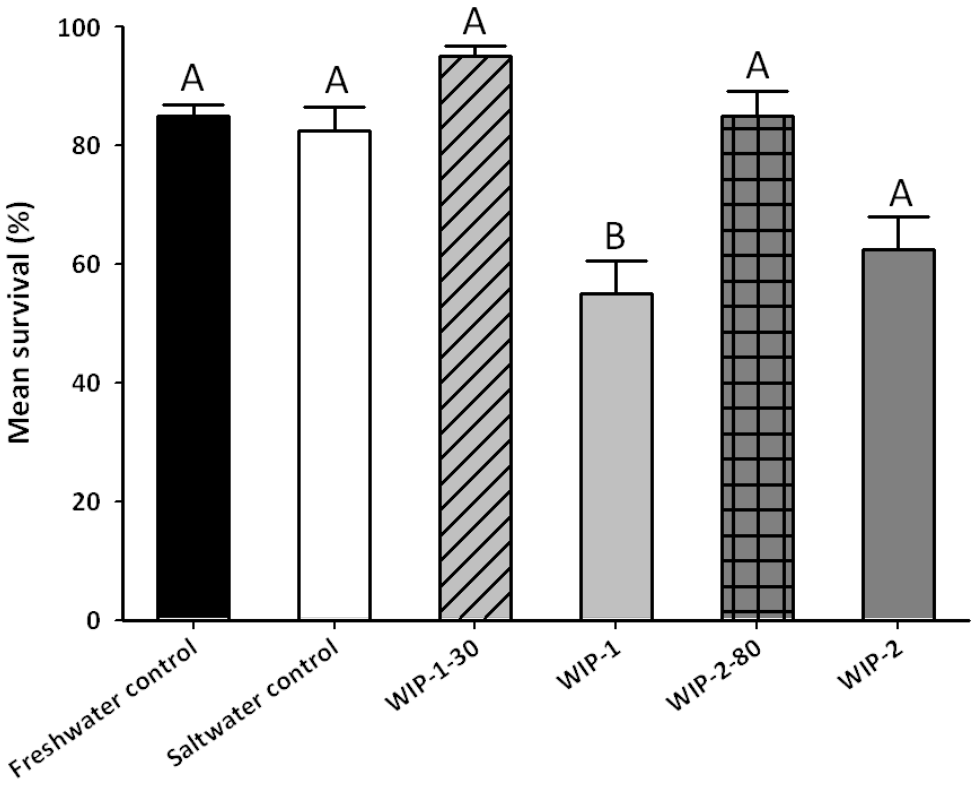


**Figure 4.1:** Mean (± SEM) survival of *Chironomus dilutus* larvae following 10-day exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined using a one-way ANOVA followed by Tukey's HSD post-hoc test (n=4, $\alpha$ =0.05) and are designated by different letters.

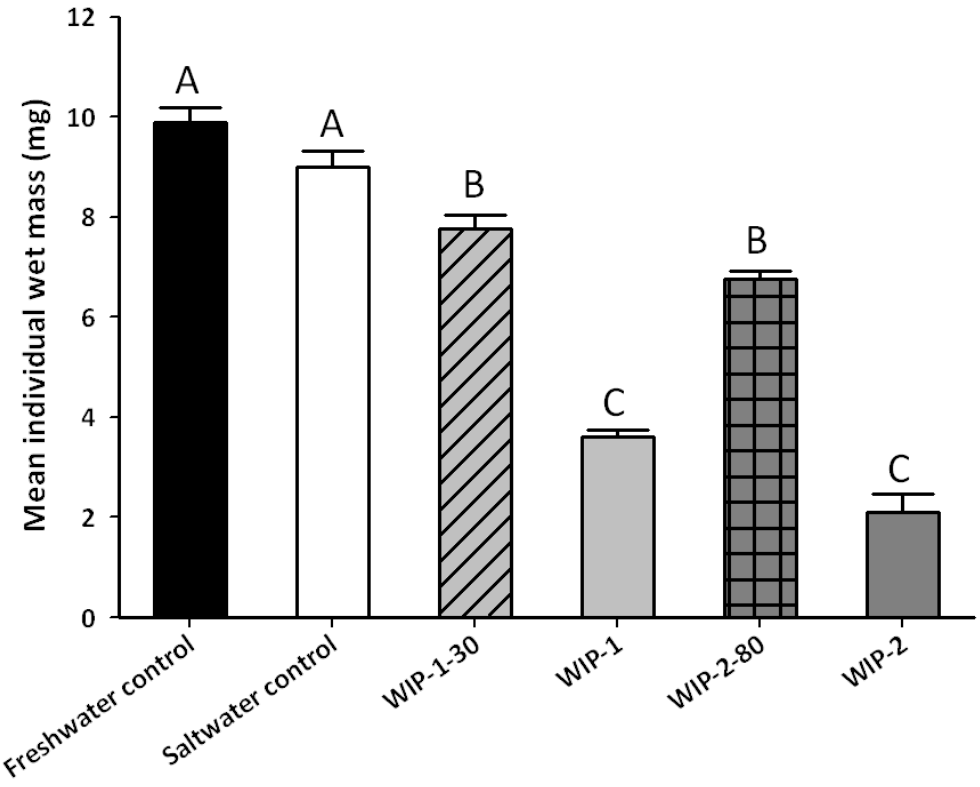


**Figure 4.2:** Mean (± SEM) individual wet mass of *Chironomus dilutus* larvae following 10-day exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD post-hoc test (n=4, $\alpha$ =0.05) and designated by different letters.

4.3.1.3 Effects of OSPW on case building and behavior

There were differences observed in the quality of recovered larval cases between freshwater controls and the other treatment groups. *Chironomus dilutus* larvae exposed to freshwater built large, intact cases that were easily handled and recovered as a single unit. In the untreated OSPW treatments, cases were difficult to recover due to their fragile structures and were small relative to the controls. Similarly, in both of the ozonated-OSPW treatments (WIP-1-30 and WIP-2-80), cases had poor structural integrity and were difficult to recover intact. Cases were also smaller than those produced by control larvae. Representative cases showing the structure of the cases from each of the treatment groups are presented in Figure 4.3.

Exposure to OSPW had a significant effect on the activity of *C. dilutus* larvae in terms of frequency of observation outside of their cases and body position relative to cases. Behavioral data were pooled within observation times and assessed by day, due to significant differences among days ($p<0.05$). The trend in activity for each treatment group is shown in Figure 4.4. For the first three days of the exposure period, larvae exposed to WIP-2 were generally more active than the freshwater controls, and the activity of larvae exposed to WIP-1 was similar to that of the controls. From Days 7 to 9, larvae in WIP-1 and WIP-2 were significantly less active outside of their cases than in any other treatment ($p<0.05$). The activity levels of the saltwater control, WIP-1-30 and WIP-2-80 treatments did not differ significantly from that of the freshwater controls, except on Day 8 when larvae in WIP-1-30 were significantly more active than those in the freshwater control ($p<0.05$).

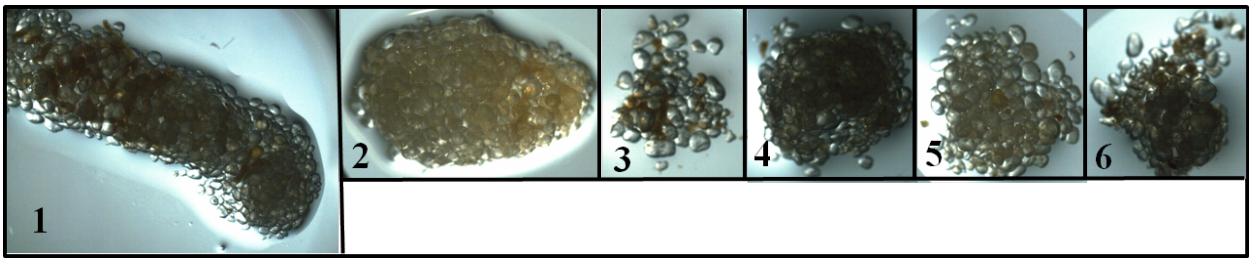


**Figure 4.3:** Representative gross morphology of *Chironomus dilutus* larval cases removed from each of the treatment groups following a 10-day exposure period. 1) Freshwater control, 2) Saltwater control, 3) WIP-1-30, 4) WIP-1, 5) WIP-2-80, and 6) WIP-2. Differences in magnification were used in the figure for 1 and 2 vs. 3-6 due to disparity between sizes of recoverable cases.

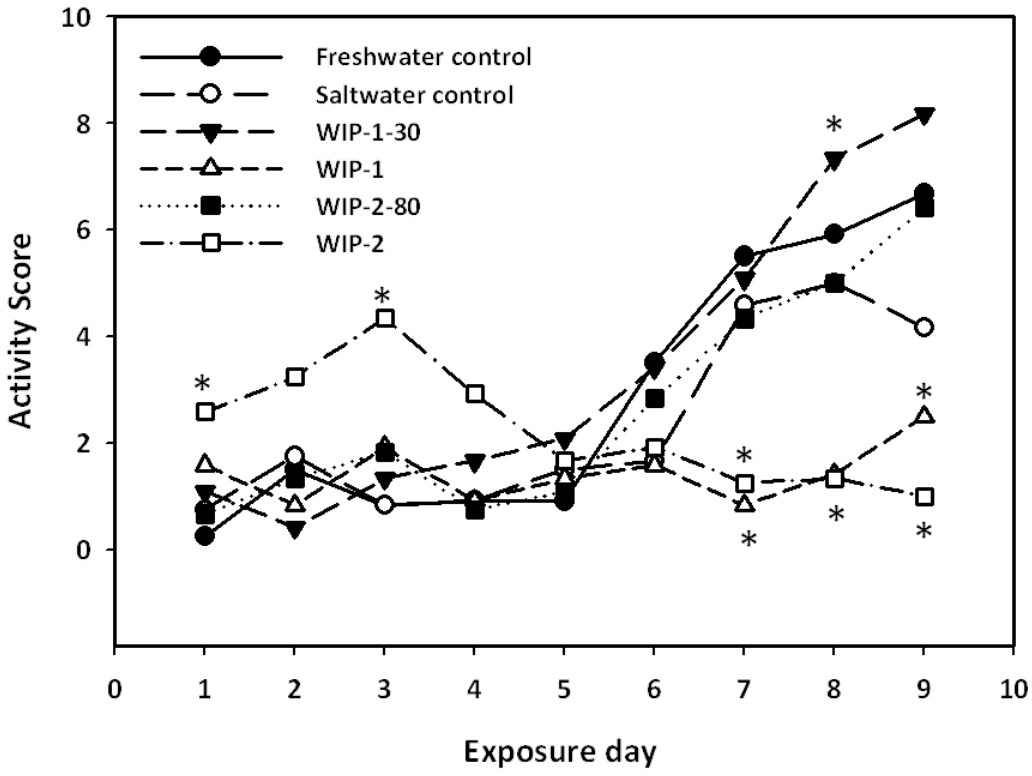


**Figure 4.4:** Trends in daily activity levels of *Chironomus dilutus* larvae over the course of a 10-day exposure to ozonated or untreated OSPW. Mean scores were determined based on three daily observations made at 11:00, 13:00, and 15:00. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's post-hoc test and are indicated by an asterisk ($\alpha$ =0.05). Measures of variance were omitted from the figure for clarity.

*4.3.2 Chronic exposure*

*4.3.2.1 Pupation and emergence*

Significant differences in rates of pupation and time-of-emergence were observed among treatments (Table 4.2). Pupation was first observed on Day 15 of the study, with the first successful emergence on Day 18. The rate of pupation in the freshwater control was 96%, while only 65% of larvae pupated in the WIP-1 treatment and 25% in WIP-2, both of which represent significantly lesser rates of pupation than in the freshwater control ($p<0.05$). Ozonation attenuated the effects of OSPW on pupation, with rates of pupation of 93% and 86% for WIP-1-30 and WIP-2-80, respectively, rates which were not significantly different from the freshwater control (Figure 4.5). The rate of pupation in the AC-WIP-1 was 78% and also not significantly different from the rate of the larvae in the freshwater control.

The rate of emergence of adults was significantly affected by exposure of larvae to WIP-OSPW. Approximately 81% of the larvae in the freshwater control reached adulthood. However, the percentage of larvae exposed to untreated OSPW that successfully emerged was significantly less ($p < 0.001$), with emergence rates of 13% and 8% for larvae exposed to WIP-1 and WIP-2, respectively. Emergence was significantly greater in ozonated-OSPW than in untreated OSPW, with emergence 75% greater in WIP-1-30 vs. WIP-1 and 48% greater in WIP-2-80 vs. WIP-2. The time-to-emergence of males was significantly delayed in WIP-1 and WIP-2 relative to freshwater controls, by an average of 13.0 d in WIP-1 and 8.4 d in WIP-2 ($p<0.01$) (Table 4.2). Time-to-emergence in AC-WIP-1 and both ozonated-OSPW treatments did not differ significantly from that of the freshwater controls. No significant delays in emergence of females were

observed in any of the treatments, but there were no successful female emergences in over half of the WIP-2 replicates. There were no significant differences in sex ratio among any of the treatment groups, with ratios of males:females ranging from 0.67:1 in WIP-1 to 1.24:1 in the freshwater control. However, significantly fewer males and females emerged in both OSPW treatment groups. The cumulative emergence over the duration of the exposure period followed a pattern of delayed emergence in the AC-WIP-1 and emergence was inhibited in untreated WIP-OSPW (Figure 4.6).

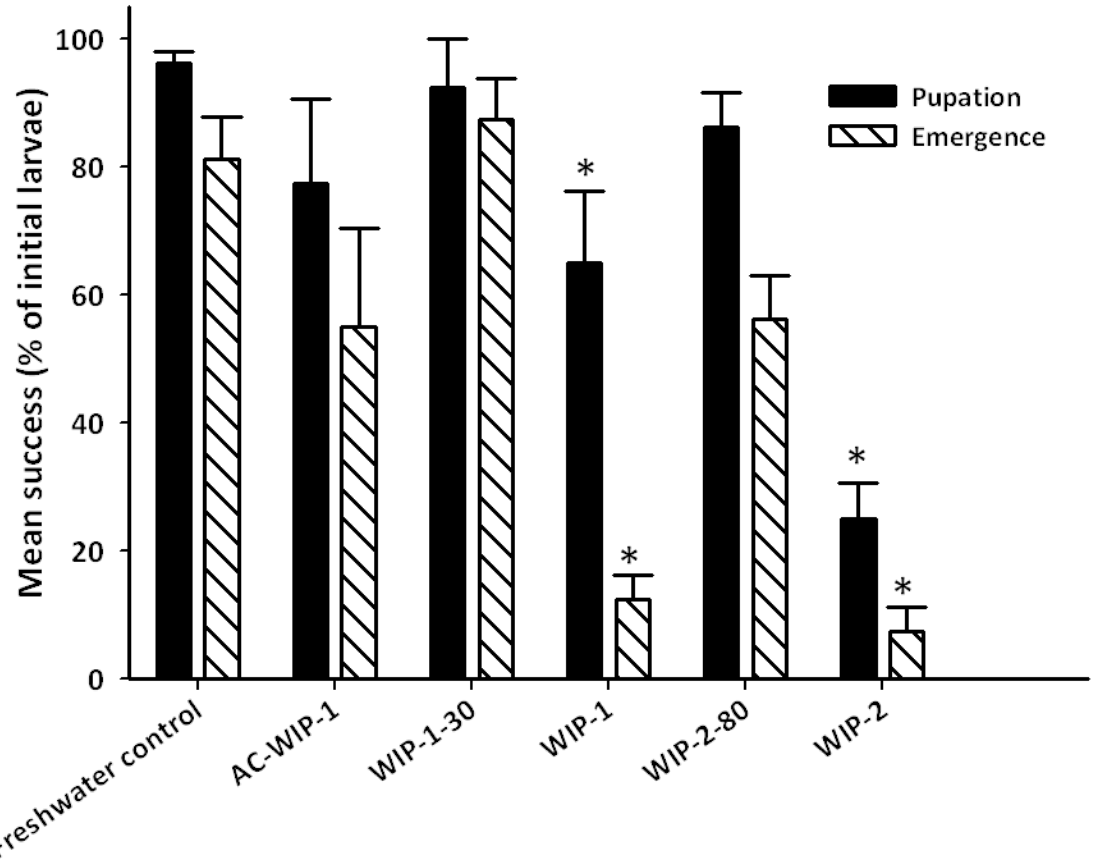


**Figure 4.5:** Mean (± SEM) pupation and adult emergence rates for *Chironomus dilutus* after chronic exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD post-hoc test (n= 4 or 8, $\alpha$ =0.05) and designated by an asterisk.

**Table 4.2:** Summary of pupation and emergence statistics for *Chironomus dilutus* following chronic exposure to ozonated or untreated OSPW. Significant differences within endpoints from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD post-hoc test or Games-Howell post-hoc test (n=4 or 8, $\alpha$ =0.05) and are designated by different letters.

| Endpoint | | Treatment Water | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Freshwater control** | **AC-treated OSPW** | **WIP-1-30** | **WIP-1** | **WIP-2-80** | **WIP-2** |
| Pupation (%) | | 96 [a] | 78 [ab] | 93 [ab] | 65 [b] | 86 [ab] | 25 [c] |
| Emergence (%) | | 81[a] | 55 [a] | 88 [a] | 13 [b] | 56 [a] | 8 [b] |
| Emergence/Pupation (%) | | 84 [a] | 71 [a] | 95 [a] | 20 [b] | 65 [a] | 32 [b] |
| Mean delay in emergence time vs. freshwater control (days) | Males | N/A | 4.7[a] | 0.4[a] | 13.0 [b] | 2.3[a] | 8.4 [b] |
| | Females | N/A | 2.6[a] | 1.2[a] | 0.5[a] | 1.1[a] | 4.1[a] |

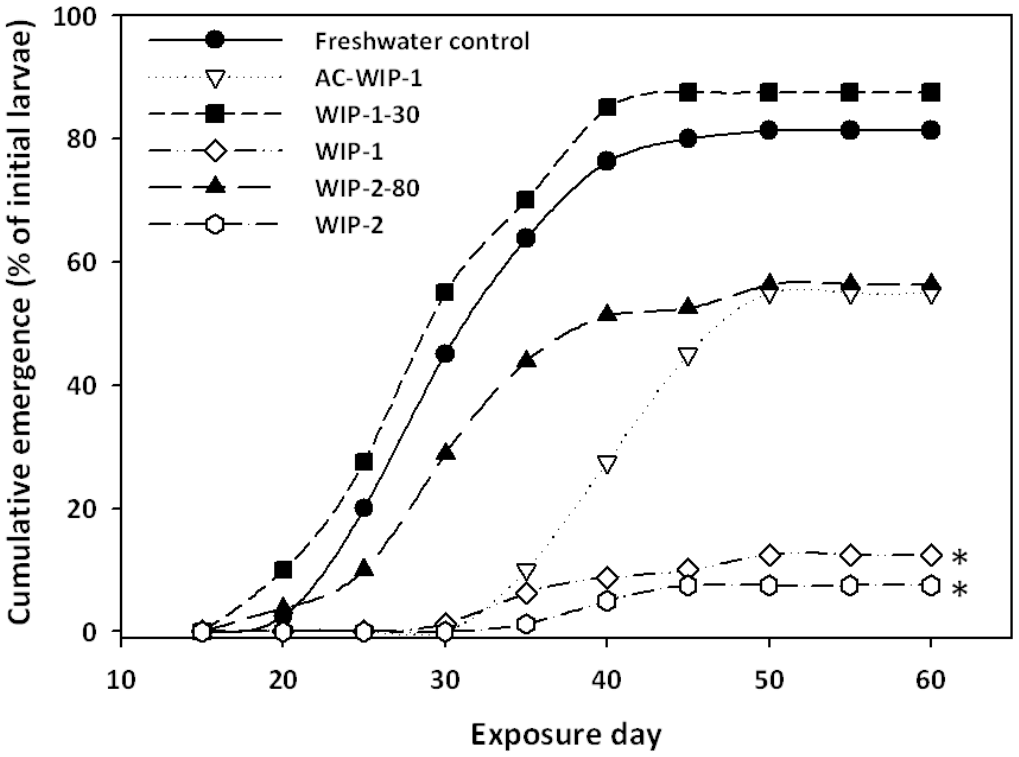


**Figure 4.6:** Cumulative emergence of adult *Chironomus dilutus* midges following chronic exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD post-hoc test (n=4 or 8, α=0.05) and designated by an asterisk. Measures of variance were omitted from the figure for clarity.

## 4.4 Discussion

Ozone is most typically applied as a treatment for municipal drinking water or wastewater, as opposed to industrial process water, and ozonation generally results in transformation products with lesser toxic potency than their parent compounds (Escher *et al.,* 2009). The popularity of ozone for municipal systems stems from its disinfecting properties, in addition to its ability to oxidize ammonia, react with and destroy cyanide and a suite of toxic organic compounds, and precipitate many metals and metal complexes (Ball *et al.,* 1997). Specific improvements in water quality and biological indices observed following treatment of wastewater with ozone included less baseline toxicity, less estrogenic potency, and less acetylcholinesterase (AChE) inhibition (Escher *et al.,* 2009; Stalter *et al.,* 2010). Application of ozone to lead/zinc mine effluent significantly improved survival of *C. dubia* and fathead minnow (*Pimephales promelas*) from 0 to 100% in 48- and 96-hr tests, respectively (Ball *et al.,* 1997). While it is possible for oxidative products of ozonation to have detrimental effects (Stalter *et al.,* 2010), application of ozone to industrial and sewage effluents can result in significant improvements in health and survival of aquatic organisms downstream (Escher *et al.,* 2009; Stalter *et al.,* 2010). For OSPW, ozonation at the applied doses significantly improved survival, growth, pupation, and emergence of *C. dilutus* exposed during both a short and chronic exposure.

### *4.4.1 Acute exposure*

Growth, development, and reproduction endpoints have been well documented as sensitive and capable of providing early warnings of perturbations with potential for

consequences at the population and community level (Azevedo-Pereira *et al.,* 2010; Giesy and Hoke, 1989; Langer-Jaesrich *et al.,* 2010; Sibley *et al.,* 1997). Lesser growth, such as that observed in this study when larvae were exposed to untreated OSPW, is consistent with that observed in both lab and field-reared *C. dilutus* exposed to untreated OSPW during larval development (Whelly, 1999). The effects on growth observed in previous studies were not as prevalent or extreme in the field-reared population compared to the lab-reared population of *C. dilutus,* and less sensitivity (in terms of inhibition of growth) was observed in *C. riparius* than in *C. dilutus* (Whelly, 1999). Impaired growth can reduce fecundity and reproductive success, which results in potential population collapse if reproduction is sufficiently suppressed (Sibley *et al.,* 1997). Inhibition of growth could potentially have population and ecosystem-level implications since chironomids generally represent an essential component of the aquatic food web in the Athabasca oil sands region (Bendell-Young *et al.,* 2000). However, field surveys of benthic invertebrates would be required to confirm whether population-level effects were occurring in natural populations.

Past work has led to predictive tools for determining how growth of individual *C. dilutus* larvae will affect proliferation of future generations. Growth reductions of 64% or greater caused delayed emergence, less adult emergence, and shifts in male:female ratios, while 90% inhibition of growth of chironomid larvae was predictive of greater lethality in the long term (Liber *et al.,* 1996). Additionally, it has been determined that a minimum larval dry mass of 0.5-0.6 mg is required for successful emergence (Sibley *et al.,* 1997). Using a relationship between wet and dry mass established in our lab with 3$^{rd}$ instar larvae of $y = 0.1186x - 0.1504$ (where y= dry mass and x= wet mass, $R^2 = 0.9995$),

an approximate fresh mass of 5.5 mg would be required for emergence and this mass was not achieved by individuals exposed to either WIP-1 or WIP-2. Based on the observed inhibition of growth, there is strong potential for longer-term mortalities and adverse reproductive effects in larvae exposed to untreated OSPW. Often, toxicants that reduce somatic cell growth and development (i.e. growth) can also negatively affect reproduction and development of gametic tissues (Sibley *et al.*, 1997), but further studies are required to determine whether this is true for *C. dilutus* exposed to OSPW.

Oil sands-derived waters have concentrations of ammonia, which can cause adverse effects on growth and survival (MacKinnon and Boerger, 1986). However, the measured concentrations of between 0.1 and 1.2 mg/L would not have adversely affected growth or survival of *C. dilutus* larvae (Liber *et al.,* 1996), so the observed effects can be attributed to other constituents in the treatments. Exposure to WIP-2 inhibited growth to a greater extent than WIP-1 (79% vs. 64% less mass than controls). Since no inhibition of growth was observed in the saltwater control and growth was significantly greater after ozonation (both WIP-1-30 and WIP-2-80), it is unlikely that salts or other inorganic fractions of OSPW were responsible for the observed toxicity (as these were unchanged by ozonation). Although WIP-2 had a lesser concentration of NAs than WIP-1, there were subtle differences in the profile of NAs (ring number and carbon number) that may explain the differences in inhibition of growth (described in more detail by Wang, 2011). For example, the most common structure in WIP-1 was the group of NAs with C=14 and Z=-6, while in WIP-2, the most common structure was C=13 and Z=-4. Also, in WIP-1, NAs with C>18 comprised ~1.4% of total NAs and, in WIP-2, this group represented ~2.3% of the total concentration of NAs. The $EC_{20}$ values for WIP-1 and WIP-2, as

determined by use of the Microtox® test, were 16.7 and 11.5 % of full strength OSPW (Wang, 2011), which corresponds to the greater inhibition of growth and lesser pupation and emergence in WIP-2 than WIP-1 reported here. Ozonation decreased concentrations of NAs in both WIP-1 and WIP-2 and resulted in attenuation of effects on both survival and growth of *C. dilutus*. This result is consistent with the hypothesis that the toxicity of OSPW is dependent not only on the total concentration of NAs, but also on the relative concentrations of specific NAs.

Changes in case building and case occupation were observed and quantified among treatments. Larval cases of chironomids are important because they provide protection from predators and possibly contaminants, are involved in respiratory functions, and provide a food source for developing larvae (Chaloner and Wotton, 1996; Halper *et al.,* 2002). Building of cases begins during the first instar (Armitage *et al.,* 1995) and disruption of this activity could represent significant potential for less survival and subsequent population dynamics. Building of cases is an energetically expensive process requiring scavenging for building materials plus input in the form of silk production. Silks produced by insects have relatively great protein content and as many as 15 different proteins can be produced by chironomid silk glands (Sehnal and Sutherland, 2008). External stressors or metabolic demands can diminish the energy stores available for this process (Mondy *et al.,* 2011; Sehnal and Sutherland, 2008). The smaller size and lesser structural integrity of cases built by larvae exposed to OSPW are consistent with the expected effects of a general stressor and associated energy demands. Altered case production was also observed in ozonated-OSPW, which suggests that constituents of OSPW that are unchanged by ozonation might be responsible, or that

ozonation failed to reduce the NAs to concentrations that would have no effect on the building of cases. Further studies are needed to examine the exact mechanisms for the observed impact of WIP-OSPW on building of cases.

In addition to differences in the structure of cases themselves, there were also changes in the way that larvae used cases, which might be indicative of the functionality of the structures. Exposure to chemical stressors, such as chloramines, caused significant increases in the proportion of *Chironomus luridus* that deserted their cases *(*Halpern *et al.,* 2002). It is possible that stress associated with exposure to untreated OSPW caused the larvae to abandon their cases and remain upon the substratum during the initial few days of exposure. Larval chironomids are prey for a number of aquatic organisms and individuals who regularly inhabit cases are less likely to be subject to predation than those that rarely or never occupy cases. In fact, time spent outside the protection of a case has been observed to be a significant predictor of rates of predation (Hershey, 1987). Based on the patterns of behavior over the course of the acute exposure, chironomids in a reclamation wetland containing untreated OSPW could suffer greater predation.

### *4.4.2 Chronic exposure*

The slightly greater survival observed during the first ten days of the chronic exposure study compared to the 10-day acute exposure might be attributed to the fact that individuals were not removed from their cases for the chronic exposure and therefore, were not required to rebuild their cases. Case building uses energy and protein, both of which could affect the size of the larvae and the rate with which they attain a size sufficient to pupate and emerge. Deficits accrued during larval instars could also affect

reproductive fitness of adults. For chironomids that construct tightly woven cases, destruction of cases can result in reduced survival, delayed emergence, smaller oocytes, and lesser protein and lipid content in egg yolks (McKie, 2004; Mondy *et al.,* 2011).

As predicted by the inhibition of growth observed following the acute exposure, chronic exposure to untreated OSPW resulted in significantly lesser rates of pupation and emergence. Less growth and emergence has been observed in *C. riparius* and *C. dilutus* when exposed to other toxicants, such as thiaclorpid (Langer-Jaesrich *et al.,* 2010), 17α-ethinylestradiol (EE2) (Dussault *et al*., 2008) and mercury (Azevedo-Pereira and Soares, 2010). Emergence in all treatment groups followed the expected pattern of males emerging several days prior to females. There were no differences in emergence of males and females among treatments, but detection of differences was limited in untreated WIP-OSPW due to lesser rates of emergence. Stressors, such as reduced food availability, tend to result in a greater proportion of males, since females emerge later and have greater requirements for energy (Liber *et al*., 1996). However, in the studies described here, there were replicates in both WIP-1 and WIP-2 that produced either zero males or zero females, so sex ratio is not a reliable indicator of exposure to WIP-OSPW.

Pupation success was significantly less in WIP-2 than WIP-1, and the rate of emergence was also less (though not statistically significant), possibly due to inherent differences in toxicity between different batches of OSPW with different profiles of NAs. While there was less pupation and adult emergence in the AC-WIP-1 relative to the freshwater control, these were not statistically significant so it is unlikely that the effects of untreated WIP-OSPW treatments can be attributed to salinity or other inorganic constituents. Nearly the entire organic fraction was removed from WIP-OSPW by treatment with activated charcoal and the response of the chronically exposed larvae was not significantly different from that of larvae exposed to the freshwater control. Similarly, ozonation of both WIP-1 and WIP-2 attenuated effects, such that pupation and emergence

of adults were not significantly different between *C. dilutus* exposed to the freshwater control and either the WIP-1-30 or WIP-2-80 treatment. Ozonation, both in this study and others, reduced the overall concentration of NAs in OSPW, as well as shifted the proportion of larger and smaller molecular weight NAs and oxidized-NAs (Martin *et al.,* 2010; Wang, 2011). As previously suggested for other organisms, these results are consistent with the organic components of OSPW, including NAs, being the major toxicants to *C. dilutus (*Herman *et al*., 1994; Rogers *et al*., 2002).

In summary, exposure to untreated OSPW causes toxicity in a model invertebrate, *C. dilutus*, in both short-term and long-term exposures. The predominant response was inhibition of growth of larvae, as measured by increase in mass, which then resulted in significantly impaired emergence of adults. A sufficient impairment of adult emergence, and associated reproductive output, paired with increased susceptibility to predation due to changes in behavior, could have population-level implications for chironomids in reclaimed wetlands if these results occur *in situ*. Treatment of OSPW with ozone attenuated effects on survival, growth, pupation, and emergence, and should therefore be considered as part of a series of treatment options for OSPW prior to release. Additional information is required to determine concentrations of ozone to minimize toxicity and prevent unintended side effects caused by residual oxidation products, as well as to better understand the way in which different components of OSPW react with ozone.

# CHAPTER 5:
# Discussion

## 5.1 General discussion

Surface mining operations in the oil sands region of Alberta have produced over one billion cubic metres of oil sands process-affected water (OSPW) (Del Rio *et al.,* 2006; Government of Alberta, 2011b) that must be treated or remediated prior to release in order to protect downstream aquatic organisms. Currently, companies are prevented from discharging into local freshwater bodies by environmental legislation and the OSPW is held within active settling basins on the companies' lease sites (Government of Alberta, 2008; Madill *et al*., 2001). The OSPW is known to contain organic acids, including naphthenic acids (NAs), which have been suggested as the primary cause of the toxicity observed in fish and other organisms (e.g. Gentes *et al.,* 2007; Kavanagh *et al.,* 2011; Nero *et al.,* 2006b). However, due to the complex nature of the organic fraction of OSPW and the limitations of current analytical tools, the specific NAs or organic compounds contributing to toxicity have yet to be identified and isolated.

Some work has been conducted on the effects of fresh and aged OSPW on fish, but only limited characterization of OSPW toxicity to invertebrates has been completed (e.g. Whelly, 1999). The work presented in this thesis helps fill an important niche by examining both the acute and chronic effects of OSPW on a model benthic invertebrate, *Chironomus dilutus.* Chironomids are ubiquitous organisms that comprise a large portion of the aquatic invertebrate biomass within the Athabasca region (Bendell-Young *et al.,* 2000) and are sensitive to a variety of toxicants while still displaying tolerance to some poor environmental conditions (Armitage, 1995). These traits made them an appropriate study organism for the research carried out for this thesis and allowed achievement of the objectives outlined in Chapter 1.

In addition to assessing the effects of OSPW itself, this research examined the effectiveness of biodegradation and ozonation as potential OSPW treatment options for oil sands companies. The first key observation was that exposure to relatively fresh OSPW caused impaired growth and emergence in chironomid larvae. Secondly, while aging of OSPW attenuated the majority of growth inhibition effects, pupation and emergence were still negatively impacted by exposure to aged OSPW. Finally, ozonation effectively attenuated the effects of exposure to OSPW on chironomid growth, pupation, and emergence and represents a promising treatment method for the large volumes of OSPW requiring reclamation.

## 5.2 Effects of OSPW on *C. dilutus*

### *5.2.1 Effects of fresh OSPW*

Untreated OSPW from West In-Pit (WIP) represented relatively fresh OSPW with a relatively great concentration of NAs and was used in the research described in chapters 2 and 4 to characterize effects of exposure to untreated OSPW. Following short-term (10 d) exposure to WIP-OSPW, chironomid larvae had significantly less fresh mass than controls. When the exposure duration was extended until adult emergence, both pupation and emergence were significantly impaired and delayed. These findings agree with those from Whelly (1999) where field and lab-derived chironomid populations had impaired survival, growth, and development following rearing in OSPW.

Additionally, preliminary observations were conducted on larval behavior and case building after pilot studies suggested that these endpoints were important. Behavior of WIP-OSPW-exposed larvae was significantly different from freshwater control larvae

on several days during the short-term exposure period. However, more work is required to elucidate the reasons for differences in the frequency of observation outside cases and body position relative to cases. As well, the cases built by larvae exposed to WIP-OSPW were very small and fragile compared to cases built by freshwater control larvae. Again, the cause of these differences was not apparent, but could potentially be related to impaired energy metabolism or interference with silk production.

### *5.2.2 Effects of aged OSPW*

Aging or natural biodegradation occurs while OSPW is retained in active settling basins and allowed to be acted upon by natural degradative processes in order to reduce toxicity. There are several experimental reclamation ponds on mining companies' lease sites that employ different strategies for addressing the process-affected water and tailings. For the work presented in Chapter 2, water was taken from three different reclamation ponds on the Syncrude Canada Ltd. lease site– Big Pit, FE5, and TPW. TPW had the greatest total concentration of NAs and was comprised of OSPW aged since 1993. FE5 had the least concentration of NAs of the waters used in this research and was comprised of mature fine tailings (MFTs) capped with OSPW in 1989. Big Pit had an intermediate concentration of NAs and was created in 1993 by capping MFTs with freshwater.

*Chironomus dilutus* larvae that were exposed to aged OSPW showed no differences in survival compared to freshwater controls. Larvae exposed to Big Pit and TPW waters were smaller than the control larvae, but were significantly larger than those larvae exposed to WIP-OSPW. Cases constructed by larvae exposed to Big Pit or TPW

water were smaller and less intact than the freshwater control cases, and the cases built by FE5-exposed larvae were smaller and more delicate still, suggesting that aging did not attenuate effects on case building activities.

Following chronic exposure of *C. dilutus* to these reclamation pond waters, there was significantly less pupation and emergence in TPW-exposed animals and emergence was delayed. These findings were comparable to those from Kavanagh *et al.* (2011) where the researchers observed impaired reproduction in fathead minnow following exposure to aged OSPW and concluded that aging did not completely eliminate toxicity. In the study presented in Chapter 2, exposure to FE5 and Big Pit waters did not result in significantly impaired pupation or emergence. However, pupation and adult emergence was less in all aged waters compared to the freshwater control and a larger sample size may have been required to detect differences statistically. A follow-up reproductive study with multiple generations would help provide further information on the effects of exposure to OSPW since the results presented in this thesis suggest that there may be reproductive consequences.

### *5.2.3 Gene expression*

Exposure of *C. dilutus* larvae for 7 days to either aged and fresh OSPW resulted in patterns of gene expression that were significantly different from those of larvae exposed to freshwater. Changes were observed in the expression of hemoglobins, estrogen-related receptor, ultraspiracle protein, and ribosomal protein genes. The abundances of transcripts of hemoglobins were significantly different in FE5-exposed larvae compared to WIP-OSPW-exposed larvae, while the abundances of the endocrine-

related receptors and ribosomal protein genes were similar between the two treatments. These results suggest that aged and fresh OSPW may be exerting toxicity via different mechanisms. Further studies of transcriptional abundance for these and other metabolic and endocrine-related genes would be beneficial in elucidating the exact mechanisms of toxicity and potentially identifying biomarkers of exposure and effect for OSPW.

### 5.3 Effects of ozonation

Ozonation is a water treatment method often used in municipal and industrial water treatment (Camel and Bermond, 1998) and since ozonation has been shown to deplete NAs concentrations and toxicity, it has been suggested as a potential option for eliminating toxicity of OSPW (Martin *et al.,* 2010; Perez-Estrada *et al.,* 2011; Scott *et al.,* 2008). It has also been reported that ozonation can target the more recalcitrant fractions of NAs that are typically inaccessible for biodegradation and persist even with extended natural aging periods. Therefore, ozonation could be a complementary treatment to biodegradation (Martin *et al.,* 2010; Perez-Estrada *et al.,* 2011). Scott *et al.* (2008) observed 95% depletion of NAs following just 130 min. of ozonation. The WIP-OSPW used for the work presented in Chapter 4 was ozonated using either 30 or 80 mg $O_3$/L , which resulted in 49% and 90% reductions in total NAs concentrations, respectively.

Exposure to ozonated-OSPW did not result in any significant effects on chironomid survival, pupation, or emergence compared to freshwater control larvae. However, larvae exposed to ozonated-OSPW had significantly less mass than the controls (20-30% smaller), but were still significantly larger than their counterparts exposed to untreated OSPW. Ozonation failed to eliminate effects on larval case structural integrity,

as animals exposed to both untreated WIP-OSPW and ozonated-OSPW built cases that were smaller than the controls and very fragile. However, larvae exposed to ozonated-OSPW did not differ from the control larvae in terms of body positioning relative to cases in the behavioral observations. Overall, ozonation of OSPW led to attenuation of the majority of the observed toxicity of OSPW and improved nearly all of the biological endpoints examined in this study.

### 5.4 Water chemistry

Oil sands process-affected water is typically quite saline and alkaline, and contains a complex mixture of inorganic and organic compounds (Holowenko *et al.*, 2002; Rowland *et al.,* 2011a). In Chapter 2, a multi-element analysis was completed using IC and ICP-MS in order to characterize the water used for the exposure assays and to attempt to identify the specific constituents that should be targeted by treatment methods in order to eliminate toxicity of OSPW. Several elements (Mn, Ni, U) were strongly, negatively correlated with survival, growth, pupation, and emergence of *C. dilutus*. In addition, total NAs concentrations were also significantly correlated with each of the endpoints assessed and are likely contributing to overall toxicity of the mixture.

In both Chapters 2 and 4, an activated charcoal treatment was used to remove much of the organic fraction of WIP-OSPW in order to assess the contribution of organic compounds to OSPW toxicity. Following such removal, there were no differences in *C. dilutus* survival, growth, pupation, and emergence rates between AC-treated OSPW and freshwater. However, delayed emergence in males was observed in AC-treated OSPW. These results suggest that an organic fraction of OSPW is likely the major driver of

toxicity, but there may be contributions and interactions with other contaminants, including metals, in the water.

Similarly, ozonation of OSPW did not result in considerable changes to water chemistry characteristics, including pH, total hardness, alkalinity, and concentrations of major ions and trace elements. One exception was uranium, which surpassed water quality guidelines in both batches of WIP-OSPW tested, but was measured at considerably lesser concentrations in ozonated, aged, and activated charcoal treated OSPW. Uranium has been shown to cause less overall growth, less survival, and delayed development in chironomids (Dias *et al.,* 2008; Muscatello and Liber, 2009), and was correlated with reduced *C. dilutus* survival, growth, pupation, and emergence in the study presented in Chapter 2. It is possible that uranium was contributing to the toxicity of OSPW and there are naturally-occurring deposits located within the Athabasca Basin (Alberta Geological Survey, 2011) so elevated uranium concentrations are not unexpected within OSPW.

Preliminary analyses of the NAs profiles of WIP-OSPW were conducted using UPLC-HRMS prior to and following ozonation (Wang, 2011). There were distinct differences between the two batches of OSPW in the degree of toxicity observed, but the NA profiles and total NAs concentrations were only subtly different from each other. It has been suggested that greater molecular weight NAs (greater than 22 carbons) are more persistent in the environment (Holowenko *et al.,* 2002) and that the number of rings and carboxylation of the NAs structure may affect toxicity by changing the degree to which the compounds can cross cell membranes (Frank *et al.,* 2009). Use of some of the emerging analyticcal techniques could hopefully help to separate NAs into more discrete

groupings and further aid in identifying which compounds should be targeted for treatment.

### 5.5 Future research

As there had been little work conducted previously on the effects of oil sands process-affected water on benthic invertebrates, especially *Chironomus* spp., the research presented in this thesis represents a basis for future studies to build upon. The results of the studies described in Chapters 2, 3, and 4 suggest that the organic fraction of OSPW is likely the major driver of toxicity, but due to the complexity of the mixture, it is not possible to confirm which components are most responsible at this time. As analytical techniques improve and it becomes possible to better fractionate NAs into more discrete clusters, the methods described in the previous chapters could be applied to determine which fractions are of the greatest concern in a future release scenario. Different approaches could also be used to remove organic or inorganic fractions prior to testing as only an activated charcoal treatment and a reconstituted salt control were used in the assays described in Chapters 2 and 4.

Future experiments could use dry body masses to make these studies more comparable to others that do not preserve the tissues for subsequent analyses since this was one comment brought up by reviewers and conference participants. Additionally, there are a number of questions raised by the results of the case building and occupation observations that could be followed up upon to determine why the behavior is being impacted by exposure to both ozonated and untreated OSPW.

One of the most important gaps not addressed satisfactorily by this research was the effect of OSPW exposure on *C. dilutus* reproduction. There was at least some degree of pupation and emergence in all treatment waters and, while there have not been sufficient field surveys for confirmation, presumably the chironomid populations found within the oil sands region are able to successfully propagate. However, given the severity of the growth, behavioral, and emergence effects observed here, the evidence suggests that reproductive impairment may be a legitimate concern for these organisms and potentially other benthic invertebrates. While a reproductive study was considered, the logistics of providing an out-breeding population over the extended period of emergence involved in the chronic exposures proved to be too much of a logistical challenge given the scope of this project. Prior to wide-scale ozonation of OSPW, a reproductive assay should be conducted to confirm that residual ozone would not interfere with hormone production or function and to complete the characterization of OSPW toxicity in *C. dilutus*.

Since sensitivities can vary between species and between laboratory and field populations, additional research could be conducted to evaluate if the results observed in *C. dilutus* are representative of effects seen in other benthic invertebrate species. Also, field trials could be used to better understand the population dynamics for chironomid lineages that have colonized the oil sands region and thus may be better adapted to the conditions of that area than the lab-reared animals used for the studies conducted here. Additional research could focus on further investigating the toxicity of OSPW in *C. dilutus* at a genomic level in order to elucidate the mechanisms of toxicity and determine

whether treatment methods, such as ozonation, can effectively attenuate toxicity without risk of toxicity from ozonation byproducts.

### 5.6 Conclusions

In the chapters of this thesis, the toxicity of OSPW to *Chironomus dilutus* was shown to manifest primarily as impaired growth and emergence. Passive aging can reduce NAs concentrations, but NAs degradation occurs slowly and does not effectively eliminate all chronic toxicity (since impacts on pupation and emergence can persist). Active ozonation of OSPW has shown promise in eliminating or significantly reducing toxicity of OSPW to *C. dilutus.* While further research on the effects of ozonation would be prudent prior to full-scale use as a treatment method, ozonation has been shown in this research to be a promising option for eliminating the toxicity of OSPW.

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