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Effectiveness of Ozonation Treatment in Eliminating Toxicity of Oil Sands Process-Affected Water to *Chironomus dilutus*

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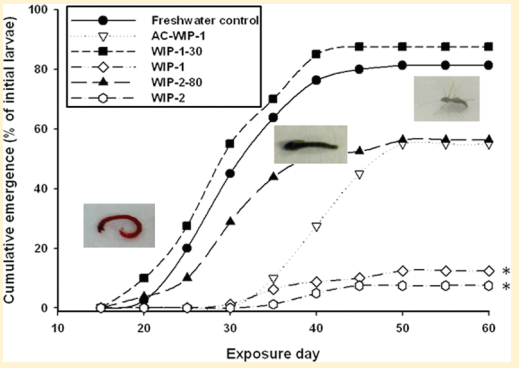
Supporting Information

ABSTRACT: Water soluble organic compounds (OCs), including naphthenic acids (NAs), are potentially toxic constituents of oil sands process-affected water (OSPW) that is generated during extraction of bitumen from Alberta oil sands. Ozonation can decrease concentrations of OCs in OSPW. However, effects of ozonated-OSPW on multicellular organisms are unknown. A 10-day and a chronic exposure of *Chironomus dilutus* to OSPW were conducted to assess effects on survival, growth, development, and behavior. Two separate batches of OSPW were treated with 30 or 80 mg ozone (O_3)/L. Wet body masses of larvae exposed to OSPW were 64 to 77% less than their respective controls ($p < 0.001$). However, both levels of ozonation significantly attenuated effects of OSPW on growth. 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 the two batches of OSPW ($p < 0.05$). Emergence was significantly less for larvae exposed to OSPW, with 13% and 8% of larvae emerging, compared to 81% in controls ($p < 0.0001$). Both levels of ozonation of OSPW attenuated effects on emergence. These results suggest that OCs degraded by ozonation causes toxicity of OSPW toward *C. dilutus,* and that ozonation attenuates toxicity of OSPW.



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.[1,2] 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 tailings ponds.[3,4] OSPW is alkaline, saline, and produced at a rate of up to 4 m^3 per cubic meter of oil sands processed,[5] 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 m^3 of OSPW are held in active settling basins[6] 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).[7] 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.[5,8,9] NAs with more rings tend to be more persistent in the

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environment.[8,9,12,13] It has been estimated that there are potentially more than 200,000 individual NA structures associated with oil sands.[10] 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.[4,8,11]

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 attenuation alone does not seem sufficient to treat the volumes of OSPW currently in containment.[5,11] 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 numbers of rings, which tend to be more resistant to biodegradation.[13] Ozonation, which reduces the total concentration of NAs and changes the relative proportions of the different fractions of NAs in OSPW,[13] also reduces toxicity as measured by the Microtox assay[14] and attenuates endocrine disrupting effects on eukaryotic cells *in vitro*.[15,16]

Toxicity of NAs and OSPW toward benthic invertebrates has not been extensively characterized, but sensitivities vary among taxa and between laboratory and field conditions.[17] Midges represent a group of ubiquitous, ecologically significant freshwater organisms[18] 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,[19] 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.[20,21] 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.

2. MATERIALS AND METHODS

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 h light:dark regime. Prior to test initiation, larvae were kept in control freshwater, which was Saskatoon, SK, municipal water that was carbon filtered, biofiltered, and aerated for 24 h prior to placement into aquaria or test vessels.

2.2. Exposure Waters. Two separate batches of OSPW were collected from the West In-Pit (WIP), an active settling basin located on the Syncrude Canada Ltd. lease site (near Fort McMurray, AB). The WIP-OSPW represents fresh, untreated process water that is regularly fed from the main extraction plant (as described by Han et al.[22]). The first batch of WIP-OSPW was collected in summer 2009 and is designated as WIP-1. The second batch of WIP-OSPW was collected in winter 2010 and is designated as WIP-2. The WIP-1 was treated with 30 mg/L ozone and is designated as WIP-1-30. The WIP-2 was treated with 80 mg/L ozone and is designated as WIP-2-80. Ozonation of WIP-OSPW was conducted at the University of Alberta (Edmonton, AB) and is described in more detail in the Supporting Information and elsewhere.[23] *C. 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 8.1 ± 0.3 mg/L, conductivity 419 ± 31 μS/cm, pH 8.35 ± 0.20, alkalinity 88 ± 7 mg/L as $CaCO_3$, and hardness 123 ± 12 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 2000 and 2500 mg/L, with sodium, bicarbonate, chloride, and sulfate the dominant ions.[24,25] 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 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 h 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 concentration 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.[13] 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 of 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 of O_3/L.[23] Treatment of WIP-1 with activated carbon had no measurable effect on the concentrations of inorganic compounds (data not shown).

2.3. Acute Exposure. *2.3.1. Effects on Survival and Growth.* The experiment was conducted under the same environmental conditions as described in Section 2.1. Effects of acute exposure to the treatments were assessed by use of a modified 10-day static-renewal assay with end points 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 × 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) were 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 exposure 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 end point was added. Representative images showing the gross structure of these cases were photographed at 18 to 22× magnification using an Olympus SZ61 zoom stereo microscope equipped with a Q-Color 5 Olympus digital camera (Olympus America Inc., Pennsylvania, PA).

2.3.2. Effects on Behavior. Behavior of larvae was assessed over the course of the 10-day exposure. To do so, observations of behavior were made three times daily. The measurement end point was amount of activity, as determined by body position relative to its case and frequency of observation outside the case. Details of the scoring methodology are provided in the Supporting Information.

2.4. Chronic Exposure. To assess effects of chronic exposure to these same treatments, ten larvae (8−9 days posthatch) were randomly assigned to each of four replicate 1-L 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. End points assessed included survival, pupation success, 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 for the acute experiment. 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.

2.5. Statistical Analysis. All statistical analyses were conducted using SYSTAT (version 12, Systat Software, Inc.). The experimental unit was the test beaker. Statistical differences were assessed by one-way ANOVA followed by Tukey's posthoc pairwise 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 posthoc test. Data with unequal variances were assessed by Games-Howell posthoc pairwise comparisons. All data are presented as mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$.

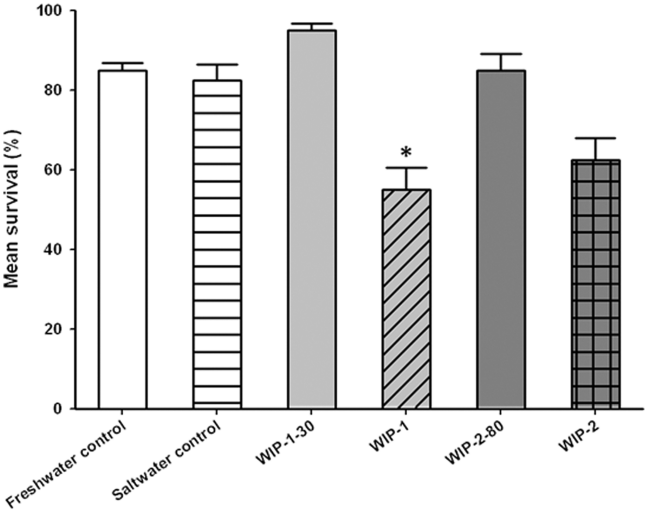

Figure 1. Mean (±SEM) survival of *C. dilutus* larvae following 10-day exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined using a one-way ANOVA followed by Tukey's HSD posthoc test ($n = 4$, $\alpha = 0.05$) and designated by an asterisk.

3. RESULTS

3.1. Acute Exposure. *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 S1. 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. Mean 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 as follows: 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.

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.[26] 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 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.

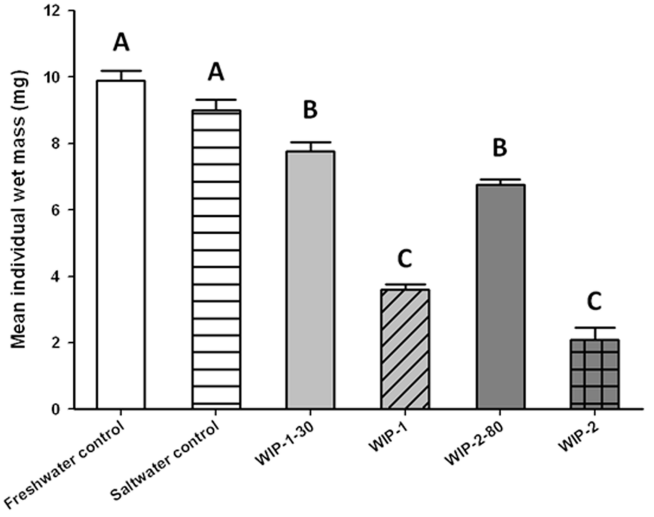

Figure 2. Mean (±SEM) individual wet mass of *C. dilutus* larvae following 10-day exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD posthoc test ($n = 4$, $\alpha = 0.05$) and designated by different letters.

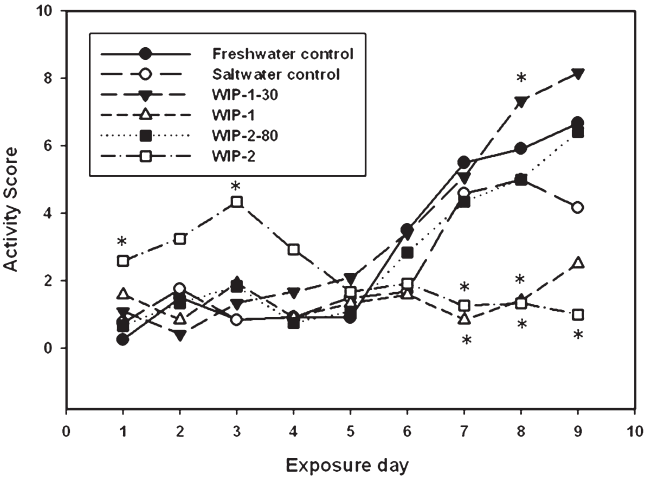

Figure 3. Trends in daily activity of *C. dilutus* larvae over the course of a 10-day exposure to ozonated or untreated OSPW. Mean scores were determined based on three daily observations made at 11:00, 13:00, and 15:00 h. Significant differences from the freshwater control are indicated by an asterisk ($\alpha = 0.05$).

Inhibition of growth was observed following exposure to untreated OSPW (Figure 2). *Chironomus dilutus* exposed to WIP-1 and WIP-2 had mean body masses that were 64% and 79% less, respectively, relative to the freshwater control following the 10-day exposure period ($p < 0.001$). The masses of larvae exposed to ozonated-OSPW were also significantly less than those of 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 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 ($p < 0.01$).

3.1.3. Effects of OSPW on Case Building and Behavior. There were differences observed in the quality of recovered larval cases between the freshwater controls and the other treatment groups. *Chironomus dilutus* larvae exposed to freshwater built intact cases which 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 or 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 images showing the structure of the cases from each of the treatment groups are presented in Figure S1.

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 3. For the first three days of the exposure 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 ($p < 0.05$). Activity in the saltwater control, WIP-1-30 and WIP-2-80 treatments did not differ significantly from that of the freshwater controls, except on Days 5 and 8 when larvae in WIP-1-30 were significantly more active than those in the freshwater control ($p < 0.05$).

3.2. Chronic Exposure. *3.2.1. Pupation and Emergence.* Significant differences in rates of pupation and time of emergence were observed among treatments (Table S2). 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 30 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 rate in the freshwater control (Figure 4). The rate of pupation in the AC-WIP-1 was 78% which was also not significantly different from the rate of larvae in the freshwater control.

The rate of emergence of adults was significantly affected by exposure of larvae to 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 control, by an average of 11.4 days in WIP-1 and 9.7 d in WIP-2 ($p < 0.01$) (Table S2). Time-to-emergence in the AC-treated OSPW 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 beakers containing WIP-2. There were no significant differences in sex ratio among any of the treatment groups with ratios of males to females ranging from 0.67:1 in WIP-1 to 1.24:1 in the freshwater control. However, significantly fewer males and females emerged

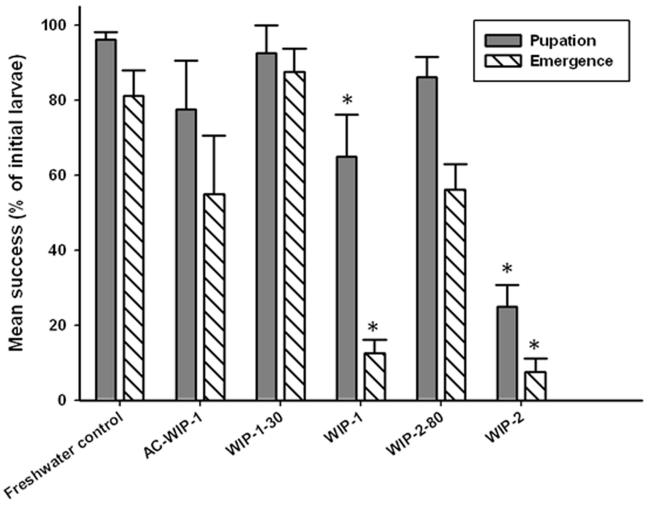

Figure 4. Mean (±SEM) pupation and emergence success rates for *C. dilutus* after chronic exposure to ozonated and untreated OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD posthoc test (n = 4 or 8, α = 0.05) and designated by an asterisk.

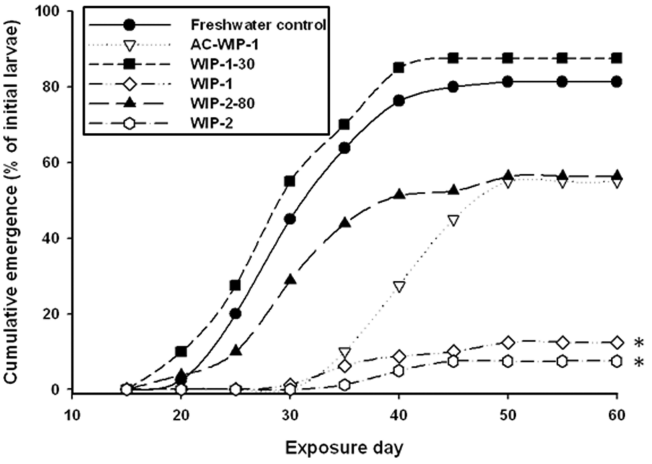

Figure 5. Cumulative emergence of adult *C. dilutus* midges following chronic exposure to ozonated or untreated OSPW. Significant differences from the freshwater control were determined by one-way ANOVA followed by Tukey's HSD posthoc test (n = 4 or 8, α= 0.05) and designated by an asterisk.

in both OSPW treatment groups (Figure S2). The cumulative emergence over the duration of the exposure followed a pattern of delayed emergence in the AC-WIP-1 and emergence was inhibited in untreated OSPW (Figure 5).

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.[27] 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.[28] Specific improvements in water quality and biological indices observed following treatment of wastewater with ozone include less baseline toxicity, less estrogenic potency, and less acetylcholinesterase (AChE) inhibition.[27,29] Application of ozone to lead/zinc mine effluent improved survival of *Ceriodaphnia dubia* and fathead minnow (*Pimephales promelas*) from 0 to 100% in 48- and 96-h tests, respectively.[28] While it is possible for oxidative products of ozonation to have detrimental effects,[29] application of ozone to industrial and sewage effluents can result in significant improvements in health and survival of aquatic organisms downstream. 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.

4.1. Acute Exposure. Growth, development, and reproduction end points have been well documented as sensitive and capable of providing early warnings of perturbations with potential for consequences at the population and community level.[20,21,30,31] 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.[17] 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 growth inhibition) was observed in *C. riparius* than in *C. dilutus*.[17] Impaired growth can reduce fecundity and reproductive success, which results in potential population collapse if reproduction is sufficiently suppressed.[21] 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.[19] 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, lesser emergence success, and shifts in male: female ratios, while 90% inhibition of growth of chironomid larvae was predictive of greater lethality in the long term.[32] Additionally, it has been determined that a minimum larval dry mass of 0.5−0.6 mg is required for successful emergence.[21] Using a relationship between wet and dry mass established in our lab with third 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 growth inhibition, 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,[21] 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.[33] 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,[32] 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 might explain differences in inhibition of growth (described in more detail by Wang, 2011).[23] 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 concentrations 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[23], 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.[34,35] Building of cases begins during the first instar[18] 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.[36] External stressors or metabolic demands can diminish the energy stores available for this process.[36,37] The smaller size and lesser structural integrity of cases built by larvae exposed to OSPW are consistent with the 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 OSPW on building of cases.

In addition to differences in the structures of cases themselves, there were also changes in the way that larvae used cases, which might be indicative of the functionality of cases. Exposure to chemical stressors, such as chloramines, caused significant increases in the proportion of *Chironomus luridus* that deserted their cases.[35] It is possible that stress associated with exposure to untreated OSPW caused the larvae to abandon their cases and remain listlessly upon the substratum during the first few days of the 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.[38] Based on the patterns of behavior over the course of the acute exposure, chironomids in a reclamation pond containing untreated OSPW could suffer greater predation.

4.2. Chronic Exposure. The slightly greater survival observed during the first ten days of the chronic exposure than in 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. Building of cases uses energy and protein, both of which could have effects on the size of the larvae and the rate with which they attain a size sufficient to pupate and emerge. These deficits 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.[37,39]

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. Lesser growth and emergence has been observed in *C. riparius* and *C. dilutus* when exposed to other toxicants, such as thiaclorpid,[31] 17α-ethinylestradiol (EE2),[40] and mercury.[41] 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 larvae exposed to untreated OSPW due to the lesser rates of emergence. Stressors, such as reduced food availability, tend to result in a larger proportion of males since females emerge later and have greater requirements for energy.[32] 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 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 profiles of NAs. While there was pupation and emergence in the AC-WIP-1, relative to the freshwater control, these were not statistically significant so it is unlikely that the effects of the untreated OSPW treatments can be attributed to salinity or other inorganic constituents. Nearly the entire organic fraction was removed from the 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.[13,23] 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*.[4,11]

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 occurr *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 that could be caused by residual oxidation products, as well as

to better understand the way in which different components of OSPW react with ozone.

■ ASSOCIATED CONTENT

Supporting Information. A description of the ozonation of OSPW and the criteria used to assess *C. dilutus* behavior; water chemistry characteristics of ozonated and untreated OSPW and control water samples; a summary of pupation and emergence statistics for *C. dilutus* following chronic exposure to ozonated or untreated OSPW; and photographs of representative *C. dilutus* larval cases removed from each of the treatment groups following the 10-day exposure period. This material is available free of charge via the Internet at http://pubs.acs.org.

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