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Advanced Analytical Mass Spectrometric Techniques and Bioassays to Characterize Untreated and Ozonated Oil Sands Process-Affected Water

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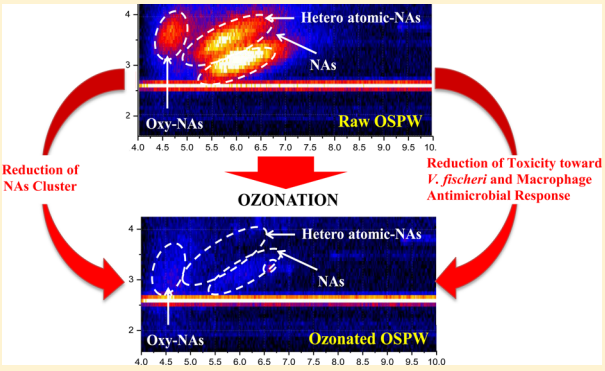
Supporting Information

ABSTRACT: Oil sands process-affected water (OSPW) is a toxic and poorly biodegradable mixture of sand, silt, heavy metals, and organics. In this study, qualitative and quantitative comparisons of naphthenic acids (NAs) were done using ultraperformance liquid chromatography time-of-flight mass spectrometry (UPLC TOF-MS), Fourier transform ion cyclotron resonance (FT-ICR) MS, and ion mobility spectrometry (IMS). The unique combination of these analyses allowed for the determination and correlation of NAs, oxidized NAs, and heteroatom (sulfur or nitrogen) NAs. Despite its lower resolution, UPLC-TOF MS was shown to offer a comparable level of reliability and precision as the high resolution FT-ICR MS. Additionally, the impacts of ozonation (35 mg/L utilized ozone dose) and subsequent NAs degradation on OSPW toxicity were assessed via a collection of organisms and toxicity end points using *Vibrio fischeri* (nonspecific), specific fish macrophage antimicrobial responses, and fish olfactory responses. Fish macrophages exposed to ozonated OSPW for 1 week showed higher production of reactive oxygen and nitrogen intermediates; however, after 12 weeks the responses were reduced significantly. Fish olfactory tests suggested that OSPW interfered with their perception of odorants. Current results indicate that the quantification of NAs species, using novel analytical methods, can be combined with various toxicity methods to assess the efficiency of OSPW treatment processes.



INTRODUCTION

The Athabasca oil sands in northern Alberta, Canada are one of the largest known crude oil reserves in the world.[1] The surface minable bitumen is extracted using large amounts of water that generates oil sands process-affected water (OSPW), which is a complex mixture of suspended solids, salts, inorganic compounds, dissolved organic compounds (naphthenic acids; NAs), and trace metals.[1−6] Recent advances in mass spectrometry including gas chromatography-time-of-flight-mass spectrometry (GC × GC-TOF-MS)[7] and liquid chromatography-orbitrap MS,[8] along with progress in ion-mobility spectrometry (IMS),[9] have allowed for the identification of NAs including aromatic carboxylic acids, tricyclic diamondoid acids, sulfur containing species,[7,10−12] and dicarboxylic acids.[13] Additionally, further elucidation of aromatic sulfur-NAs has recently been reported by West et al.[14] However, despite these advances it is still not possible to completely characterize OSPW which contains thousands of unidentified organic compounds.[10,11,15]

OSPW causes acute, subchronic, and chronic toxicity to a variety of organisms, including fish, amphibians, phytoplankton, and mammals.[16−20] Several toxicity assays for various organisms are currently available. The Microtox assay using *Vibrio fischeri* is fast, reliable, and widely used for the screening of wastewaters including OSPW.[21−23] A disadvantage of this assay is its nonspecificity and inability to extrapolate toxicity to other species. Alternatively, the fish macrophage assay is a relatively new method that can be used to correlate toxic effects, such as immunotoxicity, to other typically tested organisms.[24−26] This assay uses the production of nitrogen or oxygen reactive intermediates in cells to determine toxicity. In addition, organic contaminants such as organophosphate insecticides and surfactants (e.g., the NAs) have been shown to cause olfactory impairment to aquatic organisms.[27] Many contaminants have

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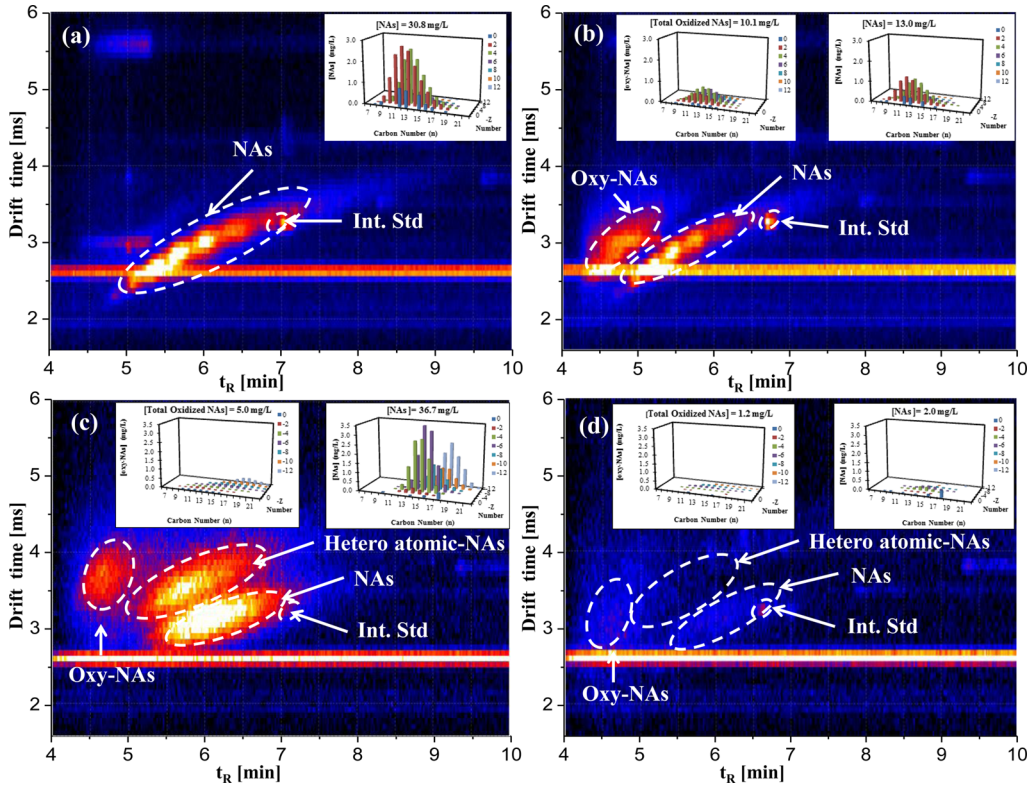

Figure 1. IMS plot of Merichem standard (a); 20 mg/L ozone-treated Merichem standard (b); untreated OSPW (c); and 20 mg/L ozone-treated OSPW (d). Insets are the profiles of NAs species estimated using UPLC-TOF MS.

been tested for their impact on fish olfaction; however, no studies have investigated the potential impact of NAs in OSPW. OSPW contains ringed organic compounds that may possibly affect olfactory tissues by eliciting a temporary or permanent reduction of olfaction function following OSPW exposure. As well, OSPW compounds may mimic naturally occurring biomolecules that can act as odorants causing behavioral changes in fish exposed to OSPW effluents. An advantage of both the macrophage and olfactometry assays is that they can be easily adapted into environmental guidelines. Environment Canada is using rainbow trout as a model organism for toxicity testing[28] and the olfactometry assay in this study used trout as a model organism.

Extensive research has been conducted to develop methods to characterize, decontaminate, and detoxify OSPW.[9,29−31] To address the above-mentioned needs and advance the characterization using analytical and toxicological tools, the aims of this study were as follows:

(1) Validation of the IMS method for qualitative analysis of raw and ozonated OSPWs and assessment of its potential as a screening method. A Merichem NA standard was also assessed which consists of aliphatic and alicyclic NAs with few aromatic NAs and no oxidized or heteroatomic NAs.[10] The Merichem NAs differ from OSPW NAs; however, they allow for the validation of treatment processes and biological and chemical assays.

(2) Correlation of the electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) and ultraperformance liquid chromatography time-of-flight (UPLC-TOF) MS methods for the validation of the UPLC-TOF MS for the quantitative analysis of individual NAs. The majority of OSPW analyses has been done by ultrahigh resolution MS methods,[32,33] so we tested whether UPLC-TOF-MS, given its lower resolution, can be used as reliably as the semiquantitative high resolution FT-ICR MS.

(3) Determination of the speciation of NAs species in Merichem standard and raw/ozonated OSPWs.

(4) Determination of whether raw/ozonated OSPWs can alter the olfactory tissues of fish and act as an odorant.

(5) Examination of the effects of raw/ozonated OSPWs on macrophage antimicrobial responses by measuring their impact on the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) using a flow through exposure apparatus.

These responses were compared with the widely used *Vibrio fischeri* and the novel olfactory data. Although the macrophage assay considering OSPW has already been published by other authors,[24,25] this assay, in combination with the other assays, will help to widen the spectrum on toxicity of untreated and treated OSPWs. To date, this is the only flow-through apparatus system designed to assess the OSPW toxicity.

MATERIALS AND METHODS

Materials and Ozonation Experiments. The experiments were performed using Syncrude West in-Pit OSPW with a pH of 8.4, collected on September 27, 2010. OSPW was stored at 4 °C in a cold room. Experiments were performed in November 2011, resulting in a storage time of 14 months. Merichem NAs mixture was provided by Merichem Chemicals and Refinery Services LLC (Houston, TX, USA) and was recovered from the extractions of petroleum distillates during oil processing. It consists predominantly of aliphatic and low condensed alicyclic NAs and has low amounts of aromatic NAs and no heteroatomic or higher oxidized NAs. OSPW was ozonated using an ozone generator (WEDECO, GSO-40, Herford,

Germany) and following the procedure described elsewhere.[9] After ozonation, the OSPW was purged for 20 min with pure nitrogen to strip residual ozone. A series of thiophene aromatic carboxylic acids were purchased from Sigma (Oakville, ON, Canada) and used without further purification. Figure S1 in the Supporting Information (SI) shows the structures of these carboxylic acids.

ESI FT-ICR MS and UPLC-TOF MS Analyses. Three samples were selected for UPLC-TOF MS and ESI FT-ICR MS analyses: raw OSPW, ozonated OSPW at 20 mg/L utilized ozone dose, and ozonated OSPW at 50 mg/L utilized ozone dose. Details about the sample preparation for analytical analyses can be found in the SI. The ESI FT-ICR MS analyses were conducted using a Bruker 9.4 T Apex-Qe FT-ICR MS (Bruker Daltonics, Billerica, MA) in the negative ion mode (details can be found in the SI). UPLC-TOF MS analyses were conducted using a Synapt G2 HDMS system (Waters, Milford, MA) (40 000 fwhm) that integrates an ion-mobility cell between the ion source and the TOF MS detector. A detailed description of the UPLC-TOF MS method is described in Wang et al.[9] The analysis of the ESI FT-ICR and UPLC-TOF MS data and the molecular formula assignments from TOF data are discussed in the SI.

Toxicity Measurements. Bioassays using *V. fischeri*, acute and subchronic exposure experiments, and RNI and ROI assays have been described elsewhere,[24,25] and detailed information can be found in the SI. Fish olfactory toxicity was conducted using juvenile *Oncorhynchus mykiss*. The experiments were approved by the University of Alberta Animal Care Committee (#7301003) and followed the Canadian Council of Animal Care guidelines (CCAC-Canada). Fish were anesthetized using 150 mg/L MS-222 (Syndel, Nanaimo, BC) buffered five parts to one with sodium bicarbonate. Upon cessation of opercular (gill flap) movements, fish were wrapped in a wet tissue, transferred to a v-shaped holder in a 15 °C water bath, and their gills were perfused with 75 mg/L of the above anesthesia for the duration of the experiments. More details of the fish olfactory toxicity tests can be found in the SI.

RESULTS AND DISCUSSION

Validation and Application of IMS. A two-dimensional (2D) separation can be achieved using IMS after UPLC, resulting in an observable cluster of ions. Considered as a qualitative analysis, IMS provides insight on the structural characteristics of a wide range of chemicals, biomolecules, and polymers.[34,35] To assign NAs-related clusters in OSPW, a 30 mg/L Merichem standard was prepared and then subjected to quantitative and qualitative analyses. The concentrations of NAs (O_2) and oxidized NAs (x = 3, 4, and 5; oxy-NAs) estimated using UPLC-TOF MS were 30.8, 0.8, 0.6, and 0.1 mg/L, respectively, which indicated that the major compounds in the Merichem mixture were NAs (O_2) (SI Figure S3). These results agreed with previous publications, showing that Merichem mixtures only contain minor traces of oxy-NAs.[36]

As shown in Figure 1a, only one cluster could be observed in the Merichem standard before ozonation. Therefore, this cluster was identified to be NAs (O_2). After ozonation with 20 mg/L utilized ozone dose, beside the NAs (O_2) cluster, another cluster showed up at a retention time of 4.4 to 5.3 min and a drift time from 2.5 to 3.5 ms (Figure 1b). The estimated concentrations of NAs (O_2), (O_3), (O_4), and (O_5) oxy-NAs, in the ozonated Merichem mixture were 13.0, 7.1, 2.4, and 0.6 mg/L, respectively, indicating that the amount of oxy-NAs increased after ozonation (SI Figure S3). Therefore, this new cluster present in the IMS spectrum was identified to be oxy-NAs.

To further explore the possibility of identifying different NAs species, an OSPW sample was injected and the IMS plot (Figure 1c) showed three different clusters: NAs, oxy-NAs, and a new cluster with a retention time from 5.0 to 6.6 min and a drift time from 3.0 to 4.0 ms. A closer examination of Figure 1c revealed that ions from the three groups were well separated and NAs (O_2) showed the most intense cluster, indicating that NAs (O_2) were the most abundant species in the untreated OSPW, confirming the results previously reported.[15] Previously, we reported that this new cluster may correspond to heteroatom related NAs such as sulfur-NAs and nitrogenated NAs.[9] The presence of nitrogenated species in OSPW has been previously reported,[8,15,37] with the majority of them detected in positive ionization mode.[15] In the present study, however, only ionization in the negative mode was used to identify and quantify the different NAs species; this may explain the incapacity to detect nitrogenated species in the OSPW samples. To verify the presence of sulfur-NAs, a mixture of thiophene aromatic carboxylic acids were used as sulfur-NAs standards (SI Figure S2a) and an OSPW sample (SI Figure S2b). The retention of the sulfur standards ranged from 4.5 to 5 min, which matched the retention time range of the new cluster, indicating that the ions of the new cluster should have similar polarity to the sulfur-NA standards. On the other hand, the drift time of the sulfur-NA standards was between 2 and 2.5 ms that was much smaller than the drift time registered by the naturally abundant cluster from OSPW. The ion mobility theory indicates that the time ions travel in the drift tube is related to its rotationally averaged cross-section area, which means that compact ions will travel more rapidly and register a shorter drift time, while more branched ions will travel more slowly and register a longer drift time in the drift tube.[34] The fact that all sulfur-NA standards used in the work travel much more rapidly than species naturally abundant in OSPW can be explained by the difference in their structures. Previous studies have pointed out that naturally abundant sulfur or aromatic related NAs have more complex spatial structures[14] compared to the commercially available thiophene aromatic carboxylic acids. Though the IMS results of sulfur-NAs standards cannot verify the compounds related to the new cluster conclusively, combined with the IMS results of Merichem and ozonated Merichem NAs, it has drawn some insights into what kind of compounds are distributed in the area of interest. With the naturally abundant NAs (O_2) and oxy-NAs well separated from the new cluster, it is rational to imply that the new cluster represents NAs with hetero atoms, such as sulfur, which are derived from the bitumen.

After ozonation with 20 mg/L utilized ozone dose, the IMS spectra of ozonated OSPW hardly showed any intense clusters, indicating the degradation of NAs (O_2) and oxy-NAs (Figure 1d). The estimated concentrations of NAs (O_2) and oxy-NAs in OSPW were 2.0 and 1.2 mg/L for 20 mg/L utilized ozone dose, and 0.5 and 0.3 mg/L for 50 mg/L utilized ozone dose, respectively (SI Figure S4). Because of the lack of commercially available standards for sulfur-NAs, their concentrations were not estimated using UPLC-TOF MS. However, the IMS spectrum of ozonated OSPW (Figure 1d) revealed that most of the heteroatom related NAs species were degraded after ozonation.

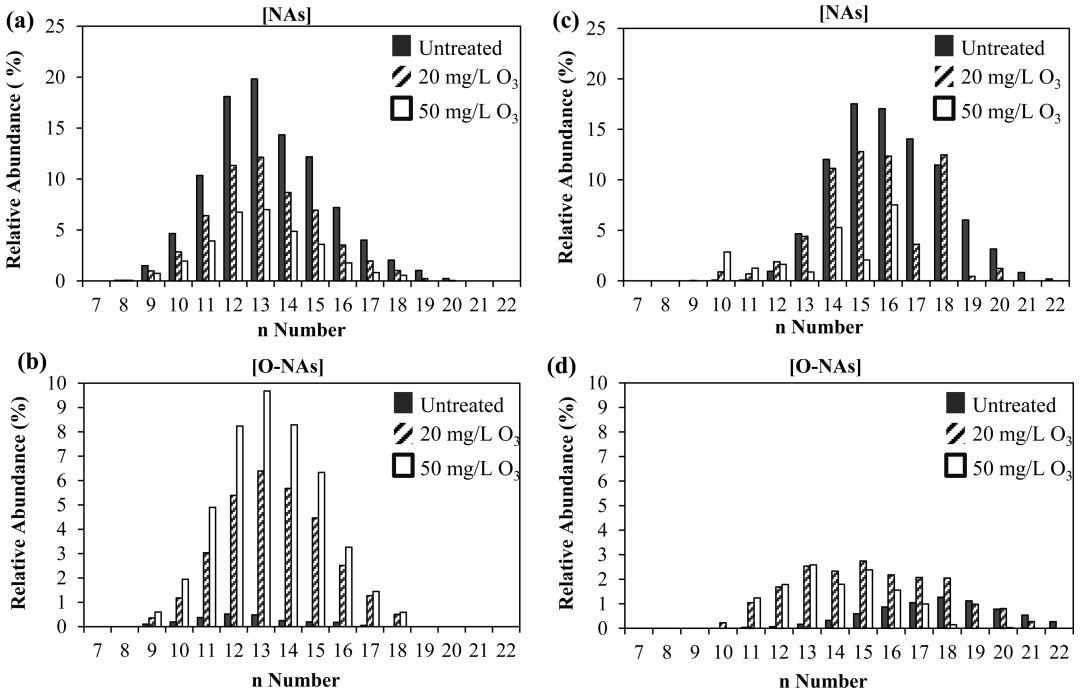

Figure 2. Relative abundance of NAs species in Merichem standard (a, b) and OSPW (c, d) based on the carbon (n) number, estimated using UPLC-TOF MS. Relative abundance of other NAs species (i.e., O_2-NAs and O_3-NAs), as well as abundances based on Z-number, can be found in the Supporting Information.

To test the variability between the different OSPW samples, a second batch of OSPW (batch 2), sampled in the same period, was analyzed using UPLC-TOF MS and IMS. As shown in SI Figure S5, the distribution of NA species changed in comparison to that of the first batch (Figure 1). The concentrations of NAs (O_2) and oxy-NAs in the untreated OSPW (batch 2) were 24.7 and 33.1 mg/L, respectively, indicating that oxy-NAs comprised 57.3% of all the NAs species (SI Figure S6). In batch 1, however, the oxy-NAs comprised 12% of all the NAs species. This suggests that OSPW from batch 2 may have been subjected to biodegradation in tailings ponds, resulting in an increase in the concentration of oxy-NAs species.[38] Although the batches are different, the reproducibility of our method is excellent with a mean standard deviation of NAs quantification generally between 5% and 7%. These results highlight the importance of characterizing the OSPW samples before and after treatment, even if the samples come from the same sampling area and during the same sampling period.

The findings reported in this study support the statement that even though considered as a qualitative analysis, IMS offers informative details on the distribution of organic compounds in a sample solution, and therefore, the use of UPLC-TOF MS combined with IMS separation can be considered to be a powerful semiquantitative method, able to identify different classes of NA species present in untreated and treated OSPWs. IMS combined with MS techniques allows the structural separation of commercial and naturally occurring NAs mixtures, without extensive sample preparation,[9,39] and the identification of the heteroatom-containing hydrocarbons as well as multiple conformational classes without wet chemical prefractionation.[40]

Speciation of Merichem and OSPW NAs Species after Ozonation. The estimated concentration of NAs (O_2) as well as oxy-NAs in the Merichem mixture, OSPW, and ozone-treated samples are shown in SI Figures S3 and S4. The quantification results were consistent with the IMS results. At a 20 mg/L utilized ozone dose, almost all the NAs species in the Merichem and OSPW samples were degraded.

In the untreated Merichem mixtures, the most abundant species were NAs (O_2) with n number ranging from 11 to 16 (Figure 2) and Z number between −2 and −4 (SI Figure S7). In untreated OSPW, the most abundance species were NAs (O_2) with n number ranging from 14 to 20 and Z being −4, −6, and −12. These results are consistent with previous publications that indicate relatively high alkyl branching and high ring number (high Z number) of OSPW NAs compared to Merichem NAs.[41]

After ozonation, an increment of the oxy-NAs was observed in the Merichem mixture (SI Figure S3). In the present study, the O-NAs (i.e., $C_nH_{2n+Z}O_3$) increased 9-fold after ozonation with 20 mg/L utilized ozone dose. This result compares well to a previous publication that reported a 7.7-fold increase in mono-oxidized NAs after ozonation.[36] In OSPW, on the contrary, the oxy-NAs decreased after ozonation. It was reported previously that oxy-NAs are formed after ozonation of OSPW.[36] Lower utilized O_3 doses result in an increase in oxy-NAs; however, they are further degraded at higher utilized O_3 doses. The present study showed that oxy-NAs in OSPW decreased with increasing ozonation, confirming that these compounds were degraded at a faster rate than that at which they were formed.[36] In general, depending on the amount of utilized O_3, an increase of oxy-NAs during the first steps of oxidation can be observed, but they are degraded at higher utilized O_3 doses.

The profiles of NAs (O_2) in the Merichem mixture and OSPW changed after ozonation, demonstrating the difference in reactivity of the different NAs species toward ozone and hydroxyl radicals. After ozonation with 20 mg/L utilized ozone dose, the median of the major species in OSPW shifted to smaller n (12 to 17) and Z numbers (−4 and −6). As the utilized ozone dosed increased to 50 mg/L, further shift to

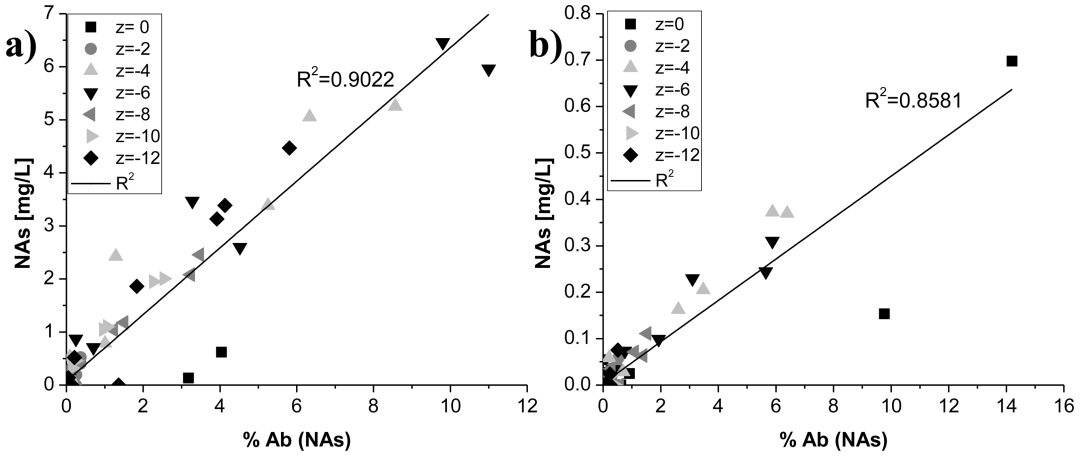

Figure 3. Correlation between %Ab (FT-ICR MS) and concentration of NAs (UPLC-TOF MS) for untreated OSPW (a) and 20 mg/L ozone-treated OSPW (b).

smaller n (11 to 15) (Figure 2) and Z number (−4) was observed (SI Figure S7). A similar distribution trend was also observed for oxy-NAs in OSPW (Figure 2 and SI Figure S8). These shifts in the distribution of NA species may be explained by the higher reactivity of NAs with higher n number due to an increment of hydrogen atoms available for abstraction.[42] High reactivity of NAs with a higher number of rings was also found due to the presence of tertiary carbons that result in an increase of the reactivity of their H atoms.[42,43]

As one of the methods that offer the highest resolutions,[11] FT-ICR MS was also used to obtain the distributions of NA species in untreated and treated OSPWs (SI Figure S9). Based on this technique, the most abundant species in untreated OSPW were the NAs (O_2) with %Ab of 95% (SI Table S1). The distribution profiles obtained using FT-ICR MS showed that most NAs (O_2) detected in untreated OSPW were the species with n ranging from 13 to 21 and Z from −4 to −12, of which the most abundant species had n number ranging from 13 to 17 and Z from −4 to −6 (SI Figure S9). This finding is consistent with the results obtained using UPLC-TOF MS and previous studies.[9,31] The distribution of O-NAs in untreated OSPW was slightly different from NAs (O_2) (SI Figure S9). Both the n and Z numbers spread wider, with n ranging from 11 to 22 and Z from −4 to −12. The most abundant species in this category were the NAs with n number ranging from 17 to 19 and Z number −12. Only a very small amount of O_2-NAs and O_3-NAs were detected in untreated OSPW. After ozonation, the center of the most abundant species shifted to the area with smaller n and Z numbers.

As discussed previously, the IMS results clearly indicated the presence of sulfur-NAs in the OSPW sample. However, due to the limitation of the analysis, a suitable internal standard that could be used to quantify sulfur-NAs for UPLC-TOF MS has not been identified so far. FT-ICR MS was used to determine the relative abundance of sulfur-NAs in raw and ozonated OSPWs as shown in SI Figure S10. The %Ab of sulfur-NAs in untreated, 20 mg/L ozone treated, and 50 mg/L ozone treated OSPWs were 1.3%, 0.04%, and below detection, respectively (SI Table S1). The relative abundances of NAs (O_2) and oxy-NAs (SI Table S4) were in good agreement with the results reported by Nyakas et al.[44]

The different reactivity of NA species may be explained by the differences in their structure. Compounds that only have C−H functional groups as ozone reactive sites react very slowly with ozone, yet there is a wide spread within the reaction rate constants. These constants can be as low as 6×10^{-3} M^{-1} s^{-1} for butyric acid,[45] 0.1 M^{-1} s^{-1} for geosmin,[46] or 8 M^{-1} s^{-1} for octanal.[47] The reactions of ozone with olefinic acids, such as acrylic, methacrylic, maleic, or muconic acid always lead to the formation of hydroxyacetaldehydes, acetic acids, oxaldehyde, and formylformic acid.[48] Nitrogen containing compounds react with ozone when the lone pair electrons of the nitrogen is accessible. Reaction rates vary between 9×10^7 M^{-1} s^{-1} for aniline[47] and 1×10^{-2} M^{-1} s^{-1} for propylamine.[45] Sulfur containing compounds in their low oxidation states such as thiols, organic sulfides, sulfoxides, and sulfinic acids have reaction rates which vary between 8 M^{-1} s^{-1} for dimethyl sulfoxide and 2.4×10^6 M^{-1} s^{-1} for the cysteine anion.[49] Ozone does not react very easily with aliphatic compounds[50] and has little reactivity with commercial NAs,[42] but due to the pH 8 of the samples (both Merichem and OSPW) the predominant reaction was •OH radical induced. This explains why, although aliphatic NAs are quite unreactive, an overall oxidation could be achieved. In previous studies, the oxo- and hydroxylated NAs degradation products were observed after the ozonation of OSPW.[36] These degradation products are more reactive than their parent NAs.

Correlation between the UPLC-TOF MS and the ESI FT-ICR MS. With advantages of the small sample volume, high precision, and accuracy on exact mass determination, various MS methods have been adopted to quantify NA-related species in either commercial standard solutions or OSPW.[7,8] To compare the performance between the high- and ultra-HRMS, a correlation between the UPLC-TOF MS and the FT-ICR MS was developed. Figure 3 shows the correlation between the % Ab determined by FT-ICR MS and the absolute concentration of NAs (O_2) determined by UPLC-TOF MS for untreated and ozone-treated OSPWs. A linear correlation with an R^2 value of 0.9022 for raw and 0.8581 for ozonated OSPW can be clearly seen. The R^2 values for each NAs group (Z-values) are shown in SI Table S2. The possible outliers on Figure 3a and b are Z = 0 NA series. Those species might be the compounds that are more resistant to ozonation. Hardly any line could be visualized on the correlation plot for 50 mg/L ozone-treated OSPW (data not shown) due to the low abundance of OSPW constituents (at 50 mg/L utilized ozone dose, almost all the NAs species were removed). The overall linear regression R^2 values between UPLC-TOF and Orbitrap (also an ultra-HRMS technique) for

Merichem standard and raw OSPW were 0.9345 and 0.7102, respectively (SI Table S2 and Figure S11), showing a good correlation between these two methods.

To compare the quantification UPLC-TOF MS with the semiquantification FT-ICR MS, the absolute concentrations of NAs (O_2) were converted to %Ab and tabulated (SI Table S1). For untreated and 20 mg/L ozone-treated OSPWs, good agreements were achieved, with 88% vs 96% for NAs (O_2) in untreated OSPW and 62% vs 76% for 20 mg/L ozone-treated OSPW. In untreated OSPW, the most abundant oxy-NAs were O-NAs with 7% determined by UPLC-TOF MS and 3% by FT-ICR MS. After ozonation, %Ab of NAs determined by UPLC-TOF MS decreased to 62% for 20 mg/L ozone-treated OSPW. For the 50 mg/L ozone-treated OSPW the correlation is no longer apparent; however, this is an artifact of the ultrahigh resolution of the FT-ICR MS being able to determine peaks in the very low concentrations (under 1 mg/L total NAs) of NAs remaining which the UPLC-TOF MS cannot detect.

Several analytical approaches have been developed to characterize NAs; most of them are semiquantitative methods.[8,9] FT-ICR MS has contributed to a better understanding of profiling environmental samples in the Athabasca oil sands region.[51,52] In the present study, UPLC-TOF MS has been proven to offer the same level of reliability, accuracy, and precision as the ultrahigh resolution FT-ICR MS method. It also offers the advantage of providing direct estimation of NAs-related species in the OSPW samples.

Impact of Ozonation on OSPW Toxicity toward *Vibrio fischeri* and Fish Olfaction. The toxic effects of untreated and ozone-treated OSPWs toward *V. fischeri* and fish olfactory were estimated at a specific utilized ozone dose (35 mg/L; intermediate value between the low and high ozone levels used during the validation experiments). The results showed that the IC_{20} value of 35 mg/L ozone-treated OSPW (SI Table S3) was higher than that of untreated OSPW (IC_{20} = 25.4 ± 2.6%). These results indicated that after ozonation the OSPW become less toxic toward *V. fischeri*, showing agreement with previous studies.[9,31] After ozonation at 35 mg/L utilized ozone dose, the estimated concentration of NAs (O_2) and oxy-NAs decreased from 24.7 and 33.1 mg/L to 2.6 and 18.4 mg/L, respectively (SI Figure S5). These findings show that, even though about 90% and 44% of NAs (O_2) and oxy-NAs were degraded after ozonation, OSPW still showed toxicity toward *V. fischeri*. The oxidation byproducts generated after ozonation and other OSPW constituents, still remaining after ozonation, may contribute to the toxic effect of OSPW toward *V. fischeri*.

The toxic effects of untreated Merichem mixture toward *V. fischeri* were higher (94.9% inhibition) than those caused by untreated OSPW (64.6% inhibition) (SI Table S3). After ozonation, the toxic effects of the Merichem mixture decreased from 94.9% to 68.6% after ozonation with 35 mg/L utilized ozone dose, agreeing with previous publications.[36] The results also showed that the reduction of the toxicity was lower in Merichem mixture compared to that observed in OSPW. This may be explained by (1) the fact that the short chain NAs in OSPW are more toxic than the NAs present in OSPW; (2) that OSPW consists of other organic compounds which are more easily degraded during ozonation than Merichem; and (3) the fact that oxy-NAs in OSPW are less toxic than the oxy-NAs in Merichem.[41]

The impact of untreated and ozonated OSPWs on fish olfaction was also estimated. Fish olfactory tissue responded to L-serine (L-ser) and taurocholic acid (TChA) in a concentration-dependent manner (Figure 4 and SI Figure S12). For both odorants, concentrations greater than 10^{-5} M evoked

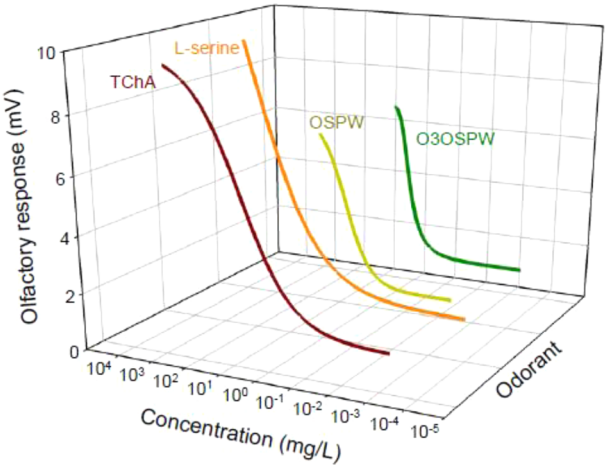

Figure 4. Electro-olfactogram (EOG) responses in millivolts (mV) from the olfactory tissue of juvenile rainbow trout in response to two second pulses of taurocholic acid (TChA), L-serine, OSPW, and ozonated OSPW (O3OSPW) at various concentrations. Curve equations can be found in Figure S13 in SI.

strong responses. However, there was a difference in response magnitude between the odorants ($F_{1,62}$ = 24.899, P = 0.002), with TChA evoking stronger response overall (mean difference: 0.920, t = 4.990, P = 0.002). Olfactory tissues also responded to OSPW and ozonated OSPW in a concentration-dependent manner (Figures 3 and SI Figure S13). Electro-olfactogram (EOG) responses were strong at and above mg/L NAs concentrations. However, OSPW and ozonated OSPW did not differ in response magnitude ($F_{1,71}$ = 0.392, P = 0.545). These data suggested that OSPW may act as an odorant regardless of ozonation.

OSPW decreased olfactory responses to TChA ($F_{3,189}$ = 4.105, P = 0.018). Specifically, exposure to 10% but not 1% reduced EOG responses (mean difference from control vs 10%: 51.3%, t = 2.656, P = 0.014; vs 1%: 24.1%, t = 1.244, P = 0.226) (SI Figure S13a). Ozonation did not ameliorate the EOG change (mean difference: 62.0, t = 3.216, P = 0.004). For the 10% exposure groups (including ozonation), the reduction in EOG occurred 4 min into exposure (mean difference from control vs 10% OSPW: 72.6%, t = 2.630, P = 0.011; mean difference from control vs 10% ozonated OSPW: 82.6%, t = 2.998, P = 0.004), with recovery occurring 28 min into exposure (mean difference from control vs 10% OSPW: 57.7%, t = 0.911, P = 0.367; mean difference from control vs 10% ozonated OSPW: 64.2%, t = 2.196, P = 0.033; not significant) (SI Figure S13a).

OSPW also reduced the olfactory responses to L-ser. Exposure to both 10% and 1% reduced EOG responses significantly (mean difference from control vs 10%: 76.7%, t = 4.717, P = 0.003; vs 1%: 63.4%, t = 2.718, P = 0.012) (SI Figure S13b). Ozonation did not ameliorate the EOG change (mean difference from control vs ozonated OSPW: 106%, t = 4.717, P < 0.001) (SI Figure S13b). Reduction in EOG response occurred at 2 min into exposure for both 10% groups (mean difference from control vs 10% OSPW: 96.0%, t = 2.813, P = 0.007; vs 10% ozonated OSPW: 136%, t = 4.178, P < 0.001), with recovery occurring immediately following exposure

cessation (mean difference from control vs 10% OSPW: 9.6%, t = 0.267, P = 0.791; vs 10% ozonated OSPW: 42.9%, t = 1.261, P = 0.212) (SI Figure S13b). Reduction in EOG response occurred at 6 min into exposure of 1% OSPW (mean difference from control vs 1%: 111%, t = 3.450, P = 0.001) with recovery occurring 30 min into the exposure period (mean difference from control vs 1%: 57.8%, t = 1.794, P = 0.078) (SI Figure S13b).

Fish olfactory tissue responded to OSPW and ozonated OSPW at concentrations in the mg/L range and above, and in a concentration-dependent manner similar to that of naturally occurring odorants (the amino acid L-serine and the bile salt taurocholic acid). This suggested that fish may be able to "smell" compounds within OSPW, which is not surprising, as NAs are ringed organic compounds that may resemble detectable biomolecules such as steroids, and as other studies have found that olfaction is highly plastic—olfactory neurons can detect synthetic chemicals fish have not evolved with (e.g., 2,4-D[53]). The data also suggested that ozonation did not affect detectability, even though NAs within ozonated OSPW may be 80-fold less (by concentration). Conceivably, ozonation may have changed the nature or toxicity of NAs (and perhaps their smell) but did not diminish their ability to evoke olfactory neuron responses. Regardless, given that fish can detect OSPW in NAs concentration ranges similar to natural odorants, if fish were to encounter a plume of OSPW released into the environment, they may actively avoid, or be attracted to, the plume. Future studies would be needed to test this conjecture, especially considering that some toxic compounds can be attractive to fish,[28] presumably because they either smell like food or indicate a food source (e.g., smell like organic decay). If ozonation were found to promote or increase an adverse response to OSPW, this would suggest that it is a beneficial treatment in consideration of OSPW release.

When fish olfactory tissue was given 30 min exposures to OSPW, regardless of dilution or ozonation, responses to an amino acid (food odor) were reduced. However, following exposure, olfactory responses returned to levels greater than those observed pre-exposure. Olfactory responses to a bile salt (hypothesized social cue) followed a similar pattern, with the exception that 1% OSPW had no effect. This suggested that the neurons responsible for detecting bile acids were not as sensitive to OSPW, which was the opposite of what was found for the effects of a herbicide.[53] Taking all the data together, the present study suggested that OSPW interfered with the perception of other odorants by likely acting as odorant(s) itself, and that within a 30 min time frame, it did not exert toxicity. Since there were increased olfactory responses following OSPW exposure, it suggested that the tissue may rapidly adapt (physiologically) to OSPW to maintain olfactory responses. The effects of longer term exposures and the cellular effects of OSPW on olfactory neurons require further investigation.

Changes of Macrophage Antimicrobial Functions after Acute and Subchronic Exposure to Raw and Ozonated OSPW. The active macrophages of fish may either be upregulated (as an inflammatory response)[54−58] or down-regulated (as a toxicity response)[70−72] in response to chemical exposures. Therefore, any results significantly lower or higher than control treatments are an indication of a potentially negative impact on the macrophage cells. Currently, the production of ROI and RNI by primary macrophages was employed as a standard eukaryotic cell toxicity assay, which has been developed and optimized in our previous studies.[20,54]

There were no statistically significant differences in the ROI and RNI production by macrophages obtained from fish after acute exposure (1 week) to raw OSPW compared to control treatments using dechlorinated water (SI Table S3). However, significantly higher macrophage ROI and RNI production by macrophages was observed for ozonated OSPW (35 mg/L utilized ozone dose) treatments for acute exposures versus both the control and raw OSPW treatments (SI Table S3). In contrast, macrophages obtained from fish after subchronic exposure (12 weeks) to both OSPW treatments exhibited significantly lower ROI and RNI production versus control treatments using dechlorinated water (SI Table S3). There were no significant differences in the production of ROI and RNI by macrophages exposed subchronically between the raw and ozonated OSPW treatments.

Ozonation is a potential remediation technique to reclaim OSPW, with reports of both complete[9,37] or partial[36,55] NA degradation. In this study, macrophages from fish exposed for 1 week to ozonated OSPW exhibited significantly higher ROI and RNI. This would be expected as it has been well established that fish macrophages synthesize and release potent antimicrobial molecules (ROI and RNI) during an inflammatory response.[56−60] In addition, it has been previously reported that OSPW, as well as other NAs sources, can induce inflammatory responses in fish.[25,61,62] However, after prolonged exposure of fish to ozonated OSPW (12 weeks), the antimicrobial responses of macrophages were significantly reduced when compared to those obtained from control fish. A similar prolonged exposure following the ozonation of OSPW has shown significantly reduced toxicity in mice after oral exposure to OSPW.[63] However, it should be noted that the immunotoxicity mechanisms differ between mammals and fish, given that the OSPW exposure has been shown to induce inflammatory responses in fish but not mice.[25,64,65] There are thousands of contaminants in OSPW, including a variety of heavy metals, which can potentially cause decreased inflammatory responses.[66−69] Given the presence of these other contaminants, it is probable that they may actually be responsible for the observed decrease in antimicrobial responses of fish macrophages. This is indicated in the reduction of ROI and RNI for raw OSPW in both 1 week and 12 week exposures, with statistically lower responses for both in the 12 week exposures (SI Table S3). However, our acute toxicity results show that exposure to ozonated OSPW had initially enhanced the ROI and RNI production by macrophages, suggesting that there may be a formation of oxidation byproducts that may initially induce an inflammatory response, but eventually degrade over a longer term exposure.[70−72] After this initial response, the toxic response of other contaminants in the OSPW may then act to reduce the synthesis of both ROI and RNI molecules as compared to control fish after 12 weeks of exposure.

■ ENVIRONMENTAL IMPLICATIONS

The complete characterization of OSPW remains one of the significant challenges facing the oil sands industry. Based on the UPLC-TOF MS results, our findings support previous work showing that ozonation reduces the concentrations of NAs, oxy-NAs, and sulfur-NAs. In addition, ozonation was capable of reducing the toxicity of OSPW toward *V. fischeri*. Although the toxicity reduction may be related to the degradation of NAs

(O_2), oxidized, and sulfur-NAs species after ozonation, the species responsible for specific toxicological effects have not been precisely identified. Furthermore, it is well documented that NA species may account for less than 50% of all the organic contaminants in OSPW,[73] and it is still unclear whether the remaining contaminants have toxic effects to organisms more complex than bacteria. Therefore, the results of this study indicate that the more complex in vitro bioassays are an appropriate model to rapidly test for acute immunotoxicity of OSPW and represent valuable tools for the evaluation of remediation process efficiencies. Additionally, these assays can be easily implemented as a potential test for environmental guidelines. The olfactory assays showed that, even after OSPW ozonation, fish could detect OSPW in flow-through water, which can affect their ability to detect other odorants. Given that this is the first time the olfaction test was used for OSPW testing, the significance of these results needs to be further elucidated with longer duration studies of both raw and ozonated OSPWs to determine their impact on fish in the potential receiving environments.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text, including NA profiles estimated by using FT-ICR MS and UPLC-TOF MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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