Environmental Science & Technology

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Sensory and Behavioral Responses of a Model Fish to Oil Sands Process-Affected Water with and without Treatment

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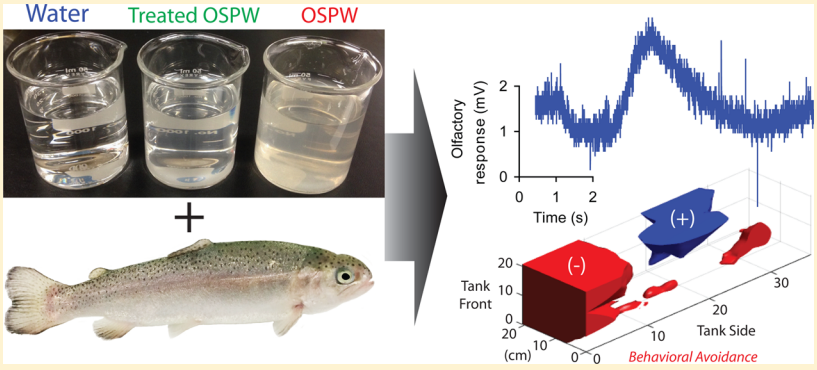
Supporting Information



ABSTRACT: If oil sands process-affected water (OSPW) is to be returned to the environment, a desire is that it not adversely affect aquatic life. We investigated whether a relevant model fish (rainbow trout, *Oncorhynchus mykiss*) could detect OSPW using its olfactory sense (smell) and whether exposure to it would result in behavioral changes. We also investigated whether ozonation of OSPW, which lowers the concentration of organic compounds attributed with toxicity (naphthenic acids), would ameliorate any observed adverse effects. We found that OSPW, regardless of ozonation, evoked olfactory tissue responses similar to those expected of natural odorants, suggesting that fish could smell OSPW. In 30 min OSPW exposures, olfactory responses to a food odorant and a pheromone were reduced to a similar degree by OSPW, again regardless of ozonation. However, olfactory responses returned within minutes of exposure cessation. In contrast, in longer (7 d) exposures, olfactory responses remained impaired, but not in fish that had received ozone-treated OSPW. In the behavioral assay, fish avoided an introduced plume of OSPW, and this response was not affected by ozonation. Taken together, our data suggest that fish smell OSPW, that they may use this sense to mount an avoidance response, and that, if they cannot avoid it, their sensory responses may be impaired, unless the OSPW has received some remediation.

INTRODUCTION

In Alberta, Canada, the oil sands industry is of public concern and scientific focus, in part because oil extraction uses fresh water, which affects water quality. The oil (bitumen) is extracted utilizing a caustic hot water method,[1,2] and the resulting oil sands process-affected water (OSPW) is held in impoundments in accordance with a zero-release policy.[3,4] The processing of one cubic meter (m^3) of oil sands produces about 4 m^3 of OSPW.[5,6] At present, 130 km^2 is covered by OSPW-containing ponds, and it is estimated that upward of one billion cubic meters of OSPW will be produced within the next 20 years. This poses a challenge in the volume of affected water that will need to be returned to the ecosystem.[7]

To return OSPW to the ecosystem, it will require remediation. The remediation of OSPW is complicated, in part owing to its complexity—it is a mixture of salts, metals, organic and inorganic compounds, and particulate matter, which is alkaline and poses a risk to aquatic life.[8−16] Correctly or incorrectly, the organic component, which includes naphthenic acids (NAs), is considered to be the primary source of toxicity,[4,17−20] despite relatively high concentrations of salts and metals.[9] NAs have been associated with immunotoxic effects,[12] steroidogenic effects,[21] increased risk of deformity,[18,22] disturbances in vasculature permeability,[23] and central nervous system depression.[24] Toxic effects on

Received: March 31, 2017
Revised: May 17, 2017
Accepted: May 19, 2017
Published: May 19, 2017

DOI: 10.1021/acs.est.7b01650
Environ. Sci. Technol. 2017, 51, 7128−7137

aquatic biota are of particular interest because of the potential release of remediated OSPW to the aquatic environment.

This study aimed to explore the effects of OSPW on the sensory and behavioral responses of rainbow trout (*Oncorhynchus mykiss*), an ecologically relevant model salmonid species. Salmonids, such as Arctic grayling (*Thymallus arcticus*) and bull trout (*Salvelinus confluentus*), were historically abundant in the oil sands region.[25] We elected to focus on the sense of smell (olfaction), as it is both highly sensitive and highly important to fishes.[26−28] Olfaction is essential to foraging, avoiding predators, mating, and often migrating (i.e., eat or be eaten and reproductive responses).[27] There is a strong body of literature on the ability of a vast number of human-sourced contaminants to impair all responses related to olfaction (reviewed in refs 26 and 28). In spite of this, there is a dearth of evidence on the effects of oil-related compounds on olfaction. This study was intended to determine (1) if fish could smell OSPW, (2) if they would behaviorally avoid it, and (3) what an unavoidable OSPW exposure would do their olfactory and behavioral responses. A secondary aim was to determine if OSPW remediation technology would ameliorate any adverse effects. We expected that OSPW would be detectable by olfactory sensory tissue as two of its major constituents, carboxylic acids (of which NAs are composed) and salt, may act as odorants (in terrestrial models[29,30] and fish[31,32]). Accordingly, we expected OSPW would evoke olfactory-mediated behaviors. Finally, we expected that OSPW ozonation would reduce biological responses because of its efficacy in removing NAs.[8,12,15,33]

MATERIALS AND METHODS

Fish. Rainbow trout embryos were obtained from the Raven Brood Trout Station (Caroline, Alberta) and were hatched December 2011 at the University of Alberta in a flow-through system using dechlorinated municipal water (pH 7.9, 172 mg/L hardness, and 125 mg/L alkalinity). Fish were fed Nu-Way Trout Grower Finisher 5 mm pellets (Unifeed, Edmonton, AB) twice daily and were kept under a 16:8 h light:dark cycle. Fish were 10.9 ± 0.8 g and 90.4 ± 2.2 mm at the time of their use (n = 184). Experiments were approved by the University of Alberta (AUP nos. 7301003 and 052).

Water. The OSPW samples were collected from an oil sands tailings pond in Fort McMurray, Alberta, Canada, on September 27, 2010, from Syncrude West in-Pit.[8] After collection, the OSPW samples were stored in high density polyethylene (HDPE) barrels at 4 °C in a cold room until further use. Fish were exposed to OSPW and ozonated OSPW (O_3OSPW) that contained 41.7 and 3.25 mg/L NAs, respectively (Tables 1 and S1). The quantification of NAs was conducted using an ultraperformance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS, Synapt G2, Waters Canada).[8,34] The ozonation experiments were carried out using a semibatch reactor as described in Wang et al.[12] Detailed analytical and ozonation procedures can be found in the Supporting Information. We also included a salinity control made to represent a concentration of NaCl similar to that of OSPW (Na and Cl were 577 and 101 mg/L, respectively, vs ranges of 555−854 and 159−365, respectively, in six other OSPW ponds[35,36]). Sodium concentration was measured using atomic absorption spectrophotometry (AAS) and chloride content was determined with a spectrophotometer at an OD of 480 nm (Ultrospec 3000; Biochrom Cambridge, UK).

Table 1. Quantification of Naphthenic Acid (NA) Species in Oils Sands Process-Affected Water (OSPW) Using UPLC-TOF MS[a]

sample	NA species	UPLC-TOF MS (mg/L)	UPLC-TOF MS Ab%[b]
OSPW	classical NAs[c]	36.7	88
	O−NAs	2.95	7
	O_2−NAs	1.16	3
	O_3−NAs	0.89	2
	total NAs	41.7	100
O_3-treated OSPW (20 mg/L dose)	classical NAs[c]	2.01	62
	O−NAs	0.61	19
	O_2−NAs	0.44	13
	O_3−NAs	0.19	6
	total NAs	3.25	100

[a]The OSPW and its chemistry is further detailed in Sun et al.[8] [b]Percent abundance (Ab%) values were estimated using NAs (O_2) and oxyNAs; S-NAs were not considered. [c]Classical NAs = $C_nH_{2n+z}O_2$; O−NAs = $C_nH_{2n+z}O_3$; O_2−NAs = $C_nH_{2n+z}O_4$; O_3−NAs = $C_nH_{2n+z}O_5$.

Chemicals. All chemicals were sourced from Sigma (Oakville, ON), and their purities were L-alanine ≥98%, L-serine ≥99%, and taurocholic acid sodium salt hydrate (TChA) ≥ 95%. Stock solutions of 10^{-2} M of L-serine and TChA were prepared and stored at 4 °C, and L-alanine was freshly prepared as a 10^{-4} M solution before use. A NaCl solution was prepared as 2 L stock solution and was the equivalent of 200% OSPW (7.16 g NaCl and 62.5 g Na_2SO_4; ≥99%). For anesthetization, tricaine methanesulfonate (Syndel; BC, Canada) was prepared in a 5 g/L stock solution (buffered 5:1 $NaHCO_3$).

Sensory Responses. Change in olfactory tissue generator potential were recorded as electro-olfactograms (EOGs), which is an indication of whether or not a compound is binding to receptors and has the potential to be an odorant. Olfactory tissue was isolated in situ and given similar exposures as the whole animal. Details of the procedure can be found in Sun et al.[8] and Maryoung et al.[37] In brief, fish were anesthetized at an induction dose of 150 mg/L tricaine methanesulfonate, placed in a holder, and a maintenance dose of 75 mg/L was perfused over their gills. To record the EOGs, Ag−AgCl electrodes filled with a 3 M solution of KCl filled glass capillary tube with a tip diameter of 70−120 μm filled with 8% gelatin in 0.6% NaCl were used. These electrodes were placed at the midline of the surgically exposed rosette raphe and a recording electrode was submerged in the water bath to measure change in membrane potential (mV). A 10 min acclimation period was provided before delivering any solutions. A computer controlled solenoid was utilized to switch from background water to 2 s pulses of test solution. Recordings were amplified using a DAM50 differential amplifier (World Precision Instruments; FL, USA), and digitized. Responses were taken as peak difference of the change in membrane potential.

An amino acid (L-serine) and a bile salt (TChA) were chosen as odorants. L-Serine was used in place of L-alanine for olfactory versus behavioral experiments as it is more commonly used in toxicology studies[28] and as it shares relevance to food and predator cues. Since amino acids share a common receptor neuron type,[27] any changes in L-serine response would be expected to correspond to changes in L-alanine response. The bile salt TChA was used as it is activates a different class of contaminant-specific receptor neurons,[38,39] and has relevance

DOI: 10.1021/acs.est.7b01650
Environ. Sci. Technol. 2017, 51, 7128−7137

to social cues.[40−42] To produce dose response curves, fish olfactory tissue was presented with serial dilutions of each odorant (10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} M), and each concentration was given three times at 2 min intervals (Figure S1).

To determine if OSPW activated olfactory tissue, OSPW and O_3OSPW were pulsed on the olfactory tissue undiluted and in serial dilutions (50, 10, 5, and 1%) as above (2 s pulses 3×, 2 min apart). To help account for individual variability and to confirm that the fish was responsive, EOGs were taken to the reference odorants prior to OSPW pulses (10^{-4} L-serine and 10^{-5} M TChA; fish with EOGs < 1.5 mV were not used).

To determine if OSPW exposure altered the detection of L-serine and TChA, fish were given 30 min or 7 d exposures to 1 and 10% OSPW, and 10% O_3OSPW. Exposures were randomized. For 30 min exposures, fish were exposed on the EOG rig (i.e., background water perfusion of the naris was replaced); for 7 d exposures, fish were exposed in 20 gallon tanks using 50% daily renewal of tank water and held at acclimation temperature (14 °C). Tank water and a NaCl solution equivalent to that of 10% OSPW exposures were included as controls (tank water, 37 and 4.43, and NaCl, 57.7 and 10.1, mg/L Na and Cl, respectively). To minimize any physiological issues owing to salinity, all exposures to 10% OSPW/O_3OSPW/NaCl were preceded by a 5 d saline acclimation (saline was increased each day to reach a 10% value). As described, 2 s pulses of 10^{-4} M L-serine and 10^{-5} M TChA were given at 2 min intervals in alternating order. Before and after 30 min exposures, odorants were pulsed until two consecutive pulses of the same odorant did not differ >5% or for a maximum of 30 min.

Behavioral Responses. Swimming activity and avoidance/attraction were measured in two 10 gallon tanks placed within a black fabric covered enclosure. Each tank had three chambers, the first of which was a small chamber to which solutions were added by peristaltic pump and in which aeration was used to facilitate mixing. Solutions exited this chamber and were rapidly diluted ≥10× upon entry into the second chamber (determined by dye calibration; Figure S2), the testing arena (36 × 21 × 19 cm, L × W × H). The third chamber collected water for drainage. Closed circuit cameras were positioned to the side and top of the test arenas, and were connected to a PC running recording software (Cyberlink PowerDirector 10; Santa Clara, CA). All trials were captured under infrared light to limit any visual influences.

For trials, rainbow trout were placed in the testing arena and allowed to acclimate for 30 min, after which solutions were pumped into the tank at 50 mL/min for 5 min (Masterflex pump; Cole-Parmer, Montreal, QC). Solutions included tank (fresh) water (negative control), 10^{-4} M L-alanine (food cue;[43]), 100% OSPW, 100% O_3OSPW and 100% salt control. Two pre-exposure regimes were also conducted where fish were given either 30 min or 7 d exposures to OSPW or O_3OSPW, before assessing their response to L-alanine. For the 30 min exposures, fish were exposed during the acclimation period, and included tank (freshwater) water (control), 10% salinity control, and 10% OSPW, or 10% O_3OSPW. In 7 d exposures, fish were exposed to the same solutions, except that 1% exposures were also carried out to OSPW and O_3OSPW. As above, all exposures to 10% OSPW/O_3OSPW/NaCl were preceded by a 5 d saline acclimation. For 7d exposures, photoperiods and feeding cycles were maintained as in the holding tanks. For 30 min exposures, owing to animal use consideration, a saline but not freshwater control was used. This was because fish from 7 d freshwater exposures would be essentially no different. For comparisons, we related data from the 7 d freshwater control to the 30 min data. Prior to testing, all fish were fasted for 24 h as satiated fish are less likely to respond to food odorants.[44]

Video was taken during the acclimation period and for 5 min following cessation of delivery (i.e., a total of 40 min). Fish position was tracked at 15 Hz using EthoVision XT (Noldus, Netherlands) in both top and side views, and the resulting coordinate data was used to generate 3D position. Inactive fish were not included in analysis (one fish). To quantify tank space use, the tank was divided into quadrants (Figure S3), with the lower front receiving the stimulus solutions. Data analysis were designed to assess the relative activity of the fish before and after stimulus injection, as well as to examine avoidance behavior. Activity was measured as the mean speed over a specified time internal $t_1 \leq t \leq t_n$. Explicitly, the path length was estimated to be $L = \Sigma_{i=2}^{n}[(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2 + (z_{i+1} - z_i)^2]^{1/2}$, and from this, the mean speed was given by $s = L/(t_n - t_1)$. To assess avoidance, we first determined what region in the tank was used by the fish over a specified time interval. Specifically, the volume of the tank was discretized onto a grid having 0.5 cm resolution. The closest the fish got to a location (X,Y,Z) in the tank was denoted by $D(X,Y,Z) = \min([(X - x_i)^2 + (Y - y_i)^2 + (Z - z_i)^2]^{1/2} | i = 1... n)$. This function was visualized using MatLab (MathWorks; Natick, MA) to plot the surface where D = 3 cm, a conservative value to reflect distance from center of mass (i.e., this would tend to slightly underestimate tank use). For analysis, we computed the closest approach function, D, for times between 10 min before injection and the injection time, and for times beginning at the injection time up to 10 min later. The proportional use of each tank quadrant was determined per time min.

Statistics. A two-way repeated measures (RM) analysis of variance (ANOVA) followed by a Holm−Sidak post hoc test was used for most comparisons. For L-serine and TChA-evoked olfactory comparisons, the factors were stimulus (chemical) and concentration. For 30 min and 7 d EOG exposures, the factors were time and stimulus. For OSPW detection, and for the 30 min and 7 d exposures, the factors were the same. For tank position data, there were three factors to consider (time, stimulus and location [quadrant]). Separate two-way ANOVAs were run for each quadrant, and as no differences were found in quadrants 2−4, only data from quadrant one (area of stimulus entry) was used. To model swimming activity responses during the L-alanine introduction in 7 d exposures, polynomial regression was used. Statistical difference was accepted at $p < 0.05$. All data are shown as mean ± SEM. All statistical procedures and graphing were performed using SigmaPlot (Systat, San Jose, CA).

RESULTS AND DISCUSSION

Our findings indicate that OSPW, regardless of ozonation, evokes sensory and avoidance responses. This is encouraging, as it suggests that fish would leave an area where an OSPW release had occurred and so toxicity would be questionable. Another finding was that fish that had received an OSPW exposure, that is, were not able to leave an area of an OSPW spill, would increase their searching of a food odorant. A key result was that ozonation reduced adverse effects of OSPW exposure. These findings are in agreement that ozonation reduces OSPW toxicity.[8,11,12,20,21]

DOI: 10.1021/acs.est.7b01650
Environ. Sci. Technol. 2017, 51, 7128−7137

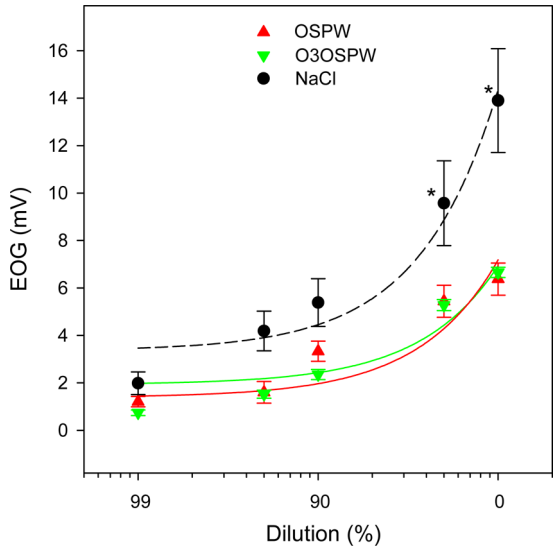


Figure 1. Responses of juvenile rainbow trout olfactory tissue given 2 s pulses to oil sands process-affected water (OSPW), ozone treated OSPW (O_3OSPW) and a saline control meant to replicate the NaCl constituents of OSPW. Tissue responses were taken as electro-olfactograms (EOGs). To show dose-dependency, first degree polynomials were fit to the raw data (OSPW, $F_{1,29}$ = 50.7, P < 0.0001, R^2 = 0.644, n = 6; O_3OSPW, $F_{1,29}$ = 233, P < 0.0001, R^2 = 0.893, n = 6; NaCl, $F_{1,34}$ = 44.8, P < 0.0001, R^2 = 0.576, n = 7). Asterisk indicates difference from OSPW and O_3OSPW.

Sensory Responses. Both OSPW and O_3OSPW evoked concentration-dependent olfactory responses ($F_{5,5}$ = 71.181, p < 0.001 and $F_{5,5}$ = 275.124, p < 0.001, respectively) that did not differ from each other (t = 0.282, p = 0.233) and were lower than the saline control (OSPW, t = 3.414, p = 2.935; O_3OSPW, t = 3 0.696, p = 3.177) (Figure 1). The concentration response relationships were typical of what would be seen to natural odorants,[45−47] suggesting that OSPW, regardless of treatment, stimulates olfactory tissue and that NAs are likely not driving its olfactory detection (O_3OSPW had ∼8-fold lower NAs than OSPW). In support of this, the salt control also evoked olfactory responses, and these responses were generally larger that the responses to OSPW. Two studies have shown that fish can "smell" salt,[31,32] and so perhaps this is primarily what drove the olfactory responses. We note, however, that if salt acted as the primary odorant, that saline control EOG responses would have been similar to OSPW. This was not the case, as OSPW evoked lower responses (i.e., salt was more stimulatory to the olfactory tissue). It is possible that other components in OSPW partially impaired the EOG response or were chelating salt so that it was less available to the tissue. There is evidence that dissolved organic matter will lower metal toxicity.[48,49] Regardless, the former of these theories suggests toxicity; the latter does not. An investigation of the interaction of organic and inorganic fraction on tissue effects remains for study.

We note that in an earlier publication,[8] we presented the same sensory data and offered that O_3OSPW evoked responses at a lower NA concentration; this was an artifact of using NA concentration and not dilution factor on the x-axis. When responses were considered simply by dilution of either OSPW or O_3OSPW, olfactory tissue responses did not differ (i.e., ozonation had no influence).

The second aim of this study was to investigate the effects of OSPW exposure on olfaction after brief (30 min) and longer term, albeit acute (7 d), OSPW exposures. Olfactory responses to an amino acid food/predator cue (L-serine) and a social cue

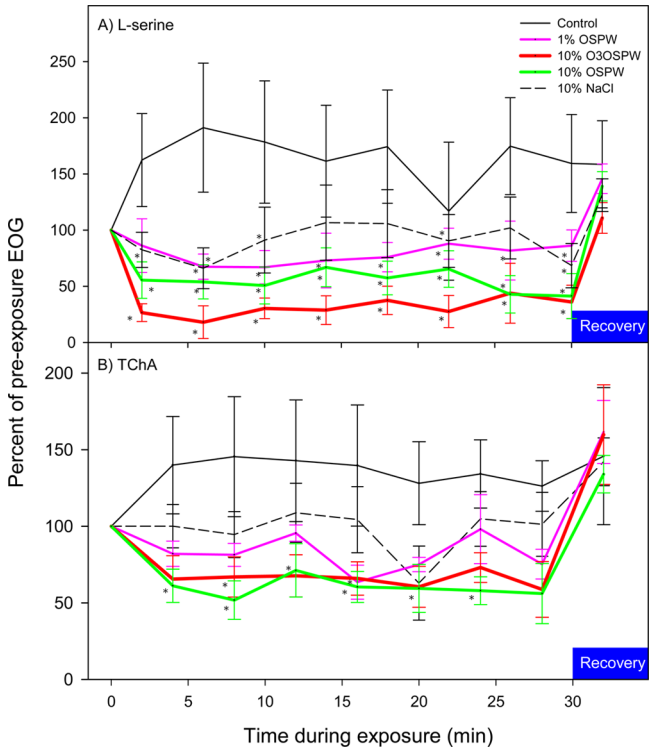


Figure 2. Olfactory tissue responses of juvenile rainbow trout to two natural odorants (the food odorant L-serine [10−4 M] and the pheromone TChA [10−5 M]) during 30 min exposures to tank water (control), oil sands process-affected water (OSPW), ozone-treated OSPW (O_3OSPW), and a saline solution meant to replicate OSPW. Tissue responses were taken as electro-olfactograms (EOGs), and responses during exposure were considered as a percent of the last stable pre-exposure value. Asterisk denotes difference from control (control, n = 4; 10% NaCl control, n = 6, 1% OSPW, n = 11, 10% OSPW, n = 6; 10% O_3OSPW, n = 6).

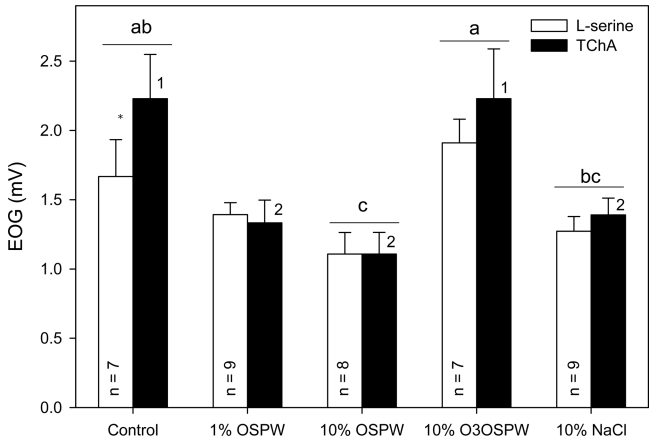


Figure 3. Olfactory tissue responses of juvenile rainbow trout to two different odorants, the amino acid L-serine (10^{-4} M) and the bile salt taurocholic acid (TChA; 10^{-5} M), following 7 d exposures to tank water (control), oil sand process water (OSPW) at 1 and 10% dilution, ozonated oil sand process water (O_3OSPW) at 10% dilution, and a saline solution equivalent to the NaCl in 10% OSPW. Asterisk denotes difference between the odorants; dissimilar numbers denote difference between treatment groups within an odorant; dissimilar letters denote difference between treatment groups.

DOI: 10.1021/acs.est.7b01650
Environ. Sci. Technol. 2017, 51, 7128−7137

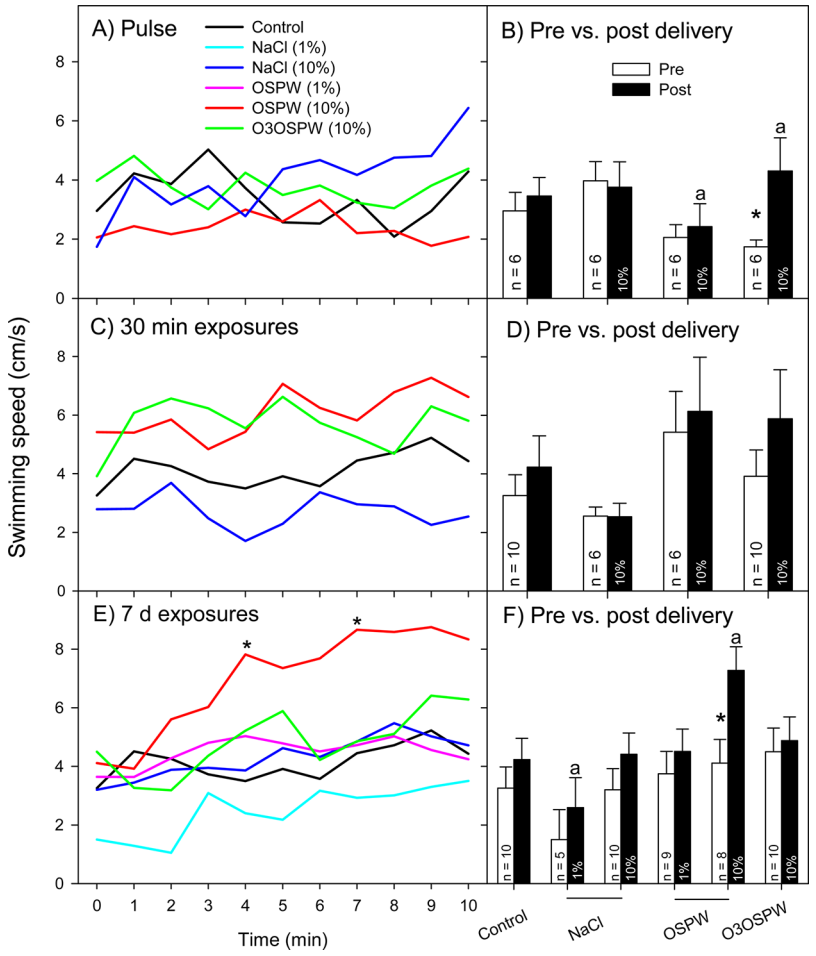


Figure 4. Swimming activity of juvenile rainbow trout following 5 min pulses of OSPW, ozonated OSPW or control water (fresh or saline) (A, B), or following 30 min (C, D) or 7 d (E, F) exposures and a pulse of the amino acid L-alanine (all delivered solutions are considered possible stimulants). Data are shown for average activity before (time zero; 30 min acclimation), and for each of 10 min following delivery of stimulants; data are also shown comparing the preto poststimulus activity. Asterisk denotes difference from freshwater control in time series data, or between preand postactivity.

(TChA) were reduced under either exposure scenario. In 30 min exposures, both L-serine and TChA EOGs were rapidly reduced (in 2−4 min) in a similar manner by 1 and 10% OSPW, and 10% O_3OSPW but recovered within 2 min of exposure cessation (Figure 2). For L-serine, the exposures reduced overall EOG responses ($F_{4,266}$ = 5.783, p = 0.002); ozonation did not rescue the responses (1% vs O_3OSPW t = 1.915, p = 0.068, and 10% vs O_3OSPW, t = 1.312, p = 0.202). Numerically, O_3OSPW did not reduce EOGs as much as OSPW (difference = 29.1%, t = 1.312, p = 0.202). The only indication of concentration-dependency was that 2 min into exposures, responses during 1% OSPW exposure did not differ from control whereas those during 10% did (t = 1.585, p = 0.118 vs t = 2.813, p = 0.007, respectively). For TChA, EOG reductions during 30 min OSPW exposure were very similar to those evoked by L-serine (Figure 2). Again, there was an overall EOG reduction from OSPW exposure ($F_{4, 241}$ = 3.095, P = 0.031), and again ozonation did not make a difference (1% OSPW vs 10% O_3OSPW, t = 2.051, p = 0.052, and 10% OSPW vs 10% O_3OSPW, t = 0.583, p = 0.566). The only difference from L-serine responses was that TChA responses were not reduced to the same extent (mean difference between 10% OSPW vs control in L-serine-evoked EOGs was 76.7% vs 51.3% for TChA-evoked EOGs).

In 7 d exposures, the general trend was similar to the 30 min exposure results, in that OSPW reduced olfactory tissue responses to either odorant (Figure 3). There was some evidence of concentration dependency, as 1% OSPW-exposed fish had responses that were no different from control (t = 2.526, p = 0.016), but responses from 10% OSPW-exposed fish were reduced (t = 3.533, p = 0.001; 1% and 10% did not differ; t = 1.143, p = 0.261). A difference from 30 min-exposed fish was that responses from ozone-treated OSPW exposed fish were no different from control, i.e. ozonation appeared to ameliorate EOG impairment (t = 0.265, p = 0.793). Interestingly, the saline control fish had responses that did not differ from fish exposed to 10% OSPW (t = 1.001, p = 0.324) and were lower than fish given 10% O_3OSPW (salt alone may have been as impairing as 10% OSPW). Although ozonation does not change alkalinity, turbidity or conductivity, ozonation can oxidize iron, manganese and sulfur to form insoluble metal oxides or elemental sulfur.[50] Therefore, the inorganic constituents of O_3OSPW likely differed from OSPW.

The brief exposure demonstrated that impairment was transient, the 7 d exposure indicated that impairment may persist. As carboxylic acids (which include NAs) and amino acids do not appear to activate the same olfactory pathways,[51] it is unlikely that responses were reduced by cross adaptation (i.e.,

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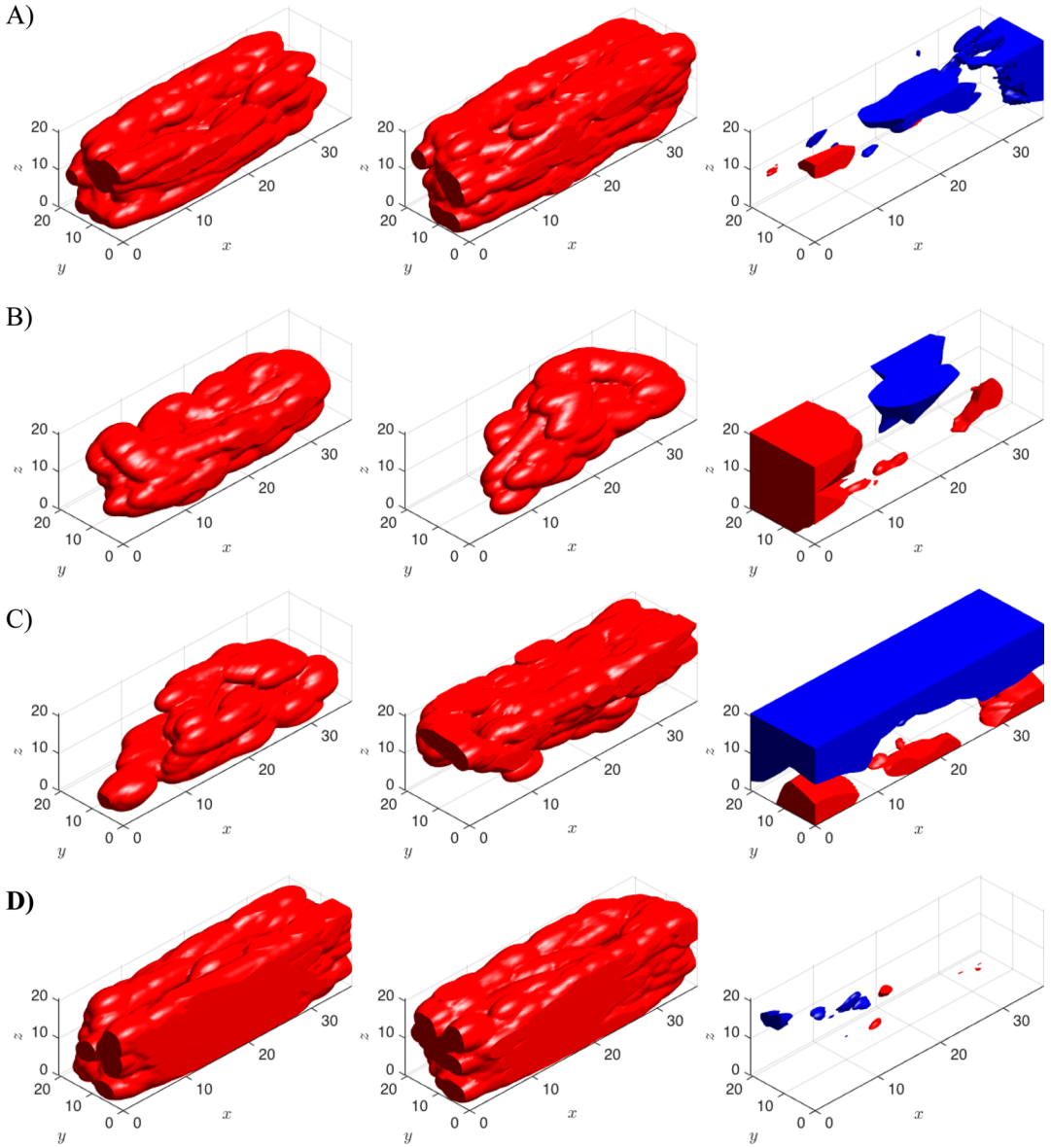

Figure 5. Example area occupancy plots which show individual fish use of the tank. The front of the tank is shown as "*z*" and "*y*", and the side by "*x*"; units are cm. The first image in the row indicates 10 min of tank use before stimulus introduction, the second indicates 10 min following, the third indicates the difference in tank area use between the two time periods, with red indicating reduced use, and blue increased use: (A) freshwater control, (B) OSPW introduction, (C) O_3OSPW introduction, and (D) L-alanine response following 7 d OSPW exposure.

lowered response because the neuronal pathway was already activated). These findings generally reflect what we know of olfactory toxicity, that a host of dissolved chemicals reduce olfactory neuron responses, but that if the exposure is discontinued within a brief period (minutes), the olfactory responses return.[28] Where we see persisting olfactory impairments have been to metals, including copper,[52] and nickel and cadmium.[53] OSPW does contain a variety of metals, including barium (64 μg/L), chromium (19 μg/L), copper (30 μg/L), and selenium (12 μg/L).[9] As suggested above, the independent effects of the inorganic fraction of OSPW should be carried out using a variety of end points.

An important finding was that ozonation ameliorated olfactory impairment in 7 d exposed fish. Intriguingly, ozonation did not appear to have much or any effect in OSPW detection or impairment during brief exposures. As ozonation reduces NAs, but not metals or salts, we suggest that metals and salts may be a factor in OSPW sensing but not toxicity. However, olfactory responses following a 7 d exposure to a saline control with similar NaCl to 10% OSPW indicated that saline-exposure alone could result in an impairment similar to untreated OSPW. Fish may experience olfactory sensitivity due to changes in the concentrations of ions such as sodium and calcium.[40]

Behavioral Responses. Fish avoided both OSPW and O_3OSPW, albeit briefly and at different times following delivery into the tanks (Figure 4). Specifically, fish avoided O_3SPW during its initial presentation (1st min; mean difference = 0.209, t = 2.81, p = 0.006), whereas fish avoided OSPW later (10th min; mean difference = 0.241, t = 3.24, p = 0.002). These changes were only apparent in the inflow area of the tank (bottom front; $F_{27,239}$ = 2.598, p < 0.001), and not the top front ($F_{27,239}$ = 0.672, p = 0.889), or back upper ($F_{27,239}$ = 0.698, p = 0.865) and lower areas ($F_{27,239}$ = 1.547, p = 0.050). These

DOI: 10.1021/acs.est.7b01650
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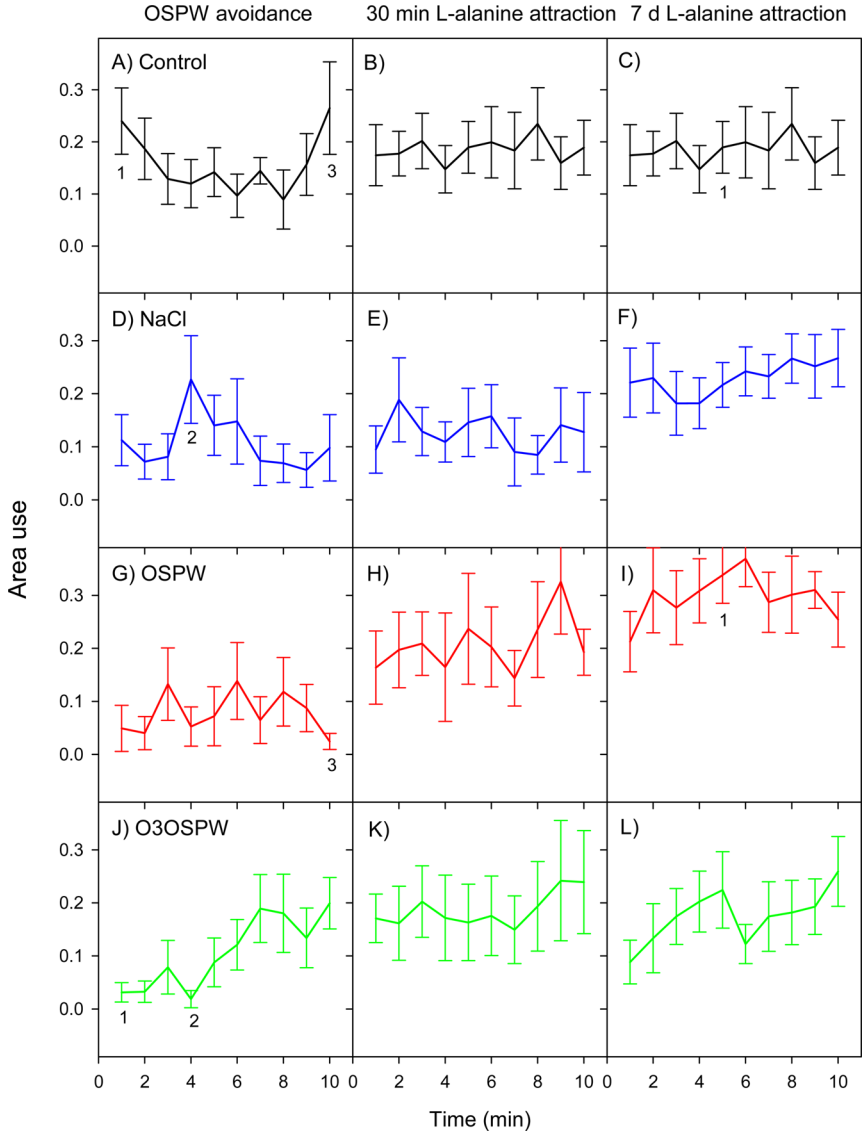

Figure 6. Changes in the occupancy of the inflow area of tanks to which control (tank water), or 10% NaCl, oil sands process-affected water (OSPW), or ozonated OSPW (O_3OSPW) were delivered. The numbers represent the fraction of the volume of the inflow quadrant occupied by the fish within 1 min. If the fish was not in the quadrant at all over the minute in question, the number would be zero; if it swam throughout the whole quadrant in that minute, the number would be one. Reduced area use indicates avoidance. Like numbers indicate difference from control.

findings are encouraging in that they suggest if an accidental release of OSPW were to occur, an advancing front of diluted OSPW may cause fish to leave. Having said this, if a slow release were to occur, there is little reason to expect an avoidance response; avoidance relies on a detectable gradient.[26]

An expectation was that swimming activity would increase as fish responded to the introduction of OSPW to their tanks; this was observed for O_3OSPW but not OSPW (swimming speed was unaffected by freshwater (p = 0.279), saline control (p = 0.734), and OSPW (p = 0.987) but was increased by ozonated OSPW (pre- vs postdelivery mean difference = 4.70 cm/s, t = 3.934, p < 0.001)) (Figure 4B). As OSPW and O_3OSPW were equally stimulatory to the olfactory tissue, perhaps the breakdown of NAs changed the efficacy of the odorants. Ozonation will lead to shorter chained NAs,[12] which perhaps makes dissolved compounds more amenable to activating odorant receptors. Alternately, perhaps increased swimming activity in the O_3OSPW-exposed fish was the result of a physiological response (e.g., toxicity), though this may not be the case, as ozonation eliminated OSPW olfactory toxicity in 7 d exposed fish.

As the data demonstrated that OSPW exposure can reduce olfactory responses to an amino acid, there was an expectation that fish would be less behaviorally responsive to an amino acid; what we found suggested the opposite, as fish given 7 d exposures to OSPW swam faster after the introduction of L-alanine. Specifically, fish given 7 d exposures to 10% OSPW (and not 1%), swimming activity was increased from freshwater control (at minutes 3−4 and 6−7), and overall (pre- vs postdelivery mean difference = 3.16 cm/s, t = 4.767, P < 0.001) (Figure 4E and F). Visual examples of tank use are provided (Figure 5). OSPW-exposed fish also spent more time in the area of L-alanine introduction at 5 min (i.e., at the cessation of delivery; mean difference = 0.282, t = 3.473, p < 0.001) (Figure 6I). In support of L-alanine stimulating swimming activity, the linear models for saline controls, 10% OSPW and 10% O_3OSPW had significant and positive slopes in the 10 min during and following L-alanine introduction (Figure S4) (in 30

DOI: 10.1021/acs.est.7b01650
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min exposures, swimming activity was unaffected by the introduction of L-alanine; overall, $F_{3,301}$ = 1.275, P = 0.305; Figure 4C, D). As this amino acid is considered to be a food cue,[43] perhaps exposed fish had become more interested in feeding. Alternately, if their olfactory tissues were irritated, perhaps fish were endeavoring to escape a plume of olfactory tissue irritating chemical. Either way, the data indicate that olfaction, or perhaps gustation or solitary chemosensory cells, were still functioning in OSPW-exposed fish.

Behavior versus Sensation. Previously, we have shown that behavioral responses are more sensitive than physiological responses in olfactory responses following contaminant exposure.[54] This likely owes to sensory integration and interpretation (cognition driving motor output). In the current study, the data argue in the same manner. Specifically, the electrophysiology data indicated that both OSPW and O_3OSPW evoked virtually identical responses; however, only OSPW was associated with an adverse effect (in 7 d exposures). While EOGs can be used to generate a wealth of sensory information, they will not tell you what the animal will do with it. Clearly the challenges of behavioral toxicology experiments, especially with respect to variation[55,56] and "animality", is worth the journey.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b01650.

Quantification methods for naphthenic acids, chemical characterization of OSPW, ozonation methods, olfactory tissue responses to amino acid and bile salt odorants, calibration of behavioral apparatus, and swimming velocity changes following amino acid introduction (PDF)

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■ ACKNOWLEDGMENTS

We sincerely thank Mike Belosevic for his support and guidance in this study. We acknowledge the financial support provided by a research grant from Natural Sciences and Engineering Research Council of Canada (NSERC) Senior Industrial Research Chair (IRC) in Oil Sands Tailings Water Treatment through the support by Syncrude Canada, Ltd., Suncor Energy, Inc., Shell Canada, Canadian Natural Resources, Ltd., Total E&P Canada, Ltd., EPCOR Water Services, IOWC Technologies, Inc., Alberta Innovates—Energy and Environment Solution, and Alberta Environment and Parks. The financial supports from the Helmholtz-Alberta Initiative (HAI) through the Alberta Environment and Parks' ecoTrust Program and a grant from Canada's Oil Sands Innovation Alliance (COSIA) and Alberta Innovates are also acknowledged. Special thanks go to Dr. Md. Shahinoor Islam for conducting the ozonation experiments. Finally, the authors acknowledge the gracious support by NSERC to M.G.D. and K.B.T.

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