Naphthenic acid fraction compounds, produced by the extraction of bitumen from oil sands, alter survival and behaviour of juvenile yellow perch (*Perca flavescens*)

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Abstract

We evaluated whether naphthenic acid fraction compounds (NAFCs) extracted from oil sand tailings adversely affect fish survival and behaviour. Following a before–after-control-impact design, we housed wild-caught juvenile yellow perch (*Perca flavescens*) in outdoor mesocosms to assess survival and behaviour under baseline conditions, then exposed fish to one of three treatments: negative control, 2 mg/L NAFC, or 15 mg/L NAFC. We performed behavioural assays (no-stimulus activity, food stimulus, and predator stimulus using a model bird) and assessed a comprehensive suite of endpoints (equilibrium losses, activity, shoaling, burst swimming, freezing, and space use). We found that exposure to 15 mg/L NAFCs substantially reduced fish survival and impaired fish equilibrium in all three behavioural tests. Furthermore, exposure to NAFCs impaired anti-predator behaviour: while the activity of control fish increased by two-fold in response to a predator stimulus, fish exposed to 2 or 15 mg/L NAFC did not change their activity levels after stimulation. No significant changes were observed in other behavioural endpoints. Overall, our findings suggest that a week-long exposure to NAFCs at concentrations commonly found in tailings ponds, constructed wetlands, and other mining-impacted waters may affect multiple facets of fish behaviour that could ultimately lead to reduced fitness in fish populations.

Key words: oil sands, naphthenic acids, fish behaviour, ecotoxicology, mesocosms

Introduction

In Canada's oil sands region, industrial operators have not been permitted to deliberately discharge effluent from the mining and extraction of bitumen, known as "oil sands process-affected water" (OSPW) (Martin 2015). Consequently, OSPW has accumulated over five decades in tailing ponds that cover over 120 square kilometres (Chow-Fraser and Rougeot 2022) and contain 1.4 trillion litres of fluid tailings (Alberta Energy Regulator 2021). The toxicity of OSPW to invertebrates, fish, amphibians, birds, and mammals is well established (reviewed by Li et al. 2017; Mahaffey and Dubé 2017), and one class of contaminants in OSPW of particular concern are the naphthenic acid fraction compounds (NAFCs; $C_nH_{2n+Z}O_xN_{\alpha}S_{\beta}$, as defined by Headley et al. (2021)). Water quality guidelines for the protection of aquatic life do not exist for NAFCs but are currently under development, as a new policy framework may soon allow the industry to release treated OSPW to the environment (Government of Alberta 2015; Alberta Energy Regulator 2017). The current study responds to science needs identified for developing standards

for OSPW release (Tanna et al. 2019) by examining how the behaviour of an important fish species in Canada's oil sands region is affected by exposure to NAFCs.

Behavioural alterations can be important sublethal endpoints for assessing the toxicity of environmental pollutants (Ford et al. 2021). Traditionally, regulatory guidelines for pollutants in aquatic ecosystems have largely been based on acute lethality thresholds and may include chronic impacts on development, growth, and reproduction, but unfortunately, risk assessments do not effectively utilize behavioural metrics to evaluate the environmental impacts of pollutants (Agerstrand et al. 2020; Ford et al. 2021). Behavioural endpoints can indicate linkages between physiological processes and population-level effects and can be influenced by several factors, including neuro-hormonal mechanisms and external stimuli (Zala and Penn 2004; Saaristo et al. 2018). As such, behavioural changes can be used to assess sensitive, sublethal responses to environmental pollutants (Scott and Sloman 2004). In addition, changes in behaviour can have immediate consequences for fitness through changes in growth, repro-

duction, and survival (Brodin and Johansson 2004; Smith and Blumstein 2008). These changes in individual fitness can in turn have important consequences for population dynamics and ecosystem functioning (Ford et al. 2021).

Though the exact mechanisms by which NAFCs may affect behaviour are not clear, chemical contaminants are known to alter fish activity in several ways. Disruption of the endocrine or chemosensory functions in fish may interfere with complex behaviours by making fish less active overall or by inhibiting their ability to detect or respond to stimuli (Saaristo et al. 2018). Both NAFC and OSPW exposure have been shown to disrupt endocrine and chemosensory pathways and thus may influence behaviour through these mechanisms (Kavanagh et al. 2012; Lari and Pyle 2017). In addition, differences in activity levels could also be attributed to changes in metabolic rate following NAFC exposure. Fish metabolism is influenced by the integration of several physiological functions and is associated with fish behaviour, and thus disruption of normal metabolic processes may have substantial influences on normal behaviour (Scott and Sloman 2004). An important receptor involved in the metabolism of aromatic hydrocarbons, which include NAFCs, is the ligand-dependent transcription factor, the aryl-hydrocarbon receptor (AhR). NAFCs may be agonists of the AhR and thus influence fish metabolism through this pathway. One study profiled the transcriptome of fish following embryonic exposure to NAFCs and found that gene networks involved in xenobiotic metabolism, drug metabolism, and xenobiotic clearance are most affected by NAFCs (Loughery et al. 2019). Marentette et al. (2017) found elevated levels of *cyp1a1* mRNA transcripts in walleye embryos exposed to fresh NAFCs, suggesting that NAFC toxicity occurs by AhR-mediated metabolism. Other studies have found that NAFCs uncouple oxidative phosphorylation in fish mitochondria, which can increase the production of reactive oxygen species that impose oxidative stress and cell apoptosis (Rundle et al. 2018, 2021). As complex behaviour changes in fish are often altered by interfering with a combination of physiological systems, NAFCs may affect any particular endpoint in fish through multiple mechanisms.

Despite the potential consequences to fish populations of contaminant-induced behavioural changes, only a limited number of studies have examined the effects of exposure to OSPW contaminants on fish behaviour. Studies investigating the olfactory responses of rainbow trout (*Oncorhynchus mykiss*) found fish can smell and avoid small concentrations of OSPW introduced as point sources, and that olfaction may be impaired if fish are exposed to OSPW and are unable to escape (Lari and Pyle 2017; Reichert et al. 2017). Moreover, embryonic exposure to OSPW causes decreased activity, alters swim speed, and impairs alarm responses in zebrafish (*Danio rerio*), and these changes in behaviour are transgenerational (Philibert et al. 2019). Only one study to date has reported changes in fish behaviour in response to NAFCs specifically, showing embryonic exposure alters swimming activity in larval fathead minnows (Reynolds et al. 2022).

The aim of the present study was to investigate the effects of NAFC exposure on multiple facets of behaviour in juvenile yellow perch (*Perca flavescens*), a common sport and food fish found in waters within the oil sands region of north-ern Alberta (Wallace and McCart 1984). We hypothesized that NAFC exposure alters swim activity, impairs anti-predator behaviour, and inhibits foraging behaviour. We chose to assess swimming activity, anti-predator behaviour, and foraging behaviour because impairments in these behaviours have direct consequences for fish survival and fitness. Typical anti-predator behaviour in juvenile yellow perch includes increased shoal cohesion, increased freezing responses, decreased time moving, increased latency to resume feeding, and decreased foraging attempts following exposure to risky cues (Mirza and Chivers 2001; Harvey and Brown 2004). Thus, we predicted that fish exposed to NAFCs would show decreased shoal cohesion, freezing, and swimming activity when responding to either a predator stimulus or a food stimulus. This study provides an important novel context for the effects of OSPW-derived NAFCs on juvenile fish behaviour and thus provides important alternate endpoints for the development of criteria and policies governing the management of OSPW in tailings ponds in Canada's oil sands region.

Materials and methods

Experimental design

Our study followed a before–after-control-impact design (Underwood 1993) (Fig. 1a). After a period of acclimatization to captivity (4 days), groups of five wild-caught fish were held in replicate mesocosms filled with clean lake water for 1 week (hereafter, "baseline phase"), then exposed to one of three NAFC treatments for a second week (hereafter, "exposure phase"). The day NAFCs were added (i.e., 22 September 2020) is referred to as "day 0" (Fig. 1a). NAFC treatments consisted of three nominal concentrations (clean lake water as a negative control, 2 mg/L NAFC, and 15 mg/L NAFC), with four mesocosms per treatment distributed over the study area in a random block design (Fig. 1b). The experiment was conducted at the QE3 Living Lab, an outdoor experimental ecotoxicology facility on the property of the Queen's University's Biological Station near Elgin, Ontario, Canada. Animal husbandry and procedures were approved by the Queen's University Animal Care Committee (Orihel 2020–1829).

In early September 2020, juvenile yellow perch were collected from Warner Lake, Ontario (9.2 ha, mean depth 2.9 m, maximum depth 6.4 m, 44°33′55″N 76°19′35″W) using baited minnow traps (Gee; 42 × 19 cm, 6.4 mm square galvanized mesh). Captured fish were placed into 16 L holding containers filled with lake water and transported to shore, where they were transferred to aerated holding tanks (300 L). Fish of similar size (6.3 g ± 1.2 g mass, 6.9 cm ± 0.7 cm fork length, mean ± SD) were selected to reduce any associated individual variation in other measures (OECD 2013). Retained fish ($n = 60$) were transferred to the 12 outdoor mesocosms (5 fish per mesocosm) and acclimatized for 4 days to reduce the effects of handling stress on fish. The mesocosms (75 cm diameter × 91 cm height; Fig. 1c) were lined with food-grade polyethylene plastic bags and filled with 60 L of filtered water from Warner Lake. Fish were fed ad libitum daily with frozen brine shrimp (*Artemia* spp.) delivered through a plastic tube to reduce disturbance and habituate fish to

Fig. 1. Study design for assessing the behavioural responses of juvenile yellow perch (*Perca flavescens*) exposed to naphthenic acid fraction compounds (NAFCs). (*a*) Experimental timeline with days of data collection for the no-stimulus activity, predator stimulus, and food stimulus behavioural tests and fish size measurements (weight and total length). (*b*) Random block layout of replicate mesocosms, each containing one of the three NAFC treatments (0 mg/L, grey; 2 mg/L, blue; 15 mg/L, green); and (*c*) the replicate mesocosms at the QE3 Living Lab.
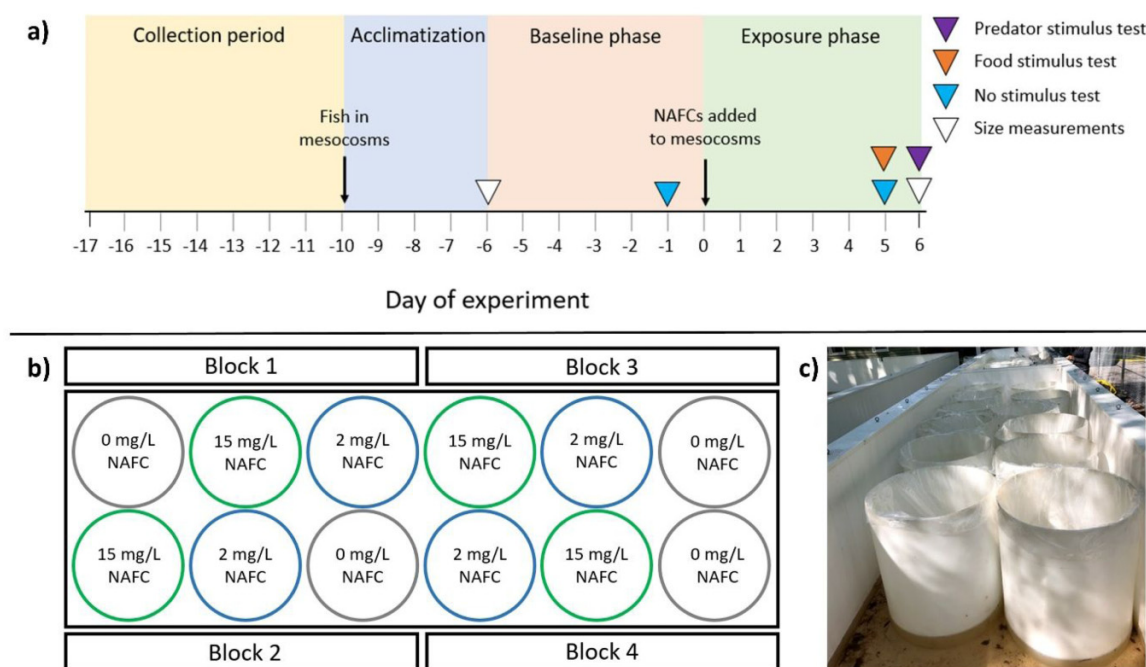
the food delivery process prior to food stimulus tests. Water temperature, pH, dissolved oxygen, conductivity, salinity, and ammonia levels in the mesocosms were measured daily using Hana (H198129) and Hach meters (HQ40d) and ammonia test strips (API). Light intensity and temperature were monitored hourly using HOBO data loggers (Onert, Bourne, Massachusetts, USA). All water parameters remained within the recommended ranges for freshwater fish throughout the experiment (Tables S1 and S2) (OECD 2013). We monitored the mesocosms daily for any mortalities, and all fish were weighed (Thermo Fisher Scientific Denver Instrument P-403, ±0.001 g) and photographed (Canon EOS 100 D) at the beginning (day −6) and end of the experiment (day 6) or on the day they were recorded as a mortality. Photographs were later analyzed using ImageJ2 to determine fork length.

Preparation of NAFCs

This experiment used an NAFC extract prepared from a large volume sample from an active tailings pond in Canada's oil sands region. The preparation and analysis of this NAFC extract have been described in detail elsewhere (Marentette et al. 2015*a*, *b*; Bartlett et al. 2017; Reynolds et al. 2022). In summary, a large volume (<1000 L) of OSPW was acidified to pH < 2 and allowed to settle for 24 h prior to centrifugation. Following centrifugation, the insoluble pellet was resuspended in 0.1 N NaOH (pH 12) to solubilize organic acids and repeatedly centrifuged to remove suspended solids and humins. The alkaline mixture was filtered through a column of diethylaminoethyl-cellulose (DEAE-cellulose) to remove

high-molecular-weight humic-like materials. The alkaline DEAE-celluose column eluent solution was then subjected to repeated liquid-liquid extractions using DCM to remove neutral organics such as PACs. The total NAFC concentration of the extract was 2.64×10^3 mg/L, as measured via negative-ion electrospray ionization high-resolution mass spectrometry (ESI-HRMS, Orbitrap) using a method similar to Headley et al. (2013). The extract was stored in darkness at pH 10 and 4 °C until diluted with lake water to nominal concentrations of 2 and 15 mg/L for this experiment. The initial pH of the NAFC treatments ranged from 8.7 to 10.0 and was titrated to 8.0 ± 0.1 with 0.1 mol/L hydrochloric acid. Throughout the experiment, pH values ranged from 7.0 to 7.8 for controls, 7.2 to 7.7 for the 2 mg/L NAFC treatments, and 7.0 to 7.7 for the 15 mg/L NAFC treatments (Table S1). Water samples from the treatment and control mesocosms were collected in amber glass bottles on day 0 (2 h after NAFC addition), day 3, and day 6 of the exposure phase, and stored at 4 °C until analysis to confirm target NAFC concentrations were achieved.

Quantification and analysis of NAFCs

NAFCs were extracted from mesocosm water samples following a previously reported procedure (Headley et al. 2002). Briefly, Biotage Isolute ENV + cartridges (Biotage, Uppsala, Sweden) were mounted in a solid-phase extraction (SPE) manifold, rinsed with 6 mL of MilliQ water, 6 mL of LCMS-grade methanol (Fisher Scientific, Hampton, NH, USA), and conditioned with 6 mL of MilliQ water. Samples were then acidi-

fied to pH < 2, and then extracted at approximately 5 mL/min under gentle vacuum (approximately 50–60 torr). Following sample loading, SPE cartridges were rinsed with 5–6 mL of MilliQ water under gravity to de-salt. Samples were eluted from cartridges with 6 mL of LCMS-grade methanol under gravity, then evaporated under gentle N_2 flow in a water bath at approximately 40 °C. Samples were reconstituted into 50:50 acetonitrile:water with 0.1% NH_4OH, then transferred to LCMS vials for analysis.

Concentrations of NAFCs were quantified using an external standard calibration method (Bauer et al. 2015) using NAFC extracts derived from OSPW (Rogers et al. 2002). This OSPW-derived NAFC mixture was originally quantified against a Kodak NA mixture via FTIR, as described by Rogers et al. (2002). Sample extracts were bracketed by calibration standards and blanks for every 10 samples. All samples were run in a single sequence batch, except where sample dilutions were required to reach the method's instrument linear calibration range between 1.0 and 100 mg/L, which were analyzed in a second batch with independent blanks and calibration brackets. The method detection limit (using 100 mL sample aliquots) was 0.10 mg/L, with an estimated extraction efficiency of 105% (rsd ≤ 4%). NAFC concentrations are reported as semiquantitative results as authentic reference standards are not available. Sample data from these non-targeted analyses were pre-processed in XCalibur version 2.2. (Thermo Fisher Scientific, Waltham, MA, USA), then formulae were assigned to accurate mass data at a level-4 identification confidence level (Schymanski et al. 2014) using Composer 1.5.6 software (Sierra Analytics, Modesto CA, USA). Formula assignment was limited to compounds between 100 and 500 m/z with a maximum tolerable mass error of ≤3 parts per million.

Quantification provided by HRMS and Orbitrap-MS should be considered semiquantitative. A lack of standardized NA or NAFCs, as well as a lack of agreement on analytical methodology, similarly limits all available methods from definitively quantifying NAFCs (Kovalchik et al. 2017). Further, there are many factors affecting ion abundances and ionization efficiency in a mass spectrometer, which will selectively promote or suppress the response factor of particular compounds and compound classes (Palacio Lozano et al. 2020). Comparisons based on spectral features, such as proportions of heteroatomic classes or particular formulae, must be interpreted only for relative trends and do not directly report absolute amounts of each.

Behavioural tests

We assessed three behaviours in assays at different time points: swim activity, foraging activity, and antipredator responses. All behavioural assays involved filming the groups of fish using overhead-mounted digital cameras (Canon HF R800; Fig. S1). Swim activity was assessed by filming the fish in their 60 L exposure mesocosms for 4 min in the absence of an introduced stimulus ("no-stimulus activity test"). A 4 min film time was chosen based on modified methods from Mathis and Smith (1993) and Chivers and Smith (1995). Videos of regular swim behaviour were recorded in the

morning (9–11 am) during both the baseline (day −1) and exposure (day 5) phases. Foraging activity was assessed by filming the fish for 4 min periods before (pre-food stimulus) and after (post-food stimulus) a food stimulus was introduced on day 5 ("food stimulus test"). The food stimulus consisted of an injection of frozen brine shrimp (2 g) delivered through a plastic tube in the same manner that food was delivered throughout the experiment. To assess anti-predator behaviour, fish from each mesocosm were transferred together from the exposure mesocosms to 60 L cylindrical test tanks at the end of the exposure phase (day 6), allowed to acclimatize for 10 min, and then filmed for 4 min periods before (pre-predator stimulus) and after (post-predator stimulus) the introduction of a predator stimulus ("predator stimulus test"). The predator stimulus was a model bird head resembling the great blue heron (*Ardea herodias*; Fig. S1), a common local predator of juvenile yellow perch. The model was mounted on a rod, introduced at the centre of the test tank in a downward motion to strike the bottom of the tank, and then immediately retracted.

Behavioural endpoints

Behavioural videos were reviewed with the display overlaid with an 11 × 11 grid, where the grid width was approximately one fish body length (Fig. S2). During each behavioural test, we recorded the number of equilibrium losses (underbelly of a fish was visible); activity, indicated by the number of grid lines crossed; shoaling, indicated by a shoaling index (see below); number of burst swim events (sudden increase in velocity); total time spent frozen (seconds); and space use, indicated by a space use score (see below). Equilibrium losses, activity, burst events, and time spent frozen were all tracked continuously for each fish within a replicate trial (Table S3).

Shoaling activity and space use were scored every 15 s during behavioural tests as per previous experiments (Mathis and Smith 1993; Chivers and Smith 1995). Shoaling was scored using an index of shoal cohesiveness, where 1 = all fish were at least one body length away from their nearest neighbour and 5 = all five fish were within one body length of each other. Space use was assessed with the grid overlay divided into two zones of equal area encompassing the centre and outer perimeter of the mesocosm, with individual fish located in the outer zone scoring 1 and fish scoring 2 if they were in the middle zone (Fig. S2a). During food stimulus tests, a second space use score was assessed, where the grid overlay was divided into three equal areas and scores ranged from 1 for each fish in the farthest zone from the food injection site to 3 for each fish in the food injection zone (Fig. S2b). The scores for each measure at 15 s intervals were summed over the pre- and post-food stimulus periods for each test and then averaged by the number of fish present in the trial to give each trial period a final score. Final shoaling scores ranged from 0 to 1, where 0 indicates no time spent shoaling and 1 indicates the majority of the trial was spent shoaling. Space use (location) scores ranged from 0 to 2, where 0 indicates no time spent in the centre of the arena and 2 indicates the majority of the trial in the centre of the arena. Space use

Fig. 2. Chemical analysis of naphthenic acid fraction compounds (NAFCs) measured in replicate mesocosms housing juvenile yellow perch (*Perca flavescens*) on days 0, 3, and 6 of the NAFC exposure phase ($n = 3$–4 per sample). Total NAFC concentrations at each sampling time. Horizontal dashed lines represent nominal treatment concentrations, while solid lines represent measured concentrations. Points and error bars are means ± SD.
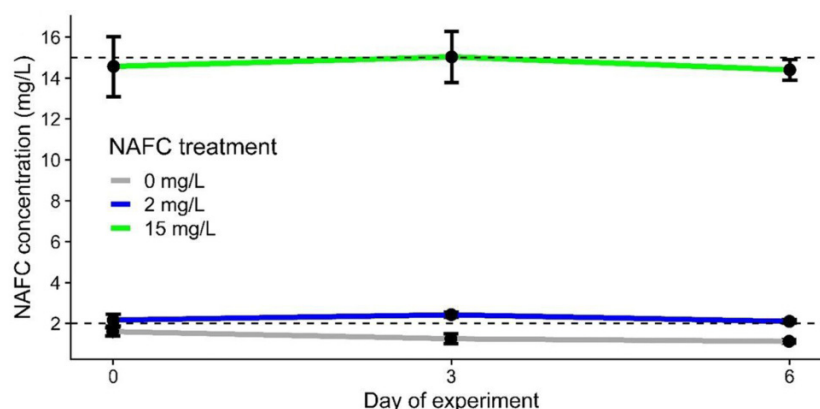
(location) scores in relation to a food stimulus ranged from 0 to 3, where 0 indicates no time spent near the food stimulus and 3 indicates the majority of the trial spent near the food stimulus. Behavioural data were extracted from the videos by an observer tracking an individual fish during each assessment, with corresponding exposure treatments unknown to minimize observer bias. To ensure behavioural observations were credited to the correct individual fish during assessments, each video was assessed multiple times, each time tracking and observing a different individual fish.

Statistical analyses

Statistical analyses were performed using R version 4.0.3 (R Core Team 2021). Model residuals were visually assessed to ensure assumptions for normality and homogeneity of variance were met. To test the influence of NAFCs on fish survival, we used generalized linear models (GLM) from the *car* package (Fox and Weisberg 2019), with proportions surviving to the end of the baseline phase and to the end of the exposure phase in each replicate and NAFC treatment as a fixed effect against a quasibinomial error distribution. In addition, we used Kaplan–Meier (K-M) survival analysis for daily survival to estimate survival probability throughout the experiment (Rich et al. 2010), followed by a log-rank test to assess differences in survival curves using the *survival* package in R (Therneau 2021). To determine the influence of NAFCs on behavioural endpoints for both the food stimulus and predator stimulus tests, we averaged responses across the fish in each mesocosm and used those mesocosm values as the experimental units in GLMs for both the pre- and post-stimulus periods, with NAFC treatment as a fixed effect. Finally, to test the influence of NAFCs on behavioural endpoints during the no-stimulus activity tests on day −1 and day 5, we used GLMs for each experimental phase with treatment as a fixed effect. For the behavioural endpoints, we used either Gaussian (grid lines crossed, shoaling and location scores, freeze time) or Poisson (equilibrium losses, burst swims) error distributions. Figures were generated using *ggplot2* (Wickham 2016).

Results and discussion

NAFCs in mesocosm water

Concentrations of total NAFCs measured in water samples from NAFC-treated mesocosms were similar to nominal treatment levels (i.e., 2.1 ± 0.2 mg/L for the 2 mg/L treatment and 13.3 ± 5.1 mg/L for the 15 mg/L treatment; mean ± SD, $n = 10$–11/treatment) and were stable over time during the exposure phase (Fig. 2). These treatment concentrations are below those reported for fresh oil sands tailings (19.7–85 mg/L; Mahaffey and Dubé 2017) and are at the low end of the range of concentrations reported for reclamation ponds (5–40 mg/L; Anderson et al. 2012; Kavanagh et al. 2013) and bitumen-affected wetlands in Canada's oil sands region (3.3–72 mg/L; Vander Meulen et al. 2021a). Background concentrations of NAFCs in natural water in control mesocosms were 1.2 ± 0.3 mg/L (mean ± SD; $n = 11$), which is comparable to values we reported previously for natural lake water (∼0.5 mg/L) (Reynolds et al. 2022; Robinson et al. 2023) and reference wetlands in Canada's oil sands region unaffected by bitumen-derived inputs (Vander Meulen et al. 2021b). The background acid-extractable compounds that were detected in natural water included substantial amounts of $z = 0$ compounds (i.e., C_9, C_{10}, C_{16}, and C_{18}) that were likely fatty acids, as well as oxygen-rich formulae (e.g., O_3-, O_4-, O_5-, and O_6-containing formulae) reflective of ubiquitous humic and/or fulvic materials, similar to previous observations from Athabasca region surface waters observed with this method (Sun et al. 2017).

The NAFC extract used for our study was composed primarily of classical NAs (O_2 species; Fig. 3) with 15–16 carbon atoms and 2–4 double bond equivalents. Although classical NAs have been implicated as a primary organic contaminant in OSPW (Morandi et al. 2015; Hughes et al. 2017), recent work has demonstrated that classical NAs are not the only cause for concern. Formulae that are more abundant in aged bitumen-derived inputs, which include compounds with oxygen-rich and/or aromatic bonding configurations (Bauer

Fig. 3. Percent abundance of different heteroatom species in naphthenic acid fraction compound (NAFC) samples collected during the exposure phase. Panel headers indicate NAFC treatment (0, 2, and 15 mg/L). In the *x*-axis labels, "Day" represents the day of the experiment (where "day 0" refers to the date of NAFC addition), and "R" indicates the replicate number.
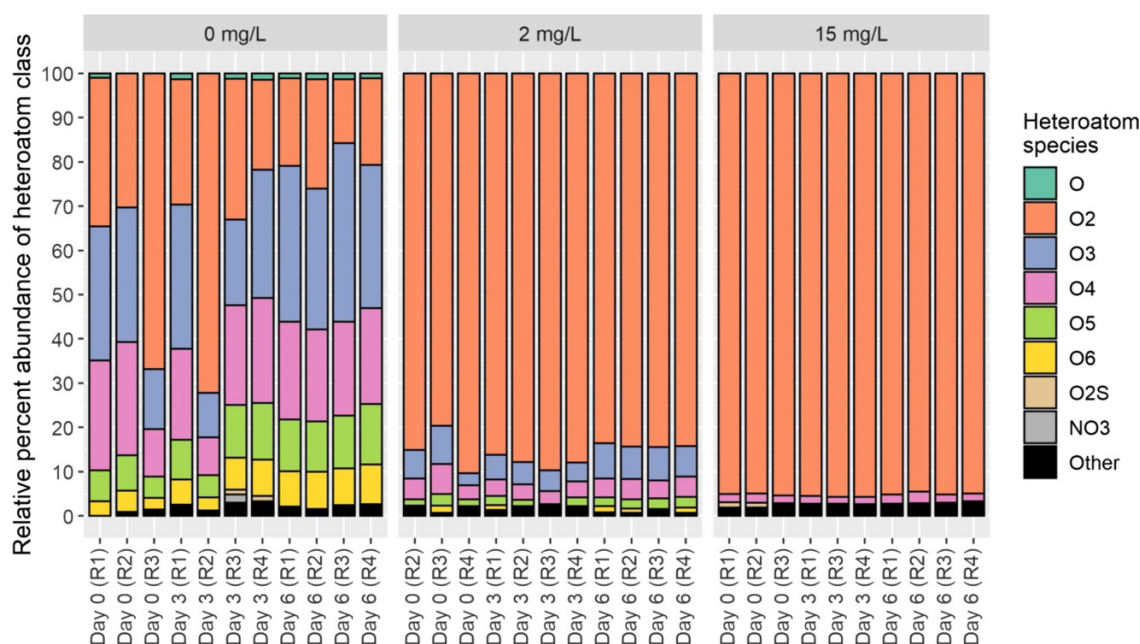
et al. 2019*b*), such as hydroxylated aldehydes and other oxidative structural moieties, can also cause deleterious effects (Sun et al. 2017; Bauer et al. 2019*a*). The molecular composition of OSPW may also include substantial amounts of O$_3$- and O$_4$-NAFCs (Vander Meulen et al. 2021*a*). The NAFC extract in this case appears to be composed primarily of classical NAs (Fig. 3), so associated toxic outcomes should be attributed to this specific compound class.

NAFC exposure reduced fish survival

Exposure to 15 mg/L NAFCs substantially reduced fish survival. Fish exposed to the 15 mg/L NAFC treatment had average survival rates of 80% during the baseline phase that decreased to 25% after 6 days of NAFC exposure (Fig. 4; Fig. S4). In contrast, during the baseline and exposure phases, control fish had average survival rates of 80% and 75%, respectively, and fish exposed to the 2 mg/L NAFC treatment had average survival rates of 85% and 70%, respectively. Over the entire experiment, survival varied with NAFC treatment (GLM, $F_{2,18} = 4.02$, $P = 0.036$) and the treatment × exposure phase interaction ($F_{2,18} = 3.88$, $P = 0.039$), which was driven by decreased survival in the 15 mg/L treatment compared to the other two groups but not between exposure phases ($F_{1,18} = 0.97$, $P = 0.34$). Survival of yellow perch did not differ between treatments during the baseline phase (GLM, $F_{2,9} = 0.072$, $P = 0.93$), but did differ significantly between NAFC concentrations during the exposure phase (GLM, $F_{2,9} = 7.25$, $P = 0.013$), with the 15 mg/L treatment resulting in decreased survival (Fig. 4). However, K-M survival curves representing the entire experimental period identified significant differences between treatments (K-M; $X^2_{(2)} = 14.3$, $P < 0.001$; Fig. S4).

Fig. 4. Daily survival rate of juvenile yellow perch (*Perca flavescens*) in replicate mesocosms ($n = 5$ fish per mesocosm on day −6; $n = 4$ mesocosms per treatment) during the baseline and NAFC exposure phases in negative control (0 mg/L) and NAFC-exposed treatments (2 and 15 mg/L NAFCs). The vertical dotted line indicates the beginning of the NAFC exposure phase on day 0. Points and error bars are daily means ± SE.
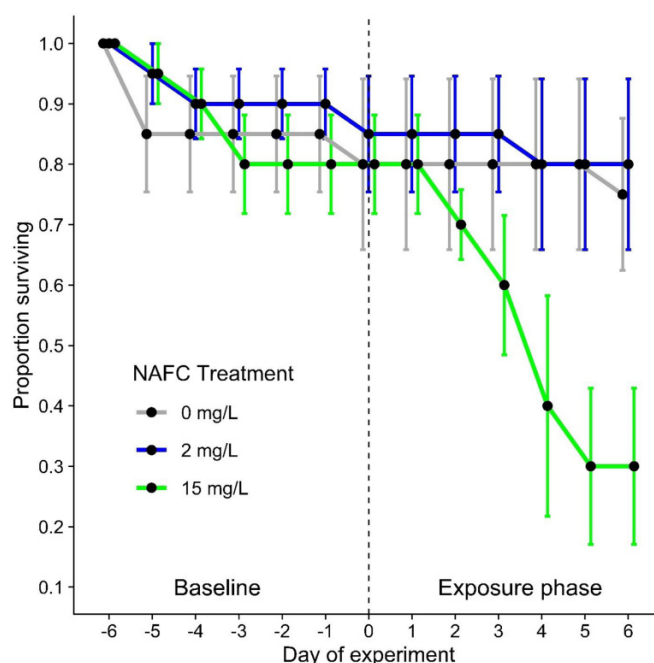
Our findings of substantially reduced yellow perch survival following exposure to 15 mg/L NAFCs provide important new context for the impacts of NAFCs on fish survival. A recent study on embryonic fathead minnows using the same NAFC extract reported an LC$_{50}$ value of 28.5 mg/L and a 10% benchmark dose value of 22.3 mg/L (Reynolds et al. 2022). Laboratory-based studies using NAFCs derived from the same settling basin (Industry A, but sampled in different years) and using the same extraction method as this study reported an EC$_{50}$ value for hatch success of 11 mg/L for walleye embryos and 23.2 mg/L for fathead minnow embryos (Marentette et al. 2015b). Another study using the same NAFC extract resulted in an LC$_{50}$ value of 32.8 mg/L for fathead minnow embryos and an LC$_{50}$ value of 51.8 mg/L for fathead minnow larvae (Kavanagh et al. 2012). Fathead minnow is a robust fish species with a higher tolerance to chemical exposure, whereas yellow perch tend to be more sensitive (Teather and Parrott 2006). A study involving young-of-the-year yellow perch reported 100% mortality in <96 h of exposure to 6.8 mg/L NAFCs isolated from surface waters of the West InPit (WIP) settling basin (Syncrude Canada Ltd.) (Nero et al. 2006). However, inconsistencies in source materials and chemical analysis methods may preclude direct comparisons of results between studies. Thus, our findings of reduced survival in yellow perch exposed to 15 mg/L NAFCs, a similar concentration to walleye LC$_{50}$ values and a much lower concentration than LC$_{50}$ values reported for fathead minnows using the same extract, highlight the differences in sensitivities between species. Comparing differences in sensitivities between fish species exposed to NAFCs allows for a more thorough understanding of how OSPW may affect aquatic communities. In addition, 15 mg/L NAFCs is at the low end of the range of concentrations reported for bitumen-affected wetlands in Canada's oil sands region (3.3–72 mg/L, Vander Meulen et al. 2021a). Our findings provide important information for establishing regulations for the safe release of NAFCs into aquatic ecosystems.

NAFC exposure impaired activity in fish

Total activity measured by the number of grid lines crossed during the observations tended to be greater in the post-stimulus periods than the pre-stimulus periods, with generally lower activity levels in the 15 mg/L treatment (Figs. 5d–5f). During the no-stimulus activity tests, there was no significant effect of treatment during the baseline phase on day −1 (GLM, $F_{2,9} = 0.53$, $P = 0.61$) or during the exposure phase on day 5 (GLM, $F_{2,8} = 0.21$, $P = 0.82$), although activity tended to be greater during the exposure phase (Fig. 5d). In the predator stimulus test, the number of grid lines crossed pre-predator stimulus did not vary significantly between NAFC concentrations (GLM, $F_{2,8} = 1.9$, $P = 0.21$), although activity levels were lower in the 15 mg/L treatment compared to the others (Fig. 5e). During the post-predator stimulus period, there was also no overall significant effect of treatment (GLM, $F_{2,8} = 3.57$, $P = 0.078$), but there was a clear stepwise pattern of lower activity level with increasing NAFC concentration (Fig. 5e). In the post-predator stimulus period of the predator stimulus test, fish crossed an average of 28 lines/min in the control

treatment, 19 lines/min in the 2 mg/L NAFC treatment, and 5 lines/min in the 15 mg/L NAFC treatment. In the food stimulus test, the number of grid lines crossed did not vary significantly between treatments, either pre-food stimulus (GLM, $F_{2,8} = 0.21$, $P = 0.82$) or post-food stimulus (GLM, $F_{2,8} = 0.71$, $P = 0.52$), while the control treatment was associated with the highest observed activity levels (Fig. 5f). Our findings are consistent with Philibert et al. (2019), who reported decreased activity following an alarm cue stimulus consistent with an anti-predator response in fish exposed to OSPW during development. Similar decreases in activity and constrained anti-predator behaviours have been observed in fish exposed to crude oil (Barron et al. 2005; Kochhann et al. 2015; Lari et al. 2016) and polycyclic aromatic hydrocarbons (PACs) (Vignet et al. 2014; Vignet et al. 2015; Johansen et al. 2017).

NAFC exposure impaired equilibrium in fish

The occurrence of equilibrium losses in yellow perch was significantly influenced by NAFC exposure in the three behavioural tests (Figs. 5a–5c), with fish in the 15 mg/L NAFC treatment being the only ones to lose equilibrium. In the no-stimulus activity test during the baseline phase, no equilibrium losses were observed, whereas fish in two out of three replicate mesocosms in the 15 mg/L treatment did lose equilibrium during the exposure phase (one twice and one seven times; Fig 5a), driving a significant effect of treatment in the exposure phase (GLM, $F_{2,8} = 10.79$, $P = 0.0053$). In the predator stimulus test, equilibrium losses significantly increased with NAFC exposure during both the pre-predator stimulus and post-predator stimulus periods (GLM, both $F_{2,8} = 5.19$, $P = 0.036$), although these equilibrium losses occurred in only one of the 15 mg/L NAFC replicate mesocosms (twice pre-predator stimulus and thrice post-predator stimulus; Fig. 5b). In the food stimulus test, equilibrium losses again occurred exclusively in the 15 mg/L treatment in both the pre-food stimulus (GLM, $F_{2,8} = 11.4$, $P = 0.0046$) and post-food stimulus (GLM, $F_{2,8} = 5.19$, $P = 0.036$) periods (Fig. 5c). Equilibrium losses have also been observed in juvenile pink salmon exposed to crude oil, which is notable as NAFCs are found in oil residues (Yang et al. 2021). Reynolds et al. (2022) observed similar equilibrium losses in recently hatched fathead minnows exposed to 21 and 29.5 mg/L of NAFCs, concentrations that were also associated with higher rates of mortality. One study suggests that loss of equilibrium in fish can serve as an alternate endpoint that can identify a larger portion of the population that is under stress and potential forthcoming mortality (Gosselin and Anderson 2020). If OSPW and NAFCs are released, fish in the Canadian oil sands region would not likely be statically exposed to contaminants as they were in this study, and thus it is relevant to understand both the immediate effects on survival and the changes in behaviour that decrease survival over time.

NAFC impacts on other behavioural endpoints

Examination of other behavioural endpoints (i.e., shoaling score [Figs. S5a–S5c], number of burst swim events [Fig. S5d–S5f], freezing time [Figs. S6a–S6c], and space use score [Figs.

Fig. 5. Number of equilibrium losses (*a–c*) and activity measured in grid lines crossed (*d–f*) of juvenile yellow perch (*Perca flavescens*) between the baseline (day −1) and NAFC exposure (day 5 or 6) phases (left column), and before and after a predator stimulus (middle column) and a food stimulus (right column). Boxplots indicate sample medians and 25th and 75th percentiles, and whiskers indicate 1.5 times the interquartile above the 75th interquartile.
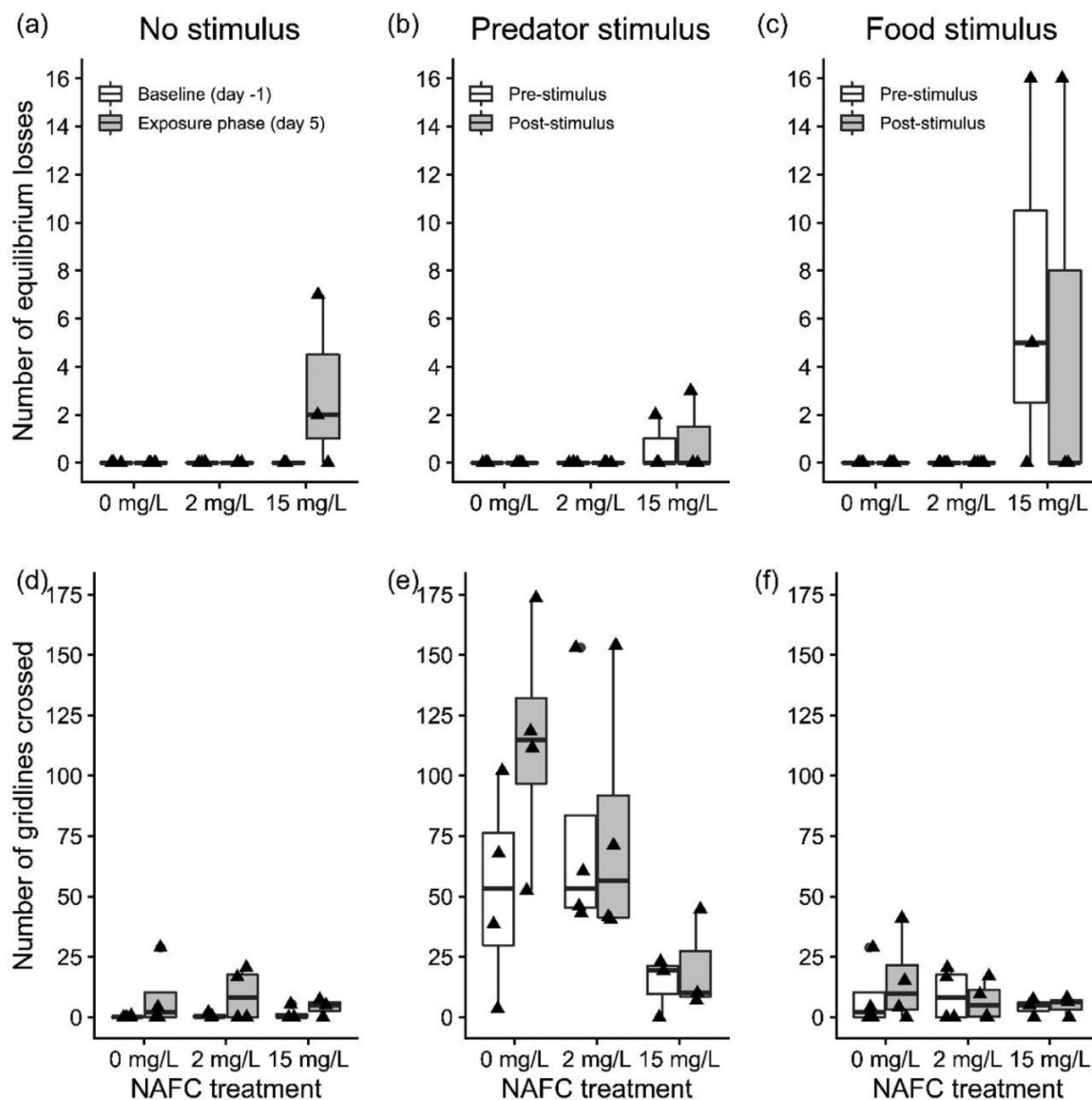
S6*d*–S6*f*]) yielded equivocal evidence that NAFC exposure caused alterations in these behaviours. In the no-stimulus activity test, fish shoaling score was independent of treatment in the baseline (GLM, $F_{2,9} = 0.13$, $P = 0.88$) and exposure (GLM, $F_{2,7} = 2.41$, $P = 0.16$) phases, with a marked decrease in shoaling in the 15 mg/L treatment during exposure (Fig. S5*a*). Similarly, during the predator stimulus test, shoaling scores were not significantly influenced by treatment in either the pre-predator stimulus (GLM, $F_{2,7} = 0.86$, $P = 0.46$) or the post-predator stimulus (GLM, $F_{2,6} = 2.47$, $P = 0.15$) periods, although there were trends towards greater shoaling scores post-predator stimulus in the two NAFC treatments compared to the controls (Fig. S5*b*). In the food stimulus test, shoaling scores differed significantly between treatments both pre-food stimulus (GLM, $F_{2,7} = 5.54$, $P = 0.036$) and post-food stimulus (GLM, $F_{2,7} = 5.09$, $P = 0.043$) periods, with these differences driven by shoaling scores that were 40%–50% lower in the shoals of fish exposed to 15 mg/L NAFCs compared to the 2 mg/L and control treatments (Fig. S5*c*). By the end of the experiment, there were notably smaller sample sizes of fish in the 15 mg/L NAFC treatment due to greater mortality. One replicate in the 15 mg/L treatment had 100% mortality, and one replicate had only one fish live to the end of the experiment, and thus this replicate could not be included in shoaling assessments during behavioural tests. In addition, observed changes in shoaling cannot be attributed to the food stimulus, as similar patterns of effect were found in both the pre- and post-food stimulus periods. Therefore, it is not conclusive if the observed change in shoaling was indeed a consequence of NAFC exposure.

The total number of burst swims did not differ significantly between NAFC treatments in any behavioural test (all $P > 0.05$; Fig. S5d). In the predator stimulus test, there were no differences in burst swims between treatments pre-predator stimulus (GLM, $F_{2,8} = 1.04$, $P = 0.39$), but there were significant differences between treatments post-predator stimulus (GLM, $F_{2,8} = 5.38$, $P = 0.033$), with the greatest number of burst swims observed in the control treatment (Fig. S5e). Fish in the food stimulus tests did not demonstrate any significant differences in burst swims between treatments in either the pre- or post-food stimulus periods (both $P > 0.05$; Fig. S5f).

Time spent frozen did not change significantly between NAFC treatments in any behavioural test (all $P > 0.05$), although times were uniformly greater during the exposure phase (175 ± 28 s, mean ± SD across all treatments) compared to the baseline phase (0 s) in the no-stimulus activity test (Fig. S6a). Freezing time during the predator stimulus test did not vary significantly between treatments in either the pre- (GLM, $F_{2,8} = 2.0$, $P = 0.0.19$) or post-predator stimulus (GLM, $F_{2,8} = 1.09$, $P = 0.38$) periods, although fish in the 15 mg/L treatment tended to spend more time frozen compared to the other groups in both sampling periods. Freezing time during the food stimulus did not vary between treatments in either period (all $P > 0.05$; Fig. S6c).

Space use as indicated by location score did not vary significantly between treatments in any behavioural test (all $P > 0.05$ (Figs. S6d–S6f), although location scores were consistently lower in the predator test (Fig. S6e) compared to the no-stimulus activity (Fig. S6d) and food stimulus (Fig. S6f) tests.

During the food stimulus test, space use and proximity to the food stimulus location did not vary significantly between treatments in either the pre-food stimulus (GLM, $F_{2,8} = 1.42$, $P = 0.29$; Fig. S7) or post-food stimulus (GLM, $F_{2,8} = 1.33$, $P = 0.32$; Fig. S7), indicating that fish did not change locations when a food stimulus was added to the mesocosm. Our findings are similar to those of Philibert et al. (2019), who found no difference in distance travelled or prey captured by fish exposed to OSPW during development. However, our findings contrast with those reported by Lari and Pyle (2017), who found that OSPW exposure impairs food searching behaviour in fish by disrupting their perception of foraging cues. The lack of response to a food stimulus that we observed may be attributed to the conditions of the behaviour tests rather than the potential effects of NAFC exposure. A lack of response to the food stimulus may have occurred because fish were fed daily and excess food was not removed from the test tanks. As such, fish had constant access to food throughout the day and may not have been motivated to respond overtly to the food stimulus during behavioural trials. As the ability to capture prey is essential for survival and is a complex behaviour that relies on visual perception, recognition, decision-making, and motor control (Muto and Kawakami 2013), future studies with adjusted methods may be warranted to further investigate if NAFCs can alter foraging behaviour in fish.

Environmental implications for wild fish

Our study experimentally manipulated the exposure of wild juvenile yellow perch to different concentrations of NAFCs in outdoor aquatic mesocosms to identify changes in fish survival and behaviour caused by NAFCs. Such survival and behavioural changes in response to NAFCs would have been impossible to isolate in a field study of wild fish in Canada's oil sands region. However, the changes in survival and behaviour we observed in yellow perch under experimental conditions could occur in wild fish under similar exposure scenarios in the oil sands region (e.g., in reclamation ponds constructed on former open-mine pits, in the case of an accidental breach of a tailings pond into a natural aquatic ecosystem, or possibly downstream of future outfalls releasing treated industrial effluent). If so, this could have implications for the health of fish populations. First, we observed a substantial reduction in survival in yellow perch exposed to 15 mg/L NAFCs. Previous studies spanning decades found exposure to OSPW in the range of 2–4 mg/L NAFCs has immunological and reproductive effects on yellow perch (van den Heuvel et al. 2012; Hogan et al. 2018). However, few studies have investigated the effects of NAFCs isolated from OSPW on yellow perch survival (Nero et al. 2006), and thus our findings provide important new information on the effects of NAFCs on yellow perch survival that can be compared to previous studies using NAFCs from the same settling basin and extraction methods (Kavanagh et al. 2012; Marentette et al. 2015b; Reynolds et al. 2022). Second, we observed that yellow perch exposed to 2 or 15 mg/L NAFCs changed fish activity in response to a predator stimulus. Changes in short-term activity can alter how fish encounter and respond to both resources and risks in the environment (Saaristo et al. 2018). Individuals that can quickly and accurately detect and assess risk are favoured by natural selection, and thus, disruption of a sensory or motor system due to contaminant exposure can have important implications for individual survival and fitness. Even small changes in fish survival can have a major impact on fish recruitment (Scott and Sloman 2004), and thus, increased susceptibility to predation, as a consequence of NAFC exposure could potentially lead to population declines. Third, we observed that yellow perch exposed to 15 mg/L NAFC experienced losses in equilibrium. If fish are unable to maintain equilibrium, this would affect their ability to forage for food, avoid predators, and reproduce with mates, among other normal activities critical for survival.

Conclusions

Currently, over 1 billion cubic meters of OSPW are stored in tailings ponds in Canada. A more comprehensive understanding of NAFC toxicity, particularly of the sublethal effects of chronic exposure to NAFCs, will help inform policy decisions regarding the safe elimination of tailing ponds from the landscape of Canada's oil sands region. We found that exposure to 15 mg/L NAFCs significantly decreased survival in juvenile yellow perch with an NAFC mixture primarily composed of classical naphthenic acids (i.e., O_2-NAFCs). We also found that exposure to either 2 or 15 mg/L NAFCs impairs fish activity when responding to a predator stimulus. Moreover, we found that exposure to 15 mg/L NAFCs significantly increased the occurrence of equilibrium losses in juvenile perch. These

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NAFC concentrations are relevant to those found in OSPW in the oil sands region. This study is the first to demonstrate that NAFCs affect juvenile fish behaviours vital to survival and performance and thus provides important new context on sublethal effects on aquatic vertebrates associated with NAFCs. Future studies should prioritize the testing of NAFCs on a variety of native fish species and across multiple life stages, as well as model the predicted consequences that NAFC-induced behaviour changes may have on wild fish populations under different exposure scenarios.

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The data are available at https://github.com/JessieReynolds/NAFCYellowPerchStudy.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/facets-2022-0176.

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