Effects of environmentally relevant residual levels of diluted bitumen on wild fathead minnows (*Pimephales promelas*)

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All authors contributed to the execution of the study. Study conception and design were led by Vince Palace. Material preparation, data collection and the majority of analyses were completed by Lauren Timlick. Additional analyses were completed by Lisa E. Peters, Sarah J. Wallace, Heather Dettman and Johanna Mason. Laboratory space, equipment and consumables for analysis were provided by R. Steven Brown and Valérie S. Langlois. The first draft of this manuscript was written by Lauren Timlick. All authors read and approved the final manuscript.

Abstract

Transportation of crude oil across North America's boreal ecozone creates the potential for spills in freshwater where less is known about the sensitivity of resident fish than for marine systems. The sensitivity of wild fathead minnows (FHM) to residual concentrations (ppb range) of the water accommodated fraction (WAF) of diluted bitumen (dilbit) was assessed by exposing them for 21 d followed by a 14 d depuration. Target concentrations were well below detection limits for GC-MS, but were estimated by dilution factor (1:100,000 and 1:1,000,000 WAF:water) to contain less than 0.0003 µg/L of polycyclic aromatic compounds. Confinement and handling stress caused by transfer of wild fish into tanks much smaller than their natural range resulted in mortality and lower body condition among all groups, but interactive effects of oil exposures still resulted in females with smaller cortical alveolar oocytes, and males with larger testicular lobe lumen sizes. Additional studies examining the compounded effects of stress and environmentally relevant oil exposures in wild fishes are needed.

Keywords: fathead minnow, diluted bitumen, freshwater, histology, *cyp1a*

Introduction

Canada has the world's third largest crude oil reserves, mostly in the form of bitumen in Alberta's oil sands region. Diluted bitumen products (dilbit) are a commonly transported crude oil consisting of 70-80% bitumen with 20-30% light oil diluent (e.g., naphtha-based condensate) added to reduce overall viscosity allowing the product to be transported through transmission pipelines (Crosby et al. 2013; Alsaadi et al. 2018a).The use of pipelines to transport petroleum across North America continues to increase (Natural Resources Canada 2017) and despite advances in spill prevention, oil spills periodically occur in aquatic environments. The efficiency and speed of oil spill cleanup operations is regulated in Canada by the Canada Energy Regulator which mandates containment and oil recovery to commence within 72 h of discovering a spill (CEPA 2015). These measures maximize oil removal from the affected waterbody in a relatively short period of time but inevitably some oil constituents, in the low ppm (Noskov, 2018) to ppb range (Agostinis, 2017), remain in the water even after a cleanup effort is completed.

Understanding how residual concentrations of oil affect aquatic biota after a spill cleanup and delineating trajectories of recovery is critical. This study focuses on the potential toxicity of minute concentrations of diluted bitumen on fathead minnows. Wild fathead minnows (FHM; *Pimephales promelas*) are used in toxicity testing because of their widespread distribution, well characterized reproductive physiology and behavior, sexual dimorphism, sequenced genome, and common use as a sentinel freshwater species (Ankley et al. 2010). Reproductive and overall health in FHM exposed to two very dilute concentrations of dilbit water accommodated fraction (WAF) were assessed using meristics, histology, and targeted gene expression. Results from this study will provide information for risk assessors regarding exposure and effects markers in wild FHM following the rehabilitation of a real-world spill site.

Materials and Methods

Adult fathead minnows, collected from a reference lake, were acclimated for 60 d in 40-L glass aquaria covered with opaque plastic sheets and cooled with external circulating water to stabilize temperature. During acclimation, 50% of the water was refreshed daily. Ammonia, dissolved oxygen, and temperature were recorded daily for the first 30 days, and then every other day when ammonia levels were maintained < 2 ppm. Beginning four days after capture, fish were fed *ad libitum* using Tetrafin™ fish flakes. Uneaten food and feces were removed from each aquarium with a low flow siphon 10 min after food was introduced.

A low-energy WAF was prepared at CanmetENERGY Devon using Cold Lake Blend (CLB) dilbit using an open wave tank approximating natural wave action where 8.97 kg of CLB was applied to the surface of continuous waves of North Saskatchewan River water (1200 L). Prior to oil addition to the water, North Saskatchewan River flood plain sediment (mesh size < 500 microns) was added to an initial concentration of 2000 ppm. Water temperature was 15°C while the air temperature was 21°C. The WAF sample was collected three hours after oil application from a port on the side of the tank. The sample was shipped on ice to Winnipeg by courier and stored in the dark at 5ºC for 2 months prior to application.

The total polycyclic aromatic compound (TPACs) content of the WAF was determined using Gas Chromatograph Mass Spectrometry (GC-MS) at ALS Laboratories (Calgary AB). Analysis followed procedures adapted from the US-EPA (1996, 2018) with the exception that samples exceeded the recommended ALS holding time by 3 days. FHM were exposed to dilutions of 1:1 000 000 ("very low") or 1:100 000 ("low") of this WAF in water obtained from a reference lake in northwestern Ontario (IISD-ELA Lake 114, Playle 1987). Scanning spectrofluorometry (Quanta-Master Fluorescence Spectrometer, PTI Ltd., London, ON, Canada) was used as a

secondary method to assess TPACs (Adams et al. 2014) using an excitation wavelength of 300 nm and emissions from 310-460 nm. All spectra were background corrected using 50:50 reference lake water and 99% ethanol (%v/%v) and exposure concentrations were estimated against dilution curves for CLB dilbit and WAFs. While volatiles (e.g. BTEX) were not measured in the exposure water, due to the extended storage time and small applied amounts concentrations are expected to be below detection limits for the majority of the exposure period (Stoyanovich et al. 2019).

After acclimation, fish were exposed to one of three exposures: reference, low exposure and very low exposure, all conducted in 40-L aquaria maintained at ~23°C. Water changes remained the same as during acclimation. After the 21-d exposure, a 2-week recovery phase began when breeding triplicates of 2 females and 1 male FHM were randomly selected from each treatment aquaria and moved to 9-L breeding chambers in a commercial zebrafish bioassay unit that provided circulating water (21-26°C, >8.0 mg/L DO, 6.5-7.5 pH). Breeding groups were isolated from external stimuli using an opaque curtain around the bioassay unit.

At the end of the recovery period, fish were anesthetized in 0.4 g/L tricaine methanesulfonate (MS-222) pH buffered (at 7.0) and sacrificed by severing the spine. Each fish was weighed, measured and dissected. Condition factor (K), liver somatic index (LSI), and hepatocyte volume index (HVI) were determined as measures of overall FHM health. Livers and gonads were weighed, and the gonads and half of each liver were fixed in 10% formalin pH buffered (at 7.0). The remaining half of the liver was flash frozen between slabs of dry ice and stored at -80 °C prior to gene expression analysis.

Histological slides were prepared at the Manitoba Agriculture Veterinary Diagnostic Services laboratory and photographed at University of Manitoba Biomedical Services. Briefly, the tissues were trimmed and embedded in paraffin and sectioned (7 μm). They were dehydrated and stained using hematoxylin and eosin (H&E) and mounted on microscope slides for analysis. Digital images were obtained and analyzed using Zeiss Zen Blue software (Carl Zeiss, Brussels). Three consecutive sections were made for each fish and from these, three non-overlapping images were analyzed.

Female reproductive potential (Reference n = 5, Very Low n = 9, Low n = 6) was assessed by the number, size, and developmental stages of oocytes (Fig. 1A-C), randomly selected from microscopic field of view (1800 x 1300 μm) using an overlaid 10x10 grid. Only cells with visible nuclei were counted and measured to ensure similar measurements along the cross sections of the oocytes.

Male reproductive health was assessed by measuring the diameter of 5 random seminiferous tubules selected from three microscopic fields of view (355 x 265 μm)and tubular lumen and their ratio (Reference n = 4, Very Low n = 7, Low n = 7; Fig. 1D) and by determining developmental stages of the testes (Ankley et al. 2006). Liver health was assessed using hepatocyte volume indexes (Leatherland and Sonstegard 1984) using nine randomly selected areas of 100 μm^2 (5700 x 4200 μm field of view) from each fish's liver (Reference n = 5m 5f, Very Low n = 7m 7f, Low n = 7m 9f).

Targeted gene expression for phase I and phase II metabolism pathways were examined as indicators of exposure to the WAF following methods described by Alsaadi et al. (2018b). Target genes were cytochrome p450 (*cyp1a*; Alsaadi et al., 2018b) and glutathione-S-transferase (*gst*; Mager et al. 2018) both normalized to the housekeeping genes 60S ribosomal protein L8 (*rpl8*) and elongation factor 1α (*ef1α;* Martyniuk and Denslow 2012). Levels of mRNA as fold changes relative to the reference tank were assessed using a CFX96 Real Time System qPCR (BioRad, Mississauga, ON CA) and all analyses were performed following the MIQE guidelines (Bustin et

al. 2009). A standard curve in duplicate with a serial dilution (1:4) of pooled cDNA, no template controls, and no reverse transcriptase controls were included for each plate and considered acceptable with an efficiency of 100 ± 10% and $R^2 > 0.985$.

Heteroscedasticity and normality of the data were confirmed using a Levene's test (homoscedasticity accepted if $p>0.05$) and by examining a Q-Q plot before using a one-way ANOVA followed by Tukey's post hoc analysis to determine differences among treatment groups. Non-parametric (e.g., oocyte counts) were assessed using Kruskal-Wallis followed by Mann-Whitney U-Test to determine differences from the reference group. All statistical analyses were performed and accompanying figures created using R Studio (2019), with statistical significance accepted at $p<0.05$.

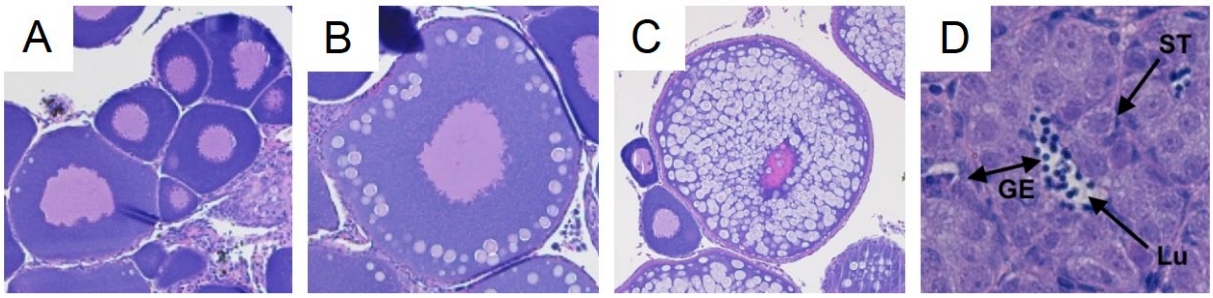

Fig. 1 Histological slides, stained with H&E, of different stages of oocytes and the structure of the seminiferous tubule in testes. Images are not to scale and all are from the reference treatment. A. Perinucleolar oocytes (identified by presence of nucleoli at the periphery of the nucleus), B. Cortical alveolar oocyte (identified by the appearance of yolk vesicles within the ooplasm), C. Vitellogenic oocyte (identified by obvious spherical yolk granules), Atretic oocytes (not shown) identified by compromised cell membrane D. Male testes with the perimeter of the seminiferous tubule (ST) and lumen (Lu) as well as the diameter of the germinal epithelium (GE) indicated in black.

Results and Discussion

The original WAF contained 26 µg/L TPACs (GC-MS analysis) but we chose to further dilute this stock to develop exposures representative of residual PACs at a spill site after cleanup and volatilization of lower molecular weight compounds. Because scanning spectrofluorometry tended to overestimate TPACs relative to GC-MS analysis, it was used only to express proportions of WAF. The only instance where TPACs in the low tank were above detection limits for this method indicated a concentration equivalent to ~4% WAF; all other points were non-detects. Early life stages of fish are most sensitive to the effects of TPACs (McKim 1977) but concentrations associated with effects are typically higher than those from the current study (e.g., <1-18 µg/L TPAC) (Carls et al., 2008; Madison et al., 2017) and the EC50 for developmental malformations in FHM embryos is 500 µg/L TPH-F (Alsaadi et al. 2018b).

There were a significant number of mortalities across all three treatments, especially during acclimation (Fig. 2). No spawning occurred among any of the groups likely because of the stress of confinement combined with prolonged reproductive energy output in this asynchronous spawning species (Unger and Sargent 1988; Divino and Tonn 2008; Pankhurst 2016). Condition factor (K), a common measure of nutritional status, was significantly lower in fish from all three treatments at the end of the study relative to reference fish (Fig. 3-A), which is common among post-spawn fish (Nash et al., 2006). Higher or unchanged K has previously been reported in fish exposed to crude oil (Kavanagh et al. 2012; Van den Heuvel et al. 2012; Raine et al. 2017; Parrott et al. 2019). Oil-exposed fish in this study tended to have lower K but the difference was not significant. It is possible that the near 50% mortality caused preferential selection for more robust

fish to remain at the time of dissection. Additional studies are required to determine if wild fish are more sensitive than laboratory cultures (Wendelaar Bonga 1997) such that low-level exposure to dilbit may exacerbate confinement and reproductive stress.

Higher liver somatic index (LSI) can be indicative of replete nutrition or exposure to contaminants and induced metabolism enzymes (Everaarts et al., 1993; Huuskonen & Lindström-Seppä, 1995) but there were no consistent differences related to oil exposure (Fig. 3-B).

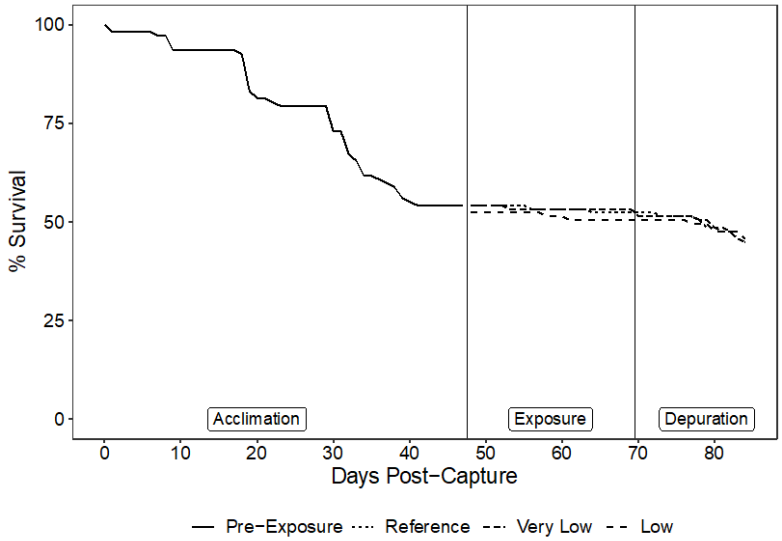

Fig 2 Cumulative percent survival of wild adult FHM during the acclimation, exposure, and depuration phases of the experiment.

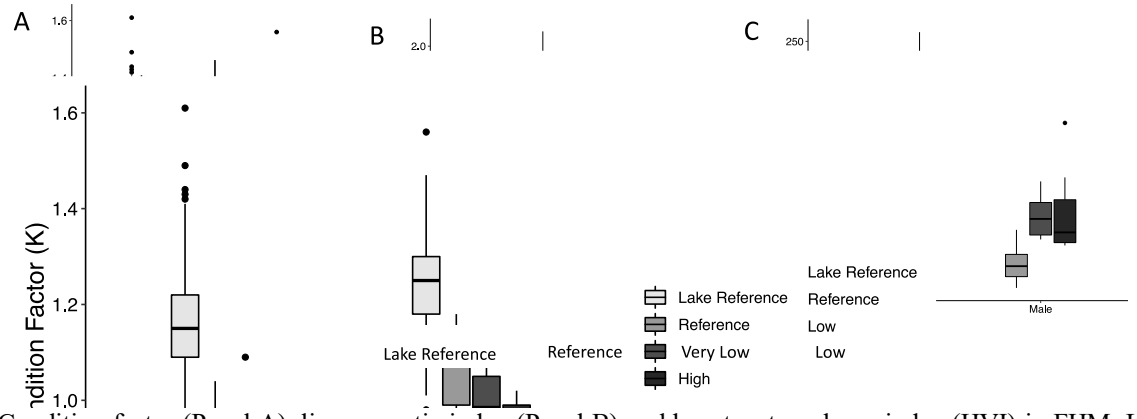

Fig 3 Condition factor (Panel A), liver somatic index (Panel B) and hepatocyte volume index (HVI) in FHM. Lake reference fish included in Panel A are fathead minnows caught from the reference lake 114 in June 2018 (N= 221F, 223M). These are representative of the average condition of free-swimming fish from 114 during the spawning season. As indicated by an asterisk (*), lake reference fish are significantly different from the reference treatment fish.

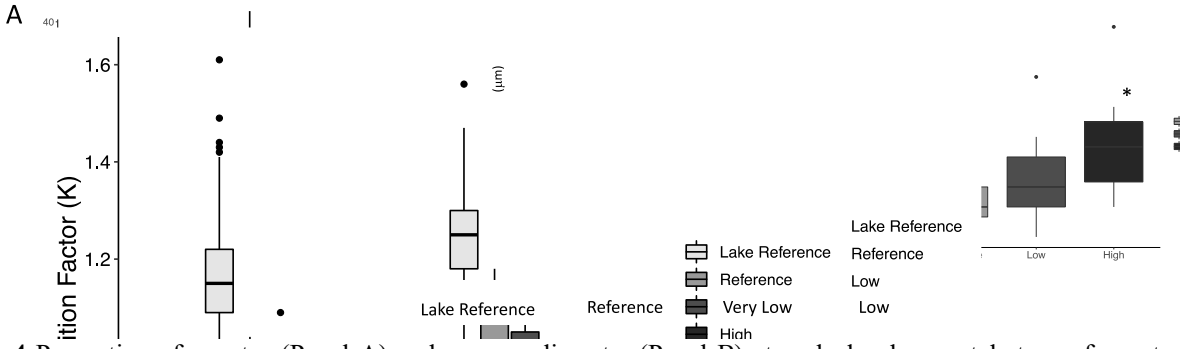

Fig 4 Proportion of oocytes (Panel A) and average diameter (Panel B) at each developmental stage of oocytes in female FHM, and the ratio of tubule to lumen area in male FHM (Panel C).

Hepatocyte volume index (HVI), indictive of liver cell size (Leatherland and Sonstegard 1984; Palace et al. 2002) was also not affected (Fig. 3-C).

There are conflicting reports regarding the impacts of petroleum products on gonad development in freshwater fish species with some studies reporting inhibited development (Kavanagh et al. 2011, Tetreault et al., 2003) while another reported opposing results in different species from the same systems (Van den Heuvel et al., 2012). Histology of ovaries and testes was used to assess reproductive potential and development in fish from this study. Perinucleolar oocytes were the most common stage among all treatments (Fig.4-A). Cortical alveolar cells from the reference treatment were significantly larger than the low treatment oocytes ($p < 0.001$) but low treatment fish had the most vitellogenic oocytes. This may be indicative of more advanced development among the low treatment but there were too few vitellogenic oocytes present in all fish to compare statistically (Fig. 4-B). More than 80% of the testicular tissue in male fish from this study comprised a severe increase in the proportion of spermatogonia (Grade 4; Ankley et al. 2006). Similar to female FHM, low treatment fish were more developed than those of the very low ($p < 0.05$) and reference ($p < 0.001$) fish (Fig. 4-C) based on the size of the lumen relative to the seminiferous tubule.

Expression of genes responsive to oil exposure *(cyp1a* and *gst*; Alsaadi et al. 2018b) and linked to embryotoxicity (Madison et al., 2017; McDonnell et al., 2019) were measured in livers of fish from this study but no significant up-regulation in either gene was detected relative to the fish from the reference tank (Fig. 6).

This study provides important information regarding responses of wild fish to very dilute ppb oil exposures after model spill cleanup in a pristine Canadian boreal lake. Studying impacts of contaminant exposures in wild fish at environmentally relevant concentrations is important because they can be more sensitive than laboratory strains (Wendelaar Bonga 1997). All wild fish from this study exhibited effects of confinement or handling stress, including high mortality, low condition and failure to spawn. Even with these effects, we were still able to detect impacts on gonad development in males and females exposed to the low oil concentrations in this study. Ongoing work in our group will continue to examine the effects of post cleanup oil exposure on wild fish health and reproduction.

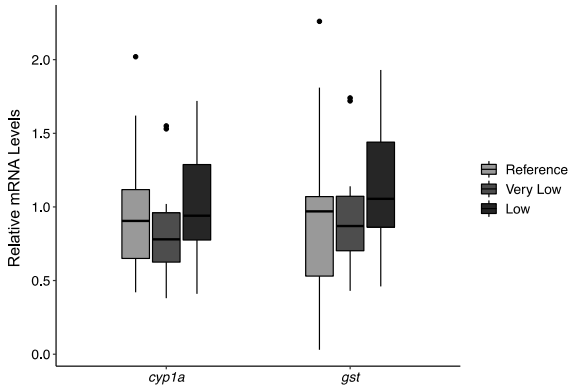

Fig 6 Relative mRNA level of *cyp1a* and *gst* in the livers of exposed FHM.

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Compliance with Ethical Standards

Study design and care regime for animals was approved under University of Manitoba Animal User Protocol #F17-010. The authors declare they have no potential conflicts of interest affecting the integrity of this work.

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