Extracts From Dated Lake Sediment Cores In The Athabasca Oil Sands Region Alter EROD Activity and Gene Expression In Avian Hepatocytes

ABSTRACT

Increases in oil sands mining operations in the Athabasca oil sands region have resulted in increased concentrations of polycyclic aromatic compounds (PAC) and heavy metals in aquatic systems located near surface mining operations. In this present study, sediment cores were collected from three lakes with varying proximity to surface mining operations to determine the differences in PAC concentrations. Sediment cores were separated into two sections – current mining (top [T]; 2000-2017) and pre-mining (bottom [B]; pre-1945) – and extracts were prepared for in vitro screening using a well-established chicken embryonic hepatocyte (CEH) assay. Concentrations and composition of PACs varied between sites with the highest ∑PACs in Saline Lake (SL), 5 km from an active oil sands mine site. The proportion of alkylated PACs was greater than parent PACs in the top sediment sections compared to the bottom. Ethoxyresorufin-*O*-deethylase (EROD) activity in CEH permitted the ranking of lake sites/core sections based on an aryl hydrocarbon receptor-mediated end point; mean EC50 values were lowest for the top cores from SL and another near-mining operations lake, WF1. A ToxChip PCR array was used to evaluate gene expression changes across 43 target genes associated with numerous toxicological pathways following exposure to T and B sediment core extracts. The two study sites with the greatest ∑PAC concentrations (Saline Lake, WF1) had the highest gene expression alterations on the ToxChip PCR array (19(T) -17 (B)/43), compared to a reference site (13(T) -7 (B)/43). The avian in vitro bioassay was useful for identifying toxicity of complex PAC extracts associated with variably contaminated sediment cores, supporting its potential use for hotspot identification and complex mixture screening.

Keywords: polycyclic aromatic hydrocarbons, Athabasca oil sands, EROD, ToxChip PCR, in vitro toxicology, environmental toxicology

INTRODUCTION

In 2018, the Alberta oil sands produced approximately 2.65 million barrels of crude oil per day (b/d) (CAPP 2018). Comprising three distinct regions in Alberta – Athabasca, Cold Lake, and Peace River – the oil sands contain approximately 170 billion barrels of proven oil, making it the third largest reserve in the world (Government of Alberta 2017; CAPP 2018). The Athabasca oil sands region (AOSR) is a geologically unique area where surface extraction of oil sands is possible through open pit mining (Conly et al. 2002). The surface minable area (SMA) accounts for only 3% of the total oil sands area (4800 km^2) but contains 20% of the potential oil. Government of Alberta projections predict an increase in production to 4.2 million b/d by the end of 2035, raising concerns regarding the potential adverse effects of mining operations on environmental health at both a local and regional level.

Studies of mining effects in the AOSR have reported increased loadings of heavy metals to rivers, an increase in the release of polycyclic aromatic compounds (PACs) to the environment, and an increase in the long range transport of PACs (Kurek et al. 2013; Jautzy et al. 2015; Alexander and Chambers 2016). PACs, a broad group that comprises polycyclic aromatic hydrocarbons (PAHs), dibenzothiophenes (DBTs), benzonaphthothiophenes (BNTs), and their alkylated derivatives are released predominantly via two emission sources: pyrogenic (e.g. forest fires, oil seeps, volcanoes) and petrogenic (e.g. petroleum upgrading and refining, burning of fossil fuels and wood, production of coke) (Page et al. 1999; Douben 2003; Ball and Truskewycz 2013; Manzetti 2013). Petrogenic PACs primarily consist of alkylated homologues, having different chemical and physical properties than unsubstituted parent PACs. Such differences

have been shown to lead to variable toxicity; in general, alkylated PACs are more toxic than unsubstituted parent PACs (Lin et al. 2015). In aquatic systems, the fate of pyrogenic PACs is generally controlled by sedimentation rate (Douben 2003). In comparison, petrogenic PACs are more prone to biodegradation, volatilization, and photochemical oxidation (Douben 2003). Carcinogenic, mutagenic, and toxic properties of PACs are of concern for exposed biota and ecosystems (White 2002; Douben 2003). The US Agency for Toxic Substances and Disease Registry ranked PAHs ninth on their list of hazardous substances (The US Agency for Toxic Substances and Disease Registry 2017).

Early-life exposure of fathead minnows (*Pimephales promelas*) to sediments containing bitumen led to decreased growth and jaw deformities (Vignet et al. 2019), and a significant decrease in survival (Droppo et al. 2019; Vignet et al. 2019). Increased ethoxyresorufin-*O*-deethylase (EROD) activity and cytochrome P450 1A4 (*CYP1A4*) mRNA expression were observed in avian hepatocytes exposed to extracts of semipermeable membrane devices deployed near surface mining operations (Mundy et al. 2019) and mining by-products, such as petcoke (Crump et al. 2017). Additionally, several studies have demonstrated the effects of PAC exposure on EROD induction in various fish and amphibian species (Pacheco and Santos 1997; Gauthier et al. 2004; Rankouhi et al. 2005). Thus, the detection of PACs in the Athabasca River and its tributaries at concentrations increasing from 0.009 µg/L upstream of mining facilities to 0.023 µg/L in winter and 0.202 µg/L in summer downstream, is of concern as these concentrations are potentially toxic (Kelly et al. 2009).

The hydrophobic nature of PACs permits preferential binding to sediments in aquatic ecosystems, where they can remain stable over long periods of time (Abdel-Shafy and Mansour 2016; Parrott et al. 2019). Lake sediment cores can provide a natural record of PAC inputs over

time, providing insight on concentration, composition, and source, as well as a greater understanding of the legacy of mining operations (e.g. in the AOSR) (Korosi et al. 2017; Thienpont et al. 2017). Evaluating the toxicity of PACs in lake sediment cores may provide greater insight into the effects of mining operations since the onset of bitumen extraction. Studies have shown that sediments from wetlands near oil sands mining operations (Parrott et al. 2019) and tailings ponds (Wayland et al. 2008) had significantly higher concentrations of alkylated PACs. Given that current research efforts place limited emphasis on testing the toxicity of alkylated PACs, DBTs, BNTs, and complex mixtures (Ball and Truskewycz 2013), evaluating such effects from sediment-derived extracts is important for assessing risks caused by oil sands operations. Parrott et al. (2019) demonstrated that higher levels of parent and alkylated PACs in sediments had more pronounced effects on growth and deformities in fathead minnows, yet there have been few studies on PACs and alkylated PACs specific to the oil sands area.

The objectives of the present study were to characterize the complex mixture of PACs in extracts prepared from lake sediment cores from the AOSR and determine their effects on EROD activity and gene expression in a well-established avian in vitro screening assay, which has been used to screen a range of priority chemicals and complex mixtures to date (Porter et al. 2014; Crump et al. 2015; Pagé-Larivière et al. 2018; Mundy et al. 2019). Sediment cores were sampled from two lakes in proximity to mining activity and one far from industrial activity. Additionally, two sediment core depths (top and bottom) were included to provide a historical (paleotoxicological) perspective in terms of temporal changes in PAC contamination from pre-mining to present day.

EXPERIMENTAL SECTION

Study sites and sample collection

This present study was conducted in the Municipality of Wood Buffalo near the city of Fort McMurray, Alberta (Figure 1). Saline Lake (SL) is directly adjacent to the Athabasca River, which flows north, within 5 km of the nearest oil sands upgrader, while WF1 is 10 km from the nearest oil sands upgrader. Conversely, Mariana Lake (ML) is approximately 120 km southwest of the nearest upgrader, adjacent to highway 63. ML was selected as a reference site due to its distance from oil sands upgraders and the prevailing wind direction, which is from the northwest. Please refer to Salat et al. (2020) for further information regarding study site selection.

Lake sediment cores were collected from SL, WF1, and ML in June 2017. Sediment cores were collected using a gravity corer and sectioned at 0.5 cm intervals using a vertical extruder. Sediments were kept in Whirl-Pak® bags at -20°C at the University of Ottawa, Ontario, Canada. Following a Before-After-Control-Impact (BACI) design (Green 1979), an equal number of sediment intervals from the top and bottom of each sediment profile were homogenized for PAC extraction. We used ^{210}Pb dates (discussed in dating sediment cores) to divide the cores into two sections: (1) a period of active mining (2000 to 2017); and (2) pre-mining (older than 1945). These two sections will be referred to as T (2000 to 2017) and B (older than 1945) respectively.

Dating sediment cores

WF1, SL and ML sediment cores were ^{210}Pb-dated using an Ortec High Purity Germanium Gamma Spectrometer (Advanced Measurement Technology Ink, Oak Ridge, TN, USA) at the University of Ottawa. Chronologies based on excess ^{210}Pb activities were constructed using ScienTissiMe software (Barry's Bay, ON, Canada) and the constant rate of supply model (Appleby and Oldfield 1978; Appleby et al. 1983). Efficiency corrections were

made using Certified Reference Material (312 and 385) from the International Atomic Energy Association (Vienna, Austria) (Salat et al. 2020).

Polycyclic aromatic compound analysis

The target analytes were the 16 US EPA priority PAHs (Table S1), benzonaphthothiophene (BNT), dibenzothiophene (DBT), and their respective alkylated forms. Sediments contained a minimum of 0.1 g TOC per interval for a total of 1 g TOC per pooled sample. Wet samples were mixed with diatomaceous earth (Thermo Scientific, Waltham, MA, USA) and centrifuged to remove water from sediments. Samples were extracted using an accelerated solvent extractor (ASE-200, Dionex Corporation, Sunnyvale, CA, USA), with 50:50 acetone:hexane (US EPA method 3540C modified for accelerated solvent extraction). Solvent and residual water were separated by liquid-liquid extraction using 3 x 10 mL hexane rinse. Samples were evaporated to 1 mL under a gentle stream of nitrogen (TurboVap, Biotage, Charlotte, NC, USA) and centrifuged to remove any remaining water. Samples were then fractionated using a 6 g silica gel column (Grade 644) for column chromatography (Fisher S7441, Hampton, NH, USA) with 35 mL 50:50 dichloromethane:hexane.

The sample fraction was then evaporated to 1 mL in hexanes under a gentle stream of nitrogen. PAC concentrations were quantified using gas chromatography (Agilent 7890B) mass spectrometry (Agilent 5977B). A 100 µL subsample was combined with 900 µL 2,2' ,4-trimethylpentane (TMP, Sigma-Aldrich, Oakville, ON, Canada), and spiked with an internal standard: 10 µL p-terphenyl (D_{14}, 98%, Cambridge Isotope, DLM-382-1). Samples were run in conjunction with a vial of 100 µL of deuterated standard mixture: Naphthalene (D_8, 99.5%), Acenaphthene (D_{10}, 99%), Phenanthrene (D_{10}, 98%), Benz[*a*]Anthracene (D_{12}, 98%), Perylene

(D_{12}, 98%) and *n*-Tetracosane (D_{50}, 98%), (Cambridge Isotope Laboratories Inc. Tewksbury, MA, USA), and PACs were subsequently recovery corrected using the deuterate standards. Mean recoveries for surrogate target compounds (+1 standard deviation) were 33.8 + 16.6 for Naphthalene (D_8, 99.5%), 42.9 + 14.4 for Acenaphthene (D_{10}, 99%), 61.9 + 12.7 for Phenanthrene (D_{10}, 98%), 53.8 + 24.7 for Benz[*a*]Anthracene (D_{12}, 98%) and 30.7 + 25.3 for Perylene (D_{12}, 98%). The remaining 900 µL of PAC extract was solvent exchanged into dimethyl sulfoxide (DMSO) via ethyl acetate for toxicity testing. TMP was reagent grade, 99.8% purity. All remaining solvents were Optima® grade from Fisher Scientific.

Preparation and dosing of chicken hepatocytes

Unincubated, fertilized white leghorn chicken eggs (n=25) were obtained from the Canadian Food Inspection Agency (Ottawa, ON) and artificially incubated until 2 d pre-hatch, at which point the embryos were euthanized by decapitation (Pagé-Larivière et al. 2018). Livers were removed and pooled, and cultured hepatocytes were prepared by collagenase digestion and filtration, as described previously (Kennedy et al. 1995; Head et al. 2006).

Cultured hepatocytes were incubated for 24 h at 37.5°C and 5% CO_2 in 48-well plates, then treated with 2.5 µL (0.5% v/v in 500 µL of medium) of the DMSO vehicle control, or serial dilutions of the "neat" extracts prepared from the lake sediment cores. A top (T) and bottom (B) core extract from each of the 3 sites were included for toxicity evaluation (n=3 wells/treatment group for cell viability and EROD activity; n=6 wells/treatment group for PCR array analysis). The following concentrations of extract were administered: 1) 1 ("neat"), 0.1, 0.01, and 0.001 (cell viability); 2) 1 ("neat"), 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, and 0.0001 (EROD activity); and 3) 1 ("neat") (PCR array). Hepatocytes were incubated for 24 h following extract administration, the medium was aspirated, and cells were either frozen at -80 °C for subsequent

RNA isolation or tested immediately for cell viability. Chicken embryonic hepatocytes (CEH) used for EROD assays were rinsed with 200 µL/well of phosphate-buffered saline-ethylenediaminetetraacetic acid prior to being flash frozen in powdered dry ice and stored at -80 °C.

Cell viability and EROD activity

We estimated the viability of CEH following exposure to serial dilutions of sediment PAC extracts with a ViaLight Plus kit (Lonza, Basel, Switzerland). The appropriate positive and negative controls were included on each plate and luminescence was measured using a Luminoskan Ascent luminometer (Thermo Fisher Scientific, Wilmington, DE, USA) as previously described (Pagé-Larivière et al. 2018). There were no effects on CEH viability following exposure to any of the sediment extract concentrations (1, 0.1, 0.01, 0.001) and therefore, none of the treatment groups needed to be excluded from the biochemical or gene expression analyses. The EROD assay was conducted using a previously described method (Kennedy et al. 1995; Head et al. 2006); EROD activity (pmol/min/mg protein) and total protein concentration were analyzed using a Beckman Coulter DTX880 multimode detector (Beckman Coulter, Brea, CA, USA). On each cell culture plate for EROD determination, a positive control consisting of 3 wells exposed to a nominal concentration of 1 nM 3,3',4,4',5-pentachlorobiphenyl (PCB126) was included to ensure the assay was responding to a known agonist.

ToxChip PCR array

For each of the six sediment core extracts, the highest non-cytotoxic concentration (1 or "neat") was selected for RNA isolation using the Qiagen RNeasy 96-kit, according to the manufacturer's instructions (Qiagen, Mississauga, ON). Two replicate wells of hepatocytes were combined prior to RNA extraction (n=3 technical replicates/treatment group) to ensure sufficient yield. Total RNA (300 ng) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen) and resulting cDNA samples were diluted with diethyl pyrocarbonate (DEPC)-water and added directly to RT^2 SYBR Green Mastermix (Qiagen) as described previously (Porter et al. 2014; Pagé-Larivière et al. 2018). Twenty five µL of the mastermix was aliquoted to each well of the 6th generation chicken RT^2 Profiler ToxChip PCR Array (Qiagen, Frederick, MD; Catalog # CAPG13982; Table S2) containing primers at pre-optimized concentrations. Each ToxChip comprised two identical sets of 43 target genes and 5 controls (2 housekeeping genes, a positive PCR control, a reverse transcription control, and a genomic DNA contamination control) on a 96-well plate.

Data analysis

PAC concentrations detected in lake sediment core sections (top [T] and bottom [B]) are reported in ng/g TOC. Simple bar graphs were used to compare ∑alkylated, ∑parent, and ∑total PACs in lake sediment cores.

Changes in hepatocyte cell viability following lake sediment extract administration were determined using a one-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison test using GraphPad Prism v5.02 (San Diego, CA, USA). EROD activity data were fit to a modified Gaussian curve and EC50 values were calculated, when possible (i.e. if maximal EROD induction was reached) (Kennedy et al. 1995; Herve et al. 2010). In cases where maximal EROD induction was not attained, $EC_{threshold}$ (EC_{thr}) was calculated, the dilution of extract that

elicited a significantly higher EROD induction than the DMSO-treated cells. A one-way ANOVA and Dunnett's Multiple Comparison test was used to calculate EC_{thr} with DMSO as the control. For both cell viability and EROD assays, data were normally distributed and therefore, parametric tests were applied.

ToxChip PCR array data were analyzed according to Mundy et al. (2019); an example of raw cycle threshold (Ct) data is available in the supplemental data (Table S3). Ct data were normalized to two housekeeping genes (elongation factor 1-alpha [*Eef1a1*] and ribosomal protein L4 [*Rpl4*]), and fold change of target gene mRNA abundance to the vehicle control was calculated using the $2^{-\Delta Ct}$ method (Schmittgen and Livak 2008). Significant fold change differences (≥1.5-fold, $p<0.05$) of sediment core extract-exposed cells compared to the DMSO control were calculated using a two-way t-test (Qiagen PCR Array Data analysisV4). Hierarchical clustering was performed in R software environment (V3.2.5; R Development Core Team) using the "heatplot.2" function and "gplots" package.

RESULTS AND DISCUSSION

Chemical analysis of PAC extracts

Top sediment core sections collected from two lakes in the SMA of the AOSR (WF1 and SL) had total sum PAC concentrations (parent and alkylated) (2007 ng/g TOC [WF1.T] and 2375 ng/g TOC [SL.T]) more than an order of magnitude higher than ML (158 ng/g TOC [ML.T]), a reference site located south of Fort McMurray (Figure 1). ∑PACs in lake sediment cores from highest to lowest was: SL > WF1 > ML. In WF1, both alkylated and parent PACs were higher at the top (WF1.T) compared to the bottom (WF1.B) of the core. Alkylated PACs comprised the majority of the total PAC profile at WF1; 85% (WF1.B) and 89% (WF1.T). The

relatively high concentration of alkylated PACs (compared to ML) in the bottom section of WF1 could reflect the presence of natural petroleum sources. Given that WF1 is located inside the SMA, the higher PAC concentrations at the bottom of the core (WF1.B) may be the result of the underlying geology (Conly et al. 2002), i.e., oil sands, which are often dominated by alkylated rather than parent PACs (Yang et al. 2014). Interestingly, the proportions of ∑BNT, ∑Chrysenes (CHY), and ∑DBT (parent and alkylated for each respectively) were higher in the top cores of all lake sediments, which indicates a shift to petrogenic sources, as discussed by Salat et al. (2020). BNT, CHY and DBT are commonly associated with oil sands mining operations and crude oils (Kelly et al. 2009; Zhang et al. 2015); higher proportions of these PACs in top versus bottom sediment sections of SL and WF1 may be the result of atmospheric deposition associated with mining operations. Within the SMA, atmospheric transport of PACs into aquatic environments has been linked to major point sources, including upgrader emissions and/or unweathered bitumen in the form of dust particles (Kelly et al. 2009; Kurek et al. 2013; Jautzy et al. 2015).

ML located south of the SMA, is not influenced by PAC sources from the SMA (i.e., upgraders and unweathered bitumen), as a result of prevailing winds and the underlying geology. ML is adjacent to a highway (i.e., highway 63), which may have altered PAC additions into the system via surface runoff. Previously, Salat et al. (2020) demonstrated an increasing petrogenic signature in upper ML sediments (early 2000s), likely associated with petroleum by-products *via* surface runoff from highway vehicular traffic. Such source information was informed based on the analysis of PACs and petroleum biomarker diagnostic ratios (Salat et al. 2020). Additionally, Markiewicz et al. (2017) demonstrated that PAH runoff from a highway (Gothenburg, Sweden) resulted in 2-6% (5.8-29kg) of annually emitted PAHs entering the stormwater system. These

PAHs were from vehicle exhaust, tire wear, lubricating oils, road surface wear and brake linings. Thus, the shift in PAC proportions in ML is likely a result of anthropogenic activity.

Parent PACs in ML.T were less concentrated (relative to ML.B), which may be attributed to changes in sedimentation rate or solvent depletion processes, including diagenesis and sedimentary mineralization of organic matter, which results in contaminant amplification in recalcitrant organic carbon at the bottom of the core (Figure 2, Table S4) (Macdonald et al. 2002). Similarly, SL.B had higher concentrations of both alkylated and parent PACs, relative to SL.T. Previous down core analysis of SL showed an increase in both alkylated and parent PACs beginning in the 1970s coeval with mining operations (Salat et al. 2020). In ML and SL, sedimentation rates were historically low, increasing towards the top of the profile. When sedimentation rates are accounted for in PAC analysis, the increases in PAC flux through time are visible (Salat et al. 2020). For the purposes of in vitro toxicity screening in the present study, we evaluated two broad core sections, which would not account for changes in sedimentation rates. However, this does not negate the fact that the results of the toxicity testing were based on the raw PAC extracts obtained from the sediment sections shown in Figure 2.

The PACs measured in this present study do not represent the entire suite of PACs or other hydrophobic chemicals that could be present in such sediment core extracts. Nevertheless, it is concerning to observe higher PAC concentrations (WF1.T) and proportions of alkylated PACs (SL.T) in more recently deposited sediment in terms of the potential to elicit toxic effects in exposed organisms (Smits et al. 2000; Douben 2003). Importantly, the concentrations of quantified PACs were variable both in terms of geographical space (i.e., between lakes) and within individual cores (i.e., top vs bottom) enabling us to determine if the biochemical and gene

expression approaches utilized were sufficient to reflect this variability in a real-world extract preparation.

Effects of sediment extracts on EROD activity in CEH

Sediment extracts from the lakes in the SMA (SL and WF1) induced EROD activity to a greater extent than those from the reference lake (ML) (Figure 3). The observed decrease in EROD activity at higher sediment core extract concentrations at SL and WF1 was expected based on earlier studies (Herve et al. 2010; Mundy et al. 2019). EROD activity findings were corroborated by the PAC concentration data; i.e., two distinct groups (impacted vs. reference). The rank order of sediment extracts based on EC_{thr} was as follows: WF1.T < SL.T = SL.B < WF1.B < ML.T = ML.B (Figure 3). EC50 values for ML could not be determined because EROD activity did not reach a maximum. This result is unsurprising as ML had very low PAC concentrations in both sediment sections and is located over 100 km from the SMA and its respective upgraders. Interestingly, EC50 values for SL were similar (SL.T = 0.0155; SL.B = 0.0194) and EC_{thr} values were identical between the two core sections even though SL.B had higher ∑PACs. This finding may be associated with the distinct composition of the PAC mixtures at different depths at SL along with fluctuations in sedimentation rate, resulting in greater deposition of sediment in recent times, which may dilute the atmospheric deposition of PACs. Conversely, for WF1, a distinct top/bottom trend was observed in both measured PAC concentrations and EROD activity. Variable EROD EC50 values were observed between the top of the sediment core (WF1.T; 0.0155) and the bottom (WF1.B; 0.0340). The clear separation between the two sediment sections based on EROD activity data was likely associated with the differences in PAC concentrations and the proportion of PACs present in the extract mixtures.

EROD activity is a well-established biomarker of Aryl hydrocarbon receptor (AhR) pathway perturbation following exposure to planar halogenated chemicals, PACs, and other complex environmental mixtures in fish, amphibians, and avian species (Smits et al. 2000; Whyte et al. 2000; Gauthier et al. 2004; Colavecchia et al. 2007; Crump et al. 2015). In particular, measuring EROD activity in CEH has been an effective screening approach for a wide range of complex mixtures to date (e.g. Crump et al. 2015; Crump et al. 2017; Crump et al. 2019; Mundy et al. 2019). Hersikorn and Smits (2011) reported higher EROD activity in wood frog tadpoles raised within young (<7 years old) wetlands formed from reclaimed oil sands process affected materials. This is in agreement with the results for WF1 in the present study, where top sediment sections (i.e., more recent) in the SMA elicited higher EROD activity in avian hepatocytes. Similarly, nesting tree swallows (*Tachycineta bicolor*) living on reclaimed wetlands had increased cytochrome P450 activity proportional to the degree of wetland contamination (Gentes et al. 2006). Furthermore, Smits et al. (2000) reported increased EROD activity in tree swallows, which was correlated with PAH concentrations in sediment samples from nearby wetlands. This highlights the potential importance sediment contamination may have on a surrounding area, effecting wild bird populations that may not be in direct contact with sediment.

Finally, EROD induction and associated AhR activation may have been influenced by additional chemicals, which were not quantified in the sediment core extracts, but that can elicit effects via this nuclear receptor. However, it is not feasible from a cost/time perspective to screen for all potential chemicals that may agonize the AhR. Rather, presenting a suite of PAC data and corresponding EROD activity data provides insight in terms of contaminant profile changes through time in lake sediments from impacted and non-impacted sites in the AOSR.

Gene expression evaluation using the chicken ToxChip PCR array

The 6th generation chicken ToxChip PCR array was used to evaluate the molecular-level effects of lake sediment core extracts in CEH on a more extensive range of toxicity pathways than that captured using the EROD assay alone. From a QA/QC perspective, the cycle threshold values of the two housekeeping genes (*EEF1A1* and *RPL4*) were invariable regardless of sediment core extract administration and the genomic DNA contamination control, positive PCR control, and reverse transcription control all passed the relevant quality control and assurance criteria (Qiagen). A complete list of all gene targets and their respective fold changes for the extracts is included in Table S5.

The top and bottom sediment core extracts from each lake clustered together with a distinctive branch separating the reference lake, ML, from both lakes located within the SMA (SL and WF1; Figure 4). This result suggests that the chemical mixture profiles were more similar, albeit at variable concentrations, within a site, as opposed to among sites and that the location of the site (i.e., proximity to SMA) was important in terms of the gene expression response. ML extracts led to the dysregulation of fewer target genes overall – 13/43 and 7/43 for ML.T and ML.B, respectively – compared to WF1 (17-19/43) and SL (18/43) (Figure 4, Table S5). Furthermore, the xenobiotic metabolism enzyme associated with AhR activation, *CYP1A4*, was the most responsive transcript in terms of sediment PAC extract exposure and thus, influenced the hierarchal clustering. For example, fold-changes in the top sediment section for *CYP1A4* were 27.3-, 26.5-, and 15.5-fold for SL, WF1 and ML, respectively (Table S5). Given the role of *CYP1A4* in PAC metabolism (Shimada and Fujii-Kuriyama 2004), this variable response among sites was expected based on the chemical and EROD activity profiles described above.

In addition to *CYP1A4*, several other transcripts from the chicken ToxChip were dysregulated in CEH exposed to sediment extracts from all lakes at the two discrete depth sections. These included genes associated with xenobiotic metabolism (aminolevulinate, delta, synthase 1 [*ALAS1*]; methionine adenosyltransferase I, alpha [*MAT1A*]), lipid homeostasis (solute carrier organic anion transporter family, member A2 [*SLCO1A2*]; stearoyl-CoA desaturase (delta-9-desaturase) [*SCD*]), and immune function (fibrinogen alpha chain [*FGA*]) (Figure 4, Table S5). The fold changes in gene expression were greater at SL and WF1 than ML in all cases (Table S5), therefore permitting a certain level of geographic discrimination (i.e., impacted vs. non-impacted). However, given the significant change at all sites, these genes may not be as useful for discriminating subtle PAC contamination status of extracts. *ALAS1* is associated with the heme biosynthesis pathway and is expressed predominately in liver cells where high concentrations are required for hemoglobin and cytochrome P450 biosynthesis (Riddle et al. 1989). Previous studies have reported *ALAS1* induction in CEH exposed to complex environmental extracts prepared from herring gull eggs (Crump et al. 2015), petcoke (Crump et al. 2017), and passive samplers deployed at variably contaminated wetlands in the AOSR (Mundy et al. 2019). *FGA* was downregulated in CEH ranging from -5.48 to -1.77-fold depending on the lake sediment core section (Figure 4, Table S5). Fibrinogen is a positive acute phase protein produced by hepatocytes, where it is converted to fibrin by thrombin during coagulation, and increased production occurs in states associated with inflammation (Gardhouse and Eshar 2016). A concordant downregulation of *FGA* was reported in CEH exposed to delayed (-11.2-fold) and fluid petcoke (-2-fold) (Crump et al. 2017) as well as extracts from three wetlands near surface mining operations (Mundy et al. 2019). Finally, *SLCO1A2* was downregulated following exposure of CEH to all sediment core extracts (-4.35 to -1.7-fold;

Table S5). *SLCO1A2* is an important organic anion transporter, mediating the uptake of numerous endogenous amphipathic substrates, including bile salts, thyroid hormones, steroid conjugates, and prostaglandin E2 (Franke et al. 2009; Eloranta et al. 2012), as well as pharmaceuticals and xenobiotics. Exposure of CEH to petcoke resulted in a concordant downregulation of *SLCO1A2* mRNA (-2.31-fold) (Crump et al. 2017).

Alternatively, for several gene targets, significant dysregulation was only observed at the two impacted lakes, SL and WF1 (e.g., heme oxygenase 1 [*HMOX1*], *CYP7B1*, liver fatty acid binding protein 1 [*LBFABP*], liver expressed antimicrobial peptide 2 [*LEAP2*], Figure 4, Table S5). *HMOX1* is induced by its substrate heme and a number of other agents that cause oxidative stress (e.g., endotoxins, heavy metals, and heat shock) (Choi and Alam 1996) . The greatest upregulation of *HMOX1* was observed following exposure of CEH to WF1.T (2.65-fold), comparable to the fold-change induction reported by Mundy et al. (2019) for CEH exposed to the most PAC-contaminated passive sampler extracts. Oxysterol 7α-hydroxylase (*CYP7B1*) was downregulated ~2-fold following exposure to sediment core extracts from SL and WF1 (Figure 4, Table S5). This cytochrome P450 enzyme plays a role in bile acid synthesis and conversion of cholesterol to bile acids (Schwarz et al. 1998). A concordant decrease in expression was observed in CEH exposed to delayed petcoke, but not fluid petcoke (Crump et al. 2017); the former having greater PAC concentrations, much like the impacted vs reference lake cores in the present study. Additionally, *CYP7B1* was downregulated in CEH exposed to herring gull and double crested cormorant egg extracts, but only for the sites with the highest organohalogen contaminant egg burdens (Crump et al. 2015; Crump et al. 2019). *LBFABP*, a gene associated with lipid homeostasis, plays an integral role in the metabolism and transport of bile acids in avian livers (Murai et al. 2009). In avian liver cells exposed to sediment core extracts from the

two impacted lakes, *LBFABP* was downregulated -2.4- to -2.8-fold. Once again, exposure of CEH to other PAC complex mixtures derived from petcoke (Crump et al. 2017) and passive samplers (Mundy et al. 2019) revealed a significant downregulation of this gene target, which was dependent upon relative PAC burdens.

Finally, two genes (transthyretin [*TTR*], thyroid hormone responsive SPOT14 [*THRSP*]) associated with the thyroid hormone pathway – critical for normal growth and development in birds – were downregulated in all lake sediment sections except ML.B (Figure 4, Table S5). Previous studies have demonstrated adverse health effects of PAH exposure on thyroid status in fish and wildlife that include: developmental deformities, decreased reproductive success, and dermatologic abnormalities (Rolland 2000). Furthermore, deer mice (*Peromyscus maniculata*) living on reclaimed mine sites in the AOSR had altered thyroid gland pathology and circulating thyroid hormone concentrations (Movasseghi et al. 2017). In avian species specifically, Gentes et al. (2007) reported altered plasma triiodothyronine and thyroxine content of thyroid glands in tree swallow nestlings that were exposed to oil sands processed materials. Thus, the dysregulation of two genes associated with the thyroid hormone pathway is concordant with previous studies that have reported associations between thyroid hormone pathway dysregulation and exposure to PAC mixtures. Furthermore, *TTR* and *THRSP* were similarly downregulated in CEH following exposure to delayed (20-fold) and fluid (3-fold) petcoke extracts, while *THRSP* (downregulated -2.3- to -4.7-fold in CEH in the present study) was also downregulated following exposure to extracts of passive samplers from a highly contaminated wetland site in the AOSR (Mundy et al. 2019).

Overall, the spatial proximity of lakes to oil sands mining operations in the AOSR was reflected in the PAC concentrations in sediment cores and the biochemical and gene expression

signatures in avian hepatocytes. For certain core samples, temporal trends were also reflected in the measured endpoints when comparing historical lake sediment extracts to those from the year 2000 onwards. The avian in vitro screening approach represents a promising method for oil sands monitoring, especially as it relates to hotspot identification (e.g., more vs less contaminated lake sediments) as well as a potential means to monitor changes in the local environment through time. Future studies should analyze sediments from specific time periods to determine a more precise chronology of shifting toxicity profiles in lakes effected by oil sands mining operations. Additionally, further evaluation of confounding variables such as sedimentation and deposition rates would improve the understanding of chemical and toxicological trends observed in impacted lakes. Our results support the use of this in vitro approach as a tool to aid in identifying areas significantly affected by oil sands mining.

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Supplementary Information

Table S1, list of PACs and abbreviations; Table S2, list of genes and their function on the chicken ToxChip PCR array; Table S3, example of raw cycle threshold data; Table S4, complete list of PAC quantification data in sediment cores; Table S5, normalized fold change of target genes following sediment extract exposure.

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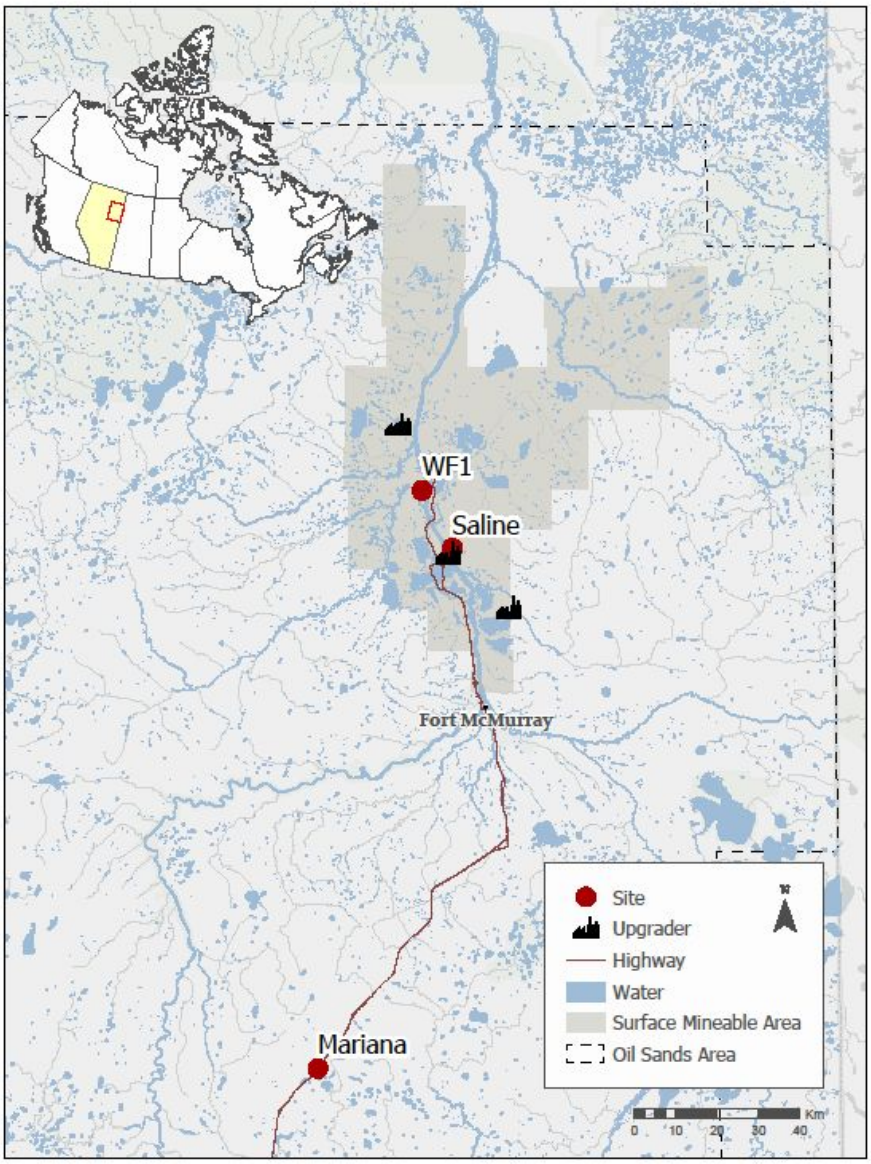


Figure 1. Map of study region with sampling locations in the Athabasca oil sands region: Saline Lake (57°04'41.81" N, 111°31'20.43" W), Mariana Lake (55°57'02.35" N, 112°01'34.97" W), and WF1 (57°12'10.14" N, 111°38'41.82" W). The map inset shows the study region circled in red.

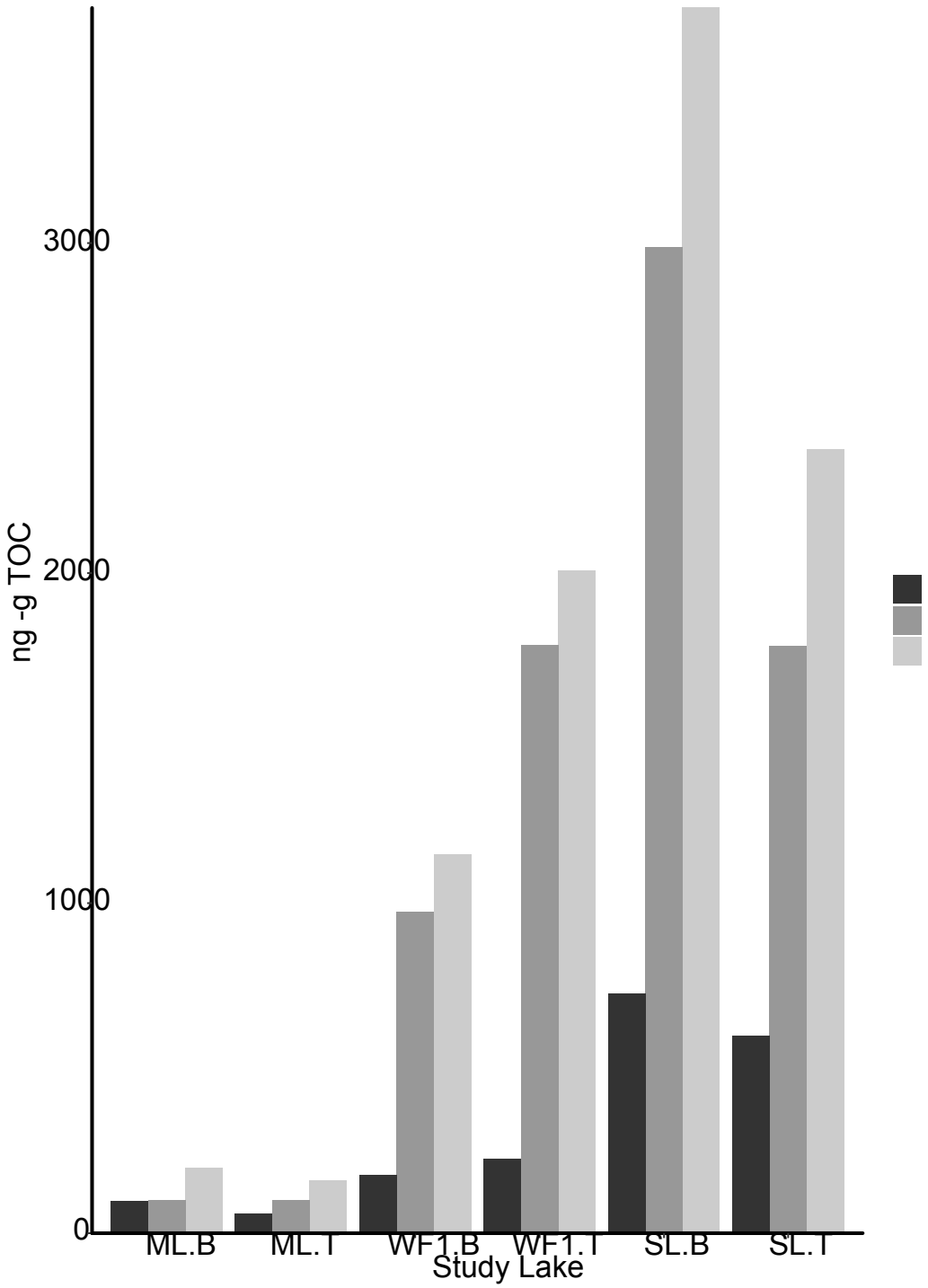
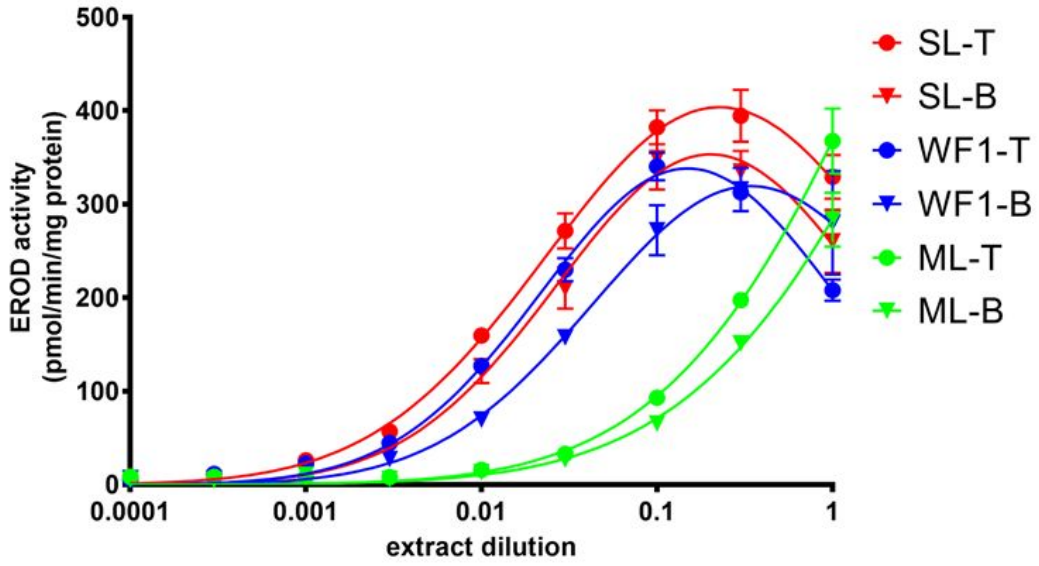


Figure 2. ∑Total PACs (light gray bar), ∑alkyl PACs (medium gray bar), and ∑parent PACs (black bar) detected in lake sediment extracts (ng g^{-1} TOC) from 2 boreal lakes in the surface minable area of the AOSR (SL, WF1) and 1 reference lake (ML).



Lake	EC50	EC threshold
SL.T	0.0155	0.01
SL.B	0.0194	0.01
WF1.T	0.0155	0.003
WF1.B	0.0340	0.03
ML.T	nc	0.1
ML.B	nc	0.1

Figure 3. Concentration dependent effects of polycyclic aromatic compound (PAC) extracts on 7-ethoxyresorufin-*O*-deethylase (EROD) activity in cultured chicken embryonic hepatocytes (CEH). Data points represent the mean value obtained from triplicate cell culture plates ± standard error. Extract dilution values are represented on a log scale for EROD activity, and the mean effect concentration 50 (EC50) and $EC_{threshold}$ (EC_{thr}) values are included for each PAC extract; nc represents EC50 and EC_{thr} values that were not calculated.

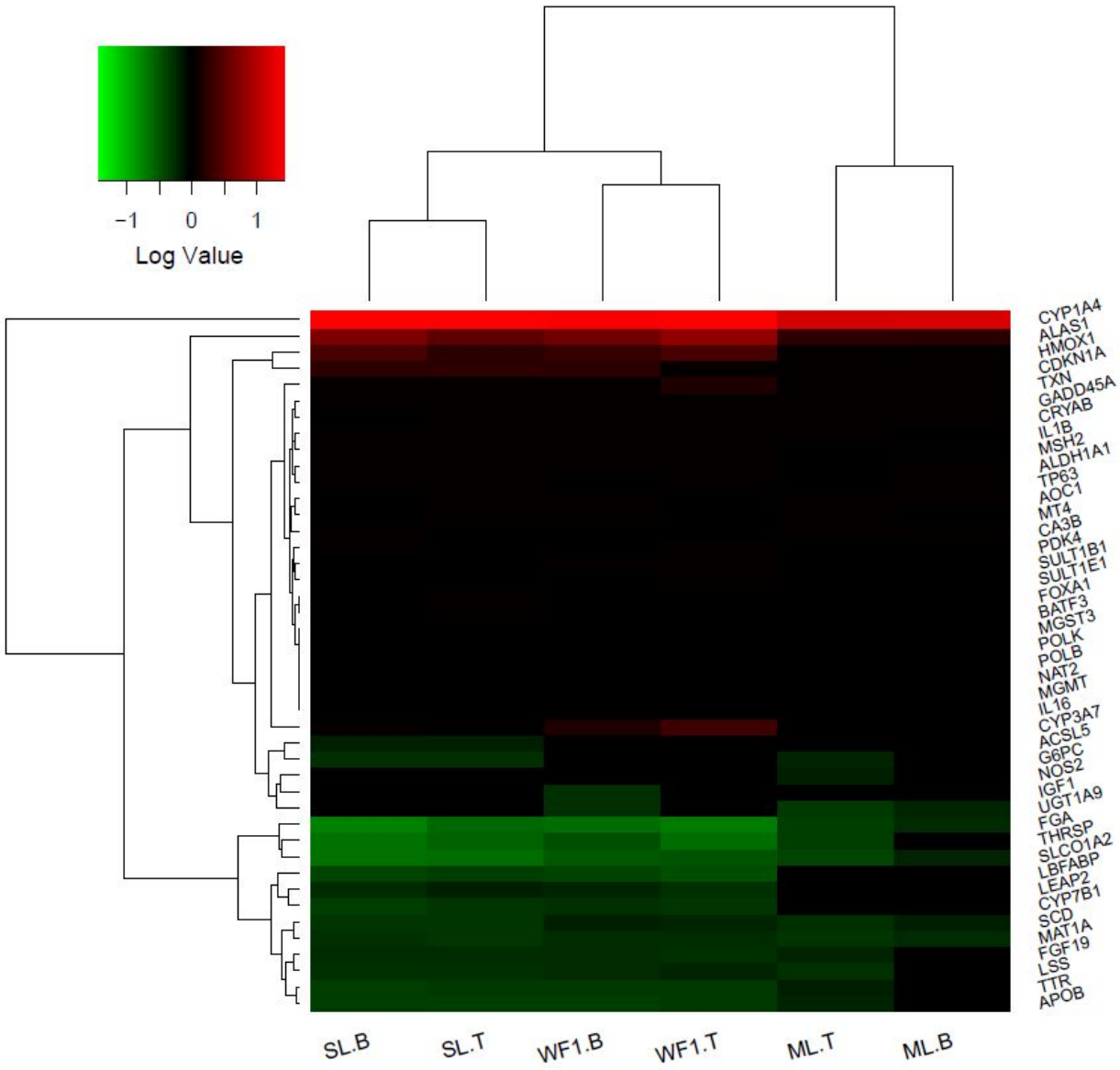


Figure 4. Heat map depicting significant fold changes of genes (≥1.5-fold, $p<0.05$) on the 6th generation chicken ToxChip PCR array clustered by polycyclic aromatic compound (PAC) extracts (1 or 'neat') generated from sediment sections at 3 variably contaminated lakes at two depths, located in the Athabasca oil sands region (AOSR). Red and green hues indicate significant up and down regulation, respectively.