Oil Sands Process-Affected Water Toxicity Attribution and Evaluating Ageing as a Remediation Strategy

by

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Abstract

Oil sands process-affected water (OSPW) is a byproduct of bitumen extraction in the surface-mining oil sands industry of Northern Alberta. OSPW contains a complex and environmentally persistent dissolved organic mixture that can be toxic to aquatic organisms. One long-term remediation strategy involves ageing of OSPW in end-pit lakes such that *in situ* natural processes will eventually allow detoxification and safe environmental re-integration of this water. Over 30 end-pit lakes are planned, but only one has been established so far, Base Mine Lake (BML), which was commissioned in 2012 at Syncrude Canada Ltd. Predicting the effectiveness of this strategy relies on an understanding of what chemicals cause toxicity in fresh OSPW, and how the chemical mixture might change over time. This investigation used chemical fractionation and ultrahigh resolution mass spectrometry combined with cytotoxicity and endocrine disruption assays to further study the toxicity of candidate chemical classes in fresh and aged OSPW samples. Real-time cell analysis with human liver carcinoma cells (HepG2) was used with OSPW samples for the first time, while the yeast estrogenic/androgenic screens were used as a standardized and comparable assay to previous studies. A chemical fraction isolated from BML 2015 containing naphthenic acids (NAs) was largely responsible for the cytotoxicity observed towards HepG2 cells, with a point of departure (IC_{10}) at 17 mg/L, similar to the estimated field concentration of NAs in BML 2015 (12.9 mg/L). A corresponding non-acid fraction, speculated to contain steroid-like chemicals, was not cytotoxic up to 12.5× above field concentrations in BML 2015. The total organic extract of BML 2017 was cytotoxic to HepG2 cells, generating an IC_{50} of 8× above field concentrations, with the point of departure marginally above 1×. Based on a series of BML water samples collected over a 4-year span, the cytotoxicity of BML extracts (i.e. toxicity per volume) decreased with ageing, while the toxic potency of the extracts (toxicity

per mass of extract) was not significantly different between years. This toxicological result was supported by mass spectral evidence whereby total *m/z* intensity of BML organics decreased in samples over time, but the relative proportion of chemical classes remained unchanged. Older OSPW aged 23 years in an experimental pond had a unique biphasic toxicity profile (time-dependent), with a toxic potency greater than BML at 24 h post exposure, but not at 60 h. This sample also had a unique distribution of chemical classes compared to BML. Estrogen and androgen receptor antagonists, but not agonists, were identified in all BML 2015 fractions. Notably, the NA and non-acidic fractions showed endocrine activity below environmentally relevant concentrations and were more potent (toxicity per mass of extract) than positive control antagonistic hormones. Overall, with BML and experimental pond samples ranging up to 23 years of ageing in the field, there was little difference in the potency of estrogen and androgen receptor antagonism between the organic extracts, and always with EC_{50}'s below environmental concentrations and similar to positive control antagonistic hormones. While organic acids, including naphthenic acids, decreased to a large extent in the aged samples, chemical classes detected in positive mode (i.e. polar organics) were less depleted and became relatively enriched in the total organic extract, suggesting that non-acid polar organics contribute to environmentally persistent estrogen and androgen receptor antagonism. Overall, decreases in cytotoxicity in BML over time, although promising for the end-pit lake strategy, are likely only due to a dilution effect. Also, this is the first report of an older experimental pond sample having a biphasic cytotoxicity profile, which, along with its chemical profile, remains to be understood. This highlights the importance of chemical composition, not only concentration, in determining risk, and warrants further research into chemical-specific regulatory limits needed to ensure environmental protection.

Preface

This thesis is original work by Ian Gault. No part of this thesis has been previously published.

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List of Abbreviations

(-) – Negative mode, acidic species

(+) – Positive mode, non-acidic species

× – Enrichment factor scale

4-HT – 4-Hydroxytamoxifen

ABC – ATP-binding cassette

AC – Activated carbon

AhR – Aryl Hydrocarbon Receptor

Anti-YAS – Yeast androgenic screen antagonism

Anti-YES – Yeast estrogenic screen antagonism

APCI – Atmospheric pressure chemical ionization

APPI – Atmospheric pressure photoionization

AR – Androgen receptor

As (III) – Arsenite

BML – Base Mine Lake

BrdU – Bromodeoxyuridine

C – Carbon

CAR – Constitutive androstane receptor

CAT – Chloramphenicol acetyltransferase

CDFA, AM – 5′-carboxyfluorescein diacetate-acetoxymethyl ester

CI – Cell index

CPRG – Chlorophenol red-β-D-galactopyranoside

DHT – 5α-dihydrotestosterone

E2 – Estradiol

EC_{50} – Treatment concentration that results in 50% of a response of the selected endpoint

EDA – Effects-directed analysis

EMEM – Eagle's minimum essential media

EPL – End Pit Lake

EQ – Equivalence

ER – Estrogen receptor

ESI – electrospray ionization

FFT – Fluid fine tailings

FL – Flutamide

FTICRMS – Fourier transform ion cyclotron resonance mass spectrometry

FTIR – Fourier transform infrared spectrometry

G – Growth Factor

GC/MS – gas chromatography mass spectrometry

GST – Glutathione s-transferase

HepG2 cells – human liver carcinoma cells

HPLC – high pressure liquid chromatography

IC_{10}/EC_{10} – Point of departure threshold for an effect

IC_{20} – Treatment concentration that inhibits the response of the endpoint tested by 20%

IC_{50} – Treatment concentration that inhibits the response of the selected endpoint by 50%

I_R – Induction ratio

JOSMP – Joint Canada-Alberta Oil Sands Monitoring Program

K_{ow} – Octanol-water partitioning coefficient

LC/MS water – Liquid Chromatography Mass Spectrometry grade water

LC_{50} –Treatment concentration that is lethal to 50% of the sample

m/z – Mass to charge ratio response in mass spectrometry

MFT – Mature fine tailings

MLSB – Mildred Lake Settling Basin

MS – Mass spectrometry

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N – Nitrogen

NA – Naphthenic acids

NCI – Normalized cell index

OD – Optical density

OECD – Organisation for Economic Co-operation and Development

OSLW – Oil sands lixiviate water

OSPW – Oil sands Process-Affected Water

OSW – oil sands water

Oxy-NAs – oxygenated naphthenic acids

PAC – Polycyclic aromatic compounds

PAH – Polyaromatic hydrocarbons

PBS – Phosphate-buffered saline

PC – Petroleum coke

PCA – Principle component analysis

PPARγ – Peroxisome proliferator-activator receptor γ

PUCA – Pull-down assay with untargeted chemical analysis

PXR – Pregnane X receptor

qPCR – Quantitative polymerase chain reaction

RAMP – Regional Aquatic Monitoring Program

RNA-seq – RNA sequencing

ROS – Reactive oxygen species

R_R – Reduction ratio

RTCA – Real-time cell analysis

S – Sulfur

SEM – Standard error of the mean

SEP – South East Pond

SPE – Solid phase extraction

SWSB – Southwest Settling Basin

T – Testosterone

TAE – Total acid extract of environment samples

TCRPs – Time-dependent cellular response profiles

TIE – Toxicity identification evaluation

TR – Thyroid receptor

TT21C – Toxicity testing in the 21st century

U_s – β-galactosidase activity

US EPA – US Environmental Protection Agency

VTG – Vitellogenin

WIP – West-In-Pit

YAS – Yeast androgenic screen

YES – Yeast estrogenic screen

Z – Impedance

Glossary of Terms

4-HT EQ – 4-hydroxytamoxifen equivalence. A parameter comparing the potency of environmental samples to the potency of 4-hydroxytamoxifen, a known antagonistic hormone of the estrogen receptor.

Active pond – A tailings pond that is currently receiving OSPW from the bitumen extraction process.

Acute toxicity – Exposure time of the experimental treatment is short and a single treatment dose.

Adverse effect potency – Potency for an adverse effect observable in a whole organism. There is sufficient mechanistic potency in a system of molecular pathways to lead to an adverse effect.

Adverse outcome pathway – *"conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment"*[1] This term is used to encompass the nuanced terminology between mode and mechanism of action.

Agonism – Defined here as the process by which a chemical binds to and activates a receptor, leading to induction of a biological response.

Antagonism – Defined here as the process by which a chemical binds to and inhibits a receptor, leading to a reduction in a biological response.

Apical endpoints – An observable adverse effect to a test organism.

Bioaccumulation – Umbrella term describing the process by which a chemical may be taken up by the organism from the contacted medium or through oral ingestion.

Biphasic toxicity profile – Described here as time-dependent toxicity, where there are two phases of the toxic effect depending on the time of exposure.

Chronic toxicity – Exposure time is over a substantial period of the test organism's life and may include chronic exposure to the treatment or multiple doses over time.

CI – Cell index is an impedance-based parameter of cell adhesion to an electrode, representing changes in cell growth, proliferation, and/or morphology.

Cytotoxicity – Toxicity to the cell *in vitro*, ultimately leading to cell death through apoptosis or necrosis. Used as an umbrella terms for multiple mechanism of action affecting cell viability or proliferation.

EC_{50} - Treatment concentration on a toxicological dose-response curve that corresponds to 50% of an effect of a tested endpoint. Used here to describe and rank endocrine activity of environmental samples.

EDA – An effects-directed analysis approach combines sequential chemical fractionation and bioassay tests to attribute a toxicological endpoint to specific chemicals within a mixture.

Endocrine activity – An exogenous substance that perturbs a mechanistic pathway of the endocrine system. Not classified as an endocrine disrupting compound unless the mechanistic potency leads to an apical, observable adverse effect in an organism.

Enrichment factor (×) – A scale that is relative to the volume of original water an organic extract is derived from, where 1× is the field concentration of organics, more than 1× a concentrate and less than 1× a dilution. Assesses environmental relevance. The counterpart to 'toxic potency,' which uses the absolute scale of toxicity per mass of extract and negates the volume of original water.

Environmental protection – a framework published by the Government of Alberta on the management of industrial effluents in Alberta. Safe release is based on a triad approach of whole effluent toxicity, chemical specific toxicity, and biological effects monitoring.

Environmental reference – Represents a negative control for environmental samples. Used here to define water from the Athabasca River. This is within the McMurray geological formation, yet is upstream of the anthropogenic influence of the oil sands industry.

EPL – An end pit lake is an artificial lake used as a reclamation or remediation strategy for industrial byproducts.

Field concentration – Term used in this investigation to signify the concentration of organics in OSPW in its original aqueous state, also represented by an enrichment factor of '1×'.

FL EQ – Flutamide equivalence. A parameter comparing the potency of environmental samples to the potency of Flutamide, a known antagonistic hormone of the androgen receptor

Heteroatomic chemical class – Technique used in complex mixtures of grouping chemical species by their number of oxygen, sulfur, or nitrogen atoms (heteroatoms), while excluding carbon and hydrogen atoms for simplicity.

IC_{50} - Treatment concentration on a toxicological dose-response curve that inhibits the response of the tested endpoint by 50%. Used here to describe cytotoxicity by RTCA.

I_R – Induction ratio in the YES/YAS assay. Calculated parameter to distinguish changes in estrogen or androgen receptor interactions compared to the solvent control

LC_{50} –Treatment concentration on a toxicological dose-response curve that kills 50% of the test sample population.

Mechanism of action - ''*A complete and detailed understanding of each and every step in the sequence of events that leads to a toxic outcome*."[1] However, in practice, and in this

investigation, mechanism of action is used to describe a specific portion of a biological pathway, not necessary the boarder scope of a common mode of action.

Mechanistic potency – Defined here as the potency to perturb a molecular pathway in an *in vitro* screen, not the potency leading to an adverse effect observable in a whole organism.

Mixture effects – When components of a mixture have different toxicological potencies in isolation compared to within a mixture. Includes synergistic or antagonistic effects.

Mode of action – "*A common set of biochemical, physiological, or behavioral responses that characterize an adverse biological response where major, but not necessarily all, linkages between a direct initiating event and an adverse outcome are understood*"[1] However, in practice, and in this investigation, mode of action is used to more broadly describe a biological pathway, as opposed to the exact mechanism of a specific portion.

Negative mode – Detection in mass spectrometry requires chemicals in their ionized form. In negative ionization mode, only chemicals that form anions can be detected, and this is typical of acidic species with a COOH functional group that readily loses a proton to become COO^-, though a negative charge can also be generated to other functional groups.

OSPW organics – The whole organic mixture in oil sands process-affected water. Referred to synonymously as bitumen-derived organics.

Persistent – Umbrella term describing a chemical that resists degradation and remains present in the environment over a significant period of time, depending on the media.

Point of departure – Point on a toxicological dose-response curve corresponding to the threshold of an observable effect. Calculated here through an EC_{10} or IC_{10} analysis.

Positive mode - Detection in mass spectrometry requires chemicals in their ionized form. In positive ionization mode, only chemicals that form cations can be detected, and this is

typical of polar non-acidic species that can readily accept a proton and become positively charged.

Recalcitrant organics – Term used to describe the OSPW organics that resist degradation and remain persistent despite having undergone a remediation treatment.

Reclamation – "*Returning disturbed land to a stable, biologically productive state*."[2]

Remediation - The process of reducing hazardous material to prevent or minimize risk to the environment.[3]

R_R – Reduction ratio in the YES/YAS assay. Calculated parameter to distinguish changes in estrogen or androgen receptor antagonism compared to the baseline level of stimulation in the agonist controls wells.

Species – Empirical formula of a chemical without any knowledge of chemical structure.

Sublethal effect – An effect that is below the threshold of lethality to an organism or cell culture.

TAE – A total acid extract, performed here at pH 2. Contains the toxicologically relevant neutral- and acid- extractable organics of oil sands-process affected water.

Toxic potency – Potency for an adverse effect defined here in the absolute scale of toxicity per mass of extract, negating the volume of original water. Counterpart to assessing environmental relevance through an enrichment factor scale (×).

Toxicity identification – The standardized form is termed a Toxicity Identification Evaluation (TIE), where components of an effluent mixture are sequentially removed to evaluate where to attribute toxicity. Here, a full TIE was not performed, though a few of the techniques were used, leading to a more rudimentary 'toxicity identification' process.

Whole OSPW – When the organics are incorporated into its real-world aqueous matrix.

1. Introduction

1.1. Oil sands in Alberta

1.1.1. Location, features, and demand for energy

Conventional sources of light oil are becoming more scarce, resulting in more exploitation of non-conventional heavy oil sources, such as the Alberta oil sands.[4,5] The oil sands industry in the Athabasca region of Northern Alberta, Canada, represents great economic potential but also great environmental disruption. The Alberta oil sands regions is the 3rd largest crude oil deposit in the world, following Saudi Arabia and Venezuela.[4] In Alberta there are three geological regions of oil sands deposits: the Athabasca, Cold Lake, and Peace River regions, covering 142,200 km^2.[2]

The size of the of the oil sands industry has increased with rising prices for crude oil, and better technologies, which together have reduced the costs of recovering bitumen from the oil sands. This growth coincides with growing global human population and demands for energy.[6] Bitumen is a highly biodegraded and poor quality viscous form of oil that is high in resins, asphaltenes, sulfur, metals, and acid content.[7] Only 8-14% of the oil sands ore is bitumen, and the extraction and upgrading process used to generate crude oil are much more laborious and energy intensive than for conventional sources of crude oil.[7] Nevertheless, a 2014 report stated that oil sands accounted for 57% of Canada's oil production, with 2.29 million barrels of crude oil produced per day, and 173 billion barrels in oil reserves.[4] The National Energy Board of Canada estimated that global energy demand will increase by 50% by 2030.[8] However, there is concern for the environmental disturbances posed by the oil sands industry in terms of its water use, waste water production and groundwater contamination, growing air emissions, and habitat disturbances, and long-term land and water restoration challenges.[5]

1.1.2. Open pit oil sands mining

While *in situ* mining technologies may be used to extract deep deposits of bitumen positioned more than 75 meters below ground, open pit mining is used for shallower deposits.[2] This process requires extensive land disturbance through clear-cutting, and removal of muskeg and overburden to access the oil sands ore. The ore is crushed into small pieces and either manually or hydraulically transported to an extraction facility where bitumen is extracted through the Clark Caustic Extraction process.[2] By this technique, the crushed ore is mixed with large volumes of water in mixing tanks with sodium hydroxide to facilitate separation. The slurry is aerated to help liberate and float bitumen from the water, stratifying the mixture into layers. The bottom layer contains the heaviest particles (sand/clay), and the middle layer is a slurry of fine particles, unrecovered hydrocarbons and process-affected water, referred to collectively as Fluid Fine Tailings (FFT). The top layer is the valuable bitumen froth which is taken for further processing, including secondary bitumen froth cleaning and dewatering (removal of OSPW).[2]

The remaining sand and FFT layers are further processed but ultimately pumped to vast containment units called tailings ponds.[2] Tailings ponds may be mined out pits, or above-ground containment structures built of sand. While tailings ponds represent a substantial liability in terms of cost, or land and hydrology disturbance, they also serve a function in recycling of water back into the bitumen extraction process. Thus, the solid and aqueous tailings are held on site to prevent environmental contamination, but also to maximize water reuse efficiency.[9] Within a tailings pond, the course solids (sand and clay) quickly settle to the bottom and contribute to the dyke structure which contains the water (**Figure 1**). The remaining FFT consists of oil sands process-affected water (OSPW) and a fine solids fraction of silt and clay that remains suspended in the water column and only slowly sinks and densifies into mature fine tailings (MFT) at the

base of the pond. Over time, the MFT dewaters and releases more OSPW to the overlying water column.[10] The bitumen extraction process is not entirely efficient, and FFT also contains unrecovered bitumen that floats to the surface of the pond over time[2] and may be skimmed away to be further upgraded.

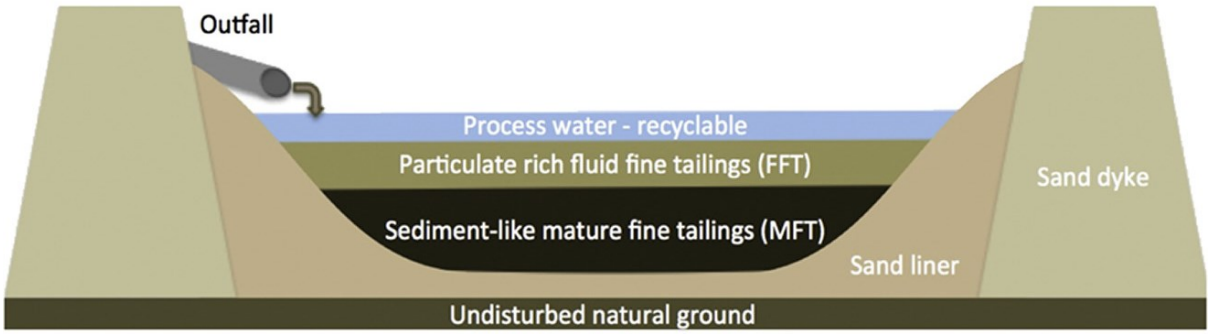

Figure 1: Profile of an Athabasca oil sands tailings pond, with course sand on the bottom layer, followed by mature fine tailings (MFT), fluid fine tailings (FFT), and OSPW on top, labeled in the source figure as 'Process water – recyclable.' Reproduced with permission from, source: Willis et al.[11] abstract image, p. 1604.

In percentages, fresh OSPW is 70-80% water, 10-20% is solid, be that sand, silt, and clay, and 1-3% is bitumen.[12] It is highly concentrated in salts due to their natural occurrence in oil sands ore, and contain heavy metals, such as Al, Mo, Se, and V, which often exceed water quality guidelines.[13,14] There is a complex mixture of dissolved organic chemicals that is persistent and has shown to be the primary toxic component of OSPW, due to amelioration in toxicity after their removal.[13,15] While the recycling process in tailings ponds reduces water withdrawal from the Athabasca River, it concurrently increases the concentration of components such as salts and heavy metals, thereby decreasing water quality of the remaining OSPW.[14] This is problematic for bitumen extraction efficiency, and scaling and corrosion of equipment.

1.1.3. Environmental challenges with open pit mining

1.1.3.1. Water management

It was estimated in 2013 that 976 million m^3 of fluid tailings, including OSPW, is stored on site in tailings ponds and this number is increasing.[16] Oil sands ore consists of 4% water per weight, further adding to OSPW inventories.[2] For every m^3 of synthetic crude oil produced after bitumen extraction and transportation, 2.5 m^3 of additional hot water is required.[2] This water ultimately originates from the Athabasca River, though 80% is recycled from tailings ponds to minimize natural water use and mitigate concern of too much water being taken from the Athabasca River,[2] particularly during sensitive seasonal times that may affect wildlife.[17]

In a 2015 report from Syncrude Canada Ltd., 37.6 million m^3 of fresh water was withdrawn from the Athabasca River that year.[18] More broadly, based on the *Surface Water Quantity Management Framework for the Lower Athabasca*[19] released in 2015, the current licensed net allocation for oil sands industries withdrawing from the Athabasca River, minus the required water returned to the river, is 392,043,101 m^3/year.[18] The estimated net water use from oil sands industries, also minus the actual water returned to the river, is 102,686,300 m^3/year, i.e. ~26% of the licensed volume. Notably, in 2011 the licensed water allocation was 645,547,643 m^3, and actual volume withdrawn was 143,483,558 m^3,[18] so the volumes have decreased with the 2015 management framework. Nevertheless, there is an input of water into the industry with minimal output, leading to further accumulation of on-site containment within tailings ponds.

At Syncrude Canada Ltd. there are multiple interconnected active tailings ponds receiving OSPW. These consist of Mildred Lake Settling Basin (MLSB), South East Pond (SEP), Southwest Settling Basin (SWSB), and Aurora. OSPW is transferred between these structures to aid in the settling process,[10] and MLSB and Aurora are the main sources of recycle water for the

industrial processes.[20] This list does not include tailings ponds from other Alberta oil sands companies. The predicted time for sedimentation of fine particles was estimated in a laboratory study to take 125 years,[21] though the rate *in situ* is largely unknown.

The National Energy Board in 2006 defined reclamation as: "*returning disturbed land to a stable, biologically productive state*."[2] Once oil companies have complied to the Alberta Environmental and Protection Act and have reclaimed the land that they have disturbed, they may be awarded a reclamation certificate as a standardized method of deeming a site as acceptably reclaimed.[2] Remediation, as defined by Alberta Environment in 2002, is the process of reducing hazardous material to prevent or minimize risk to the environment.[3]

There are three main technologies used by Syncrude Canada Ltd to aid the water recycling process through reducing and remediating FFT.[18] Firstly, Centrifuged Tailings was a project launched in 2015 where FFT is centrifuged to accelerate its sedimentation. Secondly, Composite Tailings, is a protocol where FFT is mixed with gypsum, a coagulating agent, to consolidates the fine particles and enhances their rate of sedimentation. The accumulated sediment can then be capped with soil and sand for land reclamation, while the aqueous supernatant water can be recycled for extraction. Lastly, Water Capping, is where FFT is capped with fresh water in the hopes to dilute and degrade the toxic OSPW dissolved organics.[18]

One leading remediation strategy for OSPW, termed the wet landscape or end-pit lake strategy,[22] incorporates water capping and composite tailings techniques. Here, formerly mined-out pits are used as artificial lakes, termed End Pit Lakes (EPLs), to hold OSPW. Input of Fresh OSPW is stopped and the existing OSPW is allowed to age, with additions of fresh water for dilution and coagulating factors to help sediment the fine tailings.[22] It is hoped, but not proven, that OSPW will detoxify over time through sedimentation of the FFT and biodegradation of the

dissolved bitumen-derived organics (see section 1.2.2.1). Ideally, the water will eventually be fit for safe release or hydraulic reconnection to the watershed and not adversely affect the ecosystem. However, uncertainties with this method have been highlighted by the Royal Society of Canada,[2] notably: the rate of biodegradation of the dissolved bitumen-derived organics is expected to be slow[10] but data are sparse and in a full-scale EPL, the rates of biodegradation are unknown. Furthermore, in small-scale experimental ponds under field conditions, studies have shown that aged OSPW samples still have chronic toxicity attributable to the dissolved bitumen-derived organics.[23] Still, EPLs have been used by other industries with varying degrees of success and controversy.[24]

The world's first full-scale oil sands EPL was commissioned in 2012 with the decommissioning of an active tailings pond called the West-In-Pit (WIP), at Syncrude Canada Ltd. This in-ground structure was renamed as Base Mine Lake (BML),[22] and at least 30 more EPLs are planned in the region. EPLs are considered a wet landscape reclamation strategy by the industry, and some pilot-level field research has been conducted over the past ~25 years in various experimental ponds at Syncrude Canada Ltd. to examine pond parameters that might most effectively assist in OSPW remediation, with or without the presence of FFT.[22] In other words, these experimental ponds were prior projects used to inform the construction of BML, the first full-scale EPL that is spatially separate. The age and composition of the experimental ponds vary, and there was no experimental replication. Pond 5 (1989) has a base layer of MFT and was capped with OSPW; Pond 9 (1993) only contains OSPW; Big Pit (1993) has a base layer of MFT and is capped with freshwater; and CT-Pond (1997) only contains pore-OSPW that has been released from MFT after treatment with gypsum.[10] Despite their limitations, considering that these four experimental ponds have been aged longer than BML, they may help to predict the

future water chemistry and toxicity of BML, which is physically similar but much larger and deeper than Pond 5.

The water quality in BML is important to monitor over time as it will eventually be reconnected to natural hydrological systems, and environmental exposure of wildlife and downstream human populations will occur. However, acceptance criteria for evaluation of the toxic potency or chemical concentrations in its water remain unclear, as are future risks to the regional environment.[25] The Alberta Environmental Protection agency released the *Water Quality Based Effluent Limits Procedural Manual* in 1995 to maximize environmental protection from industrial discharges into the environment.[26] Here, environmental protection is accomplished through a triad approach, comprising: whole effluent toxicity limits, chemical-specific toxicity limits, and biological monitoring. Whole effluent toxicity is typically screened with standardized acute lethality tests and offers the most practical information of the effluent. Chemical-specific limits relies on knowing the specific toxicants of concern in the effluent, and establishing concentration limits for these. Biological monitoring in the field is a prudent approach to confirm the effectiveness of the above two approaches once discharge is underway, and may include monitoring for sub-lethal effects that may affect populations and the ecosystem more broadly. At the current early stages of BML, there is a need to develop bioassays to effectively measure its toxicity over time, and to combined these with high resolution mass spectrometry measurements that can accurately monitor the associated chemicals of concern.[27]

A challenge in establishing such criteria for OSPW is its heterogeneity and chemical complexity, and how the mixture can be variable depending on its source, location and age.[28,29] Of particular concern is the organic mixture, which may never be fully characterized (section 1.2).[30] Therefore, there are inherent challenges to attribute OSPW toxicity to specific chemicals

that could then be used to set chemical-specific limits. Moreover, not knowing the chemicals responsible for toxicity, or their toxic mechanism of action, leads to uncertainty in endpoint selection for biological monitoring. These challenges, as well social and political challenges contribute to a lack of guidelines today for safe OSPW release back into the environment.[9]

1.1.3.2. Land disturbances

In 2013, it was reported that 220 km^2 of land has been cumulatively displaced by tailings ponds.[16] More broadly, the total active footprint of industrial operations as of 2016, with certified reclaimed land as the only excluded category of land, was 953 km^2,[31] out of the total 4800 km^2 that is surface minable.[32] The area of disturbed land varies between years, given industrial goals of reclaiming former mines.[18] Reclamation operations include re-introduction of soils, hydrology, flora, and fauna to facilitate a new ecosystem.

1.1.3.3. Contamination of the environment

There are various reports on the extent of ambient environmental contamination associated with byproducts or emissions from the surface mining oil sands industry. Until 2011, monitoring of the area was the responsibility of a Regional Aquatic Monitoring Program (RAMP), and this organization suggested that elevated levels of contaminants in the area were from natural erosion of bitumen and releases of natural ground water which contains bitumen-derived organics due to contact with the McMurray geological formation.[33] Delineating between anthropogenic and natural sources of contaminants has been a significant analytical challenge.[34] Nevertheless, in 2009, several reports identified much greater concentrations of polycyclic aromatic compounds (PACs)[35] and metals[36] in the Athabasca River and its tributaries than originally reported.

Furthermore, through strategic spatial sampling of snowpack and water, the oil sands industry has been identified as a major and increasing source of such contaminants. This contamination is suggested to be from stack-emissions but also from various dusts, such as from petroleum coke stockpiles.[37] It was also proposed that evaporation of PAHs from tailings ponds may be an important source of environmental contamination,[38] but a follow-up critique argued that this was unlikely,[39] and no empirical evidence for this source has been published.

Kelly et al.[35] criticized the RAMP for lacking transparency, having inconsistent, inadequate monitoring approaches, and identified that there was a need for greater scientific oversight. Initially, government and industry denied the accusations by Kelly et al.;[35] however, eventually an appointed expert scientific panel supported the contamination found surrounding the industry.[40] This acted as a catalyst for increased monitoring in the area with the disbanding of RAMP, and initiation of the Integrated Monitoring Program in 2011 by Environment Canada[5] as well as the Joint Canada-Alberta Oil Sands Monitoring Program (JOSMP) in 2012.[41]

There was suspected seepage of OSPW from tailings ponds through groundwater into the Athabasca River,[34] with particular concern for the Beaver River and McLean Creek tributaries.[33] However, analytically confirming the source of bitumen-derived organics (i.e. OSPW, or natural groundwater seepage) represents a significant challenge.[42] Headley et al.[28] used Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) to assess water samples from different locations around the oil sands, and looked at trends based on elemental chemical classes, such as the number of carbons (C), sulfurs (S), and nitrogens (N). They only used negative ionization mode in this study, which is more typical for assessing bitumen-derived organics, as negative mode allows for ionization and detection of acidic species that readily lose a proton (-). Positive mode, contrarily, can ionize and lead to detection of non-acid species that

readily gain a proton (+). They suggested that the chemical class ratio of O_nS^-:$O_nS_2^-$ has potential to differentiate between OSPW sources, while the O_n or NO_n^-:$N_2O_n^-$ ratios could differentiate between OSPW and natural sources.[28] Another study used Orbitrap mass spectrometry (MS) again in negative mode and gas chromatography paired with quadrupole time-of-flight mass spectrometry (GC×GC/QTOFMS) on a variety of water samples, and found that O_2:O_4 ratios of river samples were more similar to OSPW when closer to tailings ponds compared to farther away or upstream, suggesting a common source.[34] However, considering how many variables can influence detection—like sample preparation and ionization source, which will be explored in section 2.0—each method should be questioned, and the complicated task of source attribution should consider multiple methods.[34]

Recently, Sun et al.[42] used HPLC-Orbitrap to profile all OSPW organic chemical classes in both positive and negative mode. They profiled a total of 40 water samples up- and down-stream of industry, including ground water, surface water, river water, tributary water, lakes, and OSPW. Here, they did not observe higher O_2^-:O_4^- in potentially affected waters as found by Frank et al.,[34] and while Beaver Creek and Mclean Creek did have higher O_2^- content, other samples far upstream of industry were also high in O_2^-. The O_2^- chemical class is termed 'naphthenic acids' (NAs), and is the most studied group within the complex organic mixture (explored in section 1.2). Sun et al.[42] suggested the SO^+ chemical class as a potential indicator of anthropogenic sources, as it was detected in OSPW, Beaver Creek, and Mclean Creek, and not in any other water samples. In terms of overall environmental impact of OSPW seepage, they concluded that with the high flow of the Athabasca River, any current seepage flux from anthropogenic sources is non-detectable in the mainstem river, and that future focus should be on

smaller tributaries. Though advances have been made, more research is needed, as well as a strategy to effectively monitor the area using current knowledge and technologies.

1.1.4. Environmental regulation of open pit mining

The oil sands industry operates on a zero-discharge policy, whereby companies are prohibited by the Alberta Government from directly releasing OSPW into the environment, namely the Athabasca River.[43] This is because the bitumen-derived organics in OSPW become concentrated and have demonstrated to be acutely toxic to a variety of organisms (Section 1.3 OSPW Toxicity). This policy is in line with the *Canadian Fisheries Act* (1985) to monitor water quality and the health of aquatic species.[44] That being said, while Syncrude does not release OSPW, they state on their website that they do discharge treated sanitary sewage, "*diverted clean surface and basal water from the Aurora mine via Stanley Creek, and clean surface water from a gravel pit*."[45]

The Alberta Environmental and Protection Act requires oil companies to reclaim the land they disrupt to its equivalent state, and a number of different technologies and strategies are implemented to adhere to this act.[22] The *Tailings Management Framework*[16] and *Surface Water Quantity Management Framework*[19] were recently updated in 2015 as part of the Lower Athabasca Regional Plan. These updates limit tailings pond volume, prohibits water from being taken from the Athabasca River during low flow periods, presses industry for innovative technologies to meet these constraints, requires financial organization for unforeseen remediation problems, and sets a guideline for tailings to receive treatment in order for reclamation to occur within 10 years after a mining project is finished (i.e. when the mining of bitumen is complete

for an area).[16,19] Through the *Oil Sands Conservation Act,* oil sand mines must be approved beforehand to ensure orderly development in line with the framework.[16]

1.2. Oil sands process-affected water (OSPW)

While there are multiple constituents of concern within OSPW, the dissolved organics pose the largest problem for industry due to their toxic and persistent nature. This thesis introduction will go more in depth into some of the concerns of the organic fraction, such as their persistence, relative concentrations, bioaccumulation potential, exposure potential through water, and their analytical characterization.

The OSPW organic mixture has been described as "supercomplex."[46,47] Approximately 3000 distinct chemical formulas were detected cumulatively in both positive and negative mode with liquid chromatography high resolution mass spectrometry.[30] The detected chemicals can therefore be described by empirical formula in either ionization mode, e.g. $C_xH_yO_zS_aN_b^{+/-}$, and each chemical formula is termed a 'species'. These species are not individual chemicals, but represent the total sum of numerous structural isomers. The vast number of isomers that are present for each individual species in OSPW can be enormous, and the supercomplexity of the sample becomes apparent when partial separation of these isomers is attempted.[48] It is therefore useful to characterize OSPW organics based on heteroatomic class instead. In this way, several species ($C_xH_yO_zS_aN_b^{+/-}$) are binned into categories based on their heteroatom content and ionization mode (e.g. O_2^-, O_2^+, SO_2^-, NO^+), thereby ignoring numbers of C and H for simplicity.

Some individual isomers have been elucidated by multidimensional chromatography (e.g. GCxGC)[49] and supercritical fluid chromatography,[48] but the vast majority of isomers remain unknown. Pereira et al.[30] coupled HPLC with Orbitrap and found that the positive O_2 (non-acid)

species were chemically distinct from the negative O_2 (acid) species through differences in retention time. Initially, there was uncertainly whether other heteroatomic classes detected in both positive and negative modes are distinct or the same species that can have multiple charges;[50] however, Morandi et al.[51] were able to chemically fractionate between multiple heteroatomic classes in positive and negative mode, suggestive of distinct chemicals. Furthermore, Sun et al.[42] found the positive and negative mode data sets to not be redundant.

Among the acid-extractable organics are a prominent group of carboxylic acids detected in negative ionization mode, termed naphthenic acids (NAs) or the O_2^- class.[51] NAs are classically defined as a class of cyclic and alkyl-substituted aliphatic carboxylic acids with the formula $C_nH_{2n+z}O_2$, where n is the number of carbons, and z is the degree of unsaturation through a zero or even, negative integer.[33] NAs are surfactants due to their polar carboxylic acid moiety and non-polar hydrocarbon chain or rings.[52] While NAs were initially thought to be the most highly concentrated chemical class in OSPW, advances in analytical detection methods have found more compounds in OSPW than originally thought,[52] uncovering that NAs may compromise less than 50%,[52] 33%,[53] or even 11%[51] of the total organic content. Interestingly, more compounds were detected in positive mode than negative mode of mass spectrometry.[54]

The majority of papers attribute acute toxicity of OSPW to NAs based on work done by Mackinnon and Boerger[55] and Verbeek et al.[56] Mackinnon and Boerger used two treatments to ameliorate acute toxicity, suggesting surfactants within the polar acidic fraction to be responsible.[55] They find similarities between the Fourier transform infrared spectrometry (FTIR) profiles of commercial NAs and the organic acid fraction, yet they say further characterization is necessary to find the toxic component and do not assume that organic acids are solely composed of NAs. Similarly, Verbeek et al. looked at how detoxification can be accomplished through

extracting surfactants—not NAs specifically—from the mixture through various treatment methods, and found that 50-60% of the toxicity was attributable to the acid extractable fraction, while neutrals may account for the remainder.[56] It is only with how these pioneering papers are referenced in the 90's and 2000's that statements such as "*acute toxicity has been attributed to NAs*,"[57] among others,[58,59] start to surface. Therefore, until recently, NAs as the toxic component were more so a propagated assumption that lacked direct experimental evidence.

In fact, over time, even the term NA has become more ambiguous, with "non-classical NAs" including oxygenated NAs (oxy-NAs) with more than 2 oxygen atoms as additional carboxylic acid or hydroxyl functional groups (defined as O_x^-),[44,52,54,60] as well as aromatic NAs.[47] Recent detection of other heteroatom groups such as species with nitrogen and sulfur atoms has led to a broader focus on the total extractable organics of OSPW.[54] That being said, classical NAs are proposed to be added to the National Pollutant Release Inventory in Canada.[61]

Given the complexity of OSPW organics, their quantification is an analytical challenge due to a lack of any perfect, authentic standards to calibrate their detection.[62] In fact, any quantitative measure is only a semi-quantitative estimate of the true value.[62] Another analytical challenge for the organic fraction is that no single standardized set of experimental parameters can adequately assess all components of the complicated mixture.[54] Lastly, each OSPW sample has spatial and temporal heterogeneity between tailings pond sites, and even within each individual tailings pond.[28] This makes inter-laboratory comparisons difficult and a potential source for erroneous conclusions. Traditional estimates of NA concentration in a 2005 review ranged from 20-120 mg/L[63] depending on the source, though a more recent review in 2015[64] indicated lower concentrations with improvements in analytical methods.

1.2.1. Parameters affecting detection of species

1.2.1.1. Resolution

The traditional detection methods used for OSPW include FTIR, low resolution GC/MS, and ultraviolet spectroscopy, but have advanced to the use of high pressure liquid chromatography (HPLC) combined with different forms of high resolution mass spectroscopy such as QTOF, Orbitrap, and FTICRMS. Complete characterization of the thousands of OSPW organic species has yet to be obtained because of the complexity of the sample matrix and the number of different isomers that can exist for each species.[48] Tradition unit resolution methods have been shown to give false positives and lead to misclassifications.[62] The ultrahigh resolving power of FTICRMS (450000-650000 at *m/z* 500)[54] and high resolving power of Orbitrap (100000 at *m/z* 400)[32] can distinguish between separate ions with very high selectivity, allowing for unambiguous assignment of empirical formulas to chemicals with high mass accuracy. From here, the organic mixture can be viewed through heteroatomic class distributions.[30,51,54,65] High resolution is particularly important for samples such as OSPW that have high matrix interference from other OSPW components, as well as from fatty acids from biota,[33] and natural background levels of bitumen-derived organics in the region.[28]

1.2.1.2. Ionization source

Through FTICRMS, electrospray ionization (ESI) has been found to preferentially ionize more aliphatic, less hydrogen deficient OSPW organics, particularly O_x species.[54] ESI negative mode is typically used to evaluate classical NAs because they are weak acids that deprotonate in solution.[66] However, Barrow et al.[54] found that ESI in positive mode had a greater number of peaks, detecting O_2^+ species among other heteroatoms. Therefore, solely looking at negative

mode while excluding positive mode chemical could lead to misattribution of toxicity to particular negative mode chemical classes when others may be present. Other ionization sources such as atmospheric pressure photoionization (APPI) have been shown to detect more aromatic species with a greater degree of hydrogen deficiency, as well as preferentially detect N and S species.[54] Atmospheric pressure chemical ionization (APCI) has also been used, and was found to be slightly more sensitive than ESI in negative mode,[67] but possibly forms adducts.[68] Taken together, all of the mentioned ionization techniques offer complimentary information to more fully characterize OSPW organics, but this also leads to difficulty in comparing results.

1.2.1.3. Extraction method

The extraction method for OSPW organics can have a large impact on what species are present for detection in MS or treatment in bioassays. In fact, these methods can be geared towards which compounds in the mixture the study is looking to assess.[69] This is important to consider when conclusions are made attributing toxicity to particular chemical classes within the organic mixture, because some methods often have the aim of extracting NAs while excluding the remainder of the organics, such as Leclair et al..[70] Common extraction methods often include a combination of solid phase extraction (SPE),[61,71–73] pH-dependent liquid-liquid extraction,[30,51,53] and distillation.[59]

Relevant to this investigation, Pereira et al.[30] introduced a new detection method with high-resolution Orbitrap MS, to which a pH 2 liquid-liquid extraction on OSPW was effective at looking more broadly at the organic mixture. Similarly, Morandi et al.[51] performed 3 rounds of chemical fractionation of OSPW while using high resolution Orbitrap MS and a bioassay in order to find the most acutely toxic chemical classes. The first fractionation step was a series of

liquid-liquid extractions, yielding a neutral extract, an acid extract, and a basic extract. The neutral extract was the predominantly toxic fraction, followed by the acid extract. The basic extract largely contained species with their carbon number less than 10 and their signal intensity was very low; accordingly, this extract was not toxic and did not appear to contain constituents of concern. Therefore, an extraction method, such as a total acid extract (TAE), should theoretically contain the acidic and neutral components, and this would be an efficient means of comparing the whole organic mixture (albeit, with exclusion of the irrelevant basic component) between environmental samples in toxicological tests.

Similarly, Huang et al.[53] performed a stepwise liquid-liquid extraction from pH 12.4 to 2.0 and found that different proportions of O_x-NA species were extracted at each pH. They estimated the pKa of the O_x-NA species and found that as additional oxygen is added to the molecule the pKa increased, suggesting that O_3 species likely contains a hydroxyl group, while O_4 species could contain both hydroxyl[53] and carboxylic acid groups.[74] Importantly, dropping the pH from 12.4 to 2.0 allows for fractionation, while the reverse would extract the whole organic mixture.

Moreover, another study found that the OSPW organics were preferentially extracted based on the organic solvent used, whereby hexane extracts the greatest proportion of O_2 species, while others are more suitable for other chemical classes.[32] Notably, dichloromethane (DCM) appears to have a more holistic extract with O_2 classes as well as other oxygenated classes (O_x).

Overall, the analytical challenges of assessing OSPW organics are showcased through the different parameters that can affect detection. This knowledge is needed in order to attribute toxicity in bioassays. For example, considering the advancements in analytical method and knowledge of the complexities of the mixture, some of the conclusions from preliminary toxicity studies may have confounding variables in attributing toxicity to particular chemicals without

being able to detect other classes present. Furthermore, while there is a need to have a standardized set of methods for comparing OSPW studies, it appears as though there is no one set of parameters that can completely characterize the organic mixture. This is important to note in comparing studies in that each study is dependent on the spatial and temporal variables of the OSPW sample, the detection and extraction method used, and the bioassay performed.

1.2.2. Persistence of OSPW organics

The Persistence and Bioaccumulation Act defines a chemical as persistent in water if its half-life (the time for 50% of the chemical to degrade) is greater than 182 days.[75] The half-life of recalcitrant NAs was approximated to be 12.8-13.6 years,[10] yet another laboratory study of OSPW NAs indicated a range of half-lives from 40-240 days, depending on the particular species.[76] Therefore, while there is variation depending on the individual species, the overall mixture can be defined as persistent.[10] These aforementioned studies looked specifically at NAs, but the rate of degradation of other heteroatomic classes is unknown. Part of the uncertainty with the EPL strategy is knowing which chemical classes cause adverse effects and whether these chemicals will persist and remain in the water by the time of environment integration.

There are multiple co-occurring processes in EPLs that add to the complexity of predicting persistence of the organic profile and intensities: firstly, there is fresh OSPW originating from consolidation of MFT; then input of fresh water for dilution purposes; and then sedimentation (aided with coagulants) and potential degradation of the hydrocarbons as outputs. The unknown persistence contributes to currently unquantifiable risk in the future EPLs strategy.

1.2.2.1. Natural biodegradation

Biodegradation is the process of microbial populations breaking down hydrocarbons as a source of carbon, and there is evidence of this process occurring with OSPW NAs [10,76,77] and commercial NAs.[76,78,79] Commercial NAs are a synthesized, simplified representation of what NAs in OSPW could be like, though they can be useful under certain experimental parameters when little is known about OSPW NAs. It was found that 15-26% of commercial NAs were resistant to biodegradation under all aerobic conditions tested,[78] with resistant NAs containing more alkyl branching.[79] Here, resistant NAs could potentially accumulate over time;[78] however, commercial NAs lack some validity because they have different toxicities[80] and biodegradation rates[81] compared to OSPW NAs.

A laboratory study found that OSPW NAs were mostly recalcitrant, with degradation decreasing with higher degrees of cyclization.[76] *In situ* however, there was "*no discernible enrichment of the highly cyclic fraction.*"[10] The concentration of NAs was lower in older OSPW samples with a higher proportion of oxy-NAs, yet there was marginal change in the original parent NA signiture.[10] Oxy-NAs may be persistent intermediates of NAs and could indicate OSPW age.[10] Natural biodegradation by indigenous microbes requires aerobic conditions. If NAs sink while adsorbed to fine particles, anaerobic conditions may exist;[82] however, anaerobic biotransformation does not occur, increasing their persistence during groundwater seepage.[83] Passive, natural biodegradation of OSPW organic is desirable due to cost effectiveness, but there is a lack of experimental evidence eliciting natural biodegradation with current high resolution MS technologies. Nevertheless, other advanced treatments to degrade the organics have been explored.

1.2.2.2. Other treatment strategies

Ozonation is a promising treatment that preferentially degrades recalcitrant NAs and was found to increase the proportion of oxy-NAs by 7.7 fold.[60] Moreover, it was found to fully[84] or partially[60] remediate acute toxicity though Microtox (a common assay with bioluminescent bacteria), and to largely remediate endocrine activity in a steroidogenesis assay.[6]

Soil microorganisms immobilized in a biofilm, called bioreactors, is another explored technology. They have been shown to degrade around 40% of the liable NAs,[85] yet degradation decreases with the number of cyclic groups, similar to natural biodegradation.[76] Furthermore, the endocrine activity of isolated organic fractions were not ameliorated through the bioreactor.[71]

A promising and cost effective treatment strategy currently being implemented by Syncrude Canada Ltd. is a Petroleum Coke Treatment.[86] Extracted bitumen must be upgraded to produce synthetic crude oil, and this requires the rejection of carbon and injection of hydrogen. The rejected carbon results in a large volume of petroleum coke (PC) produced, proportional to 15% of the total bitumen extracted.[86] For every barrel of synthetic crude oil, 20 kg of PC is produced and stored onsite.[86] Activated Carbon (AC) is a known means of stripping organics from aqueous solutions, and recent studies have found that PC has similar adsorption characteristics to AC.[86] One study found that PC was able to reduce the dissolved organics and metals in OSPW depending on the dose and residence time, and that treatment ameliorated acute toxicity.[86] A pilot project was implemented in 2012, and more recently in 2018, a large scale strategy will be started to integrate the OSPW and PC by-products as a method of water remediation.[87]

1.2.3. Environmental fate

The EPL strategy is intended to enable safe release of OSPW to the environment. One of the uncertainties of this strategy is determining the environmental fate of the organics. The alkaline pH of OSPW is conducive for many of the organic and inorganic chemicals to remain soluble. The high flow rate and volume of the Athabasca River would allow for dilution, decreasing the concentration of the OSPW constituents, and buffering the difference in pH. Though to give context, integration of OSPW will account for 0.5% of the total water in Lake Athabasca, the downstream receiving basin and 8th largest lake in Canada.[9] Therefore, predicting the environmental fate of OSPW organics is important in assessing exposure.

The structure of toxicants determine their chemical and physical properties.[88] As such, the unknown structures of OSPW organics makes their environmental fate difficult to characterize, which is necessary to determine exposure. NAs do have some average properties that can help estimate their environmental fate and act as a model of the mixture. NAs are amphipathic, in that they contain both hydrophobic alkyl and hydrophilic carboxylic acid moieties.[88] This gives them surfactant properties, where they will likely distribute between the interface of sediment/organic surfaces and water.[74] During the alkaline extraction, NAs are deprotonated and soluble in water as sodium salts; they continue in this forms in tailings pond, as the pH of OSPW ranges from 7-8.[10] pKa is the acid dissociation constant, and a pKa of 3.5 indicates a carboxylic acid moiety. With a pH below 3.5 the NAs will be protonated and extractable.[53] NA as anions in OSPW will have a low volatility,[83] meaning that OSPW exposure and transport will likely be through water.

Zhang et al.[50] estimated the octanol-water partitioning coefficient (K_{ow}), an indicator of bioaccumulative potential into an organism's fat, where a chemical is said to be bioaccumulative if it has a K_{ow} value above 5000.[75] They analyzed 2114 organic species through adherence onto

polydimethylsiloxane coated stir bars, and found that most species had limited partitioning with a K_{ow} coefficient of <1. However, some NAs ranged from negligible to a coefficient of 100, and non-acid species detected in positive mode were generally more hydrophobic, with some classes such as SO^+ generating a K_{ow} of 203 000. Therefore, the majority of organics are not bioaccumulative, yet some specific classes may be, particularly non-acids, warranting further toxicological assessment. In a follow up study, Morandi et al.[89] predicted the acute toxicity of the extractable organics from a model fish based on lipid partitioning and also brought attention to the SO^+ and SO_2^+ chemical classes for being predicted as both bioaccumulative and toxic.

1.2.4. Exposure

1.2.4.1. Toxicokinetics

With a water-bound exposure pathway, intake would be through respiration, dermal contact, or ingestion for aquatic species, and ingestion or dermal contact for terrestrial species. Young et al.[90] exposed fish in the laboratory to a steady state of 3 mg/L commercial NAs for 10 days. They measured the concentration of a specific NA species with n = 13 and z = -4 as a representation of all NAs. They assigned the NA species a bioconcentration factor of around 2 at pH 8.2 for fish *in vivo*; however, once the fish was placed in an NA-free basin, 95% of the NA depurated in one day. In a follow up study, they found higher concentrations of the NA species in the gills and liver compared to muscle, in concordance with the predict exposure pathway.[91]

More recently, Zhang et al.[92] exposed Japanese medaka to OSPW and calculated the bioconcentration factors of the extractable organics and compared these values to the previous studies of predicted bioaccumulation values. Of all the candidate chemical classes, only SO^+, NO^+, and $O2^-$ were detected in the fish, and this provides a broader view of the bioaccumulative

organics compared to older experiments.[90] Therefore, the more analytically advanced studies still warrant concern for exposure to the O_2^- class, which is known to be acutely toxic, though the positive mode polar non-acid species need to also be explored, particularly because bioaccumulate species could yield concentration in the organism that surpass real-world OSPW concentrations.[51]

1.2.4.2. Biomonitoring of the surrounding area

There is concern for surrounding aquatic species, wildlife, and humans that are possibly being exposed; not only from OSPW, but from other industrial by-products and disruption. However, it is difficult to delineate a particular source, especially with general anthropogenic stress to wildlife that is not associated with contamination. Accordingly, monitoring reclaimed land surrounding industry is nuanced with conflicting reports. Hersikorn et al.[93] assessed woodland frogs by looking at their metamorphosis and thyroid hormone level. They looked at 14 reclaimed wetlands, grouped as young (<7 years old) or old (>7 years old, including Pond 9) and compared to a reference site. They found that tadpoles in young wetlands had delayed or incomplete metamorphosis, and that thyroid hormone status was altered.[93] Interesting, the old wetlands were similar to the reference site, indicating ageing as an effective remediation strategy. Though, Gentes et al.[94] assessed Tree Swallows on reclaimed wetlands and found higher thyroid hormone concentrations and that these birds had 70-72% more parasitic burden.[95]

Kavanagh et al.[96] assessed the reproductive and developmental health of a population of fathead minnows that were accidentally introduced into one of the experimental ponds, Demonstration Pond, at the time of its inception. ~14 years later, the minnows were still able to inhabit the pond, but had altered liver, gonad, and spleen indexes, altered secondary male

characteristics (such as late sexual maturity), lower concentrations of 11-ketotestosterone, less disease or parasites (suspected to be due to the inhospitable environment of the pond for other organisms), and abnormal opercula and proliferative/degenerative cells.[96]

Young et al.[91] were not able to detect their candidate NA in fish from the Athabasca River. More recently, Simmons et al.[17] surveyed the proteome of white sucker from various location around the Athabasca River and found plasma proteins unique to fish downstream of industry. These unique proteins related to metabolism, small molecule biochemistry, endocrine-related disorders, among others, though influences other than industry may play a role. There is a lack of mammalian biomonitoring with aged OSPW, though deer mice from a reclaimed habitat near the oil sands industry (so not specific to OSPW) were found with a combination of endocrine, oxidative stress, and histological endpoint alterations.[97] Mammalian toxicology will become more relevant as the EPL strategy proceeds, especially to the downstream human community of Fort Chipewyan who are concerned about environmental exposure and their own health.[98,99]

1.3. OSPW toxicity

1.3.1. Toxicity attribution

Considering the complexity of the organic mixture and the analytical challenge it represents, this makes ascribing toxicity to any one compound particularly difficult. In fact, this is one of the inhibiting factors in generating chemical-specific guidelines for safe environmental release. Toxicity has traditionally been ascribed to NAs; however, many of the earlier studies were more bound by analytical limitations. New knowledge of the relevance of other organics detected in both negative and positive mode through high resolution MS may help in this attribution.

OSPW samples used in studies are highly variable and depend on sample location, age, and treatment.[28,29] Also, the extraction methods used to isolate the organic fraction can greatly influence the composition of the OSPW treatment (section 1.2.1.3). Notably, bioassay exposures can be with OSPW, OSPW extractable organics, oil sands water generated through natural erosion,[100] organic sub-fractions,[51,70,71] commercial NAs,[101,102] and individual synthetic NAs[80] to name a few. Furthermore, there appears to be inter-species and inter-cell line variability in responses to even the same samples.[29] This makes comparison between studies challenging.

Fortunately, bioassay-based approaches allow for the assessment of complex samples that are challenging to analyze analytically.[103] One standardized strategy used by the EPA is called the Toxicity Identification Evaluation (TIE).[104] This is effective at attributing toxicity to components within a water sample, such as cationic metal, organics, oxidants, pH dependent toxicity, ammonia, or toxicants affected by filtration or aeration, through the sequential removal of each component and assessing any changes in toxicity. Activated carbon (AC) is one method used to strip water samples of the organic fraction through adsorption.

An Effects-directed analysis (EDA) is another approach, which pairs multiple rounds of chemical fractionation with chemical characterization and bioassays and can more narrowly attribute toxicity within the organic mixture of a water sample.[104,105] Morandi et al.[51] used an EDA approach to find the most acutely toxic chemical classes in OSPW. They successfully separated NAs from polar non-acid species detected in positive mode, such as O^+, O_2^+, NO^+, and SO^+, and found that NAs were in fact the most acutely toxic chemical class, but that non-acid species were also toxicologically relevant. They support NAs acting through surfactant-like narcosis (section 1.3.3) and hypothesized that the non-acid species may act through a different, unknown mechanism. Next, Morandi et al.[25] investigated the mechanisms of action of the

fractions with an *E. coli* live cell array and found no clear separation of genes affected between fractions, with general stress, protein and DNA damage as indicators. However, the *E. coli* live cell array has limitations, with other open format investigations finding other effects: for example, RNA-seq (global measuring of transcribed mRNA from genes encoding proteins)[106] on fathead minnow livers treated with WIP 2010 found oxidative stress and metabolism, apoptosis, and immune function affected. Also, multiple studies with qPCR (selective measuring of transcribed mRNA) and various OSPW find endocrine activity, among other effects (**Table 1**). This table highlights the plethora of toxic effects that are observable at the gene level, and the difficulty that exists in comparing studies due to differences in the type of OSPW sample tested.

An EDA analysis also has limitations in that there is uncertainty with which chemical class is responsible within active fractions if multiple classes exist.[27] Therefore, another technique has been used to attribute toxicity to more specific chemicals, called the Pull-down assay with Untargeted Chemical Analysis (PUCA), which identifies chemicals bound to a protein or receptor as an affinity matrix.[27] Here, 30 ligands of the peroxisome proliferator-activator receptor γ (PPARγ) were identified in OSPW, with activation less in Pond 9 than BML 2012. PPARγ has the downstream effects of adipogenesis and is associated with obesity. The ligands were largely polyoxygenated or heteroatomic chemical classes, inclusive of carboxylic and sulfonic acids.

Identifying the most toxic components in OSPW is important, with some studies even testing model compounds to identify mechanisms of action;[80,107,108] however, the realistic exposure scenario would come in the form of a multi-component mixture. Not only with mixture effects from other aqueous components of OSPW,[109] there is also evidence of chemical antagonism within the organics.[51,71] Moreover, there is evidence of chemosensitization, where organics inhibited the efflux of chemicals from the cell, thereby potentiating the effects of PAHs.[110]

Table 1: Summary of relevant toxicogenomic studies.

Author	Technique	Model	Samples	Dose	Pathways	Conclusions
Zhang 2010[111]	Microbial genome wide assay	*E. coli*	Commercial NAs	10, 100, 1000 mg/L	Pentose phosphate pathway, ATP-binding cassette, SOS response pathway	General stress response, which is conserved across species
Garcia-Garcia 2011[102]	qPCR	Mouse bone marrow derived macrophages + *in vivo*	OSPW extracts, commercial NAs	250-62.5 mg/L *in vivo*; 50-6.25 mg/L *in vitro*	Pro-inflammatory cytokines	OSPW extract immunotoxic, different response from commercial NAs
He et al. 2010[6]	qPCR	Human adreno-carcinoma cell line (H295R)	OSPW, ozonated OSPW	0 - full strength	Cyp19A (aromatase)	Increased expression
Gagne 2012[112]	qPCR	Rainbow trout hepatocytes	OSPW extracts	0.02, 0.1, 0.5%	Xenobiotic biotransformation, estrogenicity, oxidative stress, DNA repair	OSPW extracts affect suit of gene targets
He 2012[113]	qPCR	Fathead minnow (*in vivo*); Brain-Gonad-Liver (BGL) axis	OSPW, ozonated OSPW	Full strength	Steroid receptors, hormones, peptide receptors, steroidogenic enzymes, yolk precursors	Endocrine-disruption all levels BGL axis; ozonation largely attenuates
He 2012b[15]	qPCR	Fathead minnow (*in vivo*)	OSPW, ozonated OSPW, activated carbon OSPW	Full strength	Xenobiotic biotransformation, oxidative stress, apoptosis	Ozone or AC significantly attenuated all adverse effects
Wiseman 2012[106]	RNA-seq & qPCR	Fathead minnow (*in vivo*); livers	OSPW, ozonated OSPW	Full strength	Oxidative stress, apoptosis, immune function	RNA-seq & qPCR same direction of fold change; ozonated OSPW different genes affected, and to a lesser extent
Wiseman 2013 [8]	qPCR	*Chronomus dilutus* (midges)	Fresh OSPW, old OSPW	Full strength	Oxidative stress, steroid hormone receptors,	Ageing OSPW largely attenuated these effects
Reinardy 2013[72]	Vitellogenin (VTG) assay	Zebrafish	Non-aromatic, aromatic extracts	0, 0.01, 0.1, 1 mg/L	Yolk precursor	Aromatic fraction weakly estrogenic, non-aromatic non-estrogenic
Mohseni 2015[114]	qPCR	Differentiated mouse embryonic stem cells	OSPW NA extract	0.025-2.5 mg/L	Cardiac development	May cause abnormalities in the heart and nervous system

Note: qPCR is quantitative polymerase chain reaction reaction; RNA-seq is RNA sequencing; SOS pathways is a global response to DNA damage; cytokines are secreted by certain immune cells; aromatase transforms testosterone to estradiol; VTG is a yolk precursor protein and biomarker for estrogenicity in male zebrafish.

Looking at the organics in its aqueous context, a preliminary study found that salts decreased the toxicity of the organics to fish,[115] though fish have evolved mechanisms to regulate ions in the environment. More recently, whole OSPW with varying yet environmentally relevant concentrations of NAs were found to be immunomodulatory and cytotoxic to mouse macrophages.[109] Here, the isolated organic fraction by itself at the same doses did not generate a response, but had synergistic or additive interaction only when combined with the inorganic fraction of OSPW.[109] These synergistic effects suggested that the organics within its aqueous matrix are actually acutely toxic at lower doses than originally thought when tested alone, and that the inorganics warrant further investigation as a toxic component. Though importantly, mouse macrophages have not evolved to manage inorganic ions at real-world concentrations outside of their host organism, so it is sensible for the inorganics to be toxic.

That being said, this investigation maintains focus on the organic mixture, as further development of the chemical-specific limits is needed to guide safe environmental integration. Particularly due to the multiple lines of evidence that show how stripping OSPW of organics largely ameliorates the potency of the endpoint tested.[15,43,60,84,106] Therefore, this investigation was set to further investigate the organics of OSPW based on the literature, but it does not mitigate the potential of the inorganics or the mixture of organics and inorganics to hold toxicity.

1.3.2. *In vivo* laboratory studies

Focusing on mammals, Rogers et al.[116] exposed rodents acutely and sub-chronically to OSPW organics to assess risk for terrestrial wildlife. 14 days after the rodents were given a 300 mg/kg dose of OSPW organics, they demonstrated hepatotoxicity, brain hemorrhaging, cardiac necrosis, and fibrosis. A sub-chronic dose of 60 mg/kg OSPW organic over 90 days, still 10

times higher than the worst-case daily exposure, demonstrated hepatotoxicity again, and changes in blood biochemistry. Another study exposed mice to 100 mg/kg NAs via gavage and observed decreased pro-inflammatory cytokine and chemokine response in the spleen, liver, and lymph nodes after 1 week, and ozone treatment of the NAs ameliorated the effect.[117] However, overall, the doses in these mammalian studies are much higher than field concentrations.

Anderson et al.[13] exposed midges (*Chironomus dilutus*) to fresh and aged OSPW samples under acute (10 days) and chronic (until adult emergence) conditions. The masses of larvae, pupation, and rates of emergence were lower than control when exposed to fresh OSPW, while the toxicity was less potent with aged OSPW, coinciding with lower NA concentrations. This is consistent with the organic fraction being responsible for the toxicity, yet aged OSPW still retained some of the effects. Exposed midges had a similar survival responses in Wiseman et al.;[8] however, they also looked at gene expression biomarkers of oxidative stress and endocrine disruption. They found that there was disruption after treatment with fresh OSPW and that this was attenuated in aged OSPW, which is promising for ageing as a remediation strategy.[8]

There is evidence of OSPW that is aged one month being less lethal to fathead minnows, but chronic toxicity is unclear.[55] Van den Heuvel et al.[118] exposed yellow perch to aged OSPW in reclamation ponds for three and ten months, resulting in a dose-dependent relationship of NA concentration with observations of gross pathologies, fin erosion, tumors, aneurysms, proliferation of epithelial and chloride cells, and mucus in the gills. Another study placed Yellow Perch and Goldfish in reclamation ponds of aged OSPW for three weeks and a multitude of gill and liver histopathological changes were observed.[57] Furthermore, there was a positive correlation with NA concentration and frequency of deformed embryos in Yellow Perch and Japanese Medaka, possibly due to surfactant-like NAs disrupting the egg chorion.[119]

Other studies found testosterone and estradiol reduced in goldfish exposed to OSPW in reclamation ponds for 17 days relative to control, while cortisol was higher in males.[120] Explants of the gonadal tissues were taken from males and females in this study, and the basal levels of testosterone were significantly reduced after OSPW exposure. Next, goldfish were exposed for 7 days to a NAs extract from OSPW in the laboratory, but the results were not reproduced. This demonstrates the effect of the extraction procedures, in that they isolated NAs specifically, while leaving out the polar species in positive mode that may be steroid-like.[30] Lastly, one study found aromatic NAs to be more lethal than alicyclic NAs to zebrafish, narrowing toxicity.[61]

1.3.3. Cytotoxicity

Cytotoxicity is defined as toxicity to the cell *in vitro*, ultimately leading to cell death through apoptosis or necrosis.[121] However, this is ambiguous, as bioassays may evaluate different cytotoxic endpoints, such as cell viability, proliferation, or metabolic indicators, among others.

NAs are surfactants, leading to speculation by Frank et al.[74] that NAs, like other surfactants,[122] act through narcosis as the predominant mechanism of action, and this is supported by more recent studies.[51,89] Narcosis is a non-specific mechanism where the chemical integrates into the lipid bilayer of cell membranes, altering membrane fluidity, thickness, and surface tension, with potency correlated to the degree of partitioning into lipids.[59,89,122] This alters cell function and can lead to cell death; however, it is often less toxic than specific, receptor-mediated effects,[123] in that there needs to be a higher dose of exposure for an effect. Interestingly, there is variability in the cytotoxicity results (**Table 2**), with some studies showing a cytotoxic effect, or no effect on cell viability, or even enhanced cell proliferation. Therefore, more evidence is needed to clarify these results, considering that narcosis is suspected as the

main mechanism of NAs. To aid with this, impedance-based technology, such as Real-Time Cell Analysis (RTCA), may give insight into cell growth more holistically, rather than colorimetric assays that test very specific endpoints, thereby evaluating 'cytotoxicity' more broadly.

Table 2: Summary of relevant cytotoxicity studies.

Author	Technique	Model	Samples	Dose	Conclusions
Scott 2008 [84]	Microtox	*Vibrio Fischeri*	OSPW, ozonated OSPW	Not reported	IC_{20} = 23% OSPW; ozonation decrease cytotoxicity
He 2010 [6]	MTT	Human adreno-carcinoma cell line (H295R)	OSPW, ozonated OSPW	full strength to 10 000 x dilution	No cytotoxicity at any dilution used
Garcia-Garcia 2011 [102]	Trypan blue exclusion, MTT, & BrdU	Mouse bone marrow derived macrophages	OSPW extracts, commercial NAs	50-6.25 mg/L	No effect on cell viability; though low dose increased cell proliferation, and high dose decreased by 40%
Gagne 2012 [112]	Trypan blue exclusion	Rainbow trout hepatocytes	OSPW extracts	0.02, 0.1, 0.5%	Viability decrease 15-20% at 0.5% (2.5 fold full strength) for either OSPW or river water
Tollefsen 2012 [80]	Alamar blue, & CFDA, AM	Rainbow trout hepatocytes	Individual commercial NAs and mixtures	10-5-10-3 mol/L	EC_{50} = 24-89 mg/L; all individual and mixtures of synthetic NAs were cytotoxic; some differed from the concept of additivity
Sanson 2012 [29]	Alamar blue, CFDA-AM, & Neutral red	6 fish cell lines	49 OSPW samples	80%	Decrease in viability all 3 dyes, some samples decreased viability by 90%
Gagne 2013 [124]	Flow cytometry	Rainbow trout hepatocytes	Synthesized OSPW, OSLW, OSW	0-25%	Cytotoxicity at 5% OSPW (100→ 85% viable)
Lacaze 2014 [100]	Trypan blue exclusion	Rainbow trout hepatocytes	Commercial NAs, synthesized OSPW extracts, and OSLW extracts	10-100 %	18h after exposure, viability not affected

Note: Grey shading indicates a positive cytotoxic result, white indicates a negative result. IC_{20} = inhibitory concentration that inhibits a response by 20%.[84] EC_{50} = effective concentration that results in 50% of the maximal response.[80] OSLW = oil sands lixiviate water (water mechanically mixed with oil sands).[124] OSW = oil sands water (water passive in contact with oil sands). Microtox is an acute toxicity assay with bioluminescent bacteria. MTT assay is a colorimetric dye measuring metabolic activity. Trypan blue exclusion assay is a colorimetric assay measuring cell viability based on intact cell membranes. BrdU is incorporated into the DNA of only proliferating cells. Alamar blue is a colorimetric assay measuring cell viability through a reducing intercellular cytosol. CDFA, AM is a colorimetric dye measuring membrane integrity, and neutral red is a colorimetric dye that stains lysosomes of viable cells only. Flow cytometry uses laser technology to analyze chemical and physical characteristics of cells.

1.3.3.1. Real-Time Cell Analysis (RTCA)

With the increase in industrial chemicals of unknown toxicities, there is a movement towards rapid, high throughput, cost effective *in vitro* assays and away from *in vivo* assays as part of the *Toxicity Testing in the 21st Century (TT21C) paradigm*.[125] A technology supporting this paradigm is the xCelligence Real-Time Cell Analysis high-throughput system (RTCA). RTCA allows for efficient, label-free, dynamic, long term cytotoxicity assays using viable cells,[126] and avoids some of the confounding factors from colorimetric cytotoxicity tests.[127] It measures changes in impedance through a dimensionless parameter called cell index (CI), which is a representation of cell adhesion.[127] As the human cells grow in these wells, the extent of cell adhesion changes, indicating a change in cell proliferation and morphology. Treatment chemicals added to the wells can influence the cells by inducing: apoptosis or necrosis, morphological changes, or uncontrolled proliferation.[121] RTCA allows concentration-, chemical-, time-, and cell-dependent toxicological relationships to be established.[127]

Typically, with cytotoxicity assays there is only one time point selected; this neglects other possible phenomena occurring at different time points. Therefore, having a continuous, dynamic, recording of cell activity over a few days, will allow for rich, multiplex data collection generating time-dependent cellular response profiles (TCRPs).[121] TCRPs have potential to reflect the mode of action of a chemical through partially characterizing a series of physiological events through time.[121] In fact, multiple programs, such as the National Toxicology Program, the National Institutes of Health Chemical Genomics Center, and the US Environmental protection Agency (US EPA), have started implementing RTCA in their regimen to assess cytotoxicity efficiently of unknown industrial chemicals within the novel *ToxCast framework*.[127,128]

RTCA is a commonly used method in Dr. Xing-fang Li's laboratory, with other studies performed on water disinfection by-products[129] and nanoparticles,[127] among others. Recent papers have moved towards clustering similar TCRPs of a large number of unknown chemicals, which may act through a similar mode of action, and this can help the processes of prioritizing chemicals and selection of confirmatory endpoint for future assessments.[121,130] Given the largely unknown composition of OSPW organics and the variability between different samples, the high throughput capabilities of RTCA may be suitable for monitoring many OSPW samples at once or contribute to chemical-specific information within the organic mixture. In fact, Pan et al.[131] looked at developing a water quality index using RTCA as a high-through screen for environmental water samples, stating its suitability for when the responsible toxicants are unknown and when there is potential synergistic effects within the chemical mixture. To the best of my knowledge, assessment of OSPW with RTCA have not been published, therefore an initial investigation is warranted to add to the nuanced cytotoxicity data (**Table 2**).

With testing lower doses and longer exposures, the literature has shifted from assuming solely a non-specific narcosis mechanism[132] to specific receptor-mediated effects,[8] such as oxidative stress and metabolism and endocrine disruption. However, with such a complex mixture of chemicals, it is logical for there to be multiple mechanisms of action.

1.3.4. Oxidative stress

For normal cellular function, a minimal concentration of reactive oxygen species (ROS) is necessary.[8] ROS are O_2-derived free radicals produced through normal metabolism and xenobiotic exposure.[133] Antioxidant enzymes, such as Glutathione S-Transferase (GST) and Chloramphenicol Acetyltransferase (CAT), protect the cell from deleterious effects of oxidative

stress by clearing ROS. Oxidative stress arises when the capacity of the antioxidant defenses is overwhelmed, resulting in damage to mitochondria, peroxidation of lipids,[8] promotion of caspase enzymes, and activation of cell death pathways.[15]

There are multiple lines of evidence of OSPW organics changing the expression of oxidative stress-related genes (**Table 1**) and activating the Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR),[106] and Aryl Hydrocarbon Receptor (AhR),[70] all of which up-regulate protein expression for xenobiotic biotransformation. In fact, an oxidative stress-induced apoptotic mechanism has been suggested.[8,15,112] Moreover, Lacaze et al.[100] found oxidative DNA damage. Interestingly, Alharbi et al.[134] found that fresh OSPW induced oxidative stress to Japanese medaka embryos and this was not replicated in aged OSPW from Pond 9, supporting aging as an effective remediation strategy. In an acute exposure scenario, oxidative stress would likely predominate compared to endocrine disruption, while endocrine disruption may have a more pronounced effect in a chronic exposure scenario, such as in the EPL strategy.

1.3.5. Endocrine disruption

The endocrine system is a system of glands and tissues that interact through signalling molecules such as hormones.[135] It impacts cell differentiation and organ formation during development, and most tissue and organ functionality after development. Endocrine disruption can occur when an exogenous substance alters the function of this system, such as hormone synthesis, secretion, action, or metabolism.[136] Embryonic development, growth, reproduction, and overall homeostasis may be affected, along with the promotion of hormone-dependent cancers, among other adverse health effects.[135] Endocrine disruptors are challenging for risk assessments due to the vulnerability of organisms at critical stages of development and low dose

effects, disrupting the picomolar or nanomolar range that hormones operate at in a biological system.[137] While many mechanisms exist, estrogen (ER),[71] androgen (AR),[43,70] and thyroid receptor (TR)[93,94] interactions are more common in the literature.

The Organisation for Economic Co-operation and Development (OECD) created a conceptual framework in 1998 to guide the screening of endocrine disruptors, updated in 2012,[138] and 2018.[139] It consists of 5 levels with level 1 as models, level 2 as *in vitro* tests about mechanisms and pathways, and levels 3 – 5 as *in vivo* tests of increasing biological complexitiy.[138] Level 2 assays include ER or AR binding assays, or trans-activation assays, such as the yeast estrogenic and androgenic screens (YES/YAS). Trans-activation assays have the advantage of testing efficacy of an effect rather than solely ability of a chemical to bind a receptor. The higher levels include fish short-term reproduction assays, looking at endpoints such as VTG, a biomarker of xenoestrogen exposure in males, or a 21 day fish assay called the androgenized female stickleback screen measuring Spiggin, a biomarker of xenoandrogens, among others. Similarly, the US EPA[140] created a guidance document for testing endocrine activity, where a range of tests are needed to contribute to the weight of evidence in assessing endocrine activity, with not one single assay able to provide that conclusion. It appears as though, to the US EPA,[141] that a chemical may be termed as having 'endocrine activity' in an assay, but the weight of evidence framework is needed to determine if that activity will lead to an adverse outcome, to which the term 'endocrine disruption' can then be used, though this terminology is nuanced and not maintained, even in the OECD guidelines.[139]

Looking at the OSPW toxicity data, Knag et al.[101] tested commercial NAs while measuring VTG and Spiggin and found no estrogenicity or androgen antagonism at environmentally relevant doses. While these assays are biologically complex, commercial NAs act differently

from OSPW-NAs, and other OSPW organics were ignored. This shows the challenges in comparing OSPW toxicity studies and the importance of analytical methodology to compliment bioassays.

In vitro, there are multiple lines of evidence for endocrine activity among OSPW, with studies predominantly focusing on ER, AR, and steroidogenesis, though as noted previously, PPARγ interactions[27] have been observed as well as thyroid abnormalities[93,94] in biomonitoring of species in the industrial area. He et al.[6] performed a steroidogenesis assay (with the H295R human adrenal gland/cortex cell line) with varying concentration of OSPW and found a decreased concentration of testosterone (T) and an increased concentration of estradiol (E2). They also found an increase in aromatase expression (which converts T into E2) and a decrease in E2 metabolism. Next, He et al.[43] used T47D-Kbluc (estrogen-responsive) and MDA-kb2 (androgen-responsive) human breast cancer cell lines to look at OSPW modulation of ER and AR responses. They found that OSPW potentiated the estrogenic response, while the androgenic response was both potentiated and inhibited depending on the concentration of T.[43] Similarly, the organic fraction of processed water in the North Sea near Norway was found to act as a weak estrogen receptor agonist and androgen receptor antagonist through YES and YAS assays, respectively.[142]

More recently, LeClair et al.[70] fractionated OSPW-NAs into 4 fractions, measuring AhR, ER, and AR responses, and steroidogenesis. Contrary to previous findings, none of the fractions were ER or AR agonists, nor affected steroidogenesis, but there was AhR binding and ER and AR antagonism. They concluded that NAs at environmentally relevant concentrations had endocrine active capabilities, even with their OSPW sample that had been aged 17 years.

Interestingly, Yue et al.[71] performed an EDA of OSPW extracts with the YES and found estrogenic responses, contrary to Leclair et al..[70] They claimed to have narrowed the responsible species to the O_2^-, O_3^-, and O_4^- classes of carbon chain length 17-20 with 6-10 units of double bond equivalency, though this study only used negative mode MS. They found that the whole OSPW sample had too low of an estrogenic response to measure, yet when they fractionated the extracts into eight fractions an estrogenic effect was detected, suggesting chemical antagonism. Therefore, perhaps the four fractions Leclair et al.[70] used did not sufficiently isolate the OSPW organics for a response to be measured compared to the eight fractions used by Yue et al.,[71] or that their extraction included non-acids species and not only NAs. Notably, other studies have found aromatic NAs[71,143] and the O_2^+ class[30] to be chemically similar to estrogens and progesterone, respectively.

Importantly, chemical composition of processed water varies: He et al.[6,43] and Yue et al.[71] used fresh OSPW from WIP, Thomas et al.[142] used processed water from the North Sea, and LeClair et al.[70] a 17-year-old experimental OSPW pond. The limitations of LeClair et al.[70] is that they used a NA-specific extraction method, excluding other organics. Furthermore, all of the YES/YAS studies only looked through negative mode of MS, thereby excluding other unknown species that may contribute, such as the non-acidic species detected in positive mode. To my knowledge, a study has not yet compared the YES/YAS response of OSPW samples of different ages. Therefore, an EDA combining YES/YAS with OSPW samples of multiple ages and analyzed in both positive and negative mode of high resolution MS would be timely.

1.3.6. Base Mine Lake (BML) toxicity

The aforementioned toxicity data included variable OSPW samples; however, the most likely OSPW sample for environmental release and exposure to life is BML, and it is important to have toxicity data to assess how the EPL is progressing. Much of the toxicity data used in OSPW is focused on discovering mechanisms of action and isolating particular parts of the mixture to attribute toxicity. While this is needed, it is equally important to have acute and chronic toxicity assessment of the whole effluent in fundamental terms of survival and growth, as provided by Syncrude Canada Ltd. in their report[144] to the Alberta Energy Regulator (**Table 3**).

Table 3: Syncrude Canada Ltd. whole effluent Base Mine Lake (BML) toxicity over time.[1] Source: Syncrude Canada Ltd.[144] Table 4-10, p. 75.

Toxicity Test	Units	2016 Data				Historical Data (2013-2015)			
		Min	Median	Max	n	Min	Median	Max	n
Acute Toxicity									
Ceriodaphnia 7 d Survival Test - LC50	% BML water	77	>100	>100	9	72	>100	>100	21
Daphnia 48 h Static Acute Test - LC50	% BML water	>100	>100	>100	9	>100	>100	>100	24
Fathead Minnow 7 d Survival Test - LC50	% BML water	>100	>100	>100	9	63	>100	>100	21
Rainbow Trout 96 h Static Acute Test - LC50	% BML water	>100	>100	>100	9	66	>100	>100	24
Chronic Toxicity									
P. subcapitata 72 h Growth Inhibition Test - IC25	% BML water	17	60	>91	9	1.4	74	>91	18
Fathead Minnow 7 d Growth Test - IC25	% BML water	>100	>100	>100	9	54	>100	>100	21
Lemna minor 7 d Growth Test (Dry Weight) - IC25	% BML water	18	95	>97	9	2.2	58	>97	15
Lemna minor 7 d Growth Test (Frond Number) - IC25	% BML water	2.1	8	51	9	2.4	15	>97	15
Bacterial Luminescence Test (15 min) - IC20	% BML water	17	29	52	9	11	21	>91	27
Ceriodaphnia 7 d Reproduction Test - IC25	% BML water	20	39	62	9	0.14	38	77	21

Note: Water was collected from three platform stations on BML and were mixed to form one composite sample for toxicity testing. A 100% BML water dose has no volume dilution compared to the field.

[1] Provided by Warren Zubot, Syncrude Canada Ltd. (SCL)

In **Table 3**, the units are in "% BML water," in that 100% is the maximum concentration of effluent with no volume dilution. LC_{50} is the dose required to kill 50% of the population, and all of the medians for the acute toxicity tests have an LC_{50} >100% no matter the year tested. This means that the full dose of OSPW in its aqueous state is not acutely toxic as a median response and that an exact LC_{50} was not able to be calculated. However, it is important to note that the minimum values of the historical data are mostly <100%, indicating that the water was lethal to some but not all organisms. It is promising to see that in 2016 the acute toxicity observed is ameliorated in fathead minnows and rainbow trout, though chronic toxicity data is more varied.

The fish acute lethality assay is commonly used in regulatory ecotoxicology to assess industrial effluents as a standardized protocol set by the OECD.[29] Based on the *Water Quality Based Effluent Limits Procedure Manual* by Alberta Environmental Protection, environmental protection is achieved through incorporation of whole effluent toxicity limits, chemical specific toxicity limits, and biological effects monitoring.[26] Therefore, while the standardized fish acute lethality assay is passed by the whole effluent of BML, this does not incorporate chemical specific toxicity nor sub-lethal measurements. This is important considering the plethora of data in the literature showing sub-lethal effects from even aged OSPW samples to fish[23] and midges,[13] as well as chemical-specific effects. Therefore, the standardized screen for acute toxicity are environmentally realistic, valid, and promising for the EPL strategy in terms of short term exposure, but there are also limitations. Notably, an LC_{50} of >100% does not give information on the true LC_{50} value, nor does it allow for comparison of the toxic potency between samples. Furthermore, it does not provide any information about sub-lethal effects that may indirectly cause lethality, i.e. ecological death. Lastly, assessing the whole effluent does not address the chemical-specific effects of the organics, particularly when considering the chronic

exposure scenario and the potential for bioaccumulation of particular chemical classes. This knowledge could help design guidelines for OSPW management to ensure a safe release.[9]

Given how complex, variable, and dynamic the organic fraction of OSPW can be over space and time,[28,29,145] focus on whole effluent toxicity limits are more practical, and this is shown with Syncrude's data of BML. However, Bartlett et al.[146] argued that OSPW guidelines need to adapt as our knowledge and technology improves, and that emphasis should be on the composition as of the organics, not just concentration. No matter the approach, to prevent an expensive and toxic legacy for future generations, it is argued that strides towards a safe release of OSPW is needed to be implemented in this generation while the financial gains from oil sands exploitation are still being realized.[9]

1.4. Rationale for thesis research

As non-conventional oil production increases, so does the amount of OSPW generated with limited space to hold it.[59] While there is suspected seepage of OSPW from tailings ponds in certain tributaries, flux to the Athabasca River appears to be non-detectable after mixing.[42] More attention should now focus on the inevitable future when large volumes of OSPW must be released to the environment from EPLs,[22] to which there are currently no chemical nor toxicological guidelines for, and unknown risk to the surrounding environment, wildlife, and human populations. Predicting the effectiveness of this strategy relies on an understanding of what chemicals cause toxicity in fresh OSPW and how these may change over time. There are multiple studies showing that OSPW stripped of their organic component ameliorates the potency of the selected endpoints tested.[60,84] This investigation was previously set to further investigate the organic component of OSPW, but does not mitigate the potential of inorganics to

hold toxicity.[109] There are a lack of mammalian toxicological studies; as such, this investigation used a mammalian liver cell line as a model, given previously observed hepatotoxicity[116] and that exposure would be through oral ingestion resulting in first-pass metabolism.

Studies often lack the analysis of organic chemical classes in both positive (non-acidic species) and negative mode (acidic species) of high resolution mass spectrometry. By extension, more inclusive extraction methods are needed to assess the whole organic mixture, not only NAs, such as a total acid extraction used here. Syncrude Canada's BML data (**Table 3**) is promising for the EPL strategy in terms of observing a reduction in acute toxicity to the whole effluent; however, there are limitations, in that environment protection of industrial water release is not only based on whole effluent toxicity, but chemical-specific toxicity and biological monitoring. Therefore, increasing the TAE to above field concentrations will allow for assignment of unambiguous toxic potencies, thereby monitoring ageing as a remediation strategy for the organics. Moreover, by using both mg (toxicity / mass of extract) and × (toxicity / volume) scales, the impact of *in situ* fresh water dilution of BML will be evaluated.

While further investigation of the most acutely toxic class, the NAs, is needed, the polar non-acids need also be explored with novel fractionation methods. This is supported by evidence of SO^+ as a candidate OSPW-specific class for differentiating between anthropogenic and natural sources,[42] of SO^+ and NO^+ with modelled and *in vivo* evidence of being the most bioaccumulative chemicals,[92] and of SO_x^+, NO_x^+, and O_x^+ being toxicologically relevant.[51] Therefore, this investigation will address the need of attributing toxicity to chemical classes in the organic mixture, and will give inferences on endpoint selection for biological monitoring.

It is challenging comparing OSPW studies due to variability in the sample, extraction method, ionization mode, detection method, and bioassay used. Therefore, this study will be

using the same experimental parameters while measuring how toxicities change with multiple OSPW samples. As such, the standardized endocrine disruption assay, the YES/YAS,[147] was chosen to assess endocrine activity and to enable comparison to previous reports.[70,71] Furthermore, incorporating new technology, such as RTCA, which is designed for high-throughput assessment, will enable dynamic, holistic measurements of cytotoxicity, and assess a mammalian relevant mode of action though a human hepatocellular carcinoma cell line (HepG2 cells).[131] This investigation will use chemical fractionation and high resolution Orbitrap mass spectrometry combined with cytotoxicity and endocrine disruption assays to study candidate chemical classes in the organic component of various fresh and aged OSPW samples. This will add predictive value to the chronic exposure scenario by informing the chemical-specific and biological monitoring approaches needed for guidelines, thereby contributing to a safer release of OSPW and preventing an expensive and toxic legacy of the oil sands industry.

1.5. Research questions

- What chemical classes among the OSPW organics need to be monitored to allow for a safer environmental release with respect to a mammalian model?
- Is natural ageing in the End-Pit Lake remediation strategy effective at decreasing its cytotoxic potency and endocrine activity?

1.6. Hypothesis

- The O_2^- class within Base Mine Lake OSPW from 2015 is responsible for the cytotoxic effects.
- The polar non-acids (O_x^+, NO_x^+, SO_x^+) within Base Mine Lake OSPW from 2015 are responsible for the endocrine activity.
- The cytotoxic potency and the endocrine activity decreases as the *in situ* age of OSPW organics increases.

1.7. Objectives

1. To attribute toxicity to Base Mine Lake 2015 organic fractions through measuring their cytotoxicity towards human hepatocellular carcinoma cells and their endocrine activity with yeast strains genetically modified with the human estrogen and androgen receptors.
2. To measure the cytotoxicity and endocrine activity of the whole organic mixture, from field OSPW samples of different ages, to evaluate the effectiveness of natural aging in the End Pit Lake remediation strategy.

2. Methods

2.1. Sample collection of environmental samples

OSPW samples were provided by Syncrude Canada Ltd. and stored at 4°C in high-density polyethylene pails of various sizes. Considering that BML was commissioned as an EPL in 2012, this year is a time zero reference for ageing for BML. **Table 4** compares the ages of all samples tested. The aged sample used was collected in 2016 from Pond 9, which was commissioned in 1993.[10] Given that the experimental ponds were *in situ* experiments to test the effectiveness of the EPL strategy, the Pond 9 water can act as an indicator of what BML could be like in the future after an equivalent amount of ageing. Surface OSPW samples were collected with a Van Dorn water sampler from an outflow barge between 0.5 and 1 m below the surface.[2]

Table 4: Water samples tested in this investigation and years aged (if applicable).

Samples & Year Sampled	LC/MS Water	River 2017	BML 2013	BML 2015	BML 2017	Pond 9 (2016)
Years Aged *in situ*	0	0	1	3	5	23

Note: LC/MS is liquid chromatography mass spectrometry grade water, River 2017 is the Athabasca River, and BML is Base Mine Lake. Year aged *in situ* is only relevant for environmental sample of OSPW taken from Syncrude Canada Ltd.

The Athabasca River water sample was collected in 2017 by colleagues from Dr. Bill Shotyk's lab at the University of Alberta, labeled as 'River 2017.' The sample was collected from the upstream location A20e SW (**Figure 2**), and was used as an environmentally-relevant

[2] Provided by Warren Zubot, Syncrude Canada Ltd. (SCL)

natural reference to compare to the OSPW samples. Here, the river water sample was collected approximately 30 cm below the surface in an acid-cleaned 2.5 L polypropylene jug, with the cap secured below the surface to avoid contamination by the surface microlayer.[3] Optima® LC/MS water (Thermo Fisher Scientific, San Jose, CA) was used as an extraction blank for toxicity testing (Section 2.2).

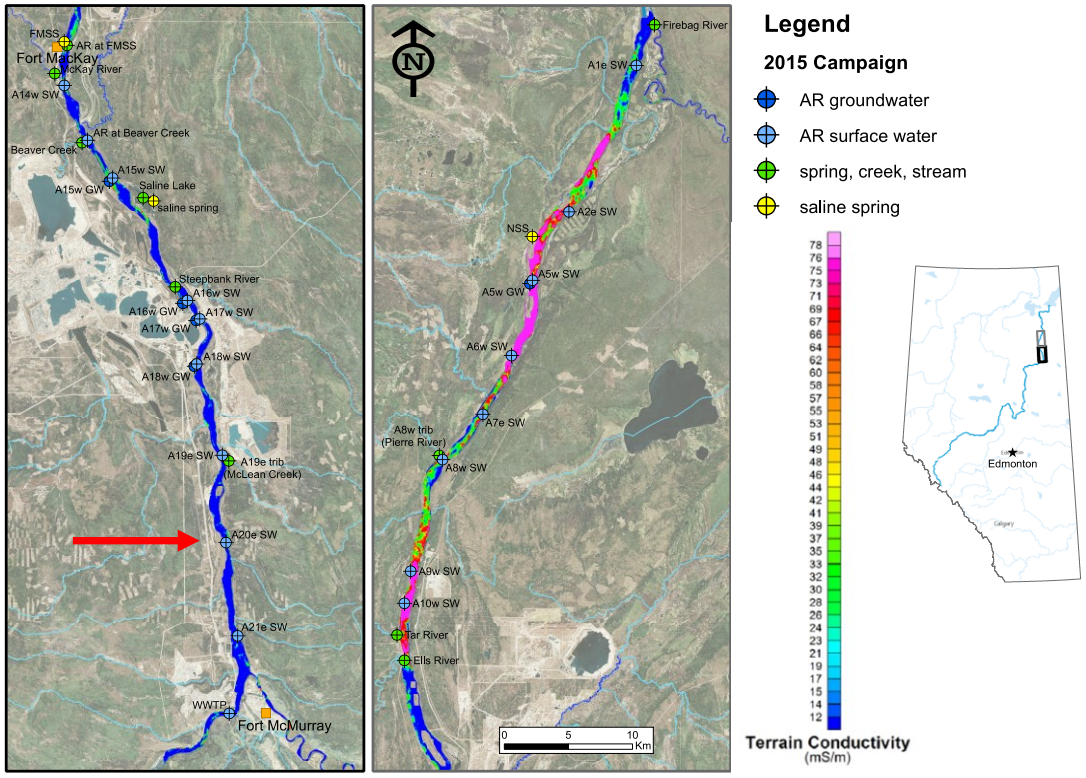

Figure 2: Sample collection location of the Athabasca River water sample, A20e SW (highlighted by red arrow), which is upstream of the oil sands industry. Reproduced with permission from, source: Gibson et al.[148] and its adapted version provided by Dr. Chad Cuss, University of Alberta.

[3] Personal communication with Dr. Chad Cuss, University of Alberta

BML water balance has been directly managed by yearly addition of freshwater,[144] resulting in approximately a 20% dilution of OSPW.[4] To manage suspended fine tailings and improve turbidity, gypsum[2] has been added as a coagulant, as well as a trial of 1520 tonnes of Alum in September 2016 to aid the process.[144] Therefore, differences between samples of BML in 2013, 2015, and 2017 cannot be attributed solely to ageing (explored in section 4.2).

2.2. Toxicity identification of BML 2015

A variety of analytical techniques were used to isolate different components of the OSPW mixture, though a full TIE standardized method (section 1.3.1) was not performed.[104] All analytical techniques performed on OSPW were done in an ultra-clean trace elements lab with rapid air turnover to minimize contamination. Once the treatments were complete, cytotoxicity was assessed by Real-Time Cell Analysis (RTCA) (ACEA Biosciences, San Diego, CA).

For preliminary toxicity identification experiments, the only sample tested was BML 2015, and initially, in its aqueous state. The first step across all treatments was vacuum filtration through a 1.2 μm G4 glass fibre filter (Thermo Fisher Scientific, San Jose, CA) to remove sediment, with one filter used per 500 mL of BML 2015 to prevent clogging. The pH of OSPW is approximately 8.0, while cell culture media is usually 7.4. To test whether the pH of OSPW had an effect on cell proliferation, one of the treatments had filtered BML 2015 adjusted to 7.4 with 1.0 N HCl (BioReagent, suitable for cell cultures, Sigma-Aldrich, Oakville, ON, Canada) and a pH meter (Orion Star A211, Thermo Fisher Scientific, San Jose, CA).

[4] Personal communication with Warren Zubot, Syncrude Canada Ltd.

Next, two methods were used to remove the organic mixture from BML 2015: activated charcoal (AC) powder (Sigma-Aldrich, Oakville, ON, Canada) and Supelclean ENVI-Carb SPE cartridges (Sigma-Aldrich, Oakville, ON, Canada). This allowed for the toxicity of the remaining components to be assessed, i.e. the inorganics and dissolved salts. For the AC-treated BML 2015, a 10% mass of AC per volume of BML 2015 was mixed in a beaker with a stir bar for 4 h. The sample was transferred to a 15 mL tube and centrifuged at 4500 rpm for 15 min with a Sorval ST 40R Centrifuge (Thermo Fisher Scientific, Langenselbold, Germany). The supernatant was then syringe-filtered through a 0.45 μm nylon filter membrane (Sigma-Aldrich, Oakville, ON, Canada) and collected in a sterilized glass bottle. For Supelclean ENVI-Carb extractions, 1 mL of distilled water conditioned the cartridge, and one SPE cartridge was used for each 1 mL of BML 2015. Each sample was collected in sterile glass bottles. All treated samples were stored at -4°C until toxicity analysis.

2.3. Effects Directed-Analysis (EDA) of BML 2015

An EDA previously performed on BML 2012 by Morandi et al.[51] and colleagues identified the most acutely toxic chemical fractions in its organic mixture. Here, we attempted to replicate these two fractions, the NAs and polar non-acids, but through a large volume SPE method and using a sample of BML 2015. The SPE fractionation procedure (**Figure 3**) separated the organic mixture into specific groupings of chemical classes with similar physical/chemical properties. This extraction was used to explore the first objective of this investigation: toxicity attribution within the organic mixture.

The SPE method was performed by Research Associate Dr. Chenxing Sun (Martin Group, UofA). An Oasis HLB SPE Cartridge (35 cc, 6 g sorbent per cartridge, 60 μm particle size,

Waters Limited, Ontario, Canada) was used, which is a universal sorbent for acidic, neutral, and basic compounds. As seen in **Figure 3**, each SPE cartridge was conditioned with 20 mL MeOH, equilibrated with 20 mL MS grade water, and then loaded with 2.1 L of BML 2015. To remove any remaining salts from the cartridge, LC/MS water was passed through, resulting in a sample of 'SPE waste.' The cartridges were then washed in turn with 40 mL MeOH:H_20 (50:50, v/v) to generate Wash 1, and then 20 mL MeOH: 2% acetic acid (55:45, v/v) to generate Wash 2.

Another eluent was collected by adding 40 mL of MeOH: 5% NH_4OH (65:35, v/v), termed Eluent 1. Eluent 1 was adjusted to pH 12 and extracted three times with 40 mL DCM, evaporated to dryness, and termed Eluent 1-Basic. The remaining aqueous sample was then adjusted to pH 2 and extracted three more times with 30 mL DCM, termed Fraction 1. Eluent-2 was generated by using 25 mL of pure MeOH to elute any remaining analytes from each SPE cartridge, and this was combined with Eluent 1-Basic to produce Fraction 2. The dry weight of each fraction was measured and allowed for an estimated field concentration of mg dried extract per L of original BML 2015 OSPW, termed as a gravimetric analysis. The fractions were dried and reconstituted in 200 μL of anhydrous ethanol (Commercial Alcohols, Toronto, ON, Canada), resulting in a 5000× concentrate. The unit, ×, is defined here as 'enrichment factor,' where 1× is the real-world concentration of organic extract in the original water sample, less than 1× is a dilution, and greater than 1× is enriched above the sample concentration. The SPE waste and unextracted BML 2015 sample were sent to a commercial laboratory, the Natural Resources Analytical Laboratory (NRAL) at the University of Alberta, for a Total Organic Carbon (TOC, liquid sample) analysis,[149] of which the methodology is not included here.

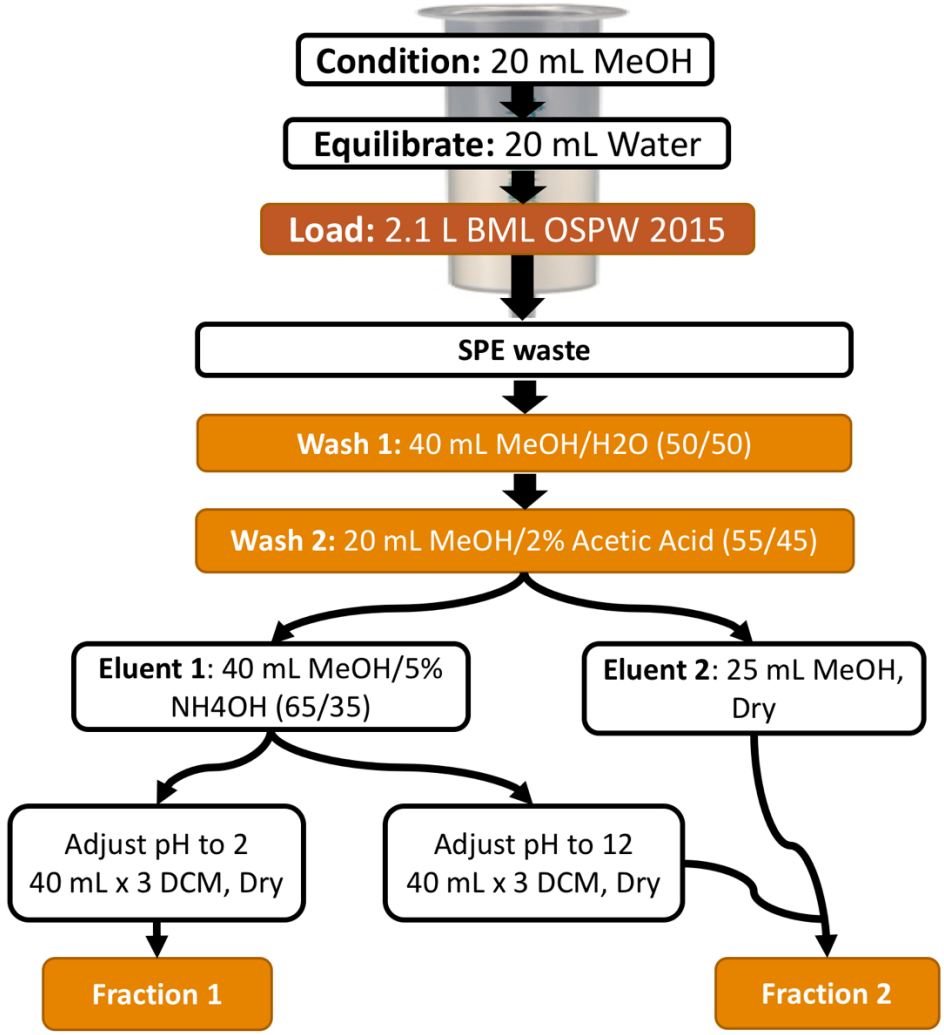

Figure 3: Multi-step SPE fractionation procedure for BML 2015 used in the current research.[5]

2.4. Total Acid Extract (TAE)

A pH-adjusted liquid-liquid extraction method was used to extract the whole organic mixture from all of the water samples tested. This method enabled testing of the second objective of this investigation: evaluating ageing as a remediation strategy of the OSPW organics from different environmental samples. As mentioned in Chapter 1, an EDA performed by Morandi et al.[51] on

[5] Fractionation method reproduced with permission from Dr. Chenxing Sun, University of Alberta

BML 2012 determined that the acutely toxic fractions in the first stage of fractionation were the neutral (pH 7) and acid (pH 2) extracted organic fractions, while the basic (pH 11) organic extract, which contained mostly short-chain carbon species, was not acutely toxic. Therefore, in an attempt to get as much of the relevant organic mixture as possible, a total acid extract (TAE) at pH 2 was performed on the following samples: BML 2013, BML 2015, BML 2017, Pond 9 2016, Athabasca River water 2017. The TAE should theoretically contain all chemicals that were present in the neutral and acid extracted organic fractions of Morandi et al.[51] An extraction control was performed with Optima® LC/MS water (Thermo Fisher Scientific, San Jose, CA) to test for non-specific toxicity from analytical reagents and materials.

First, 1 L of sample was vacuum-filtered with a G4 glass fibre filter. A pH meter was used to measure acidification of the water to pH 2 with the dropwise addition of concentrated sulfuric acid (98%) (Thermo Fisher Scientific, San Jose, CA). A liquid-liquid extraction was performed in a 2 L glass separatory funnel with 200 mL of DCM (99.5%) used as a solvent. The extraction was repeated three times with fresh DCM and the combined extracts were reduced in volume by rotary evaporation (Rotavapor R-210, Buchi, Flawil, Switzerland). The concentrated extract was transferred to a pre-weighed glass vial and brought to complete dryness by nitrogen evaporation (TurboVap® LV, Caliper LifeSciences, Hopkinton, Massachusetts). The dried organic mass was weighed and reconstituted in 200 μL of anhydrous ethanol, generating a 5000× concentrate (termed as a gravimetric analysis).

2.5. Analysis by HPLC-LTQ-Orbitrap-MS

Reversed-phase liquid chromatography was coupled with Orbitrap mass spectrometer (Orbitrap ELITE, Thermo Fisher Scientific, San Jose, CA) operating in ESI mode.

Chromatographic separation was achieved with an HPLC Accela System (Thermo Fisher Scientific, San Jose, CA), including a degasser, 600 bar quaternary pump, auto sampler, and column oven, and a C18 gold column (100 x 2.1 mm, 1.9 μm particle size, Thermo Fisher Scientific, San Jose, CA) kept at 40 ºC. The Orbitrap was set to a nominal resolving power of 240, 000 at *m/z* 400. To detect organic acids in negative ionization mode, and polar organic neutral and organic bases in positive ionization mode, two separate injections into the instrument were made. The mass range was set from *m/z* 100-500 in negative mode and from *m/z* 160-500 in positive mode. The flow rate was 0.5 mL/min and the injection volume was 3 μL in both ionization modes. The mobile phases were (A) 0.1% acetic acid in water and (B) 100% methanol. The elution gradient started with 5% B and 95% A for 1 min, then a linear ramp increasing the proportion of B to 90% at 9 min, to 99% B over 5 min, then returning to 5% B in 1 min, finishing with a 4 min hold before the next injection.[150]

To characterize the total acid extracts, heteroatomic chemical class distributions were made with Xcalibur software (Thermo Fisher Scientific, San Jose, CA), with the time range on the chromatogram set from 7-13 min for negative mode and 3-13 min for positive mode. Mass tolerance for empirical formula assignment was set to 5 ppm, while the normalized intensity range was set to 0.010-100 to filter out noise. Using Excel software (Microsoft Office, Redmond, Washington), *m/z* ratios were converted to Kendrick Mass values, and by extension, the Kendrick Nominal Mass and Kendrick Mass defect were calculated. This allowed us to generate a 'mass identifier' for each detected *m/z* ratio that had a degree of error to allow for easier comparison of a peak in the sample to the same peak detected in the blank (LC/MS-water extract). If there was a match between mass identifiers of the sample and blank, a signal greater than 3× the blank was required to be accepted as a true signal in the sample. The blank-

subtracted sample was then filtered for each heteroatomic class: e.g. O_x, NO_x, $SO_x^{-/+}$, and heteroatomic class distribution plots were created to compare each sample. All MS data was collected on the same day, but an internal standard was not used. Thus, the TAE data from these samples were not comparable to the BML 2015 fractions, which were run on a different day. By extension, to generate the heteroatomic class distributions of the fractions of BML 2015, a separate analysis was used, as the fractionation method was more complex than the TAE, involving a number of back calculations performed by Dr. Chenxing Sun (Research Associate in Martin Group, University of Alberta), which is outside the scope of this investigation.

2.6. Sample preparation for bioassays

Sample preparation for bioassays was dependent on whether the sample was prepared in aqueous or organic solvent. For toxicity testing of aqueous whole BML 2015, pH-adjusted whole BML 2015, AC-treated BML 2015, and ENVI-Carb-treated BML 2015, a powdered format of Eagle's Minimum Essential Medium Eagle (EMEM) (M0643, Sigma-Aldrich, Oakville, ON, Canada) was used, where the ratio of 9.6 g of EMEM per 1 L of water sample was maintained. The powdered EMEM was directly reconstituted with the treatment water and supplemented with 2.2 g $NaHCO_3$ per 1 L of sample (BioReagent, Sigma, Oakville, ON, Canada). This allowed for cell cultures to be exposed to a 100% dose of real-world BML 2015, with no volume dilution occurring to the field sample. For the pH-adjusted sample, the pH was adjusted to 7.4 to be suitable for cell cultures. The treated media was then sterile filtered with a syringe and 0.22 μm Millex® GV Durapore® PVDF membrane (Sigma-Aldrich, Oakville, ON, Canada) for small volumes (~5 mL), or with a vacuum and Millipore Express™ PLUS 0.22 μm filter unit (Sigma-Aldrich, Oakville, ON, Canada) for larger volumes (~500 mL). Afterwards, the media was

supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada) and 1% Penicillin/Streptomycin (P/S) (100 U/100 μg/mL, Invitrogen, Carlsbad, CA). Media formulation was prepared on the same day as cell culture treatments.

For the organic extracts in anhydrous ethanol, treatments were added directly into the premade liquid format of EMEM (30-2003™, American Type Culture Collection (ATCC), Manassas, VA) and supplemented with FBS and P/S. For RTCA, a dilution scheme of anhydrous ethanol was first tested to find an appropriate concentration range of ethanol in media (% v/v) that is sufficient to dissolve the organics but not cause any cytotoxic effects. Furthermore, a concentrated stock of TAE was required in order to reach a final concentration of 12.5× in each well. The optimized protocol used 200 μL of anhydrous ethanol, resulting in 0.25% ethanol (v/v) at the highest treatment dose (12.5×) from which further dilutions were created, maintaining the ratio of organic extract to solvent constant throughout all dilutions.

For the YES/YAS assays, the percentage of anhydrous ethanol in the positive controls was set by the supplier at 0.67% (v/v). Thus, the highest treatment dose (10×) was also prepared with a final concentration of 0.67% (v/v) ethanol, while maintaining a consistent ratio of organic extract or control hormone to solvent in each treatment dilution. Keeping the ratio of carrier solvent constant allowed for comparisons between tested environmental samples; however, the YES/YAS does have a higher ratio of solvent compared to that used in the RTCA.

2.7. Real-Time Cell Analysis (RTCA)

2.7.1. Cell culture

The human hepatocellular carcinoma cell line, HepG2 [HEPG2] (ATCC® HB-8065™, Manassas, VA) was grown in EMEM supplemented with 10% FBS and 1% P/S. HepG2 was

derived from a 15 year old adolescent male and have epithelial cell morphology with adherent properties suitable for RTCA. HepG2 was chosen because the liver is a relevant organ that would be exposed following oral exposure of OSPW, given first-pass metabolism. Also, of the few mammalian studies that exist, OSPW-NA extracts demonstrated hepatotoxicity in mice.[116] Even more warranting is that HepG2 have been used in high throughput RTCA experiments to rapidly prioritize chemicals of unknown toxicities for further testing and to give insight into potential modes of action based on similar toxicity profiles.[121]Therefore, using HepG2 as a model here will allow for easier comparisons to other RTCA data.

HepG2 cells were maintained at 37°C in 5% CO_2 and passaged twice a week into standard 10 cm x 20 mm cell culture dishes (Corning Inc., Corning, NY). To ensure cell viability and consistency between experiments, cells were only passaged a maximum of five times before a new vial was thawed. From frozen in liquid nitrogen, cryovials were placed in a water bath of 37°C for ~2 min, then thawed cells were plated into a cell culture dish with pre-warmed media for incubation. To passage the cells, Dulbecco's PBS (Thermo Fisher Scientific, Burlington, ON, Canada) was used to wash the cells, and 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Burlington, ON, Canada) was used to detach the adherent cells from cell culture dishes.

2.7.2. RTCA measurements

Cytotoxicity was measured using Real-Time Cell Analysis (RTCA) (ACEA Biosciences, San Diego, CA). RTCA consists of a 96-well plate with microelectrodes covering the bottom surface of each well. A current is passed through the wells at three different frequencies: 10 kHz, 25 kHz, and 50 kHz, measuring changes in impedance (Z) over time at the electrode-media interface

of each well.[127] Three frequencies allows for the maximization of Z, which is converted to resistance (R) by the system analyzer through the following equation:

$$Z = R + jX$$

Here, j is the imaginary component and X is the reactance. R is then converted to the unit-less parameter called Cell Index (CI) through the following equation:

$$CI = \max_{i=1,\ldots,N} \left(\frac{R_{cell}(f_i)}{R_b(f)} - 1 \right)$$

With $R_{cell}(f_i)$ as the frequency-dependent resistance when cells are attached to the microelectrode, $R_b(f)$ as a reference value for when cells are no bound, and N as the number of frequencies that Z is measured under. This means that as the $R_{cell}(f_i)$ value changes over time due to cell attachment and proliferation, so does CI.[127] Therefore, CI is a representation of cell-electrode contact (**Figure 4**).

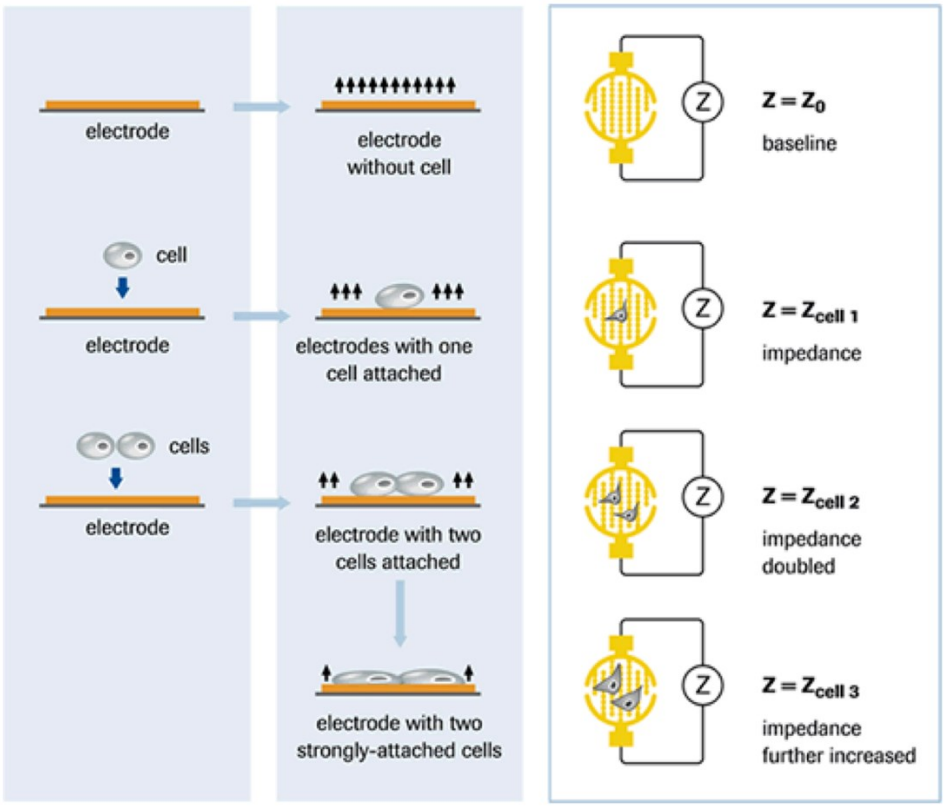

Figure 4: Schematic illustrating how cell adhesion onto a RTCA well microelectrode translates to changes in impedance (Z), and ultimately Cell Index (CI). Reproduced with permission from, source: Rotroff et al.[151] Figure 1, p. 1098.

Cell seeding density was determined through a trial RTCA experiment, where different cell densities (10,000-20,000 cells/well) were tested to achieve a CI of 1 within 20-24 h after seeding. For HepG2 cells, an optimized seeding density was determined to be 12,500 cells/well (data not shown). Once a CI of 1 was achieved, the CI values were normalized to reduce inter-well cell number variation, and this was termed the normalized cell index (NCI). The NCI allowed differences in cell growth to be attributed to the various treatments, and not to slight variations in initial cell number between wells. The NCI values collected hourly over the exposure period were plotted automatically through the RTCA system analyzer to create time-dependent cellular response profiles (TCRPs).

Negative control wells typically follow a growth curve consisting of lag phase, exponential growth phase, plateau phase, when the wells achieve confluence, and the decline phase, when cells die due to lack of nutrients or senescence.[152] Treatment effects causing apoptosis, necrosis, morphological changes, or uncontrolled proliferation can be reflected in the TCRPs.[127]

Generally, each treatment has 4 replicate wells, which are averaged for comparison to other treatments or controls within the plate. Thus, every averaged TCRP has a standard error of the mean (SEM). Measurements are taken every hour over a period of 100 h while cells are still in the incubator, generating a dynamic response profile which is visualized and analyzed with GraphPad Prism 7.

Negative control wells contained media only, allowing for maximal cell growth, while the solvent control included 0.25% (v/v) anhydrous ethanol. Arsenic (III) at a concentration of 250 μM was used as a positive control, as it is a well-studied cytotoxic chemical that leads to cell death resulting in a NCI of 0.[153] It is therefore a useful benchmark for comparison. The positive control was prepared from sodium arsenite (Sigma-Aldrich, Oakville, ON, Canada) in deionized water. Treatments generating NCIs less than the negative control, but higher than 1, are described as causing growth inhibition. Treatments generating NCI values less than 1 are described as causing cell death. 'Cytotoxic' is used here in its general sense to describe a sample that reduces the NCI below that of the negative control, be that above or below a NCI of 1.

One RTCA plate was used to test a range of doses of two different OSPW extracts, along with the negative control, the solvent control, and the positive control, as shown in the plate layout in **Figure 5**. Due to impedance measurements through the e-plate, heat is naturally generated. While water is placed in gaps between the wells to minimize this, the edge wells have less of a buffer for the additional heat, and consistently resulted in marginally reduced CI values

when compared to wells that were inside the plate and more protected. Therefore, to ensure that none of the replicates of a given treatment are influenced by the edge effect, treatments were arranged so that only one of the replicates were in the outside well position.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Blank	**12.5**	**10**	**7.5**	**5**	**0.1**	0.1	12.5	10	7.5	5	Blank	A
B	Blank	**10**	**12.5**	**10**	**10**	**0.1**	0.1	10	12.5	10	10	Blank	B
C	Blank	**7.5**	**7.5**	**12.5**	**7.5**	**0.1**	0.1	7.5	7.5	12.5	7.5	Blank	C
D	As	**5**	**5**	**5**	**12.5**	**0.1**	0.1	5	5	5	12.5	As	D
E	As	**2.5**	**2.5**	**2.5**	**0.5**	0.25%	Neg cont	2.5	2.5	2.5	0.5	As	E
F	Blank	**1.75**	**1.75**	**0.5**	**1.75**	0.25%	Neg cont	1.75	1.75	0.5	1.75	Blank	F
G	Blank	**1**	**0.5**	**1**	**1**	Neg cont	0.25%	1	0.5	1	1	Blank	G
H	Blank	**0.5**	**1**	**1.75**	**2.5**	Neg cont	0.25%	0.5	1	1.75	2.5	Blank	H

Figure 5: Example RTCA plate layout. Two OSPW extracts are tested at a time (ranging from 0.1×-12.5×), with a negative control (Neg cont), positive control (As), solvent control (0.25%), and blank wells in case a well is non-responsive and an adjustment is needed to be made to ensure 4 replicates per treatment.

To quantify the TCRPs, Inhibitory Concentration (IC_{50}) histograms were plotted over time.[129] An IC_{50} is the dose required to reduce the measured response by 50%; here, reducing the NCI. In order to have sufficient power in quantifying IC_{50} histograms over time, four replicates were used per plate, and three separate plate replicates were tested (n = 3). IC_{50} histograms were calculated through an enrichment factor scale (toxicity / volume) to assess environmental relevance, and on a mg scale (toxicity / mass of extract) to assess toxic potency.

To generate the IC_{50} histograms, the concentration (× or mg) was log transformed and normalized from 0% to 100%, where 100% is the NCI of the negative control at every time point measured. Next, a nonlinear fit with the formula of 'log (inhibitor) vs normalized response (variable slope)' was used. The generated IC_{50} values at each time point were plotted on an IC_{50} vs. time (h) graph for a total of three replicates to calculate a mean and SEM.

When an IC_{50} was not able to be calculated, yet there was still a degree of cytotoxicity, a point of departure (i.e. threshold), or IC_{10}, analysis was employed. This signifies the point at which an effect begins to be detected and the response deviates from negative control by 10%. A point of departure analysis was also conducted for samples that were able to generate IC_{50}'s for HepG2 cells, so that thresholds could be compared. For this analysis, after transformation and normalization of the data, the IC_{10} was calculated using GraphPad through a 'log(agonist) vs response – Find ECanything' non-linear regression. This regression allowed for the F value to be set to 90 (granted that it is an IC_{10}, not EC_{10} that is needed), and constraints of 0 (bottom) to 100 (top) were implemented to the normalized data.

2.8. Yeast Estrogenic and Androgenic Screen (YES/YAS)

The Yeast Estrogenic Screen (YES) and the Yeast Androgenic Screen (YAS) are part of level 2 of the OECD conceptual framework for testing endocrine disrupting chemicals.[138] The XenoScreen XL YES/YAS kit (Xenometrix, Allschwil, Switzerland) included all the necessary reagents. Here, genetically modified Baker's yeast (*Saccharomyces cerevisiae*) have the human estrogen receptor (hERα) or androgen receptor (hAR), depending on the strain, integrated into the main chromosome.[147] This allows for expression of the hERα and hAR in the cytosol for potential ligands to bind to. The yeast contain a plasmid with the lacZ reporter gene, which encodes the β-galactosidase enzyme, as well as an estrogen (YES) or androgen (YAS) response element. Once a ligand is bound to its receptor, the complex can bind to its respective response element on the plasmid, allowing for expression of the β-galactosidase enzyme. From here, the use of lyticase and a detergent, termed the lacZ reaction mixture, allows for secretion of the enzyme into the extracellular media, catalyzing the reaction of the yellow substrate, chlorophenol

red-β-D-galactopyranoside (CPRG), to its red product. This is quantified with a plate reader as an indicator of the degree of hERα or hAR agonism or antagonism.

YES and YAS strains were grown in flasks with 25mL of growth media for 24 h on an orbital shaker at 100 rpm in a 31°C incubator, and then frozen for future experiments.[147] Frozen aliquots were grown in vented T25 flasks for 48 h before the experiment, with two dilutions performed to ensure exponential growth. For positive control hormones, 100 μL of anhydrous ethanol was used to dissolve 17β-Estradiol (E2), 4-Hydroxytamoxifen (4-HT), 5α-dihydrotestosterone (DHT), and Flutamide (FL). Serial dilution of the positive controls and samples were performed based on **Figure 6**, with a maximum solvent control of 0.67%. The highest dose of positive control were in row H (**Figure 6**), where E2 was set to 6.7e-9 M, 4-HT to 2.7e-6 M, DHT to 6.7e-8 M, FL to 6.7e-9 M, and serial diluted. Agonist media only contained essential nutrients, while antagonist media also contained 3.3e-10 M E2 for YES or 3.3e-9 M DHT for YAS as a baseline level of agonism. For antagonist plates, the ability of the positive control or sample to inhibit this baseline level of agonism was measured.

YES AGONIST

	Agonist Positive Control		F1		F2		W1		W2		Vehicle Control	
	1	2	3	4	5	6	7	8	9	10	11	12
A	E2	E2	0.5x	0.5x	0.5x	0.5x	0.5x	0.5x	0.5x	0.5x	0.67%	0.67%
B	E2	E2	1x	1x	1x	1x	1x	1x	1x	1x	0.67%	0.67%
C	E2	E2	1.75x	1.75x	1.75x	1.75x	1.75x	1.75x	1.75x	1.75x	0.67%	0.67%
D	E2	E2	2.5x	2.5x	2.5x	2.5x	2.5x	2.5x	2.5x	2.5x	0.67%	0.67%
E	E2	E2	5x	5x	5x	5x	5x	5x	5x	5x	0.67%	0.67%
F	E2	E2	7.5x	7.5x	7.5x	7.5x	7.5x	7.5x	7.5x	7.5x	0.67%	0.67%
G	E2	E2	10x	10x	10x	10x	10x	10x	10x	10x	0.67%	0.67%
H	E2	E2	12.5x	12.5x	12.5x	12.5x	12.5x	12.5x	12.5x	12.5x	0.67%	0.67%

YES ANTAGONIST

	Antagonist Positive Control		F1		F2		W1		W2		Agonist Baseline	
	1	2	3	4	5	6	7	8	9	10	11	12
A	HT + E2	HT + E2	0.5x + E2	0.5x + E2	0.5x + E2	0.5x + E2	0.5x + E2	0.5x + E2	0.5x + E2	0.5x + E2	0.67% + E2	0.67% + E2
B	HT + E2	HT + E2	1x + E2	1x + E2	1x + E2	1x + E2	1x + E2	1x + E2	1x + E2	1x + E2	0.67% + E2	0.67% + E2
C	HT + E2	HT + E2	1.75x + E2	1.75x + E2	1.75x + E2	1.75x + E2	1.75x + E2	1.75x + E2	1.75x + E2	1.75x + E2	0.67% + E2	0.67% + E2
D	HT + E2	HT + E2	2.5x + E2	2.5x + E2	2.5x + E2	2.5x + E2	2.5x + E2	2.5x + E2	2.5x + E2	2.5x + E2	0.67% + E2	0.67% + E2
E	HT + E2	HT + E2	5x + E2	5x + E2	5x + E2	5x + E2	5x + E2	5x + E2	5x + E2	5x + E2	0.67% + E2	0.67% + E2
F	HT + E2	HT + E2	7.5x + E2	7.5x + E2	7.5x + E2	7.5x + E2	7.5x + E2	7.5x + E2	7.5x + E2	7.5x + E2	0.67% + E2	0.67% + E2
G	HT + E2	HT + E2	10x + E2	10x + E2	10x + E2	10x + E2	10x + E2	10x + E2	10x + E2	10x + E2	0.67% + E2	0.67% + E2
H	HT + E2	HT + E2	12.5x + E2	12.5x + E2	12.5x + E2	12.5x + E2	12.5x + E2	12.5x + E2	12.5x + E2	12.5x + E2	0.67% + E2	0.67% + E2

YAS AGONIST

	Agonist Positive Control		F1		F2		W1		W2		Vehicle Control	
	1	2	3	4	5	6	7	8	9	10	11	12
A	DHT	DHT	0.5x	0.5x	0.5x	0.5x	0.5x	0.5x	0.5x	0.5x	0.67%	0.67%
B	DHT	DHT	1x	1x	1x	1x	1x	1x	1x	1x	0.67%	0.67%
C	DHT	DHT	1.75x	1.75x	1.75x	1.75x	1.75x	1.75x	1.75x	1.75x	0.67%	0.67%
D	DHT	DHT	2.5x	2.5x	2.5x	2.5x	2.5x	2.5x	2.5x	2.5x	0.67%	0.67%
E	DHT	DHT	5x	5x	5x	5x	5x	5x	5x	5x	0.67%	0.67%
F	DHT	DHT	7.5x	7.5x	7.5x	7.5x	7.5x	7.5x	7.5x	7.5x	0.67%	0.67%
G	DHT	DHT	10x	10x	10x	10x	10x	10x	10x	10x	0.67%	0.67%
H	DHT	DHT	12.5x	12.5x	12.5x	12.5x	12.5x	12.5x	12.5x	12.5x	0.67%	0.67%

YAS Antagonist

	Antagonist Positive Control		F1		F2		W1		W2		Agonist Baseline	
	1	2	3	4	5	6	7	8	9	10	11	12
A	FL + DHT	FL + DHT	0.5x + DHT	0.5x + DHT	0.5x + DHT	0.5x + DHT	0.5x + DHT	0.5x + DHT	0.5x + DHT	0.5x + DHT	0.67%+DHT	0.67%+DHT
B	FL + DHT	FL + DHT	1x + DHT	1x + DHT	1x + DHT	1x + DHT	1x + DHT	1x + DHT	1x + DHT	1x + DHT	0.67%+DHT	0.67%+DHT
C	FL + DHT	FL + DHT	1.75x + DHT	1.75x + DHT	1.75x + DHT	1.75x + DHT	1.75x + DHT	1.75x + DHT	1.75x + DHT	1.75x + DHT	0.67%+DHT	0.67%+DHT
D	FL + DHT	FL + DHT	2.5x + DHT	2.5x + DHT	2.5x + DHT	2.5x + DHT	2.5x + DHT	2.5x + DHT	2.5x + DHT	2.5x + DHT	0.67%+DHT	0.67%+DHT
E	FL + DHT	FL + DHT	5x + DHT	5x + DHT	5x + DHT	5x + DHT	5x + DHT	5x + DHT	5x + DHT	5x + DHT	0.67%+DHT	0.67%+DHT
F	FL + DHT	FL + DHT	7.5x + DHT	7.5x + DHT	7.5x + DHT	7.5x + DHT	7.5x + DHT	7.5x + DHT	7.5x + DHT	7.5x + DHT	0.67%+DHT	0.67%+DHT
G	FL + DHT	FL + DHT	10x + DHT	10x + DHT	10x + DHT	10x + DHT	10x + DHT	10x + DHT	10x + DHT	10x + DHT	0.67%+DHT	0.67%+DHT
H	FL + DHT	FL + DHT	12.5x + DHT	12.5x + DHT	12.5x + DHT	12.5x + DHT	12.5x + DHT	12.5x + DHT	12.5x + DHT	12.5x + DHT	0.67%+DHT	0.67%+DHT

Figure 6: 96-well plate layout for YES agonists, YES antagonists, YAS agonists, and YAS antagonists. Positive controls are in M with serial dilutions made from the highest doses highlighted in row H. Environmental samples are scaled in ×, and solvent in %.

The yeast were incubated with the treatments for 18 h with a gas permeable foil and humidified atmosphere. After incubation, the lacZ reaction mixture was incorporated, followed by 1 h incubation to allow for colour development. For the first trial, the SpectraMax® M3

Microplate reader (Molecular Devices, San Jose, CA) was used in absorbance mode with optical densities (OD) measured at both wavelengths of 570 nm (OD_{570}) and 690 nm (OD_{690}). OD_{690} measures yeast growth and also functions as a correctional value for diffraction when OD_{570} is measured for color development. Growth factor (G) can be calculated via the following equation:

$$G = \frac{A_{690,S}}{A_{690,N}}$$

Where $A_{690,S}$ is the absorbance at 690 nm of the sample (S) before lysis with the lacZ reaction mixture, and $A_{690,N}$ is the same measurement of the solvent control (N). Therefore, the growth of the sample is relative to the solvent control in order to assess cytotoxicity. As sub-lethal doses are needed to assess endocrine activity, this is achieved by excluding doses that reduce the growth factor below 50% of the solvent control.

The second microplate reader measurement is performed after incubation with the lacZ reaction mixture and color has developed. β-galactosidase activity (U_s) is calculated via:

$$U_s = \frac{A_{570,S}}{A_{690,S}}$$

Where $A_{570,S}$ is the difference in absorbance of 570nm – 690nm of the sample (S) after lysis, i.e. the colorimetric value corrected for diffraction from the yeast. U_s is therefore a corrected value relative to the solvent control. Lastly, the Induction Ratio (I_R) is calculated via:

$$I_R = \frac{1}{G} x \frac{A_{570,S}}{A_{570,N}}$$

$A_{570,N}$ is the difference in absorbance of 570nm – 690nm of the solvent control (N) after lysis, i.e. the colorimetric value corrected for diffraction from the yeast. Therefore, the corrected colorimetric value of the sample is relative to the corrected colorimetric value of the solvent control and is inversely correlated with changes in growth.

Xenometrix[147] defines an agonist as when:

$$I_R\ sample \geq 10\%\ (I_R\ agonist\ control, max - I_R\ vehicle\ control)$$

Here, I_R sample is for any of the environmental organic extract treatment groups. I_R agonist control, max, is the maximum I_R of the dose-response curve of a given agonist positive control hormone, be that E2 or DHT (**Figure 6**). I_R vehicle control is only the growth media with 0.67% ethanol (v/v). Xenometrix[147] defines an antagonist as when:

$$I_R\ sample < 50\%\ (I_R\ \text{agonist baseline} - I_R\ vehicle\ control)$$

I_R agonist baseline is the 'negative control' for the antagonist plate, in that it does not contain any environmental sample, but 0.67% ethanol (v/v) and a known amount of 6.7e-9 M E2 or 6.7e-8 M DHT, depending on the assay, as a baseline level of agonism in all wells (**Figure 6**).

The data was analyzed with GraphPad Prism 7 (GraphPad Software, San Diego, CA). Here, the mg/L concentration of the environmental samples and positive controls were log transformed. For the agonist plates, I_R values were normalized from 0% to 100%, where 100% was the maximum I_R of the agonist control and 0% is the vehicle control. For the antagonist plates, I_R values were normalized from 0% to 100%, where 100% was the agonist baseline control and 0% was the vehicle control (**Figure 6**). Next, a non-linear regression was performed with a sigmoidal dose-response formula. The relative EC_{50} or IC_{50} values were calculated and compared to the environmental concentrations to assess their environmental relevance. The equivalence (EQ) factor to the corresponding positive controls for each assay were calculated to assess toxic potency:

$$Positive\ control\ equivalence = \frac{EC_{50\ sample}}{EC_{50\ positive\ control}}$$

Where $EC_{50\ \text{positive control}}$ is based on the dose-response curve of agonist (in the agonist plates) or antagonist (in the antagonist plates) positive control.

For the second trial, a number of changes were made, including switching to a Varioskan® LUX plate reader (Thermo Fisher Scientific, San Jose, CA). Previously, some of the OSPW sample doses were cytotoxic to yeast, not allowing for sub-lethal ER/AR effects to be interpreted. Furthermore, effects that were observed to be sub-lethal only measured the high end of the dose-response curve, preventing the calculation of a point of departure. Therefore, a lower and broader dilution scheme was needed.

Moreover, as only antagonists were detected and no agonists, for the next trial, only antagonists were tested. This provided space on the plate to test eight samples instead of four. These samples included: F1, F2, W1, and W2, as previously tested; and then BML 2013, BML 2017, Pond 9 2016, and River 2017 TAEs. Before running the second YES/YAS assay, a preliminary cytotoxicity test of the new samples was done to ensure that the doses were sub-lethal, ranging from 0.625× to 10× in duplicate with an exposure time from 18 to 46 h. Incorporating the cytotoxicity data, a half logarithmic dilution scheme was used for the second trial, with the cytotoxic samples having the highest dose of 3× and the non-cytotoxic samples increasing to a dose of 10× (**Table 5**). The ratio of solvent to organics remained the same throughout the dilution scheme, where 10× and all solvent controls contained 0.67%.

	BML 2013		
Field dose	1x	75.8	1.88
	Half log scale		
	(X)	mg/L	Log(mg/L)
DOSES	0.001	0.07	-1.14
	0.003	0.23	-0.64
	0.01	0.72	-0.14
	0.03	2.27	0.36
	0.09	7.19	0.86
	0.30	22.7	1.36
	0.95	71.9	1.86
	3.00	227.3	2.36
Dilution for cytotoxic samples	10.0	757.6	2.88
STOCK	1515.0	114776.4	

	BML 2017		
	1x	57.9	1.76
	Half log scale		
	(X)	mg/L	Log(mg/L)
	0.001	0.05	-1.26
	0.003	0.17	-0.76
	0.01	0.55	-0.26
	0.03	1.74	0.24
	0.09	5.49	0.74
	0.30	17.4	1.24
	0.95	54.9	1.74
	3.00	173.6	2.24
	10.0	578.5	2.76
	1515.0	87642.8	

	Pond 9 (2016)		
	1x	38.1	1.58
	Half log scale		
	(X)	mg/L	Log(mg/L)
	0.003	0.12	-0.92
	0.01	0.38	-0.42
	0.03	1.21	0.08
	0.10	3.81	0.58
	0.32	12.1	1.08
	1.00	38.1	1.58
	3.16	120.6	2.08
	10.0	381.2	2.58
	1515.0	57751.8	

	River 2017		
	1x	0.76	-0.12
	Half log scale		
	(X)	mg/L	Log(mg/L)
	0.003	0.00	-2.62
	0.01	0.01	-2.12
	0.03	0.02	-1.62
	0.10	0.08	-1.12
	0.32	0.24	-0.62
	1.00	0.76	-0.12
	3.16	2.40	0.38
	10.0	7.60	0.88
	1515.0	1151.4	

	F1		
Field dose	1x	12.9	1.11
	Half log scale		
	(X)	mg/L	Log(mg/L)
DOSES	0.001	0.01	-1.91
	0.003	0.04	-1.41
	0.01	0.12	-0.91
	0.03	0.39	-0.41
	0.09	1.22	0.09
	0.30	3.87	0.59
	0.95	12.3	1.09
	3.00	38.7	1.59
Dilution for cytotoxic samples	10.0	129.1	2.11
STOCK	1515.0	19558.7	

	F2		
	1x	2.12	0.33
	Half log scale		
	(X)	mg/L	Log(mg/L)
	0.003	0.01	-2.17
	0.01	0.02	-1.67
	0.03	0.07	-1.17
	0.10	0.21	-0.67
	0.32	0.67	-0.17
	1.00	2.12	0.33
	3.16	6.70	0.83
	10.0	21.2	1.33
	1515.0	3211.8	

	W1		
	1x	21.8	1.34
	Half log scale		
	(X)	mg/L	Log(mg/L)
	0.003	0.07	-1.16
	0.01	0.22	-0.66
	0.03	0.69	-0.16
	0.10	2.18	0.34
	0.32	6.91	0.84
	1.00	21.8	1.34
	3.16	69.1	1.84
	10.0	218.4	2.34
	1515.0	33087.6	

	W2		
	1x	1.71	0.23
	Half log scale		
	(X)	mg/L	Log(mg/L)
	0.003	0.01	-2.27
	0.01	0.02	-1.77
	0.03	0.05	-1.27
	0.10	0.17	-0.77
	0.32	0.54	-0.27
	1.00	1.71	0.23
	3.16	5.41	0.73
	10.0	17.1	1.23
	1515.0	2590.7	

Table 5: Final dilution scheme of the second YES/YAS trial of BML 2013, BML 2017, Pond 9 2016, River 2017, and BML 2015 fractions.

However, the modification of the testing scheme to include only antagonistic and not the agonists plates, resulted in the exclusion of the vehicle control. Previously, the vehicle control was the minimum absorbance value, while the 'agonist baseline control,' the control with a known amount of E2 or DHT, is the maximum absorbance value (**Figure 6**). Although the lack of a vehicle control affected the measurement scale, on the basis of the results of the first trial, the vehicle control had the same growth factor as the agonist baseline control, allowing for replacement of the vehicle control growth factor with the agonist baseline control. Furthermore, the agonist baseline control is the most similar control to the samples tested, in that all wells contain ethanol and a set concentration of positive control hormone, with the only changing

independent variable being the environmental sample. Thus, the results in absence of the vehicle control are still insightful for the ranking of endocrine activity of OSPW-impacted samples.

Due to the exclusion of the vehicle control, the formulas for data analysis that were previously used were modified for the second trial. Instead of an Induction Ratio based on the vehicle control, a 'Reduction Ratio (R_R)' was calculated based on the agonist baseline control:

$$R_R = \frac{OD690\ agonist\ baseline}{OD690\ sample} * \frac{net\ absorbance\ sample\ (OD570 - 690)}{net\ absorbance\ agonist\ baseline\ (OD570 - 690)}$$

With the R_R, a positive result is a decrease in induction from the agonist baseline control. Without the vehicle control, Xenometrix's definition of an antagonist is no longer applicable. That being said, the antagonist positive control dose-response is still present to allow for ranking potencies of samples. As such, R_R was normalized with 0% set as the highest dose of the antagonist hormone, FL or 4-HT, where the greatest degree of antagonism is known to occur, while 100% is the lowest dose of FL or 4-HT, which has negligible antagonism. Next, a non-linear regression without constraints was performed with a sigmoidal dose-response formula, with EC_{50} and EQ values calculated.

3. Results

3.1. Analysis of samples by HPLC-Orbitrap

3.1.1. BML 2015 fractions

Based on the heteroatomic chemical class distribution of unextracted BML water from 2015, a broad range of chemical classes were observed (**Figure 7**a). **Figure 7** demonstrates that the fractionation method successful isolated NAs (O_2^-) in Fraction 1 (F1) from the polar non-acids (O^+, O_2^+, SO^+, NO^+) in Fraction 2 (F2). This effectively replicated the chemical composition of the two most acutely toxic fractions in a previous EDA.[51] The first and second wash of the SPE column, Wash 1 (W1) and Wash 2 (W2), contained largely a mixture of more highly oxygenated chemical classes. The SPE waste, which washed the cartridge mainly to remove salts, contained a low abundance of all organic chemical classes. After gravimetric analysis of the dried fractions and washes, the concentration of organics (mg organics/L) was estimated in each fraction based on the volume of BML 2015 sample from which it was derived (**Table 6**). SPE waste was excluded from bioassays due to its minimal abundance of chemicals, with the TOC analysis from a commercial lab (NRAL) resulting in 0.74 mg/L (data not shown).[6] The unextracted BML 2015 sample from which the fractions were generated had a concentration of 40.0 mg/L, also from a TOC analysis (data not shown),[7] while the sum of the tested fractions, F1, F2, W1, and W2, was 38.6 mg/L, resulting in a mass balance and percent recovery of 96.4%.

[6] Provided by Natural Resources Analytical Laboratory (NRAL) at the University of Alberta
[7] Provided by Natural Resources Analytical Laboratory (NRAL) at the University of Alberta

Table 6: Field concentration of BML 2015 organic fractions after SPE fractionation. Calculated from gravimetric dry mass per volume of original BML 2015 water.

	F1	F2	W1	W2
Concentration (mg organics/L)	12.9	2.12	21.8	1.71

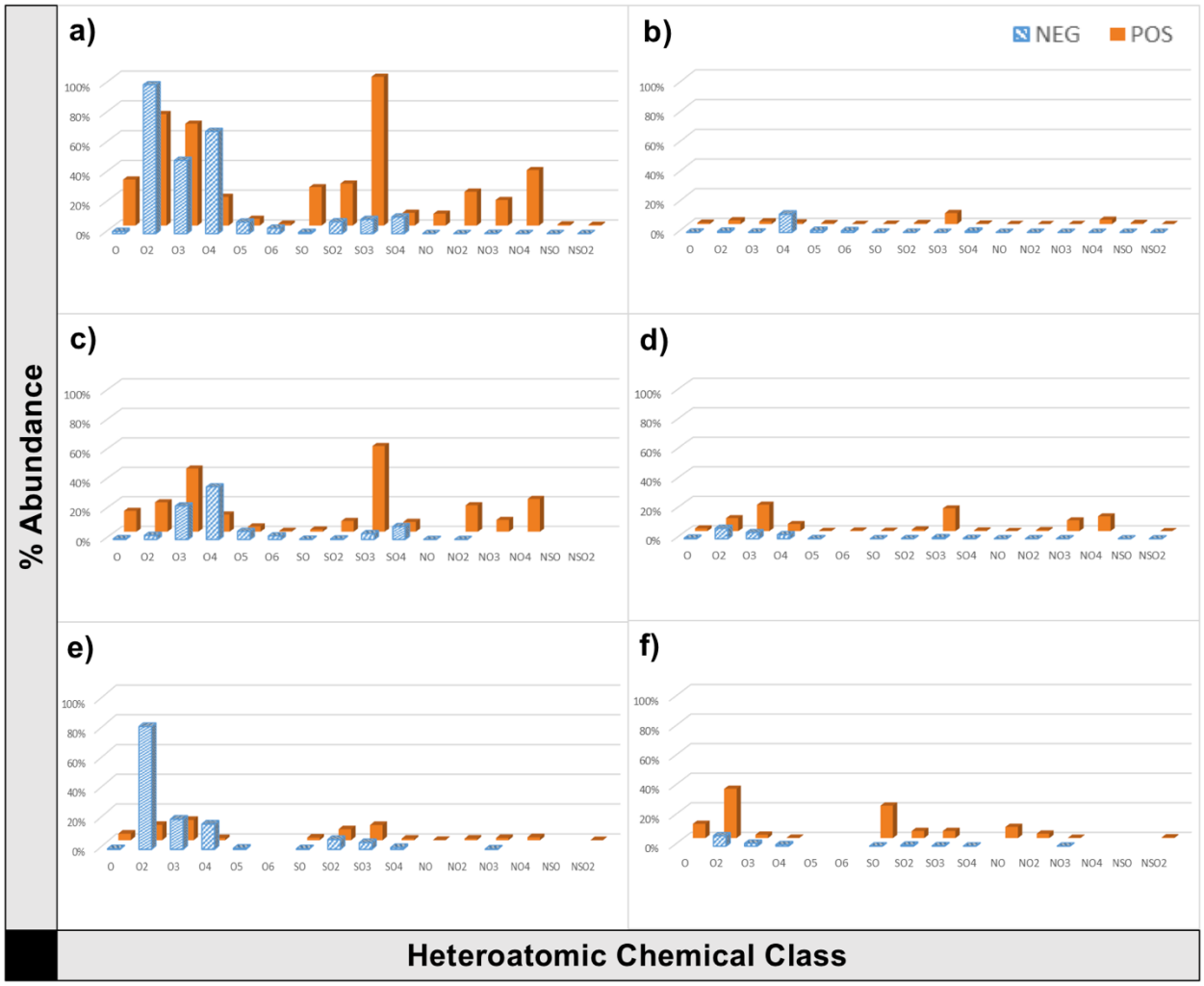

Figure 7: Heteroatomic chemical class distributions of a) unextracted BML 2015, and its fractions: b) SPE waste, c) Wash 1 (W1), d) Wash 2 (W2), e) Fraction 1 (F1), and f) Fraction 2 (F2). Here, signal intensity is normalized to the most abundant chemical class in unextracted BML 2015 depending on the ionization mode, either SO_3^+ or O_2^-.

3.1.1. Total Acid Extracts (TAEs)

After total acid extraction, gravimetric weights of the recovered dried organic masses were recorded (mg) and concentration of organics are reported per volume of water extracted. LC/MS-grade water was used as an extraction blank, and River 2017 was an environmental reference. BML 2013 had the highest concentration of all samples examined (75.8 mg/L), approximately 100-fold higher than the reference sample collected from the Athabasca River (**Table 7**). BML samples collected in later years, and experimental Pond 9, collected in 2016, had lower total organic concentrations that roughly correlated with the number of years aged in the field (**Table 7**). The mg/L concentration of BML 2015 TAE is different here from that of the TOC analysis of unextracted BML 2015 in the SPE fractionation method (section 3.1.1), though this is sensible for the different methodology used.

Table 7: Total acid extract (TAE) field concentrations and years aged for the various samples analyzed. Concentration are calculated by gravimetric dry mass per volume of sample.

	LC/MS WATER	RIVER 2017	BML 2013	BML 2015	BML 2017	POND 9 (2016)
Years Aged	0	0	1	3	5	23
Concentration (mg organics/L)	N/A	0.76	75.8	66.0	57.9	38.1

The organic concentrations based on gravimetric weights are rudimentary for a complex mixture, however, quantitative studies are challenging given the lack of authentic standards for most chemicals in the OSPW organic mixture. As an alternative, total ion intensity by HPLC-Orbitrap was examined to gain insight into the relative concentration of organics in the samples. Similar to gravimetric mass, BML 2013 had the highest total intensity in both positive and

negative mode (**Figure 8**), and intensity decreased with ageing in the other BML and experimental pond samples. Interestingly, the negative mode signal decreased to a greater extent with ageing than the positive mode signal, as seen with the increase percentage of positive mode species from 27% in BML 2013 to 57% in Pond 9 (2016) (**Figure 8**). Total intensity was much lower in the reference River sample, and after blank subtraction the only significant signal was in positive ion mode.

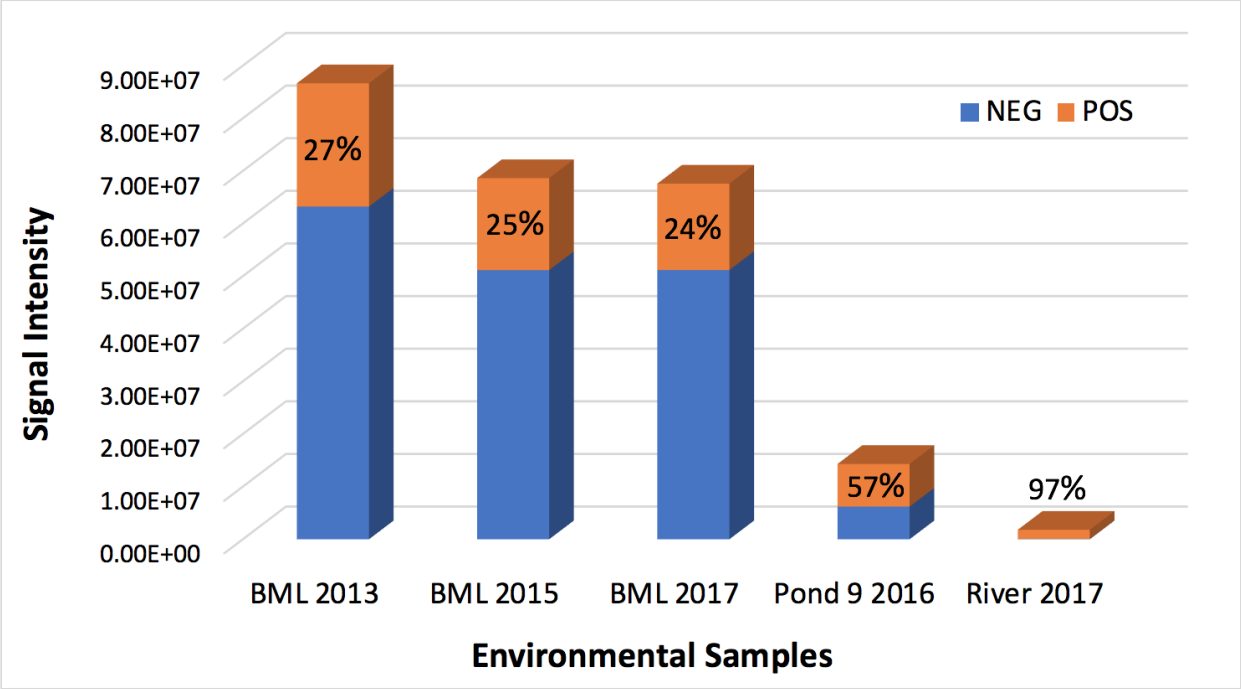

Figure 8: Total HPLC-Orbitrap raw ion intensity in negative (NEG) and positive (POS) ion modes, after blank-subtraction with LC/MS-grade water. The proportion of positive ion mode species to the total signal intensity is indicated by percentages, with the proportion of positive modes species increasing in the aged sample.

Going beyond total ion intensity, chemical class distributions of the samples were also examined. In negative ion mode, there were similar proportions of O_2^- > O_4^- > O_3^- in all BML samples (**Figure 9**a). In Pond 9 (2016), the O_4^- class was most prominent, followed by O_3^- > O_2^-,

though the overall intensity for all of these was lower than in BML (**Figure 9**a). There are minimal differences between the intensities or relative class profiles in BML 2015 and 2017, though the general trend compared to BML 2013 was a decrease in intensity over time, with the exception of the SO_3^- class, which appeared to persist over time. In positive ion mode, there were more significant differences between BML 2015 and 2017, particularly for the SO_3^+ and O_3^+ classes (**Figure 9**b). There was a wider contribution of chemical classes in all OSPW samples to the overall intensity, i.e. O_x^+, SO_x^+, and NO_x^+, though the general trend was again a decrease in intensity in water that had been aged longer in the field. Though again, positive mode classes appear to be less depleted over time compared to negative mode for Pond 9 (2016). Interestingly, SO^+ in the BML samples did not decline with age, and was still present in the very old sample from Pond 9 (2016) (**Figure 9**b).

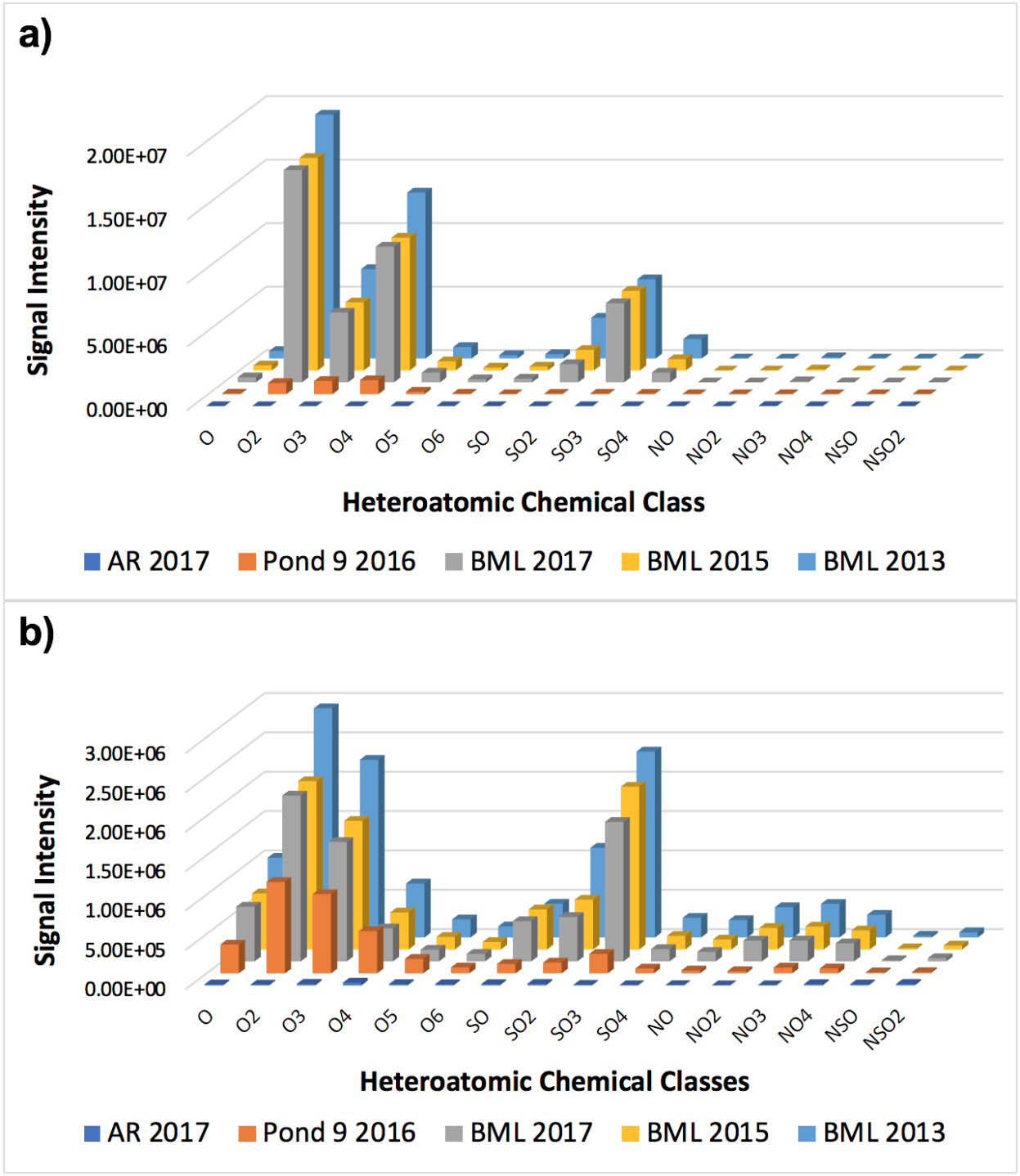

Figure 9: Heteroatomic chemical class distribution of different environmental samples in a) negative and b) positive ionization modes. The signal intensity are the raw values generated for each chemical class selected for in the figure.

To give a more representative of view of each water sample tested, the complete heteroatomic chemical class distributions were also plotted in both positive and chemical ionization classes together (**Figure 10**). The % abundance for each class was relative to the sum of the intensity of selected chemical classes included in the figure, where each ionization mode is separately normalized to 100%. The BML samples had very similar chemical class profiles between years, with $O_2^- > O_4^- > O_3^-$ and $O_2^+ \cong SO_3^+ > O_3^+$ (**Figure 10**b, c, d). This means that the signal intensity and organic mass is decreasing in BML as it ages, but the distribution of selected chemical classes appears static. Pond 9 (2016) had a unique distribution from BML, with $O_4^- > O_3^- > O_2^-$, and $O_2^+ > O_3^+ > O_4^+ > SO_3^+$ (**Figure 10**e). The natural reference from the Athabasca River was also unique, dominated by positive ion mode classes, in particular dominated by the O_4^+ class (**Figure 10**a).

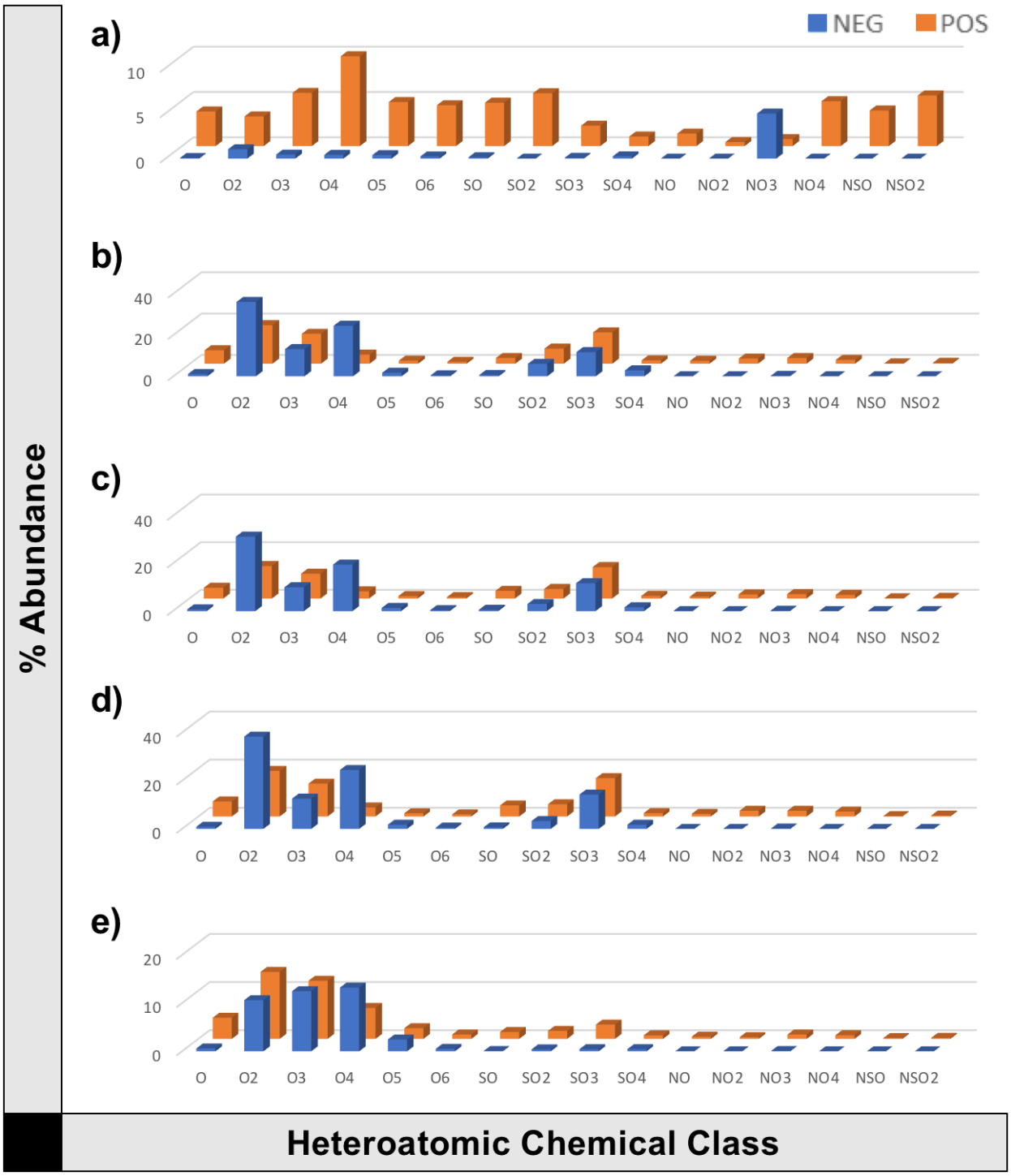

Figure 10: Heteroatomic chemical class distributions of environmental samples: a) River 2017, b) BML 2013, c) BML 2015, d) BML 2017, e) Pond 9 (2016). Blue (NEG) is negative ionization mode and orange (POS) is positive ionization mode. The % Abundance is calculated separately in each mode from the total intensity of selected chemical classes detected, excluding chemical classes that were not selected and signals without an assigned formula.

3.1. RTCA toxicity testing

3.1.1. Unextracted whole OSPW cytotoxicity by RTCA in HepG2 cells

The 100% dose of whole BML 2015 (i.e. unextracted) led to growth inhibition of HepG2 cells (**Figure 11**), however, the effect was small and an IC_{50} could not be determined. The HepG2 cells followed a typical growth pattern and treatment with whole BML 2015 resulted in a dose-response profile, with 4 replicates generating a narrow SEM between doses. To quantify the effect, an IC_{10} point of departure calculation was made, to which a near constant IC_{10} value over time of approximately 50% v/v BML 2015 water was generated (**Figure 12**e).

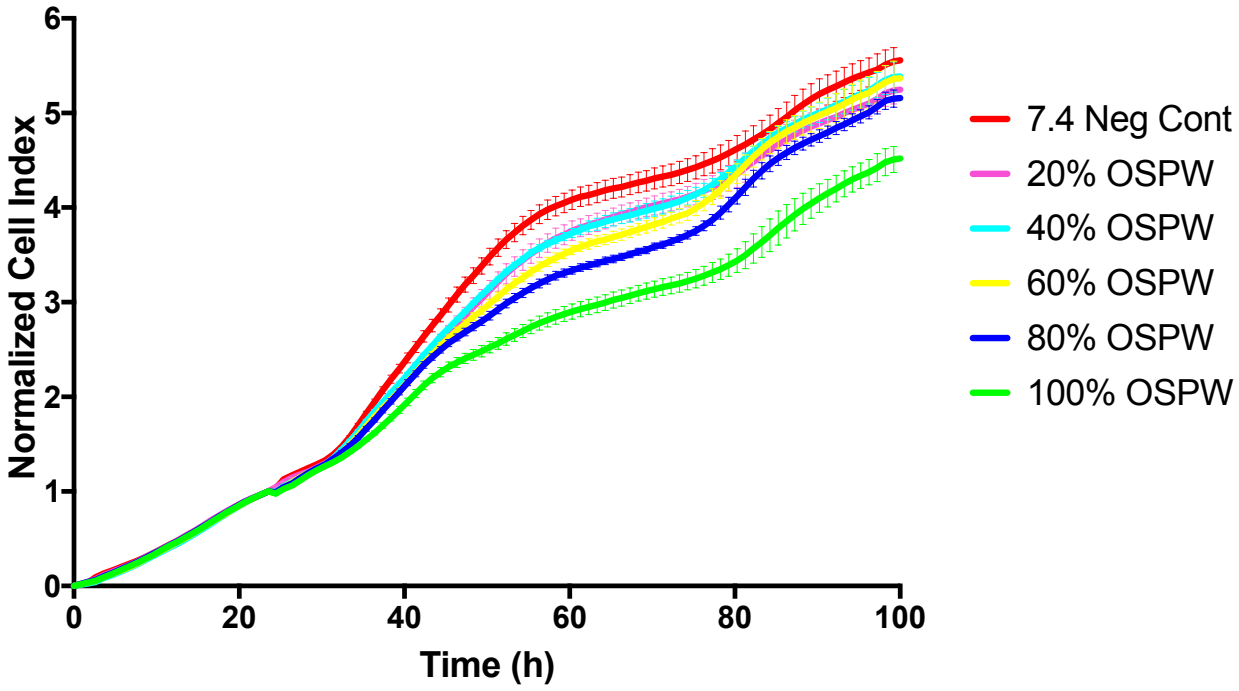

Figure 11: Cell index (CI) over time (normalized at time of cell treatment) for HepG2 cells exposed to whole BML 2015 water determined using RTCA. Powdered cell culture media was reconstituted with filtered whole BML 2015 in order to get a 100% dose with no volume dilution. Values are the mean ± SEM (n = 4 replicates within a plate).

The toxicity in BML 2015 water may be from the combined effect of the organic and inorganic components (e.g. salts and ammonia), or additionally due to the pH of the sample (pH = 8.1, typical for OSPW) which is higher than optimal for cell culture, typically pH = 7.4. As part of a rudimentary toxicity identification experiment, two of these hypothesized contributing factors were removed to look at the effect it had on the RTCA toxicity profiles. Specifically, the pH of whole BML 2015 was adjusted to 7.4, or the water was treated with AC or ENVIcarb to remove most of the organics.

In these experiments (**Figure 12**) the TCRPs had a similar degree of growth inhibition for whole BML 2015 water and all of the toxicity identification treatments. Any difference between treatments only become apparent in the point of departure (IC_{10}) analysis (**Figure 12**e), where AC and pH ameliorates the baseline level of growth inhibition in BML 2015 water at almost all time points. For the ENVIcarb treatment, a point of departure was not able to be calculated until 24 h post exposure, in that there was no growth inhibition. However, when a point of departure was able to be calculated for ENVIcarb, it was initially more cytotoxic than BML 2015 but then is ameliorated at approximately 60 h post exposure. ENVIcarb is planar, and in theory, preferably removes planar organic molecules (e.g. polycyclic aromatics), which may account for the difference compared to AC, which is less selective. With the initial data generated here, RTCA did not seem sufficiently powerful to detect changes in cytotoxicity, so further replicates of the BML 2015 treatments were not performed. Overall, this rudimentary TIE experiments was largely inconclusive. Neither organics nor the pH could explain all the toxicity, and it is likely that the salinity of the sample was responsible for some of the toxicity to HepG2 cells in the current bioassays. Given only the small level of cytotoxicity in the untreated BML sample, and

possible interference by salts and other inorganics, this direct approach to toxicity testing of OSPW was deemed ineffective.

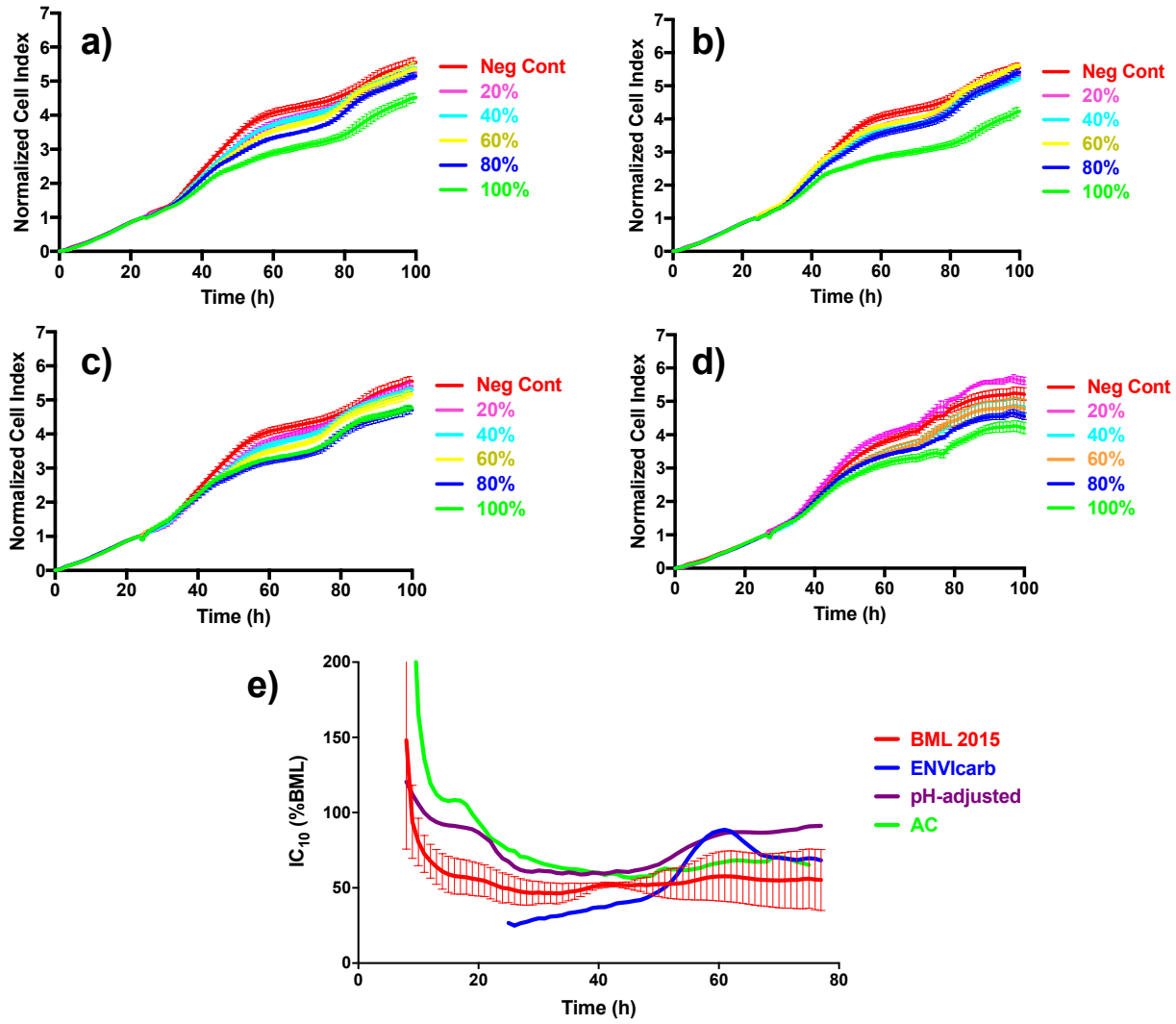

Figure 12: Toxicity identification analysis with HepG2 cells and RTCA after exposure to a) BML 2015 and its treatments: b) pH adjustment to 7.4, c) ENVIcarb treatment stripping the organics, and d) Activated Carbon treatment also stripping the organics. Values are the mean ± SEM (n = 4 replicates within a plate), indicating deviation for all treatments from negative control (Neg Cont). In e), the point of departure, or IC_{10}, is calculated across time points, where 0 h is the point of treatment. Only BML 2015 was tested in three plate replicates (mean ± SEM), while the toxicity identification treatments overall did not appear sufficiently different to warrant further replicates.

The large body of literature already attributing toxicity of OSPW to the organic mixture warranted further investigation of toxicity attribution for the organic components in the current bioassay.[15,84,106,154] Such information may guide the safe remediation and release of OSPW in the future, and the organics are the major concern for the regulators and the industry.

3.1.2. Solvent vehicle and quality control experiments

In order to test the organic extracts of OSPW by RTCA, the type and % of solvent first needed to be tested with HepG2 cells. Many toxicity studies testing OSPW organic extracts use ethanol as a solvent control.[43,51,100] A range of doses of anhydrous ethanol (100% purity) were tested to find thresholds of cytotoxicity to HepG2 cells. The percent of solvent needed to be sufficiently high to dissolve the OSPW organics and deliver them to cell culture media, but low enough not to induce cytotoxicity. Cytotoxicity became apparent at a dose of 1% v/v ethanol (**Figure 13**c). A lower dose of 0.2% ethanol showed no apparent effect, whereas a slightly higher dose of 0.3% ethanol showed some evidence for enhanced proliferation. Therefore, a final ethanol solvent composition of 0.25% was chosen to maximize solubility of the extracts. In the same experiment, two doses of As (III) were tested as positive controls for cytotoxicity, and 250 µM was selected for further experiments due to the rapid cell death induced at this dose (**Figure 13**a).

A blank TAE was then performed with Optima® LC/MS Water to test for background toxicity that might be introduced from impurities or contamination during the extraction. There was no cytotoxic effect from the extraction blank (**Figure 13**b). A relevant environmental water sample from the Athabasca River was also tested for background toxicity, and was not cytotoxic to HepG2 cells, even at doses up to 12.5× its field concentration (**Figure 13**a).

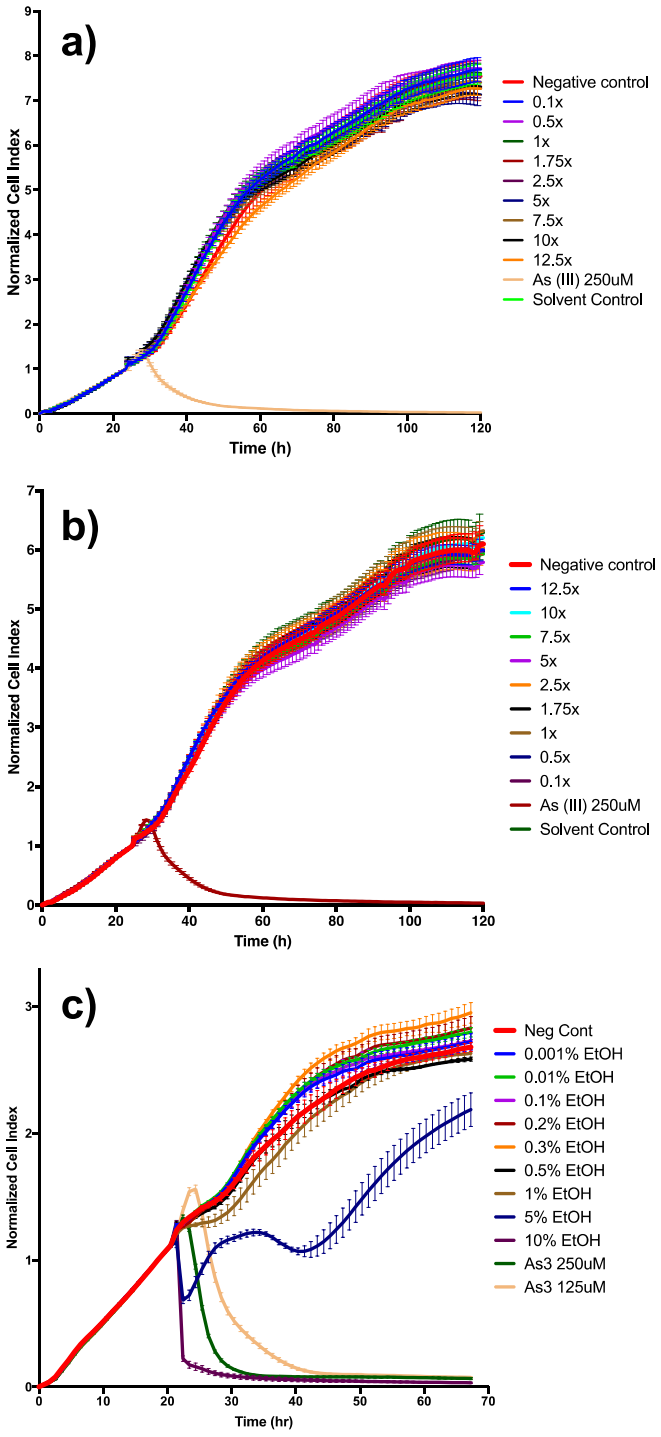

Figure 13: TCRP of HepG2 cells after treatment with the TAE of a) River 2017 and b) LC/MS grade water, and with c) anhydrous ethanol (EtOH), and arsenite (As3). Values are the mean ± SEM (n = 4 replicates within a plate),

3.1.3. Evaluating environmental TAEs of different ages by RTCA

Using TAEs of environmental OSPW samples of different ages, their relative cytotoxicity was tested by RTCA with HepG2 cells. As described above, BML 2013 was the least aged sample and had the highest gravimetric concentration of organics. Here, BML 2013 was the most cytotoxic sample, displaying dose-response cytotoxicity between 1.75× and 12.5× enrichment factors (**Figure 14**a). The SEM of 4 replicates at each dose were quite narrow, thereby allowing visual inspection of significant differences between the doses, and more quantitative analysis performed with IC_{50} and IC_{10} calculations. The 10× dose appeared to flat-line the cell growth from time of exposure, whereas the 12.5× dose likely killed the HepG2 cells, indicated by NCI falling below 1 and eventually falling to 0, similar to the As (III) positive control.

The toxicity and dose-response of BML 2015 (**Figure 14**b) was similar to BML 2013, but the highest dose (12.5×) did not kill the cells, but caused cell growth to stop, suggesting it as slightly less toxic than BML 2013. Notably, at the environmentally relevant 1× dose of BML 2015 organics, the SEM overlapped with the negative control, suggesting no significant differences at this dose. This is consistent with the whole BML 2015 water toxicity identification analysis in **Figure 12**, where removal of the organics marginally increased the point of departure value, though there was not sufficient power to know if this effect was significant. BML 2017 was even less cytotoxic than BML 2015 and BML 2013, with a steady dose-response leading to the highest dose of 12.5× almost flat-lining cell growth, though proliferation was still possible (**Figure 14**c).

Pond 9 (2016) displayed a unique biphasic response compared to all the BML samples, whereby after 24 h of exposure it was cytotoxic, but the cells appeared to recover with exposure time to an asymptote by approximately 60 h (**Figure 14**d). Pond 9 (2016) had been aged 23 yrs at the time of collection, and may predict what BML water could look like in the future.

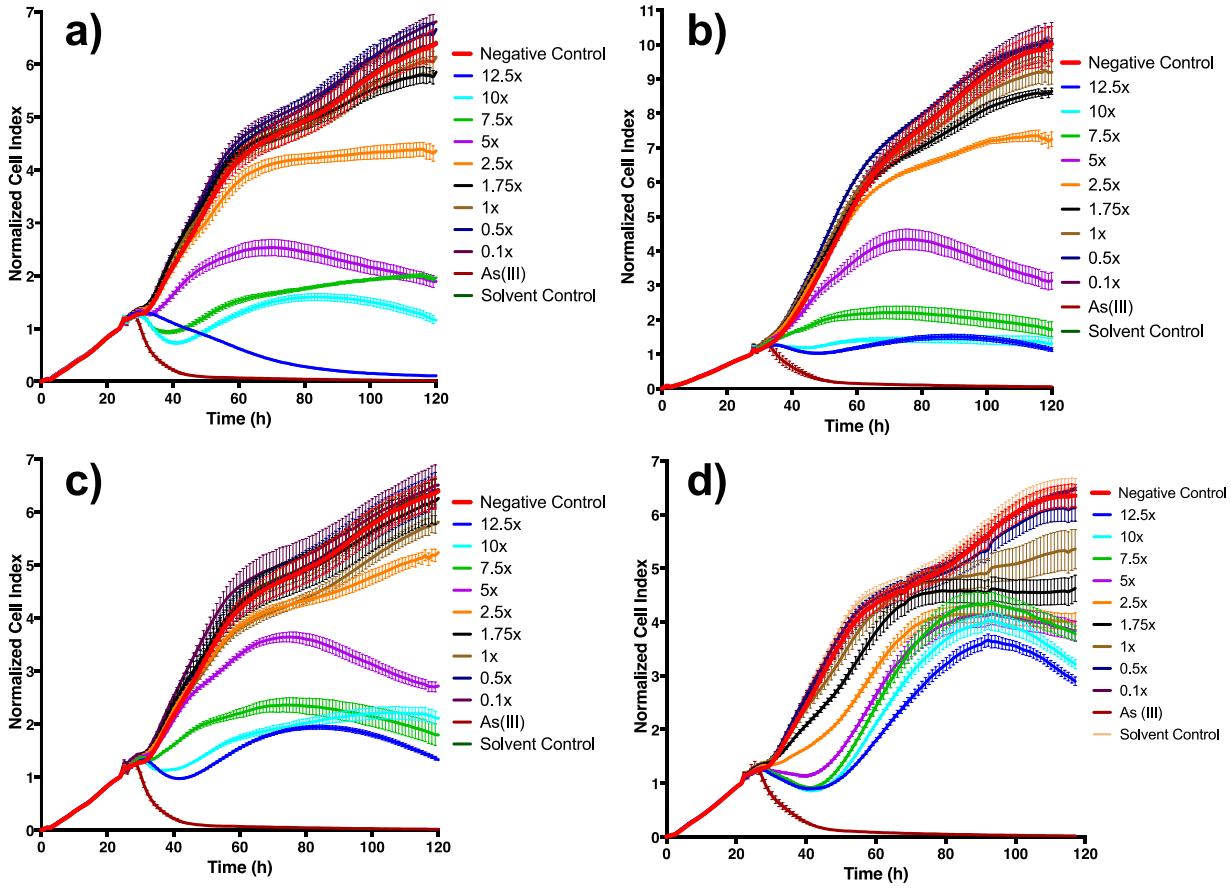

Figure 14: TCRPs of HepG2 cells after exposure to the TAE of a) BML 2013, b) BML 2015, c) BML 2017, and d) Pond 9 2016. Values are the mean ± SEM (n = 4 replicates in a plate).

To quantify and compare the different TCRPs for each sample, 50% inhibitory concentration (IC_{50}) histograms were made. IC_{50} is defined as the concentration of an inhibitor required to reduce cell growth by half; lower IC_{50}'s indicate a more potent inhibitor of cell growth. The measured response for each dose in each sample was compared to the negative control at every time point and averaged across three replicate plates to generate a SEM. In IC_{50} histograms, 0 h is the point of treatment of environmental samples.

BML samples all demonstrated a decrease of the IC_{50} over exposure time (**Figure 15**), with BML 2013 having the lowest value and therefore being the most potent. For BML, cytotoxicity

decreased progressively with the age of the environmental samples, i.e. to BML 2015 and BML 2017. Overall, the acute IC_{50} values for BML samples ranged from 6× to 10×, which is well above concentrations found in the field. On the other hand, Pond 9 (2016) displayed a unique IC_{50} profile, whereby after 24 h this water is more potent than all of the BML samples, yet by 50 h the water is less potent and less toxic than any BML sample. For Pond 9 (2016), the subsequent decrease of IC_{50} between 60 and 70 h is likely due to lack of nutrients remaining in the cell culture media or cell senescence. Up to around the first 10 h of exposure, IC_{50} values are extrapolated from the data, as the highest treatment dose is 12.5×. Moreover, at this time the cells incur inherent disturbances due to changes in cell treatment media. Therefore, less accuracy is associated with the first ~10 h post exposure.

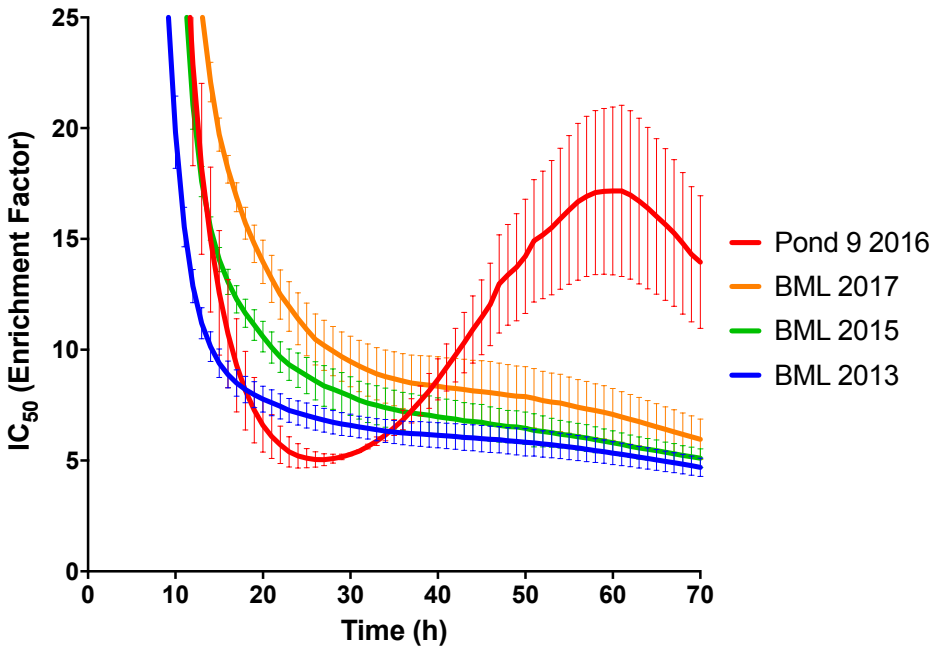

Figure 15: Temporal IC_{50} histogram with an enrichment factor (×) scale of OSPW samples of different ages towards HepG2 cells. 0 h is the point of treatment of environmental samples. Values are the mean ± SEM (n = 3) of plate replicates.

While the enrichment factor-scale is an effective means at evaluating environmental relevance of any toxic response (i.e. 1× is the concentration in the field), it is not useful for benchmarking the absolute toxic potency, typically expressed as mass of substance per unit volume (i.e. mg/L). Therefore, to compare the toxic potency of organics in each sample, enrichment factor was transformed to mg/L based on the gravimetric concentrations (**Table 7**).

All differences in toxicity described above between BML 2013, BML 2015, and BML 2017 on the enrichment factor scale, disappear when plotted on the mg/L scale (**Figure 16**). In other words, while the toxicity of the various BML water samples differs, the toxic potency of the dissolved organics in each sample is identical. Pond 9 (2016) showed the same biphasic profile after converting to mg/L, however the relative potency at 24 h is even more pronounced compared to BML samples. Some IC_{50} (mg/L) values for Pond 9 (2016) after 60 h were not able to be calculated by GraphPad (**Figure 16**), thus, all 3 replicates at that time were excluded.

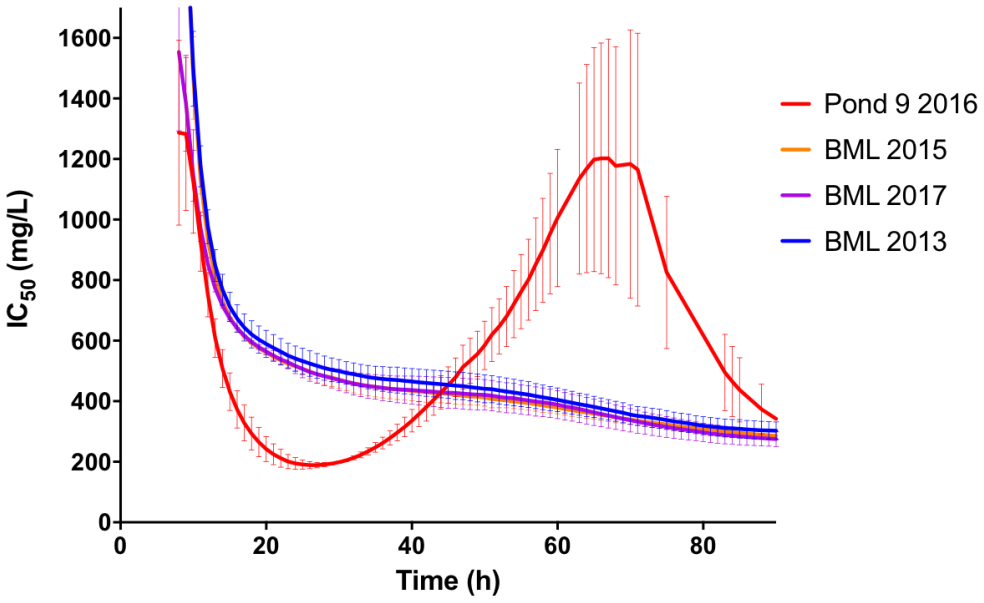

Figure 16: Temporal IC_{50} histogram with mg/L scale for OSPW samples of different ages. 0 h is the point of treatment of environmental samples. Values are the mean ± SEM (n = 3) of plate replicates.

Given the result of the toxicity identification experiment with whole BML 2015 (**Figure 12**), and the finding here that the SEM of the 1× dose of BML organics overlapped with negative control, a more confirmatory point of departure, or IC_{10}, analysis was performed, again, at each time point for all BML TAE samples (**Figure 17**). In these experiments, the point of departure approached but did not pass the field concentration of BML 2013 (75.8 mg/L), BML 2015 (66.0 mg/L), and BML 2017 (57.9 mg/L) after 100 h of exposure. However, it is important to note that at 100 h of exposure other factors such as lack of nutrients in the 96-well plate or cell senescence could contribute to an adverse effect, so IC values generally hold greater validity at earlier exposure times, such as less than 80 h. No matter, overall this suggests a lack of effect of the organics at 1×, and that only just above this point would a cytotoxic effect become observable. Interestingly again, the point of departure is not changing with ageing of BML samples, further supporting that toxic potency of the remaining organics is not changing (**Figure 17**).

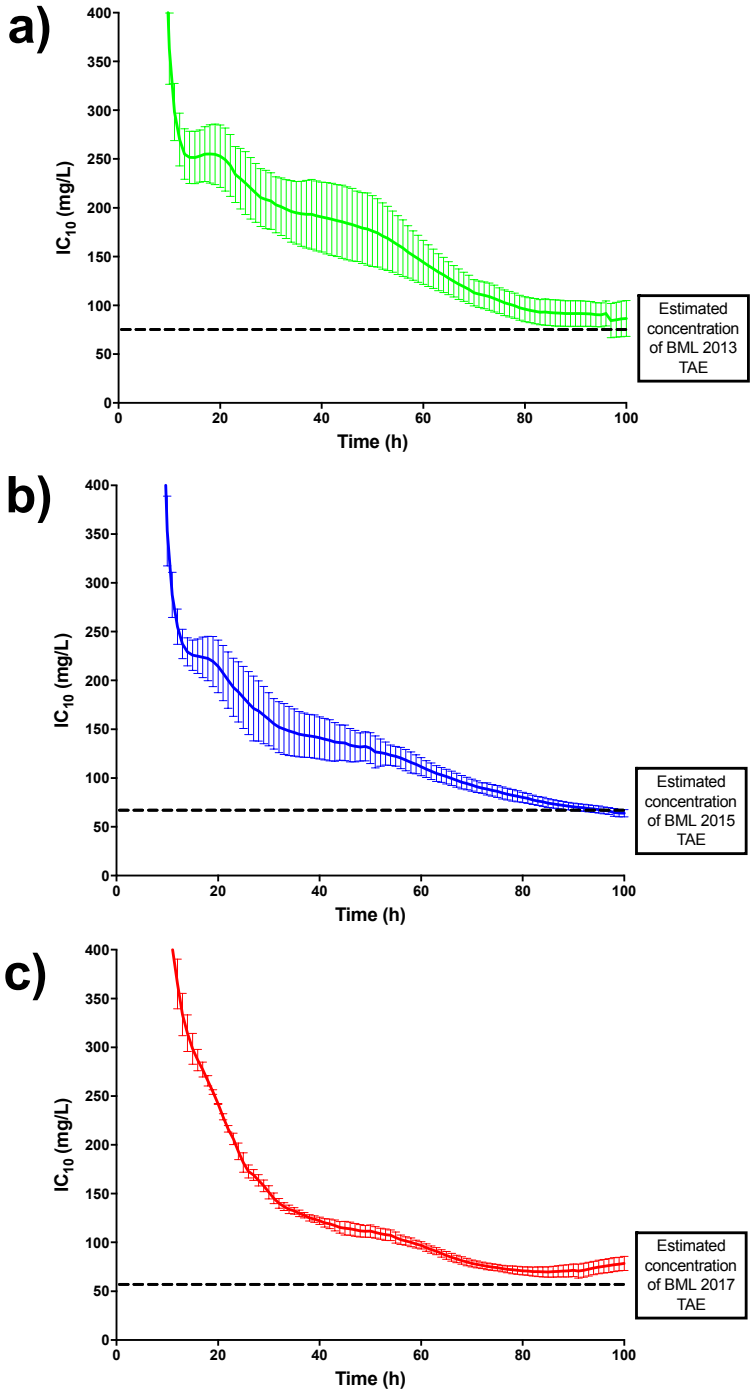

Figure 17: Point of departure (IC_{10}) over time of the TAEs of a) BML 2013, b) BML 2015, and c) BML 2017, with their corresponding field concentration indicated by the dotted line. 0 h is the point of treatment of environmental samples. Values are the mean ± SEM (n = 3) of plate replicates.

3.1.4. Attributing cytotoxicity by RTCA to certain chemical classes in BML 2015

The two fractions (F1, F2), along with the two washes (W1, W2) obtained through SPE fractionation of BML 2015 were tested by RTCA in HepG2 cells to identify chemical classes of concern. F1, containing predominantly NAs, was the most cytotoxic fraction, showing a clear dose-response, and at the highest dose (12.5×) the cell growth flat-lined (**Figure 18**), similar to unfractionated BML 2015 TAE (**Figure 14**b). The results for F2 and W1 were difficult to interpret because of enhanced proliferation compared to negative control, possibly a stress response in HepG2 cells, but evidence for cytotoxicity was generally lacking. W2 had a minor cytotoxic response, but not enough for an IC_{50} to be calculated.

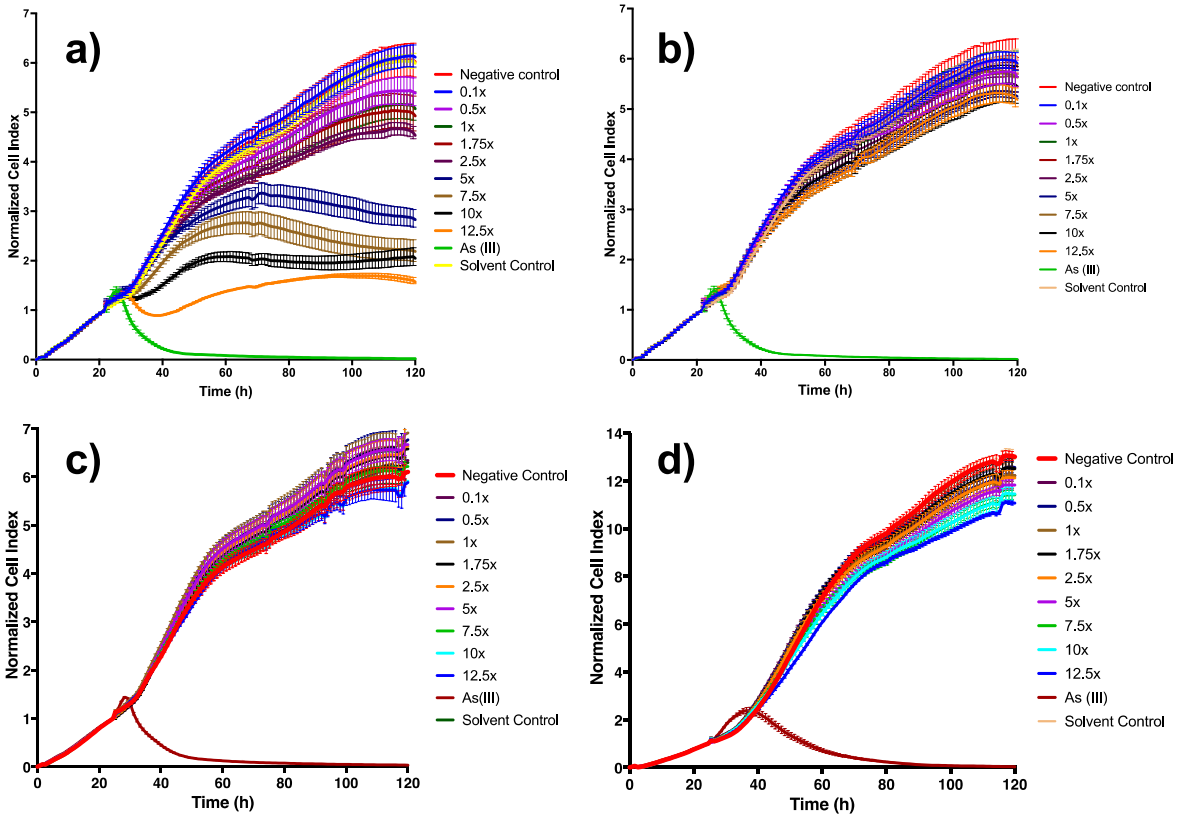

Figure 18: TCRPs of BML 2015 fractions in RTCA with HepG2 cells. a) F1 contains NAs and was the most cytotoxic fraction, b) F2 contains largely polar non-acids, and c) W1 and d) W2 are mixtures of chemical classes. Values are the mean ± SEM (n = 4 of replicates in a plate).

To assess toxicity attribution within the BML 2015 organic mixture, IC_{50} histograms of BML 2015 TAE and its fractions were compared. F1 was the only fraction where an IC_{50} could be calculated, and largely accounted for the cytotoxicity in unfractionated BML 2015 TAE organics (**Figure 19**). The balance of cytotoxicity is likely explained by minor cytotoxicity observed in the other fractions, but this could not be accounted for here.

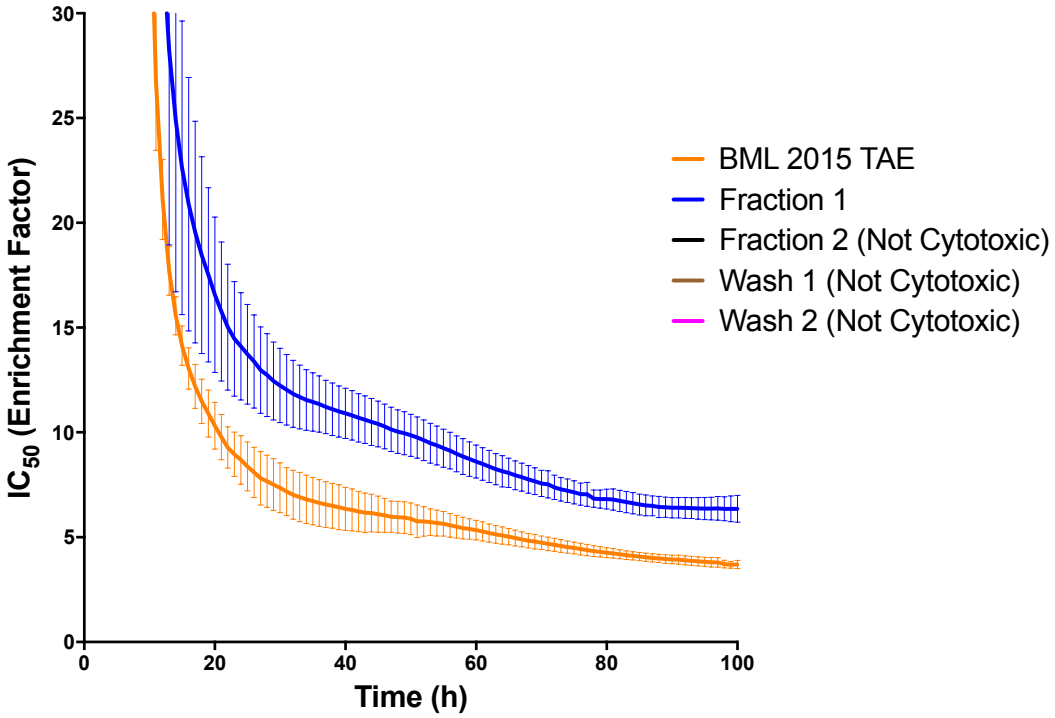

Figure 19: Temporal IC_{50} histogram with an enrichment factor scale (×) of BML 2015 TAE and its fractions towards HepG2 cells. Only F1 was able to generate an IC_{50} and largely accounted for the cytotoxicity seen in BML 2015 TAE. 0 h is the point of treatment of environmental samples. Values are the mean ± SEM (n = 3) of plate replicates.

The IC_{50} histogram in **Figure 19** was based on enrichment factor and evaluated the toxicity of the organics relative to the volume of water that the extract was originally derived from (toxicity / volume). However, it does not assess the toxic potency of the dissolved organics (toxicity / mass). Therefore, in **Figure 20** the scale was normalized to the mass of organics

(toxicity / mass), and this showed how F1 was evidently much more potent than BML 2015 TAE. This is sensible because the cytotoxic chemical class, NAs, is largely isolated from the rest of the organics, which were shown to have lower cytotoxicity.

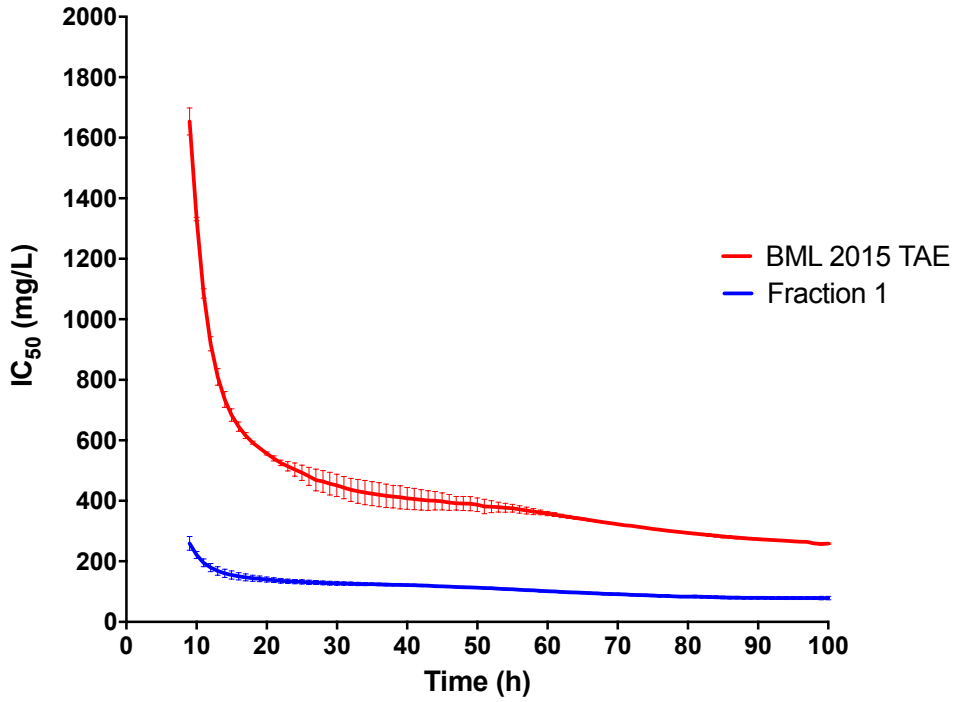

Figure 20: Temporal IC_{50} histogram on a mg/L scale of BML 2015 TAE and the cytotoxic fraction, F1, towards HepG2 cells. Values are the mean ± SEM (n = 3) of plate replicates.

Given that F1 was by far the most cytotoxic fraction in BML 2015, the IC_{10} over time was calculated as a rudimentary estimation of the point of departure (**Figure 21**). The IC_{10} approached the relevant field concentration of the F1 chemical classes in BML 2015 (12.9 mg/L), but came to a plateau at ~17 mg/L. This value will later be compared to the estimated point of departure for sub-lethal endocrine activity. The point of departure toxicity profile of F1 is similar to that of BML 2015 TAE (**Figure 17**c).

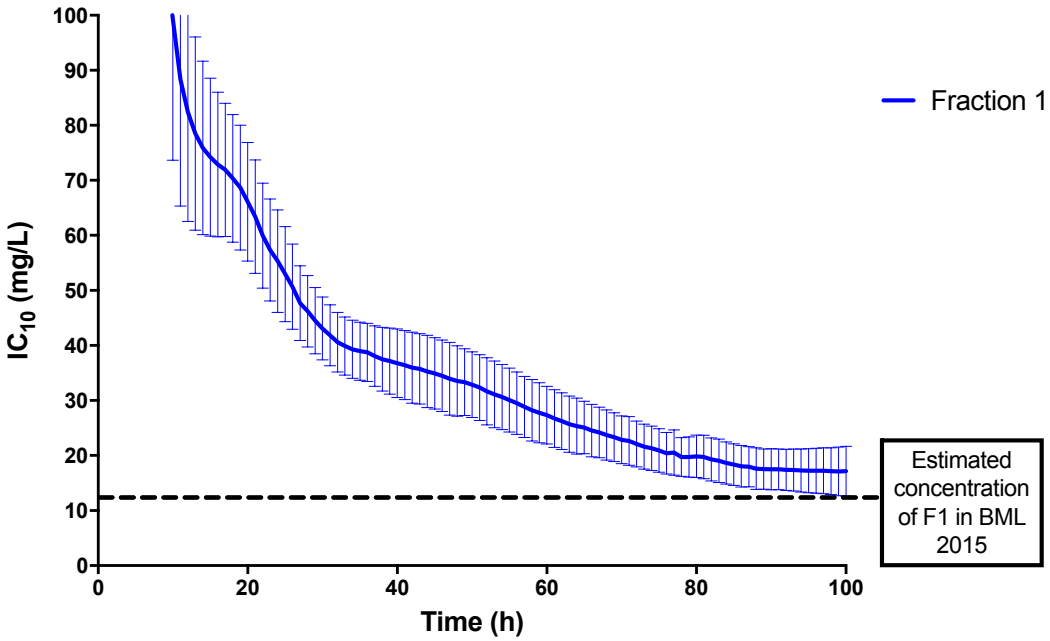

Figure 21: Point of departure over time for the F1 fraction by RTCA in HepG2 cells. Values are the mean ± SEM (n = 3) of plate replicates, and the estimated field concentration of F1 is indicated by the dotted line.

3.1. Yeast Estrogen and Androgen Screening (YES/YAS)

3.1.1. Attributing agonism and antagonism to BML 2015 fractions

Part of the YES/YAS assay incorporates a measurement of cytotoxicity (growth factor) because it is important that non-cytotoxic doses are used for accurate interpretation of the colorimetric response. Xenometrix sets growth factor criteria based on comparison of the optical density in the sample relative to solvent control, with the ratio not to drop below 50%. Using the YES antagonist experiment as an example (**Figure 22**a), F1 was cytotoxic at ~2×, F2 was cytotoxic at ~10×, while W1 and W2 were not cytotoxic. Similarly, using the YAS antagonist experiment as an example (**Figure 22**b), F1 was cytotoxic at doses greater than ~3×, F2 was not cytotoxic below 12.5×, while W1 and W2 were not cytotoxic at any dose. Doses that lead to cytotoxicity were excluded when measuring ER and AR interactions here.

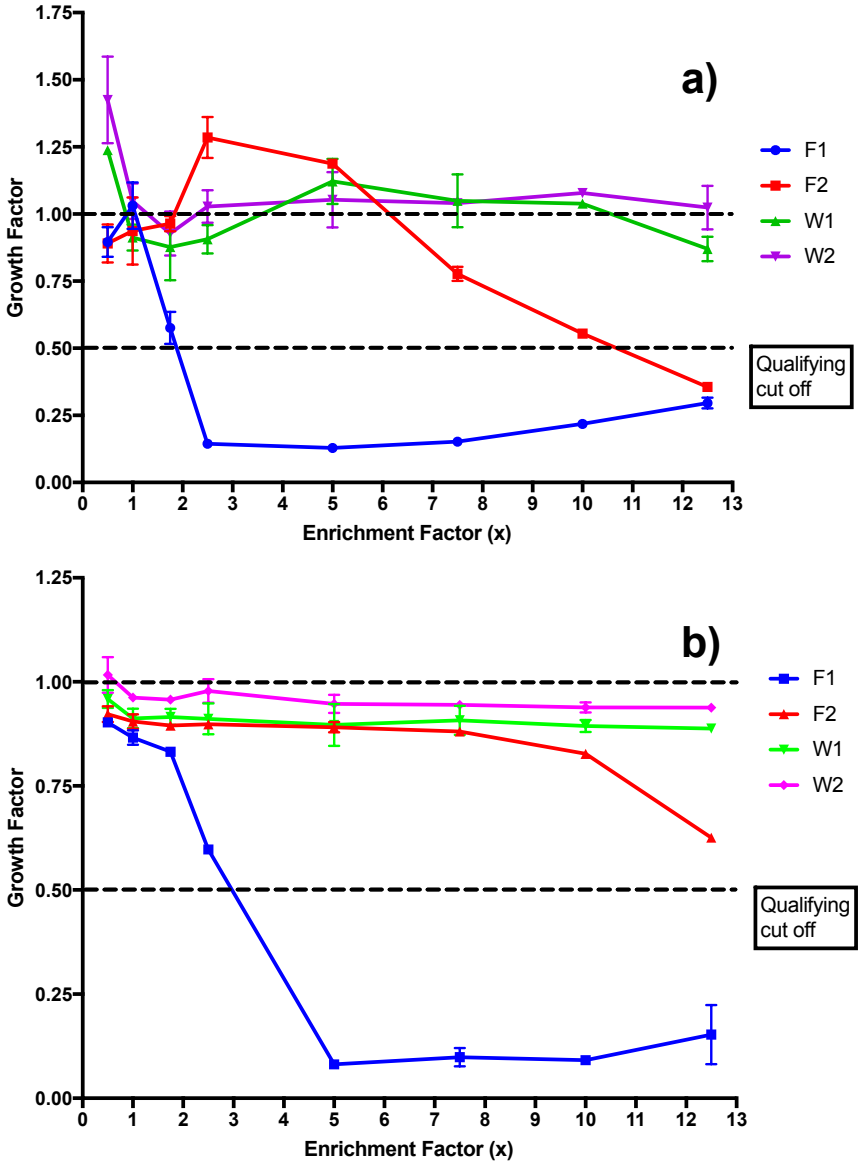

Figure 22: Growth during antagonist experiment after treatment with BML 2015 fractions for a) YES and b) YAS strains. Plate reader measured OD_{690} after 18 h of exposure. 1.00 = the solvent control growth factor. Values are the mean ± SEM (n = 2 in the plate). Cytotoxicity is categorized by Xenometrix as when the growth factor is < 50% of the solvent control and this is indicated by the dotted line at 0.50.

To identify receptor agonists in the YES/YAS assay, induction ratios were log transformed, normalized (100% = max positive control induction ratio, 0% = solvent control), and a sigmoidal dose-response curve was applied. The agonist criterion set by Xenometrix is that Induction >10%

is indicative of agonism; therefore, no estrogen or androgen agonists were detected in any BML 2015 fractions through YES (**Figure 23**a) or YAS strains (**Figure 23**b), respectively.

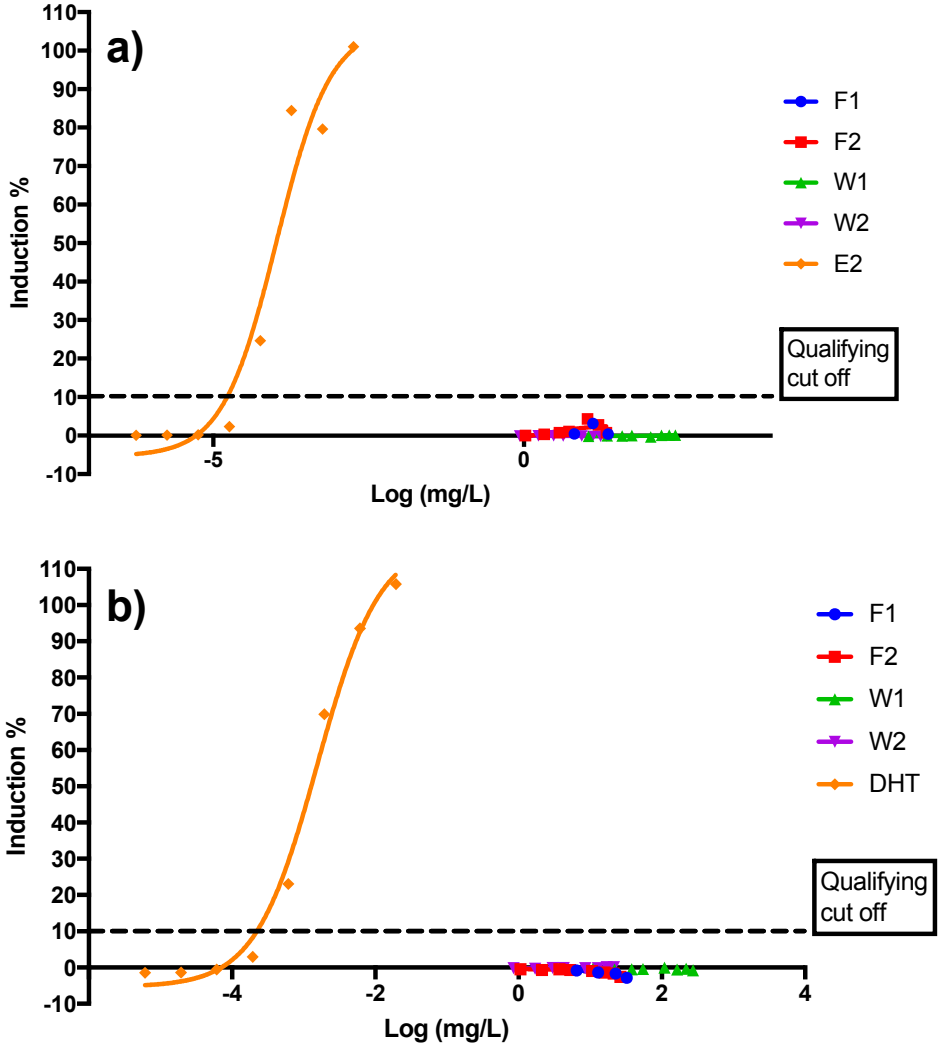

Figure 23: No agonists present in BML 2015 fractions in the a) YES or b) YAS strain. Xenometrix criterion for agonism was indicated by the dashed line. Each treatment dose was run in duplicate, though raw values were averaged earlier in the analysis process of the Xenometrix workbook, not allowing for error to be carried through to the final figures.

To screen for antagonists, the assays are repeated with fixed concentrations of positive control hormones, either E2 or DHT, in the test media of all wells. Positive results are indicated

by a decrease in Induction %, and the Xenometrix criterion for antagonism is when Induction is less than 50%. As such, ER antagonists were present throughout all BML 2015 fractions (**Figure 24**a). The cytotoxic doses of F1 were excluded, leaving only three data points, which were too few to generate a sigmoidal function, but the three available data points were nevertheless formally indicative of ER antagonist. The highest dose of F2 was also removed due to cytotoxicity, but the remaining lower doses were sub-lethal and allowed for the generation of a sigmoidal function. W1 and W2 were less potent and were able to generate full dose-response curves, with upper and lower plateaus starting to become observable (**Figure 24**a). This suggesting that ER antagonists were present through the fractions at different potencies, with F1 and F2 being the most potent.

In the YAS assay, antagonists were also present throughout the BML 2015 fractions (**Figure 24**b). The highest doses of F1 were excluded because of cytotoxicity, but four non-cytotoxic doses displayed antagonism and allowed for a sigmoidal function to be applied. Similar to the YES antagonist experiment (**Figure 24**a), F1 and F2 were the most potent, with W1 and W2 lower in potency. However, W1 seems to have greater YAS antagonism than YES antagonism, while W2 has YES antagonism, but barely passed the cut-off for YAS antagonism.

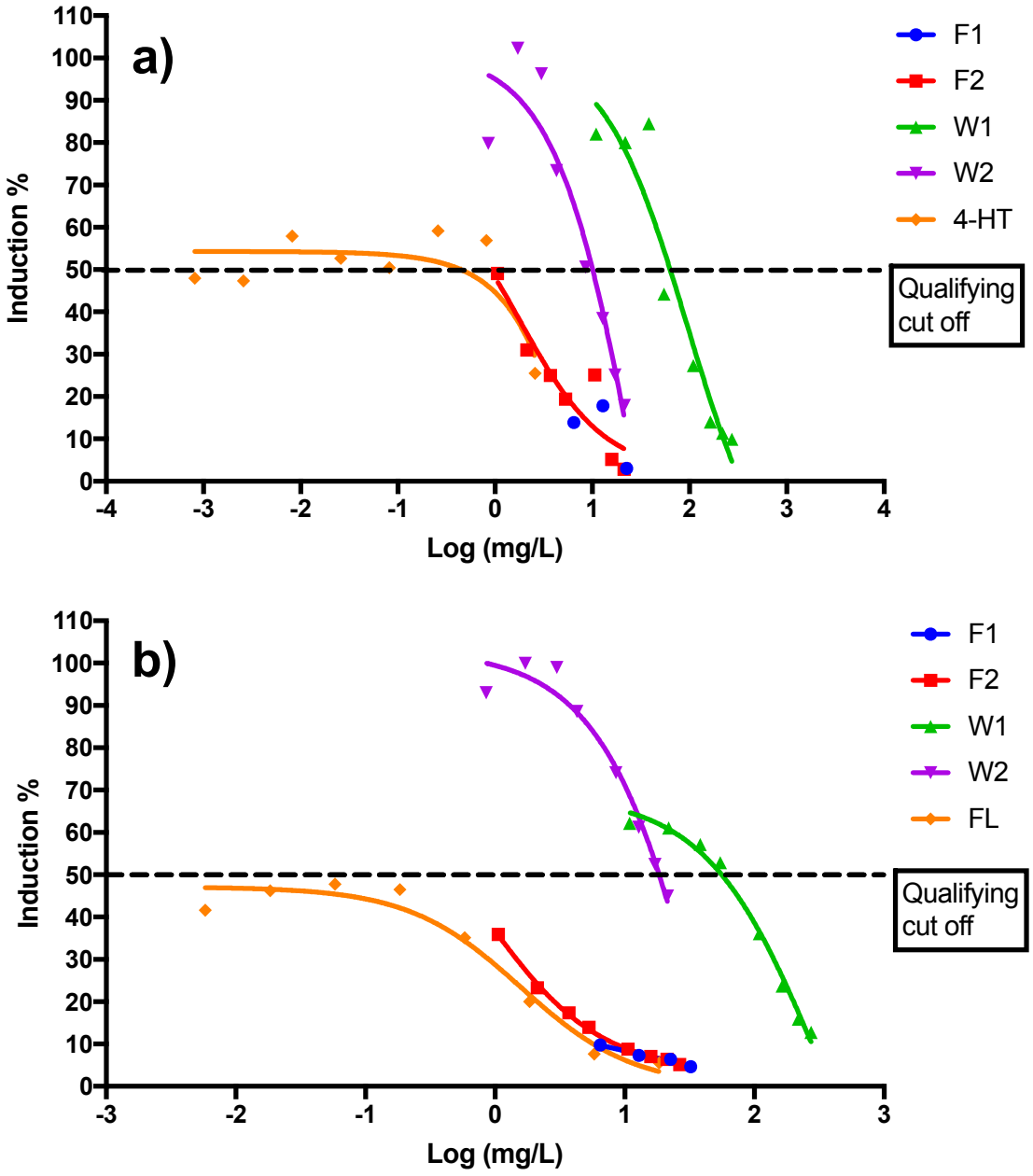

Figure 24: a) YES antagonists and b) YAS antagonists were present throughout BML 2015 fractions. Xenometrix's criterion of antagonism is indicated by the dotted line (<50%). 4-HT was a known antagonist of the ER, and FL was a known antagonist of the AR. Each treatment dose was run in duplicate, though raw values were averaged earlier in the analysis process of the provided Xenometrix workbook, not allowing for error to be carried through to the final figures.

In both YES and YAS, F1 and F2 appeared to be of similar potency to the positive control hormones: 4-HT for ER antagonism and FL for AR antagonism (**Figure 24**). However, the curves generated for all of the BML 2015 fractions were incomplete, with doses only revealing the high-end of the dose-response relationship, particularly for the most bioactive fractions, F1 and F2. Therefore, quantitative analysis was held off until the YES and YAS antagonist assays were run again at a wider and lower range of doses.

In the next trial, lessons learned were incorporated with a change in methodology, as defined in section 2.8. Also, only antagonism was screened for, as there were no agonists found. The maximum sub-lethal dose of F1 was 3×, while F2, W1, and W2 were able to go up to 10× and still be sub-lethal. In this trial, complete dose-response curves were generated with upper and lower plateaus, allowing for accurate EC_{50} measurements and for the data to be analyzed in both a mg organics/L scale for toxic potency and an enrichment factor scale for environmental relevance (**Figure 25**). On a mg/L scale, F1 and F2 were similar in potency and are both more potent than the positive control hormones for YAS antagonism (**Figure 25**a) and YES antagonism (**Figure 25**c). On an enrichment factor scale, F1 and F2 were more divergent in terms of their YAS antagonism (**Figure 25**b) and YES antagonism (**Figure 25**d), with F1 becoming comparably slightly more potent, indicating greater environmental relevance. W1 and W2 only have an effect at their highest doses and were both less potent than the positive control antagonistic hormones (**Figure 25**a, c). W2 appears slightly more potent than W1 on a mg/L scale, though it is of equal environmental relevance for YAS antagonism (**Figure 25**b). W1 appears more environmentally relevant than W2 for YES antagonism (**Figure 25**d).

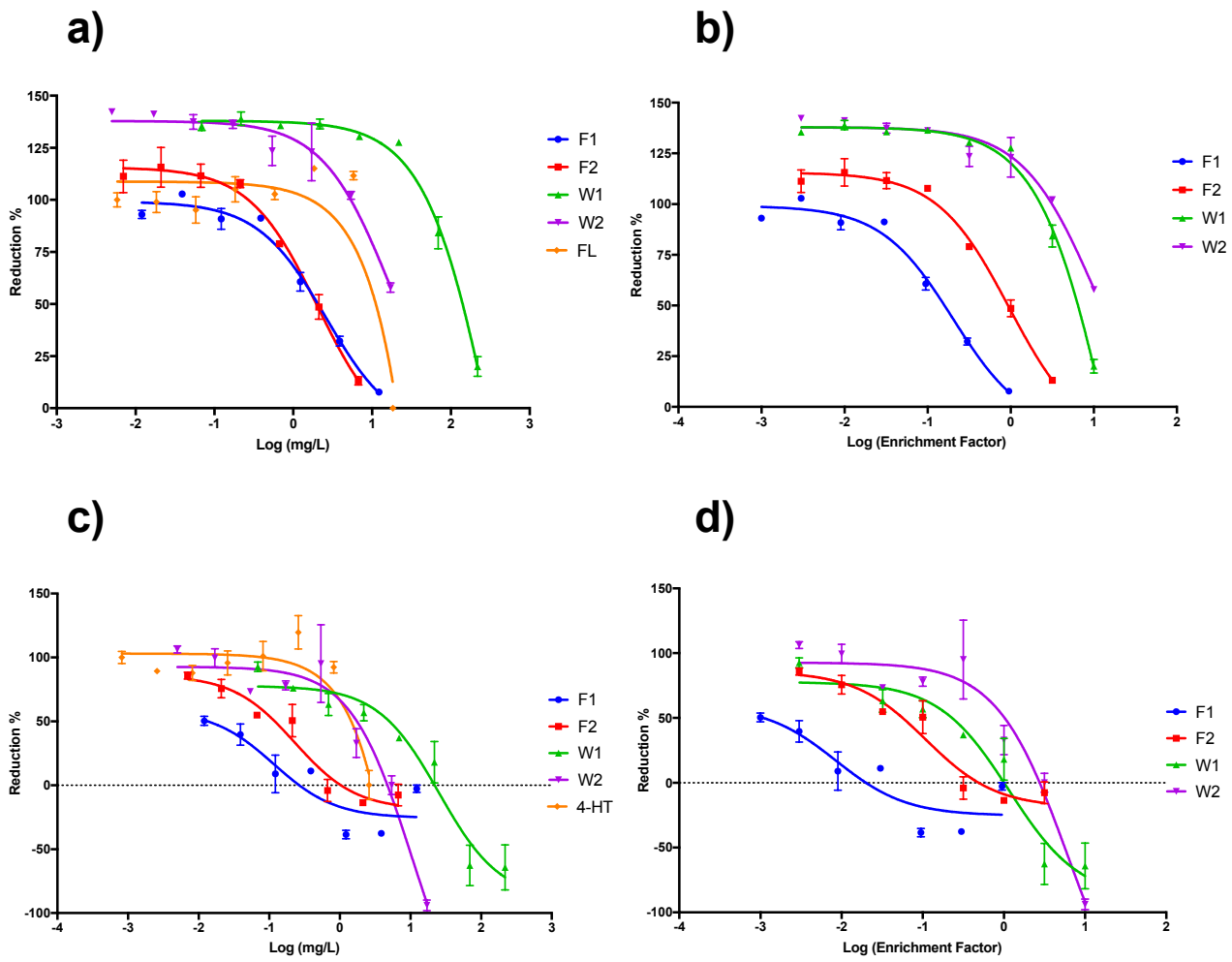

Figure 25: Trial #2 of YAS antagonism on a) a mg/L scale and b) an enrichment factor scale, as well as YES antagonism on c) a mg/L scale and d) an enrichment factor scale. Reduction % was normalized to the antagonistic positive control for the assay, where 0% was the highest dose and 100% was the lowest dose. Only on the mg/L scale can the fractions be compared to the antagonistic positive control of 4-HT for YES antagonism and FL for YAS antagonism. With the new approach in analysis, values were the mean ± SEM (n = 2 replicates within a plate).

With the full dose-response curves generated, a quantitative approach could now be taken, comparing the BML 2015 fraction EC_{50} and EC_{10} values to rank fractions in terms of potency, as well as allow for comparison of the fractions to antagonistic positive control hormones to determine equivalency (EQ). Here, an EQ value <1 means the sample was more potent than the positive control hormone, and > 1 means the sample was less potent.

However, there were issues with the EC_{50} calculations for the positive control antagonistic hormones needed to calculate EQ. Even though the dilution scheme of the positive controls were set by the company, in the first trial the lowest doses of the positive controls appeared to still be potent inhibitors compared to negative control, i.e. ~50% induction (**Figure 24**). Moreover, GraphPad was largely not able to calculate an accurate EC_{50} for the experimental 4-HT or FL treatment in the first or second YES/YAS trials.

Fortunately, the Xenometrix Workbook provided a commonly fitted value for the antagonistic hormone controls, in order to compare the experimental and theoretical values. Given the challenges, I resorted to using a 'theoretical 4-HT' EC_{50} value and 'theoretical FL' EC_{50} value. This has an inherent limitation in that the theoretical values are not generated from the same experimental conditions, but it is a start to allow for some comparison of toxic potency. Interestingly, for FL, GraphPad was able to experimentally define its EC_{50} at 1.6 mg/L, which was quite similar to the Xenometrix's theoretical EC_{50} of 2.91 mg/L, so using the theoretical values may not be too different from the experimental values. However, to remain consistent between YES and YAS, both 'theoretical EC_{50} values' of 4-HT and FL were used to determine equivalency (EQ) of the BML 2015 fractions.

With that said, EC_{50} values of the BML 2015 fractions from the second trial were compared to their field concentrations in BML 2015 to assess environmental relevance and compared to antagonistic control hormones to asses toxic potency (**Table 8**). F1, W1, and W2 were all more potent YES antagonists than YAS antagonists based on their EC_{50} values, while F2 was marginally a more potent YAS antagonist (**Table 8**). The YES and YAS antagonists were distributed amongst all the fractions, indicating that the fractionation technique did not isolate the endocrine active species into a single fraction. Without the vehicle control, categorizing these

samples as antagonist based on Xenometrix's criteria (i.e. reducing the IR of max solvent – vehicle control by 50%) was not possible (discussed in section 2.8), though compared to positive antagonistic controls, it was clear that there was a similar antagonistic response.

More specifically, F2 was the most potent AR antagonist, with an EC_{50} of 2.04 mg/L, which was slightly below its field concentration in BML 2015, and has a FL-EQ of 0.70 (**Table 8**). F1 was the most potent ER antagonist, with an EC_{50} of 0.11 mg/L, which was far below its field concentration in BML 2015, and has a 4-HT EQ of 0.09 (**Table 8**). Overall, F1 and F2 were the most potent and were the only fractions that had FL and 4-HT EQ values below 1, suggesting successful isolation of the most potent chemical classes in these two fractions. W1 had an EC_{50} above BML 2015 field concentrations, though its EC_{10} was below (**Table 8**).

The point of departure for YES and YAS antagonism of F1, F2, and W1 are all far below their estimated concentration, while W2 generated ambiguous values, suggesting less environmental relevance (**Table 8**). With the degree of isolation and separation achieved in the SPE fractionation, this allowed for generating novel toxicological information for particular chemical classes, specifically: assigning a threshold for which ER or AR antagonists effects begin to be detected below controls. F1 had a YES antagonism threshold of 0.019 mg/L and YAS antagonism threshold of 0.36 mg/L, while F2 had an YES antagonism threshold of 0.043 mg/L and YAS antagonism threshold of 0.24 mg/L.

3.1.2. Evaluating ageing through antagonism of environmental TAEs

Lessons learned from the first YES/YAS trial with BML 2015 fractions were extended here, with knowledge that no agonists were detected even in isolated fractions and that a wider and lower dilution scheme is needed for sublethal doses. To ensure that the new environmental

samples tested were sublethal, an initial cytotoxicity test with the YES strain was run; the YES strain was more sensitive than the YAS strain in the first trial (**Figure 22**). The new samples tested were BML 2013, BML 2017, Pond 9 (2016), and River 2017. Here, a range of doses were used to find the dose that decreased the growth factor of negative control by 50%, as set by Xenometrix.

BML 2013 and BML 2017 had similar cytotoxicity, causing ~50% reduction in growth factor at the dose of 5× after 18 h post exposure (**Figure 26**a). When tested with a longer exposure time of 40 and 46 h though, yeast treated with the highest dose (10×) of BML 2017 recovered, while yeast in BML 2013 did not (**Figure 26**b). Thus, BML 2013 was the most cytotoxic, and keeping the highest dose of BML samples at or below 3× should be sufficient for assessing sub-lethal effects in the YES/YAS antagonism assay. For Pond 9 (2016) and River 2017 there was minor cytotoxicity that was very close to the negative control, even up to 10× and over prolonged time as well (though River 2017 was not tested over time). Interestingly, while HepG2 cells treated with Pond 9 (2016) was cytotoxic and had a biphasic response with prolonged exposure time by RTCA (**Figure 15**), with yeast Pond 9 (2016) had a marginal effect, and the biphasic response was observed in BML 2017 (**Figure 26**b).

In **Figure 26**a, the ethanol treatment contained the corresponding dose of solvent at each dose of TAE, up to 0.67% in 10×. Therefore, ethanol did not affect yeast growth at any dose tested here. Based on this initial cytotoxicity experiment, the maximum dose for the cytotoxic samples (i.e. BML 2013 and BML 2017) was 3×, while for the non-cytotoxic samples (i.e. River 2017 and Pond 9 (2016)) it was 10×.

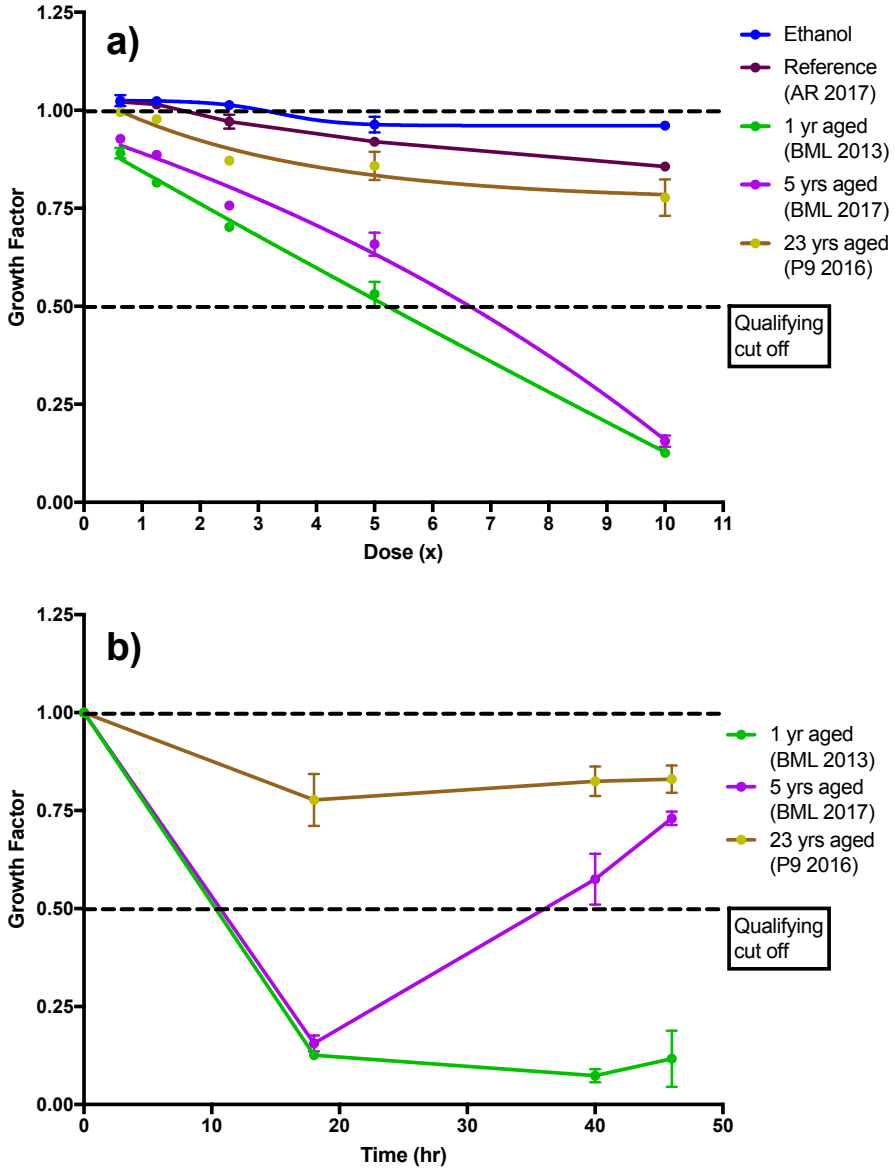

Figure 26: Growth of YES strain after treatments with TAEs of environmental samples a) over a range of doses, and b) monitoring the 10× dose over time. Values are the mean ± SEM (n = 2 replicates in a plate).

In **Figure 27**, data were plotted by enrichment factor (×) in the right panel to rank samples by environmentally relevant concentrations, and in the left panel the mg/L scale was used to compare all treatments to antagonistic positive controls. In evaluating ageing as a remediation strategy, the antagonistic effect of environmental OSPW samples of different ages were

contrasted. They were also compared to a reference sample of Athabasca River water, upstream of the oil sands industry. In this trial, full dose-response curves were generated with upper and lower plateaus (**Figure 27**). In both YES and YAS, there was a clear distinction between OSPW-impacted samples and the reference River 2017 sample, in that OSPW-impacted samples had a greater antagonistic effect and were effective at much lower doses (**Figure 27**). Secondly, on both enrichment factor (×) and mg/L scales, there were hardly any differences in the YES and YAS antagonistic effect between OSPW samples that were aged 1 year (BML 2013), 5 years (BML 2017), or 23 years (Pond 9). As a reminder, all of these doses are sub-lethal, so the BML samples had the highest dose of 1× (3× was excluded due to cytotoxicity), while Pond 9 went up to 3× (10× was excluded due to cytotoxicity). Interestingly, when comparing Pond 9 (2016) at 1× to the BML samples at 1×, Pond 9 (2016), even as the oldest sample, had the highest efficacy for YES antagonism (**Figure 27**d).

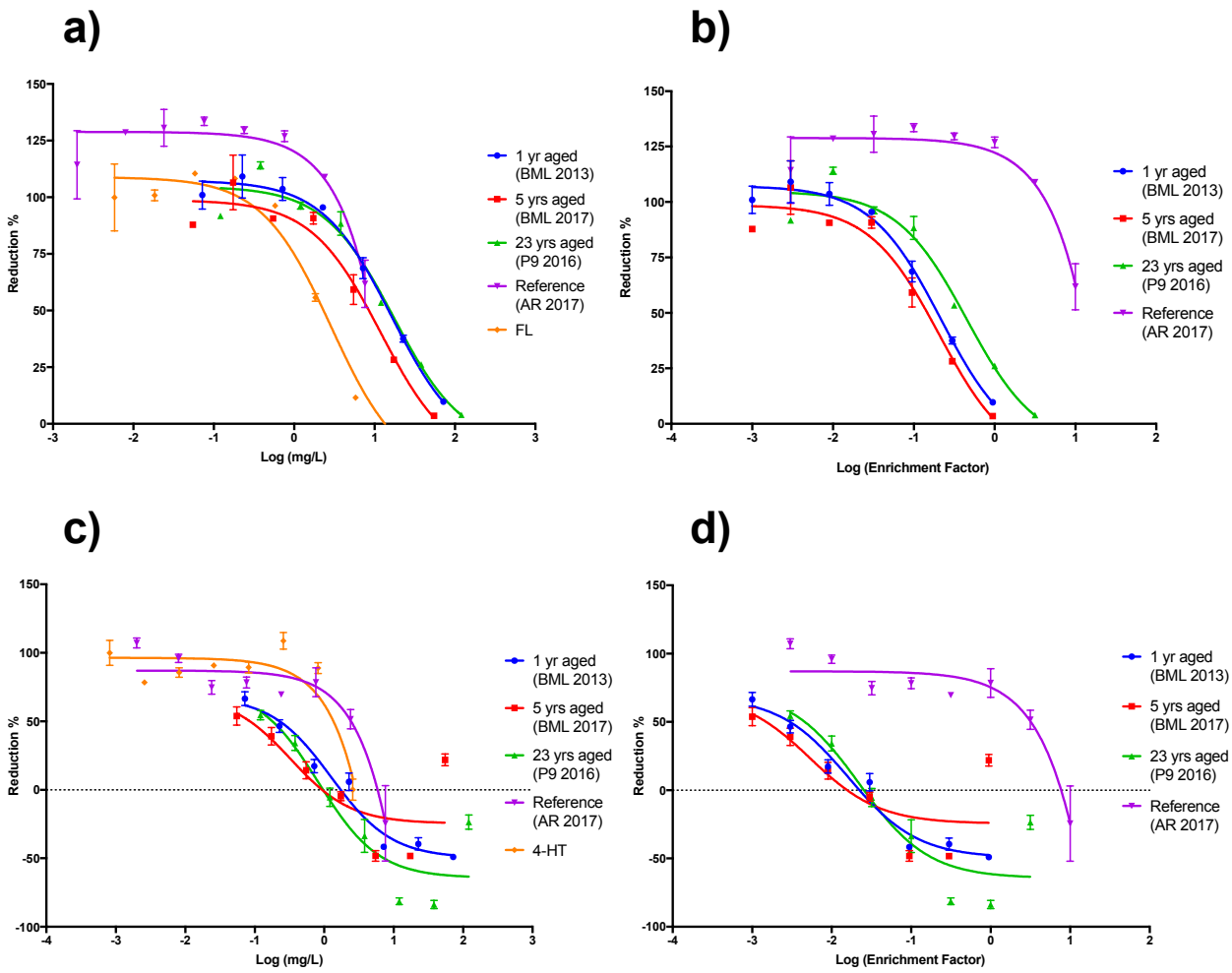

Figure 27: YAS antagonism on a) a mg/L scale and b) enrichment factor scale, as well as YES antagonism on c) a mg/L scale and d) enrichment factor scale. Reduction % was normalized to the antagonistic positive control for the assay, where 0% was the highest dose and 100% was the lowest dose. Only on the mg/L scale can the TAEs be compared to the positive control hormone of 4-HT for YES antagonism and FL for YAS antagonism. With the new approach in analysis, values were the mean ± SEM (n = 2 replicates in a plate).

While there were marginal differences among OSPW-impacted samples visually, quantitative calculations allowed for the ranking of potency (mg/L). For YES antagonism, the high to low potency decreased as follows: F1, F2, BML 2017, Pond 9 (2016), BML 2013, W2, W1, and River 2017 (**Table 8**). For YAS antagonism, the high to low potency decreased as follows: F2, F1, BML 2017, BML 2013, W2, Pond 9 (2016) = W1, and River 2017 (**Table 8**). It was

interesting that BML 2017 was consistently more potent than BML 2013, and also that the endocrine active fractions, F1 and F2, were consistently more potent than the OSPW TAEs. Even though there was minor variability between the BML and Pond 9 samples, the EC_{50} values overall were quite similar, which is surprising considering the amount of ageing between samples and the differences in chemical profiles (**Figure 9**). Also, all of the River 2017 calculations were ambiguous and far above field concentrations, showing a clear divergence in effect for all OSPW-impacted samples.

More specifically, the EC_{50} values for ER and AR antagonism of BML 2013, BML 2017, and Pond 9 (2016) were all below their field concentrations (**Table 8**). As discussed, the experimental 4-HT and FL EC_{50} values were largely ambiguous again, so the theoretical EC_{50} values were taken from Xenometrix's workbook to determine equivalence (EQ). Here, BML 2017 and Pond 9 (2016) were both more potent on a mg/L scale than 4-HT, indicated by EQ values below 1, while BML 2013 had a 4-HT of 1.01. For YAS antagonism, none of the TAEs were below 1 for FL EQ equivalence, and only the BML 2015 fractions, F1 and F2, were below 1. Overall, this highlights isolation of the most potent chemical classes in F1 and F2 for YAS antagonism, as well as for YES antagonism, though the potency of TAEs of OSPW-impacted samples are close behind. The points of departure were based on EC_{10} estimations, and considering that all of the OSPW-impacted samples had EC_{50}'s below field concentrations, their corresponding EC_{10}'s were also all below field concentrations (**Table 8**).

Table 8: Quantifying the antagonistic potency of BML 2015 fractions and environmental sample TAEs by YES/YAS.

	Field [ ] mg/L	Anti-YES EC_{50} mg/L	Anti-YES EC_{10} mg/L	EC_{50} Below field [ ]	4-HT EQ based on Anti-YES EC_{50}	Anti-YAS EC_{50} mg/L	Anti-YAS EC_{10} mg/L	EC_{50} Below field [ ]	FL EQ based on Anti-YAS EC_{50}
F1	12.9	0.11	0.019	yes	0.09	2.61	0.36	yes	0.90
F2	2.10	0.26	0.043	yes	0.21	2.04	0.24	yes	0.70
W1	21.8	25.1	2.19		20.5	370	27.8		127
W2	1.70	10.3	~$5.29e^3$		8.42	17.2	~$3.04e^5$		5.92
BML 2013	75.8	1.24	0.049	yes	1.01	16.2	2.06	yes	5.58
BML 2017	57.9	0.33	0.14	yes	0.27	11.8	1.70	yes	4.05
Pond 9 (2016)	38.1	0.83	0.26	yes	0.67	17.2	2.46	yes	5.92
River 2017	0.76	~198	~$3.39e^3$		161	~$3.81e^4$	1.80		$1.22e^4$
4-HT		1.23			1.00				
FL						2.91			1.00

Note: Field [ ] is an estimation of field concentration by gravimetric analysis. Anti-YES is YES antagonism, and Anti-YAS is YAS antagonism. '~' indicates an ambiguous measurement. F1, F2, W1, W2 were fractions of BML 2015, while BML, Pond 9, and River samples were TAEs. EC_{50}'s and EC_{10}'s (the point of departure measurement) were based on gravimetric analysis (mg/L). 4-HT and FL were known antagonistic chemicals, to which their theoretical EC_{50}'s were compared to experimental treatments to derive an equivalency value (EQ), where a ratio less than 1 was indicative of greater potency than the given antagonistic chemical.

4. Discussion

4.1. Toxicity attribution for BML 2015

4.1.1. Cytotoxicity of whole BML 2015

With acute mammalian LD_{50} assays banned in 2002 by the OECD,[155] alternative means to assess relevant mammalian toxicity and mechanisms of action are necessary, and much of the past toxicity testing of OSPW has focused on aquatic organisms, which is still allowed by the OECD. There is an urgency to move towards more *in vitro* toxicity testing and away from *in vivo* lethality testing as part of the modern toxicology '3R framework' of replacement, reduction, and refinement.[125] Although common regulatory ecotoxicology assays for industrial effluents may not yet be replaceable - such as the 96 h acute lethality test in fish - *in vitro* tests may be advantageous for prioritizing contaminants in high-throughput formats, differentiating between mechanisms of action with less subjective apical endpoints, and being more rapid and less expensive.[29] RTCA is valuable technology for assessing human cellular toxicity of mixtures in environmental water samples of relevance to human exposure.

To further advance our understanding of chemicals that attribute toxicity to the sample of BML from 2015, HepG2 cells were tested using RTCA. The samples tested included: whole BML 2015 water, whole BML 2015 water treated by adsorption with AC or ENVIcarb, or whole BML 2015 water with a pH adjustment, as well as BML 2015 TAE, an extraction control, and a reference natural water sample.

A 100% dose of whole BML 2015 water was not cytotoxic enough to generate an IC_{50} with HepG2 (**Figure 11**). However, an IC_{10} point of departure analysis demonstrated that a cytotoxic effect started to surface at approximately 50% BML 2015 water, and this remained consistent

from 20-80 h post exposure, indicating minor toxicity (**Figure 12**e). Through IC_{10} comparisons, toxicity was marginally reduced after treatment with ENVIcarb, AC, and by pH-adjustment, though this reduction did not appear statistically or biologically significant (**Figure 12**e). Therefore, under these conditions the organic content, the elevated pH, and high concentration of salts typical of all OSPW could not be delineated. However, the combined effect of a powdered media (EMEM), formulated with the optimal osmolarity for HepG2 growth, with the addition of a high salt and high pH OSPW sample as the reconstituting agent, may lead to hyperosmotic conditions and sub-optimal cell proliferation. Ultimately, with only a minor toxic effect of BML 2015, and no practical way to concentrate it without also increasing salt concentrations, the approach of testing whole OSPW by RTCA was deemed too insensitive and inappropriate to meet the objectives of the research in this thesis.

A previous study reported that the inorganic components of OSPW at field concentrations in an active Syncrude tailings pond, Aurora, was cytotoxic and immunotoxic to mammalian macrophages.[109] They argued that the effect was not due to the saltiness of OSPW, because its osmolarity was actually less than that of the cell culture media; however, they did not rule-out effects from osmolarity. Importantly, this study used a different experimental approach for exposing the cells than in the current work. Liquid media with a natural osmolarity was used with liquid OSPW added to it, and a corresponding control containing the amount of volume displaced by OSPW but with phosphate-buffered saline (PBS).[109] Here, powdered media (containing optimal salts for HepG2) was reconstituted with 100% aqueous OSPW (instead of pure water). Thus, the final osmolarity exceeded the optimal conditions for HepG2, possibly causing some toxicity. However, with this method a 100% dose of OSPW was accomplished, enabling a point of departure calculation.

Due to difficulties working with whole OSPW, the next step in the toxicity identification approach was to extract the organic mixture to assess and test its toxicity without the confounding effects of inorganics and salts, thereby minimizing any osmolarity-related effects. Notably, when testing the TAE of BML 2015, the 1× dose did not significantly reduce cell growth compared to negative control (**Figure 14**), which was further evidence that the organics did not contribute to the minor toxicity in 100% whole BML 2015. Furthermore, the calculations in **Figure 17** showed how the point of departure approached the estimated field concentration of BML 2015 (66 mg/L) by 100 h post exposure, though it did not go beyond. Overall, this confirms the initial suspicions that the organic mixture in whole BML 2015 is not responsible for the growth inhibition observed in whole BML 2015. This is also in line with Syncrude Canada Ltd.'s data of BML (**Table 3**), where the whole effluent (100%) from 2016 was generally not toxic in various acute toxicity assays. A caveat of the point of departure calculation in **Figure 17** is that at 100 h post exposure there may be inherent toxicity due to lack of nutrients in wells and natural cell senescence, whereas the more accurate data is taken between ~10-72 hours post exposure. Nevertheless, even at the 72 h time point, the conclusion is maintained that the point of departure exceeds, but approaches, the field concentrations of all BML samples.

Further exploring the dose-response of BML 2015 TAE, cytotoxicity was in fact detected near the 1.75× dose and steadily became more potent up to 12.5×, at which point cell growth was flat-lined immediately from the time of exposure (**Figure 14**b). The extraction control (TAE of 1 L of LC/MS water) demonstrated that the extraction process itself did not introduce contamination to alter cell proliferation (**Figure 13**b). Similarly, the 1 L TAE of an upstream Athabasca River water did not demonstrate any cytotoxicity (**Figure 13**a). Overall, this indicated that the bitumen-derived organics generated and concentrated through industrial processing were

responsible for the cytotoxicity observed in BML 2015 TAE. This is a novel finding considering the lack of human-relevant cytotoxicity data, especially with RTCA, which measures cell morphology, growth, and proliferation through a more holistic lens compared to colorimetric assays that have specific endpoints dependent on specific processes, like metabolism.

BML 2015 TAE had IC_{50} values over time ranging from 6× to 9× above field concentrations, depending on exposure time (**Figure 15**). Notably, Syncrude's acute toxicity data (**Table 3**) were ambiguous in stating that BML 2016 water had an IC_{50} >100%, therefore, these IC_{50} values (**Figure 15**), albeit in human cells, give new insight and data that may be used as a benchmark for monitoring remediation progress as BML ages.

Considering other literature, Sansom et al.[29] tested 49 OSPW effluents at an 80% dose (i.e. 0.8×) to 6 fish cell lines and reported a high variability in cytotoxicity depending on tested sample location, age, and treatment. Some of the OSPW samples decreased cell viability up to 90%, while others had no effect. Therefore, while here the organics at 1× did not cause an effect, experimental parameters like species used in a bioassay and type of OSPW sample or extract can have a big impact on the cytotoxic response.

For example, He et al.[6] tested whole OSPW from WIP 2007, an activate tailings pond at the time, on H295R human adrenocarcinoma cells (used for testing steroidogenesis) and found no cytotoxicity with the MTT assay. This is interesting considering that WIP 2007 was an active tailings pond and would be expected to be more concentrated and more potent than whole BML 2015 that has been aged and diluted. Garcia-Garcia et al.[102] tested a NA-specific extract from WIP 2006, with doses ranging from 6.25-50 mg/L NAs, where field concentration was 39.1 mg/L, and used 3 colorimetric cell assays on mouse bone marrow derived macrophages. Similar to He et al.,[6] they also did not find that cell viability was affected (i.e. with MTT or Trypan blue),

however, they reported that cell proliferation (BrdU assay) decreased by 40% (though not statistically significant) when dosed with 50 mg/L NAs. Considering the NA concentration in the BML 2015 sample used here was estimated to be 12.9 mg NAs/L (**Table 6**), achieving a 50 mg/L dose would require a 3.9× increase from field concentrations. Looking at the profile of BML 2015 TAE (**Figure 14**) it could be estimated that a 3.9× dose may be close to a 40% decrease in HepG2 proliferation. However, the nuances between studies may speak to the differences in the endpoints measured in the colorimetric MTT assay, Trypan blue, or BrdU proliferation assay, especially when compared to the impedance-based RTCA system, which measures cell growth more holistically and with repeated measurements over time.

Further nuancing the discussion, Fu et al.[109] used a scanning electron microscope to image mouse macrophages treated with whole OSPW at environmentally relevant doses, and the treated cells "*featured frayed plasma membrane with multiple intrusive-like structure that are indicative of membrane degeneration or/or disruption of membrane stability*." They go on to suggest oxidative stress damage due to particular gene responses. However, when they tested the organic fraction of OSPW alone, there were no changes in cell morphology, suggesting the effect was from the combination of the inorganic fraction with the organic fraction.

Overall, there were conflicting results. Attempts to reconcile these differences and a possible mechanism of action will be discussed later (section 4.2.3.1). However, it is important to note that there may be multiple mechanisms contributing to cytotoxicity, and this may depend on the source of OSPW, the dose at which the organics have their effect, and whether or not the organics are in their original OSPW matrix.

4.1.2. Cytotoxicity of BML 2015 fractions

Based on **Figure 7**, BML 2015 organics were successfully fractionated using a SPE method to re-create the most toxic fractions (i.e. NAs, and polar non-acids) based on the work of Morandi et al.[51] While Morandi et al.[51] used BML 2012, here BML 2015 was studied; thus, there has been three more years of potential biodegradation or other losses, with approximately 20% of free water was added,[8] as well as large doses of coagulating agents.[144]

In this study, fraction F1, which largely isolated the NA chemical class, had a calculated field concentration of 12.9 mg/L and IC_{50} values with HepG2 cells ranging from 7× to 10× depending on the exposure time, i.e. 24 - 100 h. Fractions F2 and W1 marginally enhanced cell proliferation, likely as a stress response. W2 had marginal cytotoxicity, though an IC_{50} could not be calculated. Therefore, the cytotoxicity of BML 2015 TAE was largely due to NAs (**Figure 19**). This is consistent with Morandi et al.,[51] amongst other studies.[55]

Interestingly, when assessing toxic potency of the remaining organics on a mg/L scale, F1 was much more potent than the BML 2015 TAE (**Figure 20**). This is because NAs only account for 20% of the TAE in terms of mass, and speaks to the successful isolation of the most acutely toxic class in the fractionation method. This result is similar to Morandi et al.[51] where their NA fraction contained only 10.8% of the original dissolved organic mass.

The degree of isolation and separation of NAs from the rest of the chemical classes, demonstrated in both positive and negative mode, allowed for a more accurate assignment of a point of departure value to NAs. The point of departure of F1, largely composed of NAs, approached their estimated field concentration in BML 2015 at 100 h post exposure, i.e. an IC_{10} of ~17 mg/L versus an estimated concentration of 12.9 mg/L in BML 2015 (**Figure 21**).

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[8] Personal communication with Warren Zubot, Syncrude Canada Ltd.

Furthermore, the point of departure for a cytotoxic effect of BML 2015 TAE (63 mg/L, **Figure 17**) also approached its estimated field concentrations (66 mg/L) at 100 h post exposure, highlighting how the majority of the cytotoxicity can be attributed to NAs in the TAE. Therefore, at only slightly above field NA doses in BML 2015 would a dissolved organic-driven toxic effect be observed in a short-term test.

Fu et al.[109] tested whole OSPW from the Aurora tailings pond (2014) with the RAW 264.7 mouse macrophage cell line. They exposed the cells for 18 h with additional OSPW acid extractable organics added to whole OSPW, based on environmentally relevant NA concentrations ranging up to 18 mg/L. When 12-18 mg/L NAs were added with the inorganic content of OSPW, they were cytotoxic to the cells, reducing cell proliferation to less than 20% of negative control in the BrdU test. Interestingly, this effect only occurred when the organics were in the matrix of whole OSPW and not when the organics were isolated by themselves, nor when whole OSPW had 0 mg/L NAs added. This finding was suggested to be due to a mixture effect from the combination of organic and inorganics, and that when the organics are in their aqueous matrix, NAs have a lower threshold for an effect than originally thought (12-18 mg/L).[109]

By comparison, the point of departure of NAs in this thesis approached 17 mg/L after 100 h of exposure, though in Fu et al.,[109] the point of departure was only ~70 mg/L at 18 h (**Figure 21**). Despite the different exposure times, which may be determined by the intrinsic parameters of the bioassay, only above 17 mg/L would NAs reach their threshold for a toxic effect. At 18 mg/L NAs and only when within a whole OSPW matrix, Fu et al.[109] observed reduced cell proliferation (i.e. 0%) and reduced cell viability (i.e. <10%). This highlights that inorganics have a profound effect on toxicity. Though, mammalian macrophages *in vitro* are outside of its host organism and would not normally need to regulate their ion gradients to environmental

conditions outside of the host organism. Therefore, it is sensible for mammalian cells to be more impacted by the inorganic content of OSPW, and this finding does not negate the pursuit of the organics as a toxic component. Moreover, BML 2015 in the current study has been aged three years and diluted with fresh water compared to fresh OSPW, so it is also sensible for this sample to be less toxic than the Aurora active tailings pond. Though, to apply their conclusion of mixture effects between the organic and inorganic fraction to this study, it would be interesting to see if the point of departure of 17 mg/L NAs (above the estimated field concentration of 12.9 mg/L) here would be potentiated if tested within a whole BML 2015 matrix.

Looking at the F2 fraction, it is interesting that it was not acutely toxic to HepG2 cells even at its highest doses of 12.5×. This is because Morandi et al.[51] found their F2 fraction, containing similar polar non-acids, was the second most acutely toxic fraction, resulting in 100% mortality of fathead minnow embryos at ~5×. To potentially explain this difference, Morandi et al.[51] used a sample of BML from 2012 while the current study used BML from 2015, and the total intensity of the species detected is known to have decreased with ageing (**Figure 8**); although the positive mode species signal intensity appeared to deplete to a lesser extent than the negative mode species. An alternative explanation could be that HepG2 cells have a different sensitivity to OSPW organics compared to developing fathead minnow embryos, or that there are intrinsic differences in the assays. The fractionation techniques used were similar in the purpose, yet different in methodology, so the particular chemical classes isolated may also be somewhat different. Interestingly, with testing the cytotoxicity of BML 2015 fractions on yeast, the sensitivities to the BML 2015 fractions changed again: F1 was classified as cytotoxic based on a reduction in growth factor beyond 50% at 2.5×, and F2 was classified as cytotoxic at 10×

(**Figure 22**). Therefore, there may be a combination of factors that lead to the differences between Morandi et al.[51] and the current study's results.

On the other hand, Hughes et al.[156] critiqued Morandi et al.[51] and argued that the polar non-acids were not relevant and that focus should still remain on classic NAs. They defended this argument by saying that there is currently no way to quantify the positive mode species, and that with an LC_{50} of 2.2× in Morandi et al,[51] polar non-acids would result in minimal adverse effects at field concentrations due to its relatively low potency compared to NAs, which had a reported LC_{50} of 0.7×. However, just because a contaminant class cannot be quantified does not make it toxicologically irrelevant. Secondly, I argue that an LC_{50} of 2.2× is indeed toxicologically relevant due to the fact that this was an acute toxicity test, and the EPL strategy inherently involves chronic exposure, and it is not clear which chemical classes may be most important in longer term exposures. Moreover, Morandi et al.[51] suggested that the polar non-acids may act through a different mechanism than NAs, possibly endocrine disruption—in which case, sub-lethal endpoints need also be assessed to prevent ecotoxicity.

Hughes et al.[157] tested the toxicity of multiple organic fractions where the % composition of classic NAs differed, and argued that the potency of individual NAs increased with carbon number. They concluded this by finding that two fractions each had the same concentration of NAs, though one fraction had 50% mortality, while the other had 100% mortality, and suggested that the latter fraction had more potent NAs chemical species. However, they only analyzed their samples in negative ion mode, so it is unknown whether positive mode species had an influence on the observed toxicities of any of their fractions. While the current study indicated the polar non-acids were even less acutely toxic than the Morandi et al.[51] findings with fathead minnow

embryos, i.e. supportive of Hughes' et al.[156] argument, the sub-lethal effects of these non-acid chemical classes still warrants testing, particularly with endocrine disruptive endpoints.

Despite differing opinions about relevance of the polar non-acids, the RTCA results in the current study continue to support NAs as being the most acutely toxic chemical class in OSPW. The human-relevant point of departure for NAs could contribute to the monitoring and regulating of OSPW as it ages, in that a value of less than 17 mg/L is needed to not be acutely toxic to human cells. That being said, this aspect of the study only tested RTCA with HepG2 cells, so the point of departure conclusion cannot be made without a greater body of research and a species sensitivity distribution. Nevertheless, this may be a useful starting point for determining other human-relevant points of departure for NAs.

4.1.3. Endocrine activity of BML 2015 fractions

All of the BML 2015 fractions demonstrated anti-estrogenic and anti-androgenic effects, yet none had any agonistic effects. I was successful in isolating the most cytotoxic chemical class, the NAs, but the ER and AR antagonists were unexpectedly distributed throughout the fractions with different potencies, though the majority of the effect was attributed to F1 and F2. In other words, the endocrine active chemicals could not be isolated by chemical class alone, and other fractionation approaches are warranted.

In the first YES/YAS trial, the polar non-acids in F2 were the most potent chemical classes, however, the dose-response curves for most fractions were incomplete, leaving overall results inconclusive. In the second YES/YAS trial with a wider and lower dose range, it became clear through the full dose-response curves that F1 and F2 were similar in their antagonistic ability of the ER and AR. Most interesting though, was that polar non-acids, while not being cytotoxic to

HepG2 cells and only marginally cytotoxic to yeast cells at high doses, were similarly potent (i.e. mg/L scale) as NAs through a sublethal endocrine active mechanism of action. As discussed in the section above, this must be considered in the overall discussion of environmental relevance of various chemical classes compared to NAs. Nevertheless, on the × scale, F2 was in fact slightly less environmentally relevant than F1. No matter, it is interesting that the EC_{50}'s of ER/AR antagonism for F1 and F2 were both below their estimated environmentally relevant concentrations. F1 was the most acutely toxic fraction yet also had a potent sublethal antagonistic ER/AR mechanism. With the very low doses of F1 and F2 needed in order to generate an upper plateau on the dose-response (similar to the agonist baseline control, i.e. negative control), this is consistent with the EC_{50} being drastically below environmentally relevant concentrations. Overall, our hypothesis of F1 and F2 having different mechanisms was not supported, however, even more concerning is that both of these fractions were endocrine active through both ER and AR antagonistic mechanisms.

To characterize these fractions further, the point of departure threshold for toxic effects were calculated. Since F1 and F2 were the most potent and relevant fractions, this novel threshold could be useful in the absence of other data for interpreting targeted water monitoring and remediation to ensure environmental protection. Based on the data generated here, F1, which is largely the NA class, had an ER antagonism threshold of 0.019 mg/L, an AR antagonism threshold of 0.36 mg/L, and a cytotoxic threshold of ~17 mg/L. Therefore, the sub-lethal effects can be detected at a much lower dose than the cytotoxic effects, albeit in different assays with different species. The ER/AR antagonism thresholds for F1 are quite potent, given the concentration of 12.9 mg/L in BML 2015. However, a caveat should be noted that there is uncertainty pertaining to the efficacy of the YES/YAS results, in that the results are scaled to the

positive control antagonistic hormone and not to the vehicle control. Notably, the point of departures generated here are specific to these experiments, and a species sensitivity distribution would be needed before implementation in any remediation strategy, as YES/YAS is only a screening test.

F2 has an ER antagonism threshold of 0.26 mg/L and an AR antagonism threshold of 0.24 mg/L (i.e. more potent than F1). It was not cytotoxic to HepG2 cells, yet it was cytotoxic to yeast at a dose of 10×. The estimated field concentration in BML 2015 is 2.10 mg/L; therefore, there is less of a difference between the current concentrations of polar non-acids in BML and the point of departure, while there is a bigger difference for F1. Therefore, reducing F2 to levels below its point of departure would be more achievable than F1 for targeted remediation.

W1 and W2 both had measurable EC_{50}'s, but for W2 an EC_{10} could not be calculated. This could mean that W1 has a clearer dose-response for antagonistic effects and at more environmentally relevant concentrations. Importantly, W2 had a lower concentration (1.70 mg/L) than W1 and required a very high enrichment factor for antagonistic effects to surface, which is not environmentally relevant. W1 had an EC_{50} above field concentrations in BML 2015, though its EC_{10} is in fact below BML field concentrations, suggesting that it may too have relevance for endocrine activity, in addition to F1 and F2. Therefore, chemical features other than molecular formula class may be important in determining the endocrine activity of OSPW organics.

4-HT and FL equivalencies were calculated by comparing the EC_{50} values of environmental samples with theoretical EC_{50} values of the known antagonistic hormones on a mg/L scale. These equivalency values give insight to the potency of environmental samples compared to known antagonists. On a mg/L scale, F1 and F2 were more potent than FL and 4-HT, while W1

and W2 were less potent (**Table 8**). Though it is important to emphasis that this is highlighting mechanistic potency in an *in vitro* screen and is not linked to apical adverse effects.

Comparing the toxicity results with the Orbitrap MS results allows some extent of toxicity attribution, though more work is needed. Among the least potent fractions, W1 contained a mixture of chemical classes (O_3^-, O_4^-, O_2^+, O_3^+, SO_3^+, NO_4^+) and while it was not cytotoxic in RTCA, it had ER/AR antagonistic potencies at environmentally relevant doses. W2 contained another mixture of chemical classes (O_2^-, O_2^+, O_3^+, SO_3^+, NO_4^+) in low abundance, had minor cytotoxicity in RTCA, and while it did have ER/AR antagonist activity, these may only surface at non-relevant environmental doses.

Considering the mixture of overlapping chemical classes between W1 and W2, this may warrant a principle component analysis (PCA) to further attribute toxicity not only to fractions, which still contain a mixture of chemical classes, but to unique chemical classes within the fractions. Through a PCA, the intensity of a particular chemical class among all fractions could be related to a given toxic effect. For example, W2 had minor cytotoxicity and contained O_2^- as one of its prominent chemical classes, among others, while W1 was not cytotoxic and did not contain O_2^- though was prominent in positive mode species. This may further support O_2^- as being the most cytotoxic class, while the positive mode species in W1 may be responsible for maintaining the ER/AR antagonism, though a PCA could attempt to confirm this.

The toxicity attribution that has been suggested here should be compared to what already exists in the literature. Yue et al.[71] used the YES assay and Orbitrap analysis in negative mode with OSPW from WIP, an active tailings pond. They suggested that O_2^-, O_3^- and O_4^- species from C_{17} to C_{20} with double bond equivalents between 6 and 10 had chemical formulas similar to estrone-like and estradiol-like compounds. This is not consistent with the current data in that

YES agonists were not detected in the BML 2015 fractions, only YES antagonists. Notably, Yue et al.[71] did not analyze in positive mode, nor did they test for YES antagonism. However, there may be chemicals in fresh OSPW, such as WIP, that are agonists but that degrade with ageing: this hypothesis will be discussed in terms of antagonism (not agonism) in section 4.2.4.

Leclaire et al.[70] tested OSPW aged 17 yrs using both YES and YAS and only found anti-estrogenic and anti-androgenic effects; however, their extraction method was specific to NAs and they only analyzed the extract in negative mode MS. Therefore, this is consistent with the current investigation in that F1, largely containing NAs, did have YES/YAS antagonism and no associated agonism. Leclaire et al.[70] generated 4 fractions with different NA contents: fractions with a higher aliphatic NA content appeared to be the most potent, with all fractions having EC_{50}'s (mg/L) ranging from 1.5 - 16 mg/L for AR antagonism. In the current investigation, the AR antagonism EC_{50} for a broader group of NAs was 2.61 mg/L (**Table 8**). Similarly in Leclaire et al.,[70] all of the fractions generated EC_{50}'s (mg/L) ranging from 0.49 - 9.57 for ER antagonism, while here, the EC_{50} for NAs was 0.11 mg/L. The main conclusion of Leclaire et al.[70] was that NAs can act through a steroid antagonism mechanism at environmentally relevant concentrations. By extension, this investigation demonstrated that the polar non-acids and NAs are both potent antagonists at environmentally relevant concentrations, with other chemical classes potentially playing a role. A caveat in comparing studies are the different OSPW samples and extraction techniques used. This highlights the importance of looking at endocrine activity and cytotoxicity of different OSPW samples (section 4.2.4) with the same extraction method and MS specifications.

The knowledge about mechanisms of action from *in vitro* studies could give insight into *in vivo* observations. For example, evidence exists of endocrine activity *in vivo* through Wiseman et

al.[8] testing fresh OSPW on *Chironomids*, or Lister et al.[120] testing goldfish in aged reclamation pond water, and these effects may be through ER or AR antagonism as opposed to agonism, which was found in an earlier study.[6] Important to note, the YES strain only contains the hERα receptor and neglect potential interacts with hERβ receptor; this may add to the disparity between YES results and tests on organisms of higher biological complexity. Also, the mechanistic potency, described here as disturbance to a molecular pathway, does not necessarily translate to potency for an adverse effect in a whole organism. Therefore, more work is needed to determine the adverse outcome pathway and link the mechanistic data to in vivo adverse effect observations.[1]

With many chemical classes associated with YES/YAS antagonism distributed throughout the fractions with different potencies, targeted remediation strategies will be challenging. That being said, NAs and the polar non-acids could be a starting point for targeted remediation of acute toxicity and monitoring. While polar non-acids from BML 2015 were not cytotoxic to HepG2 cells, their environmental relevance becomes more pressing due to their EC_{10} thresholds for ER/AR antagonism below field concentrations, an endpoint suspected to have low-dose effects.[137] This is combined with their apparent persistence with ageing (**Figure 8**), and their bioaccumulation potential.[50,89] Therefore, polar non-acids may be just as toxicologically relevant as NAs in the EPL strategy.

As mentioned, environmental protection is based on the interplay of whole effluent and chemical-specific toxicity, combined with biological effects monitoring.[26] The supercomplex mixture of OSPW organics, with their spatial and temporal variability between sources, makes setting guidelines for safe release a significant challenge. While Syncrude's data on BML suggests that BML 2016 is now largely passing standardized acute toxicity tests, they only tested

the whole effluent. The current investigation suggests chemical-specific effects for cytotoxicity and endocrine disruption with corresponding thresholds. The point of departure of F1 and BML 2015 TAE for cytotoxicity were approaching, but not passing beyond the BML 2015 whole effluent field concentration. The point of departure of F1 and F2 for YES/YAS antagonism, on the other hand, were far below the field concentrations of BML 2015. Notably, both of these assays were under acute conditions and do not account for the more environmentally relevant scenario of chronic exposure. However, the thresholds generated here could contribute to chemical-specific guidelines for NAs, polar non-acids, and the TAE overall. Furthermore, the detection of an antagonistic mechanism of action, not agonistic, should be considered when selecting endpoints for biological effects monitoring or for choosing future endocrine disruption screens. For example, the more extensive androgenized female stickleback screen may be warranted to detect antagonism in a higher order species, and this is in line with OECD endocrine disruption testing guidelines.[139] This has been performed previously by Knag et al.,[101] but they used commercial NAs, which are not a true representation of OSPW NAs.

4.2. Evaluating ageing as a remediation strategy for OSPW

4.2.1. Cytotoxicity of BML OSPW samples over time

As mentioned, there are multiple co-occurring processes occurring in EPLs that add to the complexity of assessing ageing as a remediation strategy: firstly, there is input from fresh OSPW originating from the underlying consolidating MFT, freshwater added for dilution purposes, rain water addition and evaporative losses, and sedimentation and potential degradation losses for the organics. Comparing different toxicity studies, a common caveat is the use of different OSPW samples, extractions methods, detection methods, and bioassays used. Therefore, this

investigation has the advantage of maintaining uniformity in the experiments while testing OSPW samples of different origins and ages.

RTCA is quick, high-throughput, and non-lethal, allowing for prioritization of industrial effluents, and has been suggested by Pan et al.[131] to be suitable for samples where the responsible toxicants are unknown. In initial tests with the whole effluent of BML 2015, there was only minor cytotoxicity, and with no practical way to concentrate the organics without also increasing salt concentrations. Similarly, Syncrude Canada's data monitoring the acute toxicity of the whole effluent of BML at different years is effective at generating whole effluent limits, but the reported LC_{50}'s are ambiguous (i.e. LC_{50}>100%). Therefore, a concentrate of the organic mixture through a TAE was deemed appropriate in generating more chemical-specific toxicity data, while being conducive for the RTCA framework and for calculating unambiguous IC_{50} values to monitor the effect of ageing with multiple OSPW samples at once. Here, a positive control of As (III) was used to ensure instrument functionality, as it readily caused cell death; however, it also acts as a reference for a well-studied environmental contaminant with corresponding *in vivo* toxicity data. It can therefore be a benchmark of potency and provides some relevance for the OSPW organic extracts tested. Though, translating the *in vitro* effects to biological significance is outside the scope of this investigation.

Looking at BML on an enrichment factor scale allows evaluating environmental relevance. BML 2013 was the most cytotoxic sample, with potency (×) decreasing progressively as BML aged (i.e. to BML 2015 and BML 2017). This is sensible for the toxicity to decrease with age of the sample, as the mg/L concentration of the organics decreased (**Table 7**) as well as the mass spectrometry total ion signal intensity (**Figure 8**). Moreover, this result is in line with the trend seen in Syncrude Canada Ltd.'s acute toxicity testing of BML over several years (**Table 3**).

However, when the toxicity profile is on a mg/L scale, which allows for measurements of toxic potency on an absolute scale negating the volume of original water, the differences in toxicity between BML 2013, BML 2015, and BML 2017 disappear (**Figure 16**). This means that simple dilution can account for any cytotoxicity differences between BML samples of different ages. This is furthermore supported by the similarity in heteroatomic chemical class distributions for each BML sample, despite differences in age (**Figure 10**).

Interestingly, recently Dompierre et al. 2017[158] modelled the change in volume and chemical mass in and out of BML between 2013 to 2015. They found that while the water balance remained fairly consistent over time, the slight improvements in overall water quality was largely due to dilution, described at 5-10% dilution per year. They more explicitly described the largest input in BML from fresh water from the Athabasca River, and a balanced output of water that is likely pumped back into the bitumen extraction process. Therefore, while there are uncertainties in the potential and rate of sedimentation and degradation, dilution is known to be occurring, so it is therefore sensible for the toxicity data generated here to also only see dilution as reducing toxicity. White and Liber 2018[159] found that the pore water from FFT is similar to fresh OSPW, and that this indefinite input into the overlying water creates long term reclamation challenges, with dilution having little effect on the underlying FFT.

Dilution is effective in reducing the toxic potency of most any environmental contaminant, and it is often used to reduce the concentrations of a contaminant to below the threshold of a toxic effect. Therefore, even if it is merely dilution of the toxic organics, and not their degradation, this still serves a reclamation mechanism for the EPL strategy. However, as mentioned, total OSPW stored on site in the oil sands industry is estimated to account for 0.5% of the volume of downstream Lake Athabasca,[9] Canada's 8th largest lake, so dilution of this

amount of OSPW may not be an acceptable strategy. Especially when industries are already pressed for withdrawals from the Athabasca River. Also, the rationale and goal of the EPL strategy is not for dilution, but to allow for sedimentation of solids and degradation of organics over time.[22] Continuous dilution and coagulant addition to not only BML, but the future 30 planned EPLs could represent a challenging and expensive long term reclamation strategy. This highlights the importance of calculating the IC_{50} values of the organic mixture on both × and mg/L scales, particularly with bioassays like RTCA that allow assessment of water samples with unknown contaminant mixtures. Lastly, there is urgency in starting the safe release of remediated OSPW sooner than later to prevent an expensive toxic legacy for future generations, therefore additional treatments within the EPL may be necessary to achieve the goal of a biologically productive lake connected to the watershed.

It is important to look at what the absolute IC_{50} values were: BML 2013 had an IC_{50} at ~6×, BML 2015 at ~7×, and BML 2017 at ~8×, depending on exposure time (**Figure 15**). Here, the generated IC_{50}'s for all BML samples were much higher than their real-world concentrations. However, only acute toxicity was tested here and this does not consider chronic exposure, sub-lethal mechanisms, bioaccumulation, chemosensitization, or possible mixture effects in its aqueous matrix. In comparison to Syncrude Canada Ltd.'s data, this investigation allows for true IC_{50} values to be calculated over a time period of 4 years for BML, rather than ambiguous reports of LC_{50} or IC_{25} being, e.g. >100% the whole effluent of OSPW. In terms of what concentration would allow for safe release, this cannot be concluded, though the point of departure values for all BML samples, the point at which a cytotoxic effect starts to surface, is approaching the estimated concentration at 100 h post exposure. Hopefully, this benchmark value could give insight into the monitoring of biological effects. It is important to note, however, that the EPL

strategy is only one of the strategies used for OSPW remediation, with the promising intervention of Petroleum Coke currently being implemented.[86]

4.2.2. Chemical classes in OSPW of different ages

In order to change the scale of environmental samples from enrichment factor to mg/L, the dry weight of the organics from liquid-liquid extraction was recorded and corresponded well with total intensity of ions detected with the Orbitrap method, in that the values were lower in samples with more ageing; albeit only marginal differences between BML 2015 and BML 2017. Looking at the individual heteroatomic chemical class distributions for each BML sample of different age, there were no profound changes in the relative distribution of heteroatomic groups. This supports that changes in cytotoxicity (on an enrichment factor scale) are likely only due to dilution, and not likely due to degradation of the organics. More rigorous chemical characterization and analysis would be needed to explore this conclusion further. Pond 9 (2016) had a unique distribution of chemical classes in negative mode, suggesting that through unknown processes OSPW organics can eventually change and decrease in intensity over 23 years. However, the positive modes species of Pond 9 (2016) were more persistent and actually had similar proportions to BML, which may suggest recalcitrant species only undergoing dilution.

4.2.3. Cytotoxicity of Pond 9 (2016), a highly aged sample

The cytotoxicity of Pond 9 (2016) TAE had a time-dependant biphasic effect on HepG2 cells. At ~24 h post exposure it was more potent than any of the BML samples tested, but then the cells recovered and reached an asymptote by ~60 h post exposure. This suggests a unique mode of action, and other studies are exploring how chemicals with similar RTCA profiles can

give insight into mode of action.[121,130,160] Furthermore, taking the IC_{50} histogram on a mg/L scale, the Pond 9 (2016) is less concentrated than BML, but the remaining organics are even more potent than all BML samples at 24 h, and the biphasic response is maintained and consistent, irrespective of the scale (**Figure 20**). This highlights the importance of the composition of OSPW, not solely concentration.

Looking to the literature for Pond 9 or aged OSPW, there is generally lesser toxicity associated with aged OSPW compared to fresh, but a degree of toxicity still remains in the aged samples,[13,57,96,120,161] suggesting that active treatment may be necessary for full remediation. On the contrary, Marentette et al.[162] tested NA extracts from fresh and aged OSPW for their effects in fathead minnow embryos and reported marginal differences between them. Moreover, Bartlett et al.[146] later performed a number of bioassays with fresh and aged OSPW and also reported an aged sample to be similar or more potent than fresh samples, depending on the species tested. Most literature toxicity data considers only one time point, thereby ignoring the temporal dimension, and to the best of my knowledge this investigation is the first report of the temporal biphasic effect in an aged OSPW sample. This sheds light on some of the conflicting results noted above, and future studies of OSPW remediation should consider multiple exposure-time periods in their bioassay designs.

Sansom et al.[29] exposed fish cell lines for 24 h to an 80% dose of 49 aqueous OSPW samples, including Pond 9 among other aged samples, and found that NA concentration was correlated with a decrease in cell viability. Alharbi et al.[110,134] published two papers in 2016 testing Pond 9 and BML 2012 samples while looking at ATP-binding cassette (ABC) superfamily of transporter proteins, oxidative stress, and co-exposure to PACs. These papers are most relevant to this investigation because they looked at the acidic, basic, and neutral extracts of

Pond 9, concentrated the organic mixtures, and tested these in *in vitro* assays. They reported that only the acidic fraction of BML 2012 (largely dominated by NAs) impacted survival of embryos while only the neutral or basic fractions of BML 2012 (with higher proportions of O_x^+, SO_x^+, and NO_x^+ chemical classes) had an effect on ABC proteins.[110] Interestingly, their pooled extracts of Pond 9 up to 5× did not have an effect on embryo survival or ABC proteins. Overall, their conclusions were that ageing was an effective remediation strategy. However, the embryos were exposed for 192 h, so the initial toxicity seen in the biphasic response in the results presented here may have been missed. In the current study with HepG2 cells, Pond 9 (2016) TAE had an IC_{50} of ~5× at 24 h post exposure, but the potency subsequently decreased to 17× by 60 h post exposure. Thus, the results here do not necessary contradict Alharbi et al.[110,134] Interestingly, Alharbi et al.[110,134] tested Pond 9 in 2012, while here Pond 9 sampled in 2016 was tested, so technically the 2016 sample should be less cytotoxic.

Overall, this highlights the advantage and novelty of using RTCA to test OSPW samples, in that repeated measurement of cell proliferation over time yields new information**.** Thinking in terms of a more environmentally relevant scenario – when EPLs are connected to the natural environment in the future – organisms could be constantly in contact with a diluted form of water potentially with a similar chemical profile to Pond 9, so that initial toxic effect observed after 24 h could become chronic, and in this circumstance, organisms may not be able to readily recover. This could add some clarity as to why the Alharbi et al.[110,134] papers find that 5× of a Pond 9 total extract had no effect on embryo survival or ABC proteins at a certain time point, while fish placed in aged ponds in a chronic *in situ* experiment had reproductive, developmental, and other adverse health effects.[23]

While Pond 9 (2016) is less concentrated in total dissolved organics, the remaining organics are more potent during initial phases of exposure. This may be due to decreased chemical antagonism, with the environmentally recalcitrant chemical fraction having potency and a unique mode of action. However, Pond 9 is only one of several experimental ponds made as preliminary *in situ* experiments to evaluate ageing, and BML was actually modelled after Pond 5. Pond 5 contained MFT at the bottom of the pond,[10] which may increase biodegradation due to the presence of adsorbed bacteria, but would also release organics to the overlying water as the MFT continues to densify. RTCA could be applied to all of these experimental all at once, to gain insight into differences between various remediation strategies.

Looking at the heteroatomic class distributions in **Figure 9**, Pond 9 (2016) had a unique distribution of chemical classes compared to BML, interesting given the correspondingly unique biphasic toxicity profile. In negative mode, the Pond 9 O_4^- class was most prominent, followed by O_3, then O_2, though the overall signal intensity is significantly lower than in BML. Furthermore, the positive mode species, such O^+, O_2^+, O_3^+, and O_4^+ may take up a greater proportion of the total organic mixture and is speculated here to exert a unique mode of action that is otherwise masked by antagonism in fresher samples. This hypothesis warrants fractionation of Pond 9 for confirmation.

4.2.3.1. Potential mechanisms of action based on cytotoxicity profile

In most simplistic terms, the biphasic response of Pond 9 (2016) may be from transient narcosis. With narcosis being the suspected mechanism of action for OSPW TAE, and with Pond 9 having a much lower intensity and concentration of chemicals, the cytotoxic effect may merely be transient in its effect with the cells able to recover, as narcosis is reversible.[123] A steep dose-

response curve is indicative of narcosis[51] with chemicals being integrated into cell membranes, though a transient nature of narcosis may look like chemicals partitioning out of cell membranes or not being of sufficient concentration to counter the proliferative nature of immortalized cell cultures. Similarly, Pond 9 did not have a time-dependant biphasic cytotoxicity response in yeast, but BML 2017 did, so this may support a general transient narcotic effect for OSPW organics. If so, imaging of the cell membrane after 24 h, and then at 60 h, could add to elucidating the mechanism of the biphasic toxicity profile.

That being said, for such a unique, novel, and consistent response observed in RTCA, further investigation is warranted to explore possible next steps. Specifically, studies have tried to group chemicals based on their RTCA profiles to gain insight into whether or not they have similar mechanisms of action. The closest RTCA profiles compared to Pond 9 was for an anti-mitotic subgroup, represented by a drug called monastrol.[130] Cell cycle arrest at mitosis is characterized by cell rounding and transient detachment from where the cells are attached. With cell lines that lack a robust mitotic checkpoint, such as cancer cell lines like HepG2, 'mitotic slippage' can occur.[130] This is where a subpopulation of cells escapes the initial arrest, resulting in a recovery of CI, i.e. cell growth. The authors go on to speculate the mechanism of this anti-mitotic effect, whether it is through stabilization or disruption of tubulin (for microtubules/spindle formation), motor proteins important for spindle formation, or Ca^{2+} channel modulation. However, the authors also say that the TCRPs alone are not able to differentiate between the specific mechanisms, but is more so used as a guide for prioritizing chemicals of toxicological relevance and for performing other assays to confirm the possible mechanisms. Therefore, my hypothesis is that the Pond 9 (2016) sample is anti-mitotic through some unknown mechanism(s). The

uncertainty in mechanism is especially true for OSPW samples, considering it as a mixture rather than a pure chemical.

That being said, another source reported a biphasic RTCA profile with HepG2 cells treated with vinblastine, a chemotherapy drug.[121] ACEA, the manufacturer of RTCA also demonstrated the effect of vinblastine with RTCA, but they integrated this response with a micronucleus flow assay, which allows for simultaneous detection of genotoxic substances, able to differentiate between clastogens, aneugens, and mutagens.[160] Vinblastine was shown to be an aneugen, resulting in daughter cells with an abnormal number of chromosomes. Similarly, Xi et al.[121] clustered 47 chemicals into similar modes of action to HepG2 cells based on their RTCA profiles, and used Vinblastine Sulfate as a representative compound for a biphasic response, and classified it as targeting tubulin, similar to the Abassi et al.[130] who tested monastrol.

This insight brings attention to the genotoxic potential of OSPW. There are three papers that have found a genotoxic potential for OSPW using different water sources: BML 2012,[163] several model NAs and synthetic OSPW in the lab,[100] and commercial diamondoid NAs.[164] These papers used the SOS chromotest, rainbow trout, or haemocytes of marine mussels, respectively. Therefore, no work has been done on human cells lines nor with the benefits and scope of information generated from the micronucleus flow assay, and may warrant further investigation to clarify the similarities between vinblastine, monastrol, and Pond 9 (2016). If anything, it is interesting that such a supercomplex mixture in Pond 9 can result in a toxicity profile that is so similar to aneugenic chemicals, given the potential for antagonism or synergism in mixtures. Notably, BML 2012 TAE was classified as genotoxic from 7× to 10×, while Fu et al.[109] found stress gene responses after NA treatment at the surprising low dose of 10-20 mg/L, but only

when dissolved in OSPW as a medium; therefore, the mechanism of action and potency may differ between sources and medium.

Upon further reflection of the cytotoxicity literature and of the results presented here, there may be a trend, albeit a nuanced trend, suggesting that OSPW organics are cytotoxic through a decrease of cell proliferation, and this may be the result of an anti-mitotic mechanism, be that tubulin- or aneugen-induced. The first supporting evidence for this hypothesis is that Garcia-Garcia 2011[102] did not find cell viability to be affected, i.e. with MTT or Trypan blue; however, they also tested cell proliferation through BrdU and found that that 50 mg/L NAs decreased cell proliferation by 40%, though not statistically significant. These differences in endpoint of cytotoxicity methods may be part of the reason for the varied results with cytotoxicity (**Table 2**). Secondly, the results with BML and HepG2 show a decrease in cell proliferation, with a 12.5× dose of BML 2015 flat-lining cell growth (**Figure 14**). Lastly, this hypothesis is supported with Pond 9 demonstrating a biphasic toxicity profile similar to drugs that are anti-mitotic through affecting tubulin or having aneugenic activity. As mentioned, new technology, such as the micronucleus flow assay, may be warranted to further investigate this initial hypothesis.

4.2.4. Endocrine disruption of OSPW samples over time

As mentioned in section 3.1, there is a need to look at endocrine disrupting endpoints with multiple OSPW samples of different ages. This is particularly warranted considering the variation that exists in the experimental parameters between studies, such as OSPW samples, a recurrent caveat. In this investigation however, it is interesting that OSPW-impacted samples of different ages were very similar to each other. In other words, while the cytotoxicity of BML on

an × scale decreases with ageing, the sublethal ER/AR antagonistic ability of the all OSPW samples was consistent no matter the age, be that 1, 5, or 23 yrs old.

Looking at the EC_{50} values on a mg/L scale, technically BML 2017 was the most potent, then BML 2013 and Pond 9 (2016) were similar. This is in stark contrast to the environmental control of River 2017, for which an EC_{50} value could not be calculated due to the lack of response. It is interesting reflecting on the idea developed with RTCA that dilution may play a role in the cytotoxicity differences in BML, because looking at YES/YAS antagonism, the OSPW samples are very similar on both an × and mg/L scale. With this scale transition, the Pond 9 curve shifts slightly to the left for YAS antagonism, becoming more potent and even more similar to the BML samples, though it is marginal and the shift was not observed for YES antagonism. Looking at the HPLC-Orbitrap total ion intensity of environmental samples in **Figure 8**, the negative mode species are more depleted over time compared to the positive mode species. Therefore, the relative proportion of positive mode species in the sample appears to increase over time, as indicated by the percentage values in **Figure 8**, and potentially due to greater persistence. This is in line with the chemical class distributions in both negative and positive mode (**Figure 9**). Specifically, considering how prominent O_2^-, O_3^-, O_4^-, and SO_3^- are in BML samples, these select chemical classes have almost become insignificant in Pond 9 2016. However, in positive mode, O^+, O_2^+, O_3^+ and O_4^+ represent the most prominent chemical classes in the mixture and decrease to a lesser extent. With BML 2013 and BML 2017, the chemical profile is similar, with the most obvious difference in total signal intensity and weight of total dissolved organics, yet this change did not seem to have a large impact on ER/AR antagonism.

Considering the lack of change in ER/AR antagonism over time and the increased proportion of positive mode species, ER/AR antagonism in Pond 9 may be largely maintained by positive

mode species. To support this, among the BML 2015 fractions, F1 and F2 were quite similar in terms of ER/AR antagonistic potency, but there may be a greater chance for the F1 chemicals to degrade over time, while the F2 chemical classes may persist. That being said, River 2017 mostly contained positive mode species, so some of the positive species may be natural in the environment, but even if derived or maintained from the natural environment, these chemical classes in River 2017 clearly did not have any antagonistic effects. As mentioned, chemical antagonism exists in the mixture, so the overall decrease in signal intensity may lead to isolation of endocrine active chemicals in Pond 9 (2016), leading to a greater potential for an effect. It is also important to note that many of the other SO_x^+ and NO_x^+ chemical classes remaining in Pond 9 (2016) may also contribute to the effect. Overall, the prominent chemical classes in Pond 9 (2016) may not only contribute to maintaining the ER/AR antagonism as OSPW ages, but also contribute to the unique biphasic toxicity profile seen by RTCA.

Comparing to the literature, Leclaire et al.[70] tested a 17 year old aged OSPW with YES/YAS and only found anti-estrogenic and anti-androgenic effects; however, their extraction method was NA-specific and the extract was only analyzed in negative mode MS. They generated four fractions with different NA content: fractions with a higher aliphatic NA content appeared the most potent, with all the fractions generating an EC_{50} (mg/L) ranging from 1.5 – 16.4 mg/L for YAS antagonism, and 0.5-9.6 for YES antagonism. In this investigation, the TAE of Pond 9 generated an EC_{50} (mg/L) of 17.2 for YAS antagonism and 0.83 for YAS antagonism. Firstly, these results are consistent in that YES antagonism is more potent than YAS antagonism. Secondly, the EC_{50} values are in a similar range, though the fractionated Pond sample may be more potent due to isolation of antagonists. For example, it is known that chemical antagonism exists in BML 2012,[51] and even Yue et al.[71] needed to fractionate the organic mixture in order

for endocrine activity, albeit an estrogenic (agonistic) response to be detected. Finally, the Pond 9 sample in this investigation may be less potent because it is six years older than the sample in the Leclaire et al.[70] paper.

The unchanging YES/YAS antagonism potency as OSPW ages may be impactful to the EPL strategy and the environmental protection guidelines affected it. Even though the whole effluent of BML is now passing standardized acute toxicity (**Table 3**), and the IC_{50}'s calculated here for the BML organics are above field concentrations, with points of departure surfacing at only marginally above 1×, the sub-lethal endocrine active effects have mechanistic potencies far below field concentrations no matter the age of the OSPW sample. This is surprising considering that Pond 9 (2016) has been aged 23 yrs and may be a representation of what BML could look like in the future at the point of environmental release, yet it remained just as endocrine active as fresher samples of BML. This data may add to the chemical-specific toxicity limits for sub-lethal endocrine activity, in that TAEs were tested. Biological effects monitoring is needed to test the effectiveness of the whole- and chemical- specific toxicity limits, and knowing the mechanism of action may inform the strategy used through the selection of endpoints that would typically be affected by anti-estrogens and anti-androgens, such as secondary sex characteristics.[165]

However, it is important to distinguish between mechanistic potency generated from *in vitro* tests based on disturbances to molecular pathways compared to the potency for an adverse effect at a higher level of biological complexity. The US EPA[140] in 2010 released a "*Weight-of-Evidence Guidance Document: Evaluating Results of EDSP Tier 1 Screening to Identify Candidate Chemical for Tier 2 Testing*." Here, Tier 1 screens, where YES/YAS would be categorized, are used to determine if a substance has the potential to interact with the endocrine system with specific mechanisms tested, while Tier 2 is more definitive and can determine if a

chemical can cause an adverse effect though more apical endpoint measurements.[141] It is argued that a hypothesis-driven weight of evidence framework is necessary to examine and integrate different studies from different tiers and determine endocrine activity.[141] Similarly, OECD[139] in 2018 released a "*Revised Guidance Document 150 on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption*." Here, and relevant to this investigation, a conceptual framework was made to guide tests to assess relevant endpoints for a particular mechanisms, such as for ER/AR antagonism. The endpoints are extensive, but relevant higher order tests include: the 21-day fish assay, medaka extended one-generation reproduction test, androgenized female stickleback screen (AR modality), uterotropic bioassay in rodents (ER modality), the Hershberger bioassay (AR modality), among others.[139] Answering the adverse effect potency questions is out of the scope of this research, but what can be concluded is that OSPW TAEs had endocrine activity, and even aged samples had the same mechanistic potency as fresh samples. This warrants further testing to contribute to the existing weight of evidence to assess the effectiveness of ageing as a remediation strategy for endocrine activity.

A minor caveat in comparing the RTCA and YES/YAS results, is that YES/YAS does have a higher ratio of ethanol to sample (0.67%) compared to that used in the RTCA (0.25%). This may lead to solvent-enhanced effects due to easier cell uptake of organics in the YES/YAS. However, within each bioassay, the environmental samples are comparable to each other due to equivalent ratios of solvent between doses of extracts.

Also, no ER/AR agonists were found in this investigation among the BML 2015 fractions. Based on the results in the literature of YES/YAS and OSPW, it appears as though ER agonism was only found once the sample was sufficiently fractionated and isolated, while in the whole organic mixture an agonist effect was not observable.[71] Also, some of the reports of ER agonism

are statistically significant, but appear more so as weak agonists as opposed to strong agonists.[43,72] These principles were then applied to this investigation, with the assumption that if ER/AR agonists were not found within a fractionated sample of BML 2015, then the whole organic mixtures were even less likely of leading to observable agonistic effect. Therefore, ER/AR agonism was not tested for in the TAEs of OSPW samples to assess ageing, as it was deemed unlikely to be observable.

5. Concluding remarks

5.1. Summary

In summary, analytical techniques were used on various fresh and aged OSPW samples as part of a rudimentary toxicity evaluation strategy, focusing particularly on the TAE of the dissolved organic mixture, and associated SPE-generated fractions of BML 2015. We successfully reproduced two fractions of BML 2015 that were previously found to be the most acutely toxic:[51] the NAs, and a mixture of various polar non-acids. The heteroatomic chemical class distributions of OSPW of various ages showed unique profiles for Pond 9 (2016) but very little changes in BML over time. The dry organic mass of the TAE was a useful proxy for total organics concentration, and as OSPW aged the organic mass decreased, consistent with a decline in intensity of the corresponding HPLC-Orbitrap signal.

RTCA data indicated that whole BML 2015 water had a point of departure at ~50% dilution, though it was not potent enough for an IC_{50} to be calculated. Removal of the organics or adjustment of the pH only marginally impacted the toxicity profile to HepG2 cells based on point of departure, though RTCA did not seem sufficiently powerful to detect changes in cytotoxicity between candidate toxic components of the aqueous mixture when testing directly. Alternatively, a TAE of BML 2015 demonstrated a clear dose-response, where 1× did not significantly differ from negative control. Moreover, the point of departure approached but did not exceed field concentrations (1×). Together, this suggests that the inorganic components of OSPW were largely responsible for the observed cytotoxicity of whole BML 2015 towards HepG2 cells, while the organics were only cytotoxic slightly above their field concentration for this bioassay.

Syncrude Canada Ltd.'s acute toxicity data indicated lower toxicity of BML with ageing, as BML 2016 had LC_{50} values >100%, i.e. a non-lethal effluent. However, as mentioned, the point of departure for BML 2015 TAE in RTCA towards HepG2 cells was slightly above field concentrations, and at higher doses the TAE generated IC_{50}'s of ~8×, depending on exposure time. Overall, the toxicity profile of the BML 2015 TAE compared to the extraction and environmental controls indicated that the bitumen-derived organics were cytotoxic to a human cell line.

With comparing BML samples over a 4 year span, the cytotoxic effect on an enrichment factor scale decreased as the environment sample is aged, with IC_{50}'s increasing from ~7× to 9×, depending on experimental exposure time. However, on an absolute (i.e. mg/L) scale, the toxic potency of the organics in BML remained unchanged, consistent with similar chemical profiles measured by HPLC-Orbitrap.

A TAE from a 23 year old pond, Pond 9 (2016), displayed a unique time-dependent biphasic toxicity profile, such that after 24 h of exposure it was shown to be more potent than any BML sample, but then the cells recovered, coming to an asymptote in cell growth after 60 h of exposure. Whether this was due to transient narcosis, anti-mitotic, or aneugenic mechanisms remains to be seen. The differing TCRPs makes sense given the unique heteroatomic chemical class distribution of Pond 9 (2016), notably with positive mode species being more prominent in the whole organic mixture than negative mode species, which may contribute to a unique mode of action. In an engineered yeast assay, Pond 9 (2016) did not replicate the biphasic cytotoxicity profile, though while BML 2013 maintained its cytotoxic effect over experimental time, yeast treated with BML 2017 were able to recover, resulting in what again appeared to be a biphasic toxicity profile.

Testing BML 2015 fractions demonstrated that NAs were largely responsible for the observed cytotoxicity of BML 2015 TAE to HepG2 cells. Interestingly, the polar non-acid fraction used here was not cytotoxic to HepG2 cells, while in a previous study it was found to be the second most acutely toxic fraction in BML 2012 toward fathead minnow embryos. On a mg/L scale, the isolated NAs were much more potent when isolated compared to the whole organic extract, and the point of departure of 17 mg/L approached but did not exceed the estimated environmental concentration of 12.9 mg/L. With recombinant yeast, NAs continued to be the most cytotoxic chemical class, with complete cell death occurring near 5×; however, the polar non-acids were also cytotoxic at the highest doses, above 10×. Overall, there appears to be variability between test organisms, cytotoxicity assay used, and OSPW sample. NAs, however, were consistently the most acutely toxic chemical class in OSPW no matter the experimental parameters.

Among BML 2015 fractions, no ER or AR agonists were detected, whereas antagonists were detected throughout. Most notably, the polar non-acids and the NAs were endocrine active at doses below field concentrations in BML 2015, and in fact were more potent than hormones used as antagonistic positive controls. While the fractionation method used here was designed to isolate the most acutely toxic chemical classes, it was not successful in isolating the endocrine active chemicals, as seen by the dispersed antagonism throughout the fractions. Among the least potent fractions, W2 had a slight degree of cytotoxicity, while W1 had more environmentally relevant ER/AR antagonistic potencies. Interestingly, W2 contained NAs as one of its prominent classes, while W1 was more prominent with positive mode species. This may partially continue to support NAs as the most cytotoxic class, while polar non-acids only act through endocrine active mechanisms.

Looking at the TAE of environmental samples, it was clear that all OSPW-impacted samples had ER and AR antagonistic capacity, unlike the Athabasca River environmental reference. The antagonistic EC_{50} potencies of OSPW-impacted samples of 1 year old, 5 years old, or 23 years old largely did not change, interesting given the differences in concentration and chemical class distribution. Specifically, while the intensity of the negative mode chemical classes decreased with ageing, the positive mode chemical classes seemed to be more persistent and become more prominent in the whole organic mixture. Overall, this suggests that the positive mode chemical classes, particularly, O^+, O_2^+, O_3^+, and O_4^+ may maintain the antagonistic potency of OSPW-impacted samples over time, though NAs in F1 are clearly just as potent. The point of departures and EC_{50} values for all OSPW-impacted samples were well below estimated field concentrations, indicating possible environmental relevance of these results.

5.2. Environmental significance

The cytotoxicity of whole BML OSPW towards HepG2 cells decreased with ageing, which is promising for the EPL remediation strategy. This result is particularly important given the lack of mammalian-relevant research to date. That being said, the decrease in cytotoxicity was most likely due to dilution, and this was the only indication in this thesis research that toxicity decreases with ageing of the OSPW. A recurring theme in this investigation highlights the importance of composition of the OSPW organics, not just concentration. Notably, the toxic potency of the organics of BML on a mass scale remain unchanged, indicating that active dilution may be responsible for the reduction in cytotoxicity of whole BML OSPW, rather than *in situ* removal or degradation processes. Furthermore, looking at the organic profile of BML over the years, there are no profound changes in the distribution of heteroatomic chemical

classes, only the total intensity, consistent with the constant toxic potency. Important to note, BML has undergone a multi-faceted engineered effort involving natural ageing, active dilution, and addition of coagulating agents, yet the toxic potency of the organics remained the same. This is suggestive that more aggressive means may be necessary to reduce the toxic potency of the organics, such as ozonation,[84] bioreactors,[71] or Petroleum Coke treatments.[86]

It was suggested by Bartlett et al.[146] that the inherent complex nature of OSPW calls for water quality guidelines that are not solely based on concentration, but also on composition, and that guidelines will need to adapt as knowledge and technologies improve. The approach used here, of bioassays paired with ultrahigh resolution MS, further highlights the importance of OSPW organic composition. Compared to BML, the aged sample of Pond 9 (2016) showed a drastic decrease in negative mode species and a persistence of positive mode species, with a change in not just concentration, but composition as well. Interestingly enough, this change in composition may lead to unique toxic effects, and this is the first report of a temporally biphasic toxicity profile for an OSPW sample. This may indicate that the recalcitrant organics have a unique mode of action that is only uncovered when other organics are removed with ageing. Through an RTCA literature search I suggest that this RTCA response indicates an anti-mitotic effect.[121,130] Similarly, the potency of ER/AR antagonism was maintained between OSPW samples of different ages, and this is surprising given the large range of ages tested from 1 to 23 years old. Given the persistence and increased proportion of positive mode species over time, this suggests that the positive mode species may be responsible for maintaining the endocrine disrupting effects as well as the unique, potentially anti-mitotic, biphasic mechanism.

There are varied results for OSPW cytotoxicity in the literature, though I hypothesize that OSPW organics are cytotoxic through decreasing cell proliferation via an anti-mitotic

mechanism, at least at certain concentrations that are slightly above BML. This is supported here by the unique TCRP of Pond 9 (2016) being congruent with pure chemicals of specific mechanisms, as well as from observing flatlining of cell growth from BML 2015 at 12.5× (**Figure 14**). Within the literature, ambiguity of the term 'cytotoxicity,' may contribute to the varied results in **Table 2**, in that specific endpoints such as cell viability or cell proliferation are grouped together, which may lead to conflicting results if cell proliferation is affected at certain doses, but not viability. This hypothesis requires further investigation, particularly considering that these effects may vary depending on factors such as aqueous OSPW as a matrix.

Another finding from the bioassay and mass spectrometry approach is the divergence of mechanism between BML 2015 fractions. Given the unique extent of isolation and separation achieved between the NAs and the polar non-acids, the point of departures generated here are novel for their narrow degree of toxicity attribution. The NAs continue to be the most acutely toxic chemical class and are responsible for the majority of the cytotoxic effect of BML 2015 TAE, with a point of departure approaching estimated field concentrations. While the fractionation technique isolated the acutely toxic fraction, it did not isolate the endocrine activity. Instead, the candidate species were distributed throughout the fractions at different potencies, with NAs and polar non-acids being by far the most potent chemical classes, with point of departures for ER/AR antagonism well below environmental concentrations and antagonistic hormone equivalencies. Therefore, the divergence of mechanism is highlighted with polar non-acids not being acutely toxic to a human cell line, but having an alternative and potent endocrine active mechanism of action through ER/AR antagonism. In contrast, NAs have both a potent cytotoxic effect and ER/AR antagonistic mechanism. Moreover, some endocrine active species

are in W1 and W2 fractions as well, suggesting that distinguishing chemical features other than chemical formula class may be necessary to isolate and separate these species.

Granted that endocrine disrupting compounds are problematic for regulation due to their low dose effects and potential for disruptive reproduction and development, polar non-acids may be just as toxicologically relevant as NAs in the EPL strategy, particularly when considering that positive mode species are potentially more bioaccumulative[50] and persistent. As a starting point in this pursuit, the point of departure values of NAs and polar non-acids could contribute to the chemical-specific information needed to advise safe release, as well as inform the endpoints needed to be looked at among organisms in the downstream environment during biological monitoring. For example, the point of departure of NAs for cytotoxicity was 17 mg/L, but was much lower for the sub-lethal endocrine active antagonism – 0.019 mg/L for the AR and 0.36 mg/L for the ER. Overall, both the NAs and polar non-acids may need to be targeted as part of the chemical-specific approach for environmental protection to enable safe release.

With ageing as a remediation strategy, BML 2017 is currently the closest sample in this investigation to the current status of BML. Its TAE had an IC_{50} of ~8×, while its point of departure was marginally above 1× in cytotoxicity measurements with a human cell line. Towards recombinant yeast, the growth factor was reduced by 50% at a dose of ~ 7×, and a biphasic toxicity profile surfaced for the 10× dose, where the yeast were largely killed at 18 h post exposure, though recovered by 46 h. While the IC_{50} was much higher than field concentrations, there are possible synergistic effects when the organic and inorganic components are together, though Syncrude's data indicates that the whole BML 2016 effluent is now largely passing the acute toxicity tests. Acute toxicity testing does not consider chronic exposure, sub-lethal effects, bioaccumulation, and chemosensitization, which could all lead to environmentally

relevant effects in surrounding life. Therefore, more research is needed to find the limits for environmental protection.

In assessing the effect of ageing on endocrine activity, the EC_{50}'s and point of departures for ER/AR antagonism were far below field concentrations in all OSPW samples. Determining if endocrine activity will lead to a potent adverse effect beyond scope of this investigation, but here all OSPW TAEs had endocrine activity, and mechanistic potency did not change with age. The chemical-specific thresholds found here could add to guideline development. Also, by knowing the mechanism of action, this could inform the biological effects monitoring strategy used through the selection of endpoints that would typically be affected by anti-estrogens and anti-androgens.

In terms of the EPL strategy, the fact that after 23 years of ageing, Pond 9 2016 was just as endocrine active as BML 2013 at sublethal doses is concerning, especially considering the volume of aged OSPW that is intended to be connected into the environment in the future, i.e. comprising 0.5% of the total volume of the downstream Lake Athabasca.[9] While dilution may be effective at decreasing the cytotoxicity of the whole effluent, the lack of change in toxic potency of the organics does become problematic for sublethal effects, warranting more aggressive treatments. Even though the chemical class distribution did not change between the different BML samples, there must be some unknown process for the organics to end up as Pond 9 (2016), and to correspondingly generate a unique toxicity profile by RTCA. Importantly, the biphasic toxicity profile of the aged sample itself does not signify detoxification, and further exploration of its mechanism is needed, as opposed to being assumed insignificant and safe for release.

5.3. Future direction

The predominant toxic effects may depend on the source of OSPW, the dose at which the organics have an effect, and whether the organics are extracted or present in its aqueous OSPW matrix. There is value in being able to monitor human-relevant cells through RTCA, in that it is rapid, high-throughput, inexpensive, non-lethal, suitable for unknown complex mixtures, and measures at many time points. Therefore, this technology may be effective in monitoring OSPW organics as they age, in comparing various sources, or in testing advanced treatment methods, such as Petroleum Coke. Also, by using a battery of cell lines, this could potentially screen for multiple mechanisms of action on target organs, even across species.

Of particular relevance to this investigation, RTCA technology can be used additionally to the micronucleus assay to assess genotoxicity, and this approach would give further insight into the unique biphasic profile of Pond 9 TAE that I suspect is anti-mitotic. Potentially even testing Pond 9 (2016) in its aqueous state, its organic extract, or isolated into fractions to attribute toxicity to particular chemical classes. Other aged experimental ponds could also be tested, particularly Pond 5, which is most similar to BML in its physical construction.

The potency of the YES/YAS antagonism among BML 2015 fractions and the TAE of environmental OSPW samples was a surprising result. Importantly, YES/YAS is only a level 2 trans-activational screen, as set by OECD guidelines,[139] so more research is needed through a weight of evidence framework to determine if the detected endocrine activity here can lead to an apical adverse effect. Once there is a sufficient weight of evidence, only then could the OSPW TAE be categorized as an endocrine disruptor. An attempt was made to screen for human-relevant endocrine active effects through RTCA and MCF-7 cells (data not shown), though there were issues with the approach, which impeded any conclusions from being made. However,

further exploration of human relevant endocrine activity is needed. Alternatively, more standardized and comprehensive mammalian tests are the uterotropic bioassay (estrogen modality) or Hershberger bioassay in rodents (androgen modality),[165] but due to animal use these should only be justified after *in vitro* studies are complete. While polar non-acids and NAs were the most potent classes, our fractionation technique did not isolate all of the responsible chemical classes; therefore, a new EDA for endocrine activity could be explored, or a Pull-down assay with Untargeted Chemical Analysis (PUCA) for the ER/AR. Also, ER/AR are only two receptors in the endocrine system, with *in vivo* biomonitoring studies implicating the TR,[93] PPARγ,[27] PXR and CAR,[106] and AhR.[70] Therefore, furthermore exploration of these receptors are warranted.

Implicit throughout this investigation is the importance of high resolution MS detection in both negative and positive mode, as polar non-acids may be just as toxicologically relevant as NAs. Moreover, an effective extraction method is needed to enable proper toxicity attribution in bioassays, where some methods only try to isolate the NAs. Here, only one extraction was performed for each 1 L of OSPW; therefore, more extraction replicates may be necessary to demonstrate reproducibility of the experiment. Also, a more thorough analysis of which chemical classes are the most persistent is needed in controlled experiments, as this was not the main focus of this investigation.

To prevent an expensive and toxic legacy for future Canadian generations, it is argued that technological strides towards safe release of OSPW need to be taken and implemented soon while the financial gains from oil sands exploitation are still being realized.[9] Therefore, more research on the chemical-specific toxicity limits and the toxic mechanisms of action for biological monitoring is needed, as this could help in designing guidelines for OSPW treatment and management to ensure a safe release by the many operators.[9]

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