In vitro bioassay monitoring to assess baseline conditions prior to potential discharge of treated oil sands process water in receiving aquatic environments

by

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Abstract

The Alberta oil sands is one of the largest oil reserves in the world. However, there are concerns regarding the potential environmental and human health impacts of the oil sands development. During oil sands mining, large volumes of oil sands process-affected water (OSPW) is produced, which can only be recycled and/or stored in tailings ponds as there currently are no guidelines regarding its treatment and release into the receiving environment. OSPW is a complex mixture consisting of bitumen, organic and inorganic compounds, and solid materials, which can cause acute and sub-chronic toxicity to a range of aquatic organisms including invertebrates and fish. Recently, there have been efforts in motion by the oil sands industry to treat and release OSPW into the Lower Athabasca River, which is being assessed by the Federal and Alberta governments.

This study uses a battery of seven *in vitro* bioassay tests to perform a bioanalytical assessment of untreated OSPW, surface water samples from 15 sites along the Lower Athabasca River and the effluents from two regional municipal wastewater treatment plants (WWTPs). All samples were filtered and enriched using solid phase extraction and the reconstituted extracts were used for chemical (FTIR) and bioanalyses.

The main objective of this research was to assess the current bioactivity of the Lower Athabasca River. The same battery of *in vitro* bioassays was used to investigate whether *in vitro* bioassays can differentiate untreated OSPW from other types of water samples (i.e., municipal effluents and surface waters) by determining the relevant toxicity pathways. Finally, the bioassay responses were compared to the ecological effects-based trigger (EBT) values appropriate for each assay. The tests covered five toxicity endpoints: cytotoxicity, estrogenicity, mutagenicity, oxidative stress response and xenobiotic metabolism.

Untreated OSPW showed high responses to all bioassay tests employed where 5 out of 7 signals exceeded the published EBT values. This result indicated that *in vitro* bioassays can be applied in the oil sands industry, and that all endpoints targeted in this study are relevant to this industry with cytotoxicity, estrogenicity, mutagenicity, and binding to the PPARɣ as primary toxicity indicators and oxidative stress and the induction of the AhR as secondary indicators.

Although the WWTPs were active in most bioassays, the potential toxicity risks associated with direct exposure to these effluents are likely reduced due to the mixing patterns of the river. The low bioactivity responses of samples from the Lower Athabasca River suggests that there are low toxicity risks with its current stressors, but there is the potential for increased toxicity risks due to the discharge of OSPW-related contaminants if treated OSPW is released into the river. Finally, this study demonstrates that there is a relationship between bioactivity of the river and its hydrologic conditions due to the higher responses observed in June during high-flow conditions than in August (low-flow). However, further investigation is needed to establish this relationship.

Preface

This thesis is an original work by Kia Barrow under the supervision of Dr. Maricor Arlos. No part of this thesis has been previously published.

Dedication

This work is dedicated to the resilient, unstoppable, and intelligent women who came before me and changed the way to the world looks at engineers. It is also dedicated to the confident, strong, and determined women who will come after me.

‘There have been lots of other women who had the talent and ability before me. I think this can be seen as an affirmation that we're moving ahead. And I (know) it means that I'm just one in a long line.’ – Mae Jemison.

Acknowledgements

This work would not have been possible without the love and guidance of many people. My deepest gratitude to my supervisor, Dr. Maricor Arlos for her friendship, kindness, encouragement, humor, and patience. I am truly proud and grateful for the time we have spent together. This experience has been more fulfilling than I would have ever imagined. Thank you again.

Many thanks to the members of the Arlos Lab (current and past) for their encouragement, assistance, and company in and outside of the lab. I would also like to thank Dr. Keegan Hicks, and Dr. Beate Escher for their guidance and patience, and for providing me their valuable suggestions throughout this work. I also greatly thank Dr. Rita Schlichting, and Maria König for analyzing some of my samples at UFZ, and for the late evening calls. Many thanks to the Ulrich Lab for assisting me in your lab. I would also like to thank Kristin Hynes for her support and motivation during the field sampling campaign. Special thanks to Drs. Mohamed Gamal El-Din and Pamela Chelme-Ayala for your assistance during the start up of our lab. This work would not have been possible without all of you.

Most of all, to my family and loved ones near and far, thank you for your unconditional love, motivation, and support every day.

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1. Introduction

1.1. Background

The Alberta oil sands is one of the largest oil reserves in the world, estimated to hold approximately 165 billion barrels of bitumen, with crude bitumen production rates of about 2.8 million barrels per day [1]. However, there are public and scientific concerns regarding the potential environmental and human health risks associated with the oil sands development, especially about infiltration and seepage of untreated oil sands process-affected water (OSPW) into groundwaters and subsequently into the Lower Athabasca River (LAR) [2]. Some of these health risks include the decline in fish population, reproductive impairments, changes in benthic communities, bioaccumulation of organics in organisms, hormonal effects, impacts on immunotoxicity, carcinogenicity, and developmental issues in exposed organisms including invertebrates, fish, mammals, and humans [2, 3]. Concerns have also been raised by the nearby Indigenous population who no longer believes that the fish are safe for consumption due to a noticeable decline in the fish population and health which may be attributed to the contamination of the LAR from the oil sands development and other industrial pollutants [4].

During the extraction process of oil sands mining, a ‘tailings’ by-product (i.e., a mixture of water, sand, clay, and residual bitumen) is generated [5]. Industry operators either recycle some of the water from these ponds back into the extraction process or the remaining water is stored in tailings ponds as OSPW. OSPW is a complex mixture comprising of organic compounds (e.g., naphthenic acids [NAs], polycyclic aromatic compounds [PACs]), inorganic chemicals (e.g., metals, salts), and solid materials (e.g., sand, clay)) [6]. The characterization of OSPW is challenging as the composition of OSPW varies widely due to differences in source deposits and extraction technologies among the industry [7].

There is no guideline regarding the release of treated OSPW back into the receiving environment. As a result, Alberta's tailings ponds have held over 1 trillion litres of OSPW since its operation began [8]. However, efforts are in motion by industry operators to develop technologies to sufficiently treat OSPW and therefore allow for its discharge into the LAR. The Alberta and Federal governments are working alongside the industry to establish regulations to protect the environment and human health once the treated OSPW is released [5]. The potential for the discharge of treated OSPW led to the formation of the OSPW Science Team which is mandated to (1) predict the potential impacts of the release of treated OSPW on environmental and human health, (2) establish a baseline of the environmental conditions of the LAR, and (3) identify relevant biological and ecological endpoints for toxicity testing concerning both acute and chronic toxicity [9].

Extensive research has gone into evaluating the toxic effects of untreated OSPW but it can be challenging to compare the toxic effects across studies due to the complexity of OSPW, the variability of its constituents, the variations of OSPW type (e.g., fresh, mature, fractionated) and the confidentiality of the OSPW sources [10, 11]. Significant research has focused on the toxicity of the organic fraction of OSPW. Several studies have suggested that NAs are the primary contributors to toxic effects such as endocrine disruption, reproduction issues, and acute toxicity in various organisms including fish, invertebrates, and mammals [12]. Recent studies have found that OSPW toxicity may also be attributed to other organic compounds such as PACs, but information on the contribution of these compounds to toxicity is limited [10, 11].

Assessing the environmental quality resulting from organic chemical pollution can be done via: (1) chemical analysis – a targeted and/or non-targeted approach to quantify chemical concentrations, (2) modeling – a predictive approach to estimating chemical concentrations, and

(3) bioanalytical tools - focuses on cell toxicity pathways and combined biological activity. Chemical analysis is considered the gold standard in environmental monitoring. With the recent advancement in analytical chemistry techniques and instrumentation, extremely low concentrations in complex environmental matrices can now be detected [13, 14]. This field is very active in the oil sands research area, and additional techniques are being developed to improve selectivity and sensitivity, and further reduce variabilities in measured datasets.

Fate and transport modeling is founded on the principles of mass balance. It includes the prediction of target contaminants upon their release into the receiving environments. The LAR has been modeled under various configurations (i.e., one- to two-dimensional) for several classes of water quality constituents including both organic substances (e.g., NAs, PACs) and metals (e.g., lead, arsenic, vanadium) [15]. However, for a model to be ultimately employed for prediction purposes, it requires analytical measurements to validate its applicability.

1.2. Bioanalytical tools for exposure assessment

Bioanalytical tools such as *in vitro* bioassays can be used to complement chemical analysis and modelling approaches for water quality assessment. Typically, chemical analysis is used to quantify the occurrence of individual compounds and assess whether concentrations collected at a given time are below the applicable thresholds expected to cause adverse effects on their own [16]. However, chemical identification and quantification is only the tip of the iceberg (Figure 1.1). Chemical analysis is limited by sample preparation, analytical methods and instruments, concentration of pollutant, and availability of appropriate standards. It also requires *a priori* knowledge of target compounds and their relevant transformation products. The rest of the iceberg as seen in Figure 1.1 is the unknown chemical risks that chemical analysis can not

properly identify, thus preventing the ability to observe a much bigger picture of exposure risks [16].

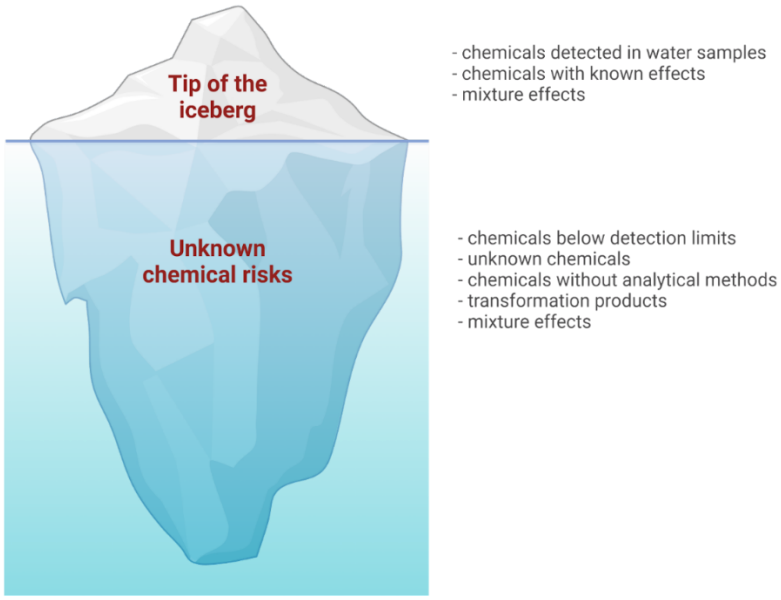

Figure 1.1. Detection of organic pollutants using chemical and bioanalytical tools. Adapted from Neale et al. (2020) [17].

Bioanalytical tools including *in vitro* bioassays can capture the whole iceberg, by measuring the cumulative effects of bioactive compounds which may be present below chemical detection limits at micro-, nano-, or pico-concentrations but act through a similar mode of action [16]. These tools are suitable for use with samples with low levels of contaminants and can be modified to increase sensitivity and automation for high-throughput screening [16]. More specifically, *in vitro* bioassays use cells to simulate the processes that occur when an organism is exposed to chemicals. These cellular processes are categorized as the toxicokinetic and toxicodynamic phases (Figure 1.2). The toxicokinetic phase refers to the uptake, distribution, and

elimination processes such as absorption, excretion, and metabolism, which link the external exposure of the contaminant to the biological target concentration [16].

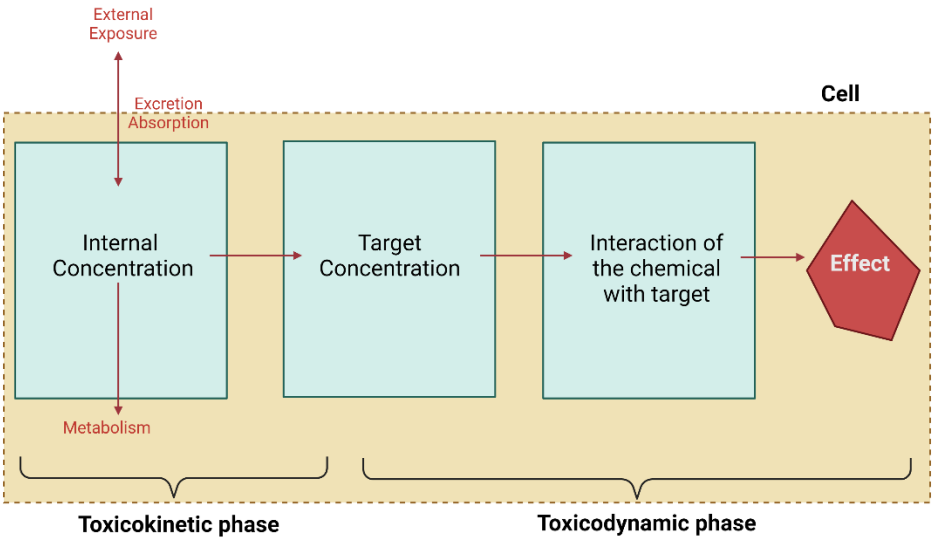

Figure 1.2. Cell toxicity pathways from exposure (toxicokinetics) to effect (toxicodynamics). The yellow region represents the cell structure. Adapted from Escher and Hermens (2002) [18].

Next, the toxicodynamic phase refers to the interactions occurring within the cell, starting with the molecular interaction between the chemical and the biological target (e.g., receptor). Eventually, this molecular interaction may induce cellular mechanisms and responses (e.g., protein signalling and activation of genes) that result in observable toxic effects. This cellular response pathway is also called a toxicity pathway, which can be identified using *in vitro* bioassays. Due to the high sensitivity of these tools, cellular responses may not always imply higher-level effects but the detection of the initiation of key events may indicate potential adverse effects [19]. This molecular initiation event is a critical assessment endpoint as cellular responses may be linked to responses at the organ level, followed by effects on the organism and a population, and ultimately may threaten the health of an ecosystem.

In vitro bioassays are not meant to replace chemical analysis as these tools can not identify the compounds responsible for the observed toxicity. Instead, they can be used as a screening technique to provide information on the prioritization of further chemical testing [16]. Therefore, combining *in vitro* bioassays with chemical analysis can provide a more comprehensive toxicological profile of a water sample.

Most studies using *in vitro* bioassays have focused on surface waters and municipal wastewaters, with recent works also targeting drinking and industrial waters. These bioassays have often been applied as a tool to monitor water quality over time and from various locations, typically to evaluate treatment efficiencies and to compare natural and engineered treatment processes of municipal WWTPs, and reclamation and drinking water treatment plants [16].

Although no standardized set of bioassays is recommended for water quality assessment as the endpoints of interest may vary, specific bioassays have become more popular based on the type of water sample (Figure 1.3). For instance, with municipal wastewaters, hormone receptor-mediated effects including estrogenicity are often employed as it is critical to ensure that the treated effluents will not threaten the reproductive health of receiving aquatic ecosystems upon discharge [20]. However, for drinking water, adaptive stress response and reactive toxicity endpoints such as genotoxicity and mutagenicity are of main concern due to the presence of disinfection by-products which may induce human health risks [16]. Surface water quality assessments are dominated by hormone receptor-mediated effects and xenobiotic metabolism, typically due to the impacts of municipal, industrial wastewater, and stormwater effluents in these environments. Therefore, as this research field progresses and more toxicity tests covering different endpoints and receptors are developed, it opens the opportunity for *in vitro* bioassays to be applied in the oil sands industry.

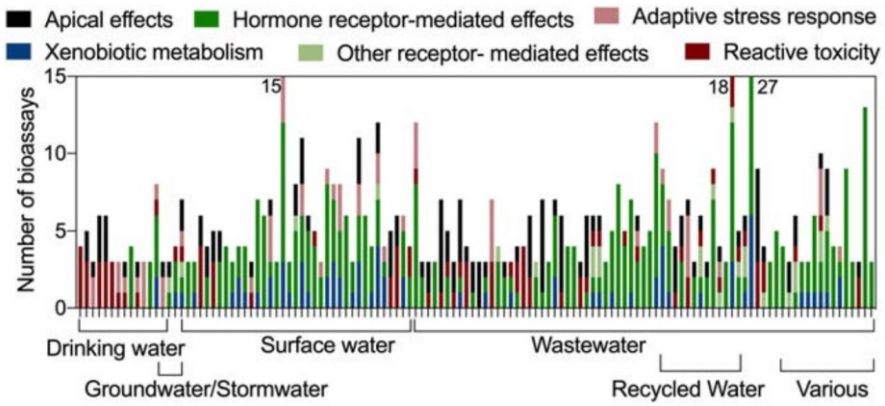

Figure 1.3. Studies published until September 2020 using *in vitro* and *in vivo* assays. Adapted from "Bioanalytical Tools in Water Quality Assessment: Second Edition" by Escher et al. (2021), p 305 [16]. Different endpoints may be used depending on the water matrices of interest.

1.3. *In vitro* bioassays in OSPW

Recent studies have used *in vitro* bioassays to investigate the potential adverse effects of OSPW (Table 1.1), but the majority of work has been done using animal models (e.g., invertebrates and fish) [10]. Most of the *in vitro* studies have employed the Microtox assay, a simple bioassay to assess the cytotoxicity of whole OSPW and/or its organic fractions, by measuring the inhibition of bioluminescence of *Aliivibrio fischeri* (formerly *Vibrio fischeri*) bacterium (Table 1.1). This assay has commonly been used in research to evaluate the efficiency of various developing treatment technologies (e.g., ozonation, oxidation) targeting the reduction of toxicity of OSPW-derived organic compounds, especially NAs [21]. More recently, the use of *in vitro* bioassays, especially with human cell lines, has been identified as a critical research gap as endpoints related to liver and immune responses in these cell lines including development, reproduction, metabolic and immune patterns of mammals can inform potential human health impacts of OSPW exposure [3].

Currently, provincial environmental monitoring programs (discussed in Section 1.5) and numerous research studies have focussed on the quality of surface water in the Athabasca River Watershed near the oil sands development. The vast majority of research has used chemical analysis to establish the contaminant profiles including OSPW-related compounds (e.g., NAs, PACs), major ions (e.g., calcium, sodium, sulphate) and inorganic chemicals (e.g., heavy metals-zinc, arsenic, iron) in the LAR and its tributaries [22, 23]. Other studies have used predictive modelling to estimate the loadings of contaminants under various discharge scenarios, including seasonal conditions, and periodic release patterns [3].

Information related to the use of *in vitro* bioassays to assess the toxicity of the surface water in the LAR is limited as most studies have focused on microbial, benthic, and fish communities to evaluate the toxicity impacts of the LAR [24, 25, 26]. A recent study analyzed the change in gene expression related to cytotoxicity, xenobiotic metabolism, estrogenicity, genotoxicity, and oxidative stress, using rainbow trout hepatocytes upon exposure to LAR surface water samples [27]. This work has further reiterated that toxicity studies using *in vitro* bioassays with human cell lines are needed to evaluate the potential adverse effects in the receiving environments regarding exposure routes and exposure concentrations [3].

Table 1.1: Published studies using *in vitro* bioassays for OSPW research. The type of OSPW (fresh, aged, treated, fractionated) are also indicated. WIP: west-in-pit, OF: organic fraction, AEOs: acid extractable organics, IF: inorganic fraction, RCW- recycle water, BMDM: bone marrow-derived macrophages, MLSB- Mildred Lake Settling Basin, PC- petroleum coke, AOP: advanced oxidation process

OSPW type	Test organism	Toxicity endpoint	Reference
Fresh & aged - fractionated	3T3-L1 preadipocytes cells	Activation of PPARɣ signalling	[28]
WIP- OF- untreated and treated using ozonation	C57BL mouse BMDM	Immunotoxicity	[29]
WIP- untreated and treated using ozonation	C57BL mouse BMDM	Immunotoxicity	[30]
Fractionated & AEOs	*Escherichia coli* strain PQ37	Cytotoxicity and genotoxicity	[31]
Untreated and treated using AOP	Goldfish primary kidney macrophage	Acute toxicity and antimicrobial response	[32]
WIP- untreated and treated using ozonation	H295R cells	Cytotoxicity and disruption of sex hormone production	[33]
Fractionated aged tailings water	H295R cells	Disruption of sex hormone production	[34]
Fractionated aged tailings water	H4IIE-*luc* cells	Cytotoxicity and binding to the aryl hydrocarbon receptor	[34]
WIP- untreated & treated using ozonation	MDA-kb2 cells	Androgenic response	[35]
Whole, OF, IF and reconstituted-OF-IF	RAW 246.7 mouse macrophage	Acute toxicity	[36]
Whole, OF, IF	RAW 246.7 mouse macrophage	Immunotoxicity	[37]
Fractionated aged tailings water	Recombinant yeast *Saccharomyces cerevisiae* cells	Induction of estrogen and androgen receptor	[34]
WIP- fractionated- untreated and biologically treated	Recombinant yeast *Saccharomyces cerevisiae* cells	Activation of estrogen receptor	[38]
WIP- Fractionated	RTgill-W1 cell line	Cytotoxicity and uptake of ionizable organic chemicals	[39]
MLSB- fractionated	*Salmonella* strains TA98 and TA100	Mutagenicity	[40]

Fractionated & AEOs	*Salmonella* strains TA98 and TA100	Cytotoxicity and mutagenicity	[31]
WIP- untreated & treated using ozonation	T47D-kbluc cells	Estrogenic response	[35]
Untreated and treated using photocatalytic degradation	THP-1 cell	Detection of immune cell activating compounds	[41]
MLSB- fractionated	*Vibrio fischeri* strain M169	Mutagenicity	[40]
RCW pond- untreated and treated using ozonation	*Vibrio fischeri* bacteria	Acute toxicity	[42]
WIP- fractionated	*Vibrio fischeri* bacteria	Acute toxicity	[43]
WIP- untreated and treated using ozonation	*Vibrio fischeri* bacteria	Acute toxicity	[44]
WIP & coke-treated	*Vibrio fischeri* bacteria	Acute toxicity	[45]
WIP, RCW pond, MLSB- untreated and treated with PC adsorption	*Vibrio fischeri* bacteria	Acute toxicity	[46]
Untreated and treated using simulated wetland	*Vibrio fischeri* bacteria	Acute toxicity	[11]
Fresh- AEOs- untreated	*Vibrio fischeri* bacteria	Acute toxicity	[47]
WIP- untreated and treated using ozonation	*Vibrio fischeri* bacteria	Acute toxicity	[30]
Untreated and treated using AOP	*Vibrio fischeri* bacteria	Acute toxicity	[32]
WIP- untreated and treated using ozonation	*Vibrio fischeri* bacteria	Acute toxicity	[48]
WIP- fractionated	*Vibrio fischeri* bacteria	Acute toxicity	[49]
Untreated and treated using coagulation/flocculation process	*Vibrio fischeri* bacteria	Acute toxicity	[50]
Untreated and treated using UV/oxidation	*Vibrio fischeri* bacteria	Acute toxicity	[21]
Whole & AEOs	*Vibrio fischeri* bacteria	Acute toxicity	[51]
Untreated and treated using photocatalytic degradation	*Vibrio fischeri* bacteria	Acute toxicity	[41]

1.4. Study objectives

This study aims to evaluate the current bioactivity of the LAR using a battery of *in vitro* bioassays. The same bioassays were applied to untreated OSPW to investigate whether *in vitro* bioassays can be employed as a monitoring tool in the oil sands industry, and to determine the relevant toxicity pathways of concern. Additionally, two municipal wastewater treatment plant effluents were included to contrast the responses of municipal and industrial wastewaters. Finally, the responses of our samples were compared to the published ecological effect-based trigger values – a threshold that differentiates between acceptable and poor water quality.

The data in this study may be used in future research to compare the bioactivity of the river before and after the potential discharge of treated OSPW and to determine any long-term effects. The outcome includes recommendations on (1) establishing the correlation between the hydrologic conditions and the pollutant concentrations of the river (2) future monitoring programs as the discharge of treated OSPW may lead to complex interactions between OSPW-related contaminants and contaminants from WWTP effluents and (3) an *in vitro* bioassay test battery with primary and secondary indicators applicable to the oil sands industry.

1.5. Study scope

A field sampling campaign was conducted to collect water samples from 15 sites along the LAR, and two municipal effluents discharging into this river. Untreated OSPW was also included in our sample set. The samples were extracted and concentrated using solid phase extraction (SPE) and then the reconstituted extracts were analyzed using chemical and *in vitro* bioassay analyses. For chemical analysis, Fourier Transform Infrared Spectroscopy (FTIR) was used as a semi-quantitative method to analyze the acid extractable organics of the samples.

The river sample sites in June align with the sampling design of the Joint Oil Sands Monitoring (JOSM) plan while the August river sites align with the Enhanced Monitoring Program (EMP). The Governments of Canada and Alberta established the JOSM plan to monitor the water quality and quantity, air quality and biodiversity of the LAR and its tributaries [52]. This monitoring program provides information related to the water quality, the distribution of contaminants and the contribution of toxic substances to the river by natural or anthropogenic activities. The JOSM study design involves the collection of water samples for the quantification of 270 water quality parameters, including nutrients, major ions, metals, mercury, cyanide, phenol, petroleum hydrocarbons (e.g., BTEX) , NAs, and PACs [52].

The EMP was later introduced, along with the OSPW Science Team, to establish the baseline environmental conditions of the LAR before the potential discharge of treated OSPW, and to assess the potential associated environmental impacts [9]. This study design involves the collection of water samples for similar chemical analysis as in the JOSM plan, in addition to data elements on the fish communities, benthic macroinvertebrates, sediment quality and grain size, and algae taxonomy [9].

In both these monitoring programs, extensive chemical analysis is performed on the surface water samples to assess the water quality, but a gap exists as they do not include the use of a battery of bioassays to evaluate the bioactivity of the samples. Even with this chemical analysis, it is likely that there will still be substances that can not be identified and/or quantified and the mixture interactions between compounds at low concentrations cannot be captured. Hence, an improved design for these monitoring programs would include *in vitro* bioassays to identify the potential exposure risks of the samples by accounting for a wide range of toxicant groups that may not be detected through chemical analysis. These bioassays may also prove to be

a more cost-effective method, as they can be used as a screening tool to direct additional chemical analysis that could identify the causative agents of the observed toxicity. This may result in a better allotment of time and resources in identifying priority chemicals relevant to the risk assessment.

1.6. Selection of the battery of *in vitro* bioassays

A battery of *in vitro* bioassays was chosen for this study based off the following criteria. The first criterion was to include toxicity pathways that cover the three classes of modes of action (MOA): non-specific, specific, and reactive toxicity, as recommended by Escher et al. (2021). A MOA refers to a common set of signs that characterize a particular adverse biological response caused by a range of biochemical processes and/or interactions between xenobiotics and an organism [16]. This criterion is important as groups of chemicals with a common MOA act together in mixtures, and a single chemical can act through different MOAs based on exposure duration and target organism. Therefore, by covering the three classes of MOAs, it is more likely to capture a wider picture of the potential adverse effects, allowing for a more comprehensive toxicity assessment of a mixture.

Secondly, a comprehensive literature review was conducted which looked at the toxicity pathways that have been previously identified as being relevant to untreated OSPW through similar and other forms of bioanalyses (Table 1.1). Next, the test battery was chosen based on the protection goal of the samples. Most of the samples used in this study (i.e., surface water and municipal effluents) have the potential to threaten aquatic ecosystem health. Therefore, an emphasis was placed on including relevant pathways that may potentially be impacted upon exposure to these samples. Moreover, it was important to also include bioassays using human

cell lines to obtain information on potential impacts on human health as there are estimated 155,000 Indigenous residents living within or adjacent to the Lower Athabasca region [4]. Ultimately, the final battery of bioassays consisted of seven *in vitro* tests (Figure 1.4).

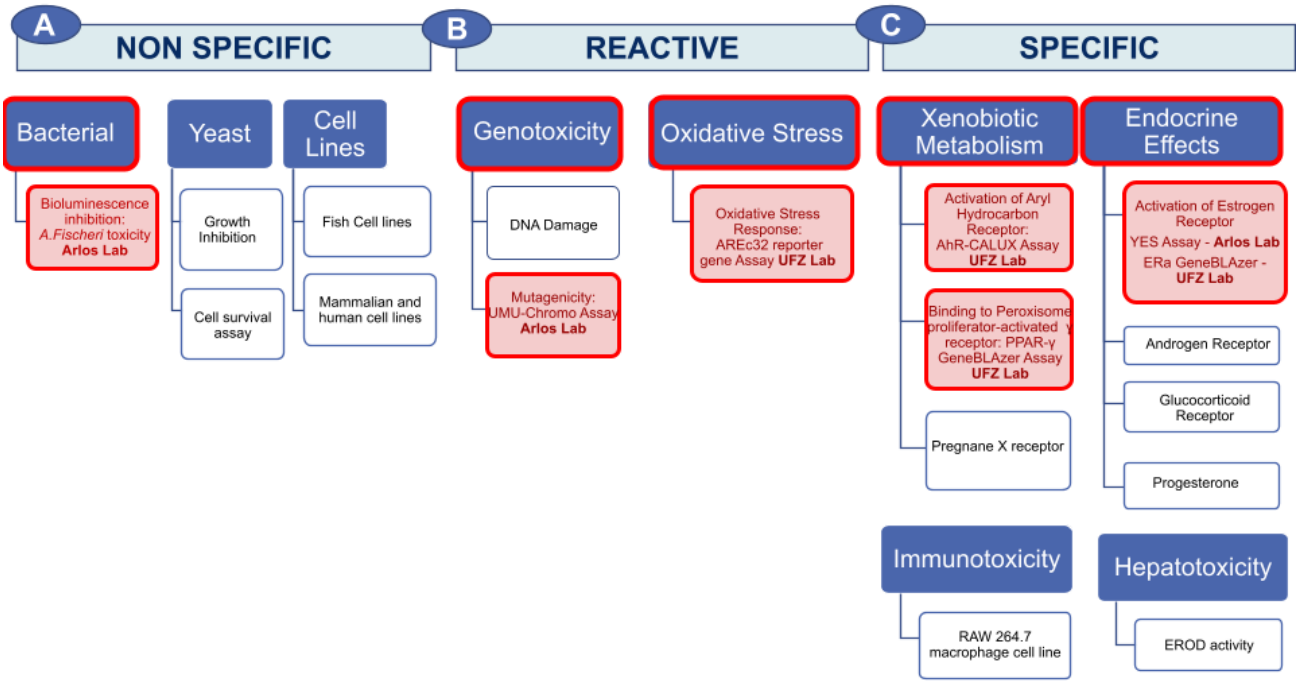

Figure 1.4. Selection of toxicity pathways. *In vitro* bioanalyses highlighted in red were run in this study based on selection criteria (please see text above). Each assay is discussed in detail subsequently below.

Non-specific toxicity includes all cytotoxic responses that lead to the dysregulation of normal cellular activity [16]. For this MOA, cytotoxicity was measured using *Aliivibrio fischeri* bacteria. This well-established method is used to determine the overall toxic effect of the mixture where cytotoxicity is calculated based on the inhibition of luminescence of the bacteria [21]. This assay is the most frequently employed bioassay in the assessment of OSPW toxicity due to its simplicity, quick results, and high sensitivity to organic compounds (Table 1.1) [10].

Reactive toxicity refers to the chemical reactions that occur between the chemical and biological molecules [16]. These MOAs include mutagenicity and oxidative stress. Mutagenicity is the effect caused by a physical or chemical agent that changes the genetic material (e.g., DNA), resulting in a higher-than-normal frequency of mutations in an organism [20]. In this study, mutagenicity is assessed using the UMU-ChromoTest assay – a method which uses genetically engineered *Salmonella typhimurium* TA1535 to measure the response to genetic damage through colorimetric evaluation. This method is based on the principle that the umuC gene is directly involved and responsible for the induction of mutagenesis [53]. This assay was included due to the known mutagenicity of PACs which are found in OSPW, and municipal WWTP effluents [31, 53]. Based on this, mutagenicity was a critical endpoint to contrast the responses between the types of water samples used in this study.

Oxidative stress response (OSR) is a type of adaptive stress response – a pathway that plays a critical role in returning a cell to homeostasis after damage by stressors [16]. Typically, the presence of electrophilic chemicals and chemicals that produce reactive oxygen species (e.g., disinfection by-products, pesticides, pharmaceuticals) releases the Nrf2 transcription factor which activates the antioxidant response element (ARE) in mammals, therefore inducing the OSR [54, 55, 56]. Escher et al. (2013) found that the OSR can be induced by a wide range of chemicals that can directly or indirectly produce reactive oxygen species, and as such the induction of the OSR may be better suited as an early warning of potential adverse effects due to its increased sensitivity [56]. The AREc32 reporter cell line was generated by Wang et al (2006) and adopted by Escher et al. (2013) for water quality assessment [57, 56]. This cell line uses breast cancer cells and allows for the luminescence measurement in response to various chemicals [56]. The AREc32 reporter gene assay was used to measure the OSR in this study.

Specific toxicity refers to all mechanisms by which specific groups of contaminants (e.g., endocrine disrupting compounds) selectively bind to a receptor or interfere with an enzyme function [16]. Typical bioassays employed for this type of toxicity target endocrine effects (e.g., activation of the estrogen receptor) and xenobiotic metabolism as in the detection of the induction of the aryl-hydrocarbon nuclear receptor (AhR) and binding to the peroxisome proliferator-activated receptor (PPAR).

Endocrine disrupting compounds (e.g., natural and synthetic hormones, alkylphenols, phytoestrogens, pharmaceuticals) are commonly found in wastewater and environmental samples and are known for their toxic effects on the hormonal systems of aquatic organisms which may lead to issues with sexual, development and behavioral patterns [58, 59, 60]. Estrogenicity is a relevant pathway for the health of both aquatic ecosystems and humans. It has been well studied that WWTP effluents are a significant source of estrogenic compounds in surface waters, therefore this endpoint was critical in distinguishing between the responses of municipal effluents and untreated OSPW [60, 61]. Moreover, recent studies have shown that OSPW exhibits estrogenic behavior and thus, estrogenicity is an important endpoint when evaluating the toxicity of OSPW-derived extracts [62]. Commonly employed assays targeting the estrogen receptor (ER) include the yeast estrogen screen (YES) and ERα-GeneBLAzer tests [58].

The YES assay uses recombinant *Saccharomyces cerevisiae* yeast which has been transfected with the human estrogen receptor and an expression plasmid carrying the reporter gene *lac-Z* encoding for the enzyme β-galactosidase. Once the yeast is exposed to estrogenic compounds, the β-galactosidase metabolizes the ONPG substrate producing a quantifiable luminescence response [61]. Although the YES assay is simple and inexpensive, there are some limitations such as its relatively high limit of detection, and potential matrix interferences from

compounds such as anti-estrogens [63]. These factors motivated the decision to also include the ERα-GeneBLAzer to compare the results of estrogenic activity.

The ERα-GeneBLAzer assay uses a mammalian cell line and is based on the quantification of β-lactamase with fluorescence measurement. This assay is more sensitive to estrogenic activity than the YES assay, and therefore has a lower limit of detection [64, 65]. This assay has been used for the analysis of estrogenic activity of treated wastewater effluents, surface waters and drinking water [64]. Based on this, it was interesting to compare the estrogenicities of the samples especially OSPW using these assays. Surprisingly, there were no similarities in the trends of the results from these two assays and possible reasons for the differences which will be discussed in Chapter 3.2.

Xenobiotic metabolism refers to biotransformation processes in cells which metabolise, detoxify or bioactivate chemicals that are not naturally found in an organism [16]. The most studied xenobiotic metabolism receptors include the AhR and the PPAR, as they can act as indicators of the presence of chemicals. The AhR is a nuclear receptor that plays an essential role in the toxicity of halogenated aromatic hydrocarbons and polychlorinated dibenzodioxins. The activation of the AhR contributes to toxicity due to the conversion of many AhR ligands to reactive intermediates that may lead to DNA damage [16]. Traditionally, the AhR was used to detect the presence of dioxin-like chemicals, but it may also be applied in the detection of environmental chemicals (e.g., polyaromatic hydrocarbons, polychlorobiphenyls) [16]. The activation of the AhR using the AhR CALUX assay was included in our battery of bioassays due to its relevancy to our samples. This assay utilizes a rat hepatoma cell line and is based on the measurement of luciferase activity.

The PPAR is a transcription factor with three isoforms: PPAR- (α, β ɣ), each of which perform different functions. Most studies on environmental water samples have focused on the PPARɣ as it is a key regulator of adipogenesis (i.e., the development of fat cells). The PPARɣ plays a significant role in regulating lipid metabolism, energy homeostasis and insulin sensitivity [58, 16]. Ligands of the PPARɣ include constituents of WWTP effluents such as pharmaceuticals (the thiazolidinedione family), environmental contaminants (e.g., triphenyl phosphate) and fatty acids that can lead to health issues including obesity [58]. A recent study found that NAs are structurally similar to fatty acids, and therefore binding to the PPARɣ is likely a sensitive target endpoint in the toxicity assessment of OSPW [28]. Due to its relevancy to the samples of this study, the PPARɣ-GeneBLAzer assay was included in the test battery.

1.7. Sample preparation

Typically, when using *in vitro* bioassays, samples must first be filtered to remove suspended particulate matter and extracted to separate the organic compounds from the salts, inorganics, and metals of a sample mixture. Although it is possible to use some *in vitro* bioassays with whole effluent samples, this approach is not suited for less polluted water samples such as highly treated wastewater effluents and surface waters, which usually need to be concentrated to detect a bioassay signal [16].

In OSPW research, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the most commonly used methods, but studies have found that each method may lead to variations in the results of NAs analysis [36]. LLE has been used since the early 19th century and is the traditional method used in research laboratories for OSPW studies [66]. This method is based on the partitioning of organic analytes from the sample into a solvent (e.g.,

dichloromethane [DCM]) that is immiscible with water [16]. This is done through vigorous shaking of the sample and once separated, the solvent layer is collected so that the procedure is repeated twice more with fresh solvent to maximize the recovery of organics from the water sample. Once the three solvent batches have been combined, the solvent is evaporated leaving behind the dried organics for resuspension into the bioassay solvent [16]. The advantage of LLE is its simplicity and its applicability in unfiltered samples; however, LLE can be time-consuming, and requires large volumes of organic solvents which can be harmful to the environment and the researcher. For instance, during OSPW extraction, DCM is typically used as the extraction solvent, however the EPA classifies DCM as a likely carcinogen to humans with the principal route of exposure being inhalation, along with skin absorption [67, 68]. Moreover, the reliability of the extraction process varies as emulsions can be formed between the two liquid phases which can reduce the extraction recovery [16, 66].

SPE was developed in the 1970s and in this method, the water sample passes through a cartridge packed with a sorbent which retains the organic compounds as the water passes through. Sorbents are typically made of three components: a copolymer mix, a hydrophilic monomer to capture polar chemicals and a lipophilic monomer to capture hydrophobic chemicals [16]. Once the water passes through the column and the cartridge has been dried, the analytes are eluted with solvents (e.g., ethyl acetate) which are then blown to dryness. Finally, the dried organics are resuspended in a final solvent (e.g., methanol) creating a concentrated extract for bioassay analysis. There are many types of SPE cartridges which use different sorbent materials and therefore have varying affinities for specific compounds in a mixture. Additionally, sorbent beds have a maximum saturable capacity which can become fully saturated and lead to the breakthrough of un-retained organics, especially with highly polluted samples [16]. Therefore,

the SPE sorbent type and size must be considered prior to sample extraction to optimize the recovery efficiency of the target organics of the mixture. SPE is advantageous as it can be automated, and a high extraction recovery can be achieved [36, 16]. On the other hand, SPE cartridges can easily become clogged thus requiring that water samples with a turbidity of at least 5 NTU be filtered using glass fibre filters prior to extraction [16].

Recent studies have compared the recovery efficiency of OSPW organics using LLE and SPE using various sorbent types. For example, Qin et al. (2019) compared the recoveries of dissolved organic carbon (DOC) and total NAs from OSPW by LLE and SPE using 5 cartridges: Oasis HLB, octadecyl silica C18, Bond Elut PPL, Isolute ENV+ and MAX. They found that the LLE recovery efficiency was 48.8% and 81.0% for DOC and total NAs respectively, while the highest SPE recovery efficiency was achieved using Oasis HLB, with values of 95.4% and 90.0% for DOC and total NAs, respectively [36]. This difference between SPE vs LLE recoveries is consistent with the findings of Bataineh et al. (2006) who compared the recovery efficiency of NAs by LLE and SPE with Oasis HLB cartridges. In this study, the authors reported that the SPE recoveries were 11-30% higher than LLE for NAs in oil sands tailings water [69]. Comparable results were also observed by Meshref et al. (2020) who compared the recovery of AEOs extracted by SPE (ENV+ cartridge) and LLE using FTIR analysis. In this study, the authors found that the SPE recoveries were 1.4× LLE recoveries and attributed these recovery differences in SPE vs LLE methods to losses of hydrocarbons and phenolic compounds due to variations in selectivity and efficiency of specific components used in extraction [66].

The high recovery efficiency for OSPW compounds using HLB cartridge was also reported by Alharbi et al. (2019) who compared the MS characterization profiles of OSPW NAs using LLE and 4 SPE cartridges including HLB. They suggested that HLB sorbents were most

efficient at extracting a wide range of compounds including OSPW NAs due to their ability to isolate acidic, basic, and neutral polar or non-polar compounds when present in their neutral form [70]. Qin et al. (2019) attributed the high NAs recovery of HLB cartridges for OSPW compounds to the higher hydrophilicity of these cartridges which allows for a higher extraction efficiency of organic matter.

Based on the high achievable extraction recovery of NAs and DOC, lower consumption rate of solvents, reduced use of harmful solvents, and quicker extraction time, SPE with Oasis HLB cartridges was used in this study to target the extraction of OSPW organic compounds, especially NAs (Figure 1.5).

1.8. Surrogate measure of NAs in the river via FTIR

This work focused on Fourier Transform Infrared Spectroscopy (FTIR) for the chemical quantification of NAs. The original method was developed by Jivraj et al. (1996) and has since been commonly used throughout the oil sands industry for measuring NAs because this method is relatively simple, quick, and inexpensive [71, 72].

NAs have the general formula $C_n H_{2n+z} O_2$, where n is the number of carbon atoms and Z is a negative integer or zero representing the hydrogen deficiency from the ring formation of the compound [36]. However, this definition of NAs is quite vague to describe a group of compounds as there are over 3000 isomers of NAs in the acidic extracts of OSPW which poses a significant challenge for their characterization and quantification [73].

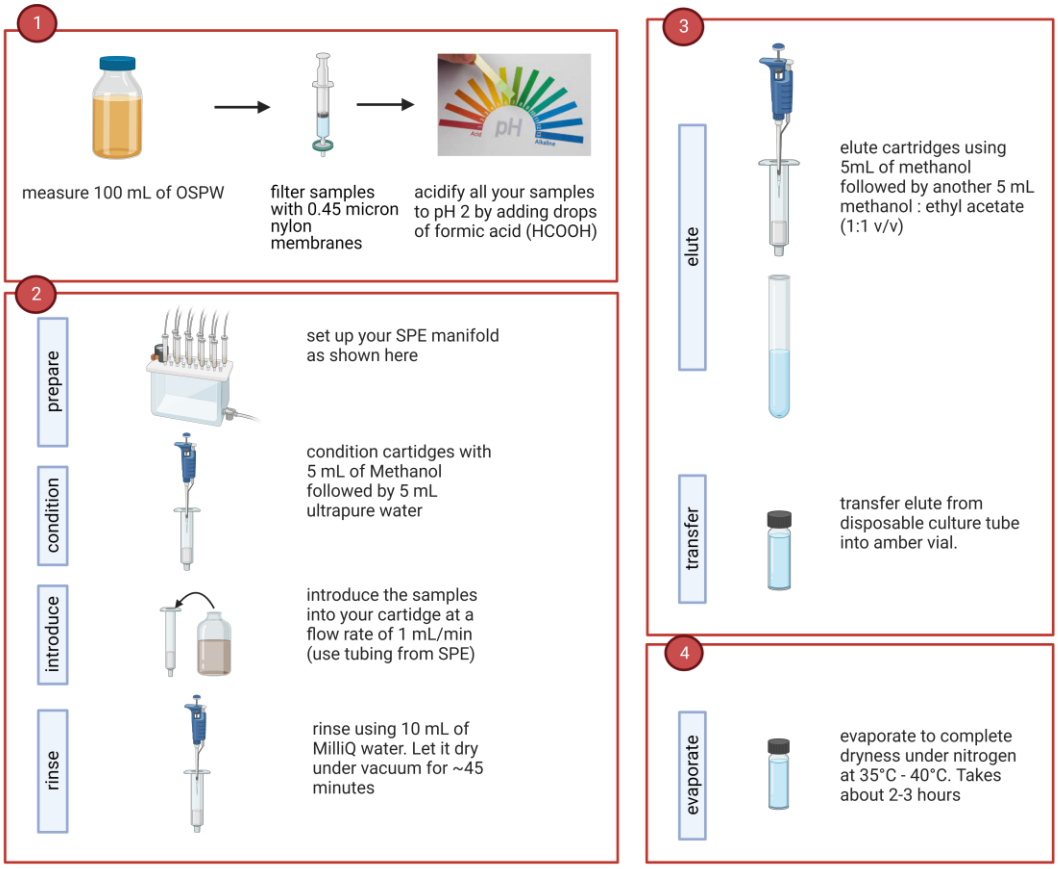

Figure 1.5. Solid phase extraction (SPE) method employed in this study to extract organic compounds from untreated OSPW. A similar approach was completed for surface water samples with a few modifications (see Chapter 2).

There is no absolute analytical method for the measurement of NAs due to complications including the lack of a set calibration standard, use of different analytical instruments, complexity of NAs, and variations in sample preparation [74]. Nonetheless, extensive research continues to target the development of a universally accepted method of the analysis of NAs as this group of compounds is thought to be the main contributors to OSPW toxicity [74].

Current NAs analytical methods include FTIR, orbitrap-mass spectrometry and gas chromatography-mass spectrometry among others, but the use of different instrument methods means that it is important to know what is being measured rather than classical NAs [74]. The

FTIR method measures the absorbance of the monomeric and dimeric forms of the carbonyl function group in carboxylic acid molecules at wavelengths 1743 cm^{-1} and 1706 cm^{-1} [72]. The drawback with this method is the possibility of detecting other organic compounds including naturally occurring fatty acids and fulvic acids [12]. Due to this low selectivity, the FTIR method is considered to be semi-quantitative, and it is more appropriate that the term 'acid extractable organics' is more appropriate when referring to the NAs quantification using this technique [71].

Calibration standards for NAs analysis can either come from commercially available NA mixtures or NA fraction compounds (NAFCs) extracted from OSPW [74].There are issues regarding the suitability of these standards since commercially available NA mixtures have a simpler composition and are dominated by classical NAs, whereas the NAFCs vary widely depending on the OSPW source [73]. In this study, commercially available NAs (from Sigma-Aldrich) were used as the calibration standard due to the consistent availability and suitability in simpler analytical techniques [74].

Site description and the sample preparation for FTIR and *in vitro* analyses are described more specifically in Chapter 2. The implications of the bioanalytical results are evaluated and the applicability of *in vitro* bioassays in the oil sands industry are then assessed in Chapter 3.

2. Methodology

2.1. Site description

Field sampling was completed in the LAR in June and August 2021 covering a total study area of ~120 km (Figure 2.1).

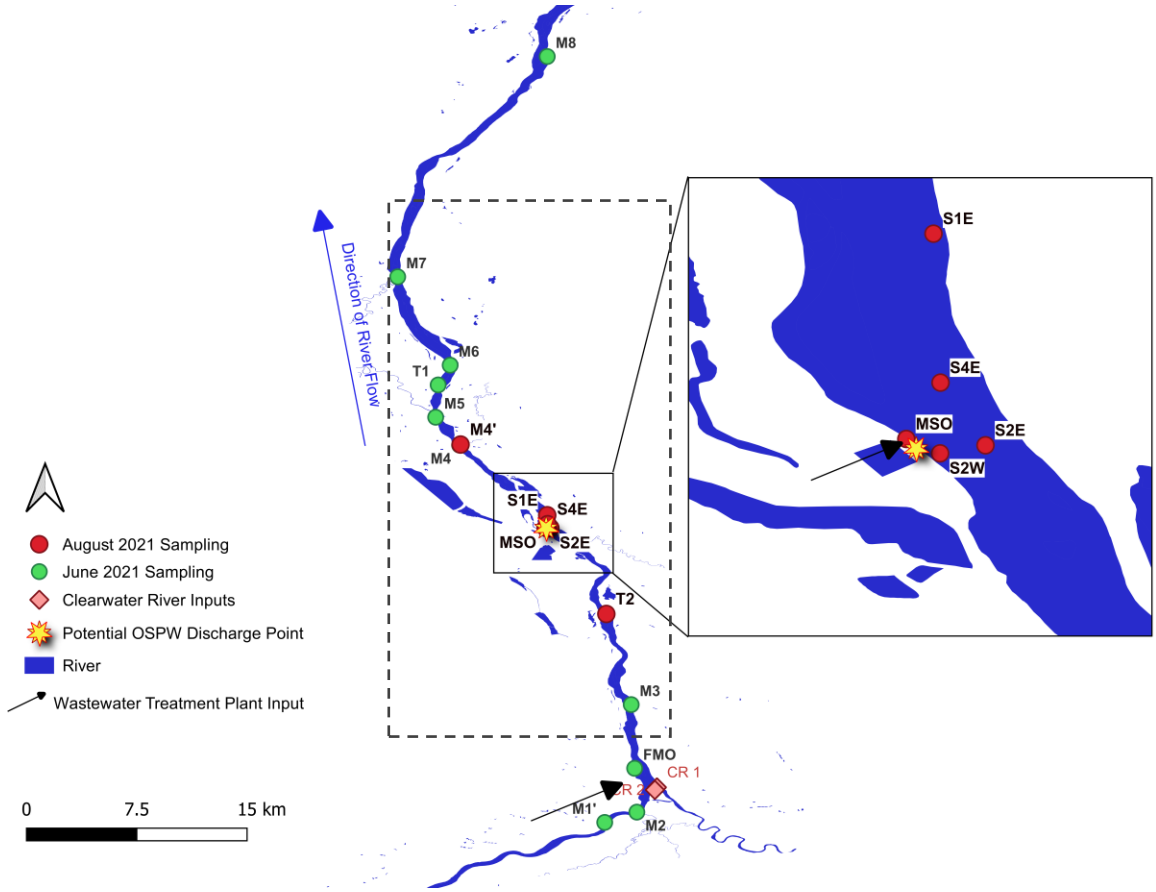

Figure 2.1. Map of the study area in the Lower Athabasca River in Alberta, Canada. Sites inside the black dashed box are inside the oil sands mineable area. FMO = Fort McMurray regional municipal wastewater treatment plant outfall. MSO = Sewage outfall. Red circles represent samples collected in August 2021. Green circles represent samples collected in June 2021.

In June 2021, samples were collected from 9 river sites located upstream, within and downstream of the oil sands mineable area. M1 and M2 are upstream of the oil sands mineable area. M3 to M7 are within the oil sands mineable area and M8 served as the downstream reference site. T1 represents a site sampled from a side stream entering the LAR. These river sites were chosen in alignment with the OSMP, which was established by federal and provincial governments to monitor the water quality of the LAR [52]. During this sampling campaign, we also collected effluent samples (FMO) from a municipal WWTP that services ~112,000 residents of the Regional Municipality of Wood Buffalo using biological nutrient removal technology followed by UV disinfection [75, 76].

In August 2021, there were 6 sampling sites along the river, which were all within the oil sands mineable area and are aligned with the Alberta Environment and Parks EMP. The purpose of the EMP is to establish the baseline environmental conditions of the LAR prior to the potential discharge of treated OSPW, therefore we conducted a smaller sampling to collect bioassay data to support this program [9]. In addition, samples were collected from a sewage outfall (MSO), that services a small population of workers from a nearby oil sands mining plant.

A key observation during field sampling was the drop in the water level of the Athabasca River from 3.3 m in June to 2 m in August (Figure 2.2). It is likely that this is a relevant factor in determining whether there are possible correlations between the concentrations of contaminants, the bioassay responses, and the hydrologic conditions. In the past, the water quality of the LAR has been highly impacted by mixing behaviors and the hydrologic conditions including annual and seasonal changes, affecting contaminant concentrations throughout the year [77].

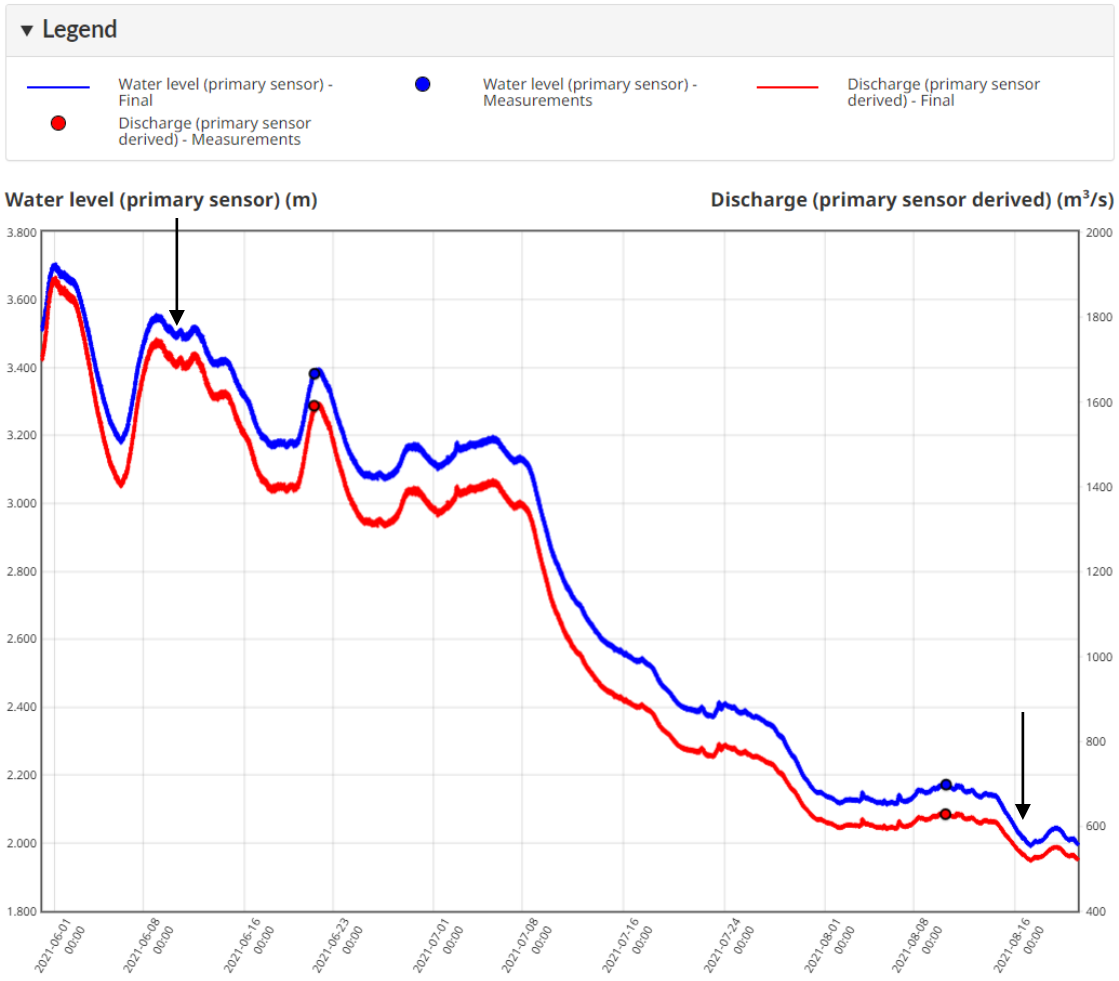

Figure 2.2. Hydrometric data for Lower Athabasca River retrieved from https://wateroffice.ec.gc.ca. Arrows denote our sampling days.

2.1.1. Mixing patterns of the Lower Athabasca River

Mixing behaviors in the LAR play a significant role during water quality assessment, especially when considering the potential impact of industrial inputs into the river. Mixing at river confluences can be rapid or delayed due to properties including the channel width, momentum ratio of the flows, the angle of intersection where the tributary joins the main stem and bed height discordance [77]. Hence the location of treated effluents with relation to sampling sites are important as the effects of these discharges can be masked due to dilution and mixing of

the river or can lead to the increase of contaminant concentrations in areas where there is delayed mixing. Water monitoring is also affected by the annual/seasonal changes of the river flow, extreme weather conditions, contributions of tributaries, differences in water quality on the east and west sides of the river and the hydrological conditions [77].

The flow rates of most tributaries are less than 2% of the LAR, thus making negligible contributions into the main stem but the Clearwater River (CR) had a flow rate of about 10% - 17% of the LAR. At the CR confluence, the width and width:depth ratio of the LAR are 850 m and 255-425, respectively. As these two parameters are >100 m and >100 respectively, it is expected for complete lateral mixing to be delayed for downstream distances greater than 100 times the confluence width (i.e., 85 km) [77]. It is possible then that mixing was not fully complete 85 km downstream from the CR (Figure 2.1).

At present, there are two point-sources (i.e., treated WWTP effluents) which deposit anthropogenic pollutants as side discharges into the LAR: FMO and MSO. Typically, vertical mixing occurs at the point of release and much more quickly than lateral and longitudinal mixing which happens at intermediate and distant fields from the point of release [78]. It is important then to consider the length of the LAR needed to achieve complete mixing for these discharges. The mixing length was calculated following the empirical equation developed by Fischer et al. (1979) for mixing in natural streams [79]. The river velocity was calculated from its flow rate, the channel depth was 3.3 m (in June) and 2 m (in August), and channel slope was 0.00014 m/m [80]. The width from the effluent to the nearest island was 200 km and 110 km for FMO and MSO, respectively, as approximated from Google Earth. Using these properties, the estimated mixing lengths were 67 km and 24 km from the point of discharge of FMO and MSO,

respectively. Although this is a long distance required for complete mixing, there may be channel bends or changes in the cross section within this distance to speed up the mixing process.

2.2. Sample collection, preparation, and extraction

2.2.1. Reagents and materials

Formic acid (88%), methanol (Optima-LC/MS grade), 10N sodium hydroxide solution, 3,5-dichlorophenol, dimethyl sulfoxide (HPLC grade) and sodium chloride were purchased from ThermoFisher Scientific, Canada. Ethyl acetate, dichloromethane and naphthenic acid were purchased from Sigma-Aldrich, Canada. Bioassay reagents are outlined in their methodologies in SI Section-B.

2.2.2. Sampling

Physical and chemical parameters (water temperature, pH, conductivity, total dissolved solids, salinity) were measured during field sampling in June 2021 using a calibrated portable multiparameter meter (Thermo Scientific Orion Star A329, Table S4). Water quality data on PACs and naphthenic acids was retrieved from Alberta Environment & Parks (AEP) (https://aws.kisters.net) for samples collected in June (not conducted on the same day as our sampling) and August sampling (done on the same day as our sampling).

Samples were collected in 1 L pre-cleaned amber glass bottles with Teflon cap tubes using a swinger sampler (Nasco Sampling B01310WA), except for the WWTPs effluents which were collected directly in the sample bottle at the outfall point. Total volume collected was 9.8 L and were allocated as follows: 1.8 L was collected for FTIR analysis, and 8 L for *in vitro* bioassays. Large sample volumes were chosen due to expected low levels of chemical

contamination in the river samples (high dilution) and therefore required high enrichment factors [16]. All samples were collected ~1m below the surface by boat except M1 and M2 which were collected on the surface by foot, and FMO and MSO which were collected directly at the outfall point (Table 2.1 for details). These sites were chosen primarily due to accessibility, and the alignment of our sites with the sampling strategies within the OSMP for the June samples and the EMP for the August samples as previously discussed. Additional samples (June 2021 only) were collected and sent for conventional water quality analysis at Natural Resources Analytical Laboratory, University of Alberta, Canada. Field sampling protocols for these parameters were followed as directed by the analytical laboratory and the results of this analysis are shown in Table S5. Another sampling campaign was conducted in October 2021 to collect additional samples of FMO and MSO for PACs analysis.

Table 2.1. Details of sampling sites

Site	Latitude (°)	Longitude (°)	Sampling description
June 2021 Sampling			
M1	56.709425	-111.441239	West, by foot
M2	56.720824	-111.405641	West, by foot
FMO	56.769322	-111.408084	Collected directly at the outfall
M3	56.839889	-111.411878	East, by boat
M4	57.127226	-111.602073	Directly below sandbar, by boat
M5	57.158124	-111.628559	Centre, by boat
T1	57.193911	-111.625830	Side stream (west), by boat
M6	57.215791	-111.61250	Centre, by boat
M7	57.313621	-111.670648	Centre, by boat
M8	57.557581	-111.504948	East, by boat
August 2021 Sampling			
T2	56.940291	-111.439454	Thalweg, by boat
S2W	57.034967	-111.504514	West, by boat
S2E	57.035504	-111.501486	East, by boat
MSO	57.035927	-111.506772	Collected directly at the outfall
S4E	57.039686	-111.504488	East, by boat
S1E	57.049622	-111.504956	Thalweg, by boat
M4’	57.127898	-111.600994	Thalweg, by boat

2.2.3. Sample preparation and extraction

A detailed outline of the sample extraction procedure can be found in SI Section-A. In summary, all samples were filtered and acidified to a pH of ~2 using formic acid. SPE using Oasis HLB cartridges was used for the extraction of organics due to its higher extraction recovery of dissolved organic carbon, NAs and volatile polyaromatic hydrocarbons [69, 36]. A manual SPE vacuum manifold was set up and the cartridges were conditioned with methanol first and then ultrapure water. Next, the samples were introduced to the cartridges using a vacuum pump. The sample volumes used for the river/WWTP samples was 800 mL per cartridge for 5 cartridges were used for bioanalysis and 900 mL per cartridge for 2 cartridges for FTIR. For the untreated OSPW, the sample volume was 100 mL. After the samples passed through, the cartridges were rinsed with ultrapure water and allowed to dry under vacuum for 1 h. Once dry, the cartridges were eluted using the appropriate solvents and the eluents were evaporated to dryness under a gentle stream of nitrogen at 35°C – 40°C. Finally, the dried SPE extracts were reconstituted into solvents specific for chemical and bioanalysis.

The dried SPE extracts of 2 × 900 mL samples for the river/WWTPs were used for FTIR analysis. The extracts were reconstituted in 5 mL dichloromethane and recombined into one vial for analysis (total of 10 mL). For OSPW samples, dried extracts were reconstituted in dichloromethane (5 mL). For *in vitro* bioassays carried out in Alberta, the dried extracts obtained from 5 × 800 mL samples were reconstituted and combined to a final volume of 1.5 mL methanol. For the OSPW, the dried extract was reconstituted in 0.5 mL methanol. For other *in vitro* bioassays carried out in Germany, the dried SPE extracts were shipped to the UFZ lab. There the dried extracts were reconstituted in methanol to an extraction factor [EF] of 1000 for samples and 500 for OSPW.

To determine extraction recoveries, SPE was performed using 2-1L ultrapure water spiked with 20 mg NAs and a 1L ultrapure water sample (blank). Additional controls (field and SPE blanks) were prepared and processed following the same protocol as the river samples. After SPE, the extracts were analyzed using FTIR to determine the acid extractable (AEO) concentrations which is a surrogate measure for NAs. The extraction recoveries ranged from 71% to 90% (mean = 79.2 ± 7.3 %, Figure 2.3), excluding recoveries <50% which had issues with preparation of the spiked samples. For the blank samples, 7 out of 11 were < LOD, with the remaining blanks having an FTIR AEO concentration of less than 1.5 mg/L.

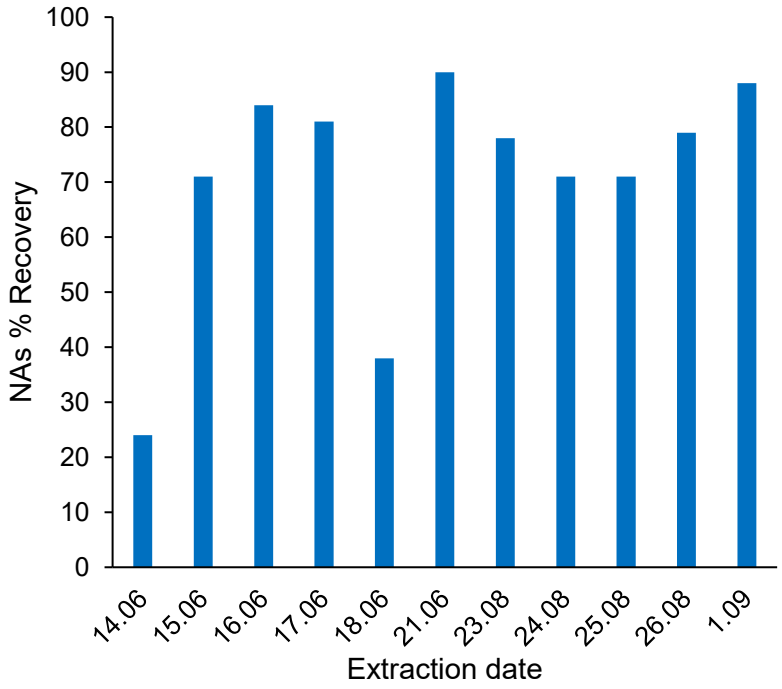

Figure 2.3. % Recovery of naphthenic acids from spiked samples. Extraction dates are the dates when SPE was conducted. Mean NAs recovery = 79.2 ± 7.3 %.

2.3. Chemical analysis

The FTIR analytical procedure follows the protocol from Jivraj et al. (1996), replacing LLE with SPE for the extraction process [72]. Briefly, the SPE dried extracts were reconstituted into dichloromethane and introduced to the KBr FTIR cell. Total peak area ratios at absorbances 1743 cm^{-1} and 1706 cm^{-1} were summed and the total AEOs concentration was calculated using a calibration curve. The calibration curve was made from dilutions of commercially available NAs (Sigma-Aldrich) in dichloromethane (see Figure 2.4 for an example).

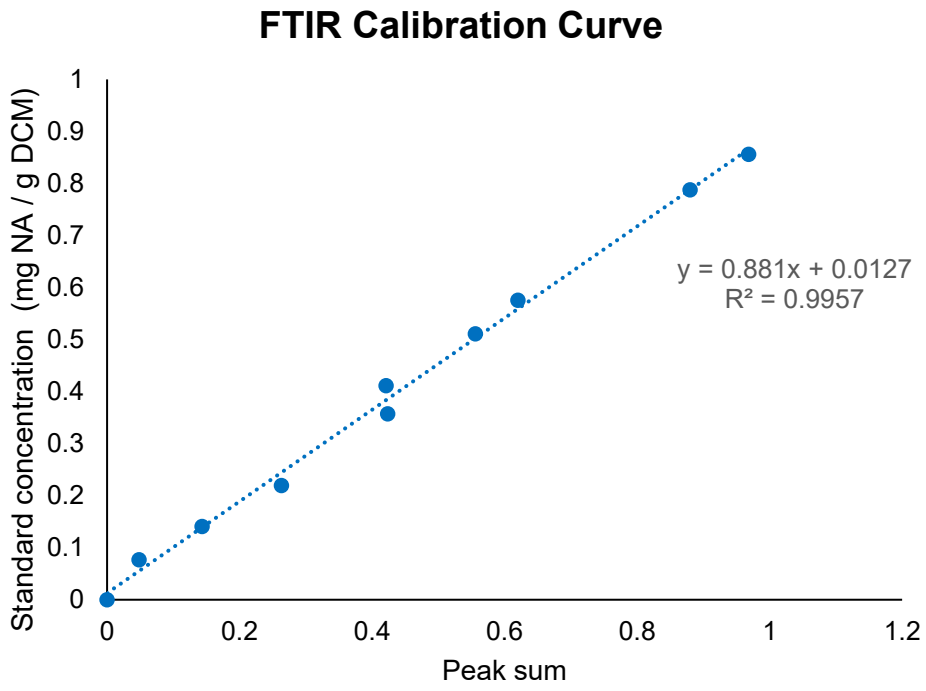

Figure 2.4. Example of calibration curve for FTIR using commercial Sigma naphthenic acids.

2.4. *In vitro* bioanalysis

Cytotoxicity was analyzed using *Aliivibrio fischeri* bacteria as well as in all bioassays with mammalian cell lines performed at UFZ in Germany. The yeast estrogen screen (YES) and ERα-GeneBLAzer mammalian gene assay were used to measure the estrogenicity. For xenobiotic metabolism, the activation of the aryl hydrocarbon receptor and the activation of the peroxisome

proliferator-activated receptor were measured using the AhR-CALUX assay and the PPAR-ɣ GeneBLAzer assay, respectively. The UMU-ChromoTest was used in this paper to measure the mutagenicity of samples. Finally, the oxidative stress response of the samples was measured using the AREc32 reporter gene assay. Sample concentrations in the bioassay were expressed as relative enrichment factor (REF). REF is based on extraction (sample enrichment) and dosing factors, and its derivation is explained in detail in Escher et al. (2021) [16].

2.4.1. Cytotoxicity

Cytotoxicity of the samples was assessed via bioluminescence inhibition using the bacteria *Aliivibrio fischeri* and with imaging methods for mammalian cell lines. Bioluminescence is directly proportional to the metabolic activity of the bacteria which may be affected upon exposure to samples, leading to cell death. All reagents were included in the BioTox™ LumoPlate™ Kits purchased from Environmental Bio-detection Products Inc (EBPI). The methodology has been adapted from ISO Standard 21338 and is outlined in SI (Section B-1).

Cell viability was quantified in parallel to the reporter gene activation in the ERα-GeneBLAzer, AhR-CALUX, PPAR-ɣ GeneBLAzer and AREc32 assays by quantifying the reduction of cell confluency during exposure using an IncuCyte S3 live cell imaging system (Essen BioScience, MI, USA) [81].

2.4.2. Estrogenicity

2.4.2.1. YES Assay

All reagents were purchased from Sigma-Aldrich, Canada. The estrogenic activity of all samples was analyzed using the YES assay. The Servos Lab (University of Waterloo) graciously

provided a recombinant yeast strain *Saccharomyces cerevisiae*. The YES assay procedure was completed using the protocol described in Arlos et al. (2016) with some modification [82]. The full procedure is outlined in SI Section B-2.

2.4.2.2. ERα-GeneBLAzer

ERα GeneBLAzer assays was conducted according to König et al., 2017 [83]. Briefly, the extracts were serially diluted in phenol red free DMEM with 2% charcoal stripped FBS. After exposing the cells to the samples for 22 h, activation of the estrogen receptor was measured using ToxBLAzer (Thermo Fisher Scientific, Schwerte, Germany). The compound 17β-estradiol (E2) was used as positive reference compounds. Details in SI Section B-3.

2.4.3. Xenobiotic metabolism

2.4.3.1. AhR- CALUX

The AhR CALUX assay developed by Brennan et al. (2015) was performed according to Neale et al. (2017) [84, 85]. Medium was 90% DMEM + GlutaMAX plus 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin. The plates were incubated at 37°C for 24 h, then luciferase production was measured using luminescence. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was used as reference compound in this assay. Details in SI Section B-4.

2.4.3.2. PPARɣ-GeneBLAzer

The PPARγ GeneBLAzer assay was performed according to Konig et al. (2017) and Neale et al. (2017) in 98% Opti-MEM supplemented with 2% charcoal-stripped FBS 100 U/mL penicillin and 100 µg/mL streptomycin [83, 85]. Reporter gene activity was quantified as for the ERα

GeneBLAzer assay. Performance of the assay was monitored using the reference compound rosiglitazone. Details in SI Section B-5.

2.4.4. Mutagenicity

Mutagenicity was analyzed using the UMU-ChromoTest; kits were purchased from EBPI. These kits use genetically engineered *Salmonella typhimurium* TA1535 to measure the mutagenic response of a cell to genetic damage. Details of the bioassay procedure can be found in the SI Section B-6.

2.5. Data analysis

A sigmoidal or linear fit was used to describe concentration-response curve (CRC) using the concentration and the measured response of the assay which can be toxicity (inhibition) or any biological effect including activation of receptors, induction of enzymes or binding to receptors (Table 2.2 for data analysis, Figure S1 for CRCs) [16]. When inhibitory or effective responses are measured, the inhibitory concentration (IC_{10}) or effective concentration (EC_{10}) was calculated which refers to the concentration that causes 10% inhibition, or 10% of the measured effect.

Typically, the models used for the CRCs allow for the calculation of a minimum and maximum response. However, there is no maximum response for some reporter gene assays such as oxidative stress response and mutagenicity. Therefore, an induction ratio (IR) defined as the ratio of signal of sample to the signal of the negative control, is used for the comparison of sample responses. [16]. In this study, the threshold concentration used was an EC with IR of 1.5 ($EC_{IR1.5}$).

After calculating the EC, the bioanalytical equivalent concentration (BEQ) of each sample was calculated using a reference compound. The BEQ was used to compare mixture effects to the effects caused by a single chemical which is known to induce the toxicity pathway being investigated [16]. After the BEQ value was calculated for each sample in each test, the values were compared to the published EBT, which is a threshold that differentiates between acceptable and unacceptable bioassay responses [86].

Table 2.2. Data analysis steps completed for each assay. OD = optical density, RLU = relative light units, IC = inhibition concentration; EC = effect concentration; YES = yeast estrogen screen assay. B = blue, G = green, E=effect. IR = induction ratio.

Assay Type	Validation	Data Analysis
Aliivibrio Fischeri toxicity assay	Positive control is 3,5-dichlorophenol. In this study, $IC_{10,15\ min} = 2.7 \pm 1.2$ mg/L.	1. Calculate % Inhibition from the raw RLU using the equation below: $$\%\ \text{Inhibition} = 1 - \frac{\text{RLU}_{\text{sample,t min}}}{\text{RLU}_{\text{sample,0}} * \frac{\text{RLU}_{\text{blank,t min}}}{\text{RLU}_{\text{blank,0}}}}$$ 2. Normalize % Inhibition from 0 - 100% 3. Complete a Ligand Binding-Sigmoidal Dose response regression using log concentration and average normalized % Inhibition (on Sigmaplot) 4. Calculate IC_{10} using parameters obtained from regression fitting
YES assay	Positive control and reference compound is 17β-estradiol (E2). In this study, $EC_{10} = 1.01 \times 10^{-10} \pm 3.65 \times 10^{-11}$ M.	1. Calculate the β-Galactosidase (β-Gal) response using the raw cell density (OD_{660}) and raw β-Gal data (OD_{420}). Note that at OD_{420} only absorbance values between 0.2 to 1.0 were included in the analysis. $$BGal\ response = \frac{1000 * slope(raw\ Bgal\ data)}{volume\ of\ cells\ plated\ (mL) * average\ OD_{660}}$$ 2. Normalize β-Gal response from 0 – 100% 3. Remove concentrations affected by cytotoxicity from the data set 4. Model the data using a 4-Parametric Logistic Equation using concentration and average normalized β-Gal response (on Sigmaplot) 5. Calculate EC_{10} using parameters obtained from regression fitting 6. Calculate the BEQ of each sample using the EC_{10} of E2

All mammalian cell lines	No positive control	1. Calculate the % cytotoxicity from the confluency data $$\%\ \text{Cytotoxicity} = 1 - \frac{\text{Confluency(sample)}}{\text{Confluency(unexposed cells)}}$$ 2. Data in the linear range up to the 30% cytotoxicity were fitted to a linear trendline with a slope and an y-intercept of 0 3. Determine the cytotoxicity IC_{10} of each sample $$IC_{10} = \frac{10}{\text{slope}}$$
ERα-GeneBLAzer; PPARɣ-GeneBLAzer	Positive control and reference compound is 17β-estradiol (E2). ERα: EC_{10} for E2 $1.3 \times 10^{-11} \pm 1.3 \times 10^{-12}$ M PPARɣ: EC_{10} for rosiglitazone $3.5 \times 10^{-10} \pm 1.3 * 10^{-10}$	1. Calculate the blue: green ratio using the following equation: $$\frac{B}{G} = \frac{\left(E_{460\ nm}(2\ h) - \left(E_{460\ nm}(0\ h, \text{unexposed cells})\right)\right) - E_{460\ nm}(2\ h, \text{cellfree})}{\left(E_{530\ nm}(2\ h) - \left(E_{530\ nm}(0\ h, \text{unexposed cells})\right)\right) - E_{530\ nm}(2\ h, \text{cellfree})}$$ 2. Calculate the % effect using the following equation: $$\%\ \text{effect} = \frac{\frac{B}{G}\text{ratio (sample)} - \frac{B}{G}\text{ratio (unexposed cells)}}{\frac{B}{G}\text{ratio (maximum)} - \frac{B}{G}\text{ratio (unexposed cells)}}$$ 3. Data with concentrations lower than the cytotoxicity IC_{10} and in the linear range up to the 30% effect were fitted to a linear trendline with a slope and a "0" y-intercept. 4. Find the EC_{10} for each sample using the following equation: $$EC_{10} = \frac{10}{\text{slope}}$$ 5. Calculate the BEQ using the EC_{10} of E2 or rosiglitazone, respectively.

		$\mathrm{BEQ_{bio}} = \frac{\mathrm{EC_{reference}}}{\mathrm{EC_{sample}}} = \frac{\mathrm{slope_{sample}}}{\mathrm{slope_{reference}}}$
AhR-CALUX & assay	Reference compound is benzo[a]pyrene (B[a]P); EC_{10} = 8.38 * 10^{-6} M.	1. Calculate the % effect using the following equation: $\%\ \mathrm{effect} = \frac{\mathrm{RLU - RLU(min)}}{\mathrm{RLU(max)-RLU(min)}}$ 2. Data with concentrations less than the cytotoxicity IC_{10} and in the linear range up to the 30% effect was fitted to a linear trendline with an y-intercept of 0 3. Find the EC_{10} for each sample using the following equation: $\mathrm{EC_{10}} = \frac{10}{\mathrm{slope}}$ 4. Calculate the BEQ using the appropriate reference compound
AREc32 reporter gene assay	Reference compound is dichlorvos; $EC_{IR1.5}$ of 7.70 * 10^{-6} M	1. Calculate the induction ratio IR $\mathrm{IR} = \frac{\mathrm{RLU\ (sample)}}{\mathrm{RLU(unexposed\ cells)}}$ 2. Data with concentrations lower than the cytotoxicity IC_{10} and in the linear range up to 3-4 was fitted to a linear trendline with a slope an y-intercept of 1 3. Find the effect concentration triggering an IR of 1.5 (50% over control) $EC_{IR1.5}$ for each sample using the following equation: $\mathrm{EC}_{IR1.5} = \frac{0.5}{\mathrm{slope}}$ 4. Calculate the BEQ using the appropriate reference compound

UMU-ChromoTest assay	Reference compound & positive control is 4-nitroquinoline 1-oxide (4-NQO). For test validation, the IR of 4-NQO at well concentration of 5.26 µM must be at least 2. In this study, IR = 8.8 ± 2.9.	1. Determine the β-Galactosidase (β-Gal) activity using the following equation: $\beta - \text{Galactosidase activity} = \frac{A_{420}\text{sample} - A_{420}\text{blank}}{A_{420}\text{negative control} - A_{420}\text{blank}}$ 2. Determine the growth factor (G) using the following equation: $\text{Growth factor} = \frac{A_{600}\text{sample} - A_{600}\text{blank}}{A_{600}\text{negative control} - A_{600}\text{blank}}$ Note: G must be greater than 0.5 for results to be considered valid 3. Find the IR by dividing the β-Gal by G. Note: For a sample to be considered mutagenic, IR must be > 1.5 4. Find the slope by fitting the data to a linear trendline with a y-intercept of 1 5. Find the $EC_{IR1.5}$ for each sample using the following equation: $EC_{IR_{1.5}} = \frac{0.5}{\text{slope}}$ 6. Calculate the BEQ using the $EC_{IR1.5}$ of 4-NQO as $EC_{reference}$.

3. Results and discussion

3.1.1. Oxidative stress response

The AREc32 assay was performed according to Escher et al. (2012) with some modifications [87]. Briefly, the extracts were serially diluted in DMEM with 10% FBS and added to a 384 well plate containing cells at a density of 8.33×10^4 cells/mL. Luciferase activity was quantified as for AhR-CALUX. tert-Butylhydroquinone (tBHQ) was the positive reference compound for AREc32. Details of the procedure can be found in the SI (Section B-7).

3.2. Chemical analysis

OSPW is a complex mixture of organic and inorganic substances with its main contaminants of concern being NAs and PACs, along with metals, phenols, salts, and petroleum hydrocarbons (e.g., benzene, toluene, and xylene) [6]. We focused our assessment primarily on the organic fraction as this is considered the main fraction responsible for the toxic effects of OSPW [88, 71]. Details are described more specifically below.

3.2.1. Acid extractable organics and Naphthenic acids

The FTIR analysis is commonly used for 'naphthenic acid' ('NA') quantification because it is a relatively simple and inexpensive method. However, this method lacks selectivity and may also account for other carboxylic acids in a mixture [71]. Hence, we used 'acid extractable organics' (AEOs) throughout this paper as a more appropriate terminology to describe NA concentrations derived via FTIR analysis (Figure 3.1).

The AEO concentrations of the municipal WWTP effluents FMO and MSO were 1.3 mg/L and 3.3 mg/L, respectively. While domestic wastewater effluents would not be a main source of NAs, it is possible that the FTIR analysis detected other organic compounds such as

natural fatty acids, acidic pharmaceuticals (e.g., diclofenac and ibuprofen), and per- and polyfluorinated alkane carboxylic acids, which can interfere with the signals produced by NAs at the wavelengths specific for carboxylic acids [73, 19]. Thus, it is possible that the results reported for FMO and MSO are not purely representative of NAs. Nonetheless, the AEO concentrations in the WWTP effluents are still an order of magnitude lower than that of the untreated OSPW (Figure 3.1). In the context of the LAR, the WWTP effluents can be seen as a relevant source of AEOs whereas the untreated OSPW is a contained source of AEOs.

The river samples collected in June showed statistically significantly higher AEO concentrations, ranging from 0.4 to 2.4 mg/L (mean = 1.0 ± 0.7 mg/L) than those collected in August where concentrations ranged from <LOD to 0.8 mg/L (mean = 0.2 ± 0.3 mg/L) (One-way ANOVA, $p = 0.027$, $\alpha = 0.05$). The AEO analysis further reveals that the highest concentrations for the June sampling were detected at sites M6 and M7 which are both located inside the oil sands mineable area. This result was expected as we suspected that the sampling sites within the oil sands disturbances would be more impacted by overland flow than the sites outside of this region. This observation further indicates that there may be a relationship between the hydrologic conditions and concentrations of contaminants in the river, suggesting that year-round monitoring is beneficial to assess temporal changes.

Recently, more advanced analytical techniques including orbitrap-mass spectrometry (orbitrap-MS) have been emerging for the quantification of NAs. It has been suggested that FTIR may overestimate the NA concentration when used for other types of waters such as ground and surface waters [89]. Therefore, we compared the data obtained for the river samples and untreated OSPW from our in-house FTIR, and orbitrap-MS analysis completed by the AEP (data

available at https://aws.kisters.net). The samples collected by AEP for NAs analysis during June and August correspond to our sampling sites labeled as: M4', S1E, S4E, S2E, S2W, and T2.

The orbitrap-MS NAs concentrations for the June sampling was <DL (4 µg/L), except T2 which had a concentration of 21.7 µg/L. The concentrations for the August samples was <DL. Given that the FTIR AEO concentrations are orders of magnitude higher than the orbitrap-MS detections, FTIR analysis likely other compounds in the mixture containing one or more carboxylic moieties.

The AEO concentration of the untreated OSPW was determined to be 52.2 ± 8.0 mg/L whereas the OSPW NAs concentration via orbitrap-MS was found to be 9.2 mg/L. The OSPW-NAs characterization profile can be found in Figure S2. The AEOs concentration reported in this study is consistent with the results of Han et. al (2009) who reported values of 50 to 77 mg/L based on FTIR analysis of various water samples including oil sands ore extracts, dyke seepage waters and active settling basins [90]. The NA concentration for OSPW is also similar to what was reported by Sun et al. (2017) who used in-line SPE-HPLC/Orbitrap analysis and found NAs concentrations of 6.34 to 29 mg/L for untreated OSPW samples collected from various active tailings ponds in the Alberta oil sands region [88].

A more detailed chemical analysis was outside the scope of this study, as we considered the FTIR approach to be an acceptable and straightforward method which lends a semi-quantitative assessment of NAs in water samples to support our bioanalytical assessment of the Lower Athabasca River.

June 2021

August 2021

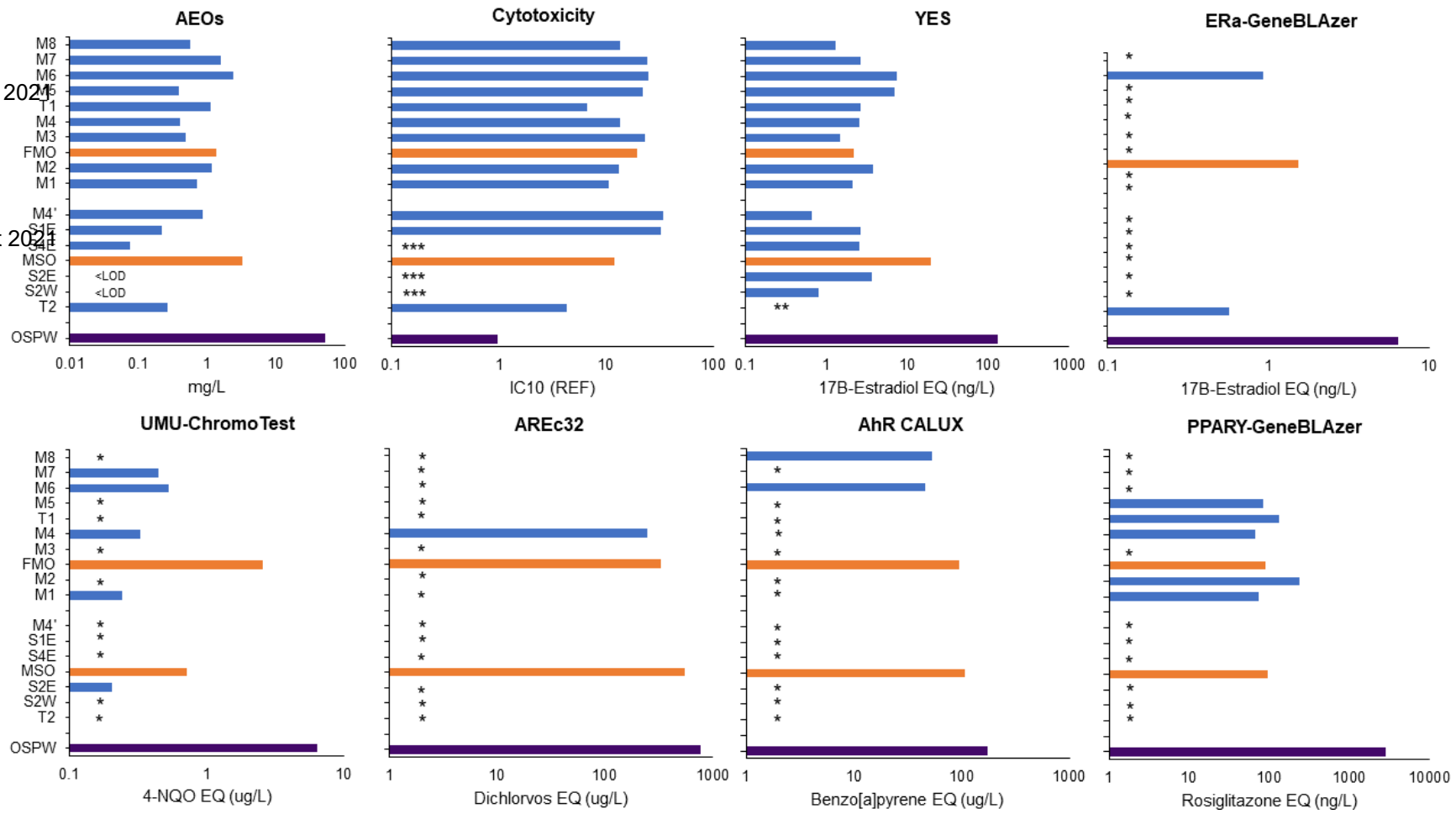

Figure 3.1. Summary of AEO concentrations and BEQ responses for bioassays for all samples (Values found in Table 3.1). Blue bars represent river samples, orange bars represent WWTP effluents and purple bars represent untreated OSPW. * = no activation; ** = not analyzed; *** = cannot be determined.

3.3. *In vitro* bioanalysis

3.3.1. Cytotoxicity

Cytotoxicity through the inhibition of bioluminescence in *Aliivibrio fischeri* has been routinely employed to evaluate OSPW toxicity and assess the efficiency of potential treatment technologies (Table 1.1) [10]. Due to the number of data available for comparison, it was deemed appropriate to employ this assay as a non-specific toxicity endpoint in our test battery.

The IC_{10} derived for all samples are shown in Figure 3.1. Overall, IC_{10} values are >10 REF for all river samples except for T1 (IC_{10} = 6.6 REF), a small side stream entering the main river. Furthermore, the mean IC_{10} values was 16.7 ± 6.6 REF and 23.4 ± 16.6 REF for the June and August river samples, respectively. Although the mean values are not statistically significant different from each other (One-way ANOVA, $p = 0.32$, $\alpha = 0.05$), the results still imply that there was slightly less cytotoxicity during August. This assumption is further supported by the IC_{10} values derived using the mammalian cell assays (Table 3.1) wherein all June river samples showed baseline cytotoxicities, while only 1/7 river samples showed cytotoxicity in August. More specifically, the mean IC_{10} values in June were 2.8 and 1.7 REF for ERα and PPARɣ assays respectively, while the August sampling IC_{10} mean values ranged from 3.1 to 8.6, respectively. Although these values are <10× the *Aliivibrio fischeri* bioluminescence $IC_{10,}$ the similarity in cytotoxicity patterns (i.e., higher in June) is supportive of the trend observed for AEOs data. Low river flows in August likely reduced the input of chemicals into the river which subsequently induced relatively lower cytotoxicity than the June samples.

The IC_{10} values were 19 REF for FMO and 12 REF for MSO. It can be expected for WWTP effluents to show some signs of cytotoxicity, as reported by previous studies [91, 53]. However, these effects observed may be minimal considering the mixing patterns and dilution

factor (DF) of the effluents in the LAR is quite high (DF>6700 and >2200 for June and August, respectively) reducing the risk of exposure risk for organisms (see SI for calculation of DFs).

Although the environmental exposure in the river sites was mostly considered to be low risk (details in Section 2.9), the results show that OSPW is acutely toxic with an IC_{10} of 0.98 ± 0.66 REF. This is not surprising as these results are comparable to what was reported by Wang et al. (2013), Gamal El-Din et al. (2011) and Fang et al. (2019) who used the *Vibrio fischeri* assay with raw OSPW, reporting IC_{20} values in the range of 0.30 REF [30, 45, 21].

The chemicals responsible for these cytotoxic effects in the samples cannot be identified solely from the data reported in this study, although it has been indicated that organic compounds, especially NAs in OSPW, contribute mostly to its toxicity [74].

3.3.2. Xenobiotic metabolism

3.3.2.1. AhR CALUX assay

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that plays a significant role in the detoxification of xenobiotics and in mediating diverse organ-specific toxic responses of naturally occurring and synthetic contaminants such as dioxins, PACs, and aryl hydrocarbons [16]. As PACs are found in OPSW, we included this endpoint in our battery selection. The AhR controls several target genes encoding for metabolic enzymes which are activated in a series of events when a contaminant enters a responsive cell and binds to the AhR. The activation of these genes can then convert ligands into reactive intermediates that can cause DNA damage [16]. The AhR activity in our samples was measured using the AhR CALUX bioassay. The sample responses are reported as Benzo[a]pyrene-EQ (B[a]P-EQ) values in Figure 3.1.

From the June sampling, only M6 and M8 activated the AhR with B[a]P-EQ values of 46.4 and 53.2 ng/L respectively, while none of the river samples from the August campaign activated this assay. The August result can be attributed to the lower concentrations of PACs during this sampling as compared to June (Table S7). These AhR results are consistent with the findings of other studies which have reported similar EC_{10} values of 2.0 to 10.7 REF (Table 3.1) for surface samples collected from the Ammer River (Germany) and the Swiss Plateau (Switzerland) that are both impacted by WWTP effluents even though these rivers are smaller than the LAR [59, 92].

The B[a]P-EQ values were 106.5 ng/L for MSO and 94.9 ng/L for FMO WWTP effluents. These values are comparable to what has been reported by Neale et al. (2017), who found that the EC_{10} values for 3 WWTP effluents ranged from 4 to 6.33 REF (Table 3.1) [59]. Ings et al. (2011) studied the exposure effects of tertiary-treated municipal wastewater effluents on the gene and protein expression in rainbow trout liver and reported that the exposure to these effluents affects stress-related proteins involved in metabolism including the AhR, which is likely due to the presence of PACs and dioxins [93]. Based on the supporting PACs data for FMO and MSO (Table S7), it is possible that PACs and other compounds such as corrosion inhibitors and pharmaceuticals contributed to the AhR activation of FMO and MSO [59].

The B[a]P-EQ value for OSPW was 172.4 ± 142.8 ng/L. Although the OSPW used in this study was not analyzed for PACs, it is likely that the relatively high response of OSPW to this bioassay can be attributed to these compounds. Studies have shown that the exposure of OSPW-derived AEOs and PACs to walleye eleuthero embryos and Japanese medaka embryos increase the expression of the cytochrome P450 (CYP) enzyme involved in Phase 1 biotransformation

[94, 95]. However, the work by Marentette et al. (2017) suggests that in addition to PACs, there may be other OSPW water soluble aromatic compounds that activate this receptor [94].

3.3.2.2. PPARɣ-GeneBLAzer assay

The peroxisome proliferator-activated receptors (PPARs) (-α, -δ, -ɣ) are ligand-activated transcription factors in the nuclear receptor family that play a role in regulation of lipid and glucose homeostasis. As the name suggests, these receptors mediate the effects of peroxisome proliferators such as fatty acids and their metabolites [16]. Of the three, PPARɣ is commonly investigated and therefore was selected in our test battery as it plays a significant role in insulin sensitivity and in the regulation of lipoprotein and glucose metabolism [16]. This receptor is also activated by tire-wear chemicals such as benzothiazole sulfonic acid, and potentially naphthenic acids that are structurally similar to fatty acids [28]. In this study, the PPARɣ-GeneBLAzer assay was used to measure the binding to the PPARɣ, and the results are shown in Figure 3.1 as rosiglitazone-EQ values.

For the river samples, 5 sites from June showed a signal for the PPARɣ with rosiglitazone-EQ values ranging from 65.7 to 233.9 ng/L while none from August activated this assay. These values compare to the findings of Neale et al. (2020) who reported BEQ values ranging from 2.4 ng/L to 790.4 ng/L for samples collected during rain events from unimpacted rivers and those impacted by WWTP effluents and agricultural streams. In this study, the authors found that diclofenac, benzothiazole sulfonic acid and the herbicide MCPA were the main contributors to the sample activities [17].

The rosiglitazone-EQ values were 89.0 ng/L for FMO and 94.8 ng/L for MSO. It is suspected that PPARɣ agonists likely to be found in wastewater effluents include phthalates,

pharmaceuticals (e.g., rosiglitazone, pioglitazone) and organotins, however further work is required to definitively determine the compounds responsible for PPARɣ activity in WWTP effluents [58]. The measurement of specific trace organic chemicals typically found in municipal effluents was outside the scope of this study, but we can postulate that these types of compounds are contributing to the activity observed for FMO and MSO.

The rosiglitazone-EQ values for OSPW were 2824 ± 590 ng/L. The high signal for untreated OSPW can be supported by the work of Peng et al. (2016) who identified 30 chemicals found in OSPW including hydroxylated carboxylic acids, oxygenated sulfonic acids or heteroatomic chemicals as compounds which activate the PPARɣ [28].

3.3.3. Mutagenicity

The UMU-ChromoTest assay which utilizes *Salmonella typhimurium* TA1535 was used to estimate the mutagenicity of our samples. This assay is a sensitive standardized method which uses the β-galactosidase activity and bacterial density to determine the induction ratio of the *umu*-C gene upon exposure to polluted water samples including industrial wastewaters [53]. Induction ratios greater than 1.5 indicate potential mutagenic activity in water samples. $EC_{IR1.5}$ values were used to derive the 4-nitoquinoline 1-oxide (4-NQO)-EQ values shown in Figure 3.1.

From the June sampling campaign, 4 samples activated this assay with 4-NQO-EQ values ranging from 0.2 to 0.5 µg/L. Of the river samples collected in August, only 1 sample (S2E) was active in this assay, with a 4-NQO-EQ of 0.2 µg/L. Another recent study by Sun et al. (2017) investigated the mutagenic activity of the Jialu River (China) which is a polluted urban river receiving reclaimed wastewater. In their study, the reported 4-NQO-EQ values range from 0.28 to 0.69 µg/L, which are comparable to this study [96].

The 4-NQO-EQ value is 2.5 µg/L for FMO and 0.7 µg/L for MSO. These results are comparable to other studies that have reported values ranging from 0.09 to 2.55 µg/L for other WWTP effluents which treat municipal wastewaters [91, 97]. It is likely that PACs (which are known carcinogens) contributed to the observed activity, and this hypothesis can be supported by the PACs data in the effluents (Table S7) [98]. Interestingly, Fang et al. (2012) reported lower PACs concentrations of about 0.18 µg/L with similar 4-NQO-EQ values around 2.13 µg/L for municipal WWTP effluents [97]. The variations between PACs concentrations and reported mutagenicity across studies have led to the uncertainty among researchers about the extent of PACs contribution and nontarget compounds to the genotoxic/mutagenic activity of a variety of effluents [97]. Thus, it is likely that the observed mutagenicity of FMO and MSO in this study can be attributed to the presence of other compounds in addition to PACs [62].

The 4-NQO-EQ value for OSPW is 6.4 µg/L. This result is comparable to the findings of Zetouni (2015) who found that the bioactivation of the neutral and acid extractable fractions of OSPW occurred at REFs of 1.5-25 (Table 3.1). The authors suggested that the reported mutagenicity was not environmentally relevant for short-time exposure times since the doses required for bioactivation were more than 1× original concentration [99]. Therefore, the long-term bioaccumulation of these compounds in aquatic organisms may be more relevant for future studies [99]. Zetouni (2015) also speculated that most PACs were removed when additional sample clean up (filtration) was completed prior to the mutagenicity analysis. Therefore, this process may have suppressed the mutagenic effects of their OSPW samples and/or there are simply other substances responsible for mutagenicity. Filtration prior to SPE is a key step for water samples containing visible particles, or more specifically with a turbidity of at least 5 NTU [16]. The clogging of cartridges is one of the main drawbacks of SPE, therefore it is advisable to

complete an extraction/bioanalysis of the suspended solids filtered out from the samples to evaluate the total mutagenic effects of a water sample.

PACs may need to be metabolically activated using mammalian metabolic enzyme preparations (S9) to simulate xenobiotic activation and detoxification in *in vitro* assays [100]. S9 is typically employed when using cell lines such as *Salmonella typhimurium* which is metabolically deficient and cannot activate some mutagens in the absence of an enzyme preparation. At present, S9 is prepared from a variety of mammalian species but this can lead to significant differences in results as different S9 systems (e.g., rat liver, hamster, human liver) are recommended for different types of assessments [100]. S9 activation was not used in this study but it is important to note that there likely would be variations in results with and without S9 enzymatic activation. An express bacterial strain P450 1A2 was run in this study for select samples and details on this can be found in SI Section B-6.

3.3.4. Oxidative stress response

The oxidative stress response (OSR) is one of the adaptive stress responses monitored in water quality assessment [16]. Typically, electrophilic chemicals, and reactive oxygen species (e.g., superoxide, hydroxyl radical) induce the OSR, and in a series of events, activates the antioxidant response element (ARE) [54, 16]. Adaptive stress responses are usually induced at lower concentrations than cytotoxicity, implying that these assays can be implemented as sensitive monitoring tools for environmental water samples [55]. Escher et al. (2012) suggested that the induction of the OSR can be classified as a non-specific mode of action considering that a large fraction of chemicals can produce reactive oxygen species directly or indirectly. Hence, the induction of the OSR is an indicator of a defence mechanism, rather than a toxic effect [87]. The

AREc32 reporter gene assay was used in this study for the measurement of the OSR. $EC_{IR1.5}$ values were used to derive the dichlorvos-EQ values for the samples (Figure 3.1).

From the June 2021 sampling, only M4 showed activity, with a dichlorvos-EQ value of 248.1 µg/L, and similar to other assays, none of the samples collected in August 2021 showed an activation for this bioassay. The response of M4 (Table 3.1) is slightly lower than the findings of Neale et al. (2017) who reported EC_{10} values ranging from 20 to 33 REF for river samples collected downstream of each of 3 WWTPs in Switzerland, during low flow conditions [59]. The difference in OSR between studies may be attributed to the differences in dilution of the WWTP effluents, and the hydrologic conditions (e.g., flow rate) during sampling.

Both WWTP effluents activated the bioassay with dichlorvos-EQ values of 333.8 µg/L and 559.7 µg/L for FMO and MSO, respectively. The EC_{10} values of these samples (Table 3.1) are comparable to with the findings of Escher et al. (2013) and Neale et. al (2017) who evaluated the OSR for various WWTP effluents (0.34 to 17.1 REF) [56, 59]. Muller et al. (2018) identified that the WWTP effluents are major sources of organic micropollutants such as transformation products, disinfection by-products, pesticides and pharmaceuticals that are known inducers of the oxidative stress response [92].

The BEQ value for OSPW was 774.7 ± 292.8 µg/L dichlorvos-EQ. The high response of untreated OSPW as compared to the river and WWTP samples is consistent with the trends seen in the other chemical and bioanalytical analyses. This further exemplifies the high toxicity of untreated OSPW.

3.3.5. Estrogenicity

Endocrine disrupting activity in environmental waters can threaten the health of aquatic ecosystems. There are several endpoints which can be targeted for endocrine disruption (e.g., androgenicity, estrogenicity), however the vast majority of work thus far has focused on estrogenic activity [64]. The YES and ERα-GeneBLAzer mammalian reporter gene assays were both used in this study to measure estrogenicity activities.

The YES assay has several advantages as this assay is simple, produces quick results and has low maintenance and consumable costs. However, as this assay is yeast-based, the presence of a yeast cell wall may affect the active transport mechanisms during the uptake of some compounds [65]. Moreover, the YES assay has a relatively higher detection and quantification limits, thus considered less sensitive than other assays [64]. This limitation was the primary reason that the ERα-GeneBLAzer assay was also run in this study with the latter being a more sensitive estrogenicity assay [86]. Note that although both assays use the same reference compound 17β-estradiol (E2), the EC_{10} values of E2 and the relative effect potency of other estrogenic chemicals in water samples vary and each assay result to different ecological EBT values. The 17β-estradiol-EQ (EEQ) values for the samples using the YES Assay and ERα-GeneBLAzer are shown in Figure 3.1.

All river samples collected in June showed estrogenic activity using the YES assay, with EEQ values ranging from 1.3 ng/L to 7.5 ng/L (mean = 3.5 ± 2.3 ng/L EEQ). Five out of six river samples collected in August have reported EEQ values ranging from 0.66 ng/L to 3.7 ng/L (mean = 2.1 ± 1.3 ng/L EEQ). By contrast, only M7 from the June river samples (0.92 ng/L EEQ), and T2 (0.57 ng/L EEQ) from the August sampling showed estrogenic activity using the ERα-GeneBLAzer.

Additionally, the highest EEQ values (YES assay) came from sites M5 and M6, which was expected considering that these sites are located within the oil sands mineable area and may be more impacted by the presence of nonhormonal estrogenic substances (e.g., aromatic naphthenic acids, alkylphenolic compounds) [62, 38].

There is not enough evidence to show if whether there is a statistically significant difference between the June and August concentrations using the YES assay (ANOVA, $p = 0.24$, $\alpha = 0.05$), as our August sample size is small. Nonetheless, the slightly higher estrogenic activity in June can be supported by the findings of Zhao et al. (2011) who used the YES assay to estimate the estrogenic activity of surface water during the wet and dry seasons along several locations of the Pearl River System (China). Overall, the authors found that there were higher estrogenic risks during the wet season however, some of the individual concentrations of xenoestrogens (e.g., 4-nonylphenol and 4-t-octylphenol), and natural and synthetic estrogens (e.g., estrone, 17β- estradiol and 17α-ethinylestradiol) varied spatially, and not seasonally [101].

The EEQ values determined for FMO and MSO (municipal WWTPs) are 2.2 and 19.9 ng/L EEQ using the YES assay, respectively. By contrast, the EEQ values determined using the ERα-GeneBLAzer is 1.53 ng/L for FMO and no activation was observed for MSO. The observed activity in FMO using both assays was expected as the presence of endocrine disrupting compounds (EDCs), such as natural and synthetic hormones, is commonly found in treated municipal wastewater effluents (albeit low if tertiary-treated) [63]. The reported estrogenicity of FMO using the YES assay is comparable to the findings of Arlos et al. (2018) who determined the effluent estrogenicity levels for another WWTP with a similar treatment train as FMO to be 3.4 ng/L [102]. The high estrogenicity of MSO from the YES assay was not expected as the population it services (employees from the oil sands operation) is small and has an approximate

male: female ratio of 3:1 [103]. On the other hand, it is possible that the high estrogenicity of MSO from the YES assay may be because this effluent comes from a sewage lagoon which lacks the treatment processes to remove endocrine disrupting compounds more efficiently. Given that MSO flow contribution is very low (<1% of the total river flow), the estrogenicity loadings into LAR environments is considered low.

The stark difference between the results of the two estrogenicity assays was also observed for untreated OSPW. More specifically, the EEQ value for OSPW is 133.8 ± 34.8 ng/L and 6.4 ± 2.5 ng/L using the YES and ERα-GeneBLAzer assays, respectively. The value reported for the YES assay is also consistent with the finding of another study which reported an EEQ value of 157.5 ng/L [38]. Regardless, our results support the work of Rowland et al. (2011) who identified that the aromatic steroidal structures in OSPW are similar to the structures of known estrogens and consequently recommended that OSPW should be monitored for its estrogenicity in future studies [62]. Gagne et al. (2012) further investigated the changes in molecular signals related to gene expression (estrogen-based) on rainbow trout hepatocytes upon exposure to river, lake, and OSPW extracts. The authors found that the OSPW elicited higher gene expression responses as compared to the other water samples [27].

As previously mentioned, both estrogenicity assays did not show similar trends for the river/WWTP samples with ERα-GeneBLAzer estrogenic activity only observed in 2 river samples whereas all river samples activated YES. This result may be due to factors such as the interferences from other endocrine-active compounds (e.g., androgens, anti-androgens, and anti-estrogens), differences in assay sensitivities, and variations in the active mechanisms of each assay.

Fernandez et al. (2007) found that the YES assay is affected by the presence of anti-estrogenic compounds in WWTP effluents which may suppress the bioassay response which was also reported by other studies [63]. However, the extent of the suppressant effect is a complex mechanism as it depends on the concentrations of the strong estrogens in the mixture [61]. Therefore, it may also be important to investigate the relationship between anti-estrogens and estrogens to confidently predict the toxicological implications of these types of samples when using the YES assay [61].

Note that similar to all the assays completed in this study, the validity of the results from both estrogenicity assays were only considered after a rigorous analysis of the assay quality controls (solvent/reagent controls, SPE blanks, cytotoxicity), which were found to be within acceptable limits. Nonetheless, we recognize that the mechanisms as to why the activation of MSO extract via YES and not with ERα-GeneBLAzer still requires further investigation (e.g., test another sampling event).

Table 3.1. Summarized results for bioassays - EC10, $EC_{IR1.5}$, IC10 (REF) values

	PPARɣ		AhR		AREc32	
	EC10	IC10	EC10	IC10	EC(IR1.5)	IC10
June						
M8	*	1.8	4.0	**	*	**
M7	*	1.9	*	**	*	**
M6	*	2.0	4.6	**	*	**
M5	1.1	2.1	*	**	*	**
T1	0.7	0.8	*	3.9	*	**
M4	1.4	2.3	*	**	6.9	**
M3	*	2.0	*	**	*	**
FMO	1.0	**	2.2	**	5.1	**
M2	0.4	1.3	*	**	*	**
M1	1.2	1.5	*	**	*	**
August						
M4’	*	**	*	**	*	**
S1E	*	5.4	*	**	*	**
S4E	*	**	*	**	*	**
MSO	0.9	3.0	2.0	**	3.0	**
S2E	*	**	*	**	*	**
S2W	*	**	*	**	*	**
T2	*	**	*	**	*	**
OSPW	0.04 ± 0.01	2.7 ± 0.3	2.3 ± 2.2	3.7 ± 1.0	2.5 ± 1.2	5.1

* = no activity, ** = no cytotoxicity, *** = cannot be determined; n.p. = not processed.

Table 3.1. continued.

	ERα		YES	UMU	Cytotoxicity
	EC10	IC10	EC10	EC(IR1.5)	IC10
June					
M8	*	1.9	16.1	*	13.3
M7	3.7	4.8	11.1	208	23.9
M6	*	1.3	3.0	100	24.8
M5	*	2.0	3.0	*	21.9
T1	*	0.8	8.3	*	6.59
M4	*	5.2	7.2	161	13.5
M3	*	2.2	13.4	*	22.7
FMO	2.2	7.8	10.6	21	19.2
M2	*	1.4	9.2	*	13.1
M1	*	1.8	16.6	385	10.6
August					
M4’	*	**	27.9	*	33.5
S1E	*	8.6	17.0	*	32.6
S4E	*	**	10.3	*	***
MSO	*	4.8	1.5	74	12.0
S2E	*	**	9.4	455	***
S2W	*	**	25.2	*	***
T2	6.0	**	n.p.	*	4.2
OSPW	0.7 ± 0.3	1.6 ± 0.04	0.2 ± 0.07	5.5 ± 3.5	1.0 ± 0.7

* = no activity, ** = no cytotoxicity, *** = cannot be determined; n.p. = not processed.

3.4. Comparison to ecological effect-based trigger values

The EBT value is an assay-specific threshold which differentiates whether a water mixture is unlikely to produce adverse effects during water quality assessment [16, 104]. *In vitro* bioassays are highly sensitive and may detect a signal in 'clean' waters especially if they have been enriched. Hence, not every bioassay response automatically implies that there will be an associated ecotoxicological risk. EBT values are unique to each bioassay and are available based on the sample type. In this study, ecological EBTs for surface water are compared to the sample responses (Figure 3.2). These EBT values have been proposed for surface waters and wastewaters with the purpose of protecting the aquatic ecosystem health and exposed aquatic organisms (Table 3.2) [104].

Table 3.2. Effects-based trigger (EBT) for surface water used for comparison to sample bioassay responses

Assay	EBT value	Units
Cytotoxicity	<20 [a]	REF
Mammalian cell line cytotoxicity	<10 [b]	REF
YES Assay	1.07 [c]	ng/L 17β-Estradiol EQ
ERα-GeneBLAzer	0.34 [c]	ng/L 17β-Estradiol EQ
AhR activation	250 [b]	ng/L benzo[a]pyrene EQ
PPAR-ɣ activation	1.2 [b]	µg/L rosiglitazone EQ
Mutagenicity	0.64 [d]	µg/L 4-nitroquinoline 1-oxide EQ
Oxidative stress response	1.4 [b]	mg/L dichlorvos EQ

[a] Threshold for chronic toxicity effects obtained from van der Oost et al. (2017). [b] EBT values obtained from Escher & Neale (2020). [c] EBT values obtained from Escher et al. (2018). [d] Predicted no-effect concentration obtained from Xu et al. (2014)

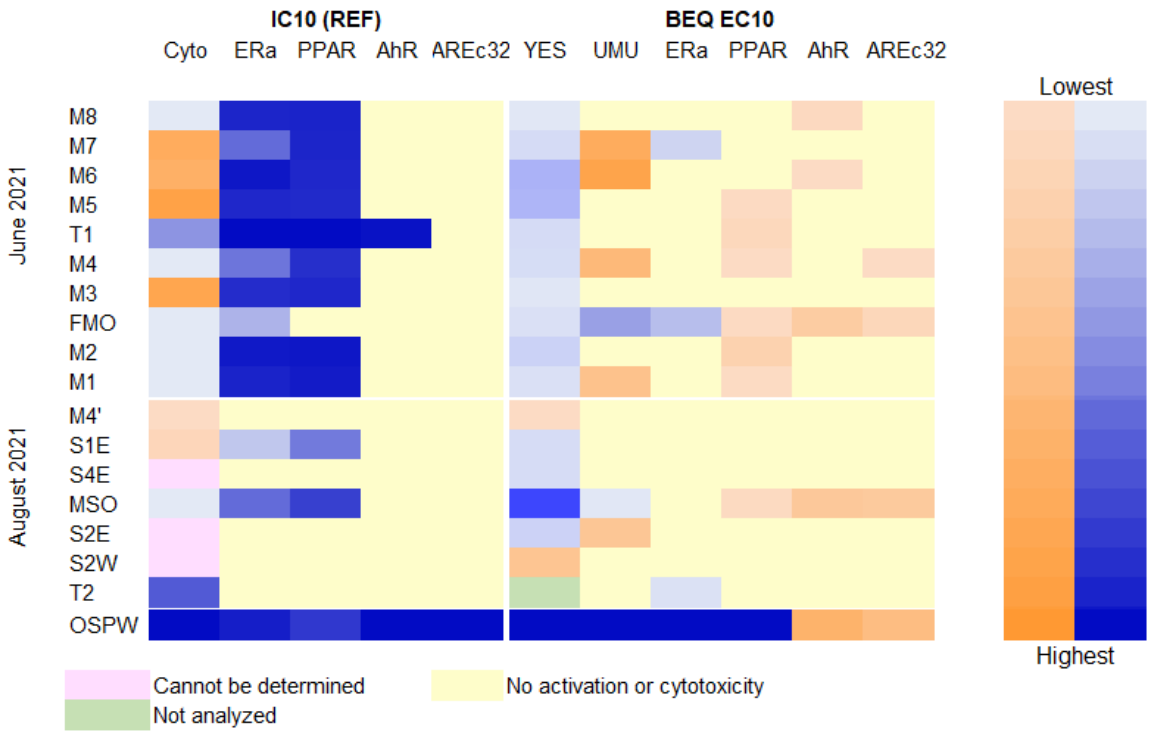

Figure 3.2. Comparison of sample responses to the EBT-IC_{10} value and ecological EBT-EQ values for surface waters (found in Table 3.2). Yellow represents samples with no activation or cytotoxicity; pink = cannot be determined; green = not analyzed; shades of orange represent responses below the EBT value; shades of blue represent responses exceeding the EBT value, with darker shades of orange and blue representing the greater responses.

For the bioluminescence cytotoxicity, the responses are compared to the threshold for chronic risks (<20 REF) as described in detail by van der Oost et al. (2017), whereas the mammalian cytotoxicity are compared to the EBT-IC_{10} = 10 threshold where any sample that has IC_{10}>10 is of acceptable water quality [105, 106]. There is no fixed EBT-EQ for mutagenicity due to a lack of data, so for this study the predicted no-effect concentration of 0.64 4-NQO µg/L from Xu et al. (2014) was used for comparison [107].

Untreated OSPW was very cytotoxic and activated all bioassays with 5/7 exceeding the EBTs. The results further imply that the pathways investigated in this study are relevant to OSPW monitoring. Even though the OSPW responses did not exceed the EBT values for the AREc32 and AhR assays, these responses were still ~3.5× larger than those of the river samples. EBT thresholds are a rapidly changing area of research which gets updated as more data becomes available. Hence the AREc32 and AhR assays may still serve as important secondary biological activity indicators.

All bioassay tests were activated by FMO, with 4 of the 7 responses exceeding the EBT values. MSO activated 6 assays with 3 responses exceeding the ecological EBT values. The relatively high responses of these samples may imply that direct exposure to these effluents could potentially lead to toxic effects. However, if we consider the dilution (DF: >2200 to >6700) and mixing patterns of the river, it may be fair to assume that these factors play essential roles in reducing the exposure risk of organisms to these effluents. The impact of the discharge of these effluents may greatly change with extreme hydrological conditions such as a drought, and may become a cause for concern, considering the demonstrated potential health risks associated with these effluents.

For the river samples, 5 out of 9 collected in June and T2 from the August sampling show the potential for chronic risks to aquatic organisms upon exposure. There is a lower risk of chronic exposure as the bioluminescence IC_{10} values are >20 REF. When cytotoxicity derived from the mammalian cell lines (ERα and PPARɣ) are used, all river samples exceeded the EBT-IC_{10} threshold (i.e., all samples have IC_{10} <10 REF), further suggesting that there might be existing risk of chronic exposures to organic substances within the LAR. Although it is outside the scope of this study, it might be useful to contrast the bioassay results here to the chemical and

other bioindicators reported in the current monitoring programs (e.g., JOSM, EMP) to fully elucidate exposure conditions. It is difficult to exactly identify what the chronic effects might be based on cytotoxicity data alone. As shown in Figure 3.2, some river sites exceeded the EBTs for other endpoints as discussed below.

All river samples collected in June activated the YES assay with responses that were 1.2 to 7 times larger than the ecological EBT-EQ value. However, for the August samples, only 3 out of 5 analyzed samples had EEQ values greater than the EBT value, with bioactivities that are 2.4 to 3.5 times larger than the EBT-EQ. Interestingly, only M7 from the June sampling showed activity with an EEQ value that was approximately 3.5 times larger than the EBT-EEQ when the ERα-GeneBLAzer assay was used. This assay was also activated by one August sample (T2), with an EEQ value that was 2 times larger then the EBT-EQ value. We reiterate the need to assess the differences in these two bioassays further as it could direct potential approaches to assess estrogenicity in the LAR.

For the AREc32, and AhR CALUX and PPARɣ-GeneBLAzer assays, the responses of the active June river samples did not exceed the EBT values while there was no activation for the August river samples. None of the river samples collected in June or August showed responses exceeding the PNECs value for mutagenicity.

Overall, we observed that there is more exceedance of the surface water EBT values from the June samples than from the samples collected in August which often did not activate the assays. These results of these bioassays are supported by the AEOs data (Figure 3.1) which indicated that the chemical contamination of the river is greater during high flow conditions (June) which may be attributed to the inputs from run-off and/or snowmelt.

4. Limitations

Although this study provided insights on the biological activity of the LAR using *in vitro* bioassays, there are limitations which should be considered when interpreting the results of this work. For instance, due to the large volumes of water samples needed to complete seven bioassays (~10 L per site for one replicate), this study primarily focused on the spatial extent (covering 120 km of river length) as opposed to collecting several replicates at one sampling location. Nonetheless, field replicates collected at the same point in space and time are valuable in capturing any variation in the storage and sampling techniques, and natural sample variability. Therefore, the combination of field and analytical replicates would increase the precision and accuracy of the sample results. Also, the samples were only analyzed once on each assay/test therefore the reported results do not include information regarding inter-assay variability (i.e., experimental mean value or spread of the data). Even though the test results were validated through QA/QC of controls (i.e., positive and negative controls, blank), the sample data does not capture potential analytical errors including variability in the bacteria/cell line or instrumentation.

In addition, the statistical analysis (i.e., One-way ANOVA test) was performed on the June vs August river samples where possible. However, the dissimilarity of the sites during these campaigns may imply that there is not a direct comparison of the mean value of the results. Therefore, it is challenging to confidently link a relationship between the observed biological activity/concentrations and the hydrologic conditions of the river. Finally, mixing patterns may play a role on the biological activity. Although the results show almost no impact in biological activity due to effluent releases (FMO and MSO), the samples were collected within the mixing zone based on a preliminary estimate of the river mixing length.

5. Conclusions and Recommendations

This study has successfully demonstrated the applicability of *in vitro* bioassays in the oil sands industry and in assessing the bioactivity in receiving aquatic environments. OSPW showed high responses to all 7 *in vitro* bioassay tests used, with 5 out of 7 signals exceeding the currently proposed EBT values for each assay. These responses indicate that all toxicity endpoints are relevant to OSPW, with primary toxicity indicators including cytotoxicity, estrogenicity, mutagenicity, binding to the PPARɣ, and secondary indicators including oxidative stress and the induction of the AhR.

The WWTP effluents activated almost all bioassays where 4 and 3 out of 7 bioactivities exceeded the EBTs for FMO and MSO, respectively. This indicates that WWTP effluents may require continuous monitoring as they activate most of the endpoints and exceeded most EBT thresholds. Most of the active June river samples did not exceed the EBT values, whereas the August river samples mostly did not activate the assays. This implies that the current bioactivity of the Lower Athabasca River is low, but this depends on the hydrologic conditions and may drastically vary during extreme flow events such as a flooding or drought. Based on challenges encountered in this study, recommendations for future studies are discussed below.

Additional toxicity pathways including immunotoxicity, and genotoxicity can be targeted to deepen the knowledge of the potential adverse toxicity effects of untreated OSPW. In this study, there were some issues in determining the estrogenic activity of the samples, hence it would be valuable to conduct another field sampling for analysis using both the YES and Erα-GeneBLAzer assays to further research the suitability of each test for these samples. Furthermore, the use of other mammalian reporter gene assays (e.g., ERα CALUX) may aid in

investigating the possible reasons for the discrepancies between the yeast and mammalian-based assay.

This study has only investigated the potential adverse toxicity effects of untreated OSPW, however with the potential future discharge of treated OSPW, it would be critical to analyze the toxicity effects of treated OSPW which may inform more relevant ecotoxicological implications in the receiving aquatic environments. Moreover, a similar battery of *in vitro* bioassays can be used in the water quality assessment of both untreated and treated OSPW such that the efficiency of the treatment technology may be evaluated.

The mixing patterns and DF of the river minimize the potential impacts of the discharge from its current stressors (i.e., WWTP effluents), and potentially treated OSPW in the future. However, the effects of these effluents in the LAR may change due to decreased river flow or increased effluent discharge. Therefore, the WWTP effluents and future treated OSPW discharge could be constantly monitored using bioanalytical tools. Additionally, two sampling campaigns were conducted which targeted different sampling sites along the LAR, with only one overlapping site (M4/M4'). Therefore, a year-round monitoring program using a consistent battery of *in vitro* bioassays on the same sampling locations would aid in developing a temporal relationship between the seasonally variable hydrologic conditions of the river and the contaminant concentrations.

In this study, a baseline of the biological activity of the river was assessed focusing on the spatial variations of the samples (upstream, within, downstream of oil sands mineable area). The LAR is a large river system affected by anthropogenic activities which may have implications on water quality monitoring especially if samples are collected in the regions of contamination input or are affected by delayed mixing. Therefore, it may be useful to include

composite and cross-river transects samples to assess the variations in the center, right and left banks of the river. Additionally, samples may be collected at different depths to compare the water quality. These samples would deepen the understanding of the mixing processes and contributions of contaminants during monitoring of the LAR.

In the future, *in vitro* bioassays can be used to support the work by the JOSM plan and EMP, by screening samples to direct where further chemical analysis should be targeted. These bioanalytical tools can be useful in the JOSM plan as they can differentiate between different groups of contaminants using a range of toxicity endpoints. This may aid in identifying the source of pollutants (e.g., natural vs industrial vs municipal effluents) along the LAR. As in the case of the EMP, *in vitro* bioassays may help in setting regulations regarding the discharge of treated OSPW by identifying groups of compounds that contribute to the observed toxicity effects. This may be a critical source of information about the ecotoxicological profile of OSPW, while extensive research is still underway regarding its chemical composition.

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Appendix A: Supplementary Information

A- Sample Extraction

Water samples were filtered onsite using glass fiber filters (VWR Glass Fiber Filters, diameter = 4.7cm, Particle retention = 1.5 µm, CA28333-129) and acidified to a pH of 2 using formic acid to extract the organic acids during solid phase extraction (SPE). The untreated OSPW was filtered using syringe filters (Basix^{TM} Syringe Filters, Nylon, diameter = 25 mm, pore size = 0.45µm) and acidified similar to the river samples. SPE using Oasis HLB was used for the extraction of organics due to its higher extraction recovery of dissolved organic carbon, naphthenic acids and volatile polyaromatic hydrocarbons (PAHs) [69, 36]. The SPE vacuum manifold (24-position Supelco Visiprep) was set up with the Oasis HLB cartridges (6 cc, 500 mg), which were first conditioned with 5 mL methanol, followed by 5 mL ultrapure water (Milli-Q system, 18.2 resistance). Samples were then introduced to the cartridges using a vacuum pump (GAST Model DOA-P704-AA). For the river/WWTP samples, the volume introduced was 800 mL per cartridge for bioanalysis (total 5 cartridges), and 900 mL per cartridge for FTIR (total 2 cartridges). For the untreated OSPW, the sample volume introduced to the cartridge was 100 mL. After the samples passed through, the cartridges were washed with 10 mL ultrapure water and allowed to dry under vacuum for 1 h. For the river and WWTP samples, the SPE cartridges were eluted with 10 mL methanol followed by 10 mL ethyl acetate whereas the OSPW SPE cartridges were eluted with 5 mL methanol then 5 mL methanol: ethyl acetate (1:1 v/v). All eluents were then evaporated to dryness under a gentle stream of nitrogen at 35°C – 40°C using a nitrogen evaporator (Organomation Associates). Finally, the dried SPE extracts were reconstituted into solvents specific for chemical and bioanalysis.

B- Methodology

1. Cytotoxicity - Aliivibrio fischeri bioluminescence inhibition assay

On the morning of the assay, the *Aliivibrio fischeri* lyophilized bacteria was reconstituted with the reagent diluent (provided by Environmental Bio-detection Products Inc [EBPI]) and equilibrated at 4 °C for at least 30 minutes. 70 µL and 45 µL from the reconstituted SPE extract of the river/WWTP samples and OSPW respectively was transferred into a test tube and evaporated to dryness using a nitrogen evaporator. The dried sample was reconstituted in 900 µL ultrapure water and 100 µL OAS Solution (provided by EBPI). The pH of the sample solution was adjusted to 7 ± 0.2, using 1N sodium hydroxide. A 96-well plate was prepared with a 1:2 serial dilution of 100 µL sample and 100 µL Sample Diluent (provided by EBPI). The final concentration in the wells ranged from 93 to 1 REF and 4.5 to 0.07 REF for the river/WWTP samples and OSPW respectively. Positive control wells were used to validate each run using 3,5-dichlorophenol (DCP) with well concentrations ranging from 67.5 to 1 mg/L. All samples and the positive control were run in duplicate. After the 96-well plate was prepared, the plate and the *Aliivibrio fischeri* bacteria were equilibrated on a chill block at 15 °C for 30 minutes. Next, 100 µL bacteria was pipetted into each well. The luminescence of the 96-well plate was measured at time intervals 0, 5, 10, 15 and 30 minutes. The plate was placed on the chill block in between measurements.

2. Estrogenicity – Yeast estrogen screen (YES)

All chemicals were purchased from Sigma-Aldrich. The GOLD solution, GOLD media and Minimal media were prepared as stock solutions and stored at 4°C prior to running the assay.

Table S1. Preparation of GOLD solution

Compound	Concentration (g/L)	Storage	Volume to make GOLD solution (mL)
Adenine hydrochloride hydrate	1.2	Room Temperature	75
L-Histidine-HCl	2.4	4 °C	50
L-Arginine-HCl	2.4	4 °C	25
L-Methionine	2.4	4 °C	25
L-Tyrosine	0.9	Room Temperature	25
L-Isoleucine	3.6	4 °C	25
L-Lysine-HCl	3.6	4 °C	100
L-Phenylalanine	3	Room Temperature	25
L-Glutamic Acid	6	Room Temperature	25
L-Aspartic Acid	4	Room Temperature	25
L-Valine	18	4 °C	25
L-Threonine	24	4 °C	25
L-Serine	45	4 °C	50
L-Leucine	3.6	Room Temperature	25
L-Tryptophan	4.8	4 °C	50
Uracil	2.4	Room Temperature	25

Table S2. Preparation of GOLD medium

Solution	Volume (mL)
20% Dextrose stock	60
10X YNB without amino acids	60
GOLD solution	110
Ultrapure water	370

Table S3. Preparation of Minimal medium

Solution	Volume (mL)
20% Dextrose stock	100
10X YNB without amino acids	100
L-Lysine-HCl	10
Ultrapure water	790

Agar solution was made by combining 78 mL ultrapure water, 10 mL-10X YNB without amino acids and 2 g of bactoagar into a glass media round bottle. The solution was autoclaved on a liquid cycle. Once the bottle was cool to touch, the following was added and mixed into the agar solution: 10 mL 20% dextrose, 1 mL L-Histidine-HCl and 1 mL L-Lysine-HCl. From one batch of agar solution, about 6 petri plates were prepared;10-15 mL of the agar solution was

poured onto each petri plate and left to solidify at room temperature. When the agar has solidified, the plate was streaked with cells from a previous stock. The streaked plate was then inverted and incubated at 30 °C for 3-4 days. The plate with the grown cells was stored at 4°C for 2 weeks until it was discarded.

Running the assay:

A colony of cells from the previously streaked plate was isolated and transferred to a 15 mL conical tube with 1 mL of GOLD medium. The tube was incubated at 30 °C, 300 rpm for 18-24 hours. The next day, a spectrophotometer was used to check the optical density at 660 nm (OD_{660}) of the cells was checked to ensure that it was approximately 1. Then, the cells were transferred to a flat bottom flask, with 9 mL of minimal media and incubated at 30 °C, 300 rpm for 18-24 hours. After incubation, 100 µL of cells was added to a microcentrifuge tube with 100 µL of 30% glycerol. This cell stock was stored at -80°C for future assays. Next, 10 mL of minimal media was added to the flask and incubated at 30 °C, 300 rpm for 4-6 hours. At the end of incubation, the seeding media was prepared using 100 µL 10mM copper (II) sulphate pentahydrate, 20 mL minimal media and cells. The cells were added to the seeding media until the OD_{660} reached 0.03 ± 0.002. Immediately after, the samples and controls were prepared in duplicates. 5 µL of the previously prepared samples were added to 2 mL amber vials and left for the methanol to evaporate. Similarly, the positive control, 17β-estradiol (E2) had been previously prepared in stock solutions in methanol. 10 µL of each concentration of E2 was added to a 2 mL amber glass vial and left to dry before the cell solution was added. Once the methanol had evaporated, 200 µL of cells was added to each vial. The amber vials were incubated at 30 °C, 300 rpm for 18-24 hours. The exposure concentrations ranged from $3.13 * 10^{-9}$ M to $2.44 * 10^{-11}$

M for E2; 66 to 0.5 REF for river/WWTP samples and 5 to 0.04 REF for OSPW. The next day, 25 µL of the exposed cells were transferred to a 96-well plate, along with 75 µL of minimal media in each well. The cell density at 660 nm was read using a plate reader (Synergy LX), with Gen 5.3.11 software programmed to a 10-minute kinetic mode read with 50 second intervals. Next, each well received 100 µL of 1:1 YPER-β-Galactosidase solution (Thermo Scientific), and the plate was read at an optical density of 420 nm using a 1-hour kinetic mode read with 50 second intervals. Blank and solvent control wells were also included in each plate to ensure that there were no sources of contamination during the bioassay. For all plates, these wells showed signals below the detection limit.

3. ERα-GeneBLAzer:

GeneBLAzer ERα-UAS-bla GripTite Cells are stably expressing the β-lactamase reporter gene. The test system is based on the GeneBLAzer® FRET Assay from Thermo Fischer Scientific, which quantifies the amount of formed β-lactamase with fluorescence measurement. The method is based on a specific substrate for the β-lactamase, whose implementation depends on the Forster resonance energy transfer (FRET). The substrate readily enters the cell, where endogenous esterases rapidly convert it into a fluorescent precursor (blue fluorescence) that is then transformed into an enzyme product by β-lactamase (green fluorescence).

5 x 10^3 cells per well were seeded in 30 µL of medium per well (Opti-MEM, 2 % csFBS, 100 U/mL penicillin, 100 µg/mL streptomycin) in black, clear bottom poly-D-lysine coated 384-well plates (Corning). Cells were treated with 10 µL/well of the dosing medium containing the samples, blanks, and the reference estradiol (E2) and incubated for 24 h. For the detection of the expression of β-lactamase, the ToxBLAzer detection reagent was prepared according to the instructions of the manufacturer and 8 µL of the reagent was added per well. Fluorescence was

read with excitation at 409 nm and emission at 460 nm (blue) and 530 nm (green) immediately after addition of the reagent (t = 0h) and after 2h of incubation at room temperature in the dark. To determine cell viability of treated cells and unexposed cells as control, confluency was measured based on phase contrast images acquired using an Incucyte Zoom S3 (Essen BioScience, Ann Arbor, Michigan, USA). Cell viability assessment using IncuCyte and the commonly used PrestoBlue cell viability reagent was compared previously for the AhR CALUX assay in Nivala et al. (2018), with IncuCyte found to be a more reliable cell viability measurement [108]. Cell viability was expressed as percentage of the control value.

4. AhR CALUX:

The rat hepatoma cell line H4L7.5c2 stably expressing the luciferase reporter gene plasmid pGudLuc7.5 containing a total of 20 XREs was used in the CALUX assays (Brennan et al., 2015; Neale et al., 2017). 3.5 x 10^3 cells per well were seeded in 30 µL of medium per well (DMEM with Glutamax, 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.4 mg/mL geneticin) in black, clear bottom poly-D-lysine coated 384-well plates (Corning). Plates were incubated for 24 h. Cells were treated with 10 µL/well of the dosing medium containing the samples, blanks or controls and incubated for 24 h. Luminescence was measured and the AHR activity potential of the samples evaluated against the reference TCDD. To measure luciferase activity, cells were washed twice with PBS and subsequently 20 µL of lysis buffer was added (25 mM Tris, 1 % Triton-X 100, 2 nM EDTA, 2 mM DTT, 10 % glycerol). After a 10 min incubation period at RT 20 µL of luciferase substrate buffer (20 mM Tricine, 2.67 mM MgSO4, 33.3 mM DTT, 0.1 mM EDTA, 0.261 mM coenzyme A, 0.53 mM ATP, 0.47 mM D-luciferin was added to each well and luminescence was read. Cell viability was determined following the same principle as for the ERα-GeneBLAzer assay.

5. PPARɣ-GeneBLAzer:

GeneBLAzer PPARγ-UAS-bla 293H cells are based on the same reporter gene system as ERα-GeneBLAzer assay and the assay was performed in a similar way except that the cells were seeded with 6.5 x 10^3 cells per well and rosiglitazone was used as reference compound.

6. UMU-ChromoTest:

The night before the assay was run, the freeze-dried bacteria was rehydrated using growth media, reagent V and 1x glucose solution (provided by EBPI). The reconstituted bacteria were incubated at 37 °C, 100 rpm for 16-18 hours. The next morning, the optical density at 600 nm (OD_{600}) of the overnight growth media was measured against a fresh medium blank to ensure that the OD_{600} was more than 0.1. Next, the overnight bacteria were diluted using fresh growth media. The inoculated bacteria were incubated at 37 °C, 100 rpm for 1.5 hours. During this time, the samples were prepared. 325 µL and 80 µL from the reconstituted SPE extract of the river/WWTP samples and OSPW respectively was transferred into a test tube. The contents of the test tube were evaporated to dryness using a nitrogen evaporator and the dried sample was reconstituted in 1.2 mL of 10% dimethyl sulfoxide (DMSO) in sterile 0.85% saline. The pH of the sample was adjusted to 7 ± 0.2 using 1N sodium hydroxide. Samples were run in duplicate with well concentrations ranging from 481 to 15 REF and 8 to 0.28 REF for the river/WWTP samples and OSPW respectively. A positive control, 4-nitroquinoline 1-oxide (4-NQO), was included in each run with well concentrations ranging from 5.26 to 0.16 µM. 4-NQO is used to validate the results of each run, where the induction ratio at the well concentration of 5.26 µM must be at least 2. The first 96 well-plate (Plate A) was prepared with samples and controls following the procedure described by ISO 13829. After the incubation of the inoculated bacteria, the OD_{600} was measured to ensure that it was at least 80% of the overnight OD_{600}. 70 µL of the

bacteria was added into all wells except the blank wells. The well-plate was incubated at 37 °C, 100 rpm for 2 hours.

Near the end of incubation of Plate A, 270 µL of growth medium was pipetted into each well of Plate B, which was then placed in the incubator at 37 °C, 100 rpm with the lid on. At the end of incubation of Plate A, 30 µL from each well was transferred to the corresponding well in Plate B. The absorbance at 600 nm of Plate B was measured using the microplate reader (Synergy LX) and then this plate was incubated at 37 °C, 100 rpm for 2 hours.

Near the end of incubation of Plate B, the ONPG powder was dissolved in a phosphate buffer (provided by EBPI) and stored in the dark at room temperature until use. The B-Buffer was brought down to room temperature. Once at room temperature, 35 µL of 2-mercaptoethanol was added to the B-Buffer. 120 µL of the B-buffer solution was pipetted into each well of Plate C, which was incubated at 37°C, 100 rpm. At the end of incubation of plate B, the absorbance at 600 nm was measured. Next, 30 µL from each well was transferred to the corresponding well in plate C, immediately followed by 30 µL ONPG solution. Plate C was incubated at 37 °C, 100 rpm for 30 minutes. Once the yellow color was developed, 120 µL of stop solution (provided by EBPI) was added to each well. Lastly, the absorbance at 420 nm of Plate C was measured.

UMU-Express P450 1A2 bacterial strain

The P450 1A2 bacterial strain was used as an alternative to S9 activation. The human cytochrome P450s are involved in the metabolism of drugs, carcinogens, mutagens, steroids, and prostaglandins. The role of the P450s is to either direct substrates for detoxification or activate substrates to produce carcinogenic or mutagenic intermediates [109]. Therefore, the P450 strain can be used to determine the mutagenic activity of a compound with significance to human

exposure. In this study, only 4 samples (M4, M4', M6, FMO and MSO) were analyzed using this bacterial strain as a confirmatory test to investigate the mutagenic potential of PAHs in our samples.

The night before the assay is run, reagents V, W, X, Y and Z (provided by EBPI) are added to the growth media prior to rehydrating the lyophilized bacteria. Once the growth media has been added to the bacteria, the bacterial solution is incubated for 14-16 hours at 37°C, 100 rpm. At the end of incubation, the optical density (OD_{600}) of the overnight growth media was measured against a fresh medium blank. The OD_{600} should reach a value between 0.15-0.20. The remaining steps follow the same procedure as described for the method without this bacterial strain.

M6 was the only river site that showed activity using this bacterial strain with a 2-aminoanthracene (2AA)-EQ value of 1.76 µg/L. On the other hand, both WWTPs showed activities of 13.71 µg/L and 11.95 µg/L 2AA-EQ for FMO and MSO respectively. OSPW did not show any activity with this strain, which we did not expect. In our plate design the OSPW was run at only well 4 concentrations ranging from 7.41 to 0.93 REF. Although we do not have the PAH concentrations for the OSPW used in this study, we know that OSPW has high PAH concentrations typically ranging from 2048 to 5252 µg/L [98]. It is possible then that the high exposure concentrations of PAHs led to a cytotoxic environment, as observed by the low growth factors ($G < 0.26$). From these results, we suggest that further analysis be conducted using a wider range of smaller well concentrations of OSPW to eliminate any interfering cytotoxicity effects.

7. AREc32 reporter gene assay:

The AREc32 cell line is a cell line, which expresses luciferase stably under the antioxidant response element-driven NRF-2 line based on the MCF7 breast cancer cells (Wang et al., 2006) [57]. 2.65 x 10^3 cells per well were seeded in 30 µL of medium per well (DMEM with Glutamax, 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin) in black, clear bottom poly-D-lysine coated 384-well plates (Corning). The assay was performed similar to AhR-CALUX except for a higher concentration of 1.9 mM D-luciferin in the luciferase substrate buffer. Luminescence was measured and the AHR activity potential of the samples evaluated against the reference tert-butylhydroquinone (tBHQ).

Table S4. Field parameters for samples collected in June 2021

Site	pH	Conductivity µs/cm	TDS ppm	Salinity psu	Water temperature °C	Air temperature °C	Humidity %	Wind speed km/h	Atmospheric pressure mm Hg	Altitude ft
M1	7.91	218.3	107.5	0.154	21.2	N/A	N/A	N/A	N/A	N/A
M2	7.82	396.5	194.5	0.242	25	N/A	N/A	N/A	N/A	N/A
FMO	7.02	1584	776.6	0.847	25	N/A	N/A	N/A	N/A	N/A
M3	8.08	183.3	90.8	0.137	17	10.6	69.9	N/A	20.99	877
M4	7.95	443.3	217.7	0.261	17.5	11.3	76	5.7	29.04	835
M5	7.95	248.4	122.2	0.168	17.1	10.9	75.8	8.2	29.05	824
T1	8.05	217.1	106.9	0.153	17.1	11.2	68.2	5.5	29.05	817
M6	8.04	223.9	110.9	0.157	17.3	11.8	72.2	8.5	29.06	811
M7	8.03	211.5	104.1	0.151	17.1	11.3	69.2	10.4	29.08	800
M8	N/A	269.8	132.7	0.178	16.9	11.4	74.8	4.7	29.1	772

Table S5. Water chemistry and physicochemical parameters of samples collected in June 2021

Site	TSS	TDS	TOC	DOC	TIC	TN	DN	Cl-	PO4-P	SO4-S	TON-N	NO2-N	NH4-N
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	mg/L	µg/L	µg/L
FMO	59.6	580.4	11.13	11.06	36.05	10.170	9.805	125.4	8	25.1	8.63	14.6	13.7
M1	245.2	152.0	14.19	14.79	18.52	0.474	0.6041	2.3	<LOD	8.2	0.115	<LOD	6.1
M2	277.6	154.0	14.94	15.43	18.48	0.518	0.6422	2.2	14	8.2	0.112	<LOD	5.4
M3	121.6	132.8	13.56	14.26	12.84	0.434	0.521	11.3	13	3.5	0.040	<LOD	5.6
M4	106.0	156.4	13.18	14.47	17.98	0.435	0.605	3.1	9	7.9	0.097	<LOD	5.6
M5	226.8	154.8	13.36	15.00	17.64	0.466	0.6509	3.4	8	7.6	0.112	1.4	5.9
M6	250.4	156.0	13.88	15.06	17.90	0.499	0.6735	3.1	13	7.8	0.114	<LOD	5.5
M7	134.0	148.8	14.01	15.20	16.56	0.472	0.6603	6.1	11	6.1	0.092	<LOD	7.3
M8	172.0	156.8	13.73	15.68	16.11	0.463	0.643	6.4	10	6.6	0.089	<LOD	6.9
T1	160.4	165.6	17.66	19.15	17.34	0.575	0.8055	2.9	10	7.9	0.102	<LOD	5.4

Table S5. continued

Sites	CaCO3 equiv.	Al	B	Ca	Cu	Fe	K	Mg	Mn	Na	Ni	P	Pb
		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
FMO	mg/L	0.031	0.189	58.047	<LOD	0.054	12.444	18.736	0.098	82.791	0.003	0.047	<LOD
M1	222.10	0.866	0.026	25.182	<LOD	0.701	1.717	7.011	0.007	7.346	0.003	0.025	<LOD
M2	91.75	0.707	0.032	25.324	<LOD	0.609	1.601	7.069	0.006	7.575	0.003	0.024	<LOD
M3	92.34	0.405	0.028	16.423	<LOD	0.637	1.184	4.930	0.007	11.070	<LOD	0.029	0.004
M4	61.31	0.416	0.023	24.429	<LOD	0.417	1.644	6.753	0.006	7.450	0.002	0.022	<LOD
M5	88.81	0.456	0.023	24.473	<LOD	0.454	1.567	6.752	0.006	7.673	0.003	0.023	<LOD
M6	88.91	0.464	0.023	24.794	<LOD	0.449	1.535	6.820	0.006	7.659	0.002	0.023	<LOD
M7	90.00	0.670	0.026	22.117	<LOD	0.656	1.449	6.080	0.008	8.808	0.002	0.028	<LOD
M8	80.26	0.620	0.025	22.910	<LOD	0.622	1.569	6.209	0.008	9.185	0.003	0.029	<LOD
T1	82.77	0.536	0.035	23.496	<LOD	0.560	1.555	6.852	0.007	9.173	0.002	0.027	<LOD
	86.89												

Table S5. continued.

Sites	**S** mg/L	**Se** mg/L	**Si** mg/L	**V** mg/L	**Zn** mg/L
FMO	25.192	<LOD	2.437	<LOD	0.032
M1	7.179	<LOD	4.412	0.003	<LOD
M2	7.107	<LOD	4.072	0.002	<LOD
M3	3.288	<LOD	3.596	0.002	<LOD
M4	6.795	<LOD	3.282	<LOD	<LOD
M5	6.740	<LOD	3.467	0.002	<LOD
M6	6.866	<LOD	3.434	0.002	<LOD
M7	5.446	<LOD	4.153	0.002	<LOD
M8	5.919	<LOD	3.990	0.002	<LOD
T1	6.946	<LOD	3.395	0.002	<LOD

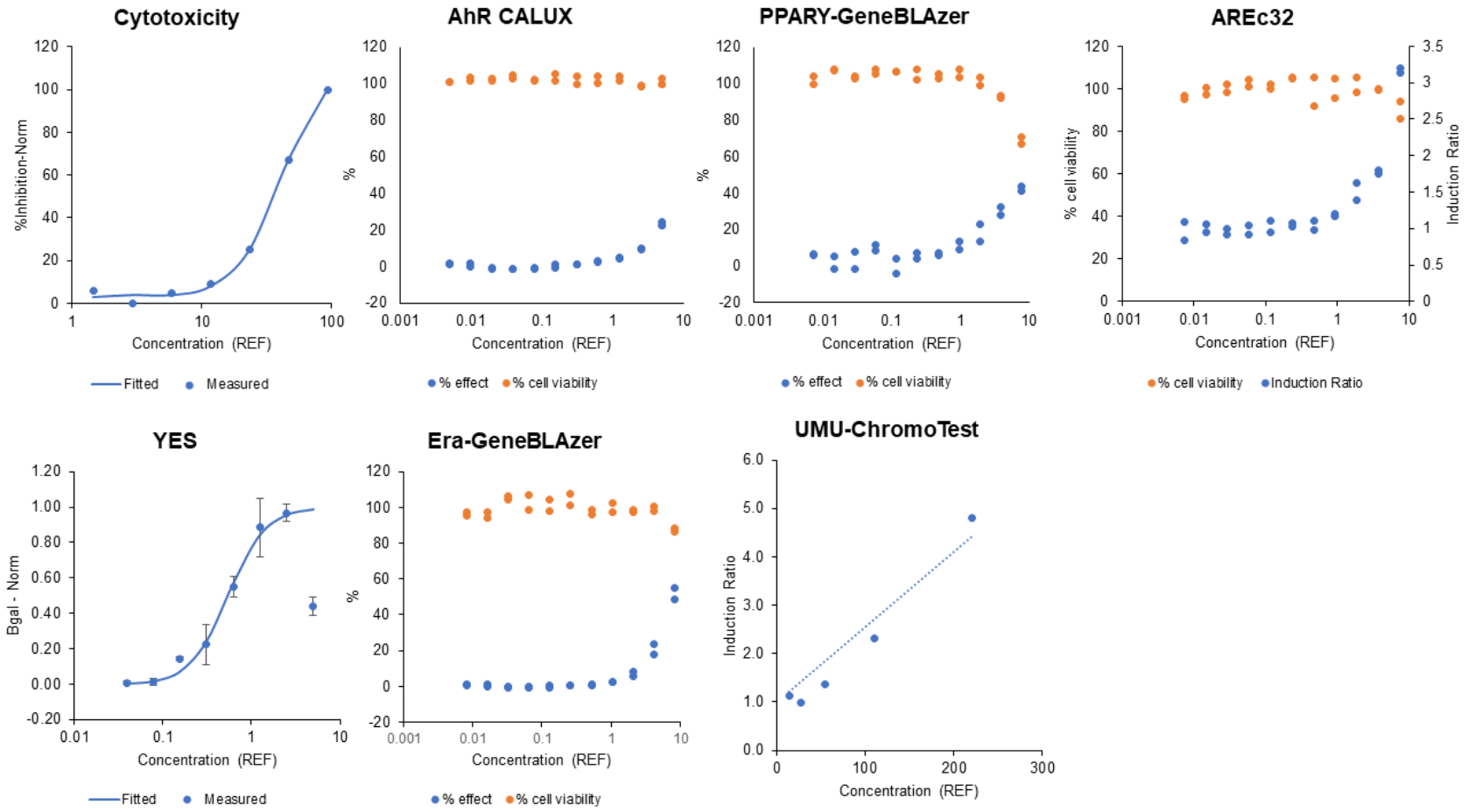


Figure S1. Examples of the concentration-response curve for each bioassay.

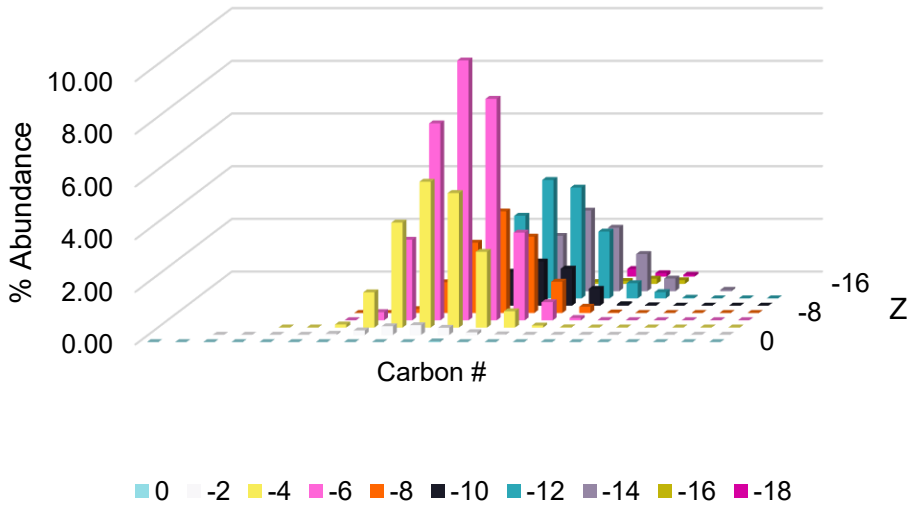


Figure S2. NA speciation of untreated OSPW. 'Z' represents the unsaturation or number of rings in each compound.

Table S6. Summarized results for bioassays – AEO concentrations & BEQ values

	AEOs (mg/L)	PPARɣ [a]	AhR [b]	AREc32 [c]	ERα [b]	YES [b]	UMU [b]	Cytotoxicity [b]
June								
M8	0.6	*	53.2	*	*	1.3	*	13.3
M7	1.6	*	*	*	0.92	2.7	0.5	23.9
M6	2.4	*	46.4	*	*	7.5	0.5	24.8
M5	0.4	83.7	*	*	*	7.1	*	21.9
T1	1.1	130.9	*	*	*	2.7	*	6.59
M4	0.4	65.7	*	248.1	*	2.6	0.3	13.5
M3	0.5	*	*	*	*	1.5	*	22.7
FMO	1.3	89.0	94.9	333.8	1.53	2.2	2.6	19.2
M2	1.2	233.9	*	*	*	3.8	*	13.1
M1	0.7	74.2	*	*	*	2.1	0.2	10.6
August								
M4’	0.9	*	*	*	*	0.7	*	33.5
S1E	0.2	*	*	*	*	2.7	*	32.6
S4E	0.1	*	*	*	*	2.6	*	***
MSO	3.3	94.8	106.5	559.7	*	19.9	0.7	12.0
S2E	<LOD	*	*	*	*	3.7	0.2	***
S2W	<LOD	*	*	*	*	0.8	*	***
T2	0.3	*	*	*	0.57	n.p.	*	4.2
OSPW	52.2 ± 8.0	2824.2 ± 589.8	172.4 ± 142.8	774.7 ± 292.8	6.4 ± 0.3	133.8 ± 34.8	6.4	1.0 ± 0.7

[a] Units = ng/L rosiglitazone-EQ. [b] Units are the same as Table 3.2. [c] Units = µg/L dichlorvos-EQ. * = no activity; *** = cannot be determined; n.p. = not processed.

C - Dilution Factor

$$Dilution\ factor = \frac{Flowrate\ of\ river}{Flowrate\ of\ WWTP\ effluent}$$

Flow rate during June sampling (m^3/s) = 1565 and 162, for LAR and CR, respectively

Flow rate during August sampling (m^3/s) = 522 and 89.8, for LAR and CR, respectively

All flow rates of the LAR and CR were retrieved from https://wateroffice.ec.gc.ca

Average daily flow of FMO = 0.233 m^3/s [110].

D - Polycyclic Aromatic Compounds (PACs)

PACs data for select river samples was obtained from AEP (retrieved from https://aws.kisters.net) and are presented in Table S7. The total PAHs concentrations in June 2021 (mean = 445.7 ± 204.6 ng/L) are statistically significantly higher than that of the samples collected in August 2021 (mean = 73.9 ± 15.7 ng/L) (ANOVA, $p = 0.001$, $\alpha = 0.05$). This supports the trend observed in the bioassay and AEOs data, where there are higher concentrations during higher flow conditions of the LAR. This trend correlates with the findings of Droppo et al. (2018) who investigated the temporal influence on contaminant transport in two tributaries of the Lower Athabasca River and found that higher daily loadings of PACs occurred in the high flow seasons (May-July) compared to low flow seasons (July to October). The authors further reported that the daily PACs loads decreased 10 to 100 times from May to October and that the highest PAC loads occurred during the spring melt [111].

Although the WWTP effluents collected in June and August were not sent for PAC analysis, we collected samples at a later date (October 2021) to assess the PAC concentration in these point sources. The total PACs concentrations are 62.8 ng/L for FMO and 151.3 ng/L for MSO. Our reported PACs concentrations are lower than numerous studies which reported PACs levels ranging from 864 to 1720 ng/L, which may be related to variations in the wastewater

treatment process and differences in influent concentrations, service populations and volume of water to be treated [112].

The untreated OSPW used in our study was not analyzed for PACs however, previous literature has reported that the PAC concentrations of oil sands tailings range from 2048 to 5252 µg/L [98].

Table S7. PACs Data for selected samples

Sample	**Alkylated PAHs (ng/L)**		**Parent PAHs (ng/L)**		**Dibenzothiophenes (ng/L)**	
	June	August	June	August	June	August
M4’	387.6	66.6	34.2	10.1	117.6	17.0
S1E	570.7	62.0	37.0	9.7	211.7	18.7
S4E	214.0	45.9	30.5	8.2	59.1	10.9
S2E	219.5	49.5	29.3	11.4	64.5	15.9
S2W	210.4	38.8	29.5	8.9	60.9	10.2
M3’	282.7	38.8	29.2	8.9	85.8	11.7
FMO*	37.2		9.2		16.5	
MSO*	85.7		20.9		44.8	

Data retrieved from Alberta Environment & Parks – Enhanced Monitoring Program. *= Samples were collected in October 2021.

Appendix B

Table S8. Sigmaplot fitting data for cytotoxicity assay

Site	R^2	Min	Max	Log (EC_{50})	Hillslope
June					
M8	0.9977	0.0345	1.1282	1.6122	2.4562
M7	0.9884	0.0482	1.0410	1.6627	4.4397
M6	0.9883	0.0626	1.0111	1.6357	5.7614
M5	0.9963	0.0606	2.5918	2.0619	2.4945
T1	0.9948	0.0647	1.4016	1.7540	1.6754
M4	0.9905	0.0392	1.0009	1.4647	3.4914
M3	0.9925	0.0785	1.1507	1.7750	4.0299
FMO	0.9942	0.0752	1.1119	1.7212	3.6843
M2	0.9914	0.0340	1.1103	1.5963	2.4742
M1	0.9997	-0.0051	3.3629	2.2825	1.1868
August					
M4’	0.9941	0.0561	1.2376	1.8376	4.5233
S1E	0.9892	0.0653	1.0177	1.7197	6.8820
S4E					
MSO	0.9975	-0.0078	1.9235	1.9444	1.4208
S2E					
S2W					
T2	0.9933	-0.0577	263.0115	5.8850	0.6127
OSPW	0.9795 ± 0.0189	0.0590 ± 0.0278	1.1023 ± 0.2766	0.2238 ± 0.1854	14.8783 ± 16.5251
3,5-DCP	0.9553 ± 0.0308	0.0288 ± 0.1696	0.9326 ± 0.0522	0.6033 ± 0.0829	11.5776 ± 14.5026

Table S9. Sigmaplot fitting data for YES

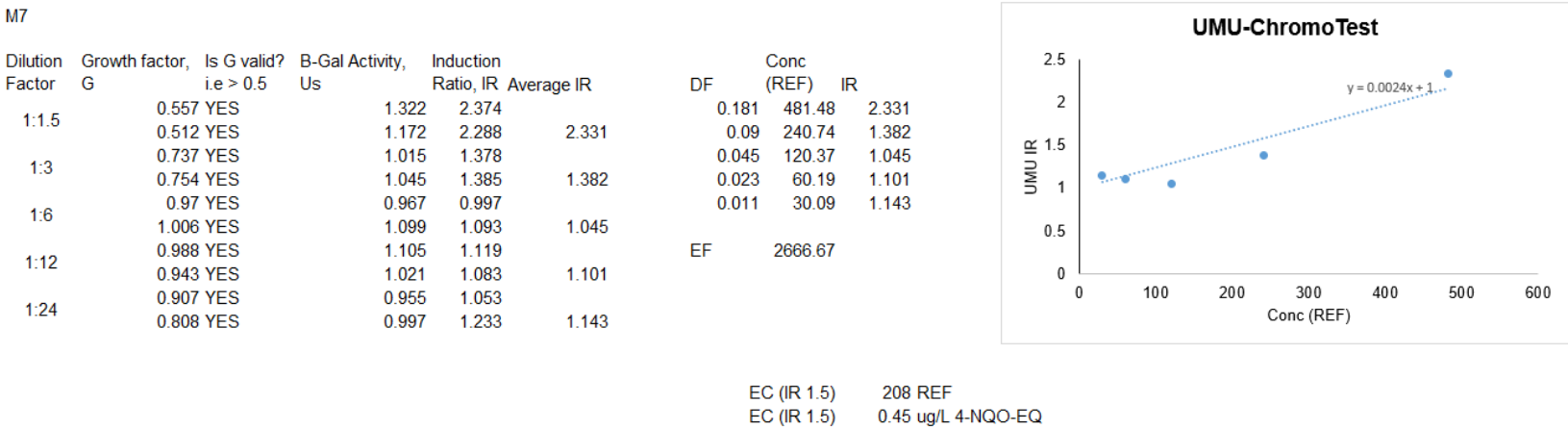
Site	R^2	EC_{50}	Hillslope
June			
M8	0.9157	25.6928	4.7037
M7	0.9795	22.1022	3.2049
M6	0.9837	8.6050	2.0764
M5	0.9704	9.5604	1.8723
T1	0.9897	9.8247	12.8544
M4	0.9984	24.8623	1.7651
M3	0.9592	24.5298	3.6442
FMO	0.9493 ± 0.0016	166.2286 ± 0.3136	17.7398 ± 21.9150
M2	0.9662	17.3330	3.4849
M1	0.9362	33.0219	3.1862
August			
M4’	0.9984	36.6218	8.0225
S1E	0.9585	34.0220	3.1647
S4E	0.9728 ± 0.0381	22.9034 ± 11.8348	2.6524 ± 0.3607
MSO	0.9221	7.1926	1.4103
S2E	0.9896	17.6823	3.4659
S2W	0.8974	38.2572	5.2411
T2	*	*	*
OSPW	0.9726 ± 0.0285	0.4800 ± 0.0877	3.8647 ± 2.5771
E2	0.9427 ± 0.0569	$3.4605 *10^{-10} \pm 1.5188 *10^{-10}$	2.3326 ± 1.4514

*- not analyzed. Min = 0 and max = 1 for all samples.

Table S10. Abundance of NA species in OSPW categorized by carbon # and Z

Carbon #	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Z	**% Abundance**																				
0	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.030	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
-2			0.005	0.005	0.005	0.005	0.040	0.170	0.330	0.370	0.270	0.100	0.020	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
-4					0.005	0.005	0.120	1.340	3.990	5.540	5.110	2.880	0.620	0.080	0.010	0.005	0.005	0.005	0.005	0.005	0.005
-6							0.005	0.310	3.060	7.480	9.870	8.410	3.330	0.690	0.090	0.010	0.005	0.005	0.005	0.005	0.005
-8							0.005	0.005	0.150	1.170	2.670	3.860	2.900	1.190	0.240	0.020	0.005	0.005	0.005	0.005	0.005
-10									0.020	0.190	0.700	1.290	1.680	1.410	0.640	0.060	0.020	0.005	0.005	0.005	0.005
-12										0.100	0.940	3.140	4.500	4.210	2.540	0.580	0.240	0.030	0.005	0.005	0.005
-14											0.040	0.540	2.100	3.060	2.410	1.410	0.480		0.050		
-16											0.005	0.030	0.110	0.070	0.110	0.200	0.140				
-18												0.005			0.290	0.130	0.070				

All abundance values reported as 0.005% were calculated as ½ detection limit.



M7

Dilution Factor	Growth factor, G	Is G valid? i.e > 0.5	B-Gal Activity, Us	Induction Ratio, IR	Average IR
1:1.5	0.557	YES	1.322	2.374	
	0.512	YES	1.172	2.288	2.331
1:3	0.737	YES	1.015	1.378	
	0.754	YES	1.045	1.385	1.382
1:6	0.97	YES	0.967	0.997	
	1.006	YES	1.099	1.093	1.045
1:12	0.988	YES	1.105	1.119	
	0.943	YES	1.021	1.083	1.101
1:24	0.907	YES	0.955	1.053	
	0.808	YES	0.997	1.233	1.143

DF	Conc (REF)	IR
0.181	481.48	2.331
0.09	240.74	1.382
0.045	120.37	1.045
0.023	60.19	1.101
0.011	30.09	1.143
EF	2666.67	

EC (IR 1.5) 208 REF
EC (IR 1.5) 0.45 ug/L 4-NQO-EQ

Figure S3. Example of data analysis for UMU-ChromoTest

M8

EF 2666.666667

	@ time = 0 min		@ time = 15 min		%Inh		% Inh - norm				%Inh Equation	DF	Conc (REF)	log Conc
									Average	STDEV				
A	51795	50822	19632	19298										
B	5588	4798	97	89	95%	95%	100%	100%	100%	0%	100%	0.035	93.33	1.97
C	12646	12720	1993	1884	58%	61%	66%	68%	67%	2%	67%	0.018	46.67	1.67
D	26529	26188	8354	8613	17%	13%	27%	23%	25%	3%	25%	0.009	23.33	1.37
E	36140	34984	13914	13426	-2%	-1%	10%	9%	9%	0%	8%	0.004	11.67	1.07
F	44474	41975	17816	16848	-6%	-6%	6%	5%	5%	1%	4%	0.002	5.83	0.77
G	49384	45836	20958	19255	-12%	-11%	0%	0%	0%	0%	4%	0.001	2.92	0.46
H	51353	50739	19657	20770	-1%	-8%	10%	3%	6%	5%	3%	0.0005	1.46	0.16
KF	0.38	0.38			min	-12%	-11%							
					max	95%	95%							

Volume of extract taken	70 uL
Dilution tube volume	1000 uL
Dilution factor	14.29
Volume taken from tube	100 uL
Volume of diluted extract in w	7 uL
Volume in well	200 uL

*** Complete a Ligand Binding - Sigmoidal-Dose response regression using log Concentration & Average % Inhibition. Use Sigmaplot**

	Coefficient
Rsquared	0.9977
min	0.0345
max	1.1282
log EC50	1.6122
Hillslope	2.4562

EC20		EC10	
y	0.2	y	0.1
y-min	0.1655	y-min	0.0655
log EC20	1.3073	log EC10	1.1253

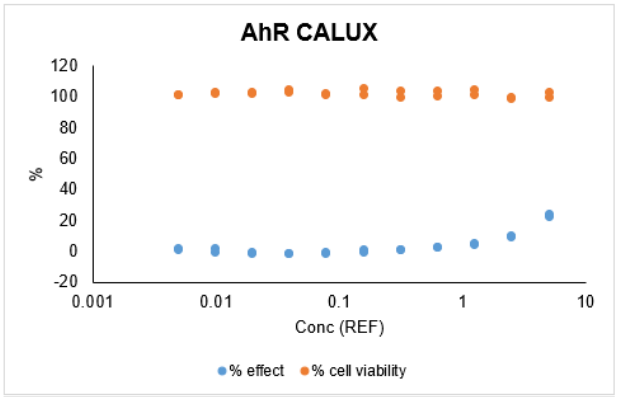
EC50	**40.945**	**REF**
EC20	**20.292**	**REF**
EC10	**13.346**	**REF**

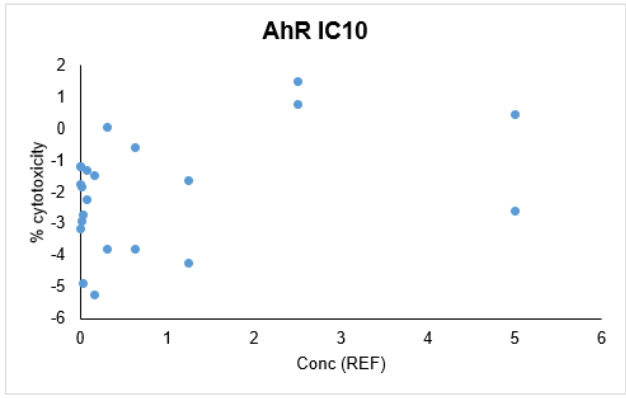
Figure S4. Example of data analysis for cytotoxicity assay

FMO

Conc (REF)	% effect	% cell viability	% cytotoxicity
5	22.57	99.57	0.43
5	24.37	102.59	-2.59
2.5	9.49	99.23	0.77
2.5	10.06	98.53	1.47
1.25	4.94	104.26	-4.26
1.25	4.41	101.64	-1.64
0.625	2.88	100.62	-0.62
0.625	2.53	103.81	-3.81
0.3125	1.38	103.81	-3.81
0.3125	1.05	99.96	0.04
0.1563	1.05	101.47	-1.47
0.1563	-0.47	105.26	-5.26
0.0781	-0.53	102.25	-2.25
0.0781	-1.56	101.33	-1.33
0.0391	-1.22	102.72	-2.72
0.0391	-1.38	104.88	-4.88
0.0195	-1.04	101.85	-1.85
0.0195	-0.51	102.92	-2.92
0.0098	1.95	103.18	-3.18
0.0098	-0.14	101.78	-1.78
0.0049	1.22	101.22	-1.22
0.0049	1.60	101.22	-1.22

IC10	no cytotoxicity (<10%)
EC10	2.23 REF
EC10	94.90 ng/L B[a]P-EQ





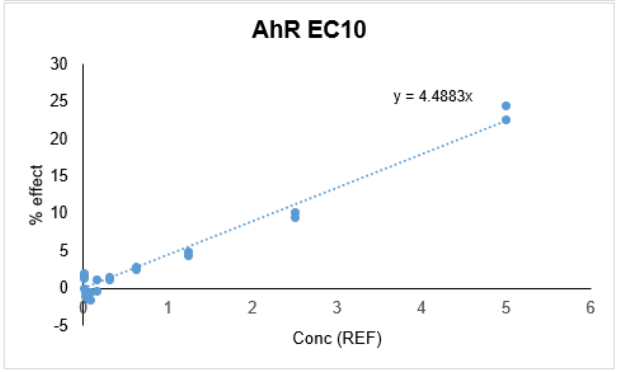
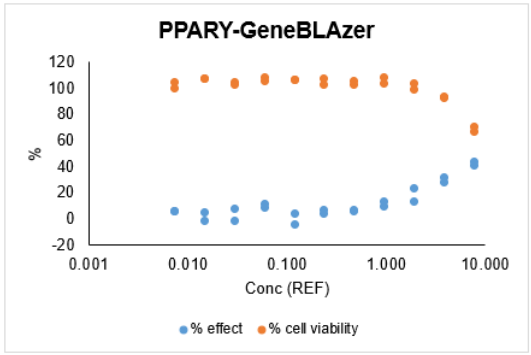


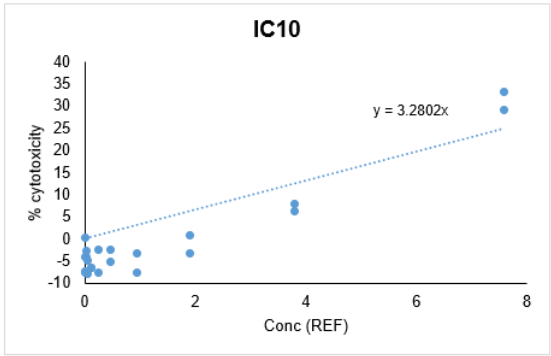
Figure S5. Example of data analysis for AhR CALUX

SSO

Conc (REF)	% effect	% cell viability	% cytotoxicity
7.576	43.95	66.82	33.18
7.576	41.19	70.85	29.15
3.788	28.05	92.08	7.92
3.788	32.21	93.66	6.34
1.894	23.11	99.18	0.82
1.894	13.77	103.41	-3.41
0.947	9.39	103.22	-3.22
0.947	13.75	107.79	-7.79
0.473	7.02	102.59	-2.59
0.473	6.23	105.27	-5.27
0.237	7.30	102.39	-2.39
0.237	4.09	107.63	-7.63
0.118	4.28	106.52	-6.52
0.118	-3.95	106.61	-6.61
0.059	11.64	105.04	-5.04
0.059	8.33	107.90	-7.90
0.030	7.83	104.20	-4.20
0.030	-1.79	102.89	-2.89
0.015	-1.57	107.42	-7.42
0.015	5.28	107.69	-7.69
0.007	6.36	104.21	-4.21
0.007	6.03	99.71	0.29

IC10	3.05 REF
EC10	0.94 REF
EC10	0.095 ug/L rosiglitazone-EQ





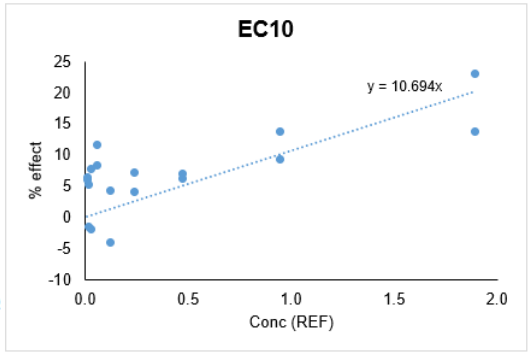
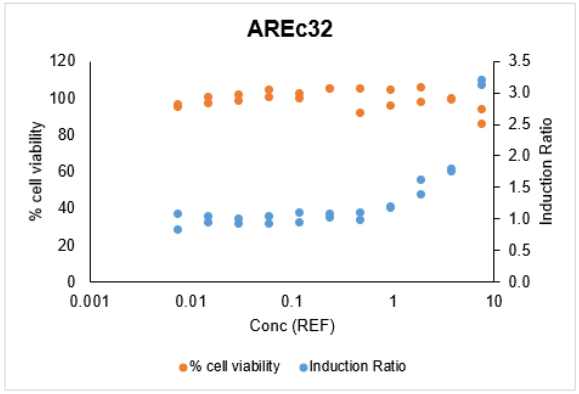


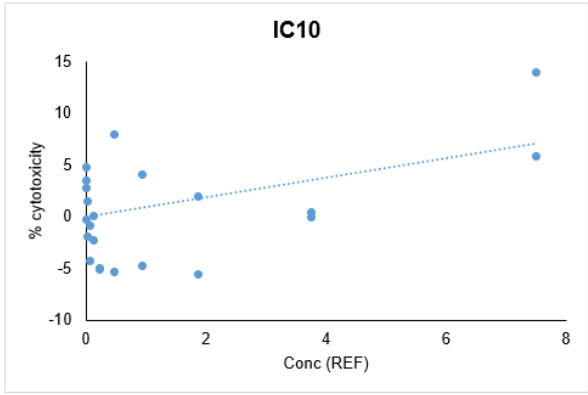
Figure S6. Example of data analysis for PPARɣ-GeneBLAzer. Concentrations highlighted were used to calculate EC_{10}.

OSPW

Conc (REF)	Induction Ratio	% cell viability	% cytotoxicity
7.5	3.132	94.158	5.842
7.5	3.206	86.080	13.920
3.75	1.758	99.560	0.440
3.75	1.804	100.045	-0.045
1.875	1.625	105.614	-5.614
1.875	1.396	98.086	1.914
0.938	1.176	95.884	4.116
0.938	1.201	104.795	-4.795
0.469	0.981	91.998	8.002
0.469	1.100	105.400	-5.400
0.234	1.023	104.934	-4.934
0.234	1.080	105.143	-5.143
0.117	1.102	99.889	0.111
0.117	0.951	102.325	-2.325
0.059	1.041	104.315	-4.315
0.059	0.923	100.849	-0.849
0.029	0.923	101.874	-1.874
0.029	1.003	98.513	1.487
0.015	0.944	97.229	2.771
0.015	1.055	100.305	-0.305
0.007	0.838	95.186	4.814
0.007	1.094	96.534	3.466

IC10	no cytotoxicity (<10%)
EC(IR1.5)	1.84 REF
EC(IR1.5)	0.92 mg/L dichlorvos-EQ





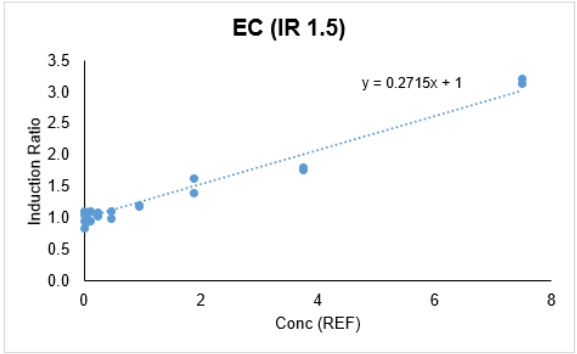


Figure S7. Example of data analysis for AREc32 reporter gene assay

OSPW Input

Volume extracted	100 mL	Volume exposed to cells	5 uL
Volume after reconstitution	0.5 mL	Cell volume	200 uL

Standard Curve	Extraction Factor	Dosing Factor	Conc (REF)	Cal Curve 1 Bgal Response Std. A	Cal Curve 2 Bgal Response Std. B	Cal Curve 1 Rel. B Gal A	Cal Curve 2 Rel. B Gal B	Average	St. Dev
1	200	0.025	5.000	2772072.009	1990979.801	0.477	0.403	0.440	0.052
2	200	0.0125	2.500	5733543.743	4270885.262	1.000	0.935	0.967	0.046
3	200	0.00625	1.250	4421373.537	4549784.161	0.768	1.000	0.884	0.164
4	200	0.003125	0.625	2949020.502	2795894.149	0.508	0.591	0.550	0.058
5	200	0.0015625	0.313	878491.618	1566801.055	0.143	0.304	0.223	0.114
6	200	0.00078125	0.156	841018.241	917571.738	0.136	0.152	0.144	0.011
7	200	0.000390625	0.078	230154.709	264032.773	0.028	0.000	0.014	0.020
8	200	0.000195313	0.039	69281.261	333842.710	0.000	0.016	0.008	0.012
			Max	5733543.74	4549784.16				
			Min	69281.26	264032.77				
			Overall Max	**5733543.74**					
			Overall Min	**69281.26**					

*Complete a Sigmoidal, Hill, 4 Parameter regression on Concentration and Ave Normalized Bgal - Measured. Exclude points with cell death. Copy Results to Sigma Plot Report - Cal Curve.

	Conc (REF)	Average Normalized Bgal - Measured	Bgal Curve
1	5.00	0.440	0.9894
2	2.50	0.967	0.9578
3	1.25	0.884	0.8464
4	0.63	0.550	0.5722
5	0.31	0.223	0.2451
6	0.16	0.144	0.0731
7	0.08	0.014	0.0188
8	0.04	0.008	0.0046

$$y = \min + \frac{(\max - \min)}{1 + (x/EC50)^{-HillSlope}}$$

Four Parameter Logistic Equation

min	0
max	1
EC50	0.542
Hillslope	2.0424
R^2	0.9927

Points excluded: 1
Rationale: cell death

EC50 (REF)	**5.42E-01**	50
EC25 (REF)	**3.17E-01**	25
EC10 (REF)	**0.185**	10

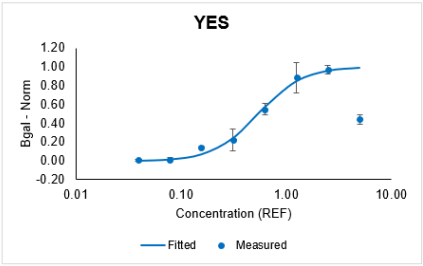
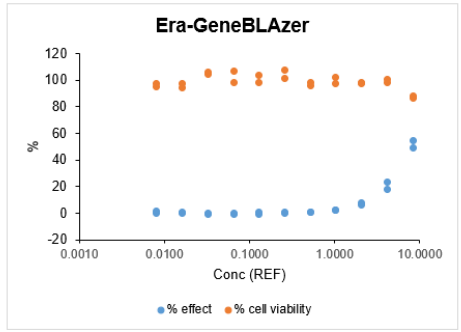


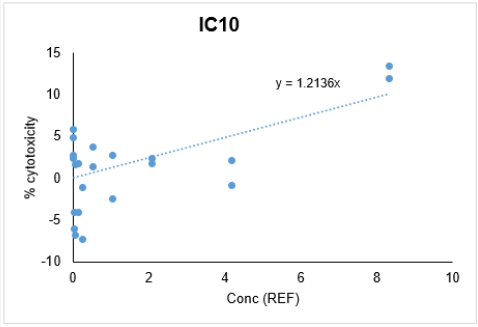
Figure S8. Example of data analysis for YES

FMO

Conc (REF)	0h_Blue	0h_Green	2h_Blue	2h_Green	B/G ratio	% effect	confluency-norm to unexposed	% cell viability	% cytotoxicity
8.3333	318	436	3388	1673	2.313	54.798	87.738	88.071	11.929
8.3333	311	433	2995	1626	2.089	48.765	86.299	86.626	13.374
4.1667	310	428	2098	1868	1.158	23.673	97.504	97.874	2.126
4.1667	309	432	1965	2085	0.939	17.771	100.447	100.829	-0.829
2.0833	314	428	1368	2130	0.575	7.952	97.946	98.318	1.682
2.0833	309	429	1228	2125	0.501	5.951	97.253	97.623	2.377
1.0417	318	429	1029	2199	0.368	2.375	102.056	102.444	-2.444
1.0417	311	432	1003	2181	0.362	2.208	96.882	97.250	2.750
0.5208	314	428	889	2107	0.310	0.801	98.236	98.609	1.391
0.5208	299	421	914	2161	0.322	1.114	95.857	96.221	3.779
0.2604	329	430	925	2182	0.309	0.777	100.755	101.138	-1.138
0.2604	314	422	928	2356	0.290	0.264	106.935	107.341	-7.341
0.1302	322	426	924	2230	0.304	0.633	97.902	98.274	1.726
0.1302	319	425	872	2338	0.262	-0.479	103.683	104.077	-4.077
0.0651	311	425	850	2151	0.282	0.045	98.050	98.422	1.578
0.0651	326	433	873	2319	0.263	-0.464	106.392	106.796	-6.796
0.0326	356	437	847	2208	0.249	-0.840	105.657	106.058	-6.058
0.0326	327	438	910	2364	0.276	-0.121	103.754	104.148	-4.148
0.0163	483	452	1013	2159	0.280	-0.010	97.266	97.635	2.365
0.0163	333	436	937	2154	0.319	1.055	93.853	94.209	5.791
0.0081	371	440	933	2195	0.290	0.263	94.847	95.208	4.792
0.0081	319	433	947	2204	0.323	1.158	96.880	97.248	2.752

IC10	8.24 REF
EC10	2.23 REF
EC10	1.53 ng/L EEQ





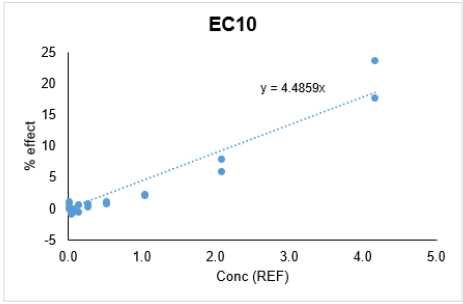


Figure S9. Example of data analysis for Erα-GeneBLAzer. Concentrations highlighted were used to calculate EC_{10}.

Table S11. PACs analysis for FMO and MSO

	FMO			MSO		
PAC compound	flag	ng/L	ng/L (RL)	flag	ng/L	ng/L (RL)
Naphthalene		2.25	0.118		4.02	0.154
Acenaphthylene	ND		0.122	ND		0.145
Acenaphthene	ND		0.136		0.375	0.292
2-Methylfluorene	ND		0.207		0.347	0.13
C2 Phenanthrenes/Anthracenes		1.8	0.156		5.24	0.051
Fluorene		0.236	0.056		0.634	0.145
Phenanthrene		0.779	0.19		2.91	0.122
Anthracene	ND		0.192		0.571	0.124
C1 Phenanthrenes/Anthracenes		0.375	0.17		7.59	0.161
Fluoranthene	NDR	1.79	0.083	NDR	1.89	0.067
Pyrene		2.21	0.081		2.48	0.065
Benz[a]anthracene		0.154	0.126		1.63	0.094
Chrysene		0.886	0.13		2.86	0.1
Benzo[b]fluoranthene	ND		0.096		0.257	0.142
Benzo[j,k]fluoranthenes	ND		0.111	ND		0.151
Benzo[e]pyrene	NDR	0.274	0.144		1.17	0.202
Benzo[a]pyrene	ND		0.152		1.03	0.214
Perylene	ND		0.15		0.314	0.216
Dibenz[a,h]anthracene	ND		0.155		0.423	0.193
Indeno[1,2,3-cd]pyrene	ND		0.149	NDR	0.31	0.125
Benzo[ghi]perylene	NDR	0.203	0.137		0.938	0.122
2-Methylnaphthalene		1.46	0.143		3.15	0.322
1-Methylnaphthalene	NDR	0.666	0.15		1.75	0.338
C1-Naphthalenes		1.46	0.143		4.9	0.322
Biphenyl		0.686	0.1		0.601	0.362
C1-Biphenyls		0.425	0.082		0.68	0.115
C2-Biphenyls		0.964	0.104		1.05	0.123
C2-Naphthalenes		2.23	0.17		4.96	0.294
1,2-Dimethylnaphthalene		0.271	0.17	ND		0.294
2,6-Dimethylnaphthalene		0.252	0.144		0.987	0.249
C3-Naphthalenes		2.29	0.177		5.57	0.307
2,3,6-Trimethylnaphthalene		0.589	0.174		2.13	0.303
2,3,5-Trimethylnaphthalene	NDR	0.367	0.179		1.19	0.312
C4-Naphthalenes		7.19	0.232		4.17	0.216
C1-Acenaphthenes		0.106	0.106	ND		0.143
C1-Fluorenes		0.848	0.207		1.53	0.13
C2-Benzofluoranthenes/Benzopyrenes	ND		0.18		2.96	0.261
1,4,6,7-Tetramethylnaphthalene	NDR	1.45	0.232		0.599	0.216

	FMO			MSO		
PAC compound	flag	ng/L	ng/L (RL)	flag	ng/L	ng/L (RL)
1,7-Dimethylfluorene		1.11	0.28	ND		0.256
C2-Fluorenes		6.97	0.28		4.83	0.256
C3-Fluorenes		22.8	1.23		9.22	0.211
Dibenzothiophene	ND		0.147		2.01	0.056
C1-Dibenzothiophenes	ND		0.167		5.97	0.198
2/3-Methyldibenzothiophenes	ND		0.167		2.2	0.198
C2-Dibenzothiophenes		2.12	0.235		15.4	0.196
2,4-Dimethyldibenzothiophene	ND		0.235		1	0.196
4,6-Dimethyldibenzothiophene	NDR	0.308	0.198		1.49	0.165
C3-Dibenzothiophenes		6.69	0.218		13.8	0.16
C4-Dibenzothiophenes		7.52	0.134		7.58	0.176
3-Methylphenanthrene	NDR	0.989	0.168		2.08	0.158
2-Methylphenanthrene		0.173	0.17		2.43	0.16
2-Methylanthracene	ND		0.163		0.8	0.154
9/4-Methylphenanthrene		0.202	0.168		1.06	0.158
1-Methylphenanthrene	ND		0.17		1.21	0.161
3,6-Dimethylphenanthrene	NDR	0.207	0.162	NDR	0.473	0.053
2,6-Dimethylphenanthrene	ND		0.156		0.683	0.051
1,7-Dimethylphenanthrene	ND		0.15		0.738	0.05
1,8-Dimethylphenanthrene	ND		0.156		0.19	0.051
C3-Phenanthrenes/Anthracenes		6.37	0.119		5.24	0.16
1,2,6-Trimethylphenanthrene	NDR	0.169	0.119		0.408	0.16
Retene	NDR	0.691	0.417		0.738	0.402
C4-Phenanthrenes/Anthracenes		12.2	0.417		31.7	0.402
C1-Fluoranthenes/Pyrenes		2.76	0.212		4.67	0.145
3-Methylfluoranthene/Benzo[a]fluorene		0.366	0.212		1.26	0.145
C2-Fluoranthenes/Pyrenes		3.14	0.091		5.71	0.137
C3-Fluoranthenes/Pyrenes		1.59	0.121		2.44	0.195
C4-Fluoranthenes/Pyrenes		0.988	0.076		0.998	0.12
C1-Benzo[a]anthracenes/Chrysenes		1.33	0.102		9.28	0.112
5/6-Methylchrysene	ND		0.101		0.319	0.111
1-Methylchrysene		0.159	0.103		1.02	0.113
C2-Benzo[a]anthracenes/Chrysenes		1.3	0.111		6.46	0.127
5,9-Dimethylchrysene		0.383	0.111		1.62	0.127
C3-Benzo[a]anthracenes/Chrysenes	ND		0.159		0.941	0.104
C4-Benzo[a]anthracenes/Chrysenes	ND		0.137	ND		0.104
C1-Benzofluoranthenes/Benzopyrenes		1.09	0.244		6.7	0.195
7-Methylbenzo[a]pyrene	ND		0.244	ND		0.195

FMO and MSO were collected in Oct 2021. RL = reporting limit; ND = not detected at reporting limit; NDR = peak detected but not meet quantification criteria, result reported represents the estimated maximum possible concentration.