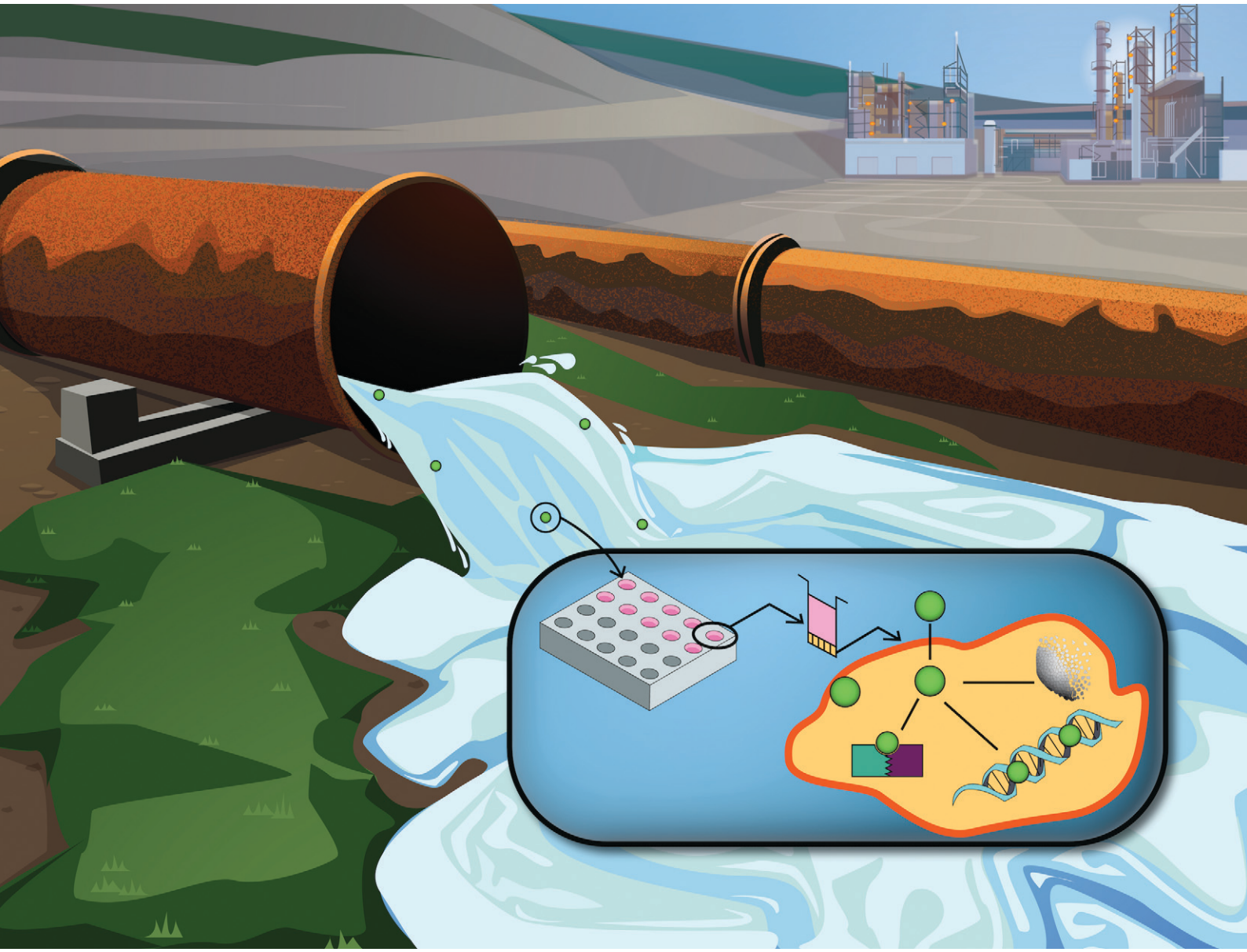
Volume 9
Number 8
1 August 2023
Pages 1973–2162

Environmental Science
Water Research & Technology

rsc.li/es-water



ISSN 2053-1400

ROYAL SOCIETY OF CHEMISTRY

PAPER
Maricor J. Arlos *et al.*
Water quality monitoring with *in vitro* bioassays to compare untreated oil sands process-affected water with unimpacted rivers

Environmental Science
Water Research & Technology

PAPER

View Article Online
View Journal | View Issue

Cite this: *Environ. Sci.: Water Res. Technol.*, 2023, **9**, 2008

Water quality monitoring with *in vitro* bioassays to compare untreated oil sands process-affected water with unimpacted rivers†

Kia Barrow,[a] Beate I. Escher,[bc] Keegan A. Hicks,[d] Maria König,[b] Rita Schlichting[b] and Maricor J. Arlos *[a]

Received 29th December 2022,
Accepted 29th May 2023

DOI: 10.1039/d2ew00988a

rsc.li/es-water

The Alberta oil sands are among the largest oil reserves in the world and generate acutely toxic oil sands process-affected water (OSPW) that is currently stored in tailings ponds. One of the future tailings management strategies considered by mining companies is to treat and release OSPW into the Lower Athabasca River (LAR), but the potential future impacts on the receiving aquatic environments are not completely understood. This study employs a battery of *in vitro* bioassays (cytotoxicity, estrogenicity, mutagenicity, oxidative stress response, and xenobiotic metabolism) to assess the (eco)toxicological effects of the mixtures of organics extracted from surface water samples at 15 sites along the LAR under high- and low-flow conditions. We also assessed the biological activity of untreated OSPW and the effluents from two municipal wastewater treatment plants (WWTPs) for comparison. Overall, the LAR samples showed little to no bioactivity responses, suggesting a low risk to the aquatic environment prior to potential OSPW discharge. Although treated OSPW was not considered in this study, the high responses observed for untreated OSPW suggest that the use of selected *in vitro* bioassay endpoints can be extended to assess the efficacy of treatment technologies that may be implemented for OSPW in the future. Treated municipal WWTP effluent extracts also activated the bioassays. However, in comparison to the proposed effect-based trigger (EBT) values for *in vitro* bioassays (after dilution is considered), the potential toxicity risks associated with direct exposure to these effluents are reduced due to the relatively low contribution to the river flow (0.01–0.04%).

Water impact

Given that the battery of *in vitro* bioassays employed in this work was used to monitor the current biological activity of the receiving environment, there is an opportunity to employ this approach to assess water quality before and after the potential discharge of complex treated industrial effluents and evaluate possible exceedances to the reported effect-based trigger values (EBTs) for aquatic environmental health after dilution.

1. Introduction

Although considered among the largest crude oil reserves in the world, the oil sands development in northeastern Alberta has raised both public and scientific concerns related to its potential impacts on environmental health, especially on nearby aquatic ecosystems.[1] In addition to the release of naturally occurring substances during the oil sands mining, the extraction of crude oil from the deposits produces large volumes of oil sands process-affected water (OSPW), which are stored in tailings ponds and recycled for re-use during the oil extraction process.[2] OSPW is a complex mixture containing bitumen, organic compounds (*e.g.*, naphthenic acids [NAs], polycyclic aromatic compounds [PACs] that also include polycyclic aromatic hydrocarbons [PAHs]), inorganic materials (*e.g.*, ammonia, salts, and metals) and solid constituents (*e.g.*, sand, silts, and clays).[2–4] Due to differences in source deposits and a wide variety of extraction processes currently employed by the industry, OSPW compositions and chemistry also vary from pond to pond.[5]

[a] *Department of Civil and Environmental Engineering, University of Alberta, 9211-116 St. NW, Edmonton, Alberta, Canada T6G 1H9. E-mail: arlos@ualberta.ca*
[b] *Department of Cell Toxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany*
[c] *Environmental Toxicology, Department of Geosciences, Eberhard Karls University Tübingen, Tübingen, Germany*
[d] *Government of Alberta, Environment and Protected Areas, Resource Stewardship Division, 4938 89th St., Edmonton, Alberta, T6E 5K1, Canada*
† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d2ew00988a

There is currently a zero-discharge policy related to the release of treated OSPW and no guidelines exist on its treatment and release into receiving environments. However, there has been a huge interest in treatment and the potential for a treated OSPW to be discharged into the Lower Athabasca River (LAR) as evident from the recent large-scale field piloting of the petroleum coke adsorption technology by one of the oil sand operators.[6] Overall, the effort is currently being assessed both by Alberta and Federal governments including the establishment of the baseline conditions of the LAR and prediction of the effects and implications of treated OSPW release into the LAR.[7,8]

Bioanalytical tools, especially *in vitro* bioassays have been gaining more traction in recent years due to their potential to support chemical analysis in water quality assessment. These tools measure the toxicity of a water sample by focusing on a particular mode of action (MOA), thus accounting for the mixture effects of known and unknown compounds. The battery of bioassays employed in monitoring ideally incorporates toxicity endpoints covering the three major classes of MOAs: non-specific, specific, and reactive.[9] Much of the recent applications of *in vitro* bioassays have been centered on municipal wastewater treatments and surface water, with a select few on industrial sites.[9] This type of work has also been attempted in the oil sands industry but the focus has been on (un)treated OSPW and less on the receiving environments.[4]

In this study, we completed a bioanalytical assessment of 15 sites in the LAR within and outside of the bitumen-rich Fort McMurray formation (Alberta) using a battery of 7 *in vitro* bioassays that cover toxicity pathways *via* non-specific, specific, and reactive toxicities. The main objective of this study is to assess the current bioactivity of the LAR and to determine the relevant toxicity pathways that differentiate river water from untreated OSPW, which will then indicate the potential for these tools in monitoring the impacts of potential future discharge(s) of treated OSPW. The final effluents of two municipal wastewater treatment plants (WWTP) were also included to contrast the mixture effects of existing point sources in the LAR and its receiving environment. We also compared the measured effects in the surface waters with the effect-based trigger values (EBT) specific to each bioassay. The assessment of the potential treatment of OSPW prior to discharge is currently an active area of research. Hence, the results from this work can be combined with future evaluations of the biological activity in the LAR that will support the development of a long-term record of chemical exposure in these systems. Such future applications include comparisons of the conditions before and after the potential discharge of treated OSPW.

2. Methodology

2.1. Site description

Field sampling was completed in the LAR in June and August 2021 covering a total river length of ~120 km (Fig. 1, site details in Table S1†). In June 2021, samples were collected from 9 river sites located upstream, within, and downstream of the oil sands mineable area (Fig. 1). The sites were selected in alignment with the Oil Sands Monitoring Program (OSMP), a program established by federal and provincial governments to monitor the water quality of the LAR.[10] During this sampling campaign, we also collected effluent samples from a municipal WWTP in Fort McMurray (FMO) that services ~107 000 residents of the Regional Municipality of Wood Buffalo using biological nutrient removal followed by UV disinfection.[11,12] 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 OSMP's Enhanced Monitoring Program (EMP) that focuses on establishing the baseline environmental conditions of the LAR prior to the potential discharge of treated OSPW.[7] At this time, samples were also 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 from 3.3 m in June to 2 m in August (Fig. S1 and S2†). 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.[13] We consider our study sites to be representative of the LAR given the thorough work during the design of OSMP and EMP.[7]

2.2. Sample collection, preparation, and extraction

2.2.1. Reagents and materials. Formic acid (88%), methanol (Optima-LC/MS grade), 10 N sodium hydroxide solution, 3,5-dichlorophenol, dimethyl sulfoxide (HPLC grade) and sodium chloride were purchased from Thermo Fisher Scientific, Canada. Ethyl acetate, dichloromethane, and naphthenic acid were purchased from Sigma-Aldrich, Canada. Bioassay reagents are outlined in their methodologies in ESI† section C.

2.2.2. Sampling. Physical and chemical parameters (water temperature, pH, conductivity, total dissolved solids, salinity, Table S2†) were measured during field sampling in June 2021 using a calibrated portable multiparameter meter (Thermo Scientific Orion Star A329). Data on PACs and NAs for both sampling campaigns were retrieved from an open data portal.[14] Samples were collected in 1 L pre-cleaned amber glass bottles with Teflon cap tubes using a swinger grab sampler (Nasco Sampling B01310WA), except for the WWTP effluents, which were collected directly in the sample bottle at the outfall. The total volume collected was 9.8 L and was 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 the expected low levels of chemical contamination in the river samples (high dilution) and, therefore, required high enrichment factors.[9] The large sample volumes also limited the number of replicates per sampling site due to logistics (*i.e.*, boat capacity, timely sample processing), so the design was focused on covering a wider spatial extent (~120

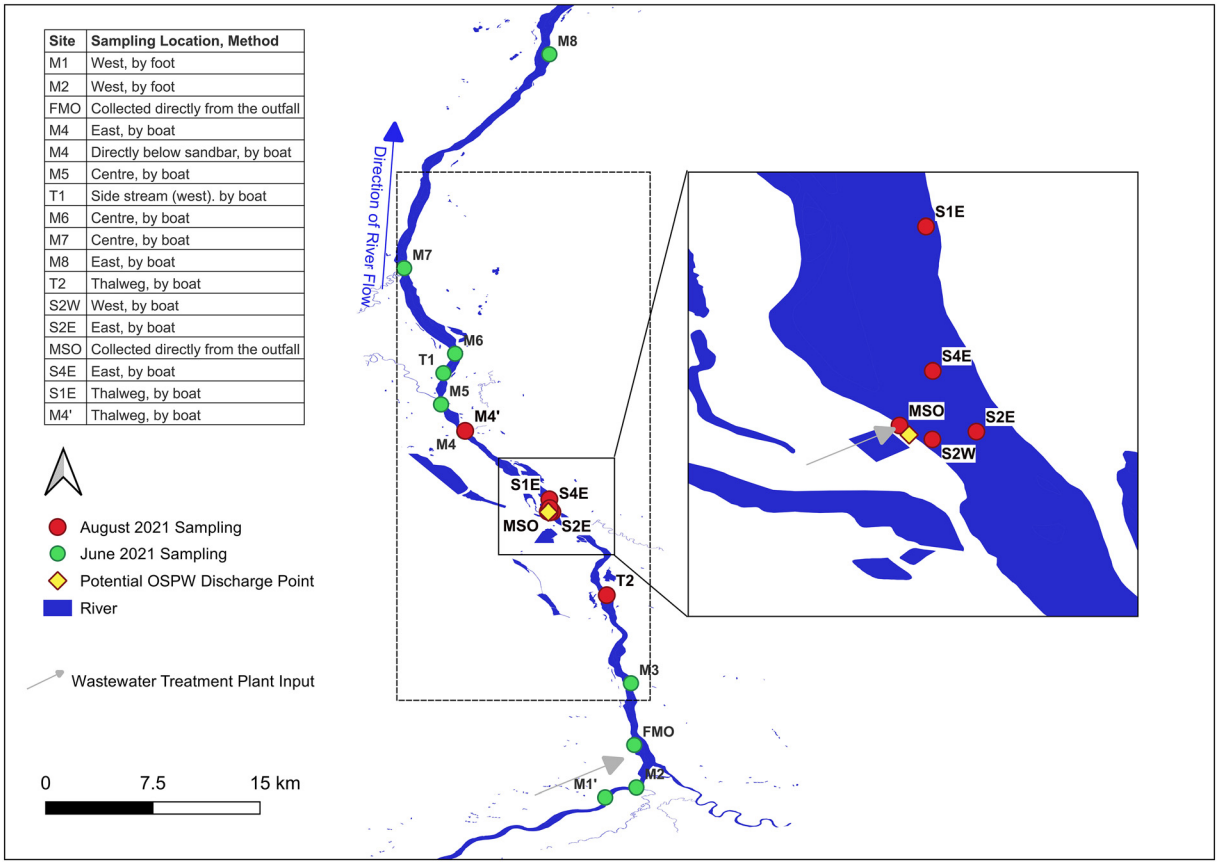


Fig. 1 A map of the study area in the Lower Athabasca River in Alberta, Canada. Sites inside the dashed box are inside the oil sands minable area. FMO = Fort McMurray regional municipal wastewater treatment plant outfall. MSO = sewage outfall. T1 = side stream.

km) to capture activity inside and outside the oil sands mineable area. All samples were collected ~1 m below the surface by boat, except for M1 and M2 that were collected on the surface by foot. 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 S3.† Another sampling campaign was conducted in October 2021 to collect additional samples of FMO and MSO for PAC analysis. Untreated OSPW was provided by a third party.

2.2.3. Sample preparation and extraction. A detailed outline of the sample extraction procedure can be found in the ESI† (section A). In summary, all samples were filtered and acidified to pH 2 using formic acid. The Oasis HLB cartridge was used due to its reported higher efficiency when extracting dissolved organic carbon, naphthenic acids and PAHs (a sub-class of PACs).[15–17] The SPE vacuum manifold was set up with the cartridges, which were conditioned with methanol and ultrapure water (MilliQ IQ 7000). The sample volumes used for the river/WWTP samples were 5 × 800 mL per cartridge for bioanalysis and 2 × 900 mL per cartridge for FTIR. The sample volume for untreated OSPW was 100 mL. After the sample introduction, the cartridges were rinsed with ultrapure water and allowed to dry under vacuum for 1 h. The cartridges were then eluted using the appropriate solvents and the eluents were evaporated to dryness under a gentle stream of nitrogen at 35–40 °C. Finally, the dried SPE extracts were reconstituted into solvents specific for chemical analysis and bioanalysis.

For FTIR analysis, the extracts (from the 2 × 900 mL samples) 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 in 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 (in dry ice). There, the dried extracts were reconstituted in methanol to an extraction factor (EF) of 1000 for samples and 500 for OSPW.

To determine the extraction recoveries, 2 × 1 L of ultrapure water spiked with 20 mg NAs and a 1 L ultrapure water sample (blank) were included in each SPE run. Additional controls (field and SPE blanks) were processed following the same protocol as the river samples. After SPE, the extracts were analyzed using FTIR to determine the acid

extractable organics (AEO) concentrations, which is a surrogate measurement for NAs. The extraction recoveries ranged from 71% to 90% (mean = 79.2 ± 7.3%, Fig. S3†). For the SPE-processed blanks, 7 out of 11 were below the LOD, with the remaining blanks having an FTIR AEO concentration of <1 mg L^{-1}.

2.3. Chemical analysis

The FTIR analytical procedure follows the protocol from Munir *et al.* (1996),[18] replacing LLE with SPE for the extraction process. Briefly, the reconstituted extracts were introduced into the KBr FTIR cell. Total peak area ratios at absorbances 1743 cm^{-1} and 1706 cm^{-1} were summed and the total AEO concentration was calculated using a calibration curve. The calibration curve was made from dilutions of commercially available NAs (Sigma-Aldrich) in dichloromethane (Fig. S4†).

2.4. *In vitro* bioanalysis

The rationale behind the selection of the battery of bioassays was reported by Barrow (2022)[19] and is also described in ESI-section B.† The cytotoxicity was analyzed using *Aliivibrio fischeri* bacteria *via* the BioTox LumoPlate kits purchased from Environmental Bio-detection Products Inc (EBPI). Directions from the kit follow the methodology based on ISO Standard 21338.[20] Cytotoxicity in all bioassays with mammalian cell lines was also assessed at UFZ in Germany. The yeast estrogen screen (YES) and ERα-GeneBLAzer mammalian gene assay were used to measure the estrogenicity using methods adapted from Arlos *et al.* (2016)[21] and Konig *et al.* (2017),[22] respectively. For xenobiotic metabolism, the activation of the aryl hydrocarbon receptor (AhR) and the activation of the peroxisome proliferator-activated receptor-γ (PPARγ) were measured using the AhR-CALUX and the PPARγ GeneBLAzer assays described by Konig *et al.* (2017)[22] and Neale *et al.* (2017),[23] respectively. The UMU-ChromoTest was used to measure the mutagenicity of samples from kits purchased from EBPI. The oxidative stress responses of the samples were measured using the AREc32 reporter gene assay according to Escher *et al.* (2012)[24] with some modifications. Sample concentrations in the bioassay were expressed in terms of the relative enrichment factor (REF). The REF is based on extraction (sample enrichment) and dosing factors (DF), and its derivation is explained in detail by Escher *et al.* (2021).[9] All details for all bioassay methods are provided in the ESI† (sections C1–4).

3. Data analysis

A sigmoidal or linear fit was used to describe the 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 (Fig. S6†). When the inhibitory or effective responses were 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 the signal of the sample to the signal of the negative control (assay diluent background), was used for the comparison of sample responses. In this study, the threshold concentration used was an EC with IR of 1.5 ($EC_{IR1.5}$).

Using the EC_{10} values, the bioanalytical equivalent concentration (BEQ) of each sample was calculated using the EC_{10} reference compound (Table 1). The BEQ values were then compared to the reported EBTs,[25] which are thresholds that differentiate between acceptable and unacceptable bioassay responses.[15] A detailed explanation of the data analysis can be found in Table S8.† Finally, given the huge variation in the flow conditions between the sampling campaigns, statistical analysis of the differences between the June (high flow) and August (low flow) sampling events was completed by pooling the sites together.

4. Results and discussion

4.1. Chemical analysis – acid extractable organics and naphthenic acids

FTIR analysis is commonly used for NA quantification because it is relatively simple and inexpensive.[26] However, it lacks selectivity and may account for other carboxylic acids in a mixture. Hence, the term 'acid extractable organics' (AEOs) is used throughout this paper as a more appropriate terminology to describe NA concentrations *via* FTIR analysis (Fig. 2).

The AEO concentrations of the municipal WWTP effluents FMO and MSO were 1.3 mg L^{-1} and 3.3 mg L^{-1}, respectively. Domestic wastewater effluents would not be a main source of NAs but the FTIR analysis could detect other organic compounds such as natural fatty acids, acidic pharmaceuticals such as diclofenac and ibuprofen, and per- and polyfluorinated alkane carboxylic acids, which can interfere with the signals produced by NAs at the wavelengths specific to carboxylic acids.[27,28] Thus, it is likely that the results reported for FMO and MSO effluents 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 (Fig. 2), which is a known source of NAs/AEOs (albeit currently contained).

The river samples collected in June showed significantly higher AEO concentrations, ranging from 0.4 to 2.4 mg L^{-1} (mean = 1.0 ± 0.7 mg L^{-1}) as compared to those collected in August with concentrations ranging from $<$LOD to 0.8 mg L^{-1} (mean = 0.2 ± 0.3 mg L^{-1}) (ANOVA, $p = 0.027$, $\alpha = 0.05$). The AEO analysis further revealed that the highest

Table 1 Summarized results of bioassay responses. OSPW = oil sands process-affected water. * = no activity, ** = no cytotoxicity, *** = cannot be determined; n.p. = not processed. See Table S8† for approaches employed in calculating bioassay responses. IC10 units are in REF. EC10 units are ng L^{-1} rosiglitazone-EQ, ng L^{-1} benzo[*a*]pyrene (B[*a*]P)-EQ. ng L^{-1} dichlorvos-EQ, ng L^{-1} estradiol-EQ, and ng L^{-1} 4-nitroquinoline 1-oxide-EQ (4-NQO-EQ) for PPARγ, AhR, AREc32, ERα/YES, and UMU assays, respectively

	PPARγ		AhR		AREc32		ERα		YES	UMU	Cytotoxicity
	EC10	IC10	EC10	IC10	EC(IR1.5)	IC10	EC10	IC10	EC10	EC(IR1.5)	IC10
June											
M8	*	1.8	4.0	**	*	**	*	1.9	16.1	*	13.3
M7	*	1.9	*	**	*	**	3.7	4.8	11.1	208	23.9
M6	*	2.0	4.6	**	*	**	*	1.3	3.0	100	24.8
M5	1.1	2.1	*	**	*	**	*	2.0	3.0	*	21.9
T1	0.7	0.8	*	3.9	*	**	*	0.8	8.3	*	6.59
M4	1.4	2.3	*	**	6.9	**	*	5.2	7.2	161	13.5
M3	*	2.0	*	**	*	**	*	2.2	13.4	*	22.7
FMO	1.0	**	2.2	**	5.1	**	2.2	7.8	10.6	21	19.2
M2	0.4	1.3	*	**	*	**	*	1.4	9.2	*	13.1
M1	1.2	1.5	*	**	*	**	*	1.8	16.6	385	10.6
August											
M4′	*	**	*	**	*	**	*	**	27.9	*	33.5
S1E	*	5.4	*	**	*	**	*	8.6	17.0	*	32.6
S4E	*	**	*	**	*	**	*	**	10.3	*	***
MSO	0.9	3.0	2.0	**	3.0	**	*	4.8	1.5	74	12.0
S2E	*	**	*	**	*	**	*	**	9.4	455	***
S2W	*	**	*	**	*	**	*	**	25.2	*	***
T2	*	**	*	**	*	**	6.0	**	n.p.	*	4.2
OSPW	0.04 ± 0.01	2.7 ± 0.3	2.3 ± 2.2	3.7 ± 1.0	2.5 ± 1.2	5.1	0.7 ± 0.3	1.6 ± 0.04	0.2 ± 0.07	5.5 ± 3.5	1.0 ± 0.7

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 sampling sites within the oil sands disturbances would likely be more impacted by overland flow than the sites outside of this region. This observation further indicates that there may be a

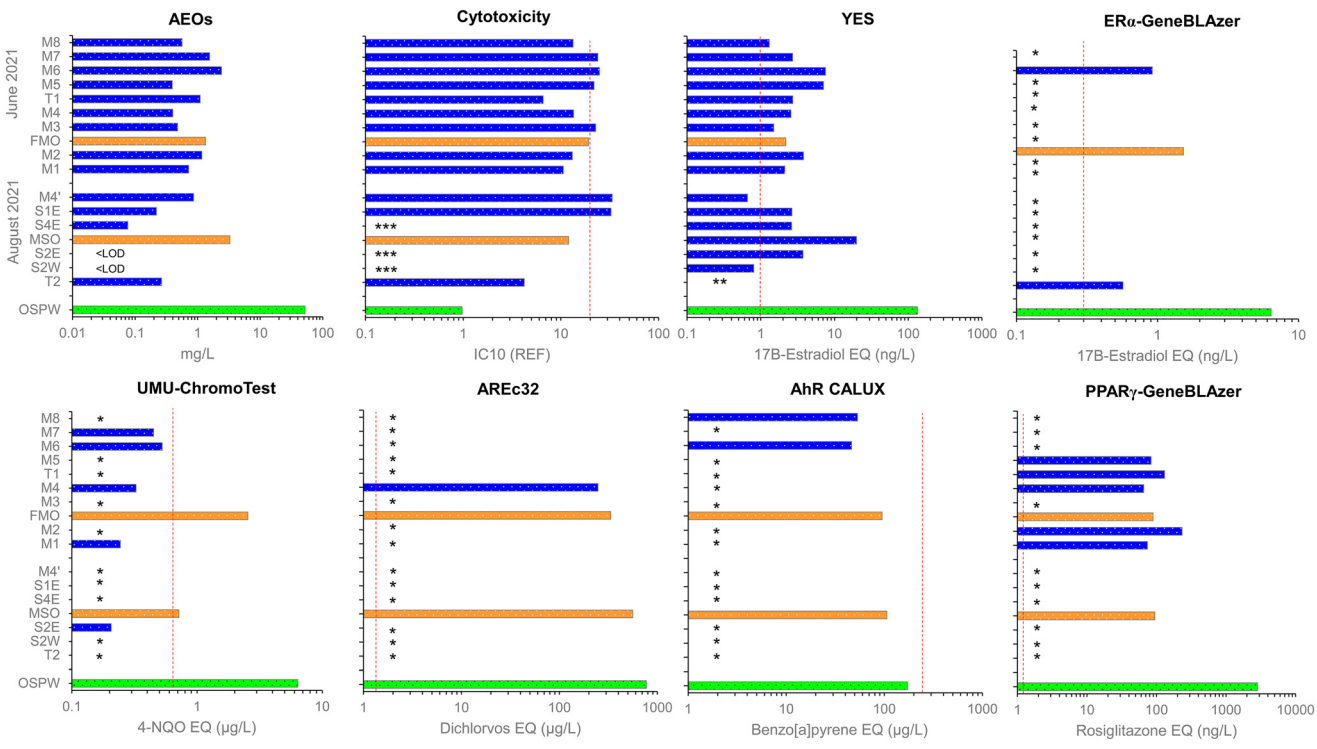


Fig. 2 Summary of AEO concentrations and BEQ responses for all samples (numerical values in Table S9,† site information in Fig. 1). Blue bars represent river samples, orange bars represent WWTP effluents and purple bars represent untreated OSPW. The red vertical line is the assay-specific effect-based trigger value (EBT). Note that exceedance analysis for the effluents and untreated OSPW does not apply since EBTs only apply to receiving environments. * = no activation; ** = not analyzed; *** = cannot be determined.

relationship between the hydrologic conditions and concentrations of contaminants in the river, further suggesting that the current year-round monitoring program continues to be beneficial in assessing temporal changes.

Due to the lack of method specificity, note once again that FTIR may overestimate the NA concentration when used for other water matrices including ground and surface waters.[29] Therefore, we compared the data obtained for the river samples and untreated OSPW from our in-house FTIR with the orbitrap-mass spectrometry (MS) analysis completed *via* the EMP.[14] The samples collected by this monitoring program for NA analysis during June and August correspond to sampling sites labeled as M4′, S1E, S4E, S2E, S2W, and T2. The NA concentrations in LAR using orbitrap-MS for the June sampling were <DL (4 μg L^{-1}), except for T2, which had a concentration of 21.7 μg L^{-1}, while the concentrations for all August samples were <DL. Given that the AEO concentrations are orders of magnitude higher than the orbitrap-MS detections, FTIR analysis detected other compounds in the mixture containing one or more carboxylic moieties. Furthermore, the AEO concentration of the untreated OSPW was determined to be 52.2 ± 8.0 mg L^{-1} while the concentration *via* orbitrap-MS was found to be 9.2 mg L^{-1} (OSPW-NAs profile in Fig. S7†). We recognize these differences but the FTIR approach is an acceptable and straightforward method that lends a semi-quantitative assessment of NAs in water samples to support our bioanalytical assessment of the LAR. The AEO concentrations reported in this study are consistent with the results obtained by Han *et al.* (2009) who reported values of 50 to 77 mg L^{-1} based on the FTIR analysis of various water samples, including oil sands ore extracts, dyke seepage waters and active settling basins.[30]

4.2. *In vitro* bioanalysis

4.2.1. Cytotoxicity. Cytotoxicity through the inhibition of bioluminescence in *Aliivibrio fischeri* is routinely employed to evaluate OSPW toxicity and assess the efficiency of potential treatment technologies.[31] Due to the amount of data available for comparison, it was deemed appropriate to employ this assay as a non-specific toxicity endpoint in this battery of tests. Overall, the IC_{10} values were >10 REF (Table 1, Fig. 2) 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 were 16.7 ± 6.6 REF and 23.4 ± 16.6 REF for the river water sampled in June and August, respectively. Although the mean cytotoxicity values for June and August samples were not statistically different (One-way ANOVA, p = 0.32, α = 0.05), the results still imply that there was slightly less cytotoxicity during August. This observation was further supported by the IC_{10} values derived using the mammalian cell assays (Table 1) wherein all river water sampled in June showed cytotoxicity (albeit lower than OSPW), 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 PPARγ 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} were 19 REF for FMO and 12 REF for MSO. WWTP effluents were expected to show cytotoxicity, as reported by previous studies.[32,33] However, the observed cytotoxicity may be minimal, considering that the mixing patterns, and dilution factors (RDF) of the effluents in the LAR are quite high (RDF > 6700 and >2200 in June and August, respectively), reducing the exposure risk for organisms (see ESI† section-D for RDF calculations).

Although the environmental exposure in the river sites was mostly considered to be low risk, the results show that untreated 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) who used the *Aliivibrio fischeri* assay with raw OSPW, reporting IC_{20} values in the range of 0.30 REF.[34]

4.2.2. Xenobiotic metabolism

4.2.2.1. 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 and PAHs.[9] Since PAHs were found in OSPW, this endpoint was included in the test selection. The AhR controls the encoding of several target genes for metabolic enzymes that are activated when a contaminant enters a responsive cell and binds to the AhR. The activation of the genes can then convert ligands into reactive intermediates that can cause DNA damage.[9] 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 (Table 1, Fig. 2).

From the river water sampled in June, only M6 and M8 activated the AhR with B[*a*]P-EQ values of 46.4 and 53.2 ng L^{-1}, respectively, while none of the river samples from August activated this assay. This result can be attributed to the observed lower concentrations of PACs and NAs during this sampling as compared to June (Table S11,† also in the online database[14]). Nonetheless, the AhR activity (Table 1) is consistent with the findings of other studies that have reported similar EC_{10} of 2.0 to 10.7 REF for surface samples collected from the Ammer River (Germany) and the Swiss Plateau (Switzerland), which are both impacted by WWTP effluents.[35,36]

The B[*a*]P-EQ values were 106.5 ng L^{-1} for MSO and 94.9 ng L^{-1} for FMO WWTP effluents. These values (Table S9†) are comparable to those reported by Neale *et al.* (2017),[35] who found that the EC_{10} values for 3 WWTP effluents ranged from

4 to 6.33 REF. Ings *et al.* (2011)[37] studied the exposure effects of tertiary-treated municipal wastewater effluents on the gene and protein expression in rainbow trout livers 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 and also other trace organic chemicals such as corrosion inhibitors and pharmaceuticals that have been shown to activate AhR.[36]

The B[*a*]P-EQ value for OSPW was 172.4 ± 142.8 ng L^{-1}. The OSPW used in this study was not analyzed for PACs, which are well-known AhR agonists.[38] However, Leclair *et al.* (2015)[39] observed that the AhR activation in H4IIE-*luc* cells may have instead been dominated by NAs, as planar and neutral compounds such as PAHs are likely present in small amounts in the OSPW fraction they investigated.

4.2.2.2. PPARγ-GeneBLAzer assay. The peroxisome proliferator-activated receptors (PPARs) (-α, -δ, -γ) are ligand-activated transcription factors in the nuclear receptor family that play a role in the 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.[40] Of the three, PPARγ is commonly investigated and was, therefore, selected in the battery of tests as it plays an important role in insulin sensitivity and the regulation of lipoprotein and glucose metabolism.[9] The receptor is also activated by tire-wear chemicals such as benzothiazole sulfonic acid, and potentially NAs which have similar structures to fatty acids.[41,42]

For the river samples, 5 sites from June showed PPARγ activation with rosiglitazone-EQ values ranging from 65.7 to 233.9 ng L^{-1}, while none from August activated this assay (Fig. 2). These values are comparable to the findings of Neale *et al.* (2020)[43] who reported BEQ values ranging from 2.4 ng L^{-1} to 790.4 ng L^{-1} for samples collected during rain events from unimpacted rivers and those impacted by WWTP effluents and agricultural streams, suggesting that runoff events including spring melt can increase PPARγ activation.

The rosiglitazone-EQ values were 89.0 ng L^{-1} for FMO and 94.8 ng L^{-1} for MSO. It is suspected that PPARγ agonists are likely to be found in wastewater effluents, including phthalates, pharmaceuticals (*e.g.*, rosiglitazone, pioglitazone) and organotins; however, further work is required to definitively determine the compounds responsible for PPARγ activity in WWTP effluents.[44] The measurement of specific trace organic chemicals typically found in municipal effluents was outside the scope of this study but we can speculate that these types of compounds may contribute to the activity observed for FMO and MSO.

The rosiglitazone-EQ values for OSPW were 2824 ± 590 ng L^{-1}. The high signal of untreated OSPW is supported by the work of Peng *et al.* (2016)[42] who identified 30 chemicals found in OSPW, including hydroxylated carboxylic acids, oxygenated sulfonic acids or heteroatomic chemicals, as compounds that activate the PPARγ.

4.2.3. 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 that 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 wastewater.[32] Induction ratios >1.5 indicate potential mutagenic activity in water samples. $EC_{IR1.5}$ values were used to derive the 4-nitroquinoline 1-oxide (4-NQO)-EQ values shown in Fig. 2.

From the June sampling campaign, this assay showed that 4 samples had 4-NQO-EQ values ranging from 0.2 to 0.5 μg L^{-1}. 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^{-1}. Another recent study by Sun *et al.* (2017)[45] 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 ranged from 0.28 to 0.69 μg L^{-1}, which are comparable to this study.

The 4-NQO-EQ value is 2.5 μg L^{-1} for FMO and 0.7 μg L^{-1} for MSO. These results are comparable to other studies that have reported values ranging from 0.09 to 2.6 μg L^{-1} for other municipal WWTP effluents.[33,46] It is likely that PACs (as known carcinogens) contributed to the observed activity, and this hypothesis can be supported by the PACs data in the effluents that include parent PAHs, alkylated PAHs, and dibenzothiophenes (Table S11†).[47] Interestingly, Fang *et al.* (2012)[46] reported lower PAH concentrations of about 0.2 μg L^{-1} with similar 4-NQO-EQ values around 2.1 μg L^{-1} for municipal WWTP effluents. The variations between PAH concentrations and reported mutagenicity across studies have led to uncertainty among researchers about the extent of the contribution of PAHs and other nontarget compounds to the genotoxic/mutagenic activity of a variety of effluents. 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.[48]

The 4-NQO-EQ value for OSPW is 6.4 μg L^{-1}. This result is comparable to the findings of Zetouni *et al.* (2017)[49] who reported that the bioactivation of the neutral and acid extractable fractions of OSPW occurred at REFs of 1.5–25.[49,50] The authors suggested that the reported mutagenicity was not environmentally relevant for short exposure times since the doses required for bioactivation were more than 1× the original concentration. Therefore, the long-term bioaccumulation of these compounds in aquatic organisms may be more relevant for future studies. It was also speculated that most PAHs were removed when additional sample clean-up (filtration) was completed prior to the mutagenicity analysis,[50] which may have suppressed the mutagenic effects of their OSPW samples or other substances responsible for mutagenicity. Filtration prior to SPE is an important step for water samples containing visible particles, or more specifically with a turbidity of at least 5 NTU.[9] The clogging of cartridges is one of the main drawbacks of SPE, and although it is outside the scope of this study, 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 (*i.e.*, bound and unbound).

PAHs may need to be metabolically activated using mammalian metabolic enzyme preparations (S9) to simulate xenobiotic activation and detoxification in the *in vitro* assays.[51] S9 is typically used when using cell lines such as *Salmonella typhimurium*, which is metabolically deficient and cannot activate some mutagens in the absence of enzyme preparation. At present, S9 is prepared from a variety of mammalian species but this can lead to significant modifications of the results as different S9 systems (*e.g.*, rat liver, hamster, human liver) are recommended for different types of assessments.[51] S9 activation was not used in this study but it is important to note that there would likely 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 ESI† section B-6.

4.2.4. Oxidative stress response. The oxidative stress response (OSR) is one of the adaptive stress responses monitored in water quality assessment.[9] Typically, electrophilic chemicals, and reactive oxygen species (*e.g.*, superoxide, hydroxyl radical) induce the OSR, and in a series of events, activate the antioxidant response element (ARE).[9] Adaptive stress responses are usually induced at lower concentrations as compared to cytotoxicity, implying that these assays can be implemented as sensitive monitoring tools for environmental water samples.[52] Escher *et al.* (2013)[52] suggested that the induction of the OSR can be classified as a non-specific mode of action as 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. 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 (Fig. 2).

From the June 2021 sampling, only M4 showed activity with a dichlorvos-EQ value of 248.1 μg L^{-1}. Similar to other assays, none of the samples collected in August 2021 showed activation for this bioassay. The response of M4 (Table S10†) is slightly lower than the findings of Neale *et al.* (2017)[35] who reported EC_{10} values ranging from 20 to 33 REF for river samples collected downstream of 3 WWTPs in Switzerland during low flow conditions.

Both WWTP effluents activated the bioassay with dichlorvos-EQ values of 333.8 μg L^{-1} and 559.7 μg L^{-1} for FMO and MSO, respectively. The EC_{10} values of these samples (Table S10†) are comparable to the findings of Escher *et al.* (2013)[52] and Neale *et al.* (2017)[35] who evaluated the OSR for various WWTP effluents (0.34 to 17.1 REF).

For OSPW, the BEQ value was 774.7 ± 292.8 μg L^{-1} for 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, further indicating the high toxicity of untreated OSPW.

4.2.5. Estrogenicity. Several endpoints can be targeted for endocrine disruption (*e.g.*, androgenicity, estrogenicity) but the vast majority of work has focused on estrogenic activity.[53] The YES and the ERα-GeneBLAzer mammalian reporter gene assays were both used in this study to measure estrogenic activities. The YES assay has several advantages including its simplicity and 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.[54] Moreover, the YES assay has relatively higher detection and quantification limits, thus being less sensitive than other assays.[53] This limitation is the primary reason that the Erα-GeneBLAzer assay was also run in this study, with the latter being a more sensitive estrogenicity assay.[55] 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 results in different ecological EBT values.

All river samples showed estrogenic activity using the YES assay, with EEQ values ranging from 1.3 ng L^{-1} to 7.5 ng L^{-1} (mean = 3.5 ± 2.3 ng L^{-1} EEQ). Five out of six river samples collected in August had EEQ values ranging from 0.7 ng L^{-1} to 3.7 ng L^{-1} (mean = 2.1 ± 1.3 ng L^{-1} EEQ). In contrast, only M7 from the river water sampled in June (0.9 ng L^{-1} EEQ), and T2 (0.6 ng L^{-1} 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 were located within the oil sands mineable area and may, therefore, be more impacted by the presence of estrogenic substances (*e.g.*, aromatic naphthenic acids, alkylphenolic compounds)[48,56] Furthermore, there was no statistical difference between the mean estrogenicity in June and August measured using the YES assay (ANOVA, $p = 0.24$, $\alpha = 0.05$), as our August sample set is small. Nonetheless, the slightly higher estrogenic activity in June (high flow conditions) can be supported by previous findings,[57] where the YES assay was used 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, although 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.[57]

The EEQ values determined for FMO and MSO (municipal WWTPs) were 2.2 and 19.9 ng L^{-1} EEQ, respectively, using the YES assay. In contrast, the EEQ value determined by the ERα-GeneBLAzer was 1.53 ng L^{-1} for FMO and activation could not be reported due to the cytotoxicity interference of the MSO extract with this assay. 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).[58] The

reported estrogenicity of FMO using the YES assay is comparable to the findings of Arlos *et al.* (2018)[59] who determined the effluent estrogenicity levels for another WWTP with a similar treatment train as FMO to be 3.4 ng L^{-1}. The high estrogenicity of MSO effluent (sewage lagoon) *via* the YES assay may be due to the lack of advanced treatment processes that can remove endocrine-disrupting compounds more efficiently. Given that MSO flow contribution is very low (<0.1% of the total river flow), the estrogenicity loadings into the LAR were considered low.

High estrogenicity for untreated OSPW was observed from both assays. The EEQ values for OSPW were 133.8 ± 34.8 ng L^{-1} and 6.4 ± 2.5 ng L^{-1} on using the YES and ERα-GeneBLAzer assays, respectively. The value reported for the YES assay is consistent with the finding of another study that reported an EEQ value of 157.5 ng L^{-1}.[56] Our results support the work of Rowland *et al.* (2011)[48] 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. Gagné *et al.* (2011)[60] further investigated the changes in molecular signals related to gene expression (estrogen-based) in 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.

As previously mentioned, both estrogenicity assays did not show similar trends for the river/WWTP samples since the ERα-GeneBLAzer assay showed activity in only 2 river samples, whereas all river samples activated YES. This difference may be due to interferences from other endocrine-active compounds (*e.g.*, androgens, anti-androgens, and anti-estrogens), differences in assay sensitivities, and/or variations in the active mechanisms of each assay. For instance, Fernandez *et al.* (2007)[61] found that the YES assay is affected by the presence of anti-estrogenic compounds in WWTP effluents that may suppress the bioassay response, which was also reported by other studies. However, the extent of the suppressant effect is a complex mechanism as it depends on the concentrations of the strong estrogens in the mixture. Therefore, it may also be important in the future 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.

The validity of the results from both estrogenicity assays was only considered after a rigorous analysis of the assay quality controls, which were found to be within acceptable limits. The QA/QC of the YES result cannot effectively determine whether the results were affected by cytotoxicity, but due to the high cytotoxicity observed from the ERα-GeneBLAzer, there were likely cytotoxicity interferences for the YES. Nonetheless, we recognize that the mechanisms regarding why there are activations of the river and MSO samples *via* YES and not with ERα-GeneBLAzer still require further investigation.

4.3. Comparison with effect-based trigger (EBT) values

The EBT value is an assay-specific threshold that differentiates whether a mixture (*e.g.*, surface water) is likely to produce adverse effects during water quality assessment.[9] *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 implies that there will be an associated ecotoxicological risk. EBT values are unique to each bioassay and have been proposed for surface waters to protect the aquatic ecosystem health and exposed aquatic organisms (Table S12†).[55]

For the bioluminescence cytotoxicity using *Aliivibrio fischeri* bacteria, the sample responses were compared to the threshold for chronic risks (<20 REF) as suggested by van der Oost *et al.* (2017).[62] The mammalian cytotoxicity was compared to the EBT-IC_{10} threshold of 10 REF where a sample with IC_{10} > 10 REF implies acceptable water quality.[55,62] There is no fixed EBT-EQ for mutagenicity due to lack of data, and for this study, we used the predicted no-effect concentration (PNEC) of 0.64 4-NQO μg L^{-1} from Xu *et al.* (2014)[63] for comparison.

To further illustrate the degree of exceedances, ratios of IC_{10} (REF) or BEQ (ng_{ref} L^{-1})-EC_{10} (Table S9†) to available EBTs[25] were further determined (Fig. 3), with values >1 suggesting exceedances. For municipal effluents and OSPW, the BEQs were further corrected to account for river dilution factors. Untreated OSPW was very cytotoxic and activated all bioassays at higher BEQs than the river samples. More specifically, the calculated BEQs are up to two orders of magnitude greater than those of the river samples, except for AREc32 and AhR. Although the responses for these assays are only ~3.5 times larger in untreated OSPW than in the river samples, AREc32 and AhR may still serve as secondary biological activity indicators.

Overall, the bioassay test results for untreated OSPW imply that the pathways investigated are highly relevant to OSPW monitoring and validate the choice of *in vitro* bioassays included in this study. Interestingly, once dilution factors were considered, it appears that OSPW may have very low adverse effects in the aquatic environment as the ratios of IC_{10} (REF) to EBT and EBT to BEQs are ≪1 (Fig. 3). Although our study did not constitute an assessment of treated OSPW, the risks of exposure to treated OSPW may be even lower, suggesting that the receiving environment (*i.e.*, LAR) can assimilate pollutants stemming from OSPW. It is currently difficult to predict what the impacts may be once the discharge of treated OSPW begins but our results can now be coupled with other OSPW-related chemical and biological monitoring data to support decisions surrounding water quality management in the area.

All tests were activated by the FMO, and 6/7 bioassay tests were activated by the MSO effluents (Fig. 3). Similar to OSPW, the potential of the municipal effluents to cause harm to aquatic environment is substantially reduced once a low-flow river dilution factor (RDF = 2200) was considered, resulting

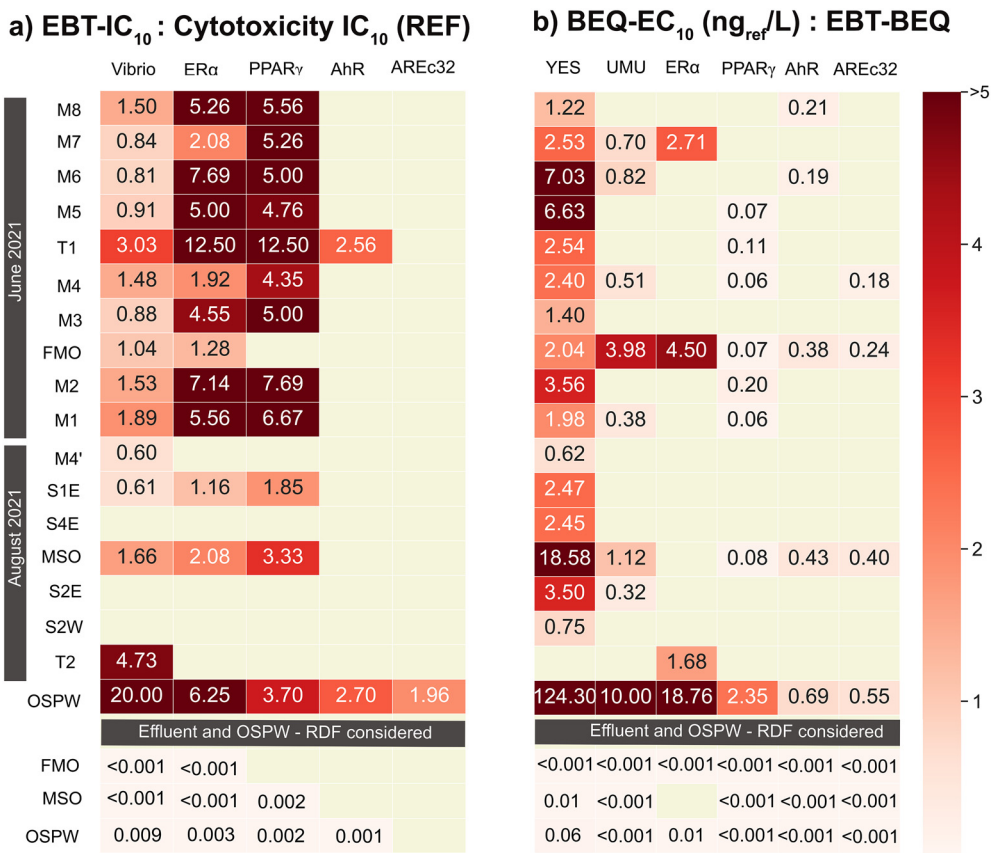


Fig. 3 Comparison of sample bioassay responses with the EBT-IC_{10} and ecological EBT-EQ values for surface waters (Table S12†). (a) The ratios of EBT-IC10 to IC10 and (b) BEQ to EBT-BEQ. If the ratios are >1, the EBT is exceeded. Closeness to the darker red region suggests a greater severity in exceedances. For current and future point sources (municipal effluents and OSPW), the river dilution factor (RDF) (*i.e.*, 2200× based on FMO flows in August [low flow]) was considered and the ratios were re-calculated. Cells without values suggest no activation in the assay or cannot be determined.

in ratios of BEQs to EBT-EC_{10} that are <0.001 (Fig. 3). The impact of the discharge of these effluents may greatly change with population growth (*i.e.*, increase in domestic sewage production) and extreme hydrological conditions (*i.e.*, droughts), which can be a cause for concern, considering the demonstrated potential health risks associated with these effluents.

For the river samples, 5/9 samples collected in June, and T2 from the August sampling showed exceedances to EBT-IC_{10} (Fig. 3). For the remaining samples, there is a lower risk of exposure as their IC_{10} values are >20 REF. When cytotoxicity derived from the mammalian cell lines (ERα and PPARγ) was 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 a 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 to the chemical results and other bioindicators reported in the current monitoring programs (*e.g.*, EMP) to fully elucidate the impact *in vivo*.

All the river samples collected in June activated the YES assay with responses that were 1.2 to 7 times larger than the ecological EBT value. However, for the August samples, only 3/5 analyzed samples had EEQ values greater than the EBT value, with bioactivities that were 2.4 to 3.5 times larger than the EBT. Interestingly, only M7 from the June sampling showed estrogenic activity with an EEQ value that was ~3 times higher than the EBT 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 greater than EBT. This reiterates the need to assess the differences between the results of the two bioassays as it could direct subsequent experiments to effectively assess estrogenicity in the LAR. For the AREc32, and AhR CALUX and PPARγ-GeneBLAzer assays, the responses of the active June samples did not exceed the EBTs while there was no activation for the August samples. None of the LAR samples collected in June or August showed responses exceeding the PNEC value for mutagenicity. Finally, we observed that there was greater 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. The results of these bioassays are supported by the AEOs data (Fig. 2), which indicated that the chemical contamination of the river is greater during high flow conditions, which may be attributed to the inputs from run-off and/or snowmelt.

5. Conclusions

This study shows that a battery of *in vitro* bioassays can be used to assess the toxicity of untreated OSPW, as it showed relatively high responses in all bioassay tests employed. These bioassay endpoints additionally provide indications of the relevant toxicity pathways for OSPW monitoring, where primary toxicity indicators include cytotoxicity, estrogenicity, binding to the PPARγ, mutagenicity, and secondary indicators include induction of the AhR and oxidative stress response. Although some assays such as estrogen screen assays have been established for a while, there are some interferences associated with the receptor binding that can highly influence the results. In this study, further investigation into the differences in estrogenicity results is needed to confirm the applicability and suitability of the YES *vs.* ERα-GeneBLAzer bioassays. Nonetheless, our study shows that the high responses of untreated OSPW further indicate the importance of sufficiently treating OSPW prior to its potential discharge into a receiving aquatic environment.

Both municipal WWTP effluents (FMO and MSO) were active in almost all bioassay tests applied, suggesting that there may be a potential toxicity risk associated with direct exposure to these effluents. Considering the mixing patterns of the LAR, these risks are likely reduced but there may be implications associated with extremely low flow conditions that give rise to low dilution events, which are only becoming more frequent with climate change.[64]

Finally, the results suggest that the LAR, with its current stressors (*e.g.*, WWTPs), has low toxicity risks, as our samples showed low bioassay responses, especially during low flow conditions. Given that the development of adequate OSPW treatment continues as an active research area, the *in vitro* bioassays employed in this study can be useful in assessing the differences in multiple stressors that may lead to increased toxicity risks in receiving environments.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This research was supported by funding from the Canada First Research Excellence Fund as a part of the University of Alberta's Future Energy Systems research initiative. Additional funding from the NSERC Discovery Grant (NSERC RGPIN-2021-02412) was also obtained to support this work. The authors would also like to acknowledge the assistance of Ania Ulrich, Petr Kuznetsov, Amy-lynne Balaberda, Mohamed Gamal El-Din, and Pamela Chelme-Ayala from the University of Alberta. We are grateful for the help provided by Kyra Jubinville (University of Alberta) and Kristin Hynes from Alberta Environment and Parks during the field sampling campaigns. We gratefully acknowledge access to the platform CITEPro (Chemicals in the Environment Profiler) funded by the Helmholtz Association for bioassay measurements and financial support of the work at UFZ from the Helmholtz POF IV Topic 9 "Healthy Planet-towards a non-toxic environment". Portions of the river water quality data was provided by the Oil Sands Monitoring Program but does not necessarily represent the official position of the Program.

References

1 J. M. Culp, I. G. Droppo, P. D. di Cenzo, A. C. Alexander, D. J. Baird, S. Beltaos, G. Bickerton, B. Bonsal, R. B. Brua, P. A. Chambers, Y. Dibike, N. E. Glozier, J. L. Kirk, L. Levesque, M. McMaster, D. C. G. Muir, J. L. Parrott, D. L. Peters, K. Pippy and J. W. Roy, *Environ. Rev.*, 2021, **29**, 315–327.
2 E. W. Allen, *J. Environ. Eng. Sci.*, 2008, **7**, 123–138.
3 H. A. Alharbi, S. B. Wiseman and J. P. Giesy, *ACS ES&T Water*, 2021, **1**, 698–706.
4 R. N. Tanna, A. D. Redman, R. A. Frank, T. J. Arciszewski, W. A. Zubot, F. J. Wrona, J. A. Brogly and K. R. Munkittrick, *Integr. Environ. Assess. Manage.*, 2019, **15**, 519–527.
5 M. C. Simair, J. L. Parrott, M. le Roux, V. Gupta, R. A. Frank, K. M. Peru, C. Ajaero, D. W. McMartin and J. V. Headley, *Sci. Total Environ.*, 2021, **772**, 145508.
6 W. Zubot, Z. An, C. Benally and M. Gamal El-Din, *J. Environ. Manage.*, 2021, **289**, 112407.
7 K. Hicks and G. Scrimgeour, *A study design for enhanced environmental monitoring of the Lower Athabasca River*, Office of the Chief Scientist, Ministry of Environment and Parks, Edmonton, Alberta, 2019.
8 Alberta Environment Parks, *A compendium of work plans to fill information gaps to inform the development of regulatory guidance documents for the safe release of treated oil sands mine waters to the Lower Athabasca River*, Government of Alberta, Ministry of Environment and Parks, 2021.
9 B. Escher, P. Neale and F. Leusch, *Bioanalytical Tools in Water Quality Assessment Second Edition*, IWA Publishing, 2021.
10 N. E. Glozier, K. Pippy, L. Levesque, A. L. Ritcey, O. Tobin, C. A. Cooke, M. Conly, L. Dirk, R. Hazwinkel and E. Keet, *Surface water quality of the Athabasca, Peace and Slave rivers and riverine waterbodies within the Peace-Athabasca delta*, Alberta Environment and Parks, Edmonton, 2018.
11 Regional Municipality of Wood Buffalo, *Wastewater Master Plan - Strategy Report Summary*, 2014.
12 Regional Municipality of Wood Buffalo, *Census 2021*, 2021.
13 C. W. Cuss, M. Ghotbizadeh, I. Grant-Weaver, M. B. Javed, T. Noernberg and W. Shotyk, *J. Hydrol.*, 2021, **597**, 125747.
14 Alberta Government, *Oil Sands Monitoring Program Data Portal*, **https://aws.kisters.net/OSM/applications/public.html?publicuser=Guest#waterdata/stationoverview**, (accessed August 2022).
15 M. Bataineh, A. C. Scott, P. M. Fedorak and J. W. Martin, *Anal. Chem.*, 2006, **78**, 8354–8361.
16 P. R. Kootstra, M. H. C. Straub, G. H. Stil, E. G. van der Velde, W. Hesselink and C. C. J. Land, *J. Chromatogr. A*, 1995, **697**, 123–129.

17 R. Qin, D. Lillico, Z. T. How, R. Huang, M. Belosevic, J. Stafford and M. Gamal El-Din, *Sci. Total Environ.*, 2019, **695**, 133532.
18 N. J. Munir, F. Betty and M. Mike, *Naphthenic acid extraction and quantitative analysis with FT-IR spectroscopy*, Syncrude Edmonton Research Center, Edmonton, 1996.
19 K. Barrow, *Master of Science Thesis*, University of Alberta, 2022.
20 ISO21338, *Water quality- Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) - Part 3: method using Freeze-dried bacteria*, International Organisation for Standardization (ISO), Geneva, Switzerland, 2007.
21 M. J. Arlos, R. Liang, M. M. Hatat-Fraile, L. M. Bragg, N. Y. Zhou, M. R. Servos and S. A. Andrews, *J. Hazard. Mater.*, 2016, **318**, 541–550.
22 M. Konig, B. I. Escher, P. A. Neale, M. Krauss, K. Hilscherova, J. Novak, I. Teodorovic, T. Schulze, S. Seidensticker, M. A. K. Hashmi, J. Ahlheim and W. Brack, *Environ. Pollut.*, 2017, **220**, 1220–1230.
23 P. A. Neale, R. Altenburger, S. Ait-Aissa, F. Brion, W. Busch, G. de Aragão Umbuzeiro, M. S. Denison, D. Du Pasquier, K. Hilscherova, H. Hollert, D. A. Morales, J. Novac, R. Schlichting, T.-B. Seiler, H. Serra, Y. Shao, A. J. Tindall, K. E. Tollefsen, T. D. Williams and B. I. Escher, *Water Res.*, 2017, **123**, 734–750.
24 B. I. Escher, M. Dutt, E. Maylin, J. Y. M. Tang, S. Toze, C. R. Wolf and M. Lang, *J. Environ. Monit.*, 2012, **14**, 2877.
25 B. I. Escher and P. A. Neale, *Environ. Toxicol. Chem.*, 2021, **40**, 487–499.
26 D. M. Grewer, R. F. Young, R. M. Whittal and P. M. Fedorak, *Sci. Total Environ.*, 2010, **408**, 5997–6010.
27 M. S. Ross, A. d. S. Pereira, J. Fennell, M. Davies, J. Johnson, L. Sliva and J. W. Martin, *Environ. Sci. Technol.*, 2012, **46**, 12796–12805.
28 P. A. Neale, J. W. O'Brien, L. Glauch, M. König, M. Krauss, J. F. Mueller, B. Tscharke and B. I. Escher, *Water Res.: X*, 2020, **9**, 100072.
29 A. C. Scott, R. F. Young and P. M. Fedorak, *Chemosphere*, 2008, **73**, 1258–1264.
30 X. Han, M. D. MacKinnon and J. W. Martin, *Chemosphere*, 2009, **76**, 63–70.
31 C. Li, L. Fu, J. Stafford, M. Belosevic and M. Gamal El-Din, *Sci. Total Environ.*, 2017, **601–602**, 1785–1802.
32 H. Dizer, E. Wittekindt, B. Fischer and P. D. Hansen, *Chemosphere*, 2002, **46**, 225–233.
33 J. Y. M. Tang, F. Busetti, J. W. A. Charrois and B. I. Escher, *Water Res.*, 2014, **60**, 289–299.
34 N. Wang, P. Chelme-Ayala, L. Perez-Estrada, E. Garcia-Garcia, J. Pun, J. W. Martin, M. Belosevic and M. Gamal El-Din, *Environ. Sci. Technol.*, 2013, **47**, 6518–6526.
35 P. A. Neale, N. A. Munz, S. Aït-Aïssa, R. Altenburger, F. Brion, W. Busch, B. I. Escher, K. Hilscherová, C. Kienle, J. Novák, T.-B. Seiler, Y. Shao, C. Stamm and J. Hollender, *Sci. Total Environ.*, 2017, **576**, 785–795.
36 M. E. Müller, B. I. Escher, M. Schwientek, M. Werneburg, C. Zarfl and C. Zwiener, *Environ. Sci. Eur.*, 2018, **30**, 20.
37 J. S. Ings, M. R. Servos and M. M. Vijayan, *Environ. Sci. Technol.*, 2011, **45**, 2368–2376.
38 J. R. Marentette, K. Sarty, A. M. Cowie, R. A. Frank, L. M. Hewitt, J. L. Parrott and C. J. Martyniuk, *Aquat. Toxicol.*, 2017, **182**, 11–19.
39 L. A. Leclair, L. Pohler, S. B. Wiseman, Y. He, C. J. Arens, J. P. Giesy, S. Scully, B. D. Wagner, M. R. van den Heuvel and N. S. Hogan, *Environ. Sci. Technol.*, 2015, **49**, 5743–5752.
40 J. L. Wilson, R. Duan, A. El-Marakby, A. Alhashim and D. L. Lee, *PPAR Res.*, 2012, **2012**, 1–7.
41 N. A. Sossalla, J. Nivala, B. I. Escher, T. Reemtsma, R. Schlichting, M. van Afferden and R. A. Müller, *Water*, 2020, **12**, 3050.
42 H. Peng, J. Sun, H. A. Alharbi, P. D. Jones, J. P. Giesy and S. Wiseman, *Environ. Sci. Technol.*, 2016, **50**, 7816–7824.
43 P. A. Neale, G. Braun, W. Brack, E. Carmona, R. Gunold, M. König, M. Krauss, L. Liebmann, M. Liess, M. Link, R. B. Schäfer, R. Schlichting, V. C. Schreiner, T. Schulze, P. Vormeier, O. Weisner and B. I. Escher, *Environ. Sci. Technol.*, 2020, **54**, 8280–8290.
44 P. A. Bain, M. Williams and A. Kumar, *Environ. Toxicol. Chem.*, 2014, **33**, 2297–2307.
45 C. Sun, W. Shotyk, C. W. Cuss, M. W. Donner, J. Fennell, M. Javed, T. Noernberg, M. Poesch, R. Pelletier, N. Sinnatamby, T. Siddique and J. W. Martin, *Environ. Sci. Technol.*, 2017, **51**, 9524–9532.
46 Y.-X. Fang, G.-G. Ying, J.-L. Zhao, F. Chen, S. Liu, L.-J. Zhang and B. Yang, *Environ. Toxicol. Chem.*, 2012, **31**, 1273–1282.
47 A. Bispo, M. J. Jourdain and M. Jauzein, *Org. Geochem.*, 1999, **30**, 947–952.
48 S. J. Rowland, C. E. West, D. Jones, A. G. Scarlett, R. A. Frank and L. M. Hewitt, *Environ. Sci. Technol.*, 2011, **45**, 9806–9815.
49 N. C. Zetouni, A. G. Siraki, M. Weinfeld, A. D. S. Pereira and J. W. Martin, *Environ. Toxicol. Chem.*, 2017, **36**, 1397–1404.
50 Z. Nikolas Cavalheiro, *Master of Science Thesis*, University of Alberta, 2015.
51 J. A. Cox, M. D. Fellows, T. Hashizume and P. A. White, *Mutagenesis*, 2016, **31**, 117–130.
52 B. I. Escher, C. van Daele, M. Dutt, J. Y. M. Tang and R. Altenburger, *Environ. Sci. Technol.*, 2013, **47**, 7002–7011.
53 F. D. L. Leusch, P. A. Neale, A. Hebert, M. Scheurer and M. C. M. Schriks, *Environ. Int.*, 2017, **99**, 120–130.
54 K. Kinnberg, *Evaluation of in vitro assays for determination of estrogenic activity in the environment*, Danish Ministry of the Environment, Danish Environmental Protection Agency, 2003.
55 B. I. Escher, S. Aït-Aïssa, P. A. Behnisch, W. Brack, F. Brion, A. Brouwer, S. Buchinger, S. E. Crawford, D. Du Pasquier and T. Hamers, *Sci. Total Environ.*, 2018, **628**, 748–765.
56 S. Yue, B. A. Ramsay, R. S. Brown, J. Wang and J. A. Ramsay, *Environ. Sci. Technol.*, 2015, **49**, 570–577.

57 J.-L. Zhao, G.-G. Ying, F. Chen, Y.-S. Liu, L. Wang, B. Yang, S. Liu and R. Tao, *J. Environ. Monit.*, 2011, **13**, 813–821.
58 M. Petrovic, *TrAC, Trends Anal. Chem.*, 2003, **22**, 685–696.
59 M. J. Arlos, W. J. Parker, J. R. Bicudo, P. Law, P. Marjan, S. A. Andrews and M. R. Servos, *Sci. Total Environ.*, 2018, **610–611**, 1103–1112.
60 F. Gagné, C. André, M. Douville, A. Talbot, J. Parrott, M. McMaster and M. Hewitt, *J. Environ. Monit.*, 2011, **13**, 3075.
61 M. P. Fernandez, M. G. Ikonomou and I. Buchanan, *Sci. Total Environ.*, 2007, **373**, 250–269.
62 R. van der Oost, G. Sileno, M. Suárez-Muñoz, M. T. Nguyen, H. Besselink and A. Brouwer, *Environ. Toxicol. Chem.*, 2017, **36**, 2385–2399.
63 J. Xu, C. Zhao, D. Wei and Y. Du, *J. Environ. Sci.*, 2014, **26**, 1961–1969.
64 D. J. Sauchyn, J.-M. St-Jacques and B. H. Luckman, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 12621–12626.