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## Adaption and application of cell-based bioassays to whole-water samples

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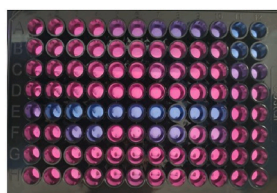
### HIGHLIGHTS

- Adaption of *in vitro* bioassays of ecologically relevant endpoints for WET.
- Freezing as a way of sample preservation is generally acceptable.
- Samples were compared in whole water form to traditional SPE.
- Samples in whole water were up to 50× more toxic than the corresponding extract.

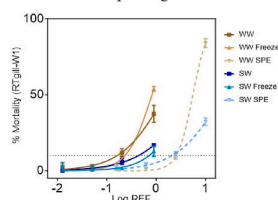
### GRAPHICAL ABSTRACT



Solid phase extraction  
Vs  
Whole water



Whole water up to 15× more toxic than  
corresponding extract



### ARTICLE INFO

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### ABSTRACT

The increasing presence of contaminants of emerging concern in wastewater and their potential environmental risks require improved monitoring and analysis methods. Direct toxicity assessment (DTA) using bioassays can complement chemical analysis of wastewater discharge, but traditional *in vivo* tests have ethical considerations and are expensive, low-throughput, and limited to apical endpoints (mortality, reproduction, development, and growth). *In vitro* bioassays offer an alternative approach that is cheaper, faster, and more ethical, and can provide higher sensitivity for some environmentally relevant endpoints. This study explores the potential benefits of using whole water samples of wastewater and environmental surface water instead of traditional solid phase extraction (SPE) methods for *in vitro* bioassays testing. Whole water samples produced a stronger response in most bioassays, likely due to the loss or alteration of contaminants during SPE sample extraction. In addition, there was no notable difference in results for most bioassays after freezing whole water samples, which allows for increased flexibility in testing timelines and cost savings. These findings highlight the potential advantages of using whole water samples in DTA and provide a framework for future research in this area.

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## 1. Introduction

In Australia, around 5 billion litres of wastewater are produced daily. This water, even after treatment, contains a complex mixture of potentially harmful chemicals (Patel et al., 2019; Schwarzenbach et al., 2006). Traditionally, the chemicals of interest were persistent organic pollutants and heavy metals (Petrović et al., 2003). However, an increase in product use has led to a growing list of contaminants of emerging concern (CEC) (Ahmed et al., 2021), which include pharmaceuticals, surfactants, antibacterial agents, pesticides, industrial chemicals, and their many transformation products (Abbas et al., 2019). The majority of CEC are unregulated and inadequately removed by existing wastewater treatment plants (WWTPs) (Ahmed et al., 2021; Melvin and Leusch, 2016). CEC pose a significant environmental risk through a variety of toxicity mechanisms, including genotoxicity, endocrine disruption and acute toxicity (Bolong et al., 2009). This has driven a need for increased monitoring and analysis of wastewater constituents.

Historically, the main focus of wastewater monitoring has been on chemical analysis (Neale et al., 2017). Chemical analysis of wastewater is important, and can determine the types and concentrations of particular chemicals in a sample, yet has limitations (König et al., 2017). For instance, it does not account for interactive toxicity in complex mixtures, chemical transformation products or bioavailability of chemicals (Brack et al., 2019). Furthermore, there is an ever increasing list of chemicals entering the environment, with over 350,000 chemicals and mixtures registered for commercial use (Wang et al., 2020). This makes it almost impossible to test and account for all contaminants potentially present in wastewater by chemical analysis alone. Alternatively, direct toxicity assessment (DTA), also called whole effluent toxicity (WET) testing, measures the overall toxicity of a water sample to exposed living organisms. While DTA bioassays provide limited information on the identity of the chemicals present, they provide information on the combined effects of complex chemical mixtures in water samples. DTA can complement chemical analysis of wastewater discharge, and in conjunction, offer a more ecologically relevant assessment (Neale et al., 2020a b; Norberg-King et al., 2018).

In most countries, industries that discharge wastewater into the environment are required to conduct DTA of wastewater to ensure it is safe for disposal into the receiving environment. The traditional DTA performed on wastewater discharge is through tests on whole organisms (*in vivo*). Not only are these *in vivo* tests expensive, low throughput and subject to ethical considerations, they are also limited to apical endpoints (mortality, development, growth and reproduction) (Prasse et al., 2015). There is a current paradigm shift towards cheaper, faster and more ethical methods that include an increased range of toxicological endpoints and higher throughput (NRC, 2007). Societal pressure has led towards greater adoption of the 3Rs principle to replace, reduce and refine the use of animals in research and monitoring. Effect-based methods (EBM) using cell-based (*in vitro*) bioassays are one such alternative that provide these benefits (Collins et al., 2008). Not only are *in vitro* bioassays high-throughput in nature, cheaper and more ethical, they can provide increased sensitivity for a range of non-apical environmentally relevant endpoints (Leusch et al., 2017). However, there are still several improvements to *in vitro* bioassays that are required in the context of DTA, including adaptation to whole water samples and establishment of *in vivo* to *in vitro* correlations (to validate ecological relevance).

Currently there are two main methods to prepare samples for *in vitro* testing of wastewaters. The first, and most common, involves extraction of contaminants from the sample, generally through solid phase extraction (SPE), to concentrate the contaminants into a carrier solvent (Abbas et al., 2019; Schirmer et al., 2001). While this approach is widely used and offers many advantages, such as enhancing the method detection limit and allowing testing to focus on organic micropollutants, there are also drawbacks. For example, contaminants can be either altered or lost all together through processing (Dayeh et al., 2002). In

addition, the extraction is focused on organic contaminants, and thus in a DTA context does not capture non-organic contaminants such as metals, metalloids or nutrients. An alternative approach is to reconstitute powdered assay media directly in the water samples of interest and test those directly in the bioassays, without having to extract and reconstitute into a carrier solvent (Dayeh et al., 2002). While this leads to a loss in method sensitivity (due to the lack of sample concentration during SPE), this drawback may not be a critical issue when testing (relatively polluted) wastewater samples. On the other hand, there is a range of benefits in exposing cells to the actual effluent sample: it minimises loss of toxicant during sample extraction and encompasses potential organic and inorganic mixture effects (Bols et al., 2005), it reduces the cost, time and environmental impacts associated labour- and solvent-intensive SPE (Dayeh et al., 2002), and it dramatically reduces volumes required for whole water testing and associated shipping costs (from 1 to 2 L down to 10–100 mL). Furthermore, whole water *in vitro* testing is more comparable to DTA testing protocols as it eliminates extensive extraction of samples (Dayeh et al., 2002; Niss et al., 2018). Therefore, for both whole water *in vitro* testing and DTA, the sample remains unaltered.

As samples provided by WWTPs often vary in delivery times, it is essential to preserve samples to minimise chemical degradation between sampling and analysis. Sample preservation enables larger batches of samples to be run simultaneously, increasing assay efficiency, and reducing costs – both particularly important in environmental monitoring programs. A common approach in water testing is to freeze water samples until analysis (Chapman and Mostert, 1990; Vanderford et al., 2011). To validate the application of cell-based assays as an alternative to DTA, it is important to determine whether freezing of wastewater samples and holding time impacts the bioassay results.

The aims of this study were therefore to 1) adapt a suite of cell-based bioassays covering a range of ecologically relevant endpoints to whole water testing (Finlayson et al., 2022a, b) test the influence of freezing and holding time on bioassay results; and 3) assess the difference in results between whole water and SPE extracts with metal and nutrient analyses to investigate the potential drivers of differential response. Wastewater and surface water samples were used as test samples.

## 2. Materials and methods

### 2.1. Reagents and chemicals

Reference compounds of chromium (as Na<sub>2</sub>CrO<sub>4</sub>, 98%), Diuron (≥98%), ethyl methanesulfonate (EMS), pentachlorophenol (PCP, 97%) and 2,3,7,8-tetrachloro-dibenzo-dioxin (TCDD, ≥95%) were purchased from Sigma-Aldrich. Methanol (MeOH; Sigma-Aldrich, ≥99.8%) was used as a carrier solvent for organic compounds and SPE extracts. Ultrapure water (18.2 MΩ cm) was used as a carrier for inorganic compounds, whole water dilutions and controls.

### 2.2. Water sampling and filtration

Secondary treated wastewater effluent samples were collected from a local conventional activated sludge WWTP, which serves a population of approximately 300,000 equivalent people. Surface water samples were collected from Loders Creek (27°58'06.1"S, 153°23'45.9"E), a local waterway with known contaminant issues.

All samples underwent a series of filtration steps to remove particulate matter and ensure sterility. Initially, 2.0 μm glass fibre prefilters (Whatman®, Merk Germany) were employed, followed by polyethersulfone (PES) filter (0.45 μm, Sterlitech USA) then 0.22 μm PES filter (Sterlitech USA). This was the final filtering step for samples undergoing SPE. Samples to be used as whole water in bioassays were subjected to an additional sterile filtration using a 0.22 μm PES syringe filter (Millex®, Merk Germany) before use. Water was then aliquoted into triple solvent (methanol, hexane/acetone (1:1) and ultrapure

water) rinsed sterile glass bottles either for direct bioassay use or SPE.

Prior to freezing, SPE was carried out on 500 mL water using an automated SPE system (SPE-03 High Efficiency Automated SPE System, Promochrom). Oasis HLB solid-phase extraction cartridges (6 cc, 500 mg; Waters) were preconditioned and eluted with equal volumes (10 mL) of methanol, hexane/acetone (1:1) and ultrapure water. Eluates were evaporated under gentle nitrogen stream, then reconstituted in 500  $\mu$ L methanol. This process resulted in a relative enrichment factor (REF) of 1000 for each sample.

To evaluate whether whole water samples could be frozen for storage, aliquots were prepared to be run “fresh” (within 2 days of collection) and after 30 days of storage at  $-20^{\circ}\text{C}$ . For stored samples, glass bottles were kept in the dark and placed at  $4^{\circ}\text{C}$  for 12 h before and after freezing to allow gradual thermal change.

### 2.3. Bioassays – cytotoxicity

To evaluate the cytotoxicity to fish cells, a resazurin assay was performed on extracts using RTgill-W1 and PLHC-1 cells (both purchased from ATCC), as previously described for sea turtle cells by Finlayson et al. (2021), with minor modifications, including testing extracts in 4-point, 4-fold serial dilutions. For whole water samples, a  $10 \times$  concentrated media formulation was produced using powdered media reconstituted in ultra-pure water: Minimum Essential Medium (MEM, M0643; Sigma-Aldrich, 96 mg/mL),  $\text{NaHCO}_3$  (22 mg/mL) and 20% fetal bovine serum (FBS) for PLHC-1 cells; L-15 medium (Leibovits, L4286; Sigma-Aldrich, 138 mg/mL), sodium bicarbonate ( $\text{NaHCO}_3$ ; Sigma-Aldrich,  $\geq 99.8\%$ , 37 g/mL) and 20% FBS for RTgill-W1 cells. Concentrated media was sterile filtered (0.22  $\mu\text{m}$ ), then added to samples at 10%, producing a final  $1 \times$  concentration of media in the tested samples, and a maximum relative enrichment factor (REF) of 0.9 in the assay for whole water samples. SPE extracts were added at 1%, resulting in a maximum REF tested of 10. Prior to experimentation, multiple trials of an 8-point, 4-fold serial dilution of the reference compound, sodium chromate, was run in both media types, for both cell lines to assess any variation between differing media types. Little to no variation was observed between different media types for either cell type (Fig. S1 and Fig. S2). All samples were tested in duplicate following a 4-point, 4-fold serial dilution, in at least two independent trials. The assay detection limit (DL) was based on the variability of the assay response with the negative control and set to 10%. An 8-point, 4-fold serial dilution of the reference compound, sodium chromate, was run in every plate for quality control.

### 2.4. Bioassays – genotoxicity

To evaluate genotoxicity, a high-throughput automated micronucleus (HiTMiN) assay using PLHC-1 cells was performed on extracts, as previously described by Johnson et al. (2022), with minor modifications, including the increase of seeded cell density to 3000 cells/well and field captures to 36 per well at  $20 \times$  magnification. For whole water, a  $10 \times$  concentrated media formulation was produced using powdered media reconstituted in ultra-pure water consisting of Minimum Essential Medium (MEM, M0643; Sigma-Aldrich, 96 mg/mL),  $\text{NaHCO}_3$  (22 mg/mL) and 20% FBS. Concentrated media was sterile filtered (0.22  $\mu\text{m}$ ), then added to samples at 10%, producing a final  $1 \times$  concentration of media in the tested samples, and a maximum REF of 0.9 in the assay for whole water samples. SPE extracts were added at 1%, resulting in a maximum REF tested of 10. Prior to experimentation, multiple trials of an 8-point, 4-fold serial dilution of the reference compound, EMS, was run in both media types to assess any variation between differing media types. Little to no variation was observed between different media types (Fig. S3). All samples were tested in duplicate following a 4-point, 4-fold serial dilution, in at least two independent trials. The assay DL was based on the variability of the assay response for the negative control (media only) and set to an induction ratio of 1.5. An 8-point, 4-fold serial

dilution of the reference compound, EMS, was run in every plate for quality control.

### 2.5. Bioassays - aryl hydrocarbon receptor activity

To assess the presence of aromatic hydrocarbons, the aryl hydrocarbon receptor chemically activated fluorescent expression (AhR CAFLUX) assay was performed on extracts as previously described by (Nagy et al., 2002). For whole water, a  $10 \times$  concentrated media formulation was produced using powdered media reconstituted in ultra-pure water, consisting of Dulbecco's Modified Eagle's Medium (DMEM, D2902; Sigma-Aldrich, 100 mg/mL),  $\text{NaHCO}_3$  (37 mg/mL), D-glucose (D-(+)-Glucose; Sigma-Aldrich,  $\geq 99.5\%$ , 35 mg/mL) and 20% FBS. Concentrated media was sterile filtered (0.22  $\mu\text{m}$ ), then added to samples at 10%, producing a final  $1 \times$  concentration of media in the tested samples, and a maximum REF of 0.9 in the assay for whole water samples. SPE extracts were added at 1%, resulting in a maximum REF tested of 10. Prior to experimentation, multiple trials of an 8-point, 4-fold serial dilution of the reference compound, TCDD, was run in both media types to assess any variation between differing media types. Little to no variation was observed between different media (Fig. S4). All samples were tested in duplicate following a 4-point, 4-fold serial dilution, in at least two independent trials. Assay DL was based on the variability of the assay response with the negative control (media only), and set to 10%. An 8-point, 4-fold serial dilution of the reference compound, TCDD, was run in every plate for quality control.

### 2.6. Bioassays – algal toxicity and photosynthesis inhibition

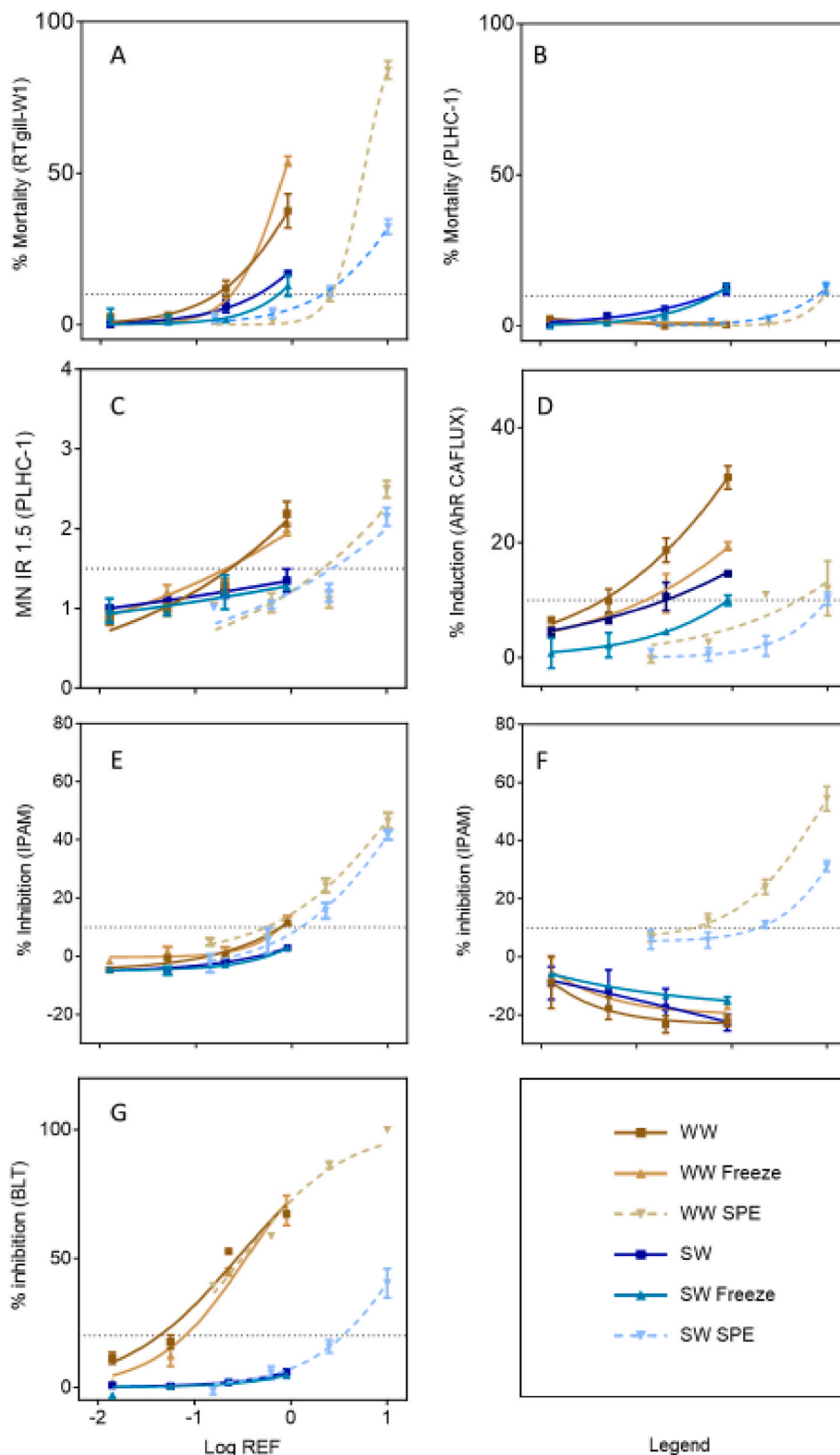
To assess algal toxicity and photosynthesis inhibition, an imaging pulse amplitude modulated (IPAM) fluorometry assay was performed on extracts as previously described by Escher et al. (2008). Measurements were taken at two timepoints of 2 h and 24 h to assess photosynthesis inhibition and potential growth inhibition, respectively. For whole water,  $10 \times$  concentrated algae was added directly to samples, or ultra-pure water for the negative control. Preliminary testing showed no difference in results for concentration-effect curves between  $1 \times$  algae and  $10 \times$  algae diluted down to  $1 \times$  (data not shown). Concentrated algae were then added directly to the sample at 10% to produce a maximum REF of 0.9 in the assay for whole water samples. SPE extracts were added at 1.75%, resulting in a maximum REF tested of 17.5. Prior to experimentation, multiple trials of an 8-point, 4-fold serial dilution of the reference compound, diuron, was run in both media types, for both time points (2 h and 24 h) to assess any variation between differing media types. Little to no variation was observed between different media types or timepoint (Fig. S5 and Fig. S6). All samples were tested in duplicate as a 4-point, 4-fold serial dilution, in at least two independent trials. The assay DL was based on the variability of the assay response with the negative control (ultra pure water) and set to 10%. An 8-point, 4-fold serial dilution of the reference compound, diuron, was run in every plate for quality control.

### 2.7. Bioassays – toxicity to bacteria

To assess bacterial toxicity, the bacterial luminescence toxicity screen (BLT-screen) was performed on extracts as previously described by van de Merwe and Leusch (2015). Minor alterations were made for the BLT-screen assay for adaption to whole water samples. A  $10 \times$  concentrated sterile experimental medium (pH = 4.0) consisted of  $\text{KH}_2\text{PO}_4$  (136 mg/mL),  $\text{NaCl}$  (282 mg/mL),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (74 mg/mL) and  $\text{KCl}$  (7.5 mg/mL) was produced. Media was sterilised by autoclave ( $121^{\circ}\text{C}$  for 20 min), then added to samples at 10% giving a final  $1 \times$  concentration of media in the tested samples. Bacteria (5  $\mu\text{L}$ ) was then added directly to the sample to produce a maximum REF of 0.88 in the assay for whole water samples. SPE extracts were added at 1%, resulting in a maximum REF tested of 10. experimentation, multiple trials of an

8-point, 4-fold serial dilution of the reference compound, PCP, was run in both media types to assess any variation between differing media types. Little to no variation was observed between different media (Fig. S7). All samples were tested in duplicate following a 4-point, 4-fold

serial dilution, in at least two independent trials. Assay DL was based on the variability of the assay response with the negative control (media only) and set to 20%. An 8-point, 4-fold serial dilution of the reference compound, PCP, was run in every plate for quality control.



**Fig. 1.** Sample concentration-effect curves comparing whole water (solid line) to SPE (dashed line) sample processing techniques with and without freezing for the battery of bioassays. (A) RTgill-W1 cytotoxicity, (B) PLHC-1 cytotoxicity, (C) PLHC-1 HiTMiN, (D) AhR CAFLUX, (E) IPAM-2h, (F) IPAM-24 h (G) BLT-screen. The dotted horizontal line represents the detection limit of the corresponding bioassay, EC<sub>10</sub> for all bioassays except the BLT-screen (EC<sub>20</sub>) and HiTMiN (IR<sub>1.5</sub>).

## 2.8. Bioassay – data analysis

For the HiTMiN assay, change in percentage of micronucleated cells was calculated by dividing the effect in the sample by the effect in the negative control to express results as an induction ratio (IR), where  $> IR_{1.5}$  was considered a significant induction.

For all other assays, a Hill equation was fitted to the concentration-effect data ( $x = \text{REF}$ ,  $y = \text{effect}$ ) by least squares regression using Microsoft Excel Solver. Sample effect was then scaled from the response of the positive control, where 100% represents the maximum effect and 0% represents the minimum effect on the concentration-effect curve of the reference compound. Respective sample  $EC_{10}$  and  $EC_{20}$  values were then derived using nonlinear regression in GraphPad Prism version 9.5.1. Differences between the whole water samples and their corresponding extracts were tested statistically for each bioassays using unpaired t-tests on biological replicates (GraphPad Prism version 9.5.1).

## 2.9. Matrix interference

To assess the potential for matrix interference in whole water, 8-point 4-fold serial dilutions of reference compound were tested in both waste and surface water samples for every assay. The only bioassay to produce any interference was the AhR CAFLUX. After the addition of TCDD to whole water samples, an increase of induction was witnessed compared to the standard positive control (in assay media). As such, the maximum induction of TCDD spiked samples was used to calculate the effect of the corresponding sample (waste or surface water).

## 2.10. Chemical analysis

Selected trace metals were measured by inductively coupled plasma-mass spectrometry (Agilent 8900) with external calibration using certified multi-element calibration standards (High Purity Standards; North Charleston, USA). Scandium, yttrium, indium and bismuth were used as internal standards and added online to the sample flow using a mixing-tee. Accuracy was evaluated by analysis of a river water certified reference material (SLRS-6 River Water; National Research Council Canada), which returned typical recoveries between 89 and 104% of the certified value.

Selected nutrients were measured by Berthelot reaction and colorimetric methods (SEAL AA500) using standard analytical methods (Baird et al., 2017).

## 3. Results and discussion

### 3.1. Cytotoxicity

A clear dose-dependent mortality was observed for all sample types in the RTgill-W1 cytotoxicity assay (Fig. 1A). For RTgill-W1 cells,

significantly higher toxicity was observed for whole water compared to the corresponding extract for both wastewater and surface water ( $p = 0.004$ ,  $t = 14.20$ ,  $df = 2$ , and  $p = 0.011$ ,  $t = 9.174$ ,  $df = 2$ , respectively). For wastewater, whole water was  $\sim 15 \times$  more toxic than the corresponding SPE extract, with  $EC_{10}$  REF values 0.16 and 2.5, respectively. For surface water, whole water was  $\sim 5 \times$  more toxic than the corresponding extract, with  $EC_{10}$  REF values 0.44 and 2.1, respectively (Fig. 1A–Table 1). The discrepancy in toxicity between the whole water and corresponding extracts is unsurprising, as SPE is targeted towards recovery of organic micropollutants only, whereas whole water will contain both organic and inorganic contaminants (Andrade-Eiroa et al., 2016). RTgill-W1 cells have previously been shown to be sensitive to inorganic compounds such as metals (Dayeh et al., 2005; Scott et al., 2021), which were present in the whole water samples tested here (see section 3.7).

In contrast, in the PLHC-1 cytotoxicity assay, a clear dose-dependent mortality was only observed for the surface water samples (Fig. 1B). Similar to the RTgill-W1 cells, the whole water was  $\sim 13 \times$  more toxic than the corresponding SPE extract, with  $EC_{10}$  REF values of 0.62 and 8.0, respectively. Contradictory to the results in the RTgill-W1 assay (where the wastewater sample produced the most mortality) the wastewater samples (both whole water and SPE) caused minimal mortality to the PLHC-1 cells (Fig. 1B–Table 1).

Overall, RTgill-W1 cells were more sensitive than PLHC-1 cells, for both whole water and corresponding extracts. For the SPE extracts, the wastewater and surface water samples were  $\sim 4 \times$  more toxic in the RTgill-W1 cells compared to PLHC-1 cells (Fig. 1A and B). However, for whole water samples, RTgill-W1 were more sensitive to the wastewater compared to the surface water. The opposite was true for the PLHC-1 cells, which were more sensitive to surface water, while the cytotoxic response to wastewater was below the detection limit with the PLHC-1 cells. These differential responses underscore the importance of employing multiple cell lines to evaluate contaminants across various sample types. A recent study has suggested that, when assessing the origin of cells from the same species, rainbow trout gill cells are more sensitive to cytotoxicity than rainbow trout liver cells, due to increased metabolic capacity (Ball et al., 2023). The study found gill cells to be more sensitive for certain compounds, while the liver cells were more sensitive to others (Ball et al., 2023). Further, when assessing different species, one study found that certain compounds elicited no detectable effect in RTgill-W1, but did in PLHC-1 cells (Kienzler et al., 2012), while another study illustrated that RTgill-W1 cells were more sensitive than PLHC-1 cells when evaluating cytotoxicity of environmental samples (Amaeze et al., 2015). Both cell lines have retained some cytochrome P450-dependent monooxygenase activities, enabling the metabolism of a range of cytotoxic and genotoxic compounds (Amaeze et al., 2015; Franco et al., 2018). With conflicting results in the literature, regarding metabolic capacities, it may be concluded that differences in sensitivity between the two cell lines may be compound-specific, and that more

**Table 1**

Summary of toxicity statistics for whole water and SPE extracts of wastewater and surface water samples tested in the BLT-Screen, IPAM, AhR CAFLUX, cytotoxicity and HiTMiN assays. REF = relative enrichment factor; IR = induction ratio.

Bioassay	Threshold value	Wastewater (fresh)	Wastewater (30d-freeze)	Wastewater SPE	Surface water (fresh)	Surface water (30d-freeze)	Surface water SPE
BLT	$EC_{20}$ REF	$0.043 \pm 0.0008$	$0.068 \pm 0.0005$	$0.058 \pm 0.0006$	BDL ( $>0.88$ )	BDL ( $>0.88$ )	$3.4 \pm 0.05$
IPAM 2 h	$EC_{10}$ REF	$0.43 \pm 0.05$	$0.75 \pm 0.2$	$0.57 \pm 0.06$	BDL ( $>0.9$ )	BDL ( $>0.9$ )	$0.89 \pm 0.04$
IPAM 24 h	$EC_{10}$ REF	Growth <sup>(a)</sup>	Growth <sup>(a)</sup>	$1.1 \pm 0.1$	Growth <sup>(a)</sup>	Growth <sup>(a)</sup>	$3.6 \pm 0.7$
AhR CAFLUX	$EC_{10}$ REF	$0.044 \pm 0.01$	$0.12 \pm 0.08$	$5.1 \pm 0.2$	$0.19 \pm 0.01$	$0.91 \pm 0.1$	$9.8 \pm 0.9$
Cytotoxicity (RTgill-W1)	$EC_{10}$ REF	$0.16 \pm 0.05$	$0.23 \pm 0.03$	$2.5 \pm 0.2$	$0.44 \pm 0.09$	$0.72 \pm 0.2$	$2.1 \pm 0.2$
Cytotoxicity (PLHC-1)	$EC_{10}$ REF	BDL ( $>0.9$ )	BDL ( $>0.9$ )	$9.5 \pm 0.07$	$0.62 \pm 0.1$	$0.65 \pm 0.1$	$8.0 \pm 0.8$
HiTMiN	$IR_{1.5}$	$0.23 \pm 0.09$	$0.21 \pm 0.01$	$2.1 \pm 0.2$	BDL ( $>0.9$ )	BDL ( $>0.9$ )	$2.6 \pm 0.2$

BDL - Below detection limit. (a) Instead of inhibition, growth promotion was detected in whole water samples with an average of  $\sim 22\%$  for both waste and surface water.

research is required in this field.

Prior research has demonstrated that RTgill-W1 cells exhibit resilience across a broad spectrum of osmotic conditions, spanning from 100 to 550 mOsm/kg (Lee et al., 2009; Scott et al., 2021; Yue et al., 2015). While osmolality could not be analysed in these samples, specific conductance (SPC) and salinity were analysed, which have been shown to be an accurate proxy for osmolality (Saoud et al., 2007; Topcu and Bayraktar, 2022; Yoo et al., 2021). Equations within these studies allowed for the calculation of osmolality from SPC and salinity, which ranged from 1.1 to 40.8 mOsm/kg, for the waste and surface water samples, respectively. These values were well under the OECD and ISO guidelines for molality in the fish cell cytotoxicity assay (290–320 mOsm/kg), indicating that neither water sample would induce osmotic stress in the current study.

The resazurin assay is a commonly used cytotoxic test assessing cellular metabolic activity (Finlayson et al., 2022). Good quantitative correlations have been shown between *in vivo* toxicity and *in vitro* cytotoxicity evaluations for a range of fish species, especially when toxicokinetic parameters and partition coefficients are taken into account (Fischer et al., 2019; Glden and Seibert, 2005; Stadnicka-Michalak et al., 2014). The toxicity of wastewater effluent has previously been assessed using the RTgill-W1 cell line (Dayeh et al., 2002). Previous studies have shown that sensitivities vary between species and cell types, especially with inorganic compounds (Finlayson et al., 2019a,b; Tong et al., 2016). The higher sensitivity of RTgill-W1 cells towards wastewater samples suggests their potential for detecting contaminants associated with industrial or domestic effluents. Integrating multiple cell lines facilitates a broader assessment of contaminant loading across a range of sample types, potentially enhancing the accuracy and reliability of water quality evaluations. Such an approach enhances the accuracy and reliability of water quality assessments, ultimately aiding in effective environmental monitoring and risk management strategies.

### 3.2. Genotoxicity

A clear dose-dependent increase in micronucleated cells was observed in all samples, apart from the surface water in whole water form (Fig. 1C). For wastewater, whole water samples were significantly more genotoxic ( $\sim 10 \times$ ) than the corresponding extract, with  $IR_{1.5}$  REF values of 0.23 and 2.15, respectively ( $p = 0.012$ ,  $t = 8.843$ ,  $df = 2$ ). For surface water, the whole water sample was just below the detection limit ( $IR_{1.5}$ ), with an average IR of 1.33 at an REF of 0.9, while the SPE extract exhibited a genotoxicity similar to the wastewater SPE extract (Fig. 1C; Table 1). This highlights the limitations of not being able to concentrate whole water, as without extrapolating the data, the REF required to surpass the detection limit could not be calculated.

Genotoxic organic and inorganic compounds have been detected in water samples using both *in vivo* and *in vitro* approaches (Gauthier et al., 1993; Stahl Jr, 1991). The micronucleus (MN) assay is one of the most common and sensitive methods to evaluate genotoxic effects through DNA damage in organisms exposed to genotoxic compounds (Poletta et al., 2009). DNA damage detected through the MN assay is irreparable, with the potential to cause catastrophic consequences within organisms (Obiakor et al., 2012). Effects of clastogens and aneugens are both detected through the micronucleus assay, with clastogenic damage resulting from chromosomal breakage and aneugenic damage from whole chromosomes (Doherty et al., 2016; Mishima, 2017). Aneugens have a non-reactive mode of action (MoA), while clastogens have a reactive MoA and considered to have no safety threshold (Mishima, 2017). Therefore, clastogens are of highest concern. The variations in sensitivities observed between whole water and extracts may be attributed to the higher concentrations of metals detected in the whole water samples compared to extracts analysed in this study (see section 3.7). Prior studies have shown metals to significantly increase micronucleation events for a range of cell types (Finlayson et al., 2019a,b;

Kopp et al., 2018). Whole water is therefore potentially a better exposure medium to determine MN induction, due to the complications associated with possible loss or transformation of contaminants in SPE extracts.

### 3.3. Aryl hydrocarbon receptor activity

A clear concentration-dependent induction was observed for both whole water and SPE extracts of the surface and wastewater samples (Fig. 1D). Significantly increased sensitivity was observed when comparing whole water to the corresponding SPE extracts for both surface water and waste water ( $p = 0.019$ ,  $t = 7.145$ ,  $df = 2$ , and  $p = 0.0028$ ,  $t = 18.79$ ,  $df = 2$ , respectively). For wastewater, whole water samples were  $\sim 100 \times$  more potent than the corresponding extract, with  $EC_{10}$  REF values of 0.044 and 5.1 respectively. For surface water, whole water samples were  $\sim 50 \times$  more toxic than the corresponding extract, with  $EC_{10}$  REF values of 0.19 and 9.8 respectively (Fig. 1D). For comparison with similar studies, AhR activity was converted into bio-analytical equivalent concentrations (BEQs) by dividing the EC of the reference compound by the EC of the sample to calculate a TCDD equivalent value. BEQs followed the same trend as the  $EC_{10}$  REF, with 2364, 1122, 110 and 39 pg TCDEQ/L for wastewater, surface water, wastewater SPE and surface water SPE, respectively. These results were comparable to similar studies investigating AhR activation associated with wastewater contaminants (Escher and Neale, 2021).

AhR activation in environmental samples is strongly correlated with aromatic hydrocarbons such as dioxins and dioxin-like compounds (Giesy et al., 2002; Koh et al., 2004). The SPE process has also been shown to produce high recovery of organic compounds and therefore, compounds that activate the AhR should theoretically be well recovered in the extract (Andrade-Eiroa et al., 2016). Yet we observed higher AhR activity in whole water samples; this is potentially due to inorganic compounds that were not recovered during the SPE process (see section 3.7). Some studies have found that inorganics, including metals such as copper and lead, in the presence of an AhR ligand, can increase the mRNA expression of cytochrome P450 1A1 (CYP1A1), an enzyme linked with metabolism of aromatic hydrocarbons (Korashy and El-Kadi, 2004; Shimada and Fujii-Kuriyama, 2004). The study concluded that metals alone did not significantly alter the CYP1A1 activity and associated protein levels, but simply amplified the response of organic AhR activators (Korashy and El-Kadi, 2004). However, the regulation of the CYP1A1 gene has been shown to involve the activation of the AhR dependent pathway through direct binding of AhR ligands to the receptor (Whitlock Jr, 1999). Similarly, Cavallini et al. (2016) reported a significant increase of mRNA expression of AhR due to exposure of metals alone. Interestingly, metals and typical AhR ligands share no similarities or structural properties, suggesting metals are indirect inducers of AhR. Therefore, some metals may be able to induce the CYP1A1 gene without binding directly to the receptor. Evaluation of AhR activity through luciferase activity found that metals, in the presence of an AhR ligand, either inhibited the activation of the receptor or had no significant effect (Chou et al., 2009). These findings promote the concept of capturing complex mixtures of both organic and inorganic contaminants to better understand the effect of wastewater samples. Discrepancies of data within the literature and the findings in this study warrant further investigation into this topic.

It is important to note that high variability was observed in the AhR assay for the wastewater SPE extract (due to high autofluorescence), which was not evident in the whole water sample for wastewater or the whole water sample and extract for surface water. There is the potential that upregulation of the signal, detected as auto-fluorescence, may be caused by cell death (Kim et al., 2020). Certain compounds within the sample may also contribute to the autofluorescence, but more research is required in this area. Whole water samples containing elevated contaminants are potentially a better matrix to assess AhR induction, due to the complications associated with autofluorescence of the SPE extract,

combined with the increased toxicity of the whole water samples due to the presence of a larger variety of chemicals and complex mixtures. However, whole water samples should be tested as soon as possible, without freezing, as freezing of samples caused a reduction in bioassay response (see section 3.6).

### 3.4. Toxicity to algae and photosynthesis inhibition

Both wastewater and surface water SPE extracts produced a clear concentration-dependent inhibition of photosynthesis for the IPAM assay at the 2 h endpoint (Fig. 1E, dashed lines). There was a comparable concentration-response of photosynthesis inhibition in the SPE extracts, with  $EC_{10}$ REF(2 h) values of 0.57 and 0.89 for wastewater and surface water, respectively. Whole water and SPE extracts also displayed very similar concentration-effect curves for wastewater (Fig. 1E), and the  $EC_{10}$ REF(2 h) values for whole water and SPE extracts were comparable for wastewater (0.43 and 0.57 REF, respectively,  $p = 0.11$ ,  $t = 2.801$ ,  $df = 2$ ; Table 1). However, the degree of photosynthesis inhibition observed from the surface water in whole water form was considerably lower to that of the wastewater, and the response of the surface whole water sample was below the detection limit of the assay. This again highlights the limitations of not being able to concentrate whole water, as without extrapolating the data, the REF required to surpass the detection limit cannot be calculated. IPAM 2 h results from the wastewater suggest that most photosynthesis blocking compounds of algae are organic chemicals recovered by SPE (such as PSII inhibiting herbicides), and that other chemical contaminants in water samples are not significant contributors to photosynthesis inhibition.

The response between the whole water and the sample extract differed considerably for the IPAM assay at the 24 h endpoint (Fig. 1F). There was a clear concentration-dependent response of reduced photosynthesis in the SPE extracts, with  $EC_{10}$  REF (24 h) values of 0.98 and 3.6 for wastewater and surface water, respectively (Fig. 1F, dashed lines; Table 1). Photosynthesis inhibition in water samples is strongly correlated with PSII inhibiting herbicides such as diuron and simazine (Knauert et al., 2008; Ricart et al., 2009). The SPE results suggest that herbicides are present in wastewater at concentrations that could affect algal photosynthesis (causing 15% inhibition at a REF of 1), while concentrations in surface water are lower and less likely to have a significant effect (<10% inhibition at a REF of 1). A diuron equivalent of 0.4  $\mu\text{g/L}$  was calculated for the wastewater sample, which is below ANZECC (2000) guidelines for diuron of 0.6  $\mu\text{g/L}$ . Results are comparable to similar studies investigating photosynthetic inhibition associated with wastewater contaminants (Tang and Escher, 2014).

Contrasting results were observed when using whole water samples, with both wastewater and surface water exhibiting a concentration-dependent decrease in photosynthesis inhibition; in other words, an increase in photosynthesis at higher REF (Fig. 1F). This equated to maximum average algal growth of ~22% for both samples in whole water form. This is likely due to the presence of nutrients in the samples (see section 3.7), which were not retained by SPE, and are known to promote algal growth and could counter-act the impact of co-occurring concentrations of herbicidal compounds (Fig. 1F).

Photosynthesis inhibitors act by competing with plastoquinone for binding sites, which alters the electron transfer, causing excitation to be emitted as fluorescence instead of driving photochemical reactions (Muller et al., 2008). This increase in fluorescence yield is detected through IPAM fluorometry, which can then be translated to a measure of photosynthesis inhibition (Schreiber et al., 2007). The differing results between the 2 and 24 h readings for the samples in whole water form are potentially a measure of both herbicidal and nutrient concentrations in the sample. It has been reported that the effect of photosynthesis inhibitors is almost instantaneous for algae (Escher et al., 2008). Therefore, the presence of herbicidal concentrations in the samples is well represented in the IPAM 2 h reading. Conversely, the observable effect of algal growth from increased nutrient loading takes considerably longer

(days) (Pedersen and Borum, 1996). Therefore, the presence of nutrients in the samples (see section 3.7) is potentially incorporated in the IPAM 24 h reading and compensating for the inhibitory effect of herbicides at this timepoint. Correlations have been shown using optical density as a measure of growth and chlorophyll fluorescence (Escher et al., 2008). This can potentially explain the results observed in the IPAM 24 h response in this study, which indicates an increase in algal growth due to nutrient loading of the whole water samples.

This difference highlights an important role for the IPAM assay in assessing toxicity of whole water samples, and the comparison can provide interesting insights into the combined inhibitory activity of herbicides and the stimulatory activity associated with nutrients. In the end, both types of contaminants are undesirable: herbicides can negatively affect primary productivity (Ricart et al., 2009), and excessive nutrients can lead to eutrophication (Wurtsbaugh et al., 2019). Application of the combined 2- and 24-h IPAM assay for testing with whole water samples can detect both outcomes simultaneously, and both upwards and downwards deviation from the baseline would be deemed indicators of risk in the receiving environment.

### 3.5. Toxicity to bacteria

Both wastewater and surface water extracts produced a clear concentration-dependent inhibition of bacterial bioluminescence in the BLT-screen (Fig. 1G). Whole water and SPE extracts displayed very similar concentration-effect curve for wastewater, and the  $EC_{20}$  values for whole water and SPE extracts were comparable for wastewater (0.043 and 0.058 REF, respectively,  $p = 0.058$ ,  $t = 3.946$ ,  $df = 2$ ; Fig. 1G; Table 1). However, the degree of luminescence inhibition observed from the surface water sample in whole water form was considerably lower to that of the wastewater, resulting in the surface whole water sample being below the detection limit of the assay. This again highlights the limitations of not being able to concentrate whole water, as without extrapolating the data, the REF required to surpass the detection limit cannot be calculated. Results from the wastewater suggest that most compounds that are toxic to bacteria are organic chemicals recovered by SPE, and that other chemical contaminants in water samples are not significant contributors to bacteria toxicity.

Naturally luminescent bacterial assays are widely used to assess basal toxicity of water samples (Daniels et al., 2018; Escher et al., 2014). Toxicity towards bacteria can have significant ecological ramifications, as these microorganisms play crucial roles in various biogeochemical cycles (Paerl and Pinckney, 1996). An extensive range of organic compounds including organochlorines such as pentachlorophenol (PCP) are known inhibitors of bacterial luminescence, have been detected in the environment and shown to have a range of human and environmental health impacts (Ge et al., 2007; Jayaraj et al., 2016; van de Merwe and Leusch, 2015). Metals have generally been shown to be much less toxic to these bacteria, compared to organic contaminants (van de Merwe and Leusch, 2015). Therefore, even with presence of metals in a water sample, toxicity of the assay will be driven by organic contaminants. For future use of this assay and dependent on potential toxicity of a sample, either SPE or whole water can be used.

### 3.6. Impact of sample freezing and holding on bioassay activity

Storage of either wastewater or surface water samples for 30-d at  $-20\text{ }^{\circ}\text{C}$  did not significantly ( $p > 0.05$ ) affect the bioassay results for cytotoxicity (Fig. 1A and B, solid lines), genotoxicity (Fig. 1C), photosynthesis inhibition or algal toxicity (Fig. 1E and F), or bacterial toxicity (Fig. 1G; Table 1), indicating that there was little to no degradation of contaminants detected by these assays during the freezing and storage process. Freezing is a recommended method for sample preservation with both organic and inorganic compounds being shown to preserve well in storage at  $-20\text{ }^{\circ}\text{C}$  (Elsner et al., 2006; Kotlash and Chessman, 1998). The AhR CAFLUX assay was the only assay in this study where

freezing the samples had a significant impact on the bioassay results for surface water ( $p = 0.031$ ,  $t = 5.534$ ,  $df = 2$ ). Samples exhibited a slight decrease in toxicity following 30-d storage at  $-20\text{ }^{\circ}\text{C}$  (Fig. 1D). This was evident with a decrease in  $\text{EC}_{10}$  between pre and post frozen samples, by a factor of 2.2 and 2.8 for surface and whole water samples, respectively (Table 1). While most chemicals have been shown to preserve well in frozen waters, it is possible that tannins or dissolved organic carbon (DOC) may precipitate upon thawing, then not re-solubilise into the sample (Bonifácio et al., 2022). There is then the potential that concentrations of metals or other contaminants with high affinity to the precipitate may be impacted. Correlations have been made between an increased loss of contaminant loading in water and higher DOC after a freeze-thaw cycle (Fellman et al., 2008). This may potentially explain the sample potency reduction in the AhR CAFLUX assay after a freeze-thaw cycle. However further research is required to investigate this.

### 3.7. Contribution of inorganic contaminants to the bioassay response

The bioanalytical equivalent associated with measured inorganic contaminants was estimated using the “iceberg modelling” approach, using the mass load of the detected contaminants and the corresponding relative effect potency relative to the reference compound (Neale et al., 2020a,b). Due to limited availability of relevant  $\text{EC}_{50}$  data in the literature from available relative bioassays and due to low activity in the surface water sample, iceberg modelling was only produced for the RTgill-W1 cytotoxicity assay and BLT-screen for the wastewater sample. All supporting information for the iceberg modelling calculations can be found in Table S1.

For the 18 detected metals within the sample (Table 2), reliable and relative (assessing metabolic activity) data for the RTgill-W1 cytotoxicity assay and BLT-screen could only be found for nine and ten metals, respectively. Up to 30.7% of the RTgill-W1 cytotoxicity observed in the wastewater sample could be explained by these nine metals, with manganese accounting for the majority (26%) of the cytotoxic effect, followed by copper (3%). In most cases where data was available, the concentrations of detected metals were considerably lower than the reported RTgill-W1  $\text{EC}_{50}$  values (Dayeh et al., 2005; Scott et al., 2021).

**Table 2**

Summary and comparison of selected metals and nutrients detected in samples in either whole water or SPE form. All concentrations reported are  $\mu\text{g/L}$ .

Contaminant	Wastewater	Surface water	Wastewater SPE	Surface water SPE
<b>(Metal)</b>				
Al	12.97	0.7760	0.0088	0.0145
V	0.3330	0.1690	0.0002	0.0002
Cr	1.959	0.1830	0.0035	0.0008
Mn	69.39	56.09	0.0316	0.0072
Fe	54.62	32.01	0.1070	0.0252
Co	0.4370	0.3170	0.0016	0.0009
Ni	1.525	1.039	0.0045	0.0038
Cu	9.991	10.05	0.0119	0.0338
Zn	11.08	5.437	0.0319	0.0269
As	1.922	0.7120	0.0023	0.0025
Se	0.5160	0.1830	0.0018	0.0031
Mo	0.3280	1.1380	0.0001	0.0001
Cd	0.0160	0.1830	0.0000	0.0001
Sb	0.1480	0.1720	0.0002	0.0002
W	0.0210	0.0290	0.0001	0.0001
Pb	0.0640	0.1770	0.0001	0.0002
U	0.0550	0.0460	0.00001	0.00001
<b>(Nutrient)</b>				
$\text{NOx-N}$	23.44	59.76	1.320	1.748
$\text{PO}_4^{3-}\text{-P}$	2639	16.53	BLR (<0.3)	BLR (<0.3)
$\text{NH}_3\text{-N}$	3321	220.5	1.753 <sup>a</sup>	BLR (<0.4)

BLR= Below limit of reporting.

<sup>a</sup> Sample blanks were run for all sample types, returning results below limit of reporting.

This fits well with the narrative that individual compounds on their own are unlikely to explain elucidated effects and that it is complex mixtures that need to be evaluated.

Toxicity of individual metals to bacteria were sourced for the BLT-Screen specifically, but also from Microtox  $\text{EC}_{50}$  values due to the reported concordance between the two assays (van de Merwe and Leusch, 2015). Up to 19.3% of activity observed in the BLT-Screen could be explained by these ten metals with the majority (20%), explained by three metals (copper (10%), chromium (4%) and zinc (4%)). Among the identified metals with available data, concentrations tended to be notably lower than the reported BLT-Screen or Microtox  $\text{EC}_{50}$  values (Hsieh et al., 2004; van de Merwe and Leusch, 2015). Again, this highlights the importance of considering complex mixtures when evaluating the toxicity of a water sample.

There is the possibility that metals may potentiate organic chemical toxicity and reduce biodegradation of organic contaminants (Sandrin and Maier, 2003), which may explain several of the results observed here. Firstly, the vast increase of sensitivity when using whole water samples for a range of endpoints compared to the corresponding extract could be explained by the potentiation of present metals on the organic contaminants in the sample. Secondly, it could explain the increase in observed results for the wastewater sample compared to the surface water sample due to the greater concentration of metals leading to increased potentiation of organics. Thirdly, this possible potentiation could explain the vast increase of activity for samples in whole water form compared to the corresponding extract observed in the AhR CAFLUX assay, however, further research is required in this field. Nevertheless, the inclusion of metals through possible mixture effects and potentiation of organics is an important insight and highlights the benefit of using whole water samples for toxicity assessments of water.

## 4. Conclusions and future directions

In this study, we present an ethical, representative, high-throughput, and cost-effective alternative to traditional DTA methodologies for the analysis of wastewater and surface water samples covering a diverse range of endpoints, both using whole water samples and SPE extracts. Encouragingly, these findings indicate a potential shift away from reliance on whole organism-based approaches for monitoring wastewater and other environmentally significant water samples. However, further research is needed to address certain areas, such as the optimisation and refinement of whole water sample processing techniques, as well as the comprehensive evaluation of complex contaminant mixtures found in wastewater. It is important to acknowledge that chemical analysis alone can only provide an estimation of the potential environmental risks, given the presence of low concentrations of individual chemicals and the possibility of unknown or infrequently measured active substances contributing to sample toxicity. Therefore, there remains a risk of underestimation when relying solely on chemical analysis and SPE in complex wastewater samples.

For some assays and endpoints (e.g., BLT and IPAM 2 h), which are strongly driven by organic contaminants, our study shows little difference between whole water or SPE. This suggests that most relevant contaminants are organic compounds that are well retained by SPE, and both whole water or SPE extracts could thus be used. This will be dependent on study objectives, contaminant loading of water and any logistical constraints. For other assays (RTgill-W1 cytotoxicity, HiTMiN, AhR CAFLUX and IPAM 24 h), disparities can arise when comparing results obtained from whole water and SPE extracts. These differences are likely attributed to the removal or alteration of critical chemical components during the SPE process, such as inorganic contaminants. There is therefore an argument to be made for the application of whole water samples in an *in vitro* DTA alternative bioassay battery, and whole water samples may be preferred despite the lower detection limits associated with this testing format.

Detection limits for each assay were converted to equivalency values

for comparison with ecoEBT values, finding in most cases the detection limit for *in vitro* assays was higher than the ecoEBT value. However, due to dilutions associated with mixing zones, detection limits for whole water samples being lower than effect-based trigger bioanalytical equivalent concentration (EBT-BEQ) (Table S2), may not be of such concern. The presence of wastewater effluent release has been successfully recorded at concentrations as low as 0.2% in the mixing zone (Lawrence, 2010). A conservative estimate of effluent dilution at  $10 \times$  into receiving environments, suggests that a lower detection limit compared to EBT-BEQ is still relevant. However, it is worth noting that if significant bioassay activity is observed at an REF of 0.9, then a more accurate environmentally relevant risk can potentially be determined. Freezing for 30 days had minimal impact on the toxicity of the two sample types tested in this study (except with the AhR CAFLUX assay), so long term storage, and running samples in larger batches is a possibility (although the length of possible storage needs to be investigated further). However, individual samples may respond differently to a freeze-thaw cycle, due to the inherently varying complex mixtures in different water samples. The impact of freezing on water toxicity should therefore be validated on a case-by-case basis.

Accurate assessment of contaminant loading in water samples is critical for effective environmental monitoring and safeguarding human and ecological health. The present study highlights the potential advantages of utilising whole water for the evaluation of environmental and wastewater samples. One plausible explanation for the observed increase in effect of whole water samples is the removal of metals, nutrients and some organic compounds during the SPE process. However, it is important to acknowledge that the filtration step prior to both the SPE and whole water process involved in the procedure may inadvertently eliminate certain contaminants from the water (e.g., those bound to particulate matter), potentially affecting the overall assessment of sample composition. Future investigations should aim to identify and quantify the specific contaminants present on the filter to determine whether their inclusion in the sample could enhance the accuracy of the analysis.

While certain inorganic compounds may not directly bind to specific receptors transfected within cells used in *in vitro* bioassays, there is evidence of heightened induction of samples with increased inorganic contaminant loads (Korashy and El-Kadi, 2004). This phenomenon is also observed when assessing protein expression or mRNA associated with the relevant receptors. The findings presented in this study for the AhR assay support the notion that even though individual inorganic compounds may not exhibit direct receptor binding, their presence can influence the expression and activity of the target receptors. However, this study only presents one endpoint to support that notion and warrants further experimentation. Further, when assessing additional endpoints such as cytotoxicity, genotoxicity, or photosynthesis inhibition there is the potential to underestimate the contaminant load of a sample by not encompassing all potential contaminants, which may be removed through the extraction process of SPE.

In summary, the findings of this study highlight the benefits of using whole water samples in the assessment of environmental and wastewater samples. This approach offers a more thorough assessment of contaminants and cost-effectiveness compared to traditional SPE methods, while also reducing the reliance on whole organism-based assays. Further research and development in this field will contribute to a more comprehensive understanding of water contamination and its potential environmental impacts.

#### CRedit authorship contribution statement

**Matthew Johnson:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kimberly Finlayson:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Jason P. van de Merwe:** Writing – review & editing, Visualization, Supervision,

Project administration, Funding acquisition, Conceptualization. **Fred-eric D.L. Leusch:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2024.142572>.

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