Wastewater treatment efficacy evaluated with *in vitro* bioassays

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**Abstract**

Bioassays show promise as a complementary approach to chemical analysis to assess the efficacy of wastewater treatment processes as they can detect the mixture effects of all bioactive chemicals in a sample. We investigated the treatment efficacy of ten Australian wastewater treatment plants (WWTPs) covering 42% of the national population over seven consecutive days. Solid-phase extracts of influent and effluent were subjected to an *in vitro* test battery with six bioassays covering nine endpoints that captured the major modes of action detected in receiving surface waters. WWTP influents and effluents were compared on the basis of population- and flow-normalised effect loads, which provided insights into the biological effects exhibited by the mixture of chemicals before and after treatment. Effect removal efficacy varied between effect endpoints and depended on the treatment process. An ozonation treatment step had the best treatment efficacy, while WWTPs with only primary treatment resulted in poor removal of effects. Effect removal was generally better for estrogenic effects and the peroxisome proliferator-activated receptor than for inhibition of photosynthesis, which is consistent with the persistence of herbicides causing this effect. Cytotoxicity and oxidative stress response provided a summary parameter of all bioactive chemicals including transformation products and removal was poorer than for specific endpoints except for photosynthesis inhibition. Although more than 500 chemicals were analysed, the detected chemicals explained typically less than 10% of the measured biological effect, apart from algal toxicity, where the majority of the effect could be explained by one dominant herbicide, diuron. Overall, the current study demonstrated the utility of applying bioassays alongside chemical analysis to evaluate loads of chemical pollution reaching WWTPs and treatment efficacy.

1. Introduction

Wastewater treatment plant (WWTP) effluent is a major contributor to chemical pollution in the aquatic environment (Eggen et al., 2014; Neale et al., 2017b). A National Wastewater Monitoring Program aligned the collection of wastewater influent and effluent samples from around Australia with the 2016 Australian Census to improve understanding of public health and the release of chemicals from WWTPs (O’Brien et al., 2019). These samples have been used to investigate the consumption of illicit drugs, alcohol and tobacco (O’Brien et al., 2017), to refine excretion factors for codeine and methadone (Thai et al., 2019), to determine the relationship between sociodemographic factors and diet, drug and lifestyle biomarkers (Choi et al., 2019), to assess the prevalence of gout in Australia (Ahmed et al., 2020), to calibrate passive samplers for a range of pharmaceuticals and illicit drugs (Mckay et al., 2020) and to estimate the release of UV filters in wastewater effluent (O’Malley et al., 2020). To date, the research has focused on chemical analysis, but the mixture of thousands of chemicals present in complex samples, such as wastewater, means that targeted chemical analysis cannot detect all chemicals present nor can it accurately predict the toxicity and thus the environmental risk of these complex mixtures (Escher et al., 2020).

Bioanalytical tools (i.e., *in vitro* bioassays and well plate-based *in vivo* assays) have been proposed as a complementary approach to chemical analysis as they can detect the mixture effects of all active chemicals in a sample, including transformation products.
and unknown chemicals. While \textit{in vitro} bioassays are recommended for water quality monitoring (Brack et al., 2019), they have also been applied to evaluate treatment efficacy by wastewater, advanced water and drinking water treatment processes (e.g. Escher et al., 2011; Escher et al., 2014; Conley et al., 2017; Houtman et al., 2018). Surface waters receiving wastewater effluent have previously been shown to induce effects in both \textit{in vitro} and \textit{in vivo} assays (e.g. Harth et al., 2018; Sonavane et al., 2018), highlighting the need for effective wastewater treatment to remove biological effects.

The current study aimed to determine the biological equivalent loads in some of the Australian National Wastewater Monitoring Program (O’Brien et al., 2019) influent and effluent samples and evaluate effect removal efficacy using a battery of bioassays (Jia et al., 2015). The bioassay test battery included assays indicative of different stages of cellular toxicity pathways, including xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses as well as cytotoxicity, and has previously been applied to monitor wastewater and surface water extracts (Escher et al., 2014; König et al., 2017). The important xenobiotic metabolism receptors aryl hydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor gamma (PPARγ) were included in the current study because chemicals can stimulate transformation processes in cells to metabolise, detoxify or bioactivate chemicals (Omiecinski et al., 2011). Hormonal pathways are essential for growth, development and reproduction, with endocrine disrupting chemicals able to interfere with hormone action (Bergman et al., 2012). Hence, activation of the estrogen receptor (ER) and the glucocorticoid receptor (GR) by the wastewater extracts were also evaluated. Adaptive stress response pathways are activated in cells after damage by stressors, including chemicals (Simmons et al., 2009), with an assay indicative of the oxidative stress response included. In addition to the cell-based bioassays, an assay indicative of photosystem II (PSII) inhibition of green algae was included to detect the presence of PSII-inhibiting herbicides and other algal toxicants.

While many studies have estimated wastewater mass loads (e.g. Phung et al., 2017; Beckers et al., 2018) or per-capita mass loads (O’Malley et al., 2019; O’Malley et al., 2020), water quality assessment with \textit{in vitro} bioassays has typically relied on bioanalytical equivalent concentrations. An understanding of the per-capita effect load in wastewater influent and effluent is important as the population density of major Australian cities, as well as cities worldwide, has rapidly increased over the last few decades (Coffee et al., 2016). Since different chemicals may be used in consumer products and pharmaceuticals in different regions, the effect loads should allow for a better comparison between regions, as well as between different wastewater treatment processes.

Bioanalysis was complemented with a target screening analysis of 503 organic micropollutants, namely pharmaceutical and personal care products (PPCPs), industrial compounds and pesticides, on 7-day pooled extracts. The mixture effects quantified by the \textit{in vitro} bioassays were translated into effect loads by relating them to the per-capita flow in the considered WWTP. The removal efficacy of effect loads by the WWTPs was compared with chemical removal efficacy and iceberg modelling was used to determine the contribution of known chemicals to the biological equivalent load.

2. Materials and methods

2.1. Sample collection

Influent (I) and effluent (E) composite samples were collected from ten WWTPs in Australia for seven consecutive days around Census day 2016 (August 9, 2016). The studied WWTPs covered approximately 42% of the Australian population (over 9 Mio people). Further information about sample collection can be found in O’Brien et al. (2019). Two WWTPs (P1, P2) had only primary treatment, five WWTPs (S1, S2, S3, S4, S5) had secondary treatment and three WWTPs had a tertiary treatment step, either UV (T1), UV plus chlorination (T2) or ozonation plus UV plus chlorination (T3). Average influent and effluent per-capita flow data are provided in Table S1 in the Supplementary Material.

2.2. Sample enrichment

All samples were enriched using solid-phase extraction (SPE) with Oasis HLB cartridges (400 mg sorbent) (Waters, Rydalmere, Australia). Prior to SPE, the samples were filtered with 0.2 μm regenerated cellulose filters (Phenomenex, Lane Cove, Australia). The cartridges were conditioned with 7 mL methanol, 7 mL ethyl acetate and 7 mL ultrapure water, then between 19.0 and 23.6 mL of sample was enriched per cartridge (Table S2). Ultrapure water blanks and conditioning controls were also included (SPE blanks). After drying, the cartridges were eluted with 7 mL methanol and 7 mL ethyl acetate and the extracts were blown down to dryness and immediately resuspended in 500 μL of methanol. This gave sample enrichment factors ranging from 37.2 to 47.2. The methanolic extracts were then run in the cell-based bioassays (Section 2.4). For chemical analysis and the algae assay, the seven daily extracts were pooled to give a single influent and effluent extract for each WWTP. The algae assay required a larger volume of extract than the cell-based bioassays, so it was only possible to analyse the pooled extracts.

2.3. Chemical analysis

For chemical analysis, the pooled extracts were evaporated and then reconstituted with an appropriate volume (69–79 μL) of methanol to give an enrichment factor of 40. Twenty-five microlitres of the methanol extracts were then added to 1 mL ultrapure water to mimic the concentration of the original water sample (enrichment factor = 1). Twenty-five microlitres of an internal standard mix containing 40 isotope-labelled compounds at 40 ng/mL in methanol and 5 μL of a 2 M formate buffer pH 3.5 were added to the reconstituted water samples. Calibration standards were prepared at twelve levels ranging from 1 to 5000 ng/L by adding 25 μL of the respective analyte solution, 25 μL of internal standard mix and 5 μL of buffer into 1 mL of water from a pristine stream. Five hundred and three chemicals (Table S3) were analysed by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) with the method described in detail in Neale et al. (2020) and Section S1 of the Supplementary Material. The concentrations reported are referring to water concentrations after SPE, which facilitates a direct comparison with the bioanalysis data and is inherently consistent for quantification of treatment efficacy but does not account for SPE recovery, which will be affected by the SPE sorbent and the sample blow down steps. Oasis HLB typically has good recovery (>80%) of neutral chemicals with a log octanol-water partition coefficient (log \(K_{ow}\)) between 1 and 5, but is less suitable for very polar chemicals and charged chemicals (Koke et al., 2018; Osorio et al., 2018).

2.4. Bioanalysis

The extracts were analysed in five reporter gene bioassays (Table 1) that were indicative of xenobiotic metabolism (activation of AhR, binding to PPARγ), hormone receptor-mediated effects (activation of ERz and GR) and adaptive stress response pathways (oxidative stress response). All reporter gene assays were run in a
2.5. Data evaluation

All bioassay data were evaluated using linear concentration-effect curves. Inhibitory concentrations leading to a 10% reduction of cell viability (IC\textsubscript{10}) were deduced from the cell viability in all reporter gene cell lines. Only concentrations < IC\textsubscript{10} were used for further evaluation. The concentration causing 10% effect (EC\textsubscript{10}) was derived from linear concentration-effect curves up to 30% effect for assays indicative of xenobiotic metabolism, receptor-mediated effects and the combined algae test. The effect in the oxidative stress response assay was expressed as the effect concentration causing an induction ratio of 1.5 (EC\textsubscript{IR1.5}) using linear concentration-effect curves up to an induction ratio of 4. Further information about bioassay data evaluation can be found in Escher et al. (2018b). The EC values were expressed in units of REF, which takes into consideration sample enrichment by SPE and dilution in the assay. A REF of 10 means the sample needs to be enriched 10 times before 10% effect or an induction ratio of 1.5 is observed. The EC values were converted to bioanalytical equivalent concentrations for bioanalysis (BEQ\textsubscript{bio}) (Equation (1)). BEQ\textsubscript{bio} were expressed as benzo[a]pyrene equivalent concentrations (B[a]P-EQ) for AhR CALUX, rosiglitazone-EQ for PPAR\textgamma GeneBLAzer, 17β-estradiol equivalent concentrations (EEQ) for ER\textalpha and 17β-estradiol-EL (EEQ) for ARES32. In the combined algae test, we calculated diuron equivalent concentrations (DEQ\textsubscript{chem}) for diuron inhibition and 24h growth-inhibition-DEQ\textsubscript{chem} for overall toxicity (Glauch and Escher, 2020). The limit of detection (LOD) for each bioassay is provided in Table 1. The cytotoxicity was expressed as toxic units T\textsubscript{U}\textsubscript{bio} (Equation (2)) that was combined into one value averaged over all cell lines (n), because the IC\textsubscript{10} values were similar in the different cell lines (Escher et al., 2019).

\begin{equation}
\text{BEQ}_{\text{bio}} = \frac{\text{EC (ref)}}{\text{EC (sample)}}
\end{equation}

\begin{equation}
\text{TU}_{\text{bio}} = \frac{\sum_{i=1}^{n} \text{IC}_{10} \text{ (sample)}}{n}
\end{equation}

2.6. Bioanalytical equivalent load and mass load

As each WWTP differed in size, we normalised BEQ\textsubscript{bio} to bioanalytical equivalent load for bioanalysis (BEL\textsubscript{bio}, Equation (3)), the toxic units for cytotoxicity to toxic unit loads (T\textsubscript{U}\textsubscript{bio}) and chemical concentration (C\textsubscript{i}) in ng/l to mass load (ML, Equation (5)) to better compare between WWTPs. BEL\textsubscript{bio} and ML, both in

| Assay name | Endpoint | Method reference | Reference compound | EC value | Limit of detection (LOD) | Bioanalytical equivalent load BEL |
|------------|----------|------------------|-------------------|----------|------------------------|----------------------------------|
| AhR CALUX  | Activation of AhR | Brennan et al. (2015) | TCDD\textsuperscript{b} | EC\textsubscript{10} (5.95 ± 0.23) × 10\textsuperscript{-11} | 2.05 × 10\textsuperscript{-12} | Benzo[a]pyrene-EQ (B[a]P-EL) |
| PPAR\textgamma GeneBLAzer | Binding to PPAR\textgamma | König et al. (2017) | Rosiglitazone | EC\textsubscript{10} (4.29 ± 0.15) × 10\textsuperscript{-10} | 5.87 × 10\textsuperscript{-10} | Rosiglitazone-EL |
| ER\textalpha GeneBLAzer | Activation of ER\textalpha | König et al. (2017) | 17β-Estradiol | EC\textsubscript{10} (1.19 ± 0.03) × 10\textsuperscript{-9} | 5.96 × 10\textsuperscript{-12} | 17β-Estradiol-EL (EEQ) |
| GR GeneBLAzer | Activation of GR | König et al. (2017) | Dexamethasone | EC\textsubscript{10} (4.71 ± 0.16) × 10\textsuperscript{-9} | 9.42 × 10\textsuperscript{-11} | Dexamethasone-EL (DEX-EL) |
| ARE\textsubscript{32} | Oxidative stress response | Wang et al. (2006); Escher et al. (2012) | Butylhydroquinone (BHQ) | EC\textsubscript{IR1.5} (2.58 ± 0.03) × 10\textsuperscript{-8} | 5.46 × 10\textsuperscript{-7} | Dichlorvos-BEL |
| Combined algae test | 2h-PSII inhibition | Glauch and Escher (2020) | Diuron | EC\textsubscript{10} (7.70 × 10\textsuperscript{-6}) | 1.63 × 10\textsuperscript{-6} | 2h PSII inhibition-diuron equivalent load (DEQ\textsubscript{chem}) |
| | 24h-PSII inhibition | Glauch and Escher (2020) | Diuron | EC\textsubscript{10} (6.15 ± 0.27) × 10\textsuperscript{-9} | 2.50 × 10\textsuperscript{-9} | 24h PSII inhibition-DEQ\textsubscript{chem} |
| | 24h-growth inhibition | Glauch and Escher (2020) | Diuron | EC\textsubscript{10} (7.64 ± 0.79) × 10\textsuperscript{-8} | 3.75 × 10\textsuperscript{-8} | 24h growth inhibition-DEQ\textsubscript{chem} |

\textsuperscript{a} Reference compound for bioassay quality control.
\textsuperscript{b} Reference compound for BEL.
\textsuperscript{c} EC\textsubscript{10} from Neale et al. (2017a).
\textsuperscript{d} EC\textsubscript{IR1.5} from Escher et al. (2013).
units of ng/person/day, and TULbio, in units of Lbioassay/person/day, were calculated using per-capita flow in units of L/person/day based on the flow data acquired from each WWTP (Table S1) and the catchment populations as described in Tscharke et al. (2019).

\[
\text{BELbio} = \text{BEQbio} \times \text{per - capita flow} \quad (3)
\]

\[
\text{TULbio} = \text{TUbio} \times \text{per - capita flow} \quad (4)
\]

\[
\text{ML} = C_i \times \text{per - capita flow} \quad (5)
\]

BELbio and TULbio were used to calculate removal efficacy. BELbio removal efficacy was calculated using Equation (6) based on the BELbio values of the influent (BELbio, influent) and corresponding effluent sample (BELbio, effluent) and analogously for cytotoxicity TULbio (Equation (7)).

\[
\text{BELbio removal (\%)} = \frac{\text{BELbio, influent} - \text{BELbio, effluent}}{\text{BELbio, influent}} \quad (6)
\]

\[
\text{TULbio removal (\%)} = \frac{\text{TULbio, influent} - \text{TULbio, effluent}}{\text{TULbio, influent}} \quad (7)
\]

In the case of activation of AhR, the SPE blank samples induced a response in the assay, so BELbio was calculated using blank-corrected BEQbio values. Only samples that had a BEQbio value of 50% or higher than the BEQbio of the SPE blanks were used to calculate removal.

Removal efficacy was also calculated for the analysed chemicals in the pooled samples based on the ML in the influent (MLinfluent) and the ML in the effluent (MLeffluent) (Equation (8)). If a chemical was below the LOD in the effluent, but the same chemical was present in the influent, removal was calculated using half the LOD (O’Malley et al., 2020).

\[
\text{ML removal (\%)} = \frac{\text{MLinfluent} - \text{MLeffluent}}{\text{MLinfluent}} \quad (8)
\]

2.7. Iceberg modelling

The bioanalytical equivalent load from chemical analysis (BELchem) was calculated using the ML of each detected chemical in the pooled samples and its relative effect potency (REP) relative to the reference chemical (Equation (9)). REP, was calculated using the EC value of the assay reference compound and the EC value of the detected chemical, with EC values for the majority of detected chemicals published in Neale et al. (2020). All EC and REP values used for iceberg modelling are provided in Table S5.

\[
\text{BELchem} = \sum_{i=1}^{n} \frac{\text{ML}_i \times \text{REP}_i}{100} \quad (9)
\]

BELchem was only calculated for the bioassays indicative of xenobiotic metabolism, adaptive stress responses and the combined algae assay as the chemical analysis did not target chemicals active in ERα and GR. BELchem was compared to the corresponding average BELbio value for the influent and effluent of each WWTP. This is analogous to iceberg modelling using BEQbio and bioanalytical equivalent concentration from chemical analysis (BEQchem) (Neale et al., 2018). The contribution of each detected chemical i to BELchem was calculated using “tip of the iceberg” modelling based on Neale et al. (2020) (Equation (10)).

\[
\% \text{contribution of } i \text{ to BELchem} = \frac{\text{REP}_i \times \text{ML}_i}{\text{BELchem}} \times 100\% \quad (10)
\]

3. Results and discussion

3.1. Bioanalysis

Influent and effluent extracts collected over a 7-day period for each WWTP were run in the bioassays, with all EC values shown in Fig. S2 and Table S2. Example concentration-effect curves for plant S4 are provided in Fig. S1. The assays indicative of activation of ER and binding to PPARγ were the most responsive in the wastewater influent samples (Fig. S2). The ERα assay is highly sensitive to estrogeneric compounds, such as estrone, 17β-estradiol and 17α-ethinylestradiol, which are commonly present in wastewater influent and effluent (Murk et al., 2002; Leusch et al., 2014; Hamilton et al., 2016). Further, many pharmaceuticals, such as bezafibrate, diclofenac, indomethacin and telmisartan, are active in PPARγ GeneBLazer (Neale et al., 2017a, 2020). Oxidative stress response was the least responsive assay (with highest EC values), while the response in activation of GR was often masked by cytotoxicity in the influent samples due to the much higher burden of non-specifically acting compounds. In any case, most samples induced a response at a REF less than 1, indicating a high effect burden in the wastewater influent extracts. The variation in EC values between WWTPs and over the 7 days was fairly minimal, with no clear trends observed (Fig. S2). The same was true for cytotoxicity with IC10 values (Fig. S3). Therefore, the daily extracts could be combined prior to testing in the algae assay. The algae assay was the only assay run in a 96-well plate format and therefore had a higher volume requirement, so it was only possible to analyse the pooled extracts.

Wastewater treatment resulted in reduction of effect for most WWTPs, with similar EC values in all effluents for most assays. The exceptions were WWTPs P1 and P2, which both applied primary treatment only, where high estrogeneric and PPARγ activity remained after treatment. Excluding these WWTPs, the EC values in effluent extracts were typically greater than REF 1, indicating the samples needed to be enriched to detect an effect. The EC values in effluent were in a similar range as previously observed in the literature (e.g., Escher et al., 2014; Volker et al., 2017; Nivala et al., 2018). It should be noted that effluents from Australian WWTPs are discharged into a mixing zone specified in their operating licence (ARMCANZ & ANZECC, 2007) and thus the effects are expected to be even lower outside the mixing zone due to dilution. The effect outside of the mixing zone was not assessed in the current study, but could be compared with available effect-based trigger values to determine if the effect in the receiving environment was acceptable or not (Escher et al., 2018a).

In addition to the WWTP extracts, SPE blanks were run in all bioassays. These SPE blanks did not induce a response in the assays up to the highest tested REFs, apart from activation of AhR, where the SPE blanks had an EC10 of REF 1.7 to 1.8 (Table S2). The cause of the contamination is unknown but may be related to the solvents used for conditioning or elution. In any case, any wastewater effluent extracts that had the same effect as the SPE blanks were excluded from further analysis.

All EC values were converted via BEQbio and TUbio to BELbio and TULbio to compare effect and cytotoxicity loads between the WWTPs and to calculate removal efficacy (Table S6). Seven-day averages were calculated to simplify the figures and for the following discussions because there was little variation in effects and cytotoxicity during the 7 days (Figs. S2 and S3). The variation in BELbio
between WWTPs was much smaller than BEQbio as it was normalised based on the average per-capita flow. This is demonstrated in Fig. 1A, where there was less variability between the 17β-estradiol equivalent load (EELbio) in the influent samples for all plants compared to EEQbio, EELbio in the influent (Fig. 1B). Figure were also compared with the expected excretion of estrogenic compounds. The average excretion of estrone, 17β-estradiol and 17α-ethinylestradiol in units of µg/person/day from Laureson et al. (2014) was converted to the 17β-estradiol equivalent load for chemical analysis (EELchem) using REP values from Konemann et al. (2018). This gave an EELchem of 9.87 µgE2/person/day (9870 ngE2/person/day), which is in a similar range as the influent EELbio values in the current study (Fig. 1B).

Consequently, the BELbio was calculated for all bioassays and averaged over the 7 days (Fig. 1C) but the BELbio for each day separately can be seen in Table S6. As can be seen in Fig. 1C, there was little variation in the average influent BELbio values for the 10 WWTPs with coefficients of variation ranging from 17 to 65% for the different bioassays and there was no evident relationship between influent quality and treatment type. This means the influent quality per-capita was very similar across Australia and therefore the different plants can be directly compared.

### 3.2. Effect and chemical removal

The efficacy of effect removal in each WWTP based on BELbio is summarised in Fig. 2 averaging over the 7 days, with removal for each WWTP for each day provided in Fig. S4 and Table S7. Cytotoxicity TULbio were averaged between all cell lines. The average removal efficacy for plants with secondary or tertiary treatment (WWTPs S1, S2, S3, S4, S5, T1, T2, T3) ranged from 39% for AhR activity and 41% for 2h PSII inhibition to 93% for estrogenic activity and PPARγ activity and cytotoxicity TULbio were reduced by 42%–84%. Removal was much lower in WWTPs P1 and P2, which had only primary treatment, with on average 16% removal of AhR activity and 11% removal of estrogenic activity. Herbicides detected by algal toxicity had only 18% removal in WWTP P1 and were not removed in WWTP P2. For PPARγ activity and oxidative stress response the removal was negative with -5% and -11%, respectively, and cytotoxicity TULbio also increased slightly from influent to effluent (Fig. 2). Apparent negative removal may stem from fluctuations in input because samples were taken at the same time and do not represent the same water parcel. Given there were hardly any variations in effect during the 7 days in the influent, these should be minor fluctuations. Certain chemicals may also form during treatment if they are excreted as conjugates that are not or less active in the in vitro assays and cleaved back to the bioactive parent chemical in the effluent. This has been hypothesised for the pharmaceutical carbamazepine and its glucuron conjugate (Vieno et al., 2007).

Variable removal of AhR activity during wastewater treatment has been reported in the literature, with 13–90% removal observed (Jalova et al., 2013; Nivala et al., 2018). Similar PPARγ removal efficacy has also been observed previously, with between 69 and >94% removal reported (Bain et al., 2014; Nivala et al., 2018). Up to 99.7% removal of estrogenic activity was observed, which fits with previous studies where between 80 and >99% removal was reported (e.g. Jugan et al., 2009; Jalova et al., 2013; Escher et al., 2014; Hamilton et al., 2016; Houtman et al., 2018; Nivala et al., 2018). Removal of GR activity could only be calculated for WWTP T1, with most samples either cytotoxic or inactive in GR GeneBlazer. Between 67 and 69% removal of GR activity was observed. Poor to moderate removal of GR activity has previously been reported, with between -7 and 66% removal efficacy observed (Bain et al., 2014; Roberts et al., 2015; Houtman et al., 2018). Oxidative stress response removal varied between WWTPs and over time, with between 10 and 90% removal efficacy observed (average of 60%) in all plants except P1 and P2. Fewer studies have considered oxidative stress response removal during wastewater treatment processes, though between 61 and 85% removal has previously been reported (Volk et al., 2017; Nivala et al., 2018). Algal toxicity is driven by herbicides, which are typically poorly removed by secondary treatment as was observed previously with 48% removal of 2h PSII inhibition-DEQ and 38% removal of 24h PSII inhibition-DEQ by secondary treatment (Escher et al., 2008). Ozoneation also improved treatment efficacy to over 80% (Escher et al., 2009), which is consistent with our findings for T3. Cytotoxicity was not removed after primary treatment, though it was often well removed after secondary and tertiary treatment, which aligns well with the removal of chemicals. Stalter et al. (2011) also found cytotoxicity was well removed by secondary and tertiary wastewater treatment using a rat pituitary cell line assay.

For comparison with effect load the chemical concentrations reported in Table S5 were converted to mass loads ML (Table S8). The sum of ML in the influent was very similar for all WWTPs and ranged from 0.13 to 0.34 g/person/day, which was consistent with the little variation of effect loads of all influents. A detailed analysis of the chemical removal is beyond the scope of the present work and will be forthcoming in a study by other authors. The removal efficacy of all chemicals detected in the WWTP influent are provided in Table S8 and summarised in Fig. 2 with each grey dot representing one chemical. Removal efficacy varied between WWTPs and for different chemicals but aligned well with the BELbio and TULbio removal efficacy results (Fig. 2) although the differences
between individual chemicals were evidently much larger than between different bioassays.

3.3. Iceberg modelling

Iceberg modelling serves to compare the measured effect of the wastewater sample with the predicted mixture effect of the detected chemicals and was only performed for AhR, PPARγ, oxidative stress response and the combined algae assay. Since no natural and pharmaceutical hormones were included in the chemical analysis, iceberg modelling was not meaningful for ER and GR. In these assays, a small number of highly potent chemicals typically explains most of the effect (e.g. Leusch et al., 2010; Schriks et al., 2010; Jia et al., 2016; Konemann et al., 2018), which means that it is sufficient to perform bioanalysis to evaluate the removal efficacy for estrogrenic chemicals. In addition, no iceberg modelling was performed for cytotoxicity.

An average BELbio value was calculated for the influent and effluent samples for iceberg modelling (Table S9) to compare with BELchem derived from the chemical analysis of the pooled extracts. BELchem was calculated using effect data for reporter gene assays provided in Neale et al. (2020). An additional 15 active chemicals contributed to BELchem for the three assays in the current study, with effect data collected from the Tox21 database or the literature (Neale et al., 2017a). The effect data for 13 herbicides and 12 non-herbicides in the combined algae test were from Glauch and Escher (2020). The average percent contribution of known chemicals to BELchem for each assay is shown in Fig. S5. In each bioassay there were a few dominant chemicals, but they differed from assay to assay. The pharmaceutical telmisartan and the herbicide diuron dominated the B[a]P-ELchem and telmisartan was also the main contributor to rosiglitazone-ELchem. In the case of the oxidative stress response phytoestrogen daidzein dominated in influent while industrial compound 2-benzothiazolesulfonic acid dominated in effluent. The DEQchem in the combined algae assay was dominated by diuron. This analysis of chemicals contributing to BELchem is equivalent to the widely used toxic unit approach for chemical analysis, where literature ecotoxicity data is used to scale and sum the detected concentrations (Massei et al., 2018; Kandie et al., 2020). Here we can additionally compare the BELchem with the BELbio to estimate which fraction of the overall effect can be explained by the detected chemicals.

Thirty-four chemicals contributed to B[a]P-ELchem for activation of AhR, with industrial compound 2,4-dichlorophenol having the overall highest contribution to B[a]P-ELchem but only in WWTP T2 (1.1 μgB[a]p/person/day). Telmisartan and diuron were the dominant contributors to B[a]P-ELchem and because they were hardly removed by secondary treatment, there was no change in their B[a]P-ELchem during treatment except for WWTP T3 which had additional ozonation (Fig. 3). The same applied to the antifungal climazol and 2-benzothiazole sulfonic acid. In contrast, the phytoestrogens daidzein and genistein, which contributed substantially to B[a]P-ELchem in the influent, were well degraded and hardly detectable in the effluents, apart from WWTPs P1 and P2 which lacked secondary treatment.

Comparing B[a]P-ELchem to the corresponding average B[a]P-ELbio value, only up to 0.56% of the activation of AhR load could be explained by the 34 detected active chemicals, with a similar fraction explained for both influent and effluent samples (Table S9). For comparison, with 40 active chemicals, up to 2.8% of the activation of AhR response could be explained by known chemicals in surface water samples from Germany (Neale et al., 2020). We had effect data for 72% of the 503 targeted chemicals but the majority was inactive, with only 69 (19%) having EC50 values. Given that there are likely to be tens of thousands of chemicals present in a water sample and even if only 19% activate AhR, it is conceivable that the detected chemicals explain only a low fraction of the effect. The detected AhR-active chemicals had REP values from 7.10^-7 to 6.10^-4, indicating that the targeted chemicals were of very low potency, hence chemicals such as polycyclic aromatic hydrocarbons, which were not analysed and would be expected to be present in very low concentrations, but which have very high REP values, could have contributed substantially to the mixture effects.

Only 19 of the detected chemicals contributed to rosiglitazone-ELchem in the PPARγ assay, but they had much higher REP values than the detected chemicals in AhR. Telmisartan had the highest REP of 0.003 and was a major contributor to rosiglitazone-ELchem in most WWTPs, with little difference in rosiglitazone-ELchem between the influent and effluent for most WWTPs due to poor removal of telmisartan (Fig. S6). The herbicide 2,4-dichlorophenoyxacetic acid had the highest contribution to rosiglitazone-ELchem but only in the influent of WWTP T2 because of its very high concentration despite a low REP, value of 6.10^-4. Between 0.16 and 9.3% of BELbio could be explained by detected chemicals in PPARγ GeneBLAzer (Table S9). A much larger fraction of the effect could be explained in the effluent (maximum 9.3%, average 5.0%) compared to the influent samples (maximum 0.71%, average 0.39%). This suggests that unknown chemicals that activate PPARγ were better removed than known activators of PPARγ but it is also possible that individual contributors fell below the analytical limit of detection.
The detection limit in the effluent but still contributed to mixture effects. The fraction explained by known chemicals was higher than observed in previous studies (e.g. König et al., 2017; Neale et al., 2018) and can be explained by the high load of the potent PPARγ activator telmisartan, which was present at 384–1533 µg/person/day in all samples except for WWTP T3 effluent. Telmisartan is used to treat high blood pressure and was among the top 10 most commonly prescribed pharmaceuticals based on daily dose in Australia in 2015 (Pharmaceutical Benefits Advisory Committee, 2016).

Forty-six of the detected chemicals contributed to dichlorvos-ELchem in the oxidative stress assay, with daidzein (REP 0.41) having the greatest contribution in the influent samples (Fig. S7). Chemicals such as daidzein, genistein (REP 0.49) and the alkaloid piperine (REP 0.39) primarily contributed to dichlorvos-ELchem in the influent samples, but had low to no input to dichlorvos-ELchem in the effluent samples due to their good removal efficacy (>98%) except for in WWTPs P1 and P2 (between -26 and 39% removal for daidzein, genistein and piperine in P1 and P2). In contrast, the dichlorvos-ELchem was often similar in the influent and effluent for poorly removed compounds such as 2-benzothiazolesulfonic acid, diuron and the pharmaceutical mebendazole. Dichlorvos-ELchem could only explain between 0.0004% and 0.20% of dichlorvos-ELbio with little difference between the influent and effluent samples (Table S9). According to the literature (Martin et al., 2010), a large fraction of chemicals can activate the oxidative stress response. Three hundred and fifty-seven of the targeted chemicals had experimental data and 24% thereof were active. As for AhR, it is likely that many chemicals present but not targeted may cause the large observed oxidative stress response. The low fraction explained is consistent with other studies also finding that known chemicals could typically explain less than 1% of the observed effect in water samples (Escher et al., 2013; Tang et al., 2014; Neale et al., 2015).

In the case of algal toxicity, there were fewer herbicides detected than in European WWTP effluents (Glauch and Escher, 2020) but the effects were still dominated by herbicides with only a minor contribution by biocide triclosan, which is well removed by secondary and tertiary treatment and has a lower REP than most herbicides, i.e., REP_triclosan was 0.002–0.004 for inhibition of photosynthesis after 2h and 24h, respectively (Glauch and Escher, 2020). By a large margin, diuron dominated the effect and explained between 70 and 100% of the DELchem for 2h PSII inhibition (Fig. 4) and 24h PSII inhibition (Fig. S8) and somewhat less for 24h growth inhibition (Fig. S9). The other herbicides made up the rest of the effect, apart from 24h growth inhibition where triclosan contributed up to 40% of DELchem due to the REP_triclosan of 0.13 for this endpoint. The DELchem explained on average 48% of the DELbio for 2h PSII inhibition (Table S9) and 58% for 24h PSII inhibition but less than 10% for 24h growth inhibition, where many other chemicals can contribute to the effect and the herbicides are of lower relative potency. This is consistent with previous work on advanced water treatment (Tang and Escher, 2014), where in WWTP effluent between 47 and 73% of algal toxicity could be explained by the combined action of atrazine, diuron, hexazinone and simazine.

### 3.4. Implications

The study presented here is unique because it covered WWTPs at major cities, capturing 42% and more than 9 Mio of the Australian population of 23,401,892 people at Census day 2016. Thanks to sampling during a population Census, a very precise population estimate was possible and hence the measured concentrations could be converted to per-capita mass and bioanalytical equivalent loads in units of ng/person/day. This was an excellent basis for direct comparison between treatment efficacy of different plants with respect to mixture effects. However, even if no flow and population data are available, bioanalytical equivalent concentrations are still very useful to compare plants and to evaluate if the overall effects in the effluent are safe for the receiving waters after dilution (Jarosova et al., 2014). Further, after applying a dilution factor appropriate to the circumstances of a given WWTP, the expected contribution of effect to surface water could be predicted and the resulting BEQ in surface water compared with existing
surface water effect-based trigger values (Escher et al., 2018a). Effluents from Australian WWTPs are discharged into a mixing zone, but effects both within and outside the mixing zone were not evaluated in the current study.

The removal of mixture effects was clearly related to the treatment type, with poorest removal for primary treatment. It is interesting to note that both cytotoxicity and oxidative stress response typically had the lowest removal efficacies, even after ozonation. Oxidative stress response is a rather general adaptive stress response often occurring just prior to cytotoxicity. Biodegradation and ozonation will not necessarily mineralise chemicals but transform them with the transformation products often being more hydrophilic and hence less cytotoxic but still contributing to the mixture effects (Escher and Fenner, 2011). Therefore, these fairly non-specific bioassays are important to estimate the overall toxic potency.

“Tip of the iceberg” modelling showed that the detected chemicals contributing to BELchem were often abundant chemicals with moderate to high potency, such as telmisartan, which had an average total daily load of 18 kg. Together with its high potency in PPARγ, it was the dominant risk driver for this endpoint and also important for AhR. Diuron, despite a total load of less than 1 kg per day, dominated the algal toxicity due to its very high potency and contributed substantially to AhR activation. 2-Benzothiazolesulfonic acid (11 kg per day) and daidzein (11 kg per day) were both drivers for activating the oxidative stress response. The mixture risk drivers changed after treatment with recalcitrant chemicals playing a more important role. Telmisartan and diuron were poorly removed in most WWTPs, so they remained important before and after treatment.

4. Conclusions

The current study aimed to determine per-capita biological equivalent loads in influent and effluent samples collected during the 2016 Australian Census and to evaluate effect removal efficacy using a bioassay test battery covering different stages of cellular toxicity pathways, as well as algal toxicity. While bioanalytical equivalent concentrations were rather variable between the WWTP influents, translating into bioanalytical equivalent loads reduced the variability. Effect removal efficacy was poorest for primary treatment, with good removal of estrogenic activity and PPARγ activity by secondary and tertiary treatment. However, even secondary treatment did not completely reduce algal toxicity, which can be explained by herbicides being rather persistent to biodegradation and only well removed by ozonation. With the exception of algal toxicity, the detected chemicals could only explain a small fraction (<10%) of the observed effect. The study demonstrates how both bioanalytical equivalent loads and mass loads can be applied in a complementary way to provide a better comparison of removal efficacy between WWTPs.

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.

Acknowledgements

The authors thank Jenny John and Rita Schlichting (UFZ) for eluting the SPE cartridges and contributing to the bioassays. We would also like to thank the QAEHS staff members who assisted in developing and conducting the wastewater sampling campaign, as well as the various wastewater treatment plant operators, including Hunter Water, Logan City Council, Melbourne Water, Sydney Water and Urban Utilities, who provided both samples and associated data. The sample collection was supported by an Australian Research Council Linkage Project (LP150100364). We gratefully acknowledge access to the platform CITEPro (Chemicals in the Terrestrial Environment Profiler) funded by the Helmholtz Association.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wroa.2020.100072.

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