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Investigating (anti)estrogenic activities within South African wastewater and receiving surface waters: Implication for reliable monitoring☆

Edward Archer, PhD [a], Gideon M. Wolfaardt, PhD [a, b, *], Johannes H. van Wyk, PhD [c], Nico van Blerk, MSc [d]

[a] Department of Microbiology, University of Stellenbosch, Stellenbosch, 7602, South Africa
[b] Department of Chemistry and Biology, Ryerson University, Toronto, ON, M5B 2K3, Canada
[c] Department of Botany and Zoology, University of Stellenbosch, Stellenbosch, 7602, South Africa
[d] Scientific Services, East Rand Water Care Company (ERWAT), Kempton Park, 1631, South Africa

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ABSTRACT

Natural and synthetic steroid hormones and many persistent organic pollutants are of concern for their endocrine-disrupting activities observed in receiving surface waters. Apart from the demonstrated presence of estrogen- and estrogen-mimicking compounds in surface waters, antagonistic (anti-estrogenic) responses originating from wastewater effluent have been reported but are less known. Estrogenicity and anti-estrogenicity were assessed using recombinant yeast estrogen receptor binding assays (YES/YAES) at ten South African wastewater treatment works (WWTWs) and receiving rivers in two separate sampling campaigns during the summer- and winter periods in the area. Four WWTWs were then further investigated to show daily variation in estrogenic endocrine-disrupting activities during the treatment process. Although estrogenicity was notably reduced at most of the WWTWs, some treated effluent and river water samples were shown to be above effect-based trigger values posing an endocrine-disrupting risk for aquatic life and potential health risks for humans. Furthermore, estrogenicity recorded in samples collected upstream from some WWTW discharge points also exceeded some calculated risk trigger values, which highlights the impact of alternative pollution sources contributing towards endocrine disrupting contaminants (EDCs) in the environment. The YAES further showed variable anti-estrogenic activities in treated wastewater. The current study highlights a variety of factors that may affect bioassay outcomes and conclusions drawn from the results for risk decision-making. For example, mismatches were found between estrogenic and anti-estrogenic activity, which suggests a potential masking effect in WWTW effluents and highlights the complexity of environmental samples containing chemical mixtures having variable endocrine-disrupting modes of action. Although the recombinant yeast assay is not without its limitations to show endocrine-disrupting modulation in test water systems, it serves as a cost-effective tier-1 scoping assay for further risk characterisation and intervention.

1. Introduction

There is growing evidence that wastewater treatment works (WWTWs) have varying success in removing organic micro-pollutants from the major wastewater types (domestic, industrial and agricultural) that consequently lead to the contamination of surface waters. The causes of such incomplete removal include a variety of biotic- and abiotic factors, such as the physiochemical properties of the pollutants, climatic conditions, the type of treatment technologies, their treatment capacities and source of wastewater, as well as the level of WWTW maintenance and management thereof. These factors lead to the large variety in removal efficiencies of organic pollutants by WWTWs that have been reported by studies around the world (Petrie et al., 2014;

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* Corresponding author. Department of Microbiology, University of Stellenbosch, Stellenbosch 7602, South Africa.
E-mail addresses: earcher@sun.ac.za (E. Archer), gmw@sun.ac.za (G.M. Wolfaardt), jhvw@sun.ac.za (J.H. van Wyk).

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Kanaujiya et al., 2019). Along with the continuous strive to improve water treatment technologies, there should be a concurrent development of analytical techniques with increased sensitivity (coupled with expanding databases) to offer new avenues to assess the efficiency of WWTWs to mitigate harmful discharge of emerging contaminants into environmental waters.

Apart from the potential lethal toxicity risk that organic pollutants may pose upon wildlife and human health, it has become evident that several micro-pollutant are also able to modulate physiological pathways such as endocrine system pathways in one way or another at concentrations regularly detected in surface waters (Archer et al., 2017b). Compounds that interfere with gonadal endocrine system pathways have been the major point of interest (Jia et al., 2019; Sabir et al., 2019) where the presence of such endocrine-disrupting contaminants (EDCs) in surface waters have been proposed to contribute towards various non-communicable diseases and developmental/reproductive disorders (Soto and Sonnenschein, 2010; Zarean and Poursafa, 2019). Due to the fact that the endocrine system is fairly conserved among vertebrates (including humans), it has been proposed that similar long-term effects might be observed in humans sharing the same environment. However, it remains difficult to extrapolate the possible effect that many untested environmental pollutants may have on wildlife and humans, as these compounds do not impose an immediate toxic outcome. It appears possible that a variation of inorganic- and organic compounds (including their breakdown products) in water bodies may yield several mixture interactions that are not yet fully understood. For this reason, effect-based biomonitoring methods such as *in vitro* bioassays and *in vivo* bio-indicator organisms offer a valuable approach to assess the net endocrine-modulation in test matrices or to show functionality of chemical stressors. However, disadvantages associated with *in vivo* experimentation, such as ethics, cost and time warrants the consideration of using high-throughput screening (HTS) bioassays to show potential risk triggers in environmental matrices (Villeneuve et al., 2019).

This study was conducted in South Africa, a semi-arid country facing rapid urbanisation and population growth that outpace infrastructure development (Oranje et al., 2020), thus offering a suitable proxy for other developing countries facing similar challenges. A consequence of such population dynamics is increased risk of surface water pollution due to insufficient sanitation services, along with a higher demand for treated water, food and health services – all being negatively impacted by pollution. For instance, the higher demand for food production leads towards an increased pesticide- and pharmaceutical use on crops and livestock respectively, while the unhealthy living conditions in the rapidly growing informal or low-cost housing schemes may lead to an increased reliance on pharmaceuticals. Moreover, rapid population growth and urbanisation also directly leads towards an increased presence of natural and/or synthetic steroid hormones within sewage. These factors, combined with the demonstrated recalcitrance of various organic- and inorganic micro-pollutants that are considered as EDCs place severe strain on WWTWs to lower the pollutant burden 41fq its discharge, especially in countries without enough financial resources to upgrade or expand existing systems to meet rising urban development and population growth.

The aim of this study was to use a recombinant yeast receptor binding assay to assess the load and persistence of pollutants in sewage that show agonistic- and/or antagonistic binding affinity to the human estrogen receptor alpha (hER-α). The seasonal and daily variation of estrogenicity within WWTW influent and effluent, as well as the associated river waters located upstream and downstream of the WWTWs were compared and considered in the context of potential adverse health risks associated with the measured estrogenic concentrations. The influence of compounds which may antagonise estrogen-mediated receptor binding (anti-estrogens) in the bioassay was also investigated to further elaborate on the potential endocrine disrupting risk which treated wastewater may pose on surface waters spanning over multiple modes of actions.

2. Materials and methods

2.1. Study site and sampling procedure

The study was done at ten WWTWs located in the City of Ekurhuleni Metropolitan area (Gauteng Province, South Africa). These WWTWs are shown to be compliant with the South African Department of Water and Sanitation (DWS) Green Drop certification scheme that assesses the chemical-, microbial- and physical compliance of the country's WWTWs (http://dwa.gov.za). Although the DWS Green Drop programme is considered outdated in the country, the company that manages the WWTWs still use the DWS-Green Drop compliance criteria as internal quality performance guidelines for all their treatment facilities. The main types of primary and secondary treatment processes are shown in Table 1, where all the WWTWs use chlorine disinfection before discharge.

The study area experiences its highest rainfall during the summer period (December–February), with a colder but drier climate during winter (June–August). The ten WWTWs varied in their operating capacities, treatment processes and loading of the receiving wastewater (Table 1). All the WWTWs were sampled during a summer (2015/16) and winter (2016) period for five consecutive days (Monday to Friday). The use of flow- and/or volume-proportional composite sampling at the WWTWs was not possible, as all the treatment facilities did not have the required apparatus for this and thus, grab sampling was deemed appropriate

Table 1
Information of the sample sites during the current study.

	Load ratio (Domestic:Industrial)	Capacity (ML/day)	Treatment Type
WWTW-1	70:30	105.0	PST, BF + AS (BNR)
WWTW-2	60:40	50.0	PST, AS (BNR)
WWTW-3	100:0	1.0	PST, AS (BNR)
WWTW-4	100:0	19.0	AS (BNR)
WWTW-5	80:20	35.0	PST, AS (BNR)
WWTW-6	70:30	15.0	BF
WWTW-7	83:17	36.0	BF (2-stage)
WWTW-8	96:4	83.0	BF (x 3), AS (BNR, x 1), MP
WWTW-9	100:0	10.8	PST, AS (BNR)
WWTW-10	96:4	170.0	PST, AS (BNR)

Abbreviations: *BF* = biological filtration, *AS* = activated sludge, *BNR* = biological nutrient removal, *PST* = primary settling tank, *MP* = maturation pond.

to avoid sampling bias. Grab samples (200 mL) were collected daily from both the influent (after screening and de-gritting) and effluent (after chlorination). The samples were transported on ice to the laboratory, acidified to pH 3 using hydrochloric acid (HCl) and stored at 4 °C until the final day of sampling was completed. The final 5-day grab samples were then pooled together to obtain a semi-composite sample of each WWTW influent and effluent location for the study periods. For both the summer 2015/16 and winter 2016 campaigns, grab samples of river water (200 mL) were collected on a single day of the week at locations upstream- and downstream from the respective points of WWTW discharge (between 50 m and 100 m), except for the downstream sample of WWTW-2 that was collected approximately 5 km from the treated effluent discharge due to logistical reasons. The collected river water samples were acidified to pH 3 using HCl and kept on ice during transportation and further processed on the same day of sampling. Four WWTWs were selected for further monitoring during another summer sampling period (December 2016/17) over seven consecutive days (Monday to Sunday). Grab samples (200 mL) of both influent- and effluent wastewater were collected each morning, followed by immediate sample processing, giving a 7-day grab sample for each of the four WWTWs. No rainfall was recorded at any of the study sites during the summer 2015/16 and winter 2016 sampling campaigns, whereas high rainfall was recorded for the whole study region during the summer sampling period of 2016/17.

2.2. Extraction procedure

Water samples were extracted using solid-phase extraction (SPE) and Oasis HLB cartridges (6 cc, 200 mg; Waters Corporation, Milford, MA, USA). The cartridges were conditioned with 4 mL methanol, followed by 4 mL of ultrapure water and allowed to pass through the column under gravity. After conditioning, the water samples (200 mL) were passed through using a vacuum manifold (Supelco Visiprep™, Sigma-Aldrich) at a flow rate of 5 mL/min and allowed to run dry for a minimum period of 30 min. The dried SPE cartridges were eluded with 6 mL MeOH (HPLC-grade; Sigma-Aldrich, USA) under gravity and then dried under a gentle stream of nitrogen. The evaporated samples were then re-suspended in 400 μL MeOH, giving a 500x concentrated water sample. All samples were stored at −20 °C in 2 mL amber glass vials until the bioassays were performed.

2.3. Yeast estrogen screen (YES)

The yeast-based screen followed the protocol described by Sohoni and Sumpter (1998). Briefly, *Saccharomyces cerevisiae* that were stably transfected with the human estrogen receptor alpha (hER-α) gene and a plasmid containing an estrogen response element-linked *lac*-Z gene was used. Successful binding of ligands (steroids and other EDCs) to the hER-α in the yeast activates the expression of the *lac*-Z reporter gene that encodes for the enzyme β−galactosidase, which then metabolises chlorophenol red galactopyranoside (CPRG) in the assay medium from a yellow to a red colour in a dose-dependent manner.

The assay medium was prepared as described by Sohoni and Sumpter (1998). The yeast was incubated in assay medium (without CPRG) for 48 h at 26 °C on an orbital shaker. The concentrated water extracts (500x) were serially diluted and 10 μL was spiked into sterile 96-well flat-bottomed plates with low-evaporation lids (Costar, 3370, Sigma-Aldrich, USA) and the MeOH allowed to evaporate completely. The incubated yeast culture was then included into new assay medium containing CPRG at a concentration of 8×10^5 cells/mL, whereby 200 μL of the seeded assay medium was then added to each well in the assay plate. For the raw wastewater (influent) extracts, the serial dilutions and addition of the seeded assay medium resulted in the samples being 12.5-times to 0.39-times concentrated in the assay. For the treated effluent and river water samples, serial dilutions of the sample extracts resulted in the samples being 50-times to 6.25-times concentrated in the assay. The reason for the different sample extract concentrations were due to high levels of cytotoxicity of the 50-times and 25-times concentrated raw wastewater samples in the assay and were thus excluded for response calculations. All samples were analysed in triplicate in the same assay plate, and each assay was repeated twice. A standard curve for the steroid hormone 17β-estradiol (E_2; CAS 50-28-2; Sigma-Aldrich, USA) was included for each assay plate in 12 serial dilutions, ranging from 1.0 to 2700.0 ng/L. Blank wells were also included in each assay plate containing only assay medium without any hormone spike or water sample extracts. The assay plates were incubated on a shaker for 72 h at 30 °C in the dark.

2.4. Yeast anti-estrogen screen (YAES)

The YAES was performed for the treated wastewater effluent samples during the winter 2016 and summer 2016/17 sampling campaigns in the same manner as described for the YES, with minor modifications. Each well in the 96-well assay plate was first spiked with a submaximal E_2 concentration of 450 ng/L prior to addition of the serially-diluted concentrated water extracts. Apart from an E_2 standard curve as used in the YES, the YAES contained a positive control of the estrogen receptor antagonist tamoxifen (TAM; CAS 10540-29-1; Sigma-Aldrich, USA) in 12 serial dilutions (0.9−1860.0 mg/L in the assay). The blank wells were separated into two sets; one set containing assay medium and yeast with a submaximal E_2 spike (6 wells) and one set containing blank wells with only the assay medium and yeast (6 wells). The assay plates were then allowed to incubate on a shaker for 72 h at 30 °C in the dark.

2.5. Calculations

Upon the 72 h of incubation, the YES and YEAS assay plates were measured for colour change using a microplate absorbance reader (iMark™, Bio-Rad). The absorbance was measured at 570 nm for colour change of CPRG caused by steroid hormone-mediated β−galactosidase production, and 620 nm for turbidity change and cytotoxicity. The threshold for cytotoxicity in the samples were determined using equation (1):

$$\text{Cytotoxicity} = \text{Median Blank}_{620\text{nm}} - (3 \times \text{stdev Blank}_{620\text{nm}}) \quad (1)$$

To correct for turbidity in the wells, a corrected absorbance (CA) was calculated for each sample in the assay using equation (2):

$$\text{Corrected absorbance (CA)} = (\text{OD}_{570\text{nm}} - [\text{OD}_{620\text{nm}} - \text{Blank}_{620\text{nm}}]) \quad (2)$$

where OD_{570nm} and OD_{620nm} refers to the optical density of the sample measured at 570 nm and 620 nm respectively, and $Blank_{620nm}$ refers to the median optical density measured for the blank wells in each assay plate at 620 nm. Water samples were only considered for further analysis if the corrected absorbance was above a detection threshold using equation (3):

$$\text{Detection} = \text{Median Blank}_{\text{CA}} + (3 \times \text{stdev Blank}_{\text{CA}}) \quad (3)$$

For the YES, the CA of water samples above the detection threshold of the assay were then log-transformed and expressed as a percentage of the maximum log-absorbance value calculated in

the E_2 standard curve using equation (4):

$$\text{Log \% max } E_2(\text{sample/standard curve}) = (\log - CA_{\text{sample}}/\log - CA_{E2\ \max}) \times 100 \quad (4)$$

A non-linear calibration curve was constructed for the E_2 standard curve of each individual assay plate by plotting the calculated log % max E_2 of the E_2 dilution series against its known concentration (in ng/L). An E_2-equivalent concentration (EEQ; ng/L) for each water sample was calculated from the generated trend-line of the calibration curve and corrected for their dilution factors to obtain a final EEQ concentration of each water sample (in ng/L). To compensate for the variation in daily flow rates within- and between the WWTWs that were used during the study, the calculated EEQ values were then used to estimate EEQ mass loads (g/day) in the raw and treated effluent wastewater samples using equation (5).

$$\text{Mass load (g/day)} = EEQ_{\text{inf/eff}} \times FR_{\text{inf/eff}} \times 1/1000 \quad (5)$$

where $EEQ_{inf/eff}$ refers to the EEQ concentration (in ng/L) of the samples in the YES for influent and effluent wastewater samples, and $FR_{inf/eff}$ refers to the flow rate (ML/day) of the influent and effluent wastewater for each WWTW during the period of the sampling campaigns. To estimate the reduction of estrogenic responses at the WWTWs, an EEQ mass load reduction estimate (%) from each raw wastewater sample and the corresponding treated effluent sample was calculated using equation (6):

$$\text{EEQ mass load reduction (\%)} = (ML_{\text{inf}} - ML_{\text{eff}}) \Big/ ML_{\text{inf}} \times 100 \quad (6)$$

where $ML_{inf/eff}$ refers to the mass loads (in g/day) of the samples calculated from EQ. (5) for influent and effluent wastewater samples at the various WWTWs.

For the YAES, the CA of the water extracts were compared with the CA measured for the blank wells containing the submaximal E_2 spike to evaluate a percentage change of the sample extracts from the submaximal E_2 spike. Samples that were above the E_2 spike threshold (>100%) were considered to contain estrogenic analytes which may suppresses the anti-estrogen response, whereas samples below the E_2 spike threshold (<100%) were considered to contain analytes which significantly supress the binding of E_2 to the hER-α in the assay, therefore showing an anti-estrogenic response. The samples that successfully supressed E_2-mediated receptor binding were then considered for estimation of a tamoxifen-equivalent concentration (TAM-EQ, μg/L) that as calculated in a similar manner as for the EEQ calculations in the YES, with the exception of rather using tamoxifen in the standard curve as opposed to E_2.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (v.5.00, GraphPad, USA). Variation between individual samples were assessed using an unpaired *t*-test. For the determination of significant variation between sampling days and study sites, a one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons *post hoc* test was performed. Significant variance was shown for a range of P-values (ranging from below 0.05 to below 0.0001).

3. Results and discussion

3.1. Influent wastewater

Raw influent wastewater showed varying levels of estrogenic activity during the sampling campaigns, which ranged from 4.4 to 45.4 ng/L EEQ during summer periods (2015/16 and 2016/17; Tables S1 & S2) and 6.6–31.5 ng/L EEQ during the winter 2016 sampling period (Table S1). The highest levels of estrogenicity during summer 2015/16 was estimated for WWTWs 2, 7 and 10 (Table S1) and during winter 2016, the highest concentrations were observed for WWTWs 1, 3 and 5 (Table S2). The EEQ mass load estimations showed that WWTWs 1, 2, 8 and 10 had the highest levels during summer 2015/16, and WWTWs 1, 2, 5 and 10 during winter 2016 (Fig. 1). As it is expected that the highest potency of estrogenic responses in the YES will be from natural- and/or synthetic steroids, the WWTWs which showed the highest EEQ loads were also those that primarily receive domestic sewage, apart from WWTWs 1 and 2 that also receives a relatively high proportion of industrial wastewater. The contribution of industrial wastewater on estrogenic endocrine-disrupting responses in a bioassay has been shown previously, which included the identification of trace amounts of phthalates, phenols and pesticides that all have been characterised to interfere with gonadal endocrine system pathways (Mahomed et al., 2008).

For the four selected WWTWs that were additionally screened during summer 2016/17, the EEQ (ng/L) estimations within raw wastewater did not differ significantly between sampling days for either WWTWs 2, 9 or 10 (ANOVA, $P > 0.05$; Table S2). However, EEQ (ng/L) estimations for WWTW-8 showed a significant variation in estrogenicity for the Tuesday and Wednesday sampling days compared to the weekend days (ANOVA, $P < 0.05$; Tukey, $P < 0.001$; Table S2). When the variation in daily flow rates at the WWTWs were accounted for, a more pronounced variation in EEQ mass loads (g/day) between sampling days were recorded for the WWTWs (Fig. 2). For WWTW-2, a significant variation was calculated between the Tuesday and Sunday sample (ANOVA, $P = 0.033$; Tukey, $P < 0.05$; Fig. 2; Table S3). WWTW-8 showed the highest rate of variations between sampling days, with a significant increase in estrogenic loads that were calculated from Tuesday towards the weekend period (ANOVA, $P < 0.05$; Tukey, $P < 0.05$; Fig. 2; Table S3) and a high estrogenic load that was still recorded for the Monday sample, but decreased significantly on the following day (ANOVA, $P < 0.0001$; Tukey, $P < 0.0001$; Fig. 2; Table S3). For WWTW-9, a significant decrease was observed from Monday to Friday, along with an increase over the weekend period (ANOVA, $P < 0.0001$; Tukey, $P < 0.05$; Fig. 2; Table S3). For WWTW-10, the same trend was observed as for WWTW-9, with a more pronounced decrease towards Friday, followed by an increase over the weekend period (ANOVA, $P < 0.0001$; Tukey, $P < 0.01$; Fig. 2; Table S3). These results propose that higher weekday/weekend variations were shown for those WWTWs that receive wastewater from domestic sources, where the total estimated EEQ mass loads calculated for these WWTWs (WWTW-10 > WWTW-8 > WWTW-2 > WWTW-9; Fig. 2) were in association with each plant's estimated population size (Table S4). These results further suggest that the amount of estrogenic EDC loads are directly influenced by the *de facto* and *de novo* population being served by the sewage plants. The results highlight the fact that effect-based monitoring in wastewater treatment systems should not just be a single periodic event, as significant variation in estrogenic compounds entering WWTWs may occur over time (Fig. 2).

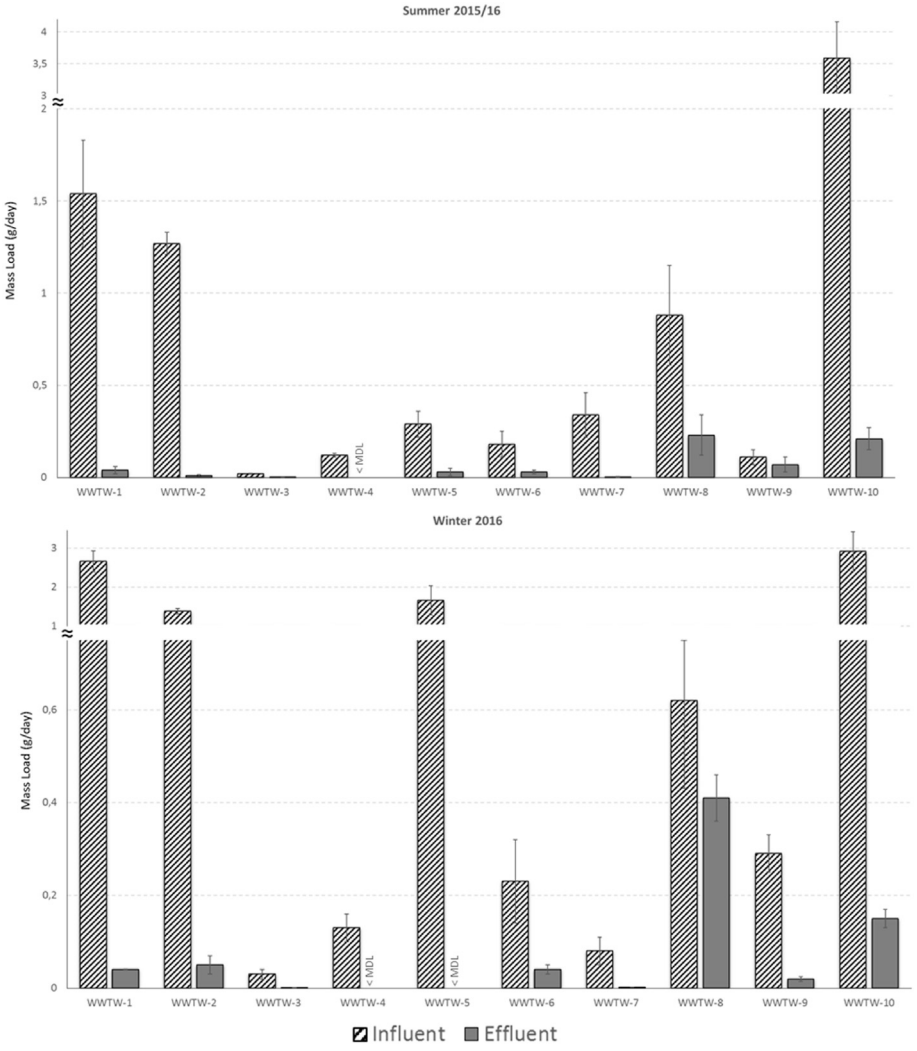

Fig. 1. Mass load (g/day) of estrogen equivalent concentrations (EEQ) measured using the yeast estrogen screen (YES) for the various WWTWs during sampling campaigns in summer 2015/16 (December) and winter 2016 (June).

The EEQ mass load estimates in raw wastewater influent between the two summer sampling campaigns (2015/16 and 2016/17) did not differ significantly for WWTW-8 (*t*-test, $p > 0.05$), but were significantly different for WWTWs 2, 9 and 10 (*t*-test, $p < 0.05$; Table 2). Higher loads were estimated during summer 2015/16 for WWTW-2 as opposed to higher loads which were recorded during summer 2016/17 for WWTW-9 and WWTW-10. It is worth to consider the high rainfall pattern which was experienced during the summer 2016/17 sampling that may explain these variations between sampling periods. However, only WWTW-10 showed a two-fold higher average daily flow rate of receiving wastewater during summer 2016/17 (Table S4). This higher influx of wastewater could be explained by the diversion of raw wastewater from WWTWs 7 and 8 to this works during large runoff surges, along with potential groundwater infiltration into aging sewage pipelines and/or illegal wastewater connections that are not clearly identified. As most surface urban stormwater runoff are not necessarily channelled to WWTWs in the country, and rather discharged directly into surface waters, the higher influx of wastewater at WWTW-10 alone may not necessarily be due to higher rainfall that was recorded in this area during the summer 2016/17 period. Moreover, although the larger influx of wastewater at WWTW-10 was recorded, the estimated EEQs (ng/L) in raw wastewater did not differ significantly between the two summer sampling campaigns (*t*-test, $p > 0.05$; Tables S1 and S2) and a high level of EEQ mass load reduction was still maintained during summer 2016/17 (Table 2).

3.2. Treated effluent and reduction estimates

EEQ estimations in treated wastewater effluents from the various WWTWs varied between 0.3 and 6.9 ng/L during summer 2015/16 (Table S1), 0.2–4.9 ng/L during winter 2016 (Table S1), and 0.2–3.0 ng/L during summer 2016/17 (Table S2). Mass loads of the estimated EEQs (compensating for flow variations between the WWTWs) ranged between 0.0004 and 0.2 g/day during summer 2015/16, and 0.001–0.41 g/day during winter 2016 (Fig. 1; Table 3), and 0.01 to 0.60 g/day during summer 2016/17 (Fig. 2; Table 2). The estimated EEQs from the current study correlates well to similar monitoring studies done elsewhere in the world. For example, EEQs estimated in two Canadian WWTW effluents ranged from 1.0 to 24.0 ng/L (Arlos et al., 2018) and 0.5–18.0 ng/L in WWTW effluent from 75 European WWTWs (Jarošová et al., 2014b). Although high EEQs were estimated in the study by Jarošová et al. (2014b), it should be noted that final effluents of only two of the 75 WWTWs sampled showed EEQs above 10 ng/L, with most ranging between

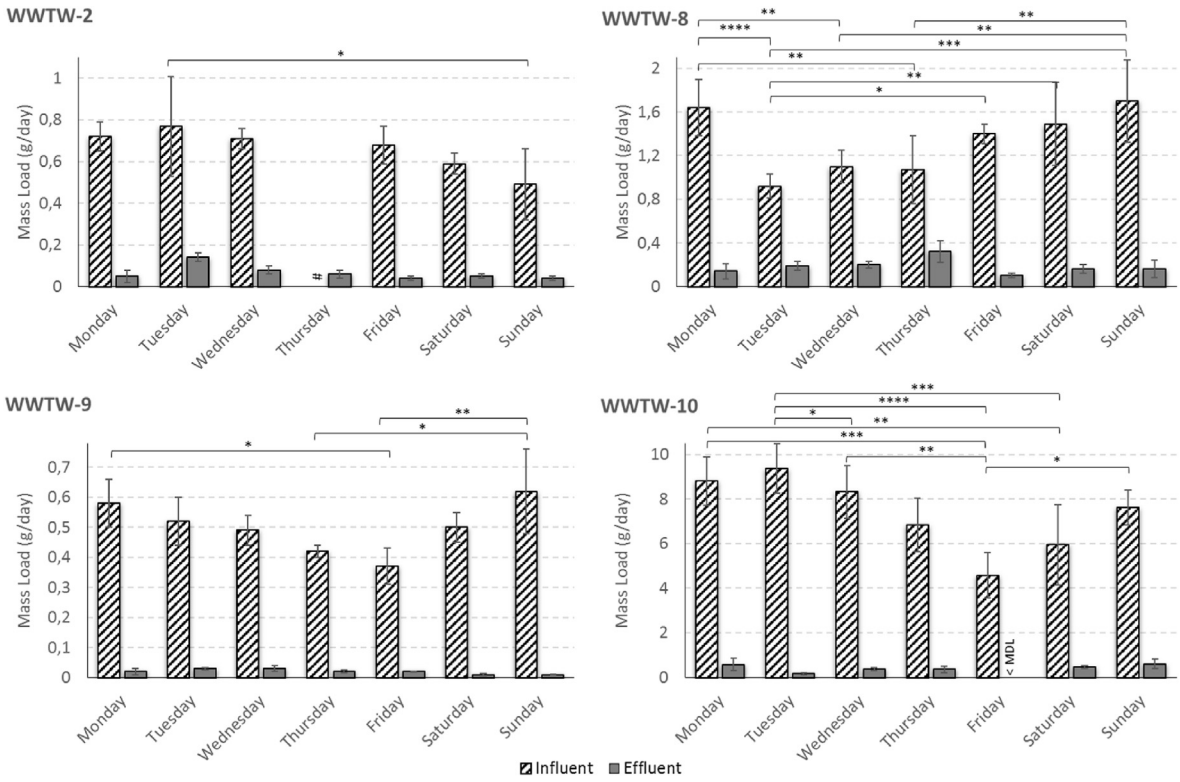

Fig. 2. Daily variation in mass loads (g/day) of estrogen equivalent concentrations (EEQ) estimated for the various WWTWs during the summer 2016/17 sampling campaign (December). Statistical significant variation between sampling days are shown as: **** = P < 0.0001; *** = P < 0.001; ** = P < 0.01; * = P < 0.05. # – sample was lost during processing.

Table 2
Mass loads (g/day) estimated using EEQ concentrations in the YES for both raw- and effluent wastewater samples at the various WWTWs. Abbreviations: <MDL - samples below the detection limit of the YES; n.s - not screened.

Site	Summer 2015/16 (December)			Winter 2016 (June)			Summer 2016/17 (December)		
	Influent	Effluent	Reduction (%)	Influent	Effluent	Reduction (%)	Influent	Effluent	Reduction (%)
WWTW-1	1.5 ± 0.3	0.04 ± 0.02	97	2.7 ± 0.3	0.04 ± 0.001	98	n.s		
WWTW-2	1.3 ± 0.06	0.01 ± 0.004	99	1.4 ± 0.07	0.05 ± 0.02	96	0.7 ± 0.1	0.07 ± 0.04	89
WWTW-3	0.02 ± 0.003	0.004 ± 0.0001	98	0.03 ± 0.01	0.003 ± 0.0002	98	n.s		
WWTW-4	0.1 ± 0.01	< MDL	–	0.1 ± 0.03	< MDL	–	n.s		
WWTW-5	0.3 ± 0.07	0.03 ± 0.02	90	1.7 ± 0.4	< MDL	–	n.s		
WWTW-6	0.2 ± 0.07	0.03 ± 0.01	83	0.2 ± 0.1	0.04 ± 0.01	83	n.s		
WWTW-7	0.3 ± 0.1	0.003 ± 0.001	99	0.1 ± 0.03	0.002 ± 0.0001	98	n.s		
WWTW-8	0.9 ± 0.3	0.23 ± 0.1	74	0.6 ± 0.2	0.4 ± 0.05	34	1.3 ± 0.3	0.2 ± 0.1	86
WWTW-9	0.1 ± 0.04	0.07 ± 0.04	36	0.3 ± 0.04	0.02 ± 0.005	93	0.5 ± 0.1	0.02 ± 0.01	96
WWTW-10	3.6 ± 0.6	0.21 ± 0.06	94	2.9 ± 0.5	0.15 ± 0.02	95	7.4 ± 1.7	0.4 ± 0.2	95

1.0 and 4.0 ng/L.

The EEQ mass load estimates (g/day) were considered as a more useful measure to determine the reduction of the calculated estrogenic responses at the various WWTWs, as this compensates for variable daily flow rates within and between the respective WWTWs. Most of the WWTWs showed high EEQ mass balance reduction (>75%) during the sampling campaigns (Tables 2, S2 & S3) with the exception of WWTW-9 that was estimated at 36% during summer 2015/16 and WWTW-8 that was estimated at 34% during winter 2016 (Tables 2 and S1) as well as variable reduction between sampling days during summer 2016/17 (Table S2). This WWTW diverts its raw wastewater into three separate bio-trickling modules and one activated sludge module, suggesting that such a treatment may not be suitable for the removal and/or reduction of estrogenic pollutants. The inadequate reduction of estrogenicity at WWTW-9 could also be explained by the WWTW that experienced operational challenges during the summer 2015/16 sampling. A new treatment system was in the process of being installed whereby the old (decommissioned) treatment system had to be revamped to act as a back-up treatment system and was reported to not perform well during the upgrading period.

It should be noted that the hydraulic retention times of the WWTWs were not included for the EEQ mass load reduction calculations during the current study. Therefore, the reduction estimations should be considered semi-quantitative as a raw influent and treated effluent sample taken during the same day does not reflect the time taken for the treatment of the influent sample before its effluent product was analysed in parallel. It is also noted that grab sampling poses a constraint in the sampling results of the current study. However, daily grab samples were taken for both the WWTW influent and effluent over a period of five days and pooled together on the final day during summer 2015/16 and winter 2016,

and daily grab samples were taken at the same period during seven-consecutive days during summer 2016/17. As flow- or time-proportional composite sampling was not possible for all the WWTWs, this sampling method was deemed adequate to avoid sampling bias. Regardless, the EEQ reduction estimates from the current study shows good agreement with similar WWTW studies using the YES, along with studies showing the removal profiles of known estrogenic EDCs (Arlos et al., 2018; Chen et al., 2018; Manickum and John, 2014). Although the level of estrogenicity was indeed well removed for most WWTWs, the levels which will be discharged into recipient waters should still be considered for risk characterisation, which may add to the pollutants that are also present within the surface water system.

Estimating the exact source of estrogenicity within waste- and surface waters may prove difficult due to the vast amount of known- and unknown chemicals having multiple modes of endocrine-disrupting actions that may present a variation of mixture interactions in bioassay outcomes. For this reason, the current study did not include chemical analysis of known estrogenic micro-pollutants and merely reported on the net (anti)estrogenic responses of the chemical mixture in the test waters that shows affinity to interfere with hER-α binding. However, we do acknowledge that information on known estrogenic stressors may assist with the explanation of effect-based monitoring outcomes. Although several pesticides, pharmaceuticals, personal care products and industrial by-products have been established as estrogenic EDCs, estrogenicity within waste- and surface waters are primarily attributed to the synthetic steroid hormone ethinylestradiol (EE_2), and secondary by natural estrogen steroid hormones (Tanaka et al., 2000), merely due to steroid hormones being more potent ER agonists than the estrogen-mimicking EDCs. For example, plasticizers such as bisphenol-A (BPA) and phthalates are known to bind to the hER in a dose-dependent manner, although at far lower potencies than E_2 and EE_2 (Bistan et al., 2012). As mentioned before, some phenols, phthalates and pesticides at the μg/L level has been recorded previously in the province where the current study was done that originated from industrial runoff sources (Mahomed et al., 2008). Moreover, levels of phthalates have also been detected at concentrations reaching mg/L levels in South African surface waters (Fatoki et al., 2008), along with various pharmaceuticals and personal care products (PPCPs) of which many have been shown as potential EDCs (Archer et al., 2017b). These studies points to the potential of both known- and unknown estrogen agonists that can exert an additive mixture effect towards a net estrogenic response in bioassays such as the YES. Even though it is stated that estrogenic responses from a bioassay may be a cause of complex chemical mixtures from various origins in test waters, some studies have aimed at correlating the concentrations of steroid hormones with observed EEQs generated from assays such as the YES. A study by Truter et al. (2016) compared levels of E_2 and EE_2 with estimated EEQ values using the YES. Within a surface water sampling site, concentrations of E_2 ranged between 3.9 and 30.8 ng/L, for EE_2 between 1.4 and 10.8 ng/L, and for EEQ in the YES between 10.2 and 43.0 ng/L. From these results, the combined load of the major estrogen analytes (E_2 and EE_2) did correlate well with the EEQs generated by the YES. However, in another study, the same trend was not observed for comparisons between the concentrations of known steroid hormones and EEQ estimates in various WWTW effluents (Aerni et al., 2004). Although the current study did not analyse the levels of steroid hormones, concentrations of E_2 from South African WWTW effluents have been shown to range from 1.0 to 20.0 ng/L (Manickum et al., 2011), which are in line with the EEQs that were estimated during the current study.

The bioassay used for the current study expresses a response from a mixture of micro-pollutants in the test water sample. Therefore, analytes that do not necessarily bind to steroid receptors in an agonistic manner, but interfere with normal steroid receptor binding (such as anti-estrogens) may influence the results from the assay. For this reason, the presence of anti-estrogenicity was measured in treated wastewater to investigate such a masking effect. The estimated TAM-EQ concentrations ranged from 3.7 to 78.7 μg/L in the treated wastewater from the ten WWTWs during the winter 2016 sampling campaign (Table S6), and 0.7–10.0 μg/L for four WWTWs during the summer 2016/17 sampling campaign (Table S7). The results from the YAES indicated a masking effect by estrogenic compounds present in the water samples, whereby estrogenicity in the YAES decreased in the concentrated water extracts in a dose-dependent manner (Figs. S1 and S2). This is represented by a significant deviation above 100% of the E2 spike in the YAES for a 75-times concentrated sample of WWTW-2, WWTW-6, WWTW-9 and WWTW-10, whereby the deviation was less in the 50-times and 25-times concentrated samples (Fig. S1). This may be attributed to the estrogenicity calculated for these WWTWs in the YES during the same sampling periods (Fig. S3). Higher estrogenicity was associated with lower anti-estrogenicity for most of the WWTW effluent samples (Figs. 3 and S3).

As for the vast amount of potential estrogenic EDCs that exist, pollutants contributing to anti-estrogenicity may vary between the locations, which are influenced by the types of industry and domestic activities/density in the area, as well as different agricultural practices and types of crops necessitating different pesticide applications. Although the identification of anti-estrogenic EDCs are less known, some compounds such as the bisphenol antiseptic hexachlorophene (HCP), the synthetic vitamin menadione (K3) and the pesticide/disinfectant pentachlorophenol (PCP) have all shown affinity to antagonise ER binding *in vitro* (Jung et al., 2004), whereas persistent pollutants such as 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) can cause tissue-specific estrogenic signalling inhibition *in vivo* (Yoshida et al., 2019). Anti-estrogenicity has also been identified for several pesticides, fragrances and UV filters, all with demonstrated recalcitrance to biodegradation (Hernandez Leal et al., 2010; Westlund and Yargeau, 2017). Furthermore, degradation and disinfection processes may create undesired by-products which may lead to anti-estrogenicity in treated wastewater (Wu et al., 2009).

3.3. River water

Although the source of estrogenicity in surface waters are often

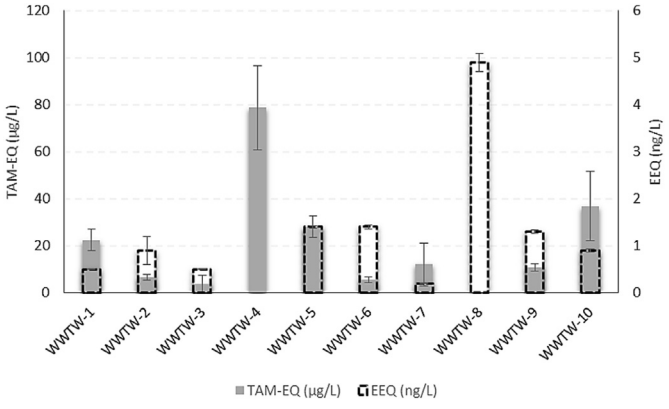

Fig. 3. Anti-estrogenicity calculated as tamoxifen-equivalent concentrations (TAM-EQ; grey bars; 1° y-axis; μg/L) and estrogenicity calculated as estradiol-equivalent concentrations (EEQ; dashed bar; 2° y-axis; ng/L) for treated wastewater effluent at the various WWTWs during the winter 2016 sampling campaign.

proposed to primarily originate from WWTW effluent discharge, the EEQ estimates for river water located upstream from seven of the 10 WWTWs showed higher concentrations than downstream water samples (Table S1). This was particularly shown for WWTW-9, where the average upstream EEQ during the winter 2016 sampling was 18.9 (±0.2) ng/L as opposed to a calculated EEQ of 15.1 (±0.9) ng/L for the raw influent during the same period (Table S1). The treated discharge of WWTW-9 showed an average EEQ concentration of 1.3 (±0.03) ng/L during the same sampling period and subsequently, the total estrogenicity was lowered downstream from the WWTW discharge (EEQ = 11.3 ng/L) in comparison to the upstream sample. Although we do acknowledge that a semi-composite of treated effluent was compared with a single grab sampling event for the river water, this finding still suggest that substantial pollution occurs upstream from the WWTW and that the observed estrogenicity downstream from the WWTW may not necessarily be associated with WWTW effluent alone in the study area. The river system associated with WWTW-9 has been reported to experience extreme pollution pressures from peri-urban communities located upstream from the WWTW, including direct sewage disposal into the river system through stormwater drainage.

For the other sampling locations, the river system associated with WWTW-1 also passes through informal settlements and industrial areas upstream of the plant. The high loads of estrogenicity illustrated during the current study for the upstream river water samples are therefore indicative of alternative human pollution activities other than waste from treated WWTW discharge. More alarming, the river system associated with WWTW-2 serves as a main feed into a reservoir of a nature reserve that provides around 8% of the drinking water supply to a city with a population exceeding 2.1 million. This drinking water resource has been previously shown to be highly impacted by industrial pollutants and known EDCs (Aneck-Hahn et al., 2009; Barnhoorn et al., 2015). The estimated EEQs from this downstream river sample was higher than EEQs estimated for WWTW-2 effluent during the summer 2015/16 sampling campaign (Table S1). We previously reported similar results for this river, showing an extensive list of pharmaceuticals and personal care products at higher concentrations in downstream river water compared to treated wastewater discharge, as this downstream sample is located nearly 5 km from the WWTW discharge with substantial human activities further downstream (Archer et al., 2017a). The same trend was shown in the current study for WWTWs 1, 3, 6, 7 and 9 during summer 2015/16, and WWTWs 1, 3, 6 and 9 during winter 2016 sampling. Although it is difficult to establish where these increased estrogenic responses are originating from other than WWTW discharge, it should be noted that the study areas were located among various human activities such as industrial areas, agricultural lands and informal/peri-urban settlements. The latter is of specific interest, as many peri-urban areas in the country are not necessarily supplied with adequate municipal sanitation services (such as flush toilets, water distribution systems and waste bins) and consequently, leads to substantial disposal of human waste products (sewage, greywater and solid waste) into surrounding rivers and groundwater via urban runoff and illegal dumping.

The YES received wide recognition as a bioassay to estimate the net affinity of analytes in surface water samples that bind to the hER-α in other studies. Estimated EEQs were recorded to range between 0.1 and 26.5 ng/L in Europe (Cargouët et al., 2004; Matthiessen et al., 2006; Plutzer et al., 2018; Brettschneider et al., 2019), 0.07–6.4 ng/L in Asia (Ra et al., 2011; Xiao et al., 2017) and 7.1–23.0 ng/L in Australia (Uraipong et al., 2018). The higher levels of estrogenicity of the current study are in agreement with estimated EEQs in another South African river system receiving treated domestic wastewater discharge and surrounded by agriculture and mining activities, ranging between 10.2 ng/L during winter, 14.2 ng/L during spring, and 43.0 ng/L during summer (Truter et al., 2016). The reliance on these rivers for potable water at various scales – from small communities to large cities, highlights the need to establish the environmental risk through a refined weight-of-evidence approach.

3.4. Risk characterisation

Despite the moderate to high level of EEQ reduction that was estimated for most WWTWs during the current study (Table 2), it should not be assumed that the residual EEQ loads in wastewater discharge are without risk to the receiving environment. Conventional risk assessment approaches focus on acute or chronic lethal toxicity endpoints (Hernando et al., 2006). However, such an approach of risk characterisation does not consider the complex mixture of interactions amongst environmental pollutants within a test water system, nor do they consider sub-lethal toxicity endpoints such as endocrine disruption on vertebrate sentinel organisms. The YES offers a viable option that indicates the net estrogenic potential of a water sample to modulate hormone receptor binding. It is therefore feasible to compare EEQ values with toxicological studies that were done on sentinel aquatic organisms in a similar manner as conventional risk characterisation studies. For example, a lowest-observed-effect-concentration (LOEC) of 10 ng/L has been proposed from a study showing intersexuality in fish after chronic exposure to E_2 (Metcalfe et al., 2001) along with a concentration of 5 ng/L E_2 that is shown to induce the production of vitellogenin (VTG) in male fish (Brion et al., 2004), which is an established biomarker of endocrine disruption (Mortazavi et al., 2019). Any pollutant in the water samples which mimics an E_2 mechanism of action (expressed as EEQs in the YES) could thus yield the same effect.

As expected, nearly all of the EEQ estimates of raw influent WWTW samples were either above the 5 or 10 ng/L level (Tables S1 and S2), as several micro-pollutants are known to mimic an estrogen response similar to E_2 (Archer et al., 2017b; Mckinlay et al., 2008). For treated effluent wastewater, none of the sampling sites showed levels above the proposed LOEC of 10 ng/L and only EEQ estimations for WWTW-9 were above the 5 ng/L level during the summer 2015/16 sampling, and WWTW-8 EEQs close to this level during the winter 2016 sampling (Fig. 4; Table S1) – both of which showed low EEQ mass balance reduction during the respective sampling campaigns (Table 2). Furthermore, predicted no-effect concentrations (PNEC) of 1.0 ng/L and 2.0 ng/L E_2 has also been proposed as a baseline limit which may modulate fish reproduction and thus serves as a trigger value of hazard identification (Young et al., 2002; Caldwell et al., 2012). The EEQ estimations for treated effluent samples showed that WWTWs 5, 6, 8 and 10 exceeded these PNECs during the various sampling campaigns (Fig. 4, Table S1; Fig. S4, Table S2). Such trigger values thus confirm that residual pollutants that persist through wastewater treatment may still contribute towards environmental health risks, even for WWTWs that showed moderate-to high reduction of estrogenicity during their treatment processes.

Overall, the estimated river water EEQs showed more pronounced environmental risks factors than treated wastewater effluent, whereby EEQ estimates for both the up- and downstream river water at WWTW-1 and WWTW-9 even exceeded or were close to the 10 ng/L level during summer 2015/16 and winter 2016 (Fig. 5; Table S1). Moreover, EEQs of surface water samples located upstream from the discharge of WWTW-1 were also above the

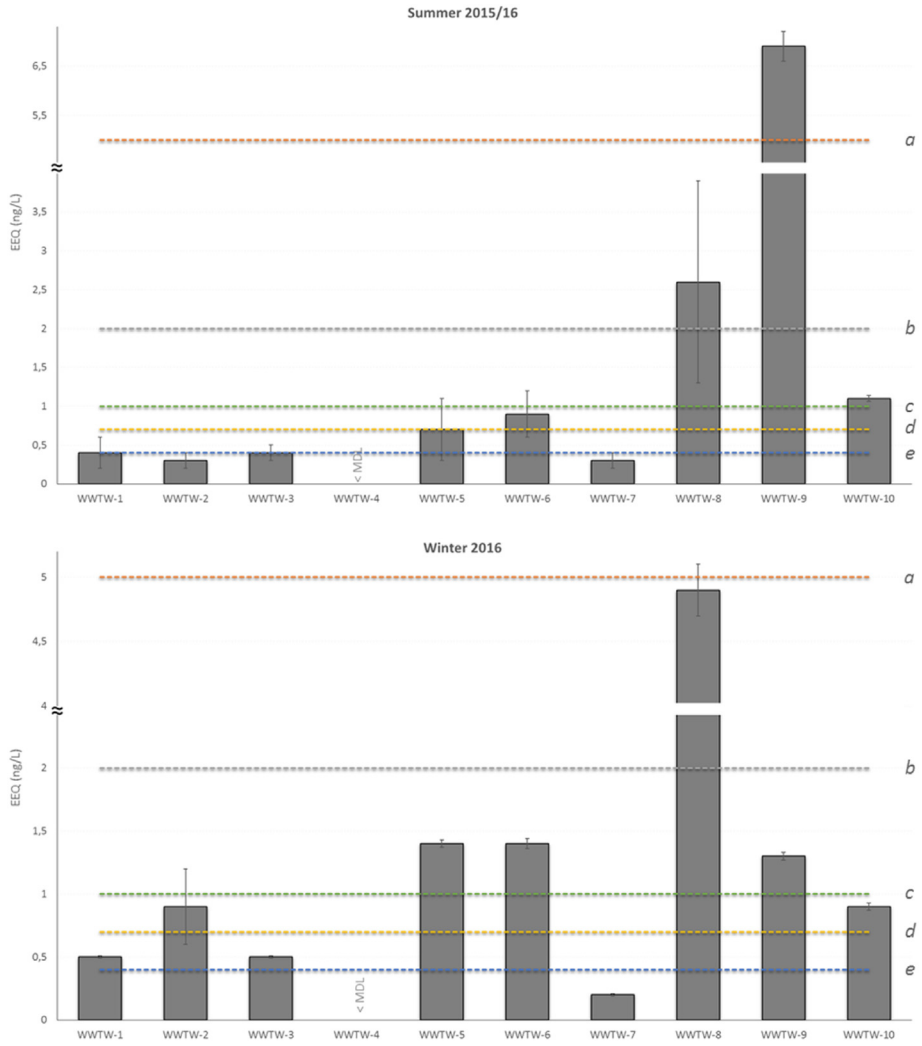

Fig. 4. E_2-equivalent concentrations (EEQ; ng/L) measured at the treated effluent samples from the 10 WWTWs during summer 2015/16 and winter 2016. [a] Concentration of E_2 (5 ng/L) showing increased VTG production in fish (Brion et al., 2004); [b] Predicted no-effect concentration (PNEC, 2 ng/L) to modulate fish reproduction (Caldwell et al., 2012); [c] Long-term PNEC (1 ng/L) for impaired reproduction in fish (Young et al., 2002); [d] Estimated trigger value (0.7 ng/L) for risk in drinking water (Genthe et al., 2013); [e] EEQ trigger value (0.4 ng/L) of effluent on long-term fish exposure (Jarošová et al., 2014a).

5 ng/L level during both sampling campaigns (Fig. 5; Table S1), but not for the treated effluent (Fig. 4; Table S1), thus highlighting the extent of pollution in some river systems at locations above WWTW discharge. The results for surface waters located up- and downstream of the discharge of WWTWs 1, 3, 4, 7, 8 and 10 may even suggest that the WWTW discharge could contribute to the lowering of estrogenic loads in the surface waters. However, this should not be a universal assumption, as many surface water locations did, however, show an increased EEQ in surface waters located downstream from WWTW discharge (Fig. 5).

More refined effect-based trigger values (EBTVs) have also been proposed to serve as sensitive markers that suggest that further risk intervention should be done in a tiered approach or to identify the particular chemical stressors in the test water system. An estimated E_2 trigger value of 0.7 ng/L for drinking water standards have been proposed in a South African research institution (Genthe et al., 2013) along with an EEQ-derived EBTV of 0.4 ng/L for long-term exposure to effluent-impacted surface waters (Jarošová et al., 2014a). Several WWTW effluent and river water samples were above these sensitive risk indicator levels (Figs. 4 and 5; Tables S1 and S2). Although such EBTVs are mostly relevant for the assessment of drinking water resources that may impact public health, most surface waters such as these that were tested during the current study all feed to drinking water extractions and reservoirs at some point and thus may impact the quality and safety of potable water resources for the surrounding communities and highlights the need for further risk intervention.

4. Conclusions

The results presented here discussed three interrelated themes, namely (1) the incidence of estrogenicity within raw wastewaters originating from areas having multi-faceted demographics, (2) evaluation of the transfer and fate of (anti)estrogenicity from treated wastewater to receiving surface waters, and (3) to refine bioassay outcomes for risk characterisation, with particular reference to reported effect-based trigger values (EBTVs) and endocrine-disrupting outcomes in sentinel aquatic organisms. The calculated EEQ and TAM-EQ values using the YES and YAES correlate well with existing literature and highlight the value of flow-proportional EEQ mass load estimations as an approach to evaluate WWTW treatment performance and comparisons between

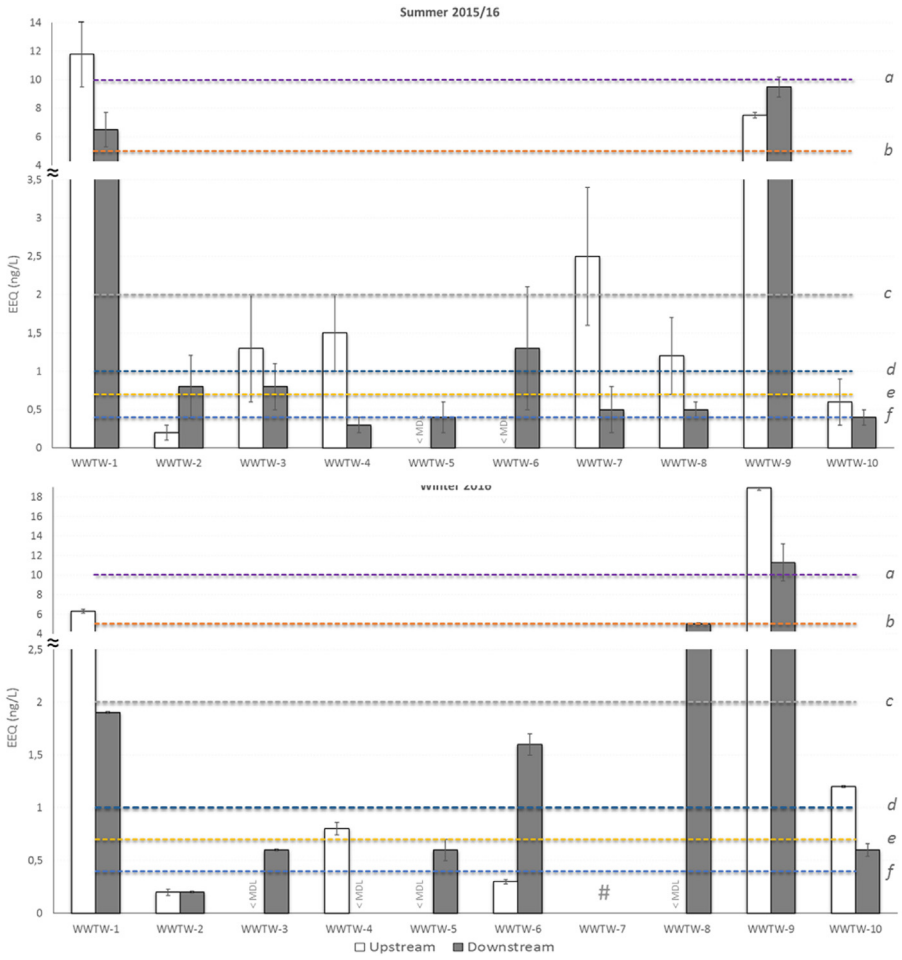

Fig. 5. E_2-equivalent concentrations (EEQ; ng/L) measured for river water samples located upstream and downstream of the WWTW discharges during summer 2015/16 and winter 2016. [a] lowest-observed-effect-concentration (LOEC; 10 ng/L) of chronic E_2 exposure causing intersexuality in fish (Metcalfe et al., 2001); [b] Concentration of E_2 (5 ng/L) showing increased VTG production in fish (Brion et al., 2004); [c] Predicted no-effect concentration (PNEC, 2 ng/L) to modulate fish reproduction (Caldwell et al., 2012); [d] Long-term PNEC (1 ng/L) for impaired reproduction in fish (Young et al., 2002); [e] Estimated trigger value (0.7 ng/L) for risk in drinking water (Genthe et al., 2013); [f] EEQ trigger value (0.4 ng/L) of effluent on long-term fish exposure (Jarošová et al., 2014a).

WWTWs having variable sizes. Even though a notable reduction of estrogenicity by the WWTWs was measured, the effluent EEQs reported here remain a concern because of the risk for adverse outcomes to the aquatic environment and, by extension, human health.

The study highlights the complexity associated with interpreting bioassay outcomes such as the YES and YAES when multiple biotic- and abiotic factors may influence the results. Furthermore, the complex composition of environmental samples containing both inorganic and organic micro-pollutants poses challenges when endpoints such as estrogenicity and anti-estrogenicity are assessed using a single bioassay, where masking of agonistic and antagonistic substances by one-another and other complex chemical mixture interactions are proposed. We acknowledge that using the YES and YAES alone for risk characterisation does have limitations, whereby the bioassay only considers an (anti)estrogenic response based on ligands that interfere with the hER-α and thus do not consider other pathways of endocrine system modulation. Regardless, the YES and YAES has tremendous potential to serve as first-tier, cost-effective bioassays to point to potential public- and environmental health risks that should be followed-up using a battery of effect-based monitoring bioassays and chemical analyses to understand the total extent of health risks in test waters.

Despite the evidence presented here implicating WWTWs as a source of estrogenic EDC discharge into surface waters, the results also suggest additional pollution sources that also need to be prioritised when risk assessment and mitigation strategies are done. Clearly, the relative contribution of these alternative sources will show great regional variation, and with building evidence of the possible negative effect of other micro-pollutants not discussed here, it is imperative to develop (or rather refine) techniques such as YES and YAES to become affordable tools that provide realistic indication of health risks, even in remote areas. Various factors may indeed contribute to the higher level of EEQs that were estimated in surface waters located above WWTW discharge, such as illegal dumping by waste controllers, pollution of solid-, sewage- and greywater waste from informal and peri-urban communities, aging and insufficient wastewater infrastructure to cope with population densification, agricultural runoff, insufficient environmental law enforcement, and/or poor municipal governance. Moreover, most conventional WWTW technologies are not necessarily designed to eliminate the vast range of recalcitrant and emerging pollutants that are continuously being identified. These challenges may even be of greater concern in low and middle-income countries, and thus a stark reminder of the hurdles in the way of achieving sustainability of freshwater resources that not only impacts environmental resilience, but also domestic, agricultural and industrial growth. Effective means should be found, in a non-alarmist way, for using

the growing body of evidence to show the diversity in causes for the deterioration of surface water quality, to inform decision makers and the broader society. It is evident from the current study that WWTWs are not the sole source of micro-pollutant discharge into freshwater ecosystems. New thinking in the planning and implementation of appropriate interventions such as decentralised treatment systems, altering societal behaviour and improving the science-policy interface on freshwater conservation thus need to be incorporated.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Edward Archer: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Gideon M. Wolfaardt:** Conceptualization, Resources, Data curation, Writing - original draft, Supervision, Funding acquisition. **Johannes H. van Wyk:** Conceptualization, Validation, Resources, Writing - original draft, Supervision. **Nico van Blerk:** Validation, Investigation, Resources, Writing - review & editing, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114424.

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