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RESEARCH ARTICLE

Estrogenic, androgenic, and glucocorticoid activities and major causative compounds in river waters from three Asian countries

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Abstract

Estrogen, androgen, and glucocorticoid receptors (ER, AR, and GR) agonist activities in river water samples from Chennai and Bangalore (India), Jakarta (Indonesia), and Hanoi (Vietnam) were evaluated using a panel of chemical-activated luciferase gene expression (CALUX) assays and were detected mainly in the dissolved phase. The ER agonist activity levels were 0.011–55 ng estradiol (E2)-equivalent/l, higher than the proposed effect-based trigger (EBT) value of 0.5 ng/l in most of the samples. The AR agonist activity levels were < 2.1–110 ng dihydrotestosterone (DHT)-equivalent/l, and all levels above the limit of quantification exceeded the EBT value of 3.4 ng/l. GR agonist activities were detected in only Bangalore and Hanoi samples at dexamethasone (Dex)-equivalent levels of < 16–150 ng/l and exceeded the EBT value of 100 ng/l in only two Bangalore samples. Major compounds contributing to the ER, AR, and GR agonist activities were identified for water samples from Bangalore and Hanoi, which had substantially higher activities in all assays, by using a combination of fractionation, CALUX measurement, and non-target and target chemical analysis. The results for pooled samples showed that the major ER agonists were the endogenous estrogens E2 and estriol, and the major GR agonists were the synthetic glucocorticoids Dex and clobetasol propionate. The only AR agonist identified in major androgenic water extract fractions was DHT, but several unidentified compounds with the same molecular formulae as endogenous androgens were also found.

Keywords Androgens · CALUX · Estrogens · Glucocorticoids · River water

Introduction

Surface water pollution caused by direct discharge of untreated municipal wastewater remains a serious issue in many lower income countries, including in Asia. According to the 2013–2017 statistics from FAO's Global Information System on Water and Agriculture (AQUASTAT, FAO), the percentage of untreated municipal wastewater in India and Vietnam was 71% and 90%, respectively. A wide range of chemicals of domestic, industrial, agricultural, and medical origins can be directly discharged along with municipal wastewater, and multiple classes of contaminants including fecal sterols, plasticizers, pharmaceuticals, and various pesticides have been detected at high levels in river water and sediment from major Vietnamese cities in a few screening studies based on gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–time-of-flight MS (LC–ToFMS) databases (Chau et al. 2018; Duong et al. 2014). However, most studies on organic contaminants in river water of lower income Asian countries targeted only

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compounds with established routine analytical methods, such as persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and limited sets of pharmaceutical and personal care products (PPCPs) (Babut et al. 2019; Mitra et al. 2019; Ngo et al. 2021; Sharma et al. 2019), and potential adverse effects of non-routinely monitored chemicals remain overlooked. For comprehensive risk assessment of complex contaminant mixtures in surface water in lower income countries, evaluation of the overall toxic effects and identification of priority contaminants for further monitoring are needed.

Municipal wastewater is known to contain endogenous hormones and synthetic chemicals capable of affecting nuclear hormone receptor–mediated transcription. Among these endocrine-disrupting chemicals, estrogens (or estrogen receptor (ER) agonists) have been extensively studied since their early reported association with fish feminisation (Tyler and Filby 2011). Frequently detected ER agonists in municipal wastewater treatment plants (WWTPs) include not only endogenous estrogens (17β-estradiol, E2; estriol, E3; estrone, E1) and synthetic estrogens (e.g., the contraceptive 17α-ethinylestradiol, EE2) but also other chemicals such as alkylphenols, bisphenols, and phtalates (Jobling et al. 2009). Endogenous and synthetic androgens (or androgen receptor (AR) agonists) have also been found in WWTP effluents and may cause masculinisation effects in specific fish species in effluent-impacted waters (Fan et al. 2011; Thomas et al. 2002). Recently, the occurrence of glucocorticoid receptor (GR) agonists, including endogenous hormones such as cortisol as well as synthetic compounds such as the anti-inflammatory drugs prednisolone and dexamethasone (Dex), in wastewater has received increasing attention (Fan et al. 2011; Isobe et al. 2015; Shen et al. 2020; Weizel et al. 2018) considering their involvement in metabolism, stress response, and immune system regulation of aquatic species (Leatherland et al. 2010). Hormone receptor–mediated activities have been evaluated in an increasing number of studies on surface water in Australia, Europe, Japan, and the USA (Conley et al. 2017; Daniels et al. 2018; de Baat et al. 2019; Hashimoto et al. 2007; Scott et al. 2014; Simon et al. 2022) but remain largely neglected in studies on water contamination in lower income countries. Activity contribution of well-known hormones and hormone-like drugs has often been assessed, but very few studies have conducted comprehensive effect–directed identification of hormone-disrupting chemicals (Hashmi et al. 2018; Thomas et al. 2002; Zwart et al. 2020).

In view of the lack of information on overall hormone disrupting effects of complex water contaminant mixtures in untreated wastewater-impacted rivers in Asia and on the effect contribution of non-routinely monitored contaminants in general, the present study aimed to investigate hormone receptor–mediated activities and primary contributing contaminants in river water from a number of Asian cities. Specifically, river water samples from several major cities in India, Indonesia, and Vietnam were evaluated for ER-, AR-, and GR-mediated activities for the first time. Identification of major compounds contributing to the detected activities was conducted for pooled samples by using effect-directed analysis. The activities of well-known endogenous hormones and potential contribution of synthetic chemicals were examined.

Materials and methods

Collection and pretreatment of water samples

Surface water samples were collected from various rivers in Hanoi (HN, $n=7$), Vietnam, January 2014), Chennai (CN, $n=11$) and Bangalore (BG, $n=7$), India, and Jakarta (JA, $n=10$), Indonesia in 2014–2015. The sampling locations in Hanoi included Kim Nguu, To Lich, and Nhue Rivers; in Chennai, Buckingham Canal, Adyar and Kortalaiyar Rivers; in Bangalore, Vrishabhavathi River; in Jakarta, Sunter River, Kali Petukangan, and Cakung Drain (Table S1). The samples were collected into solvent-prewashed amber glass bottles or polypropylene bottles, transported to the laboratory, and then either immediately filtered and solid-phase-extracted as detailed below, or stored at − 20 °C until analysis.

Each water sample (100 ml) was passed through a pre-baked multilayer glass fiber filter (Whatman GMF150, 1 µm, GE Healthcare, Japan). A small volume of methanol (≤ 5% of the water volume) was used to rinse the sample bottle and then filtered together with the sample. The filter was dried under vacuum for 15 min, and then compounds in the suspended phase (SP) were extracted by successive sonication with distilled acetone, dichloromethane, and hexane (20 ml each). The filtrate (50 ml portion) was loaded on methanol- and MilliQ water-prewashed solid-phase extraction cartridges (Oasis HLB Plus LP, Waters, USA) and then compounds in the dissolved phase (DP) were extracted by eluting with 6 ml of LC/MS-grade methanol. Both SP and DP extracts were then solvent-exchanged into bio-analytical-grade dimethyl sulfoxide (DMSO, 100 µl for SP, 250 µl for DP) for subsequent analyses.

Reference chemicals for bioassays

E2 and tamoxifen (Tam) were used as reference agonist and antagonist for ER, with 5α-dihydrotestosterone (DHT) and flutamide (Flu) for AR, and Dex and mifepristone (RU486) for GR, respectively. E2 (purity > 98%), Dex (> 97%), Tam (> 99%), Flu (> 99%), and RU486 (> 98%) were obtained from Sigma-Aldrich (Japan) whereas DHT (> 95%) was

from Wako (Japan). All compounds were dissolved in bioanalytical-grade DMSO.

Measurement of ERα-, AR-, and GR-mediated activities

Agonist and antagonist activities for ERα, AR, and GR were evaluated using respective chemical-activated luciferase gene expression (CALUX) assays (Sonneveld et al. 2005; van der Linden et al. 2008) based on recombinant U2OS human osteosarcoma cell lines (BioDetection Systems b.v., The Netherlands). In agonist assays, cells were exposed to various sample doses, and the luciferase fold induction levels were compared with the maximum induced by the reference agonist. Doses inducing ≥ 5% of the maximal level induced by the reference agonist were considered exerting significant agonist activities, and the activity levels were calculated as reference agonist equivalents (E2EQ, DHTEQ, or DexEQ) from the reference dose–response curve. In antagonist assays, cells were co-exposed to the reference agonist at 50%-effective concentration (EC_{50}) and the sample extract at various doses, and the luciferase fold induction levels were compared with that induced by the positive control (reference agonist at EC_{50} and DMSO). No significant antagonist activities (≥ 10% inhibition) were found for any extracts. All activity values were reported as the average of at least three measurements. Details on cell culture and assay procedures, and performance validation requirements were described elsewhere (Suzuki et al. 2013). The observed fold induction levels and EC_{50} values of the reference chemicals were well within the acceptable ranges in all assay iterations.

Identification of major ER, AR and GR agonists

Identification of major compounds causing the ER, AR, and GR agonist activities detected by CALUX assays was conducted using effect-directed analysis on representative pooled samples prepared from extracts with high activities. Briefly, each pooled extract was LC-fractionated into 35 fractions, which were then solvent-exchanged into DMSO and subjected to CALUX assays to identify active agonist fractions. The major ER and AR agonist fractions were analyzed using LC–ToFMS for suspect screening of known estrogens and androgens, whereas the major GR agonist fractions were target-analyzed for glucocorticoids using LC–tandem mass spectrometry (LC–MS/MS). A fraction with no agonist activity was also analyzed for each fraction series, and only compounds not detected in this "baseline" fraction were considered possible causative compounds for the activities of agonist fractions. Compounds that could be identified using authentic standards were quantified, and their contribution to the activities of the respective fractions was evaluated as ratios between the calculated reference agonist equivalent levels—chemical concentrations multiplied by relative potency factors (REPs)—and the CALUX-measured levels.

The fractionation instruments consisted of a LC system (LC-2000 series, Jasco, Japan) with a Synergi Polar-RP column (4 µm, 10 × 250 mm, Phenomenex, USA) and a CHF122SC fraction collector (Advantec, Japan). The mobile phase consisted of a mixture of acetonitrile (10%) and variable proportions of methanol and MilliQ water, all solvents being of LC/MS grade. The content of methanol was 60% at 0–10 min, increased to 75% at 10–14 min, and then was held for 46 min. The column temperature was 40 °C and the flow rate was 4 ml/min. For reference, the retention times of several estrogens, androgens, and glucocorticoids in these conditions were determined using an UV detector (UV-2075 Plus, Jasco, Japan). The fraction timing and the elution points of the standards are given in Table S2. Before fractionation, a series of blank run was conducted to check for contamination until no UV peaks could be observed.

Screening of the ER and AR agonists in selected fractions was conducted using a 1290 Infinity LC (Agilent, USA) with a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm, Agilent, USA), and a 6530 Q-TOF MS (Agilent, USA) operating in electron spray ionization (ESI) mode. The injection volume was 5 µl. The mobile phase was a mixture of methanol and MilliQ water at a flow rate of 0.3 ml/min at 40 °C. The content of methanol was 10–80% at 0–10 min, increased to 99% at 10–15 min, stayed at 99% for 5 min, and then was reset to 10% and maintained for 5 min. The settings of the ToFMS were as follows: desolvation gas temperature 350 °C, sheath gas temperature 325 °C, capillary voltage 3500 V, nozzle voltage 1000 V, *m*/*z* range 100–1500 u. ER agonists were screened in negative ionization mode, and an aqueous solution of ammonium was introduced post-column (final concentration 10 mM) to enhance the formation of deprotonated $[M–H]^-$ ions. AR agonists were screened in positive ionization mode, and formic acid was added to the aqueous mobile phase (0.1%) to improve the formation rate of protonated $[M+H]^+$ ions. Under these conditions, the detection limits of the endogenous estrogens E2, E3, E1, and the androgen DHT were 0.58, 1.0, 0.31, and 0.94 ng/l water-equivalent, respectively. Molecular features (*m*/*z* peaks) on the chromatogram were assigned using Mass Hunter and putative chemical formulae were calculated as $[M+H]^+$ or $[M–H]^-$ ions. Molecular features matching those of known steroids were investigated as "suspects," and external standards were used to verify the retention times on the LC–ToFMS chromatograms and to calculate the concentrations of the confirmed compounds. Suspected compounds with no matching standards were examined using MS/MS product ion spectra for comparison with databases such as National Institute of Standards and Technology mass library (NIST2017) and MassBank (https://massbank.eu/MassBank).

Glucocorticoids in GR agonist fractions were analyzed using LC–MS/MS, because of the poor sensitivity of the ToFMS for synthetic glucocorticoids. The targets included endogenous compounds (cortisol and cortisone) as well as antiallergic drugs (prednisolone, Dex, betamethasone, betamethasone 17-valerate, betamethasone dipropionate, clobetasol propionate, clobetasone butyrate, and difluprednate). The analytical instruments consisted of a Prominence LC (Shimadzu, Japan) and a Qtrap 5500 tandem mass spectrometer (Sciex, USA) operating in ESI mode. Other instrumental details were described by Isobe et al. (2015). Quantification was done also using external standards.

Results and discussion

Hormone disrupting activities in surface water

ER, AR, and GR agonist activities were detected mostly in the dissolved phase rather than in the suspended phase of the river water samples (Fig. 1). Estrogenic activities in the DP extracts were in the range of 0.13–55 ng E2EQ/l, at least an order of magnitude higher than in the SP extracts (0.011–2.1 ng E2EQ/l). Similarly, androgenic activities were detected in most DP extracts (< 2.1–110 ng DHT/l) but not in SP extracts (< 0.74 ng DHT/l). GR agonist activities were detected in only DP extracts of six samples from Hanoi and four samples from Bangalore at (up to 74 and 150 ng DexEQ/l, respectively), but not in any SP extracts (< 3.5 ng DexEQ/l). The results indicate that the majority of the extractable ER, AR, and GR agonists in river water were partitioned in the dissolved phase.

The estrogenic, androgenic, and glucocorticoid activity levels varied in general within an order of magnitude for different sampling locations in each city. All activities in the samples from Chennai and Jakarta were substantially lower than in those from the other cities, and possible reasons are the sampling locations in Chennai being less populated and high dilution during the rainy season in Jakarta. To understand the extent of contamination, the activity levels measured in the present study are compared with those reported for surface water by a number of recent studies (Table 1). Disregarding possible differences caused by specific assays employed, the estrogenic levels in Bangalore and Hanoi rivers (up to 55 and 31 ng E2EQ/l, respectively) were higher than the E2EQ range reported for surface water in European countries (up to 1.1 ng, Simon et al. 2022), in the same range as those found in WWTP effluents in Japan (up to 49 ng/l, Hashimoto et al. 2007), China (up to 33 ng/l, Guo et al. 2019), and WWTP influents in Finland (up to 42 ng/l, Välitalo et al. 2017) but did not reach as high as the upper ranges reported for WWTP influents in Japan (up to 120 ng/l, Hashimoto et al. 2007) and Australia (up to 122 ng/l, Bain et al. 2014), and US streams (up to 116 ng/l, Conley et al. 2017). AR- and GR-mediated activities are less commonly studied, and the androgenic activities in the present study (up to 110 ng DHT/l) were similar to those reported for WWTP influents in Finland (up to 67 ng DHT/l) and hospital wastewater in The Netherlands (86 ng/l, van der Linden et al. 2008), but lower than the levels detected in Australian WWTP influents (up to 350 ng DHT/l, Bain et al. 2014). The GR agonist activities in Hanoi rivers (up to 74 ng DexEQ/l) were higher than the levels reported for surface water in The Netherlands, and comparable to those found in WWTP effluents in Japan and Australia (Table 1), whereas the levels detected in Bangalore rivers (up to 150 ng DexEQ/l) were as high as those reported for hospital wastewater in The Netherlands (96 ng/l, van der Linden et al. 2008) and Switzerland (162 ng/l, Macikova et al. 2014). Considering the lack of municipal wastewater treatment in lower income countries, hormone receptor–mediated activities in river water samples of this study reaching the levels of WWTP influents in more developed countries are expected results and reflect the direct discharge of untreated

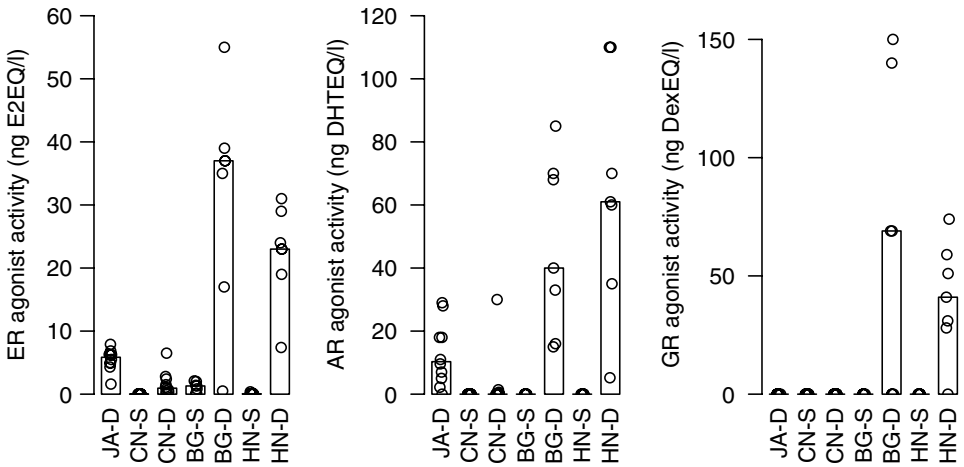

Fig. 1 ER, AR, and GR agonist activities in suspended (S) and dissolved (D) phases of river water samples from Jarkarta (JA), Chennai (CN), Bangalore (BG), and Hanoi (HN). Circles and bars represent individual values and medians, respectively, non-detects are plotted as zero

Table 1 ER, AR, and GR agonist activities in river water (dissolved phase) from India, Indonesia, and Vietnam in comparison with reported values for surface water from other countries

Countries (area)	ER agonist (ng E2EQ/l)	AR agonist (ng DHTEQ/l)	GR agonist (ng DexEQ/l)	Reference
Indonesia (Jakarta)	1.6–7.9 (5.9)	< 2.1–29 (10)	< 20	This study
India (Chennai)	0.13–6.5 (0.95)	< 3.9–30 (< 3.9)	< 22	This study
India (Bangalore)	0.50–55 (37)	15–85 (40)	< 22–150 (69)	This study
Vietnam (Hanoi)	7.4–31 (23)	5.2–110 (61)	< 16–74 (41)	This study
China (Beijing WWTP effluents)	10–33			Guo et al. (2019)
Japan (WWTP influent)	10–120			Hashimoto et al. (2007)
Japan (Osaka WWTP effluent)			7.0–78 (32)	Suzuki et al. (2015)
Australia (various)	< 0.1–6.5	< 7		Scott et al. (2014)
Australia (WWTP influent)	28–122	30–350	37–85	Bain et al. (2014)
Europe (14 countries)	< 0.02–1.1			Simon et al. (2022)
Finland (WWTP influent)	0.45–42	ND–67		Välitalo et al. (2017)
Netherlands (various land use)	ND–1.6		ND–27	de Baat et al. (2019)
Switzerland (Baden)			ND–30	Macikova et al. (2014)
Serbia (Novi Sad)	0.014–0.67	ND–2.3	ND	König et al. (2017)
USA (various streams)	ND–116 (0.74)	ND–4.8 (ND)	ND–43 (ND)	Conley et al. (2017)
USA (Arizona)	< 0.005–0.8		9–170	Daniels et al. (2018)

Values between parentheses are medians (if available)

residential wastewater containing various endogenous hormones from human waste.

Risk assessment of surface water is conventionally done for specific contaminants by comparing their individual concentrations with respective guideline values. However, there are no official guideline values for in vitro effect levels, which are measures of the combined effects of the whole mixtures of chemicals extractable from the samples. Instead, the use of effect-based trigger (EBT) values as the highest effect levels with acceptable environmental risk was proposed, and EBT values have recently been developed for risk assessment of surface water using specific CALUX assays (Escher et al. 2015; van der Oost et al. 2017). The surface water EBT value for estrogenic activity with ER-CALUX (0.5 ng E2EQ/l, van der Oost et al. 2017) was derived as the 5th percentile hazard concentration of a species sensitivity distribution (SSD) of E2EQ-converted toxicity data of environmentally relevant estrogenic compounds. This EBT value was exceeded in the majority of the river water samples in this study, with the exception of three samples from Chennai and one from Bangalore. The EBT value for androgenic activity (3.4 ng DHTEQ/l) can be converted from the testosterone-EQ value derived for the ARGeneBLAzer assay using SSD analysis of chemicals in the Australian Guidelines for Water Recycling (Escher et al. 2015). All detectable androgenic activity levels in this study were above this EBT value. These estrogenic and androgenic activities indicate possible ecological risks from ER and AR agonists in most of the sampling locations. The EBT value for GR-CALUX (100 ng DexEQ/l, van der Oost et al. 2017) was derived from the lowest-observed effect concentration of Dex for increased gonadal somatic index in fathead minnow. Only BG1 and BG2 contained GR agonist activities higher than the EBT value.

Identification of major ER, AR, and GR agonists

Major compounds contributing to the detected CALUX activities in DP extracts were identified for only Hanoi and Bangalore sample sets, because of the low activities in all samples from Chennai and Jakarta. A representative pooled extract was prepared for each set by combining extracts with high activities. The pooled extracts were fractionated individually to find major ER, AR, and GR agonist fractions for subsequent chemical identification, as described in the "Materials and methods" section.

Estrogenic activities and major ER agonists in pooled DP extract fractions

ER-CALUX measurement results of Hanoi and Bangalore pooled extract fraction series (HN and BG) showed very similar activity patterns, with F3 + 4 and F9 + 10 as the major groups of estrogenic fractions (Fig. 2). The measured E2EQ levels of these fractions accounted for 67% and 18% of the total of all HN fractions, and 28% and 64% of all BG fractions, respectively. Estrogenic activity was also detected in F6 (9.7% and 4.5% of the total of HN and BG fractions, respectively) and a few other minor fractions (together contributing < 5% of the total).

The major estrogenic fractions F3 + 4 and F9 + 10 coincided with the predetermined elution points of the endogenous estrogens E3 and E2, respectively (Table S2). E3 was detected in HN-F3 + 4 and BG-F4, confirmed and quantified against external standard using LC–ToFMS analysis. Similarly, E2 was identified and quantified in HN-F9 and BG-F9 + 10. Based on their calculated E2EQ levels, E3 contributed 99% and 37% of the measured EQEQ levels in HN-F3 + 4 and BG-F4, and E2 contributed 96% and 77% in HN-F9 and BG-F9 + 10, respectively (Table 2). These results indicate that E3 and E2 were the major ER agonists in water samples from Hanoi and Bangalore, together accounting for respectively 80% and 56% of the total E2EQ levels of all HN and BG fractions. As the most important source of these endogenous hormones is human waste (Liu et al. 2009), their large contribution to the estrogenic activities of river waters reflects the heavy contamination by residential wastewater.

The minor estrogenic fraction F6 corresponded to the elution point of bisphenol A (BPA) (Table S2). Although BPA was indeed detected in both HN- and BG-F6 using LC–ToFMS analysis, its estrogenic activity contribution was negligible due to low potency (Table 2). The endogenous estrogen estrone (E1) was detected in HN-F15 and BG-16, also consistent with its predetermined elution point, and contributed 60% and 46% of the estrogenic activities in these fractions, respectively. $[M–H]^-$ features of synthetic estrogens such as EE2 and diethylstilbestrol, and well-known ER agonists such as alkyl phenols and BPA analogues were not found in the LC–ToFMS data of any estrogenic fractions. Further search for other ER agonists in minor estrogenic fractions was not attempted.

Androgenic activities and major AR agonists in pooled DP extract fractions

The androgenic activity patterns of HN and BG fraction series were also similar, with three major groups of androgenic fractions: F11 (HN) or F12 (BG), F18 (HN) or F17 + 18 (BG), and F21 (HN and BG) (Fig. 2). The

Table 2 ER, AR, and GR agonists identified in major agonist fractions of pooled dissolved phase extracts

Compound	REP [b]	Hanoi pooled extract (concentration factor = 1770[a])				Bangalore pooled extract (concentration factor = 483[a])			
		Fraction number	Concentration (ng/ml)	Calculated/measured refEQ[c] (ng/ml)	Calculated/measured refEQ[d] (%)	Fraction number	Concentration (ng/ml)	Calculated/measured refEQ[c] (ng/ml)	Calculated/measured refEQ[d] (%)
ER agonists									
Estriol (E3)	0.12	3 + 4	100	12/13	92	4	6.8	0.82/2.2	37
Bisphenol A (BPA)	3.7×10^{-5}	5	44	0.0016/1.8	< 1	5	68	0.0025/0.51	< 1
Estradiol (E2)	1.0	9	2.5	2.5/2.6	96	9 + 10	5.5	5.5/7.2	76
Estrone (E1)	0.016	15	5.6	0.090/0.15	60	16	2.2	0.036/0.079	46
AR agonists									
Dihydrotestosterone	1.0	18	7.2	7.2/9.5	76	18	3.9	3.9/10	39
GR agonists									
Dex/betamethasone	1.0	7	16	16/24	67	7	9.7	9.7/18	54
Clobetasol propionate	26	21	0.88	23/24	96	21 + 22	3.3	86/110	78

All concentration and activity values are normalized to the pooled extract volume (before fractionation)

[a]Water sample volume / extract volume ratio

[b]Relative potency factor (mass-based): E3, E1, and BPA vs E2 from Sonneveld et al. (2006), clobetasol propionate vs Dex from Suzuki et al. (2015)

[c]Reference agonist equivalent activity values

[d]Activity ratios relative to CALUX-measured activities of the respective fractions

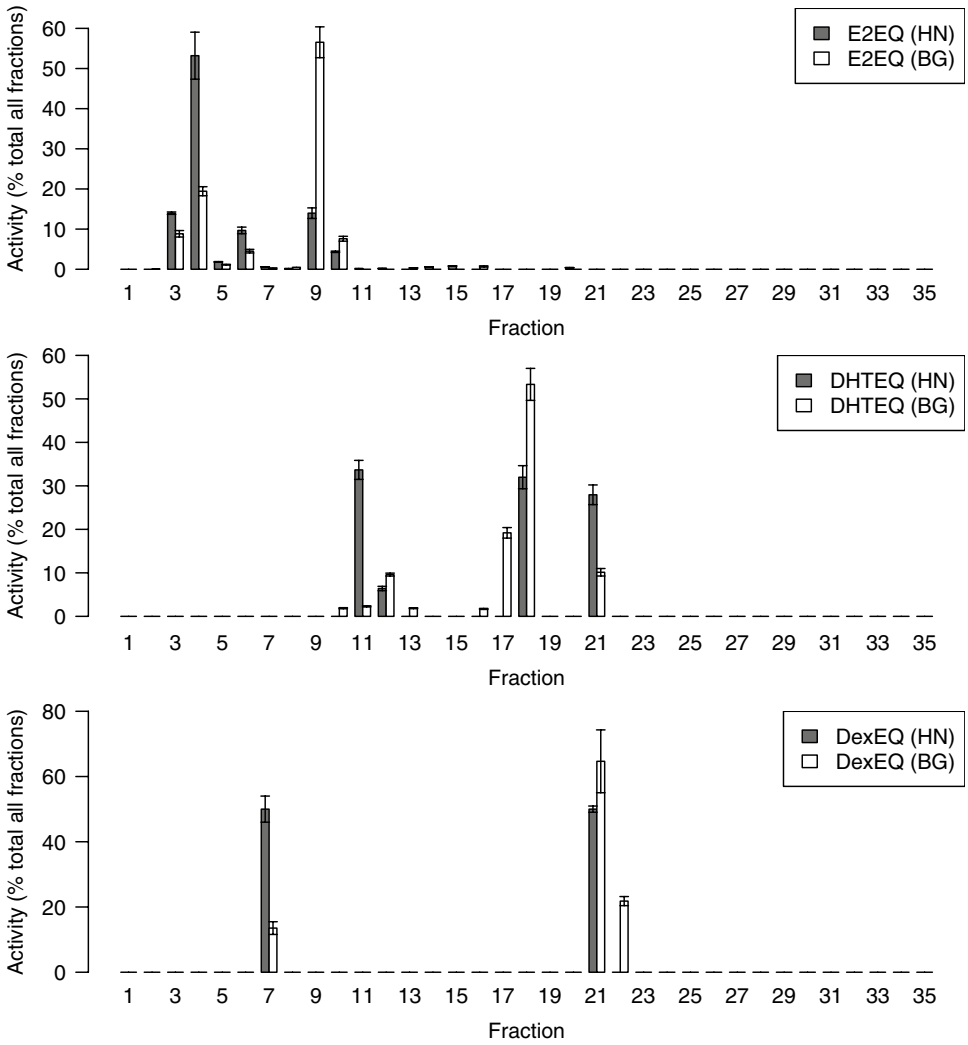

Fig. 2 CALUX-measured ER, AR, and GR agonist activities (top, middle, and bottom) in Hanoi (HN) and Bangalore (BG) pooled dissolved phase extract fractions (relative to the total of all fractions). Values are shown as mean ± SD of three measurements

measured DHTEQ levels of these fractions accounted for 34%, 32%, and 28% of the total level of all HN fractions, and 9.6%, 72%, and 10% of all BG fractions, respectively. F18 coincided with the predetermined elution point of the endogenous androgen DHT (Table S2), and its presence in this fraction was confirmed using LC–ToFMS analysis. DHT was a major androgenic activity contributor of F18, explaining 77% (HN) and 39% (BG) of the CALUX-measured DHTEQ levels (Table 2). The LC–ToFMS data obtained for the major androgenic fractions were screened for endogenous androgens, which were detected at relatively high concentrations in surface water from the UK (Thomas et al. 2002) and China (Liu et al. 2011). Several compounds were found with molecular features matching the $[M+H]^+$ ions of endogenous androgens (Table S3). However, identification and activity evaluation of these compounds were not possible due to the unavailability of standards, as many anabolic steroids are controlled substances in Japan. Nandrolone-type synthetic androgens (nandrolone and trenbolone) were not detected in any major androgenic fractions. A recent study in the Netherlands also found major androgenic fractions corresponding to unknown androgens when conducting effect-directed analysis of AR agonists in WWTP influent and effluent samples (Zwart et al. 2020).

Unknown androgen-related compounds detected in the major androgenic fractions included the following: compound A1 ($[M+H]^+$ $m/z = 287.2000$, tentative molecular formula $C_{19}H_{26}O_2$) in HN-F11 and BG-F12, compound A2 ($m/z = 287.2161$, $C_{19}H_{28}O_2$) in BG-F17, compound A3 ($m/z = 291.2312$, $C_{19}H_{30}O_2$) in BG-F18, and a pair of compounds A4/A5 ($m/z = 289.2166$, $C_{19}H_{28}O_2$) in both HN- and BG-F21. The retention times in LC–ToFMS and MS/MS product ion spectra from precursor $[M+H]^+$ are shown in Figs. S1, S2, S3, S4, S5, S6, S7, S8, and S9. Compound A1 was found to have the same molecular formula as the endogenous androgen 4-androstenedione (androst-4-ene-3,17-dione), but its retention time on the LC–ToFMS chromatogram did not match that of the authentic standard (Fig. S1). The MS/MS product ion spectrum from precursor $[M+H]^+$ of A1 was different from the spectra of 4-androstenedione

and boldenone, an anabolic steroid with the same molecular formula (Figs. S2 and S3). However, their spectra shared common fragment ions, suggesting some structural similarities. Other known androgens with the formula of $C_{19}H_{26}O_2$ include the endogenous 5-androstenedione and the synthetic anabolic steroid 1-androstenedione. Compound A2 was an isomer of the endogenous androgen testosterone, with different retention times and MS/MS spectrum (Figs. S4 and S5). The best match found for A2 using MS/MS spectra search against NIST2017 was 5β-androst-1-en-17β-ol-3-one (Fig. S6), a metabolite of boldenone. Compound A3 was an isomer of DHT, but not androsterone (Fig. S7). A3 could not be identified using MS/MS spectra search due to the low intensity of the precursor ion. The best matches for compounds A4 and A5 in NIST2017 were the endogenous androgens androstanediones (5α/5β-androstane-3,17-diones) (Fig. S9). 5α-androstanedione was reported to be a relatively potent androgen and major androgenic activity contributor in UK surface water (Thomas et al. 2002). Although causative compounds for major androgenic fractions F11/12 and F21 could not be identified, possible contribution from synthetic androgens warrants further studies on androgenic contaminants in surface water of lower income countries.

GR agonist activities and major GR agonists in pooled DP extract fractions

Only two fraction groups with significant GR agonist activities were found for both HN and BG fractions series: F7 and F21 (F21 + 22 for BG series) (Fig. 2). Target LC–MS/MS analysis of glucocorticoids showed that F7 contained Dex and/or betamethasone (coeluting isomers) (Table 2). The calculated DexEQ of HN-F7 and BG-F7, assuming that these fractions contained only the slightly more potent isomer Dex (REP of betamethasone = 0.82, Suzuki et al. 2015), accounted for 67% and 54% of the respective CALUX-measured levels. HN-F21 and BG-F21 + 22 were found to contain clobetasol propionate (Table 2) which explained most of the measured GR agonist activity (96% and 78%, respectively). The major GR agonist activity contribution of synthetic glucocorticoids such as clobetasol propionate and Dex in water samples from Hanoi and Bangalore could be expected considering their much higher potency compared with endogenous glucocorticoids (Suzuki et al. 2015). Clobetasol propionate and Dex were previously reported as the most important DexEQ contributors in surface water from Czech and Switzerland (Macikova et al. 2014). Clobetasol propionate was also a major GR agonist in water from Japanese WWTPs (Suzuki et al. 2015). Depending on the glucocorticoid usage patterns in the catchment areas, substantial DexEQ contribution from other synthetic glucocorticoids such as betamethasone valerate and triamcinolone actinide was also reported (Schriks et al. 2013; Suzuki et al. 2015).

Conclusions

ER and AR agonist activities were frequently detected in water samples from several rivers in Bangalore and Chennai (India), Jakarta (Indonesia), and Hanoi (Vietnam), with substantially higher levels in Bangalore and Hanoi rivers, where GR agonist activities were also detected. Effect-directed analysis of pooled samples showed that primary contributing estrogens, androgens, and glucocorticoids in the Bangalore and Hanoi rivers were similar. High ER and AR agonist activity contribution from endogenous estrogens (E2 and E3) and androgen (DHT) rather than industrially produced chemicals reflected the river water pollution in these cities with residential wastewater. In contrast, the major glucocorticoids identified were the anti-inflammatory drugs dexamethasone and clobetasol propionate rather than endogenous hormones. Several unknown isomers of endogenous androgens may also have contributed to the AR agonist activities. Although the sample set of present study may not reflect recent contamination levels, the high activity contribution of endogenous estrogens and androgens, most likely originated from human waste, implies that the overall estrogenic and androgenic activities in surface water will not diminish without significant improvement of the wastewater treatment systems in the investigated cities. Considering the high activity levels found in the present study compared with the effect-based trigger (EBT) values for surface water, further monitoring and risk assessment of the water contamination by ER, AR, and GR agonists in lower income countries are necessary.

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Author contribution N. M. Tue, L. H. Tuyen, P. H. Viet, A. Sudaryanto, A. Subramanian, S. Tanabe, and T. Kunisue planned the study and conducted the sampling. N. M. Tue and L. H. Tuyen performed the sample pretreatment and bioassays. G. Suzuki developed the fractionation method. N. M. Tue and H. Matsukami performed the LC–ToFMS analysis. All authors contributed to the data interpretation, manuscript preparation, and approval.

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Declarations

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