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Evaluation of mixture effects of endocrine active substances in wastewater using CALUX reporter-gene assays

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ABSTRACT

Endocrine active substances (EAS), which are commonly used in pharmaceuticals and personal care products, are released into surface water mainly through WWTP effluents and have been shown to cause adverse effects in aquatic organisms. In wastewater, a variety of EAS with different hormonal activities is present, which can lead to additive effects or mask an endocrine activity. To investigate hormonal combination effects, with a focus on estrogen and androgen-modulators, influent samples from municipal and hospital wastewater treatmenr plants were spiked with 17α-ethinylestradiol, toremifene, 17α-methyltestosterone and bicalutamide and analyzed using *in vitro* reporter gene CALUX assays. All wastewaters caused endocrine activities in human cells, which were modified by adding one or several endocrine active substances. As expected, estrogenic activity was reduced in presence of the anti-estrogenic toremifene and androgenic activity decreased with the anti-androgen bicalutamide. In general, substance addition caused a similar trend in altered endocrine activities; however, their intensities differed between the wastewaters. Our results indicate that masking effects, leading to a suppressed biological signal, are of significant importance in the assessment of complex water samples, and combination effects rather than single substances determine the final biological effect. This emphasizes the need of effect-based tools in the assessment of water samples.

1. Introduction

Increasing amounts of micropollutants in surface waters are a threat to the aquatic ecosystem, drinking water quality and finally to human health (Bergmann et al., 2011; Kidd et al., 2014; Maletz et al., 2013). Organic micropollutants comprise pharmaceuticals and personal care products (PPCP), steroid hormones, surfactants, industrial chemicals and pesticides. Organic trace substances reach the aquatic environment mainly through wastewater treatment plant (WWTP) effluents, resulting in ecotoxicologically relevant concentrations in surface waters (Luo et al., 2014; Umweltbundesamt, 2015). Endocrine active substances (EAS) can be synthetic hormones, which are commonly used as active agents in PPCP, natural hormones, which are produced and excreted by humans, e.g. estrogens and androgens, or industrial chemicals. They can affect the endocrine system of organisms and differ in their biodegradation behavior, chemical and physical properties, mobility and toxicological relevance. Because of their high bioactive potential, pharmaceuticals have a high toxicological risk even at very low concentrations (Escher et al., 2011). EAS occur in WWTP influents in concentrations below 1 µg/l (Luo et al., 2014). In WWTP effluents EAS are usually measured in much lower concentrations below 10 ng/l, but can reach 100 ng/l and cause endocrine-related adverse health effects in wildlife and human (Gies, 2006; Kidd et al., 2014; Luo et al., 2014; Kunz et al., 2017). National and international directives, e. g. the EU water framework directive (WFD), demand the evaluation of potentially harmful chemicals to achieve a good quality of water bodies (European Parliament and Council, 2000; Oberflächengewässerverordnung, 06/2016). Recently, the estrogenic compounds estrone, 17β-estradiol and 17α-ethinylestradiol were added to the *watch list* as supplement to the WFD, and throughout Europe environmental quality standards (EQS) for estrogens and other micropollutants in surface waters have been discussed (European Parliament and Council, 2015). However, in contrast to estrogenic substances, there is only little known about effects of androgens in the environment (Bellet et al., 2012; Vulliet et al., 2007). Also studies on mixture effects of EAS in environmental samples, especially antagonistic activities, in complex matrices like wastewater are still rare and require more consideration (Gehrmann et al., 2018; Ihara et al., 2015; Kunz et al., 2017).

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Besides additive and synergistic mixture effects, antagonistic effects can occur, leading to a suppressed biological signal, which is called masking effect (Shi et al., 2016). Investigations of wastewater treatment processes showed androgenic and estrogenic effect changes in different treatment steps. Studies of hospital and urban WWTPs have revealed that influents show estrogenic as well as androgenic activities, which, in most cases, can be reduced or eliminated during advanced treatment (Chang et al., 2011; Gehrmann et al., 2018; Itzel et al., 2017; Neale et al., 2017). However, after ozone treatment of hospital wastewater there are documented cases of atypical increasing estrogenic activities (Bieling, 2011; Maletz et al., 2013; Nafo et al., 2012). For urban wastewater treatment such effects are not documented. In another study of hospital wastewater anti-androgenic effects were detected in influent samples and were slightly reduced by membrane bioreactor and ozone treatment, while anti-estrogenic effects increased (Itzel et al., 2018). Due to higher amounts of active ingredients of pharmaceuticals in hospital wastewater, antagonistic combination effects are a probable explanation.

Effect-based analysis with bioassays is a useful tool to determine the presence of bioactive substances based on the measured biological effect. Sensitive cell-based bioassays are available for the detection of EAS, allowing the determination of activities as sum parameter for environmental samples (Escher et al., 2014; Hecker and Hollert, 2011). This is of particular advantage in samples with unknown compounds and mixtures, in which combination effects are probable to occur. In this study, CALUX assays (BioDetection Systems, Amsterdam, NL) were chosen to measure estrogenic and androgenic effects. For the detection of estrogenic effects two cell lines were compared: genetically modified osteosarcoma cells (U2OS ERα) and breast cancer cells (T47Dluc) (Legler et al., 1999; Sonneveld et al., 2005). In U2OS ERα cells only the estrogen receptor α is analyzed, while T47Dluc cells possess the estrogen receptors α and β. Furthermore, T47Dluc cells cover glucocorticoids, and not only estrogens, thereby including a different pathway (Brinkmann et al., 2014).

The aim of this study is to address the relevance of endocrine combination effects in real water samples, which are complex mixtures of known and unknown chemicals. Up to now, the common way to analyze environmental water samples covers only a fraction of trace substances due to restrictions by target analyses or sample enrichment to reach higher sensitivity levels of biological and instrumental methods (Dopp et al., 2019). To measure a net toxicity of a sample it is important to keep samples unchanged to avoid loss of (toxic) substances. During sample enrichment, e.g. by solid phase extraction, and target analysis substances and information gets lost. But because of very low concentrations of trace substances in environmental samples and wastewater treatment plant effluents such applications are necessary. However, WWTP influents are a complex mixture with usually high biological potentials, even without enrichment. So, in this study influent samples of WWTPs were used as model water for complex mixtures to overcome methodical restrictions. Model substances were selected to investigate additive as well as inhibitory effects of estrogen- and androgen-modulators. It was expected that masking effects contribute significantly to the overall toxicological effect of wastewater samples.

2. Materials and methods

2.1. Test substances and chemicals

Test substances were selected that are known to inhibit or activate the hormone-regulated pathways in humans and are of increasing environmental relevance. Four endocrine active components of pharmaceuticals were chosen for this purpose, which are used in cancer therapy or treatment of hormonal disorder (Law et al., 2014; Moltmann et al., 2007). 17α-Ethinylestradiol (EE2) is a synthetic steroid hormone with an estrogenic activity of natural estrogens. It is classified as highly toxic to aquatic organisms and known to cause adverse effects in the aquatic environment. Toremifene (Tor) is a non-steroidal selective estrogen receptor modulator (SERM). It is an anti-estrogenic agent in breast cancer therapy, but can also act estrogenic (Law et al., 2014). 17α-Methyltestosterone (MT) is a synthetic androgen, which is mainly metabolized to 17β-estradiol and dihydrotestosterone in the liver. Its androgenic potential is 2.5 times higher than of testosterone (Law et al., 2014). Bicalutamide (Bic) is a non-steroidal anti-androgenic agent. Its consumption has risen in recent years, thus leading to an increased input to the environment and bearing potential risks (Bergmann et al., 2011).

EE2 (> 99%) and MT (> 99%) were purchased from Sigma Aldrich Chemie GmbH (Munich, Germany), Tor (≥98%) and Bic (≥98%) from VWR GmbH (Darmstadt, Germany). Stock solutions of the substances were prepared in dimethyl sulfoxide (DMSO, > 99%, Sigma Aldrich) at the highest soluble concentration and stored for up to six months below −15 °C in the dark.

The reference substances of the CALUX assays 17β-estradiol (E2, ≥98%), flutamide (Flu, ≥ 99%) and tamoxifen (TMX, ≥ 98%) were purchased from Sigma Aldrich, the reference substance dihydrotestosterone (DHT ≥ 98%) from VWR. Stripped fetal calf serum, lysis solution, and substrate mix were purchased from BioDetection Systems e.V. (BDS; Amsterdam, the Netherlands).

2.2. Mixtures of endocrine active substances

The selected test substances were spiked at the half maximal effective concentration (EC_{50}) of the individual substances. The EC_{50} value was chosen to clearly distinguish the signal of the test substances from effects of the influent samples. The concentrations of the test substances were derived from a dose-response curve in CALUX (chemically activated luciferase gene expression) assays using U2OS AR, U2OS ERα and T47Dluc cells (Table 1). The selected test substances were investigated individually and as equipotent mixtures. Mixtures contained EE2 and toremifene, MT and bicalutamide or all four substances. The final test concentrations are listed in Table 1.

Table 1
Overview on test substances with a specific endocrine effect, the respective cell lines as well as test concentrations (EC_{50}) derived from a dose-response relationship in CALUX assays. ER: estrogen receptor, AR: androgen receptor.

Test substance	CALUX system	Cell line(s)	Measured effect	Derived test concentration (EC_{50}) [ng l^{-1}]
17α-ethinylestradiol	ER CALUX	U2OS ERα, T47Dluc	ER activation	1.8
toremifene	ER-anti CALUX	U2OS ERα, T47Dluc	ER inhibition	5×10^4
17α-methyltestosterone	AR CALUX	U2OS AR	AR activation	3.9×10^2
bicalutamide	AR-anti CALUX	U2OS AR	AR inhibition	4×10^4

2.3. Sampling and sample preparation

To investigate combination effects in complex mixtures, WWTP influents were used as matrix for the selected test substances with known endocrine activities.

Random samples of untreated wastewater (influent) were taken from two municipal (M1, M2) and two hospital (H1, H2) WWTP after the screening stage, and filtered the same day over a filter circle (ash free; Schleicher & Schuell GmbH, Dessel), a 5 µm syringe filter (PALL Acrodisc, Supor Membran) and finally a 0.2 µm cellulose acetate syringe filter (VWR) for sterile filtration. Aliquots were stored in glass vials for up to six months below −15 °C in the dark.

Spiked influent samples were prepared in sterile glass vials just before each test. The stock solution of each test substance in DMSO was diluted in ultrapure water and in a last step the diluted substances were added to the different influent samples alone or combined. The spiked samples were 10-fold diluted in the test to reach the desired substance concentration and a 1:10 dilution of the influents. The final DMSO concentration in the bioassays was below 0.01%, except for samples containing toremifene, which had a DMSO concentration of 0.1%. In this case, a solvent control was measured in addition.

2.4. Cell culture maintenance

Genetically modified human breast cancer cells (T47Dluc) and osteosarcoma (U2OS) ERα cells (both with a pERE-TATA-Luc reporter gene) were used for the determination of estrogenic agonist and antagonist effects in the ER or ER-anti CALUX reporter gene assay. U2OS AR cells (pHRE-TATA-Luc reporter gene) were used to measure androgenic agonist and antagonist activities in the AR or AR-anti CALUX assay. All cell lines were purchased from BDS. Cells were cultured according to van der Burg et al. (2010) at 37 °C (± 1 °C), 5% CO_2 and 95% humidity in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) with phenol red (c.c.pro GmbH, Oberdorla, Germany), supplemented with 7% fetal calf serum (Thermo Fisher, Braunschweig, Germany), 0.5% gentamycin (c.c.pro GmbH), and 1% non-essential amino acids (c.c.pro GmbH).

2.5. Cytotoxicity measurement

To determine possible cytotoxic effects of the influents and the test substances the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983) was performed with U2OS AR, U2OS ERα and T47Dluc cells before application of the CALUX assays with these cell lines. The cultivation and exposition of the cells was performed the same way as in the CALUX assays to ensure equal test conditions. The negative control in the MTT assay was cell culture medium (supplemented DMEM/F12 medium without phenol red), the positive control was 25% DMSO. The individual test substances were tested in several concentrations to determine the highest non-cytotoxic concentration before measuring the dose-response curves in the CALUX assays. The dilution of the influent samples in the test was 1:10. After 24 h of exposure, the cells were stained with MTT, lysed and the absorbance measured at 595 nm. The viability of the cells was calculated in relation to the negative control. Only samples without cytotoxic effects, i.e. cell viability > 70% according to DIN EN ISO 10993–5:2009–06, were considered for the determination of endocrine effects.

2.6. CALUX assays

The estrogenic potential of the samples was measured with the ER CALUX, the androgenic potential was determined with the AR CALUX (Sonneveld et al., 2005). The inhibition of the estrogenic and androgenic receptor, respectively, was measured using the antagonistic CALUX test procedure (van der Burg et al., 2010a, 2010b). Briefly, cells were seeded in supplemented DMEM/F12 medium without phenol red with a density of 10^4 cells/100 µl and incubated overnight. Then, cells were exposed for 24 h (± 10 min) to samples and reference substances, respectively. A concentration series of E2 (2.7×10^{-3} - 27 ng/l) was used as reference in the ER CALUX, DHT (0.29–8.7×10^3 ng/L) was used in the AR CALUX. For the antagonistic tests, the exposure medium was spiked with 1.6 ng/l E2 for the ER-anti CALUX or 87 ng/l DHT for the AR-anti CALUX. A concentration series of TMX (20–2×10^6 ng/L) was used as reference in the ER-anti CALUX, flutamide (1.4×10^2 - 1.4×10^6 ng/L) was used in the AR-anti CALUX. The influent samples were spiked with test substances just before the exposure. Test substances were added to each of the four wastewaters individually or mixed. Final substance concentrations are given in Table 1. The final dilution of the wastewaters was 1:10. Influent samples, spiked influent samples, standards including positive control (reference substance), negative control (cell culture water) and solvent control (DMSO) were applied on each test plate in triplicate. After exposure, cells were lysed and the lysate transferred into a white plate. Directly after substrate addition the luminescence was measured using a microplate reader (Tecan GENios, Tecan Group Ltd., Maennedorf, Switzerland). The output was given in relative light units (RLU).

2.7. Calculations

The dose-response relationships, EC_{50} values of the test substances and the standards, and equivalent concentrations (EQ) of the (spiked) influents were calculated with a calculation sheet provided by BDS. The sigmoidal curve was derived from a four-parameter logistic function. The limit of detection (LOD) and limit of quantification (LOQ) are fixed quality parameters in the CALUX assays. In the ER CALUX they are 0.2 (LOD) and 0.7 (LOQ) ng EEQ/l, in the ER-anti CALUX 1486 and 4829 ng TMX-EQ/l, in the AR CALUX 1.2 and 4.1 ng DHT-EQ/l and in the AR-anti CALUX 8839 and 27621 ng Flu-EQ/l. For each test plate individual LODs and LOQs were determined, which were less or equal to the fixed quality values.

To evaluate endocrine effects of the single test substances compared to mixtures, relative transcriptional activities were calculated based on the OECD guidelines TG455 (OECD, 2016a) and TG458 (OECD, 2016b). Agonistic effects were related to the maximum response of the standard substance (positive control, PC), while antagonistic effects were related to the response of the agonistic spike (negative control, NC). First, all values were normalized by subtracting the mean value of the vehicle control from each well. Then, normalized values of the samples were divided by the mean value of the respective normalized control (control = 100%) to gain relative activities of the samples.

Statistical analysis was performed with GraphPad Prism 8.1 (GraphPad Software, Inc., La Jolla, CA, USA). To assess differences in the mean of relative activities, RM (repeated measures) two-way ANOVA (1st factor WWTPs, 2nd factor substances) was applied with Tukey's multiple comparison test (95% confidence interval). A normal distribution was approved using the Shapiro-Wilk normality test. Significance levels are classified into $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), $p \geq 0.05$ (not significant, n. s.).

3. Results

3.1. Estrogenic effects in U2OS ERα cells

In U2OS ERα cells the four tested wastewaters had relative estrogenic activities of 24% (H2, < LOQ, 0.28 ng EEQ/l), 27% (H1, < LOQ, 0.38 ng EEQ/l), 31% (M2, < LOQ, 0.63 ng EEQ/l) and 44% (M1, 0.86 ng EEQ/L). An inhibition of the estrogen receptor by the influents was not detected (Table 2). As suspected, the influent samples spiked with the agonist EE2 caused estrogenic activities, which were significantly higher than those of the original samples, reaching relative activities up to 77% (Fig. 1a). The addition of Tor to the wastewaters

Table 2
Change of estrogenic effects in U2OS ERα cells by endocrine active substances.

Endocrine activity	Influent origin	Relative activity of Inf only [%]	Inf only vs. Inf + EE2	Inf + EE2 vs. Inf + EE2 + Tor	Inf only vs. Inf + Tor	Inf + Tor vs. Inf + EE2 + Tor	Inf only vs. Inf + EE2 + Tor	Inf only vs. Inf + EE2 + Tor + Bic + MT
ER activation	**M1**	44	↑*	↓***	↓***	(↑)	↓***	↓***
	M2	31	↑***	↓***	↓**	(↑)	↓*	↓*
	H1	27	↑***	↓***	↓**	(↑)	↓*	↓*
	H2	24	↑***	↓***	↓***	(↑)	↓**	↓**
ER inhibition	**M1**	–	–	↑***	↑***	(↑)	↑***	↑***
	M2	–	–	↑***	↑***	(↑)	↑***	↑***
	H1	–	–	↑***	↑***	(↑)	↑***	↑***
	H2	–	–	↑***	↑***	(↑)	↑***	↑***

Estrogen receptor (ER) activation and inhibition of the unspiked influents, and after substance addition to the influent samples from two municipal (M1, M2) and two hospital (H1, H2) WWTP spiked with: EE2 = 17α-ethinylestradiol, Tor = toremifene, MT = 17α-methyltestosterone, Bic = bicalutamide. Increase or decrease of estrogenic effects indicated with arrows, significance levels indicated by asterisks. Inf = influent.
***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, arrow in brackets: $p \geq 0.05$ not significant, dash: no effect change/effect below minimum.

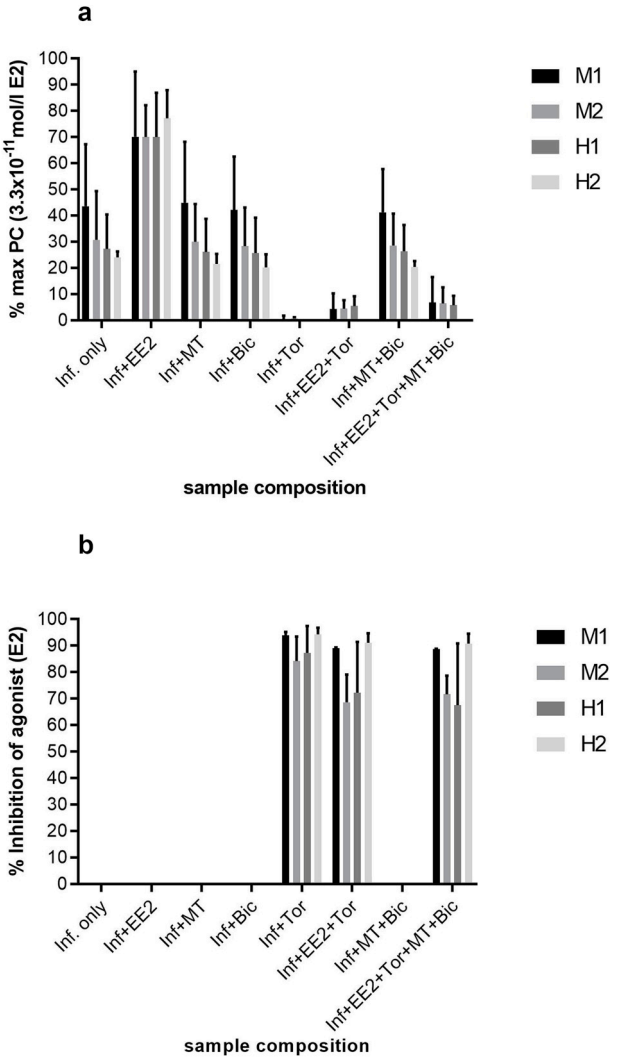

Fig. 1. Estrogenic agonistic (a) and antagonistic (b) relative activities in U2OS ERα cells caused by influent samples from two municipal (M1, M2) and two hospital (H1, H2) WWTP spiked with endocrine active substances. Mean and standard deviation of 3 independent replicates. In case of values below minimum columns are missing in the diagram. Inf = Influent, EE2 = 17α-ethinylestradiol, MT = 17α-methyl-testosterone, Bic = bicalutamide, Tor = toremifene, E2 = 17β-estradiol, PC = positive control E2.

significantly reduced and almost eliminated the estrogenic activity. The combined addition of EE2 and Tor as well as the addition of all four test substances caused a significant reduction of the initial estrogenic activity to below 7%. Bic and MT had no significant effect on the measured estrogenic activities of the influents. In all influents the addition of Tor led to antagonistic estrogenic activities in the cells of 86%–98% (Fig. 1b). The antagonistic effect was reduced slightly in combination with EE2.

3.2. Estrogenic effects in T47Dluc cells

In T47Dluc cells the four tested wastewaters had relative estrogenic activities of 19% (H2, EEQ < LOD), 38% (M2, < LOQ, 0.40 ng EEQ/l), 45% (H1, < LOQ, 0.36 ng EEQ/l) and 61% (M1, < LOQ, 0.45 ng EEQ/l). Regarding the relative activities, except for wastewater H2 the original influents caused higher estrogenic activities in T47Dluc cells than in U2OS ERα cells (Table 2, Table 3), while EEQ values were higher in U2OS ERα than in T47Dluc cells. This may seem unexpected, but can be explained by different maximum effect levels. To reach the maximum effect level in T47Dluc cells a lower concentration of E2 is necessary than in U2OS cells, resulting in a higher relative activity at lower E2 concentrations. An inhibition of the estrogen receptor by all influents was also detected with relative antagonistic activities of 4% (M1, 203 ng TMX-EQ/l), 6% (M2, 197 ng TMX-EQ/l), 14% (H1, 629 ng TMX-EQ/l) and 38% (H2, 2246 ng TMX-EQ/l). The addition of the single test substances or substance mixtures had a weaker influence on the initial activity in T47Dluc cells than in U2OS ERα cells. In wastewater M1 the estrogenic activity was barely changed when spiked with EE2, and in the other three samples there was a slight increase of the estrogenic activity (Fig. 2a). The estrogenic activity was reduced in all wastewaters when spiked with toremifene, whereas the combination with EE2 led again to an increase of the estrogenic activities, but not reaching the level of EE2 alone. The addition of toremifene to the influent samples caused an increase of the estrogen antagonistic activity (Fig. 2b). The effects of the test substances on the measured activities in wastewater differed significantly between the different wastewaters. The mix of EE2 and toremifene increased the anti-estrogenic activity of the samples H1, H2, and M1, but to a lower extent than toremifene. In wastewater M2 the this mixture eliminated the anti-estrogenic activity.

Table 3
Change of estrogenic effects in T47Dluc cells by endocrine active substances.

Endocrine activity	Influent origin	Relative activity of Inf only [%]	Inf only vs. Inf + EE2	Inf + EE2 vs. Inf + EE2 + Tor	Inf only vs. Inf + Tor	Inf + Tor vs. Inf + EE2 + Tor	Inf only vs. Inf + EE2 + Tor	Inf only vs. Inf + EE2 + Tor + Bic + MT
ER activation	**M1**	61	(↑)	(↓)	↓**	(↑)	(↓)	(↓)
	M2	38	(↑)	(↓)	↓*	↑***	(↑)	(↑)
	H1	45	(↑)	(↓)	↓**	(↑)	(↓)	(↓)
	H2	19	(↑)	(↓)	(↓)	(↑)	(↓)	(↓)
ER inhibition	**M1**	4	(↑)	↑**	↑**	(↓)	(↑)	(↑)
	M2	6	(↓)	↑*	↑*	↓***	(↓)	(↓)
	H1	14	(↓)	↑**	↑**	(↓)	(↑)	(↑)
	H2	38	(↓)	(↑)	(↑)	(↓)	(↑)	(↑)

Estrogen receptor (ER) activation and inhibition of the unspiked influents, and after substance addition to the influent samples from two municipal (M1, M2) and two hospital (H1, H2) WWTP spiked with: EE2 = 17α-ethinylestradiol, Tor = toremifene, MT = 17α-methyltestosterone, Bic = bicalutamide. Increase or decrease of estrogenic effects indicated with arrows, significance levels indicated by asterisk. Inf = influent.
***p < 0.001, **p < 0.01, *p < 0.05, arrow in brackets: p ≥ 0.05 not significant.

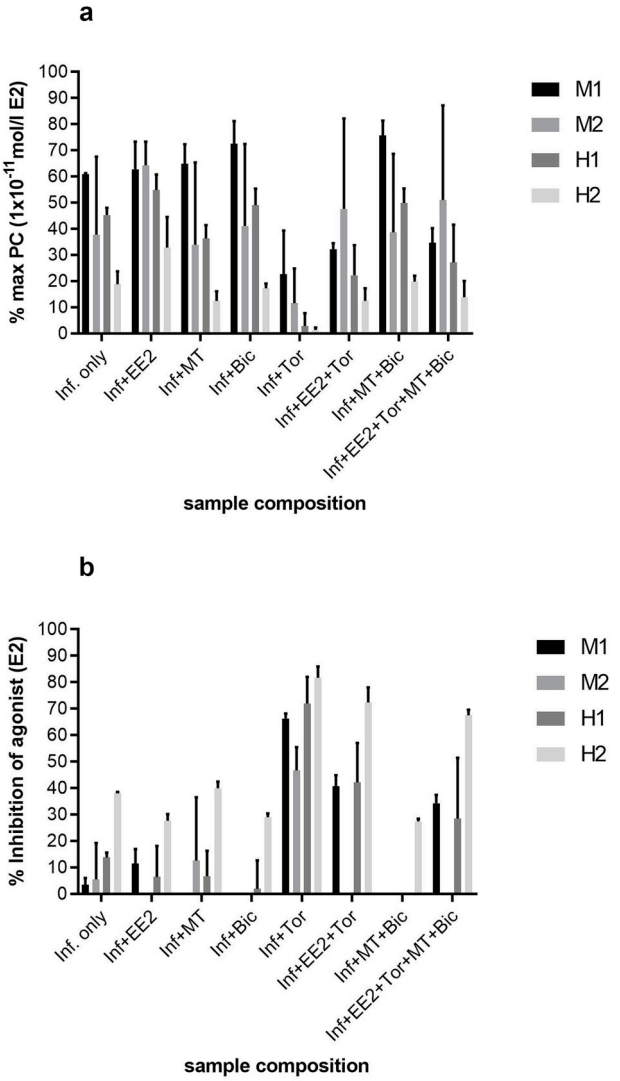

Fig. 2. Estrogenic agonistic (a) and antagonistic (b) relative activities in T47Dluc cells caused by influent samples from two municipal (M1, M2) and two hospital (H1, H2) WWTP spiked with endocrine active substances. Mean and standard deviation of 3 independent replicates. In case of values below minimum columns are missing in the diagram. Inf = Influent, EE2 = 17α-ethinylestradiol, MT = 17α-methyl-testosterone, Bic = bicalutamide, Tor = toremifene, E2 = 17β-estradiol, PC = positive control E2.

3.3. Androgenic effects in U2OS AR cells

In the AR CALUX in all four tested wastewaters androgenic activities were detected, with the highest relative activity of 6% in M1 (7.94 ng DHT-EQ/l). Androgenic antagonist activities were detected in three influents with levels of 35% (H2, 56413 ng Flu-EQ/l), 20% (H1, 31938 ng Flu-EQ/l) and 11% (M2, 11700 ng Flu-EQ/l). Influent samples spiked with the agonist MT caused androgenic activities which were significantly higher than those of the original influents (Table 4, Fig. 3a). The addition of Bic to this mixture resulted in a slight to significant reduction of the activity caused by MT. The low androgenic agonist activity of the influent was lowered only in influent M1 when Bic was added. In all influents, the addition of Bic led to an increase of the androgenic antagonistic activity by 42–47% (Fig. 3b). The antagonistic activities measured in the influents H1, H2 and M2 were removed by adding MT to the samples. The combined addition of bicalutamide and MT to the wastewaters led to an elimination of the measured activity in sample M2 (lowest anti-androgenic activity), while the antagonistic activities of the hospital influent samples did not change. EE2 and Tor had no significant effect on the anti-androgenic activity. The addition of all four test substances led to a slight decrease (H2) or removal (H1) of the anti-androgenic activity compared to the activity caused by the samples spiked with Bic, and to a slight increase in wastewater M2.

4. Discussion

4.1. Masking effect of estrogen antagonist

The comparison of four different endpoints in the CALUX assay revealed that municipal as well as hospital WWTP influents cover several endocrine activities but with different intensities. These may be changed in combination with other endocrine substance. Independent from the influent matrix, the results approve the occurrence of masking effects in the presence of antagonistic substances. The masking effect is very distinct in U2OS ERα cells when applying estrogen modulators. After addition of toremifene the estrogenic effect was lowered, although E2 was still in the sample. If only the agonistic assay was applied, this information would suggest a minor concentration of estrogens in the sample. Only the ER-anti CALUX assay revealed the anti-estrogenic effect, which is relevant for the mixture toxicity. Here, in case of estrogen receptor modulators, the antagonistic effect dominated over the agonistic effect, because toremifene masked the effects of E2. These results confirm the suggestion of Ihara et al. (2014) that anti-estrogenic compounds in wastewater can suppress the effect of estrogenic substances.

Table 4
Change of androgenic effects in U2OS AR cells by endocrine active substances.

Endocrine activity	Influent origin	Relative activity of Inf only [%]	Inf only vs. Inf + MT	Inf + MT vs. Inf + MT + Bic	Inf only vs. Inf + Bic	Inf + Bic vs. Inf + MT + Bic	Inf only vs. Inf + MT + Bic	Inf only vs. Inf + EE2+Tor + Bic + MT
AR activation	**M1**	6	↑***	(↓)	(↓)	↑**	↑*	↑*
	M2	3	↑***	(↓)	(↓)	↑*	↑*	↑*
	H1	1	↑***	(↓)	(↓)	↑***	↑***	↑**
	H2	–	↑***	↓***	(↓)	(↑)	(↑)	↑**
AR inhibition	**M1**	–	(↓)	(↑)	(↑)	↓***	(↓)	(↓)
	M2	11	(↓)	(↑)	(↑)	↓**	(↓)	(↓)
	H1	20	↓**	↑**	(↑)	(↓)	(↑)	(↓)
	H2	35	(↓)	(↑)	(↑)	(↓)	(↑)	(↑)

Androgen receptor (AR) activation and inhibition of the unspiked influents, and after substance addition to the influent samples from two municipal (M1, M2) and two hospital (H1, H2) WWTP spiked with: EE2 = 17α-ethinylestradiol, Tor = toremifene, MT = 17α-methyltestosterone, Bic = bicalutamide. Increase or decrease of estrogenic effects indicated with arrows, significance levels indicated by asterisk. Inf = influent.
***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, arrow in brackets: $p \geq 0.05$ not significant, dash: effect below minimum.

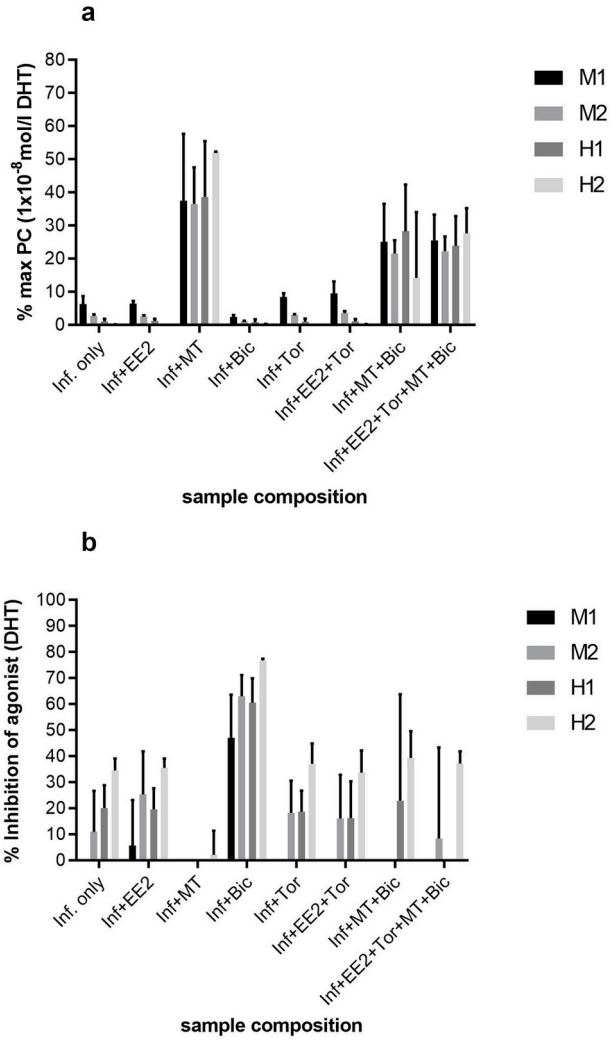

Fig. 3. Androgenic agonistic (a) and anti-androgenic (b) relative activities in U2OS AR cells caused by influent samples from two municipal (M1, M2) and two hospital (H1, H2) WWTP spiked with endocrine active substances. Mean and standard deviation of 3 independent replicates. In case of values below minimum columns are missing in the diagram. Inf = Influent, EE2 = 17α-ethinylestradiol, MT = 17α-methyl-testosterone, Bic = bicalutamide, Tor = toremifene, DHT = dihydrotestosterone, PC = positive control DHT.

4.2. Masking effect of androgen agonist

Moreover, not only antagonists can mask agonists, but also agonistic compounds may cover an antagonistic activity. This case is evident in the effects of the androgen modulators on the androgen receptor: The androgenic response of methyltestosterone dominates the anti-androgenic activity of bicalutamide, although to a lower extent as observed for the estrogen modulators. The bioassay reveals a net effect of all the substance activities. In the AR and AR-anti CALUX this is shown in relative activities of the substance mix, which lie between the effect levels of the single substances.

4.3. Matrix effects of complex water samples

Although mostly a similar trend of the activities after substance addition can be observed, the intensity of the activation and inhibition, respectively, differs between the influents. So, in addition to the known and quantified activity of target EAS, the activities of unknown EAS need to be considered when assessing mixture samples. This is of major importance regarding regulatory limits. Often limit values relate to concentrations of single chemicals based on their known toxicological potential. Mixture effects are rarely considered. As shown in this study, each complex sample can have a different endocrine potential, though some substance concentrations are the same. An overall assessment can be reached by analyzing such samples using bioassays in addition to chemical analyses.

4.4. Choice of cell line depends on research question

The ER CALUX assay was applied with two cell lines, resulting in different responses in U2OS ERα cells and T47Dluc cells. Different responses when analyzing estrogenic and anti-estrogenic effects in these cell lines can be explained by their different origins and cellular pathways. These aspects can explain the differences in the CALUX response. Interestingly, in T47Dluc cells it was possible to measure both estrogenic and anti-estrogenic effects in the same sample. The application of T47Dluc cells may complicate the assessment of the estrogenic potential of a specific substance, but at the same time it may enable the assessment of metabolites, as has been shown by Brinkmann et al. (2014). However, one should keep in mind the glucocorticoids when using T47Dluc cells. When considering bioanalytical tools for the assessment of mixtures also other bioassays should be considered based on the research question and desired application. In addition to human cell lines, yeast cells can be used for the detection of endocrine effects (Gehrmann et al., 2018; Hettwer et al., 2018). CALUX assays are more sensitive than yeast-based assays and provide human cell lines, but yeast cells can be applied with contaminated samples and do not need sterile conditions (Gehrmann et al., 2018). Hence, for the choice of a

bioassay and a cell line a well-defined research question is essential.

4.5. Effect-directed analysis needs more consideration in water monitoring

In hospital wastewater different water compositions and endocrine effects are expected due to a variety of pharmaceutical agents (Gehrmann et al., 2018; Itzel et al., 2017). In the tested samples, the endocrine effects did not differ between municipal and hospital wastewater, but the hospital influent samples developed higher antagonistic effects. Especially androgenic and antagonistic substances need more consideration, since up to now they are scarcely investigated and not regulated, though they contribute to the overall effect (Altenburger et al., 2018). This emphasizes the importance of further investigations on sensitive effect-based analysis tools in addition to instrumental chemical analyses, resulting in effect-directed analysis for the identification of toxicity drivers and the monitoring of surface waters (Brack et al., 2016; Snyder and Leusch, 2018; Dopp et al., 2019). This is of particular importance during treatment steps, which eliminate specific substances, e. g. by oxidative processes, resulting in a different mixture composition and changed mixture effects. In addition to chemical analysis of compounds of concern, a panel of bioassays can cover many toxic effects to adequately assess environmental samples.

5. Conclusions

Results of the present study led to the conclusion that masking effects are of significant relevance in the assessment of water samples with complex matrices like influent samples. It is recommended to test for both antagonistic and agonistic effects with different cell systems, to gain the best possible characterization of a sample's endocrine potential. Furthermore, a specific EAS may cause effects of different magnitude in dependency of the sample matrix. So, it is very important to take into account combination effects in environmental samples. Effect-based tools are the best choice to overcome these analytical challenges, and thus are an important supplement to chemical analysis.

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