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The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters

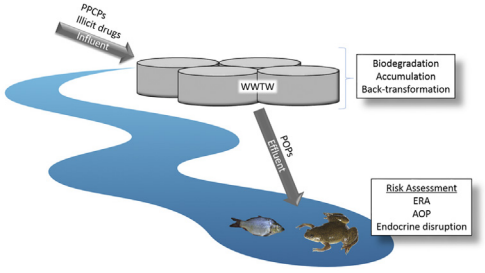
Edward Archer [a, d, *], Bruce Petrie [b], Barbara Kasprzyk-Hordern [b], Gideon M. Wolfaardt [a, c]

[a] Department of Microbiology, University of Stellenbosch, Stellenbosch 7602, South Africa
[b] Department of Chemistry, University of Bath, Bath BA2 7AY, UK
[c] Department of Chemistry and Biology, Ryerson University, Toronto, ON M5B 2K3, Canada
[d] Department of Botany and Zoology, University of Stellenbosch 7602, South Africa

HIGHLIGHTS

- A list of ECs and metabolites persist in the aqueous phase of surface waters.
- High levels of illicit drugs in wastewater indicates drug abuse within the area of study.
- Conjugate or metabolites of PPCPs might be back-transformed during WWTW processes.
- Persistent ECs may accumulate in WWTW effluent, leading to environmental risk.
- Concentrations of ECs in surface waters provide a link towards possible endocrine disruption within aquatic vertebrates.

GRAPHICAL ABSTRACT



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ABSTRACT

A large number of emerging contaminants (ECs) are known to persist in surface waters, and create pressure on wastewater treatment works (WWTW) for their effective removal. Although a large database for the levels of these pollutants in water systems exist globally, there is still a lack in the correlation of the levels of these pollutants with possible long-term adverse health effects in wildlife and humans, such as endocrine disruption. The current study detected a total of 55 ECs in WWTW influent surface water, 41 ECs in effluent, and 40 ECs in environmental waters located upstream and downstream of the plant. A list of ECs persisted through the WWTW process, with 28% of all detected ECs removed by less than 50%, and 18% of all ECs were removed by less than 25%. Negative mass balances of some pharmaceuticals and metabolites were observed within the WWTW, suggesting possible back-transformation of ECs during wastewater treatment. Three parental illicit drug compounds were detected within the influent of the WWTW, with concentrations ranging between 27.6 and 147.0 ng L^{-1} for cocaine, 35.6–120.6 ng L^{-1} for mephedrone, and 270.9–450.2 ng L^{-1} for methamphetamine. The related environmental risks are also discussed for some ECs, with particular reference to their ability to disrupt endocrine systems. The current study propose the potential of the pharmaceuticals carbamazepine, naproxen, diclofenac and ibuprofen to be regarded as priority ECs for environmental monitoring due to their regular detection and

* Corresponding author.
E-mail address: earcher@sun.ac.za (E. Archer).

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persistence in environmental waters and their possible contribution towards adverse health effects in humans and wildlife.

1. Introduction

There is growing evidence that a variety of pharmaceuticals and personal care products (PPCPs) persist in natural freshwater resources (Kasprzyk-Hordern et al., 2009; Bahlmann et al., 2014; Petrie et al., 2014; Blair et al., 2015). These organic pollutants are considered a part of emerging contaminants (ECs), which enter water systems from various sources, such as human excretion (sewage), wrongful disposal, leeching from landfill, drain water, or from industries. Even though it has been reported that these ECs are typically present at low environmental concentrations (ng/l to μg/l range), it is still unclear whether the levels of these compounds present in environmental waters can cause undesired physiological effects in wildlife and humans.

Research has shown that several regularly-used PPCPs may mimic or alter different vertebrate endocrine system pathways, which are collectively referred to as endocrine-disrupting contaminants (EDCs) (Schlumpf et al., 2001; Boberg et al., 2010; Veldhoen et al., 2014). Several PPCPs have been shown to be persistent or pseudo-persistent during wastewater treatment, thereby posing potential risk when discharged in environmental waters (Kasprzyk-Hordern et al., 2009; Verlicchi et al., 2012; Petrie et al., 2014). The prioritisation of these ECs for risk assessment is difficult, because concentrations in environmental waters show huge variation (Verlicchi et al., 2012; Petrie et al., 2014; Matongo et al., 2015), and their effects at sub-lethal concentrations are not yet well established. Studies reporting on the detection of PPCPs in South African water systems have only increased in the past few years (Agunbiade and Moodley, 2014/2016; Amdany et al., 2014; Madikizela et al., 2014; Matongo et al., 2015). These studies focussed on the detection of several classes of PPCPs and the plasticizer bisphenol-A, with concentrations regularly surpassing the μg L^{-1} level in WWTW effluent and environmental waters. However, with the vast majority of ECs shown to be present in wastewater and environmental waters on a global scale, their fate and environmental effects within WWTWs and environmental waters are still poorly described.

Apart from the ubiquitous detection of PPCPs in South African surface waters, the detection of illicit drugs and other drugs of abuse are poorly investigated. Although illicit drug usage is shown to be on the rise in South Africa (Dada et al., 2015), the sources of information for drug abuse in the country are largely limited to law enforcement and treatment centre data. However, these sources may underestimate the extent of drug abuse. A promising approach to estimate illicit drug use include sewage epidemiology, which estimates drug consumption through the detection of illicit drugs and their metabolites in wastewater (Baker and Kasprzyk-Hordern, 2013).

The present study focused on the daily loads and fate of ECs and metabolites in the aqueous phase of a WWTW influent and effluent, as well as in surface waters in a river system located upstream and downstream of the plant. This study is a first to verify the detection and fate of a large list of PPCPs, metabolites, and illicit drugs at a South African WWTW. A further aim was to correlate the persistence of selected ECs detected during the study with data showing modulation of wildlife reproductive and thyroid systems, with reference to established adverse outcome pathway (AOP) frameworks.

2. Materials and methods

2.1. Chemicals and materials

The study included the screening for 90 ECs including 38 deuterated internal standards for the method development. All the reference standards were supplied by the Department of Chemistry at the University of Bath (Bath, UK; see Petrie et al., 2016 for further details). All chemicals were prepared at either concentrations of 0.1 or 1 mg mL^{-1} in the relevant solvents and stored in the dark at −20 °C. All glassware were deactivated using dimethylchlorosilane (DMDCS) in toluene (5% v/v) to limit the sorption of basic chemicals to glass surfaces. Both the MeOH and toluene used for experimentation were obtained from Sigma-Aldrich (99%, HPLC grade).

2.2. Study site and sampling procedures

The site of study was at a WWTW situated in the Gauteng Province of South Africa (Fig. 1; Fig. S1). Treated wastewater effluent is discharged into a nearby river, which joins other streams that eventually feeds into a major dam that supplies approximately 6% of the total municipal drinking water to the surrounding communities.

Sampling was done over five consecutive days during the month of July 2015 (Monday to Friday). Influent and effluent samples were taken each day at 60 min intervals (100 mL h^{-1}) from the WWTW influent (after grit screens) and effluent (after chlorination), upon which a final composite sample (100 mL) was obtained for each sampling location per day. The influent and effluent samples were taken concurrently and not matched to the hydraulic retention time of the WWTW. Grab samples of river surface water (100 mL) were taken at a location upstream (100 m) and downstream (3.5 km from the point of discharge) of the WWTW (Fig. 1). All samples were transported on ice from the sampling sites to the laboratory and were kept cold (±4 °C) and in the dark until analyte extraction, which was done within a maximum time of 10 h.

2.3. Extraction procedure

The collected water samples were adjusted to a pH of 7 (±0.2) and filtered using 0.45 μm pore size PTFE filters prior to solid-phase extraction (SPE). Each 100 mL water sample from the various locations were split into two 50 mL samples to allow for duplicate extraction of each locality and between each day of sampling. Each sample included 50 ng of each of the deuterated PPCP internal standard and was mixed well before extraction. The water samples were extracted using Oasis® HLB (3 cc, 60 mg) SPE cartridges (Waters; Microsep, Johannesburg, South Africa). The cartridges were conditioned with 2 mL methanol, followed by 2 mL of ultrapure water (Millipore) at a flow rate of 1 mL min^{-1}. After conditioning, the water samples (50 mL) were passed through the SPE cartridges at a flowrate of 5 mL min^{-1} and allowed to run dry for a minimum period of 15 min. The dried cartridges were kept frozen (−20 °C) until all sampling were completed, from which the cartridges were then sent to the University of Bath (United Kingdom) for chemical analysis within 10 days of extraction. Each cartridge

was individually wrapped with foil and placed frozen in a polystyrene pack lined with ice packs. Upon arrival at the University of Bath, the cartridges were dried and eluted with 4 mL MeOH using a manifold at a flow rate of 1 mL min^{-1}. The extracts were dried under a gentle stream of nitrogen using a TurboVap evaporator (Caliper, UK, 40 °C, N_2, <5 psi) and the evaporated samples were re-suspended in 500 μL of a H_2O:MeOH (80:20) solvent, giving a 100× concentrated sample, and transferred to polypropylene MS vials (Waters, Manchester, UK) for chromatography.

2.4. Liquid chromatography – mass spectrometry

A Waters Acquity UPLC system coupled to a Xevo Triple Quadrupole Mass Spectrometer (UPLC/TQD-MS; Waters, Manchester, UK) was used following the method described by Petrie et al. (2016). Two separate chromatography methods were used for the quantification of acidic and basic compounds, as further described in the supplementary information (Fig. S2). In essence, both methods used a reversed-phase BEH C18 column (150 × 1.0 mm, particle size – 1.7 μm; Waters, Manchester, UK) coupled with a 0.2 μm (2.1 mm) in-line column filter. Mobile phase flow rates and injection volumes were maintained at 0.04 mL min^{-1} and 15 μL respectively for both methods. Argon was used as the collision gas, and nitrogen as the desolvation and nebulising gas. Solvent blanks containing H_2O:MeOH (80:20) were inserted after each ten samples, and two solvent blanks after quality control (QC) samples of the internal standards. The corrected recoveries of the analysed compounds are shown in the supplementary information (Table S1).

2.5. Calculations

The mass loads of the target analytes in the aqueous phase at both the influent and effluent of the WWTW during the sampling period (g day^{-1}) were determined using Equation (1):

$$\text{Mass Load}\left(g \cdot day^{-1}\right) = (Inf \text{ or } Eff * FR * 1/1000) \quad (1)$$

where *Inf* and *Eff* refers to the concentration (in ng L^{-1}) of the analytes detected at the influent and effluent wastewater samples, and *FR* refers to the mean flow rate of the plant (ML day^{-1}) during each sampling day. The ability of the WWTW to remove the detected compounds in the aqueous phase were determined by calculating the percentage removal efficiency (RE %) between influent and effluent wastewater during the sampling period using Equation (2):

$$RE(\%) = ((Inf - Eff)/Inf) * 100 \quad (2)$$

where *Inf* refers to the mass loads (g day^{-1}) of the analytes detected at the influent sample site of the plant, and *Eff* refers to the mass loads (g day^{-1}) of the analytes detected at the effluent sample site of the plant.

2.6. Statistical analysis

Statistical analyses were performed using Statistica (version 13.0). Concentrations of the ECs detected at the WWTW influent and effluent, as well as water samples located upstream and downstream of the plant during the sampling period were compared using a repeated measure mixed-model ANOVA with the sampling day as a random factor. Significant differences were recorded as $p < 0.05$.

3. Results and discussion

3.1. Analysis of WWTW samples

A total of 55 ECs were detected in wastewater influent, and 41 ECs in effluent samples, which represented 19 classes of PPCPs, human indicators, illicit drugs and metabolites (Fig. 2; Table S2). The human indicators contained chemicals which are associated with endogenous products of human metabolism or which are used for population equivalent estimates. Although flow data (ML day^{-1}) from each day was incorporated to calculate the mass loads of the ECs within the WWTW during the sampling period, the variation in the mass loads of the ECs between sampling days may be attributed to several factors, such as the variation in the daily human usage of these compounds, pollution events from surface water sources leading towards the plant, the retention time of wastewater within the plant, or the overall performance of the treatment processes within the sampling period.

The WWTW sampled in the current study showed varying removal efficiencies of the detected ECs (Table S3) and conforms to similar removal data of PPCPs reported in other studies (Verlicchi et al., 2012; Bahlmann et al., 2014; Petrie et al., 2014; 2016). By comparing the average mass loads of the ECs at the influent and effluent, it was calculated that 28% of all detected ECs were removed by less than 50%, and 18% of all ECs were removed by less than 25% (Fig. 3). A significant increase in final effluent concentration of the pharmaceutical metabolite desvenlafaxine (from venlafaxine; $p = 0.039$), were measured compared to influent wastewater (negative mass balance). Also, a significant average negative mass balance was observed for the antibiotic azithromycin ($p = 0.001$; Fig. 3). Negative mass balances were also calculated based on the average mass load concentrations for the pharmaceutical metabolites 10,11-dihydro-10-hydroxycarbamazepine and *O*-desmethyltramadol, as well as the parental compound tramadol (Fig. 3). However, statistical analysis deemed these negative mass balances non-significant due to the variation between sampling days ($p > 0.05$).

Two potential explanations for the occurrence of negative mass balances for ECs in WWTWs have been postulated, namely: i) persistent ECs accumulate in aggregates, leading to subsequent dissolution through biotic or abiotic processes, and/or ii), metabolites and/or conjugate forms of ECs are not detected at the WWTW influent and are subsequently back-transformed or de-conjugated into parental compounds through either biotic or abiotic processes within the WWTW (Verlicchi et al., 2012; Blair et al., 2015). For example, conjugate forms of ethynyl-estradiol (EE_2), carbamazepine and diclofenac have been shown to be de-conjugated by bacterial cultures (Vieno et al., 2007; Lee et al., 2012; Aris et al., 2014), while metabolites of the antibiotic sulfamethoxazole were shown to be transformed to its parental compound by photolytic processes (Bonvin et al., 2013). Although not all metabolites and conjugate forms of ECs can be regarded to be easily transformed within wastewater, it is possible that the perceived recalcitrance of some compounds in the current study at the WWTW (such as carbamazepine, tramadol, sulfamethoxazole and NSAIDs) may partly be caused by a combination of biotic and/or abiotic events leading towards the transformation of parental compounds and their metabolites.

The recalcitrance of certain ECs (such as tramadol) is well-known. Tramadol, is rapidly metabolised in the liver by desmethylation enzymatic activities encoded by the Cytochrome P450 gene (CYP2D6), which give rise to its two primary metabolites *O*-desmethyltramadol (ODT) and *N*-desmethyltramadol (NDT) (Ardakani and Rouini, 2007). Only 10–30% of the parental tramadol is excreted in sewage, and hence, a large amount of the primary or

secondary metabolites will also be prevalent in wastewater (Ardakani and Rouini, 2007). It is also noteworthy to mention that levels of the enzyme CYP2D6 may vary significantly between human individuals and sex. Taken that the WWTW screened during the current study receives wastewater from public, domestic and industrial sources, the daily levels of ECs (such as tramadol and its primary metabolites) in wastewater may vary greatly according to the *de facto* population contributing sewage of the WWTW. Partition coefficients for tramadol (log Kow 3.01) and its metabolites ODT and NDT (log Kow 2.45; EPI Suite v4.11, KOWWIN, v1.68 estimate) are shown to be high, which is indicative of a tendency towards sorption onto organic constituents within soil, sediment and/or sludge. Therefore, tramadol will tend to bio-accumulate or bio-concentrate in organisms (such as microbes) within the water body. Such association with microbial aggregates and solids may protect these compounds from degradation until release. Floc breakup in the sludge and other forms of decay may then lead to association-disassociation events that may explain the large variation in metabolite levels.

Apart from the possible transformation of parental and/or metabolite ECs in wastewater, a number of factors could also potentially contribute to the attenuation and biodegradation of ECs in WWTWs. These include climatic conditions, physiochemical properties of the ECs, the hydrological retention time of the WWTW, and the microbial activity within the plant during the sampling period. The latter will include the properties of the extracellular polymeric substances (EPS) produced by microbial biofilms to absorb organic and inorganic pollutants (Writer et al., 2011; Petrie et al., 2014). It is possible that, unless some of these ECs are fortuitously co-metabolised, the energy that can be gained by microorganisms via degrading them at the low, environmentally relevant concentrations would not warrant the energy input required for enzyme production for further biodegradation. Furthermore, it was shown that environmentally relevant concentrations of the antibiotics phenazone, amoxicillin, and erythromycin can affect the initial adhesion of bacteria onto surfaces (Schreiber and Szewzyk, 2008), and that a specialist degradative strain of yeast had a lesser habitat range than non-degradative strains in soil (Barratt et al., 2003). Admittedly speculative, it is possible that such a shift, together with the availability of labile nutrients at relatively high concentrations may favour generalists to dominate the microbial community at the expense of specialist degraders, causing ECs to pass through unaltered.

3.2. Detection of illicit drugs at the WWTW

During the current study, eight ECs classified as illicit drugs, their metabolites, and a drug precursor were identified in wastewater influent (Fig. 2). An increase in the loads of breakdown products and precursors of amphetamine-type stimulants (ATS) were observed at the WWTW (pseudoephedrine > norephedrine > amphetamine > methamphetamine) (Fig. 2). The ATS mephedrone was detected only at influent samples, ranging from 36 to 121 ng L^{-1} and calculated at an average load of 3.1 g day^{-1} during the sampling period. Similar to the ATS drugs, higher levels in the breakdown products of cocaine was observed in the wastewater influent (benzoylecgonine > cocaethylene > cocaine) (Fig. 2). The drug-precursor and nasal decongestant, (pseudo)ephedrine, was detected at an average concentration of 6321 ng L^{-1} (269.4 g day^{-1}) (Table S3). Norephedrine was detected at an average concentration of 1519 ng L^{-1} (65.1 g day^{-1}). In comparison, the levels of ephedrine and norephedrine ranged between 8.7 and 1979.5 ng L^{-1} (median load of 16.5 g day^{-1}) and 15.0–99.9 ng L^{-1} respectively at the influent of six

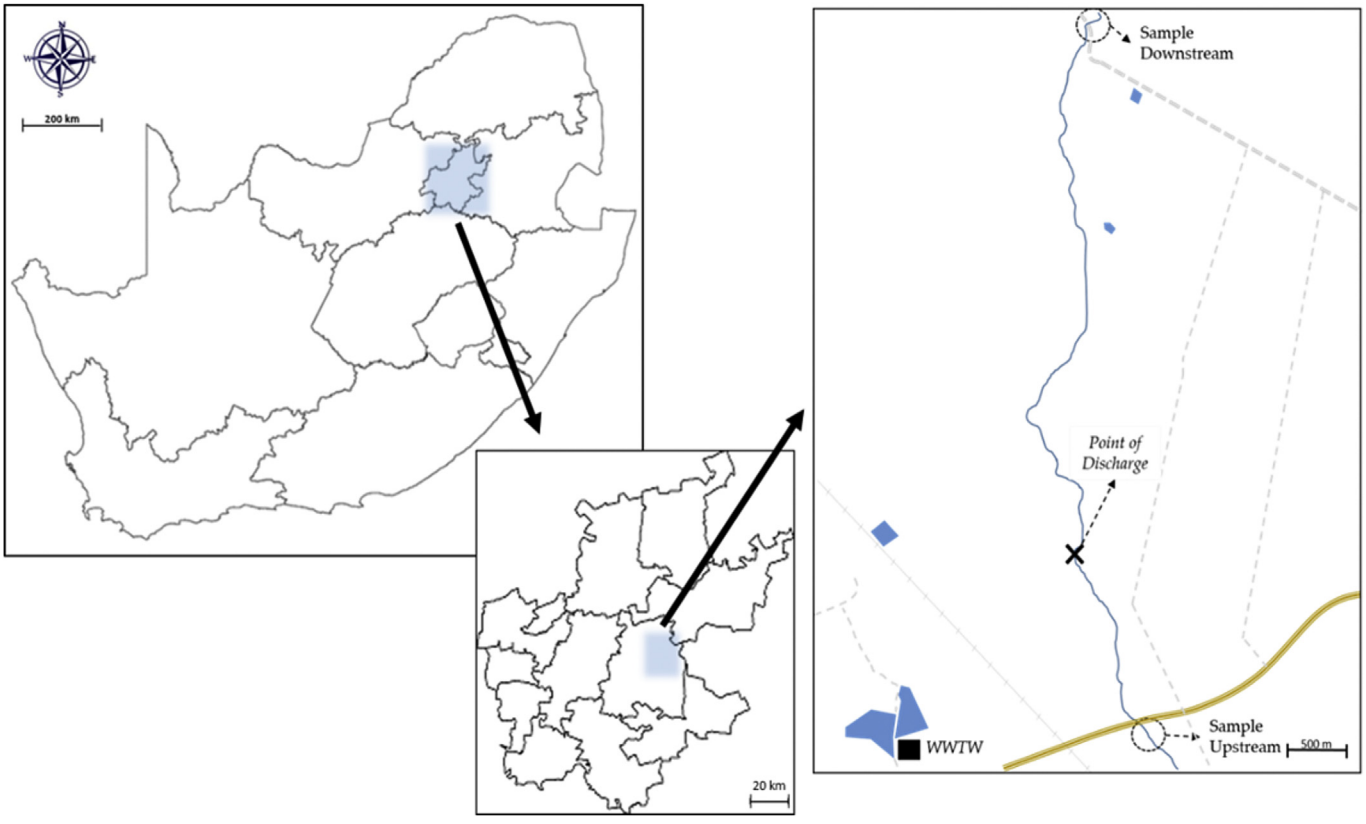

Fig. 1. Location map of the WWTW, point of discharge into the receiving waters, and sampling locations upstream and downstream from the plant.

WWTWs in the UK (Baker and Kasprzyk-Hordern, 2013). These levels were noticeably lower than reported in the current study. Although (pseudo)ephedrine is generally used as an over-the-counter nasal decongestant, a report by the South African Department of Social Development mentioned that South Africa is one of the largest importers of (pseudo)ephedrine in the world (CDA, https://www.cda.gov.za), which explains the high levels of the compound detected in the current study.

It is worthy to mention that the sources of ATS drugs in surface waters might vary. Methamphetamine is shown to be metabolised to amphetamine by de-methylation enzymes from the CYP2D6 gene (De la Torre et al., 2012), whereas amphetamine is also an active ingredient in medication for the treatment of attention deficit hyperactivity disorder (ADHD). Therefore, the presence of certain compounds could not be attributed to a specific source of exposure or drug abuse. The levels detected for methamphetamine (271–450 ng L^{-1}; 15.2 g day^{-1}), cocaethylene (186–225 ng L^{-1}; 8.8 g day^{-1}), and (pseudo)ephedrine (283–16640 ng L^{-1}; 269.4 g day^{-1}) in the WWTW influent (Tables S2 and S3) were detected at much higher levels than reported for UK WWTWs (Petrie et al., 2014). The current study therefore highlights the usage of sewage samples to indicate the trends of drug abuse within a community, and may therefore serve as a valuable tool in epidemiological studies.

3.3. ECs in environmental waters

A total of 40 ECs were detected in surface waters located upstream and downstream of the plant during the sampling period (Table 1). The levels of diclofenac, ibuprofen, ketoprofen, sulfamethoxazole, and bezafibrate were also analysed in other South African surface waters, and were detected at higher concentrations than in the current study (Agunbiade and Moodley, 2016). However, the average concentrations of the measured ECs in surface water during the current study showed that 30 of the 40 detected ECs (75%) were higher than found in UK surface waters (Petrie et al., 2014; Petrie et al., 2016). Although methylparaben, bisphenol-A, nicotine, cotinine, caffeine, and 1,7-dimethylxantine were removed with moderate to high efficiency by the WWTW (Fig. 2), the average concentrations of these compounds were calculated to be higher in downstream samples compared to the WWTW effluent (Table S2). However, only the levels of nicotine were found to be significantly higher in downstream samples compared to WWTW effluent samples during the sampling period ($p < 0.05$), mainly due to large variations of the other EC levels during the sampling period and also due to composite samples which were taken at the WWTW effluent and grab samples taken at the downstream site. In contrast, by comparing the average levels of ECs at the upstream and downstream sampling sites, it was shown that 26 out of the 40 detected ECs in surface waters (65%) were found to be two-fold or higher in downstream samples, with codeine detected higher than 10 fold (Table 1). The concentrations of ECs in surface waters are known to not only fluctuate on a seasonal or daily basis, but also in distance from the plant (Vieno et al., 2005). The higher levels of some PPCPs detected at downstream samples may therefore largely be attributed to the distance between the WWTW effluent and downstream sampling points (3500 m from the point of discharge), and fluctuations in the concentrations of the detected compounds between sampling days could also be attributed to variations in river water flow rates between sampling days.

The fact that human indicator compounds (such as nicotine) were detected at higher average levels in the downstream samples relative to the WWTW effluent samples (Table S2) indicates human activities further downstream from the WWTW, which could have re-introduced some ECs (such as methylparaben, codeine and bisphenol-A) back into the water system after the levels of the pollutants were lowered at the plant. The direct discharge and/or illegal dumping of sewage and other waste products may have been a contributing factor to these observations. This might also explain the high fold-increase in codeine further downstream of the plant (Table 1), which is regarded as the most abused over-the-counter drug in the country. However, the difference in mode of sampling should also be pointed out; composite samples were taken at the WWTW, whereas grab samples were taken for the river water, which should be corrected in future studies. Some industrial (such as a brick manufactory) and agricultural practices (poultry and other livestock) are present between the point of discharge of the WWTW and the downstream sample taken in the study. The presence of PPCPs and some metabolites point to other waste sources and human activities further downstream from the WWTW.

3.4. Environmental risk of the detected pollutants

The demonstrated presence of a vast mixture of ECs in WWTW effluent and river water in the current study emphasises the need to consider potential associated environmental risks. Conventional methods for environmental risk assessment (ERA) includes acute- and/or chronic toxicity data based on the most sensitive organism or combination of organisms within a given ecosystem to determine a predicted no-effect concentration (PNEC) of an environmental pollutant. This value is then compared to predicted- or measured environmental concentrations (PEC or MEC respectively) to obtain a risk quotient (RQ) of the EC of interest. An RQ value exceeding 1.0 is then regarded as an environmental risk. Table S4 reports on RQs determined for some ECs which were detected in WWTW effluent and river water during the current study. The MECs show that 5 out of the 41 detected ECs in WWTW effluent, and 4 of the 40 detected ECs in environmental surface waters posed an environmental risk (RQ > 1; Table S4). These ECs included diclofenac, sulfamethoxazole, clarithromycin, codeine, and nicotine, with clarithromycin only showing an environmental risk for WWTW effluent water (RQ > 1), although still of environmental relevance (RQ = 0.8; Table S4).

Although conventional ERA is valuable to show toxicity risks of ECs found in environmental waters, a few limitations exist. These models only consider lethal toxicity on an *in vivo* level as a risk endpoint and hence, the consequences of pollutants triggering sub-lethal toxicity on physiological pathways (from molecular to cellular level) are highly underestimated. The possibility of PPCPs to modulate molecular and/or cellular pathways (such as those involved in endocrine system function) at concentrations well below lethal toxicity therefore makes such outcomes more ecologically relevant for risk assessment. To assist with the understanding of toxicity mechanisms leading towards an observed adverse outcome on a population level, an adverse outcome pathway (AOP) framework has been proposed (Ankley et al., 2010). This conceptual framework is aimed towards using existing toxicological knowledge to establish a relationship between biological events on cellular-to organism level (termed key events, KEs) through key event relationships (KERs), which is initiated by a molecular initiating event (MIE) (Villeneuve et al., 2014; Margiotta-Casaluci et al., 2016). The KERs are dependent on a weigh-of-evidence approach to show the relationship between established KEs. The downstream KEs through biological complexity subsequently lead towards an observed adverse outcome (AO) which can be used for environmental regulatory decision-making (Ankley et al., 2010). Although the AOP framework is not directly constructed to be chemical specific, nor to serve as a risk assessment

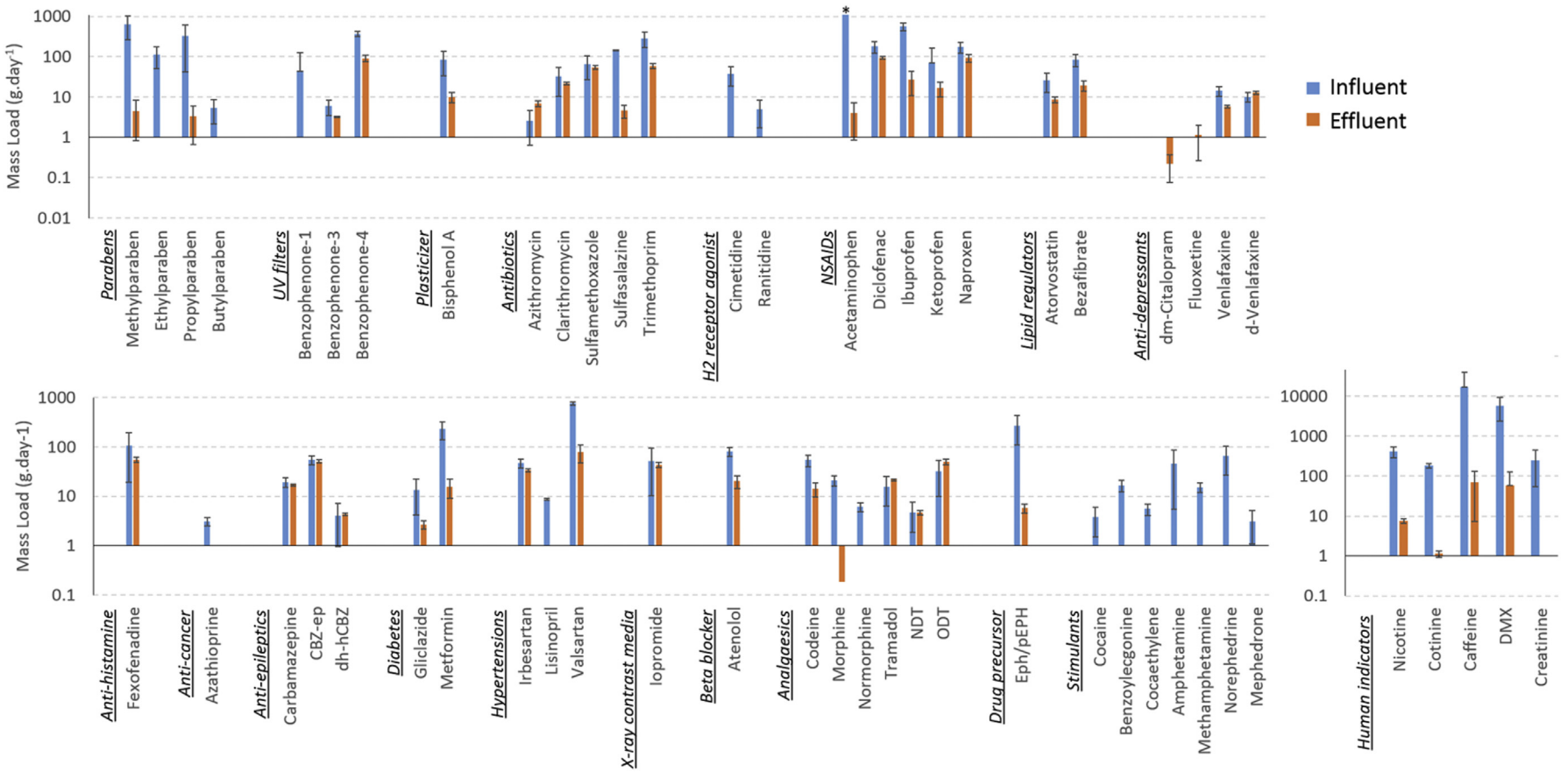

Fig. 2. Mass loads (g day^{-1}) for the detected compounds at the WWTW during the sampling period. Values are expressed on a logarithmic scale. The standard deviations shows variation between sampling days. *NSAIDs*: non-steroidal anti-inflammatory drugs, *EPH/pEPH*: ephedrine/pseudoephedrine, *dh-hCBZ*: 10,11-dihydro-10-hydroxycarbamazepine, *CBZ-ep*: carbamazepine-10,11-epoxide, *DMX*: 1,7-dimethylxantine, *NDT*: N-desmethyltramadol, *ODT*: O-desmethyltramadol. * Mass load of acetaminophen = 10,530 (±3216) g day^{-1}.

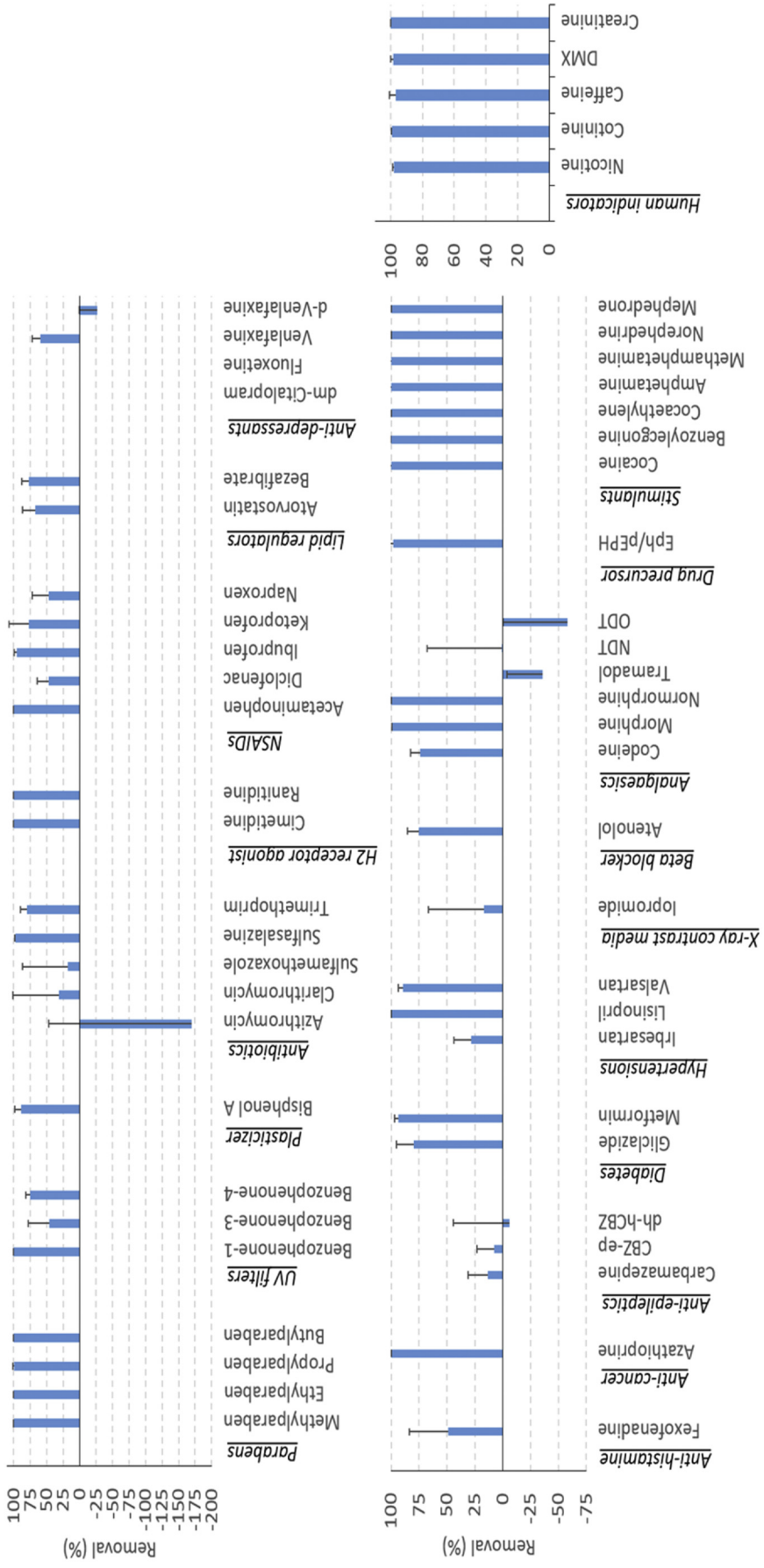

Fig. 3. Removal efficiencies (%) of the detected compounds at the WWTW during the sampling period. Standard deviations indicate variation in between sampling days. *NSAIDs*: non-steroidal anti-inflammatory drugs, *EPH/pEPH*: ephedrine/pseudoephedrine, *dh-hCBZ*: 10,11-dihydro-10-hydroxycarbamazepine, *CBZ-ep*: carbamazepine-10,11-epoxide, *DMX*: 1,7-dimethylxantine, *NDT*: N-desmethyltramadol, *ODT*: O-desmethyltramadol.

tool, the correlation of established AOP networks to EC monitoring studies (such as the current study) can therefore show the possibility of ECs in environmental waters to modulate certain MIEs or KEs. As a result, RQ values can be generated for certain MIEs and KEs (RQ_{MIE}/RQ_{KE}) in a similar way as conventional ERA. Such risk predictions can therefore serve as early warning systems to prioritise ECs for their potential to contribute towards possible detrimental health effects in the environment.

Several studies have suggested that organic pollutants can modulate endocrine system pathways of vertebrate species at low concentrations which are regularly detected in the environment. *In vitro* studies have shown, for example, that the plasticizers bisphenol-A, disinfection products (such as parabens), and UV filters can induce MIEs such as agonistic binding to the human oestrogen receptor (hER), which has further been shown to lead towards the proliferation of breast cancer cells (Schlumpf et al., 2001; Boberg et al., 2010). Exposure of fish to paraben and UV filter compounds have been reported to induce MIEs and KEs such as increased expression of mRNA transcripts of the oestrogen receptor-α (ERα) and protein vitellogenin (VTG) in the liver of male fish, which can be linked to further downstream KEs such as increased levels of plasma VTG in the body (Inui et al., 2003; Barse et al., 2010). The protein VTG is a precursor of egg yolk in oviparous animals, and serves as a common biomarker to show oestrogenic endocrine disruption in aquatic organisms due to its direct KERs with circulating androgen- and oestrogen hormone levels (Jones et al., 2000; AOP-Wiki, KE219 & KE285). The relevant MIEs and KERs leading towards increased VTG production has been well documented to be directly linked with KEs on organismal level such as fecundity and spawning, which can eventually have an effect on population trajectories (AOP-Wiki, AOP25). Although it has been shown in the current study that parabens and UV filters are effectively-to moderately removed in the WWTW (Fig. 2), methylparaben, benzophenone-4, and benzophenone-3 were still detected in river water downstream of the plant (Table 1), albeit well-below reported levels to modulate KEs as shown in laboratory studies (Inui et al., 2003; Barse et al., 2010) ($RQ_{KE} \ll 1$; Table S4).

Exposure of other pharmaceuticals, such as the NSAIDs diclofenac and ibuprofen have also been shown to modulate KEs such as elevated plasma oestradiol levels and induction of VTG in male fish, either through upregulation of upstream MIEs (such as increased aromatase activity) or other molecular targets (Hong et al., 2007; Han et al., 2010; Gröner et al., 2017; Table S4). Although the concentrations to modulate these observed estrogenic endpoints are higher than the environmental concentrations for ibuprofen in the current study, the concentration of diclofenac in WWTW effluent and river water samples regularly exceeded the 1000 ng L^{-1} value previously reported to modulate VTG production in fish (Hong et al., 2007; $RQ_{KE} > 1$; Table S4). Although an increase in VTG production has been shown for diclofenac at 1000 ng L^{-1}, Gröner et al. (2017) mentioned that such a concentration, however, does not impair population-relevant endpoints such as survival and hatching success. On the contrary, both ibuprofen and naproxen have been reported to cause decreased egg fertilisation in fish at a concentration of 100 ng L^{-1} (Nesbitt, 2011), which therefore reflects upon modulation of an established KE of fecundity and spawning (AOP-Wiki, KE78). The concentrations of ibuprofen and naproxen within WWTW effluent and river water during the current study were well above the threshold to potentially modulate such a KE ($RQ_{KE} > 1$; Table S4), and therefore indicates a potential environmental risk to impair fecundity and spawning, which can ultimately lead to a decline in fish populations (AOP-Wiki, AO360). Whether the concentrations of these NSAIDs is sufficient to induce further downstream KEs, or can be extrapolated to other vertebrate species need further investigation.

Apart from NSAIDs showing reproductive endocrine disruption in aquatic organisms, *in vivo* exposure of 500 ng L^{-1} carbamazepine in water has been shown to cause significant reduction in plasma 11-ketotestosterone (11-KT) concentrations in male fish (Galus et al., 2013a), of which the upstream MIE causing this endpoint is still unknown. 11-KT is the primary teleost androgen necessary for normal reproductive functioning, and hence, also influence the reproductive success of fish populations. The average concentration of carbamazepine in the WWTW effluent of the current study was close to the 500 ng L^{-1} threshold which can cause such a reduction in fish steroid hormone levels (Table S4). Although the modulation of circulating 11-KT is not shown to lead towards altered oestradiol hormone synthesis through aromatase enzyme activities (such as the case with circulating testosterone levels), the modulation of circulating 11-KT levels in fish can still be regarded as a potential KE to lead towards reproductive dysfunction in male fish. Furthermore, no studies have been conducted to report on the potential endocrine disrupting effect of the primary metabolites of carbamazepine, which was shown to be detected at higher concentrations within WWTW effluent and river water (Fig. 2; Table 1), and removed to a lower extent in the WWTW (Fig. 3). We therefore propose that this compound and its metabolites need further monitoring for its potential to cause detrimental effects in aquatic vertebrates, especially due to its demonstrated recalcitrance and potential to modulate androgen-controlled endocrine pathways.

Apart from the potential of ECs to modulate reproductive endocrine system pathways, studies showing modulation of the thyroid endocrine system have also been reported. Exposure of the NSAID ibuprofen to tadpoles of the American bullfrog (*Rana catesbeiana*) at concentrations ranging from 1500 to 15 000 ng L^{-1} have been shown to potentiate triiodothyronine (T3)-induced mRNA transcription of thyroid hormone receptors (Veldhoen et al., 2014). In the same study, exposure to ibuprofen alone resulted in increased mRNA transcripts for enzymes such as thyroxine 5-deiodinase (*dio3*) in a tail fin tissue assay, which is necessary to regulate thyroid hormone homeostasis (Veldhoen et al., 2014). Although the levels of ibuprofen observed in the present study was below these concentrations in both the WWTW effluent and river water, the levels of other NSAIDs, such as naproxen (average concentration of 2296 ng L^{-1} in WWTW effluent; Table S2) and diclofenac (average concentration of 2326 ng L^{-1} in WWTW effluent; Table S2), fell within the concentration range to possibly alter such MIEs, given that these NSAIDs yield the same mechanism of endocrine disruption as ibuprofen. It has been shown that diclofenac (but not naproxen) can antagonistically bind to the thyroid hormone receptor-β (thrβ) in a human reporter assay, and also inhibit T3-induced vasodilation of rat mesenteric arteries (Zloh et al., 2016). Also, it is important to note that cross-talk between endocrine system pathways exist. For example, Nelson and Habibi (2016) demonstrated an increased production of VTG and upregulation of ERα following a treatment of T3 in female goldfish (*Carassius auratus*). Therefore, the potential of NSAIDs to alter both thyroid- and gonadal endocrine system pathways can be linked to several KEs and KERs, given that the concentrations and bioaccumulation of these pollutants are sufficient within the organisms to exert such effects. However, relatively little has been done to assess the impact of NSAIDs on whole-life cycles within aquatic organisms, and should receive more attention in future studies, especially due to their ease of purchase, their regular usage, and moderate persistence in the aqueous phase at WWTWs as shown in the current study. These results therefore indicate the importance to regard NSAIDs as priority environmental EDCs in future studies.

Another complication which arise in establishing ECs as an environmental risk is the complex range of interactions that are found in environmental waters. Several ECs generally accumulate

Table 1
Mean concentrations (ng L^{-1}) of detected PPCPs, metabolites, illicit drugs and human indicator compounds at sampling localities located upstream and downstream of the WWTW. Standard deviation indicate variation between sampling days for the compounds. Abbreviations: NSAIDs: non-steroidal anti-inflammatory drugs, EPH/pEPH: ephedrine/pseudoephedrine, dh-10-hCBZ: 10,11-dihydro-10-hydroxycarbamazepine, CBZ-ep: carbamazepine-10,11-epoxide, DMX: 1,7-dimethylxantine, NDT: N-desmethyltramadol, ODT: O-desmethyltramadol.

	Upstream		Downstream		Fold change		Upstream		Downstream		Fold change
	Average	Stdev	Average	Stdev			Average	Stdev	Average	Stdev	
Parabens						*Anti-epileptic*					
Methylparaben	58.7	29.2	146.1	107.3	2.5	Carbamazepine	157.1	11.5	279.5	24.1	1.8
Propylparaben	31.8	17.4	136.7	76.8	4.3	CBZ-ep	398.8	27.9	752.2	69.4	1.9
						dh-hCBZ	22.7	1.58	56.9	8.9	2.5
UV filters						*Diabetes*					
Benzophenone-3	56.2	1.7	64.3	6.0	1.1	Gliclazide	43.2	2.4	53.9	22.3	1.3
Benzophenone-4	441.1	23.8	1076.5	389.6	2.4	Metformin	73.3	7.2	174.6	81.7	2.4
Plasticizer						*Hypertensions*					
Bisphenol-A	239.0	72.1	396.4	208.1	1.7	Irbesartan	311.1	28.7	554.4	120.0	1.8
						Valsartan	263.7	24.6	924.7	50.2	3.5
Antibiotics											
Azithromycin	24.6	0	6.4	3.4	0.3						
Clarithromycin	76.2	13.4	235.5	66.1	3.1	*Anti-depressants*					
Sulfamethoxazole	757.4	83.2	1013.2	294.2	1.3	Fluoxetine	34.4	22.1	109.2	125.6	3.2
Sulfasalazine	37.6	3.4	53.0	13.0	1.4	Venlafaxine	35.4	3.7	94.6	19.6	2.7
Trimethoprim	383.0	42.2	898.7	303.0	2.4	Desvenlafaxine	50.0	7.5	174.9	53.8	3.5
NSAIDs						*Analgaesics*					
Acetaminophen	20.8	4.5	63.7	76.1	3.1	Codeine	11.3	6.7	128.9	65.4	11.5
Diclofenac	467.4	176.2	1461.5	508.7	3.1	Tramadol	97.7	11.2	299.9	73.2	3.1
Ibuprofen	153.3	39.5	312.1	204.6	2.0	NDT	16.0	9.9	74.0	8.1	4.6
Ketoprofen	642.2	0	330.3	319.0	0.5	ODT	207.6	32.2	577.3	149.8	2.8
Naproxen	224.3	31.1	1112.8	518.3	5.0						
						Drug precursor					
Lipid regulators						Eph/pEPH	38.8	8.0	80.4	28.4	2.1
Atorvostatin	74.0	5.2	150.6	55.7	2.0						
Bezafibrate	54.9	8.3	234.4	116.8	4.3	*Stimulants*					
						Amphetamine	27.1	22.6	37.0	22.6	1.4
Antihistamine											
Fexofenadine	368.4	36.7	887.0	172.0	2.4	*Human indicators*					
						Nicotine	154.3	78.7	245.5	67.6	1.6
X-ray contrast media						Cotinine	25.5	3.3	31.7	11.7	1.2
Iopromide	265.8	11	598.3	235.4	2.3	Caffeine	812.2	146.3	2077.5	259.7	2.6
						DMX	479.4	357.6	957.6	728.6	2.0
Beta-blockers											
Atenolol	156.2	34.43	272.0	154.6	1.7						

in complex mixtures of varying concentrations (including other types of organic and inorganic pollutants), in which the modulating effects of such mixtures may differ from observed effects of the individual pollutants. For example, a study by Galus et al. (2013b) showed a mixture of environmentally relevant concentrations of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine (500 ng L^{-1} for each compound) significantly altered embryo production, oocyte development, and fecundity in female zebrafish (*Danio rerio*), while the individual compounds exposed to the fish species at the same concentrations did not yield the same results (Galus et al., 2013a). Although investigations of PPCP mixture effects generate a more ecologically relevant scenario, it is more feasible to identify the KEs and KERs which are modulated by individual compounds, which may ultimately lead to a better understanding of the health risks in environmental waters. The fact that some ECs in the current study showed low removal from the WWTW, as well as the possibility to cause adverse effects on vertebrate endocrine system pathways (such as carbamazepine, UV filters, plasticizers, parabens and NSAIDs) highlights the importance to further monitor these priority ECs to limit their impact on both the aquatic ecosystem and contamination to drinking water resources.

4. Conclusions

The current study aimed to provide a link between the monitoring of ECs in WWTWs and environmental waters with possible adverse health consequences in wildlife. Although most ECs were shown to be notably reduced in WWTW effluent, some persisted, and were even detected at higher concentrations in effluent (as shown for some pharmaceutical metabolites and parental compounds). It is therefore important to report on the fate of both parental ECs as well as their metabolites and/or conjugate forms in surface waters to elucidate the possible negative mass balances observed in WWTWs and recalcitrance of pollutants in environmental waters.

Drawing definite conclusions regarding the health impact which these pollutants may cause when entering environmental waters is no simple task, considering that these pollutants are present in complex mixtures with varying physiochemical properties, as well as their varying affinities to modulate a range of molecular and cellular pathways in wildlife species. It is therefore clear that there is a need for more eco-toxicological assessment on the sub-lethal effects of ECs and polluted water systems into identifying MIEs, KEs and KERs which certain ECs can modulate to advance current risk assessment approaches.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.01.101.

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