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Shimko Katja M. (Orcid ID: 0000-0001-5644-117X)

Thevis Mario (Orcid ID: 0000-0002-1535-6451)

A PILOT WASTEWATER-BASED EPIDEMIOLOGY ASSESSMENT OF ANABOLIC STEROID USE IN QUEENSLAND, AUSTRALIA

Katja M. Shimko[a,b], Jake W. O'Brien[b], Leon Barron[a], Hasan Kayalar[a,b], Jochen F. Mueller[b], Ben J. Tscharke[b], Phil M. Choi[b], Hui Jiang[b], Geoff Eaglesham[b], Kevin V. Thomas[b]

[a]King's Forensics, School of Population Health & Environmental Sciences, Faculty of Life Sciences & Medicine, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UNITED KINGDOM

[b]Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Cornwall Street, Woolloongabba QLD 4102, AUSTRALIA

Corresponding author: Jake W. O'Brien. Email: j.obrien2@uq.edu.au; Tel.: +61 (0) 7 32749009

Author's ORCID: Katja M. Shimko: 0000-0001-5644-117X; Jake W. O'Brien: 0000-0001-9336-9656; Leon Barron: 0000-0001-5986-3853; Hasan Kayalar: not available; Jochen F. Mueller: 0000-0002-0000-1973; Ben Tscharke: 0000-0002-3292-3534; Phil Choi: 0000-0002-0535-8197; Hui Jiang: not available; Geoff Eaglesham: 0000-0002-2418-0777; Kevin V. Thomas: 0000-0002-2155-100X

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Highlights:

- Quantitative LC-ESI-MS/MS analysis of anabolic steroids and hormones in wastewater
- Determination of 17 steroids and hormones at ng L^{-1} level in wastewater
- Nine endogenous hormones quantified in 24-h composite wastewater samples in Australia (3-104 mg day^{-1}1000 $individuals^{-1}$)
- The anabolic steroid stanozolol detected in a wastewater influent sample

Abstract

Anabolic-androgenic steroids are synthetic compounds prohibited due to their performance enhancing characteristics. The use of these substances is known to cause health-related issues, which highlights the importance of being able to evaluate the scale of consumption by the general population. However, most available research on the analysis of anabolic steroids is focussed on animals and athletes in connection with doping. The potential of wastewater-based epidemiology as an intelligence tool for the assessment of community level use of anabolic steroids is presented herein. A liquid chromatography tandem mass spectrometry method was developed for the analysis of ten anabolic-androgenic steroids and 14 endogenous hormones in influent wastewater. The validated method was applied to sixteen 24-hour composite wastewater influent samples that were collected over a period of five years from two wastewater treatment plants in Queensland, Australia. Nine investigated compounds were found to be present at concentrations between 14-611 ng L^{-1} which translated into 3-104 mg excreted per 1000 individuals per day. It was concluded that the developed analytical method is suitable for the analysis of AAS in wastewater matrix. Additionally, both the inclusion of metabolites and further investigation into deconjugation by enzymatic hydrolysis would aid in understanding and evaluating community anabolic steroid use. For the first time, this study presents the application of wastewater-based epidemiology on anabolic-androgenic steroids in Australia.

Keywords: Anabolic-androgenic steroids, Endogenous hormones, Wastewater analysis, LC-MS/MS, Solid-phase extraction

1. Introduction

Exogenous anabolic-androgenic steroids (AAS) are synthetically derived compounds structurally related to the male hormone testosterone. Due to their muscle growth and strength-enhancing properties, they are known to be misused among bodybuilders and other athletes[1,2]. Several complications are associated with the use of AAS, including serious health-related problems such as heart failure and hypertrophy, as well as major mood disorders accompanied by violent and aggressive behaviour[3-5]. These substances are prohibited by the World Anti-Doping Agency (WADA) because of their performance-enhancing properties[6] and are classified as Schedule 4 substances in the Poisons Standard under The Therapeutic Goods Act 1989 in Australia[7]. In addition, AAS could potentially be harmful to wildlife in receiving environments, if released as endocrine disrupting chemicals, as demonstrated for related hormones[8]. Therefore, the ability to detect, identify and quantify a broad range of AAS in wastewater would assist in evaluating human consumption as well as potential environmental exposure from the release of wastewater effluents.

Most available research on AAS analysis has focused on biological matrices such as animal or human urine, blood, plasma and hair, often in the context of doping. In these cases, analysis is often conducted on biological samples of individuals and usually on professional, 'in-competition' athletes[9-16]. For doping control analyses, WADA utilises gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)[17], which is costly and labour-intensive due to hydrolysis and derivatisation steps. More commonly used and arguably more flexible techniques for measuring a large range of doping compounds are gas chromatography (GC) or liquid chromatography (LC) coupled to either high-resolution mass spectrometry (HRMS) or tandem mass spectrometry (MS/MS)[18,19]. The latter has the advantage of avoiding complicating derivatisation steps. However, one of the main challenges associated with the detection and identification of illicit drug residues, especially in wastewater, is the limited information available on their human pharmacokinetic profiles and hence the expected target metabolites of some parent analytes[20].

Wastewater-based epidemiology (WBE) is a non-invasive technique that can provide spatial and temporal information on the use of and exposure to chemicals within the general population[19,21,22]. It has previously been applied to detect and quantify chemicals such as prescription, over-the-counter and illicit drugs, personal care products and environmental

contaminants in wastewater samples collected from the influent of wastewater treatment plants (WWTPs) and has been used for monitoring purposes[23-28]. WBE has the potential for being an intelligence gathering tool for authorities[25] and data can be compared nationally and internationally for prevention and rehabilitation purposes[23,29-31]. For the successful application of WBE, the selection of the most suitable biomarker for analysis is pivotal. There are multiple factors to take into consideration which include stability (e.g., in-sewer and in-sample)[32], transformation due to microbial activity[33], specificity to compound of interest and human metabolism[21,34].

One clinical study investigated multiple AAS and showed that most are excreted in their conjugated form[35]. Nevertheless, it has also been demonstrated that conjugated hormones can be deconjugated by acid-catalysed solvolysis[36]. As wastewater samples are often acidified after collection[25,37], this might have an impact on the occurrence of free steroids and hormones in these sample matrices.

The aim of this work was to further investigate the application of WBE for the assessment of AAS use at a community level. Quantitative LC-MS/MS methods to simultaneously determine AAS, hormones and metabolites were developed, optimised and validated. The validated methodology was applied to sampling campaigns in two catchments; i) for four days during a body building event and ii) in an urban centre of more than 100,000 inhabitants over a time-period of five years with one sample per season. This is the first study to focus on AAS in Australian wastewater and provides an important reference point for future WBE studies on a global level.

2. Experimental

2.1. Materials, chemicals and reagents

A detailed account of materials, chemicals and reagents used in the experimental can be found in the Supplementary Information (S.I.). Compounds of interest are shown in Table 1. 5α-dihydrotestosterone-D_3 (5α-DHT-D_3) 100 µg mL^{-1} in methanol, androstene-3,17-dione-2,3,4-$^{13}C_3$ (99.9%) 100 µg mL^{-1} in acetonitrile and dehydroepiandrosterone-D_5 (DHEA-D_5) 100 µg mL^{-1} in methanol were purchased from Cerilliant (Round Rock, TX, USA). Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) supplied the two isotopically labelled standard solutions 19-nortestosterone (nandrolone) (16,16,17-D_3, 98%) 100 µg mL^{-1} in methanol and testosterone (16,16,17-D_3, 98%) 100 µg mL^{-1} in methanol. Epitestosterone-

D_3 was purchased from the National Measurement Institute (NMI) (North Ryde, NSW, AU). Lichrosolv grade methanol was added (by weight using a five-digit balance and accounting for specific gravity) to certified reference materials that were received as powders to prepare stock solutions. Stock solutions of all analytical standards (1000 µg mL^{-1} native standards and 100 µg mL^{-1} isotopically-labelled standards) were used to prepare working standard solutions in Lichrosolv grade methanol with concentrations of 10 µg mL^{-1} and 100 µg L^{-1}, which were subsequently stored at -20 ^{o}C in the dark to minimise degradation. Water was purified to 18.2 MΩ cm^{-1} using a Milli-Q ultrapure water system and filtered using a 0.22 µm mesh (Millipore, Bedford, USA).

2.2. Sample collection

Twenty four-hour composite samples of wastewater influent were collected by refrigerated autosamplers (4 ^{o}C) operating in flow proportional mode at the inlet of two WWTPs in Queensland, Australia. Samples (see *2.7.*) were collected in high-density polyethylene (HDPE) bottles that had been pre-cleaned with 2 × 4 mL aliquots of methanol followed by 2 × 4 mL Milli-Q water. The samples were acidified on-site with 2 M hydrochloric acid (HCl) to adjust to ≤pH 2 before on-site freezing at -20 ^{o}C to reduce microbial degradation of analytes. All wastewater influent samples were transported back to the laboratory where they were stored in the Australian Environmental Specimen Bank in the dark at below -20 ^{o}C until analysis.

2.3. Solid-phase extraction optimisation

Three commercially available SPE cartridges (details can be found in the S.I.), hydrophilic-lipophilic balanced (Oasis HLB), mixed-mode strong cation-exchange (Oasis MCX) and Sep-Pak AccellPlus CM (hydrophilic, weak cation-exchanger), were assessed for the recovery of analytes of interest, as well as their variability using two different elution methods of i) 100% methanol and ii) 5% ammonium hydroxide in methanol. As a pilot experiment, the three different sorbents were investigated using 10 mL ultrapure (Milli-Q) water spiked with native compounds at a concentration of 0.2 µg L^{-1}. Two of the most suitable sorbents and their elution conditions were selected for further optimisation. Analyte recoveries were evaluated by spiking 10 mL aliquots of pooled wastewater influent samples

with either 0.2 µg L^{-1} native compounds before extraction (concentration factor = 50) or 200 µL extract with 10 µg L^{-1} native compounds (theoretical 100% recovery, n = 3 each). Recoveries were determined by comparing the response of the analyte spiked into the sample before extraction to the response of the analyte spiked into the extract. In this study, HLB was compared to MCX and to a combination (stacking) of HLB and MCX (HLB/MCX). HLB cartridges were eluted with methanol, whereas MCX and HLB/MCX cartridges were eluted with methanol and 5% NH_4OH in methanol.

2.4. Optimised sample preparation

Acidified (pH2 adjusted with HCl) influent wastewater samples were defrosted and homogenised before 10 mL aliquots were spiked with isotopically labelled standards. Concentrations were as follows: 0.4 µg L^{-1} for DHEA-D_5 and 0.1 µg L^{-1} for 5α-DHT-D_3, androstene-3,17-dione-2,3,4-$^{13}C_3$, epitestosterone-D_3, nandrolone-D_3 and testosterone-D_3. DHEA-D_5 was spiked at a higher concentration due to its low sensitivity. Subsequently, the samples were filtered using 0.2 µm regenerated cellulose (RC) filters and subjected to SPE.

The optimised SPE method involved conditioning MCX 3 mL x 60 mg cartridges with 3 mL methanol followed by 2 x 1.5 mL Milli-Q water at pH 2. The cartridges were then loaded with 10 mL of spiked and filtered sample and washed with 3 mL Milli-Q and 70:30 Milli-Q:methanol, both at pH 2 (acidified with HCl). Vacuum at ≤25 kPa was applied for 30 minutes until the sorbent was dry, after which the compounds were eluted into polypropylene (PP) capped test tubes using 1 mL methanol followed by 2 mL 5% NH_4OH in methanol. The extracts were concentrated using a gentle stream of N_2 at 40 ^oC until 300 µL remained and transferred into a 2 mL amber vial. The PP capped test tubes were rinsed twice with 300 µL methanol, which was also transferred to the vial. The extracts were then further concentrated to dryness and reconstituted in 200 µL of 90:10 mobile phase A (0.2 mM ammonium fluoride (NH_4F) in Milli-Q water):mobile phase B (95:5 (v/v) methanol/water). This was achieved by first adding 40 µL 50:50 A:B for an increased dissolution of organic matter, to which an additional 160 µL of aqueous mobile phase A was added using positive displacement pipettes. The final extracts were vortexed and stored at -20 ^oC until analysis.

2.5. Method development and instrumental analysis

Direct infusion was used to select and optimise analyte transitions (instrument and parameters for analysis can be found in the S.I. Table S3). Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit cell potential (CXP) were optimised for the individual transitions. Negative mode was also investigated (see Table S2 for further details). Electrospray ionisation source temperatures were evaluated for optimisation of ionisation efficiency.

Three different analytical columns were assessed for separation efficiency of the target analytes: Kinetex® Biphenyl 100 Å 50 x 2.1 mm 2.6 µm, Kinetex® F5 100 Å 50 x 2.1 mm 2.6 µm and Kinetex® XB C_{18} 100 Å 50 x 2.1 mm 1.7 µm (Phenomenex, Lane Cove West, NSW, AU). Multiple mobile phase compositions and additives were investigated for peak shape and ionisation efficiency. Additives for the mobile phase A tested included: 0.2 mM NH_4F, 0.1% acetic acid and a combination of both. Different compositions of mobile phase B assessed were 50:50 (v/v) methanol/acetonitrile, 95:5 (v/v) methanol/water and 100% methanol. Mobile phase A was selected as 0.2 mM NH_4F in Milli-Q water, mobile phase B as 95:5 (v/v) methanol/water.

Mobile phase B was set to 50% at the beginning of the gradient and increased linearly to 95% until 8.50 min (see S.I. Table S3 for analysis parameters). This was kept constant until 10.91 min where the concentration of mobile phase B was then decreased to 50% and equilibrated until the end of the analytical run at 14.10 min. Acquired data was processed utilising MultiQuant 3.0.2 software (Sciex).

2.6. Method validation and stability assessment

The method, including sample preparation, was validated according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines[38]. Methodology was evaluated for specificity, linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), recovery and matrix effect by spiking and analysing pooled acidified wastewater influent. Calculations for analytes with an isotopically labelled analogue were based on the area ratio of native to isotopically labelled standard. All calculations for compounds for which an isotopically labelled standard was not available were based on peak area. For overall method linearity, a matrix-matched calibration curve was made up by spiking pooled wastewater with different concentrations of

native analytes (N = 9, ranging from 0.002–1 µg L^{-1}) and a fixed concentration of six labelled internal standards (as described in *2.3.*) and subjected to the extraction procedure. A second calibration curve in 90:10 mobile phase A:mobile phase B was prepared in the same manner to test instrumental method linearity. Linearity for each analyte was determined using a weighting of 1/x. The range was determined for each analyte where $R^2 \geq 0.99$ and $N \geq 5$. Accuracy and precision were calculated at three different concentrations (0.05 µg L^{-1}, 0.2 µg L^{-1} and 0.4 µg L^{-1}) with at least three determinations at each concentration. LOD and LOQ were determined for each analyte at the lowest available concentration from the calibration curve at which both quantifier and qualifier (with an ion ratio of ≤30%) were detected. The standard deviation of the measured response (n = 7) was multiplied by 3.3 (LOD) and 10 (LOQ) which was then divided by the slope of the matrix-matched calibration curve. The relative matrix effect (%) was assessed by dividing the slope of the matrix-matched calibration curve by the slope of the calibration curve in 90:10 A:B multiplied by 100. In addition, concentration dependent matrix effects were determined by dividing the background corrected response of the analyte spiked into the extract after SPE (0.2 µg L^{-1}, n = 6) with the response of the analyte spiked at the same concentration into 90:10 A:B.

A 24-hour stability assessment of all analytes in acidified wastewater influent was conducted to determine if stability is of concern for the target analytes. A volume of 1 mL wastewater influent was spiked with native standards (10 µg L^{-1}) and internal standards (5 µg L^{-1} and 20 µg L^{-1} for DHEA-D_5) and directly filtered into an amber vial (n = 3). This was done immediately before the sample was put into the LC autosampler, set to 22 °C, and injected (to analyse sample at t_0). Additionally, 1 mL of unspiked, filtered wastewater influent was analysed. A calibration curve in ultrapure water at pH 2 (HCl acidified) was prepared and analysed to determine the initial concentration and subsequently the percentage of initial concentration (%). Samples were analysed every hour for the first six hours, after which they were injected every 2-3 hours. Photodegradation was excluded from this study as the samples were in amber vials and analysed in a closed autosampler.

2.7. Targeted analysis of AAS

Acidified wastewater influent samples from two different wastewater treatment plants (WWTPs) in Australia were selected for targeted analysis of AAS and hormones of interest. For WWTP A, four consecutive days were selected based on the occurrence of a bodybuilding event. The first day was the day before the event, the next two during the event

and the fourth day was after the event. The four samples were extracted and analysed in triplicate as described in section *2.4.* A matrix-matched calibration curve (external calibration) was generated by pooling samples of nine consecutive days from WWTP A, including samples from the four days selected for targeted analysis.

A second wastewater treatment plant (WWTP B) was chosen based on its coastal and metropolitan features. One 24-hour composite sample per season (February, May, August and November) was randomly selected for three different years (2013, 2015 and 2017). The same aliquots of all twelve samples were pooled to create a matrix-matched calibration curve. Samples were analysed in triplicate.

It is generally not possible to obtain wastewater influent free of analyte that can be used to generate a matrix-matched calibration curve. Due to limited volume availability of influent wastewater samples, multiple 24-hour composite samples from the same WWTP were pooled to create sufficient volume for a nine-point matrix-matched calibration curve, as the matrix was assumed to be sufficiently similar within the same WWTP. Both external matrix-matched calibration curves were prepared by spiking with native standards at nine concentrations ranging from 0.002 – 0.4 µg L^{-1} and internal isotopically labelled standards at a concentration of 0.1 µg L^{-1} (0.4 µg L^{-1} for DHEA-D_5). For the six compounds with a labelled analogue, the isotope dilution method was used for quantitation. This was achieved by spiking 10 mL of the sample with internal standards at the same concentration as in the external calibration curve. Peak area ratios of internal standard in the sample and calibration curve were used to calculate the concentration of the analyte in triplicate samples. The concentrations of all other analytes were calculated using their response in sample compared to their response in the external matrix-matched calibration curve. The calculation subtracted the background concentration of analytes. All samples, including calibration curves, were subjected to the extraction procedure. For quality control purposes, 10 mL of the pooled wastewater influent from each WWTP was filtered and extracted. This involved both background subtraction of any analyte present in the pooled sample for calibration accuracy, but also to check whether any SRM transitions interfered with those of the isotopically-labelled standards (for the latter, none were present). In addition, 10 mL of ultrapure water at pH 2 was filtered, extracted and analysed to show if any contamination occurred during the preparation or analysis procedure. Ultrapure water containing 0.2 mM NH_4F (mobile phase A) was analysed between each sample set and no carry-over was observed. Analytes identified by their quantifier ion were confirmed using retention time and a second transition (qualifier ion) with an ion ratio of ≤30%.

Excretion mass loads of analytes 1000 individuals^{-1} were estimated using concentration, flow and population data, using the method presented by Lai *et al*[39], based on an approach by Zuccato *et al*[24]. The equation used was as follows: normalised excretion mass load = (concentration x flow)/(number of people 1000^{-1}). Flow and population data were available for every sample collected from WWTP A and were provided by the wastewater treatment plant authorities. Exact flow data was not available for the chosen dates of WWTP B, therefore an average flow was calculated based on data collected from multiple years. This was deemed acceptable, as the daily flow rates were quite consistent (%RSD = 8).

3.

4. Results and discussion

3.1. Method development and optimisation

Of the SPE cartridges tested, MCX was the best as it demonstrated acceptable analyte recovery (80-120%) and showed lower variability (Figure 1) and ion suppression than HLB (further details in S.I.). Sep-Pak AccellPlus CM had very poor recoveries (<20%) for the compounds of interest.

Comparison of the LC columns found that the best separation of all analytes of interest was achieved on the Kinetex® XB C_{18} column (example chromatogram in Figure 2) when compared to F5 and biphenyl stationary phases (see S.I. for further details). Best peak shape and ionisation efficiency were achieved with addition of 0.2 mM NH_4F to mobile phase A and using 95:5 (v/v) methanol/water as mobile phase B. During compound parameter optimisation, it was decided to exclude oxymetholone from further analysis due to poor ionisation efficiency and peak width (>30 seconds).

3.2. Method validation and stability assessment

A total of 24 steroids and hormones were subjected to method validation of which seven (four AAS and three hormones) did not fulfil the guideline requirements. These compounds were boldenone, equilin, mestranol, oxandrolone, stanozolol, trenbolone and estriol. Stanozolol showed an insufficient accuracy of 63% at a concentration of 0.3 µg L^{-1} (data not shown), whereas trenbolone and estriol demonstrated accuracies of 20% and 50%, respectively, at 0.05 µg L^{-1}. It should be emphasised however, that the accuracy for estriol at 0.05 µg L^{-1} was determined near the LOQ of 0.04 µg L^{-1}. Mestranol demonstrated

unsatisfactory linearity (R^2 < 0.99). Boldenone, equilin and oxandrolone also did not pass method validation due to insufficient linearity, which may have been caused by interferences observed (see below) and these analytes were therefore excluded from targeted analysis. However, the other analytes that did not pass method validation were included in the analysis for semi-quantitative purposes. Precision for all analyte retention times were within an acceptable range (0.00-0.16 %RSD). Recoveries were satisfactory with values between 77–117% with the exception of drostanolone and progesterone, which had recoveries of 66% and 68%, respectively. All analytes that passed method validation had accuracies of 84–109% across all three concentrations, with acceptable relative standard deviations of <1–7%. Exceptions were 17α-ethynylestradiol, 17β-estradiol and estrone, which had %RSD values of up to 15%. Relative matrix effects varied greatly between compounds, although all were affected by ion suppression as opposed to enhancement. Compared to other LC-MS methods that were used to investigate AAS in matrix, this method shows similar to increased precision and lower LODs/LOQs leading to increased sensitivity [26,28]. This shows that the developed method is suitable for the intended purpose of analysing AAS in wastewater. The method performance data for each analyte is listed in Table 1.

Selectivity

Method validation highlighted the possibility for the occurrence of interfering compounds during analysis. Several monitored transitions exhibited interferences due to shared structural similarity and could therefore not be validated with the applied method. Both selected reaction monitoring (SRM) transitions chosen for boldenone suffered from matrix interference. An unknown interfering compound detected in wastewater influent had a retention time (3.72 min) very similar to that of boldenone (3.68 min). Subsequently, three additional transitions were added to the analysis (*m/z* 287.1 → 269.1, *m/z* 287.1 → 199.1 and *m/z* 287.1 → 91.1), which again were all shared by the unknown compound, but with different ion ratios compared to boldenone. The interference was not seen in ultrapure water spiked with native boldenone, but was detected in all unspiked, extracted wastewater influent samples showing the highest intensity of all analytes detected. The identification of the interfering compound was outside the scope of this study. All three transitions selected for equilin (*m/z* 269.1 → 211.1, *m/z* 269.1 → 157.1 and *m/z* 269.1 → 152.1) were also shared with interfering compounds in wastewater influent in a similar way. Transitions for oxandrolone were subject to the same issue. Some transitions had interferences which were

minor and could therefore still be used as qualifier ions without any issues. Analyte transitions affected were DHEA *m/z* 271.1 → 253.1, fluoxymesterone *m/z* 337.2 → 181.1, methandienone *m/z* 301 → 149, progesterone *m/z* 315.1 → 97.1, stanozolol *m/z* 329.1 → 121.1 and tetrahydrogestrinone *m/z* 313.1 → 159.1. All analytes that passed method validation had selective quantifier ion transitions.

Stability assessment

The majority of analytes did not demonstrate significant degradation over a 24-hour period at 22 ^{o}C in acidified and filtered wastewater influent. Interestingly, all four hormones analysed in negative ESI mode showed a slight decline in initial concentration (≥15%) over the 24-hour time period (S.I., Figure S2). In-sewer degradation was outside the scope of this study.

3.3. Occurrence of AAS in Queensland wastewaters over 2013-2017

3.3.1. WWTP A

Analysis of 24-hour composite influent wastewater samples from four consecutive days revealed eight hormones and metabolites to be present in all samples at concentrations ranging between 15–611 ng L^{-1} (Table 2). Estimated excreted mass loads are presented in Figure 3. These eight compounds were 5α-dihydrotestosterone, androstenedione, epitestosterone, estriol, estrone, progesterone, testosterone and β-estradiol. No other hormones were detected in any of the four samples. Estriol was determined to be semi-quantitative due to an accuracy value of 50% at 0.05 µg L^{-1}, however it is to be noted that estriol was found to be present at concentrations of up to 0.6 µg L^{-1} relating to the highest mass load (76–104 mg day^{-1}1000 $individuals^{-1}$). Accuracy values for estriol at this level were satisfactory (see Table 1). Epitestosterone (6–7 $mg^{-1}day^{-1}$1000 $individuals^{-1}$), progesterone (6–11 mg day^{-1}1000 $individuals^{-1}$), testosterone (5–7 mg day^{-1}1000 $individuals^{-1}$) and β-estradiol (3–6 mg day^{-1}1000 $individuals^{-1}$) showed the lowest mass load values in this catchment. The testosterone/epitestosterone ratio (T/E ratio) during all four days was ~1:1 suggesting no significant population scale use of exogenous hormones, according to WADA requirements[17]. No AAS were detected in the samples collected during the bodybuilding

event. Further research may show that metabolites and/or transformation products may be more reliable target compounds but was beyond the scope of this work. Also, due to their moderate hydrophobicity, AAS and metabolite concentrations might be higher in biosolids, which has been investigated for similar compounds[40,41] but requires further research. The matrix of WWTP A differed from the matrix observed in other analysed pooled wastewater influent samples. The signal-to-noise ratio for all peaks analysed was decreased due to a higher general background level. The increased background might be due to WWTP A having about half the population size, but roughly only 25% of the flow rate compared to the wastewater treatment plant from which samples were pooled and analysed for method validation and the sample preparation experiments.

3.3.2. WWTP B

Analysis of twelve wastewater influent samples from WWTP B covering three years and four seasons revealed that steroids, metabolites and hormones were detected at concentrations ranging from 2 to 204 ng L^{-1}. These included 5α-dihydrotestosterone, androstenedione, DHEA, epitestosterone, estriol, estrone, progesterone, stanozolol, testosterone and β-estradiol (Table 2). The AAS stanozolol was detected in one of the samples but could not be quantified (<LOQ). The detection of stanozolol is significant, as it shows that WBE may emerge as a tool for monitoring trends in the use of these chemicals. Stanozolol was targeted as the parent compound only and the conjugated metabolites might be higher in abundance. However, the deconjugation in sewer is unknown. Therefore, enzymatic hydrolysis could be investigated when targeting the parent compound to deconjugate the analyte, increasing the concentration of the free parent compound.

The mass load of androstendione was calculated to be 26-66 mg $day^{-1}1000$ $individuals^{-1}$, which correlates to excretion values found in the literature[42]. When transforming those values into excretion mass loads (assuming 50:50 women:men in the catchment), values ranging between 18 and 62 mg $day^{-1}1000$ $individuals^{-1}$ were determined, which are consistent with the excretion mass loads as stated above. It is important to note that there are uncertainties when calculating consumption and/or excretion mass loads, including sampling[43], flow measurement, excretion data, number of people contributing to the wastewater samples[44] and potential for in-sewer instability[45]. In addition, the wastewater influent sample from November 2013 was considered an outlier due to very low levels and was excluded from excretion mass load calculations.

5. Conclusion

A selective and sensitive analytical method for AAS, hormones and metabolites in influent wastewater was developed and validated for 17 compounds of interest. In application of the method, nine investigated compounds were quantified in multiple 24-hour composite samples from two Queensland wastewater treatment plants. These were found to be present at concentrations of between 14 and 611 ng L^{-1} translating to excretion mass loads of 3 to 104 mg day^{-1}1000 $individuals^{-1}$. The quantified compounds were 5α-dihydrotestosterone, androstenedione, dehydroepiandrosterone, epitestosterone, estrone, progesterone, testosterone, β-estradiol, and semi-quantitative for estriol. Furthermore, the AAS stanozolol was detected in one wastewater influent sample. Future studies could investigate the addition of deconjugation steps to increase the concentration of parent drugs in wastewater samples. In addition, AAS metabolites or transformation products could be assessed for suitability within the WBE approach. The latter presents great research potential, as the information available in the literature on metabolites and transformation products is scarce. Finally, as steroids and hormones are known to be moderately hydrophobic, an additional investigation into biosolids may be worth pursuing. This first study in Australia will serve as an important reference point for global level WBE-based assessment of AAS.

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Table 1. LC-ESI-MS/MS method performance data for steroids and hormones in pooled wastewater influent using ICH guidelines[38].

Analyte	MRM transitions[a]	ESI mode	t_r (min) ± %RSD	%Average Recovery ± SD	%Accuracy ± %RSD (N = 3)			LOD (ng L^{-1})	LOQ (ng L^{-1})	Linearity (R^2)	Range (µg L^{-1})		%Relative matrix effect[b,d]	%Matrix effect ± SD[c,d]
	m/z		(n = 9)	(n = 6)	(n = 7)	(n = 6)	(n = 3)	(n = 7)	(n = 7)					(n = 6)
			0.2 µg L^{-1}	0.2 µg L^{-1}	0.05 µg L^{-1}	0.2 µg L^{-1}	0.4 µg L^{-1}							0.2 µg L^{-1}
5α-Dihydrotestosterone*	**291.1 → 255.1** 291.1 → 159.1	+	5.46 ± 0.06	104 ± 2	90 ± 5	91 ± 2	92 ± 1	3.0	9.1	0.995	(0.003-1)	(N = 9)	-32	-31 ± 6
17α-Methyltestosterone	**303.1 → 109.1** 303.1 → 267.1	+	5.06 ± 0.12	83 ± 3	99 ± <1	96 ± 3	95 ± <1	0.4	1.1	0.998	(0.002-1)	(N = 9)	-26	-17 ± 5
19-Norethindrone	**299.1 → 231.1** 299.1 → 115.1	+	4.04 ± 0.12	88 ± 4	89 ± 3	101 ± 4	97 ± 1	8.3	25.3	0.998	(0.013-1)	(N = 7)	-33	-23 ± 6
Androstenedione*	**287.1 → 97.1** 287.1 → 109.1	+	3.97 ± 0.12	111 ± 2	94 ± 3	98 ± 2	100 ± 1	3.3	10.0	0.996	(0.003-1)	(N = 9)	-32	-32 ± 3
Boldenone	**287.1 → 121.1** 287.1 → 135.1	+	3.68 ± 0.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
D(-)-Norgestrel	**313.1 → 245.1** 313.1 → 109.1	+	5.03 ± 0.06	78 ± 3	95 ± <1	95 ± 4	96 ± <1	0.3	0.8	0.999	(0.006-1)	(N = 8)	-33	-19 ± 5
Dehydroepiandrosterone*	**271.1 → 213.1** 271.1 → 253.1	+	4.71 ± 0.15	117 ± 3	92 ± 3	98 ± 3	101 ± 2	6.4	19.5	0.993	(0.006-1)	(N = 7)	-28	-36 ± 14
Drostanolone	**305.1 → 215.1** 305.1 → 269.1	+	6.50 ± 0.10	66 ± 3	96 ± 2	96 ± 5	98 ± 4	1.1	3.4	0.999	(0.013-1)	(N = 8)	-52	-28 ± 1
Epitestosterone*	**289.1 → 97.1** 289.1 → 109.1	+	5.38 ± 0.06	106 ± 1	93 ± 1	96 ± 1	97 ± 2	0.5	1.5	0.997	(0.002-1)	(N = 9)	-27	-21 ± 4
Equilin	**269.1 → 211.1** 269.1 → 157.1	+	3.62 ± 0.13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.970	n.d.	(N = 5)	-49	n.d.
Fluoxymesterone	**337.2 → 241.1** 337.2 → 181.1	+	3.61 ± 0.09	93 ± 3	93 ± 2	97 ± 3	95 ± <1	1.7	5.3	0.999	(0.013-1)	(N = 7)	-29	-28 ± 4
Mestranol	**311.2 → 121.1** 311.1 → 159.1	+	7.18 ± 0.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.999	n.d.	(N = 5)	-77	n.d.
Methandienone	**301.1 → 121.1** 301.1 → 149.1	+	4.24 ± 0.16	92 ± 3	93 ± 1	97 ± 3	96 ± 1	0.8	2.3	0.998	(0.006-1)	(N = 8)	-27	-25 ± 6
Nandrolone*	**275.1 → 109.1** 275.1 → 239.1	+	3.87 ± 0.08	100 ± 1	96 ± 1	95 ± 1	98 ± 1	0.6	1.9	0.997	(0.006-1)	(N = 8)	-32	-26 ± 2
Oxandrolone	**307.1 → 229.1** 307.1 → 271.1	+	3.88 ± 0.16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.972	n.d.	(N = 5)	-60	n.d.
Progesterone	**315.1 → 109.1** 315.1 → 97.1	+	6.13 ± 0.08	68 ± 6	86 ± 1	97 ± 6	99 ± 1	0.4	1.3	0.998	(0.002-1)	(N = 9)	-50	-28 ± 1
Stanozolol	**329.1 → 203.1** 329.1 → 121.1	+	6.40 ± 0.10	109 ± 6	81 ± 4	88 ± 6	106 ± 6	2.2	6.8	0.990	(0.05-1)	(N = 5)	-78	- 81 ± 2
Testosterone*	**289.1 → 97.1**	+	4.47 ± 0.15	99 ± 1	88 ± 1	95 ± 1	96 ± <1	0.5	1.4	0.998	(0.002-1)	(N = 9)	-27	-21 ± 4

	289.1 → 109.1													
Tetrahydrogestrinone	**313.1 → 241.1**	+	5.82 ± 0.05	77 ± 5	96 ± <1	100 ± 6	98 ± 2	0.3	0.9	0.999	(0.002-1)	(N = 9)	-46	-33 ± 1
	313.1 → 159.1													
Trenbolone	**271.1 → 199.1**	+	3.32 ± 0.13	80 ± 5	20 ± 1	95 ± 6	98 ± 1	0.5	1.5	0.999	(0.006-1)	(N = 8)	-43	-34 ± 3
	271.1 → 227.1													
17α-Ethynylestradiol	**295.1 → 145.1**	-	4.05 ± 0.10	92 ± 13	<LOQ[1]	91 ± 14	91 ± 2	23.7	71.7	0.992	(0.025-1)	(N = 6)	-42	-40 ± 17
	295.1 → 143.1													
17β-Estradiol	**271.1 → 145.1**	-	3.90 ± 0.08	96 ± 11	84 ± 8	101 ± 12	100 ± 3	4.2	12.6	0.999	(0.004-1)	(N = 9)	-41	-47 ± 7
	271.1 → 183.1													
Estriol	**287.1 → 171.1**	-	1.15 ± 0.00	96 ± 5	50 ± 8	105 ± 5	97 ± 1	14.7	44.4	0.996	(0.015-1)	(N = 9)	-48	-45 ± 4
	287.1 → 145.1													
Estrone	**269.1 → 145.1**	-	3.89 ± 0.08	95 ± 15	85 ± 7	109 ± 15	102 ± 1	10.5	31.8	0.991	(0.011-1)	(N = 9)	-38	-35 ± 5
	269.1 → 143.1													

SD = standard deviation, RSD = relative standard deviation
n = replicate number at same concentration, N = replicate number at different concentrations
n.d. = not determined
LOD = limit of detection, LOQ limit of quantification
*Calculations based on area ratio due to use of isotopically labelled analogue
[a] Transitions in bold font represent the quantifier ion for each analyte. The second transition represents the qualifier ion.
[b] Defined as: ((slope of matrix-matched calibration curve/slope of extract solvent calibration curve)*100)-100
[c] Defined as: ((background corrected response of analyte spiked into extract/response of analyte spiked into extraction solvent)*100)-100
[d] LC-ESI-MS signal suppression (-) or enhancement (+)
[1] To satisfy ICH guidelines, %Accuracy (96) and %RSD (11) were determined at a third concentration (0.3 µg L^{-1}, n = 6)

Table 2. Concentrations of steroids and hormones in wastewater influent from two WWTPs in ng L^{-1}.

Compound	WWTP A				WWTP B											
	Day 1	Day 2	Day 3	Day 4	2013				2015				2017			
		(n = 3)			Feb	May	Aug	Nov	Feb	May	Aug	Nov	Feb	May	Aug	Nov
17α-Ethynylestradiol*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17α-Methyltestosterone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19-Norethindrone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5α-Dihydrotestosterone	152-168	117-133	162-165	180	59-61	65-66	70-72	-	89-92	70-72	78-80	91-93	88-95	77-80	82-83	108-112
Androstenedione	192-196	165-170	141	169-175	160-161	162	158-159	<LOQ	203-204	146-147	146-147	183-185	119-121	173-175	156-157	122-123
D(-)-Norgestrel	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dehydroepiandrosterone	-	-	-	-	97-106	118-123	133-139	<LOQ	116-129	95-97	96-98	134-137	115-116	111-115	110-119	111-113
Drostanolone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epitestosterone	36-37	34	30	37-39	14-15	15	16	-	18	15	15-16	18	16	21-22	23-24	19
Estriol*	549-611	382-469	514-562	561-609	62-67	71-75	84-87	<LOQ	102-108	82-85	91-97	97-101	118-122	149-155	194-197	176-179
Estrone	158-190	116-124	69-79	78-83	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ -48	46-47	46-49	47-49	48-50
Fluoxymesterone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mestranol*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methandienone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nandrolone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Progesterone	46-48	35	30-32	59-63	45	28	33	2	29	33	32-33	32	20	14-15	10-11	18-19
Stanozolol*	-	-	-	-	-	-	-	-	-	-	-	-	-	<LOQ	-	-
Testosterone	40-41	28-30	26-27	33-34	14	15	19	<LOQ	22	17	18	20	14	19-20	20	15
Tetrahydrogestrinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trenbolone*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Estradiol	31-37	23-26	15-19	23-24	<LOQ	<LOQ	<LOQ	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

- = not detected (<LOD)

* did not pass method validation – included for semi-quantitative purposes

Boldenone, equilin and oxandrolone were excluded due to selectivity issues

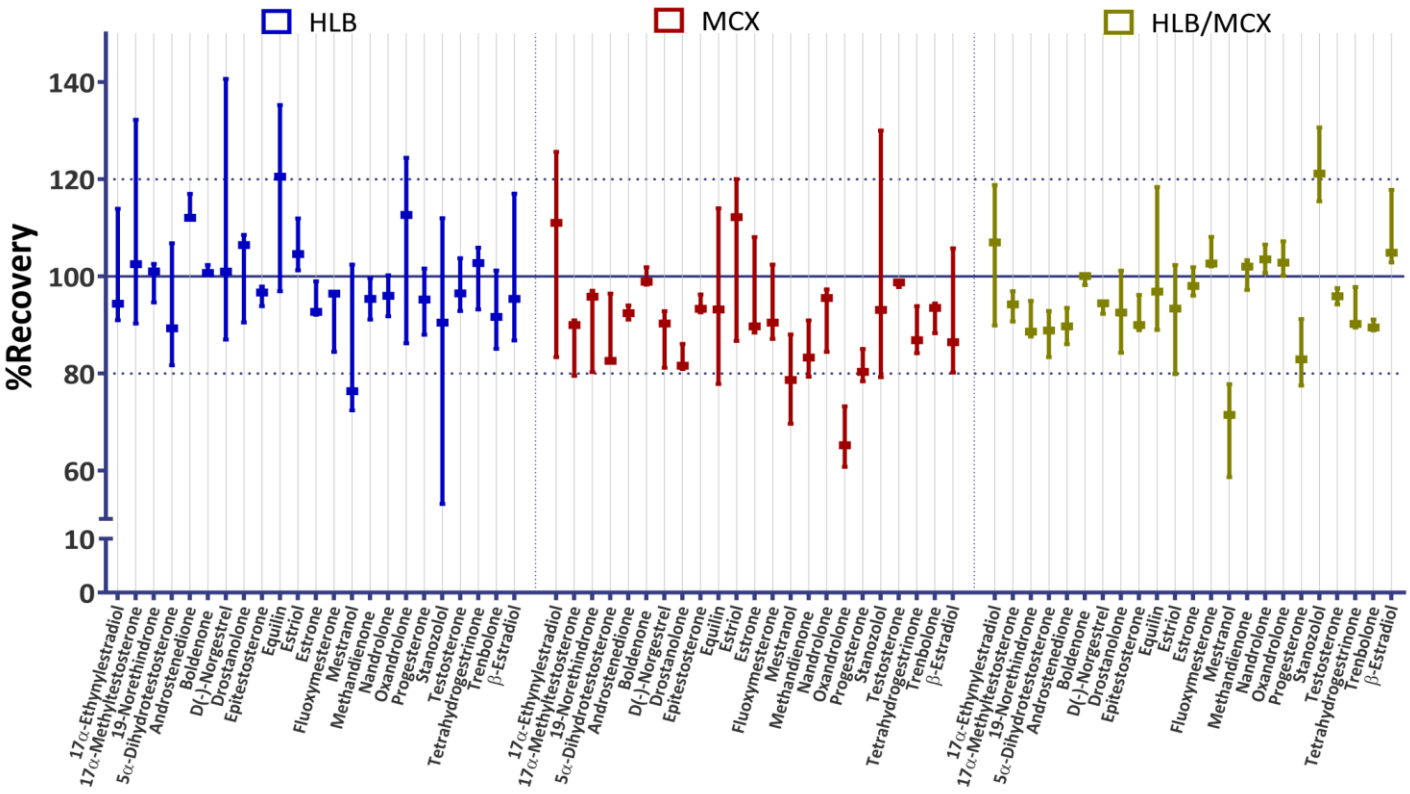

Figure 1. Performance comparison of different SPE sorbents showing min to max and median of n = 3. Graph depicts recovery values (%) for analytes and each sorbent investigated.

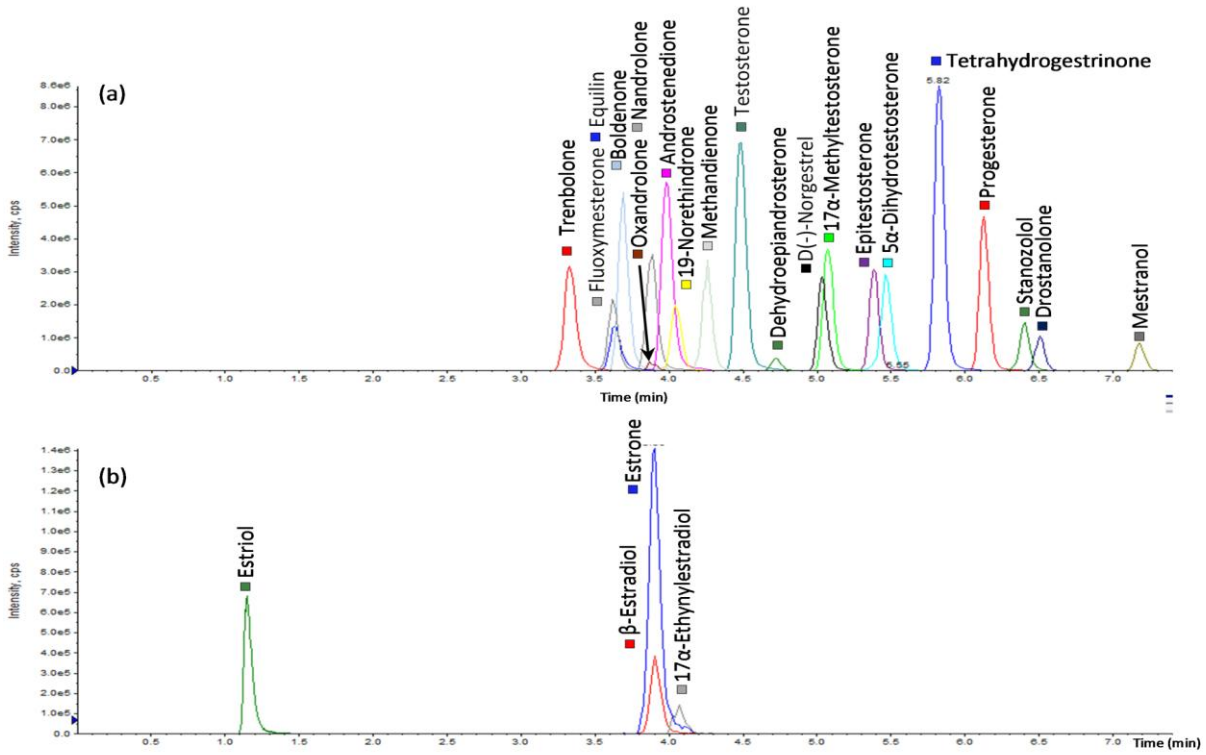

Figure 2. Extracted ion chromatogram (XIC) showing quantifier ion transitions for AAS and hormones of interest in ultrapure water at a concentration of 10 µg L^{-1}. (a) Compounds analysed in positive ESI mode. (b) Hormones analysed in negative ESI mode.

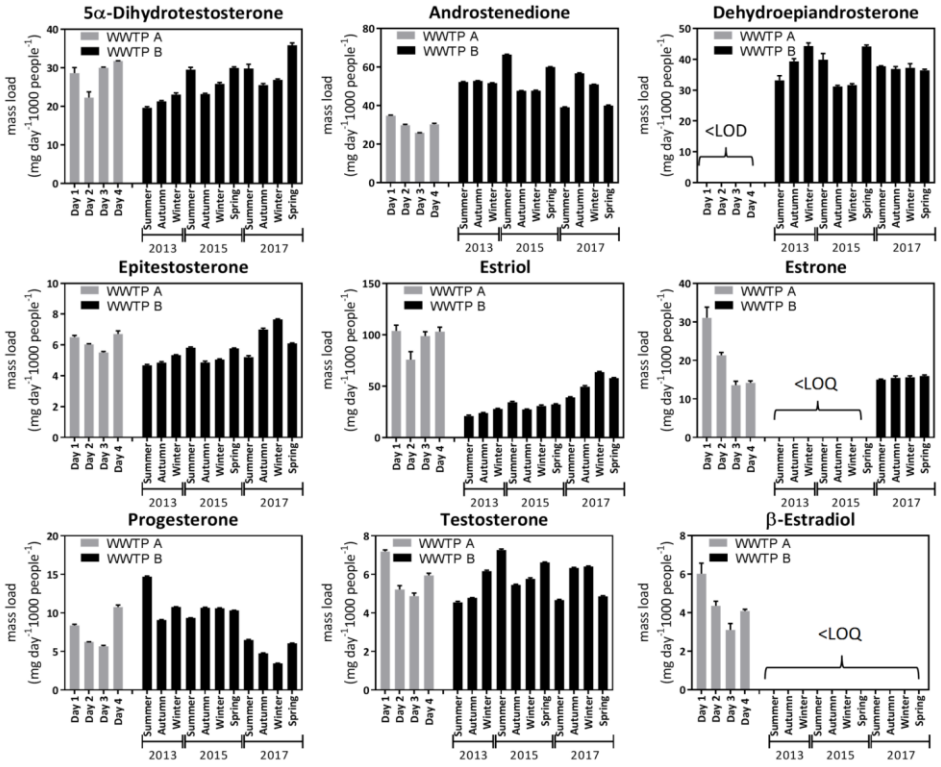

Figure 3. Calculated excreted mass load in mg day^{-1}1000 people^{-1}. Bars that are not present indicate a concentration below LOQ. Bars in grey represent excretion mass load values for samples collected from WWTP A. Black bars depict excretion mass load values from samples collected from WWTP B. Error bars show standard deviation (n = 3).