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In-sewer stability assessment of anabolic steroids and selective androgen receptor modulators

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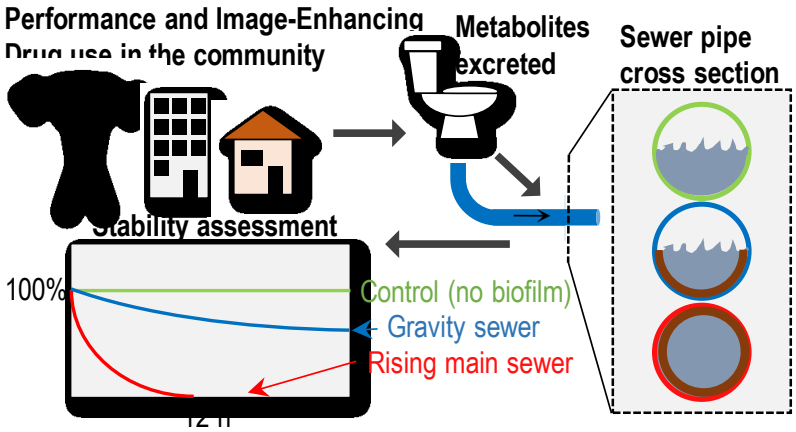

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Abstract

Wastewater-based epidemiology is a potential complementary technique for monitoring the use of performance- and image-enhancing drugs (PIEDs), such as anabolic steroids and selective androgen receptor modulators (SARMs), within the general population. Assessing in-sewer transformation and degradation is critical for understanding uncertainties associated with wastewater analysis. An electrospray ionisation liquid chromatography mass spectrometry method for the quantification of 59 anabolic agents in wastewater influent was developed. Limits of detection and limits of quantification ranged from 0.004 – 1.56 µg/L and 0.01 – 4.75 µg/L, respectively. Method performance was acceptable for linearity (R^2>0.995, few exceptions), accuracy (68-119%), and precision (1-21%RSD), and applicability was successfully demonstrated. To assess the stability of the selected biomarkers in wastewater, we used laboratory-scale sewer reactors to subject the anabolic agents to simulated realistic sewer environments for 12 hours. Anabolic agents, including parent compounds and metabolites, were spiked into freshly collected wastewater that was then fed into three sewer reactor types: control sewer (no biofilm), gravity sewer (aerobic conditions), and rising main sewer (anaerobic conditions). Our results revealed that while most glucuronide conjugates were completely transformed following 12h in the sewer reactors, 50% of the investigated biomarkers had half-lives longer than four hours (mean residence time) under gravity sewer conditions. Most anabolic agents were likely subject to biofilm sorption and desorption. These novel results lay the groundwork for any future wastewater-based epidemiology research involving anabolic steroids and SARMs.

Keywords: in-sewer degradation, performance- and image-enhancing drugs (PIEDs), wastewater analysis, wastewater-based epidemiology (WBE), sewage

Synopsis

Fate of 59 anabolic agents was investigated using laboratory-scale sewer reactors to understand biomarker transformation and sorption in sewage systems.

1 Introduction

Performance- and image-enhancing drug (PIED) use is an emerging global public health issue[1-3]. Several negative side-effects on mental and physical health have been reported, e.g., depression, aggressive behaviour, liver toxicity, and heart issues[4-7]. Their use is not restricted to professional and amateur athletes, and easy access through online markets assists in misuse of these drugs within the general population[1]. The prevalence of PIED use, in particular anabolic agent use, among the general community is currently only being estimated and monitored via survey, anti-doping testing, and seizure data (e.g., for a review see[8]). These approaches are single point-in-time measurements with small sample sizes and additional limitations, including participation, honesty, and knowledge about the substances that are consumed (i.e., dosages and purity), or testing of a specific subpopulation.

Wastewater-based epidemiology (WBE) is a widely used tool to estimate chemical consumption (e.g., for estimating illicit drug consumption within the general population), that may have a potential application in PIED monitoring[9-12]. For a chemical to be a suitable health biomarker for WBE, the chemical must meet a range of criteria including:

1) being excreted via urine in consistent amounts;

2) having a unique source from human metabolism;

3) being detectable in wastewater; and

4) being stable in wastewater[13].

Respectively, these criteria allow researchers to 1) back-calculate excreted mass loads as the loads will be proportional to the user population, and metabolites will be soluble in the water phase; 2) know the appropriate target metabolite, that the drugs have passed through the human body and not from other sewer inputs; 3) ensure that the target analyte is present in high enough concentrations amenable to analysis and; 4) know that the amount in the sample reflects total use or if it reflects part of total use due to degradation in the sewer network[13].

Criteria 1) and 2) have been assessed through anti-doping testing and research related to this field. However, thresholds and ratios of anabolic agents and their metabolites cannot be assessed and

applied to wastewater analysis as they are for anti-doping testing of individuals. Studies that have investigated synthetic anabolic agents in wastewater, criterion 3), often focussed on analysing parent chemicals as opposed to metabolites[10-12, 14]. Backe et al analysed steroid metabolites in wastewater, but could not confirm if detected biomarkers such as boldenone were excreted naturally or formed in the sewer[9]. Criterion 4 has been assessed for in-sample stability of anabolic steroids only (post-collection)[9, 10, 12], but not for selective androgen receptor modulators (SARMs). No studies have thus far investigated the stability of both anabolic steroids and SARMs, two chemically very diverse subgroups belonging to the group of anabolic agents within PIEDs[15], in the sewage system. The in-sewer stability and detectability, especially of SARMs, requires investigation as it is the first step to determine whether WBE is a suitable approach for monitoring PIED use.

In-sewer degradation is dependent on physical and chemical properties of the wastewater, as well as the in-sewer bioactivity and wastewater residence time[16, 17]. Wastewater is transported through a network of infrastructure, flowing both under gravity (gravity sewers (GS)) and under pressure (rising main sewers (RM)). Gravity sewers are usually partially filled with wastewater under aerobic conditions, while RM are pressurised pipes that are completely filled and under anaerobic conditions. Biofilm is present on the inside of the pipes and the ratio of biofilm area can vary at different points in the network due to pipe diameter and water level. Biofilm-area-to-wastewater-volume ratio (A/V ratio) is dependent on the size of the pipes, as well as the level of wastewater flowing through. The hydraulic retention times (HRTs) and A/V ratios vary in sewage systems with dynamic flows and wastewater conditions. Resident times and A/V ratios can be controlled in laboratory-scale sewer reactors, which can be used to estimate the in-sewer stability of biomarkers.

The aim of this study was to assess the suitability of 59 anabolic agent biomarkers for WBE applications by determining their in-sewer stability, using laboratory-scale sewer reactors, under rising main sewer, gravity sewer and control reactor (no biofilm) sewer conditions. This would improve our understanding of some of the uncertainties associated with monitoring community use of anabolic agents through WBE.

2 Materials and methods

2.1 Materials and reagents

Details on biomarkers investigated in this study and where analytical standards and reagents were sourced can be found in Table 1 and in the Supplementary Information (SI).

Table 1. Biomarkers investigated, how the metabolites are referred to in this study, LogP values (predicted using the Molinspiration property calculation service (http://www.molinspiration.com)), analytical method, and their spiking level in this study in µg/L. Analytical method A: mobile phase A – 0.4 mM ammonium fluoride (NH_4F) in 95:5 ultrapure water/methanol (v/v); mobile phase B – 0.4 mM NH_4F in 95:5 methanol/ultrapure water (v/v). Analytical method B: mobile phase A – 0.1% acetic acid in 95:5 ultrapure water/methanol (v/v); mobile phase B – 0.1% acetic acid in 95:5 methanol/ultrapure water (v/v). N/A: not available.

Biomarker	Metabolite name	CAS number	LogP	Analytical method	Spiking level (µg/L)
Steroids and hormones					
Boldenone		846-48-0	3.22	A	5
Boldenone glucuronide		827019-65-8	1.39	B	5
17β-Hydroxy-5β-androst-1-en-3-one	Boldenone M1	10529-96-1	3.41	A	10
4-Chloro-androst-4-en-3α-ol-17-one	Clostebol M1	51348-73-3	3.58	A	13.3
2α-Methyl-5α-androstan-3α-ol-17-one	Drostanolone M1	6961-54-2	3.9	A & B	10
2α-Methyl-5α-androstan-3α-ol-17-one glucuronide	Drostanolone M1 glucuronide	361432-78-2	2.08	B	8
Estrone		53-16-7	3.24	A	8
Fluoxymesterone		76-43-7	2.76	A	8
9α-Fluoro-17α-methyl-androst-4-en-3α,6β,11β,17β-tetra-ol	Fluoxymesterone M1	148505-57-1	2.03	A	10
9α-Fluoro-17,17-dimethyl-18-nor-androst-4,13-diene-11β-ol-3-one	Fluoxymesterone M2	3863-16-9	3.53	A	10
1α-Methyl-5α-androstan-3α-ol-17-one	Mesterolone M1	3398-67-2	3.9	A	10
Methyl-1-testosterone		65-04-3	3.85	A	10
Metandienone		72-63-9	3.67	A	5
17-Epimetandienone	Metandienone M1	33526-40-8	3.67	A	10
6β-Hydroxymetandienone	Metandienone M2	33526-41-9	2.75	A	10
17β-Methyl-5β-androst-1-ene-3α,17α-diol (Epimetendiol)	Metandienone M3	132830-78-5	4.04	A & B	10
Methasterone		3381-88-2	4.35	A	8
2α,17α-Dimethyl-5α-androstane-3α,17β-diol	Methasterone M1	1173998-58-7	4.54	A & B	10

Biomarker	Metabolite name	CAS number	LogP	Analytical method	Spiking level (µg/L)
Metenolone		153-00-4	3.95	A	5
1α-Methylene-5α-androstan-3α-ol-17-one	Metenolone M1	3398-66-1	3.74	A	13.3
Methylstenbolone		6176-38-1	4.4	A	5
Methyltestosterone		58-18-4	3.69	A	10
17α-Methyl-5α-androstane-3α,17β-diol	Methyltestosterone M1	641-82-7	4.06	A & B	10
17α-Methyl-5β-androstane-3α,17β-diol	Methyltestosterone M2	641-84-9	4.06	A & B	10
Nandrolone		434-22-0	3.0	A	8
19-Norandrosterone		1225-01-0	3.18	A	10
19-Norandrosterone glucuronide		294213-86-8	1.35	B	10
19-Noretiocholanolone		33036-33-8	3.18	A	13.3
19-Noretiocholanolone glucuronide		294213-87-9	1.35	B	10
17α-Ethyl-5α-estrane-3α,17β-diol	Norethandrolone M1	6961-15-5	4.32	A & B	10
17α-Ethyl-5β-estrane-3α,17β-diol	Norethandrolone M2	31658-50-1	4.32	A & B	10
17α-Hydroxyethyl-5β-estrane-3α,17β-diol	Norethandrolone M3	1245704-40-8	3.08	A	10
Progesterone		57-83-0	3.81	A	5
Oxandrolone		53-39-4	3.72	A	15
17-Epioxandrolone	Oxandrolone M1	26624-15-7	3.72	A	10
Testosterone		58-22-0	3.25	A	5
Androstenedione		63-05-8	3.06	A	10
Androsterone		53-41-8	3.43	A & B	13.3
Androsterone glucuronide		1852-43-3	1.6	B	15
Epitestosterone		481-30-1	3.25	A	10
Etiocholanolone		53-42-9	3.43	A & B	13.3
3'-Hydroxy Stanozolol	Stanozolol M1	125709-39-9	4.33	A	10
3'-Hydroxy Stanozolol glucuronide	Stanozolol M1 glucuronide	361432-14-9	2.16	A & B	5
Trenbolone		10161-33-8	2.63	A	10
17-Epitrenbolone	Trenbolone M1	80657-17-6	2.63	A	10
Dehydrochlormethyltestosterone (DHCMT)		2446-23-3	4.0	A	8
6β-Hydroxy-dehydrochlormethyltestosterone	DHCMT M1	25486-01-5	3.09	A	10
SARMs and other anabolic agents					
Andarine		401900-40-1	2.6	A & B	2
Cardarine		317318-70-0	5.85	A & B	2

Biomarker	Metabolite name	CAS number	LogP	Analytical method	Spiking level (µg/L)
GW 501516 Sulfone	Cardarine M1	1206891-27-1	4.29	A & B	2
GW 501516 Sulfoxide	Cardarine M2	1206891-26-0	4.26	A & B	10
Clenbuterol		37148-27-9	2.79	A	2
Enobosarm		841205-47-8	2.93	A	8
Ligandrol		1165910-22-4	3.29	A & B	5
Stenabolic		1379686-30-2	4.78	A	5
Ethyl N-(5-nitro-2-methylthiophene)-3-aminomethylpyrrolidine-1-carboxylate	Stenabolic M2	N/A	2.46	A	10
N-[(4-Chlorophenyl)methyl]-5-nitro-2-thiophenemethanamine hydrochloride	Stenabolic M6	1384516-10-2	3.56	A & B	10
Testolone		1182367-47-0	3.02	A & B	5
YK-11		1370003-76-1	4.61	A	9

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2.2 Instrument method (LC-MS/MS)

Analytes of interest were optimised by direct infusion into a Sciex QTRAP® 6500+ mass spectrometer (MS) to determine declustering potential, collision energy, and collision exit cell potential for individual precursor/product transitions (Table S1.). Positive and negative electrospray ionisation (ESI) modes were investigated for all compounds. The optimised MS parameters were: temperature 530 °C, curtain gas 30 psi, IonSpray voltage 4500 V and -4500 V, and Ion Source Gas 1 and 2, 80 psi.

The Shimadzu Nexera liquid chromatography (LC) conditions were: flow rate 0.4 mL/min, oven temperature 45 °C, autosampler temperature 4 °C, and injection volume 8 µL. The column used was a Kinetex® 1.7 µm C18 100 Å 100 x 2.1 mm, with a SecurityGuard™ ULTRA C18 2.1 mm guard column (Phenomenex, Lane Cove West, NSW, AU). The time program was as follows: 0-0.5 min 20% B, 0.5-1.5 min linear increase to 45% B, 1.5-15 min linear increase to 75% B, 15-16 min linear increase to 100% B, 16-19.9 min held at 100% B, 19.9-20 min linear decrease to 20% B, and finally 20-24 min held at 20% B. To obtain optimal sensitivity for each biomarker, two separate LC methods (method A, method B) were required.

2.2.1 Method A

The aqueous mobile phase for method A was 0.4 mM ammonium fluoride (NH_4F) in 95:5 ultrapure water/methanol (v/v). Mobile phase B was 0.4 mM NH_4F in 95:5 methanol/ultrapure water (v/v). Method A contained 54 out of 59 analytes, in addition to 14 isotopically labelled standards (Table 1, Table S2). For compounds which were analysed in both methods, for simplicity, we used the calculated concentrations based on this method for the in-sewer experiments.

2.2.2 Method B

Mobile phases for method B were: 0.1% acetic acid in 95:5 ultrapure water/methanol (v/v) and 0.1% acetic acid in 95:5 methanol/ultrapure water (v/v). Twenty-six out of 59 analytes and three internal standards were analysed with method B.

2.3 Instrument method performance

Prior to analysis of the in-sewer samples, performance of the instrument methods including linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) was assessed, following The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines[18], to ensure method applicability. The instrument method performance assessment was employed as a proof of concept to determine if the methods are suitable for direct injection analysis of steroids and SARMs spiked into wastewater influent, and to investigate criterion 3), i.e., detectability of these biomarkers in wastewater matrix. Raw wastewater influent was collected and immediately preserved with hydrochloric acid (HCl) to pH 2. Calibration solutions in HCl-preserved (pH 2) and filtered (0.2 µm Regenerated Cellulose) raw wastewater were prepared at 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, and 20 µg/L (20% methanol (v/v)). Weighting of 1/x was applied to determine linearity. Precision and accuracy for each compound were determined at low, medium and high-level spikes (n=8), by dividing the standard deviation by the mean then multiplying with 100 and by dividing the mean by the expected value and multiplying with 100, respectively. Low level spikes were 0.08, 0.31 and 1.25 µg/L for method A, and 0.08, 0.63 and 1.25 µg/L for method B. Medium and high-level spikes for both methods were 5 µg/L and 10 µg/L, respectively. LOD and LOQ were determined by multiplying the standard deviation of the response (n=8) with 3.3 and 10, respectively, and dividing that value by the slope of the calibration curve.

Subsequently, a second performance assessment experiment was conducted to determine accuracy and precision for analytes with LOQs lower than 0.08 µg/L for method A. Calibration solutions ranging from 0.0025 – 20 µg/L (N=14) were prepared in HCl-preserved and filtered wastewater and 20% methanol (v/v). Low level accuracy and precision were determined at 0.01 (n=8) and 0.04 µg/L (n=7). Method performance was acceptable for linearity (R^2>0.995, few exceptions), accuracy (68-119%), and precision (1-21 %RSD) (details in SI and Table S3).

2.4 Quality assurance and control

A calibration series in HCl-preserved and filtered wastewater influent ranging from 0.08 – 20 µg/L (N=9) was run at the beginning of each batch (20% methanol (v/v)). One calibration solution was analysed every 10 injections. Acidified ultrapure water spiked with 7 µg/L isotopically labelled standards was injected every 20 samples and served as a blank (BLK). The equivalent to a non-extracted side spike (NESS) was prepared by fortifying HCl-acidified ultrapure water with 5 µg/L native analytes and 7 µg/L internal standards (7.4% methanol (v/v)). A spike (SPK) was also prepared every 20 samples to calculate analyte recovery. The SPK was prepared by dividing a wastewater sample into two aliquots and fortifying one with 5 µg/L natives (10% methanol (v/v)). Recovery was calculated by subtracting the concentration of the analyte in the wastewater sample from the concentration of the SPK and dividing that by the NESS. Duplicates (DUP) were prepared every 8-12 samples.

2.5 Sewer reactor study

2.5.1 The sewer reactors

To investigate the stability of anabolic agents in realistic sewer environments, laboratory sewer reactors with demonstrated representative biological activity of real sewers were used in this study[19]. These comprised of a control reactor (CR), a gravity sewer reactor (GS), and a rising main reactor (RM). The GS and RM reactors have been operated for multiple years under aerobic and anaerobic conditions, respectively. Each reactor has a volume of 0.75 L, and the RM reactor has a small buffer container over the lid with a volume of 70 mL to prevent entry of oxygen during to wastewater sampling. Mature biofilms were cultivated inside the reactors showing strong biological activities, with resulting A/V ratios of 50 m^2/m^3 in the GS and 72.5 m^2/m^3 in the RM reactor. To maintain the reactors, they are fed with domestic sewage (typical sewage parameters; see SI) through a peristaltic pump (Masterflex 7520-47) every 6 hours. Homogeneous distribution in reactors was ensured by continuous mixing (250 rpm) with a magnetic stirrer. The CR was subjected to regular cleaning to ensure the absence of biofilm.

2.5.2 Study design

Batch tests were conducted to measure the biotic and abiotic transformation of biomarkers in sewer reactors. A total of three isolated experiments (week 1, week 2 and week 3) were conducted, each in triplicate (day 1, day 2, and day 3), for the purpose of separating parent compounds and metabolites, as well as glucuronides from their non-glucuronidated forms. Where possible, stereoisomers were also separated into different experiments. Generally, parent analytes and glucuronides were spiked in week 2 (day 1, 2 and 3). Metabolites were spiked in week 1 (day 2 and 3), as well as week 3 (day 1), and cardarine M1 was spiked in week 3 (day 1, 2 and 3; Table S2). The spiked wastewater was completely drained after each experimental day and was replaced 3-4 times between consecutive experimental days. Between different experimental weeks, wastewater was replaced over 20 times over 5 days. Acesulfame (stable under all conditions) and paracetamol (unstable in GS and RM) were included in the instrument method as an additional quality control, as these biomarkers have been investigated in the literature, occur at measurable levels in wastewater[16], and have previously been proposed as control chemicals[20].

Before each batch test, newly collected domestic wastewater (3L at room temperature) was spiked with the investigated biomarkers (for spiking levels see Table 1). After rapid mixing, the wastewater was fed into the three drained reactors through a peristaltic pump. Subsequently, samples were taken from each reactor at fixed time intervals of 0, 0.5, 1, 2, 4, 6, 8, and 12 hours after spiking. After collection, 1 mL was filtered, HCl-preserved to pH 2 in 2 mL glass amber vials, and frozen immediately at -80°C until sample preparation for analysis (around 4 months). Biological conditions in the GS and RM were monitored by measuring the dissolved sulfur species, methane, pH and temperature. The sulfide and methane production rates are standard parameters indicative of biological activitiy in sewers. They were determined by measuring changes in dissolved sulfide and methane in the first hour HRT after spiking (see SI).

2.5.3 Sample preparation

Samples were defrosted, and 250 µL was aliquoted into a glass amber vial, spiked with internal standards to a final concentration of 7 µg/L (2.7% methanol (v/v)), and vortexed. This was then divided into two separate vials with glass inserts, one for analysis using method A, one for method B. The vials were kept at -80°C until analysis.

Isotopically labelled standards were available for 14 of the 59 analytes. To adjust for potential variations in the instrument run such as injection volume, available isotopically labelled standards were allocated to all analytes in this study. Isotopically labelled standard allocations were determined by either structural similarity or retention time (Table S2.).

2.6 Statistical analysis

Sewer reactor data were fitted using three kinetic models – zero order kinetics (simple linear regression), first order kinetics (one-phase exponential decay/association), and exponential two-phase decay via MatlabR2015b (Higham, D. J., & Higham, N. J. (2016). MATLAB guide. Society for Industrial and Applied Mathematics; equations in SI). In order to make sure that the generated model parameters were meaningful, a non-negativity condition was set on the final concentrations. Additionally, we set a maximum of 300% of mass reduction for the fast reaction fraction of the two-phase model. This reduction in the degrees of freedom enabled us to generate comparable results across the three model orders. To select the model order, we used the combination of the regression coefficient and the root mean square error (RMSE) of the model. In other words, the model with the highest R^2 and the lowest RMSE was selected as the one describing the data the best. Additionally, the confidence interval of each model parameter, using an alpha of 0.05 was calculated using the QR decomposition of Jacobian matrix, degrees of freedom, and the RMSE.

The curves (Figure 1.) were plotted in accordance with the best-fit regression model of zero order kinetics, 1st order kinetics, and two-phase decay using GraphPad Prism (version 8.4.1).

LogP values were predicted using the Molinspiration property calculation service (http://www.molinspiration.com).

3 Results and discussion

3.1 LC-MS/MS methods

3.1.1 Method A and B

Separation of 57 analytes was achieved, including structural and stereoisomers, parents and metabolites. Only two compounds, methyltestosterone M1 and norethandrolone M2, could not be separated on the C18 column as they share the same exact mass, multiple reaction monitoring (MRM) transitions, and retention time. Therefore, these metabolites were quantified together in this study and their results should be interpreted accordingly. It was possible to separate these two analytes on a phenyl-hexyl column, but this column did not sufficiently separate stereoisomers (data not shown).

3.1.2 Quality assurance and control

Mean recoveries (n=4) ranged from 42-166%, with 47 analytes being within the acceptable range of 80-120%. Mean duplicate differences ranged from 0.8-14.5% across all 59 biomarkers (Table S4). For the majority of biomarkers with recoveries below 80% and above 120%, internal standards of other native chemicals were used, which may have had different matrix effects as they were not exact matches. QC accuracy during the batches was between 79-115%. No carryover was observed in the instrument blanks. Instrument performance, including sensitivity, remained stable throughout all runs.

3.2 In-sewer study

3.2.1 Biological activity

During the study, the sewer reactors presented strong biological activities under natural temperature conditions (week 1: 22.1±0.6°C; week 2: 21.9±0.6°C; week 3: 21.4±0.4°C). Wastewater pH remained consistent during the batch tests (week 1: CR 7.63±0.13, GS 7.29±0.09, RM 7.17±0.08;

week 2: CR 7.50±0.18, GS 7.13±0.08, RM 7.06±0.08; week 3: CR 7.58±0.18, GS 7.27±0.08, RM 7.16±0.08). Stronger activities of sulfate reducing bacteria and methanogens were found in the RM reactor as indicated by the evident sulfide and methane productions (sulfide: week 1: 5.56; week 2: 4.68; week 3: 5.29 mgS/L/h; methane: week 1: 25.86; week 2: 23.10; week 3: 19.94 mgCOD/L/h). These activities were similar to the biological conditions in diverse sewer systems, including sewer reactors, pilot sewer systems, and real rising main pipelines[19, 21]. Measured production of dissolved sulfide and methane were lower in the GS reactor (sulfide: week 1: 1.70; week 2: 1.70; week 3: 1.76 mgS/L/h; methane: week 1: 4.02; week 2: 4.38; week 3: 4.91 mgCOD/L/h). This could be attributed to the presence of oxygen and the transfer of H_2S and methane from the wastewater phase to air. No activities were detected in the biofilm-absent CR.

3.2.2 Initial concentrations in the reactors

Generally, analyte concentrations in the t_0 sample in all three reactor types differed from the spiked theoretical concentrations (Figure S1). The t_0 sample is the first sample that was collected immediately after feeding the spiked wastewater into the reactors. The mean biomarker concentrations (n=3) of t_0 in each reactor type were divided by the theoretical spiked concentrations and expressed as CR%, GS% and RM%. Mean and median (N=59) were 78% and 82% for CR, 67% and 70% for GS, and 51% and 49% for RM. In addition to the instrument method variability, the mean initial concentration in the CR (and both GS, RM reactors) would have likely been influenced by sorption to the suspended solids/particulate matter[22]. Furthermore, the additional decline in % of initial concentration in wastewater from the GS and RM reactors, compared to the biofilm-free reactor, may not be a result of immediate transformation or degradation but could instead be the result of potential increased matrix suppression with increasing biofilm and/or rapid sorption to the biofilm. The latter is supported by the fact that most of these analytes are moderately hydrophobic and have logP values >2.5. Further support is that no significant initial decrease in concentration of fluoxymesterone M1 was observed for CR, GS and RM wastewater, which has the lowest logP value

285 (2.03) of all non-glucuronidated compounds investigated and is therefore the least likely to rapidly
286 adsorb to the biofilm and/or particulate matter to a large extent.

287 Regressions of logP value and CR%, GS% and RM% were explored to investigate if a high logP
288 value could have led to a stronger adsorption at time 0 to the biofilm and/or particulate matter (logP
289 and RM% correlation, Figure S2). R^2 values were 0.402 (CR%), 0.344 (GS%) and 0.421 (RM%). This
290 indicates a weak to moderate association between logP value and adsorption potential, but also
291 suggests that other factors may be contributing. For example, stenabolic, stenabolic M2, and
292 stenabolic M6 showed lower calculated concentrations than predicted through the correlation
293 trendline, with predicted values of 29%, 66%, and 49%, and measured initial concentrations of 8%,
294 45%, and 13%, respectively. Due to their molecular structures, stenabolic and its metabolites may be
295 unstable in the wastewater matrix. This is consistent with their degradation in the biofilm-absent CR
296 over 12h.

297 Interestingly, when grouping the steroids by their molecular structure, 3, 17-hydroxy steroids
298 (N=8) had the lowest initial concentrations in all three reactor types, with means (and medians) of
299 71% (75%) CR, 62% (65%) GS and 44% (42%) RM. 3-hydroxy, 17-oxo steroids (N=9) had higher means
300 (and medians): 80% (83%) CR, 70% (74%) GS and 48% (49%) RM. The 3-oxo, 17-hydroxy steroid group
301 (N=20) had the highest initial concentration means (and medians) of 91% (98%) CR, 78% (84%) GS and
302 63% (66%) RM reactor. LogP values within the three groups averaged at 3.81, 3.51 and 3.45,
303 respectively.

304 ### 3.2.3 Carryover between sewer stability experiments and transformation between analytes

305 Some metabolites only spiked in week 3, day 1, were detected in day 2 and 3 samples from
306 the GS and RM reactors. Generally, this only occurred in wastewater sampled from the reactors where
307 biofilm was present. A possible explanation for this is that sorption and the slow desorption of analytes
308 to and from the biofilm may have occurred. Contamination was excluded as a possibility, as the same
309 wastewater was fed into the three reactors and wastewater from the CR did not contain measurable
310 concentrations of the anabolic agents when they were not spiked (for analytes not already present in

311 raw wastewater). Furthermore, concentrations of non-spiked metabolites in the GS and RM increased
312 from the first to the last measurements for the detected metabolites, suggesting that desorption
313 occurred at a faster rate than degradation or transformation in unspiked wastewater at low levels,
314 e.g., norethandrolone M3 concentrations increased from 0.09 to 0.53 µg/L in the GS and from 0.16 to
315 0.76 µg/L in the RM in week 3, day 2 (spiking level week 3, day 1: 10 µg/L). It is important to note,
316 however, that quantified concentrations were low (<10%) compared to the spiked concentration
317 levels in this study, with the largest maximum carryover percentages in week 3, day 2 samples
318 observed to be methyltestosterone M2 (GS: <LOQ to 0.87 µg/L (max. 8.7%); RM: <LOQ to 0.89 µg/L
319 (max. 8.9%)) and fluoxymesterone M1 (GS: <LOQ to 0.12 µg/L (max. 1.2%); RM: 0.14 to 0.87 µg/L (max.
320 8.7%)), both spiked at 10 µg/L in week 3, day 1. While this suggests sorption/desorption of biomarkers,
321 it is not likely to have had a significant impact on trends of subsequent experiments. One exception,
322 however, is etiocholanolone which increased in the GS from 2 to 5.2 µg/L (max. 39% carryover) and in
323 the RM from 1.8 to 3.9 µg/L (max. 29%). In this case, it is difficult to estimate whether the increase
324 was caused by desorption from the biofilm or transformation of a different analyte, such as a
325 glucuronide, into etiocholanolone. As this biomarker is endogenous and readily found in wastewater,
326 it would have been present in the reactors in previous experiments. A similar trend was found in
327 samples from week 2 where this analyte was not spiked. This will have likely had an impact on the
328 stability data of etiocholanolone in the sewer reactors. Based on these observations, we would
329 recommend future in-sewer stability experiments consider sorption/desorption.

330 Many parent compounds that were spiked and investigated in week 2 were also detected in
331 samples from week 3, day 1, in few cases above their LOQ. In this case, rather than desorption from
332 the biofilm, the low levels of parent analytes were likely a result of transformation from the spiked
333 metabolites. This is supported by samples from week 1, day 2&3, where very low levels of parents
334 were detectable, despite never have being spiked into the reactors and not being present in the blank
335 wastewater sample. While it is important to be aware of the possible transformation of the
336 metabolites into the parents, the spiked levels of the metabolites are high, while detected levels of

337 the parent biomarkers were very low (<5%). For the biomarkers investigated in this study, this will
338 likely not lead to false positives of parents when analysing wastewater, unless the metabolites are
339 present at very high levels, but should be taken into consideration when interpreting results.

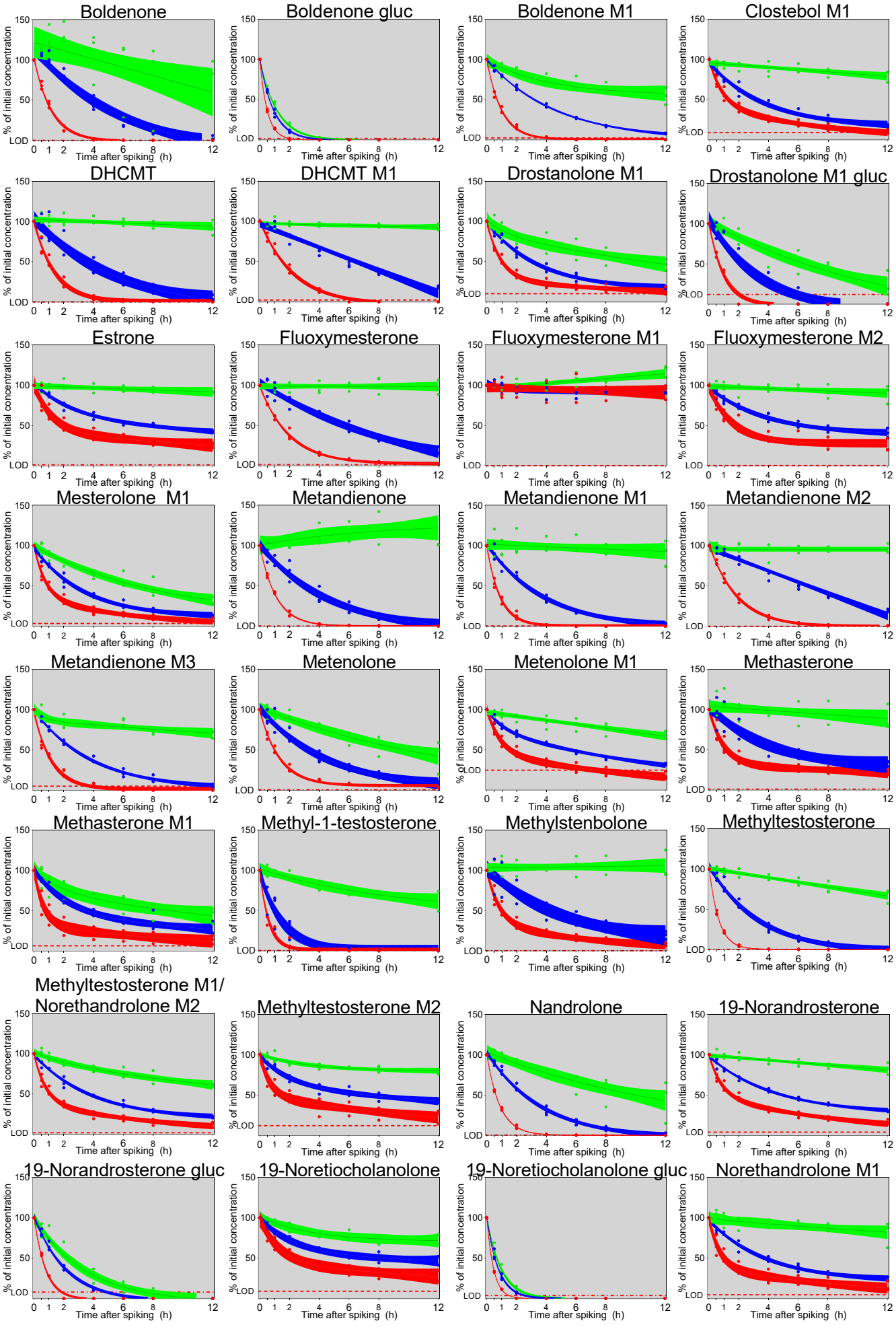
340 An exception was methasterone M1, which was spiked in week 1, and while its concentration
341 in all three reactor types decreased, the concentration for its non-spiked parent methasterone
342 increased according to first order kinetics for CR and GS, from 0.07 to 1.77 µg/L and 0.09 to 0.3 µg/L,
343 respectively (mean, n=3). The mean concentration increase between the CR, GS and RM was
344 significant (one-way ANOVA, $p < 0.05$), and the concentration increase was greatest in the CR and least
345 in the RM. This indicates that with decreasing quantities of biofilm either i) higher concentrations of
346 the parent are being formed from the metabolite, or ii) less of the parent compound is being
347 adsorbed/transformed upon formation.

348 ### 3.2.4 Stability of metabolites and parent analytes

349 All biomarkers were most stable over 12h in the CR, less stable in the GS reactor, and least
350 stable in the RM reactor (exception etiocholanolone, Figure 1). This is in agreement with previous
351 studies on different compounds[16, 19, 23]. The results of the control biomarkers acesulfame (stable in all
352 reactor types) and paracetamol (stable in the CR, greater than 10% loss in <3 hours in the GS and RM
353 reactors) were consistent with results found in the literature[16].

354 The in-sewer stability for chemicals previously investigated in the literature generally follows
355 a linear regression or first-order kinetics[16, 20, 23], whereas many biomarker concentration curves
356 investigated in this study follow a two-phase decay regression where the first fast phase may be a
357 combination of sorption and transformation and the second phase may be a combination of
358 desorption and transformation (for the model of best fit for each biomarker, and equation parameters
359 see Table S5.). Briefly, out of a total of 61 biomarker concentration curves for each reactor type (183
360 total), 24 (CR), 5 (GS), and 4 (RM) curves best fit a linear regression; 26 (CR), 39 (GS), and 32 (RM) best
361 fit first order kinetics; and 11 (CR), 17 (GS), and 25 (RM) best fit two-phase decay. This shows that 18%,
362 53%, and 29% of all concentration curves follow a linear, first order, and two-phase regression,

363 respectively. In addition, it demonstrates an increase in the number of analytes following two-phase
364 kinetics with increasing biofilm. This suggests that sorption and desorption may play a role in in-sewer
365 anabolic agent stability, while this may not have been the case for many previously investigated
366 chemicals. It should be noted, however, that the lack of two-phase decay data may also be a result of
367 previous studies focussing on zero and first-order kinetics and not investigating two-phase decay
368 behaviour.


Boldenone
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Boldenone M1
Clostebol M1
DHCMT
DHCMT M1
Drostanolone M1
Drostanolone M1 gluc
Estrone
Fluoxymesterone
Fluoxymesterone M1
Fluoxymesterone M2
Mesterolone M1
Metandienone
Metandienone M1
Metandienone M2
Metandienone M3
Metenolone
Metenolone M1
Methasterone
Methasterone M1
Methyl-1-testosterone
Methylstenbolone
Methyltestosterone
Methyltestosterone M1/ Norethandrolone M2
Methyltestosterone M2
Nandrolone
19-Norandrosterone
19-Norandrosterone gluc
19-Noretiocholanolone
19-Noretiocholanolone gluc
Norethandrolone M1
% of initial concentration
Time after spiking (h)
LOD

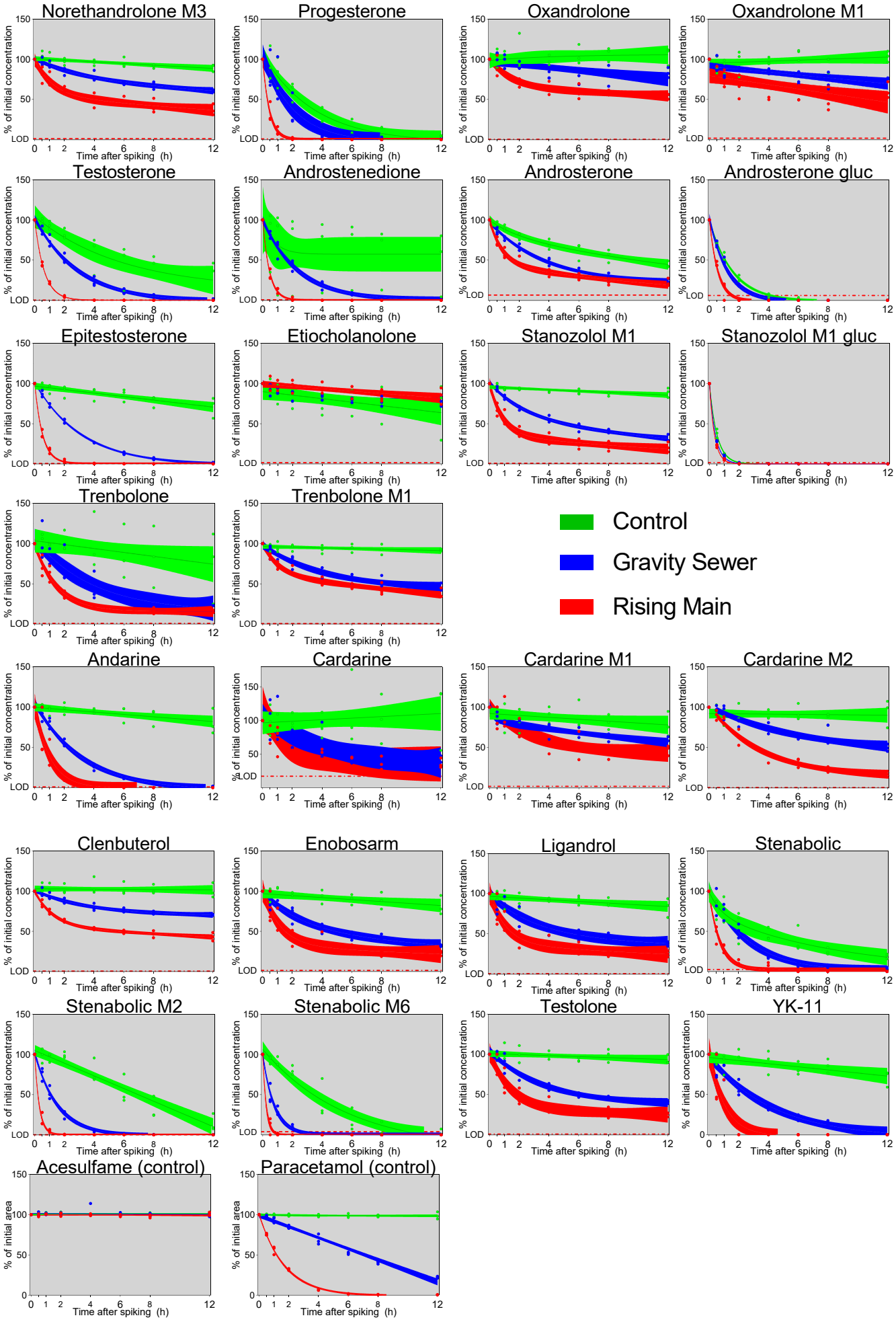
Norethandrolone M3
Progesterone
Oxandrolone
Oxandrolone M1
Testosterone
Androstenedione
Androsterone
Androsterone gluc
Epitestosterone
Etiocholanolone
Stanozolol M1
Stanozolol M1 gluc
Trenbolone
Trenbolone M1
Andarine
Cardarine
Cardarine M1
Cardarine M2
Clenbuterol
Enobosarm
Ligandrol
Stenabolic
Stenabolic M2
Stenabolic M6
Testolone
YK-11
Acesulfame (control)
Paracetamol (control)
% of initial concentration
% of initial area
Time after spiking (h)
LOD
Control
Gravity Sewer
Rising Main

Figure 1. Regression models for degradation of anabolic agents in control, gravity sewer, and rising main lab-scale sewer reactors. Horizontal axes show the time after spiking in hours; vertical axes show percent of initial concentration. Models were plotted according to the best fit of linear, first-order, and two-phase decay regressions. Error bands are the 95% confidence interval.

3.2.5 Steroid glucuronides

Glucuronides fully transformed or degraded in all three reactor types within 4-6 hours. Exceptions were 19-norandrosterone glucuronide (8-12h) and drostanolone M1 glucuronide (8-12h). Both are 3-*O*-glucuronides with 5α configuration. 3-*O*-glucuronides have previously been shown to be more stable than 17-*O*-glucuronides, and α conjugates more stable than those with β configuration[24]. This correlated with the findings in our study. While the concentration of the glucuronides decreased, the concentration of their non-glucuronidated forms increased in all three reactors (CR>GS>RM; Table S6). Due to logistical reasons, boldenone was spiked during the same experiment as its glucuronide. An increase in boldenone's initial concentrations was observed in the CR and GS. However, the percentage of transformation was not calculated, as the cause of this increase could not be determined, i.e., formation through sources other than glucuronide deconjugation.

3.2.6 Half-lives under aerobic conditions

Time until 10%, 50%, and 90% transformation was determined (Figure 2). Anabolic agents were sorted into three groups based on their mean half-lives in the GS, **a)** ≥ 4h, **b)** 4-2h, and **c)** ≤ 2h (Table 2.). Gravity sewer half-lives were chosen, because the majority of sewage systems consist of high proportions of gravity sewer pipelines[25]. Hydraulic residence times between WWTP catchments can vary greatly, therefore, the mean HRT of 4 hours, from a European study including 25 WWTPS from 11 countries, was selected[25].

Table 2. Biomarkers categorised into three groups by half-lives in hours.

Group A (≥ 4h)		**Group B (4-2h)**		**Group C (≤ 2h)**	
Analyte	**t_{50} (h)**	**Analyte**	**t_{50} (h)**	**Analyte**	**t_{50} (h)**

Estrone	7.8	Boldenone	3.6	Boldenone gluc	0.6
Fluoxymesterone	5.5	Boldenone M1	3.2	Methyl-1-testosterone	1.3
Fluoxymesterone M1	6.9	Clostebol M1	3.6	19-Norandrosterone gluc	1.4
Fluoxymesterone M2	6.9	Drostanolone M1	3.5	19-Noretiocho-lanolone gluc	0.5
Metandienone	6.7	Drostanolone M1 gluc	2.2	Progesterone	1
Methasterone	5.5	Mesterolone M1	3	Stanozolol M1 gluc	0.3
Metenolone M1	5.7	Metandienone	2.8	Testosterone	2
Methylstenbolone	4.5	Metandienone M1	2.5	Androstenedione	1.8
Methyltestosterone M1 /Norethandrolone M2	4.1	Metandienone M3	2.7	Androsterone gluc	0.9
Methyltestosterone M2	7.2	Methasterone M1	3.6	Stenabolic	1.8
19-Norandrosterone	5.6	Metenolone	3.2	Stenabolic M2	1
19-Noretiocholanolone	7.5	Methyltestosterone	2.2	Stenabolic M6	0.6
Norethandrolone M1	4.4	Nandrolone	2.4		
Norethandrolone M3	22.8	Epitestosterone	2.1		
Oxandrolone	25.5	Trenbolone	2.7		
Oxandrolone M1	23	DHCMT	2.7		
Stanozolol M1	5.1	Andarine	2.1		
Androsterone	4.2	YK-11	2.6		
Etiocholanolone	81.3				
Trenbolone M1	9.3				
DHCMT M1	6.5				
Cardarine	5.7				
Cardarine M1	15.6				
Cardarine M2	10.3				
Clenbuterol	77.1				
Enobosarm	4.8				
Ligandrol	5.7				
Testolone	6.8				

It is important to point out, that while HRTs in real sewage systems may be longer than 4 hours, sewer reactors generally overestimate degradation and transformation degree of biomarkers when compared to real sewer systems[23, 26]. One reason for this is that the biofilm-area-to-wastewater-volume ratio is higher in the reactors than in most of the sewer network. This indicates that, under real-world conditions, the half-lives may be longer than determined in this study, and the values here should be viewed as conservative, or the maximum likely degradation. Based on these results, it can be estimated that most of these biomarkers will be detectable and quantifiable in wastewater influent, if sufficiently sensitive methods are used. This is supported by the fact that, despite our

404 results indicating complete degradation of testosterone and progesterone in the GS and RM reactors,

405 they have been analysed quantitatively in wastewater in multiple countries[11, 12, 27].

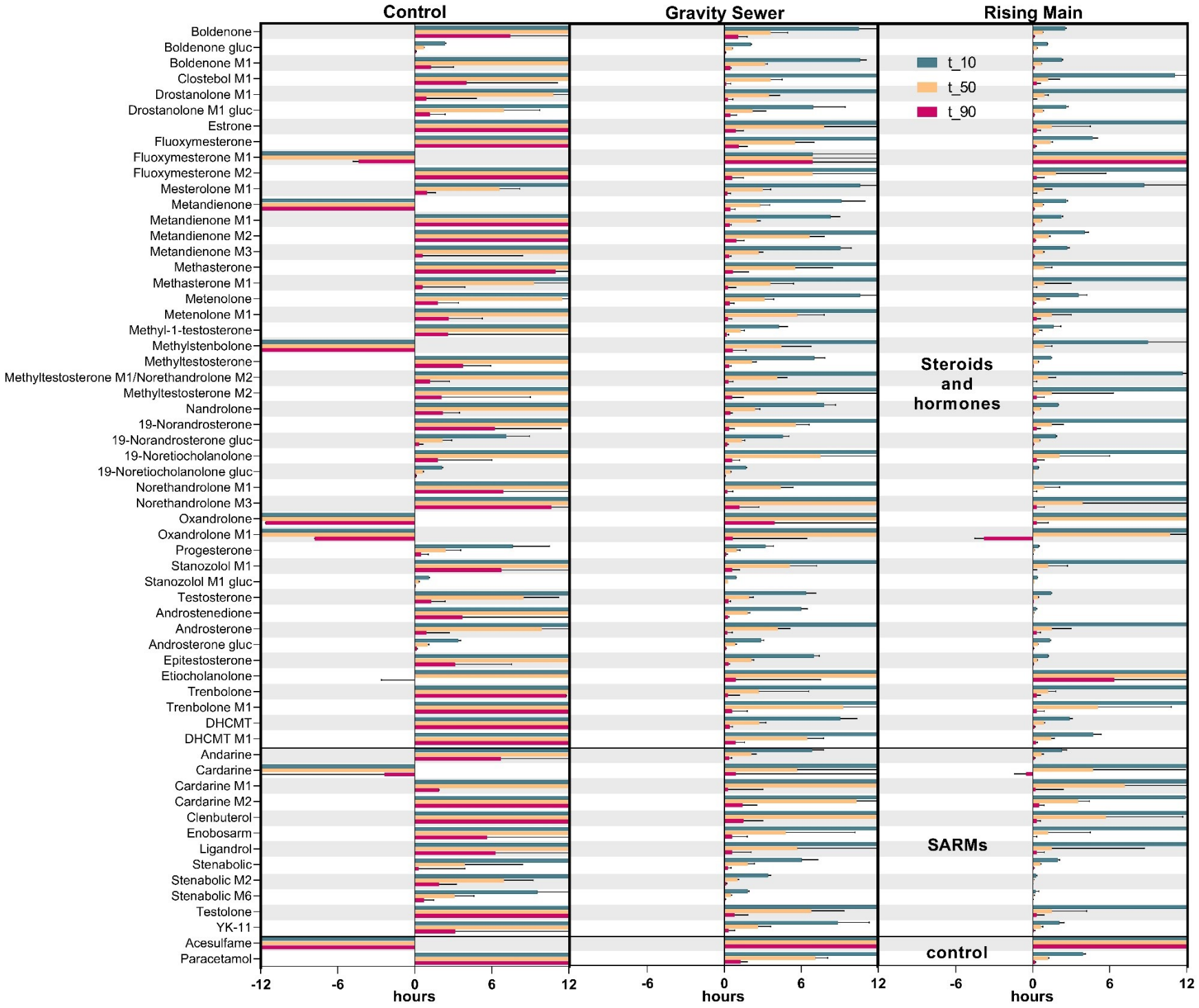
Control
Gravity Sewer
Rising Main
t_10
t_50
t_90
Steroids and hormones
SARMs
control
Boldenone
Boldenone gluc
Boldenone M1
Clostebol M1
Drostanolone M1
Drostanolone M1 gluc
Estrone
Fluoxymesterone
Fluoxymesterone M1
Fluoxymesterone M2
Mesterolone M1
Metandienone
Metandienone M1
Metandienone M2
Metandienone M3
Methasterone
Methasterone M1
Metenolone
Metenolone M1
Methyl-1-testosterone
Methylstenbolone
Methyltestosterone
Methyltestosterone M1/Norethandrolone M2
Methyltestosterone M2
Nandrolone
19-Norandrosterone
19-Norandrosterone gluc
19-Noretiocholanolone
19-Noretiocholanolone gluc
Norethandrolone M1
Norethandrolone M3
Oxandrolone
Oxandrolone M1
Progesterone
Stanozolol M1
Stanozolol M1 gluc
Testosterone
Androstenedione
Androsterone
Androsterone gluc
Epitestosterone
Etiocholanolone
Trenbolone
Trenbolone M1
DHCMT
DHCMT M1
Andarine
Cardarine
Cardarine M1
Cardarine M2
Clenbuterol
Enobosarm
Ligandrol
Stenabolic
Stenabolic M2
Stenabolic M6
Testolone
YK-11
Acesulfame
Paracetamol
-12
-6
0
6
12
hours

Figure 2. Time until 90% (t_90), 50% (t_50), and 10% (t_10) transformation in the control, gravity, and rising main sewer reactors for anabolic agents. Bars with negative hour values represent an increase in initial concentration over 12h, positive values represent a decrease in initial concentration. Note that the x axis has been set at a maximum value of 12h as estimates beyond this are extrapolation only.

3.3 Implications for wastewater analysis suitability of biomarkers

This study investigated biomarker stability over a period of 12h in a non-equilibrated system. It is difficult to estimate the amount of time required to reach equilibrium in our lab-scale reactors. In real sewage pipelines it is possible that an equilibrium is present, and that there is a constant rate of sorption and desorption. Therefore, the initial decline in concentration over the first couple of hours observed in this study may not be as pronounced in actual sewers. If the desorption rate is larger than the rate of degradation/transformation, then there is an increased chance in detecting these biomarkers in collected wastewater samples as supported by our results. Furthermore, shorter catchment HRTs, increase the possibility of detecting and quantifying these biomarkers. Therefore, upstream sampling, i.e., sampling closer to potential sources, will likely improve the detectability of anabolic agents and their transformation may be more reflective of the results in the CR.

In other studies biomarkers were determined to be stable when i) <20% loss was observed over 12h[28], ii) <10% loss was observed >12h, or iii) $R^2 < 0.3$ or regressions did not significantly deviate from zero[23], these criteria are likely not completely transferrable to biomarkers affected by sorption/desorption. It should, however, be noted that these criteria were applied to determine if back-calculation for consumption from measured concentrations was possible. We consider that for many biomarkers investigated in this study, precise back-calculations to estimate steroid and SARM use may not be advisable, especially for biomarkers in groups B and C. Furthermore, comparing concentrations of these chemicals across different sewage treatment plants presents a great challenge, as the number, length, and other characteristics of gravity and rising main sewer pipelines

and HRTs can vary significantly. Nevertheless, wastewater analysis has great promise for qualitative analysis to determine which anabolic agents are being used in the community, and trends over time. This could be used to assess seasonal and annual differences, and potential patterns in use of specific steroids or SARMs within the same population.

Supporting Information.

Tables (6), figures (2), equations (3), and explanatory text, as mentioned in the manuscript

4 Acknowledgements

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Supplementary Information (SI) for

In-sewer stability assessment of steroids and selective androgen receptor modulators

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2 Figures, 6 Tables and 3 equations;

Materials and methods

Lichrosolv grade methanol and analytical grade hydrochloric acid (32%) were purchased from Merck Pty Ltd (Highway Bayswater, VIC, AU) and 0.2 µm RC filters from Agilent (Mulgrave, VIC, AU). Ammonium fluoride was sourced from Sigma-Aldrich (Castle Hill, NSW, AU). Water was purified to 18.2 MΩ cm^{-1} using a Milli-Q ultrapure water system and filtered using a 0.22 µm filter (Millipore, Bedford, MA, USA).

Andarine, clenbuterol (hydrochloride), ligandrol, stenabolic, and YK-11 were purchase from Cayman Chemical (Ann Arbor, MI, USA). D_3-boldenone, boldenone glucuronide (K salt), boldenone M1, d_9-clenbuterol (hydrochloride), clostebol M1, dehydrochlormethyltestosterone, dehydrochlormethyltestosterone M1, drostanolone M1, drostanolone M1 glucuronide (Na salt), fluoxymesterone M1, fluoxymesterone M2, mesterolone M1, metenolone M1, metandienone M1, metandienone M2, metandienone M3, methasterone, methasterone M1, methyl-1-testosterone, methyltestosterone M1, methyltestosterone M2, d_3-methyltestosterone M2, 19-norandrosterone, d_4-19-norandrosterone, 19-norandrosterone glucuronic acid (Na salt), 19-noretiocholanolone, d_4-19-noretiocholanolone, 19-noretiocholanolone glucuronic acid (Na salt), norethandrolone M1, norethandrolone M2, norethandrolone M3, oxandrolone M1, stenabolic M2, stenabolic M6, stanozolol M1, stanozolol M1 glucuronide, androsterone, d_4-androsterone, androsterone glucuronide, etiocholanolone, d_5-etiocholanolone, trenbolone M1, 17α-methyltestosterone, oxandrolone, testosterone, and d_3-epitestosterone were sourced from National Measurement Institute (North Ryde, NSW, AU). Nandrolone was supplied by British Pharmacopoeia Commission Laboratory (Teddington, Middlesex, UK). Sigma-Aldrich (Castle Hill, NSW, AU) provided boldenone, estrone, metandienone, progesterone and trenbolone.

Androstenedione, $^{13}C_3$-androstenedione, and epitestosterone were sourced from Cerilliant (Round Rock, TX, USA). D_3-Nandrolone and d_3-testosterone were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Fluoxymesterone was supplied by LGC (Luckenwalde, Germany). Cardarine M1, cardarine M2, methasterone M1, metenolone, methylstenbolone, testolone, d_5-stanozolol M1, d_5-trenbolone, d_5-trenbolone M1 were purchased from Toronto Research Chemicals Inc. (North York, ON, CA). Enobosarm was sourced from Selleck Chemicals Llc (Houston, TX, USA).

Instrument method (LC-MS/MS)

Optimised MS parameters: temperature 530 °C, curtain gas 30 psi, IonSpray voltage 4500 V and -4500 V, and Ion Source Gas 1 and 2, 80 psi.

Table S1. Optimised MS conditions of each MRM transition including ESI polarity, precursor and selected product ions, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP).

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
Method A							
13C3-Androstenedione	+	290.0	112.0	90	10	30	17
	+	290.0	100.0	90	10	28	12
17a-Methyltestosterone	+	303.2	267.1	100	10	24	16
	+	303.2	227.1	100	10	27	14
	+	303.2	109.1	100	10	33	18
19-Norandrosterone	+	259.3	241.3	120	10	18	20
	+	277.3	241.4	50	10	19	14
	+	259.3	145.3	120	10	26	20
19-Noretiocholanolone	+	259.3	241.4	140	10	15	15
	+	259.3	145.3	140	10	27	17
	+	259.3	201.4	140	10	21	25
Andarine	+	442.2	400.1	130	10	21	25
	+	442.2	208.2	130	10	27	12
	+	442.2	190.2	130	10	31	11
Androstenedione	+	287.3	97.0	80	10	26	15
	+	287.3	109.1	80	10	29	15
	+	287.3	251.1	80	10	23	15
Androsterone	+	273.3	255.4	165	10	17	14
	+	273.3	147.3	165	10	27	17
	+	273.3	105.3	165	10	43	24
Boldenone	+	287.2	121.1	58	10	30	15
	+	287.2	135.1	58	10	20	22
	+	287.2	269.1	58	10	14	16
Boldenone M1	+	289.3	271.3	110	10	17	17
	+	289.3	187.3	110	10	25	23
	+	289.3	201.3	110	10	22	24

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
Clenbuterol	+	277.2	203.1	60	10	22	12
	+	277.2	259.2	60	10	14	15
	+	277.2	132.2	60	10	39	14
Clostebol M1	+	305.3	269.2	120	10	13	17
	+	305.3	287.2	120	10	12	19
	+	305.3	251.2	120	10	19	15
d3-Boldenone	+	289.9	272.0	180	10	15	16
	+	289.9	137.9	180	10	20	22
	+	289.9	120.9	180	10	30	16
d3-Epitestosterone	+	291.9	108.9	100	10	32	13
	+	291.9	96.9	100	10	28	15
d3-Methyltestosterone M2	+	273.9	191.9	140	10	23	28
	+	273.9	178.0	140	10	24	19
	+	273.9	111.9	140	10	24	25
d3-Nandrolone	+	277.9	241.9	100	10	24	13
	+	277.9	108.9	100	10	34	12
d3-Testosterone	+	291.9	108.9	90	10	35	14
	+	291.9	96.9	90	10	31	14
d4-19-Norandrosterone	+	262.9	245.0	150	10	18	15
	+	262.9	189.0	150	10	26	23
	+	262.9	144.9	150	10	27	20
d4-19-Noretiocholanolone	+	262.9	245.0	150	10	16	14
	+	262.9	205.0	150	10	22	12
	+	262.9	189.0	150	10	22	11
d4-Androsterone	+	277.0	259.0	140	10	18	15
	+	277.0	160.9	140	10	25	22
	+	277.0	146.9	140	10	25	17
d5-Etiocholanolone	+	277.9	260.0	125	10	15	16
	+	277.9	220.0	125	10	21	25
	+	277.9	203.9	125	10	21	26
d5-Stanozolol M1	+	350.2	97.0	210	11	52	22
	+	350.2	91.1	210	11	105	12
	+	350.2	77.1	210	11	105	12
d5-Trenbolone	+	276.2	258.1	125	10	28	15
	+	276.2	232.1	125	10	31	13

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
	+	276.2	204.1	125	10	30	11
d5-Trenbolone M1	+	276.2	258.1	150	10	28	16
	+	276.2	230.0	150	10	31	13
	+	276.2	204.1	150	10	32	12
d9-Clenbuterol	+	285.8	203.9	70	10	22	24
	+	285.8	132.9	70	10	40	19
	+	287.8	205.8	60	10	22	21
Dehydrochlormethyltestosterone	+	335.3	317.3	90	10	16	19
	+	317.2	155.2	165	10	32	20
	+	335.2	155.2	90	10	35	18
Dehydrochlormethyltestosterone M1	+	333.2	315.1	155	10	15	19
	+	351.3	315.1	50	10	15	18
	+	333.2	155.1	155	10	34	20
Drostanolone M1	+	287.4	269.4	136	10	17	17
	+	287.4	145.3	136	10	24	20
	+	287.4	121.3	136	10	27	20
Epitestosterone	+	289.3	97.3	80	10	30	12
	+	289.3	109.3	80	10	31	16
	+	289.3	253.3	80	10	24	15
Estrone	-	269.3	145.3	-125	-10	-47	-10
	-	269.3	143.3	-125	-10	-69	-16
	-	269.3	159.3	-125	-10	-46	-10
Etiocholanolone	+	273.2	255.2	100	10	15	16
	+	273.2	215.2	100	10	21	26
	+	273.2	199.2	100	10	21	24
Fluoxymesterone	+	337.4	241.3	80	10	33	15
	+	337.4	223.3	80	10	33	14
	+	337.4	181.3	80	10	40	18
Fluoxymesterone M1	+	337.4	95.1	125	10	27	15
	+	337.4	317.2	125	10	16	19
	+	337.4	299.1	125	10	19	20
Fluoxymesterone M2	+	319.3	281.1	130	10	25	15
	+	319.3	225.1	130	10	31	25
	+	319.3	299.2	130	10	25	20
Cardarine	+	454.1	257.0	185	10	40	14

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
	+	454.1	188.1	185	10	59	22
	+	454.1	172.1	185	10	90	19
Cardarine M1	+	486.1	257.2	160	10	40	16
	+	486.1	256.2	160	10	65	13
	+	486.1	188.2	160	10	65	12
Cardarine M2	+	470.2	256.9	45	10	30	24
	+	470.2	255.9	45	10	20	25
	+	470.2	187.9	45	10	64	22
Ligandrol	+	339.3	199.2	145	10	40	11
	+	339.3	170.2	145	10	71	10
	+	339.3	152.2	145	10	65	17
Mesterolone M1	+	287.3	269.3	145	10	17	15
	+	287.3	161.3	145	10	22	19
	+	287.3	147.3	145	10	22	18
Metandienone	+	301.3	149.3	58	10	21	18
	+	301.3	121.3	58	10	34	15
	+	301.3	173.3	58	10	23	23
Metandienone M1	+	283.3	121.3	150	10	29	18
	+	301.3	121.3	60	10	32	19
	+	301.3	149.4	60	10	21	18
Metandienone M2	+	299.2	281.1	85	10	12	20
	+	299.2	147.1	85	10	25	17
	+	299.2	121.1	85	10	28	19
Metandienone M3	+	269.3	105.3	85	10	34	11
	+	269.3	201.3	85	10	25	25
	+	269.3	161.4	85	10	29	21
Methasterone	+	319.4	283.4	155	10	21	18
	+	319.4	301.4	155	10	19	18
	+	319.4	229.4	155	10	26	20
Methasterone M1	+	285.4	175.3	130	10	23	11
	+	285.4	161.3	130	10	23	9
	+	285.4	91.3	130	10	65	14
Metenolone	+	303.4	187.3	115	10	28	11
	+	303.4	83.2	115	10	25	21
	+	303.4	205.3	115	10	23	12

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
Metenolone M1	+	285.2	267.2	145	10	17	15
	+	285.2	121.1	145	10	28	20
	+	285.2	119.1	145	10	35	15
Methyl-1-T	+	303.2	201.2	120	10	24	24
	+	303.2	145.1	120	10	34	20
	+	303.2	91.1	120	10	67	14
Methylstenbolone	+	317.4	201.3	130	10	25	12
	+	317.4	145.3	130	10	34	16
	+	317.4	91.2	130	10	70	10
Methyltestosterone M1	+	271.3	161.2	80	10	24	25
	+	271.3	147.2	80	10	24	21
	+	271.3	135.2	80	10	25	21
Methyltestosterone M2	+	271.2	175.1	125	10	25	26
	+	271.2	189.1	125	10	23	27
	+	271.2	109.1	125	10	26	24
Nandrolone	+	275.2	239.1	90	10	23	15
	+	275.2	109.1	90	10	34	17
	+	275.2	213.1	90	10	27	25
Norethandrolone M1	+	271.2	175.3	120	10	21	20
	+	271.2	147.3	120	10	21	18
	+	271.2	121.2	120	10	24	19
Norethandrolone M3	+	287.3	243.3	95	10	18	21
	+	287.3	147.3	95	10	28	17
	+	287.3	121.3	95	10	28	19
Enobosarm	+	407.3	390.1	60	10	16	23
	+	390.3	370.1	140	10	18	22
	+	390.3	187.1	140	10	18	22
Oxandrolone	+	307.2	271.1	50	10	18	15
	+	307.2	229.1	50	10	23	13
	+	307.2	253.1	50	10	21	14
Oxandrolone M1	+	307.3	289.4	80	10	14	17
	+	289.3	229.4	160	10	22	28
	+	289.3	135.3	160	10	26	22
Progesterone	+	315.2	97.1	105	10	26	15
	+	315.2	109.1	105	10	28	15

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
	+	315.2	79.0	105	10	69	12
Testolone	+	394.2	223.1	120	10	15	12
	+	396.2	225.1	115	10	15	13
	+	394.2	170.1	120	10	39	20
Stenabolic	+	438.2	125.2	100	10	30	13
	+	440.2	127.2	100	10	30	11
	+	438.2	89.2	100	10	100	40
Stenabolic M2	+	314.2	268.1	70	10	18	15
	+	314.2	142.0	70	10	26	18
	+	314.2	221.1	70	10	26	12
Stenabolic M6	+	283.3	125.1	60	10	18	20
	+	283.3	89.2	60	10	69	13
Stanozolol	+	329.1	107.1	100	10	51	16
	+	329.1	203.1	100	10	45	24
	+	329.1	121.1	100	10	47	14
Stanozolol M1	+	345.1	97.0	90	10	51	13
	+	345.1	97.0	260	10	51	12
	+	345.1	121.0	90	10	50	17
Stanozolol M1 gluc	+	521.0	345.0	90	10	32	20
	+	521.0	121.0	90	10	77	18
	+	521.0	97.0	90	10	80	15
Testosterone	+	289.3	97.3	92	10	27	17
	+	289.3	109.3	92	10	31	13
	+	289.3	123.3	92	10	32	14
Trenbolone	+	271.3	227.3	100	10	31	13
	+	271.3	199.3	160	10	46	6
	+	271.3	165.3	160	10	46	6
Trenbolone M1	+	271.3	253.3	155	10	28	14
	+	271.3	199.3	155	10	32	23
	+	271.3	165.3	155	10	70	20
YK-11	+	356.9	324.9	140	10	17	18
	+	357.0	307.1	140	10	26	18
	+	357.0	267.1	140	10	27	15
Method B							
19-Norandrosterone gluc	-	451.3	113.2	-140	-10	-35	-18

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
	-	451.3	273.3	-140	-10	-55	-24
	-	451.3	275.3	-140	-10	-40	-23
19-Noretiocholanolone gluc	-	451.3	113.2	-150	-10	-37	-13
	-	451.3	85.3	-150	-10	-36	-10
	-	451.3	273.3	-150	-10	-55	-16
Andarine	-	440.4	261.4	-105	-10	-25	-11
	-	440.4	150.5	-105	-10	-36	-21
	-	440.4	107.4	-105	-10	-69	-17
Androsterone	+	273.4	105.4	165	10	43	24
	+	273.4	147.4	165	10	27	17
	+	273.4	255.5	165	10	17	14
Androsterone gluc	-	465.3	113.3	-135	-10	-36	-13
	-	465.3	447.3	-135	-10	-30	-20
	-	465.3	157.3	-135	-10	-35	-15
Boldenone gluc	-	461.3	113.3	-160	-10	-34	-12
	-	461.3	85.3	-160	-10	-33	-11
	-	461.3	157.2	-160	-10	-32	-18
Clenbuterol	+	277.3	132.3	60	10	39	14
	+	277.3	203.2	60	10	22	12
	+	277.3	259.3	60	10	14	15
d5-Etiocholanolone	+	278	204	125	10	21	26
	+	278	220.1	125	10	21	25
	+	278	260.1	125	10	15	16
d9-Clenbuterol	+	285.9	133	70	10	40	19
	+	285.9	204	70	10	22	24
	+	287.9	205.9	60	10	22	21
Drostanolone M1	+	287.5	121.4	136	10	27	20
	+	287.5	145.4	136	10	24	20
	+	287.5	269.5	136	10	17	17
Drostanolone M1 gluc	-	479.5	113.2	-170	-10	-37	-13
	-	479.5	85.2	-170	-10	-36	-12
	-	479.5	157.2	-170	-10	-36	-14
Etiocholanolone	+	273.3	199.3	100	10	21	24
	+	273.3	215.3	100	10	21	26
	+	273.3	255.3	100	10	15	16
Cardarine	+	454.2	172.2	185	10	90	19
	+	454.2	188.2	185	10	59	22
	+	454.2	257.1	185	10	40	14
Cardarine	-	452.1	138.2	-75	-10	-30	-12
	-	452.1	394.1	-75	-10	-20	-21
	-	452.1	123.2	-75	-10	-55	-19
Cardarine M1	+	486.2	188.3	160	10	65	12

Analyte	ESI polarity	Precursor Ion *(m/z)*	Selected Product Ion *(m/z)*	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
	+	486.2	256.3	160	10	65	13
	+	486.2	257.3	160	10	40	16
Cardarine M1	-	484.2	426.3	-65	-10	-22	-16
	-	484.2	170.3	-65	-10	-36	-21
	-	484.2	122.4	-65	-10	-44	-18
Cardarine M2	+	470.3	188	45	10	64	22
	+	470.3	256	45	10	20	25
	+	470.3	257	45	10	30	24
Cardarine M2	-	468.2	212.2	-45	-10	-19	-11
	-	468.2	154.2	-45	-10	-35	-16
	-	468.2	139.2	-45	-10	-40	-16
Ligandrol	-	337.4	267.4	-110	-10	-14	-12
	-	337.4	170.4	-110	-10	-35	-21
	-	337.4	239.4	-110	-10	-25	-10
Metandienone M3	+	269.1	213.2	85	10	22	13
	+	269.4	161.5	85	10	29	21
	+	269.4	201.4	85	10	25	25
Methasterone M1	+	285.5	91.4	130	10	65	14
	+	285.5	161.4	130	10	23	9
	+	285.5	175.4	130	10	23	11
Methyltestosterone M1	+	271.4	135.3	80	10	25	21
	+	271.4	147.3	80	10	24	21
	+	271.4	161.3	80	10	24	25
Methyltestosterone M2	+	271.3	109.2	125	10	26	24
	+	271.3	175.2	125	10	25	26
	+	271.3	189.2	125	10	23	27
Norethandrolone M1	+	271.3	121.3	120	10	24	19
	+	271.3	147.4	120	10	21	18
	+	271.3	175.4	120	10	21	20
Norethandrolone M2	+	271.3	121.2	70	10	21	20
	+	271.3	135.3	70	10	24	25
	+	289.3	271.3	115	10	13	17
Testolone	-	348.1	321.1	-90	-10	-13	-17
	-	348.1	127.3	-90	-10	-26	-16
	-	348.1	145.2	-90	-10	-15	-14
Stenabolic M6	+	283.4	89.3	60	10	69	13
	+	283.4	125.2	60	10	18	20
Stanozolol M1 gluc	-	519.4	343.3	-145	-10	-51	-18
	-	519.4	113.2	-145	-10	-31	-13
	-	519.4	175.3	-145	-10	-29	-14

Study design

Table S2. Experiment spiking schedule and level for anabolic agents, and isotopically labelled internal standards used during instrumental analysis.

Analyte	**Spiking level**	**Time point spiked (N=3)**									**Isotopically labelled internal standard for LC analysis**
	(µg/L)	Week 1			Week 2			Week 3			
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
Andarine	2				x	x	x				d_5-Trenbolone
Androstenedione	10		x	x				x			$^{13}C_3$-Androstenedione
Androsterone	13.3		x	x				x			d_4-Androsterone
Boldenone	5				x	x	x				d_3-Boldenone
Boldenone M1	10		x	x				x			d_3-Epitestosterone
Cardarine	2				x	x	x				d_3-Methyltestosterone M2
Cardarine M1	2							x	x	x	d_5-Trenbolone M1
Cardarine M2	10		x	x				x			d_5-Trenbolone
Clenbuterol	2				x	x	x				d_9-Clenbuterol
Clostebol M1	13.3		x	x				x			d_5-Etiocholanolone
Drostanolone M1	10		x	x				x			d_3-Methyltestosterone M2
Enobosarm	8				x	x	x				d_3-Testosterone
Epitestosterone	10		x	x				x			d_3-Epitestosterone
Estrone	8				x	x	x				$^{13}C_3$-Androstenedione
Etiocholanolone	13.3		x	x				x			d_5-Etiocholanolone
Fluoxymesterone	8				x	x	x				d_3-Boldenone
Fluoxymesterone M1	10		x	x				x			d_5-Trenbolone
Fluoxymesterone M2	10		x	x				x			d_5-Etiocholanolone
Ligandrol	5				x	x	x				d_3-Testosterone
Mesterolone M1	10		x	x				x			d_3-Methyltestosterone M2
Metandienone	5				x	x	x				d_3-Boldenone
Metandienone M1	10		x	x				x			d_4-19-Noretiocholanolone
Metandienone M2	10		x	x				x			d_5-Trenbolone
Metandienone M3	10		x	x				x			d_3-Methyltestosterone M2

Methasterone	8				x	x	x				d_3-Methyltestosterone M2
Methasterone M1	10	x	x	x							d_3-Methyltestosterone M2
Metenolone	5				x	x	x				d_3-Testosterone
Metenolone M1	13.3		x	x				x			d_5-Etiocholanolone
Methyl-1-testosterone	10				x	x	x				d_3-Testosterone
Methylstenbolone	5				x	x	x				d_3-Methyltestosterone M2
Methyltestosterone	10				x	x	x				d_3-Testosterone
Methyltestosterone M1	10		x	x				x			d_3-Methyltestosterone M2
Methyltestosterone M2	10		x	x				x			d_3-Methyltestosterone M2
Nandrolone	8				x	x	x				d_3-Nandrolone
19-Norandrosterone	10		x	x				x			d_4-19-Norandrosterone
19-Noretiocholanolone	13.3		x	x				x			d_4-19-Noretiocholanolone
Norethandrolone M1	10		x	x				x			d_3-Methyltestosterone M2
Norethandrolone M2	10		x	x				x			d_3-Methyltestosterone M2
Norethandrolone M3	10		x	x				x			d_3-Methyltestosterone M2
Oxandrolone	15				x	x	x				d_3-Testosterone
Oxandrolone M1	10		x	x				x			d_3-Epitestosterone
Progesterone	5				x	x	x				d_3-Testosterone
Stanozolol M1	10		x	x				x			d_5-Stanozolol M1
Stanozolol M1 gluc	5				x	x	x				d_5-Stanozolol M1
Stenabolic	5				x	x	x				d_3-Methyltestosterone M2
Stenabolic M2	10		x	x				x			d_5-Trenbolone
Stenabolic M6	10		x	x				x			d_3-Epitestosterone
Testolone	5				x	x	x				d_3-Boldenone
Testosterone	5				x	x	x				d_3-Testosterone
Trenbolone	10				x	x	x				d_5-Trenbolone
Trenbolone M1	10		x	x				x			d_5-Trenbolone M1

Dehydrochlormethyl-testosterone	8				x	x	x				d_3-Boldenone
Dehydrochlormethyl-testosterone M1	10		x	x				x			d_5-Trenbolone
YK-11	9				x	x	x				d_3-Testosterone
19-Norandrosterone gluc	10				x	x	x				d_5-Etiocholanolone
19-Noretiocholanolone gluc	10				x	x	x				d_5-Etiocholanolone
Androsterone gluc	15				x	x	x				d_5-Etiocholanolone
Boldenone gluc	5				x	x	x				d_5-Etiocholanolone
Drostanolone M1 gluc	8				x	x	x				d_5-Etiocholanolone
Stanozolol M1 gluc (method B)	5				x	x	x				d_5-Etiocholanolone

Results and discussion

Instrument performance assessment

Table S3. Instrument method performance assessment values including quantifier transition (MRM), ESI mode, LOD, LOQ, accuracy, precision, and linearity.

Analyte	MRM	ESI	LOD	LOQ	%Accuracy ± %RSD						Linearity
	m/z				Low spike				Medium spike	High spike	(R^2)
			(µg L^{-1})	(µg L^{-1})	0.01 µg L^{-1}	0.04 µg L^{-1}	0.08 µg L^{-1}	0.31 µg L^{-1}	0.63 µg L^{-1}	1.25 µg L^{-1}	5 µg L^{-1}	10 µg L^{-1}	
			n = 8	n = 8	n = 8	n = 7	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	N ≥ 5
Method A													
Andarine	442 → 400	+	0.01	0.03	103 ± 15	98 ± 4	80 ± 4				103 ± 2	100 ± 2	0.9986
Androstenedione	287 → 97	+	0.03	0.08			95 ± 11				103 ± 2	102 ± 2	0.9992
Androsterone	273 → 255	+	0.43	1.29						114 ± 9	103 ± 3	96 ± 4	0.9980
Boldenone	287 → 121	+	0.01	0.03		98 ± 8	99 ± 4				101 ± 2	101 ± 2	0.9995
Boldenone M1	289 → 187	+	0.13	0.38				109 ± 11			99 ± 3	102 ± 4	0.9995
Cardarine	454 → 257	+	0.02	0.05			68 ± 9				103 ± 4	94 ± 3	0.9944
Cardarine M1	486 → 257	+	0.01	0.03	105 ± 6	104 ± 4	76 ± 4				108 ± 2	104 ± 2	0.9976
Cardarine M2	470 → 257	+	0.01	0.02	110 ± 5	106 ± 3	75 ± 3				103 ± 3	101 ± 2	0.9958
Clenbuterol	277 → 203	+	0.01	0.02		96 ± 5	90 ± 3				105 ± 1	103 ± 1	0.9996
Clostebol M1	305 → 269	+	0.54	1.63						116 ± 11	102 ± 5	99 ± 5	0.9970
Drostanolone M1	287 → 269	+	0.27	0.81						103 ± 6	99 ± 5	102 ± 6	0.9997
Enobosarm	407 → 390	+	0.05	0.15				100 ± 5			100 ± 3	100 ± 4	0.9991
Epitestosterone	289 → 97	+	0.03	0.10			109 ± 11	99 ± 7			102 ± 2	100 ± 2	0.9999
Estrone	269 → 145	-	0.07	0.22				89 ± 8			97 ± 3	100 ± 1	0.9979
Etiocholanolone	273 → 255	+	0.11	0.32				69 ± 15			103 ± 7	101 ± 3	0.9949
Fluoxymesterone	337 → 241	+	0.12	0.35				97 ± 12			106 ± 4	102 ± 3	0.9971
Fluoxymesterone M1	337 → 95	+	0.02	0.05		90 ± 6	97 ± 6				98 ± 5	101 ± 6	0.9995
Fluoxymesterone M2	319 → 281	+	0.02	0.05			86 ± 7				102 ± 2	102 ± 2	0.9998

Analyte	MRM	ESI	LOD	LOQ	%Accuracy ± %RSD						Linearity
	m/z				Low spike					Medium spike	High spike	(R^2)
			(µg L^{-1})	(µg L^{-1})	0.01 µg L^{-1}	0.04 µg L^{-1}	0.08 µg L^{-1}	0.31 µg L^{-1}	0.63 µg L^{-1}	1.25 µg L^{-1}	5 µg L^{-1}	10 µg L^{-1}	
			n = 8	n = 8	n = 8	n = 7	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	N ≥ 5
Ligandrol	339 → 199	+	0.01	0.03	98 ± 11	92 ± 4	93 ± 4				98 ± 1	100 ± 2	0.9989
Mesterolone M1	287 → 269	+	0.10	0.31				68 ± 14			99 ± 2	97 ± 1	0.9987
Metandienone	301 → 149	+	0.01	0.04		83 ± 5	93 ± 5				98 ± 1	100 ± 2	0.9984
Metandienone M1	301 → 149	+	0.02	0.07			72 ± 12				102 ± 3	100 ± 3	0.9994
Metandienone M2	299 → 281	+	0.004	0.01		114 ± 7	97 ± 9				100 ± 3	99 ± 1	0.9999
Metandienone M3	269 → 201	+	0.08	0.23				94 ± 8			99 ± 2	101 ± 2	0.9997
Methasterone	319 → 283	+	0.03	0.09			104 ± 11				95 ± 1	95 ± 2	0.9942
Methasterone M1	285 → 175	+	0.12	0.35				97 ± 12			97 ± 3	97 ± 1	0.9994
Metenolone	303 → 187	+	0.02	0.05			91 ± 7				102 ± 2	101 ± 3	0.9997
Metenolone M1	285 → 121	+	1.57	4.75							105 ± 9	106 ± 5	0.9924
Methyl-1-testosterone	303 → 201	+	0.01	0.03		101 ± 6	104 ± 3				98 ± 1	99 ± 1	0.9997
Methylstenbolone	317 → 201	+	0.005	0.014		104 ± 6	104 ± 2				96 ± 1	97 ± 1	0.9998
Methyltestosterone	303 → 109	+	0.02	0.05			88 ± 7				96 ± 2	99 ± 1	0.9992
Methyltestosterone M1	271 → 161	+	0.06	0.19				95 ± 6			97 ± 2	98 ± 1	0.9991
Methyltestosterone M2	271 → 175	+	0.59	1.79						100 ± 14	99 ± 5	97 ± 3	0.9963
Nandrolone	275 → 109	+	0.02	0.06			101 ± 7				103 ± 2	103 ± 2	0.9997
19-Norandrosterone	259 → 241	+	0.21	0.63						106 ± 5	105 ± 4	104 ± 4	0.9993
19-Noretiocholanolone	259 → 241	+	0.67	2.04							100 ± 4	100 ± 4	0.9958
Norethandrolone M1	271 → 175	+	0.15	0.44				111 ± 13			97 ± 1	98 ± 1	0.9995
Norethandrolone M2	271 → 135	+	0.08	0.25				80 ± 10			95 ± 3	98 ± 3	0.9999
Norethandrolone M3	287 → 243	+	0.08	0.24				71 ± 11			110 ± 3	104 ± 2	0.9930
Oxandrolone	307 → 271	+	0.05	0.16				99 ± 5			103 ± 3	103 ± 3	0.9992
Oxandrolone M1	289 → 229	+	0.11	0.32				75 ± 14			109 ± 4	106 ± 3	0.9990
Progesterone	315 → 97	+	0.01	0.02		102 ± 6	73 ± 4				100 ± 1	100 ± 2	0.9989

Analyte	MRM	ESI	LOD	LOQ	%Accuracy ± %RSD						Linearity
	m/z				Low spike				Medium spike	High spike	(R^2)
			(µg L^{-1})	(µg L^{-1})	0.01 µg L^{-1}	0.04 µg L^{-1}	0.08 µg L^{-1}	0.31 µg L^{-1}	0.63 µg L^{-1}	1.25 µg L^{-1}	5 µg L^{-1}	10 µg L^{-1}	
			n = 8	n = 8	n = 8	n = 7	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	N ≥ 5
Stanozolol M1	345 → 97	+	0.03	0.10			117 ± 11	104 ± 6			99 ± 1	98 ± 1	0.9998
Stanozolol M1 gluc	521 → 345	+	0.06	0.19				112 ± 5			109 ± 2	107 ± 3	0.9971
Stenabolic	438 → 125	+	0.01	0.03	95 ± 4	105 ± 5	78 ± 4				92 ± 2	97 ± 2	0.9970
Stenabolic M2	314 → 268	+	0.01	0.04	88 ± 21	107 ± 2	102 ± 5				96 ± 1	98 ± 2	0.9994
Stenabolic M6	283 → 125	+	0.05	0.14				94 ± 5			101 ± 2	102 ± 2	0.9974
Testolone	394 → 223	+	0.02	0.07			87 ± 10				100 ± 6	98 ± 4	0.9980
Testosterone	289 → 97	+	0.01	0.03		116 ± 4	94 ± 4				102 ± 1	102 ± 1	0.9999
Trenbolone	271 → 227	+	0.04	0.12			75 ± 21	107 ± 7			97 ± 2	98 ± 3	0.9992
Trenbolone M1	271 → 253	+	0.04	0.12			79 ± 19	103 ± 4			102 ± 1	102 ± 1	0.9992
DHCMT	317 → 155	+	0.01	0.02		99 ± 14	98 ± 4				98 ± 1	98 ± 2	0.9999
DHCMT M1	333 → 155	+	0.20	0.59						102 ± 5	99 ± 4	98 ± 2	0.9987
Method B													
19-Norandrosterone gluc	451 → 113	-	0.64	1.93							104 ± 4	106 ± 4	0.9988
19-Noretiocholanolone gluc	451 → 113	-	0.23	0.68					102 ± 11		101 ± 5	104 ± 4	0.9978
Andarine	440 → 261	-	0.01	0.02			110 ± 3				106 ± 4	103 ± 7	0.9970
Androsterone	273 → 255	+	1.48	4.47							91 ± 10	95 ± 9	0.9633
Androsterone gluc	465 → 113	-	0.57	1.73							99 ± 4	98 ± 4	0.9951
Boldenone gluc	461 → 113	-	0.07	0.20					106 ± 3		99 ± 3	100 ± 3	0.9979
Cardarine	454 → 257	+	0.01	0.04			113 ± 4				100 ± 3	94 ± 3	0.9866
Cardarine	452 → 138	-	0.01	0.02			118 ± 2				100 ± 3	96 ± 2	0.9929
Cardarine M1	486 → 257	+	0.02	0.06			108 ± 7				98 ± 1	98 ± 3	0.9974
Cardarine M1	484 → 426	-	0.02	0.06			116 ± 7				98 ± 3	99 ± 3	0.9988
Cardarine M2	470 → 256	+	0.02	0.06			115 ± 6				99 ± 2	98 ± 2	0.9978
Cardarine M2	468 → 212	-	0.01	0.03			112 ± 4				100 ± 2	99 ± 3	0.9986

Analyte	MRM	ESI	LOD	LOQ	%Accuracy ± %RSD						Linearity
	m/z				Low spike				Medium spike	High spike	(R^2)
			(µg L^{-1})	(µg L^{-1})	0.01 µg L^{-1}	0.04 µg L^{-1}	0.08 µg L^{-1}	0.31 µg L^{-1}	0.63 µg L^{-1}	1.25 µg L^{-1}	5 µg L^{-1}	10 µg L^{-1}	
			n = 8	n = 8	n = 8	n = 7	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8	N ≥ 5
Clenbuterol	277 → 203	+	0.01	0.03			100 ± 4				98 ± 2	99 ± 2	0.9983
Drostanolone M1	287 → 269	+	0.24	0.72					119 ± 10		99 ± 3	94 ± 5	0.9957
Drostanolone M1 gluc	479 → 113	-	0.62	1.88						82 ± 18	93 ± 7	99 ± 6	0.9969
Etiocholanolone	273 → 255	+	0.25	0.77					83 ± 15		98 ± 2	101 ± 3	0.9974
Ligandrol	337 → 267	-	0.12	0.37					105 ± 6		97 ± 7	100 ± 4	0.9980
Metandienone M3	269 → 213	+	0.11	0.35					103 ± 5		99 ± 2	98 ± 3	0.9985
Methasterone M1	285 → 161	+	0.26	0.78					110 ± 11		100 ± 2	97 ± 2	0.9979
Methyltestosterone M1	271 → 135	+	0.10	0.29					99 ± 6		97 ± 2	98 ± 2	0.9988
Methyltestosterone M2	271 → 109	+	0.44	1.32						105 ± 10	100 ± 4	98 ± 3	0.9952
Norethandrolone M1	271 → 175	+	0.11	0.33					104 ± 5		98 ± 2	96 ± 3	0.9988
Norethandrolone M2	271 → 135	+	0.17	0.51					102 ± 8		98 ± 2	97 ± 3	0.9975
Stenabolic M6	283 → 125	+	0.05	0.15			114 ± 16		102 ± 5		102 ± 5	99 ± 3	0.9967
Stanozolol M1 gluc	519 → 343	-	0.08	0.24					106 ± 4		101 ± 5	102 ± 5	0.9981
Testolone	348 → 321	-	0.05	0.16					95 ± 3		98 ± 3	100 ± 2	0.9992

Linearity (R^2) for all analytes in acidified and filtered wastewater was >0.995, with the exception of cardarine (method A: 0.994 & method B: 0.986 for positive ionisation; 0.992 for negative ionisation), methasterone (0.994), metenolone M1 (0.992), norethandrolone M3 (0.993), etiocholanolone (method A: 0.994), and androsterone (0.963). Limits of detection and limits of quantification ranged from 0.004 – 1.56 µg/L and 0.01 – 4.75 µg/L, respectively. Accuracies were 68 – 119%, and the precision (%RSD) range was 1 – 21% across all spiking levels and both methods. Data for each analyte can be found in Table S3.

Both LC methods demonstrated satisfactory performance and applicability for the detection and quantification of 59 anabolic agents in wastewater influent. Only cardarine showed <70% accuracy (68%) near the LOQ (method A), whereas all other compounds had satisfactory accuracies of 70 – 130% at or near their LOQs. At low, medium and high concentration levels, all analytes had RSD values below 20%, with the exception of trenbolone and stenabolic M2 (method A) which had an RSD of 21% at the low spike. The performance of the instrument methods was evaluated before the in-sewer samples were analysed, and it was concluded that both LC-MS/MS methods were suitable for the intended purpose. It should be noted that slightly different results may be obtained if this experiment was repeated with wastewater from a different WWTP, as background noise and interferences may differ.

Typical parameters of the feeding raw sewage[1]

The real sewage is weekly collected from a pump station in Brisbane (Australia). Wastewater in this residential area is the typical domestic sewage with pH 7.5, low sulfide (<3 mgS/L), 10 – 30 mgS/L sulfate, low methane (<5 mgCOD/L), 180 – 200 mg/L SCOD with 50 mgCOD/L acetate and 10 – 20 mgCOD/L propionate as the major VFAs contents, 200 – 400 mg/L TSS, and 180 – 380 mg/L VSS. The collected fresh sewage is stored in a cold room under 4°C to minimise biological reactions. The feeding sewage is heated by a thermoregulator in a water bath to room temperature before entering the reactor.

Statistical analysis

Equations applied are as follows:

For zero order kinetics (eq1):

$$\frac{C_t}{C_0} = -k_0 \times t_i + e$$

For first order kinetics (eq2):

$$\frac{C_t}{C_0} = f_f \times e^{-k_f t_i}$$

For two-phase kinetics (eq3):

$$\frac{C_t}{C_0} = f_f \times e^{-k_f t_i} + f_s \times e^{-k_s t_i}$$

Parameters:

C_t: concentration at time t

C_0: concentration at time zero

k_0: the rate of degradation for zero order degradation

e: intercept of the zero-order degradation

f_f: the fraction of compound degraded during the fast reaction

k_f: the rate of fast degradation

f_s: the fraction of compound degraded during the slow reaction

k_s: the rate of slow degradation

t_i: time

In equations eq1, eq2, and eq3 the fraction C_t/C_0 was replaced by 90, 50, and 10 for 10, 50, and 90% reduction, respectively. During the next step those equations were solved for t_i in order to estimate the transformation times.

Quality assurance and control

Mean recoveries (n=4) ranged from 89-118%, exceptions being drostanolone M1 (141%), drostanolone M1 gluc (134%), fluoxymesterone M1 (42%), fluoxymesterone M2 (121%), mesterolone M1 (122%), metandienone M3 (169%), methyltestosterone M2 (125%), androsterone (123%), etiocholanolone (137%), cardarine M1 (143%), cardarine M2 (166%), and YK-11 (133%), and the mean duplicate differences ranged from 0.8-14.5% across all biomarkers (Table S4).

Table S4. QAQC results for each biomarker. Recovery mean % was calculated by subtracting the concentration of the analyte in the wastewater sample from the concentration of the spiked sample and dividing that by the non-extracted side spike. QC accuracy is the accuracy of the 10 µg/L calibration solution reinjected during the run compared to the calibration curve.

Analyte	Recovery mean % ±SD (n=4)	Duplicate difference mean % (n=7)	QC Accuracy %
Boldenone	110±11	1.4	101
Boldenone gluc	101±4	3.1 (n=3)	95
Boldenone M1	104±10	3.4	101
Clostebol M1	103±10	3.3	99
Drostanolone M1	141±8	6.4	107
Drostanolone M1 gluc	134±11	14.0 (n=5)	79
Estrone	104±12	3.8	95
Fluoxymesterone	100±13	6.8	91
Fluoxymesterone M1	42±7	5.5	115
Fluoxymesterone M2	121±8	4.3	107
Mesterolone M1	122±13	3.4	103
Methyl-1-testosterone	109±9	3.5 (n=6)	105
Metandienone	115±6	3.8	101
Metandienone M1	134±18	3.0	105
Metandienone M2	93±6	2.3	100
Metandienone M3	169±34	7.6	106
Methasterone	107±6	3.6	109
Methasterone M1	118±11	6.1 (n=5)	104
Metenolone	112±9	5.2	98
Metenolone M1	103±8	3.2	102
Methylstenbolone	110±4	4.7	106
Methyltestosterone	103±8	2.8	99

Methyltestosterone M1/Norethandrolone M2	116±12	2.7	107
Methyltestosterone M2	125±14	4.0	103
Nandrolone	102±7	2.1	100
19-Norandrosterone	104±12	4.2	106
19-Norandrosterone gluc	108±8	5.7 (n=4)	97
19-Noretiocholanolone	106±12	4.1	105
19-Noretiocholanolone gluc	105±6	1.7 (n=4)	95
Norethandrolone M1	115±11	3.6	108
Norethandrolone M3	107±8	4.0	99
Progesterone	117±10	4.6 (n=5)	100
Oxandrolone	111±31	7.5	82
Oxandrolone M1	117±18	6.5	102
Testosterone	105±7	0.8	101
Androstenedione	102±4	4.0 (n=6)	106
Androsterone	123±6	7.6	97
Androsterone gluc	108±13	3.6 (n=3)	95
Epitestosterone	109±11	5.6	101
Etiocholanolone	137±30	5.0	101
Stanozolol M1	92±17	2.0	101
Stanozolol M1 gluc	116±8	5.7 (n=4)	100
Trenbolone	114±11	2.4	105
Trenbolone M1	101±16	3.2	98
DHCMT	113±16	3.1	98
DHCMT M1	95±7	2.4	100
Andarine	106±5	4.2	92
Cardarine	96±28	7.8	112
Cardarine M1	143±23	14.5	105
Cardarine M2	166±31	9.8	95
Clenbuterol	104±5	1.7	104
Enbosarm	105±6	6.1	96
Ligandrol	103±8	7.3	100
Stenabolic	101±11	9.3	111
Stenabolic M2	97±23	6.3 (n=6)	101
Stenabolic M6	105±8	5.5 (n=4)	103
Testolone	89±6	7.0	96
YK-11	133±11	8.1 (n=6)	n.a.

Cardarine had a NESS value of 1.28 µg/L which was 74% lower than the theoretical spiked value. To investigate this, additional experiments were conducted. To assess how filtering and the methanol content affected recovery of cardarine, solutions of 2.5 µg/L were prepared in filtered and acidified wastewater with 10%, 20%, 30%, 50% and 100% MeOH. An additional vial was prepared, where unfiltered wastewater (with 20% MeOH) was fortified to 2.5 µg/L and subsequently filtered. The experiments confirmed that the MeOH content, as well as the filtering process, had an impact on the concentration of cardarine in wastewater. Calculated concentrations for cardarine were as follows: 10% – 1.3 µg/L, 20% (%MeOH in calibration solutions) – 2.4 µg/L, 30% - 4.1 µg/L, 50% - 4.6 µg/L and 100% - 4.6 µg/L, which translates to recoveries of 52%, 96%, 164%, 184%, and 184%, respectively. This suggests that solubility is a contributing factor for this biomarker and results should be interpreted with caution when analysing an aqueous matrix. Another significant amount of cardarine was lost through filtering, as the concentration after filtering (with 20% MeOH) was 0.9 µg/L (vs 2.5 µg/L). This is likely due to its high logP value (5.85) and therefore, having a higher affinity to the particulates and/or filter than the aqueous wastewater. Overall, around 50% was lost through only having 10% MeOH as opposed to the 20% in the calibration, and 63% was lost due to filtration. All other analytes (except glucuronides) were investigated also, and no notable losses were observed with differences in methanol content or the filtering process.

In addition, a subsequent in-sample stability experiment (currently unpublished) revealed that wastewater preservation with HCl led to a 98% lower initial concentration of cardarine than the theoretical spiked value, whereas the initial concentration in unpreserved and sodium metabisulfite preserved wastewater was within the expected range. It is possible that the HCl-preserved wastewater had a pH value slightly below 2.14, which, according to ChemAxon prediction (as reported by DrugBank (https://go.drugbank.com/drugs/DB05416, accessed 29/07/2021)), is the strongest basic pKa of cardarine, whereas the pH of the calibrations series solutions may have been slightly above this pH level. As samples were preserved with HCl, this is likely a significant contributing factor, explaining a portion of the 92% decrease in initial concentration for cardarine in this study.

It is difficult to estimate which of these three factors played the largest role in the loss or lower detected concentrations of cardarine, as these are preliminary findings. If this biomarker were to be used in future experiments, losses due to filtering, methanol content in the sample, and pH values should be assessed in more detail.

Initial concentrations in the reactors

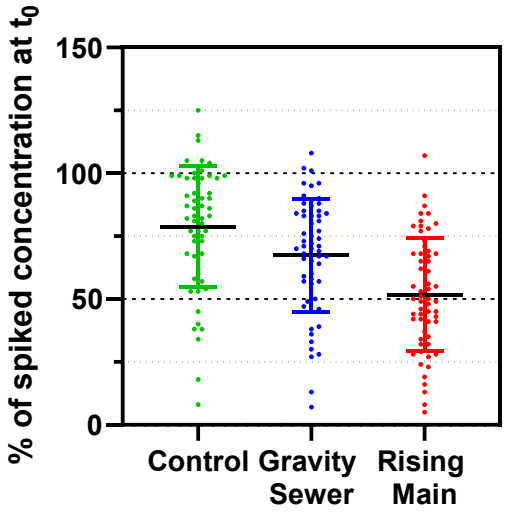

Figure S1. Scatter dot plot of measured concentration of analytes in t_0 as a percentage of the spiked theoretical concentration in all three reactor types with mean (black line) and standard deviation.

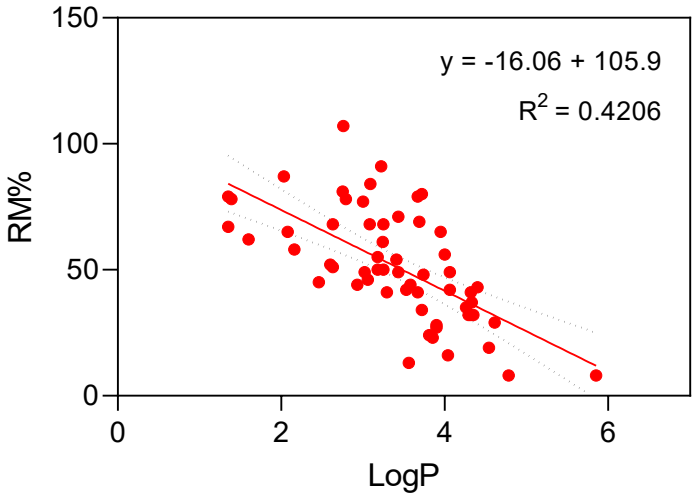

Figure S2. Scatter plot of anabolic agent (N=59) logP value vs their concentration at t_0 divided by the theoretical concentration at which they were spiked (RM%). Red trend line represents a simple linear regression. Black dotted lines represent the 95% confidence interval of the slope.

Stability of metabolites and parent analytes

Table S5. Values for parameters of zero order, first order and two-phase equations for all biomarkers in the control reactor, gravity sewer reactor and rising main reactor. Bolded values are from the model of best fit. For equations and parameter descriptions, please refer to the "*Statistical analysis"* section.

Analyte	**Control reactor**								**Gravity sewer reactor**								**Rising main reactor**							
	Zero order		First order		Two-phase				Zero order		First order		Two-phase				Zero order		First order		Two-phase			
	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s
Boldenone	*-5.165*	*121.9*	**121.4**	**-0.040**	*-49.2*	*-1.056*	*148.6*	*-0.042*	*-9.795*	*100.4*	**116.1**	**-0.233**	*2.7E+07*	*-0.104*	*-2.7E+07*	*-0.104*	*-6.746*	*56.9*	**100.0**	**-0.906**	*-5.2E+05*	*-1.223*	*5.2E+05*	*-1.223*
Boldenone gluc	*-6.633*	*56.0*	**100.0**	**-0.971**	*4.6E+06*	*-0.669*	*-4.6E+06*	*-0.669*	*-6.175*	*51.4*	**100.0**	**-1.111**	*4.4E+05*	*-0.835*	*-4.4E+05*	*-0.835*	*-4.811*	*39.1*	**100.0**	**-1.999**	*100.0*	*-1.998*	*0.0*	*-0.158*
Boldenone M1	*-3.471*	*92.2*	**95.8**	**-0.050**	*8.4*	*-2.264*	*91.7*	*-0.043*	*-7.765*	*86.0*	**99.5**	**-0.216**	*2.2*	*-67.222*	*97.8*	*-0.212*	*-6.270*	*53.0*	**99.9**	**-1.019**	*64.3*	*-1.335*	*35.8*	*-0.652*
Clostebol M1	**-1.396**	**95.6**	*96.0*	*-0.016*	*5.8*	*-42.690*	*94.2*	*-0.013*	*-6.480*	*82.3*	**92.3**	**-0.169**	*27.5*	*-1.107*	*72.3*	*-0.124*	*-6.294*	*68.9*	*89.1*	*-0.315*	**54.1**	**-1.195**	**45.6**	**-0.136**
Drostanolone M1	*-4.402*	*94.0*	*96.7*	*-0.067*	**15.4**	**-0.841**	**86.3**	**-0.051**	*-6.716*	*83.2*	**95.3**	**-0.184**	*60.8*	*-0.411*	*38.6*	*-0.067*	*-5.635*	*63.9*	*88.2*	*-0.387*	**71.2**	**-1.095**	**28.1**	**-0.066**
Drostanolone M1 gluc	*-6.745*	*97.7*	**101.5**	**-0.102**	*-8.3E+06*	*-0.162*	*8.3E+06*	*-0.162*	*-9.082*	*83.1*	**105.6**	**-0.340**	*9.1E+05*	*-0.181*	*-9.1E+05*	*-0.181*	*-6.307*	*52.3*	**100.0**	**-0.880**	*9.1E+05*	*-0.636*	*-9.1E+05*	*-0.636*
Estrone	*3.824*	*98.7*	**99.9**	**-0.007**	*-4.8*	*-0.656*	*103.2*	*-0.011*	*-4.727*	*90.3*	*95.3*	*-0.083*	**45.2**	**-0.319**	**56.0**	**-0.024**	*-5.009*	*72.8*	*82.0*	*-0.147*	**52.8**	**-1.210**	**46.4**	**-0.052**
Fluoxymesterone	**-0.029**	**98.8**	*98.9*	*0.000*	*98.3*	*0.002*	*0.0*	*2.822*	*-7.302*	*97.8*	**104.6**	**-0.134**	*1.1E+07*	*-0.062*	*-1.1E+07*	*-0.062*	*-7.466*	*69.3*	**98.9**	**-0.489**	*89.6*	*-0.594*	*10.5*	*-0.131*
Fluoxymesterone M1	**1.483**	**96.5**	*96.8*	*0.014*	*98.0*	*0.008*	*0.0*	*2.823*	*-0.753*	*98.3*	*99.0*	*-0.008*	**17.3**	**-0.299**	**84.4**	**0.007**	**-0.496**	**97.2**	*97.3*	*-0.005*	*7.0*	*-1.396*	*93.8*	*-0.001*
Fluoxymesterone M2	*-0.721*	*98.9*	**98.7**	**-0.006**	*98.9*	*-0.007*	*0.0*	*2.829*	*-4.720*	*87.7*	*92.3*	*-0.088*	**35.2**	**-0.472**	**63.5**	**-0.039**	*-5.258*	*73.1*	*84.3*	*-0.170*	**56.8**	**-0.959**	**42.8**	**-0.051**
Mesterolone M1	*-5.732*	*94.5*	**99.4**	**-0.104**	*9.7*	*-0.624*	*91.5*	*-0.089*	*-6.985*	*80.9*	**94.6**	**-0.212**	*38.1*	*-0.633*	*61.2*	*-0.138*	*-6.128*	*63.0*	*91.6*	*-0.487*	**64.1**	**-1.287**	**35.4**	**-0.147**
Methyl-1-testosterone	*2.177*	*116.3*	**101.6**	**-0.047**	*-3.4*	*-43.398*	*103.4*	*-0.049*	*-7.015*	*61.1*	**100.8**	**-0.542**	*-5.1E+05*	*-0.929*	*5.1E+05*	*-0.928*	*-6.411*	*53.3*	**100.0**	**-1.410**	*18.2*	*-51.339*	*81.8*	*-1.335*
Metandienone	*1.807*	*102.5*	**103.5**	**0.001**	*-8.7E+05*	*-0.060*	*8.7E+05*	*-0.060*	*-8.401*	*86.6*	**101.8**	**-0.254**	*9.2E+05*	*-0.156*	*-9.2E+05*	*-0.156*	*-6.495*	*55.1*	**99.9**	**-0.878**	*99.8*	*-0.884*	*0.2*	*0.014*
Metandienone M1	**-0.731**	**101.7**	*100.4*	*-0.006*	*-38.8*	*-0.099*	*138.3*	*-0.024*	*-8.331*	*84.3*	**100.9**	**-0.278**	*4.6E+06*	*-0.200*	*-4.6E+06*	*-0.200*	*-6.016*	*50.5*	**100.0**	**-1.024**	*15.6*	*-58.052*	*84.4*	*-0.939*
Metandienone M2	*-0.081*	*96.7*	**97.4**	**-0.001**	*4.3*	*-3.310*	*95.8*	*0.001*	**-6.981**	**96.6**	*102.4*	*-0.126*	*7.4E+05*	*-0.053*	*-7.4E+05*	*-0.053*	*-7.684*	*68.3*	**100.6**	**-0.568**	*-5.5*	*-62.175*	*105.5*	*-0.598*
Metandienone M3	*-2.118*	*92.9*	*93.6*	*-0.027*	**14.3**	**-1.923**	**86.0**	**-0.017**	*-7.866*	*83.0*	**99.0**	**-0.252**	*4.6*	*-1.581*	*95.4*	*-0.241*	*-6.512*	*55.9*	**100.0**	**-0.858**	*23.0*	*-63.181*	*77.0*	*-0.598*
Methasterone	**-1.268**	**103.9**	*103.4*	*-0.013*	*-4.7E+04*	*-0.028*	*4.7E+04*	*-0.028*	*-6.033*	*91.2*	**97.8**	**-0.121**	*90.2*	*-0.200*	*10.4*	*0.073*	*-5.042*	*66.9*	*81.8*	*-0.220*	**67.2**	**-1.475**	**32.7**	**-0.034**
Methasterone M1	*-4.539*	*91.0*	*96.3*	*-0.075*	**26.0**	**-0.503**	**74.4**	**-0.044**	*-5.890*	*84.3*	*95.0*	*-0.159*	**45.3**	**-0.468**	**54.6**	**-0.082**	*-5.219*	*60.2*	*86.0*	*-0.423*	**67.5**	**-1.542**	**31.9**	**-0.080**
Metenolone	*-5.048*	*99.2*	**100.4**	**-0.061**	*101.8*	*-0.072*	*0.0*	*2.820*	*-7.869*	*87.6*	**98.8**	**-0.216**	*96.6*	*-0.235*	*3.1*	*0.013*	*-6.598*	*61.8*	**97.5**	**-0.637**	*86.4*	*-0.922*	*13.7*	*-0.109*
Metenolone M1	**-2.487**	**96.6**	*97.4*	*-0.030*	*3.8*	*-52.209*	*96.2*	*-0.029*	*-5.337*	*86.6*	*91.9*	*-0.105*	**24.0**	**-0.867**	**75.3**	**-0.074**	*-5.740*	*71.6*	*85.5*	*-0.215*	**53.2**	**-1.123**	**46.5**	**-0.087**

Analyte	Control reactor								Gravity sewer reactor								Rising main reactor							
	Zero order		First order		Two-phase				Zero order		First order		Two-phase				Zero order		First order		Two-phase			
	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s
Methylstenbolone	**0.227**	**103.1**	*103.2*	*0.001*	*-4.5*	*-9.245*	*104.5*	*0.000*	*-7.018*	*90.8*	**99.9**	**-0.155**	*96.1*	*-0.189*	*5.2*	*0.051*	*-6.166*	*64.1*	*91.5*	*-0.456*	**61.1**	**-1.687**	**38.8**	**-0.149**
Methyltestosterone	**-2.835**	**100.6**	*101.2*	*-0.033*	*1.2E+06*	*-0.004*	*-1.2E+06*	*-0.004*	*-8.568*	*82.5*	**102.3**	**-0.331**	*7.8E+04*	*-0.234*	*-7.8E+04*	*-0.234*	*-5.330*	*43.9*	**100.0**	**-1.585**	*99.0*	*-1.613*	*1.0*	*-0.246*
Methyltestosterone M1/Norethandrolone M2	*-3.561*	*95.6*	*97.2*	*-0.049*	**17.1**	**-0.396**	**83.2**	**-0.033**	*-6.503*	*85.5*	**95.2**	**-0.156**	*38.1*	*-0.478*	*61.5*	*-0.095*	*-6.017*	*65.9*	*88.2*	*-0.355*	**62.2**	**-1.155**	**37.2**	**-0.114**
Methyltestosterone M2	*-1.698*	*96.6*	*96.7*	*-0.020*	**12.6**	**-0.627**	**88.1**	**-0.009**	*-4.467*	*86.9*	*91.0*	*-0.080*	**37.9**	**-0.482**	**60.9**	**-0.030**	*-4.985*	*70.6*	*80.0*	*-0.159*	**49.8**	**-1.465**	**49.6**	**-0.066**
Nandrolone	*-5.085*	*100.4*	**101.1**	**-0.053**	*100.7*	*-0.065*	*1.3*	*0.190*	*-8.569*	*83.9*	**103.3**	**-0.299**	*1.4E+06*	*-0.195*	*-1.4E+06*	*-0.195*	*-5.995*	*50.0*	**100.0**	**-1.156**	*-3.9E+05*	*-1.293*	*3.9E+05*	*-1.293*
19-Norandrosterone	**-1.503**	**99.4**	*99.5*	*-0.017*	*99.3*	*-0.022*	*0.8*	*0.184*	*-5.638*	*87.8*	**93.9**	**-0.113**	*33.9*	*-0.473*	*66.1*	*-0.066*	*-5.827*	*71.0*	*84.8*	*-0.223*	**51.0**	**-1.265**	**48.5**	**-0.101**
19-Norandrosterone gluc	*-8.414*	*80.4*	**100.2**	**-0.323**	*3.6E+06*	*-0.202*	*-3.6E+06*	*-0.202*	*-8.111*	*71.1*	**101.4**	**-0.506**	*1.5E+07*	*-0.312*	*-1.5E+07*	*-0.312*	*-5.681*	*46.6*	**100.0**	**-1.274**	*164.0*	*-1.104*	*-64.0*	*-0.877*
19-Noretiocholanolone	*-2.466*	*96.2*	*97.9*	*-0.034*	**12.4**	**-0.486**	**88.0**	**-0.020**	*-4.427*	*87.8*	*92.2*	*-0.081*	**40.2**	**-0.452**	**59.5**	**-0.027**	*-5.182*	*74.8*	*84.2*	*-0.150*	**50.0**	**-0.989**	**49.1**	**-0.057**
19-Noretio-cholanolone gluc	*-6.243*	*52.1*	**100.0**	**-1.087**	*8.3E+06*	*-0.837*	*-8.3E+06*	*-0.837*	*-5.745*	*47.5*	**100.0**	**-1.355**	*3.4E+05*	*-1.032*	*-3.4E+05*	*-1.032*	*-4.611*	*37.3*	**100.0**	**-5.194**	*100.0*	*-3.216*	*0.0*	*0.752*
Norethandrolone M1	*-1.492*	*99.3*	*98.8*	*-0.012*	**5.9**	**-0.595**	**94.8**	**-0.008**	*-6.109*	*84.8*	**93.4**	**-0.142**	*40.4*	*-0.474*	*58.3*	*-0.077*	*-5.535*	*65.1*	*84.4*	*-0.303*	**61.6**	**-1.344**	**37.9**	**-0.090**
Norethandrolone M3	**-1.020**	**100.9**	*100.6*	*-0.010*	*100.9*	*-0.012*	*0.0*	*2.816*	*-3.326*	*95.5*	*97.1*	*-0.045*	**26.5**	**-0.309**	**74.1**	**-0.017**	*-4.493*	*79.9*	*85.4*	*-0.098*	**43.6**	**-0.853**	**55.6**	**-0.035**
Progesterone	*-8.748*	*85.3*	**104.6**	**-0.307**	*1.3E+07*	*-0.203*	*-1.3E+07*	*-0.203*	*-8.169*	*71.9*	**100.2**	**-0.715**	*-1.7*	*-47.749*	*101.7*	*-0.727*	*-4.603*	*37.3*	**100.0**	**-5.203**	*135.8*	*-2.225*	*-35.8*	*-1.891*
Oxandrolone	*0.582*	*99.6*	**98.1**	**0.007**	*-2.7E+05*	*-0.021*	*2.7E+05*	*-0.021*	**-1.857**	**97.3**	*98.3*	*-0.023*	*60.0*	*-0.175*	*44.2*	*0.045*	*-3.410*	*86.8*	*88.9*	*-0.054*	**33.2**	**-0.763**	**66.4**	**-0.017**
Oxandrolone M1	**0.643**	**95.0**	*95.6*	*0.006*	*-5.6E+04*	*-0.016*	*5.7E+04*	*-0.016*	**-1.792**	**91.2**	*91.6*	*-0.023*	*94.1*	*-0.036*	*0.0*	*0.920*	**-2.755**	**79.5**	*82.4*	*-0.052*	*48.8*	*-0.677*	*49.1*	*0.014*
Testosterone	*-6.694*	*95.2*	**99.9**	**-0.082**	*100.6*	*-0.089*	*0.0*	*0.484*	*-8.388*	*79.1*	**101.7**	**-0.363**	*2.3E+06*	*-0.256*	*-2.3E+06*	*-0.256*	*-5.295*	*43.6*	**100.0**	**-1.583**	*8.1*	*-52.643*	*91.9*	*-1.496*
Androstenedione	*-2.949*	*79.6*	**100.4**	**-0.029**	*29.1*	*-2.353*	*71.0*	*-0.027*	*-7.920*	*73.3*	**100.6**	**-0.384**	*-2.8E+03*	*-0.544*	*2.9E+03*	*-0.536*	*-4.627*	*37.6*	**100.0**	**-8.219**	*100.0*	*-39.279*	*0.0*	*-1.881*
Androsterone	*-4.497*	*93.0*	*96.0*	*-0.069*	**14.0**	**-0.652**	**85.7**	**-0.054**	*-6.174*	*84.2*	**93.2**	**-0.149**	*37.7*	*-0.570*	*61.5*	*-0.086*	*-5.369*	*70.9*	*82.9*	*-0.191*	**53.3**	**-1.217**	**46.2**	**-0.074**
Androsterone gluc	*-7.294*	*62.7*	**100.0**	**-0.678**	*110.6*	*-0.635*	*-10.8*	*-0.340*	*-6.874*	*57.7*	**100.3**	**-0.804**	*8.7E+05*	*-0.544*	*-8.7E+05*	*-0.544*	*-5.011*	*40.7*	**100.0**	**-1.718**	*8.9E+05*	*-1.117*	*-8.9E+05*	*-1.117*
Epitestosterone	**-2.180**	**96.9**	*97.2*	*-0.023*	*4.3*	*-4.289*	*95.7*	*-0.022*	*-8.307*	*80.0*	**100.9**	**-0.330**	*4.6E+05*	*-0.243*	*-4.6E+05*	*-0.243*	*-4.935*	*40.4*	**100.0**	**-1.913**	*96.2*	*-1.800*	*3.8*	*-0.719*
Etiocholanolone	**-2.181**	**89.9**	*91.0*	*-0.030*	*43.2*	*-0.417*	*56.7*	*0.031*	*-1.575*	*92.4*	*93.0*	*-0.020*	**16.2**	**-0.783**	**83.1**	**-0.006**	**-1.437**	**99.2**	*99.4*	*-0.016*	*100.2*	*-0.020*	*0.0*	*2.817*
Stanozolol M1	**-0.831**	**95.6**	*95.9*	*-0.011*	*23.3*	*-0.251*	*76.6*	*0.012*	*-5.641*	*88.5*	*95.2*	*-0.114*	**39.4**	**-0.459**	**61.5**	**-0.056**	*-5.221*	*67.0*	*81.8*	*-0.228*	**62.3**	**-1.192**	**37.0**	**-0.058**
Stanozolol M1 gluc	*-4.822*	*39.2*	**100.0**	**-2.055**	*100.4*	*-2.043*	*-0.4*	*-0.711*	*-4.429*	*35.8*	**100.0**	**-2.474**	*100.0*	*-40.807*	*0.0*	*-2.295*	*-4.154*	*33.4*	**100.0**	**-6.053**	*100.0*	*-35.703*	*0.0*	*-2.858*
Trenbolone	*-2.432*	*103.6*	**100.0**	**-0.009**	*103.6*	*-0.042*	*0.0*	*1.139*	*-7.178*	*90.5*	*99.4*	*-0.170*	**70.5**	**-0.422**	**30.8**	**-0.032**	*-6.002*	*67.8*	*92.6*	*-0.364*	**78.6**	**-0.925**	**21.2**	**-0.028**

Analyte	Control reactor								Gravity sewer reactor								Rising main reactor							
	Zero order		First order		Two-phase				Zero order		First order		Two-phase				Zero order		First order		Two-phase			
	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s	k_0	e	f_f	k_f	f_f	k_f	f_s	k_s
Trenbolone M1	**-0.426**	**96.5**	*96.6*	*-0.005*	*5.0*	*-4.457*	*95.0*	*-0.003*	*-4.363*	*89.8*	*93.8*	*-0.074*	**31.4**	**-0.449**	**68.0**	**-0.034**	*-4.509*	*81.6*	*86.3*	*-0.093*	**37.0**	**-0.988**	**62.5**	**-0.045**
DHCMT	**-0.729**	**102.6**	*102.0*	*-0.006*	*-15.4*	*-0.275*	*115.4*	*-0.019*	*-8.529*	*91.0*	**99.8**	**-0.254**	*116.9*	*-0.198*	*-17.6*	*-0.089*	*-6.976*	*60.4*	**100.0**	**-0.802**	*10.4*	*-54.245*	*89.6*	*-0.723*
DHCMT M1	*-0.417*	*97.6*	*97.6*	*-0.004*	**3.9**	**-47.800**	**96.1**	**-0.002**	**-7.160**	**96.3**	*102.9*	*-0.133*	*4.1E+06*	*-0.069*	*-4.1E+06*	*-0.069*	*-8.116*	*71.5*	**101.3**	**-0.490**	*5.1E+07*	*-0.307*	*-5.1E+07*	*-0.307*
Andarine	**-1.473**	**99.9**	*100.1*	*-0.016*	*-6.6E+06*	*-0.076*	*6.6E+06*	*-0.076*	*-8.470*	*81.1*	**102.6**	**-0.339**	*1.2E+06*	*-0.228*	*-1.2E+06*	*-0.228*	*-5.922*	*50.1*	**100.0**	**-1.010**	*18.2*	*-54.042*	*81.8*	*-1.084*
Cardarine	*1.156*	*96.7*	**93.8**	**0.018**	*96.6*	*-0.052*	*4.4*	*0.250*	*-5.747*	*94.9*	*93.5*	*-0.096*	**68.7**	**-0.264**	**33.9**	**0.004**	*-6.115*	*90.2*	**85.0**	**-0.113**	*50.9*	*-0.851*	*49.0*	*-0.029*
Cardarine M1	**-1.183**	**92.3**	*93.2*	*-0.016*	*12.0*	*-2.753*	*88.2*	*-0.009*	*-3.026*	*88.8*	*90.4*	*-0.044*	**16.3**	**-2.473**	**83.7**	**-0.033**	*-4.549*	*87.3*	**91.6**	**-0.084**	*88.8*	*-0.179*	*8.6*	*0.120*
Cardarine M2	**-0.169**	**92.2**	*92.5*	*-0.003*	*21.6*	*-1.233*	*79.2*	*0.020*	*-4.232*	*95.9*	**98.9**	**-0.066**	*68.6*	*-0.148*	*32.8*	*0.012*	*-7.106*	*85.7*	**99.1**	**-0.193**	*92.9*	*-0.264*	*8.7*	*0.035*
Clenbuterol	*-0.115*	*103.0*	**100.1**	**-0.003**	*-3.7*	*-11.050*	*103.7*	*-0.003*	*-2.532*	*95.4*	*96.3*	*-0.032*	**25.7**	**-0.325**	**74.4**	**-0.005**	*-4.077*	*81.3*	*85.4*	*-0.082*	**42.9**	**-0.826**	**56.9**	**-0.024**
Enbosarm	**-1.235**	**97.0**	*97.8*	*-0.015*	*3.7*	*-2.064*	*96.3*	*-0.013*	*-5.251*	*85.5*	*91.9*	*-0.107*	**46.0**	**-0.378**	**52.5**	**-0.041**	*-5.105*	*68.3*	*82.1*	*-0.195*	**63.4**	**-1.153**	**35.4**	**-0.043**
Ligandrol	**-1.057**	**96.6**	*97.3*	*-0.012*	*6.4*	*-1.560*	*94.3*	*-0.007*	*-4.906*	*86.5*	*92.3*	*-0.095*	**55.3**	**-0.318**	**44.4**	**-0.016**	*-4.835*	*69.1*	*79.4*	*-0.160*	**59.8**	**-1.059**	**39.4**	**-0.041**
Stenabolic	*-6.212*	*81.5*	*93.7*	*-0.163*	**13.9**	**-5.666**	**86.1**	**-0.144**	*-8.156*	*77.5*	**100.6**	**-0.381**	*100.9*	*-0.383*	*0.0*	*0.423*	*-5.686*	*48.0*	**100.0**	**-1.173**	*78.3*	*-1.584*	*21.7*	*-0.455*
Stenabolic M2	**-7.877**	**104.8**	*108.8*	*-0.124*	*2.7E+06*	*-0.004*	*-2.7E+06*	*-0.004*	*-7.349*	*63.3*	**99.9**	**-0.672**	*1.8E+04*	*-0.496*	*-1.8E+04*	*-0.495*	*-4.031*	*32.4*	**100.0**	**-9.179**	*100.0*	*-6.118*	*0.0*	*2.702*
Stenabolic M6	*-9.275*	*93.0*	**108.2**	**-0.249**	*5.1E+05*	*-0.114*	*-5.1E+05*	*-0.114*	*-5.710*	*47.3*	**100.0**	**-1.265**	*24.8*	*-52.821*	*75.2*	*-1.090*	*-3.710*	*29.6*	**100.0**	**-10.560**	*1.2E+04*	*-3.283*	*-1.2E+04*	*-3.281*
Testolone	**-0.624**	**101.0**	*100.4*	*-0.006*	*56.1*	*-0.016*	*44.5*	*0.005*	*-5.207*	*91.0*	**97.4**	**-0.098**	*76.0*	*-0.235*	*27.3*	*0.023*	*-5.206*	*72.2*	*85.7*	*-0.179*	**66.9**	**-0.842**	**33.7**	**-0.025**
YK-11	**-1.927**	**96.1**	*98.5*	*-0.024*	*-5.2E+06*	*-0.057*	*5.2E+06*	*-0.057*	*-7.876*	*81.1*	**98.7**	**-0.258**	*97.9*	*-0.251*	*0.0*	*2.776*	*-5.907*	*49.4*	**100.0**	**-1.110**	*6.6E+05*	*-0.837*	*-6.6E+05*	*-0.837*
Paracetamol	*-0.112*	*99.8*	**100.0**	**-0.002**	*0.4*	*-0.563*	*99.6*	*-0.002*	**-6.865**	**98.8**	*104.0*	*-0.118*	*3.1E+07*	*-0.046*	*-3.1E+07*	*-0.046*	*-7.492*	*65.5*	**100.3**	**-0.586**	*-1.9E+06*	*-0.889*	*1.9E+06*	*-0.889*
Acesulfame	*0.081*	*100.6*	**100.1**	**0.001**	*-0.5*	*-2.618*	*100.5*	*0.001*	*-0.089*	*101.6*	**100.8**	**0.000**	*-1.3*	*-43.282*	*101.3*	*-0.001*	**-0.028**	**99.8**	*99.9*	*0.000*	*100.3*	*-0.002*	*0.0*	*2.821*

Steroid glucuronides

Table S6. Initial (t_0) concentrations of glucuronides, maximum measured concentrations of deconjugated biomarker, and maximum deconjugation of glucuronides (%) in CR, GS, and RM. The percent of initial glucuronide concentration that was transformed was determined at the maximum value observed for its non-glucuronidated form over 12h.

Glucuronide	**DRO M1***	**19-NA***	**19-NE***	**ADS***	**STZ M1***
t_0 concentration [CR] (μg/L)	5.8	9.8	9	12.9	3.3
t_0 concentration [GS] (μg/L)	5.2	8.4	7	10	2.2
t_0 concentration [RM] (μg/L)	5.2	7.9	6.7	9.4	2.2
Maximum measured concentration of non-glucuronide [CR] (μg/L)	0.9	4.7	5.6	2.1	2.9
Maximum measured concentration of non-glucuronide [GS] (μg/L)	0.6	2.9	4.3	1.9	2.2
Maximum measured concentration of non-glucuronide [RM] (μg/L)	0.4	1.9	3.1	1.6	1.4
t_0 concentration transformed [CR]	16%	48%	62%	16%	88%
t_0 concentration transformed [GS]	12%	35%	61%	19%	100%
t_0 concentration transformed [RM]	8%	24%	46%	17%	64%

*DRO M1: Drostanolone M1, 19-NA: 19-Norandrosterone, 19-NE: 19-Noretiocholanolone, ADS: Androsterone, STZ M1: Stanozolol M1

1. Li, J., Gao, J., Thai, P.K., Sun, X., Mueller, J.F., Yuan, Z., and Jiang, G., *Stability of Illicit Drugs as Biomarkers in Sewers: From Lab to Reality.* Environ. Sci. Technol., 2018. **52**(3): p. 1561-1570.