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Occurrence and fate of androgens, estrogens, glucocorticoids, and progestagens in two different types of municipal wastewater treatment plants

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Chemicals and materials

High purity standards of 28 natural and synthetic steroids were purchased from Dr. Ehrenstorfer GmbH (Germany), Supelco (USA), Riedel-de Haën (RDH, Germany), Sigma-Aldrich (USA), Cambridge Isotope Laboratories Incorporation (Massachusetts, USA), CDN Isotopes (Quebec, Canada), TCR (North York, Canada), Cerilliant (USA), ACROS and Sigma (St. Louis, MO, USA). These steroids include 14 androgens: androsta-1,4-diene-3,17-dione (ADD), 4-androstene-3,17-dione (AED), androsterone (ADR), 17α-boldenone (17α-BOL), 17β-boldenone (17β-BOL), 5α-dihydrotestosterone (5α-DHT), epi-androsterone (EADR), 4-hydroxy-androst-4-ene-17-dione (4-OHA), methyl testosterone (MT), 19-nortestoserone (19-NT), testosterone (T), 17α-trenbolone (17α-TBL), 17β-trenbolone (17β-TBL), and stanozolol (S); 4 estrogens: diethylstilbestrol (DES), 17β-estradiol (E2), estrone (E1), and 17α-ethynyl estradiol (EE2); 5 glucocorticoids: cortisol (CRL), cortisone (CRN), dexamethasone (DEX), prednisone (PRE), and prednisolone (PREL); and 5 progestagens: ethynyl testosterone (ET), medroxyprogesterone (MP), 19-norethindrone (19-NTD), norgestrel (NGT), and progesterone (P); and their corresponding internal standards testosterone-16,16,17-d3 (T-d3), stanozolol-d3 (S-d3), 17β-estradiol-2,4,16,16-d4 (E2-d4), estrone-2,4,16,16-d4 (E1-d4), cortisol-d2 (CRL-d2), progesterone-d9 (P-d9).

Methanol, ethyl acetate and hexane were all HPLC grade purchased from Merck Corporation (Shanghai, China) or CNW Technologies (Dusseldorf, Germany). Formic acid was obtained from Tedia company (Tedia, USA). The cartridges used for solid

phase extraction (SPE) were Oasis HLB cartridges (N-vinylpyrrolidone-m-divinylbenzene copolymer, 500 mg, 6 mL) that were obtained from Waters Corporation (Milford, MA, USA). Glass fiber filters (GF/F, pore size 0.7 μm) were supplied by Whatman (Maidstone, England) and pyrolyzed at 450 ^{o}C for 4 h prior to use. Neutral silica gel (100-200 mesh, Qingdao, China) was Soxhlet extracted with dichloromethane for 48 h and baked at 160 ^{o}C for 16h prior to use. Anhydrous sodium sulfate was baked at 450 ^{o}C and stored in a sealed desiccator. HPLC grade water was obtained from a Milli-Q water purification system (Millipore, Watford). Stock solutions of chemicals (100 mg/L) were prepared in methanol and stored at −18 ^{o}C for later use. Working standard solutions were prepared weekly. All glassware was hand-washed with detergent and tap water, rinsed with Milli-Q water, and baked at 450 ^{o}C for at least 4 h before use.

Sample extraction and analysis

Sample extraction and cleanup

The water samples and sludge samples were analyzed according to our previous method.[1] Solid phase extraction (SPE) was used to extract water samples. One liter of water samples was filtered through glass fiber filters (Whatman GF/F, 0.7 μm effective pore size, UK). Exactly 100 μL each of 1 mg/L of E1-d4, E2-d4, T-d3, S-d3, CRL-d2 and P-d9 was added to each sample as the internal standards. Solid phase extraction cartridges (Oasis HLB, 6 mL and 500 mg each) were preconditioned each with 10 mL of methanol followed by 10 mL of HPLC grade water. The filtered water

samples passed through the SPE cartridges at a flow rate of 5 to 10 mL/min. The sample bottle was rinsed twice with two aliquots of 50 mL of 5% (v/v) methanol in HPLC grade water, which passed through the cartridge. Then the cartridges were dried under the vacuum for 2 h, and the target compounds were eluted from the cartridges using 12 mL of ethyl acetate. The extracts were dried and re-dissolved in 1 mL of methanol. Each final extract was then filtered through a 0.22 μm membrane filter into a 2 mL amber glass vial for further cleanup.

Ultrasonic extraction was used to extract sludge samples. Three parallel freeze-dried sludge samples (0.5 g), spiked with 100 ng of E1-d4, E2-d4, T-d3, S-d3, CRL-d2 and P-d9 as internal standards, were put into 30 mL glass centrifuge tubes. To volatilize the solvent from the sludge samples, the tubes were put into the fume hood for 4 h with foil loosely caped, manually mixed well, and then kept in 4 ^{o}C overnight. The sludge sample was extracted with 10 mL of ethyl acetate in an ultrasonic bath for 15 min and then centrifuged at 3500 rpm for 10 min. The supernatant was transferred into 100 mL pear-shaped flask by a glass pipette. The extraction process was repeated twice using 10 mL and 5 mL of the above extraction solution. Then all 25 mL extract from each sample was combined, evaporated to dryness via rotary evaporation, redissolved in 1 mL of methanol, and passed through 0.22 μm filter for further cleanup.

In this study, self-made silica gel cartridge (18 cm×1 cm i.d.), which had been extracted by dichloromethane for 48 h, was used for further cleanup. The glass cartridge (self-made) was filled with glass wool (CNW), 1.0 g silica gel and 0.5 cm of

anhydrous sodium sulfate from bottom to top. Each extract (240 μL) was added to the silica cartridge, which was preconditioned with 5 mL of methanol, 5 mL of ethyl acetate/methanol (90:10, v/v), and 5 mL of hexane. After the cartridge was rinsed with 6 mL of hexane, the target compounds were eluted with 6 mL of ethyl acetate/methanol (90:10, v/v). The eluate was then dried and reconstituted in 240 μL. Before analysis, 100 μL of that concentrated solution was dried and reconstituted in a buffer for the RRLC/MSMS analysis. For negative mode, the buffer was methanol/water (50:50, v/v), whereas for positive mode, the buffer was methanol/water-0.01% formic acid (60:40, v/v).

Instrumental analysis

The target compounds were analyzed by RRLC-MS/MS with electrospray ionization (ESI). Liquid chromatography was performed on an Agilent 1200 series RRLC system (Agilent Technologies) equipped with a degasser, a binary pump, an auto sampler and a column oven. The chromatographic separation was performed on an Agilent Zorbax SB-C18 (100 mm × 3 mm, 1.8 μm) column with its corresponding pre-column filter (2.1 mm, 0.2 μm). The column oven temperature was set to 40 ^{o}C and the injection volume was 10 μL. Two gradient elution programs were applied for two groups of steroids (Group I: estrogens; Group II: androgens, progestagens, and glucocorticoids), with a flow rate at 0.3 mL/min (Group I) and 0.35 mL/min (Group II), respectively. Mass spectrometry was performed using an Agilent 6460 Triple Quadrupole detector which was operated with ESI in both negative and positive

modes (Agilent Corporation, USA). The quantitative analysis of the target compounds was performed in multiple reaction monitoring (MRM) mode. Nitrogen gas was used as the drying and collision gas. Multiple reaction monitoring (MRM) parameters for the target compounds and internal standards are listed in Table S2.

Estrogens (Group I) were analyzed in the ESI (-) mode. The mobile phase used in the analysis was (A) Milli-Q water and (B) acetonitrile, with a linear gradient from 50% to 100% B in 10 min, post time 5 min. The MS operating conditions were set as follows: gas temperature, 350 ^{o}C; gas flow, 8 mL/min; nebulizer pressure, 50 psi; sheath gas flow, 12 L/min; sheath gas temperature, 350 ^{o}C; nozzle voltage, -2000 V; and capillary voltage, 3500 V.

Androgens, progestagens, and glucocorticoids (Group II) were analyzed in the ESI (+) mode. The mobile phase was: (A) water containing formic acid (0.01%, v/v) and (B) methanol, with the following elution program: from 60% to 80% B in 15 min, then from 80% to 60% B in 0.5 min, post time 5 min. The MS operating conditions were set as follows: gas temperature, 350 ^{o}C; gas flow, 3 mL/min; nebulizer pressure, 40 psi; sheath gas flow, 12 L/min; sheath gas temperature, 350 ^{o}C; nozzle voltage, 2000 V; and capillary voltage, 3500 V.

Calibration curves were constructed for androgens from 1.0 to 2000 μg/L (standard concentration levels at 1.0, 5.0, 10, 50, 100, 200 μg/L or 10, 50, 100, 200, 1000, 2000 μg/L according to the detected concentrations level) and for other targets from 1.0 to 200 μg/L (standard concentration levels at 1, 5, 10, 50, 100 and 200 μg/L), and excellent linearity was achieved in these concentration ranges with the correlation

coefficients higher than 0.99 for all validation batches.

Using the optimized extraction and instrumental methods, good recoveries were achieved for all target compounds in matrix spiked samples of surface water, wastewater and sludge samples (Table S3). The limit of detection (LOD) and limit of quantitation (LOQ) for each target compound were calculated based on the signal-to-noise ratio (SNR) near the target peak. LOD is defined as three times of SNR, and LOQ is ten times of SNR. The LOQs for the target analytes in the influent, effluent, surface water, and freeze-dried sludge samples were 0.05-4.8, 0.02-1.63, 0.03-0.80 ng/L, and 0.3-6.9 ng/g, respectively (Table S3). Both intra- and inter-day precision of the RRLC-MS/MS instrument were examined. For the intra-day precision, a standard solution (10 μg/L of each compound) was injected successively seven times. The R.S.D. was in the 0.6-11.6% range for all compounds. For the inter-day experiment, five of the standard solutions (10 μg/L of each compound) were performed on five different days over one month interval. In this case the R.S.D. was less than 14.1%.

Mass Balance and aqueous phase removal rate calculation

Mass balance is an instructive approach to estimate mass flows of a target analyte entering and leaving a wastewater treatment plant both in treated wastewater and sludge forms. Considering the low volatilization, the losses of steroids into the air are negligible in this study. The basic mass balance equation is shown in the following equation: [2]

$M_{Inf} = M_{Eff} + M_{Sludge} + M_{Loss}$ (Eq-1)

where M_{Inf} and M_{Eff}, are the mass load of a target analyte in influent and final effluent (g/d), respectively; M_{Sludge} represents the mass load in dewatered sludge (g/d); M_{Loss} stands for the loss mass of the analyte during the whole WWTP treatment which is mainly caused by the sum contribution of sorption and degradation in each process. According to equation (Eq-1),

$M_{Loss}\% = (M_{Inf} - M_{Eff} - M_{Sludge})/ M_{Inf} \times 100\%$ (Eq-2)

And the mass percentages for each compound in final effluent and dewatered sludge samples were calculated by $M_{Eff}/M_{Inf}\%$ and $M_{Slu}/M_{Inf}\%$, respectively.

Aqueous phase removal rate is employed to estimate quantitatively the removal efficiency of a target analyte in each treatment process of WWTP and can be expressed by the following equation:

$R_{Aqueous}\% = (M_{Inf\text{-}i} - M_{Eff\text{-}i})/ M_{Inf} \times 100\%$ (Eq-3)

where $M_{Inf\text{-}i}$ and $M_{Eff\text{-}i}$ are the mass load of a target analyte in influent and effluent (g/d) of the selected unit treatment process (i), respectively; M_{Inf} represents the mass load in influent (g/d); $R_{Aqueous}\%$ stands for the removal rate of the target analyte in selected unit treatment process in aqueous phase.

In each unit treatment process, the total mass load of each target analyte can be calculated using the following equation:

$M_{Total} = C_{Dissolved} \times Q /10^6 + C_{Adsorbed} \times Q \times C_{TSS}/10^9$ (Eq-4)

Where M_{Total} (g/d) is the total mass of a target analyte in the unit treatment process; $C_{Dissolved}$ (ng/L) and $C_{Adsorbed}$ (ng/g) represent the concentrations of the target analyte

in the aqueous and suspended solids phase; Q (m^3/d) stands for the water flow; C_{TSS} (mg/L) is the concentration of total suspended solids. When the actual concentration is below the limit of detection (LOD), LOD/$\sqrt{2}$ is used for calculation.

Estimation of steroids in influent based on serving population

The primary source of steroids in influent of municipal WWTPs is from human excretion urine and feces. Based on published data, the concentration of steroids in feces is far less than that in urine and could be negligible.[3-5] Thus, the daily excretion mass of urine can be used to estimate the source of steroids in WWTP influents. According to previous studies on excretion of steroids from human, data of estrogens and androgens are easily available from the literature[3, 5] to predict the human daily excretion of estrogens and androgens. Considering the detected concentrations and potencies in environment and excretion data availability,[3] ten natural steroids (five androgens (4-Androstene-3,17-dione, androsterone, 5α-dihydrotestosterone, epi-androsterone, and testosterone), three estrogens (17β-estradiol, estrone and estriol), one glucocorticoid (cortisol) and one progestagen (progesterone)) are selected as the representatives to estimate the mass loading into the WWTPs.

As for those steroids such as testosterone and progesterone with only concentration data available, the daily excretion can be calculated by 24-h urine volume. The normal range for 24-h urine volume is 800-2000 mL (with a normal fluid intake of about 2 liters per day). Here, geometric mean 1265 mL for urine volume per day was used for calculation. Since the concentration of progesterone in pregnant women urine is not

available to our investigation, it was estimated by estrogen concentration level in the same stage of pregnancy.[5, 6] There is a significant variation for the quantity of each steroid excreted by different sections of the population. Therefore, in this study, five sections (pregnant females, menstrual females, menopausal females, females taking hormone replacement therapy (HRT) and males) were taken into account (Table S4).

The estimated concentrations of a selected steroid in influent by serving population can be calculated using the following equation:

$$C_i=(\sum P_i \times P_T \times U_i)/Q \qquad \text{(Eq-5)}$$

Where C_i (ng/L) is the estimated concentration of a target analyte (i) in influent; P_i is the percentage of people in different section, P_T stands for the total serving population of the selected WWTP; U_i (μg/d) represents the mass of the target analyte excretion from urine of different section people each day; Q (m^3/d) stands for the water flow.

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Table S1 Parameters of Plant A and Plant B.

Process	Water flow m^3/d	TSS mg/L	COD mg/L	BOD_5 mg/L	TP mg/L	NH4-N mg/L	pH	Conductivity ms/cm
Plant A								
Influent	74400	150	170-180	70-80		16	8.1	
Grit chamber	74400							
Anoxic	74400	3000						
Anaerobic	74400	3000						
Aerobic	74400	3000						
Final effluent	19680	10	20	15		3	6.7	60
Return sludge	67200	10000						
Plant B								
Influent	96000	175	185	110	2.7	23		
Grit chamber	96000	3000						
Oxidation ditch	96000	3000						
Secondary clarifier	96000							
Final effluent	170800	16	22	10	0.8	35		
Return sludge		4000						

TSS, total suspended solids; COD, chemical oxygen demand; BOD_5, 5d biochemical oxygen demand; TP, total phosphorus.

Table S2 Details of the androgens, estrogens, glucocorticoids, progestagens and their multiple reaction monitoring (MRM) parameters in RRLC-MS/MS (androgens, glucocorticoids and progestagens under positive ionization mode, estrogens under negative ionization mode) based on Liu et al. (2011).[1]

Compound	Abbreviation	Supplier	M. W. [b]	CAS	R.T. [c]	Precursor ion	Product ions	Fragmentor (volts)	Collision energy (volts)
Androgens									
Androsta-1,4-diene-3,17-dione	ADD	TCR	284.4	897-06-3	5.438	285.2	121.1	105	21
							77.1	105	61
4-Androstene-3,17-dione	AED	Dr. Ehrenstorfer	286.4	63-05-8	7.129	287.2	109.1	135	25
							97.1	135	21
Androsterone	ADR	Dr. Ehrenstorfer	290.4	53-41-8	12.114	291.2	273.2	90	5
							255.2	90	13
17α-boldenone	17α-BOL	CERILLIANT	286.4	27833-18-7	7.601	287.2	269.2	85	5
							121.1	85	25
17β-boldenone	17β-BOL	Dr. Ehrenstorfer	286.4	846-48-0	6.956	287.2	135.1	90	9
							121.1	90	21
5α-dihydrotestosterone	5α-DHT	Dr. Ehrenstorfer	290.4	521-18-6	10.458	291.2	273.2	115	9
							255.2	115	13
Epi-androsterone	EADR	ACROS	290.4	481-29-8	9.820	291.2	273.2	90	5
							175.2	90	9
4-Hydroxy-androst-4-ene-17-dione	4-OHA	TRC	302.4	566-48-3	10.256	303.2	257.2	110	13
							55.1	110	53
Methyl testosterone	MT	Dr. Ehrenstorfer	302.4	58-18-4	10.253	303.2	109.1	140	29
							97.1	140	25
19-Nortestoserone	19-NT	Dr. Ehrenstorfer	274.4	434-22-0	7.238	275.2	109.1	130	45
							55	130	61
Testosterone	T	Dr. Ehrenstorfer	288.4	58-22-0	8.565	289.2	109.1	135	25

							97.1	135	21
Testosterone-16,16,17-d3 (I.S. [a])	T-d3	CERILLIANT	291.4	77546-39-5	8.501	292.2	109.1	135	25
							97.1	135	29
17α-trenbolone	17α-TBL	CERILLIANT	270.4	80657-17-6	6.405	271.2	253.2	150	20
							115	150	96
17β-trenbolone	17β-TBL	Dr. Ehrenstorfer	270.4	10161-33-8	6.411	271.2	165.1	140	69
							128	140	69
Stanozolol	S	Dr. Ehrenstorfer	328.5	10418-03-8	13.259	329.3	81.1	215	73
							54.1	215	100
Stanozolol-d3 (I.S.)	S-d3	CERILLIANT	331.5	88247-87-4	13.202	332.3	81.1	220	53
							54.1	220	89
Estrogens									
Diethylstilbestrol	DES	Riedel-de Haen	268.4	56-53-1	4.738	267	251.1	163	17
							237.1	163	21
17β-estradiol	E2	Dr. Ehrenstorfer	272.4	50-28-2	3.719	271	183.0	204	33
							145.0	204	30
17β-estradiol-2,4,16,16-d4 (I.S.)	E2-d4	CDN	276.4	66789-03-5	3.713	275	187.0	219	25
Estrone	E1	Riedel-de Haen	270.4	53-16-7	4.643	269	145.1	148	33
							143.1	148	57
Estrone-2,4,16,16-d4 (I.S.)	E1-d4	Cambridge	274.4	53866-34-5	4.638	273	147.2	168	37
17α-ethynyl estradiol	EE2	Dr. Ehrenstorfer	296.4	57-63-6	4.170	295	159.0	170	34
							145.0	170	38
Glucocorticoids									
Cortisol	CRL	Dr. Ehrenstorfer	362.5	50-23-7	3.701	363.2	121	170	24
							91	170	72
Cortisol-d2 (I.S.)	CRL-d2	CDN isotopes	364.4	79037-25-5	3.695	365.2	122	165	24

							91.1	165	76
Cortisone	CRN	Sigma	360.5	53-06-5	3.228	361.2	163	155	20
							105	155	48
Dexamethasone	DEX	Dr. Ehrenstorfer	392.5	50-02-2	4.760	393.2	147	125	28
							91	125	72
Prednisone	PRE	Dr. Ehrenstorfer	358.4	53-03-2	3.131	359.2	147	120	28
							91	120	72
Prednisolone	PREL	Dr. Ehrenstorfer	360.4	50-24-8	3.729	361.2	343.1	135	4
							147	135	20
Progestagens									
Ethynyl testosterone	ET	Dr. Ehrenstorfer	312.4	434-03-7	8.514	313.2	109.1	135	25
							97.1	135	21
Medroxyprogesterone	MP	Dr. Ehrenstorfer	344.5	520-85-4	10.640	345.2	123.1	145	25
							97.1	145	29
19-Norethindrone	19-NTD	TRC	298.4	68-22-4	7.260	299.2	109.1	130	29
							77.1	130	73
Norgestrel	NGT	Sigma	312.4	6533-00-2	9.579	313.2	91.1	135	61
							77.1	135	77
Progesterone	P	Dr. Ehrenstorfer	314.4	57-83-0	12.715	315.2	109.1	130	25
							97.1	130	21
Progesterone-d9 (I.S.)	P-d9	TCR	323.5	15775-74-3	12.527	324.3	113.1	125	29
							100.1	125	25

[a] I.S., internal standard; [b] molecular weight; [c] retention time (min).

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Table S3 Recoveries and detection limits of steroids in influent, effluent and sludge samples by RRLC-MS/MS based on Liu et al. (2011).

Compound	Effluent			Influent			Surface water			Sludge		
	100 ng/L	LOD [a] (ng/L)	LOQ [b] (ng/L)	100 ng/L	LOD (ng/L)	LOQ (ng/L)	100 ng/L	LOD (ng/L)	LOQ (ng/L)	100 ng/g	LOD (ng/g)	LOQ (ng/g)
Androgens												
Androsta-1,4-diene-3,17-dione	111±1.2	0.08	0.28	112±5.1	0.17	0.55	111±4.8	0.05	0.16	112±14.6	0.30	0.99
4-Androstene-3,17-dione	112±1.2	0.11	0.37	107±2.8	0.28	0.95	110±2.9	0.05	0.16	87.2±6.5	0.47	1.56
Androsterone	80.6±4.7	0.40	1.33	80.8±14.0	0.82	2.72	113±11.0	0.04	0.14	82.6±5.8	0.70	2.32
17α-boldenone	113±3.9	0.11	0.38	99.2±6.2	0.14	0.47	111±3.3	0.05	0.18	121±9.7	0.41	1.36
17β-boldenone	114±1.2	0.11	0.38	113±1.9	0.18	0.60	107±2.8	0.06	0.19	101±6.2	0.63	2.10
5α-dihydrotestosterone	123±2.8	0.39	1.30	130±2.8	0.90	3.01	143±3.4	0.04	0.13	122±10.5	0.70	2.32
Epi-androsterone	103±5.8	0.26	0.87	133±33.8	0.27	0.91	119±11.4	0.04	0.14	62.6±15.0	0.83	2.76
4-Hydroxy-androst-4-ene-17-dione	104±3.4	0.17	0.56	92.1±3.2	0.22	0.72	96.7±3.1	0.08	0.25	94.0±7.3	0.25	0.83
Methyl testosterone	94.2±2.8	0.07	0.24	88.5±2.4	0.24	0.79	99.2±1.8	0.01	0.04	77.2±3.1	0.37	1.24
19-Nortestoserone	103±4.0	0.31	1.03	90.8±4.0	0.31	1.03	101±3.2	0.10	0.33	76.5±4.3	0.58	1.95
Testosterone	103±1.7	0.11	0.37	105±2.6	0.26	0.88	103±1.8	0.05	0.18	98.9±1.5	0.46	1.52
17α-trenbolone	77.7±2.0	0.09	0.31	67.2±2.8	0.16	0.54	93.3±4.8	0.11	0.36	100±5.4	0.62	2.07
17β-trenbolone	109±0.5	0.15	0.50	63.2±5.3	0.33	1.09	94.2±3.9	0.20	0.68	91.6±3.6	0.64	2.14
Stanozolol	99.3±0.6	0.01	0.02	113±2.0	0.02	0.05	102±1.0	0.01	0.03	100±2.1	0.08	0.27
Estrogens												
Diethylstilbestrol	60.7±3.4	0.16	0.52	44.0±2.9	0.63	2.09	96.2±6.2	0.14	0.46	76.3±5.7	0.59	1.96
17β-estradiol	106±11.7	0.29	0.95	139±31.5	1.44	4.81	107±7.6	0.24	0.80	94.9±2.3	0.98	3.26
Estrone	89.1±15.4	0.05	0.17	104±7.4	0.20	0.68	113±3.8	0.09	0.30	103±4.0	0.10	0.34
17α-ethynyl estradiol	94.3±5.7	0.49	1.63	94.5±7.9	0.72	2.38	96.3±3.3	0.19	0.64	69.1±7.9	0.80	2.66
Glucocorticoids												
Cortisol	102±2.3	0.14	0.47	105±7.8	0.41	1.37	97.2±3.0	0.06	0.20	100±9.4	1.66	5.54

Cortisone	78.4±1.5	0.12	0.38	133±5.4	0.27	0.89	90.6±1.3	0.07	0.24	70.3±4.7	0.58	1.95
Dexamethasone	122±4.3	0.25	0.83	200±11.2	0.45	1.50	116±2.6	0.04	0.13	116±4.0	2.06	6.86
Prednisolone	114±4.1	0.29	0.97	100±1.1	0.39	1.31	112±6.6	0.03	0.09	65.6±3.7	1.48	4.93
Prednisone	99.1±2.5	0.10	0.32	172±4.9	0.35	1.18	100±4.2	0.05	0.18	107±6.6	0.84	2.79
Progestagens												
Ethynyl testosterone	93.9±6.8	0.09	0.30	116±5.4	0.18	0.59	99.5±2.7	0.04	0.13	138±7.6	0.54	1.81
Medroxyprogesterone	117±7.5	0.04	0.15	133±5.2	0.12	0.40	110±2.1	0.04	0.13	137±4.2	0.38	1.28
19-Norethindrone	94.9±6.5	0.21	0.71	103±4.6	0.42	1.40	93.3±1.5	0.02	0.08	105±6.6	1.92	6.39
Norgestrel	100±6.3	0.03	0.10	115±5.6	0.31	1.03	96.8±2.8	0.04	0.12	109±2.9	0.90	2.99
Progesterone	98.6±0.3	0.08	0.27	110±2.2	0.09	0.29	102±1.0	0.05	0.17	113±2.4	0.42	1.39

[a] LOD, limit of detection; [b] LOQ, limit of quantitation.

Table S4 Masses of selected steroids in human urine per day (μg/d).

	Males	Females			
		Menstrual	Menopausal	Menopausal on HRT	Pregnant
Population %	50	35.6 [a]	13.4 [a]	2.0 [b]	1.0 [a]
Estrogens (μg/d)					
17β-estradiol [b]	1.8	3.2	1.0	56.0	393
Estriol [c]	1.5	8.1	2.8		24078
Estrone [b]	2.6	11.7	1.8	28.4	550
Androgens [c] (μg/d)					
4-Androstene-3,17-dione	3.7				
Androsterone	3340			1570	
5α-dihydrotestosterone	14.1				
Epi-androsterone	229				
Testosterone	56.7			6.8	
Progestagens (μg/d)					
Progesterone	16.1 [d]	127 [d]	37.5 [d]	214 [d]	260 [e]
Glucocorticoids (μg/d)					
Cortisol [c]	251			167	

[a] Estimated by Guangdong Statistical Yearbook in 2009 (http://www.gdstats.gov.cn/tjnj/table/4/c4_1.htm); [b] Johnson, A.C., Williams, R.J. (2004); [c] Referred by Liu et al.(2009); [d] Estimated based on the data from Gadzala-Kopciuch et al. (2009); [e] Estimated by Gadzala-Kopciuch et al. (2009) and Leslie et al. (2000).

Table S5 Concentrations (dissolved ng/L; adsorbed ng/g) of steroids in different treatment stages of Plant A.

Compound		Influent	Grit chamber effluent	Anoxic effluent	Anaerobic effluent	Aerobic effluent	Final effluent	Return sludge	Dewatered sludge
Androgens									
Androsta-1,4-diene-3,17-dione	dissolved	121±23.6 [a]	100±0.5	4.2±0.1	3.9±0.2	3.3±0.1	3.5±0.2		
	adsorbed			20.5±0.5	15.3±1.2	130±5.5		17.7±1.1	11.6±2.7
4-Androstene-3,17-dione	dissolved	31.1±5.1	33.0±0.5	4.7±0.3	4.9±0.2	4.7±0.0	5.5±0.3		
	adsorbed			16.0±1.5	13.4±0.8	34.3±0.4		14.3±0.4	7.9±0.1
Androsterone	dissolved	229±10.6	260±9.7	N.D. [b]	N.D.	N.D.	N.D.		
	adsorbed			N.D.	N.D.	N.D.		N.D.	N.D.
17β-boldenone	dissolved	18.8±3.6	17.3±0.7	1.5±0.0	1.6±0.2	1.6±0.2	1.6±0.0		
	adsorbed			5.2±0.3	N.D.	8.7±1.2		N.D.	N.D.
5α-dihydrotestosterone	dissolved	N.D.	N.D.	N.D.	22.1±0.7	N.D.	N.D.		
	adsorbed			295±7.3	527±23.1	252±24.7		276±50.4	460±4.4
Epi-androsterone	dissolved	1368±283	1430±38.1	18.4±1.6	9.0±1.2	7.8±0.1	N.D.		
	adsorbed			324±14.6	615±76.7	307±3.6		345±5.3	431±39.7
Methyl testosterone	dissolved	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
	adsorbed			4.4±0.1	6.6±1.6	4.9±0.6		6.5±0.1	5.7±0.6
Testosterone	dissolved	8.9±1.0	10.5±0.2	1.5±0.2	1.4±0.1	1.3±0.1	1.5±0.2		
	adsorbed			5.1±0.4	5.3±0.5	7.3±0.2		5.3±0.3	N.D.
17α-trenbolone	dissolved	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
	adsorbed			8.6±1.6	12.1±1.0	5.1±0.7		6.5±0.5	6.6±0.8
Stanozolol	dissolved	1.3±0.0	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0		
	adsorbed			N.D.	N.D.	N.D.		N.D.	N.D.
Estrogens									

17β-estradiol	dissolved	23.9±9.6	21.9±10.6	9.5±2.4	10.1±1.8	16.0±1.7	4.8±1.5		
	adsorbed			61.2±11.6	67.8±16.8	11.4±0.3		90.7±5.9	48.9±11.4
Estrone	dissolved	36.3±11.6	43.8±2.9	15.2±1.9	9.0±3.3	9.0±0.0	8.7±0.3		
	adsorbed			10.5±1.0	11.6±0.1	1.3±0.1		3.2±0.2	8.9±3.5
Glucocorticoids									
Cortisol	dissolved	130±3.1	156±11.8	10.1±2.0	15.7±1.6	9.8±2.6	4.5±0.7		
	adsorbed			N.D.	N.D.	N.D.		N.D.	N.D.
Cortisone	dissolved	61.5±20.7 [b]	57.3±2.8	3.3±1.5	2.9±1.1	1.8±0.0	1.8±0.1		
	adsorbed			N.D. [c]	N.D.	N.D.		N.D.	N.D.
Prednisolone	dissolved	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
	adsorbed			N.D.	N.D.	6.0±0.5		N.D.	N.D.
Progestagens									
Medroxyprogesterone	dissolved	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
	adsorbed			2.1±0.1	N.D.	N.D.		N.D.	N.D.
Norgestrel	dissolved	28.3±7.2	27.6±8.0	15.1±2.6	12.3±0.9	9.7±0.4	11.0±0.9		
	adsorbed			< LOD [c]	N.D.	N.D.		N.D.	N.D.
Progesterone	dissolved	12.2±1.9	15.0±0.0	2.7±0.2	1.4±0.3	0.9±0.1	1.1±0.1		
	adsorbed			19.7±1.1	13.8±0.2	96.3±3.3		N.D.	N.D.

[a] mean ± standard deviation (n=3, replicate samples at the same time); [b] N.D.: not detected; [c] <LOD: below the limit of detection.

Table S6 Mass flux (g/d) of steroids in different treatment stages of Plant A.

Compound		Influent	Grit chamber effluent	Anoxic effluent	Anaerobic effluent	Aerobic effluent	Final effluent	Return sludge	Dewatered sludge	M_{Eff}/M_{Inf}%	M_{Slu}/M_{Inf}%	Loss%
Androgens												
Androsta-1,4-diene-3,17-dione	dissolved	9.00	7.44	0.31	0.29	0.25	0.07					
	adsorbed			4.58	3.41	29.02		13.17	0.08			
	total	9.00	7.44	4.89	3.71	29.26	0.07	13.17	0.08	0.8	0.9	98.3
4-Androstene-3,17-dione	dissolved	2.31	2.46	0.35	0.36	0.35	0.11					
	adsorbed			3.57	2.99	7.66		10.64	0.06			
	total	2.31	2.46	3.92	3.36	8.01	0.11	10.64	0.06	4.8	2.6	92.6
Androsterone	dissolved	17.04	19.34	0.00	0.00	0.00	0.00					
	adsorbed			0.00	0.00	0.00		0.00	0.00			
	total	17.04	19.34	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0	100
17β-boldenone	dissolved	1.40	1.29	0.11	0.12	0.12	0.03					
	adsorbed			1.16	0.00	1.94		0.00	0.00			
	total	1.40	1.29	1.27	0.12	2.06	0.03	0.00	0.00	2.1	0.0	97.9
5α-dihydrotestosterone	dissolved	0.00	0.00	0.00	1.64	0.00	0.00					
	adsorbed			65.84	117.63	56.25		205.34	3.22			
	total	0.00	0.00	65.84	119.27	56.25	0.00	205.34	3.22	N.A.[a]	N.A.	N.A.
Epi-androsterone	dissolved	101.79	106.39	1.37	0.67	0.58	0.00					
	adsorbed			72.32	137.27	68.52		256.68	3.02			
	total	101.79	106.39	73.69	137.94	69.10	0.00	256.68	3.02	0.0	3.0	97.0
Methyl testosterone	dissolved	0.00	0.00	0.00	0.00	0.00	0.00					
	adsorbed			0.98	1.47	1.09		4.84	0.04			
	total	0.00	0.00	0.98	1.47	1.09	0.00	4.84	0.04	N.A.	N.A.	N.A.
Testosterone	dissolved	0.66	0.78	0.11	0.10	0.10	0.03					

	adsorbed			1.14	1.18	1.63		3.94	0.00			
	total	0.66	0.78	1.25	1.29	1.73	0.03	3.94	0.00	4.5	0.0	95.5
17α-trenbolone	dissolved	0.00	0.00	0.00	0.00	0.00	0.00					
	adsorbed			1.92	2.70	1.14		4.84	0.05			
	total	0.00	0.00	1.92	2.70	1.14	0.00	4.84	0.05	N.A.	N.A.	N.A.
Stanozolol	dissolved	0.10	0.09	0.09	0.09	0.09	0.02					
	adsorbed			0.00	0.00	0.00		0.00	0.00			
	total	0.10	0.09	0.09	0.09	0.09	0.02	0.00	0.00	20.0	0.0	80.0
Estrogens												
17β-estradiol	dissolved	1.78	1.63	0.71	0.75	1.19	0.09					
	adsorbed			13.65	15.14	2.55		67.49	0.34			
	total	1.78	1.63	14.36	15.89	3.74	0.09	67.49	0.34	5.1	19.1	75.8
Estrone	dissolved	2.70	3.26	1.13	0.67	0.67	0.17					
	adsorbed			2.33	2.58	0.28		2.40	0.06			
	total	2.70	3.26	3.46	3.25	0.95	0.17	2.40	0.06	6.3	2.2	91.5
Glucocorticoids												
Cortisol	dissolved	9.67	11.61	0.75	1.17	0.73	0.09					
	adsorbed			0.00	0.00	0.00		0.00	0.00			
	total	9.67	11.61	0.75	1.17	0.73	0.09	0.00	0.00	0.9	0.0	99.1
Cortisone	dissolved	4.58	4.26	0.25	0.22	0.13	0.04					
	adsorbed			0.00	0.00	0.00		0.00	0.00			
	total	4.58	4.26	0.25	0.22	0.13	0.04	0.00	0.00	0.9	0.0	99.1
Prednisolone	dissolved	0.00	0.00	0.00	0.00	0.00	0.00					
	adsorbed			0.00	0.00	1.34		0.00	0.00			
	total	0.00	0.00	0.00	0.00	1.34	0.00	0.00	0.00	N.A.	N.A.	N.A.
Progestagens												

Medroxyprogesterone	dissolved	0.00	0.00	0.00	0.00	0.00	0.00					
	adsorbed			0.47	0.00	0.00		0.00	0.00			
	total	0.00	0.00	0.47	0.00	0.00	0.00	0.00	0.00	N.A.	N.A.	N.A.
Norgestrel	dissolved	2.11	2.05	1.12	0.92	0.72	0.22					
	adsorbed			0.02	0.00	0.00		0.00	0.00			
	total	2.11	2.05	1.12	0.92	0.72	0.22	0.00	0.00	10.4	0.0	89.6
Progesterone	dissolved	0.91	1.12	0.20	0.10	0.07	0.02					
	adsorbed			4.40	3.08	21.49		0.00	0.00			
	total	0.91	1.12	4.60	3.18	21.56	0.02	0.00	0.00	2.2	0.0	97.8

[a] not available.

Electronic Supplementary Material (ESI) for Journal of Environmental Monitoring

Table S7 Concentrations (dissolved ng/L; adsorbed ng/g) of steroids in different treatment stages of Plant B.

Compound		Influent	Aerated grit channel effluent	Oxidation ditch effluent	Secondary clarifier effluent	Final effluent	Dewatered sludge
Androgens							
Androsta-1,4-diene-3,17-dione	dissolved	248±6.7 [a]	240±2.1	3.5±0.9	2.4±0.1	2.8±0.1	
	adsorbed			9.3±0.5			7.9±0.0
4-Androstene-3,17-dione	dissolved	59.3±1.3	62.6±0.4	3.2±0.5	2.5±0.0	2.8±0.0	
	adsorbed			7.7±0.2			6.7±0.1
Androsterone	dissolved	224±15.2	215±15.2	N.D. [b]	N.D.	N.D.	
	adsorbed			N.D.			N.D.
17β-boldenone	dissolved	15.9±1.2	15.6±1.5	1.9±0.1	1.8±0.5	1.8±0.5	
	adsorbed			N.D.			N.D.
5α-DHT	dissolved	64.4±40.9	86.5±25.7	N.D.	N.D.	23.1±1.0	
	adsorbed			140±5.4			173±0.3
Epi-androsterone	dissolved	934±61.4	910±27.1	N.D.	N.D.	14.2±0.6	
	adsorbed			146±32.0			176±5.4
Methyl testosterone	dissolved	N.D.	N.D.	N.D.	N.D.	N.D.	
	adsorbed			4.6±0.2			4.6±0.0
Testosterone	dissolved	6.9±0.1	7.0±0.0	2.3±0.1	1.3±0.0	1.9±0.0	
	adsorbed			4.0±0.2			5.5±0.1
17α-trenbolone	dissolved	N.D.	N.D.	N.D.	N.D.	N.D.	
	adsorbed			4.2±0.3			3.7±0.1
Stanozolol	dissolved	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0	
	adsorbed			N.D.			N.D.
Estrogens							
17β-estradiol	dissolved	12.0±2.9	25.3±13.1	6.5±2.4	9.2±3.0	4.1±0.4	

	adsorbed			10.4±0.1			12.9±0.5
Estrone	dissolved	29.5±8.2	22.0±1.8	1.6±0.1	1.5±0.1	1.5±0.1	
	adsorbed			0.3±0.0			1.0±0.1
Glucocorticoids							
Cortisol	dissolved	123±2.7	135±9.8	N.D.	N.D.	N.D.	
	adsorbed			N.D.			N.D.
Cortisone	dissolved	48.2±1.7 [b]	40.8±0.2	N.D.	N.D.	N.D.	
	adsorbed			N.D.			N.D.
Prednisolone	dissolved	N.D.	N.D.	N.D.	N.D.	2.2±0.1	
	adsorbed			4.3±0.4			4.4±0.2
Progestagens							
Norgestrel	dissolved	35.3±2.0	31.5±3.7	5.0±1.2	4.2±0.6	5.9±0.8	
	adsorbed			N.D.			N.D.
Progesterone	dissolved	4.3±0.1	4.6±0.2	1.4±0.7	0.8±0.0	1.0±0.0	
	adsorbed			N.D.			N.D.

[a] mean ± standard deviation (n=3, replicate samples at the same time); [b] N.D.: not detected.

Table S8 Mass flux (g/d) of steroids in different treatment stages of Plant B.

Compound		Influent	Aerated grit channel effluent	Oxidation ditch effluent	Secondary clarifier effluent	Final effluent	Dewatered sludge	M_{Eff}/M_{Inf}%	M_{Slu}/M_{Inf}%	Loss%
Androgens										
Androsta-1,4-diene-3,17-dione	dissolved	23.81	23.04	0.34	0.23	0.48				
	adsorbed			2.68			0.16			
	total	23.81	23.04	3.01	0.23	0.48	0.16	2.0	0.7	97.3
4-Androstene-3,17-dione	dissolved	5.69	6.01	0.31	0.24	0.48				
	adsorbed			2.22			0.13			
	total	5.69	6.01	2.52	0.24	0.48	0.13	8.4	2.3	89.3
Androsterone	dissolved	21.50	20.64	0.00	0.00	0.00				
	adsorbed			0.00			0.00			
	total	21.50	20.64	0.00	0.00	0.00	0.00	0.0	0.0	100
17β-boldenone	dissolved	1.53	1.50	0.18	0.17	0.31				
	adsorbed			0.00			0.00			
	total	1.53	1.50	0.18	0.17	0.31	0.00	20.3	0.0	79.7
5α-dihydrotestosterone	dissolved	6.18	8.30	0.00	0.00	3.95				
	adsorbed			40.32			3.46			
	total	6.18	8.30	40.32	0.00	3.95	3.46	63.9	56.0	-19.9
Epi-androsterone	dissolved	89.66	87.36	0.00	0.00	2.43				
	adsorbed			42.05			3.52			
	total	89.66	87.36	42.05	0.00	2.43	3.52	2.7	3.9	93.4
Methyl testosterone	dissolved	0.00	0.00	0.00	0.00	0.00				
	adsorbed			1.32			0.09			
	total	0.00	0.00	1.32	0.00	0.00	0.09	N.A.	N.A.	N.A.
Testosterone	dissolved	0.66	0.67	0.22	0.12	0.32				

	adsorbed			1.15			0.11			
	total	0.66	0.67	1.37	0.12	0.32	0.11	48.5	16.7	34.8
17α-trenbolone	dissolved	0.00	0.00	0.00	0.00	0.00				
	adsorbed			1.21			0.07			
	total	0.00	0.00	1.21	0.00	0.00	0.07	N.A.	N.A.	N.A.
Stanozolol	dissolved	0.12	0.12	0.12	0.12	0.20				
	adsorbed			0.00			0.00			
	total	0.12	0.12	0.12	0.12	0.20	0.00	167	0.0	-66.7
Estrogens										
17β-estradiol	dissolved	1.15	2.43	0.62	0.88	0.70				
	adsorbed			3.00			0.26			
	total	1.15	2.43	3.62	0.88	0.70	0.26	60.9	22.6	16.5
Estrone	dissolved	2.83	2.11	0.15	0.14	0.26				
	adsorbed			0.09			0.02			
	total	2.83	2.11	0.24	0.14	0.26	0.02	9.2	0.7	90.1
Glucocorticoids										
Cortisol	dissolved	11.81	12.96	0.00	0.00	0.00				
	adsorbed			0.00			0.00			
	total	11.81	12.96	0.00	0.00	0.00	0.00	0.0	0.0	100
Cortisone	dissolved	4.63	3.92	0.00	0.00	0.00				
	adsorbed			0.00			0.00			
	total	4.63	3.92	0.00	0.00	0.00	0.00	0.0	0.0	100
Prednisolone	dissolved	0.00	0.00	0.00	0.00	0.38				
	adsorbed			1.24			0.09			
	total	0.00	0.00	1.24	0.00	0.38	0.09	N.A.	N.A.	N.A.
Progestagens										

Norgestrel	dissolved	3.39	3.02	0.48	0.40	1.01				
	adsorbed			0.00			0.00			
	total	3.39	3.02	0.48	0.40	1.01	0.00	29.8	0.0	70.2
Progesterone	dissolved	0.41	0.44	0.13	0.08	0.16				
	adsorbed			0.00			0.00			
	total	0.41	0.44	0.13	0.08	0.16	0.00	39.0	0.0	61.0

[a] not available.

Table S9 Percentages of selected steroids in dissolved and adsorbed phase of different stages by estimation and measurement in Plant A.

Table S10 Percentages of selected steroids in dissolved and adsorbed phase of different stages by estimation and measurement in Plant B.

Tables S9-10 listed in excel.