

Fate and levels of steroid oestrogens and androgens in waste stabilisation ponds: quantification by liquid chromatography–tandem mass spectrometry

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Abstract The capacity for removing wastewater-borne endocrine disrupting chemicals (EDCs) was investigated for two wastewater treatment plants (WWTPs) incorporating waste stabilisation ponds (WSPs) as the principal treatment technology. Samples were analysed for a number of steroidal oestrogens and androgens using liquid chromatography–tandem mass spectrometry (LC/MS/MS). Removal efficiency for steroid androgens was high for both WWTPs (93–100%) but WSP treatment was observed to be less effective for removing steroid oestrogens, particularly oestriol. This was attributed to the fact that oestriol is more stable under UV light than the other compounds studied.

Keywords Endocrine disrupters; liquid chromatography–tandem mass spectrometry; steroid oestrogens and androgens; waste stabilisation ponds; wastewater treatment

Introduction

Endocrine disrupting chemicals (EDCs) have, over the past two decades, emerged as a group of biologically active contaminants of concern in the aquatic environment. Increasingly large quantities of these compounds have been entering aquatic ecosystems mainly as a result of human activities; with municipal wastewater representing one of the main sources of EDCs to the natural environment (Forrez *et al.*, 2009). This continuous aqueous disposal of xenobiotic EDCs is of particular importance given that our current level of understanding regarding the relative sensitivities of different organisms to these chemicals remains limited (Jobling *et al.*, 2004). Consequently, there is ongoing concern regarding the environmental consequences of EDCs discharged to the aquatic environment from wastewater treatment plants (WWTPs). Prior research has demonstrated that EDCs can interact with the endocrine systems of numerous aquatic species, and it is now generally accepted that these compounds contribute toward the disruption of developmental and reproductive functionality in a range of biological taxa (Tyler *et al.*, 1998) even at trace level concentrations (<1 ng L⁻¹) (Jobling *et al.*, 2004).

A number of EDCs are responsible for most of the oestrogenic and androgenic character of domestic wastewater, including the natural oestrogens (17 β -oestradiol, oestrone, oestriol), synthetic oestrogen (17 α -ethynylloestradiol), as well as testosterone, its metabolites (e.g., dihydrotestosterone) and other naturally occurring androgens (androstenedione, androsterone, etiocholanolone) (Desbrow *et al.*, 1998; Kirk *et al.*, 2002; de Mes *et al.*, 2005). Research in the U.K., for example, has shown that most WWTP effluents are oestrogenic and has also identified a probable link between this wastewater oestrogenicity and reproductive anomalies in wild fish populations in the receiving environment (Desbrow *et al.*, 1998). Whilst the majority of research effort has been focused on oestrogenic steroid hormones, androgens such as testosterone can also elicit both odorant and pheromonal responses in fish at extremely low concentrations (Moore and Scott, 1991). It is therefore important to increase our understanding regarding the levels and fate of oestrogenic and androgenic compounds during various wastewater treatment process and also to determine which EDCs are present in wastewaters and at what concentration. The need for such information is of even greater importance regarding Australian wastewaters due to limited studies on fate and levels of these compounds in wastewater systems and the environment (Leusch *et al.*, 2006; Williams *et al.*, 2007; Coleman, 2008a, 2008b; Holmes, 2008).

Waste stabilisation ponds (WSPs) are a simplistic and non-intensive wastewater treatment technology, with various WSP configurations employed worldwide to treat a range of different contaminants. WSPs have no additional energy requirements for aeration and circulation, and due to their reliance on ‘solar power’ for wastewater treatment, they are operationally simple and cost-effective (Oswald, 1995). Advantages of WSP treatment are both numerous and well recognised, and include: low capital and operational costs; minimum maintenance requirements; effective pathogen removal; and a capacity to withstand hydraulic and organic shock-loading (Oswald, 1995; Maynard *et al.*, 1999). There are few published reports regarding the concentrations of EDCs in Australian WWTPs in general (Leusch *et al.*, 2005; 2006; Tan *et al.*, 2008; Williams *et al.*, 2007; Coleman *et al.*, 2008a, 2008b) and even fewer accounts internationally of EDC dynamics following WSP treatment in particular (Servos *et al.*, 2005; Gomez *et al.*, 2007; Williams *et al.*, 2007; Holmes, 2008). This paper presents results from a study investigating the removal of 12 steroid oestrogens and androgens along two WWTPs employing waste stabilisation ponds as the treatment method.

Materials and methods

Description of WWTPs

Wastewater samples for this study were collected from two WWTPs in Australia. Details of the two WWTPs (A and B) are provided in Table 1.

Sampling protocol

Grab samples (1–2 L) were taken from the two WWTPs at various stages along the treatment train. Details of the sampling location at each WWTP are presented in Table 2. Grab samples were collected in duplicate and stored on ice in the dark during transport to the laboratory. Samples were pre-treated and analysed for steroidal oestrogens and androgens as outlined below.

Table 1. Details of the two wastewater treatment plants studied

WWTP	Treatment Processes	Population equivalent	Inflow (m ³ d ⁻¹)
A	Pond-based with alum dosing; pre- and post-chlorination; stream discharge	5,500	621
B	Pond-based without alum dosing	5,000	227

Table 2. Sampling program and sample details for the two WWTPs

Sample label	Sample location and description
A1	Raw effluent
A2	Primary effluent – Pond 1
A3	Secondary effluent – Pond 2
B1	Raw effluent
B2	Primary effluent – Pond 1
B3	Secondary effluent – Pond 2

Sample preparation

All samples were pre-treated by filtering and extracting before analysis. Samples were spiked with isotopic standards of oestrogens and androgens prior to filtration. This involved filtering 1 or 2 litres of sample through GF/F filter paper (Whatman[®]; 0.7 μm pore size) before extracting using solid phase extraction (SPE). For the SPE procedure, HLB cartridges (12 cc) from Oasis were used along with a Visiprep 24-port SPE vacuum manifold. The cartridges were conditioned with 5 ml of acetone:hexane (1:1), 5 ml methanol and 5 ml Milli-Q[®] water. The filtered wastewater samples were then passed through the cartridges. All cartridges were dried under vacuum using the vacuum trap for 2–3 hrs. The cartridges were then eluted using 2 × 5 ml methanol. Each sample was dried down to 0.5 mL in methanol before analysis by high performance liquid chromatography–tandem mass spectrometry (LC/MS/MS).

Analysis using LC/MS/MS

Analysis was carried out by isotopic dilution LC/MS/MS. Compounds analysed included five oestrogenic hormones (17β-oestradiol (E2), 17α-oestradiol (α-E2), oestrone (E1), oestriol (E3), 17α-ethinyloestradiol (EE2)) and seven androgenic hormones (androstenedione (An), androsterone (A), etiocholoanolone (E),

testosterone (Te), testosterone propionate (TP), dihydrotestosterone (DHT), 17 β -trenbolone (β -Tr)). The LC/MS/MS instrument included the Agilent LC series 1200 system coupled with an Applied Biosystems API 4000 Q-Trap mass spectrometer. The LC column used in the analysis was the Luna C18, 150 mm, 5 μ m, 100A column (Biolab) with security guard cartridges C18 4 mm \times 3 mm (Biolab). Mobile phases were methanol and Milli-Q[®] water; both containing 0.1% formic acid. Atmospheric pressure chemical ionisation was used as the ionisation source in the mass spectrometer. Quantification was carried out using the Applied Biosystems Analyst 1.5 software. Statistical analyses and graphical representations were formulated using PRISM 4.03 (GraphPad Software, San Diego, CA, USA).

Results and discussion

Figures 1 and 2 show the levels of oestrogens and androgens detected in all samples.

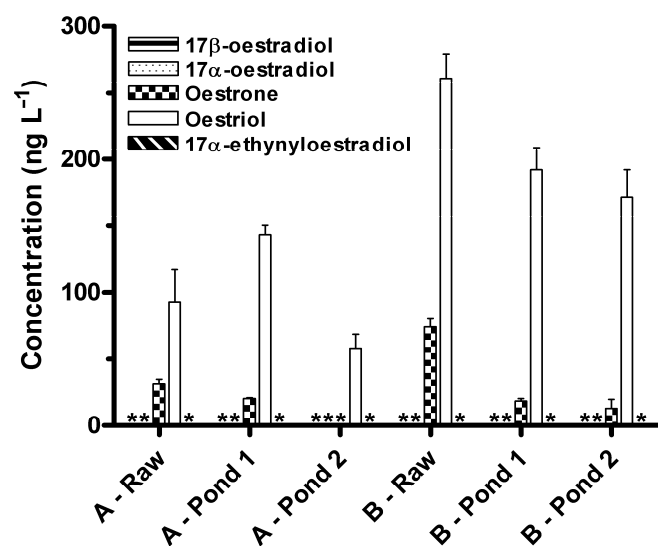


Figure 1. Fate and levels of oestrogens through the two waste stabilisation ponds. Asterisks denote EDC concentrations below the analytical limit of detection (see Table 3 for individual detection limits).

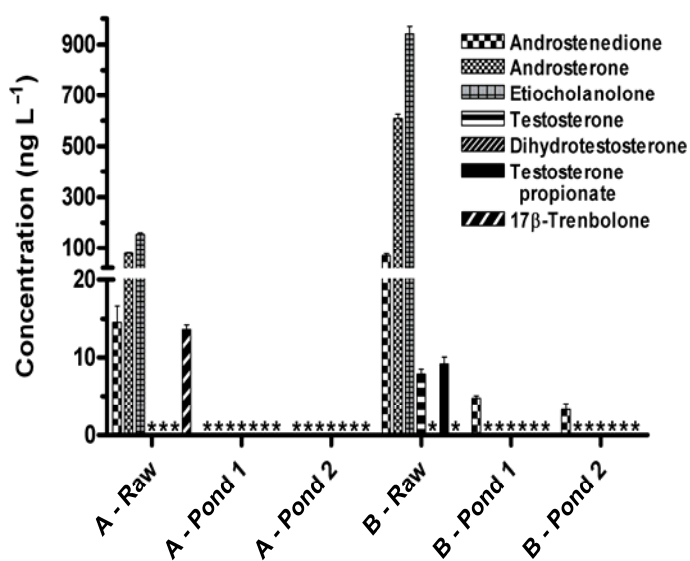


Figure 2. Fate and levels of androgens through the two waste stabilisation ponds. Asterisks denote EDC concentrations below the analytical limit of detection (see Table 3 for individual detection limits).

It can be seen in Figure 1 that the oestrogens E2, α -E2 and EE2 were below the limits of detection in all samples (0.1–1 ng L⁻¹). E1 was found in all samples except Pond 2 (after secondary treatment) in WWTP A and E3 was detected in all samples. The natural oestrogens E2, α -E2, E1 and E3 are naturally excreted by humans, either unconjugated or primarily as inactive glucuronide or sulphate conjugates; however, these conjugates can be rapidly cleaved and metabolised into their steroidal active parent compounds by enzymes. The absence of E2 and α -E2 in the samples was thought to have been mainly due to the fast degradation of oestradiol to oestrone (Servos *et al.*, 2005). E2 was also undetected in previous studies (Lee *et al.*, 2005; Tan *et al.*, 2008) or was found in the low ng L⁻¹ range (Hu *et al.*, 2007) with a maximum concentration of 16 ng L⁻¹ (Braga *et al.*, 2005). Batch experiments by Ternes *et al.* (1999) showed that E2 is degraded fast and easily to E1. There are no reports to date of α -E2 being present in Australian wastewaters. The main active compound of the contraceptive pill, EE2, has not been detected in previous studies (Synder *et al.*, 2007; Tan *et al.*, 2008) or if detected, the concentrations were again in the very low ng L⁻¹ range. The occurrence of E3 in wastewater has been reported in several studies at up to 318 ng L⁻¹ (Kim *et al.*, 2007) whereas it remained undetected in other studies (Synder *et al.*, 2007). E1 is generally—apart from the less potent E3—the most abundant steroidal oestrogen in wastewater, with concentrations reported in the range of 15–54 ng L⁻¹ (Ternes *et al.*, 1999; Braga *et al.*, 2005; Tan *et al.*, 2008). According to Johnson and Sumpter (2001), the most likely pathways for E1 to occur in wastewater are the degradation of the glucuronide and

sulfonide conjugates and of E2 in the sewer system. As summarised by Johnson and Sumpter (2001), E1 has been identified as the most environmentally important oestrogen, despite its lower potency compared to E2, as it is detected more frequently and in higher concentrations than E2; however, E2 and EE2 can also play a minor but still important role in the oestrogenic activity of wastewaters. Oestriol appears to give less cause for environmental concern, since it is generally released in low concentrations into the environment and shows a relatively low potency compared to other steroidal hormones (Johnson and Sumpter, 2001).

All of the androgens were detected at some stage except the testosterone metabolite DHT (Figure 2). The absence of DHT was possibly due to the relatively high limit of detection for DHT (12–25 ng L⁻¹) or the fact that it is not commonly found in wastewater. Following a literature survey, no previous reports of DHT in wastewater were found. As suggested by Kirk *et al.* (2002), the main pathway for androgens entering domestic sewage networks is most likely through human excretion. Concentrations of androgens in wastewater are expected to be much higher than for oestrogens due to the higher excretion rates in humans; however, results from this study and also others (Kim *et al.*, 2007; Synder *et al.*, 2007) suggest that androgens are removed effectively during secondary treatment, and that the levels of Te, E and A in secondary effluent are below the current limits of detection. During this research, the most potent androgen—DHT—could not be detected in any samples. It is interesting to note that β -Tr was detected in the raw sample of WWTP A (Figure 2). β -Tr is a metabolite of trenbolone acetate which is administered as a growth promoter to beef cattle. To our knowledge, this is the first report of trenbolone being detected in a WWTP and its presence here was most likely the result of agricultural run-off, since this WWTP is located in a beef cattle producing area.

Table 3 shows the percentage removal efficiencies of the WSPs at each of the WWTPs. 100% removals were reported for all of the androgens studied except androstenedione in WWTP B which yielded a removal rate of 95%. Removal rates for E1 and E3 were lower, although 100% removal was observed by Pond 2 of WWTP A for E1. It is interesting to note the negative removals of E3 in Pond 1 of WWTP A (Table 3). It is hypothesised that the increased levels of E3 in Pond 1 may have been a result of the liberation of previously adsorbed E3 (in the raw wastewater) being released back into solution where it was then able to be detected. It is possible that the majority of E3 in raw effluent was bound to the solid fraction and was therefore unable to be detected in the filtered samples. It should also be mentioned that because these samples were all taken at the same time, there may have been temporal differences in the concentration of E3 entering the WWTP such that samples taken from Pond 1 may not have been representative of the upstream raw influent wastewater on that day. Servos *et al.* (2005) reported on oestrogenic EDC removals (E2 and E1) in four primary-level WSPs receiving predominantly municipal wastewaters. Average (median) removal performances for E2 and E1 were relatively high at 97 and 93% respectively; although the authors commented that E1 removal efficiency was more highly variable and ranged from a very effective 98% to situations where the final effluent concentrations exceeded that of the influent.

Gomez *et al.* (2007) noted that WSPs with long retention times had high removals of oestrogenicity (90–95%), whereas trickling filters—despite being effective at removing organic load (BOD)—were less effective in removing oestrogenicity (42%). A similar trend was also noted by Servos *et al.* (2005) who reported generally very high removal of oestrogenic compounds in WSPs with hydraulic retention times >150 days. This improved EDC removal performance in WSPs compared with trickling filters (TFs) could be due to differences in photolytic potential between the enclosed TFs and open-air WSPs. WSPs are well known for their UV disinfection capabilities, so it follows that they might also be effective photo-oxidisers of EDCs. Previous work by Coleman *et al.* (2000; 2004; 2005) has shown that the natural oestrogens E2, E1 and E3 and the synthetic oestrogen EE2 are completely degraded by UV light, with all oestrogenic activity being removed. Zhang and Zhou (2008) reported on photodegradation of EDCs and found that the degradation of E1 and E2 under natural sunlight followed pseudo-first-order kinetics, with a degradation rate constant of 0.01 h⁻¹. It has also been shown that the presence of humic substances and high oxygen

concentrations in WSPs can lead to increased photo-oxidative potential through the formation of reactive oxygen species (Davies-Colley *et al.*, 1999). Jürgens *et al.* (2002) also reported that oestrogenic EDCs can be degraded in anaerobic sediments; suggesting another possible mechanism for EDC removal in WSPs.

Table 3 Mean removal efficiencies of the various EDCs across the monitored WSPs (SD in parentheses)

EDC	Sample location			
	A2 – 1° Pond 1	A3 – 2° Pond 2	B2 – 1° Pond 1	B2 – 2° Pond 2
Androstenedione	100 [†]	100 [†]	93 (0) ^{***}	95 (2) ^{***}
Androsterone	100 [†]	100 [†]	100 [†]	100 [†]
Etiocholanolone	100 [†]	100 [†]	100 [†]	100 [†]
Testosterone	ND ^a	ND ^a	100 [†]	100 [†]
Dihydrotestosterone	ND ^b	ND ^b	ND ^c	ND ^c
Testosterone propionate	ND ^d	ND ^d	100 [†]	100 [†]
17β-Trenbolone	100 [†]	100 [†]	ND ^e	ND ^e
17β-oestradiol	ND ^f	ND ^f	ND ^f	ND ^f
17α-oestradiol	ND ^g	ND ^g	ND ^g	ND ^g
Oestrone	34 (10)	100 [†]	75 (5) ^{***}	82 (11) ^{***}
Oestriol	-58 (33)	36 (5)	26 (1) [*]	34 (13) ^{**}
17α-ethinyloestradiol	ND ^h	ND ^h	ND ^h	ND ^h

[†] 100% removals inferred since EDC concentrations were below analytical detection limits

Asterisks denote a significant removal (1-way ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

ND – not detected in raw wastewater samples (below analytical limits of detection)

^a Limit of detection 0.5 ng L⁻¹; ^b Limit of detection 25 ng L⁻¹; ^c Limit of detection 12 ng L⁻¹; ^d Limit of detection 0.5 ng L⁻¹;

^e Limit of detection 1.0 ng L⁻¹; ^f Limit of detection 0.2 ng L⁻¹; ^g Limit of detection 1.0 ng L⁻¹; ^h Limit of detection 0.2 ng L⁻¹

It is important to note that because samples for this research were collected during the Australian winter (June), the measured removal performance of both WSP systems may represent the lower end of possible treatment efficiency (due to ambient temperatures being unfavourable for optimal EDC biodegradation and levels of sunlight also being at their annual minimum, thereby reducing the likely extent of UV photolysis). Removal rates for E3 in both ponds were significantly lower than for E1 (Table 3). This may be due to the fact that E3 does not absorb UV light as well as the other oestrogens. Previous research by Coleman *et al.* (2005), for example, has shown that E3 degrades at a much slower rate under UV light compared to other oestrogens. Williams *et al.* (2007), following a survey of a number of WWTPs across three states, reported that Australian WWTPs are generally capable of removing in excess of 92% of the oestrogenic activity of influent wastewater. Although Williams *et al.* (2007) did report highly variable removal efficiencies for steroidal oestrogens in WWTPs incorporating WSPs as a treatment method (0.9–24% for E1; 47–54% for E2; 25–72% for EE2), the overall effectiveness of WSPs for removing oestrogenic activity (expressed as E2 equivalents) was quite high (92–99%) due to the variable oestrogenic potencies of E1, E2 and EE2. Coleman *et al.* (2008a, 2008b) investigated several WWTPs in New South Wales, Australia for removal of oestrogenic activity and reported removal rates of up to 99% for AS processes and 87% for a membrane bioreactor process.

Although the presence of androgens in wastewater has been known for some time (Shore *et al.*, 1993), information regarding the effectiveness of various treatment processes on attenuating the androgenic activity of effluents is, alongside that of oestrogens, comparatively limited (Kirk *et al.*, 2002; Leusch *et al.*, 2006; Tan *et al.*, 2007; Coleman *et al.*, 2008a, 2008b). The lack of information on fate and levels of androgens through WWTPs is especially so for WSP treatment, such that this paper constitutes the first known report of WSP treatment efficacy for a number of steroid androgens. de Mes *et al.* (2006) stated that anaerobic conditions during wastewater treatment generally result in longer half-lives for oestrogenic compounds than do aerobic conditions. This was also found to be the case for E2 degradation by Lee and Liu (2004), whereby E2 was more persistent during anaerobic degradation than under aerobic conditions. This could be a possible advantage for facultative WSPs. Since oestrogenic compounds (E1, E2 and EE2) are known to effectively

adsorb to organic fractions during AS treatment (Andersen *et al.*, 2003; de Mes *et al.*, 2005), WSPs could serve as sedimentation basins for removal of suspended solids–EDC complexes given their recognised efficiency for physical sedimentation and solids separation.

The results of multiple studies show that adsorption onto suspended solids (organic and colloidal), aerobic and anaerobic biodegradation, abiotic chemical degradation (e.g., hydrolysis), and volatilisation are the primary removal mechanisms for EDCs during wastewater treatment processes (de Mes *et al.*, 2005). Holthaus *et al.* (2002) reported good capacities for adsorption of steroid oestrogens (E2 and EE2) to suspended riverine sediment fractions. Similarly, Williams *et al.* (2007) also reported higher sorption coefficients for both E2 and EE2 adsorbing to sediment than for E1 and also found that sorption coefficients increased with increasing organic carbon content of the solid phases. Williams *et al.* (2007), through performing experiments under both biotic and abiotic conditions, demonstrated the importance of biological degradation processes for EDC removal. Given that WSPs (facultative and maturation ponds) can support a diverse range of biological interactions between resident bacteria, algae, protists and metazoans (Hussainy, 1979; Cauchie *et al.*, 2000), and considering that these biotic communities have long been recognised as primary facilitators of the overall ‘waste stabilisation’ process, it is likely that these organisms play a measurable role in the fate of EDCs within the pond environment. So far, however, there has been no research effort investigating the contribution of these communities to EDC removal in WSPs. More research is therefore required in order to elucidate the contributions these organisms make to EDC dynamics in WSPs.

Conclusions

E1 and E3 were the main component of oestrogenic steroids in analysed wastewater samples. All seven androgens were detected at some point in the withdrawn samples except DHT; an observation in agreement with previous studies. Results showed that WSPs were effective for the removal of a number of androgenic steroids (93–100% removal); however, WSP treatment was observed to be less effective for removing steroid oestrogens—particularly E3. The poor removal of E3 was attributed to photolysis being a primary mechanism for its removal in ponds and the fact that E3 is less susceptible to degradation by UV light. The few studies that have assessed EDC dynamics in WSPs have provided only limited insights into the mechanisms behind effective EDC removal in these environments, and as such, removal processes for EDCs in WSPs remain poorly understood. Based on research into other wastewater treatment processes, however, potential removal mechanisms for EDCs in WSP environments could include: particulate adsorption and sedimentation; photo-oxidation (both direct UV photolysis and indirect chemical oxidation through DOM-derived reactive oxygen species) and metabolic biological oxidation (aerobic and anaerobic). Based on previous findings, it is likely that sorption of EDCs to both inorganic and organic suspended solids may be an important factor governing the fate of these compounds in WSPs. It has also been suggested that biomass sorption may represent another pathway for EDC removal in WSPs; however, the direct contribution of phytoplankton and other such pond biota toward EDC removal is unclear. Further research is needed to assess the importance of biomass adsorption for EDC removal in WSPs, particularly given that pond environments can at times support high plankton biomass densities. It is suggested that additional research effort is required to determine both the mechanisms responsible for EDC removal, as well as the temporal dynamics (seasonality) of EDC treatment efficiency in WSPs.

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