Fate of oestrogens during anaerobic blackwater treatment with micro-aerobic post-treatment

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Abstract The fate of oestrone (E1), 17β-oestradiol (E2) and 17α-ethynyloestradiol (EE2) was investigated in a concentrated blackwater treatment system consisting of an UASB septic tank, with micro-aerobic post-treatment. In UASB septic tank effluent a (natural) total concentration of 4.02 mg/L E1 and 18.69 mg/L E2, comprising the sum of conjugated (>70% for E1 and >80% for E2) and unconjugated forms, was measured. During post-treatment the unconjugated oestrogens were removed to below 1 mg/L. A percentage of 77% of the measured unconjugated E1 and 82% of E2 was associated with particles >1.2 μm in the final effluent implying high sorption affinity of both compounds. When spiking the UASB septic tank effluent with E1, E2, EE2 and the sulphate conjugate of E2, removal in the micro-aerobic post-treatment was 99% for both E2 and EE2 and 83% for E1. The lower removal value for E1 was a result of (slow) deconjugation during the treatment, and in the final effluent still 40% of E1 and 99% of E2 was present in conjugated form. The latter was the result of incomplete deconjugation of the spiked E2(3S) in the post-treatment system.

Keywords Blackwater; micro-aerobic post-treatment; oestrogens; source separation; UASB septic tank

Introduction

Much research has indicated the significant contribution of two natural hormones oestrone (E1) and 17α-oestradiol (E2) and the synthetic hormone 17α-ethynyloestradiol (EE2) to the oestrogenic character of domestic wastewater influents and effluents (Onda et al., 2003). Humans excrete oestrogens mainly in urine as glucuronide or sulphate conjugates, to increase their solubility in water (Williams and Stancel, 1996). Although they do not exhibit any oestrogenic potency in this form, microbial enzymes can cleave these conjugates back to their original active form. Sulphate conjugates are more stable than glucuronide conjugates (D’Ascenzo et al., 2003).

Anaerobic biodegradation of oestrogens is very slow or does not occur (de Mes et al., 2007a, b) and up to 60% is associated with sludge (Kunst et al., 2002). All three compounds can potentially be removed under aerobic conditions (Ternes et al., 1999a; Layton et al., 2000).

Current research investigated the fate of E1, E2 and EE2 during anaerobic pre-treatment with micro-aerobic post-treatment of concentrated blackwater (Figure 1). The blackwater-stream is more concentrated than domestic wastewater or sewage, enabling implementation of anaerobic digestion for energy recovery. Since oestrogens are excreted with urine and faeces, substantially higher oestrogen concentrations are expected in blackwater compared with conventional sewage. The anaerobic pre-treatment was employed in an UASB septic tank, which was demonstrated to be a suitable reactor configuration for the treatment of concentrated blackwater (Kujawa-Roeleveld et al., 2005). The downflow hanging sponge (DHS) (Tandukar et al., 2005) reactor was shown to be efficient in the removal of remaining COD and satisfactory for nitrogen removal from...
anaerobically treated domestic sewage. Also, long employed sludge retention times may have a positive influence on the removal of oestrogens (Clara et al., 2005).

Materials and methods

Research into the behaviour of oestrogens during the different treatment steps consisted of two parts: (1) measurements of “naturally” occurring E1, E2 and EE2 during anaerobic treatment and micro-aerobic post-treatment of concentrated blackwater; and (2) removal of E1, E2 and EE2 during micro-aerobic post-treatment with the oestrogens E1, E2, EE2 and sulphate conjugate of E2(3S)-spiked UASB septic tank effluent. Spiking was applied to get a better insight into the fate of all researched oestrogens; no naturally occurring EE2 was detected in the system during the first part of the research.

Pre-treatment in an UASB septic tank

The UASB septic tank was designed as a one-person equivalent and blackwater was collected by vacuum toilets. The used system is described in detail by Kujawa-Roeleveld et al. (2005). Prior to this research, it was operated for 339 days before the effluent was spiked with oestrogens. Measurements of natural occurring oestrogens started after 401 days of operation.

Post-treatment in a DHS reactor and sand filter

Two cylindrical Plexiglas DHS reactors contained three sponge sections each of 10 sheets of reticulated polyurethane 10–20-mm thick foam-sponges (Recticel, Buren, the Netherlands), with the same diameter as the DHS, a specific surface area of 500 m²/m³, a density of 19–22 kg/m³, 9–36 pores per cm² and a pore size of 2.5 mm (Elmitwalli, 2000). The distance between the sponge sections was 100 mm and the total height of the DHS was 1 m with an inner diameter of 150 mm. Openings (d = 50 mm) 2 cm above the bottom of the reactor ensured inflow of air. Recirculation (21 times the influent flow rate) was applied to guarantee a sufficient hydraulic load over the sponges.

The sand filter, with a surface of 0.8 m², contained 25 kg of gravel at the bottom and 25 kg of sand, divided into three layers. The first layer contained sand particles >2 mm, followed by a layer of 0.5–1 mm and again >2 mm at the top. The post-treatment unit was in operation for 234 days prior to the oestrogen experiment with spiked UASB septic
tank effluent being started. After 296 days of operation, measurements in unspiked wastewater commenced.

Measurements in the treatment system with non-spiked blackwater

The naturally occurring oestrogen concentrations in UASB septic tank effluent (Ia) were determined twice, during the non-spiked measurements and additionally prior to the spiking experiment, where the conjugated amounts were also measured (according to the methodology described in the next section).

E1, E2 and EE2 measurements commenced in semi-composite samples composed of seven grab samples (200 mL) taken every other day over a period of two weeks, at sampling points Ia, IV and V (Figure 1). Two samples of 120 mL from the composite sample, referred to as samples A and B, were taken for analysis, concentrated 400 and 1,200 times, respectively. Samples were filtered over a glass fibre filter (GF/C, Whatman), to prevent clogging of the SPE disks (SDB-X, Varian, the Netherlands). All filters were extracted with acetone:methanol (vv 1:1), and followed the same procedure as the filtrates. After SPE, an extra silica gel clean up was applied (Ternes et al., 1999b).

In order to increase the volatility, completely evaporated samples were derivatised for 1 hour at 60°C with 25 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MFSTFA; Sigma), containing 4 mg 1,4-dithioerythritol (Merck) and 2 mg ammonium iodide per mL. After derivatisation the fluid was evaporated under a gentle stream of N2-gas and reconstituted in petroleum ether.

Volumes of 2 μL of the derivatised extracts or standard solutions were injected in a GC-MS Interscience system (Breda, the Netherlands) consisting of a Trace GC 2000 gas chromatograph equipped with a PTV injector operated in splitless mode, an AS 2000 sampler and a Polaris Q ion-trap mass spectrometer (Thermofinnigan, Breda, the Netherlands). Compounds were separated on a 50 m × 0.25 mm I.D. low bleed-MS column coated with a 0.25 nm film of CDP-Sil 8 CB (95% dimethyl–5% phenyl polysiloxane; Varian Chrompack, the Netherlands). Helium was used as carrier gas at a constant flow of 1 mL/min. The injector, ion source and mass transfer line temperature were 250°C, 250°C and 300°C, respectively. The temperature programme was (Noppe 2006): initial temperature 30°C, first ramp with 30°C/minute to 130°C, second ramp with 2°C/minute to 268°C, on hold for 10 minutes, then the last ramp with 20°C/minute to 300°C and hold for 5 minutes to clean out the column. Data processing was performed using Xcalibur 1.4 software using the molecular masses from Table 1 (Thermofinnigan, Interscience).

Experiment with spiked anaerobic pre-treated blackwater

UASB septic tank effluent was daily collected in an 8-L Erlenmeyer flask, freshly spiked with oestrogens (E1, 710 μg/L; E2, 1,930 μg/L; EE2, 2,170 μg/L; and E2(3S), 486 μg/L), placed on a magnetic stirrer (300 rpm, Heidolph MR3001K) and continuously pumped with a peristaltic pump to the DHS plant. Prior to sampling, the plant was fed with spiked wastewater for 3 days. Semi-composite samples consisted of eight samples, collected

**Table 1** General conditions during GC-MS analysis and quantification masses as determined in derivatised standards

<table>
<thead>
<tr>
<th>Name compound</th>
<th>Retention time (min)</th>
<th>MW</th>
<th>MW derivates</th>
<th>m/z used for quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrone (E1)</td>
<td>28.80</td>
<td>270.4</td>
<td>414</td>
<td>218, 257, 342, 414</td>
</tr>
<tr>
<td>17β-oestradiol (E2)</td>
<td>27.30</td>
<td>288.4</td>
<td>416</td>
<td>129, 285, 326, 401, 416</td>
</tr>
<tr>
<td>17α-ethylnoestradiol (EE2)</td>
<td>30.90</td>
<td>296.4</td>
<td>440</td>
<td>285, 300, 425, 440</td>
</tr>
</tbody>
</table>

MW, molecular weight
over the course of a day with the last one taken in the morning prior to refreshing the influent. The samples were collected during 3 days at sampling points Ib, II, III and V (Figure 1). Daily, 50 mL of semi-composite sample was freeze-dried, extracted with acetone:methanol (vv 1:1, Acros) and concentrated by solid phase extraction (SPE) with C18 discs (Varian). After complete evaporation of the extract under a gentle stream of nitrogen gas the sample was reconstituted in 1 mL methanol and measured on an HPLC with a C18 column of 2 × 10 cm (d = 5 mm) and a mobile phase of acetonitrile (65%) with UV (200 nm) and fluorescence (extinction, 230 nm; emission, 310 nm; cut-off filter, 305 nm). The method is extensively described in de Mes et al. (2006). Additionally, an enzyme solution (Helix promatia; Sigma) with glucuronidase and sulphatase was added to another 50 mL of the semi-composite samples according to Huang and Sedlak (2001) to determine the fraction of conjugated oestrogen forms.

The pH, redox potential, O2 concentration, temperature were monitored. Volatile fatty acids (VFA), total, particulate, colloidal and soluble COD (CODtot, CODSS, CODcol and CODsol) were determined according to Elmitwalli (2000). Ammonium (NH4-N), nitrate (NO3-N), nitrite (NO2-N) were measured according to ISO11773 and 13395 methods.

Results

Pilot-plant performance

The process parameters for the operation of the UASB septic tank are summarised in Table 2. Stability of the system was confirmed by low effluent VFA concentrations over the whole operational period of 339 days. Removal efficiencies obtained for the UASB septic tank were similar to those reported by Kujawa-Roeleveld et al. (2005). Recorded temperatures were, on average, 25°C in the UASB septic tank and 22°C in the micro-aerobic post-treatment. The redox potential was −356 ± 57 mV in the UASB effluent and increased to 62 ± 62 mV in the sand filter effluent. DO levels ranged from 2.79 ± 1.15 in the DHS1 effluent to 3.05 ± 1.19 in the sand filter effluent.

After the first DHS, the particulate fraction of COD increased (Figure 2a) while the colloidal fraction decreased. Particulates produced in the first DHS were for the most part removed in the second DHS and almost completely retained in the sand filter. Only a small amount of colloidal COD was still present in the final effluent of the system. The concentration of soluble COD did not significantly change over the whole treatment sequence and the observed brown colour of the effluent is among other things caused by the presence of humic acids produced during biological treatment of blackwater.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD influent (g/L)*</td>
<td>29.2 ± 7.7</td>
</tr>
<tr>
<td>CODtot effluent (mg/L)</td>
<td>2,591 ± 520</td>
</tr>
<tr>
<td>Flow rate (L/d)</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>Organic loading rate (g/L/d)</td>
<td>0.66</td>
</tr>
<tr>
<td>HRT (d)**</td>
<td>49</td>
</tr>
<tr>
<td>SRT (d)**</td>
<td>164 ± 59</td>
</tr>
<tr>
<td>Average TS in UASB (g/L)</td>
<td>2,413 ± 161</td>
</tr>
<tr>
<td>Average VS in UASB (g/L)</td>
<td>1,457 ± 173</td>
</tr>
<tr>
<td>VFA effluent (mg/L)</td>
<td>155 ± 97</td>
</tr>
<tr>
<td>%COD removal</td>
<td>91</td>
</tr>
</tbody>
</table>

*Calculated on the basis of recorded contributions: 1 × faeces (200 mL or 138 g), 78.3 g COD; 1 × urine (200 mL), 2.56 g COD; 1 × flushing, 0.5 L; and toilet paper is 13.5 g COD/person/d, measured influent values were 31.4 ± 3.4 g/L (n = 3)

**Calculation of total amount sludge in UASB (g VS) divided by the amount discharged (g VS/d) plus the amount discharged with the effluent (g VS)
The blackwater produced in the current research is about 30 times more concentrated compared with the combined municipal wastewater in dry weather conditions. The effluent soluble COD of treated blackwater (1,325 $\pm$ 241 mg/L) corresponds to the effluent values for activated sludge systems treating municipal wastewater, which is typically in the range of 30–50 mg/L under dry weather conditions, multiplied with factor 30 (Roeleveld and Loosdrecht, 2002).

A large fraction of ammonium (60%) was nitrified (Figure 2b). Based on ammonium, nitrate and nitrite measurements over the DHS reactors a total N-removal by denitrification of 486 $\pm$ 48 mg N/L is calculated.

**Measurements in the treatment sequence with non-spiked blackwater**

The detected oestrogen concentrations in the pilot plant are presented in Figure 3. The concentration of oestrogens in the UASB septic tank effluent (Ia) were 1.23 $\mu$g/L for E1 without enzyme addition and 4.02 $\mu$g/L with the enzymatic deconjugation step and for E2 respectively 3.81 $\mu$g/L and 18.69 $\mu$g/L (Figure 3a). The fractions of conjugated E1 and E2 (70 and 80%, respectively) are therefore significant. The sum of total E1 and E2...
was 22.71 µg/L; in the range of the expected concentrations for total oestrogens in concentrated blackwater, i.e., 21 and 42 µg/L when a low toilet flushing volume of respectively 1 and 0.5 L is applied (de Mes and Zeeman, 2003). This indicates that no degradation of oestrogens took place in the UASB septic tank. This was also found in anaerobic long-term batch experiments with anaerobic sludges of various origins (data not shown). In the UASB septic tank effluent at least 70% of E1 and 80% of E2 were present in conjugated form, indicating an incomplete deconjugation under anaerobic conditions.

A significant fraction of unconjugated E1 and E2 in the effluents of the UASB septic tank, settler and a sand filter was associated with the colloidal and suspended material (particles < 1.2 µm; Figure 3b). No EE2 was detected in any of the samples. The low observed overall removal of approximately 50% for both E1 and E2 during the post-treatment could be caused by simultaneously occurring deconjugation of conjugated oestrogens. In this experiment no conjugated oestrogens were determined (no extra enzyme addition step was applied), but more than 70% for E1 and 80% for E2 of the naturally available E1 and E2 was found to be conjugated in the UASB septic tank effluent as mentioned earlier. Assuming similar conjugation percentages and complete deconjugation during treatment, removal percentages would be 85 and 81% for E1 and E2, respectively.

Experiment with spiked anaerobic pre-treated blackwater

The measured concentrations of E2 and EE2 in the influent tank to DHS1 (Ib) after spiking with the oestrogens mentioned including a conjugated E2 were generally significantly lower as determined based on the known spiked amounts (Figure 4a). Constant stirring in

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**Figure 4** (a) Spiked concentrations and measured average oestrogen concentrations in the influent tank of the micro-aerobic post-treatment (Ib). Measurements of: (b) E1 and E1 enz (enz means after enzymatic deconjugation); (c) E2 and E2 enz; and (d) EE2 and EE2 enz in the pilot plant fed with spiked influent
the influent storage tank enhanced biodegradation by the introduction of oxygen. A depletion of 99 and 38% for E2 and EE2 respectively was measured. The mean E1 concentration was 5% higher, probably as a result of E2 conversion. However, the depleted amount of E2 was substantially higher than the increased amount of E1. E1 was most probably also degraded in the influent storage tank. Also, increased values of E1 and E2 in the influent are expected to be caused by a cleavage of E2(3S) under (micro-)

The overall removal throughout the whole treatment plant was highest for E2 and EE2 (Table 3). The lower removal percentage for E1 can be explained by the slow deconjugation of the added E2(3S), for which a half-life of 2.5 days was reported in wastewater under continuous agitation at 20°C (D’Ascenzo et al., 2003). After the slow deconjugation into E2 during the treatment, E2 was rapidly converted into E1 under aerobic conditions, before further oxidation occurred (D’Ascenzo et al., 2003; de Mes et al., in preparation – a). These findings indicate that the presence of conjugates influences overall observed removal of oestrogens. At present, little attention is paid to the deconjugation rate under different redox conditions, and this deserves more attention in future research.

As follows from Figures 4b, c and d, the addition of enzymes to the 50 mL samples before analysis, for deconjugation of conjugated oestrogens, did not always result in higher total oestrogen concentrations. The enzyme solution was added to unfiltered samples, and possibly degradation during its 20 hours reaction time at the recommended temperature of 37°C occurred. In the final effluent of the sand filter, the conjugates’ contribution was at least 40 and 99% for E1 and E2, respectively (Figures 4b, c and d). The high value for E2 indicates an incomplete deconjugation of the stable E2(3S) conjugate over the post-treatment system.

**Conclusions**

- E1 and E2 are the main oestrogens detected in anaerobic pre-treated blackwater, with E2 being dominant:
  - the sum of measured E1 (4.02 μg/L) and E2 (18.69 μg/L), conjugated and unconjugated, concentrations from the UASB septic tank effluent were within the range of the calculated values for concentrated blackwater;
  - no anaerobic biodegradation of oestrogens took place.
- E2 and EE2 were removed to a high degree (>99%) in spiked UASB septic tank effluent during the micro-aerobic post-treatment consisting of two DHS reactors and a sand filter in series of E1 83% was removed.
- The unconjugated naturally occurring amounts of E1 and E2 were respectively 2.80 ± 1.66 μg/L and 1.00 ± 0.28 μg/L after anaerobic treatment and 1.37 ± 1.45 μg/L for E1 and 0.65 ± 0.78 μg/L in the final effluent of the micro-aerobic system.

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E2</th>
<th>EE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHS1 (II)</td>
<td>89</td>
<td>44</td>
<td>98</td>
</tr>
<tr>
<td>DHS2 (III)</td>
<td>−90</td>
<td>51</td>
<td>73</td>
</tr>
<tr>
<td>SF (V)</td>
<td>22</td>
<td>97</td>
<td>86</td>
</tr>
<tr>
<td>Overall removal</td>
<td>83.1</td>
<td>99.2</td>
<td>99.9</td>
</tr>
</tbody>
</table>

Table 3 Removal of estrogens (in %) over the different treatment steps and overall removal over the whole post-treatment pilot plant

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• The largest part of the naturally occurring E1 (>70%) and E2 (>80%) in UASB septic tank effluent is present in conjugated form; in the final effluent after micro-aerobic post-treatment of spiked UASB effluent, respectively 40% of E1 and 99% of E2 were present in conjugated form.

• In the UASB septic tank effluent 53% of naturally measured unconjugated E1 and 25% of E2 was associated with particles larger than 1.2 \( \mu \text{m} \). In the final effluent of the sand filter, this was 77% of unconjugated E1 and 82% of E2.

Acknowledgements
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