Oestrogen removal from biological pretreated wastewater within decentralised sanitation and re-use concepts

T.Z.D. de Mes*, A.M. Urmenyi**, A.A. Poot***, M. Wessling**, M.H.V. Mulder** and G. Zeeman*

*Wageningen University, Department of Agrotechnology and Food Sciences, Sub-department of Environmental Technology, P.O. Box 8129, 6700 EV Wageningen, The Netherlands (E-mail: *Titia.demes@wur.nl*)

**University of Twente, Sustainable Technology / Membrane Technology Group, Dept. TNW, P.O. Box 217, 7500 AE, Enschede, The Netherlands (E-mail: *a.m.urmenyi@utwente.nl*)

***University of Twente, Polymer Chemistry and Biomaterials, Dept. TNW, P.O. Box 217, 7500 AE, Enschede, The Netherlands

Abstract Two parallel researches were performed; one focused on the fate of oestrogens in the biological treatment systems within decentralised sanitation and re-use concepts (DESAR), the second related to the development of a suitable specific removal method. A new affinity membrane was developed using antibodies as specific binding sites for hormone removal. It was found that, especially in anaerobic treatment, the core technology in DESAR, the removal is insufficient and therefore an additional separation method is required. The affinity membrane with antibodies was found to be a suitable additional method, though in the current system it only removes one selected compound. Future research will focus on making this method more feasible in practise.

Keywords Adsorption; alternative sanitation systems; degradation; DeSaR; membrane application; oestrogens

Introduction

Oestrone (E1), 17β-oestradiol (E2) and 17α-ethynyloestradiol (EE2) (Figure 1) are selected for research, as they are the main contributors to the oestrogenic character of domestic wastewater (Desbrow *et al.*, 1998; Routledge *et al.*, 1998; Körner *et al.*, 2001; Onda *et al.*, 2003). These oestrogens are not always fully removed in current sewage treatment plants (STPs), leading to feminisation of male fish, amongst others when discharged into water bodies (Purdom *et al.*, 1994; Hansen *et al.*, 1998; Jones *et al.*, 2000; Vethaak *et al.*, 2002). Both E1 and E2 are natural oestrogens, excreted mainly in urine by males as well as females. EE2 is the main oestrogen used in contraceptives, the most prescribed drug worldwide (Williams and Stancel, 1996).

The present research focusses on decentralised sanitation and re-use (DESAR) concepts, based on separation of wastewater streams at the source (Zeeman and Lettinga, 2001). Within these concepts, blackwater (faeces + urine) is separately collected from greywater, resulting in a more concentrated wastestream, also containing the main fraction of oestrogens. Another possibility within DESAR is separate collection of urine. In the case of urine separation, a value of 170 μg/L total oestrogens in pure urine is estimated and for blackwater a vacuum toilet system of 43 μg/L is used while in a combined system the maximum estimated value is 1 μg/L (calculation based on Blok and Wösten, 2000; Johnson and Williams, 2004). Re-use of the nutrient rich wastestreams demands an adequate treatment method guaranteeing
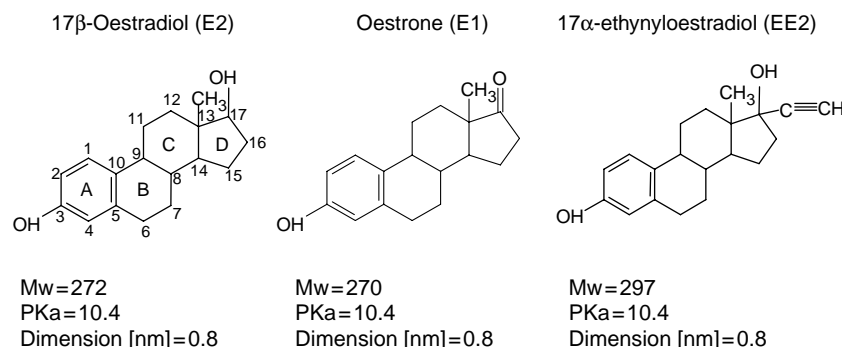
Figure 1 Chemical structures physical–chemical properties of E2, E1 and EE2

"risk-free" quality. Therefore, an understanding of the behaviour of oestrogens in DESAR applied treatment systems is required. Besides all the known advantages of DESAR, such as creating opportunities for more sustainable collection, transportation and treatment of domestic waste(water) (Zeeman and Lettinga, 2001), it is also an effective permanent measure to prevent oestrogens to enter the environment.

Anaerobic digestion is considered as the core technology (Kujawa-Roeleveld *et al.*, 2005) for the treatment of blackwater. In general, urine is not subjected to biological processes, but either used directly in agriculture (Simons and Clemens, 2003) or subjected to physical chemical processes for nutrient recovery (Wilsenach and Loosdrecht, 2004). In both situations, additional removal of oestrogens is needed. The present research evaluates possible (post)treatment processes for the removal of selected oestrogens. On the other hand, a more specific way of removing these compounds using a membrane with immobilised antibodies is researched as a possible treatment step. This membrane could either be applied after the biological treatment steps of black water or for the treatment of urine. As the latter contains the highest concentration, artificial urine with added E2 was studied to test the concept.

Behaviour of oestrogens in treatment systems

Natural oestrogens are metabolised in the liver, where they are made more water-soluble for excretion in the urine, by forming conjugates with either sulphate or glucuronide (Williams and Stancel, 1996). These conjugates are biologically inactive (Ingerslev and Halling-Sorensen, 2003), but bacteria such as *E. coli*, which produce the enzyme β-glucuronidase, can hydrolyse glucuronide conjugates back to their original form (Ternes *et al.*, 1999; Legler, 2001). The hydrolysing of glucuronide conjugates also takes place in sewers and during sewage treatment. Only a very small amount of natural hormones is excreted in faeces, contrary to the synthetic EE2 of which 30% is excreted in faeces and 22–50% in urine (Reed *et al.*, 1972). During biological treatment, oestrogens are removed by sorption on sludge and biodegradation.

The biodegradation follows first order reaction kinetics (Equation (1)) in which C_t is the concentration of the oestrogen at time t, C_0 is the initial concentration and k is the degradation constant. From this equation, the half-life ($t_{1/2}$) can be calculated according to Equation (2) and the adsorption can be described according to the Freundlich isotherm (Equation (3)), in which C_s is the concentration in the solid phase, C_w is the concentration in the liquid phase and K_f and $1/n$ are the sorption coefficient and constant, respectively:

142

$$C_t = C_0 e^{-kt} \tag{1}$$

$$t_{1/2} = \frac{\ln 2}{k} \tag{2}$$

$$C_s = K_f C_w^{1/n} \tag{3}$$

Since the half-life of oestrogens is quite long, especially under anaerobic conditions, a substantial amount will be present in the effluent and should be removed. Under aerobic/anoxic conditions, E2 will be readily oxidised to E1 in the presence of activated sludge. So, in the first instance, only the fate of E1 and EE2 has been investigated in the biological part of the experiment. Theoretically, under anaerobic conditions, E1 can be reduced to E2. Since the core technology of DESAR is anaerobic digestion, we investigated the possibility of using antibodies as highly specific affinity binders to remove and/or separate oestrogenic compounds, with E2 as the model compound, from water solutions.

Methods

Experimental set-up

Biological degradation and adsorption of oestrone and 17α-ethynyloestradiol. The experiments were performed in 1-L serum bottles covered with aluminium foil to prevent any photocatalytic degradation. The set-up is shown in Figure 2. In the experiments with E1 and activated sludge under anaerobic, anoxic and aerobic conditions, the serum bottles were not capped. In the experiment with EE2 and activated sludge the anaerobic and anoxic conditions were maintained by flushing with nitrogen after sample taking and the bottles were capped. Pressure build up in the anaerobic and aerobic batch is prevented by a hollow needle. Anoxic conditions were created by adding a stock solution of $NaNO_3$ (Merck, the Netherlands) in a concentration of 0.6 mg N/g VSS/h. The environmental conditions, pH, redoxpotential, O_2 concentration and temperature were monitored over the course of the test period. The volatile suspended solids (VSS) content was measured at the beginning and end of the experiment, according to standard method 2540E (APHA/AWWA, 1998). The sludge was collected from the activated sludge oxidation ditch of the STP of Bennekom, the Netherlands and was sieved and incubated by aeration overnight prior to use.

First order reaction constants were calculated for E1 and EE2 in activated sludge under anaerobic, anoxic and aerobic conditions, using the least square method. In the EE2
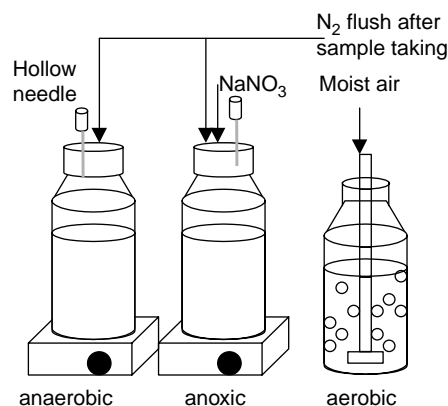
Figure 2 Set-up of the degradation experiments for E1 and EE2 with activated sludge under anaerobic, anoxic and aerobic conditions

143

experiment it was also researched if a second load of EE2 or the addition of substrate, by adding acetic acid (NaAc trihydrate from Acros, the Netherlands and applied in a concentration of 950 mg COD/L), could enhance the degradation constants. In both experiments the adsorption constants were determined according to the Freundlich isotherm using the aerobic batch.

Removal of 17β-oestradiol by an affinity membrane

Affinity membrane preparation. EVAL membranes were prepared by an immersion precipitation technique using dimethyl sulphoxide (DMSO) as solvent and 1-octanol (Aldrich) as additive (Avramescu *et al.*, 2002). EVAL membranes were used as a support material for the binding of anti-17β-estradiol antibodies (MP Biomedicals). The surface modification of the membranes followed the reaction scheme in Figure 3. The preparation and characterization of the affinity membrane is described in more detail in Urmenyi *et al.* (2005).

First, the hydroxyl groups were activated with glutaraldehyde. The obtained EVAL-GDA membranes were further reacted with bis hydrazide polyethylene glycol. The prepared EVAL-Hz membranes were characterised qualitatively by X-ray photoelectron spectroscopy (XPS) and quantitatively by hydrazide group determination. The free aldehyde groups that might be still present on the membrane surface were blocked by reaction with a 0.2 M ethanolamine solution. Afterwards, the membranes were rinsed with distilled water and PBS buffer for storage purposes. Before using them in the immobilization reaction, the membranes were washed several times with distilled water and acetate buffer. The antibody was oxidised at room temperature and the final concentration of oxidised antibody was found to be approximately 2 mg/mL acetate buffer. The oxidised antibody (Ab) was immobilised onto the membrane at pH = 5.2 acetate buffer solution at 4 °C. After 72 h, the excess of oxidised antibody was removed; the membranes were rinsed with PBS buffer and could be stored for several weeks at 4 °C. The quantity of immobilised antibody was determined by UV-VIS adsorption as a difference in concentration from the initial solution and after the reaction was performed. The concentration of the antibody can be estimated by UV-VIS at $\lambda = 280$ nm using a molar extinction of $A_{280} = 1.4$ for 1 mg/mL in a cuvet of 1 cm.

Adsorption isotherms. A well-defined quantity of membranes was kept for 24 h at a constant temperature (25 °C) with different concentrations of E2 solutions dissolved in a salt mixture similar to urine (22.59 g/L NH_4HCO_3; 0.68 g/L Na_2HPO_4; 0.14 g/L $CaCl_2$; 0.84 g/L K_2SO_4; 4.68 g/L NaCl; 2.24 g/L KCl). The E2 concentration at equilibrium was determined using ELISA (MP-Biomedicals). The amount of solute adsorbed per unit membrane mass at the equilibrium state can be calculated from Equation (4), in which $E2_{ads}$ is the quantity of E2 adsorbed onto the membrane (mg/mg), C_0 is the initial concentration (ng/L), C_e is the equilibrium concentration (ng/L) and V_0 is the volume of
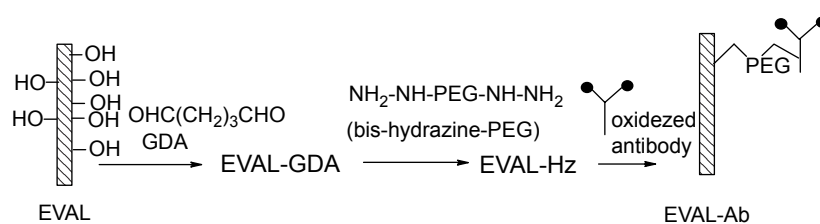

Figure 3 Reaction scheme for EVAL monoclonal antibody membrane preparation

solution used for the experiments:

$$E_{2ads} = \frac{V_0(C_0 - C_e)}{M_s} \tag{4}$$

Selectivity determination of the EVAL-Ab. A known quantity of membranes was kept for 24 h at a constant temperature (25 °C) with a solution of 1.5 µg/L E2 to which various quantities of stock of E1 solution in methanol were added to reach E1 concentrations of 1.5, 2, 2.5, 3 and 3.5 µg/L respectively, dissolved in a salt mixture similar to urine. The E1 and E2 concentrations at equilibrium were determined using ELISA. The selectivity of the membrane was calculated according to Equation (5) in which $[E1]_{ads}$ and $[E2]_{ads}$ are quantity of adsorbed E1 and E2, respectively (mg ads/mg membrane), and $[E1]_0$ and $[E2]_0$ are initial concentrations of E1 and E2, respectively [M]:

$$\alpha_{E2/E1} = \frac{[E2]_{ads}/[E1]_{ads}}{[E2]_0/[E1]_0} \tag{5}$$

Dynamic adsorption filtration performance. A stack of nine membranes was placed in a dead-end filtration cell and the dynamic adsorption capacity was evaluated at constant flow-rate by determining the E2 concentration in fractionated fixed volumes of permeate. The flow rate was 120 L/h/bar/m^2 membrane frontal area.

Membrane regeneration. The used membranes were soaked in 3 mL methanol to remove bind E2 from the membrane. After 20 min, the membranes were washed with demi water and were re-used in static adsorption experiments.

Analytical determination

To prevent any adsorption to labware, only bottles and centrifuge tubes of glass were used. Oestrone (CAS 63-16-7) and 17α-ethynyloestradiol (CAS 57-63-6) were obtained from Sigma-Aldrich, The Netherlands. A stock solution was prepared in methanol (all solvents are purchased HPLC grade at Acros, the Netherlands) and added to the serum bottles in a concentration of 5 mg/L. Samples (40 mL) were taken at set times and E1 and EE2 were determined in both liquid and solid phase. After centrifugation (10 min at 3,500 rpm, with a Labofuge 400 from Heraeus instruments), 30 mL of liquid phase was taken out the centrifuge tube for further clean up. The surplus liquid was removed from the centrifuge tube, and the solid phase was extracted four times with acetone (Ace): methanol (MEOH)(v:v, 1:1). Each time, the slurry was sonificated (transsonct 460/H from Elma) for 10 min and then centrifuged for 10 min at 3,500 rpm. The extract was collected in a clean sample bottle using Pasteur pipettes. Sample clean up was carried out by solid phase extraction (SPE) with C18 disks and used according to the manufacturers instructions (Varian, The Netherlands). The oestrogens were eluted from the C18 disks using 4 × 5 mL acetonitril. After complete evaporation, the sample was reconstituted with 1.5 mL methanol and measured on an HPLC with a C18 chromospere column of 2 × 10 cm with an external diameter of 6 mm and a C18 pre-column of 1 cm, external diameter 6 mm. The samples were placed in a Marathon sampler. The mobile phase consisted of acetonitrile (60%) and demineralised water (40%) at a flow rate of 0.4 mL/minute, pumped with a Gynotek high precision pump, model 480 with an Elite degassing system from Alltech. The compounds were detected with a programmable absorbance UV-detector; spectroflow 783 from Kratos Analytical (200 nm). Experiments with EE2 were also detected with a programmable fluorescence detector from Hewlett

Packard, HP1046A, which was operated in series with the UV-detector (extinction 230 nm; emission 310 nm). The sample volume was 10 μl and the system was flushed with 30 μl methanol between the different samples.

With regard to the affinity membrane experiments, E2 and E1 were analysed with the ELISA kit (MP-Biomedicals), according to Goda *et al.* (2000).

Results and discussion

Results of biological degradation of oestrone and 17α-ethynyloestradiol

The environmental conditions of the experiment are summarised in Table 1. The temperature in the aerobic batch is lower due to aeration. The degradation constant k and the half-life are assumed to be linear with the VSS and are standardised for 1 g VSS/L as shown in Table 2. The total concentrations in time are shown in Figure 4.

E1 is approximately ten times faster degraded under aerobic conditions compared to EE2. No degradation of EE2 was found under anaerobic and anoxic conditions, while E1 is degraded. However, in the EE2 experiments, closed bottles were used, while the E1 experiment was carried out in uncapped bottles. Therefore, it can not be excluded that trace amounts of oxygen were present in the anoxic and anaerobic batches.

A second load of EE2 to the aerobic sludge reduced the half-life to 4.5 d and the addition of substrate reduced the half-life further to 1.2 d. Also, with the addition of NaAc, the denitrifying sludge appeared able to convert EE2, leading to a half-life of 8.3 d in the anoxic batch. This indicates co-metabolism for EE2 under aerobic and anoxic conditions.

Published data on the conversion of E1 and EE2 tend to vary considerably. The trend in literature is the lower the initial oestrogen concentration, the higher the conversion rate. In batch tests with activated sludge and initial concentrations of 500 ng E1/L, the standardised k-value is 162 L/g SS/d (half-life 6 min) (Joss *et al.*, 2004). In the same research, the k-value for EE2 was 8 L/g SS/d (half-life 2 h), at an initial concentration of 100 ng EE2/L. In research where higher initial concentrations were applied, 20–25 mgL of both E1 and EE2, similar results were obtained as in this research; a k-value of 0.57 L/g SS/d for E1 (half-life 1.2 d) and no degradation for EE2 (Shi *et al.*, 2004). Applying an initial concentration of 1 mg/L gave a k-value of 3.9 L/g SS/d (half-life 4.3 h) (Ternes *et al.*, 1999), which is in between the previous mentioned ones. The cause of this phenomenon is unknown, but could be due an abiotic process resulting in removal of a fixed quantity of oestrogens. This fraction is larger when applying less oestrogen on a relatively large amount of sludge.

Limited information is available on the degradation of E1 and EE2 under anoxic and anaerobic conditions. Joss *et al.* (2004) found a k-value for E1 under anaerobic conditions of 10 ± L/g SS/d (half-life 2 h) and also a reduction of E1 to E2, which is significantly faster with a k-value of 52 ± L/g SS/d (half-life 20 min). They even found a degradation of EE2 using membrane bioreactor sludge under anaerobic conditions, resulting in a

Table 1 Environmental conditions in batches

Experiment		Oxygen (mg/L)	Temperature (°C)	Redox potential (mV)	pH	VSS_{begin} (g/L)	VSS_{end} (g/L)
E1	Anaerobic	n.a.	21.7–26.4	−160 to −212	6.98–7.13	2.30	1.90
	Anoxic		23.4–26.6	−124	7.48–8.10		1.76
	Aerobic	7.7	20.5–23.6	61 to 174	5.48–8.00		1.47
EE2	Anaerobic	n.a.	22.3–30.2	−127 to −356	5.97–6.51	1.99	1.67
	Anoxic		22.9–30.2	−102 to −266	6.87–7.92		1.47
	Aerobic	7.4–9.8	20.2–28.1	30 to 254	5.35–7.48		1.17

n.a. = not available.

Table 2 Calculated k-values for the batch experiments, including, first E1/EE2 load, second EE2 load and substrate addition

Experiment		First E1/EE2 load		Second EE2 load		Substrate addition	
		k-value (L/gVSS/d)	Half-life (d)	k-value (L/gVSS/d)	Half-life (d)	k-value (L/gVSS/d)	Half-life (d)
E1	Anaerobic	0.034	43	Not researched for E1			
	Anoxic	0.055	26				
	Aerobic	1.09	1.2				
EE2	Anaerobic	No degradation		No degradation		No degradation	
	Anoxic					0.08	8.3
	Aerobic	0.088	12.5	0.15	4.5	0.58	1.2

T.Z.D. de Mes *et al.*

k-value of 1.5 L/g SS/d (half-life 11 h). The explanation given by the authors for degradation was the presence of compounds that can act as an oxidator, such as Fe^{3+}.

Joss *et al.* (2004) report degradation of EE2 of 1.2 ± 0.3 L/gSS/d at an initial concentration of 100 ng/L under anoxic conditions. Kjølholt *et al.* (2004) found 0.17 ± 0.17 L/g SS/d at an initial concentration of 500 ng/L, but with substrate addition. Both also found a degradation of E1, 30 ± 10 L/g SS/d and 14 ± 5 L/g SS/d for Joss *et al.* (2004) and Kjølholt *et al.* (2004), respectively. The trend in both researches is the same, degradation is fastest under aerobic conditions, followed by anoxic, and is slowest under anaerobic conditions, though all values obtained are much higher compared to the results of the present research.

A personal communication with Dr. Joss elucidated that degradation constants for EE2 in the batches with and without (control) sludge hardly differ (respectively, $k = 1.2 \pm 0.3$ and $k = 1 \pm 0.5$). EE2 in the latter case might as well be completely persistent as was found in this research.

For both compounds the maximum fraction adsorbed to the sludge was approximately 60%, but in most cases it was around 50%. Parameters for the Freundlich isotherm give a K_f-value of 933 L/kg for EE2 and a 1/n of 0.76. Values for E1 are in the same range, 954 L/kg for the K_f-value and 0.75 for 1/n. The isotherm was prepared using the division of the hormone over the liquid and solid phase during the aerobic batch test, which is
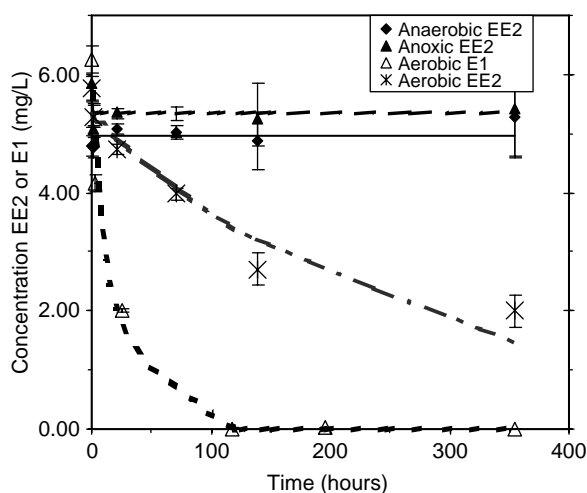

Figure 4 Concentrations of EE2 in time in the anaerobic, anoxic and aerobic batch, compared with the concentrations of E1 over time in the aerobic batch. The calculated values, obtained with the least-square method are shown as well.

147

carried out with active biomass and, therefore, the hormone is not in equilibrium. In earlier research it was shown that 90% of the equilibrium is reached in a few minutes (Lai *et al.*, 2000), which is significantly shorter than the half-life. Also, for the adsorption of EE2 to be as active as inactivated sludge, nearly the same K_f value of 480 L/kg was found (Clara *et al.*, 2004). Experiments conducted on the sorption of oestrogens to sediment resulted in lower adsorption coefficients: K_f of 54 L/kg for E1 and 52 kg/L for EE2 (Lai *et al.*, 2000). The latter is a logical result of the lower organic matter content in sediments compared to sludge. In other experiments conducted with activated sludge, a K_f of 900 ± 100 L/kg was calculated for E1 (Joss *et al.*, 2004), which is in line with the findings of our experiment.

Removal of 17β-oestradiol by an affinity membrane

The prepared EVAL membrane had the following characteristics: water permeability = 1,750 L/h/ bar/m^2, pore size = 0.278 μm, porosity = 80%, swelling degree = 8%, BET area = 9.7 m^2/g. The presence of the spacer was semi-quantitatively determined by XPS measurements. Hydrazine quantification yielded a surface concentration of $1.855 \pm 0.2 \times 10^{-10}$ mol/cm^2, concluding that the EVAL membrane is fully covered with a brush of spacer molecules. The oxidised antibody was attached to the membrane's surface via hydrazide groups. The antibody up-take calculated from the experimental UV-Vis data was approximately 4.67×10^{-12} mol/cm^2. The water permeability drops at 680 L/h/ bar/m^2 due to the presence of the antibody.

Adsorption isotherm experiments revealed that antibody EVAL membranes could adsorb up to 90×10^{-8} mg/mg membrane of E2 from synthetic urine solutions. The adsorbed quantity represents only 1% of the available sites.

An antibody can bind one or more ligands with a structure similar to the molecule which induces the immune response. This phenomenon, the so-called cross-reactivity of antibodies, could hamper or even obstruct the selective adsorption of E2. This is expected to happen as the oestrogenic effect in wastewater is caused by a mix of E1, E2 and EE2. E1 and E2 are both oestrogenic compounds with very similar chemical structures and physical–chemical properties. Note also that in Table 1 molecular weights differ only with two units. Taking into account all these similarities, we expect that the antibody will interact to a certain extent with both compounds (see Table 3).

When both compounds are present, the antibody is discriminating between the two, clearly preferring the E2. Even so, the overall concentration is also favouring a certain interaction between oestrone and the antibody. Upon increasing the concentration of oestrone, a competition between the two compounds takes place that reduces the quantity of adsorbed E2. We may expect that since the membrane is even able to discriminate between E2 and E1, it will indeed act as a specific binding material for E2 in quite a variety of solutions.

Dynamic adsorption filtration experiments. In practice, removal of compounds from complex mixtures is carried out in a dynamic non-equilibrium regime. The requirement is that the hormone concentration into the permeate should be close to zero. When the binding sites are occupied, the concentration of the hormone in the permeate is equal to the feed concentration.

Table 3 Selectivity data for 17β-oestradiol versus oestrone

$[E2]_0/[E1]_0$	1	0.75	0.6	0.5	0.43
$\alpha_{E2/E1}$	3.45	2.49	2.49	2.26	2.51

In order to fulfil the above-mentioned requirement, the hormone solution and the antibody-immobilised membrane need to be long enough in contact to allow local equilibrium. In consequence, in dynamic filtration operations, the removal of hormones depends on flow conditions. Using a stack of nine membranes, the flux of the hormone solution was $120 \, L/bar/h/m^2$. Considering the total adsorption capacity, as determined by adsorption isotherms, one can predict the amount of hormone adsorbed based on the assumption of complete hormone removal from the solution to the adsorptive interface. We calculate the adsorbed E2 onto membranes theoretically, considering that the membrane capacity determined from adsorption experiments is $90 \times 10^{-8} \, mg$ E2 per mg membrane. Theoretically, a volume of approximately $70 \, L$ waste water/m^2 membrane can be passed through the membrane before the concentration in the permeate (C_p) equals the concentration in the feeding solution (C_o). In practice, in the permeate, one can find an E2 concentration of approximately $30–40 \, ng/L$ and, in consequence, a larger volume is passed through the membrane before the E2 concentration in the permeate reaches such values that $C_p/C_o = 1$. The starting adsorption results of the antibody immobilised membrane and the re-used membrane are almost identical. However, in time a loss of activity (approximately 10%) was observed. We obtained 99% E2 removal from hormone solutions, close to the theoretical value.

Final discussion and conclusions

In conclusion, a maximum amount of 60% of oestrogens can be expected to be sorbed on sludge during treatment of blackwater in the anaerobic stage. In the anaerobic stage, E1 is reduced to E2 and no proven degradation of EE2 was found. Therefore, the amount of oestrogens in the effluent of the anaerobic stage is at least 40% of the incoming amount. Removal can be expected in a post-treatment step, although denitrifying conditions do not significantly enhance the degradation of E1 and EE2 in comparison to anaerobic conditions under unfed conditions. Under addition of substrate, the batch under anoxic conditions seems to be able to convert EE2, indicating cometabolism. Aerobic conditions are most optimal for the degradation of E1 and EE2 and might even be enhanced by using sludge under fed conditions and/or with a longer sludge retention time.

With respect to the membrane research, the main conclusion is that antibody modified EVAL membranes are indeed capable of removing natural hormone from artificial urine. Though, working with monoclonal anti-bodies made the membrane specific towards E2, but in practice it is desired to remove all three compounds in one step. Therefore, further research will focus on the use of a less specific type of antibody for the removal of E1, E2 and EE2 present both in the influent and the effluent.

References

APHA/AWWA (1998). *Standard Methods for the Examination of Water and Wastewater*, Washington, D.C. USA.

Avramescu, M.E., Sager, W.F.C., Mulder, M.H.V. and Wessling, M. (2002). Preparation of ethylene vinyl alcohol copolymer (EVAL) membranes suitable for ligand coupling in affinity separation. *J. Membrane. Sci.*, **210**(1), 155–173.

Blok, J. and Wösten, M.A.D. (2000). Origin and fate of natural estrogens in the environment (in Dutch) RIWA, Haskoning.

Clara, M., Strenn, B.E.S. and Kreuzinger, N. (2004). Adsorption of bisphenol-A, 17b-estradiole and 17a-ethinylestradiole to sewage sludge. *Chemosphere*, **56**, 843–851.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P. and Waldock, M. (1998). Identification of estrogenic chemicals in STW efflluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.*, **32**(11), 1549–1558.

Goda, Y., Kobayashi, A., Fukuda, K., Fujimoto, S., Ike, M. and Fujita, M. (2000). Development of the ELISAs for detection of hormone-disrupting chemicals. *Wat. Sci. Technol.*, **42**(7–8), 81–88.

Hansen, P.D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M. and Blaise, C. (1998). Vitellogenin – a biomarker for endocrine disruptors. *Trends Anal. Chem*, **17**(7), 448–451.

Ingerslev, F. and Halling-Sorensen, B. (2003). *Evaluation of analytical chemical methods for detection of estrogens in the environment*, EPA Denmark.

Johnson, A.C. and Williams, R.J. (2004). A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environ. Sci. Technol.*, **38**(13), 3649–3658.

Jones, P.D., Coen, W.M.d., Tremblay, L. and Giesy, J.P. (2000). Vitellogenin as a biomarker for environmental estrogens. *Wat. Sci. Technol.*, **42**(7–8), 1–14.

Joss, A., Andersen, H., Ternes, T., Richle, P.R. and Siegrist, H. (2004). Removal of estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: Consequences for plant optimization. *Environ. Sci. Technol.*, **38**, 3047–3055.

Kjølholt, J., Stuer-Lauridsen, F., Blicher, T.D., Andersen, H.R., Hansen, M., Ingerslev, F. and Halling-Sørensen, B. (2004). *Degradation of estrogens in sewage treatment processes*. Environmental Protection Agency, Denmark.

Körner, W., Spengler, P., Bolz, U., Schuller, W., Hanf, V. and Metzger, J.W. (2001). Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. *Environ. Toxicol. Chem.*, **20**(10), 2142–2151.

Kujawa-Roeleveld, K., Fernandes, T., Wiryawan, Y., Tawfik, A., Visser, M. and Zeeman, G. (2005). Performance of UASB septic tank for treatment of concentrated black water within DESAR concept. *Wat. Sci. Technol.*, **52**(1–2), 307–313.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D. and Lester, J.N. (2000). Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. *Environ. Sci. Technol.*, **34**(18), 3890–3894.

Legler, J. (2001). *Development and application of in vitro and in vivo reporter gene assays for the assessment of (xeno-) oestrogenic compounds in the aquatic environment*, Department of Toxicology, Wageningen University.

Onda, K., Nakamura, Y., Takatoh, C., Miya, A. and Katsu, Y. (2003). The behavior of estrogenic substances in the biological treatment process of sewage. *Wat. Sci. Technol.*, **47**(9), 109–116.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R. and Sumpter, J.P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.*, **8**, 275–285.

Reed, M.J., Fotherby, K. and Steele, S.J. (1972). Metabolism of ethynyloestradiol in man. *J. Endocrinol.*, **55**(2), 351–361.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M. and Sumpter, J.P. (1998). Identification of estrogenic chemicals in STW effluent: 2.In vivo responses in trout and roach. *Environ. Sci. Technol.*, **32**(11), 1559–1565.

Shi, J.H., Suzuki, Y., Nakai, S. and Hosomi, M. (2004). Microbial degradation of estrogens using activated sludge and night soil-composting microorganisms. *Wat. Sci. Technol.*, **50**(8), 153–159.

Simons, J. and Clemens, J. (2003). The use of separated human urine as mineral fertilizer. In: GTZ 2nd International Symposium on ecological sanitation " ecosan-closing the loop" ed. Luebeck, Germany (www.gtz.de/ecosan/english/symposium2-proceedings-eng.htm).

Ternes, T.A., Kreckel, P. and Mueller, J. (1999). Behaviour and occurrence of estrogens in municipal sewage treatment plants II. Aerobic batch experiments with activated sludge. *Sci. Total Environ.*, **225**(1–2), 91–99.

Urmenyi, A.M., Poot, A.A., Wessling, M. and Mulder, M.H.V. (2005). Affinity membranes for hormone removal from aqeous solutions. *J. Membrane. Sci.*, **259**, 91–102.

Vethaak, A.D., Rijs, G.B.J., Schrap, S.M., Ruiter, H., Gerritsen, A. and Lahr, J. (2002). *Estrogens and xeno-estrogens in the aquatic environment of the Netherlands; occurence, potency and biological effects*, RIZA Institute for Inland Water Management and Waste Water Treatment, RIKZ Institute for Coastal and Marine Management, Almere, 292.

Williams, C.L. and Stancel, G.M. (1996). Estrogens and progestins. In: *The Pharmacological Basis of Therapeutics*, Hardman, J.G., Limbird, L.E., Molinoff, P.B, Ruddon, R.W. and Goodman-Gilman, A. (eds). The McGraw-Hill Companies, USA, pp. 1411–1440.

Wilsenach, J.A. and Loosdrecht, M.C.M.V. (2004). Effects of seperate urine collection on advanced nutrient removal processes. *Environ. Sci. Technol.*, **38**(4), 1208–1215.

Zeeman, G. and Lettinga, G. (2001). Objectives of DESAR–Decentralised Sanitation and Reuse. *Berichte aus Wassergüte und Abfallwirtschaft Technische Universität München*, **161**, 2–17.