Field study using two immunoassays for the determination of estradiol and ethinylestradiol in the aquatic environment

Therese Hintemann\textsuperscript{a}, Christian Schneider\textsuperscript{b}, Heinz F. Schöl\textsuperscript{c}, Rudolf J. Schneider\textsuperscript{d,\*}

\textsuperscript{a}Department of Plant Nutrition, INRES-Institute of Crop Science and Resource Conservation, University of Bonn, Karlrobert-Kreiten-Str. 13, D-53115 Bonn, Germany
\textsuperscript{b}Olympus Life and Material Science Europa (Irish Branch), O’Callaghan’s Mills, Lisnemeehan, Co. Clare, Ireland
\textsuperscript{c}Institute of Environmental Geochemistry, University of Heidelberg, Im Neuenheimer Feld 236, D-69120 Heidelberg, Germany
\textsuperscript{d}BAM-Federal Institute for Materials Research and Testing, Department of Analytical Chemistry, Reference Materials, Division of BioAnalytics, Richard-Willstaetter-Str. 11, D-12489 Berlin, Germany

Article info

Article history:
Received 27 September 2005
Received in revised form 10 March 2006
Accepted 24 April 2006

Keywords:
Estradiol
Ethinylestradiol
Sewage treatment plant
Surface water
Immunoassay

Abbreviations:
PBS: phosphate-buffered saline
ELISA: enzyme-linked immunosorbent assay
CMO: carboxymethyloxime
E2: 17\textbeta-estradiol
EE2: 17\alpha-ethinylestradiol
POD: horseradish peroxidase

ABSTRACT

The effluent of four sewage treatment plants (STP) and eight surface water samples from the river Rhine in Germany and two smaller rivers were monitored for the hormones estradiol (E2) and ethinylestradiol (EE2). The studied STPs are using different treatment processes. Two facilities include an activated sludge treatment, one is a constructed wetland, and one is just an aerated lagoon. For analysis of E2 and EE2 in the aquatic environment two immunoassays have been developed allowing a very cost-effective screening for both hormones in environmental samples. Detection limits could be established at 0.05 ng L\textsuperscript{-1} for E2 and 0.01 ng L\textsuperscript{-1} for EE2, taking a 50-fold enrichment into account. Median concentrations for E2 and EE2 in effluent samples were 12 and 1.8 ng L\textsuperscript{-1}, in surface water 4.0 and 0.7 ng L\textsuperscript{-1}, respectively. The highest estrogen concentrations were found in the effluent of the lagoon, equipped with very basic means of wastewater treatment.

© 2006 Elsevier Ltd. All rights reserved.

1. Introduction

Observations of abnormalities in the aquatic environment more than 30 years ago (Blaber, 1970; Smith, 1971) initiated first research activities regarding endocrine disruption. With the advent of reproductive failure in freshwater fish populations, observed in the early 1990s (Bern, 1991; Sumpter et al., 1994) these efforts were intensified. Today there is a still growing public concern about occurrence of estrogenic compounds in the environment (European Commission,...
2004). Recently it has been shown that steroidal estrogens can bioaccumulate in fish, mainly in the bile and in both ovaries and testes of fish exposed to contaminated water (Larsson et al., 1999; Gibson et al., 2005). Regarding feminization of rainbow trout strongest estrogenic has been established for the synthetic estrogen ethinylestradiol (EE2) at a concentration of 0.1 ng L$^{-1}$ (Länge et al., 2001) and for the endogenous estrogen estradiol (E2) at levels from 1 to 10 ng L$^{-1}$ (Routledge et al., 1998). Recent environmental concentrations of E2 and EE2 measured in sewage treatment plant (STP) effluents cover a concentration range of 0–15 ng L$^{-1}$ for E2 and 0–2 ng L$^{-1}$ for EE2 (Aerni et al., 2004; Komori et al., 2004; Pawlowski et al., 2004; Servos et al., 2005). Although both compounds are biodegraded in river water (Jürgens et al., 2002), they can be regarded as “pseudopersistent” in habitats continuously affected by STP discharge (Sumpter and Johnson, 2005), yet on concentration levels in the ppt range.

The very low concentrations of E2 and EE2 found in the aquatic environment require sophisticated methods for quantitation. In past years the search for adequate techniques was focused on instrumental methods, such as LC-MS, GC-MS and tandem MS or MS$^n$ methods. Unfortunately mass spectrometric methods are associated with expensive instrumentation and require highly trained personnel, thus impeding application of the methods on a broader base. For a widespread determination of E2 and EE2 in the environment less complex and more cost-effective methods are required. Therefore we have developed two immunoassays facilitating the easy and precise quantitation of both estrogens in the aquatic ecosystem. In contrast to other immunoassays described in literature for screening of E2 and EE2 (Goda et al., 2000; Huang and Sedlak, 2001; Coille et al., 2002; Majima et al., 2002; Valentini et al., 2002; Hanselman et al., 2004), the development of our assays was initially designed to enable low-cost quantitations in environmental samples. Applicability of our assays for environmental monitoring was evaluated in a field study involving samples taken from several surface waters and effluent samples from different STP.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical or biochemical grade and were used as received. Horseradish peroxidase (EIA grade) was obtained from Roche (Mannheim, Germany), Guardian$^\text{TM}$ (an enzyme stabilizer) was from Perbio (Bonn, Germany). Tetramethylbenzidine (TMB) and Tween$^\text{TM}$-20 were purchased from Serva (Heidelberg, Germany), sodium azide was from VWR (Darmstadt, Germany), ultrapure water was obtained by running deionized water through a Milli-Q water purification system from Millipore (Schorn, Germany). 1,3,5(10)estratrien-3,17-diol-6-one-6-carboxymethylxime, EE2, E2 and all buffer salts were supplied by Sigma-Aldrich (Taufkirchen, Germany). All metabolites of E2 and EE2 were from Steraloids (London, UK). Sephadex columns were from Amersham Biosciences (Freiburg, Germany). Buffers and the TMB-based substrate have been described elsewhere (Schneider et al., 2004). Microwell plates used were transparent with 96 flat-bottom wells possessing high binding capacity (Maxi-Sorp$^\text{TM}$) from Nunc (Roskilde, Denmark). Washing steps were performed using an automatic plate washer (Flexiwash, Asys Hitech, Eugendorf, Austria). Absorbance was measured at 450 nm and referenced to 650 nm with a plate reader (Vmax, Molecular Devices, Munich, Germany). Filters were obtained from Macherey-Nagel (Düren, Germany) and Schleicher & Schuell (Dassel, Germany). SPE cartridges used were Strata$^\text{TM}$-X purchased from Phenomenex (Aschaffenburg, Germany), silica gel was from Merck (Darmstadt, Germany).

2.2. Antiserum production

Both, E2 and EE2 antisera were produced in New Zealand White rabbits in cooperation with Eurogentec (Herstal, Belgium) using BSA conjugates of the respective 6-carboxymethylxime of each steroid. Primary immunizations were performed intradermally at 10 multiple sites by injecting the immunogen emulsified in Freund’s complete adjuvant. Booster injections were administered 2, 4 and 8 weeks after priming using Freund’s incomplete adjuvant. Final bleedings were taken after 2 months for the E2 antiserum and after 3 months for the EE2 antiserum.

2.3. Preparation of enzyme conjugates

The POD conjugate of E2 was synthesized using modifications of a method described by Schneider and Hammock (1992). Hapten (2.5 μmol) E2-6-CMO (3.75 mg), and 2.5 μmol of N-hydroxysuccinimide and N,N-dicyclohexylcarbodiimide (1.16 and 2.04 mg, respectively) were dissolved in 120 μL anhydrous N,N-dimethylformamide (DMF) and stirred at room temperature overnight. The mixture was centrifuged at 1750 g for 10 min, 60 μL of the DMF supernatant was added dropwise to 0.6 mL stirred peroxidase solution (6.8 mg POD in 0.6 mL 0.13 M NaHCO$_3$). Stirring at room temperature was continued for 3 h. To purify the conjugate TBS buffer (pH 7.5, 0.1 M Tris, 0.15 M NaCl) was used for elution of the enzyme conjugate on a Sephadex G-25 column, fractions were collected in a microtiter plate and their optical density (O.D.) measured at 405 nm. Fractions of highest O.D. values were pooled, mixed with an equal amount of Guardian$^\text{TM}$, aliquoted and stored at 4°C. Synthesis of the EE2 conjugate followed a different route described elsewhere (Schneider et al., 2005).

2.4. ELISA procedures

The direct competitive ELISA format using sequential saturation was adapted for both assays (Zettner and Duly, 1974). The E2 antiserum was diluted 1:10,000 in coating buffer (50 mM sodium carbonate buffer, pH 9.6) the respective EE2 antiserum 1:50,000. Transparent microtiter plates were coated separately using 200 μL per well. Plates were covered with Parafilm$^\text{TM}$ to prevent evaporation. After overnight incubation at 20 °C, the plates were washed three times with PBS 1 (10 mM PBS pH 7.6, containing 150 mM NaCl and 2.85% Tween$^\text{TM}$-20) using the automatic plate washer. For construction of the calibration curves E2 and EE2 stock solutions were prepared in methanol and then further diluted with ultrapure water containing 5% methanol to obtain standard solutions. After the three-cycle
washing step the plates had standards added (100 μL per well) and were shaken at room temperature for 30 min. This was followed by addition of the related enzyme conjugate in PBS 2 (dilution 1:50,000 for E2-POD and 1:50,000 for EE2-POD, 100 μL per well), the plates were shaken at room temperature for 10 min, followed by a second three-cycle washing step. Finally, substrate solution was added (200 μL per well) and incubated for 30 min. The enzyme reaction was stopped by addition of sulfuric acid (50 μL per well, 1 M).

All determinations were at least made in triplicate on different plates. The mean values of optical densities were standardized according to Eq. (1) and plotted against the logarithm of the concentration of the respective analyte. A calibration curve was produced by fitting the data to a four-parametric logistic equation (4PL) (Dudley et al., 1985).

\[ Y_N = \frac{Y}{A} \]

where \( Y_N \) is the standardized optical density, \( Y \) the optical density, \( A \) the O.D. for an infinitely small analyte concentration ("blank") derived from the 4PL.

2.5. Determination of cross-reactivity

The relative sensitivity of both ELISAs towards common metabolites of both steroids was determined by assaying a dilution series of several structurally related substances in water. Cross-reactivity was calculated as the ratio of molar concentrations at the inflection points (midpoints) of the corresponding calibration curves and expressed in percentage relative to E2, and EE2, respectively (see Eq. (2)).

\[ CR = \frac{C_{\text{standard}}}{C_{\text{test}}} \times 100\% \]

where \( CR \) is the cross-reactivity, \( C_{\text{standard}} \) the molar E2 concentration at the graphs inflection point (EE2 concentration, resp.) and \( C_{\text{test}} \) the molar concentration of the cross-reacting compound at the concentration value of the inflection point of the respective curve.

2.6. Water samples

Water samples were collected in brown glass bottles, stored at 4 °C and analyzed within 24 h. Eight surface water samples were taken at the river Rhine, its tributary Ahr and at the smaller river Agger. Water quality according to the saproby system at the sampling sites is in second class, only samples taken from the river Ahr ranged from first to second class. All surface water samples, apart from the first sampling site of the river Ahr (Ahr at Blankenheim), are affected by STP (MUNLV, 2005). Seven effluent samples were taken from different STPs. The investigated plants are located in rural regions not influenced by any industrial input. Process characteristics include mechanical and biological treatments, consisting of nitrification and denitrification, using activated sludge in plants B and C, as well as a constructed wetland (plant A) and a lagoon (plant D). Capacities of the four STP differ significantly, ranging from 150 population equivalents in plant A to 20,000 in plant C (Table 1).

All sites were sampled twice in January and February 2005 when air temperatures were between −5 °C and +5 °C. Dissolved organic carbon (DOC) was determined in all samples with a Continuous Flow Analyser (AutoAnalyzer, Bran & Luebbe, Norderstedt, Germany) after removal of inorganic carbon by acidification and purging with nitrogen.

The DOC content in the STP effluents ranged from 4.2 to 10 mg L⁻¹, whereas DOC in the surface waters was in the range of 1.0 mg L⁻¹ (close to the springs of the river Ahr at Blankenheim) to 16.9 mg L⁻¹ (River Agger at Lohmar). No correlation could be found between the DOC and the measured hormone concentrations.

2.7. Sample preparation and analysis

For ELISA measurements, 500 mL of surface water or effluent sample were filtered using filter paper (Macherey-Nagel, ref. No. 619) and glass-fiber filter GF 8 (Schleicher & Schuell, ref. no. 370120) followed by solid-phase extraction on Strata™-X cartridges optimizing two methods described in literature (Quintana et al., 2004; Zühlke et al., 2005). With respect to the weak acidity of E2 and EE2, samples were adjusted to pH 7.

<table>
<thead>
<tr>
<th>Plant code</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification</td>
<td>Constructed wetland</td>
<td>Treatment plant</td>
<td>Treatment plant</td>
<td>Lagoon</td>
</tr>
<tr>
<td>Population equivalents</td>
<td>150</td>
<td>4500</td>
<td>20,000</td>
<td>150</td>
</tr>
<tr>
<td>Average daily flow [m³ day⁻¹]</td>
<td>12</td>
<td>3200</td>
<td>3500</td>
<td>21</td>
</tr>
<tr>
<td>Wastewater Primary treatment</td>
<td>Residential, rural</td>
<td>Residential, rural</td>
<td>Residential, rural</td>
<td>Residential, rural</td>
</tr>
<tr>
<td>Secondary treatment</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Aeration</td>
</tr>
<tr>
<td>Tertiary treatment</td>
<td>Aeration</td>
<td>Biological treatment (activated sludge)</td>
<td>Biological treatment (activated sludge)</td>
<td>Aeration, seasonal discharge</td>
</tr>
<tr>
<td></td>
<td>Yes, sedimentation</td>
<td>Yes</td>
<td>Yes, sedimentation</td>
<td>No</td>
</tr>
</tbody>
</table>
Representative samples of tap water, surface water and effluent samples from sewage treatment plants (STP) were spiked separately with 3.0, 6.0 and 9.0 ng L\(^{-1}\) of E2 and EE2. Each aliquot was analyzed for E2 and EE2 by ELISA (Table 2). Concentration results were plotted against spike levels and interpolated using weighted linear regression (Table 3). Recovery rates were calculated as the slope of each regression line. In tap water recovery rates for E2 and EE2 were 94% and 110%, in surface water 115% and 151%, respectively, in STP effluent 113% for E2 and 125% for EE2.

### Table 2 – Results of the determination of spiked aliquots

<table>
<thead>
<tr>
<th>Spiking level (ng L(^{-1}))</th>
<th>0</th>
<th>3.0</th>
<th>6.0</th>
<th>9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water (ng L(^{-1}))</td>
<td>0.03±0.01</td>
<td>2.79±1.00</td>
<td>5.69±0.30</td>
<td>8.53±2.00</td>
</tr>
<tr>
<td>Surface water (ng L(^{-1}))</td>
<td>1.35±0.14</td>
<td>4.90±0.16</td>
<td>8.18±0.25</td>
<td>9.46±5.10</td>
</tr>
<tr>
<td>STP effluent (ng L(^{-1}))</td>
<td>0.90±0.20</td>
<td>5.43±0.33</td>
<td>8.15±2.98</td>
<td>11.0±0.34</td>
</tr>
<tr>
<td><strong>EE2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water (ng L(^{-1}))</td>
<td>2.44±0.03</td>
<td>6.40±0.78</td>
<td>8.77±0.51</td>
<td>14.9±2.80</td>
</tr>
<tr>
<td>Surface water (ng L(^{-1}))</td>
<td>1.33±0.05</td>
<td>5.87±0.05</td>
<td>18.9±8.56</td>
<td>17.3±2.42</td>
</tr>
<tr>
<td>STP effluent (ng L(^{-1}))</td>
<td>0.65±0.11</td>
<td>4.44±0.02</td>
<td>7.26±0.60</td>
<td>11.49±1.17</td>
</tr>
</tbody>
</table>

\(^*\)Representative samples of tap water, surface water and effluent samples from sewage treatment plants (STP) were spiked separately with 3.0, 6.0 and 9.0 ng L\(^{-1}\) of E2 and EE2.

### Table 3 – Parameters of the linear regression for the determination of the recovery rates of E2 and EE2 determined in tap water, surface water and effluent samples from sewage treatment plants (STP)

<table>
<thead>
<tr>
<th></th>
<th>Intercept [ng L(^{-1})]</th>
<th>Slope [x 100%]</th>
<th>R</th>
<th>p</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td>0.03±0.01</td>
<td>94.3±4.8</td>
<td>1</td>
<td>&lt;0.0001</td>
<td>0.049</td>
</tr>
<tr>
<td>Surface water</td>
<td>1.38±0.13</td>
<td>115±4.5</td>
<td>0.9997</td>
<td>0.0156</td>
<td>0.629</td>
</tr>
<tr>
<td>STP effluent</td>
<td>1.12±0.20</td>
<td>113±4.4</td>
<td>0.9929</td>
<td>0.0071</td>
<td>2.203</td>
</tr>
<tr>
<td><strong>EE2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td>2.44±0.03</td>
<td>110±7.8</td>
<td>0.9954</td>
<td>0.0046</td>
<td>0.958</td>
</tr>
<tr>
<td>Surface water</td>
<td>1.33±0.04</td>
<td>151±2.4</td>
<td>0.9998</td>
<td>0.0002</td>
<td>0.985</td>
</tr>
<tr>
<td>STP effluent</td>
<td>0.69±0.11</td>
<td>125±3.6</td>
<td>0.9989</td>
<td>0.0011</td>
<td>1.154</td>
</tr>
</tbody>
</table>

The intercept value gives the back-calculated hormone concentration in the sample, the slope corresponds with the recovery rate, \(R\) is the correlation coefficient and the \(p\) is probability that \(R\) is zero.

Using 0.1 M NaOH, thusly decreasing retention of humic acids on the sorbent. The retention behavior of the steroid hormones was not modified by this pH shift. SPE cartridges were conditioned using 8 mL of methanol followed by 8 mL of distilled water containing 0.5% of methanol. Samples containing the same percentage of methanol were passed through the cartridges at a flow rate of 5.5 mL min\(^{-1}\) with the aid of a vacuum manifold. Cartridges were sequentially washed using 10 mL of ultrapure water, 10 mL of methanol/water (1:1, v/v), 10 mL acetone/water (1:2, v/v) and 10 mL of hexane. After drying of the solid phase under nitrogen, estrogens were eluted from the sorbent using 7 mL of methanol. The solvent of the eluate was completely blown off by a gentle stream of nitrogen and the residue resuspended in 500 μL of a hexane/acetone mixture (65:35, v/v). This extract was further purified on a silica gel column (500 mg) using 8 mL of the same mixture as mobile phase. Afterwards this eluate was again completely reduced to dryness and the residue dissolved in 500 μL of methanol. For the ELISA measurement the final extract was diluted to a methanol content of 5%. Recovery rates for E2 and EE2 in tap water, surface water and STP effluent were established by spiking aliquots of representative samples separately with 3.0, 6.0 and 9.0 ng L\(^{-1}\) of E2 and EE2. Each aliquot was extracted and treated as described above, all extractions were performed in duplicate. Additionally, two non-spiked aliquots were extracted, resulting in eight extracts for tap water, surface water and STP effluent, respectively. All extracts were analyzed for E2 and EE2 by ELISA (Table 2). Concentration results were plotted against spike levels and interpolated using weighted linear regression (Table 3). Recovery rates were calculated as the slope of each regression line. In tap water recovery rates for E2 and EE2 were 94% and 110%, in surface water 115% and 151%, respectively, in STP effluent 113% for E2 and 125% for EE2.

3. Results and discussion

3.1. Immunoassays

Both ELISAs, for E2 and EE2, have been optimized with regard to sensitivity by tracer dilution, antibody concentration and incubation time. Calibration curves for both analytes were constructed in the concentration range of 0.001–1,000 ng L\(^{-1}\). Representative calibration curves and the corresponding precision profiles for each assay are shown in Fig. 1a and b. The detection limit (LOD) and the quantitation limit (LOQ) are calculated using signal-to-noise ratios (S/N) of 3 and 10, respectively. For the E2 assay LOD was 0.05 ng L\(^{-1}\) and LOQ 0.36 ng L\(^{-1}\) whereas the EE2 assay showed a LOD of 0.01 ng L\(^{-1}\) and a LOQ of 0.12 ng L\(^{-1}\).

Analytical ranges are calculated using the respective precision profiles and a maximum CV (relative error, obtained...
from dividing each standard deviation by the respective mean) of 20% (Ekins, 1981). The analytical working range for the E2 assay was 0.28–590 ng L$^{-1}$ and 0.07–2570 ng L$^{-1}$ for the EE2 assay, respectively. All parameters given take into account the 50-fold enrichment performed with all samples.

Both assays exhibit excellent selectivity. Cross-reactivity of the E2 antiserum for typical metabolites such as estrone, estriol and the sulfate and glucuronide conjugated at ring position 17 is below 1% (ref. Eq. (2) (Fig. 2)). Merely sulfate or glucuronide conjugated at ring position 3 lead to cross-reactivity of 9% and 25%, respectively. Cross-reactivity of the E2 ELISA for EE2 is below 0.3%. The EE2 antiserum exhibits a very similar pattern and has been tested earlier using two different ELISA formats, results having been discussed elsewhere (Schneider et al., 2004, 2005).

Fig. 1 – (a) Calibration curve of estradiol (solid line, squares) with standard deviations indicated as error bars ($n = 3$) and corresponding precision profile (dashed line). Signal intensity is shown in standardized optical density. The detection limit (LOD) and the quantitation limit (LOQ) are calculated using signal-to-noise ratios of 3 and 10, respectively and taking into account a 50-fold enrichment. (b) Calibration curve of ethinylestradiol (solid line, squares) with standard deviations indicated as error bars ($n = 3$) and corresponding precision profile (dashed line). Signal intensity is shown in standardized optical density. The detection limit (LOD) and the quantitation limit (LOQ) are calculated using signal-to-noise ratios of 3 and 10, respectively and taking into account a 50-fold enrichment.

Fig. 2 – Chemical structures of 17β-estradiol and 17α-ethinylestradiol.
Table 4 – Concentrations of E2 and EE2 determined in surface water and effluent samples from sewage treatment plants (STP)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sampling month (all 2005)</th>
<th>E2 (ng L(^{-1}))</th>
<th>EE2 (ng L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahr at Blankenheim</td>
<td>January</td>
<td>2.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Ahr at Dümpelfeld</td>
<td>January</td>
<td>6.8</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>4.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Agger at Lohmar</td>
<td>January</td>
<td>9.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>3.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Rhine at Bonn</td>
<td>January</td>
<td>4.1</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>3.9</td>
<td>0.8</td>
</tr>
<tr>
<td>STP A</td>
<td>January</td>
<td>3.8</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>3.1</td>
<td>3.3</td>
</tr>
<tr>
<td>STP B</td>
<td>January</td>
<td>9.5</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>STP C</td>
<td>January</td>
<td>12</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>12</td>
<td>0.4</td>
</tr>
<tr>
<td>STP D</td>
<td>January</td>
<td>51</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>15</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* sample lost during preparation.

3.2. Water samples

Concentrations of both estrogens determined in surface waters and STP effluents appeared to be in a range similar to the ones described in literature (Aerni et al., 2004; Komori et al., 2004; Pawlowski et al., 2004; Servos et al., 2005), details are shown in Table 4. Median concentration for E2 and EE2 found in surface waters were 4.0 and 0.7 ng L\(^{-1}\), respectively. The first sampling site of the river Ahr (Ahr at Blankenheim) is close to its four springs located in the middle of a small town. Possible reasons for the contamination of the spring with both hormones can be livestock and/or human sources combined with special geological and hydrological conditions at the site. The watershed of the river Ahr, especially the drainage area of the springs, is in a rural area and has a karstic aquifer, where surface and ground water are often interconnected (MUNLV, 2004). These conditions facilitate an input of hormones (Wicks et al., 2004) analogously to pesticide findings in karstic springs.

Slightly elevated values found in the samples Agger and Ahr (Ahr at Dümpelfeld) can be explained by STP discharge upstream of these sampling sites. The concentrations determined in the river Rhine are lower than in its smaller tributary Ahr and in the river Agger, possibly due to a higher dilution, and correspond at both times very well with the E2 and EE2 amounts found by Pawlowski et al. (2004).

In samples taken from STP effluents median concentrations for E2 and EE2 of 11.7 and 1.8 ng L\(^{-1}\) have been observed. Plants B and C employ activated sludge as a secondary treatment enabling the use of Johnson and Williams’ model for predicting effluent concentration of E2 and EE2 in these plants (Johnson and Williams, 2004). For plant B, a concentration range of 0.3–1.7 ng L\(^{-1}\) for E2 and of 0.1–0.3 ng L\(^{-1}\) for EE2 can be estimated. Parameters of plant C would lead to a concentration range of 0.7–4.1 ng L\(^{-1}\) for E2 and of 0.3–0.7 ng L\(^{-1}\) for EE2. In this model a fixed removal rate for the steroids is assumed. The removal rate obviously varies between STPs and is highly dependent on both, the duration of biological treatment and, hence, sludge age, and the amount of biomass present (Holbrook et al., 2002). Therefore deviations to these model predictions could either result from discrepancies in removal rates or from overestimations in our measurements. Such false-positive measurements due to matrix effects have been reported before (Ruppert et al., 1992). Because both investigated STPs are situated in rural areas the influence of livestock, which is not included in the model mentioned above, could also contribute to higher E2 concentrations.

The model is based on mean removal rates for estrogens in activated sludge plants, consequently it cannot be adequately applied to predict effluent concentrations in plant A (constructed wetland) and plant D (lagoon). E2 values in plant A covered a range from 3.1 to 3.8 ng L\(^{-1}\) and from 1.6 to 3.3 ng L\(^{-1}\) for EE2. In plant D E2 values of 14 and 51 ng L\(^{-1}\) were determined, EE2 values were 1.8 and 3.1 ng L\(^{-1}\). Reports on quantitation of steroidal hormones in constructed wetlands and lagoons are rather scarce. Divergent design concepts of wetlands described in literature and insufficient information add to complicate a direct comparison of estrogen concentrations. Nevertheless Kolodziej et al. (2003) measured in a similar constructed wetland, which is surface-flow engineered and receiving effluents of a preceding STP, E2 concentrations in a range from 0.3 to 4.1 ng L\(^{-1}\) which is comparable to the concentrations found in this study in plant A. In contrast to the constructed wetland examined by Kolodziej et al. plant A receives influents directly from the sewage system without surface-flow and features a dense vegetation of reed (Phragmites australis). Measurements of E2 in a plant of similar size and design have been described in a study by the UFZ (Centre for Environmental Research) Leipzig-Halle (Braun et al., 2003). Unfortunately the inapty high detection limit for EE2 impeded quantitations in the effluent. The lagoon sampled in our study employs a very basic equipment for sewage treatment. The primary treatment consists only of a simple grid, secondary treatment has been implemented by manually controlled air bubbles arbitrarily adjusted by staff members. E2 values measured at this plant are significantly elevated compared to plants A–C, this can be explained by the non-optimized treatment process in the lagoon. Especially the complete absence of a controlled nitrification step might be causative for high concentrations of E2. The importance of optimized nitrifying and denitrifying conditions for elimination of both E2 and EE2 has been thoroughly studied at STPs employing conventional activated sludge treatments (Andersen et al., 2003). Results of this study might also be indicative for improvement of the lagoon presented here.

Evaluating the results the very low air temperature during the sampling period has to be considered because the microbial activity and thus the estrogen removal rate is decreasing with lower temperatures (Bringolf and Summerfelt, 2003). Therefore higher estrogen concentrations during the winter months in STP effluents and consequently in surface waters with effluent intake are likely.
4. Conclusions

In this work, E2 and EE2 concentrations of surface waters and STP effluents were determined. Concentrations of both analytes were in good agreement with data reported in earlier studies. In addition to these studies we have determined steroid concentrations in a typical constructed wetland and a lagoon. Based on two highly sensitive ELISAs and an optimized SPE procedure a new and affordable methodology facilitating the easy and precise quantitation of both estrogens in environmental samples has been developed. Compared to typical instrumental methods or other immunoassays consumption of time and resources has been significantly reduced, thus providing an opportunity for more frequent screenings of the environment for endocrine disruptors such as E2 and EE2. If monitoring of the elimination of hormones during the wastewater treatment process should be required in the future, immunoassays could be a valuable tool for this purpose because of the relative ease of automation and the possibility of running ELISA tests on site due to robust instrumentation, fast measurements and a low demand of skilled personnel.

Acknowledgements

The authors thank the local authorities for kindly providing wastewater samples. C. Schneider thanks the German Federal Environmental Foundation (DBU) for a Ph.D. scholarship.

T. Hintemann and C. Schneider contributed equally to the study.

REFERENCES


