Estrogenic Activity and Steroid Hormones in Swine Wastewater through a Lagoon Constructed-Wetland System

NANCY W. SHAPPELL,* ¹ LLOYD O. BILLEY,¹ DEAN FORBES,¹ TERRY A. MATHENY,¹ MATTHEW E. POACH,⁵ GUDIGOPURAM B. REDDY,¹ AND PATRICK G. HUNT⁵

Biosciences Research Laboratory, USDA-ARS, 1605 Albrecht Boulevard, Fargo, North Dakota 58105, North Carolina Agricultural & Technical State University, Greensboro, North Carolina 27411, and Coastal Plains Soil, Water, and Plant Research Center, USDA-ARS, 2611 West Lucas Street, Florence, South Carolina 29501

Anaerobic lagoons and treatment wetlands are used worldwide to treat wastewater from dense livestock production facilities; however, there is very limited data on the hormonal activity of the wastewater effluent produced by these treatment systems. The objectives of this experiment were to measure (1) the hormonal activity of the initial effluent and (2) the effectiveness of a lagoon-constructed wetland treatment system for producing an effluent with a low hormonal activity. Wastewater samples were taken in April, July, and November 2004 and July 2005 from a lagoon-constructed wetland system at a swine farrowing facility. Estrogenic activity (in vitro E-screen assay), 17 β-estradiol (E2), and testosterone concentrations (LC/MS–MS) were measured. A high correlation was found between estradiol equivalents determined by E-screen and LC/MS–MS (R² = 0.82). Nutrient removal was measured to ensure that the wetlands were functioning in a manner similar to literature reports. Nutrient removals were typical for treatment wetlands: TKN 59–75% and orthophosphate 0–18%. Wetlands decreased estrogenic activity by 83–93%. Estrone was the most persistent estrogenic compound. Constructed wetlands produced effluents with estrogenic activity below the lowest equivalent E2 concentration known to have an effect on fish (10 ng/L or ~37×10⁻¹² M).

Introduction

Public awareness of the correlation between aquatic pollution and alligators with abnormal gonad development and altered sex hormone concentrations (1) spurred research in the field of environmental endocrine disruption. Whereas some sources of endocrine disruption (ED) have been anthropogenic (2), such as detergents, pesticides, plasticizers, and pharmaceuticals, other sources of ED have been naturally occurring compounds, such as estrogen and testosterone released from livestock waste and poultry litter (3). The potential environmental endocrine-disrupting capacity of livestock waste in various forms and from several species was evaluated by Lange et al. (4). When animal waste is applied to fields as fertilizer, best management practices are used to reduce nitrogen and phosphorus contamination of surface and groundwater. These management practices need to be evaluated in light of their capacity to reduce environmental ED. The objectives of this experiment were to measure (1) the hormonal activity of the initial effluent and (2) the effectiveness of a lagoon-constructed wetland treatment system for producing an effluent with a low hormonal activity.

Methods for evaluating estrogenic ED include both in vivo and in vitro bioassays. Whereas in vivo assays provide information about the organismal response to environmental samples, the in vitro assay provide no such information. Literature reports of in vivo assays include exposure of fish to environmental samples and subsequent analyses for the presence of female-specific proteins (5), production of intersex gonads, and reproductive efficiency. However, there is a shortage of peer-reviewed, published literature on the long-term exposure of fish to estradiol and its effect on long-term production and the F1 generation. In vitro assays, which are much less time-intensive, include both transfected and nontransfected cell-based assays. One of the most commonly used assays is the yeast estrogen screening assay (YES) that uses yeast cells transfected with the human estrogen receptor gene (6). Two other transgenic assays use human cell lines transfected with estrogen-receptor-responsive elements (MVLN and HGELN cells) (7). Acellular assays of ED, such as the competitive estrogen receptor binding assay (8), provide even more limited information about EDs, because receptor binding can have many cellular consequences. The estrogen screen using MCF-7 human mammary epithelial cells (E-screen) (2) has some advantages over the in vitro estrogenicity assays cited above. First, this assay uses nontransfected mammalian cells; therefore, the receptor number in each cell is physiologically relevant. Second, the assay requires not only receptor binding but also an integrated series of responses, which culminate in cellular proliferation. Third, because the cell is intact, modulation by other compounds in the sample, natural feedback responses to the compounds, or both can also occur. We used the E-screen in conjunction with LC/MS–MS analyses for detection of the predominant estrogenic and androgenic compounds in this experiment.

Experimental Procedures

System Description and Sampling. The swine farrowing facility at North Carolina Agricultural & Technical State University, Greensboro, NC, was the study system, housing ~100 sows and 15 boars in 2004–2005. The manure-handling system was composed of a manure pit, primary (lagoon 1) and secondary (lagoon 2) anaerobic lagoons (total surface area of 1.6 acres), four constructed wetland cells, and a storage pond (Figure 1). Manure was flushed with “gray” water through the barns into the manure pit and entered into the primary and secondary lagoons. Water from either the secondary lagoon (lagoon 2, April–July) or primary lagoon (lagoon 1, August–November) was pumped into the wetland cells (for complete wetland description, see ref 9). Each 40 m × 11 m cell had a central aerated pond section (20 m by 11 m, 75 cm deep, aeration rate of 70 LPM) with a marsh region on each end (10 m by 11 m, ~15 cm deep). Marsh

¹ North Carolina Agricultural & Technical State University.
² Biosciences Research Laboratory, USDA-ARS.
³ Coastal Plains Soil, Water, and Plant Research Center, USDA-ARS.
⁴ Corresponding author phone: (701)239-1233; fax: (701)239-1430; e-mail: shappeln@fargo.ars.usda.gov.
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plant composition was predominantly broadleaf cattails (*Typha latifolia, L.*) with some American bulrushes (*Schoenoplectus americanus*). The pond section of two cells was covered with floating mats planted with bulrushes (referred to as “covered”), whereas the pond section of the two “open” cells consisted of duckweed (*Lemna sp*) and algae. Holding capacity of each cell was ~130 m³ of wastewater/cell. The wetlands were loaded at a rate of 10 to 20 kg N of ha⁻¹ d⁻¹ from the lagoon in operation. Wetland effluent was pumped to the storage pond and eventually completed the circuit to flush the barns.

The wastewater system was sampled in April, July, and November 2004 in a preliminary study. Residence time for the wetlands averaged 36 days for April and July 2004, 22 days for November 2004, and 50 days for July 2005, as determined from wetland volume and inflow rate. Duplicate 1-L samples were collected from all components, from the manure pit through the wetland outlets. The lagoon and storage pond samples were composites taken at the surface from each of the four sides. Composite samples were collected from the influent to the covered wetland cells and the influent to the open wetland cells at the point of the entry using automated tipping buckets. Separate composite wetland effluent samples (covered and open) were collected in the same manner. In a follow-up study, four sets of weekly samples were collected in July 2005. These collections were done as described for 2004 with the exception that a submersible pump was used to mix the contents of the manure pit for 15 min prior to sampling. Samples were frozen and stored at −20 °C until analyses.

**Sample Extraction, E-Screen Evaluation and LC/MS–MS Analyses.** Methods for sample extraction, E-screen analysis using MCF-7 cells, and cell culture were as described by Shappell (10). Proliferative responses of all samples were confirmed as estrogenic through coincubation with estrogen receptor antagonist ICI 182,780 (Tocris, Ellisville, MO). In no case were samples found to stimulate proliferation in the presence of the E₂ receptor antagonist. Limit of quantitation was 1 ×...
TABLE 1. E-Screen Evaluation of 2004 Samples

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>manure pit</td>
<td>843</td>
<td>858</td>
<td>ND b</td>
</tr>
<tr>
<td>lagoon 1</td>
<td>6.4</td>
<td>11.5</td>
<td>147</td>
</tr>
<tr>
<td>inlet wetland open</td>
<td>3.1</td>
<td>1.4</td>
<td>92.1</td>
</tr>
<tr>
<td>inlet wetland covered</td>
<td>2.4</td>
<td>2.2</td>
<td>104.1</td>
</tr>
<tr>
<td>outlet wetland open</td>
<td>1.8</td>
<td>2.8</td>
<td>7.5</td>
</tr>
<tr>
<td>outlet wetland covered</td>
<td>3.6</td>
<td>9.2</td>
<td>5.5</td>
</tr>
<tr>
<td>storage pond</td>
<td>2.9</td>
<td>0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>lagoon 2</td>
<td>3.0</td>
<td>3.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* pM estradiol equivalents, mean of two extractions. ** No data.

The concentrations of estrogenic activity in the primary lagoon (mean of 9 pM) was ~1% of the pit activity for the same period, and from lagoon to wetlands, E2Eqs were further decreased (~50% to ~3 pM). This was probably the result of two factors: mixing wastewater from lagoon 1 with wastewater from lagoon 2 prior to going to the wetlands and photolysis and microbial degradation in an above-ground holding tank placed between the lagoons and release into the wetlands. In November 2004 the estrogenic activity of the primary lagoon was substantially higher (~150 pM), most likely a reflection of increased microbial degradation and photolysis due to seasonal changes in environmental temperatures and angle, intensity, and duration of sunlight. During the fall, the rate of N application across the wetlands was increased from 10 to 20 kg of N ha\(^{-1}\) (with a resultant average flow rate of ~9470 L d\(^{-1}\)) using lagoon 1 as the sole source of inlet wastewater. The higher application rate and higher estrogenic activity of the lagoon wastewater resulted in an increase in estrogenic activity of the influent (~100 pM). The higher influent concentrations allowed for more accurate assessment of the wetland’s capacity to decrease the estrogenic activity. There were no significant differences (12%) between influents for the covered and open wetland cells for E2Eqs. Mean estrogenic activity of wetland effluent was only 7% of the influent activity (8 and 6 pM E2Eqs for open and covered effluent, respectively). In a comprehensive technical report published by the Environment Agency of England and Wales (13) the “lowest observable effect concentration” or LOEC of estradiol was proposed as 10 ng/L or 36.7 pM and the “predicted no effect concentra-
tion” or pNEC was 1 ng/L or 3.67 pM. These values were established through assessment of fish reproductive capacity in the presence of estradiol, with the goal of population sustainability. The E2Eqs of wetland effluents never reached the LOEC by E2Eq and were typically closer to the pNEC values.

The chlorine, orthophosphate (PO\(_4\)), and TKN concentrations for the November 2004 samples are presented in Figure 2 for comparison with E2Eqs. Differences between influent values for open and covered cells were minimal for the nutrients also (13% for TKN, 7% for orthophosphate, and 0% for chlorine). As water passed through the wetland cells, PO\(_4\) appeared to decrease (19 and 16%, open vs covered). This decrease in PO\(_4\) concentration was similar to the mean of 22% reported for the same constructed wetland system/site in 2000 and 2001 (14). The lack of difference in chloride concentrations from the lagoon to the wetland outlets implies that decreases observed for PO\(_4\) and TKN were not a result of dilution. Total Kjeldahl N concentrations were reduced to the greatest extent (74 and 75%, open vs covered). The decrease in TKN was similar to that found for E2Eqs (~75% vs ~93% reduction, respectively). Literature values for TKN reduction across wetlands were similar to these data for swine wastewater treated by constructed wetlands (in North Carolina, >50%, typically >75% over a range of N loading concentrations (15); in Mississippi, 82% removal loading at 12.5 kg of N ha\(^{-1}\) d\(^{-1}\) (16)). Raman et al. (17) compared the estrogen content to the N and PO\(_4\) content of various manures under various storage conditions, but they did not follow the degradation in a lagoon system. To date, only one report has attempted to assess correlations of treatment plant removal of TKN, total phosphorus, and estrogenic compounds (18).

This group reported a mean decrease from influent to effluent of 68% TKN, 81% total phosphorus, and 37% E2Eq by YES (88% E\(_1\), 65% E\(_2\)). They also found a weak correlation between waste treatment plants with active nitrification processes and increased efficiency in removal of TKN, estrogenic compounds, and their activities. The nitrification efficiency of this constructed wetland system is not as effective as most municipal wastewater systems studied.

2004 LC/MS—MS Analyses. While LC/MS—MS analyses of samples from 2004 confirmed the presence of estrone (E\(_1\)), E\(_2\), and estriol (E\(_3\)) in some samples, most were below the limits of detection or quantification. As expected, no testosterone or other androgenic compounds were found due to the low number of mature boars housed in the facility. Using the E-screen, the estrogenic activity of these compounds relative to 17β-estradiol were E\(_2\) = 1.0, E\(_3\) = 0.1, and E\(_1\) = 0.01 (2, 7). Of the compounds with the highest estrogenic

FIGURE 2. β17-Estradiol equivalents (E2Eq) by E-screen and nutrient values of wastewater in November 2004. E-screen and nutrient analyses were performed on the same samples post particulate settling. Lowest observed effect concentration of estradiol (LOEC) cited as 10 ng L\(^{-1}\) or ~37 pM.
activity, E₂ and E₃ were found in high concentrations only in the pit (900–1700 pM for E₂, 200–900 pM for E₃, Supporting Information, Figure 1). Whereas the elevated E₂Eq value found in the covered wetland outlet sample from July appeared incongruous (Table 1), the results were confirmed by LC/MS–MS analysis where estradiol was found at 12 pM (data not shown). One possibility is that nesting waterfowl were present in this wetland cell during this period and their feces contributed to a higher E₂ concentration (19).

Whereas estrone has lower estrogenic activity, it was found in highest relative concentration in 70% of the samples collected. This relationship is presented clearly in Figure 3, where the concentrations are given across the wetlands from the November 2004 sampling. Estrone has been well-documented to be the primary degradation product of estradiol in manure (20), soils (21, 22), and sewage (23, 24), and its formation is relatively rapid. Another possibility would be the deconjugation of estradiol metabolites in the wetlands, causing a rise in estrogen concentrations similar to that for municipal sewage reported by D’Ascenzo (25). Our results indicate seasonal differences may exist, and data presented by Kolodziej et al. (26) also reported the unpredictability of estrogenic constituents in dairy lagoon waste across seasons. These findings indicate a need for intensive repeated sampling at the same site to delineate seasonal effects on agricultural wastewater handling systems.

2005 E-Screen and Nutrient Analyses. During sampling in 2004, the inability to control several factors within the manure-handling system became evident. One factor was a change in the source of wetland influent, necessitated by a drop in N content in the secondary lagoon. Another factor was the number of flush cycles, which had to be decreased during periods of limited rainfall. Sampling in 2005 was performed on a weekly basis, in July, to limit the effects of environmental changes as much as possible and to determine inherent system variability within a month. The July 2005 sample compositions are reported in Figure 4. A second pit sampling was included in this data set. After the contents of the pit were released to the primary lagoon, the barn was flushed with gray water, and a second sample was taken (designated postflush). The storage pond sampling was omitted. Although the postflush pit sample was consistently higher than the preflush pit sample, ~57% higher across parameters (E₂Eq, TKN, NH₄, and PO₄), the differences were not statistically significant (Pr > F ranging from 0.42 to 0.87). The higher values could be accounted for by two factors. First, the manure in the postflush sample was relatively fresh, having limited opportunity for microbial degradation, and second, the contents were contained in ~40% the volume of the preflush sample (mean 719 L preflush and 288 L postflush). In contrast to the PO₄ decrease seen across the wetlands in November 2004, the concentration of PO₄ did not change in the July 2005 samples. The failure to observe a decrease in effluent orthophosphate in 2005 may have been due to a release of PO₄ from decomposing organic matter, a decrease in the adsorption capacity of the soil, or both. Decreases in TKN across wetlands were equal to reductions in NH₄⁺ concentrations because concentrations of both parameters were essentially identical. July 2005 decreases in TKN across wetlands were somewhat less than those found in November 2004 (mean 66% decreased for covered cells...
Table 2. Statistical Analysis of Wastewater for November, 2004 and July 2005 Sampling

<table>
<thead>
<tr>
<th>Compartment</th>
<th>E-screen (E₂ Eq pM)</th>
<th>Estradiol (pM)</th>
<th>Estriol (pM)</th>
<th>Estrogen (pM)</th>
<th>TKN mg/L</th>
<th>NH₄ mg/L</th>
<th>NO₃ mg/L</th>
<th>PO₄ mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manure pit</td>
<td>1213 ± 551</td>
<td>565 ± 430</td>
<td>7364 ± 4258</td>
<td>1475 ± 1228</td>
<td>185 ± 115</td>
<td>86 ± 46</td>
<td>0.8 ± 1.25</td>
<td>110 ± 63</td>
</tr>
<tr>
<td>Lagoon 1</td>
<td>60 ± 55</td>
<td>11 ± 8</td>
<td>298 ± 305</td>
<td>32 ± 30</td>
<td>71 ± 12</td>
<td>48 ± 21</td>
<td>0.1 ± 0.07</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Pit vs Lagoon</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Inlet open</td>
<td>40 ± 33</td>
<td>11 ± 11</td>
<td>265 ± 333</td>
<td>19 ± 23</td>
<td>67 ± 8</td>
<td>55 ± 11</td>
<td>0.05 ± 0.08</td>
<td>55 ± 6</td>
</tr>
<tr>
<td>Inlet covered</td>
<td>41 ± 37</td>
<td>2 ± 3</td>
<td>285 ± 385</td>
<td>33 ± 41</td>
<td>65 ± 11</td>
<td>56 ± 7</td>
<td>0.04 ± 0.10</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>Inlet open vs covered</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Outlet open</td>
<td>6 ± 3.5</td>
<td>8 ± 6.8</td>
<td>57 ± 11.5</td>
<td>15 ± 10.3</td>
<td>22 ± 4.7</td>
<td>16 ± 7.1</td>
<td>0.6 ± 1.09</td>
<td>53 ± 10.4</td>
</tr>
<tr>
<td>Outlet covered</td>
<td>3 ± 1.8</td>
<td>5 ± 7.6</td>
<td>27 ± 7.4</td>
<td>10 ± 3.9</td>
<td>25 ± 8.2</td>
<td>21 ± 9.0</td>
<td>0.7 ± 0.82</td>
<td>50 ± 10.6</td>
</tr>
<tr>
<td>Outlet open vs covered</td>
<td>0.10</td>
<td>0.01</td>
<td>0.10</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inlet</td>
<td>41 ± 33.0</td>
<td>6 ± 9.1</td>
<td>275 ± 339</td>
<td>26 ± 32.2</td>
<td>66 ± 9.0</td>
<td>55 ± 9.0</td>
<td>0.04 ± 0.04</td>
<td>54 ± 54.0</td>
</tr>
<tr>
<td>Outlet</td>
<td>5 ± 3.1</td>
<td>6 ± 7.0</td>
<td>42 ± 18.0</td>
<td>13 ± 7.8</td>
<td>23 ± 6.5</td>
<td>18 ± 8.0</td>
<td>0.61 ± 0.91</td>
<td>52 ± 10.0</td>
</tr>
<tr>
<td>Inlet vs Outlet</td>
<td>0.01</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>0.10</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* First two lines in set are means ± standard deviations; third line, p value; n = 5 sampling events. * Levels of significant differences by least significant difference (LSD). * Not significant.

and 59% for open cells), but similar to literature values cited above. The decreases in estrogenic activity from the manure pit to the primary lagoon and across the wetlands were similar to the decreases found in 2004 (96 vs 99% decrease from pit to lagoon 1, and 83 vs 93% decrease across wetlands for 2005 vs 2004, respectively). The application rate of N was 2-fold higher in November 2004 than in July 2005, and the resultant decrease in November 2004 was 2-fold higher than that in July 2005, and the resultant decrease in July 2004. The inverse relationship between the decreases found in 2004 (96 vs 99% decrease from pit to lagoon 1, and 83 vs 93% decrease across wetlands for 2005 vs 2004, respectively). Androgenic compounds were not detected.

2005 LC/MS–MS Analyses. Results of the 2005 sample analyses by LC/MS–MS analysis (Figure 5) were similar to those found in 2004 (Table 1). Again, no testosterone or its metabolites were detected. The manure pit preflush yielded very low E₁, E₂, and E₃ levels (Table 2). However, the concentrations fell to <4% of the pit values, similar to what was seen in April and July 2004 data (lagoon values often fell below the limits of quantification; there were no detectable levels). Fine et al. (27) reported similar ratios of E₁ > E₃ > E₂ from single samplings of lagoons at two swine farrowing facilities. Sarmah et al. (28) reported the same order of magnitude of values for E₂ and E₁, whereas E₃ was not detected in sewage effluent from one “piggery”. They also evaluated sewage effluent from seven dairy farms for estrogenic metabolites. Estriol was not detected in any sample, but in all four out of seven farms with detectable E₁ and E₂, estrone was present in greater concentrations. The profile of estrogenic compounds across the wetlands in July 2005 mirrored results seen in November 2004, whereas the magnitude differed as initial influent concentrations were decreased in 2005 as a result of a lower rate of N application.

Statistical Analysis of Compartmental Differences. For statistical evaluation of estrogenic activity and nutrients across the wastewater compartments, data from November 2004 and July 2005 were pooled. As would be expected, the concentrations of all parameters were lower in the lagoon than in the manure pit (P = 0.10 level or less, Table 2). Statistical analysis of the data from November 2004 and July 2005 for all parameters using sampling dates as replicates (Table 2). Therefore, the mean data for all inlet samples was used in subsequent analyses. Because the source of wastewater to the wetland inlets was lagoon 1, no differences would be expected between parameters from these sources, and none were found except for the E-screen (not shown). This parameter was on the border of significance having 60 pM E₂ Eq for...
significance difference in the $E_2$ by $E$-screen and in the $P$ equal to the minimal LSD of 19 for lagoon vs 40 for the inlet mean, with the actual difference.

**FIGURE 6.** Comparison of $\alpha$ of the sigmoidal shape of the $E_2$ receptor binding curve. In high concentrations, conjecturing that this was a reflection overestimated at low concentrations and underestimated at equivalents were calculated as 0.1 for estriol and 0.01 for estrone. Inset presented on Y axis is log scale.

Comparison of $E$-$MS$ versus $E$-screen $E_2$Eqs for the July 2005 data is presented in Figure 6 (mean of 4 samplings). Good agreement between the two methods was observed. The correlation coefficient was $R^2 = 0.82$ (see Figure 1, Supporting Information). Korner et al. (29) reported similar agreement between $E$-screen and GC/$MS$-$MS$ analyses for $E_2$Eqs of sewage treatment plant effluents, typically with results falling within an order of magnitude of each other. Agreement of $E_2$Eqs assessed by YES assay and $MS$-$MS$ analysis has varied. Raman et al. (30) found good correlation ($R^2 = 0.94$) between $E_2$Eq values obtained by YES versus the combination of $GC/MS$-$MS$ values for $E_2$ and enzyme-linked immunosorbent assay for $E_1$ (activity of $E_1$ corrected to 0.5 $E_2$Eq for YES). These authors reported the YES assay overestimated at low concentrations and underestimated at high concentrations, conjecturing that this was a reflection of the sigmoidal shape of the $E_2$ receptor binding curve. In fact, one of the weaknesses of the YES assay is that it does reflect receptor binding in a transfected cell, not an integrated physiological response in an "unaltered" cell, with its endogenous receptor number unmodified. Aerni et al. (31) reported YES $E_2$Eqs were within an order of magnitude of $E_2$Eqs from $GC/MS$-$MS$ ($R^2 = 0.6$). Le Guevel and Pakdel (32) observed similar differences in the YES assay and another “natural” cell line (human uterine Ishikawa cells) for $E_2$ and $E_1$. For these authors, estrone $E_2$Eqs were 0.5 by YES and ~0.1 by untransfected cellular assessment (alkaline phosphatase).

Matthiessen et al. (20) found less agreement of $E_2$Eq by YES assay versus $GC/MS$-$MS$ on stream samples with potential livestock waste contamination, but this may have been a reflection of the lower estrogenic activity of the samples.

The combination of the sensitivity of the $E$-screen with identification of estrogenic compounds by $LC/MS$-$MS$, allowed identification of the specific source of estrogenic activity in later stages of waste treatment as estrone. Although significant research has been published on the reproductive effects of the synthetic estrogen ethinyl estradiol (EE2) on fish in vivo, similar data on $E_1$ is very limited. Because estrone is the natural estrogenic compound most commonly found in both municipal and animal sewage effluent, future research should focus on establishing its LOEC and pNEC, as well as development of treatment practices that decrease its persistence.

This study is the first to show that constructed wetlands can be effective in decreasing estrogenic activity of applied swine wastewater. Similar to the work by Nichols et al. (33) in which plantings of grass filter strips were reported to decrease estradiol runoff postapplication of poultry litter, this system uses “nature” to ameliorate the endocrine-disrupting impact of agricultural practices. Through modification of husbandry facilities, the impact on the environment can be minimized. This swine facility not only conserved water usage by using recirculated gray water, but also produced a “product” that had decreased N loads and was below the proposed LOEC set by Wales and England and often below their proposed predicted NEC. Further research is needed to establish the seasonal efficacy of such a treatment system and the potential application in large concentrated feeding operations, where typical housing units may hold thousands of animals.

**Acknowledgments**

The authors recognize the excellent technical skills of Grant Harrington in operation of the $LC/MS$-$MS$. Dr. Heldur Hakk was our pioneer in method development for quantification of steroids by $LC/MS$-$MS$. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

**Supporting Information Available**

Detailed $LC/MS$-$MS$ conditions and pit data from 4/04 and 7/04 and correlation between $E$-screen and $LC/MS$-$MS$
estradiol equivalents. This material is available free of charge via the Internet at http://pubs.acs.org.

**Literature Cited**


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