

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

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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 (E_2), and testosterone concentrations (LC/MS–MS) were measured. A high correlation was found between estradiol equivalents determined by E-screen and LC/MS–MS ($R^2 = 0.82$). 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 E_2 concentration known to have an effect on fish (10 ng/L or $\sim 37 \times 10^{-12}$ 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 effects on egg 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 primary 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 \times 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

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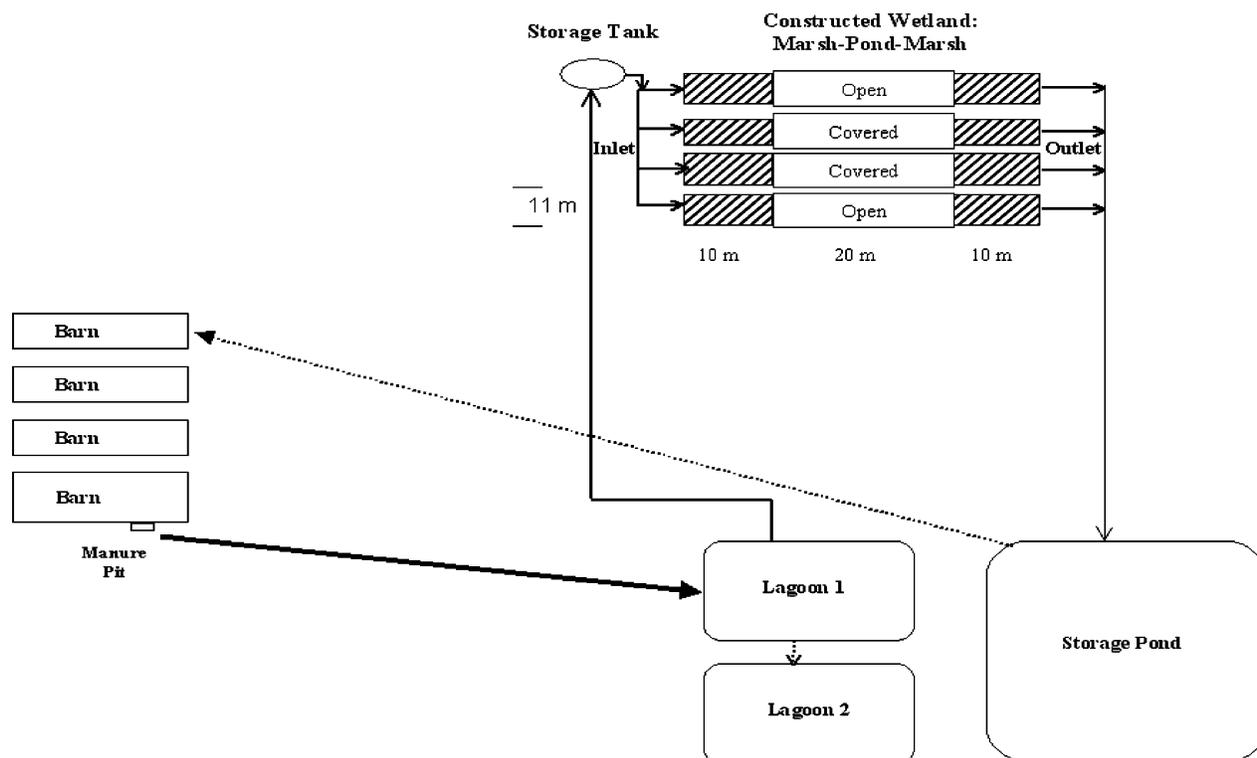


FIGURE 1. Diagram of swine wastewater handling system at North Carolina Agricultural & Technical State University, Greensboro, NC. Use of lagoon 2 was dependent on environmental factors, such as rainfall.

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 $\sim 130 \text{ m}^3$ of wastewater/cell. The wetlands were loaded at a rate of 10 to 20 kg N of $\text{ha}^{-1} \text{ d}^{-1}$ 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 $-20 \text{ }^\circ\text{C}$ 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). Briefly, samples were thawed, shaken, and allowed to settle for 1 h, then 250 mL was removed for solid-phase extraction on a cartridge containing a mixture of hydrophobic and hydrophilic packing (OASIS HLB, Waters, Milford, MA). Extracts were eluted with a series of organic

solvents, taken to dryness under N_2 , and resuspended for E-screen and LC/MS–MS analysis. Samples were tested by E-screen over a wide range of concentrations (15-fold concentrated to 128-fold diluted).

For LC/MS–MS analyses, extracts were diluted 1:1 with acetonitrile fortified with deuterated internal standards (20 $\text{pg}/\mu\text{L } d_4\text{E}_2$, 10 $\text{pg}/\mu\text{L } d_2\text{T}$) and protein-precipitated. Estradiol and its metabolites were analyzed by LC/MS–MS using a quadrupole-time-of-flight (Q-TOF) mass spectrometer (Waters, Beverly, MA) from a 10- μL injection of this preparation, whereas a solvent exchange was performed before analysis of androgens (see Supporting Information for details).

Ammonia, Total Kjeldahl N, Orthophosphate, and Chlorine Analysis. Acidified water samples (2005 collections) were analyzed for ammonia-N, total Kjeldahl nitrogen (TKN), and orthophosphate (PO_4) using EPA methods 350.1, 351.1, and 365.4, respectively (11). Samples from 2004 were not acidified, but were stored frozen at $-20 \text{ }^\circ\text{C}$, shaken after thawing, and allowed to settle for 1 h. The supernatant was then analyzed using the methods listed above, with the exceptions that ammonia-N was not assayed and chlorine was assayed by EPA method 325.1. Analyses were performed on automated analyzers (Technicon Instruments Corp., Tarrytown, NY and Bran+Lubbe Corp., Buffalo Grove, IL.)

Statistical Analysis. Data were analyzed by analysis of variance (ANOVA), regression, and least significant difference (LSD) using sampling dates as experimental replication (12). Estradiol equivalents of 2004 and 2005 data were compared on the basis of the assay: E-screen or LC/MS–MS.

Results and Discussion

2004 E-Screen and Nutrient Analyses. E-screen validation and sample extraction efficiency were previously reported (10). Proliferative responses of all samples were confirmed as estrogenic through cocubation with estrogen receptor antagonist ICI 182,780 (Tocris, Ellisville, MO). In no case were samples found to stimulate proliferation in the presence of the E_2 receptor antagonist. Limit of quantitation was $1 \times$

TABLE 1. E-Screen Evaluation of 2004 Samples^a

component	4/19/04	7/12/2004	11/23/2004
manure pit	843	858	ND ^b
lagoon 1	6.4	11.5	147
inlet wetland open	3.1	1.4	92.1
inlet wetland covered	2.4	2.2	104.1
outlet wetland open	1.8	2.8	7.8
outlet wetland covered	3.6	9.2	5.5
storage pond	2.9	0.6	2.1
lagoon 2	3.0	3.7	0.1

^a pM estradiol equivalents, mean of two extractions. ^b No data.

10⁻¹² M 17 β-estradiol equivalents (E₂Eqs) of extract applied to cells or 6 × 10⁻¹⁴ M E₂Eqs of original sample (as concentrated up to 15-fold). In July 2004, the samples from the storage pond and the effluent from the open wetland were toxic to cells at environmental concentrations, as assessed by cellular proliferation in the presence of added estradiol. Toxicity was not unexpected, as environmental samples represent a complex mixture of organic compounds. The estrogenic activity of these samples was evaluated on dilutions for which sample toxicity was minimal. Similarly, sample complexity resulted in matrix effects during LC/MS–MS analyses, substantially reducing detection of estrogens, testosterone, and their metabolites. Acetonitrile precipitation of the sample prior to LC/MS–MS analysis resulted in ~50% loss of internal standard signal (*d*₄E₂ or *d*₃T). Matrix effects were also evaluated by quantification of standards in the presence and absence of sample devoid of endogenous steroid. The matrix appeared to reduce ionization and subsequent detection. Often, environmental samples with low concentrations of steroids yielded peak areas below the lowest standards and were, therefore, below the limits of quantification. In contrast, when samples or standards were assayed in the presence of cell culture media, peak areas increased, indicating an increase in the ionization potential.

Estradiol equivalents for 2004 samples are presented in Table 1. The mean E₂Eq for April and July in the manure pit samples was 850 pM. The concentration 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 decreased 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 d⁻¹ (with a resultant average flow rate of ~ 9470 L day⁻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 E₂Eq. Mean estrogenic activity of wetland effluent was only 7% of the influent activity (8 and 6 pM E₂Eq 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-

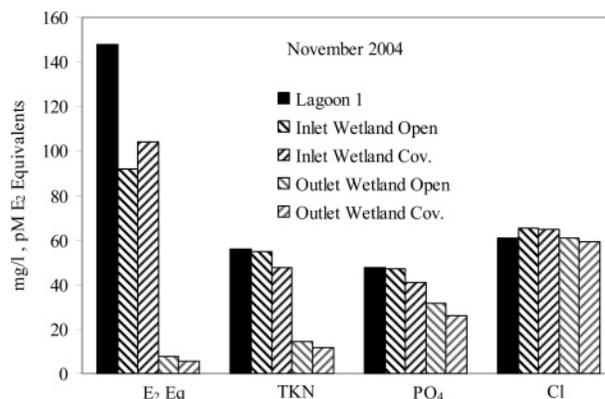


FIGURE 2. β17-Estradiol equivalents (E₂Eq) by E-screen and nutrient values of wastewater in November 2004. E-screen and nutrient analyses were performed on the same samples post particulate settling. Lowest observed effect concentration of estradiol (LOEC) cited as 10 ng L⁻¹ or ~ 37 pM.

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 (PO₄), 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, PO₄ appeared to decrease (19 and 16%, open vs covered). This decrease in PO₄ 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 PO₄ 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 E₂Eq (~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 PO₄ 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% E₂, 65% E₁). 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 (E₁), E₂, and estriol (E₃) 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 17 β-estradiol are E₂ = 1.0, E₃ = 0.1, and E₁ = 0.01 (2, 7). Of the compounds with the highest estrogenic

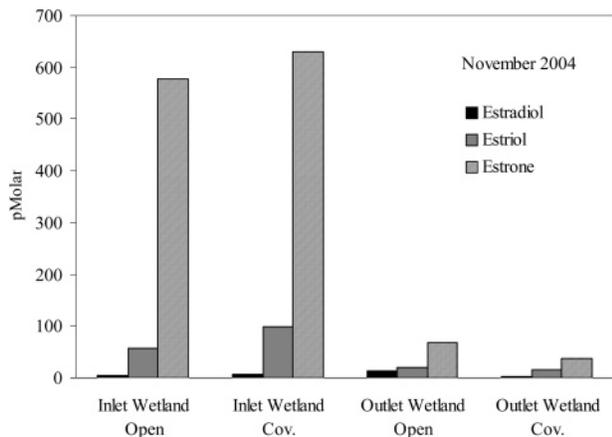


FIGURE 3. Wetland reduction of the concentration of estrogenic compounds in wastewater. Rate of N application was 20 kg of N ha⁻¹ d⁻¹. Samples from November 2004 were analyzed by LC/MS–MS.

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

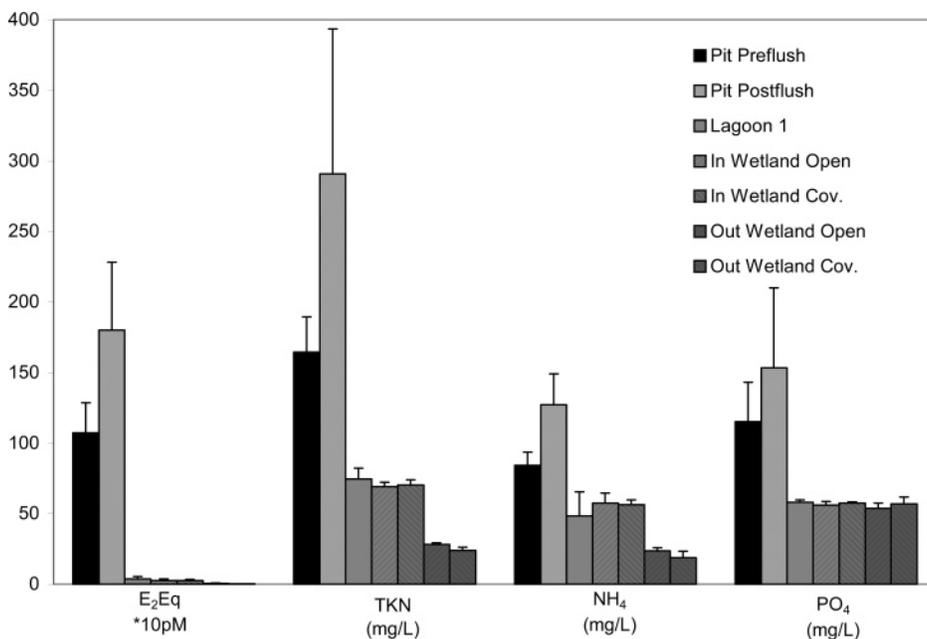


FIGURE 4. β 17-Estradiol equivalents (E₂Eq) by E-screen and nutrient values of wastewater from weekly sampling in July 2005. Values are mean ± S.E. Higher variability in pit samples was most likely a result of high particulate matter. Nutrient analyses were performed on samples that were acidified at the time of sampling without particulate removal.

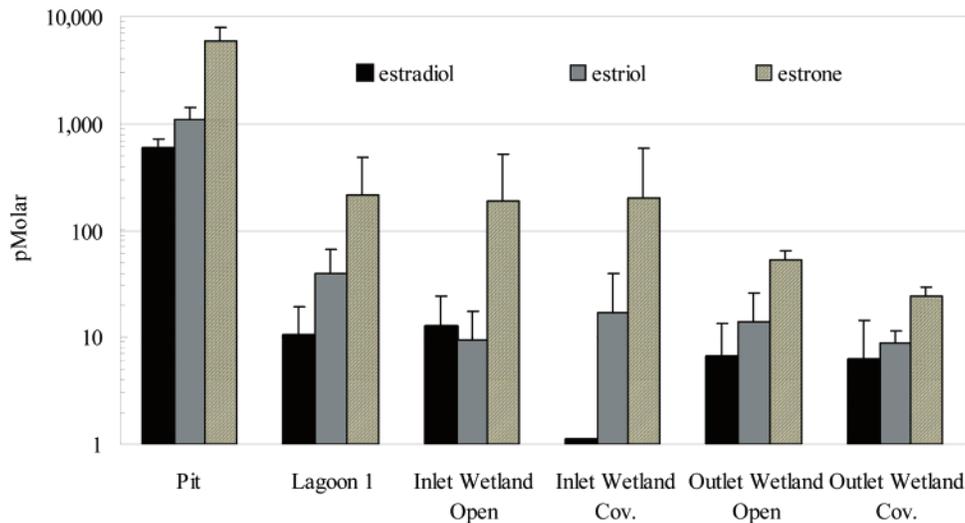


FIGURE 5. LC/MS-MS analyses of estrogenic compounds of wastewater from weekly sampling in July 2005. Values are mean \pm S.E. Androgenic compounds were not detected.

TABLE 2. Statistical Analysis of Wastewater for November, 2004 and July 2005 Sampling^a

compartment	E-screen (E ₂ Eq pM)	estradiol (pM)	estrone (pM)	estriol (pM)	TKN mg/L	NH ₄ mg/L	NO ₃ mg/L	PO ₄ mg/L
manure pit	1213 \pm 551	565 \pm 430	7364 \pm 4258	1475 \pm 1228	185 \pm 115	86 \pm 46	0.8 \pm 1.25	110 \pm 63
lagoon 1	60 \pm 55	11 \pm 8	298 \pm 305	32 \pm 30	71 \pm 12	48 \pm 21	0.1 \pm 0.07	55 \pm 7
pit vs lagoon ^b	0.05	0.05	0.05	0.10	0.10	0.10	0.10	0.10
inlet open	40 \pm 33	11 \pm 11	265 \pm 333	19 \pm 23	67 \pm 8	55 \pm 11	0.05 \pm 0.08	55 \pm 6
inlet covered	41 \pm 37	2 \pm 3	285 \pm 385	33 \pm 41	65 \pm 11	56 \pm 7	0.04 \pm 0.10	53 \pm 6
inlet open vs covered ^b	NS ^c	NS ^c	NS ^c	NS ^c	NS ^c	NS	NS ^c	NS ^c
outlet open	6 \pm 3.5	8 \pm 6.8	57 \pm 11.5	15 \pm 10.3	22 \pm 4.7	16 \pm 7.1	0.6 \pm 1.09	53 \pm 10.4
outlet covered	3 \pm 1.8	5 \pm 7.6	27 \pm 7.4	10 \pm 3.9	25 \pm 8.2	21 \pm 9.0	0.7 \pm 0.82	50 \pm 10.6
outlet open vs covered ^b	0.10	NS ^c	0.01	0.10	NS ^c	NS ^c	NS ^c	NS ^c
inlet	41 \pm 33.0	6 \pm 9.1	275 \pm 339	26 \pm 32.2	66 \pm 9.0	55 \pm 9.0	0.04 \pm 0.04	54 \pm 54.0
outlet	5 \pm 3.1	6 \pm 7.0	42 \pm 18.0	13 \pm 7.8	23 \pm 6.5	18 \pm 8.0	0.61 \pm 0.91	52 \pm 10.0
inlet vs outlet ^b	0.01	NS ^c	0.01	0.10	0.01	0.01	0.05	NS ^c

^a First two lines in set are means \pm standard deviations; third line, *p* value; *n* = 5 sampling events. ^b Levels of significant differences by least significant difference (LSD). ^c 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 2004 E₂Eq influent concentration exceeded the 2005 E₂Eq influent concentration by more than 3-fold. The values for 2005 reflect the mean of four samplings. Again, the wetland effluent E₂Eqs were all < LOEC, and six of eight samples were < pNEC for estradiol.

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 profiles of the trio of estrogenic compounds nearly identical to those found in July 2004. The inverse relationship between relative estrogenic potency of the compound and its concentration was repeated. In pit samples, estradiol was present in the lowest concentration, followed by E₃, and both were overwhelmed by E₁ (~600, ~1100, and ~6000 pM, respectively). In the primary lagoon, the order of compounds remained, but the concentrations fell to 2-4% of the pit values, similar to what was seen in April and July 2004 data (lagoon values often fell below the limits of quantification; therefore, 2004 data not shown). 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).

No significant differences in concentration were found between covered and open inlet values for any parameter 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 E₂Eq for

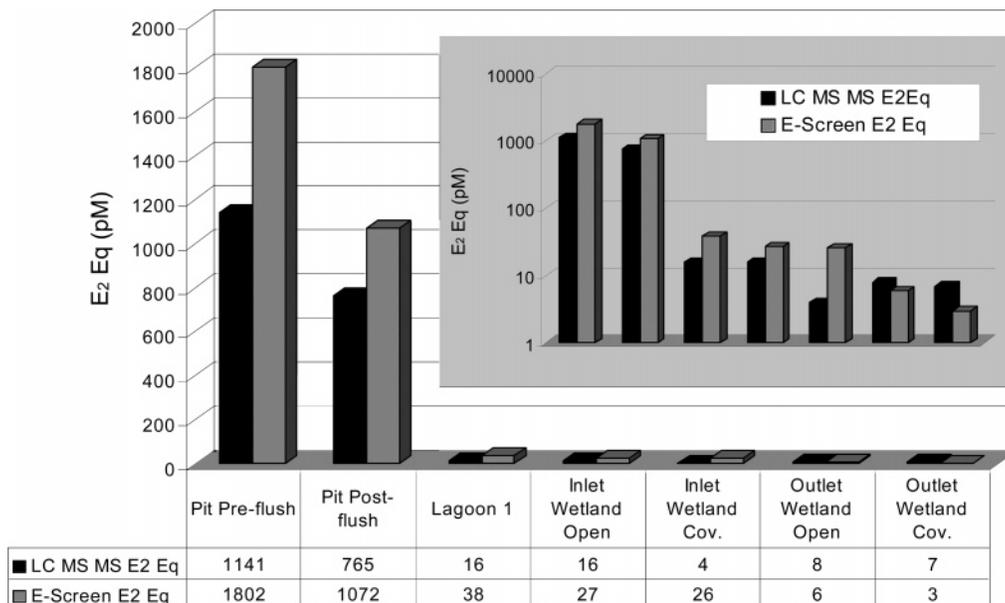


FIGURE 6. Comparison of β 17-estradiol equivalents of swine wastewater by LC/MS–MS versus E-screen for July 2005 sampling. Estrogenic equivalents were calculated as 0.1 for estriol and 0.01 for estrone. Inset presented on Y axis is log scale.

lagoon vs 40 for the inlet mean, with the actual difference equal to the minimal LSD of 19 for $P = 0.10$. There was a significant difference in the E_2Eq by E-screen and in the concentrations of estrone and estril between outlet samples from wetlands with the open pond versus wetlands with the covered pond. As wastewater moved through the wetlands, estrogenic compounds were significantly decreased as quantified by E-screen or by LC/MS–MS ($P \leq 0.10$). One exception was estradiol, which was already present in concentrations near the limits of detection at the inlet. Of the nutrient parameters, the concentration of all nitrogen parameters was significantly decreased by the wetlands, whereas orthophosphate concentration remained unchanged.

Comparison of LC/MS–MS versus E-screen E_2Eqs 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_2Eqs of sewage treatment plant effluents, typically with results falling within an order of magnitude of each other. Agreement of E_2Eqs assessed by YES assay and MS–MS analysis has varied. Raman et al. (30) found good correlation ($R^2 = 0.94$) between E_2Eq 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_2Eq 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 not 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_2Eqs were within an order of magnitude of E_2Eqs 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_2Eqs were 0.5 by YES and ~ 0.1 by untransfected cellular assessment (alkaline phosphatase). Matthiessen et al. (20) found less agreement of E_2Eq by YES assay versus LC/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 (EE_2) 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.

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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

- (1) Guilette, L. J., Jr.; Gross, T. S.; Masson, G. R.; Matter, J. M.; Percival, E. H.; Wood, A. R. Developmental abnormalities in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **1994**, *102*, 260–688.
- (2) Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernandez, M. F.; Olea, N.; Serrano, F. O. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **1995**, *103* (Suppl 7), 113–122.
- (3) Nichols, D. J.; Daniel, T. C.; Moore, P. A., Jr.; Edwards, D. R.; Pote, D. H. Runoff of estrogen hormone 17 β -estradiol from poultry litter applied to pasture. *J. Environ. Qual.* **1997**, *26*, 1002–1006.
- (4) Lange, I. G.; Daxenberger, A.; Schiffer, B.; Witters, H.; Ibarreta, D.; Meyer, H. H. D. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. *Anal. Chim. Acta* **2002**, *473*, 27–37.
- (5) Tilton, F. W.; Benson, H.; Schlenk, D. Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. *Aquat. Toxicol.* **2002**, *61*, 211–224.
- (6) Routledge, E. J.; Sumpter J. P. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **1996**, *15*, 241–248.
- (7) Gutendorf, B.; Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **2001**, *166*, 79–89.
- (8) Tremblay, L.; Fisher, P.; Leusch, F. Evaluation of the potential sodium monofluoro (1080) and fluorocitrate to bind to the estrogen receptor. *Australas. J. Ecotoxicol.* **2004**, *10*, 77–83.
- (9) Reddy, G. B.; Hunt, P. G.; Phillips, R.; Stone, K. C.; Grubbs, A. Treatment of swine wastewater in marsh-pond-marsh constructed wetlands. *Water Sci. Technol.* **2001**, *44* (11–12), 545–550.
- (10) Shappell, N. W. Estrogenic activity in the environment: municipal wastewater effluent, river, ponds, and wetlands. *J. Environ. Qual.* **2006**, *35*, 122–132.
- (11) Kopp, J. F.; McKee, G. D. *Methods for chemical analysis of water and wastes*; Rep. EPA-600/4-79020. Environmental Monitoring and Support Lab, Office of Research and Development, U.S. Environmental Protection Agency: Cincinnati, OH, 1983.
- (12) SAS Institute, 1999. The SAS system for Windows. Release 8.02. SAS Institute: Cary, NC.
- (13) Young, W. F.; Whitehouse, P.; Johnson, I.; Sorokin N. *Proposed predicted no effect concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters*. Environment Agency R & D Technical Report P2-T04./1; England and Wales Environment Agency: Bristol, 2002; pp 93–95.
- (14) Poach, M. E.; Hunt, P. G.; Reddy, G. B.; Stone, K. C.; Johnson, M. H.; Grubbs, A. Swine wastewater treatment by marsh-pond-marsh constructed wetlands under varying nitrogen loads. *Ecol. Eng.* **2004**, *23*:165:176.
- (15) Hunt, P. G.; Szogi, A. A.; Humenik, F. J.; Rice, J. M.; Matheny, T. A.; Stone, K. C. Constructed wetlands for treatment of swine wastewater from an anaerobic lagoon. *Trans. Am. Soc. Agric. Eng.* **2002**, *45*, 639–647.
- (16) McCaskey, T. A.; Britt, S. N.; Hannah, T. C.; Eason, J. T.; Payne, V. W. E.; Hammer D. A. Treatment of swine lagoon effluent by constructed wetlands operated at three loading rates. In *Constructed Wetlands for Animal Waste Management*; Dubow, P. J., Reaves, R. P., Eds.; Purdue Research Foundation: West Lafayette, IN, 1994, pp 23–33.
- (17) Raman, D. R.; Williams, E. L.; Layton, A. C.; Burns, R. T.; Easter, J. P.; Daugherty, A. S.; Mullen, M. D.; Sayler, G. S. Estrogen content of dairy and swine wastes. *Environ. Sci. Technol.* **2004**, *38*, 3567–3573.
- (18) Servos, M. R.; Bennie, D. T.; Burnison, B. K.; Jurkovic, A.; McInnis, R.; Neheli, T.; Schnell, A.; Seto, P.; Smyth, S. A.; Ternes T. A. Distribution of estrogens, 17 β -estradiol and estrone in Canadian municipal wastewater treatment plants. *Sci. Total Environ.* **2005**, *336*, 155–170.
- (19) Cockrem, J. F.; Rounce, J. R. Faecal measurements of oestradiol and testosterone allow the non-invasive estimation of plasma steroid concentrations in the domestic fowl. *Br. Poult. Sci.* **1994**, *35*, 433–443.
- (20) Matthiessen, P. D.; Arnold, A. C.; Johnson, T. J.; Pottinger, T. G.; Pulman, K. G. T. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. *Sci. Total Environ.* **2006**, *367*, 616–630.
- (21) Colucci, M. S.; Bork, H.; Topp, E. Persistence of estrogenic hormones in agricultural soils: I. 17 β -estradiol and estrone. *J. Environ. Qual.* **2001**, *30*, 2070–2076.
- (22) Lee, L. S.; Strock, T. J.; Sarmah, A. K.; Suresh, P.; Rao, C. Sorption and dissipation of testosterone, estrogens, and their primary transformation products in soils and sediment. *Environ. Sci. Technol.* **2003**, *37*, 4098–4105.
- (23) Lee, H. B.; Liu, D. Degradation of 17 β -estradiol and its metabolites by sewage bacteria. *Water Air Soil Pollut.* **2002**, *134*, 353–368.
- (24) Onda, K.; Nakamura, Y.; Takatoh, C.; Miya, A.; Katsu, Y. The behavior of estrogenic substances in the biological treatment process of sewage. *Water Sci. Technol.* **2003**, *47*, 109–116.
- (25) D'Ascenzo, G.; Di Corcia, A.; Gentili, A.; Mancini, R.; Mastro-pasqua, R.; Nazaari, M.; Samperi, R. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. *Sci. Total Environ.* **2003**, *302*, 199–209.
- (26) Kolodziej, E. P.; Harter, T.; Sedlak, D. L. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. *Environ. Sci. Technol.* **2004**, *38*, 6377–6384.
- (27) Fine, D. D.; Breidenbach, G. P.; Price, T. L.; Hutchins, S. R. Quantitation of estrogens in ground water and swine lagoon samples using solid-phase extraction, pentafluorobenzyl/tri-methylsilyl derivatizations and gas chromatography-negative ion chemical ionization tandem mass spectrometry. *J. Chromatogr., A* **2003**, *1017*, 167–185.
- (28) Sarmah, A. K.; Northcutt, G. L.; Leusch, F. D. L.; Tremblay, L. A. A survey of endocrine disrupting chemicals (EDCs) in municipal sewage and animal waste effluents in the Waikato region of New Zealand. *Sci. Total Environ.* **2006**, *355*, 135–144.
- (29) Korner, W.; Spengler, P.; Bolz, U.; Schuller, W.; Hanf, V.; Metzger, J. W. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. *Environ. Toxicol. Chem.* **2001**, *20*, 2142–2151.
- (30) Raman, D. R.; Layton, A. C.; Moody, L. B.; Easter, J. P.; Sayler, G. S.; Burns, R. T.; Mullen M. D. Degradation of estrogens in dairy waste solids: Effects of acidification and temperature. *Trans. ASAE* **2001**, *44* (6), 1881–1888.
- (31) Aerni, H. R.; Kobler, B.; Rutishauser, B. V.; Wettstein, F. E.; Fischer, R.; Giger, W.; Hungerbühler, A.; Marazuela, M. D.; Peter, A.; Schönenberger, R.; Vögeli, A. C.; Suter, M. J. F.; Eggen, R. I. L. Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. *Anal. Bioanal. Chem.* **2004**, *378*, 688–696.
- (32) Le Guevel, R.; Pakdel, F. Assessment of estrogenic potency of chemicals used as growth promoter in in-vitro methods. *Human Reprod.* **2001**, *16*, 1030–1036.
- (33) Nichols, D. J.; Daniel, T. C.; Edwards, D. R.; Moore, P. A., Jr.; Pote, D. H. Use of grass filter strips to reduce 17 β -estradiol in runoff from fescue-applied poultry litter. *J. Soil Water Conserv.* **1998**, *53*, 74–77.

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