Estrogen Concentrations in Dairy and Swine Waste Storage and Treatment Structures in and around Tennessee

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Abstract. Naturally occurring estrogens in animal wastes may cause negative environmental impacts in some watersheds. However, there is little published data regarding the concentrations of these estrogenic compounds in full-scale animal waste treatment and storage structures, thus making risk assessment difficult. To address this knowledge gap, the research described in this paper explores estrogen concentrations in 19 animal waste storage and treatment structures at dairy and swine production facilities in and around Tennessee.

Samples have been collected from eight dairy and eleven swine facilities, representing a range of waste storage and treatment structures typical of Tennessee, and much of the southeastern US. The 17β-estradiol concentrations in all samples have been assayed in triplicate serial dilutions by means of an enzyme linked immunosorbent assay (ELISA), all samples have, or will be, assayed for conventional manure constituents, including total solids, volatile solids, ash, total Kjeldahl nitrogen (TKN), total phosphorus, potassium, and chemical oxygen demand. Concentrations of 17α-estradiol, 17β-estradiol, and estrone are being assayed by gas-chromatography mass-spectroscopy (GC-MS), and total estrogenicity is periodically checked with a recombinant yeast reporter assay. At the time of this writing, the GC-MS dataset was incomplete, so that this paper is focused on the 17β-estradiol ELISA data.

Several tentative conclusions can be drawn from the preliminary data presented here, as follow: (1) 17β-estradiol concentrations are highest in swine finishing hoop structure solids and swine farrowing pit slurries, with concentrations in excess of 20,000 ppt (parts per trillion) observed in both systems. Average 17β-estradiol concentrations in swine farrowing and finishing lagoons were less than 4000 ppt. A similar trend was seen in dairy systems, where 17β-estradiol concentrations in dry stacks averaged 10,000 ppt, while concentrations in lagoons and holding ponds were below 2000 ppt. (2) The mass-ratio of 17β-estradiol to TKN followed a different trend than did the raw 17β-estradiol concentrations. For example, swine farrowing lagoons had the greatest ratio (ca. 25 ppm), while swine fishing hoop structure solids and swine farrowing pit slurries had ratios four times smaller. We believe that the mass-ratio of 17β-estradiol to application-rate-limiting macronutrients is a better predictor of the estrogen emission risk than is the raw 17β-estradiol concentration in the waste. (3) There is spatial heterogeneity in 17β-estradiol distribution within most structures studied. In 9 of the 11 liquid systems studied, a positive correlation was observed between 17β-estradiol and depth; in dry-stack dairy systems, older parts of the stack had lower 17β-estradiol concentrations than did newer parts of the stack.

Keywords. estradiol, estrone, lagoon, dry-stack, holding-pond, water-pollution, manure, livestock-waste, animal-waste
Introduction

Estrogens are a broad group of steroidal hormones that are present in fish, birds and mammals. 17β-estradiol (designated E2) is the most potent, naturally occurring estrogenic compound (Nichols et al., 1998) and estrone (E1) is the ketone form of 17β-estradiol (Arcand-Hoy et al., 1998). 17β-estradiol and estrone are emitted into the environment through animal manure (Monk et al., 1975; Shore et al., 1993) and municipal wastewater (Routledge et al., 1998). The possibility that emissions of endogenous estrogens could cause environmental problems has been increasingly acknowledged. Belfroid et al. (1999) reported that women excrete 2-12 µg of 17β-estradiol and estrone per person each day. In some places, the amount of estrogens present in wastewater treatment facility effluent can be enough to result in the feminization of male fish (Purdom et al., 1994; Sumpter et al., 1995). It has been shown (Routledge et al., 1998; Sumpter et al., 1995) that male fish begin to produce female blood proteins when exposed to water containing estrogens with a concentration of between 10 and 100 ppt (parts per trillion).

Previous work in our lab (Dyer, 2001), has demonstrated the potential for biologically relevant concentrations of 17β-estradiol to be present in runoff from plots receiving dairy manure at rates sufficient to meet crop N requirements. Furthermore, Dyer’s work suggested that mass application rates of 17β-estradiol and estrone correlate with mass losses of these compounds in runoff, at least during the first runoff event after application. Specifically, Dyer observed 17β-estradiol in runoff when 17β-estradiol application rates exceeded 5.5 g ha⁻¹. While scattered reports of 17β-estradiol and estrone concentrations in animal wastes exist in the literature (Nichols et al., 1998; Nichols et al., 1997; Knight 1980; Monk et al., 1975), there is no comprehensive survey of these compounds in the concentrated animal wastes typical of storage and treatment structures at full-scale animal production systems. Such data is needed to make an estimate of the estrogen-pollution risk associated with various waste types, and to mitigate those risks if needed. The development of such a data set is the purpose of the work described herein.

Safety Emphasis

Aqueous manure slurry presents a drowning hazard and a pathogen-exposure hazard to research personnel. The safe collection of samples from the middle of holding ponds and lagoons was facilitated by the design and development of a remotely piloted vehicle (RPV) manure sampling boat that permitted collection of 1-L samples from the center of large waste lagoons, without requiring research personnel to embark in small boats. The RPV relied on off-the-shelf components used in radio-controlled scale-model boats. Details of the design and performance of the RPV will be forthcoming.

Materials & Methods

Sampling Protocols

Stored manure from eight dairy and eleven swine facilities were tested for estrogen concentrations during the winter months of a one-year period. Three wet dairy systems – lagoons and holding ponds – and five dry stacks with varying moisture content were sampled. Five wet swine farrowing systems – four pits and one lagoon – three wet swine finishing systems – all lagoons – and three dry swine finishing systems – hoop structure – were sampled.
To observe the spatial heterogeneity in each system, multiple samples were collected from each system, as described below:

**Lagoons and holding ponds**

Samples were collected from non-agitated holding ponds and lagoons. If the farm used a two-stage lagoon system, then samples were collected from the primary lagoon only. In each liquid storage system, a total of eight samples were collected. Four 1-L samples were collected along a depth profile in the center of the liquid storage area, using the RPV. The top and bottom samples were taken 0.3 m (1 ft) from the surface and from the bottom; the others were taken at 1/3 and 2/3 the total depth. Four additional samples were collected from surface locations: one sample from each corner of the area, if accessible. When the influent pipe was readily accessible, one sample was collected from the area immediately adjacent to the pipe, instead of from the closest corner to the pipe. The surface samples were collected with a 500-mL polyethylene dipper (14-242-5, Fisherbrand, USA).

**Pits under slatted floor**

Samples were collected from non-agitated pits under slatted floors in swine rearing facilities. When possible, samples were collected close to the pit outlet. Four 1-L samples were collected along a depth profile in the pit, using a remotely-triggered water sampler (JT-1, LaMotte Co., Chestertown, MD). The top and bottom samples were taken 0.3 m (1 ft) from the surface and from the bottom; the others were taken at 1/3 and 2/3 the total depth.

**Hoop structures**

For each hoop structure two representative locations were identified in the dunging area. At each of these locations, 500-g samples were collected from the top, middle, and bottom of the material, by means of a shovel and portable scale. The two samples from each depth were combined to form a composite sample for that depth. Samples were placed in plastic bags for acidification and transport, and were stored in plastic bottles when returned to the laboratory.

**Dry-stacks – solid**

Dry stacks were divided into new and old regions, and the age of the manure in the new and old regions was estimated, through conversation with the owner/operator of the facility. Three representative sample locations were identified within the old and the new areas. At each of these three locations, a 500-g sample from the top, middle, and bottom of the stack was taken using a shovel (sometimes assisted by a front-end loader); then the three samples were combined to form a composite sample for that depth. Samples were placed in plastic bags for acidification and transport, and were stored in plastic bottles when returned to the laboratory.

**Dry stacks – semisolid**

Sampling locations were determined as described for dry-stack solid systems above. Samples were collected with a 1-L container attached to the end of a 3-m (9-ft) pole. An upward-opening door allowed semisolid materials to push up through the sampler as the sampler moved down through the stack. As the sampler was raised, the door shut, thereby collecting a sample from the desired depth. If the sampler was unable to reach the bottom of the area then the deepest depth possible was collected and the depth was noted. Samples were placed in plastic bags for acidification and transport, and were stored in plastic bottles when returned to the laboratory.
**General sample handling procedures**

Immediately after each sample was collected, its pH was determined with colorimetric pH paper. Next, approximately 25 mL of 4N H$_2$SO$_4$ was added to the sample, to acidify the sample to a pH of approximately 2. The pH was checked again, and more acid was added if necessary. Samples were then placed in a cooler filled with ice and transported back to the lab, which took anywhere from 1 to 36 h.

Samples were extracted in triplicate. In the case of liquid samples, a 10-mL sub-sample was placed in a 40-mL glass vial, along with 10 mL of deionized water, approximately 100 µL of 6N NaOH, and 10 mL of ethyl ether. In the case of dry samples, a 5-g sub-sample was placed in 40-mL glass vial, along with 5 mL of deionized water, 3 drops of 6N NaOH, and 5 mL of ethyl ether. A four-step serial dilution (1:1, 1:3, 1:9, 1:27) was preformed for each triplicate sample to ensure that the samples would be in the range of the assay plate. For each structure, a blank, spike blank (100 ppb 17β-estradiol, 500 ppb estrone, in DI water), and three matrix spikes (100 ppb 17β-estradiol, 500 ppb estrone, in one of the samples) were assayed on an ELISA plate.

Wet and dry samples were shaken for two hours on a vertical shaker. One mL of ether was extracted from each sample and blown down with N$_2$ gas. Pellets and original samples were refrigerated at 4°C to prevent degradation. Dry and wet samples were tested for 17β-estradiol by using an ELISA immunoassay (Assay Design, Inc., Ann Arbor, MI), per the manufacturers instructions. Estrone, 17α-estradiol, and 17β-estradiol levels were also assayed with the GC-MS per the methods reported in Raman et al., 2001.

Samples were analyzed for total solids (TS), volatile solids (VS), ash, total Kjeldahl nitrogen (TKN), total phosphorus (TP) content and potassium (K) content, using Standard Methods (American Public Health Association et al., 1995).

**Results & Discussion**

A total of 111 samples were collected at eight different types of facilities representing a cross-section of swine and dairy production units typical of Tennessee, and much of the southeastern US (Table 1, following page).

At the time of this writing, all samples had been analyzed for 17β-estradiol by ELISA methods, and all had been run for TS, VS, ash, and TKN. However, GC-MS data for 17α-estradiol, 17β-estradiol, and estrone, as well as the TP and K data, were not fully completed at the time of this writing, and will not be discussed further. Because the ELISA data has not been fully cross-correlated with GC-MS results, the following results must be considered preliminary in nature.
### Table 1. Summary of sample collection locations.

<table>
<thead>
<tr>
<th>ID Code</th>
<th>Description</th>
<th>Number of structures sampled</th>
<th>Number of samples taken per structure</th>
<th>Total No. samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFIL</td>
<td>swine finishing lagoon</td>
<td>3</td>
<td>8 (4 surf. locations + 4 depths in middle)</td>
<td>24</td>
</tr>
<tr>
<td>SFIH</td>
<td>swine finishing hoop structures</td>
<td>3</td>
<td>3 (composite of 2 locations at 3 depths)</td>
<td>9</td>
</tr>
<tr>
<td>SFAL</td>
<td>swine farrowing lagoon</td>
<td>1</td>
<td>8 (4 surf. locations + 4 depths in middle)</td>
<td>8</td>
</tr>
<tr>
<td>SFAP</td>
<td>swine farrowing pit</td>
<td>4</td>
<td>4 (1 location; 4 depths)</td>
<td>16</td>
</tr>
<tr>
<td>DDM</td>
<td>dairy dry-stack storages – semisol</td>
<td>3</td>
<td>6 (composite of 3 old locations at 3 depths + composite of 3 new locations at 3 depths)</td>
<td>18</td>
</tr>
<tr>
<td>DDS</td>
<td>dairy dry-stack storages – solid</td>
<td>2</td>
<td>6 (composite of 3 old locations at 3 depths + composite of 3 new locations at 3 depths)</td>
<td>12</td>
</tr>
<tr>
<td>DHP</td>
<td>dairy holding ponds</td>
<td>2</td>
<td>8 (4 surf. locations + 4 depths in middle)</td>
<td>16</td>
</tr>
<tr>
<td>DLA</td>
<td>dairy lagoons</td>
<td>1</td>
<td>8 (4 surf. locations + 4 depths in middle)</td>
<td>8</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>19</strong></td>
<td></td>
<td><strong>111</strong></td>
</tr>
</tbody>
</table>

**Spike matrix samples**

Figure 1 (following page) illustrates the ELISA spike matrix results plotted against the ELISA raw sample results. If the 17β-estradiol ELISA kits were responding perfectly linearly, with zero cross-reactivity to estrone, then all points should have fallen on the line marked theoretical – a line with an intercept of 100,000 ppt, and a slope of unity (the x-axis range is only 1/10th of the y-axis range in Figure 1). The best-fit line through the data shows a slope of 0.68, and an intercept around 175,000 ppt, perhaps reflecting significant cross-reactivity with estrone. The spike matrix results actually overestimate the uncertainty of the method, because the spike matrix concentrations used were on the high end of the ELISA kit detection range, even at the highest dilution (1:27). Since the uncertainties associated with ELISA readings is greatest at either end of the measurement range, the spike matrix results had a high degree of uncertainty associated with them. The decision to select relatively high spike matrix concentrations was made to accommodate the GC-MS assay, more than the ELISA assay method. In the future, a wider dilution range will be used for the spike matrix samples to avoid this introduction of uncertainty.
Figure 1. Spike matrix results regressed on the measured concentrations in the samples prior to spiking. The large variations seen are caused by the spike concentrations being at the high end of the measurement range, where measurement errors are inherently large. Fit curve is close to theoretical curve; the increased intercept of fit curve probably reflects cross-reaction of test with estrone. See text for additional discussion.

17β-estradiol concentrations in manure slurries and solids

The 17β-estradiol concentrations determined by ELISA in the waste holding and treatment systems are summarized by type in Figure 2. The large error bars reflect the broad range of concentrations observed within each grouping, not large measurement errors. Since each ELISA measurement was made in triplicate, coefficients of variation were computed for each of the 111 samples. The coefficient of variation within triplicate samples was typically 10% or less, suggesting that the ELISA method was highly repeatable.
The average 17β-estradiol concentrations illustrated in Figure 2 must be interpreted with caution for several reasons. First, 17β-estradiol may be rapidly converted to estrone in the environment (Raman et al., 2001; Ternes et al., 1999). For this reason, the estrone concentrations must be considered before assigning an overall estrogenicity to each of the samples. Second, estrogen concentration alone is not a good metric for establishing the estrogen-pollution-potential of a particular waste. This is because other investigators (Dyer, 2001; Nichols et al., 1997) have shown that runoff estrogen quantities are correlated to the mass application rates of estrogen in animal manure.

The mass application rate of estrogen (e.g. mg m⁻²) depends on the mass application rate of manure, and on the estrogen concentration of the manure. Since manure application rates are typically set to achieve a particular mass application rate of N or P, the estrogen-pollution-potential of a particular waste may be defined by the ratio of estrogen to N or P. Since the TP data was incomplete at the time of this writing, only the 17β-estradiol to N ratios (as TKN) will be considered here. TKN concentrations varied significantly among the systems studied (2500 ± 3000 ppm, n = 111). The TKN concentrations were moderately well correlated with total solids concentrations ($r^2 = 0.66$) for the entire dataset, but poorly correlated with 17β-estradiol concentrations as assayed by ELISA ($r^2 = 0.14$). By dividing the 17β-estradiol concentration in each sample by the TKN concentration for that sample, it was possible to find the ratio of 17β-estradiol to TKN, and then to average these ratios for each of the eight systems studied, as shown in Figure 3:
Figure 3 demonstrates the wide (order of magnitude) variation in 17β-estradiol to TKN ratios. To put these values in context, consider that Dyer’s work (2001) can be interpreted to suggest that 17β-estradiol application rates of 5.5 g ha⁻¹ and greater will cause biologically relevant concentrations of 17β-estradiol in runoff. If a typical N application rate is 200 kg N ha⁻¹, then the critical [E2]:N ratio will be 28 ppm (5.5 g ha⁻¹/200 kg ha⁻¹). In the systems studied, only the SFAL structures had ratios this high. However, as with the other 17β-estradiol data presented herein, it must be noted that GC-MS cross-correlations are not complete, and that estrone concentrations are not yet available for the entire dataset. This preliminary data demonstrates that significant differences may exist in the estrogenic pollution potential of various wastes, and that some of these wastes may have 17β-estradiol to N ratios sufficiently high to cause concern. The data also suggests that if only 17β-estradiol is considered, most waste systems in TN have 17β-estradiol to N ratios considerably lower than that thought to produce 17β-estradiol in runoff.

**Impact of sample location on 17β-estradiol concentrations**

17β-estradiol was not distributed evenly in most of the structures studied. For example, in 9 of the 11 wet systems, 17β-estradiol concentrations were positively correlated with depth (Table 2, following page). This correlation may in part reflect the observed correlation between 17β-estradiol concentrations and total solids ($r^2 > 0.5$ for half of the farms studied). This idea is given further credence by the one system that showed a clear negative correlation between depth and 17β-estradiol concentration: it was the only highly crusted lagoon of the three. Perhaps more importantly, this spatial heterogeneity implies that care needs to be exercised when sampling waste storage structures with the intent to assign a representative 17β-estradiol concentration to the waste within the structure.
While depth strongly correlated with observed $17\beta$-estradiol concentration in wet systems, manure age correlated (negatively) with $17\beta$-estradiol concentrations in dry systems (Figure 4). Specifically, old samples in the two solid dry stack systems had $17\beta$-estradiol concentrations that were 31 and 89% lower than those from new samples. In contrast, old samples in the three

Table 2. Correlations between sample $17\beta$-estradiol concentration and sample depth for all 11 wet systems. SFIL – swine finishing lagoon; SFAL – swine farrowing lagoon; SFAP – swine farrowing pit; DHP – dairy holding ponds; DLA – dairy lagoons.

<table>
<thead>
<tr>
<th>Farm ID</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SFIL-1</td>
<td>0.52</td>
<td>0.27</td>
</tr>
<tr>
<td>SFIL-2</td>
<td>-0.45*</td>
<td>0.20</td>
</tr>
<tr>
<td>SFIL-3</td>
<td>0.69</td>
<td>0.48</td>
</tr>
<tr>
<td>SFAL</td>
<td>0.82</td>
<td>0.67</td>
</tr>
<tr>
<td>SFAP-1</td>
<td>0.91</td>
<td>0.84</td>
</tr>
<tr>
<td>SFAP-2</td>
<td>0.77</td>
<td>0.59</td>
</tr>
<tr>
<td>SFAP-3</td>
<td>0.68</td>
<td>0.46</td>
</tr>
<tr>
<td>SFAP-4</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>DHP-1</td>
<td>0.77</td>
<td>0.60</td>
</tr>
<tr>
<td>DHP-2</td>
<td>0.81</td>
<td>0.65</td>
</tr>
<tr>
<td>DLA</td>
<td>0.83</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* See text for discussion.

Figure 4. Impact of age on average $17\beta$-estradiol concentrations in dairy dry storage systems, showing loss of $17\beta$-estradiol in DDS systems contrasting with minor losses or even increases in measured $17\beta$-estradiol concentration in DDM systems. Percent changes, as well as approximate age difference between new and old, are listed for each system. DDM – dairy dry-stack storages – semisolid; DDS – dairy dry-stack storages – solid.
semisolid dry stack systems exhibited minor losses or even slight increases in measured 17β-estradiol concentrations when compared to new samples. These results could reflect differences in 17β-estradiol conversion rates between anaerobic and aerobic systems, but further study is warranted to test this hypothesis.

**Conclusion**

Several tentative conclusions can be drawn from the preliminary data presented here, as follow: First, average 17β-estradiol concentrations ranged from 1400 to 41,000 ppt in the waste storage structures studied. The highest average concentrations were observed in swine finishing hoop structure solids, while the lowest average concentrations were observed in dairy holding ponds. Second, the mass-ratio of 17β-estradiol to TKN followed a very different trend than did the raw 17β-estradiol concentrations, with swine farrowing lagoons having the greatest ratio (ca. 28 ppm), and swine fishing hoop structure solids having ratios four times smaller, even though they had the highest raw 17β-estradiol concentrations. We argue that the mass-ratio of 17β-estradiol to application-rate-limiting macronutrients is a better predictor of the estrogen emission risk than is the raw 17β-estradiol concentration in the waste. Third, there is large spatial heterogeneity in the distribution of 17β-estradiol within most structures studied. For example, in 9 of the 11 liquid systems studied, a positive correlation was observed between 17β-estradiol and depth; in solid dry-stack dairy systems, older parts of the stack had lower 17β-estradiol concentrations than did newer parts of the stack. (This reduction in 17β-estradiol was not observed in semisolid dairy dry-stacks, perhaps suggesting a role for aerobic activity in 17β-estradiol degradation.)

It is difficult to assess the estrogen pollution potential of any of the systems studied without the added information that will be forthcoming from the GC-MS estrone data. Once this data is available, and once a second set of 111 samples have been collected and run, it should be possible to make some more defensible statements regarding the estrogen pollution potential from concentrated animal wastes.

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