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Transport of Steroid Hormones, Phytoestrogens, and Estrogenic Activity across a Swine Lagoon/Sprayfield System

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Abstract

The inflow, transformation, and attenuation of natural steroid hormones, phytoestrogens, and estrogenic activity was assessed across the lagoon/sprayfield system of a prototypical commercial swine sow operation. Free and conjugated steroid hormones (estrogens, androgens, and progesterone) were detected in urine and feces of sows across reproductive stages, with progesterone being the most abundant steroid hormone. Excreta also contained phytoestrogens indicative of a soy-based diet; particularly daidzein, genistein, and equol. During storage in barn pits and the anaerobic lagoon, conjugated hormones dissipated, and androgens and progesterone were attenuated. Estrone and equol persisted along the waste disposal route. Following application of lagoon slurry to agricultural soils, all analytes exhibited attenuation within 2 days. However, analytes including estrone, androstenedione, progesterone, and equol remained detectable in soil at two months post-application. Estrogenic activity in the yeast estrogen screen and T47D-KBluc *in vitro* bioassays generally tracked well with analyte concentrations. Estrone found to be the greatest contributor to estrogenic activity across all sample types. This investigation encompasses the most comprehensive suite of natural hormone and phytoestrogen analytes examined to date across a lagoon/sprayfield system, and provides global insight into the fate of these analytes in this widely used waste management system.

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ASSOCIATED CONTENT

Supporting Information Available: This information is available free of charge via the Internet at <http://pubs.acs.org>.

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Keywords

Animal feeding operations; manure; lagoon; soil; effluent; estrogen; androgen; progesterone; isoflavones; conjugated hormones; isoflavones; yeast estrogen screen; T47D-KBluc

INTRODUCTION

With hundreds to thousands of livestock housed in confinement, a single animal feeding operation (AFO) may generate waste loads similar to that of a small city.¹ On swine AFOs in the United States (US), a common waste management method is the lagoon/sprayfield system, in which manure is flushed from barns into open-pit anaerobic lagoons. Wastewater (“slurry”) from lagoons is applied to agricultural fields as a fertilizer, providing an economical waste disposal strategy.² Among the numerous contaminants of concern associated with AFO waste are manure-borne steroid hormones^{3–5} and phytoestrogens,^{6, 7} which are considered endocrine disrupting compounds (EDCs). The detection of hormones^{8–11} and phytoestrogens¹² in water bodies adjacent to AFOs has implicated manure land application as a potential source of EDCs to the aquatic environment.

While synthetic hormones are widely administered to certain livestock (e.g. cattle) as growth promoters,¹³ the use of these substances is prohibited in US swine production. Therefore, all steroid hormones in swine excreta are endogenous to the livestock, and the initial mass loading of these analytes into swine AFO waste is a function of gender and reproductive status.¹⁴ For instance, pregnant sows are known to have two peaks in circulating estrogens: a transient spike at approximately day 25–35 of pregnancy, followed by a steady rise that begins at approximately day 60–70 and peaks immediately prior to parturition at approximately day 110–115. Following parturition, estrogen levels drop and remain low until estrus begins again.^{15, 16} Progesterone remains elevated throughout the entire sow gestational period, gradually declining in the days leading up to parturition.^{15, 16} Excreted hormones in sow urine and feces are shown to closely parallel the corresponding plasma levels,^{17, 18} and may be present in either free or conjugated (sulfate or glucuronide) forms.

Comparatively, phytoestrogens are endogenous to forage crops (e.g. soybeans and red clover), and are present in AFO waste as a result of dietary intake. Phytoestrogens include isoflavones and coumestans, and are defined as plant-based chemicals that may mimic or modulate the actions of natural estrogens.¹⁹ A relatively high proportion of ingested phytoestrogens and their primary metabolites may be excreted in urine and feces.²⁰ Accordingly, phytoestrogen-rich diets are demonstrated to increase estrogenic activity in manures.^{21, 22}

Following excretion by livestock, the environmental fate of these analytes is shaped by sorption, transformation, and degradation during waste storage and disposal. In a previous study,³ we reported the abundance and distribution of steroid hormones, phytoestrogens, and estrogenic activity in the anaerobic lagoon of a prototypical commercial swine sow AFO. In the current study, we expanded our analysis by re-examining this dataset in the context of analyte transport across the entire barn/lagoon/sprayfield system. Analyte inflow into the lagoon was assessed by quantifying concentrations in urine and feces from sows in four

defined reproductive stages, and in the barn pits that temporarily store waste before it is flushed into the lagoon. Attenuation in sprayfield soil was then studied over the course of two months following lagoon slurry land application. In all units of the farm, both free and conjugated forms of steroid hormones were quantified. Feed samples were additionally analyzed in order to assess phytoestrogen intake by sows. Analyte concentrations were quantified using liquid chromatography-tandem mass spectrometry (LC/MS-MS), and corresponding estrogenic activity of all samples was assessed using the yeast estrogen screen (YES) or T47D-KBluc *in vitro* bioassays.

MATERIALS AND METHODS

Field Site

The study site is a commercial sow breeding to farrowing AFO, located in southeastern North Carolina, with a waste management system prototypical of swine AFOs in the Southeastern and Midwestern US. The system can effectively be divided into four operational units, based on the movement of waste through the facility (Google Earth™ image of the site and diagram of the waste flow are provided in Supporting Information, Figures SI-1 and SI-2):

1) Barns—This AFO holds approximately 2500 sows, which are housed according to reproductive status. Sows in the breeding barn (B) are artificially inseminated upon estrus. In the heat check barn (H), sows are up to 28 days post-insemination, but have not yet had pregnancy confirmed. In the two gestation barns (G), sows are confirmed to be between 30 and 114 days pregnant. Finally, sows are moved to the farrowing barn (F) near the end of pregnancy, and remain there through parturition and approximately 14 days of lactation. After weaning, offspring are transferred to an offsite nursery facility, and sows are cycled back to the breeding barn.

2) Barn pits—Sow urine and feces falls through slotted floors into underground pits, which are filled with slurry from the lagoon. This slurry/excreta mixture, referred to herein as “barn flush”, is periodically (once every 1–2 weeks) flushed into the lagoon.

3) Lagoon—The anaerobic lagoon measures approximately 139 × 94 meters, with a total capacity of approximately 50 million liters. Waste in the lagoon consists of two phases: a wastewater (“slurry”) phase, and a bottom sludge phase.

4) Sprayfield—Lagoon slurry is applied during the growing season to onsite agricultural fields. Applications are carried out using spray irrigation under the guidelines of a nutrient management plan.²³

Sample Collection

Handling and storage of samples prior to extraction was designed to maximize sample collection efficiency and preserve analyte integrity. High density polyethylene (HDPE) and amber glass were utilized to collect liquid samples (urine, barn flush, lagoon). Both container types are demonstrated to have negligible impacts on integrity of steroid hormone

and other EDCs.²⁴ Likewise, analytes are not expected to leach or transfer from solid matrices (feces or soil) to plastic bags under the conditions used.

Urine and feces—Urine and feces were collected from between 5 and 13 sows in each barn on September 22, 2008; May 26, 2010; and March 22, 2011. Sows were sampled at random within each barn; urine and feces were not necessarily collected from the same sows. The number of samples varied depending on the number of available animals in each barn, with total numbers as follows: Breeding: 21 urine, 18 feces; Heat Check: 14 urine, 18 feces; Gestation: 28 urine, 30 feces; Farrowing: 22 urine, 18 feces. Urine was collected in new 1-liter HDPE wide neck bottles (Fisher Scientific, Waltham, MA), and feces was collected in new plastic bags. All samples were stored on ice immediately upon collection. Upon return to North Carolina State University (NCSU), urine was stored at 4°C and feces was stored at –20°C.

Barn flush—A 4-liter grab sample was collected from the effluent pipe of each barn on September 9, 2009; June 2, 2010; and April 6, 2011. Samples were collected in baked, solvent-rinsed amber 4-liter glass jugs (Fisher Scientific, Waltham, MA), stored on ice immediately upon collection, and stored at 4°C at NCSU.

Lagoon—Lagoon slurry and sludge were collected on June 15, 2009; April 14, 2010; and February 9, 2011. 24 slurry samples and 8 sludge samples were collected from a defined transect of the lagoon on each date, for a total of 72 slurry samples and 24 sludge samples. More details on the sampling protocol are in Supporting Information; see also Yost et al.³ Samples were collected in new 1-liter HDPE wide neck bottles (Fisher Scientific), placed on ice upon collection, and stored at 4°C at NCSU.

Soil—Soil sampling was conducted on one sprayfield, measuring approximately 20 hectares, with soil type ranging from sand to sandy loam. Soil was initially collected on February 9, 2011, prior to the land application of slurry. At this time, the field had recently been planted with wheat, and slurry had not been applied for several months. Sampling recommenced after slurry application on February 17, 2011. Samples were collected at 1, 2, 4, 5, 6, 7, 8, 13, 18, 21, and 61 days post-application from three coordinates across a transect of the field (see Figure SI-1). At each coordinate, soil was collected from the top 1 cm of the field, and a 15 cm soil core was also collected. Soil was collected in new plastic bags, stored on ice, and transferred to –20°C at NCSU. Rainfall data for this sampling period is provided in Figure SI-4.

Sow Diet—Single samples of gestation feed (fed to sows in breeding, heat check, and gestation barns) and farrowing feed (fed to sows in the farrowing barn) were acquired from the farm managers on March 22, 2011. Both types of feed are dry pellets (photo in Supporting Information, Figure SI-3).

Sample Processing and Solid Phase Extraction

Processing and extraction proceeded within 24 hours of sample collection. Analytes were extracted from filtered liquids using solid-phase extraction (SPE), and freeze-dried solids

were extracted using accelerated solvent extraction followed by SPE. Barn flush and lagoon samples were centrifuged, and aqueous and solid fractions extracted separately. Details on processing and extraction are provided in Supporting Information. To prevent potential bias from sample splits, LC/MS-MS and bioassay analyses were performed using aliquots of the same extract.

LC/MS-MS

The detailed LC/MS-MS procedure is provided in the Supporting Information. All analytes detected on the farm are listed in Table 1, and the complete list of analytes included in the LC/MS-MS methodology is provided in Table SI-1. Analytes included four natural estrogens, and associated sulfate and glucuronide conjugates (12 estrogen species total); four natural androgens, and associated conjugates (8 androgen species total); two natural gestagens; six phytoestrogens; and one mycoestrogen. Eight synthetic hormones, while not expected to be present in the waste, were additionally included for completeness.

YES Assay

Estrogenic activity of urine, feces, barn flush, and lagoon sample extracts were assessed using the YES assay (detection limit ~30 ppt EEQ). Details on assay procedure and data analysis are provided in the Supporting Information. In this assay, 17 β -estradiol (E2 β) served as dose-response standard, and the estrogenic potency of each sample was reported in terms of E2 β equivalents (EEQ). EEQ was calculated as the ratio of the concentration of E2 β that elicited half-maximal activity in the assay (EC_{50E2 β}) to the concentration factor (CF) of sample extract that elicited half-maximal activity in the assay (CF₅₀):

$$EEQ = EC_{50E2\beta} / CF_{50} \quad (\text{Equation 1})$$

CF refers to the fold concentration of extract relative to the original sample. For instance, extracting 500 ml of sample to yield 1 ml of extract would result in an extract with a CF of 500.

T47D-KBluc Assay

The estrogenic activity of many soil extracts was below the detection limit of the YES assay. Therefore, the T47D-KBluc assay (detection limit ~0.3 ppt EEQ) was instead used to quantify estrogenic activity in soil. Details on the assay procedure and data analysis are provided in Supporting Information. As with the YES, E2 β served as dose-response standard in the assay, and results were reported as EEQ. EEQ was calculated as the ratio of EC_{50E2 β} to the dilution factor of sample extract that elicited half-maximal activity in the assay (DF₅₀), adjusted to the CF of sample extract:

$$EEQ = EC_{50E2\beta} / (DF_{50} * CF) \quad (\text{Equation 2})$$

Estimated Potencies

Estimated potency (EP) represents estrogenic activity that is predicted based upon the analyte composition of a sample. Calculation of EP was based upon the relative estrogenic potency (REP) of detected analytes (Table 1). To determine REP, stock solutions of each analyte were prepared in ethanol at concentrations up to 1 g/l, and were assessed in the YES and/or T47D-KBluc assays alongside an E2 β standard. REP of each analyte (i) was calculated as follows (EC₅₀s determined using molar concentrations):

$$REP_i = EC_{50E2\beta} / EC_{50i} \quad (\text{Equation 3})$$

EP of each sample was then calculated by multiplying the concentration of each analyte by its respective REP, and summing these potency-adjusted values:

$$EP = \sum REP_i C_i \quad (\text{Equation 4})$$

where REP_i is the REP of a particular analyte, and C_i is the concentration of that particular analyte in a sample.

Data Presentation

In order to present a global assessment of analyte transport across the barns/lagoon/sprayfield, data across sampling rounds were pooled for analysis. Lagoon data, presented in our previous study as three individual rounds of sampling,³ are composited herein. For barn flush and lagoon samples, only the total concentrations (calculated based on the adjusted sum of aqueous and solid phase concentrations in each sample) are discussed in this analysis. See Supporting Information for details on this calculation.

Statistical Analysis

For urine and feces, ANOVA ($\alpha = 0.05$) followed by Tukey's post-test was used to determine the relationship between sow reproductive status and analyte concentrations. For all samples, bioassay-derived EEQs were compared to EPs using linear regression. Assessment was performed using GraphPad Prism version 6 for Mac OS X (GraphPad software, La Jolla, CA).

RESULTS AND DISCUSSION

Analytes in Sow Feed

LC/MS-MS analysis of the sow feed (Table SI-3) indicated that genistein (GEN) and daidzein (DAI) were the predominant phytoestrogens in the sow diet, with formononetin (FOR), biochanin-A (BIO), equol (EQU), and coumestrol (COU) present at lower levels. Because the diet is open formula, detailed information regarding feed ingredients was not available. However, as GEN and DAI are the major isoflavones in soybeans,²⁵ results suggest that soy is the primary source of phytoestrogens in this feed.

Analytes in Urine and Feces

While natural steroid hormones and phytoestrogens were ubiquitous across samples, synthetic hormones were not detected at all, and α -zearalanol (a mycoestrogen) was not detected in any sample collected in this study. Figure 1 summarizes analyte levels and EEQ in excreta of sows from four defined reproductive stages. Concentration and detection frequency of individual analytes are provided in Table SI-4 (urine) and SI-5 (feces).

As observed in Figure 1, relative hormone levels in sow urine and feces generally followed the rank order of progesterone (P4) > total estrogens > total androgens. Estrogens included E2 β , estrone (E1), 17 α -estradiol (E2 α) and estriol (E3). Androgens included testosterone (T), 11-ketotestosterone (11-KT), epitestosterone (EPI), and androstenedione (AN). Conjugated species included sulfate and/or glucuronide forms of E2 β , E1, E3, AN, and T. In urine, both sulfate and glucuronide species were present (Table SI-4); while, in feces, conjugated species were limited to the sulfate form (Table SI-5). E1 and AN were the most abundant estrogen and androgen species in the sow waste, respectively.

Phytoestrogens were also highly abundant in sow urine and feces, with total phytoestrogen concentrations exceeding that of steroid hormones by an order of magnitude or more (Figure 1). EQU was the most abundant phytoestrogen in urine, followed closely by DAI and GEN (Table SI-4). In feces, EQU was elevated several orders of magnitude above other species (Table SI-5). FOR, COU, and BIO were less abundant overall. Unlike the other phytoestrogen analytes, EQU is not present as an endogenous chemical in plants, but rather is formed as a product of DAI metabolism by fecal bacteria. Taken together, these data suggest that dietary DAI and GEN are attenuated in the sow during digestion, with EQU concomitantly formed as a metabolite. In support of this observation, sows have been shown to harbor intestinal bacteria that mediate the DAI to EQU transformation.²⁶ Furthermore, GEN is reported to be readily degradable by intestinal bacteria.^{27, 28}

Because production of reproductive hormones is tied closely to gestation,^{15, 16} excreted hormone levels were expected to differ between sow between the four sequential barns. In urine, estrogens and EEQ peaked in the gestation and farrowing barns, reflecting the increase in circulating estrogen during gestation. However, these trends were not statistically significant (ANOVA results in Tables SI-4), likely due to biological variability within the sampling cohort in each barn. Urinary P4 as well as several androgen species dropped significantly at farrowing, while urinary phytoestrogens did not exhibit any significant trends with respect to reproductive status. In feces, conversely, EEQ and the majority of hormone and phytoestrogen analytes were significantly elevated in the farrowing barn relative to other barns (ANOVA results in Tables SI-5). This accumulation of analytes may be due to the slow rate of fecal elimination by lactating sows, which is known to occur due to a high fat diet and *ad libitum* feeding.

Radiotracer studies indicate that swine excrete estrogens primarily through urine.²⁹ Here, on a parts-per-billion basis, we found that analyte concentrations were uniformly higher in feces versus urine (Figure 1; Tables SI-4 and SI-5). Because we did not measure total daily analyte excretion by the sows (i.e. over a 24 hour period), we are unable to directly compare

our results to those studies; however, our results clearly indicate that sow urine and feces both contain considerable levels of steroid hormones and phytoestrogens.

Analyte Transport across Operational Units

When concentrations are compared across the operational units of the AFO, it is evident that the transformation and attenuation of select analytes occurs during waste storage. Figure 2A presents the molar ratios of free and conjugated hormones across the operational units of the AFO, while Figure 2B–D summarize the concentrations of hormone and phytoestrogen species as well as EEQ. See Table SI-6 and SI-7 for average analyte concentrations and detection frequencies in barn flush and lagoon, respectively.

One trend that is apparent in this dataset is the dissipation of conjugated hormones during waste storage (Figure 2A). Much interest has been raised regarding the persistence of conjugates in AFO waste, as failure to quantify these species may result in underestimation of total hormone loads. Conjugated hormones have been reported to persist longer in anaerobic versus aerobic environments,³⁰ with one study reporting that up to 95% of the estrogen load in AFO lagoons was present in conjugated form.³¹ Comparatively, we find here that while conjugated species encompassed approximately 33% of steroid hormones in urine and 7% of steroid hormones in feces (taking both estrogens and androgens into account), conjugated species comprised only 0.04% and 0.01% of total steroid hormones in the barn flush and lagoon slurry, respectively. Of the numerous conjugated hormones identified in urine/feces (Table SI-4 and SI-5), only E1-3-S, E2 β -17-S, and AN-S were detected in barn flush and/or lagoon (Tables SI-6 and SI-7). The depletion of conjugates is presumably attributable to enzymatic hydrolysis by fecal bacteria in the waste stream, which has been demonstrated to occur in municipal waste systems.^{32, 33} It appears here that hydrolysis begins in the barn pits, as the raw excreta is stored in a pit filled with lagoon slurry.

Another trend evident in Figure 2A is the attenuation of P4 and androgen mass loads relative to steroidal estrogens. While P4 was the most abundant steroid hormone in urine and feces, concentrations of P4 in barn flush and the lagoon were lower than steroidal estrogens by several orders of magnitude. Androgens were similarly attenuated, with AN essentially the only androgen species detected in barn flush and lagoon. (See Figure 2C for concentrations.) These observations contribute to a growing body of evidence indicating that P4 and androgens are more labile than steroidal estrogens under environmental conditions. This has been documented in a variety of matrices, including soil microcosms,^{34, 35} composted poultry manure³⁶, cattle feedlot soil,³⁷ and municipal treatment plants,^{38, 39} and is speculated to be driven in part by the relative stability of the estrogen aromatic A-ring.⁵ To our knowledge, this is the first report of androgen and P4 attenuation during anaerobic lagoon storage. The depletion of androgens is consistent with the recent identification of bacterial strains in swine manure that putatively utilize T and AN as primary carbon sources.⁴⁰

Although the attenuation of total estrogens appears to be less than that of androgens or P4 (Figure 2A), it is evident that the composition of estrogen species shifts during waste storage (Figure 2B). In the barn flush and lagoon, E1 persisted at higher levels relative to other

steroidal estrogen species. This trend is remarkably similar to that reported by Zheng et al. in a dairy cattle AFO,⁴¹ and is likely due to the metabolic formation of E1 from E2 β and/or E2 α . Indeed, batch experiments find that E2 β and/or E2 α will undergo biotically-mediated reversible transformation under anaerobic conditions, resulting in formation of E1 with minimal net loss of total estrogens.^{42, 43} E1 is almost invariably reported to be the most abundant estrogen in AFO lagoons, regardless of livestock species,^{31, 41, 44} suggesting the widespread nature of this trend.

Finally, phytoestrogen concentrations are presented in Figure 2D. Although other studies have measured high levels of EQU in swine wastewater,^{6, 7} this is the first assessment of phytoestrogen transport across an entire AFO lagoon/sprayfield system. DAI and GEN appear to be attenuated in barn flush and the lagoon, while EQU persisted at far higher levels relative to other phytoestrogens along the waste disposal route. Given that fecal bacteria persist in anaerobic lagoons,⁴⁵ it is plausible that EQU-producing bacteria may mediate the formation of this compound not only in the intestine, but also during lagoon storage. While further studies are needed to verify the prevalence of this trend, it is possible that EQU persistence and/or formation along AFO waste disposal routes may be a widespread occurrence, much like the formation of E1.

Analyte Attenuation in Soil

Over the course of this study, cumulative steroidal estrogen concentrations in lagoon slurry averaged 11.5 $\mu\text{g/l}$, cumulative phytoestrogens averaged 79.9 $\mu\text{g/l}$, and EEQs averaged 5.42 $\mu\text{g/l}$ (Table SI-7c). These concentrations vastly exceed those affecting aquatic species. Predicted-no-effect-concentrations in fish have been placed at 1 ng/l for E2 β and 3–5 ng/l for E1,⁴⁶ while phytoestrogens may induce reproductive and behavioral effects in fish at concentrations as low as several hundred ng/l.^{47, 48} As well, some aquatic invertebrates may be susceptible to endocrine disruption at concentrations of estrogenic EDCs similar to those affecting fish.^{49, 50} To protect these populations, it is therefore critical that slurry-borne steroid hormones and phytoestrogens are retained and attenuated in sprayfield soil.

Figure 3 presents analytes and EEQ in the top 1 m of sprayfield soil, measured over a 2-month period following slurry application. Concentrations are provided in Table SI-8. As soil type and topography were similar across the sprayfield, the range in concentrations is likely indicative of field-scale variability ($n = 3$). Prior to slurry application, soil from the top 1 cm of the field was found to have residual levels of hormone analytes including E1 [from non-detect (ND) to 4 $\mu\text{g/kg}$; av 1.4 $\mu\text{g/kg}$], AN [from .049 to .140 $\mu\text{g/kg}$; av .086 $\mu\text{g/kg}$], and P4 [from .2 to .36 $\mu\text{g/kg}$; av .263 $\mu\text{g/kg}$] (dry mass concentrations). These are likely residues from a prior land application of lagoon slurry, which would have occurred during the previous growing season.

As expected based upon abundance in the lagoon, E1 was the most abundant steroid hormone in soil following slurry application (Figure 3A). On the day after slurry application (Day 1), E1 in the top 1 cm of soil was present at levels ranging from 11 to 49 $\mu\text{g/kg}$ [av 28.3 $\mu\text{g/kg}$]. By Day 2, E1 was attenuated from these initial levels [7.6 to 14 $\mu\text{g/kg}$; av 11.9 $\mu\text{g/kg}$], but then appeared to plateau and remain relatively stable over the course of the next 12 days. Further attenuation occurred between Days 13–18, with E1 dropping to .54 – 1.4

µg/kg [av .833 µg/kg] on Day 18. This was coincident with a series of rainfall events between days 8–18 (Figure SI-4), which may have liberated analytes from soil or stimulated microbe-mediated degradation. However, E1 remained detectable through the duration of the sampling season, with final concentrations at day 61 ranging from .041 to .990 µg/kg [av .387 µg/kg].

While E1 was detected in all soil samples across the sampling period, other estrogen species were scarce in comparison. E2β and E2α occurred at a lower frequency of detection, and at concentrations approximately an order of magnitude lower than E1. This perhaps indicates that E2β and E2α were rapidly transformed to E1 or non-detected metabolites in the aerobic environment of the soil, as has been demonstrated to occur in laboratory batch experiments.^{34, 51} E3 was also not initially detected following land application, but appeared in soil between Days 4–8. This suggests formation of E3 as a metabolite of other estrogen species, as has been documented in laboratory batch experiments.⁵²

Of the other steroid hormones, P4 and AN were detected in all samples throughout the duration of the study, with 11KT present at a lower levels (Figure 3B). E1-3-S, E2β-17-S, AN-S were also ubiquitous (Figure 3C), although at low levels relative to free species. At the end of the sampling period, AN, P4, E1-3-S, and AN-S remained in soil at detectable levels.

Levels of EQU and (to a lesser extent) DAI rose in soil following land application (Figure 3D). EQU was present in the top 1 cm of soil at levels ranging from ND to 1.3 µg/kg [av .760 µg/kg] before slurry application, and rose to 63 – 250 µg/kg [av 151 µg/kg] on the day following slurry application. EQU levels fluctuated considerably between samplings, but remained at average concentrations of at least 12 µg/kg until Day 18. At the end of the sampling period, EQU was still present at a level of .33 – 9 µg/kg [av 3.74 µg/kg]. Conversely, antecedent levels of several phytoestrogens (BIO, FOR, and COU) did not increase following slurry application, and are likely present in soil due to input from plants, rather than manure.

Throughout the two month sampling period, manure-borne analytes and EEQ in the 15 cm soil core were approximately an order of magnitude lower than respective levels in the top 1 cm of soil, with many analytes not detected at all in the soil core (Table SI-8). This magnitude of attenuation suggests that these analytes were largely retained within the upper level of the soil following slurry application.

Bioassays versus LC/MS-MS

As demonstrated in Figures 1–3, EEQs exhibited the same overall trends as estrogenic analytes in the sow waste stream, closely paralleling steroidal estrogen concentrations throughout the system. Linear regression of EEQs versus EPs across all samples (Figure 4) yielded a strong correlation ($R^2 = 0.8344$; $p < 0.0001$), intersecting near the origin (x -intercept = 0.02062, y -intercept = -0.01919). This correlation between measured and predicted values further indicates that the analytes in the LC/MS-MS analysis accounted well for the estrogenic activity of these samples in the YES and T47D-KBluc, and supports the utility of these bioassays for tracking estrogenic analytes in field scenarios.

However, concordance between EEQ and EP varied between sample types. For instance, strong correlation was observed between EEQ and EP for urine ($R^2 = 0.7906$; $p < 0.0001$), while no linear relationship was present for feces ($R^2 = 0.01913$; $p = 0.2334$). Likewise, strong correlation was observed between EEQ and EP for barn flush aqueous phase samples ($R^2 = 0.7031$; $p < 0.0001$), while no relationship was observed for the barn flush solid phase samples ($R^2 = 0.003672$; $p = 0.8331$). In the lagoon, linear correlation was modest yet significant between EEQ and EP for slurry aqueous phase ($R^2 = 0.3197$; $p < 0.0001$), slurry solid phase ($R^2 = 0.1049$; $p = 0.0062$), sludge aqueous phase ($R^2 = 0.2103$; $p = 0.0242$), and sludge solid phase ($R^2 = 0.2117$; $p = 0.0237$) samples. For soil, good linear correlation was observed between the two values ($R^2 = 0.5095$; $p < 0.0001$).

Overall, the best agreement was observed when analyte concentrations spanned several orders of magnitude (e.g. urine), while weaker correlation was observed over narrow concentration ranges (e.g. lagoon). This is anticipated, as both LC/MS-MS and *in vitro* bioassays have an inherent margin of error in their quantifications. However, the poor linear relationship between EEQ and EP for feces is likely due to matrix interference in the YES assay, which may have increased measurement error. We observed that fecal extracts often appeared to be hindered by toxic and/or antagonistic factors, with some extracts unable to fully saturate receptor activation (data not shown). This is a contrast to other sample types, which generally had strong curve fits and no evidence of interference in the bioassays. It is unclear why a poor linear relationship between EEQ and EP was observed for barn flush solid extracts, as no matrix interference was observed for these samples.

Using REPs from the YES and T47D-KBluc bioassays (Table 1), the average contribution of each analyte to the EP was calculated (Table SI-9). Results indicate that E1 was the principle analyte contributing to estrogenic activity across all units of the AFO. E1 was responsible for an average of 66% (urine), 43% (feces), 69% (barn flush), 94% (lagoon slurry), 84% (lagoon sludge), and 94% (soil) of the calculated EP. E2 β , which is more potent but less abundant relative to E1, was found to be the second greatest contributor to estrogenic activity. Interestingly, phytoestrogens appeared to substantially enrich the estrogenic activity of urine and feces samples, but not samples in the distal units of the farm. In urine, for instance, GEN and EQU accounted for an average of 22% and 5.3% of the calculated EP, respectively; and in feces, EQU accounted for 37% of the calculated EP. Comparatively, EQU accounted for just 3.1% of the total barn flush EP, 0.23% of the total lagoon slurry EP, and 0.17% of soil EP on average. Thus, the attenuation of phytoestrogens during waste storage appears to curtail the relative estrogenic significance of these analytes.

Implications

This investigation examines the fate and transport of a highly comprehensive suite of natural hormone analytes across a prototypic swine AFO, and is one of very few studies to assess phytoestrogens in this type of system. Hormone fate and transport observed here corresponded well with trends that have been documented in other waste management systems, including: 1) recalcitrance of steroidal estrogens to anaerobic degradation, with E1 likely formed during waste storage; 2) lability of P4 and androgens relative to estrogens; and 3) dissipation of conjugated hormones. Our observation that EQU persists and may be

formed along the waste disposal route corroborates previous observations on the abundance of EQU in AFO wastewater.^{6, 7} *In vitro* bioassays were generally an effective means of tracking these analytes, with estrogenic activity primarily driven by potent steroidal estrogens (i.e. E1).

Detection of hormones and phytoestrogens in soil prior to slurry application and at two months post-application suggest that these analytes persist longer than expected based on short laboratory half-lives (e.g. hormone half-lives reviewed by Lee et al.⁵). Indeed, several studies indicate that hormones may form stable residues with soil particles, resulting in sorption-limited degradation and persistence over time.^{52, 53} Sorbed analytes could be transported via soil erosion, which is increased in agricultural environments; or could be dissolved and mobilized by rainfall. Unfortunately, our field site does not have drains, wells, or adjacent surface waters, and thus we were unable to directly monitor the mobility of these analytes. Other field studies indicate that manure-borne steroid hormones may migrate from agricultural soil into surface water,^{8, 10, 11} although considerable attenuation in soil is generally observed.^{54–56} Tile drains, which are common in the Midwest, may facilitate hormone mobility from soil.⁵⁷ Some studies also find that hormones may leach from AFO soil into groundwater,^{58, 59} with enhanced transport occurring through preferential flow pathways.⁶⁰ Comparatively, data at our field site suggest that analytes had limited downward mobility in soil. Overall, it is likely that analyte mobility from AFO soil is highly dependent upon site-specific variables. In order to improve the estimation of sprayfield runoff in field scenarios with limited data, a subset of data from our study is being used to develop a predictive Bayesian network model.⁶¹

As the fate and transport of manure-borne steroid hormones and phytoestrogens is characterized, risk managers will be better poised to predict and determine the potential environmental impacts of these compounds. Results from this study may be used towards developing a global understanding of the fate and offsite movement of these analytes, and ultimately increase insight into the impact of agricultural practices on local and regional watersheds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Average analyte concentrations $\pm$ standard error (SEM) of the mean in A) urine and B) feces of sows from four defined reproductive stages. Concentrations of hormones and phytoestrogens (LC/MS-MS results) are shown on the left y-axis, while the corresponding EEQ (YES assay results) is shown on the right y-axis. For estrogens, androgens, and phytoestrogens, the concentrations shown here represent the sum of all relevant species detected, including both free and conjugated forms of estrogens and androgens. All concentrations are in parts-per-billion ($\mu\text{g/l}$ urine and $\mu\text{g/kg}$ dry mass feces).

Figure 2.

Analyte transport across the AFO. Figure 2A depicts molar ratios of free and conjugated estrogens, androgens, and P4 across the major operational units of the AFO, shown as a fraction of the total steroid hormone load. Figure 2B–D depicts average analyte concentrations + SEM in each unit of the AFO (in parts-per-billion; $\mu\text{g/l}$ or $\mu\text{g/kg}$ dry mass). Lines connect analytes that were detected in all units of the farm. Shown are: B) Concentrations of steroidal estrogens, with free and conjugated forms of each estrogen species summed. EEQ (YES assay results) is shown as a dashed line alongside estrogen concentrations; C) Concentrations of P4 and androgens, with free and conjugated forms of each androgen species summed; and (D) Concentrations of phytoestrogens. For barn flush, lagoon slurry, and lagoon sludge, total concentrations are shown (see Tables SI-6c and SI-7c for values).

Figure 3.

Concentrations of A) free estrogens and EEQ, B) progesterone and free androgens, C) conjugated hormones, and D) phytoestrogens in the top 1 cm of agricultural sprayfield soil over a two month period following slurry land application. All concentrations are $\mu\text{g/kg}$ dry mass. Floating bars show the range in concentrations detected on each day (minimum to maximum), $n = 3$.

Figure 4. Linear regression of EEQ (YES and T47D-KBluc assay results; Equations 1–2) and EP (calculated based on LC/MS-MS results; Equation 4) of all samples collected across the course of this study. Each data point represents an individual sample, with the black line representing the linear regression across all samples.

Table 1

All analytes detected on the AFO, abbreviations used in text, and corresponding relative estrogenic potencies in the YES and T47D-KBluc assays.

Analytes	Type of Compound	Abbrev.	REP (YES)*	REP (T47D-KBluc)*
17 β -estradiol	Natural estrogen	E2 β	1	1
Estrone	Natural estrogen	E1	0.47	0.61
17 α -estradiol	Natural estrogen	E2 α	0.029	0.0076
Estriol	Natural estrogen	E3	0.0076	0.059
Testosterone	Natural androgen	T	0.00003	0.0000065
Androstenedione	Natural androgen	AN	0.0000018	No activity
Epitestosterone	Natural androgen	EPI	0.000005	
11-Ketotestosterone	Natural androgen	11KT	0.000028	0.0000056
Progesterone	Natural gestagen	P4	No activity	No activity
Estrone-3-sulfate	Conjugated estrogen	E1-3-S	0.0014	0.013
Estradiol-3-sulfate	Conjugated estrogen	E2 β -3-S	0.000057	
Estradiol-17-sulfate	Conjugated estrogen	E2 β -17-S	0.0000079	0.00000042
Estriol-3-sulfate	Conjugated estrogen	E3-3-S	0.0000062	
Estriol-17-sulfate	Conjugated estrogen	E3-17-S	0.000038	
Estrone-3-glucuronide	Conjugated estrogen	E1-3-G	0.00053	
Estradiol-17-glucuronide	Conjugated estrogen	E2 β -17-G	0.012	
Estriol-3-glucuronide	Conjugated estrogen	E3-3-G	0.000043	
Testosterone sulfate	Conjugated androgen	T-S	0.00000097	
Androsterone sulfate	Conjugated androgen	AN-S	0.000012	0.000023
Testosterone glucuronide	Conjugated androgen	T-G	0.0000013	
Daidzein	Phytoestrogen	DAI	0.00000059	0.000019
Genistein	Phytoestrogen	GEN	0.00015	0.000048
Equol	Phytoestrogen	EQU	0.00023	0.000061
Coumestrol	Phytoestrogen	COU	0.009	0.000091
Formononetin	Phytoestrogen	FOR	0.0000011	0.0000013
Biochanin A	Phytoestrogen	BIO	0.000026	0.000029

* All detected analytes were tested in the YES assay, but only analytes detected in soil were tested in the T47D-KBluc assay. A subset of REPs in the YES assay were originally presented in Yost et al.³