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Comprehensive Assessment of Hormones, Phytoestrogens, and Estrogenic Activity in an Anaerobic Swine Waste Lagoon

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Supporting Information

ABSTRACT: In this study, the distribution of steroid hormones, phytoestrogens, and estrogenic activity was thoroughly characterized within the anaerobic waste lagoon of a typical commercial swine sow operation. Three independent rounds of sampling were conducted in June 2009, April 2010, and February 2011. Thirty-seven analytes in lagoon slurry and sludge were assessed using LC/MS-MS, and yeast estrogen screen was used to determine estrogenic activity. Of the hormone analytes, steroidal estrogens were more abundant than androgens or progesterone, with estrone being the predominant estrogen species. Conjugated hormones were detected only at low levels. The isoflavone metabolite equol was by far the predominant phytoestrogen species, with daidzein, genistein, formononetin, and coumestrol present at lower levels. Phytoestrogens were often more abundant than steroidal estrogens, but contributed minimally toward total estrogenic activity. Analytes were significantly elevated in the solid phases of the lagoon; although low observed log K_{OC} values suggest enhanced solubility in the aqueous phase, perhaps due to dissolved or colloidal organic carbon. The association with the solid phase, as well as recalcitrance of analytes to anaerobic degradation, results in a markedly elevated load of analytes and estrogenic activity within lagoon sludge. Overall, findings emphasize the importance of adsorption and transformation processes in governing the fate of these compounds in lagoon waste, which is ultimately used for broadcast application as a fertilizer.

INTRODUCTION

With increased awareness over recent decades of the potential for steroid hormones and other endocrine disrupting compounds (EDCs) to impact the reproductive physiology of aquatic species,[1,2] there has grown concern over the persistence of these compounds in human and livestock waste effluents. In particular, animal feeding operations (AFOs) have been implicated as potentially major sources of these compounds into the aquatic environment, because of the relatively minimal treatment that waste from these operations receives.[3,4] On swine AFOs in the U.S., a widely used method of waste management involves flushing livestock excreta into large, open-air anaerobic basins, termed lagoons. The wastewater (slurry) in the lagoon consists of an aqueous phase with a relatively high volume of suspended particulates. During holding in the lagoon, a settling process takes place in which the majority of solids deposit into a bottom sludge layer. Lagoon slurry is ultimately applied onto crop fields for its nutrient value, which is done throughout the year under the guidelines of a nutrient management plan.[5] Sludge is allowed to accumulate for many years into the life of the lagoon, at which point it may be removed and land-applied.[6] In both cases, no additional treatment is generally performed on the waste prior to land application.

Of the hormones known to occur in the waste of swine operations, steroidal estrogens have received the most attention as potential contaminants of concern. These compounds, which are produced naturally by livestock and eliminated in urine and feces,[7] are potent EDCs and have a well-established linkage with the development of intersex and other reproductive dysfunctions in exposed aquatic organisms.[8] Steroidal estrogens have been found to persist at appreciable levels in AFO lagoons,[3,9,10] and several studies have demonstrated the mobility of these compounds via surface runoff and leaching following the land application of lagoon slurry.[11−13] Other

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endogenous steroid hormones, including androgens and progesterone, have also been detected in swine waste,[7,14] although the occurrence and toxicological implications of these compounds have been considerably less studied. While it is known that both estrogens and androgens are often eliminated in urine as sulfate or glucuronide conjugates, very few studies have examined the persistence of conjugated hormones in AFO waste lagoons.[10] Although conjugated hormones have little to no biological potency, they are readily hydrolyzable to the free and active form,[15] and recent evidence indicates that hormone conjugates may have enhanced mobility and persistence in the environment relative to free steroid hormones.[16] Finally, another subclass of EDCs that has been largely understudied in waste lagoons is plant-derived phytoestrogens, which are estrogenic compounds that occur naturally in many forage crops and may enter the AFO waste stream via dietary intake by livestock.[17−19] Although the potency of phytoestrogens is considerably weaker than steroidal estrogens, multiple studies indicate the potential of these compounds to affect endocrine function in exposed aquatic organisms.[20−22] Notably, synthetic hormones (e.g., trenbolone) are widely administered in the cattle industry as growth promoters, and are commonly detected in cattle waste;[23] however, these pharmaceuticals are prohibited in US swine production, and are thus not discussed here.

Because all compounds in the lagoon carry an intrinsic risk of off-site transport following land application, it is important to understand the fate and occurrence of these EDCs within this widely used waste management system. To date, most studies of this topic have focused on limited suites of analytes. Few have quantified concentrations of analytes in whole AFO slurry (including suspended solids), and no studies to our knowledge have quantified analyte loads in anaerobic lagoon sludge. Here, the estrogen, androgen, progesterone, hormone conjugate, and phytoestrogen load of an anaerobic lagoon on a prototypical North Carolina commercial swine sow AFO is assessed in three independent rounds of sampling over a two year period. Within each round of sampling, analysis is made with regard to (1) aqueous versus solid phase concentrations, (2) spatial distribution in slurry and sludge, and (3) slurry versus sludge concentrations. Analytes were quantified using liquid chromatography-tandem mass spectrometry (LC/MS-MS). Additionally, because estrogen receptor activation is of interest as the principle mode of action of many of these analytes, estrogenic activity in the lagoon was determined using the yeast estrogen screen (YES).

MATERIALS AND METHODS

Sample Collection. All samples were collected from a single lagoon that receives waste from barns housing approximately 2500 breeding, gestating, or lactating sows. See Supporting Information for more details on the field site. Sampling was conducted on June 15, 2009, April 14, 2010, and February 9, 2011. Eight coordinates on the lagoon were chosen in order to create a representative cross-section of the site: 3 locations near the outflow pipes from the barns, 4 in the middle of the lagoon, and 1 at the far end of the lagoon. These same coordinates were sampled on all three dates. At each coordinate, 1-L samples were collected at 3 different depths of slurry (0.15 m below the surface, 0.6 m below the surface, and 0.15 m above the level of the sludge) using a horizontal beta water sampler (Wildlife Supply Company, Yulee, FL). Immediately prior to slurry sample collection, temperature, dissolved oxygen, and pH were measured using a Hydrolab multiparameter water quality instrument (Hach Hydromet, Loveland, CO) (Supporting Information Table SI-1). Sludge samples were then collected from a level of 0.3 m below the sludge/slurry interface, using a specialized sludge sampler that was constructed by the NCSU Department of Biological and Agricultural Engineering. In sum, a total of 24 slurry samples and 8 sludge samples were collected on each sampling trip. All samples were transferred to new 1-L HDPE wide neck bottles (Fisher Scientific, Waltham, MA), stored on ice immediately following collection, and moved to storage at 4 °C upon return to North Carolina State University.

Sample Processing and Solid-Phase Extraction. Sample processing proceeded within 24 h of collection. Fifty milliliter aliquots of unprocessed slurry samples were reserved for quantification of total suspended solids, which was performed by the NCSU Environmental and Agricultural Testing Service (EATS) (Supporting Information Table SI-1). All samples (slurry and sludge) were then centrifuged at 13 500 × g for 30 min at 4 °C to separate the bulk of the solids from the aqueous fraction of each sample. The pelleted solids were transferred to fresh containers for freeze-drying, and the aqueous fractions were centrifuged repeatedly at 13 500 × g for 15 min intervals at 4 °C until no further pellet was observed. After no more solids could be removed, the aqueous fraction of each sample was sequentially filtered through 2.0 and 1.2 μm pore size glass fiber filters (Millipore, 47 mm diameter), and lagoon solids were freeze-dried to dryness. Volume of filtered liquid and dry mass of solid were measured and recorded for each sample. Steroid hormones and phytoestrogens were then extracted from filtered liquids using solid-phase extraction (SPE), and from freeze-dried solids using accelerated solvent extraction (ASE) followed by SPE. For extraction method details, see Supporting Information. Fifty milliliter aliquots of filtered liquid from each sample were reserved for analysis of dissolved organic carbon (DOC), and 50 mg aliquots of freeze-dried solids were reserved for analysis of percent organic carbon (%OC), performed at NCSU EATS using high temperature combustion (Supporting Information Table SI-1). Recovery analysis (Supporting Information) indicated strong recovery of analytes from both aqueous and solid phases (Supporting Information Table SI-2).

LC/MS-MS. Quantification of analytes using LC/MS-MS was performed on all extracts at the U.S. Geological Survey (USGS) Organic Geochemical Research Laboratory (OGRL) in Lawrence, KS. Detailed LC/MS-MS procedure is provided in Supporting Information. The suite of analytes included four natural estrogens, and their associated sulfate and glucuronide conjugates (12 estrogen species total); four natural androgens, and associated conjugates (8 androgen species total); two natural progestagens; six phytoestrogens; and one mycoestrogen. Eight synthetic hormones, while not expected to be present in the lagoon, were additionally included for reference. All analytes detected in the lagoon are listed in Table 1, and a complete list of analytes is provided in Supporting Information Table SI-2. Supporting Information Table SI-2 shows the subset of the compounds associated with each analytical method along with a summary of compound information. LC/MS/MS systems, analytical columns, and mobile phases used are shown in Supporting Information Table SI-3.

YES Assay. The YES assay was performed on all extracts according to the method by Routledge and Sumpter[24] and modified as described in Chen et al.[25] 17β-Estradiol (E2β)

Table 1. List of All Analytes Detected in the Lagoon, Corresponding Abbreviations Used in the Text, and Relative Estrogenic Potencies (REP) in the YES Assay

analytes detected in lagoon	type of compound	abbreviation	REP
17β-estradiol	natural estrogen	E2β	1
estrone	natural estrogen	E1	0.47
17α-estradiol	natural estrogen	E2α	0.029
estriol	natural estrogen	E3	0.0076
androstenedione	natural androgen	AN	0.0000018
11-ketotestosterone	natural androgen	11KT	0.000028
progesterone	natural progestogen	P4	no activity
estrone-3-sulfate	conjugated estrogen	E1−3-S	0.0014
17β-estradiol-17-sulfate	conjugated estrogen	E2β-17-S	0.0000079
androsterone sulfate	conjugated androgen	AN-S	0.000012
genistein	phytoestrogen	GEN	0.00015
daidzein	phytoestrogen	DAI	0.00000059
formononetin	phytoestrogen	FOR	0.0000011
coumestrol	phytoestrogen	COU	0.00899
equol	phytoestrogen	EQU	0.00023

served as dose−response standard, and the estrogenic activity of each sample was reported in terms of E2β equivalents (EEQ) (Supporting Information equation SI-1). See Supporting Information for laboratory-specific details on the assay procedure and data analysis. LC/MS-MS and YES analyses were performed using aliquots from the same sample extracts to eliminate potential bias from sample splits.

Calculation of "Total" Analyte Levels and EEQs. Extracts of lagoon liquids and solids were analyzed separately using LC/MS-MS and the YES assay, allowing partitioning of analytes between aqueous and solid phases of the lagoon to be observed. To estimate analyte levels and EEQs within whole slurry and sludge samples, "total" analyte concentrations and EEQs were then calculated. To make this calculation, aqueous phase analyte concentrations and EEQ of each slurry and sludge sample were adjusted to the total volume of liquid in the raw sample, and solid phase analyte concentrations and EEQ were adjusted to the dry mass of solids in each sample. Adjusted aqueous and solid phase concentrations were then summed to calculate "total" values for each sample.

Estimated Potencies. To compare YES-derived EEQs to estrogenicity that would be predicted based on analyte composition, an estimated potency (EP) was calculated for each sample. The EP is the potency-adjusted sum of all estrogenic analytes detected in a sample, and it assumes that the response to these compounds in the YES assay is dose additive. To determine the EP, the relative estrogenic potency (REP) of each steroid hormone, hormone conjugate, or phytoestrogen was first derived in the YES assay (Supporting Information equation SI-2), using starting concentrations ranging up to 100 mg/L. All REPs were empirically determined for this study and are listed in Table 1. EPs were calculated by multiplying the total concentration of each analyte by its respective REP, and summing these products for each lagoon sample (Supporting Information equation SI-3).

Partitioning Ratios. Within each sampling round, particle−water partitioning ratios (K_D) were calculated for each analyte in the lagoon as a ratio of solid phase to aqueous phase concentration (Supporting Information equation SI-4). This calculation was performed for both slurry and sludge. From these values, the organic carbon−water partitioning ratio (K_{OC}) was then calculated for each analyte by normalizing K_D to the mass fraction of organic carbon in each sample (Supporting Information equation SI-5). Calculated log K_{OC} values were then compared to the predicted log K_{OC} of each analyte, which were determined using the EPI Suite software KOCWIN version 2.00 molecular connectivity index method.[26]

Statistical Analysis. Statistical analysis was performed using SigmaPlot (Systat Software, San Jose, CA). Within each round of samples, 3-way ANOVA (α = 0.05) was used to determine the relationship between slurry analyte concentrations/EEQs and phase (i.e., solid or liquid), slurry depth, and location in the lagoon. For sludge, which was only measured at a single depth, 2-way ANOVA was used to determine the relationship between sludge analyte concentrations/EEQs and phase and location. To compare slurry and sludge within each round of samples, average slurry analyte concentration in each phase (aqueous or solid) was compared to the respective concentration in sludge at the same location in the lagoon using paired t test. To compare YES and LC/MS-MS results within each round of samples, EEQ was compared to EP using paired t test. No statistical comparisons were performed between different sampling rounds, due to the time frame between sampling and that the likelihood that it does not represent a repeated measure.

RESULTS AND DISCUSSION

Spatial and Phase Distribution of Analytes. Assessment of slurry using three-way ANOVA indicates that depth and location within slurry have little to no effect on analyte concentrations or EEQs. Within each sampling round, ANOVA results (Supporting Information Table SI-12) demonstrate that there was occasionally a significant correlation between location or depth and concentration of individual analytes or EEQs in slurry; however, these correlations were isolated and infrequent, and no coordinates in the slurry were consistently associated with elevated or depressed analyte levels. Overall, results indicate that slurry analyte composition can be considered homogeneous at the time of each sampling, regardless of depth or distance from the barn outflow pipes. Similarly, ANOVA results for sludge (Supporting Information Table SI-13) did not indicate any significant relationship between analyte concentrations and location in the lagoon. Slurry and sludge data within each round of samples are therefore presented herein as average values.

Conversely, ANOVA indicated that phase (aqueous or solid) was a significant variable affecting analyte concentrations in both slurry and sludge in all rounds of sampling. As discussed in more detail below, the majority of analytes were found to be highly and significantly elevated in the solid phase relative to liquid.

Slurry Analyte Composition (LC/MS-MS). Figure 1 depicts a graphic of aqueous phase and solid phase analyte concentrations, and Supporting Information Table SI-4 provides a list of average concentrations ± coefficient of variation (CV) in slurry. Calculated total analyte concentrations ± CV are provided in Supporting Information Table SI-6. Of the 37 analytes assessed, 15 of these compounds were detected in the lagoon slurry (Table 1). In all three rounds of sampling, the natural estrogens estrone (E1), E2β, 17α-estradiol (E2α),

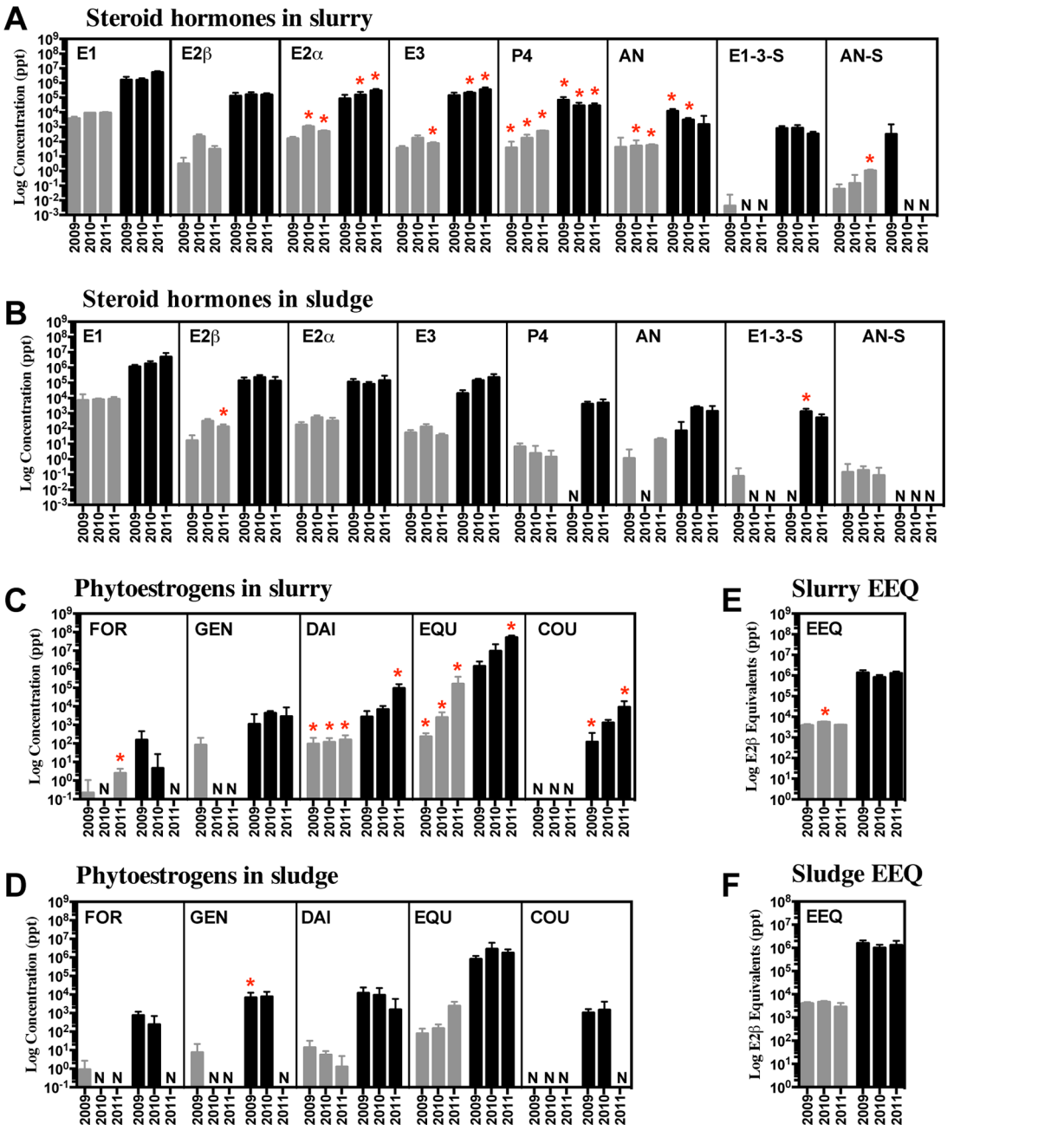

Figure 1. Mean ± standard error of the mean (SEM) analyte concentrations (LC/MS-MS results) and EEQs (YES results) in slurry and sludge across three rounds of sampling. Aqueous phase concentrations (in ng/l; gray bars) are shown alongside solid phase concentrations (in ng/kg; black bars); both are in parts per trillion (ppt). Significant difference between slurry and sludge is indicated with an asterisk; placement of the asterisk indicates the greater concentration. Concentrations and *p*-values are provided in Supporting Information. N = non-detect.

and estriol (E3) were found to be ubiquitous (Figure 1A). E1 was by far the most abundant of these compounds, occurring at total concentrations averaging 5632 ng/L, 10277 ng/L, and 12858 ng/L in June 2009, April 2010, and February 2011, respectively. Levels of the other estrogen species in both aqueous and solid phases averaged at least an order of magnitude lower relative to E1. Of the androgens assessed in this study, androstenedione (AN) was detected in all slurry samples (average total concentrations of 50−70 ng/L), while testosterone (T) and epitestosterone were not detected at all. Progesterone (P4) was the only progestogen detected, occurring at average total concentrations of 116−516 ng/l. Conjugated hormones were also pervasive, albeit at far lower levels in relation to free hormones. Estrone-3-sulfate (E1-3-S) was detected in all slurry samples, and androsterone sulfate (AN-S) was detected in most slurry samples, but average total concentrations of these hormone conjugates never exceeded 1 ng/l. Notably, a small subset (3 slurry samples) from April 2010 contained both the androgen 11-ketotestosterone and the conjugate 17β-estradiol-17-sulfate in addition to the suite of steroid hormone analytes listed above, but this was the only detection of these two additional compounds throughout the duration of this project (Supporting Information Table SI-4). Nine other natural hormone conjugates included in the analysis (Supporting Information Table SI-2) were not detected at all. There was no detection of free or conjugated synthetic

hormones, which are not used in U.S. swine production and were included in the analysis only for reference.

The magnitudes and relative abundance of steroidal estrogens observed here are consistent with previous studies of swine wastewater[10] and are likely indicative of the biotic and abiotic transformations of these compounds known to occur during lagoon storage. In addition to being one of the principle steroidal estrogens in fresh swine excreta, E1 is the primary metabolite of bacteria-mediated E2β and E2α oxidation,[27] and has been demonstrated to increase in concentrations along livestock waste disposal routes.[28] Numerous studies have reported E1 to be the predominant steroidal estrogen occurring in swine AFO waste,[9,10,19,29−31] as well as one of the most commonly detected steroidal estrogens in surface waters[8,32] and groundwater[29,33] at AFO-impacted sites. However, several studies of swine lagoons similar to our field site[10,29,31] determined relatively high concentrations of E3, and reported the relative abundance of estrogens to be E1 > E3 > (E2α) > E2β. This is contrary to findings at our study site, where levels of E3 were not particularly high in relation to other steroidal estrogens. Androgens are also known to also undergo biotic and abiotic transformations, with AN produced as a metabolite of T oxidation.[34,35] The absence of T and pervasiveness of AN observed here may be indicative of this transformation process.

The low level of conjugated hormones observed here is consistent with Hutchins et al.,[10] who reported that estrogen conjugates were more abundant in cattle and poultry waste relative to the lagoons of swine sow and swine finisher operations. Zitnick et al.[36] speculated that the trend observed by Hutchins at al. could be due to the design of many swine barns, including the barns at our field site, which allow urine and feces to fall directly into underground pits that have been backfilled with lagoon slurry; the slurry/excreta mixture sits in the pits for up to a week or more before being flushed into the lagoon. This design allows fecal bacteria to immediately begin mediating the hydrolysis of hormone conjugates, consequently diminishing the levels of these compounds in the lagoon slurry. Sulfate conjugates are more recalcitrant to hydrolysis compared to glucuronide conjugates,[10,15] so it is unsurprising that only sulfate-conjugated hormones are detected here.

Plant-derived phytoestrogens were also ubiquitous in slurry (Figure 1C), often occurring at higher levels than steroidal estrogens. The isoflavone metabolite equol (EQU) was by far the most abundant phytoestrogen species, with total slurry concentrations averaging 1750, 11570, and 193355 ng/L in June 2009, April 2010, and Feb. 2011, respectively. Daidzein (DAI) was the second most abundant phytoestrogen, detected in all slurry samples, albeit at concentrations (average 61−223 ng/l) that were 1 to 3 orders of magnitude lower than EQU. Genistein (GEN), coumestrol (COU), and formononetin (FOR) were present at total concentrations in the low ng/l range, while biochanin-A (BIO) was not detected at all. The mycoestrogen α-zearalanol was also not detected at all. To our knowledge, the fate of FOR, BIO, DAI, and COU have not been previously reported in AFO waste disposal systems, and thus this study marks the first report of these compounds in a swine lagoon. EQU concentrations observed here are somewhat lower than those reported by Burnison et al.,[18] who measured EQU at levels between 6.9 and 16.6 mg/L in sow waste from a commercial swine farm in Canada. Similarly, Furuichi et al.[19] measured EQU levels between 0.94 and 1.1 mg/L in untreated swine waste, and additionally reported that GEN levels in the waste were below the limit of detection. Unlike FOR, BIO, DAI, GEN, and COU, EQU is not present in plants, but rather is formed in the intestine as a product of DAI metabolism by fecal bacteria. DAI is prevalent in soybeans,[37] which are typically a staple of the sow dietary formula. Multiple strains of EQU-producing bacteria have been identified in swine feces,[38] and these strains may likely persist in the lagoon.[39] The overwhelmingly high levels of EQU relative to other phytoestrogens may indicate that bacteria mediate formation of this compound not only in vivo, but also during lagoon storage. To further investigate this trend, studies in our laboratory are underway to quantify the mass loading of steroid hormones and phytoestrogens from urine and feces on this sow AFO.

Overall, the same suite of analytes was observed within similar concentration ranges in all three sampling rounds, indicating that the flux of analytes through the lagoon system remains relatively consistent over time. The major exception is EQU, which increased in a defined gradient between the three samplings. Aqueous and solid phase EQU concentrations in Feb. 2011 were 2 orders of magnitude greater than those observed in June 2009. Because the three samplings were spread over an almost two year period, it is difficult to ascribe a cause to these temporal differences; for instance, as the sow diet is open formula, it is possible that variations in the diet over time could have influenced phytoestrogen levels in the lagoon. Sample collections did not coincide with major weather events, and dilution due to rainfall is unlikely to have affected the levels greatly, as the levels of suspended solids did not vary considerably between rounds of samples (Supporting Information Table SI-1). It is worth noting, however, that slurry temperature was progressively lower between the three rounds of sampling, averaging 27 °C, 20 °C, and 8 °C in June 2009, April 2010, and February 2011, respectively (Supporting Information Table SI-1); suggesting a possible negative relationship between temperature and concentrations of this analyte. The highest average concentrations of E1 and P4 were also observed in February 2011. Other studies of AFO waste[19,31] have noted higher estrogen levels in cool months relative to warm months, with one study[31] suggesting decreased photolysis and inhibition of bacteria-mediated degradation processes as a possible explanation for this trend. Here, however, it is imprudent to conclude that our observations over an almost two-year period are connected to temperature. Assessment of analyte levels over the course of a shorter time span would be necessary in order to make this correlation.

Sludge Analyte Composition (LC/MS-MS). Figure 1 depicts a graphic of sludge analyte concentrations, and Supporting Information Table SI-5 provides a list of average concentrations ± CV. Calculated total analyte concentrations are provided in Supporting Information Table SI-6. The same suite of analytes detected in slurry was also found in sludge. However, when analyte concentrations in slurry aqueous and solid phases are compared to the respective concentrations in sludge aqueous and solid phases using paired t test, it is evident that levels of many compounds (e.g., E2α, E3, AN, P4, AN-S, and phytoestrogens) in these separate phases were often significantly lower in sludge compared to slurry (Figure 1; Supporting Information Table SI-7). This suggests attenuation during sludge storage. In contrast, significant attenuation in sludge was not observed for E1 or E2β, suggesting that these steroidal estrogens are comparatively recalcitrant to anaerobic degradation. This observation is supported by findings of Zheng et al.[40] and Czajka et al.[41] that steroidal estrogens are

relatively stable over time in the anaerobic environment, and corroborates the observation by Combalbert et al.[30] that degradation of estrogens was not occurring under anaerobic conditions in the sludge of a swine waste treatment plant. Studies of municipal effluents have similarly reported that androgens (including T and AN) and P4 have higher removal efficiencies compared to steroidal estrogens.[42,43] Of note, %OC of sludge solids (av 24%) was considerably lower than that of slurry solids (av 41%) across all rounds of sampling, indicating decomposition of organic material in the sludge (Supporting Information Table SI-1).

Partitioning Ratios. With the exception of AN-S, concentrations of all analytes in slurry and sludge were found to be elevated several hundred- to several thousand-fold in the solid phase relative to the aqueous phase, with some compounds (COU, FOR, GEN, E1-3-S) often occurring exclusively within the solid phase of many samples (Figure 1 and Supporting Information Tables SI-4 and SI-5). For the majority of these analytes, the association with solids is expected based on the moderately lipophilic nature of steroid hormones (low K_{OW} = 2.5−3.9)[4] and phytoestrogens (log K_{OW} = 2.5−3.9)[44] and is generally in accordance with the few other studies that have examined the steroid hormone content of lagoon solids.[10,14,29,30,45] However, solid phase elevation of E1-3-S was not an expected result given the hydrophilic nature of this compound (log K_{OW} = 0.95).[26] Likewise, the solid phase elevation of E3 (log K_{OW} = 2.5) is in contrast to other studies[10,29] that reported minimal association of E3 with lagoon solids. It therefore cannot be ruled out that these compounds were not stable prior to sample extraction, despite efforts to keep samples at 4 °C, process samples quickly, and minimize aeration. If this were the case, it would have resulted in an underestimation of aqueous phase concentrations of these compounds.

Predicted and observed log K_{OC} values in each sampling round are provided in Supporting Information Table SI-8, and summarized in Figure 2. For some compounds, observed log

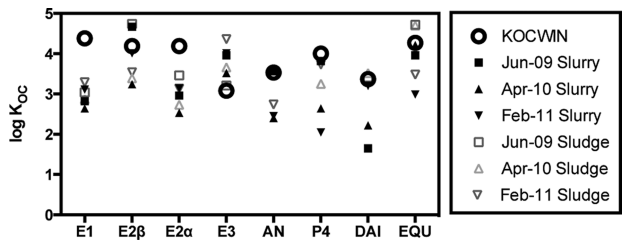

Figure 2. Log K_{OC} values for analytes estimated using EPI Suite KOCWIN software, and the corresponding mean log K_{OC} values calculated in lagoon slurry and sludge across the three rounds of sampling.

K_{OC} could not be calculated because the analyte was detected only the solid phase (i.e., E1-3-S, GEN, COU, FOR) or only in the aqueous phase (i.e., AN-S) of most samples. For the remaining analytes, log K_{OC} observed in the lagoon were almost always less than the respective values predicted using KOCWIN, indicating that the analytes may have enhanced solubility in the aqueous phase of the lagoon slurry and sludge. The exception to this trend was E3, for which observed log K_{OC} were consistently elevated above the predicted value. Temperature is expected to affect partitioning coefficients, and the KOCWIN prediction is based on partitioning at 25 °C. However, our observed trend was consistent across all rounds of sampling; which, as discussed above, spanned a wide range of lagoon temperatures. As recovery of analytes was generally high (greater than 70%) for the majority of analytes from both aqueous and solid phases (Supporting Information Table SI-2), partitioning ratios are unlikely to have been affected by the extraction process. One explanation for enhanced solubility could be the association of analytes with DOC in the aqueous phases of the lagoon, which ranged on average from 243 to 511 mg C/L in slurry to 219−725 mg C/L in sludge (Table SI-1). Steroidal estrogens have been shown to have an affinity for DOC in sewage sludge and manure,[46] and it is known that DOC can limit the sorption of hydrophobic compounds to suspended solids.[47] Another factor could be colloidal organic carbon (COC), which was not measured in this study, but is known to associate with steroidal estrogens in aquatic environments and waste effluents.[36,45,48] Association with DOC/COC has been indicated to limit the sorption of swine manure-borne estrogens to soil.[36]

Overall, sorbed analytes contributed appreciably to the total load of these compounds in slurry: on average, 16−36% of the total slurry steroid hormone load and 31−78% of the total slurry phytoestrogen load were found within the suspended solids. By definition, lagoon sludge contains a much larger mass of solids (av = 40% by mass, wet weight) compared to slurry (av = 0.8% by mass, wet weight). This large volume of solids, in concert with the aforementioned persistence of analyte species in the anaerobic environment, results in a markedly elevated total load of steroid hormones and phytoestrogens within the sludge. A comparison of the distribution of total analyte loads in the slurry and sludge is presented in Figure 3, and total concentrations ± CV are provided in Supporting Information Table SI-6. As can be seen in Figure 3, the accumulation of analytes in sludge was particularly evident for steroidal estrogens; which, as discussed above, demonstrated a greater degree of persistence in sludge relative to the other analytes.

Estrogenic Activity (YES Assay). Figure 1E-F depicts a graphic of aqueous and solid phase EEQs in lagoon slurry and sludge, and Supporting Information Table SI-9 and Table SI-10, provides a list of average EEQs and EPs ± CV in slurry and sludge. Trends in estrogenic activity generally mirrored the trends in analyte concentrations described above. As with analytes, a significant elevation of EEQs was observed in the solid phases of the lagoon relative to the aqueous phases (ANOVA results provided in Supporting Information Tables SI-11 and SI-12). With the exception of lagoon liquids in April 2010, paired t test indicated that EEQs in the separate aqueous and solid phases were not significantly attenuated in sludge relative to slurry (Figure 1 and Supporting Information Table SI-7). As a result of these factors, total EEQ loads were found to be highly elevated in sludge relative to slurry, as can be observed in Figure 3E.

YES analysis of individual compounds indicated that all analytes except P4 exhibited estrogenic activity at the concentration ranges tested (Table 1). As has been observed in other in vitro assays,[49] phytoestrogens in the YES assay exhibited estrogenic potency that was up to several orders of magnitude lower than that of steroidal estrogens. A graphic demonstrating concordance of total EEQs and EPs within each sampling season is provided in Figure 3E, and a statistical comparison of EEQs and EPs using paired t test is presented in Supporting Information Tables SI-9 and SI-10.

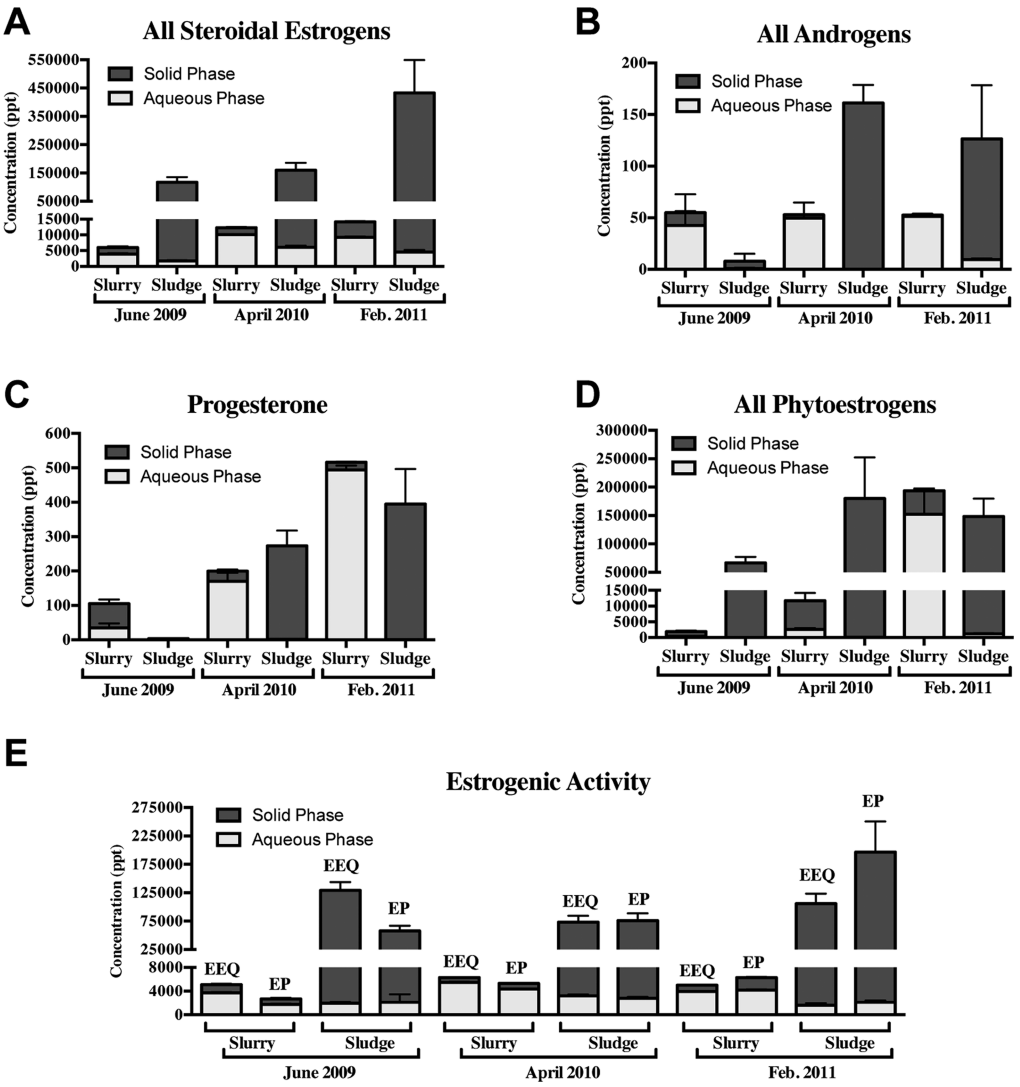

Figure 3. Total analyte concentrations and estrogenic activity (mean ± SEM) in slurry and sludge across the three rounds of sampling, showing the relative distribution of analytes and estrogenic activity between the aqueous and solid phases of each type of sample. (A) All free and conjugated estrogens (E1, E2β, E2α, E3, E1-3-S, E2β-17-S), (B) all free and conjugated androgens (AN, 11KT, AN-S), (C) progesterone, (D) all phytoestrogens (FOR, DAI, GEN, EQU, COU), and (E) EEQs/EPs.

In general, there was good agreement between EEQ and EP, although differences were evident between rounds of sampling. In June 2009, EEQs tended to be greater than their respective EPs. This difference was statistically significant in the slurry aqueous phase ($p < 0.001$), sludge aqueous phase ($p < 0.001$), and sludge solid phase ($p = 0.002$), but not for slurry solids ($p = 0.468$) from this sampling round. This trend, which has been observed in other studies of swine wastewater,[19,30] suggests that compounds not detected by LC/MS-MS analysis are contributing to total estrogenic activity in the lagoon. Greater concordance was observed in April 2010: EEQ was significantly greater than EP in the slurry aqueous phase ($p < 0.001$), but a significant difference was not observed for these values in slurry solid phase or in sludge. Conversely, EEQs in February 2011 were consistently less than corresponding EPs, indicating that activity in the YES may have been inhibited. This difference was significant in slurry aqueous ($p = 0.005$) and solid phases ($p < 0.001$) but not in sludge. No signs of toxicity to the yeast were observed (data not shown), making it unlikely that cytotoxicity contributed to this trend. However, it is noteworthy that the highest average levels of phytoestrogens, particularly EQU, were observed in February 2011 (Figure 1 and Supporting Information Tables SI-4, SI-5, and SI-6). It is feasible that high levels of phytoestrogens, which have low potency (Table 1) and low estrogen receptor binding affinity,[50] may have effectively inhibited activity in the YES by competing with potent estrogens for receptor binding sites. A recent study of phytoestrogens and estrogenic activity in cattle manure observed a similar trend.[17] However, this correlation is speculative. Overall, calculated EPs indicate that analytes quantified in this study accounted for 54%, 86%, and 134% of the total EEQ in slurry; and 45%, 103%, and 180% of the total EEQ in sludge; in June 2009, April 2010, and February 2011, respectively. This range of agreement between bioassay and analytical data is similar to what has been reported in other studies of swine waste.[19,30]

A table listing percent contribution of each individual analyte to the EP is provided in Supporting Information (Table SI-11). Overall, E1 was found to be by far the major analyte contributing to estrogenic activity in the lagoon. When all rounds of sampling are considered, E1 accounted for 81−95% of the calculated EP in slurry, and 78−97% of the calculated EP

in sludge. E2β was the second greatest contributor, accounting for 4.5 - 19% of the EP in slurry, and 2.4−21% of the EP in sludge. Despite the abundance of EQU, this weakly estrogenic compound was responsible for only 0.011−0.44% of the EP in slurry, and 0.023−0.47% of the EP in sludge. This is comparable to the contributions made by E2α and E3, which have low potency compared to other steroidal estrogens. Overall, analytes other than E1 and E2β cumulatively accounted for just 0.33−0.84% of the calculated EP.

Implications. To our knowledge, this study represents the most rigorous sampling and most comprehensive suite of analytes undertaken to date in an AFO anaerobic lagoon. Results emphasize the important contribution of suspended solids to the total steroid hormone and phytoestrogen load in lagoon slurry, and may be the first to demonstrate the prodigious load of EDCs and estrogenic activity within the sludge of this system. The tendency of these compounds, in particular, E1 and E2β, to persist at high levels in anaerobic sludge could be an important consideration when sludge from these operations is used for land application. Aerobic treatment or composting of sludge prior to broadcasting could be warranted in order to mitigate the potential risk of offsite transport of these compounds. Androgens and progesterone occurred at lower overall levels in the waste relative to estrogens and seem more prone to degradation in sludge. This and many other studies find that E1 is present at considerable levels in AFO waste, and is the major contributor to estrogenic activity in this waste stream. E1 is among the most potent natural estrogens; while the YES-derived estrogenic potency of E1 observed here was roughly half that of E2β, E1 has been found to have an REP of up to 0.8 in a fish model.[51] Conjugated hormones do not seem to be a major EDC of concern here, which is consistent with previous data from swine sow AFOs;[10] however, given the accumulation of E1-3-S in the solid phase of the lagoon, the potential underestimation of E1-3-S because of analyte instability prior to extraction should not be discounted. Meanwhile, phytoestrogens, EQU in particular, were detected at substantial levels, but the toxicological significance of these compounds in the milieu with potent steroidal estrogens appears to be minimal. Nevertheless, given the abundance of phytoestrogens found here and in other[18,19] studies of livestock waste systems, the environmental risk and potential offsite transport of these compounds from AFOs warrants further investigation.

■ ASSOCIATED CONTENT

Supporting Information

Additional details on methods and equations used, as well as tables of analyte concentrations, EEQs, lagoon physiochemical characteristics, and statistical results are supplied as supporting documents. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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