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ORGANIC COMPOUNDS IN THE ENVIRONMENT

Effect of Composting on the Fate of Steroids in Beef Cattle Manure

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In this study, the fate of steroid hormones in beef cattle manure composting is evaluated. The fate of 16 steroids and metabolites was evaluated in composted manure from beef cattle administered growth promotants and from beef cattle with no steroid hormone implants. The fate of estrogens (primary detected as estrone), androgens, progesterone, and the fusarium metabolite and implant α-zearalanol was monitored in manure compost piles. First-order decay rates were calculated for steroid half-lives in compost and ranged from 8 d for androsterone to 69 d for 4-androstenedione. Other steroid concentration data could not be fit to first-order decay models, which may indicate that microbial processes may result in steroid production or synthesis in composting systems. We demonstrate that composting is an effective strategy to remove steroid hormones from manure. Total steroid hormone removal in composted beef cattle manure ranged from 79 to 87%.

The environmental occurrence of endocrine-disrupting compounds, such as steroid hormones, is a public health concern due to their observed reproductive impacts on aquatic organisms, including abnormal expression of secondary sex characteristics (Seki et al., 2006) and abnormal gonadal development resulting in intersex fish (Tetreault et al., 2011). A primary route for the introduction of steroid hormones into the environment is biosolids and wastewater from municipal and agricultural sources. Agricultural sources, including wastewater lagoons at concentrated animal feeding operations (Fine et al., 2003; Hutchins et al., 2007; Zheng et al., 2008; Bartelt-Hunt et al., 2012), land application of manure to agricultural fields (Nichols et al., 1997; Finlay-Moore et al., 2000; Dutta et al., 2010; Gall et al., 2011), and runoff from animal feedlot surfaces (Mansell et al., 2011; Bartelt-Hunt et al., 2012), constitute an important source of steroid hormones to the environment.

Concentrated animal feeding operations in the United States generate an estimated 1.2 to 1.37 billion tons of manure each year (USEPA, 2004). Livestock manure typically contains endocrine-disrupting compounds because all livestock produce endogenous steroids, and some, such as beef cattle, are also routinely administered steroid hormones as growth promotants (Kolok and Sellin, 2008). The benefits of land application of manure include increased soil productivity, increased soil organic matter, improved water infiltration, and reduced potential for soil erosion. Furthermore, land-applied manure constitutes a source of valuable fertilizer nutrients. Despite these benefits, overapplication of manure to agricultural fields can result in water quality impairment in adjacent surface waters that can include nutrients, pathogens, and other trace level contaminants (Burkholder et al., 2007).

Strategies such as manure composting may reduce the concentration of trace organic compounds present in manure. Composting is a self-heating aerobic process in which manure

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Abbreviations: APPI, atmospheric pressure photoionization; MRM, multiple reaction monitoring.

is piled into windrows and turned occasionally to achieve thermophillic temperatures (Hakk and Sikora, 2011). Manure composting has been demonstrated to reduce the concentration of nutrients and veterinary pharmaceuticals present in manure (Kim et al., 2012; Derby et al., 2011; Arikan et al., 2009; Ramaswamy et al., 2010; Bao et al., 2009). A limited number of studies have reported the influence of composting on steroid hormone concentrations in manure. Hakk et al. (2005) reported a decrease in the amount of water-soluble 17β-estradiol and testosterone extracted from poultry litter after composting. A later laboratory composting study demonstrated that at least 85% of 17β-estradiol in poultry litter was degraded or mineralized, with the primary metabolite being estrone (Hakk and Sikora, 2011). Derby et al. (2011) documented the occurrence of 17β-estradiol and estrone during a field study of swine manure composting and determined that estrone was the dominant estrogen present in swine manure. This study found that total estrogenicity in the compost decreased by nearly 80% over the study period (Derby et al., 2011). To our knowledge, there are no other studies that have examined the influence of composting on steroid hormone occurrence in other types of animal manures, such as beef cattle or dairy manure. In addition to 17β-estradiol, estrone, and testosterone, the behaviors of additional endogenous or exogenous steroid hormones or metabolites have not been evaluated in animal manure composting systems. To better characterize the effect of composting on levels of steroid hormones in manure, we conducted a field study in which beef cattle manure containing synthetic and endogenous steroid hormones was composted for a 76-d period. The concentration of 16 steroid hormones and metabolites in the manure was measured over time, and the influence of composting on the occurrence of steroid hormones in beef cattle manure was evaluated.

Materials and Methods

Manure Collection

Beef cattle manure was collected from confined animal pens at the University of Nebraska-Lincoln Haskell Agricultural Laboratory in Concord, Nebraska. Heifers were divided equally into two groups: a treatment group in which animals were administered subcutaneous implants and feed additives containing steroid hormones and a control group with no synthetic hormone administration. In the treated group, the animals received an implant containing 36 mg of α-zeralanol. After 35 d, the same animals received an implant containing 140 mg of trenbolone acetate and 14 mg of 17β-estradiol benzoate. Animals in the treated group also received 0.45 mg of melengestrol acetate per animal per day via feed from Day 7 to the end of the study period. Additional information regarding the generation of beef cattle manure can be found in Bartelt-Hunt et al. (2012). At the conclusion of the feeding period, the animals were removed from the pens, and all soil and manure was mechanically scraped from the feedlot pens down to the clay layer. The soil and manure scraped from the pens holding cattle treated with growth promotants was held separately from that generated in the control pens. Three compost piles were created from each manure source for a total of six compost piles, and each pile contained approximately 5000 kg of manure and soil. Alfalfa hay was added to the compost piles at approximately 8% by weight to serve as a bulking agent and to increase the C:N ratio. Initially, water was added to the compost piles, and the moisture content in the compost piles was 25 to 45% by weight. The piles were turned as necessary to introduce oxygen into the compost piles.

Sampling

The compost piles were monitored for oxygen and temperature using a Compost Pro datalogger according to the manufacturer's instructions. Moisture content was determined gravimetrically. Each pile was sampled every 2 wk for the concentration and types of steroid hormones present in the manure. Manure samples were obtained by taking six 100-g subsamples from the center of each pile. These subsamples were mixed together in a stainless steel bucket, and a 250-g composite sample was collected in a foil-lined zipper bag and stored frozen at −20°C for hormone analysis. A 100-g sample was collected for moisture content analysis.

Sample Extraction and Steroid Hormone Analysis

Steroid hormones were extracted and analyzed using a newly developed instrumental method (Snow et al., 2013), which used liquid chromatography–tandem mass spectrometry with atmospheric pressure photoionization (APPI). All reagents used in steroid analysis were purchased from Fisher Scientific in the highest purity available (Optima, Thermofisher Scientific). Pure steroid standards, including 17β-estradiol, estrone, estriol, testosterone, 4-androstenedione, androsterone, 17β-trenbolone, and progesterone, were purchased from Sigma-Aldrich or Acros Chemicals, and 17α-trenbolone was obtained from Hayashi Pure Chemical Industries. Internal standards included testosterone-d_3 (Sigma Aldrich) and $^{13}C_6$–estradiol (Cambridge Isotopes).

Compost samples were extracted using microwave-assisted solvent extraction (Snow et al., 2013; Camel, 2000; Labadie et al., 2007; Matejícek et al., 2007). Briefly, 2 to 3 g of sample was weighed into a 10-mL Teflon microwave digestion vessel and mixed with 1 mg of butylated hydroxytoluene and 5 mL of high purity methanol. Twenty-five nanograms of testosterone-d_3, $^{13}C_6$–estradiol, and 17α-methyltestosterone (surrogate) was added by pipette, and the contents were vortexed before microwaving in a CEM MARS Xpress microwave at 1000 W for 10 min. Surrogate recovery averaged 97 ± 23% over all samples. No clean-up by solvent or solid-phase extraction was used.

Quantification of steroids in manure extracts used liquid chromatography tandem mass spectroscopy with APPI and multiple reaction monitoring (MRM) on a Quattro Micro triple quadrupole mass spectrometer (Waters Corp.) with an IonSabre APCI/APPI source (Snow et al., 2013). The method uses a gas-phase selective ionization with a toluene dopant compound and is optimized for simultaneous detection of estrogens, androgens, and steroid-like compounds using positive ion MRM. Minimal sample processing and cleanup was required, and reduced matrix interferences were observed in comparison to methods using electrospray ionization (Snow et al., 2013). A Thermo HyPurity C18 column (250 × 2 mm, 5 μm, 50°C) was used for gradient separation at a

flow rate of 0.35 mL min^{-1}. The gradient consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol), with 0 to 3 min at 50% B, 3 to 14 min at 65% B, and 14 to 20 min at 95% B, with a return to initial solvent conditions for the last 10 min of the gradient (30 min total). Instrument control, data acquisition, and evaluation used MassLynx 4.0 software (Waters Corp.). Identification of target compounds was accomplished by comparing the retention times for the respective MRM transition in a sample with that of a standard analyzed under the same conditions (Table 1). Retention times were considered to match if they were within ±5% of the compound observed in the calibration standards. $^{13}C_6$–Estradiol was used as the internal standard for all estrogens and resorcyclic acid lactones, and testosterone-d_3 was used for quantification of androgens, melengesterol acetate, and progesterone. Laboratory reagent blanks, fortified blanks, fortified matrix samples, and duplicates were each prepared and analyzed at a rate of at least 5%, or 1 for every 20 field samples. Average recovery in fortified blanks (3 g clean sand) spiked at 8.3 ng g^{-1} ranged between 73 ± 13% for 4-androstenedione and 163 ± 94% for estriol, with the average recovery of most compounds between 85 and 100%. Using APPI allowed the method to be optimized for androgens and estrogens rather than having two separate ion acquisition methods as required for electrospray ionization. Further details of method development and validation are provided in Snow et al. (2013). The effect of the compost matrix was monitored through the preparation of fortified compost samples (matrix) spiked with 8.3 ng g^{-1} of each analyte. Recovery of steroids, after subtracting unfortified duplicate matrix samples, averaged between 52 ± 74% for α-zearalenol and 119 ± 58% for 17α-trenbolone, with recovery for most compounds averaging between 90 and 110%. The range between laboratory-analyzed sample duplicates was concentration dependent, although it averaged between 0.2 and 5.8 ng g^{-1}. Method detection limits averaged near 0.2 ng g^{-1} determined by repeated extraction and analysis of a low-level fortified blank (Table 1). Further details of the extraction and analysis are given in Snow et al. (2013).

Results

The temperature and oxygen content in each pile was monitored over the 76-d composting period (Fig. 1). The compost piles had temperatures ranging from approximately 33°C at the start of the study to a maximum of 61°C by Day 14. Thermophilic temperatures (≥40°C) were achieved by Day 6 and sustained for approximately 3 wk. The oxygen content within each pile was also measured. The oxygen content within each pile ranged from below detection limits to ambient (20%), whereas the time-weighted average oxygen content of the compost piles was 10.2%.

Table 1. Mass transitions, retention times, method detection limits, and recovery in the validation test for determination of steroid hormones in manure samples using microwave-assisted solvent extraction and liquid chromatography–tandem mass spectrometry with atmospheric pressure photoionization.†

Analyte	Mass transition (precursor > product ion)	Retention time	MASE MDL‡	Average recovery
		min	ng g^{-1}	%
Estriol	288 > 146	7.60	0.09	103.6
11-Ketotestosterone	303 > 121	8.86	0.18	87.2
β-Zearalanol	305 > 189	9.34	0.22	85.4
Androstenedienedione	285 > 121	9.58	0.16	109.9
β-Zearalenol	303 > 285	9.65	0.52	77.2
17β-Trenbolone	271 > 199	9.89	0.29	121.1
α-Zearalanol	305 > 189	10.29	0.12	56.1
17α-Trenbolone	271 > 253	10.29	0.14	95.9
17β-Estradiol	255 > 159	10.52	0.34	51.8
17α-Ethynylestradiol	279 > 133	10.60	0.19	94.2
4-Androstenedione	287 > 97	10.60	1.87	185.5
α-Zearalenol	303 > 285	10.68	0.60	110.8
Estrone	271 > 133	10.68	0.49	61.8
17α-Estradiol	255 > 159	11.00	0.20	71.1
Testosterone	289 > 97	11.21	0.26	50.9
17α-Hydroxyprogesterone	331 > 97	11.55	0.07	110.7
Epitestosterone	289 > 109	12.58	0.54	135.7
Melengestrol acetate	397 > 337	14.08	0.71	81.8
Progesterone	315 > 97	14.23	0.59	61.0
Androsterone	273 > 255	14.79	0.58	109.6
Internal standards and surrogates				
17β-Estradiol-$^{13}C_6$	261 > 159	10.52		
Testosterone-d5	294 > 100	11.20		
α-Methyltestosterone	303 > 97	12.10		

† Data from Snow et al. (2013).

‡ Microwave-assisted solvent extraction method detection limit.

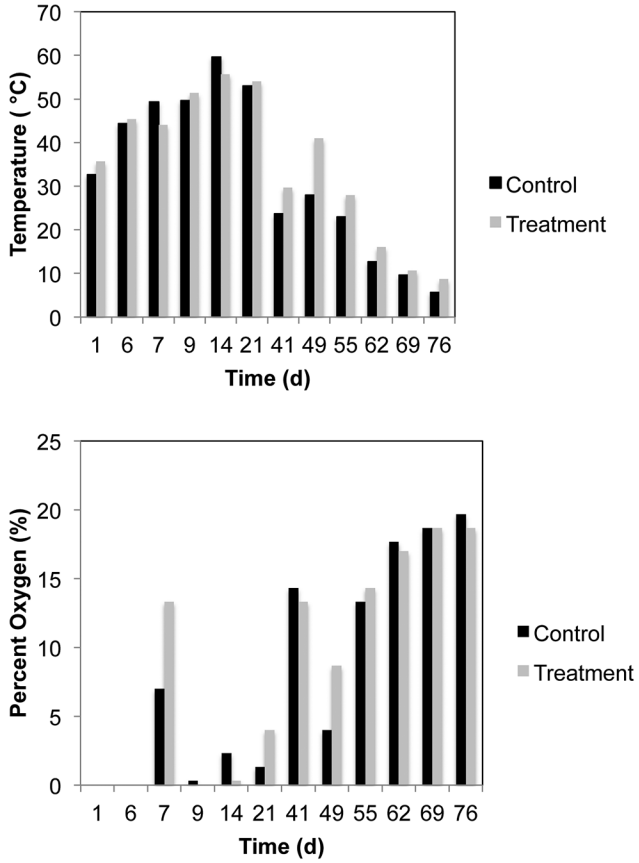

Fig. 1. Temperature and oxygen content in the compost piles.

Table 2 shows the initial steroid hormone concentrations detected in the initial control manure collected from unimplanted animals and treatment manure collected from animals receiving growth promotants and feed additives. Testosterone, 4-androstenedione, androsterone, 17β-estradiol, estrone, and progesterone were detected in treatment and control manures at concentrations ranging from 1.1 to 69 ng g^{-1} dry weight compost. Four additional compounds were detected in the treatment manure only: 17α-estradiol, 17β-trenbolone, α-zearalanol, and melengesterol acetate. Five compounds (estriol, 17α-trenbolone, α-zearalenol, β-zearalenol, and 17α-hydroxyprogesterone) were not detected in either the control or treatment manure. The total initial steroid concentration in the treatment manure (317 ng g^{-1} dry weight) was higher than that measured in the control manure (104.6 ng g^{-1} dry weight). We believe that the higher initial steroid concentrations in the treatment manure are related to the exogeneous steroids administered to these animals (Bartelt-Hunt et al., 2012). It has been demonstrated previously that levels of 17β-estradiol are higher in cattle implanted with trenbolone acetate implants (Henricks et al., 1982).

Figures 2 and 3 show the occurrence of steroids in compost piles as a function of time for the control manure and treatment manure, respectively. For the treatment and control manure samples, the concentration of androsterone decreased over time and was not detected in compost samples at the end of the 76-d study period. 4-Androsterone was also detected in treated and control compost piles at approximately 10 ng g^{-1} dry weight compost, and the concentration remained relatively

Table 2. Initial steroid hormone concentration in compost piles.

Steroid hormone	Concentration	
	Control	Treatment
	——— ng g^{-1} (dry wt. basis) ———	
Testosterone	1.3 ± 0.60	2.3 ± 1.2
4-Androstenedione	12.5 ± 1.9	14.6 ± 2.3
Androsterone	35.9 ± 10.6	69 ± 23
17α-Estradiol	ND	4.7 ± 1.4
17β-Estradiol	1.1 ± 0.49	7.3 ± 2.4
Estrone	24 ± 8.0	150 ± 62
Estriol	ND†	ND
17α-Trenbolone	ND	ND
17β-Trenbolone	ND	52 ± 15
α-Zearalenol	ND	ND
β-Zearalenol	ND	ND
α-Zearalanol	ND	4.6 ± 2.5
Melengesterol acetate	ND	1.4 ± 0.35
Progesterone	29.8 ± 8.9	11.3 ± 2.2
17α-Hydroxyprogesterone	ND	ND

† Not determined.

constant over the study period. In the treated compost pile, the concentration of estrone was 150 ng g^{-1} dry weight compost initially but decreased to less than 10 ng g^{-1} dry weight compost by 9 d. In the control compost pile, the initial concentration of estrone was 24 ng g^{-1} dry weight compost, and estrone concentrations decreased more gradually, with a final average estrone concentration of 10 ng g^{-1} dry weight compost by the end of the study period. All other estrogens were detected at concentrations ≤7 ng g^{-1} dry weight compost in the treated and control compost piles. Progesterone was detected initially in the treatment and control compost piles at concentrations of 11 and 30 ng g^{-1}, respectively. In the control compost piles, the progesterone concentration decreased from 30 ng g^{-1} initially to approximately 10 ng g^{-1} by Day 72. In the treated compost pile, progesterone concentrations remained relatively constant over the study period. Limited detections of 17α-hydroxyprogesterone were found in the control and in the treatment compost over the study period. The fusarium metabolite and implant α-zearalanol was initially detected in the control compost at a concentration of 5 ng g^{-1} and was not initially detected in the treatment compost.

Discussion

Estrone was the dominant estrogen detected in the control and treated manure compost piles at the start of the experiment. 17β-Estradiol was detected in the compost initially at average concentrations ranging from 1.1 to 7.3 ng g^{-1} dry weight compost but was not routinely detected in subsequent sampling events. In a previous publication by the authors, the concentration of 17β-estradiol in the manure and cattle feedlot surface soil that was used to generate the compost piles in the current study was reported to range between 1.2 and 19 ng g^{-1} dry matter (Bartelt-Hunt et al., 2012). This indicated that degradation of 17β-estradiol occurred after the manure and soil was scraped from the pens but before the initial sampling event for the compost piles. Results from this study agree with a previous study of swine manure composting

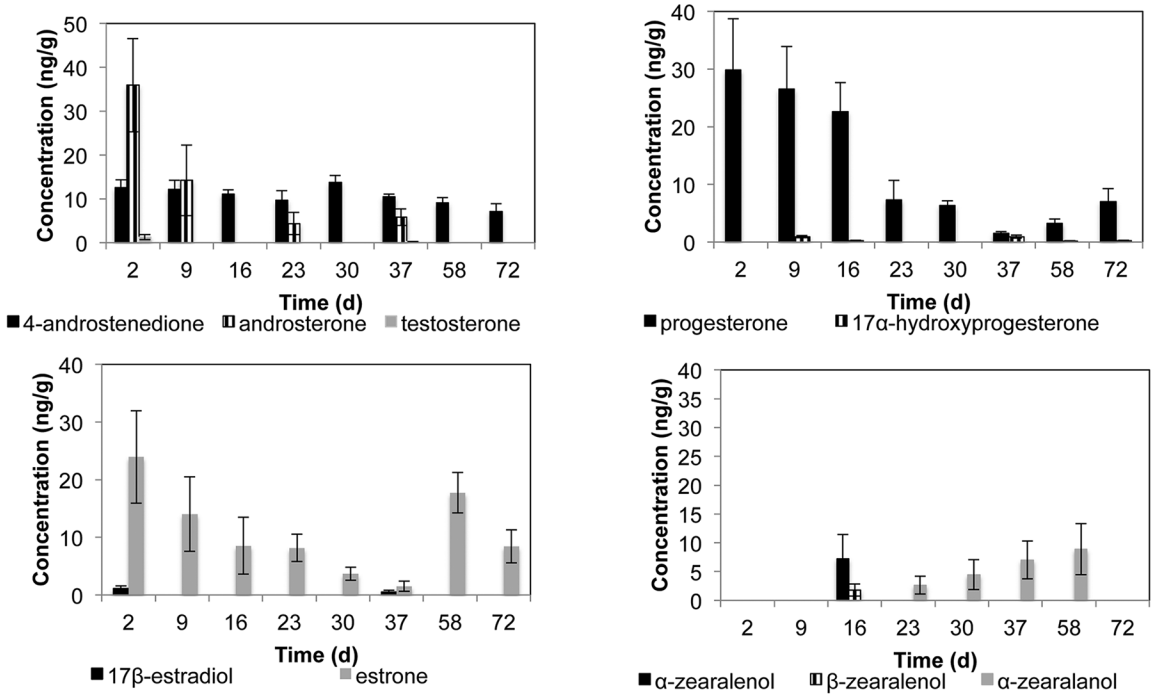

Fig. 2. Concentration of steroid hormones in composting piles containing manure from animals not treated with growth promotants. Concentrations expressed in ng g^{-1} dry material. Error bars represent ±1 SE.

published by Derby et al. (2011), which found estrone to be the dominant estrogen in swine manure compost piles. In the current study, the final estrone concentration was 95 and 36% of the initial estrone concentration present in the starting manure in the treated and control compost piles, respectively. In the compost piles containing manure from treated animals, estrone concentrations decreased gradually over the first 30 d, whereas in the control compost piles, estrone decreased substantially until Day 9 and then remained at concentrations <10 ng g^{-1} dry weight compost. These results are similar to those observed by Derby et al. (2011) in a study of swine manure composting, which found that estrone concentrations decreased until Day 36 and then remained relatively constant at a concentration of 2 ng g^{-1}. Taken together, these results indicate that composting processes do not completely degrade or mineralize estrone.

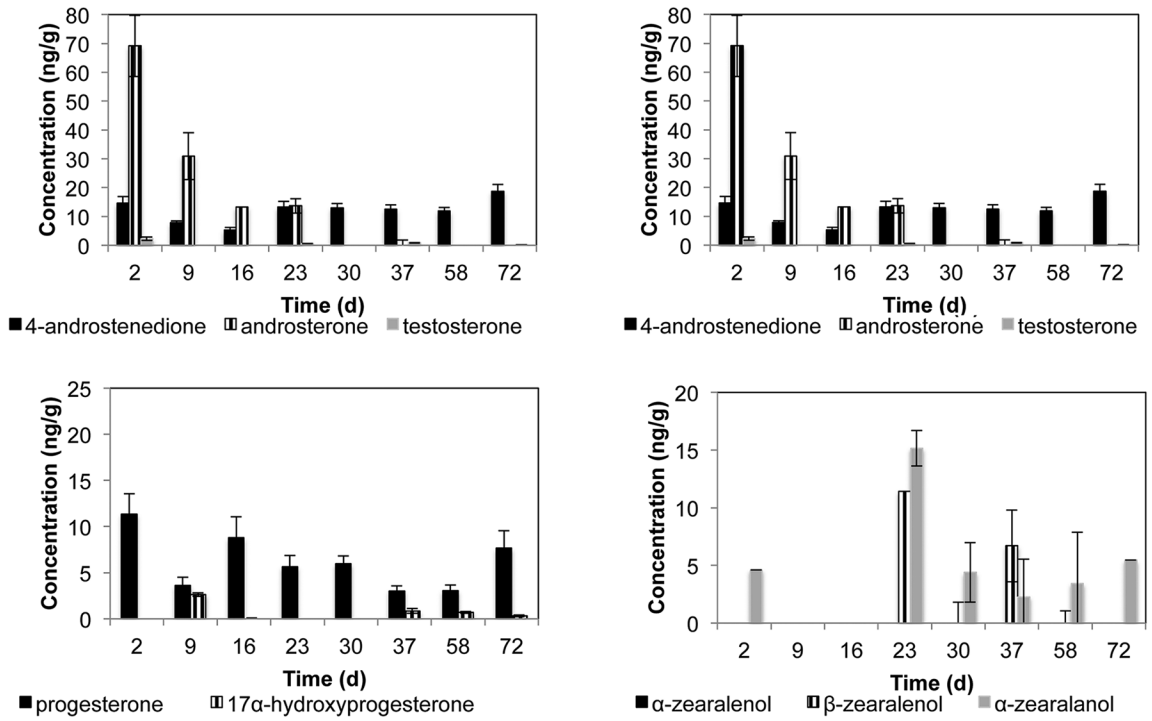

Fig. 3. Concentration of steroid hormones in composting piles containing manure from animals treated with growth promotants. Concentrations expressed in ng g^{-1} dry material. Error bars represent ±1 SE.

Testosterone was detected in the starting manure in the compost piles at relatively low concentrations (<2 ng g^{-1} dry weight compost). This is in contrast to a previous study that reported initial testosterone concentrations in poultry litter compost piles of 115 ng g^{-1} compost dry weight (Hakk et al., 2005). In the present study, the androgens 4-androstenedione and androsterone were detected more frequently and at higher concentrations than testosterone in the compost piles. Androsterone was completely degraded in the control and treated compost piles by 37 and 30 d, respectively. In contrast, 4-androstenedione was more recalcitrant and 4-androstenedione concentrations remained relatively stable over the 76-d study period in the treated and control manures. Although testosterone has been previously shown to degrade during poultry litter manure composting (Hakk et al., 2005), to our knowledge previous studies have not evaluated the fate of testosterone metabolites in manure composting. Because these metabolites are still endocrine active, the fate of testosterone metabolites in composted manure is of environmental and public health importance.

A first-order decay model was used to estimate steroid hormone dissipation half-lives in manure. Steroid concentration data that fit a first-order decay model ($R^2 \geq 0.5$) is presented in Fig. 4. The predicted half-lives for steroids in composted control manure are: 69 d for 4-androstenedione (k = 0.011 d^{-1}), 9.9 d for androsterone (k = 0.0712 d^{-1}), 9.9 d for estrone (k = 0.0744 d^{-1}), and 23 d for progesterone (k = 0.0306 d^{-1}). Calculated half-lives in composted treatment manure were 7.7 d (k = 0.0903 d^{-1}) for androsterone and 35 d (k = 0.0297 d^{-1}) for progesterone. For androsterone and progesterone, concentrations measured in control and treatment manure could be modeled using a first-order decay equation, and the predicted half-lives generally agreed well between control and treatment manures. Limited half-life estimates have been reported for steroids in manure composting. Hakk et al. (2005) reported decay rates of 0.01 d^{-1} for 17β-estradiol and 0.015 d^{-1} for testosterone in composted poultry manure. For other compounds, first-order decay equations did not fit steroid degradation data collected in this study. This may imply that, in addition to steroid degradation, other microbial processes occurring during manure composting result in steroid production or synthesis. Previous work has suggested that microbial and reversible transformation pathways of many steroids and related compounds can lead to in situ production of parent compounds or to the release of a previously unextractable steroid fraction (Mansell et al., 2011; Bartelt-Hunt et al., 2012).

This study is the first to report the fate of progesterone in animal manure composting. In the compost piles containing manure from control animals, progesterone concentrations decreased by 76% to less than 10 ng g^{-1} dry weight compost by Day 23 but remained relatively constant from Day 23 to Day 72. In the treated compost piles, the initial progesterone concentration was lower (11.3 ng g^{-1} dry weight compost), and progesterone concentrations remained relatively stable over the 76-d study period. A corresponding

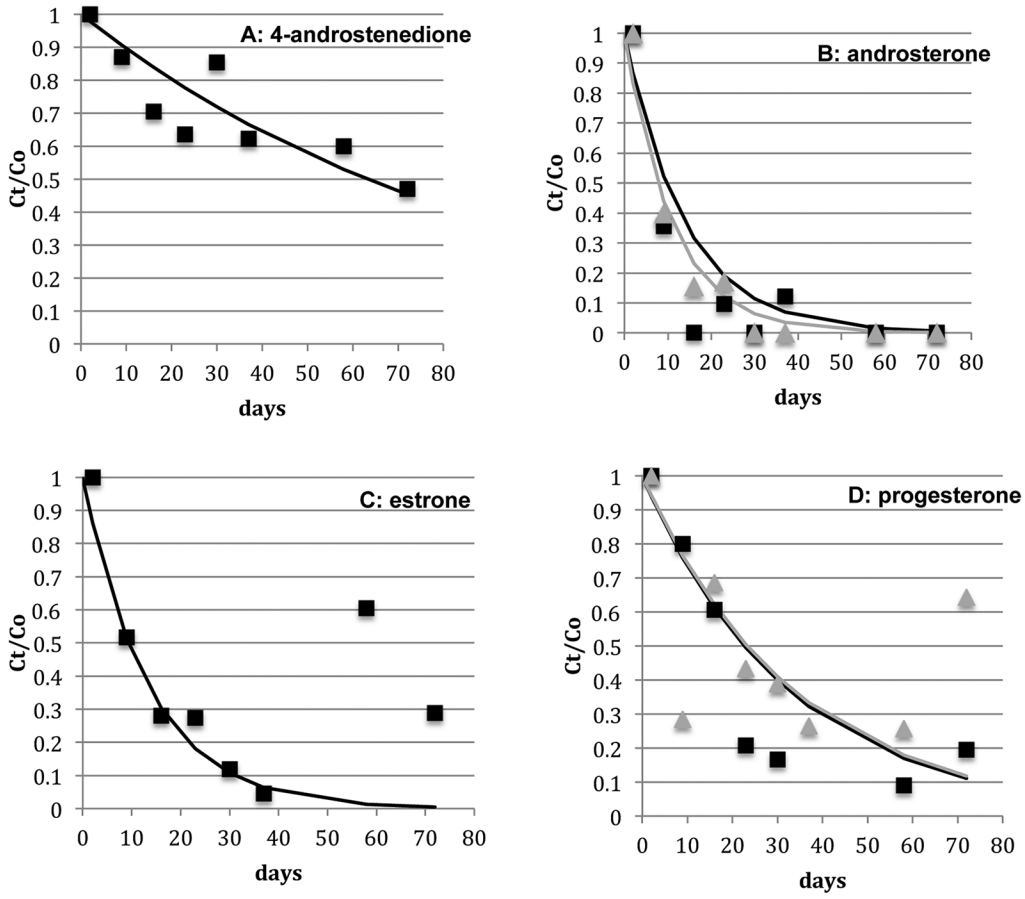

Fig. 4. Degradation of steroid hormones in control (squares) and treatment (triangles) manure. Lines represent first-order decay model results.

increase in 17α-hydroxyprogesterone was not observed with decreases in progesterone concentrations, indicating that 17α-hydroxyprogesterone may not be a primary metabolite formed from progesterone degradation in compost. Previous studies have documented 4-androstenedione and androstanedienedione production from progesterone by soil bacteria, and bacterial mechanisms for the production of testosterone via progesterone conversion to 17α-hydroxyprogesterone and androstenedione have been identified (Carlström, 1967; Wadhwa and Smith, 2000; Jenkins et al., 2004). It is possible that the stable 4-androstenedione concentrations observed in compost piles in this study may be due to oxidation of androstenedione and/or formation of 4-androstenedione from progesterone. Two recent studies of steroid occurrence in cattle feedlot runoff also hypothesized that the presence of androgens in runoff may be due to progesterone degradation in manure or feedlot soils (Mansell et al., 2011; Bartelt-Hunt et al., 2012).

α-Zearalanol was administered as a growth promotant to cattle in the treated group; however, α-zearalanol, α-zearalenol, and β-zearalenol were detected starting on Day 16 in the control compost piles and starting on Day 23 for the treated compost piles. As in a previous study by the authors, we hypothesize here that the occurrence of α-zearalanol in manure is likely due to excretion from implanted cattle and/or due to the metabolism of zearalenone produced by *fusarium* mold, which is commonly found in fermented corn (Bartelt-Hunt et al., 2012). The sporadic detection of these compounds in samples obtained from treated and control manure compost piles makes it difficult to evaluate their specific source or their persistence in compost. In compost piles containing manure from control animals, α-zearalanol was detected on Day 23, and the concentration increased gradually through Day 58. This study provides the first documentation of the occurrence of the growth promotant α-zearalanol in compost and provides initial evidence that concentrations of α-zearalanol may increase during composting.

Taken together, the total amounts of steroids present in the treated and control manures at the end of the 76-d study period were 42 and 23 ng g^{-1} dry weight compost, respectively. This represents an overall reduction in steroid concentration of 79% (treatment manure) and 87% (control manure).

This study represents the first field study to evaluate the fate of steroid hormones during beef cattle manure composting. Results from this study generally agree with previous studies conducted on swine and poultry litter composting. The primary estrogen detected in the cattle manure compost piles was estrone. Estrone concentrations decreased initially and then remained relatively stable throughout the remainder of the study period. Testosterone was not detected in the initial cattle manure samples, but other androgens, including 4-androstenedione and androsterone, were detected in cattle manure compost samples. 4-Androstenedione was found to persist in cattle manure composting throughout the 76-d study period. Additional steroid hormones detected in this study that were not previously evaluated in animal manure composting included progesterone, 17α-hydroxyprogesterone, α-zearalanol, α-zearalenol, and β-zearalenol. Our results indicate that although parent steroid concentrations may decrease during animal manure composting, the occurrence and behavior of steroid and other metabolites should be evaluated to more fully understand the potential biologic activity of composted animal manures. When summed together, steroid hormone concentrations in beef cattle manure decreased by up to 87% after composting. Although some individual steroids were found to remain stable over the study period, overall, composting is a viable manure management strategy to reduce the concentrations of biologically active steroid hormones in manure before land application of animal manure.

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