

# TRANSPORT OF STEROID HORMONES IN THE VADOSE ZONE AFTER LAND APPLICATION OF BEEF CATTLE MANURE

S. J. van Donk, S. Biswas, W. L. Kranz, D. D. Snow, S. L. Bartelt-Hunt, T. L. Mader,  
C. A. Shapiro, D. P. Shelton, D. D. Tarkalson, T. C. Zhang, S. Ensley

**ABSTRACT.** *Few studies have been conducted to investigate the potential contamination of groundwater from manure-borne steroid hormones. The objective of this study was to monitor leaching of steroid hormones at the bottom of 2.4 m deep percolation lysimeters installed in plots treated with beef cattle manure. Soil samples were also collected from surrounding field plots. Treatments consisted of application of manure from two treatment methods (stockpiling and composting) or no manure application. Laboratory analyses of manure, soil, and leachate samples used liquid chromatography and tandem mass spectrometry to identify 17 steroid hormones and metabolites. Progesterone, estrone,  $\beta$ -zearalenol, and 4-androstenedione were detected at varying concentrations in both composted (1.6 to 8.4 ng g<sup>-1</sup> dry weight) and stockpiled (3.7 to 11.4 ng g<sup>-1</sup> dry weight) cattle manure. Steroid hormones were detected in only 5% of the leachate samples, with the greatest detected being progesterone (20 ng L<sup>-1</sup>) from a lysimeter in a plot treated with stockpiled cattle manure. Steroid hormones or metabolites were detected in 10% of the soil samples. The majority of detections (74%) were for soils sampled within the top 1.2 m. The steroid detected most frequently in the soil samples (4%) was 17 $\beta$ -estradiol, with a maximum concentration of 4.3 ng g<sup>-1</sup> in a plot treated with composted cattle manure. No synthetic steroids were detected in any soil or leachate samples. The low detection of steroid hormones in the soil and leachate samples suggests that while some hormones may move through silt loam soil, most are readily degraded or adsorbed after manure application.*

**Keywords.** *Beef cattle, Leachate, Lysimeter, Manure management, Steroid hormones, Vadose zone.*

A variety of naturally occurring steroid hormones are regularly excreted by beef cattle, while additional steroid hormone and androgenic compounds have been regularly used as growth promoters by the cattle industry (USEPA, 2004). Hormones and related compounds act as growth promoters to improve beef cattle growth rates, feed efficiencies, and lean muscle

mass. Economic advantages of using hormone treatments have been estimated at between \$15 and \$40 per animal (Raloff, 2002). Almost 96% of cattle placed in U.S. feedlots receive hormone supplements at least once during their life time (USDA, 2000). The treatments are administered via feed additives and small implants placed subcutaneously behind the ear. Among commonly used implants, three contain natural steroids (estradiol, testosterone, and progesterone) and two contain synthetic or semi-synthetic growth promoters (zeranol and trenbolone acetate). Melengestrol acetate (MGA), a synthetic progestagen, may be given as a feed additive (Kolok and Sellin, 2008).

With more than 2.5 million head (~18% of the U.S. total) of cattle in feedlots at any one time, Nebraska is the state with the second largest number of cattle on feed in the nation (USDA, 2011). Approximately 3 million Mg of cattle manure are generated from concentrated animal feeding operations (CAFOs) in Nebraska and are annually applied to agricultural land (USEPA, 2001; USDA, 2011). Benefits of land application of manure include nutrient substitution for purchased fertilizer, increased crop productivity and soil organic matter, improved water infiltration, and reduced soil erosion (USEPA, 2004). Depending on manure age and storage conditions, both natural and exogenous steroid hormones and growth promoters may be contained in manure applied to cropland. Due to the extent of the use of exogenous steroids in cattle production, high levels of steroid hormone residues may enter the environment when manure is applied to soil.

Steroid hormones may be categorized as androgens, es-

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The authors are **Simon J. van Donk, ASABE Member**, Assistant Professor, West Central Research and Extension Center, University of Nebraska-Lincoln, North Platte, Nebraska; **Sagor Biswas**, Graduate Student, and **William L. Kranz, ASABE Member**, Associate Professor, Haskell Agricultural Laboratory, University of Nebraska-Lincoln, Concord, Nebraska; **Daniel D. Snow**, Research Associate Professor, School of Natural Resources, University of Nebraska-Lincoln, Lincoln, Nebraska; **Shannon L. Bartelt-Hunt**, Assistant Professor, Department of Civil Engineering, University of Nebraska-Lincoln, Omaha, Nebraska; **Terry L. Mader**, Professor, **Charles A. Shapiro**, Professor, and **David P. Shelton, ASABE Member**, Professor, Haskell Agricultural Laboratory, University of Nebraska-Lincoln, Concord, Nebraska; **David D. Tarkalson, ASABE Member**, Research Soil Scientist, USDA-ARS Northwest Irrigation and Soils Research Laboratory, Kimberly, Idaho; **Tian C. Zhang**, Professor, Department of Civil Engineering, University of Nebraska-Lincoln, Omaha, Nebraska; and **Steve Ensley**, Clinician, College of Veterinary Medicine, Ames, Iowa. **Corresponding author:** Simon van Donk, West Central Research and Extension Center, 402 West State Farm Rd., North Platte, NE 69101; phone: 308-696-6709; e-mail: [simon.vandonk@unl.edu](mailto:simon.vandonk@unl.edu).

trogens, and progestagens. Among the endogenous androgens, 4-androstenedione is the precursor of testosterone and forms the metabolites androsterone and 11-ketotestosterone (Mayer et al., 1955). The synthetic androgen implant trenbolone acetate is normally hydrolyzed in the body of cattle to active 17 $\beta$ -trenbolone before being oxidized to trendione and after reduction excreted as 17 $\alpha$ -trebolone (Khan et al., 2008). During metabolism within cattle, the free natural estrogen 17 $\beta$ -estradiol (also applied as implant) is oxidized to estrone and from estrone to 17 $\alpha$ -estradiol by reduction (Mellin and Erb, 1965, 1966). Among the progestagens, the feed additive MGA is a semi-synthetic progestagen, whereas 17 $\alpha$ -hydroxyprogesterone is derived from endogenous progesterone. Depending on the conditions, the estrogen agonist and implant zeranol ( $\alpha$ -zearalanol) may be metabolized in soil to form  $\beta$ -zearalanol,  $\alpha$ -zearalenol, and/or  $\beta$ -zearalenol (Kleinova et al., 2002).

Steroid hormones have been measured in manure from treated cattle. A study conducted in Nebraska detected 17 $\alpha$ -trenbolone and MGA in fresh manure excreted by treated heifers at maximum concentrations of 55 and 6.5 ng g<sup>-1</sup>, respectively (Bartelt-Hunt et al., 2012). From an earlier study in Germany, Schiffer et al. (2001) found trenbolone metabolites at concentrations ranging from 5 to 75 ng g<sup>-1</sup> and MGA at concentrations ranging from 0.3 to 8 ng g<sup>-1</sup> in manure from treated cattle. The extent to which steroid hormones associated with manure, soils, and sediment directly affect organisms is not well documented, although these materials are generally considered to be sources or reservoirs of biologically active compounds to aquatic environments. Recent research suggests that some estrogens, androgens, and progestagens occurring in surface water may originate from runoff impacted indirectly by livestock manure (Kolpin et al., 2002; Soto et al., 2004; Kolok et al., 2007; Shore, 2009).

In laboratory experiments exposing fish to low concentrations of diluted cattle manure, Sellin et al. (2009) determined that there were significant reproductive effects due to steroids released to the water column, although no significant differences in effects were found between manure from implanted and non-implanted cattle. Concentrations of manure-bound steroids were not determined in this study; however, aqueous concentrations of estrone and estradiol released from the manure averaged up to 740 ng L<sup>-1</sup>. Until the bioavailability of sediment-bound steroids becomes evident, the potential effects of soil or sediment bound compounds must be evaluated in terms of mobility and persistence in aquatic environments. Regardless of sources, steroids have been linked with the abnormal endocrine disrupting functions during critical growth stages of aquatic life (mainly fish, turtles, alligators, frogs, etc.) and possibly humans (Colborn et al., 1993; Matthiessen, 2003; Guillette et al., 2000; Falconer et al., 2006).

Groundwater may also be impacted by livestock waste, often evidenced by excess nitrate levels in the groundwater of fertilized fields. Ammonia and chloride may leak from wastewater holding facilities (Bartelt-Hunt et al., 2011; Bradford et al., 2008). Few studies have been conducted to investigate the occurrence and sources of steroid hormones

in groundwater. Shore et al. (1995) indicated that both testosterone and estrogen can leach from soil at varying rates and reach streams through subsurface and surface flow. Peterson et al. (2000) reported the presence of 17 $\beta$ -estradiol at concentrations that ranged from 6 to 66 ng L<sup>-1</sup> in spring water discharged from a mantled karst aquifer in a region with intense poultry and cattle feeding activities in northwest Arkansas. In a similar study, Wicks et al. (2004) detected 17 $\beta$ -estradiol at concentrations ranging from 13 to 80 ng L<sup>-1</sup> from eight springs that drain the Ozark Plateau Aquifer in the karstic basin near the Missouri-Arkansas border and concluded that the aquatic organisms living in the basin may be exposed to a 17 $\beta$ -estradiol concentration above 25 ng L<sup>-1</sup> nearly 60% of the time.

Kolodziej et al. (2004) detected steroid hormones in 7 of 26 samples from shallow (7 to 10 m) groundwater monitoring wells at concentrations significantly below levels detected in the lagoons (six detections were <10 ng L<sup>-1</sup>; all seven detections were <20 ng L<sup>-1</sup>) in the northeastern San Joaquin Valley of central California. In this study, no pattern of occurrence could be established, as no steroid hormones were detected in the deep aquifer well nor in samples from the tile drainage system. Due to sporadic detection in tile drains, it was suggested that steroid hormones are generally adsorbed or degraded over a depth of 10 to 100 m when dairy wastewater infiltrates groundwater. Moreover, the sporadic detections were believed to result from preferential flow paths in the vadose zone.

Arnon et al. (2008) reported the transport of steroid hormones from an earthen unlined waste lagoon to groundwater at a dairy farm at the southern section of the Israeli Coastal Aquifer, where the soil had high clay content in the upper section (0 to 6 m) and was predominantly sandy below 8 m. Testosterone (~0.03 ng g<sup>-1</sup>) and estrogen (~0.06 ng g<sup>-1</sup>) were detected in the deep sediment profile under the waste lagoon over the depth of 30 m. Results from this study supported the idea of preferential flow as the predominant transport mechanism and suggested that leachate from dairy farm operations can percolate through the vadose zone and into groundwater.

In a recent study, Bartelt-Hunt et al. (2011) investigated the presence of steroid hormones, their metabolites, and veterinary pharmaceuticals in lagoon water and shallow (2 to 15 m depth) groundwater samples at swine and beef cattle CAFOs in Nebraska. These facilities were considered highly impacted, with a history of elevated concentrations of chloride, nitrate, and ammonia in the groundwater. Four steroid hormones were detected in the lagoon and groundwater at both cattle and swine facilities. Estrone was detected at concentrations ranging from 40 to 390 ng L<sup>-1</sup> in groundwater near cattle facilities, and testosterone was detected at a concentration of 30 ng L<sup>-1</sup> in groundwater near swine facilities. It was concluded that the inconsistent detection of steroids in groundwater in this study provides some evidence that these compounds are strongly attenuated in the subsurface due to either biodegradation or sorption to soil. Mansell and Drewes (2004) also found that the mobility of estrogens (17 $\beta$ -estradiol and estriol) and androgens (testosterone) in subsurface systems was low. They

found that the concentrations of estriol and testosterone were  $<0.6 \text{ ng L}^{-1}$  and  $17\beta$ -estradiol was  $<2 \text{ ng L}^{-1}$  in water samples from groundwater monitoring wells or shallow lysimeters after travelling through 1.5 m of porous media. Thus, site-specific variations in sediment characteristics, manure handling, and hydrology may partially explain the variability in fate and transport of commonly used steroid hormones in the environment.

In light of these studies, the environmental fate of steroid hormones and growth promoters excreted by feedlot cattle under different manure management and application strategies remains relatively unexplored (USEPA, 2001; USGS, 1999). These gaps in knowledge impede the development of effective management strategies to mitigate potential negative impacts from the use of hormones in animal production. Research has shown that many associated inorganic and organic compounds applied to soil with manure can leach to groundwater (Casey et al., 2003, 2004; Tolls, 2001). However, information on hormone transport in soils under field conditions is lacking. The objective of this study was to determine the extent of vertical movement of steroid hormones through the vadose zone in field plots treated with composted and stockpiled manure. This information is critical in determining if there are environmental risks and the effects of agricultural management practices that will minimize risks associated with land application of manure. Given the number of variables that could affect the results (type of cattle, type of implants, length and type of manure storage, level and frequency of manure application, precipitation, soil type, etc.), it should be pointed out that this was more an observational study than a controlled experiment.

## MATERIALS AND METHODS

Manure used for this study had been generated and stored at a University of Nebraska-Lincoln (UNL) owned feedlot located at the Haskell Agricultural Laboratory near Concord in northeast Nebraska in 2007. Forty-eight heifers were assigned randomly in three feeding pens and treated with 36 mg of  $\alpha$ -zearalanol (Ralgro) on day 1 (the first day the heifers were in the feeding pens) (June 2007) and with 140 mg of trenbolone acetate and 14 mg of  $17\beta$ -estradiol benzoate (Revalor-H) implants on day 36 (July 2007). Both implants were purchased from Intervet, Inc. (Merck Animal Health, Summit, N.J.). The heifers also received 0.45 mg of MGA (MGA 200 Premix, Pharmacia and Upjohn Co., Peapack, N.J.) in their daily feed ration from day 7 until day 112 (September 2007). Animal waste, accumulated on the feedlot surface, was removed using a box scraper and placed in six separate piles that were either composted (three piles) or stockpiled (three piles). The average weight of the piles was 3600 to 4000 kg. The manure was placed on a concrete pad under a roof to prevent rainfall from entering the manure piles. Water and corn silage were added to each composted pile as a source of organic matter to initiate the composting process. There was a one-time application of 379 L water to each compost pile. Corn silage was added to each composted pile with a manure:silage

ratio of 21:1 (weight basis). During the composting process, manure was spread on the concrete pad, and water and corn silage were added before mixing it uniformly and piling it using a skid loader. The manure was composted or stockpiled for three months. The composted piles were turned weekly for the first four weeks in October 2007 and then biweekly for the next eight weeks to facilitate proper aeration during the composting process. Stockpiled and composted manure samples were collected for analysis in April 2008. They were obtained by hand from the bottom, top, center, and outer edge of each pile, mixed in a bucket and combined into composite samples, and frozen before sending to the laboratory for analysis. After manure sample collection, two composite piles (one of composted and one of stockpiled manure) were prepared by mixing and combining three piles of each manure treatment before shipping to North Platte, Nebraska, where the remainder of the study was conducted.

The field study was conducted from April 2008 to July 2011 at the UNL West-Central Research and Extension Center (WCREC) near North Platte, Nebraska ( $41^{\circ} 10' \text{ N}$ ,  $100^{\circ} 45' \text{ W}$ , 861 m elevation above sea level) on a Cozad silt loam soil (coarse-silty, mixed, mesic Fluventic Haplustoll). Soils were characterized in a previous study (Aga et al., 2005). Average percent organic matter and pH are summarized in table 1 for reference. The average water content of the soil is  $0.29 \text{ m}^3 \text{ m}^{-3}$  at field capacity and  $0.11 \text{ m}^3 \text{ m}^{-3}$  at the wilting point (Klocke et al., 1999). The climate at North Platte, Nebraska, is semi-arid, with an average annual precipitation of 508 mm and a reference crop evapotranspiration of 1403 mm. On average, about 80% of the annual precipitation occurs during the growing season, which extends from late April to mid-October (USDA, 1978).

The experimental field at WCREC was instrumented with monolithic percolation lysimeters installed near the center of each field plot ( $625 \text{ m}^2$  per plot). Each lysimeter contained an undisturbed soil core with a diameter of 0.9 m and a depth of 2.4 m. The lysimeters had stainless steel porous extractors installed at the bottom, which allowed the extraction of leachate from unsaturated soil using a vacuum pump system (Klocke et al., 1993). The leachate samples represented water that was leached from the crop root zone. The lysimeters had been used successfully for several nitrate-leaching experiments (Klocke et al., 1993; Klocke et al., 1999; Tarkalson et al., 2006). In our study, they were used to determine the amounts and types of hormones leaching below the crop root zone.

A randomized block design with three treatments and three replications (nine plots with lysimeters) was used for this study. The treatments consisted of stockpiled manure, composted manure, and a control (no manure, commercial

**Table 1. Average soil properties of the experimental field at the West-Central Research and Extension Center, North Platte, Nebraska, as reported by Aga et al. (2005).**

Depth (m)	Organic Matter (%)	pH
0.0-0.15	1.14	7.3
0.15-0.30	0.82	7.8
0.30-1.80	0.54	8.0
1.80-2.40	1.03	8.2

fertilizer). Manure was surface applied to the lysimeters and adjacent field areas in April 2008 at an application rate of 128 Mg ha<sup>-1</sup> of stockpiled manure and 106 Mg ha<sup>-1</sup> of composted manure to deliver 112 kg N ha<sup>-1</sup> of plant-available nitrogen for the first year based on a mineralization factor of 0.15 to satisfy the nitrogen requirements of winter wheat (Koelsch and Shapiro, 1997). The control plots received surface-applied commercial nitrogen fertilizer (urea) to match the manure N availability of 112 kg N ha<sup>-1</sup> of plant-available nitrogen. Additionally, potassium bromide (solution concentration 1.66 g Br L<sup>-1</sup>) was surface applied as a conservative tracer at an application rate of 168 kg ha<sup>-1</sup> to each lysimeter plot two months after manure application (June 2008). Bromide concentrations were determined in leachate to identify the vertical transport rate beneath each plot.

Winter wheat was planted by hand in the lysimeters and the surrounding field in the fall of 2007 and 2008, while soybeans were planted in the spring of 2010 and 2011. In 2008, the winter wheat crop was not irrigated due to the relatively high precipitation in the spring (fig. 1). Sprinkler irrigation was used once in 2009 and four times in 2010. The cropping history and amount of irrigation water applied are given in table 2.

During late spring and summer, precipitation was measured using four rain gauges located adjacent to the study plots. For the rest of the year, precipitation data were used from a High Plains Regional Climate Center (HPRCC; www.hprcc.unl.edu) weather station, located less than 1.5 km west of the study site. For water-equivalent data from snow, data from the WCREC dryland farm, which is located 4 km south of the study plots, were used. Using these three data sources, a precipitation record was constructed for 2008-2011 (fig. 1).

During the growing season, soil water content was measured 12 times in 2008, ten times in 2009, two times in 2010, and two times in 2011 using a neutron probe (CPN Hydroprobe, Boart Longyear Co., Martinez, Cal.). Two aluminum access tubes were installed in each of the nine plots: one in the lysimeter and one in the adjacent plot. Soil water content readings were recorded at 0.15, 0.46, 0.76,

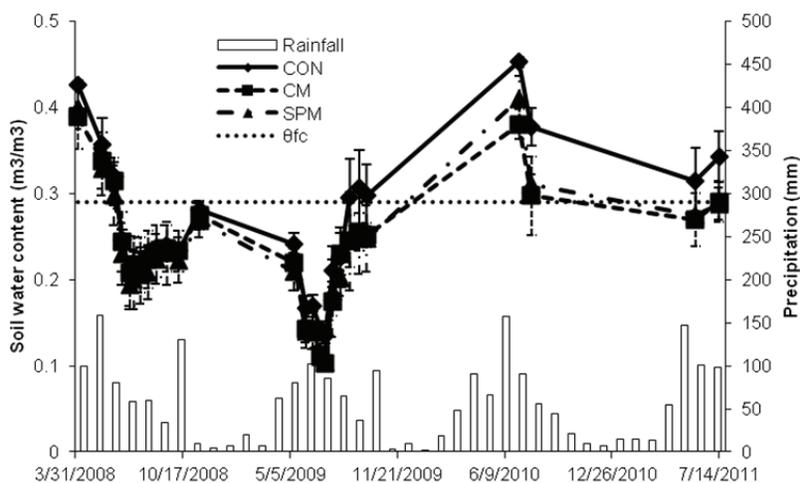
**Table 2. Cropping history and irrigation applied during study period.**

Year	Crop	Irrigation
2008	Winter wheat	None
2009	Winter wheat	51 mm on Sept. 8
2010	Soybean	25 mm on Aug. 10
		25 mm on Aug. 26
		25 mm on Aug. 31
		25 mm on Sept. 2
2011	Soybean	None

1.07, 1.37, 1.68, 1.98, and 2.29 m below the soil surface and converted to volumetric soil water content using a locally developed calibration curve.

Leachate samples were collected from each plot in 2008-2011. Sampling was conducted 11 times from May to December in 2008, four times in 2009 (May, June, September, and October), once in 2010 (July), and once in 2011 (July). Soil samples were taken adjacent to but outside the lysimeter from each plot at six depths (0.0-0.15 m, 0.16-0.30 m, 0.31-0.60 m, 0.61-1.20 m, 1.21-1.80 m, and 1.81-2.40 m) on 15 April 2008 (pre-trial, just before the manure was applied). After manure application, soil samples were taken at the same depths on five more dates: 23 September 2008 (after winter wheat harvest), 21 April 2009, 27 July 2009 (after winter wheat harvest), 27 October 2009, and 21 July 2011. To reduce sampling errors created by spatial variability, five subsamples were taken from each depth. The subsamples were mixed, and one composite sample was collected for each depth. All leachate and soil samples were immediately frozen and stored until laboratory analysis.

Manure, soil, and leachate samples were analyzed for 17 steroid hormones and their metabolites (table 3). The method details are reported elsewhere (Snow et al., 2013), and the hormones and metabolites were selected according to implants used as well as the endogenous steroid hormones and metabolites likely to occur in cattle manure (Shore and Shemesh, 2003; Hanselman et al., 2003). Additionally, leachate samples were analyzed for bromide at the EPA National Environmental Research Laboratory in Ada, Oklahoma, using flow injection colorimetric methods on an autoanalyzer (Quikchem 8500, Lachat Instruments, Love-



**Figure 1. Average soil water content ( $\pm$ SD) in nine lysimeters and monthly precipitation during the study period (CON = control, CM = composted manure, SPM = stockpiled manure, and  $\theta_{fc}$  = water content at field capacity).**

**Table 3. Seventeen steroid hormones and metabolites analyzed in this study, their instrument detection limits (IDL), method detection limits (MDL), and recoveries for water samples using on-line solid phase extraction (SPE) and soil samples using microwave-assisted solvent extraction (MASE). Instrumental analysis is by liquid chromatography and tandem mass spectrometry (LC/MS/MS) with atmospheric pressure photoionization (APPI). Spiked analyte concentrations are 5 ng L<sup>-1</sup> for on-line SPE and 0.5 ng g<sup>-1</sup> for test soil.**

Category	Steroid Hormone or Metabolite	IDL (pg)	Online SPE MDL (ng L <sup>-1</sup> )	Online SPE MDL Average Recovery (%)	MASE MDL (ng g <sup>-1</sup> )	MASE MDL Average Recovery (%)
Androgens	Testosterone	16.0	3.2	100.9	0.07	110.7
	11-ketotestosterone	16.2	3.4	116.6	0.09	103.6
	4-androstenedione	15.2	1.3	70.2	0.12	56.1
	Androsterone	48.6	2.4	94.3	0.34	51.8
	17 $\alpha$ -trenbolone <sup>[a]</sup>	9.4	3.2	85.4	0.16	109.9
	17 $\beta$ -trenbolone <sup>[a]</sup>	18.2	4.5	93.9	0.29	121.1
<i>Fusarium</i> metabolites	$\alpha$ -zearalanol	33.3	9.0	82.6	0.54	135.7
	$\beta$ -zearalanol	33.5	6.9	195.1	0.59	61.0
	$\alpha$ -zearalenol	43.6	2.0	41.5	0.71	81.8
	$\beta$ -zearalenol	37.6	4.0	115.4	0.58	109.6
Estrogens	17 $\alpha$ -estradiol	21.0	2.7	28.0	0.18	87.2
	17 $\beta$ -estradiol	23.7	4.2	55.6	0.52	77.2
	Estrone	35.1	4.9	88.8	0.60	110.8
	Estriol	62.5	7.5	69.4	1.87	185.5
Progestagens	Melengestrol acetate <sup>[a]</sup>	13.4	2.8	59.5	0.2	71.1
	Progesterone	21.0	2.7	72.7	0.26	50.9
	17 $\alpha$ -hydroxyprogesterone	26.6	3.6	95.0	0.22	85.4

<sup>[a]</sup> Synthetic.

land, Colo.). Standard quality control practices were followed, including analyses of duplicates, reagent blanks, and fortified blanks.

Steroid hormones were analyzed in lysimeter water samples using on-line solid phase extraction (SPE) liquid chromatography and tandem mass spectrometry (LC/MS/MS) with an automated extraction system (Symbiosis Environ, Spark Holland, Emmen, The Netherlands) and liquid chromatograph tandem mass spectrometer system (Quattro Micro, Waters Corp., Milford, Mass.) with an atmospheric pressure photoionization (APPI) source. Briefly, a 25 mL portion of the sample was syringe-filtered into a precombusted amber vial and fortified with internal standards (testosterone-d<sub>3</sub> and <sup>13</sup>C<sub>6</sub>-estradiol) and surrogate (17 $\alpha$ -methyltestosterone) along with 10  $\mu$ L of formic acid. Twenty milliliter aqueous samples were then auto-extracted using Prospekt 2/Symbiosis 2.0  $\times$  10 mm Oasis HLB solid phase extraction cartridges (Waters Corp.). Each cartridge was eluted directly to a Waters 2695 HPLC followed by a Micromass Quattro Micro triple-quadrupole mass spectrometer. A mixture of toluene and methanol was used as the dopant to improve ionization efficiency (Snow et al., 2013).

Microwave-assisted solvent extraction (MASE) was used for manure and soil samples (Camel, 2000; Labadie et al., 2007) and is described in detail elsewhere (Snow et al., 2013). Briefly, 2 to 3 g of sample were weighed into a 10 mL Teflon microwave digestion vessel and mixed with 1 mg of butylated hydroxytoluene and 5 mL of high-purity methanol (Labadie et al., 2007; Matejcek et al., 2007), 25 ng of internal standards (testosterone-d<sub>3</sub> and <sup>13</sup>C<sub>6</sub>-estradiol) and 17 $\alpha$ -methyltestosterone (used as a surrogate compound to monitor recovery) was added by pipette, and the contents were vortexed prior to microwaving (MARS Xpress, CEM Corp., Matthews, N.C.) at 1000 W for 10 min. Surrogate (17 $\alpha$ -methyltestosterone) recovery averaged 97%  $\pm$ 23% over all solid samples.

Instrumental detection and quantification of steroids from on-line extraction and MASE extracts utilized multiple reaction monitoring (MRM) with argon collision gas. A HyPurity C18 column (250  $\times$  2 mm, 5  $\mu$ m, 50°C, Thermo Scientific, Waltham, Mass.) was used for gradient separation at a flow rate of 0.35 mL min<sup>-1</sup>. The gradient consisted of solvent A (0.1% formic acid water) and solvent B (0.1% formic acid 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 to that of a standard analyzed under the same conditions. Retention times were considered to match if they were within  $\pm$ 5% of the standards. Except for the corrective effect of isotope dilution, analyte concentrations were not adjusted for recovery in samples. The instrument detection limits, method detection limits, and average recoveries for on-line SPE and MASE methods for determining steroid hormone concentrations are given in table 3, determined from the variability in extraction and analysis of eight replicates of fortified matrix (USEPA, 1986). The low recovery for 17 $\alpha$ -estradiol at 5 ng L<sup>-1</sup> may have resulted in greater uncertainty at low levels for this compound but was not observed in water fortified at higher (>10 ng L<sup>-1</sup>) levels (Snow et al., 2013). The apparently high recovery of  $\beta$ -zearalanol at 5 ng L<sup>-1</sup> (table 3) also did not occur at higher concentrations (>10 ng L<sup>-1</sup>) but may lead to overestimation of concentrations in samples near the detection limit.

Statistical analysis was conducted to determine the difference in average soil water content in the soil profile among the four study years and among the three manure treatments using PROC GLIMMIX in SAS statistical software (SAS, 1990). Level of significance ( $\alpha$ ) was chosen as

0.05. This statistical analysis was performed for four dates of soil water measurement, each one in the month of July in each of the four study years.

However, it was impossible to run any standard statistical analyses for the individual steroid and metabolite concentrations in leachate and soil samples due to the low frequency of detection. Thus, the occurrence (presence) of steroids in leachate and soil samples was reported in terms of percentage or frequency of detection to understand the trend of their occurrence among the manure treatments.

## RESULTS AND DISCUSSION

### MANURE

The two manure treatments differed primarily in their moisture content, with composted manure having a higher moisture content (21.5%) than stockpiled manure (14.2%) (table 4). Eleven of the 17 steroids and metabolites were detected at or above the quantification limit 0.5 ng g<sup>-1</sup> (dry manure basis, table 5). Among these eleven, 4-androstenedione,  $\beta$ -zearalenol, estrone, and progesterone were detected in both composted and stockpiled manure. Other compounds, including androsterone, 17 $\alpha$ -trenbolone,  $\beta$ -zearalanol, 17 $\beta$ -estradiol, 17 $\alpha$ -estradiol, estriol, MGA, and 17 $\alpha$ -hydroxyprogesterone were below the detection limits. Low concentrations of testosterone, 17 $\beta$ -trenbolone, and  $\alpha$ -zearalanol were detected only in composted manure, whereas  $\alpha$ -zearalenol was detected only in stockpiled manure.

Among the exogenous steroid hormones and their me-

**Table 4. Chemical and physical properties of stockpiled and composted manure, measured shortly before application to lysimeters and adjacent field plots in April 2008.**

Manure Source	pH	OM <sup>[a]</sup> (%)	OC <sup>[b]</sup> (%)	C:N Ratio <sup>[c]</sup>	MC <sup>[d]</sup> (%)	Org. N <sup>[e]</sup> (%)
Stockpiled	7.7	13	7.6	11.5	14.2	0.6
Composted	7.8	14	8.1	10.6	21.5	0.7

<sup>[a]</sup> Organic matter (%) = 100 – Ash.

<sup>[b]</sup> Organic carbon (%) = %OM  $\times$  0.58.

<sup>[c]</sup> C:N ratio = ratio of total organic carbon to total nitrogen.

<sup>[d]</sup> Moisture content determined by gravimetric method.

<sup>[e]</sup> Organic N = total N – ammonium N.

**Table 5. Average ( $\pm$ SD) concentrations (ng g<sup>-1</sup>, dry weight) of detected steroid hormones and metabolites in manure samples ( $n = 3$ ). The remaining six steroid hormones or metabolites listed in table 3 were not detected in either composted or stockpiled manure.**

Steroid Hormone or Metabolite	Stockpiled Manure <sup>[a]</sup>	Composted Manure <sup>[b]</sup>
Testosterone	ND <sup>[c]</sup>	5.8 (5.2)
4-androstenedione	5.3 (1.9)	3.9 (2.8)
17 $\beta$ -trenbolone	ND	9.8 (8.8)
$\alpha$ -zearalenol	1.9 (3.3)	ND
$\beta$ -zearalenol	11.4 (3.8)	4.5 (4.5)
$\alpha$ -zearalanol	ND	1.1 (2.0)
17 $\alpha$ -estradiol	<0.5	ND
Estrone	3.7 (1.0)	8.4 (7.5)
Melengestrol acetate	<0.5	<0.5
Progesterone	4.1 (1.1)	1.6 (1.0)
17 $\alpha$ -hydroxyprogesterone	<0.5	ND

<sup>[a]</sup> Sampling date was 9 April 2008.

<sup>[b]</sup> Sampling date was 14 April 2008.

<sup>[c]</sup> ND = no detection.

tabolites, 17 $\beta$ -trenbolone was detected only in composted manure samples at an average concentration of 9.8 ng g<sup>-1</sup> (dry weight) but was not detected in soil or leachate samples following application of this manure to the aerobic soil. We hypothesize that a major contributing factor for not detecting 17 $\beta$ -trenbolone in soil or leachate can be its short half-life of 5 to 15 h in soils (Khan and Lee, 2010). In contrast, Schiffer et al. (2001) reported a much longer half-life of 267 and 257 days for 17 $\alpha$ -trenbolone and 17 $\beta$ -trenbolone, respectively. However, unlike the current study, the above study was conducted with liquid manure under anaerobic conditions in a storage pit. Thus, piling manure under aerobic conditions may be an effective way to reduce the concentration of trenbolone metabolites compared to anaerobic digesters such as storage pits. The size and moisture level of a manure pile may influence the portion of the pile that is aerobic and thus influence trenbolone degradation.

Pottier et al. (1981) reported that beef cattle mainly excrete 17 $\alpha$ -trenbolone along with small amounts of 17 $\beta$ -trenbolone and trendione, and the conversion among these metabolites in beef cattle is reversible. Thus, in our study, 17 $\alpha$ -trenbolone in the manure likely was transformed to trendione and 17 $\beta$ -trenbolone while in storage before application to the lysimeters and field plots. The occurrence and fate of the intermediate trendione was not evaluated in this study due to unavailability of commercial standards. Data on trendione in manure could have provided additional information about the persistence of trenbolone metabolites in the soil. Khan et al. (2008) found that trendione degraded more slowly and had almost five times greater sorption affinity with soil than the other two metabolites: 17 $\alpha$ -trenbolone and 17 $\beta$ -trenbolone.

Melengestrol acetate concentration was below the quantification limit (0.5 ng g<sup>-1</sup>) in all manure samples taken after storage in April 2008 (table 5). This result is consistent with previous investigations of the environmental persistence of this feed additive. Using the same manure sources as in this study, Bartelt-Hunt et al. (2012) found that the average concentration of MGA in manure decreased from 6.5 ng g<sup>-1</sup> in June 2007 to 2.8 ng g<sup>-1</sup> in September 2007 during storage as stockpiled manure. Considering this degradation pattern, it was unlikely that any MGA would be detected in April 2008 when the composted and stockpiled manure were applied to the lysimeters and surrounding plots.

Detection of testosterone in composted manure may reflect enzymatic microbial transformation of progesterone via 17 $\alpha$ -hydroxyprogesterone and 4-androstenedione to testosterone (Carlström, 1967; Wadhwa and Smith, 2000). Mansell et al. (2011) hypothesized that the presence of androgens in cattle feedlot runoff may be due to this progesterone degradation pathway. As heifers would not be expected to excrete significant quantities of testosterone in urine or feces, testosterone remains an unexplored source of androgens in livestock waste. In general, testosterone, 17 $\alpha$ -hydroxyprogesterone, and 17 $\alpha$ -estradiol were below the method quantification limit.

Among the estrogens, estrone was the most prominent form in both manure treatments, compared to the other two metabolites of 17 $\beta$ -estradiol: 17 $\alpha$ -estradiol and estriol (not detected). Bartelt-Hunt et al. (2012) also found high, but decreasing, concentrations of estrone in manure (25.6 ng g<sup>-1</sup> dry weight in June 2007, seven days after excretion to 11.0 ng g<sup>-1</sup> dry weight in July 2007, 46 days after excretion). In contrast, the average concentration of 17 $\alpha$ -estradiol was increasing: below the method detection limit seven days after excretion and 8.5 ng g<sup>-1</sup> (dry weight) 46 days after excretion.

Although zeranol ( $\alpha$ -zearalanol) has been used as a growth promoter by the beef cattle industry for several decades, the metabolic transformation of  $\alpha$ -zearalanol and excretion from cattle is not well documented. While  $\alpha$ -zearalanol was present in composted manure, the concentrations of two of its metabolites ( $\alpha$ -zearalenol and  $\beta$ -zearalenol) were greater in stockpiled manure. Identifying the exact sources of these compounds in our study was challenging, as  $\alpha$ -zearalanol and three of its metabolites ( $\beta$ -zearalanol,  $\alpha$ -zearalenol, and  $\beta$ -zearalenol) found in the environment can form from the metabolism of zearalenone produced by commonly found *Fusarium* fungus in fermented corn (Hartmann et al., 2007). As corn silage was added to the composted manure piles and fed to the animals, corn can also be identified as a potential source of these mycotoxins in the manure.

The occurrence and concentrations of steroid hormones in manure and manure-associated soils measured in this study is consistent with previous studies, further indicating they serve as sources and reservoirs for these compounds to the environment. As discussed earlier, the potential biological significance of these levels can only be adequately evaluated after determining the mobility and persistence in the environment. The occurrence of a given compound at concentrations above the method quantification limit indicates that it is available to be mobilized but does not provide an indication of the biological significance.

## LEACHATE

A total of 142 leachate samples were collected for steroid analysis over the study period from April 2008 to July 2011. Because of occasionally insufficient leachate, often associated with lower soil water content, not all lysimeters were sampled every time. The average soil water content over the eight depths is given in figure 1. Statistical analysis was performed for four dates of soil water measurement: one date in July in each of the four study years. There was a significant ( $p \leq 0.05$ ) difference in average soil water content among the four years; the greatest soil-water content was observed in July 2010 (0.40 m<sup>3</sup> m<sup>-3</sup>) and the lowest in July 2009 (0.11 m<sup>3</sup> m<sup>-3</sup>).

Among the three manure treatment plots, there was no significant difference ( $p \leq 0.05$ ) in average soil water content in July 2008. In July 2009 and July 2011, the average soil water contents of the composted and stockpiled manure treatment plots were significantly lower than that of the control treatment plot. In July 2010, there was a significant difference in soil water content among all three manure

treatments, with the highest soil water content in the control treatment and the lowest in the composted manure treatment.

The relative Br concentrations ( $C/C_0$  = detected Br concentration divided by applied Br concentration) indicate a transport time of 12 to 16 months before detecting Br in the leachate samples above background concentrations (fig. 2). The maximum Br concentration occurred in the stockpiled manure treatment and was less than 2% of the applied concentration. It required 220 to 260 mm of water (leachate) for Br to reach the bottom of the lysimeters. After this amount of drainage, Br concentrations were much greater than background levels. Klocke et al. (1999) found that a similar amount of drainage (180 to 210 mm) was needed to drain out a chloride tracer using four lysimeters in the same field. The total amount of drainage in our study was greatest in the control treatment, which was associated with its higher soil water content from the later part of 2009 to the end of the study (fig. 1).

Mass balance analyses indicated that an average of 31% (53 kg ha<sup>-1</sup>, SD = 16 kg ha<sup>-1</sup>) of the applied Br leached through a lysimeter installed in a plot receiving stockpiled manure between June 2008 and July 2011 (fig. 3b). For the control and the composted manure treatments, this was 17% (29 kg ha<sup>-1</sup>, SD = 10 kg ha<sup>-1</sup>) and 18% (30 kg ha<sup>-1</sup>, SD = 15 kg ha<sup>-1</sup>) of the applied amount, respectively (figs. 3c and 3a).

Detectable levels of steroids were found in only 5% of the leachate samples. Only five of the 17 steroids or metabolites were detected in the leachate samples, including 11-ketotestosterone (oxidized from testosterone),  $\alpha$ -zearalanol, estrone, progesterone, and MGA (fig. 3). There was no detection of any steroids or metabolites in the leachate from plots treated with composted manure throughout the study period (fig. 3a).

Traces of estrone (5 ng L<sup>-1</sup>) and  $\alpha$ -zearalanol (12 ng L<sup>-1</sup>) were detected in October 2008 (after 127 mm of drainage) and November 2008 (after 139 mm of drainage) in leachate from lysimeters treated with stockpiled manure long before Br was detected in the leachate samples above background levels in September 2009 (fig. 3b). The Br concentration curve indicated that the average transport time in the stock-

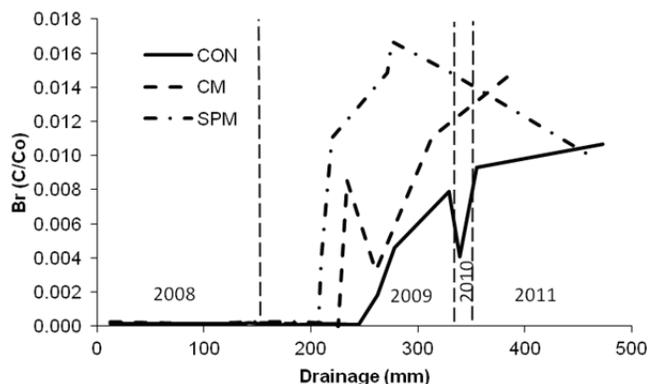


Figure 2. Relative bromide concentration in leachate as a function of drainage from the lysimeters (CON = control, CM = composted manure, and SPM = stockpiled manure). Bromide was applied to the lysimeters in June 2008.

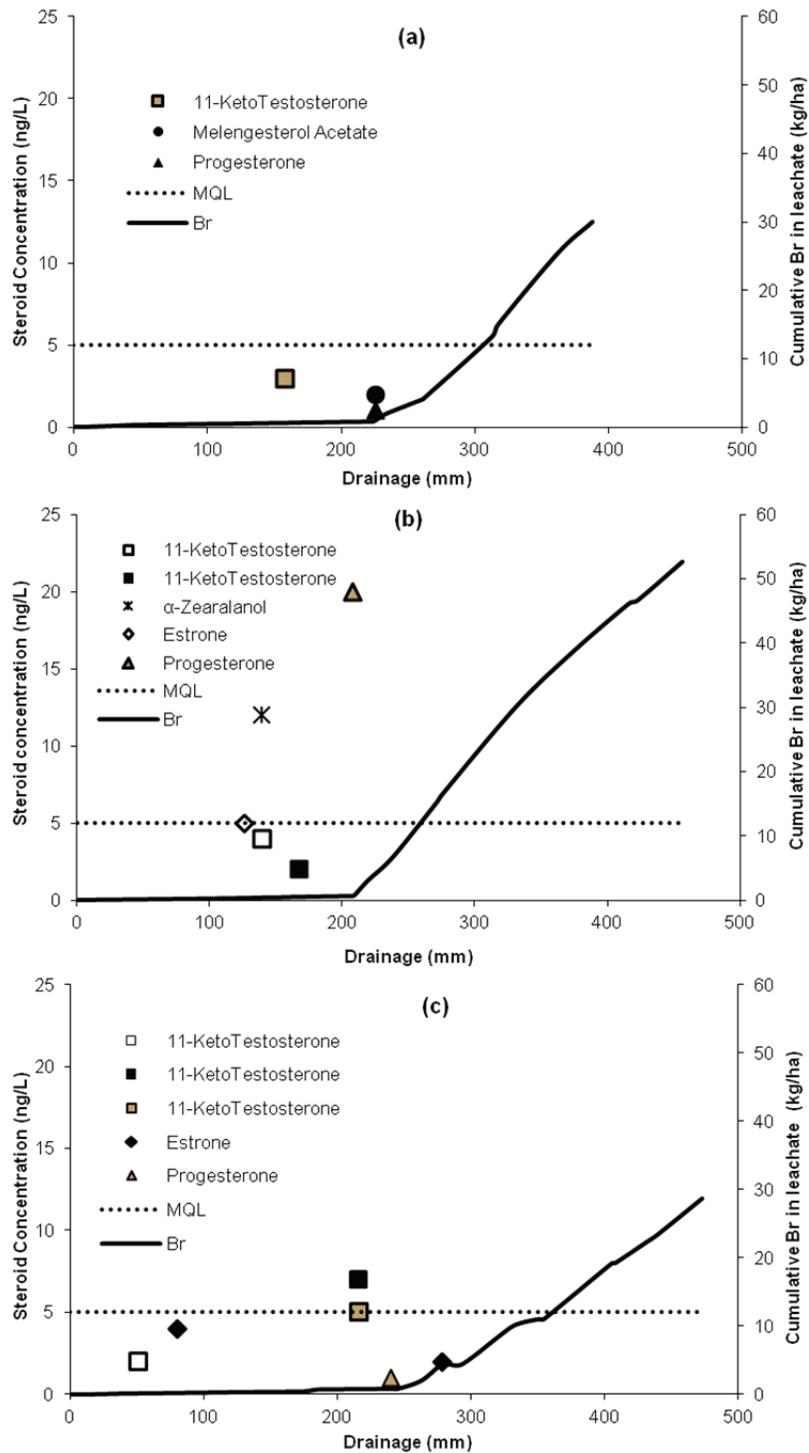


Figure 3. Steroid hormone concentration and cumulative bromide mass found in leachate from percolation lysimeters in (a) composted manure, (b) stockpiled manure, and (c) control treatments. Steroid MQL (method quantification limit) = 5 ng L<sup>-1</sup>. White symbols are data from replication 1, black symbols are data from replication 2, and gray symbols are data from replication 3.

piled manure treatment was approximately 16 months (June 2008 to September 2009) with 220 mm of drainage. Progesterone (20 ng L<sup>-1</sup>) was detected after 207 mm of drainage in May 2009, again before Br, also in the stockpiled manure treatment (fig. 3b).

The metabolite 11-ketotestosterone was detected two times in the control treatment at concentrations of 7 and 5 ng L<sup>-1</sup> in May 2009 samples, where Br was present in the

leachate samples after 260 mm drainage in September 2009 (fig. 3c), although the concentrations are near the method quantification limit for this compound. The fact that steroid hormones were detected in leachate before bromide may be explained by preferential flow through the soil profile. Limited detections of these compounds in leachate samples can be explained by their short half-life (ranging from a few hours to a few days), hydrophobic nature, and high

sorption affinity to soil in the vadose zone (Johnson et al., 2006; Casey et al., 2003, 2004).

## SOIL

A total of 324 soil samples were collected from nine field plots (3 treatments × 3 replications) at six different soil depths in 2008 (two sampling events), 2009 (three sampling events), and 2011 (one sampling event). A summary of the steroid hormone analysis of soil samples is given in table 6 with steroid concentrations, soil depths, and detection dates.

The pretreatment soil samples, collected in April 2008 before manure application to the lysimeters and adjacent field plots, showed the presence of androsterone in one plot (plot 310) at concentrations of 1.4 ng g<sup>-1</sup> (0.16-0.30 m soil depth) and 0.6 ng g<sup>-1</sup> (0.31-0.60 m soil depth), and α-zearalanol and α-zearalenol were detected in another plot (plot 342) at concentrations of 2.0 ng g<sup>-1</sup> (0.31-0.60 m soil depth) and 1.3 ng g<sup>-1</sup> (1.81-2.40 m soil depth), respectively (table 6). The presence of both zearanol-related *Fusarium* metabolites (α-zearalanol and α-zearalenol) before manure application may be explained by metabolism of zearalenone from the previous year's crop, whereas androsterone may have come from wildlife (Bartelt-Hunt et al., 2012).

Following manure application in April 2008, approximately 10% of the soil samples had detectable levels of steroids. Most of these (nearly 80%) were detected in samples collected in September 2008. There was a nearly equal frequency of detections in each treatment, although the types of hormones or metabolites were different. Most (74%) of the detections occurred within the top 1.2 m of the 2.4 m soil sampling depth (table 6). The hormones 11-ketotestosterone, 17α-trenbolone, 17β-trenbolone, β-zearalanol, progesterone, 17α-hydroxyprogesterone, and MGA were not detected in any soil samples above the method quantification limit of 0.5 ng g<sup>-1</sup>.

Several endogenous hormones were detected at various depths in control plots. The endogenous hormones testosterone (0.6 ng g<sup>-1</sup>), 4-androstenedione (0.6 ng g<sup>-1</sup>), 17α-estradiol (0.7-1.0 ng g<sup>-1</sup>), estrone (1.4 ng g<sup>-1</sup>), and estriol (0.8 ng g<sup>-1</sup>) and the mycotoxin α-zearalenol (1.1 ng g<sup>-1</sup>) were detected in control plots at several depths down to 1.8 m. Possible sources of these endogenous hormones and mycotoxin include wildlife and residues from earlier manure application. The detection of 17α-estradiol in all three control plots (one at 0.61-1.2 m depth) and 4-androstenedione in one of the control plots (at 1.21-1.8 m depth) was unexpected but may be explained by previous cattle manure application in 2002 and 2003 (Aga et al., 2005) at this location followed by macropore transport (preferential flow).

The hormones 4-androstenedione (1.4 ng g<sup>-1</sup>), α-zearalanol (4.9 ng g<sup>-1</sup>), β-zearalanol (1.1 ng g<sup>-1</sup>), 17β-estradiol (0.7-4.3 ng g<sup>-1</sup>), and estrone (1.3 ng g<sup>-1</sup>) were present in the soil receiving the composted manure treatment; 4-androstenedione was detected within the top 0.15 m of soil, where organic matter content was highest (1.14%, table 1). According to studies by Lee et al. (2003) and Mashtare et al. (2011), estrone and 17β-estradiol have similar logK<sub>oc</sub> values, ranging from 3.14 to 3.22 in soils. Because estrone is also a microbial degradation product of 17β-estradiol and has been found to be more persistent in soils and aquatic systems (Casey et al., 2003; Colucci et al., 2001), it is likely that transformation and persistence are the controlling factors in the soil profile. Estrone was detected at the 0.16-0.30 m depth in both the control and composted manure treatments in 2008. In the stockpiled manure treatment, it was detected deeper in the soil profile, but only after a few years, in 2011 (table 6).

Estrone (0.8 and 1.3 ng g<sup>-1</sup>), α-zearalanol (2.8 ng g<sup>-1</sup>), 17β-estradiol (0.9-2.3 ng g<sup>-1</sup>), and estriol (6.7 ng g<sup>-1</sup>) were

**Table 6. Detected hormone concentration in soil (ng g<sup>-1</sup>) for various depths (cm, in parentheses) and sampling dates. Pretreatment (April 2008) samples are shown in *italics*. Blanks (-) indicate hormone not detected. Seven hormones, not listed here, were not detected in any soil samples.**

Steroid Hormone or Metabolite	Control (no manure) Plots			Composted Manure Plots			Stockpiled Manure Plots		
	Plot 308	Plot 337	Plot 340	Plot 310	Plot 323	Plot 342	Plot 321	Plot 336	Plot 341
Testosterone	-	0.6 (0-15) 23 Sept. 2008	-	-	-	-	-	-	-
4-androstenedione	-	-	0.6 (121-180) 23 Sept. 2008	-	1.4 (0-15) 23 Sept. 2008	-	-	-	-
Androsterone	-	-	-	<i>1.4 (16-30), 0.6 (31-60) 15 Apr. 2008</i>	-	-	-	-	-
α-zearalanol	-	-	-	-	-	<i>2.0 (31-60) 15 Apr. 2008</i>	2.8 (0-15) 23 Sept. 2008	-	-
α-zearalenol	-	-	1.1 (16-30) 23 Sept. 2008	4.9 (0-15) 23 Sept. 2008	-	<i>1.3 (181-240) 15 Apr. 2008</i>	-	-	-
β-zearalenol	-	-	-	-	1.1 (16-30) 23 Sept. 2008	-	-	-	-
17α-estradiol	0.7 (16-30) 27 Oct. 2009	1.0 (31-60) 23 Sept. 2008	0.9 (61-120) 23 Sept. 2008	-	-	-	-	-	-
17β-estradiol	0.6 (181-240) 21 July 2011	-	-	0.7 (16-30), 0.7 (31-60) 23 Sept. 2008	-	4.3 (16-30), 2.0 (121-180) 23 Sept. 2008; 0.6 (31-60) 21 July 2011	1.6 (16-30), 1.5 (31-60) 23 Sept. 2008	2.3 (181-240) 23 Sept. 2008	0.9 (61-120), 1.2 (121-180) 23 Sept. 2008
Estriol	0.8 (0-15) 27 July 2009	-	-	-	-	-	6.7 (121-180) 23 Sept. 2008	-	-
Estrone	1.4 (16-30) 23 Sept. 2008	-	-	-	1.3 (16-30) 23 Sept. 2008	-	1.33 (181-240) 21 July 2011	-	0.8 (121-180) 21 July 2011

detected in the soil receiving the stockpiled manure treatment. Like 17 $\beta$ -estradiol, estriol was not detected in manure samples, but it was detected in soil samples from both the control (0-0.15 m) and stockpiled manure (1.21-1.80 m) treatments at varying concentrations. Lai et al. (2000) found a log $K_{oc}$  value of 3.5 for estriol in river sediment, which was equivalent to that of other estrogens in soil sediment. It may be assumed that the chemical fate and transport of estriol will be similar to that of other estrogens.

Microbial transformation of parent compounds in soil fertilized with both the composted and stockpiled manure may explain the occurrence of some compounds, while persistence is a better explanation for others. For example, estrone is known to be more resistant to microbial degradation and was detected at relatively high concentrations in both composted and stockpiled manure. Estriol is known to be a degradation product of estrone but is typically not as persistent as estrone. Similar conversions must have occurred for  $\alpha$ -zearalenol and  $\alpha$ -zearalanol, which were not present in the manure samples but were detected in the soil treated with composted and stockpiled manure. In addition, manure applied before this study may have been responsible for the presence of  $\alpha$ -zearalenol and  $\alpha$ -zearalanol, although these compounds can also be produced as metabolic products of bacteria and fungi.

The limitations of this study should be recognized, and the results should not be generalized too much. This study was conducted under a specific set of conditions, and steroids may behave differently under other conditions. A single manure application during the four-year duration of the study is atypical in normal crop production practice. Normal practice with regular manure application may increase the mass loading of steroid hormones to the vadose zone and groundwater. Moreover, a different row crop, such as corn, with a higher N requirement would have required more manure and hence created a potential for more steroids in the soil-water system. On the other hand, a soil with a higher organic matter content might have resulted in even less mobility of hormones and metabolites and more attachment to soil or sediment (Lee et al., 2003).

Another factor that may have affected the results of this study was the source and management of manure. In particular, larger piles of stockpiled manure may have a greater portion of the pile under anaerobic conditions, resulting in less oxidation of steroids and metabolites. Soil texture, microbial community, depth of water table, topography, and occurrence of preferential flow can play an important role in the movement of these steroids in the environment.

## CONCLUSIONS

Few studies have investigated the direct impact of steroid hormones from cattle manure on groundwater, prompting this study to determine the extent of vertical hormone movement through the vadose zone. Leachate and soil samples were collected during the four-year duration of the study. Steroid hormones were found in only 5% of the leachate samples. Several steroid hormones or metabolites were detected in 10% of the soil samples. Most (74%) of

these were in the top half (top 1.2 m) of the 2.4 m soil sampling depth. No detectable levels of synthetic steroids were present in any of the soil or leachate samples.

As the concentrations and occurrence of steroid hormones and their metabolites in manure and manure-associated soils measured in this study are consistent with previous studies, this study indicates that land-applied cattle manure can serve as a source and reservoir for these compounds to the environment. However, leaching through the soil profile may occur slowly and seems to be an unlikely source of these chemicals in groundwater.

The low frequency of detection of hormones in leachate samples is consistent with previous investigations indicating rapid degradation and/or adsorption depending on soil type (Hildebrand et al., 2006; Bradley et al., 2009). Earlier detections of several steroids before the tracer Br may be due to preferential flow (Kjaer et al., 2007; Wicks et al., 2004; Peterson et al., 2000). However, a few steroids that can be more persistent in the soil-water system, such as estrone or progesterone, along with other organic and inorganic compounds, may be a threat to groundwater. Additional research is required for different soils, climates, and agricultural practices to identify and understand the overall movement mechanisms of steroid hormones through the vadose zone.

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