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Mass Balance Approaches to Characterizing the Leaching Potential of Trenbolone Acetate Metabolites in Agro-Ecosystems

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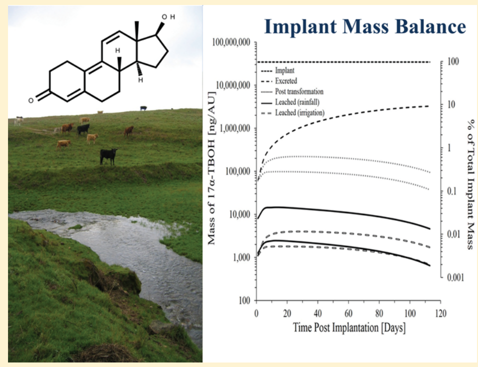
S *Supporting Information*

ABSTRACT: Several studies have documented the occurrence and fate of trenbolone acetate (TBA) metabolites in soil and water. However, considerable uncertainty still exists with respect to TBA risk in agro-ecosystems because limited data are available to quantify excretion, transformation, and leaching processes. To address these uncertainties, we used experimental mesocosms and a mass balance approach to estimate the TBA metabolite leaching potential from manure excreted by implanted (40 mg TBA, 8 mg 17β-estradiol) beef cattle. Manure sample analysis indicates that over 113 days, a maximum of 9.3% (3,200 μg/animal unit [AU]) of the implant dose was excreted as 17α-trenbolone (17α-TBOH), and <1% was excreted as 17β-trenbolone (65 μg/AU) or trendione (3 μg/AU). While most (>97%) of the total excreted mass of 17α-TBOH transforms to uncharacterized products, 0.3–0.6% (100–220 μg/AU) of the implant dose accumulates on land surfaces and is available for subsequent transport. During rainfall or irrigation events, a maximum of 0.005–0.06% (1.6–22 μg/AU 17α-TBOH) or 0.005–0.012% (1.8–4 μg/AU 17α-TBOH) of the dose leached into runoff, respectively. Leaching potentials peak at 5–30 days postimplantation, suggesting that targeted timing of implantation and irrigation could minimize steroid leaching during rainfall and irrigation events.



INTRODUCTION

A major environmental challenge of large-scale animal agriculture is managing manure and preventing manure-derived contaminants from affecting aquatic ecosystems. Animal agriculture generates 1.3 × 10^{12} kg/yr of manure in North America and 13 × 10^{12} kg/yr globally.[1] During runoff events, contaminants, including nutrients, pathogens, steroids, and veterinary pharmaceuticals, are subsequently leached from manure and can transport to receiving waters. These contaminants are leading sources of surface water quality impairment[2] and are implicated in eutrophication,[3] aquatic toxicity,[4] and disease outbreaks.[5]

Observations of endocrine disruption within aquatic vertebrates, including population-scale effects, have been linked to steroid hormones associated with animal agriculture.[6−10] For example, trenbolone acetate (TBA) is a potent synthetic androgen that is widely used as a growth promoter.[11] Annually, TBA production and use likely exceeds 5000–10 000 kg, implying that it is one of the most pervasive environmental steroids.[12] Dominant TBA metabolites likely to transport to aquatic environments include 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), and trendione (TBO).[12−14] When exposed to fish, 17α-TBOH and 17β-TBOH are capable of fecundity reduction and sex reversal at low concentrations (e.g., 10–30 ng/L).[7,15,16] Agricultural runoff from TBA-implanted cattle manure can exhibit androgenic activity, although it is unclear if this bioactivity is attributable to TBA metabolites.[8,10,17−19] Once excreted, these metabolites are mobilized in runoff and detected in surface waters, even at concentrations exceeding the lowest observed effects concentrations.[7,15,20−25] Considering their potency and widespread use, characterizing the mobilization and transport of TBA metabolites in agro-ecosystems is critical to evaluating their ecosystem risk.

In agriculture, "leaching" typically describes nutrient/contaminant mobilization from soils. Because the mechanisms (e.g., dissolution, partitioning, and desorption) governing steroid mobilization from soils and manure are identical, we use "leaching" to describe mass transfer processes that mobilize steroids from manure, even if leachate solutions subsequently transport as runoff. Therefore, the leaching potential is governed by three processes: steroid excretion, attenuation (e.g., sequestration and microbial transformation) within manure, and subsequent mass transfer to aqueous phases. For endogenous steroids, such as estradiol or testosterone, these processes (particularly excretion and attenuation) are partially characterized,[26−28] but for TBA metabolites and other exogenous contaminants, these processes remain poorly characterized, particularly mobilization and transport processes.

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Table 1. Observed 17α-TBOH Leaching during Rainfall Including 17α-TBOH Mass and Concentrations, The Maximum Rainfall Intensity (Max. I), the Average Rainfall Intensity (Avg. I), Storm Duration (Dur), and the Rainfall Depth[a]

rainfall event	17α-TBOH leached (ng)	normalized 17α-TBOH leached (ng/cm^2)	17α-TBOH manure concentration (ng/g-dw)	max. I (cm/h)	avg. I (cm/h)	dur. (hr)	depth (cm)
6/6/11	170 ± 50	0.29	39.3	0.64	0.25	14	3.6
10/4/11	14 ± 4	0.024	4.6	0.25	0.17	7	1.2
1/19/12	5 ± 3	0.008	7.4	0.06	0.04	11	0.4
2/29/12	49 ± 8	0.083	27.5	0.29	0.18	3	0.4
3/16/12	94 ± 2	0.16	[b]	0.33	0.15	2	0.8
12/2/12	1,800 ± 800	3.05	29.1	1.85	1.66	2	5.0
r			0.77[c]	0.98[c]	0.98[c]	−0.12	0.72[c]

[a]Storm variables are presented only for the time period manure was exposed to rainfall. Pearson correlations (r) between 17α-TBOH and each independent variable are included. Confidence intervals represent 95% confidence intervals (n = 3, except 10/4/11: n = 4). [b]Concentration not measured. [c]$P < 0.001$.

Fundamentally, the leaching potential is a function of the steroid mass on the land surface: as concentrations increase, higher mass transfer rates to the aqueous phase are expected. TBA metabolite concentrations excreted in manure should vary with time because their blood serum concentrations spike immediately after TBA implantation and subsequently drop by 2 orders of magnitude over 140 days.[29] This suggests that manure concentrations and subsequent ecological risks resulting from leaching also vary with time even if manure production rates are constant.[25] Furthermore, TBA metabolite transformation has mostly been evaluated in systems more characteristic of confined animal agriculture like manure piles, anaerobic liquid manures, and agricultural soils, with a wide range in persistence (hours to many months) observed .[13,25,30,31] These scenarios, however, are unrepresentative of aerobic surface environments typical of rangelands or pastures and equilibrium partitioning processes observed in agricultural soils may not accurately describe nonequilibrium leaching and transport processes occurring during relatively short irrigation and rainfall events.[32,33] To identify specific agro-ecosystems (e.g., rangelands, CAFOs), or practices (e.g., flood irrigation) that pose TBA metabolite risks to receiving waters, leaching and mass transfer processes should be evaluated independently of subsequent transport. Therefore, our goal was to use a mass balance approach to characterize the mobilization potential of TBA metabolites derived from manure after TBA implantation, by (1) characterizing TBA metabolite excretion and transformation in manure, (2) quantifying subsequent leaching during rainfall and irrigation events, and (3) modeling the leaching potential under different rangeland and irrigated pasture scenarios.

■ MATERIALS AND METHODS

TBA Implantation. Hereford/Angus cross heifers and steers (steer calves [n = 3] or yearling heifers [n = 4], 145−350 kg, 6−18 months old) were implanted with Revalor G (40 mg TBA, 8 mg estradiol, used for rangeland cattle) at the University of California Sierra Foothills Research and Extension Center (NAD 83 UTM 10S-645602E, 4345995N), Browns Valley, CA. Animals were penned in a covered barn for manure collection from a precleaned concrete floor. Each implant released TBA over 100−110 days per manufacturer estimates, and when needed, animals were reimplanted following manufacturer protocols. Animals were handled in accordance to guidelines prescribed by the University of California, Davis Animal Care and Use Committee.

Metabolite Excretion. We collected manure from three newly implanted steers (i.e., no previous implants; 145−190 kg) to quantify TBA metabolite excretion. Steers were penned for 24 h, and 50 mL homogenized samples were collected and frozen on days 0, 1, 2, 3, 4, 7, and every subsequent 10th day postimplantation for 113 days. Excreted manure also was collected and weighed daily to measure production. For analysis, TBA metabolites were extracted in triplicate by placing 5 g wet weight (g-ww) samples and methanol (25 mL) into 50 mL conical centrifuge tubes. The tubes were then shaken (1 min), sonicated (10 min), and centrifuged (3500 rpm, 10 min), and the supernatant was decanted into 1 L amber glass bottles.[24,25] Each step was repeated three times. The combined supernatant (∼75 mL) was diluted to 1 L with deionized water, spiked with 100 ng 17β-TBOH-d_3 isotopic standard in methanol and immediately loaded onto 6 mL C-18 solid phase extraction (SPE) cartridges (Restek, Bellefonte, PA).

Processing and analytical methods are described in the Supporting Information (SI) and elsewhere.[24,25] QA/QC measures included field blanks (i.e., rain/irrigation water or manure from nonimplanted cattle) and laboratory spikes (100 ng TBA metabolites in 1−4 L aqueous or 5 g-ww manure samples). All blanks were at or below method limits of detection (i.e., <0.5−1 ng/L). 17β-TBOH-d_3 recovery averaged 81 ± 6% in all samples (n = 279), and reported concentrations were corrected using 17β-TBOH-d3 recoveries, but not spike recoveries. In manure samples, 17α-TBOH, 17β-TBOH, and TBO spike recoveries were 125 ± 7%, 107 ± 9%, and 33 ± 9% (n = 4), respectively, the latter indicating that TBO recovery from manure was low. In aqueous samples, 17α-TBOH, 17β-TBOH, and TBO spike recoveries were 105 ± 7%, 94 ± 6%, and 80 ± 35% (n = 10). All samples were collected in triplicate and reported confidence intervals represent 95% confidence intervals unless otherwise noted.

Metabolite Transformation. We estimated TBA metabolite transformation rates in fresh manure by measuring concentrations daily over 5 days. Samples (5 g-ww) were incubated (T = 1, 19, or 33 °C) in the dark or in direct sunlight in the field, protected from wind to minimize desiccation, then extracted and processed as previously described.

Metabolite Leaching. In arid regions like the western U.S., flood irrigation is commonly used to produce forage for livestock.[34] While irrigation events usually last 6−12 h, we measured TBA metabolite concentrations during simulated 3 h irrigation events (due to sampling limitations) to define the mass transfer dynamics. Irrigation water supplied by the

Browns Valley Irrigation District from Collins Lake (pH = 7.6, TOC = 5.7 ± 0.1 mg/L) was slowly applied at a constant rate of 8 L/h to 9 mesocosms filled with 1−2 L fresh manure samples of varying interfacial area (i.e., 120, 600, or 1200 cm^2). For all mesocosms, the leachate pooled ∼1 cm above the manure, overflowed into amber glass containers (4 L), and was collected at 30 min intervals (see SI for detailed descriptions, Figure S1). Within the 120, 600, and 1200 cm^2 mesocosms, the estimated hydraulic retention time was 0.9, 4.5, and 9 min, respectively. Each sample was immediately pressure filtered (0.7 μm AP40 filters, Millipore, Billerica, MA) and processed as previously described.

We also measured TBA metabolite leaching during six natural rainfall events that occurred between January 2011 and December 2012 (Table 1). Fresh manure (1 kg-ww, $n = 3$) was suspended ∼15 cm above the bottom of 12 L stainless steel pots and exposed to rainfall (SI Figure S1). Samples were confined within aluminum screen cylinders (23 cm diameter, 5 cm height) that were open on the top to allow rainfall to impact the manure surface but prevented extensive rainfall-induced sample erosion. The screens also maintained a near constant bulk interfacial area (590 cm^2/kg-ww; top area and sides) exposed to rainfall. After each rainfall event, which lasted from 2 to 14 h, the leachate was immediately collected, filtered, spiked with 17β-TBOH-d_3, loaded onto SPE cartridges, and transported to the laboratory for processing.

■ RESULTS AND DISCUSSION

Metabolite Occurrence. To evaluate TBA metabolite mass excretion, we measured concentrations for 113 days after implantation. 17α-TBOH concentrations peaked at 64 ± 9 ng/g dry weight (g-dw; 1 g-ww = 0.17 ± 0.01 g-dw, $n = 27$, SI) 24 h after implantation (Figure 1). After 48 h, measured concentrations dropped to ∼40 ng/g-dw and steadily decreased to 10 ± 1 ng/g-dw over 113 days. The reduction in manure concentration after 48 h was linear with time ($R^2 = 0.88$) and was modeled as follows:

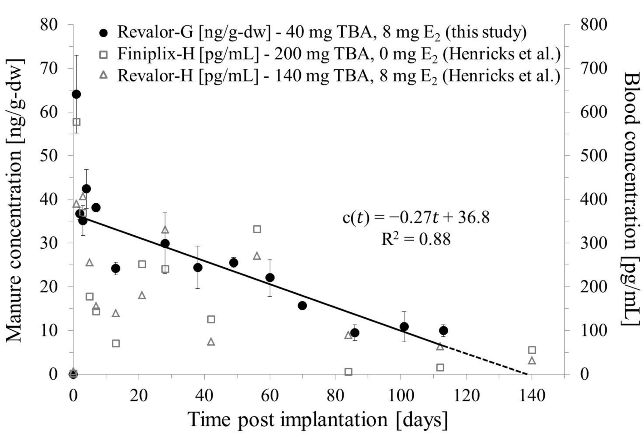

Figure 1. Measured 17α-TBOH manure concentrations (ng/g-dw, closed circles, 40 mg TBA, 8 mg estradiol, primary-y axis) and reported 17β-TBOH blood serum concentrations (pg/mL, open squares, 200 mg TBA, 0 mg estradiol; open triangles, 140 mg TBA, 8 mg estradiol; secondary-y axis; Henricks et al. 1997)[29,35] from cattle implanted with various formulations. The best fit regression line describes the manure concentrations from 2 to 113 days (solid line; eq 1). After 137 days, the manure concentration is expected to be below detection levels (dotted line). Error bars represent 95% confidence intervals.

$$C_m(t) = -0.27t + 36.8 \tag{1}$$

where $C_m(t)$ is the concentration of 17α-TBOH (ng/g-dw) in fresh manure at t days postimplantation. Using eq 1, the 17α-TBOH concentration is expected to reach nondetect levels at 137 days, although samples were not analyzed after 113 days. These trends are similar to reported TBA metabolite concentrations in blood, which spiked immediately after implantation and decreased through 140 days (Figure 1).[29,35] 17β-TBOH was detected through 7 days postimplantation at 1.7−3.8 ng/g-dw, and was only detected sporadically afterward at 1.0−3.9 ng/g-dw. Similarly, TBO was detected twice at 1.3 and 2.5 ng/g-dw through 4 days postimplantation.

The manure production of the juvenile steers ranged from 0.6 to 1.8 kg-dw/day/AU (animal unit) over 113 days, was consistent with reported estimates,[27] and was modeled as follows:

$$m(t) = 0.0086t + 0.94 \tag{2}$$

where $m(t)$ is the daily manure excretion (kg-dw/AU). The product of eqs 1 (in μg/kg-dw) and 2 describes the mass excretion:

$$s(t) = -0.0023t^2 + 0.064t + 34.71 \tag{3}$$

where $s(t)$ is the estimated 17α-TBOH (μg/d/AU) mass excreted at t days post implantation. Because $s(t)$ in eq 3 is the derivative of the cumulative excreted steroid mass (S_t; i.e., $(d/dt)S_t = s(t)$), eq 3 can be integrated to the following:

$$S_t = -0.0008t^3 + 0.031t^2 + 34.74t + 26.23 \tag{4}$$

where S_t is the cumulative 17α-TBOH mass excreted (μg/AU) at t days postimplantation. For example, at 113 days postimplantation, the total 17α-TBOH mass excreted was ∼3200 μg/AU, which accounts for 9.3% of the total TBA mass in a 40 mg implant. At 137 days (i.e., the estimated no-detect point), ∼3400 μg/AU, or 9.8% of the total implant mass, is predicted to be excreted. We estimate that 65.5 μg/AU of 17β-TBOH and 2.8 μg/AU of TBO also were excreted in fresh manure, which collectively account for ∼2% of the detected TBA metabolite mass and 0.2% of the implant dose. Equations 3 and 4, while sensitive to estimates of the TBA metabolite concentration and manure production (i.e., eqs 1 and 2), describe the total TBA metabolite mass discharged to the environment and represent an upper bound to potential mass leaching.

Overall, the excretion data were similar with reported values. Schiffer et al. reported an initial mass ratio of 17α-TBOH, 17β-TBOH, and TBO of approximately 94:4:2, respectively,[13] while Webster et al. reported the same ratio as 87:13:0.[25] However, consistent with agricultural practice, cattle used in this study and Webster et al. were implanted intravenously in the ear,[25] while animals studied by Schiffer et al. were implanted intramuscularly.[13,36] In this study, the same ratio, averaged across 113 days, was approximately 98:2:0. Bartelt-Hunt et al. reported a 17α-TBOH:17β-TBOH ratio of 99:1.[21] Schiffer et al. also estimated that 8% of the total implant dose is excreted as 17α-TBOH at 8 weeks.[13] At 8 weeks, we estimated 17α-TBOH excretion at 1900 μg/AU, accounting for 5.6% of the dose.

Metabolite Transformation. To evaluate transformation in excreted manure, TBA metabolite concentrations were measured over time in 5 g-ww samples as a function of temperature and sun exposure. When incubated at 1 and 33 °C,

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initial 17α-TBOH sample concentrations were 53 ± 5 ng/g-dw but dropped to 22 ± 6 ng/g-dw and 5 ± 4 ng/g-dw, respectively, after 5 days. For the 19 °C treatment, which was a separate trial, initial concentrations were 11 ± 0.3 ng/g-dw but dropped to 4 ± 2 ng/g-dw after 5 days (Figure 2).

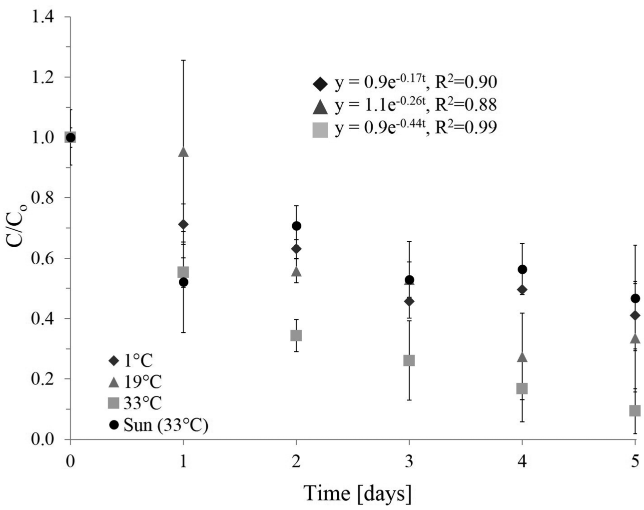

Figure 2. Observed 17α-TBOH transformation in manure as a function of time. Initial 17α-TBOH concentrations were 53.1 ng/g-dw except the 19 °C samples (10.9 ng/g dw). The data were linear following natural log transformation, and estimated first-order rate constants for samples incubated at 1, 19, and 33 °C were 0.17, 0.26, and 0.44/day. Rate constants were not estimated for sunlit samples. Error bars represent 95% confidence intervals of triplicate analysis.

Concentrations followed apparent first-order transformation kinetics with 17α-TBOH rate constants at 1, 19, and 33 °C of 0.17 ± 0.08/d, 0.26 ± 0.09/d, and 0.44 ± 0.07/d, corresponding to half-lives of 4.1, 2.7, and 1.6 days, respectively (Figure 2). Reported half-lives for TBA metabolites vary widely depending on environmental conditions, but for aerobic soils, values range from <0.2−25 days, and our data are especially similar to ∼0.2−3.3 day half-lives reported for aerobic agricultural soils.[13,25,30,31] Therefore, transformation was likely aerobic, although the redox state of manure samples was not measured.

For sunlit samples (33 °C average air temperature), initial 17α-TBOH concentrations were 53 ng/g-dw but dropped to 24 ± 9 ng/g-dw over 24 h with little further loss evident, thus precluding estimation of transformation rates (Figure 2). While aqueous TBA-metabolite photolysis has been demonstrated, we do not expect that photolysis or subsequent reversion processes affected this data.[12,37] Most of the TBA metabolite mass in manure is beneath the surface and is therefore "protected" from sunlight, while sample homogenization procedures also would have diluted any reversion effects of near-surface photoproducts. In sunlit samples, transformation was likely inhibited by low moisture conditions. The initial moisture content was 84 ± 2% in both incubated and sunlit samples. After 24 h, the sunlit samples were qualitatively similar in texture and appearance to oven-dried samples, with no similar drying observed for dark incubated samples. Therefore, in dried samples, 17α-TBOH is stable for at least five days, suggesting increased persistence of TBA metabolites in arid conditions, although we did not assess whether the moisture content affected subsequent leaching. Consistent with these observations, low moisture conditions (e.g., air-dried samples) are reported to increase 17α-TBOH persistence in soils.[31,38]

In all treatments, 17β-TBOH and TBO concentrations fluctuated considerably through time. Concentrations of 17β-TBOH ranged from 75 to 185% of initial concentrations (2.4 ng/g-dw), while TBO was 62−155% of initial concentrations (i.e., 0.8 ng/g-dw), suggesting complex transformation dynamics for these metabolites. Microbially mediated interconversion of 17α-TBOH, 17β-TBOH, and TBO occurs in soil and aqueous systems.[30,31,39] While TBO is an intermediate product in these interconversions, the conversion of TBO to 17α-TBOH is often unfavorable.[35] While concentrations were low and near the limit of detection in some cases, the conversion of 17α-TBOH to TBO and, subsequently, 17β-TBOH likely accounts for increased TBO and 17β-TBOH concentrations.

Metabolite Leaching. We used mesocosms containing fully submerged manure samples with a defined bulk interfacial surface area to quantify leaching (mass transfer from solid phases to aqueous phases) during simulated irrigation. Consistent with data for other manure-derived contaminants and theory,[40−44] mass leaching increased with both increasing interfacial area (i.e., 120−1200 cm^2) and higher initial 17α-TBOH concentrations. After 3 h of irrigation (24 total L), the area-normalized 17α-TBOH mass leaching was 0.052 ± 0.001 ng/cm^2 or 0.15 ± 0.001 ng/cm^2 for initial concentrations of 24 or 63 ng/g-dw, respectively (Figure 3). Although suspended solids concentrations were not measured, few particles were observed on filters during sample processing, suggesting that the generation of suspended solids from manure during irrigation, and thus the transport of solids-associated steroids, is low. Other TBA metabolites were infrequently detected in leachate. In samples with 1.4 ± 0.2 ng/g-dw 17β-TBOH initially, 17β-TBOH was sporadically detected at 1.2−1.8 ng/L, while TBO was not detected. Therefore, we believe that the ecological risk associated with TBA use derives primarily from 17α-TBOH mobilization although elevated concentrations of

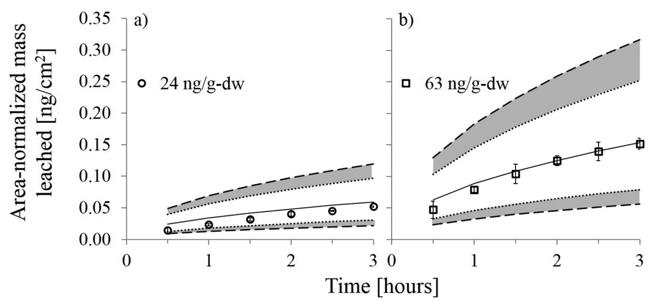

Figure 3. Modeled (lines) and observed (open circles/squares) 17α-TBOH mass leached from simulated irrigation runoff experiments. Separate experiments were conducted to assess leaching at different initial 17α-TBOH concentrations: (a) 24 ng/g-dw and (b) 63 ng/g-dw. The observed mass leached was normalized to the bulk interfacial manure/water surface area (i.e., ng/cm^2) to facilitate direct comparisons with the model. Inputs for modeled data were independent of the observed leaching data. The dashed lines represent the minimum and maximum output of the Monte Carlo simulation (n = 10 000 iterations). The shaded region between dashed and dotted lines represents the outer 5% of the probability distribution, where the probability of being different from the average modeled leaching (solid line) is $P < 0.05$. The unshaded region between the dotted lines represents the middle 95% of the probability distribution.

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17β-TBOH and TBO could occur by metabolite interconversion in some cases.

The highest leaching potential occurs when manure is fully submerged, thereby exposing maximal interfacial area. With a density of 1.0 kg-ww/L (n = 28, SI Table S1) and assuming a cylindrical geometry (3 cm depth, 10.3 cm radius for a 1 kg-ww sample), the estimated bulk surface area is ~860 cm^2. Therefore, we predict that 44 and 129 ng of 17α-TBOH could leach from a fully submerged 1 kg-ww sample over 3 h with initial concentrations of 24 and 63 ng/g-dw, respectively. As adult animals excrete ~40 kg-ww/d (6.8 kg-dw) of manure daily,[27] and assuming complete submersion, we predict 1200–5200 ng/AU of 17α-TBOH leaching over 3 h of irrigation (see SI Figure S2 for leaching comparison between 17α-TBOH and N, P, total organic carbon, total coliforms, and *E. coli*).

To evaluate leaching during rainfall, 1 kg-ww samples were exposed to six rainfall events (Table 1). The 17α-TBOH mass in rainfall leachate (0.3–1.4 L per event) ranged from 5 to 1,800 ng/kg-ww (9–1300 ng/L in leachate) and was most correlated with rainfall intensity (r = 0.98, P < 0.001) and depth (r = 0.72, P < 0.001). Surprisingly, dilution effects from larger storms were unimportant relative to the increased mass leaching potential because leachate concentrations consistently increased as both rainfall depth and storm intensity increased (Table 1). Similar to irrigation leaching, the mass leached was correlated with the initial 17α-TBOH concentration (r = 0.77, P < 0.001). Relative to irrigation, we believe that the increased leaching during rainfall is a result of rainfall induced erosion and particle generation.[45,46] As surface particles erode, additional interfacial area is exposed, thereby increasing mass leaching potential. While all rainfall leachate samples were filtered, we did not quantify TBA metabolites on suspended solids. Because TBA metabolites are moderately hydrophobic (i.e., K_{oc} = 2.72–3.38),[32,33] it is likely that some mass of TBA metabolites was retained within the suspended solids and available for subsequent transport, although we did not assess that transport process in this study.

The area-normalized leaching of 17α-TBOH during rainfall ranged from 0.008 to 3.1 ng/cm^2 (Table 1). For simulated irrigation experiments, the area-normalized mass leached ranged from 0.052 to 0.15 ± 0.001 ng/cm^2. While the maximum normalized rainfall leaching was over 20 times higher than observed data for irrigation, these comparisons are complicated by different initial concentrations. Normalizing by both interfacial area and initial concentration, the normalized irrigation leaching was 0.0022–0.0023 g-dw/cm^2 (i.e., [ng/cm^2]/[ng/g-dw]) while the normalized rainfall leaching was 0.001–0.10 g-dw/cm^2. As a first approximation, the data suggest that with the exception of low intensity rainfall events, the leaching potential is higher, by as much as 1.7 orders of magnitude, during rainfall events compared to irrigation events, which is consistent with data reported for other agricultural contaminants, including *Cryptosporidium*, phosphorus, and nitrogen.[44,47,48]

Modeled Leaching. To describe irrigation leaching dynamics, we applied a mechanistically derived one-dimensional diffusion model developed for contaminant diffusion from sediments[49] to TBA metabolite leaching from manure:

$$L(t) = \left(\frac{4D}{\pi f}\right)^{1/2} \varphi C_w t^{1/2} \tag{5}$$

where $L(t)$ is the area-normalized mass leached (ng/cm^2), C_w is the aqueous equilibrium concentration (ng/cm^3) in manure, D is the steroid diffusivity (cm^2/s), f is the dissolved fraction of TBA metabolites (unitless), φ is the porosity (unitless), and t is the exposure contact time (s). Each model parameter is easily measured, estimated, or obtained from published literature (see SI Table S1 for parameter estimation and physical/chemical properties of the manure), and we note that these parameters were all derived independently of the irrigation leaching data.[50−52]

We used a Monte Carlo simulation (n = 10 000 iterations) to evaluate the expected bounds of eq 5, test the null hypothesis that there was no statistical difference between observed and modeled leaching, and simplify model constants. The model bounds were assessed by selecting a random value within the measured, calculated, or reported range for each variable (SI Table S1) to solve eq 5 for any input concentration (e.g., 24 and 63 ng/g-dw). We then plotted the average, maximum, minimum, and the outer 5% of all iterations and compared this to the observed irrigation leaching data (Figure 3). Somewhat surprisingly, we observed excellent agreement and detected no statistical differences between the observed and modeled data (P = 0.58 and 0.71 for 24 and 63 ng/g-dw, respectively; see SI for statistical procedures, Figure S3), suggesting that TBA metabolite leaching from manure under irrigation conditions is a diffusion-dominated process. Because the observed and modeled leaching were independent, eq 5 was used to model 17α-TBOH leaching dynamics.

On the basis of the Monte Carlo analysis, the average product of $(4D/\pi f)^{1/2}\varphi$ (from eq 5), which is a constant for a given contaminant within a given media (e.g., soil or manure), was 0.0065 ± 0.0014 cm/s$^{0.5}$ (see SI for derivation). Because the manure concentration (C_m; ng/g-dw) is typically reported instead of the equilibrium aqueous concentration (C_w; ng/cm^3), we computed C_w as a function of C_m for each iteration. The equilibrium concentration can be expressed as follows: C_w = (0.0034 ± 0.0007 g-dw/cm^3) × C_m, where C_m is the average manure concentration at any time postimplantation (SI Figure S4). We assumed that irrigation flow does not affect interfacial area and "clean" irrigation water maintains a near maximum concentration gradient. Therefore, eq 5 can be simplified and the area-normalized mass leached during irrigation (L_i) can be described by a single variable C_m:

$$L_i(t) = 2.21 \times 10^{-5} C_m t^{1/2} \tag{6}$$

where the factor 2.21 × 10^{-5} (g-dw/cm^2/s$^{0.5}$) is the product of 0.0034 (g-dw/cm^3) and 0.0065 (cm/s$^{0.5}$). Webster et al.[25] developed the following expression to describe the concentration of 17α-TBOH on agricultural surfaces, normalized per AU, at any time postimplantation:

$$C_m(t) = \frac{1}{M_t} \sum_0^t S_t \exp\left(-kt \Big|_t^0\right) \tag{7}$$

where S_t is the total predicted metabolite mass excreted/AU (eq 4), k is the first-order rate constant for postexcretion decay (Figure 2), and M_t is the cumulative manure mass excreted/AU by time t (integral of eq 2). While 17α-TBOH excretion decreases through time, mass accumulation on the land surface peaks at ~30 days postimplantation because of increased manure production (eq 2, SI Figure S5). Combining eqs 6 and 7 yields the following:

$$L_{\mathrm{i}}(t_x, t_y) = 2.21*10^{-5}\left(\frac{1}{M_{t_x}}\sum_{0}^{t} S_{t_x}\exp(-kt|_{t_x}^{0})\right)t_y^{1/2} \qquad (8)$$

where L_{i} is the area-normalized mass leached from samples as a function of time postimplantation (t_x, days), and the time the sample is exposed to water (t_y, s; M_t and S_t are expressed only in terms of t_x). Multiplying eq 8 by the estimated interfacial area yields the total mass leached per AU at t_x days postimplantation following t_y seconds of irrigation (seconds were used for consistency with diffusivity units [i.e., cm^2/s]; Figure 4). Under a worst-case scenario of fully submerged manure (860 cm^2/kg-ww interfacial area or 5060 cm^2/kg-dw based on the 83 ± 1% moisture content, $n = 27$) and a long (12 h) irrigation period with slow transformation ($k = 0.17$/d) rates, a maximum leaching potential of ~4700 ng/AU is predicted at 30 days postimplantation, which corresponds to the time of maximum mass accumulation on the land surface (Figures 5, SI S5). As the 17α-TBOH mass on the land surface decreases with reduced excretion, the leaching potential decreases (Figures 4, 5, and SI S5). On average, the predicted leaching potential of 17α-TBOH from fully submerged manure during a 6−12 h irrigation event on any day postimplantation (t = 1−113 days) with relatively slow transformation ($k = 0.17$/d) is 3100 ± 300 ng/AU. As transformation rates increase (k = 0.44/d) at higher temperatures, the average leaching potential decreases to 1400 ± 150 ng/AU (Figure 5).

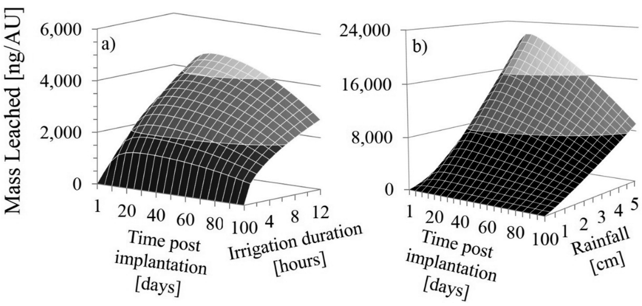

Figure 4. Modeled leaching of 17α-TBOH (ng/animal unit [AU]) from manure accumulated on the land surface following 1−12 h of irrigation (a; eq 8, $k = 0.17$/d) or 0−5 cm of rainfall (b; eq 10; k = 0.17/d) at any time postimplantation. Note different y-axis scales.

While eqs 5−8 mechanistically describe leaching via diffusion, empirical data (eqs 1 and 2) were used as inputs; therefore, the accuracy of the diffusion model is strongly tied to the accuracy of the empirical expressions describing TBA metabolite concentrations (eq 1) and daily manure production (eq 2). The regression model describing the 17α-TBOH concentrations fits the data well (Figure 1), and measured manure production and apparent first-order transformation rate constants were consistent with reported data,[27,31] all suggesting that model inputs and therefore outputs were relatively robust. Also, model inputs were easily obtained or estimated, indicating that this method can be easily extended to estimate leaching dynamics of other manure-derived steroids, antibiotics, or other pharmaceuticals.

For rainfall events, we estimated the average and maximum rainfall intensity, duration, and rainfall depth using the five nearest NOAA rain gages (SI Table S2). 17α-TBOH leaching during rainfall was highly correlated with rainfall intensity, depth, and manure concentration (i.e., $r > 0.7$; Table 1). As no mechanistic approaches were identified as suitable for these

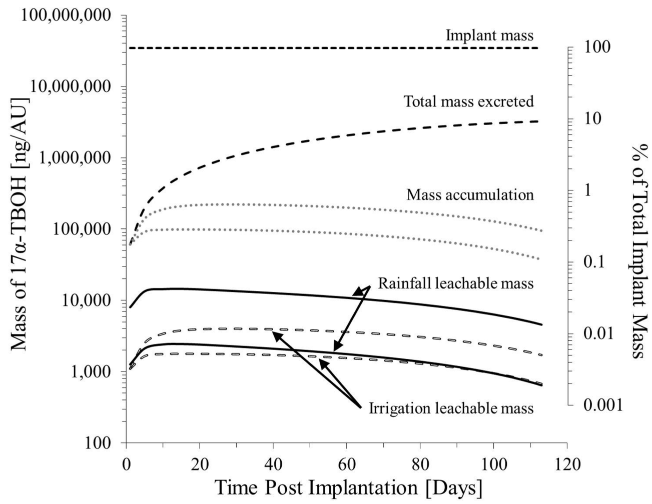

Figure 5. Mass balance of 17α-TBOH (40 mg TBA dose) as a function of time postimplantation in ng/AU (primary y-axis) and % of the total implant mass (secondary y-axis). The "implant mass" is the total equivalent mass of 17α-TBOH in a 40 mg implant. The "total mass excreted" describes 17α-TBOH mass excreted in fresh manure (eq 4), while "mass accumulation" accounts for subsequent transformation and describes the accumulated steroid mass on the land surface (eqs 2 and 7). The "rainfall leachable mass" range describes expected leaching during 1−5 cm rainfall events (eq 7), while the "irrigation leachable mass" similarly represents expected mass leaching for a 9 h irrigation event (eq 8). The upper and lower lines for mass accumulation and irrigation leachable mass account for low and high transformation rates (i.e., $k = 0.17$/d and $k = 0.44$/d), respectively.

data, we evaluated three different empirical regression models based on rainfall depth, intensity, and manure concentration to approximate the TBA metabolite leaching during rainfall events. The following power-law relationship, consistent with approaches used to describe leaching during rainfall events,[53] was deemed most reasonable (R^2 = 0.79; see SI for model comparisons and selection):

$$L_{\mathrm{r}} = 11.34(DC_{\mathrm{m}})^{1.147} \qquad (9)$$

where L_{r} is the 17α-TBOH mass leached during rainfall events (ng/kg-dw), D is the rainfall depth (cm), and C_{m} is the 17α-TBOH concentration in manure (ng/g-dw). The residuals of the model were normally distributed (Shapiro-Wilk normality test, P = 0.43), and the data were linear when log−log transformed (i.e., log L_{r} and log ($D*C_{\mathrm{m}}$)) both suggesting model validity. Conceptually, a power model is reasonable because the mass leached should converge on zero as either rainfall depth or concentration approach zero. While the correlation coefficient between mass leached was greater with intensity than with rainfall depth (i.e., r = 0.98 vs r = 0.72, Table 1), we chose this model over others because rainfall depth is easier to estimate a priori for a particular event than maximum hourly rainfall intensity. We incorporated eq 9 into eq 7 to obtain the following:

$$L_r(t_x, D) = 11.34\left(D\frac{1}{M_{t_x}}\sum_{0}^{t} S_{t_x}\exp\left(-kt\left|\begin{matrix}0\\ t_x\end{matrix}\right.\right)\right)^{1.147} \qquad (10)$$

where $L_r(t_x, D)$ is the 17α-TBOH mass leached from manure (ng/kg-dw) as a function of time postimplantation (t_x, days) and the rainfall depth (cm), and all other variables are as described before. Multiplying eq 10 by the manure production

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yields the total mass leached (ng/AU) at t_x days post-implantation following a rainfall event of D cm (Figure 4).

For rainfall events, leaching dynamics appear to be more sensitive to manure concentrations, as opposed to the total mass accumulation on the land surface as for irrigation (Figure 5). Due to differences in kinetic rates, TBA metabolite concentrations in manure were highest at low temperatures, thus low temperatures also promote higher leaching potentials. In Central California, a majority of precipitation falls as rain from October to April. Assuming 1 °C temperatures during this period, the maximum leaching potential occurs at 20 days post implantation, peaking at 22 000 ng/AU during a 5 cm event. During warmer periods (i.e., 33 and 19 °C) with faster transformation, the leaching potential peaks at 10 100−15 300 ng/AU at 5 and 10 days postimplantation, respectively, for the same rainfall event. The average leaching potential for large storms (5 cm), regardless of temperature, was 10 300 ± 1300 ng/AU; while for small storms (1 cm), the leaching potential averaged 1600 ± 200 ng/AU (Figure 5).

Mass Balance Implications. Relative to the total implant mass (3.46×10^7 ng 17α-TBOH equivalent), our data indicate that 9.3% (3.24×10^6 ng/AU) is excreted as TBA metabolites onto the land surface over 113 days (Figure 5). Following transformation within manure, a maximum of 0.28−0.64% (9.86×10^4 to 2.21×10^5 ng/AU) of the total excreted mass accumulates on the land surface as 17α-TBOH. Considering subsequent mobilization from this mass, we expect 0.0048−0.030% (1.66×10^3-1.03×10^4 ng/AU) of the total implant dose to leach during 1−5 cm storm events, or 0.00410−0.0090% (1.40×10^3-3.10×10^3 ng/AU) during a 9 h irrigation event. While exact values remain uncertain, no observed effects levels for TBA metabolites are likely near 1 ng/L.[12,54,55] This suggests that a minimum of 1400−10 300 L/AU of dilution water is needed to maintain concentrations below the no-effects levels during irrigation and rainfall events. While experiments were targeted to conditions specific to rangelands and pastures (i.e., animals implanted with 40 mg TBA implants), the relative rates of excretion, transformation, and leaching should apply to animals implanted with different doses in different agro-ecosystems. For example, for cattle implanted with Revalor-200 (200 mg TBA, 20 mg estradiol, for confined animal use), we could expect an average of ∼51 000 ng/AU 17α-TBOH leaching from a 5 cm rainfall event. Although eqs 8 and 10 were derived without complete mechanistic characterization, both are powerful tools for predicting TBA metabolite leaching in different agricultural systems (e.g., pastures and feed lots) under varying operating conditions (e.g., different stocking densities, irrigation duration, etc.).

Equations 8 and 10 were developed to explicitly describe the leaching potential of a single irrigation event relative to the implantation date and implicitly assume that leaching is independent of previous events. For example, while ∼4000 ng of 17α-TBOH can leach during irrigation events that occur on *either* 30 or 31 days postimplantation (Figure 5), 8000 ng is unlikely to leach following irrigation events that occur on *both* 30 and 31 days postimplantation because leaching is partially dependent on mass accumulation from previous days. However, these equations can be used to describe leaching from events in series given enough time lapse between events. At any time period postimplantation for any kinetic rate, <5% of the mass on the land surface is older than 5−9 days. Therefore, after 5−9 days, leaching events are largely independent of previous events and accurate predictions of leaching potential can be derived. This is particularly relevant to pasture systems where irrigation events generally occur at regular intervals of 10−14 days, depending on temperature.

During summer months, when half-lives are relatively short (e.g., 38 h at 33 °C), transformation processes greatly reduce the leaching potential for TBA metabolites during irrigation. Following implantation, the average concentration of 17α-TBOH in fresh manure is 22 ng/g-dw (Figure 1). Accounting for transformation (eq 7, assuming 33 °C, k = 0.44/d), the average 17α-TBOH concentration drops to 4 ng/g-dw. Therefore, transformation reduces the leachable mass by 80%. Removing cattle from pastures 38 h prior to irrigation would further reduce the leachable mass by 50%. In contrast, when cattle have direct access to receiving waters and direct manure-water contact occurs, the leaching potential increases by >500% because manure is completely submerged for extended periods of time with no opportunity for transformation. Using an average concentration of 22 ng/g-dw of 17α-TBOH, up to 4900 ng/d/AU can leach into receiving waters (Figure 1, eq 8).

Our results indicate that on average, the potential for TBA metabolite mobilization is highest during rainfall. While management might be targeted to managing stormwater runoff instead of irrigation runoff, mobilization risk and transport risk need to be evaluated independently. For example, during irrigation, the highest concentration of TBA metabolites is likely contained within the "first flush", which is consistent with expectations for diffusion-limited mechanisms and concurrent with high subsurface infiltration. Conversely, during rainfall events, higher mobilization occurs during intense rainfall when rainfall rates exceed infiltration rates, thereby facilitating overland transport in storm runoff. Depending on surface and subsurface characteristics (e.g., organic carbon content), the risk associated with TBA use can vary considerably depending on the route of subsequent environmental transport. To effectively evaluate the ecological risk of TBA use in agro-ecosystems and the effect of management practices, additional studies should evaluate the subsequent fate and transport of TBA metabolites in surface and subsurface systems after leaching occurs.

■ ASSOCIATED CONTENT

Supporting Information

Information related to leaching mesocosms, model parameter estimation, statistical analyses, and background information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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