**Supporting Information for:**

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

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**Contents: 19 pages, 5 Figures, 2 Tables.**

**Materials and Methods**

*Chemicals*: 17α-TBOH (17α-hydroxyestra-4,9,11-trien-3-one), 17β-TBOH (17β-hydroxyestra-4,9,11-trien-3-one), and TBO (estra-4,9,11-trien-3,17-dione) were obtained from Steraloids (Newport, RI). Deuterated 17β-TBOH (d3-17β-hydroxyestra-4,9,11-trien-3-one) was obtained from the BDG Synthesis (New Zealand) and was used for isotope dilution recovery correction for all TBA metabolites. HPLC-grade solvents were obtained from Fisher (Pittsburgh, PA). Derivatization grade N-methyl N-trimethylsilyl-triflouroacetimide (MSTFA) and $I_2$ (99.999% purity) were obtained from Sigma Aldrich (Milwaukee, WI). Complete descriptions for other experimental details can be found in Parker et al. (2012) and Webster et al (2012).[1, 2]

*Irrigation Leaching Mesocosms:* Irrigation leaching mesocosms consisted of aluminum trays of three different areas (i.e., 120, 600, or 1200 $cm^2$) filled with 1-2 L manure samples evenly distributed across the bottom of each tray. Irrigation water was applied at a rate of 8 L/hr through PVC pipes in series to each tray and the distance between the manure surface and the spout was approximately 10 cm for each mesocosm. The total demand to the pipe system was 72 L/hr (9 trays x 8 L/hr), but the total flow through the pipe system was ~100 L/hr. The excess water flowed out of the system through an overflow pipe, which was the highest elevation of the system at ~25 cm above each tray. Because water continuously flowed out of the overflow pipe, the water level at this point (i.e., ~25 cm) effectively fixed the pressure head throughout the entire system, and because we controlled the flow to each tray through individual valves, the flow to each tray was constant throughout the duration of the experiment, despite fluctuations in pressure from the feed irrigation water (Figure S1).

*Sample processing and analysis:* All samples were extracted onto solid phase extraction (SPE) cartridges. The flow through each sample was < 10 mL/minute for both loading and elution steps. If necessary, SPE cartridges were stored in a 1°C refrigerator prior to elution and analysis. SPE cartridges were eluted with methanol:water (9 mL, 95:5 v/v). The eluent was dried, resuspended in dichloromethane:methanol (12 mL, 95:5 v/v), purified using Florasil cartridges (6 mL, Restek), and dried to ~1 mL. The eluent was transferred to 2 mL vials, dried under nitrogen, derivatized using MSTFA-$I_2$ (50 µL; 1.4 mg $I_2$/mL MSTFA), and immediately dried again to remove residual iodine. Finally, extracts were resuspended in MSTFA (100 µL), heated at 60°C (40 min), and cooled to room temperature prior to analysis. Samples were analyzed by GC/MS/MS (Agilent 6890N, Santa Clara, CA, USA; Waters Quattro Micromass spectrometer, Milford, MA, USA).

In addition to TBA metabolites, irrigation leaching samples were analyzed for ammonia, nitrite, nitrate, orthophosphate, total coliforms, *E. coli*, and total organic carbon using standard approaches. Following filtration with a 0.7 µm glass fiber filter sample (see text), aliquots of the 4 L leachate samples were filtered through an additional 0.45 µm glass fiber vacuum filter. TOC samples were acidified to pH = 2 using phosphoric acid and analyzed using a Shimadzu TOC combustion analyzer. Ammonia, ammonium, nitrite, nitrate, and orthophosphate were analyzed using flow injection analysis on a Lachat Quickchem 8500 auto analyzer. Total Coliform and *E. coli* samples were collected in sterilized 120 mL plastic vessels purchased from IDEXX Laboratories. MPN concentrations were determined using the 'Colilert' defined substrate method from IDEXX. The IDEXX 51 well trays were filled with 100 mL of diluted sample (1:100,000 dilution) mixed with the 'Colilert' synthetic media, sealed using the Quanti-tray$^{TM}$ sealer, and incubated at 35 °C for 24 hours. Positive detect wells were counted and compared with standard Quanti-tray$^{TM}$ MPN tables.

## Results and Discussion

*Diffusion Model Parameter Estimation*: Using mechanistic approaches first derived for sediment systems, we assumed manure could be modeled as saturated porous media and used the following one-dimensional diffusion model to describe the mass flux of TBA metabolites from manure:

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

where L(t) is the area normalized mass leached ($ng/cm^2$), D is the effective diffusivity of TBA metabolites in manure ($cm^2/s$), f is the dissolved fraction of TBA metabolites, φ is the porosity ($V_{voids}/V_{total}$), $C_w$ is the aqueous equilibrium concentration of steroids in manure ($ng/cm^3$), and t is time (s).[3] A description of how each variable was estimated is described below.

D ($cm^2/s$): The effective diffusivity of 17α-TBOH in fresh-manure was estimated from the free diffusivity in water ($D_w$). We used three empirical formulas to estimate $D_w$ in water at a minimum (T = 0°C) and maximum (T = 37°C) temperature. These formulas are as follows:

$$D_w = \frac{14.0(10^{-5})}{\mu^{1.1} V^{0.6}}$$ (2; Othmer and Thakar, 1953)[4]

$$D_w = \frac{7.4(10^{-8})(2.6\,M)^{0.5} T}{\mu V^{1/3}}$$ (3; Wilke and Chang, 1955)[5]

$$D_w = \frac{13.26(10^{-5})}{\mu^{1.4} V^{0.589}}$$ (4; Hayduk and Laudie, 1974)[6]

where μ is the viscosity (centipoise), V is the molar volume ($cm^3/g$), and T is temperature (°K). We assumed that fresh manure was fully saturated with water and used the following equation to estimate the effective diffusivity:

$$D = D_w \varphi^{\frac{4}{3}}$$ (5; Millington and Quirk, 1961)[7]

$\varphi$ (%): We estimated $\varphi$ by oven drying 5 ml of fresh manure at 105° C for 24 hours and dividing the difference between the wet and dry manure (i.e., the volume of water assuming 1 g = 1 $cm^3$) by the total volume (i.e., 5 ml). The water content of fresh manure ranged from 0.81-0.86 and was 0.83 ± 0.01 (± 95% confidence interval) on average (n = 27).

f (%): The dissolved fraction of TBA metabolites in manure was calculated as follows:

$$f = \frac{1}{1+rK_D} \qquad (6)$$

where $K_D$ is the solids to water partitioning coefficient ($cm^3$/g), which was estimated using the 17α-TBOH partitioning coefficient to organic carbon (i.e., $K_{oc}$)[8] and the fraction of organic carbon ($f_{oc}$). We estimated $f_{oc}$ based on the fraction of organic matter ($f_{om}$) in manure using ASTM method D2974-07a (loss on ignition) and using the Van Bemmelen factor (i.e., $f_{oc}$ = 58% $f_{om}$). The fraction of organic matter in dried fresh-manure ranged from 0.77-0.85 and was 0.81 ± 0.01 on average (n = 27).

r is the ratio of solids to water (g/$cm^3$) and was calculated as follows:

$$r = \rho \frac{1-\varphi}{\varphi} \qquad (7)$$

where $\rho$ is the dry manure density (g/$cm^3$), which we estimated by oven drying 5 ml of wet manure at 105° C for 24 hours and dividing the dried mass by the total volume (i.e., 5 ml). The density of dry manure ranged from 0.14-0.21 g/$cm^3$ and was 0.18 ± .01 g/$cm^3$ on average (n = 27) and the density of wet manure ranged from 0.90-1.17 g/$cm^3$ and was 1.03 ± 0.01 g/$cm^3$ on average (n = 28).

$C_w$: The concentration gradient between the irrigation water and the aqueous equilibrium concentration within the manure was considered the driving force for leaching during irrigation. The concentration gradient was the difference between the equilibrium concentration in the manure and the concentration of the irrigation water. Because we never detected TBA metabolites within the provided irrigation water, the concentration gradient was simply the equilibrium concentration of TBA metabolites within the manure. We estimated the aqueous concentration in manure using the following mass balance:

$$S_t = C_w V_w + C_s M_s \quad (8)$$

where $S_t$ is the total metabolite mass excreted in a sample (ng), $C_w$ is the aqueous equilibrium concentration of TBA metabolites within the sample (ng/cm$^3$), $V_w$ is the sample volume of water in manure (cm$^3$), $C_s$ is the solid concentration of metabolites (ng/g-dw), and $M_s$ is the mass of solids in manure (g-dw). For a 1 g-dw sample, the previous mass balance can be written as:

$$C_m = C_w V_w + C_s \times 1 \quad (9)$$

where $C_m$ is the TBA metabolite concentration in manure (ng/g-dw). It should be noted that $C_m$ and $C_s$ have the same units (i.e., ng/g-dw) but do not represent the same parameter. $C_m$ describes the total mass of TBA metabolites, both dissolved and sorbed, in fresh manure that has been dried and $C_s$ describes the mass of TBA metabolites that are sorbed to solids resulting from equilibrium partitioning. $K_D$ and $C_w$ were substituted into $C_s$ (i.e., $K_d$*$C_w$ = $C_s$) and the equation was rearranged and solved for $C_w$ as follows:

$$C_w = C_m \left(\frac{1}{V_w + K_D \times 1}\right) \quad (10)$$

This form is advantageous for substitution into the diffusion model because the $C_m$ is often reported in the literature while $C_w$ is not. The volume of water in 1 g-dw equivalent of fresh manure (~5.88 g-ww) can be described as follows:

$$V_w = 1\ g(dw) \times \frac{1\ g(ww)}{(1-\varphi)\ g(dw)} \times \frac{\varphi\ g\ H_2O}{1\ g(ww)} \times \frac{1\ cm^3\ H_2O}{1\ g\ H_2O} \quad (11)$$

Each grouping represents a unit conversion. For example, in the second group, 1 g-ww of manure is equivalent to 1-φ g-dw of manure (~0.17 g-dw), and in the third grouping, 1 g-ww of manure is equivalent to φ g of water (~0.83 g water), etc. Therefore, the aqueous concentration of TBA metabolites in manure can be written as follows:

$$C_w = C_m \left( \frac{1}{\frac{\varphi}{(1-\varphi)} + K_D \times 1} \right) \qquad (12)$$

We used a Monte Carlo simulation (n = 10,000, see below) to estimate $C_w$ based $C_m$ ranging from 0-70 ng/g-dw (4-64 ng/g-dw was observed in experiments) by selecting random values from within the measured, calculated, or reported range of each independent variable (Table S1). The average product of $\left( \frac{1}{\frac{\varphi}{(1-\varphi)} + K_D \times 1} \right)$ was 0.0034 ± 0.0007 g-dw/cm (± stdev; Figure S4). Therefore, we simplified the mass balance to describe $C_w$ as follows:

$$C_w = 0.0034 C_m \qquad (13)$$

This mass balance was used to simplify the 1-D diffusion model.

*Monte Carlo Statistical Analysis:* Within the diffusion model, $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ is a constant for a given contaminant within a given media. We used the Monte Carlo simulation described within the text to determine whether the observed value of $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ obtained from the simulated leaching experiment was statistically different from that of the average of the model. We estimated $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ from the each simulated-irrigation leaching experiment by dividing the area normalized mass leached (L) at each time point by $C_w$ and $t^{1/2}$. For 17α-TBOH sample concentrations of 24 and 63 ng/g-dw, the average value of $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ was 0.0055 and 0.0070 cm/s$^{0.5}$, respectively. Based on the Monte Carlo simulations, the average value was 0.0065 cm/s$^{0.5}$. We created a cumulative probability distribution describing the probability that a randomly generated value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$, from each iteration, was less than a particular value of x, namely 0.0055 and 0.0070 cm/s$^{0.5}$. The cumulative probability ($P_c$) that a random value is ≤ x ranges from 0 < P ≤ 1 and can be described as follows:

$$P_c = \frac{\#\ of\ values \leq x}{10{,}000} \qquad (14)$$

For example, if x is the maximum value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ for all 10,000 iterations (i.e., 0.01 cm/s$^{0.5}$), the cumulative probability that a randomly chosen value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ is ≤ x is 1.0000 (i.e., P = 10,000/10,000). Conversely, if x is the minimum value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ for all 10,000 iterations (i.e., 0.003 cm/s$^{0.5}$), the cumulative probability that a randomly chosen value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ is ≤ x is 0.0001 (i.e., P = 1/10,000). Therefore, the cumulative probability that a randomly chosen value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ is less than the average value (i.e., 0.0065 cm/s$^{0.5}$) is 0.5 since 50% of the observations are below and 50% of the observations are above this value. Therefore, the probability that any value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ is statistically different from the average can be described as follows:

$$P = 2(0.5 - |0.5 - P_c|) \qquad (15)$$

For example, for a particular value x with $P_c = 0.5$ (i.e., x = the mean value), P = 1.0000, and we would fail to reject the null hypothesis (i.e., there is no difference between x and the mean value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$). For $P_c < 0.025$ or $P_c > 0.975$ (i.e., the upper and lower 2.5% of all 10,000 observations), P < 0.05, and we would reject the null hypothesis in favor if the alternative hypothesis (i.e., there is a difference between x and the mean value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$). For 0.0055 and 0.0070 cm/s$^{0.5}$, $P_c$ = 0.2884 and 0.6461, respectively. Therefore, the probability that there is no difference between the estimated value of $\left(\frac{4D}{\pi f}\right)^{1/2}\varphi$ from both leaching experiments and the mean value from all 10,000 iterations (i.e., 0.0065 cm/s$^{0.5}$) is P = 0.5768 (for 24 ng/g-dw) and P = 0.7078 (for 63 ng/g-dw). Because there was no statistical difference between the observed and modeled leaching (i.e., $P > 0.05$), and because the observed and modeled leaching were independent (i.e., no leaching data was used to build the model), the diffusion model was deemed appropriate to describe 17α-TBOH leaching.

*Rainfall Leaching Model:* Rainfall leaching was highly correlated with rainfall intensity, TBA metabolite concentration, and rainfall depth (see main text). Because rainfall leaching is inherently correlated with both the sample concentration as well as some rainfall metric, each model contains two independent variables describing a particular storm event and the concentration. More variables were not used to prevent over-parameterization of the model. Given the absence of available mechanistic approaches, we evaluated three empirical regression models to describe the mass of TBA metabolites that could leach (ng/kg-dw) during rainfall events ($L_r$):

$$\log(L_r(I, C_m)) = 1.16I + 0.022C_m - 1.99 \qquad (16);\ R^2 = 0.97$$

$$L_r = 54.26(IC_m)^{1.115} \qquad (17);\ R^2 = 0.88$$

$$L_r = 11.34(DC_m)^{1.147} \qquad (18);\ R^2 = 0.79$$

where I is the maximum hourly rainfall intensity (cm/hr), $C_m$ is the TBA metabolite concentration in manure (ng/g-dw), and D is the rainfall depth (cm). While each model is empirical, we evaluated the models based on two criteria: does the model make physical sense, especially at the environmental limits of each independent parameter, and which model is the most parsimonious. Because the mass leached during rainfall events varied across three orders of magnitude (i.e., 5-2500 ng), we log transformed the leaching data to prevent extreme events from driving the multiple linear regression model (equation 16). While equation 16 accounted for the greatest variability within the dataset, the mass leached is always greater than 0 ng, regardless of the value the rainfall intensity and concentration. It is also particularly sensitive to high rainfall intensities, especially when concentrations are low. For example, at 0.1 ng/g-dw, but the intensity is 2.5 cm/hr, the mass leached is 8 ng. For a 5 cm/hr intensity, the mass leached is 6,500 ng/g-dw. The model is less sensitive when concentration is high but intensity is low. Equation 16 over estimates the mass that can leach when both concentration and intensity are low, which is of particular concern since the average concentration of TBA metabolites falls relatively quickly following implantation (see text). While equation 16 fit the data well, it did not make physical sense for low values of intensity and concentration and was therefore removed for model consideration.

Power-law relationships have been used to describe contaminant leaching during rainfall events.[7] Therefore, we developed two power models to describe leaching based on the concentration and intensity or the concentration and rainfall depth. In order to ensure that the mass leached approached zero ng as either independent variable approached zero, we generated a single composite variable that was the product of the maximum hourly rainfall intensity and the sample concentration. While both models (equations 17 and 18) approach 0 ng leached as either independent variable approaches zero, the rainfall intensity in equation 17 is more difficult to estimate accurately than rainfall depth. While the intensity can be estimated from depth using SCS storm curves based on the storm type (e.g., I, Ia, II, and III) and storm duration (e.g., 6, 18, or 24 hour storm), we considered equation 18 to be more parsimonious despite the lower $R^2$

value because it measures the leachable mass directly. Equation 18 was therefore deemed more suitable than the equations 16 and 17 because: 1) the mass leached approaches zero as either rainfall depth or concentration approach zero, and 2) rainfall depth is easier to estimate *a priori* for a particular event than rainfall intensity.

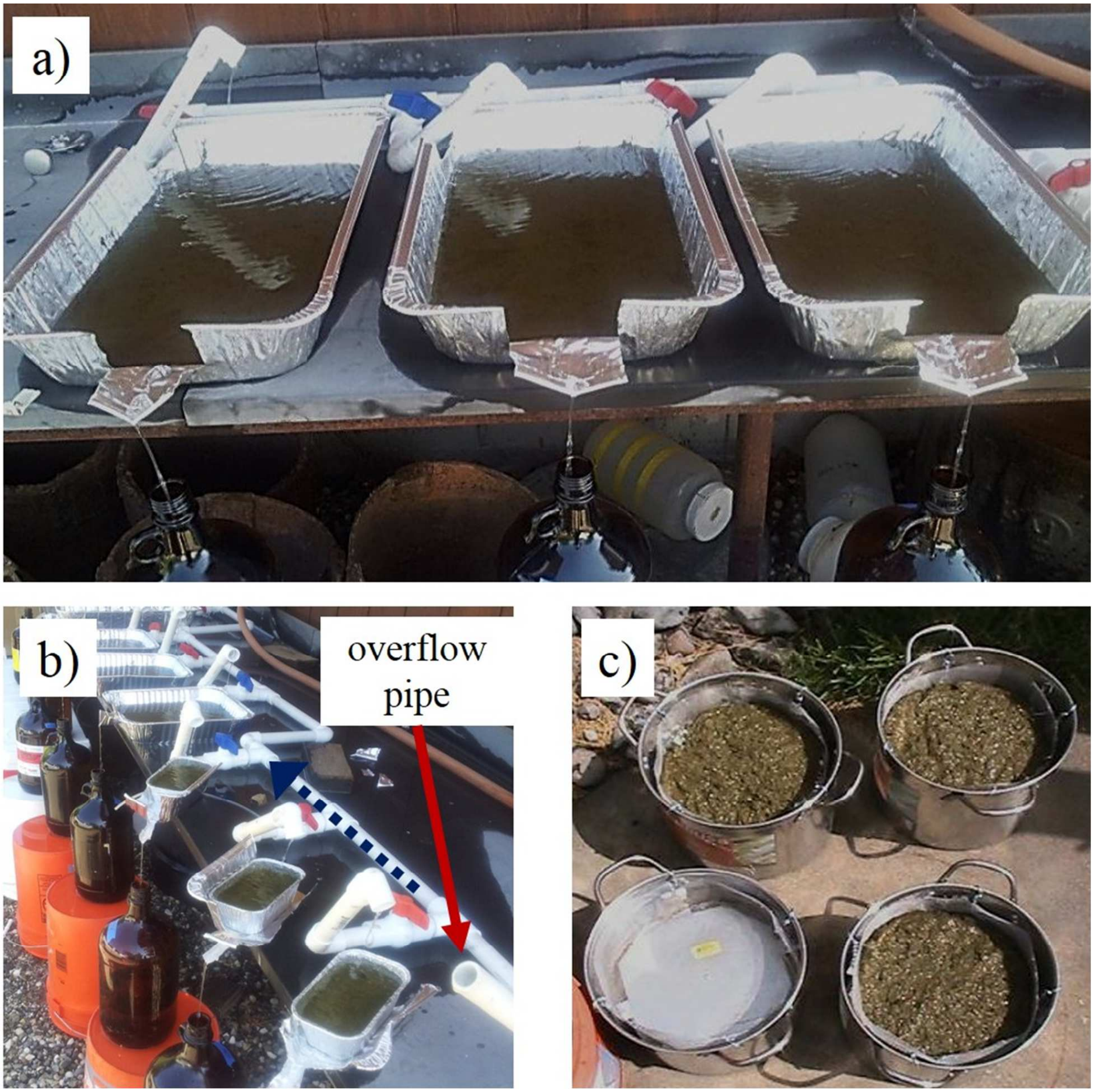


**Figure S1**. Irrigation (a and b) and rainfall (c) leaching mesocosms. Irrigation water was applied to each tray (1,200 cm2 [a] and 120 and 600 $cm^2$ [b]) at a rate of 8 L/hr (as seen in [a] and [b]). The leachate from each mesocosm overflowed directly into 4 L amber glass bottles. The pressure head of the system was fixed by an overflow pipe (b) which was the highest elevation of the piping system. The direction of flow was from the bottom right to the top left (b). Rainfall mesocosms consisted of 12 L stainless steel pots (c). Samples were placed within aluminum screen cylinders (23 cm diameter, 5 cm height, seen in all 4 pots) that were open to the top to allow rainfall to impact the manure surface but prevent rainfall induced erosion of the sample. The screens also maintained a near constant top interfacial area (410 $cm^2$/kg-ww) exposed to rainfall. Screens and samples were suspended on a stainless steel plates (seen in lower left of [c]). After each rainfall event, leachate was collected from the bottom of each mesocosm.

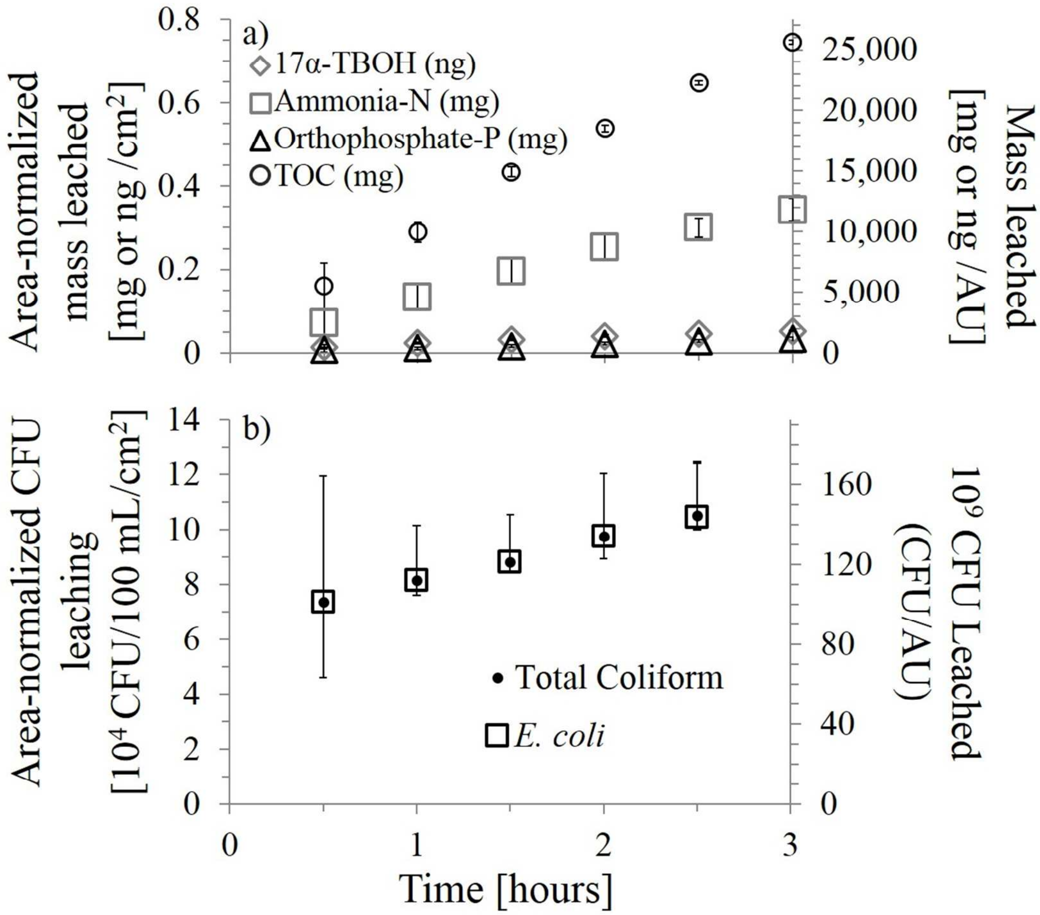


**Figure S2.** Observed leaching of (a) 17α-TBOH, N, P, and TOC, and (b) total coliforms and *E. coli* from manure during simulated irrigation leaching experiments. Nitrite and nitrate were below the limits of detection and not presented. All data were collected from the same experiment, and the 17α-TBOH data corresponds to the data presented in Figure 3a (see text). Note the differences in units within and between the figures. The primary y-axis represents the normalized (both area and concentration) leaching and the secondary y-axis represents that mass that can leach from 40 kg-ww of manure, which is the mass excreted from a single adult animal unit (AU) over 24 hours, assuming complete manure submersion (i.e., 860 $cm^2$/kg-ww x 40 kg-ww/AU = 34,400 $cm^2$ interfacial area). In (b), concentrations were multiplied by the entire sample volume (4,000 mL) as well as the total submerged interfacial area to obtain an estimate of the coliform forming units (CFU) that can leach from samples. Initial concentrations for contaminants except 17α-TBOH (24 ng/g-dw) were not extracted from the manure samples and measured directly, thereby precluding the use of the diffusion model described in equation 1. Error bars represent 95% confidence intervals.

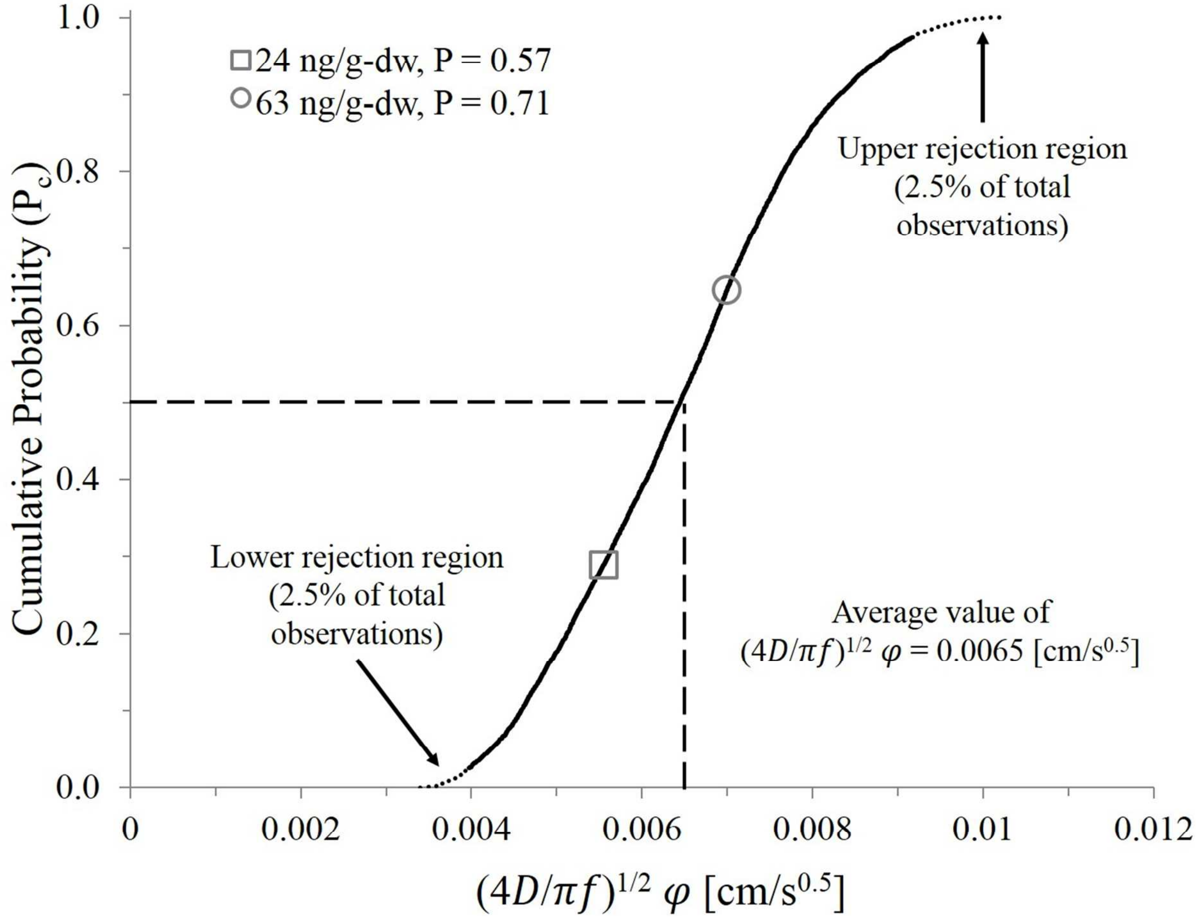


**Figure S3.** Cumulative probability distribution of $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ generated from the Monte Carlo analysis (n = 10,000 iterations). The outer 5% of all iterations (i.e., the greatest 250 ($P_c > 0.95$) and smallest 250 ($P_c < 0.025$) values of $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$) are indicated by the dotted lines and were used to define a rejection region. If $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ fell within this rejection region, the null hypothesis (i.e., there is no difference between the observed value and the average) was rejected at $P < 0.05$. The probability (P) that the observed value of $\left(\frac{4D}{\pi f}\right)^{1/2} \varphi$ is statistically different from the average for is indicated for both simulated irrigation leaching experiments.

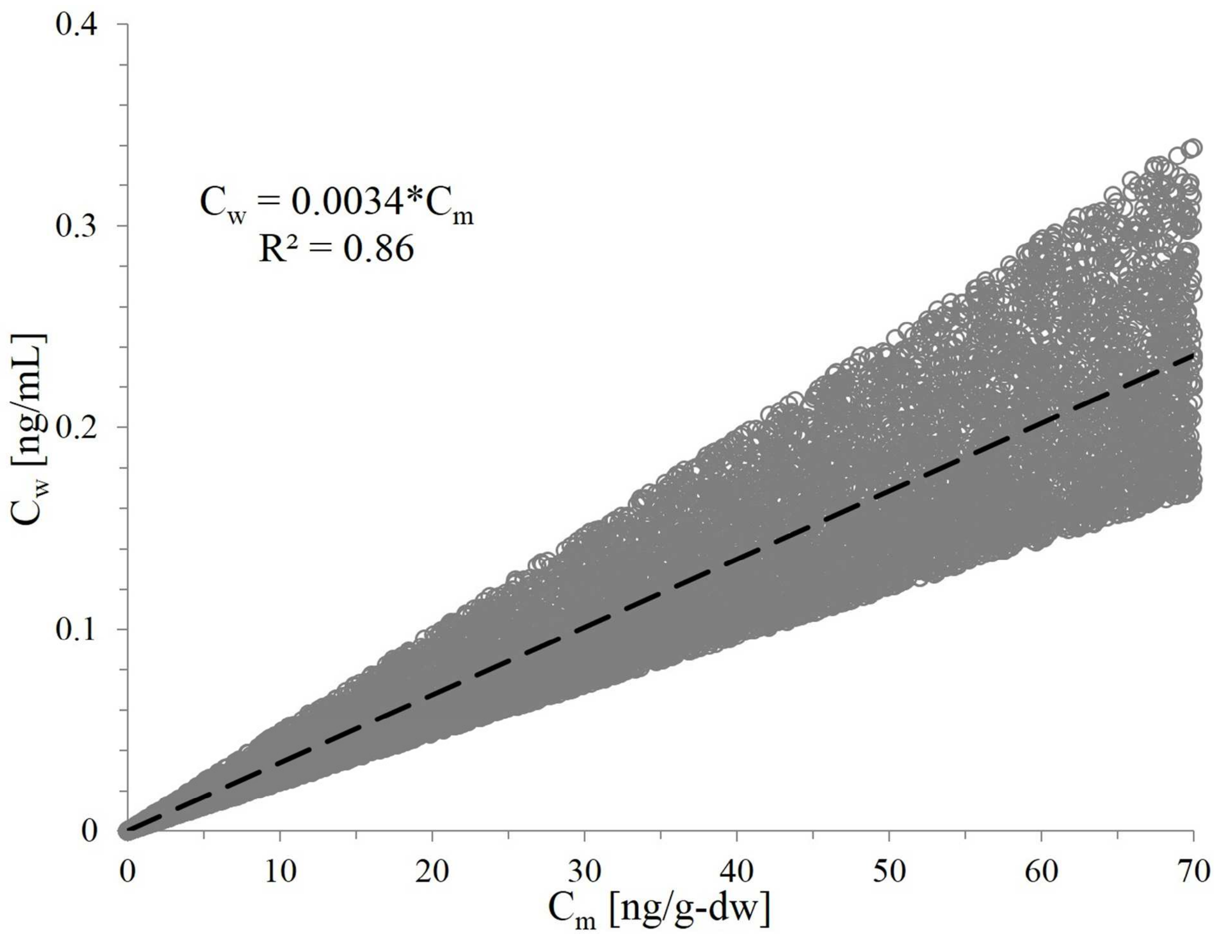


**Figure S4.** Relationship between the total 17α-TBOH concentration in manure ($C_m$; ng/g-dw) and the corresponding aqueous equilibrium concentration ($C_w$; ng/mL). Using a Monte Carlo simulation (n = 10,000 iterations), values of $C_m$ ranging from 0-70 ng/g-dw (we observed 4-64 ng/g-dw in actual samples) were generated. For each $C_m$ value, the corresponding $C_w$ value was estimated from the total mass balance described previously (equations 8-12). The slope of the line is the average product of $\left(\frac{1}{\frac{\varphi}{(1-\varphi)}+K_D\times 1}\right)$ in equation 12.

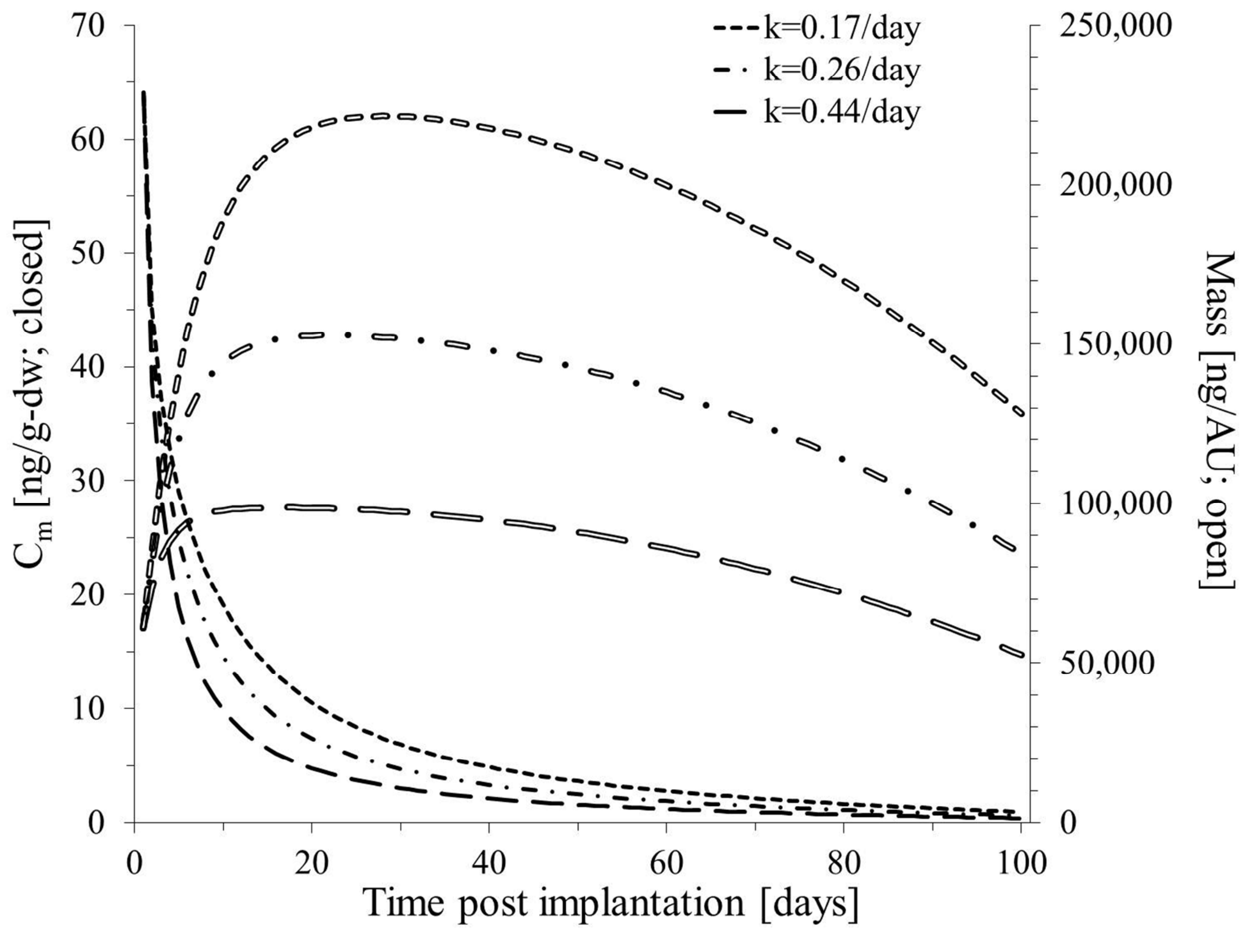


**Figure S5.** From animals implanted with 40 mg TBA, closed lines (primary y-axis) represent the average concentration of 17α-TBOH in manure excreted onto the land surface through t days post implantation. The average manure concentration is a function of the first-order transformation rate constant, which was 0.17, 0.26, and 0.44/d at 1, 19, and 33°C, respectively. These values represent environmentally realistic average seasonal temperature ranges for rangelands and pastures. The mass of 17α-TBOH that can accumulate on the land surface (closed lines; secondary y-axis) is a function of both the 17α-TBOH concentration (ng/g-dw) and the manure production per animal unit (AU; g-dw/AU; equation 2 of main text). The mass of 17α-TBOH on the land surface peaks around 30 days post implantation.

**Table S1**. Range of measured, derived, or estimated values for diffusion model independent variables.

| Parameter | Min | Max | Source |
|---|---|---|---|
| †$D_w$ ($cm^2/s$) | 2.4E-06 | 9.3E-06 | *Hayduk and Laudie (1974)[4] |
| D ($cm^2/s$) | 1.8E-06 | 7.6E-06 | Derived |
| †$\rho$ (kg-dw/$m^3$) | 0.14 | 0.21 | Measured |
| †$\varphi$ (unitless) | 0.81 | 0.86 | Measured |
| r ($g/cm^3$) | .032 | .035 | Derived |
| f (unitless) | 0.13 | 0.07 | Derived |
| †$K_{oc}$ ($cm^3/g$) | 447 | 832 | Khan et al. (2009)[8] |
| †$f_{om}$ (unitless) | 0.77 | 0.85 | Measured |
| $f_{oc}/f_{om}$ (unitless) | 0.58 | 0.58 | Van Bemmelen factor |
| $f_{oc}$ (unitless) | 0.45 | 0.49 | Derived |
| $K_d$ ($cm^3/g$) | 199 | 410 | Derived |

*values from Othmer and Thakar (1953)[2] and Wilke and Chang (1955)[3] fell within the range of Hayduk and Laudie (1974)[4]

†variables randomized in Monte Carlo analyses.

**Table S2.** Five NOAA rain gages were used to estimate the rainfall depth and intensity at SFREC. The gage location, distance to SFREC, inverse squared distance, and the weight (derived from inverse distance weighting) are included.

| City | northing | easting | Distance to SFREC (km) | $1/D^2$ | Weight |
|---|---|---|---|---|---|
| Auburn | 4313330 | 666199 | 37.7 | 7.0E-04 | 0.09 |
| Oroville | 4371916 | 618857 | 37.9 | 7.0E-04 | 0.09 |
| Beale | 4333028 | 635136 | 15.3 | 4.3E-03 | 0.58 |
| Yuba City | 4328589 | 623622 | 26.8 | 1.4E-03 | 0.19 |
| Emigrant Gap | 4349751 | 697562 | 52.7 | 3.6E-04 | 0.05 |
| SFREC | 4344609 | 645113 | -- | -- | -- |
| | | | Σ | 7.4E-03 | 1.00 |

**Supplemental References**


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