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Title: Surface and Subsurface Attenuation of Trenbolone Acetate Metabolites and Manure-Derived Constituents in Irrigation Runoff on Agro-ecosystems

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Trenbolone acetate (TBA) is a potent androgenic steroid used to increase weight gain, and thus economic value, of beef cattle prior to slaughter. While several studies have qualitatively evaluated TBA metabolite transport based on their presence/absence in storm runoff, studies have not evaluated spatial and temporal dynamics of TBA transport in rangelands and irrigated pastures. Concentrations of TBA metabolites were measured before and after treatment on surface and subsurface experimental plots (i.e., 3-5 m). Over short time scales (2-75 minutes), surface partitioning was likely responsible for observed attenuation, but it was clear that non-equilibrium processes facilitated the rapid transport of at least some contaminant fraction. This work has implications for best management practices for agricultural runoff and identifies potential opportunities for optimization.

Surface and Subsurface Attenuation of Trenbolone Acetate Metabolites and Manure-Derived Constituents in Irrigation Runoff on Agro-Ecosystems.

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Abstract

Although studies have evaluated the ecotoxicity and fate of trenbolone acetate (TBA) metabolites, namely 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), and trendione (TBO), their environmental transport processes remain poorly characterized with little information available to guide agricultural runoff management. Therefore, we evaluated TBA metabolite transport in representative agricultural systems with concurrent assessment of other manure-derived constituents. Leachate generated using manure from TBA-implanted cattle was applied to a subsurface infiltration plot (4 m) and surface vegetative filter strips (VFSs; 3, 4, and 5 m). In the subsurface experiment, 17α-TBOH leachate concentrations were 36 ng/L but decreased to 12 ng/L in initial subsurface discharge. Over 75 minutes, concentrations linearly increased to 23 ng/L ($C/C_o = 0.32\text{-}0.64$). In surface experiments (n = 4), 17α-TBOH leachate concentrations ranged from 11-150 ng/L, remained nearly constant with time, but were attenuated by ~70-90% after VFS treatment with no statistical dependence on VFS length. While attenuation clearly occurred, observation of a highly mobile fraction of all constituents in both surface runoff and subsurface discharge suggest these treatment strategies may not always be capable of achieving threshold discharge concentrations. To attain no observed adverse effects levels (NOAELs) in receiving waters, concurrent assessment of leachate concentrations and available dilution capacities can be used to guide target treatment performance levels for runoff management. Dilution is usually necessary to achieve NOAELs, and receiving waters with less than 70-100 fold dilution capacity are at highest risk for steroidal endocrine disruption.

Introduction

Trenbolone acetate (TBA) is a synthetic steroid hormone, which pervasive in beef cattle production, that promotes weight gain and thus economic value of cattle. However, observations of endocrine disruption in surface waters affected by agricultural runoff have led to concerns over TBA use, in part due to the potent endocrine activity of the TBA metabolites 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), and trendione (TBO).[1-4] While the causative agents responsible for these observations remain unclear, these steroids may contribute to potential ecological hazards of growth promoter use in agro-ecosystems. [1-3, 5, 6] Trace concentrations (e.g., 5-100 ng/L) of 17α-TBOH and 17β-TBOH can induce irreversible phenotypic sex reversal upon embryonic exposure and significant fecundity reduction in fish if exposures occur during reproductive periods.[7-10] Although no observed adverse effects levels (NOAELs) are not reported for TBA metabolites, a reasonable NOAEL estimate, based on estimates derived for other steroids, is ~1 ng/L in receiving waters.[8, 11, 12]

Limited data exist that predict TBA metabolite fate and transport in rangeland and pasture systems. While several studies have evaluated transformation kinetics and documented TBA metabolite occurrence in runoff from feedlot and tile-drained systems, [13-19] fate and transport can be differentially affected by system characteristics (e.g., bare soil vs. crops or natural vegetation) and management practices (e.g., CAFO lagoon vs. manure amended soils). For example, using soil columns, Schiffer et al. observed ~82-92% attenuation of 17β-TBOH after ~10 pore volumes, with subsequent high steroid recoveries upon solvent extraction of the soils indicating the importance of hydrophobic partitioning.[20] Given their moderate affinities for organic carbon (log K_{oc} = 2.5-3.6),[21-24] equilibrium partitioning estimates suggest that the transport of TBA metabolites and other steroids should be dominated by sequestration to immobile organic phases and soils.[25-30] However, some studies report far higher mobility and concentrations that cannot be reconciled with equilibrium partitioning estimates. While Schiffer et al.[20] observed substantial attenuation, 17β-TBOH breakthrough occurred nearly simultaneously with chloride, indicating rapid transport and similar to observations for estradiol and testosterone in soil columns.[31] Despite partitioning predictions in soils, testosterone, 17β-estradiol, and TBA metabolites are detected at relatively high concentrations (e.g., 160 ng/L) in ground water, the vadose zone, and surface waters.[14, 15, 32, 33] They also rapidly transport through the vadose zone to shallow (<3 m) tile drain systems after storms and can exhibit long-term (many weeks) persistence in heterogeneous aquatic systems.[14, 15] Collectively, these observations suggest that multiple processes affect fate and transport, including possible associations with colloids or dissolved organic matter that potentially facilitating transport or the existence of kinetic limitations to partitioning at low, environmentally relevant concentrations. These data indicate that steroids do retain unexpected mobility and persistence in the environment and that their fate is not always dominated by equilibrium hydrophobic partitioning.

Relative to transport, more data are available that characterize TBA metabolite fate processes (e.g., photolysis, sorption, transformations) in laboratory and field studies.[13, 19, 22, 34-37] Some studies qualitatively evaluate transport based on the presence/absence of TBA metabolites in surface runoff or subsurface discharge, [2, 14-16, 18, 19, 30, 38, 39] but no attempts were made to decouple TBA metabolite leaching and transport processes, both of which can strongly influence subsequent concentrations. This issue also confounds the extrapolation of laboratory data generated using synthetic solutions to field scales where leaching dynamics and non-equilibrium conditions strongly affect steroid occurrence.[20] To optimize agricultural runoff management, the relative contributions of leaching and transport processes should be evaluated independently.

For example, if leaching dominates, adequate strategies to minimize the risks associated with TBA use might involve optimizing irrigation practices to minimize the leaching potential. Conversely, if transport dominates, management practices designed to inhibit transport by sequestering TBA metabolites on immobile phases should be implemented.

Given these complex behaviors and seemingly contradictory observations, investigating the TBA metabolite transport potential in agro-ecosystems is necessary to understand agricultural discharges of steroids to the aquatic environment and develop treatment strategies to attenuate potential hazards. Previously, we evaluated leaching processes using manure excreted by TBA-implanted animals during both rainfall and irrigation events and developed models to predict leaching potential under rainfall and irrigation scenarios.[40] Therefore, our objective was to evaluate plot-scale subsurface and surface transport of TBA metabolites in agro-ecosystems with a focus on runoff management strategies. Specific study objectives were to: 1) quantify the transport potential of TBA metabolites in plot-scale surface runoff and subsurface discharge; 2) compare TBA metabolite attenuation to that of other manure-derived constituents (i.e., total ammonia, orthophosphate, nitrate, nitrite, dissolved organic carbon (DOC), total coliforms, and *E. coli*); and 3) evaluate management strategies to control the transport of steroids and other manure-derived constituents in agro-ecosystems.

Materials and Methods

Site description

Subsurface and surface plot-scale transport studies were conducted at the University of California Sierra Foothills Research and Extension Center (SFREC) near Browns Valley, CA. Soils were classified as Typic Haploxeralfs and Mollic Haploxeralfs with clay loam surface textures and clay subsoils. Soils generally extended to depths of 0.75-1.5 m above basic metavolcanic (greenstone) bedrock. Local vegetation on rangelands included naturalized annual grasses and forbs and native perennial grasses with a savanna/woodland over-story composed primarily of evergreen and deciduous oak trees. Vegetation on vegetative filter strips (VFSs) consisted of introduced forage species (e.g, perennial ryegrass, fescue, and red and white clover).

We evaluated subsurface transport of TBA metabolites and other manure constituents using a soil trench within SFREC's "Lewis-1" watershed (NAD 83 UTM 10S-645581E, 4348683N).[41] This 34.6 ha watershed has not been grazed in >10 years and consists of blue-oak savanna with an average grade of ~24%. The soil trench was constructed in 1999 and consists of a vertical cut through the soil profile to bedrock. Aluminum collection trays (1 m horizontal width) were placed at various soil horizons (i.e., bottom of the A (8 cm), AB (30 cm), Bt (65 cm), and C (110 cm) horizons; Figure 1) to capture lateral subsurface discharge. [41] During precipitation, most flow discharges from the AB horizon (60%) compared to other horizons, which individually discharge 10-17% of the flow.[41] Preferential flow through the AB horizon is a result of lower clay content as well as the presence of well-formed spheroidal soil aggregates and larger pores (e.g. decayed root channels).[41]

We evaluated overland transport of TBA metabolites and other manure-derived constituents on VFSs of different lengths (i.e., 3, 4, 5 m length x 2 m width) constructed at SFREC in 2006 (NAD 83 UTM 10S-645107E, 4344613N; Figures 1, S1). The average grade was ~6%, and each VFS was bordered with 2 mm thick x 45 cm wide aluminum sheeting buried to 30 cm below the soil surface to prevent water exchange. Each VFS was equipped with a downgradient sample collection trough constructed of concrete and aluminum where runoff volume was measured. Of the eighteen VFSs available, we selected three based on their low infiltration rates and lack of preferential flow paths to evaluate transport in surface runoff following application of a TBA-metabolite containing leachate solution.

Leachate Generation

Leachate solutions representative of agricultural runoff were generated using manure collected from Hereford/Angus cross heifers and steers (steer calves [n = 3] or yearling heifers [n = 4], 145-350 kg, 6-18 months old) that were implanted with Revalor G (40 mg TBA and 8 mg estradiol, for use on rangeland cattle) as previously described.[40] Briefly, manure was collected over 24-72 hours, stored onsite in a closed container, then immediately used in experiments to generate leachate. When needed, animals were re-implanted following manufacturer protocols. Animals were handled in accordance to guidelines prescribed by the University of California, Davis Animal Care and Use Committee. To generate leachate solutions, 20-40 L (20-40 kg) of manure from TBA implanted heifers or steers was added to a 1,500 L tank. In general, nutrient concentrations in this leachate were similar to published concentrations (0.05-7.2 mg NH_3-N/L, 0.17-4.29 mg PO_4-P/L, 1.0×10^5 - 1.8×10^6 CFU/100 mL) in agricultural runoff and to runoff concentrations from prior experiments conducted on irrigated pasture at SFREC (Figure S2).[42-47] The tank was placed upslope of the soil trench or VFS system and filled with irrigation water (Browns Valley Irrigation District; pH = 7.6, DOC = 5.7 ± 0.1 mg/L). Prior to experiments, the solution was allowed to equilibrate for 24 hours (Figures 1, S1). When applied, leachate water was drawn from ~0.3 m above the bottom of the tank to minimize the application of settled solids at the bottom of the tank.

Subsurface Discharge

On May 24, 2013, the soil 4 m upslope of the soil trench was pre-saturated for 30 minutes with “clean” irrigation water. This step was necessary to promote subsurface discharge and to conserve the limited volume of leachate solution available to investigate transport processes. After a 30 minute drying period, the leachate solution was evenly applied to the test plot using a 1.5 m perforated pipe positioned 4 m above the trench at a rate of ~15 L/min. Over the 4 m travel distance, the wetting front spread 8.5 m laterally. The estimated wetted area was 19.5 m^2 for a bulk application rate of 4.6 cm/hr (1.8 cfs/ac), which is near average for central California irrigation practices, but high for natural rainfall.[45] At this application rate, the saturated retention time of the system was ~2 minutes, and the average discharge flow rate in the AB horizon was 0.63 L/min. While no surface runoff was generated, discharge from the A horizon occurred near the end of the experiment and was estimated as 5% of the AB horizon flow. Flow was only collected from the AB horizon. The discharge collected with the sampling tray of the AB horizon accounted for ~4.5% of the total flow applied. Concurrent leachate and discharge samples from the AB horizon were collected in 4 L amber glass bottles at 15 minute time intervals for 75 minutes, an experimental period also used in VFS studies to facilitate direct comparison. For both soil trench and VFS studies, the available tank volume defined the application period and thus the sampling period, as runoff ceased when the tank emptied.

Samples were immediately pressure filtered (0.7 µm AP40 filters, Millipore, Billerica, MA, USA) on site, and a 3 L subsample was used for 17α-TBOH, 17β-TBOH, and TBO analysis.[18, 19] Of the remaining volume, 95 mL was vacuum filtered (0.45 µm GB-140, Advantec MFS Inc., Dublin, CA, USA) and split for nutrient (i.e., total ammonia (NH_3)-N, nitrate-N, nitrite-N, and orthophosphate (PO_4)-P; 20 mL) and DOC (75 mL) analysis (see [40] for nutrient and DOC analysis). After field processing, subsamples were transported to the laboratory on ice. For TBA metabolites, upon arrival to the laboratory (~3 hours after sample collection) each subsample was subsequently split into 1 L aliquots for triplicate analysis and spiked with 1 mL of 100 μg/L (i.e., 100 ng) of 17β-TBOH-d_3 internal standard in methanol, then extracted with 6 mL C-18 solid phase extraction (SPE) cartridges (Restek, Bellefonte, PA, USA; Q < 10 mL/min). SPE cartridges were stored at 1 °C prior to steroid analysis. Nutrient and TOC samples were stored at 1 °C and analyzed within 24 hours.

Surface Runoff

We evaluated the transport of TBA metabolites and other manure constituents on VFS on four different dates (24 May, 31 May, 8 June, and 3 July, 2012). All experiments were conducted similarly, with the exception of the experimental duration and the number of samples collected. The data were consistent among all experiments, but only the most comprehensive dataset (July 3) is presented here (see SI for data on other trials). To promote surface runoff generation, each VFS was pre-saturated for 30 minutes with "clean" irrigation water prior to leachate application. Leachate (prepared as previously described) was applied simultaneously and uniformly to the three VFS plots through a perforated pipe (Figure 1). The application rate over the 75 minute trial was 4 L/min. The resulting area-normalized irrigation rates for the 3, 4, and 5 m VFS were 4.0 cm/hr (1.6 cfs/ac), 3.0 cm/hr (1.2 cfs/ac), and 2.4 cm/hr (0.9 cfs/ac) respectively, which are typical for central California.[45] Average runoff rates for the 3, 4, and 5 m VFSs were 1.7, 1.4, and 1.0 cm/hr, respectively, suggesting ~40% infiltration and 60% runoff during these trials. Once runoff began (at 2, 3, and 5 minutes for 3, 4, and 5 m VFSs, respectively), 4 L samples were collected from the sampling port of the common header pipe and each VFS collection trough at 15 minute intervals (Figure 1). Prior to pressure filtration, a 100 mL subsample was collected for total coliform and *E. coli* analysis (see [40] for analysis procedures). The remaining sample was split into subsamples and processed for TBA metabolites, nutrients, and DOC analysis as described.

In addition to aqueous samples, we collected 10 cm soil cores (n = 9) throughout each VFS after leachate application following the last trial to estimate the mass of TBA metabolites sorbed to surface soils. The above ground biomass was removed from each core, which were homogenized, immediately placed on ice, and transported to the laboratory. Within 24 hours, samples (n = 6, ~100 g-dry weight) were sonicated in methanol (100 ml), rinsed with deionized water (100 ml), and centrifuged (3,600 rpm, 10 minutes). The supernatant was decanted into 4 L amber glass bottles. This process was repeated 3 times, after which, the supernatant was diluted to 4 L. The samples were then spiked with 1 mL of 100µg/L 17β-TBOH-d3 and loaded onto SPE cartridges.

Sample Analysis

TBA metabolites were processed, derivatized, and analyzed by GC/MS/MS (Agilent 6890N Gas Chromatograph, Santa Clara, CA, USA; Waters Quattro Micromass spectrometer, Milford, MA, USA) for 17α-TBOH, 17β-TBOH, and TBO.[18, 19, 40] QA/QC measures included field blanks (i.e., irrigation water), background samples (i.e., runoff prior to leachate application), and laboratory spikes (100 ng/L of 17α-TBOH, 17β-TBOH, and TBO) analyzed identically to other samples. With the exception of TBO, all field blanks and background samples were at or below the limit of detection (i.e., 0.5 ng/L 17α-TBOH and TBO and 1 ng/L for 17β-TBOH), suggesting no sample contamination. TBO was not detected in irrigation water, but TBO analysis in surface runoff was complicated by a co-eluting interference precluding quantification. The average 17β-TBOH-d_3 recovery for samples was low but consistent at 45 ± 7%, (n = 142), and reported concentrations were corrected using 17β-TBOH-d3 recoveries, but not spike recoveries. While matrix interferences of the leachate solution (~60 mg/L DOC) were likely responsible for low recoveries of the isotopic standard, 17α-TBOH, 17β-TBOH, and TBO 100 ng/L spike recoveries were 112 ± 19%, 102 ± 13%, and 92 ± 34%, respectively (n = 10).

Results and Discussion

Subsurface Discharge

We evaluated subsurface transport and attenuation processes of manure constituents by comparing leachate and discharge concentrations from the AB soil-horizon through time. Given the rapid transport of water (approximately 3 cm/s linear velocities in these systems) in both the shallow subsurface system and VFS plots (described later), we expect that contaminant attenuation arises primarily from partitioning mechanisms, as there existed insufficient hydraulic retention time for transformation mechanisms to affect concentrations. In the subsurface experiment, average leachate concentrations of 17α-TBOH and 17β-TBOH were 36 ± 5 ng/L and 3 ± 2 ng/L, respectively. TBO was detected once at 5 ng/L. 17β-TBOH was not detected in subsurface discharge, while TBO was detected once in discharge at 3 ng/L. 17α-TBOH was detected in all discharge samples, linearly increasing from an initial concentration of 12 ± 2 ng/L (65% removal; i.e., 1- C/C_o) to 23 ± 5 ng/L (32% removal) over 75 minutes (Figure 2). Based on this trend, complete breakthrough (i.e., $C/C_o = 1$) was expected to occur near 160 minutes. Thus, the sorption capacity under these conditions was expected to be exhausted after 160 minutes, after which, the mass leached/applied will equal the mass transported. Using a mass balance approach, estimates for 17α-TBOH soil concentration at 160 minutes range from 0.7-3.4 ng/kg (Table 1, see SI for calculation). By dividing the soil concentration by the aqueous concentration (36 ng/L), we estimated that a partitioning coefficient (K_D) range of 0.02-0.09 L/kg (Table 1) in this system, which is 100-500 times smaller than the K_D calculated using soil physical and chemical properties (10 L/kg, Table 2). This suggested that <1% of the total sorption capacity was used under experimental conditions and that 17α-TBOH attenuation within the shallow subsurface is dominated by non-equilibrium partitioning. We speculate that in agro-ecosystems receiving continual animal waste applications, a higher fraction of sorption capacity will be used, but similar partitioning mechanisms and non-equilibrium conditions might still be expected.

Similar to 17α-TBOH, the transport of nutrients and DOC was rapid and coincided with the initial discharge (Figure 2). For total ammonia, leachate concentrations were 7 ± 1 mg NH_3-N/L. Initial subsurface discharge concentrations were 2.1 ± 0.1 mg NH_3-N/L (70% removal) and increased linearly throughout the experiment to 5.9 ± 0.3 mg NH_3-N/L (16% removal). Complete breakthrough was expected to occur near 90 minutes. For orthophosphate, leachate concentrations were 2.5 ± 0.4 mg PO_4-P/L, and initial runoff concentrations were 0.8 ± 0.1 mg PO_4-P/L (68% removal). After increasing linearly through time, complete breakthrough was reached near 70 minutes (0% removal). For DOC, the average leachate (61 ± 6 mg-C/L) and runoff (61 ± 4 mg-C/L) concentrations were identical ($P = 0.92$) throughout the experiment. However, as with the other constituents, discharge concentrations increased linearly through time and reached complete breakthrough at 30 minutes, suggesting likely partitioning and DOC exchange during the trial (Figure 2). Using estimated breakthrough times for ammonia (90 minutes), orthophosphate (70 minutes), and DOC (30 minutes), the estimated increase in soil concentration of each constituent upon exhausting the sorption capacity was 0.07-0.32 mg NH_3-N/kg, 0.02-0.07 mg PO_4-P/kg, and 0.04-0.18 mg C/kg (see SI for calculation). We did not estimate system partitioning coefficients for ammonia, orthophosphate, or dissolved carbon due to the non-linearity of nutrient isotherms and the possibility of DOC exchange.

Several studies report rapid transport of agricultural steroids through soils, [20, 25-27, 31, 48] potentially due to mechanisms like hydrophobic partitioning to colloids. [20, 23, 48-50] However, the estimated fraction of 17α-TBOH associated with DOC was low (i.e., $F_c = 0.2\%$, Table 2, see SI), suggesting negligible colloidal transport, although we recognize that K_{oc} can be poorly correlated with steroid absorption to organic colloids.[49] Rapid transport also has been attributed to non-equilibrium partitioning, as time scales for hydrophobic equilibrium may reach 24 hours. [25-27] Thus, attenuation via partitioning to soils may be somewhat ineffective in systems with relatively short (e.g. minutes-hours) hydraulic retention times. Furthermore, partitioning is affected by rates of advective transport. If advective transport dominates (i.e., increasing Peclet number [Pe]), attachment efficiency on solid surfaces decreases, especially when $Pe > 20$.[51] For 17α-TBOH, the estimated Pe was >100, suggesting that advective transport dominated at this scale. Although transport of 17α-TBOH and other constituents were clearly retarded in the shallow subsurface, high advective transport rates and short hydraulic retention times likely best explain the observed data.

Surface Runoff

We evaluated surface transport and attenuation processes of steroids, nutrients, DOC, and coliforms on VFS systems using similar methodologies. Although the same VFS were used in all 4 trials, TBA metabolites were not detected in runoff during the saturation processes immediately prior to leachate application, indicating no analyte carryover between trials (Figure 3). On average, 17α-TBOH and TBO leachate concentrations were 34 ± 3 and 2 ± 1 ng/L, respectively, while 17β-TBOH was only sporadically detected in both leachate and runoff at concentrations <2 ng/L. 17α-TBOH concentrations were statistically greater in leachate than on

VFS (One-way ANOVA, F (19, 29) = 21.5, P < 0.001) but were not statistically different (based on Tukey's HSD post-hoc test) in runoff among all VFS (Figure 3). Little effect of VFS length was evident at these scales, as average runoff concentrations of 17α-TBOH were 8 ± 3, 11 ± 2, and 9 ± 1 ng/L from 3, 4, and 5 m VFSs, respectively. Similarly, 17α-TBOH removal (i.e., 1-C/C_o) performance for the VFS plots averaged 76 ± 8%, 69 ± 7%, and 72 ± 3% from 3, 4, and 5 m VFSs, respectively (Figure 3). Little data are published on steroid removal efficiencies on VFS; however, these observations are similar to reported 17α-TBOH removal in medium-scale irrigated pasture experiments (83%) and 17β-estradiol removal on 3.1 m VFS (79%).[47, 52] During both high and low volume/intensity rainfall events, we observed 95% removal, which was relatively constant throughout the events, of 17α-TBOH using the same VFSs.[47] These data coupled with the data presented within suggests that VFS are highly effective at attenuating TBA metabolites and steroid hormones transported in agricultural runoff.

Similar to subsurface experiments, we expected 17α-TBOH concentrations in surface runoff to increase with time until complete breakthrough occurred. For all VFSs, partial breakthrough of 17α-TBOH occurred rapidly ($C/C_o \approx 0.25$ in initial samples), indicating the existence of a highly mobile fraction of 17α-TBOH mass, again likely equilibrium limited or DOC-associated (78 mg/L DOC in leachate). Control and management of this mobile fraction is expected to be especially challenging, as its rapid transport and apparently limited interaction with treatment system materials may place an upper bound on the effectiveness of sequestration-based treatment strategies. However, unlike subsurface observations, concentrations in VFS runoff were surprisingly constant over time, especially for the 4 and 5 m VFSs (Figures 3, 4, S2, and S3), indicating slower saturation of attenuation capabilities and more efficient and consistent steroid attenuation on the VFSs relative to the subsurface system. We suspect this is a result of the increased productivity and complex heterogeneous environments on the VFS O horizon compared to the subsurface AB horizon. VFSs are irrigated and actively maintained throughout summer months and are characterized by year-round vegetative growth, unlike the soil trench watershed where vegetative growth is limited by seasonal aridity. Thus, biological activity and production of near-surface organic matter is likely substantially higher in VFS systems. Given the short hydraulic retention time (2-5 minutes), photolysis or microbial transformation processes, which occur on hours-days time scales, probably have minimal contribution to observed attenuation.

Physical filtration, sorption, subsurface infiltration, and deposition are typically cited as the primary mechanisms of nutrient (N and P), pathogen, herbicide, and veterinary antibiotic removal from VFSs.[44, 53-56] Because source leachate concentrations were near constant throughout the duration of the experiment and leachate was continuously applied, infiltration cannot explain observed concentration decreases. Furthermore, because of the tank design, the discharge of settlable solids in applied leachate was minimized, thus precluding sedimentation as a major removal mechanism. Instead, sequestration (either through sorption or filtration) within aboveground biomass or O-horizon constituents likely explained TBA metabolite removal. The presence of surface duff, biofilms, and an active rhizosphere may facilitate filtration and/or increase the hydrophobic partitioning capacity and increase attenuation potential in VFSs.[57] To verify TBA metabolite loss via soil partitioning, we extracted soil samples collected before and after leachate application on July 3. However, TBA metabolites were not detected in these samples, possibly because soils were extracted 24 hours after samples collection. With half-lives as short as 4 hours in warm, moist conditions, transformation prior to extraction may have occurred,[35] limiting our ability to directly probe the importance of soil partitioning. Additional analysis of near surface soils and organic materials is necessary to characterize removal mechanisms for TBA metabolites and other runoff constituents from VFSs.

Concentrations of total ammonia, orthophosphate, and DOC within the irrigation water that was used to make the leachate solution were 0.022 ± 0.005, 0.011 ± 0.005, and 1.5 mg/L, respectively, but increased considerably in leachate to 7 ± 1 mg NH_3-N/L and 5 ± 1 mg PO_4-P/L, and 78 ± 10 mg C/L, respectively. Nitrate and nitrite were not detected in any samples. Approximately 350 mg NH_3-N/kg-ww (wet weight manure), 250 mg PO_4-P/kg-ww, and 3900 mg-C/kg-ww leached into the irrigation water during the 24-hour contact time. Total coliforms and *E. coli* were not detected in irrigation water, and while *E. coli* was not detected in runoff from VFSs prior to leachate application, an average of 4.0×10^6 CFU/100 mL of total coliforms was present prior to trials. For total coliforms and *E. coli*, leachate concentrations increased to 8.7×10^7 and 7.9×10^7 CFU/100 mL, respectively, following contact with manure (Figure S5). Similar concentrations of ammonia, orthophosphate, and coliforms have been reported in agricultural runoff (0.05-7.2 mg NH_3-N/L, 0.17-4.29 mg PO_4-P/L, 1.0×10^5 - 1.8×10^6 CFU/100 mL) suggesting that the leachate is representative of agricultural runoff.[42-46] Compared to 17α-TBOH, nutrients and DOC occurrence in VFS runoff followed similar spatial and temporal dynamics (Figures 3, S3, and S4). Average ammonia and orthophosphate runoff concentrations on 3, 4, and 5 m VFSs were 1 ± 1 mg NH_3-N/L and 2 ± 1 mg PO_4-P/L for all three VFSs while DOC concentrations were 50 ± 9, 48 ± 2, and 48 ± 3 mg/L, respectively. Removal efficiencies for the 3, 4, and 5 m VFSs thus averaged 81 ± 10%, 85 ± 2%, and 87 ± 1% for total ammonia, 56 ± 9%, 60 ± 2%, and 60 ± 1% for orthophosphate, 32 ± 9%, 38 ± 3%, and 38 ± 4% for DOC, 49%, 37%, and 39% for total coliforms, and 41%, 50%, and 41% for *E. coli* (see Figure S5 for asymmetrical confidence intervals for total coliforms and *E coli*), respectively. On all VFSs, removal was greatest for total ammonia (84 ± 5%) followed by 17α-TBOH (72 ± 3%), orthophosphate (59 ± 4%), *E. coli* (44%), total coliforms (42%), and DOC (37 ± 4%). These removal efficiencies are consistent with previous studies examining attenuation of manure-derived constituents on VFS.[42-46, 58]

Because flow rates were similar on all VFS, mass loadings (i.e., ng/m^2 or mg/m^2) on the 4 and 5 m VFSs were 75% and 60% of the 3 m VFS mass loading. Therefore, we expected higher removal efficiencies on longer VFS given their longer hydraulic retention times and larger potential soil-water interfacial areas. However, removal efficiencies were surprisingly consistent for nutrients and DOC across these length scales (83 ± 3%, 59 ± 4%, and 38 ± 5% for ammonia, orthophosphate, and DOC, respectively; Figures 3, 4) not only for the July 3 trial, but also in the other trials (Figures S2-4). Therefore, for a given event, removal performance is independent of loading. Between events, removal efficiencies were statistically different (One-way ANOVA, F (3, 14) = 39.1, $P < 0.001$), varying from 68-88% for 17α-TBOH. While it is reasonable to expect performance to decrease with higher leachate concentrations, performance was independent of initial concentration. Surprisingly, the VFS treatment efficiency was statistically identical when 17α-TBOH leachate concentrations were either 11 or 150 ng/L (88 or 83% removal, respectively) and when leachate concentrations were either 34 or 112 ng/L (72 or 68% removal, respectively), although removal was statistically lower in the latter group (Figure 4). Although suspended solids were not measured directly, performance was the lowest when the suspended solids content was qualitatively highest (i.e., 31 May, Figure 4), suggesting a potential inverse relationship between suspended solids and removal. Thus, optimizing removal might focus upon suspended solids, DOC and colloid reduction in runoff. Conversely, if transport was limited by partitioning kinetics, increasing system hydraulic retention times (i.e. increased length or decreased slope) should be an effective management strategy.

Transport Potential

In all experiments, 17α-TBOH was easily the most abundant TBA metabolite in leachate, with 17β-TBOH and TBO detected in leachate samples at concentrations below 5 ng/L, even when 17α-TBOH concentrations exceeded 150 ng/L. While it seems reasonable to assume negligible contributions of 17β-TBOH and TBO to ecosystem risk, metabolite interconversion occurs readily.[13, 20, 35] While 17β-TBOH and TBO were detected at low concentrations in this study and in our previous work, both have been detected in agricultural runoff at concentrations up to 270 and 35 ng/ L, respectively. [14-16, 40] In those systems (i.e., CAFOs and manure fertilized fields), TBA metabolites accumulated in surface soils, and microbial processes formed 17β-TBOH and TBO from 17α-TBOH.[35, 59] Given the similar chemical properties of TBA metabolites, their environmental fate and transport behavior also should be conserved and we expect that these 17α-TBOH observations are valid for all TBA metabolites and even other steroids with similar properties.

Vegetative filter strips are widely used to manage contaminants in agricultural runoff, particularly for sediment and nutrient control. Our results indicate that VFSs were effective at attenuating 17α-TBOH over ~1 hour time scales, with an average 78% removal from surface runoff from all four experiments (Figures 3, 4, S2, S3). It is commonly perceived that a single large strip is better than a small one; however, our results clearly indicate that small VFSs are highly effective, at least over short time scales and could be deployed within pastures near manure “hotspots” (i.e., cattle congregation points) to control contaminant transport near sources. Nichols et al. observed a ~20% increase in 17β-estradiol removal when VFS lengths were increased from 3 to 18 meters, suggesting that removal was limited by equilibrium kinetics.[52] While an 18 m VFS may be impractical, multiple shorter treatment systems in series might prove to be equally effective management strategies.

While most 17α-TBOH mass was retarded within the VFS, approximately 20% of the mass was readily mobile, a trend also observed for nutrients and DOC (Figures 3, S2-4). After VFS treatment, 17α-TBOH runoff concentrations ranged from 1-43 ng/L. While NOAELs for aquatic vertebrates remain unclear, a reasonable estimate for TBA metabolites is 1 ng/L.[8, 11, 12] Therefore, concurrent dilution and attenuation are typically necessary to achieve NOAELs, and a clear relationship exists between treatment system performance and the dilution required to attain NOAELs (Figure 5). For example, 17α-TBOH concentrations of 350 ng/L are reported for CAFO runoff.[19] Without attenuation, a dilution factor (the ratio of receiving water to runoff volumes or flows) of 350 is required to attain 1 ng/L NOAELs in receiving waters. Using a VFS with 80% removal efficiency, the required dilution factor is reduced proportionally, from 350 to 70, representing a substantial savings in the necessary dilution capacity. Ideally, treatment processes can attenuate concentrations to NOAELs without dilution, which should be true for runoff concentrations below 5 ng/L and VFS performance of ~80% (Figure 5). The reality of non-point source pollution is that dilution often plays a critical role in mitigating ecosystem risk. Thus, understanding leachate concentrations, VFS performance, and receiving-water flows can identify risky periods or conditions, particularly when dilution volumes are low, where concentrations are most likely to exceed NOAELs. In general, first-order streams, wetlands, ephemeral pools, and other small waters with dilution capacities <70-100 are at the greatest risk from endocrine disrupting steroids. While treatment efficacy must increase as dilution capacity decreases to meet concentration-dependent performance goals, this relationship does not apply to mass-dependent performance goals like TMDLs. Future research should attempt to further develop these management relationships while optimizing non-point source pollution treatment strategies for steroids and other problematic contaminants.

Conclusion

In both surface and subsurface experiments, 17α-TBOH, ammonia, orthophosphate, DOC, and coliforms were present within initial runoff samples, indicating that some of this constituent mass was highly mobile and traveled at or near the velocity of water. While retardation was clearly evident for all constituents, our data suggests that non-equilibrium partitioning processes likely facilitated the rapid transport of these constituents through these systems. Comparatively, however, contaminant breakthrough occurred more rapidly within the subsurface compared to the surface VFS, and we attribute the increased removal within the surface to the increased near surface biologic productivity and organic matter. While this data suggests that subsurface transport can increase the risk of aquatic ecosystem exposure to endocrine disrupting compounds, dominant processes responsible for the observed attenuation remain unclear, and additional mechanistic characterization of removal processes is needed.

Our results indicate that the transport potential of TBA metabolites through VFSs increased with increasing leachate concentrations, although removal efficiency may depend on other factors such as TSS. 17α-TBOH removal efficiency was similar among all four VFS experiments (68-88% removal). Previously, we reported that 17α-TBOH leaching potential peaked between 5 and 30 days post-implantation, with a 14,400 and 4,000 ng/animal unit leaching potential during a 5 cm rainfall event and a 9 hour irrigation event, respectively.[40] If

20% of 17α-TBOH mass is readily mobile and unlikely to be attenuated during treatment, 800-2,900 ng/AU is available for transport to receiving waters. Depending on rainfall and irrigation rates, the runoff or discharge concentrations will vary and will be system-dependent, but can be estimated and compared against desired exposure concentrations in receiving waters to assess the ecological risks to particular receiving waters. Therefore, to fully characterize the risks associated with TBA use, or the risks of other steroids and manure-derived contaminants in agro-ecosystems, we recommend the simultaneous evaluation of leaching, transport, and hydrologic characteristics as an appropriate strategy for prioritizing the implementation of runoff treatment technologies and selecting effective best management practices.

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Electronic Supplementary Information

Additional material regarding experimental methods, soil partitioning calculations, and experimental data (Figures S1-S5) can be found within the Supplementary Information (SI).

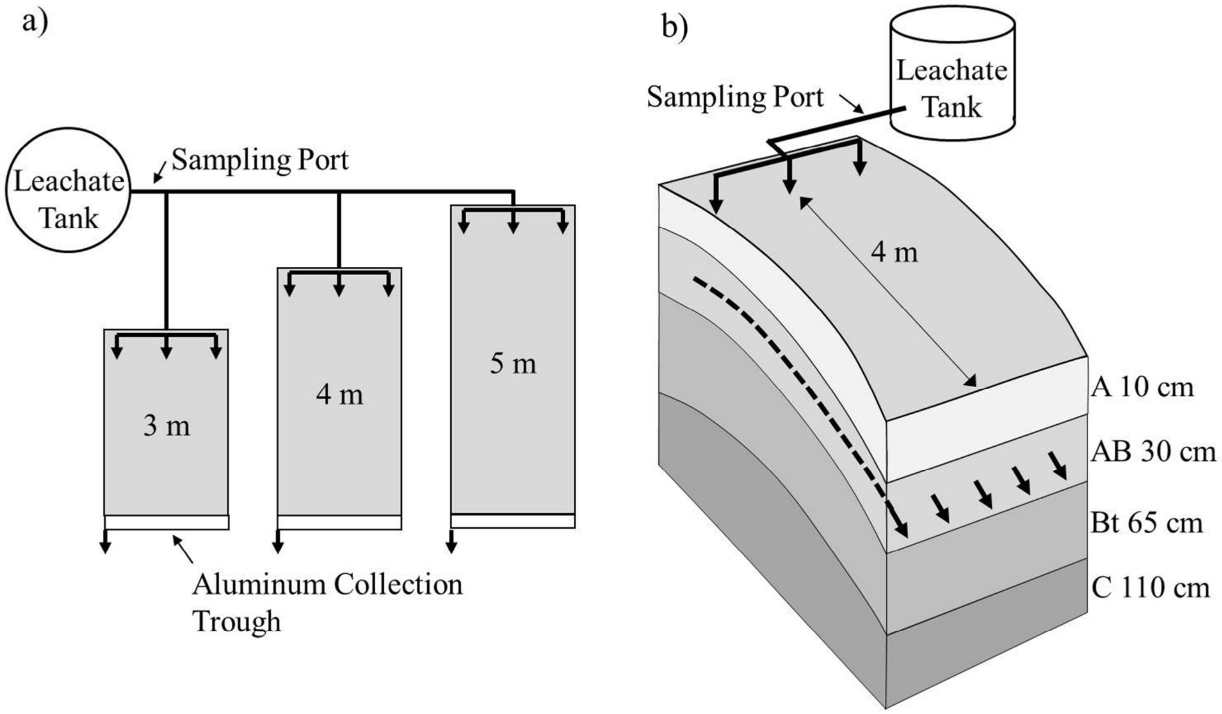

Figure 1. Schematic diagram of (a) vegetative filter strips of 2 m width x 3, 4, and 5 m length. Leachate was applied uniformly across each VFS and runoff was collected in aluminum trays. The same leachate application system was used for subsurface experiments (b). Aluminum trays (width = 1 m) were placed at different soil horizon interfaces to capture discharge. The horizon name and depth are listed.

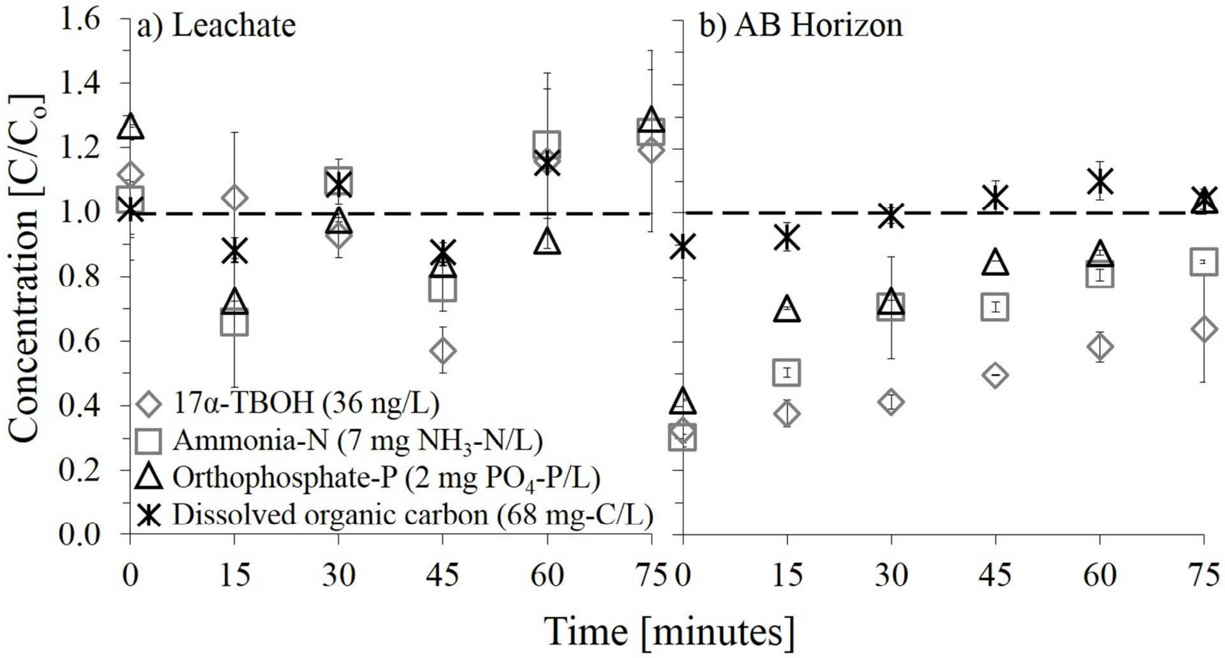

Figure 2. Normalized concentrations of measured constituents in (a) leachate and (b) subsurface discharge from the AB soil horizon after 4 m of transport in the vadose zone. All concentrations were normalized to the average leachate concentration (specific values provided in the legend for each constituent). For leachate and runoff samples, t = 0 minutes corresponds to the moment AB horizon discharge first occurred (hydraulic retention time = 2 minutes). Error bars represent 95% confidence intervals (n = 3).

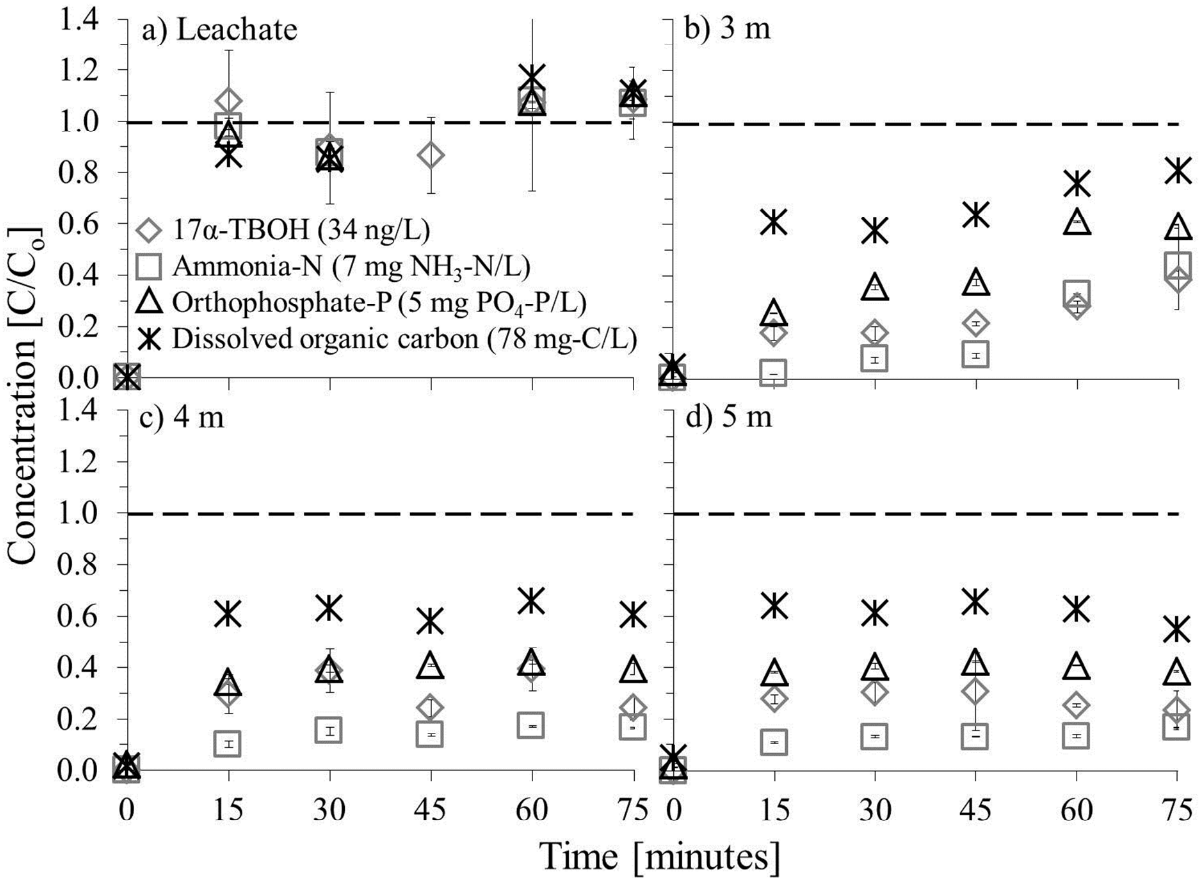

Figure 3. Relative concentrations of manure-derived constituents in (a) leachate, and surface runoff from (b) 3 m, (c) 4 m, and (d) 5 m vegetative filter strips. All concentrations were normalized to the average leachate concentration (provided in the legend). Values reported at t = 0 represent background concentrations. The first runoff samples were collected at t = 15 minutes. Error bars represent 95% confidence intervals (n = 3) and are not present for DOC.

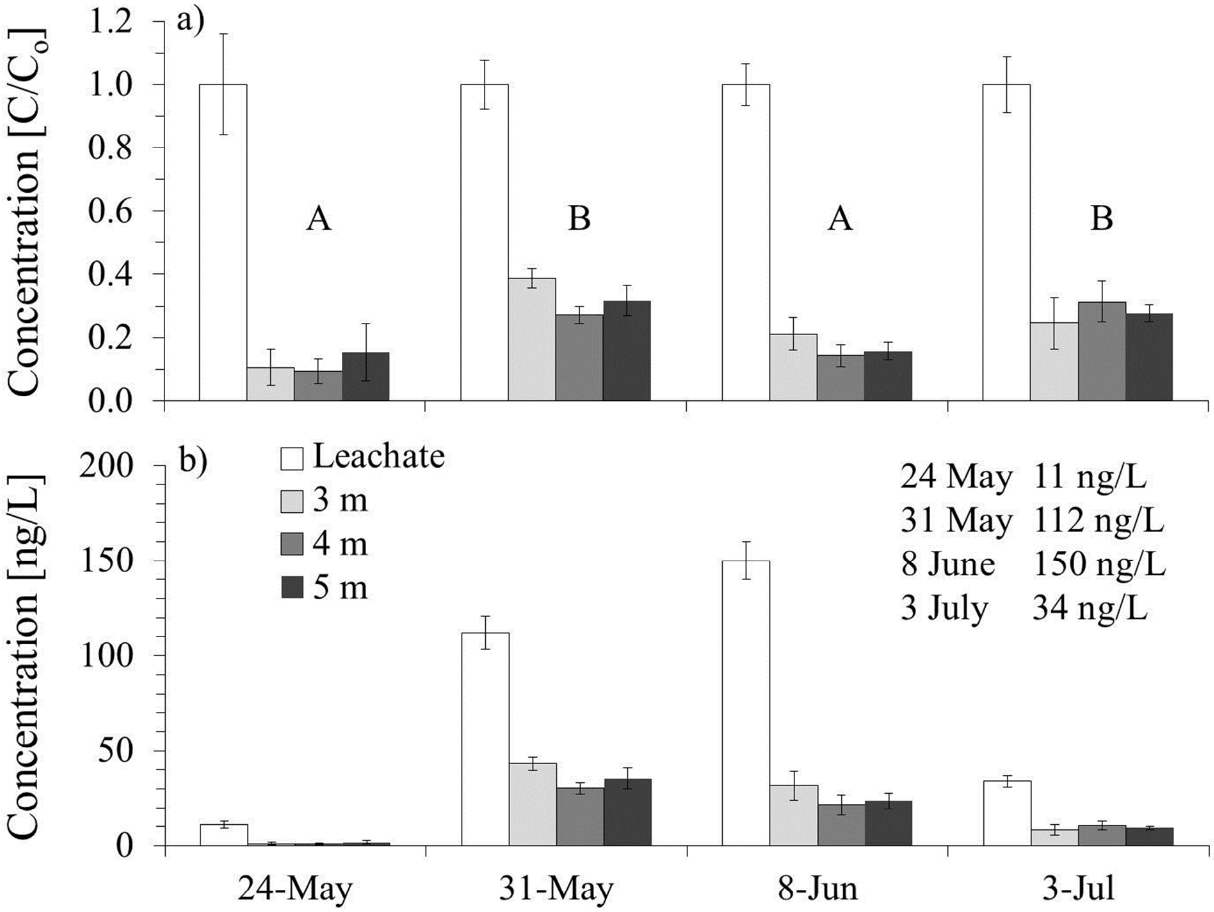

Figure 4. Average (a) normalized runoff concentrations (i.e., C/C_o) and (b) actual runoff concentrations of 17α-TBOH during four vegetative filter strip (VFS) runoff experiments. Relative concentrations were normalized based on the average contaminant concentration within leachate (provided in legend). The average normalized-concentrations were statistically different based on Tukey's HSD test following a One-way ANOVA (F (3, 14) = 39.1, $P < 0.001$) if events did not share letters (i.e., A, B) with other events (a). Error bars represent 95% confidence intervals (n = 6-30).

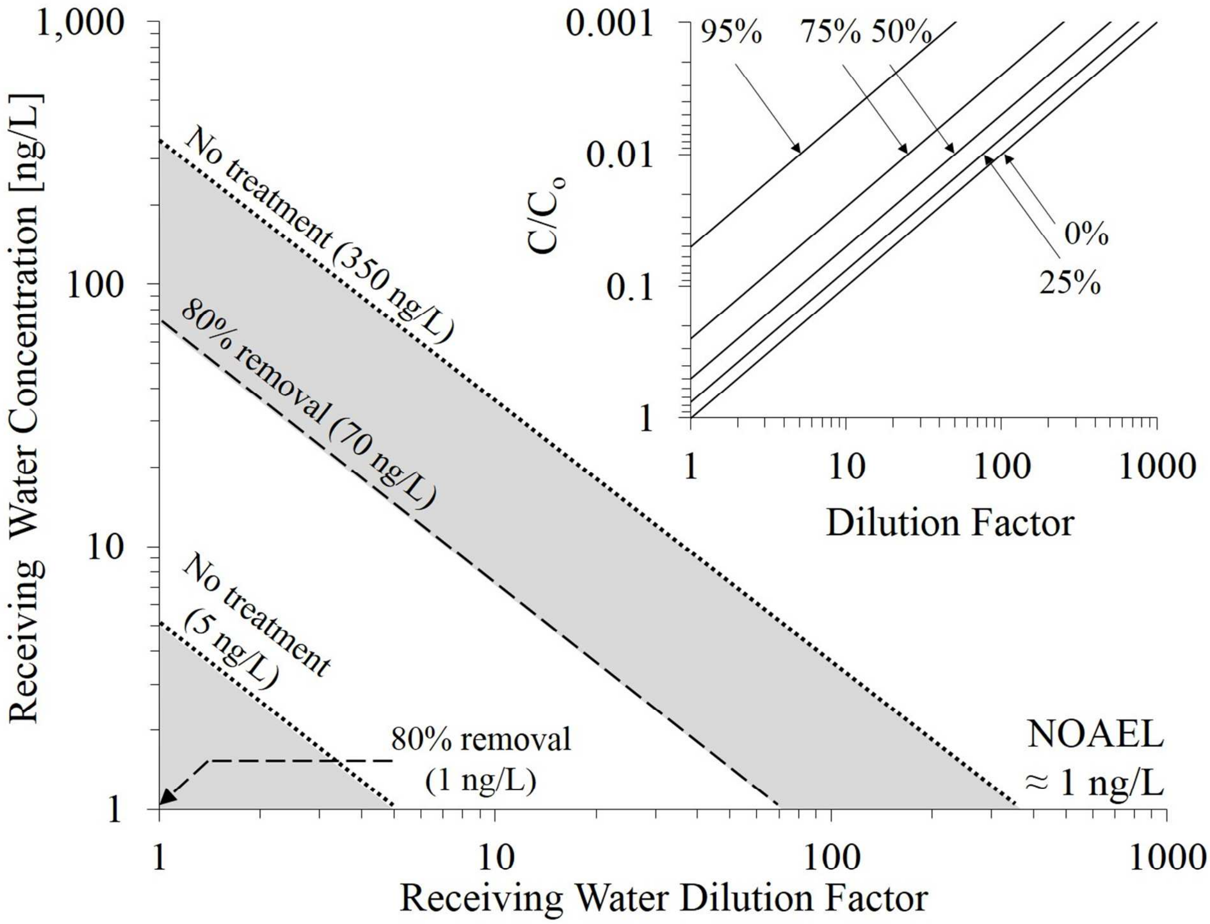

Figure 5. Receiving water concentration of 17α-TBOH as a function of runoff concentration, dilution capacity, and treatment performance. Dotted lines represent initial concentrations and dashed lines represent post-treatment concentrations in runoff assuming ~80% removal efficiencies. The horizontal line represents an assumed no observed adverse effects level (NOAEL) of 1 ng/L. Dilution likely is required to achieve receiving water concentrations of 1 ng/L when pre-treatment runoff concentrations exceed 5 ng/L. (Insert) Predicted concentrations (i.e., C/C_o, y-axis) in receiving waters as a function of treatment system performance (percent removal, lines) and the available dilution capacity of the receiving water (x-axis).

Table 1. Parameter values used to calculate the partitioning coefficient (K_D) between 17α-TBOH and soils and the mass of ammonia, orthophosphate, and dissolved organic carbon (DOC) within the AB horizon of the soil trench under three different scenarios. Soil concentrations represent the constituent mass sorbed at exhaustion.

Scenario	**A**	**B**	**C**
Flow (L/min)	15	5.4	0.63
AB soil volume (L)	5,850	5,850	1,200
AB soil mass (kg, based on ρ_b = 1.5 kg/L)	8,780	8,780	1,800
17α-TBOH soil concentration (ng/kg)	3.4	1.2	0.7
17α-TBOH system K_D (L/kg)[a]	0.09	0.03	0.02
17α-TBOH sorption capacity used[b]	0.9%	0.3%	0.2%
Ammonia soil concentration (mg NH_3-N/kg)	0.32	0.12	0.07
Orthophosphate soil concentration (mg PO_4-P/kg)	0.07	0.03	0.02
DOC soil concentration (mg C/kg)	0.18	0.06	0.04

ρ_b = bulk density

[a] see SI for calculation

[b] system K_D:calculated K_D (based on soil properties, Table 2) ratio

Table 2. Physical and chemical properties of the soils at subsurface (AB horizon, pH = 5.7) and surface (vegetative filter strips, VFS, pH = 6.8) experimental sites. Abbreviations include bulk density (ρ_b), porosity (φ), cation exchange capacity (CEC), fraction of soil organic carbon (f_{oc}), 17α-trenbolone (17α-TBOH) soil partitioning coefficient (K_D), estimated 17α-TBOH retardation factor (R), and the fraction of 17α-TBOH dissolved in water (F_D), sorbed to soil (F_S), and sorbed to dissolved organic carbon (F_C). See SI for parameter calculation.

Soil	% Sand	% Silt	% Clay	ρ_b [g/cm^3]	φ [-/-]	CEC[a] [meq/100 g]	f_{oc} [%]	K_D[b] [L/kg]	R[c] [-/-]	F_D [%]	F_S [%]	F_C [%]
AB	35	56	9	1.5	0.47	10.0/11.5	1.7	10	33	5.5	94.3	0.2
VFS	27	58	15	2	0.29	24.0/22.2	2.9	17	119	1.2	98.7	0.1

[a] CEC calculated by summing Ca^{2+}, Mg^{2+}, Na^+, and K^+ (left of slash) and extracting NH_4^+ (right of slash)

[b] K_D estimates based on the product of f_{oc} and K_{oc} ($10^{2.77}$ L/kg)[22]

[c] Estimated from ρ_b, φ, and K_D.

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