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RATES AND PRODUCT IDENTIFICATION FOR TRENBOLONE ACETATE METABOLITE BIOTRANSFORMATION UNDER AEROBIC CONDITIONS

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Abstract: Trenbolone acetate metabolites are endocrine-active contaminants discharged into the aquatic environment in runoff from agricultural fields, rangelands, and concentrated animal feeding operations. To investigate the environmental fate of these compounds and their biotransformation mechanisms, the authors used inocula from a variety of different water sources and dosed biologically active microcosms with approximately 1400 ng/L of trenbolone acetate metabolites, including 17β-trenbolone, trendione, and 17α-trenbolone. To investigate aerobic biotransformation rates and interconversions between known trenbolone acetate metabolites, gas chromatography–tandem mass spectrometry was used to measure concentrations and assess product distributions as a function of time. High-resolution liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to characterize novel transformation products and potential transformation pathways. Kinetic analysis yields observed half-lives of approximately 0.9 d, 1.3 d, and 2.2 d for 17β-trenbolone, trendione, and 17α-trenbolone, respectively, at 20 °C, although colder conditions increased half-lives to 8.5 d and biphasic transformation was observed. Relative to reported faster attenuation rates in soils, trenbolone acetate metabolites are likely more persistent in aqueous systems. Product distributions indicate an enzymatic preference for biotransformation between trendione and 17β-trenbolone. The LC-MS/MS characterization indicates dehydrogenation products as the major detectable products and demonstrates that major structural elements responsible for bioactivity in steroids are likely retained during biotransformation. *Environ Toxicol Chem* 2015;34:1472–1484. © 2015 SETAC

Keywords: Trenbolone acetate Product characterization Aerobic biotransformation Rate constant

INTRODUCTION

Agricultural runoff and municipal wastewater effluent are major contributors to the occurrence of endocrine-active steroidal contaminants capable of inducing potential adverse effects on aquatic organisms in surface waters [1–8]. Given its large geographic footprint and limited treatment, agricultural runoff may be of special concern as a source of endogenous and exogenous steroids. For example, most beef cattle in the United States are implanted with trenbolone acetate, a potent synthetic steroid and anabolic growth promoter [9]. Trenbolone acetate metabolites excreted by cattle include 17α-trenbolone (representing >95% of the known trenbolone acetate metabolite mass), 17β-trenbolone, and trendione, although >90% of the trenbolone acetate implant dose remains uncharacterized [10,11]. Thus, in addition to normal excretion of endogenous steroids from vertebrates, animal agriculture can act as point or nonpoint sources of metabolites of synthetic pharmaceuticals (such as trenbolone acetate) to aquatic environments.

Given their potency, which is usually much higher than that of endogenous steroids, steroid pharmaceuticals may be especially important contributors to ecosystem risks. Exposure to trenbolone acetate metabolites can significantly reduce fecundity in fish at 10 ng/L to 30 ng/L concentrations for 17α-trenbolone and 17β-trenbolone, respectively; and trenbolone acetate metabolites can induce partial to complete sex reversal at similar concentrations [9,12,13]. Trenbolone acetate metabolites can alter female fish behavior at low concentrations [14], and genotoxic trends in fish populations also are reported, although at substantially higher exposure concentrations [15].

Synthetic steroids usually are more environmentally persistent than their endogenous counterparts [4,6] and often are selected specifically for attributes such as reduced potential for in vivo enzymatic degradation (thus increasing their bioactivity), which also may affect their environmental fate. Data for endogenous estrogens and androgens indicate that steroid-degrading microorganisms utilize highly site- and ring-specific enzymes for biotransformation, a selectivity that can limit their utility for synthetic steroids whose structures diverge from those of endogenous steroids at key reaction centers [16,17]. Mass balance calculations suggest that up to 80 μg/d of 17α-trenbolone is excreted per implanted animal, potentially leading to concentrations of several thousand nanograms per liter in agricultural runoff and indicating the importance of subsequent environmental fate processes in governing their ecological risk [11,18].

The available biotransformation data for trenbolone acetate metabolites are limited to agricultural soils and suggest half-lives near 4 h to 12 h for 17α-trenbolone and 17β-trenbolone, with longer (1–4 d) half-lives observed for trendione [18–21]. In contrast to expectations derived from Michaelis-Menten kinetic models, some biotransformation data suggest that trenbolone acetate metabolite transformation rates exhibit concentration dependence, demonstrating slower transformation rates at

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higher concentrations [19,21]. Also, similar to data observed for estrogens, biologically mediated reactivity within families of metabolites, such as interconversions between more potent 17-hydroxy and less potent 17-keto steroids mediated by hydroxysteroid dehydrogenase, can preserve the steroid backbone and conserve bioactive functional groups despite transformation [22]. Because biotransformation data in aqueous systems or characterizations of transformation products beyond 17α-trenbolone, 17β-trenbolone, or trendione are lacking, these limitations merit additional investigation of trenbolone acetate's metabolite fate.

To address these limitations, the objective of the present study was to use kinetic modeling to determine biotransformation rates of trenbolone acetate metabolites in aerobic batch microcosms inoculated from different sources. Two different incubation temperature regimes and sampling campaigns were conducted to estimate the dependence of kinetic model results on seasonal and temperature variation. We also characterized the potential for interconversions between known trenbolone acetate metabolites to occur and used liquid chromatography–high-resolution tandem mass spectrometry (LC-HRMS/MS) for additional biotransformation product identification and characterization.

EXPERIMENTAL PROCEDURES

Chemicals

Trenbolone acetate metabolites (17α-trenbolone, 17β-trenbolone, and trendione) were purchased from Steraloids. We used 17β-trenbolone-16,16,17-d3 as an isotopic standard for quantitation (BDG Synthesis). High-performance liquid chromatography (HPLC)–grade methanol, acetone, and dichloromethane were purchased from Fisher Scientific. All other chemicals were purchased from Sigma Aldrich. Following published protocols, *N*-methyl-*N*-(trimethylsilyl)-trifluoro-acetamine and iodine (99.999% pure) were used to derivitize samples prior to gas chromatographic (GC) analysis [18,23]. Fluorescein diacetate (3′6′-diacetyl-fluorescein), fluorescein disodium salt, and sodium pyrophosphate were used in microbial activity measurements (see Supplemental Data and Adam and Duncan [24]). Glassware was silanized with dichlorodimethylsiloxane. To address nutrient limitations, a phosphate-buffered mineral medium was prepared, which contained 0.85 g/L KH_2PO_4, 2.175 g/L K_2HPO_4, 3.34 g/L $Na_2HPO_4 \cdot H_2O$, 0.05 g/L NH_4Cl, 0.275 g/L $CaCl_2$, 0.225 g/L $MgSO_4 \cdot 7H_2O$, and 0.002 g/L $FeCl_3 \cdot 4H_2O$.

Inocula sources and sampling

All studies used to quantify biodegradation constants used inocula gathered from Steamboat Creek (39°25′08.25″N, 119°44′25.91″W), a mixed-use urban agricultural watershed in Reno, Nevada, USA. Inocula were gathered in April 2012 and January 2013, respectively, representing seasonally dependent "active" and "dormant" inocula from the same site. Studies examining the possible influence of inocula source on transformation product formation were conducted by collecting inocula from 1) wastewater effluent from the secondary clarifiers at the Truckee Meadows Water Reclamation Facility in Sparks, Nevada; 2) the Sparks Marina in Sparks, Nevada; 3) the Truckee River at Mayberry Park in Reno, Nevada (39°30′09.30″N, 119°53′49.95″W); 4) a pond in Idlewild Park in Reno, Nevada (39°31′17.69″N, 119°49′55.90″W); and 5) Steamboat Creek.

Mixed culture microbial inocula were obtained by agitating the top 3 cm of sediment at the sediment–water interface and collecting the resulting high-turbidity solution. Inocula were immediately transported to the laboratory and allowed to acclimate to experimental conditions for up to 12 h prior to use. Inocula were characterized by measuring total organic carbon (TOC) and total carbon using a Shimadzu Total Organic Carbon Analyzer (TOC-V_{CSH}). Inorganic carbon was calculated as the difference between total carbon and TOC.

Microcosm design

Biotransformation studies were conducted as batch microcosms (in triplicate) in 73-mL amber glass vials filled with a 1:1 mineral medium and inocula mixture. To allow for mixing and gas exchange, each microcosm had approximately 1 cm of headspace with approximately 65 mL to 68 mL liquid volume. Trenbolone acetate metabolites (17α-trenbolone, 17β-trenbolone, or trendione) were added to microcosms by spiking 100 μL of a 1-g/L trenbolone acetate metabolite standard in methanol to obtain nominal concentrations of 1400 ng/L for all kinetic studies. Control microcosms were prepared identically, though autoclaved (121 °C, 15 psi) for 2 h and cooled to room temperature prior to trenbolone acetate metabolite addition [19]. Microcosms were loosely covered with aluminum foil and incubated in the dark as they were gently shaken (100 rpm) to promote gas exchange and maintain aerobic conditions at 20 °C in a climate-controlled lab. Sample evaporation was negligible at these experimental conditions. Seasonal microcosms using January inocula also were incubated at 5 °C in a walk-in refrigerator or at 35 °C in a large incubator to investigate temperature effects.

Experiments employed sacrificial analysis of microcosms at 0 d, 1 d, 3 d, 6 d, 10 d, and 15 d after incubation. For analysis, microcosms were spiked to 100 ng/L with 17β-trenbolone-d3 as an isotopic standard; then, the pH of each sample was recorded, and 3 mL were removed from each sample for qualitative sterility analysis using fluorescein diacetate hydrolysis analysis [24]. Next, the remaining approximately 65 mL of each sample were extracted onto preconditioned (following manufacturer protocols) Restek C-18 solid phase extraction cartridges [18]. If necessary, solid-phase extraction cartridges were stored at −18 °C prior to processing. Additional experimental details and results are found in Supplemental Data.

Gas chromatography–tandem mass spectrometry (GC-MS/MS) analysis

The GC-MS/MS analysis of trenbolone acetate metabolites is described in detail elsewhere [18,23]. Briefly, elution of solid-phase extraction cartridges was performed with a 95:5 (v/v) methanol:water mixture in three 3-mL aliquots at 1 mL/min to 5 mL/min. The eluent was dried in a vacuum oven at 25 °C, resuspended in 1 mL of a 95:5 (v/v) dichloromethane:methanol solution, transferred to silanized GC vials, and dried with nitrogen. Derivitization was performed by adding 50 μL *N*-methyl-*N*-(trimethylsilyl)-trifluoro-acetamine-I_2 (1.44 mg I_2/mL *N*-methyl-*N*-[trimethylsilyl]-trifluoro-acetamine) to each vial, vortexing, and then drying under N_2. Samples were resuspended in 100 μL *N*-methyl-*N*-(trimethylsilyl)-trifluoro-acetamine, capped prior to incubation (40 min) at 60 °C, then cooled and analyzed by GC-MS/MS with isotopic dilution analysis. At these microcosm volumes, the method limit of quantification was 14 ng/L, and quality assurance and quality control metrics (near quantitative spike recovery, precision generally within 10%, no false positives detected in blanks) are

similar to those we reported in detail for concurrent studies [11,25,26]. Samples that failed either to meet this limit of quantification or to meet a signal-to-noise ratio of 10 were excluded from quantitative analysis. Control microcosms were examined for sterility and mass conservation; data sets without sterile controls were excluded from analysis.

Transformation products

To facilitate identification, microcosms for transformation product studies were sometimes spiked to 5 μg/L or 20 μg/L. All other experimental conditions and sample processing were identical to those described above. Samples (2 mL in amber glass HPLC vials) collected for LC-HRMS/MS analysis were usually analyzed directly by partially evaporating (~90%) samples with a gentle stream of nitrogen gas, then adding HPLC-grade methanol to make a 90:10 (v/v) methanol:water solution for analysis. In addition to direct analysis, solid-phase extraction (described above) was sometimes employed to concentrate samples where methanolic extracts were adjusted to the same 90:10 (v/v) methanol:water composition. The LC-HRMS/MS analysis employed an LTQ-Orbitrap XL (Thermo-Electron) mass spectrometer to characterize products using conditions and criteria described elsewhere [27,28].

Kinetic modeling

To maintain consistency and comparison with published data for trenbolone acetate metabolites and other steroids, we applied not only the standard pseudo–first order decay model [19,22,29–31] but also 2 biphasic models to estimate transformation rates to account for observed biphasic characteristics. April and January inocula data ($t = 5\,^{\circ}C$, 20 °C, and 35 °C) were used in this biphasic analysis, which is an analysis similar to those published by Stowe et al. [32,33] for polychlorinated biphenyl (PCB) kinetic rate modeling under conditions where residual contaminants inhibited transformation rates. Biotransformation of the cyanobacterial hepatotoxin microcystin also has exhibited biphasic kinetics arising from the existence of multiple communities of microcystin-decaying microorganisms: one utilizing rapid direct metabolism at high concentrations and another responsible for slower cometabolism at low concentrations [34–36]. Other examples of systems that diverge from exponential decay or exhibit biphasic characteristics include biotransformation of trenbolone acetate metabolites and estrogens in soils [21,37], PCB transformation [32,33], and the Michaelis-Menten and Monod kinetic models.

Kinetic models included the pseudo–first order model (model 1); a "non-zero asymptote" model (model 2), which assumes the presence of both a rapidly degrading contaminant pool and a persistent residual; and a "double-exponent" model (model 3), which assumes that the persistent residual also decays [32,33].

$$C = C_1 e^{-k_1 t} \quad (1)$$

$$C = C_2 e^{-k_2 t} + C_3 \quad (2)$$

$$C = C_4 e^{-k_4 t} + C_5 e^{-k_5 t} \quad (3)$$

In the 3 models, C represents the observed metabolite concentration at time t and k_1, k_2, k_4, and k_5 all represent the corresponding first-order rate constants for the different models. In model 1, C_1 represents the initial metabolite concentration. For model 2, C_2 represents the labile metabolite concentration, and C_3 represents a residual recalcitrant concentration. In model 3, C_4 and C_5 represent the concentrations amenable to rapid or slow transformation, respectively. For models 2 and 3, summing C_2 and C_3 or C_4 and C_5 yields initial metabolite concentrations.

RESULTS AND DISCUSSION

Aerobic microcosms

Microbial activity measurements, including pH, TOC, and fluorescein diacetate analysis, indicate limited or no microbial activity in controls relative to biologically active microcosms where microbial activity increased during the experiments (Figures 1D and 2D; Supplemental Data, Figures S1 and S2). As a result of autoclaving, pH in control microcosms was generally slightly higher than for active microcosms, with the difference in pH between aerobic and control samples being statistically significant for data taken at 0 d, 1 d, 6 d, and 10 d after incubation. The Steamboat Creek inocula contained approximately 7 mg/L TOC and 13 mg/L inorganic carbon initially, which was subsequently diluted with mineral medium. Thus, TOC levels were initially low (<10 mg/L) in microcosms, but methanol addition (100 μL in spikes) increased TOC levels to 390 ± 18 mg/L (Supplemental Data, Figure S1). Consistent with Yang et al. [31], preliminary experiments conducted with and without glucose addition as a carbon source demonstrated no significant effect on transformation rates; thus, no manipulation of labile organic carbon was used.

For autoclaved controls with April inocula, 17α-trenbolone, 17β-trenbolone, and trendione exhibited 85% to 100%, 77% to 95%, and 69% to 110% recoveries of initial 1400 ng/L concentrations over 15 d, respectively (Figure 1). Observed slight deviations between nominal and observed concentrations in controls are consistent with normal variations in method performance. Over the 15 d, 17α-trenbolone controls were generally stable, whereas controls for 17β-trenbolone and trendione exhibited losses, up to 30% in one case, although usually smaller. Although sorption, abiotic transformation, or residual enzymatic activity may explain slight losses in controls, the phenomenon was not observed in other trials. However it is quite consistent with similar, though unexplained, losses observed for trenbolone acetate metabolites in autoclaved soil–water systems [19].

In biologically active microcosms spiked with 1400 ng/L 17α-trenbolone at 20 °C (Figure 1A), 17α-trenbolone concentrations in initial samples (extracted ~2 h after trenbolone acetate metabolites were spiked) were not significantly different from controls. Over the next 6 d, concentrations decreased to 170 ng/L, with no further decreases apparent to 15 d. Lag phases were not observed for any trenbolone acetate metabolite, indicating that inherent biotransformation capabilities existed for these steroids in the inocula. Consistent with results reported for estrogens and trenbolone acetate metabolites in soil systems, interconversions between trenbolone acetate metabolites were important aspects of transformation [19,22,29,37]. In the 17α-trenbolone microcosms, trendione concentrations increased to 170 ng/L over the first 3 d, corresponding to 12% of the mass dosed, then decreased. 17β-Trenbolone was first observed at 3 d (36 ng/L) and increased to 89 ng/L at 15 d, a 7% mass yield. No similar trenbolone acetate metabolites were observed in any 17α-trenbolone controls, indicating that these interconversions were biologically mediated.

In microcosms spiked with 1400 ng/L 17β-trenbolone (Figure 1B), a rapid 25% loss relative to controls apparently occurred over the initial 2-h period after 17β-trenbolone spiking but prior to extraction (processing period for 0 d samples). In the

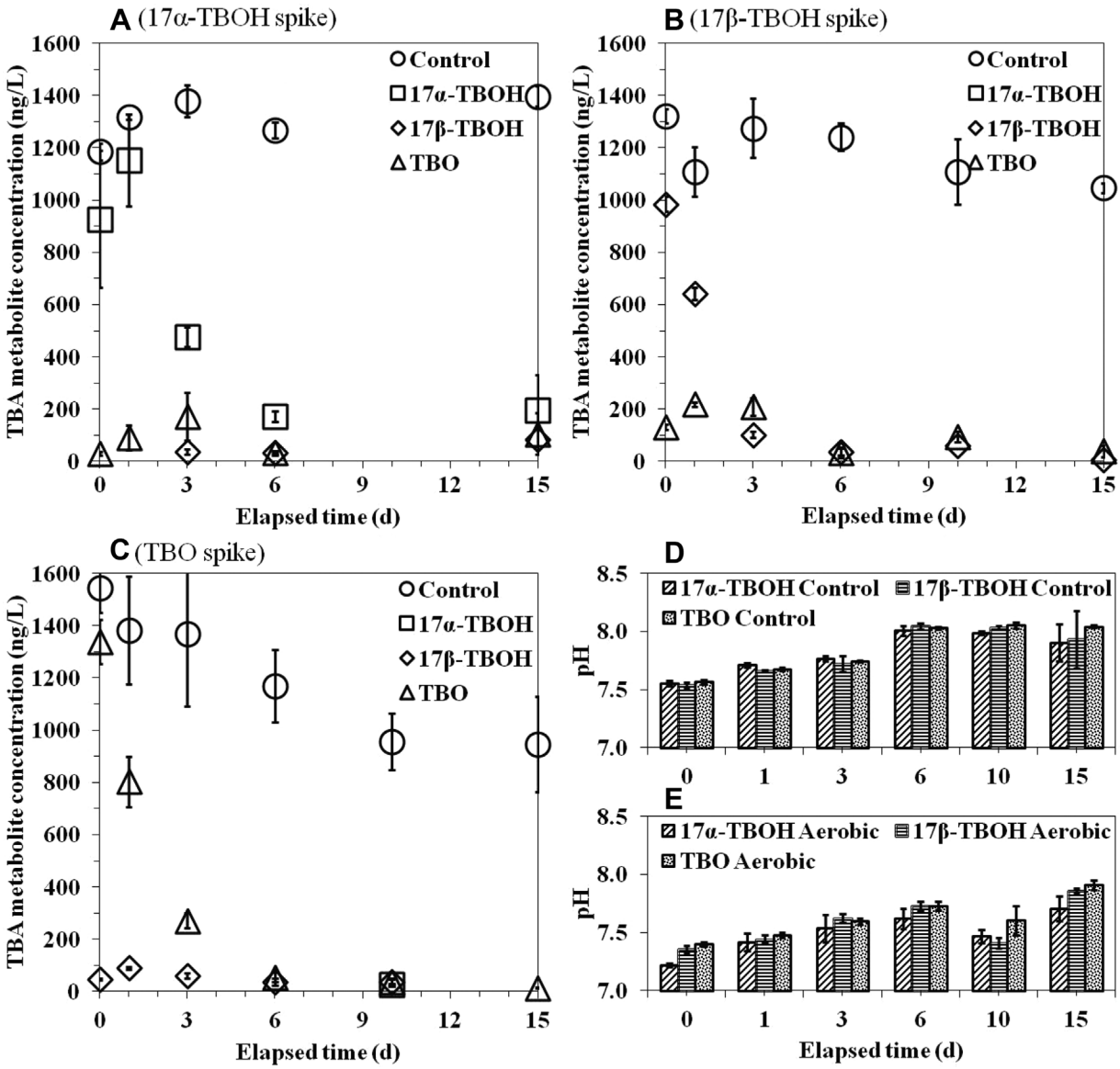

Figure 1. Aerobic biodegradation of (**A**) 17α-trenbolone, (**B**) 17β-trenbolone, and (**C**) trendione at 20 °C with April inocula. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a nominal concentration of 1400 ng/L. (**D**) pH data corresponding to control samples from (**A**) through (**C**). (**E**) pH data corresponding to aerobic samples from (**A**) through (**C**). Error bars represent standard deviations of triplicate samples. TBA = trenbolone acetate; TBO = trendione; TBOH = trenbolone.

same samples, 120 ng/L of trendione was present with no similar trendione detections observed in controls. Although not statistically significant, similar trends of rapid initial attenuation relative to controls were observed for 17α-trenbolone and trendione microcosms. This phenomenon cannot be explained by sorption to particles or other abiotic loss processes because it was not observed in controls. The present data likely indicate that enzymatic activity, such as 17-steroid dehydrogenase, can induce rapid transformations (i.e., conversion of the 17β-hydroxy to a 17-keto) of 5% to 25% of the dosed mass over sub–2-h time scales; and they are consistent with results we have observed for steroid spikes in field studies [18]. Over the next 3 d, 17β-trenbolone concentrations decreased to 120 ng/L and continued to slowly degrade to 14 ng/L by 15 d, representing a 99% overall metabolite loss. Trendione concentrations were 220 ng/L, a 16% yield, after 1 d and decreased to 34 ng/L by 15 d.

In biologically active samples spiked with 1400 ng/L trendione, concentrations decreased to 14 ng/L over 15 d (Figure 1C). Conversion to 17β-trenbolone (90 ng/L) occurred within the first day, which then decreased slowly to no-detect concentrations at 15 d. In these samples, 17α-trenbolone was detected only once at 26 ng/L after 10 d of trendione incubation. Together with the 17β-trenbolone data, these observations indicate that steroid transformations are dominated initially by interconversion between 17β-hydroxy and 17-keto species and likely exhibit steric or enzymatic inhibition for the 17-keto to 17α-hydroxy conversion. These observations are consistent with reports for estrogens, where preferences for 17-keto to the 17β isomer instead of the 17α isomer pathway are observed, and agree with trenbolone acetate metabolite data for soils [19,29].

Because trendione conversion to 17α-trenbolone or 17β-trenbolone represents C17 reduction, these reactions should be inhibited by aerobic conditions, whereas the reverse reaction (converting 17α-trenbolone or 17β-trenbolone to trendione) might be favored by aerobic conditions. When focusing on aerobic biotransformation of trenbolone acetate metabolites in agricultural soils, Khan et al. [19] reported

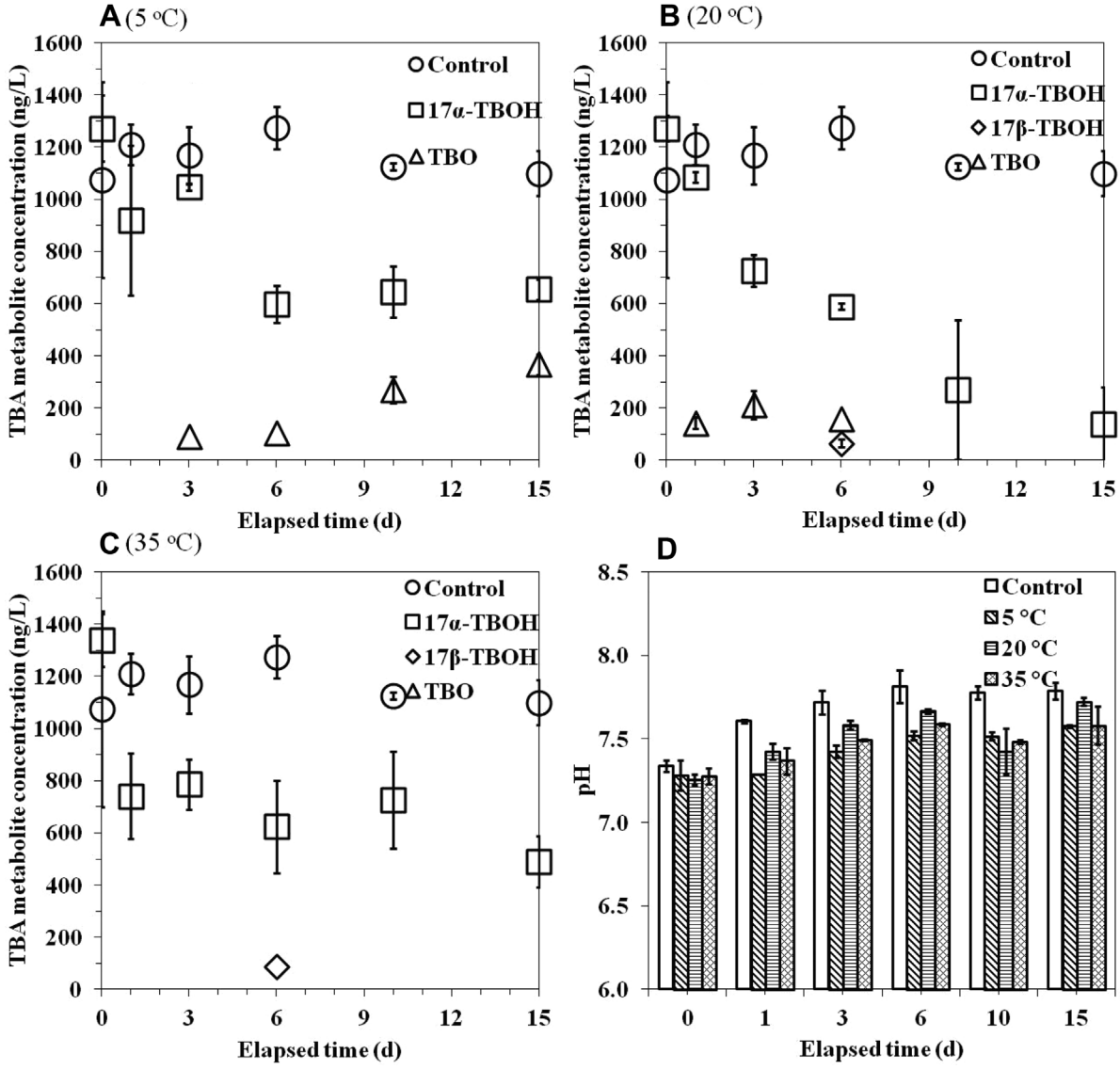

Figure 2. Aerobic biodegradation of 17α-trenbolone with January inocula at (**A**) 5 °C, (**B**) 20 °C, and (**C**) 35 °C. Hollow symbols represent sterile control samples (autoclaved). All samples were spiked to a concentration of 1400 ng/L. (**D**) pH data corresponding to (**A**) through (**C**). Error bars represent standard deviations of triplicate samples. TBA = trenbolone acetate; TBO = trendione; TBOH = trenbolone.

conversion of 17α-trenbolone and 17β-trenbolone into trendione at approximately 40% and 60% yields, respectively. Consistent with these expectations, although the differences were small, less 17α-trenbolone and 17β-trenbolone persisted in microcosms spiked with trendione compared with the trendione concentrations observed after spiking with 17α-trenbolone and 17β-trenbolone (Figure 1). Relative to published soil data, the lower maximum trendione yield observed (16%) implies that the aqueous conditions or inoculum used likely enhanced subsequent trendione transformation to uncharacterized products.

Experimental conditions

In addition to a number of preliminary trials, select experiments were repeated using inocula collected during different seasons or from different source waters. For example, 17α-trenbolone experiments were repeated with Steamboat Creek inocula collected in January. Biologically active microcosms with this inocula at 20 °C exhibited a 17α-trenbolone decrease to 140 ng/L (90% loss) over 15 d (Figure 2). Maximum trendione concentrations were 210 ng/L, a 12% yield, whereas 17β-trenbolone was detected only once, at 6 d (65 ng/L). The present data indicate slower 17α-trenbolone transformation with January inocula, but similar trends in overall loss and metabolite interconversion were evident between all inocula collected from this source.

Incubation temperature was varied in some microcosms. The January inocula described above also were incubated at either 5 °C or 35 °C. In 5 °C samples, 17α-trenbolone concentrations decreased to 920 ng/L after 1 d and to 600 ng/L over 6 d with no further transformation evident after 6 d (Figure 2A). Trendione was detected at 3 d and steadily increased to 370 ng/L at 15 d, corresponding to a 26% yield, although no corresponding 17α-trenbolone loss was observed. 17β-Trenbolone was not detected, perhaps implying some temperature dependence for the trendione to 17β-trenbolone pathway. In 35 °C samples, 17α-trenbolone concentration rapidly decreased to 740 ng/L after 1 d (Figure 2C), which is statistically similar to the loss observed at 5 °C, then transformation unexpectedly ceased. Trendione was not detected, and 17β-trenbolone was observed only once at low concentration, also consistent with the substantial inhibition of biotransformation at this experimental condition.

Kinetic modeling

All 3 models were evaluated with the data, and their accuracy was determined by comparing R^2 values, which were calculated by subtracting from 1 the ratio of the model's residual sum of squares to the data's total sum of squares. Half-lives are only reported for model 1, and all other model parameters (C_1–C_5 and k_1–k_5) are presented in Table 1.

No trenbolone acetate metabolite exhibited complete removal to no-detect levels in these 15-d experiments, particularly for 17α-trenbolone, the most common trenbolone acetate metabolite, whose concentrations stabilized (i.e., no further transformation was apparent) in many experiments. Biphasic behavior (i.e., variation in kinetic rates with time or concentration) suggests that initial rates observed at higher concentrations may not always accurately represent subsequent data. The pseudo–first order decay model and associated half-life concepts implicitly assume that attenuation rates are independent of the experimental time scale or concentration present, and this assumption can result in overestimation of transformation rates and underestimation of environmental persistence. In addition to inhibitions arising from multiple pools of degradable contaminants, system characteristics such as sorption to particles, oxygen concentrations, and nutrient availability can inhibit biotransformation [37], although we do not believe these factors explain the present data.

The double-exponent model exhibited the best fit, with 2 exceptions: the 35 °C (17α-trenbolone) data set was better estimated by the non-zero asymptote model, and the trendione (20 °C) data set was equally well estimated by all models. Models were evaluated with and without accounting for observed slight losses in controls; this analysis indicated that any control losses were negligible. Despite the goodness-of-fit for the double-exponent model, in some cases the "residual" concentration (C_3 or C_5) was negligible relative to the labile concentration, implying that the standard exponential decay model was appropriate (Table 1). As the double-exponent model often explained the data well, it is reasonable to conclude that slow cometabolic or other biphasic processes can inhibit trenbolone acetate metabolite transformation at low concentrations and sometimes a persistent residual is maintained.

The present data clearly indicate that biotransformation rates exhibit stereoselectivity for steroids. Similar to reported results for 17α-estrogens [38], slower transformation is expected for 17α-trenbolone in particular. Under identical conditions, a 2-fold difference in transformation rates was evident for 17α-trenbolone (0.27 d^{-1}) compared with 17β-trenbolone (0.57 d^{-1}), implying that the most common trenbolone acetate metabolite, 17α-trenbolone, also is the most persistent. With respect to interconversion, because trendione is the only intermediate product for 17α-trenbolone and 17β-trenbolone interconversion, rates of 17-keto reduction should dominate overall rates of transformation between known high-potency trenbolone acetate metabolites, consistent with reported results for other steroids [21,37,39,40]. Inocula collection also affected kinetics, likely because of different microbial community composition and relative activity; kinetic rates for April inocula were nearly twice the values for January inocula (0.27 d^{-1} vs 0.15 d^{-1}). Similarly, slower rates (0.099 d^{-1}) and concentration stabilization were observed during temperature manipulations. The present data suggest approximately 2-fold changes in transformation rates at temperature extremes, implying increased persistence under both colder (winter-like) conditions and warmer (summer-like) conditions, and are consistent with temperature-dependent results for estrogens [29] and trenbolone acetate metabolites in soils [21].

Half-lives in aqueous systems for 17α-trenbolone, 17β-trenbolone, and trendione (2.2–4.1 d, 0.9 d, and 1.3 d, respectively), which likely underestimate trenbolone acetate metabolite persistence, were compared with reported half-lives in soils (0.18–0.33 d, 0.18–0.31 d, and 0.54–2.0 d, respectively) [19]. Although these comparisons are always complicated by differing experimental conditions, in general, dissipation rates in soils were faster than those observed for aqueous systems [19]. This difference may arise from larger,

Table 1. Biotransformation kinetic parameters estimated for models 1, 2, and 3

Trenbolone acetate metabolite	Inocula	Temp. (°C)	C_1 (ng/L)	k_1 (d^{-1})			$t_{1/2}$ (d)	R^2
Model 1: Pseudo–first order								
17α-Trenbolone	January 2013	5	1400	0.081			8.5	0.26
		20	1400	0.17			4.1	0.90
		35	1400	0.099			7.0	0.04
17α-Trenbolone	April 2013	20	1400	0.32			2.2	0.55
17β-Trenbolone			1400	0.78			0.9	0.77
Trendione			1400[a]	0.54[a]			1.3[a]	0.99[a]
Model 2: Non-zero asymptote	Inocula	Temp. (°C)	C_2 (ng/L)	k_2 (day^{-1})	C_3 (ng/L)			R^2
17α-Trenbolone	January 2013	5	750	0.43	650			0.60
		20	1340	0.15	60			0.88
		35	730	2.0	670			0.74
17α-Trenbolone	April 2013	20	1290[a]	0.38[a]	110[a]			0.57[a]
17β-Trenbolone			1220	0.57	180			0.53
Trendione			1380[a]	0.55[a]	20[a]			0.99[a]
Model 3: Double-exponent	Inocula	Temp. (°C)	C_4 (ng/L)	k_4 (day^{-1})	C_5 (ng/L)	k_5 (day^{-1})		R^2
17α-Trenbolone	January 2013	5	400[a]	11[a]	1000[a]	0.036[a]		0.64[a]
		20	70[a]	8.6[a]	1330[a]	0.16[a]		0.91[a]
		35	590[a]	2600[a]	810[a]	0.025[a]		0.79[a]
17α-Trenbolone	April 2013	20	1330	0.32	70	0.32		0.55
17β-Trenbolone			1400[a]	0.78[a]	0.2[a]	0.015[a]		0.77[a]
Trendione			1360[a]	0.56[a]	40[a]	0.12[a]		0.99[a]

[a]These parameters reflect the best-fit model for a given data set.

C_1 = initial metabolite concentration; C_2 = labile metabolite concentration; C_3 = residual recalcitrant concentration; C_4 = concentration amenable to rapid transformation; C_5 = concentration amenable to slow transformation; k_1, k_2, k_4, k_5 = corresponding first-order rate constant for the different models; $t_{1/2}$ = half-life.

more diverse, and more active microbial communities in soils facilitating transformation and the contributions of sorption and abiotic mineral-promoted transformation to dissipation rates in soils. Approximately 20% of any steroid mass would typically remain dissolved in a typical soil–water subsurface system [20], and the higher soil transformation rates suggest that infiltration of agricultural runoff can be an effective mechanism for enhancing attenuation rates.

Transformation products

To characterize the long-term fate of trenbolone acetate metabolites, a major objective of the present experiments was to investigate biotransformation products, as structural conservation during transformation also implies the potential conservation of bioactivity. Therefore, focusing largely on 17α-trenbolone and trendione, we investigated the presence of novel biotransformation products and examined any effects of inocula source on products. To facilitate the identification of potential low-yield products, some microcosms were spiked at higher concentrations (up to 20 μg/L), although solid-phase extraction, which concentrated solutions by approximately 35-fold, was sometimes used to facilitate detection. Also, without available standards for products and because of uncertainties arising from matrix and structural effects on ionization efficiency, this analysis was not considered quantitative but qualitative only [27]. Because we cannot estimate yields, we largely attempted to verify the presence of biotransformation products and comparisons of concentration as a function of time were limited to single products.

Consistent with expectations and published data, interconversions between 17α-trenbolone, trendione, and 17β-trenbolone accounted for most of the detectable transformation products (Table 2). For 17α-trenbolone and 17β-trenbolone microcosms, trendione was usually the dominant detectable product. However, other transformation products were evident, particularly mass-to-charge ratio (*m/z*) – 2-Da products consistent with microbially mediated dehydrogenation. For example, in an *m/z* 269 scan (consistent with trendione formation at *m/z* 269.1530) of a 17α-trenbolone microcosm, we see not only the expected formation of trendione at 9.15 min but also the formation of 3 other *m/z* 269 peaks in the chromatogram, including the dominant (by area count) 6.88-min peak, whose MS/MS spectra also were all consistent with trendione fragmentation (Figure 3). Their mass accuracy and conserved MS/MS fragments indicate that all of these peaks were 17α-trenbolone transformation products. We note that with this analysis we cannot differentiate *m/z* 269 $[M+H]^+$ ions from the $[M+H\text{-}H_2O]^+$ ions expected for *m/z* 285 monohydroxy products, although relative retention times (an indication of polarity) can be used to facilitate product assignments, with earlier elution times expected for hydroxy products [27].

By analogy to testosterone biotransformation products observed using manure-borne bacteria under aerobic conditions, where *m/z* 288 testosterone eluted at 9.02 min and the more polar *m/z* 286 1,2-dehydrotestosterone product eluted at 7.82 min [31], we propose that the major 6.88-min peak (1.02 min before 17α-trenbolone) is an $[M+H]^+$ ion representing a 17α-trenbolone 1,2-dehydrogenation product. Although it is impossible to conclusively identify the structure without a standard or nuclear magnetic resonance analysis, 1,2-dehydrogenation of 3-keto steroids mediated by 1,2-dehydrogenase is a common microbial transformation pathway observed in aquatic

Table 2. Characteristics of proposed biotransformation products of 17α-trenbolone, 17β-trenbolone, and trendione

Compound	Retention time (min)	Proposed product ion	Observed mass (Da)	Exact mass (Da)	Comments
17α-Trenbolone (parent)	7.90	$[M+H]^+$	271.1684	271.1698	Orbitrap mass calibration consistently had a low bias of ~0.0014 Da for accurate mass assessment
Trendione	9.15	$[M+H]^+$	269.1528	269.1542	Confirmed by pure standard, strong literature support; preferred product
Trendione analog or dehydrogenation product	9.23	$[M+H]^+$	269.1528	269.1542	Unknown coeluting trendione analog or 17α-trenbolone dehydrogenation product
Dehydrogenation product	6.88	$[M+H]^+$	269.1528	269.1542	Potential dehydrogenation product
Monohydroxytrendione product	5.29	$[M+H\text{-}H_2O]^+$	269.1528	269.1542 or 285.1491	We propose a monohydroxy structure of the dehydrogenation product above or a monohydroxytrendione product
Coeluting hydroxylated products	Near 3–4	$[M+H\text{-}H_2O]^+$	271.1684	287.1647	A family of early eluting, presumably mono- or, more likely, dihydroxylated products
17β-Trenbolone (parent)	8.15	$[M+H]^+$	271.1684	271.1698	
Trendione	9.15	$[M+H]^+$	269.1528	269.1542	Preferred product
Dehydrogenation product	6.82	$[M+H]^+$	269.1528	269.1542	Potential dehydrogenation product
Monohydroxytrendione product	5.09	$[M+H\text{-}H_2O]^+$	269.1528	269.1542 or 285.1491	Analogous product to 17α-trenbolone products above
Trendione (parent)	11.05	$[M+H]^+$	269.1528	269.1542	These analyses used a slower chromatography gradient for improved separation
17β-Trenbolone	9.15	$[M+H]^+$	271.1684	271.1698	Preferred product
17α-Trenbolone	8.75	$[M+H]^+$	271.1684	271.1698	Minor relative to 17β-trenbolone
Trenbolone dehydrogenation product	10.05	$[M+H]^+$	267.1371	267.1385	Dehydrogenation product, possible 1,2- or 6,7- or 15,16-dehydro location; preferred and most persistent dehydro product of trendione
Trenbolone dehydrogenation product	6.75	$[M+H\text{-}H_2O]^+$	267.1371	267.1385	Dehydrogenation product, possible 1,2- or 6,7- or 15,16-dehydro location
Trenbolone dehydrogenation product	13.64	$[M+H]^+$	267.1371	267.1385	Dehydrogenation product, possible 1,2- or 6,7- or 15,16-dehydro location
17β-Trenbolone dehydrogenation product	8.07	$[M+H]^+$	269.1528	269.1542	Potential secondary dehydro product of 17β-trenbolone formed from trendione
17β-Trenbolone dehydrogenation product	5.90	$[M+H\text{-}H_2O]^+$	269.1528	269.1542	Potential secondary dehydro product of 17β-trenbolone formed from trendione

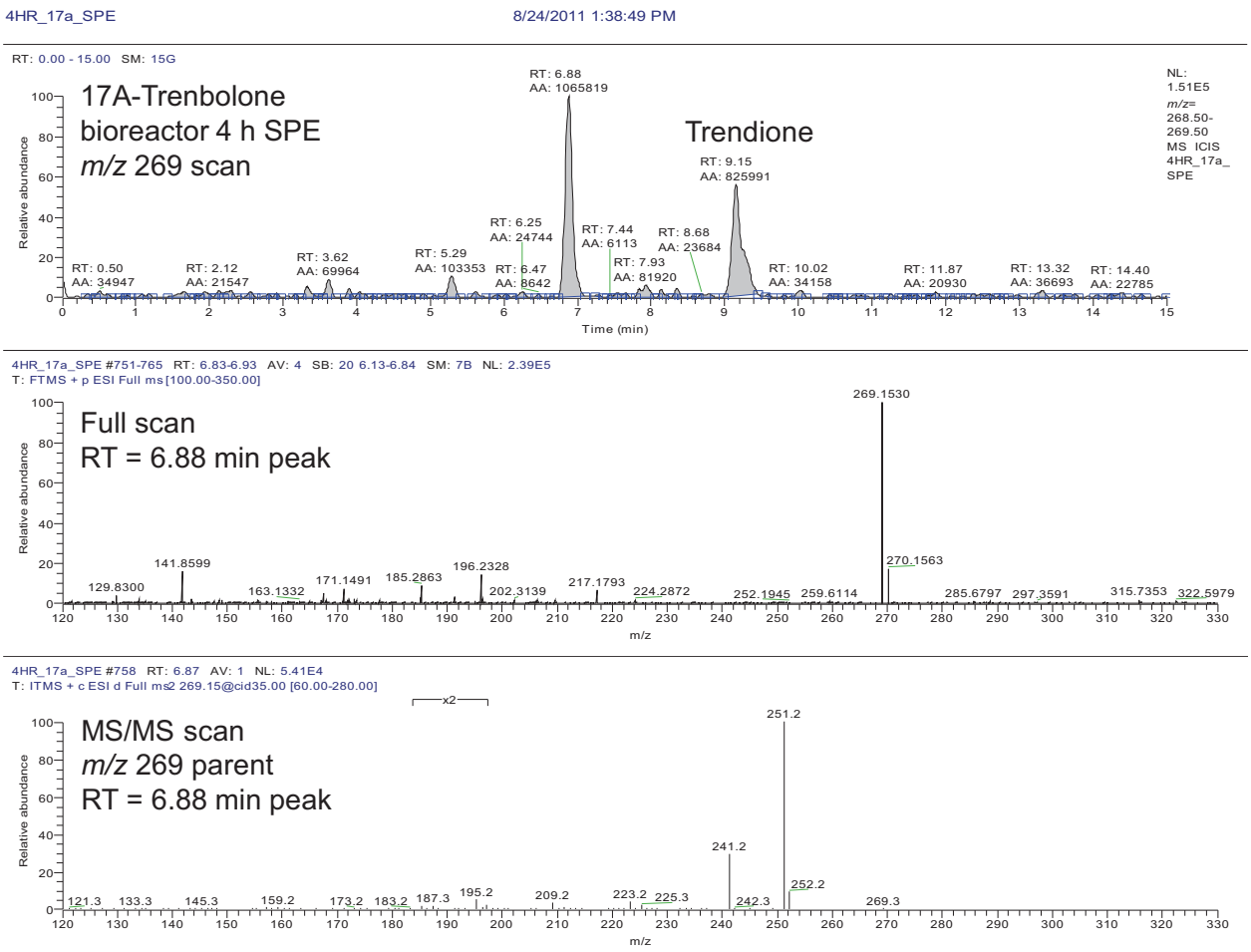

Figure 3. Representative liquid chromatogram, high-resolution mass spectrometric full-scan mass spectrum, and tandem mass spectrometric spectra for a solid-phase extraction extract from a biologically active 5000 ng/L 17α-trenbolone microcosm after 4 h of incubation. The peak at 9.15 min is trendione and consistent with the expected mass-to-charge ratio of 269.1530 mass, although we propose that the dominant peak at 6.88 min represents a $[M+H]^+$ dehydrogenation product of 17α-trenbolone. In addition, we propose that the more polar 5.29-min peak represents a monohydroxy-dehydrogenation product ($[M+H-H_2O]^+$ ion), and we also note the presence of a product coeluting with trendione at 9.23 min. *m/z* = mass-to-charge ratio; MS/MS = tandem mass spectrometry; RT = retention time; SPE = solid-phase extraction.

environments and is consistent with expectations based on steroid metabolism [16,31,41]. Other possibilities include 6,7- or 15,16-dehydrogenation [16]. As a result of its earlier elution time and because the *m/z* 269 product at 5.29 min often grows with time as later eluting *m/z* 269 products decrease, we propose that the 5.29-min peak is an $[M+H-H_2O]^+$ ion of a monohydroxy product of either trendione or the presumptive 1,2-dehydrotrenbolone product. In conjunction with the formation of electrophilic moieties resulting from 1,2-dehydrogenation or 6,7-dehydrogenation, subsequent hydroxylation in the A or B ring is most reasonable. We also note that mass spectra for these *m/z* 269 products are more similar to results reported for A ring– and B ring–hydroxylated metabolites of 17β-trenbolone and unlike the D ring–hydroxylated metabolites reported by Pottier et al. [42] for in vivo metabolites in cattle. Although all 3 of these unknown *m/z* 269 products exhibit similar MS/MS fragments as trendione, relative abundances of MS/MS fragments are substantially different from those observed for trendione (Figure 4). In particular, the 9.23-min peak observed to coelute with trendione may be a structural analog or isomer forming from trendione or may represent yet another dehydrogenation product of 17α-trenbolone of similar polarity to trendione.

Because dehydration of monohydroxy photoproducts of trenbolone is possible to elicit product-to-parent reversion [28], we also compared retention times and MS/MS spectra of these biotransformation products to those observed for monohydroxytrendione photoproducts. This analysis suggests that any biotransformation products were distinct from presumptive 5-hydroxytrendione, 10-hydroxytrendione, or 12-hydroxytrendione photoproducts capable of reversion; and no evidence of product-to-parent reversion was observed for these biotransformation products. Besides the *m/z* 269 products, other 17α-trenbolone products observed include a family of coeluting *m/z* 287 monohydroxy ($[M+H]^+$ ions) or dihydroxy ($[M+H-H_2O]^+$ ion) products, present in most samples near 3 min to 4 min. With the exception of the *m/z* 269 peak coeluting with trendione, similar biotransformation products were observed for 17β-trenbolone; and these product characterizations also were consistent with a number of similar 17α-trenbolone biotransformation experiments conducted at several different concentrations with different inocula sources, including those reported below (Supplemental Data, Figure S2).

To improve separation, subsequent LC-HRMS/MS analyses employed a slower chromatography gradient, lengthening elution times by approximately 1.8 min for trendione. This analysis was used for 17α-trenbolone and trendione microcosms (1400 ng/L, 20 °C) that used 5 different inocula sources, with a focus on trendione biotransformation because, similar to

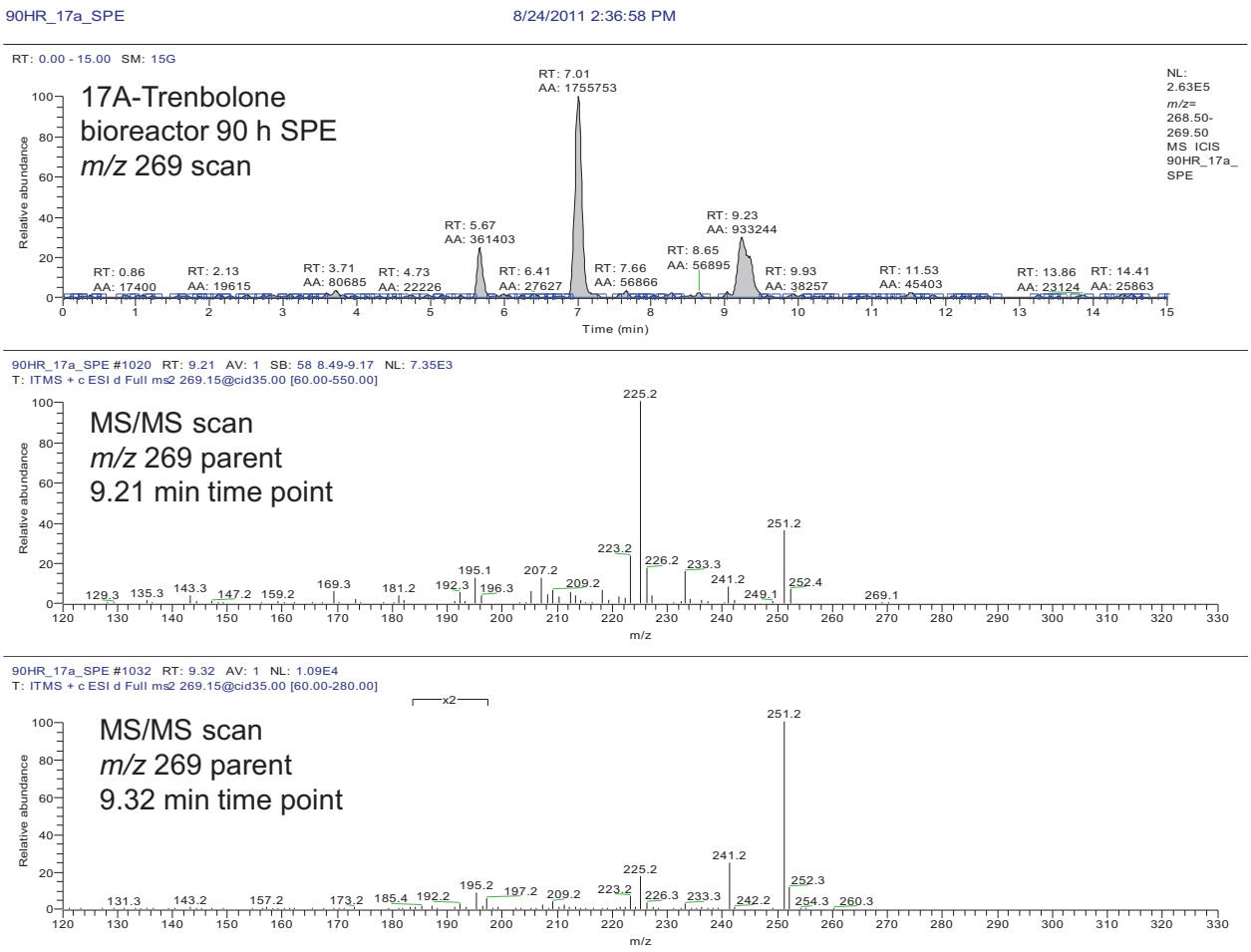

Figure 4. Representative liquid chromatogram and tandem mass spectrometric (MS/MS) spectra for a solid-phase extraction extract from a biologically active 5000 ng/L 17α-trenbolone microcosm after 90 h of incubation. The MS/MS spectra are presented both for the expected trendione product (mass-to charge ratio [*m/z*] 269.1530, 9.21 min MS/MS spectra) and for the coeluting structural analog or 17α-trenbolone dehydrogenation product (*m/z* 269.1530, 9.32 min MS/MS spectra). Note the conservation of MS/MS fragments for both products, although at different abundances. RT = retention time; SPE = solid-phase extraction.

estrone, it probably is the most stable and environmentally relevant long-term steroidal product. Over the 13-d trial period, trendione peak area decreased by only 51% in the least active inocula (Truckee River) microcosms, to decreases of well over 99% in the most active inocula (Truckee Meadows Water Reclamation Facility) composed of mixed liquor from the aeration basins at the regional wastewater-treatment facility. In fact, in the Truckee Meadows Water Reclamation Facility samples, about 50% of the trendione transformed within the initial 2-h solid-phase extraction processing interval, suggesting that very rapid and efficient intrinsic steroid transformation capabilities exist in wastewater-treatment plants.

Consistent with GC-MS/MS results, interconversion to 17β-trenbolone dominated trendione biotransformation, with only limited 17α-trenbolone formation apparent. Usually, 17α-trenbolone peak areas were only 10% to 15% of 17β-trenbolone peak areas (Supplemental Data, Figure S3). For example, after 13 d, Idlewild inocula were dominated by 17β-trenbolone formation, with final 17β-trenbolone peak areas higher than residual trendione peak areas; and similar results were observed for Truckee River inocula samples (Supplemental Data, Figure S2). In these 2 systems, limited overall trenbolone acetate metabolite degradation was evident over the 13 d, with peak areas for trendione and 17β-trenbolone at d 13 accounting for 83% to 96% of initial (0 d) trendione peak area. In contrast, after 2 d in the Truckee Meadows Water Reclamation Facility samples, only trace amounts of any known trenbolone acetate metabolites were evident. Sparks Marina and Steamboat Creek inocula were intermediate between these extremes. Efficient transformation was typical for Steamboat Creek inocula, with $<10\%$ of the initial mass remaining after 13 d. These comparisons suggest that the 1-d to 8-d half-lives estimated for trenbolone acetate metabolites with Steamboat Creek inocula may be among the faster rates expected for typical aerobic receiving waters, although we anticipate that agricultural runoff will be especially microbially active because of its high suspended solids concentration.

Novel trendione biotransformation products were observed with all inocula. In fact, every sample after day 0 contained 2 distinct *m/z* 267.1371 products, 1 at 6.75 min and 1 at 10.05 min, consistent with trendione dehydrogenation and with dehydrogenation products observed for 17α-trenbolone and 17β-trenbolone (Figure 5). We also propose that these products are 1,2-dehydrotrendione species. In fact, after 13 d in the very efficient Truckee Meadows Water Reclamation Facility–inoculated microcosms, these *m/z* 267 dehydrogenation products were still readily detected at similar peak areas to day 5 samples and were the only detectable transformation products. The present data may indicate that steroidal dehydrogenation products are more persistent than any known trenbolone acetate metabolites such as trendione, 17α-trenbolone, or 17β-trenbolone. Similar to results observed for

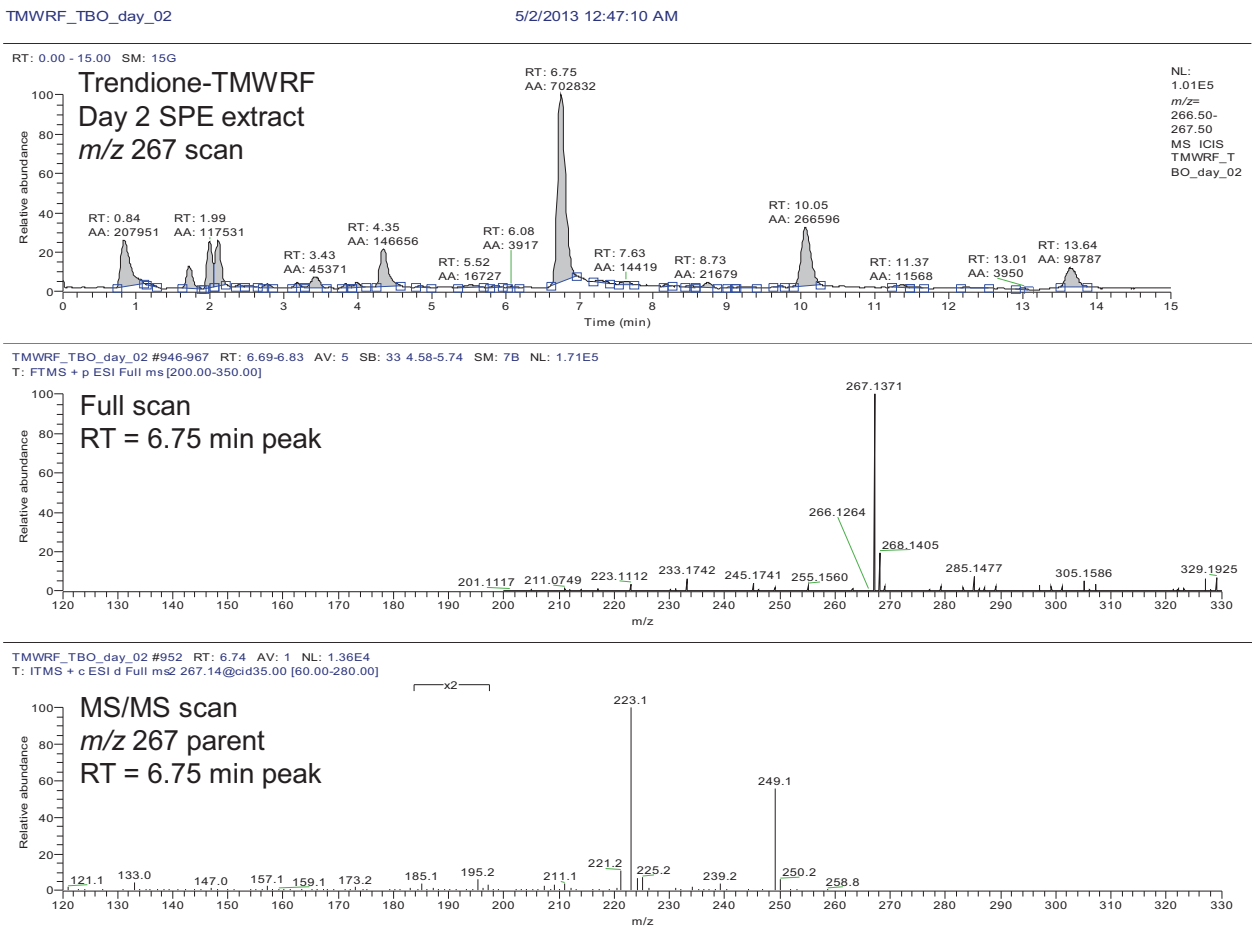

Figure 5. Representative liquid chromatogram, high-resolution mass spectrometric full-scan mass spectrum, and tandem mass spectrometric (MS/MS) spectra for a solid-phase extraction extract from a biologically active 1400 ng/L trendione microcosm after 2 d of incubation. Three mass-to-charge ratio 267.1371 transformation products are observed; we propose that the 10.05-min peak is a $[M+H]^+$ ion for a 1,2-dehydrotrendione product, whereas the 6.75-min peak is a $[M+H\text{-}H_2O]^+$ ion for a monohydroxy species. The final peak at 13.64 min is present in only a few samples and represents another uncharacterized stereoisomer or structural analog. Note the conservation of MS/MS fragments for these products with other trenbolone acetate metabolite products, and all 3 products in the present study exhibited near identical full-scan and MS/MS spectra. *m/z* = mass-to-charge ratio; RT = retention time; SPE = solid-phase extraction; TMWRF = Truckee Meadows Water Reclamation Facility.

17α-trenbolone phototransformation, where a less polar, late eluting *m/z* 271 photoproduct was observed [27], a third *m/z* 267 product was observed at 13.64 min in the Truckee Meadows Water Reclamation Facility samples only, 2.6 min after the trendione parent eluted (Figure 5). Thus, biotransformation also can potentially result in the formation of less polar stereoisomers or structural analogs, although the formation of more polar products was favored. Although numerous rearrangements to less polar structures are possible conceptually [41], the literature provides few clear explanations for products with these characteristics.

Two *m/z* 269.1530 products were detected in some trendione microcosms (Figure 6). In light of the 17α-trenbolone results, we propose that these *m/z* 269 products, 1 at 8.07 min and 1 at 5.90 min, are most consistent with $[M+H]^+$ and $[M+H\text{-}H_2O]^+$ dehydrogenation products of 17β-trenbolone, respectively. These are likely secondary products formed as trendione interconverts primarily to 17β-trenbolone, followed by dehydrogenation. More polar, poorly resolved hydroxylated products also were observed.

Product yield is a major uncertainty in this analysis, and it is certainly possible that these proposed products exist only at minor concentrations in these samples; but this is an issue we cannot easily address without standards. For most detections, peak areas for dehydrogenation products were substantially less than those observed for trenbolone acetate metabolite interconversion products, potentially suggesting low yields, although we note that our limited analyses of pure standards of other similar trenbolone acetate metabolite transformation products have exhibited poor ionization and very low peak areas with this LC-HRMS/MS analysis [27]. Thus, we have no real insight into potential yields of these proposed products.

Environmental implications

Because 17α-trenbolone accounts for 95% of the total excreted trenbolone acetate metabolite mass, it is the most environmentally relevant of the known trenbolone acetate metabolites [10,11,42]. The present study reports higher trenbolone acetate metabolite persistence, with half-lives of a few days for 17α-trenbolone, in aquatic systems relative to studies performed in agricultural soils, where half-lives of only a few hours were reported, although we note that such results can be very system-specific [19]. Soil systems should thus be more efficient at attenuating trenbolone acetate metabolites relative to the higher persistence and reduced transformation rates expected for dark, turbid aquatic systems excepting the efficient transformation expected in the photic zone [28,43]. Comparing the known trenbolone acetate metabolites,

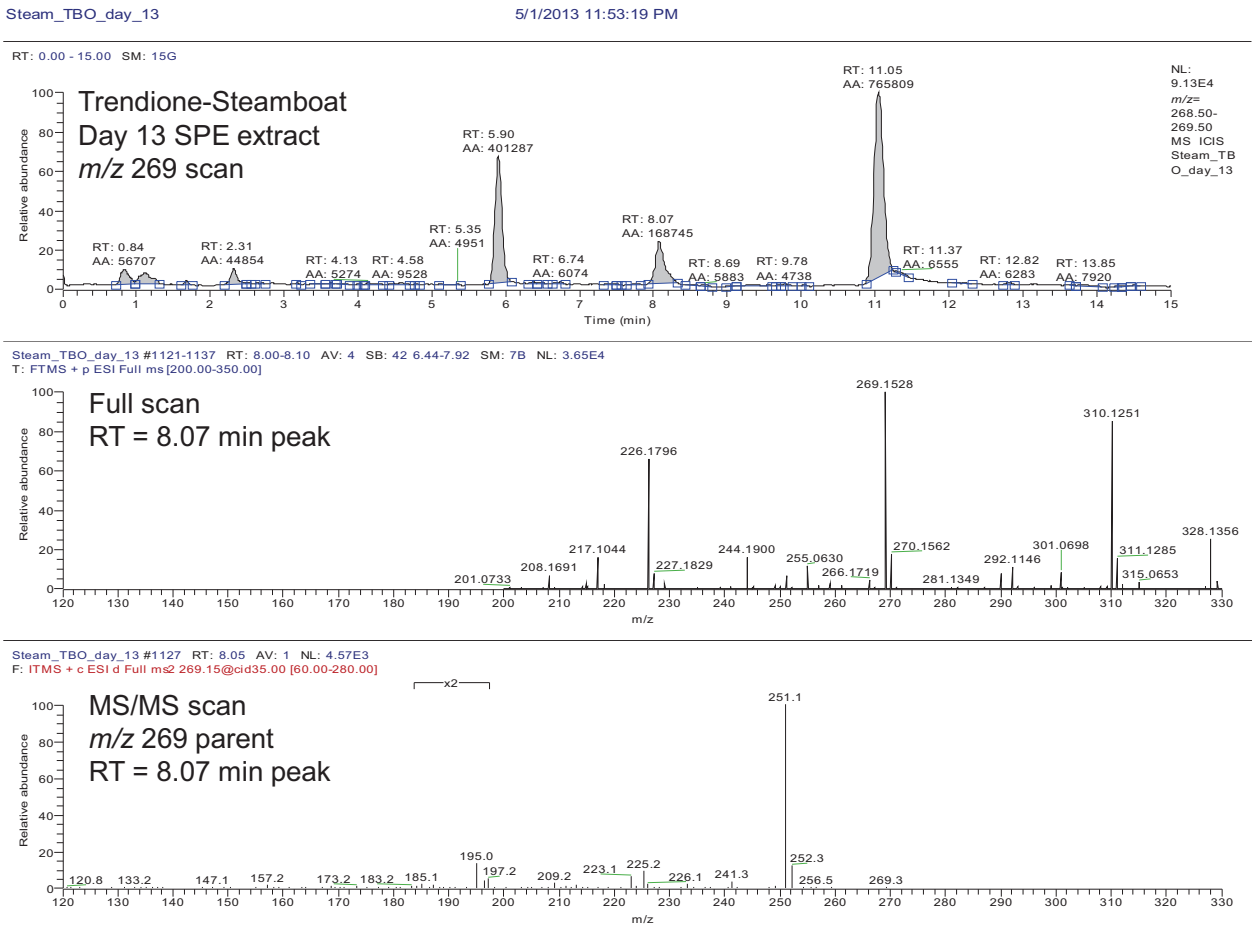

Figure 6. Representative liquid chromatogram, high-resolution mass spectrometric full-scan mass spectrum, and tandem mass spectrometric spectra for a solid-phase extraction extract from a biologically active 1400 ng/L trendione microcosm after 13 d of incubation. With this chromatography gradient, the peak at 11.05 min is trendione, and we propose that the 8.07-min and 5.90-min mass-to-charge ratio 269.1528 peaks are consistent with $[M+H]^+$ and $[M+H\text{-}H_2O]^+$ dehydrogenation products of 17β-trenbolone, respectively. *m/z* = mass-to-charge ratio; MS/MS = tandem mass spectrometry; RT = retention time; SPE = solid-phase extraction.

17α-trenbolone was the most persistent compound; and it is clear that stereochemistry governs transformation rates as relatively small structural differences have unexpectedly large effects on contaminant fate. The present data are consistent with other observations indicating unique fate outcomes for 17α-trenbolone, as receptor binding alone would predict greatly reduced potency for 17α-trenbolone relative to 17β-trenbolone [42], yet unexpectedly similar potency for 17α-trenbolone to 17β-trenbolone is observed in fish exposures [12]. 17α-Trenbolone exhibits far more rapid product-to-parent reversion kinetics from photoproducts [28], and both sorption [20] and biotransformation (the present study) outcomes exhibit substantial stereoselectivity within trenbolone acetate metabolites.

Metabolite interconversion patterns observed in well-studied estrogen systems were usually consistent with the results we observed for trenbolone acetate metabolites. Interconversion pathways for steroids are probably well conserved and widespread, including a preference for C17 ketone to 17β conversion over 17α conversion [29,38]. These pathways arise from the activity of promiscuous, stereospecific enzymes in receiving waters and soils that preferentially discriminate against 17α conversions [16,17] and likely similarly govern the transformations of the entire suite of potential steroidal contaminants in the aquatic environment excepting sterically hindered substrates such as ethinyl estradiol or progestins.

More importantly, although we cannot estimate product yields with our approaches, the present data indicate that a number of closely related transformation products that conserve key steroidal structural attributes should be present in receiving waters (Figure 7). In fact, many published studies imply the conservation of bioactivity, and even potential increases in certain aspects of bioactivity, after transformation of steroids and other pharmaceuticals [12,16,28,29,31]. For example, Yang et al. [31] documented the formation of 1,2-dehydrotestosterone, also known as boldenone, on the incubation of testosterone with manure-borne bacteria. This transformation would actually be expected to conserve and even increase bioactivity [44], as there exist many transformations that can retain and even increase certain aspects of bioactivity in steroids [45]. Thus, proposed metabolic pathways such as that presented in the environmental risk assessment for trenbolone which demonstrate hydrogenation and dehydrogenation products [41] should really be interpreted more accurately in the context that these structural modifications do not necessarily reduce overall mixture bioactivity.

Observation of conserved structure may be particularly important when considering the ecological relevance of exposure to mixtures of closely related contaminants that retain key functional groups and may play an important role in resolving any discrepancies observed between concurrent use of chemical analysis and bioanalytical approaches for sample

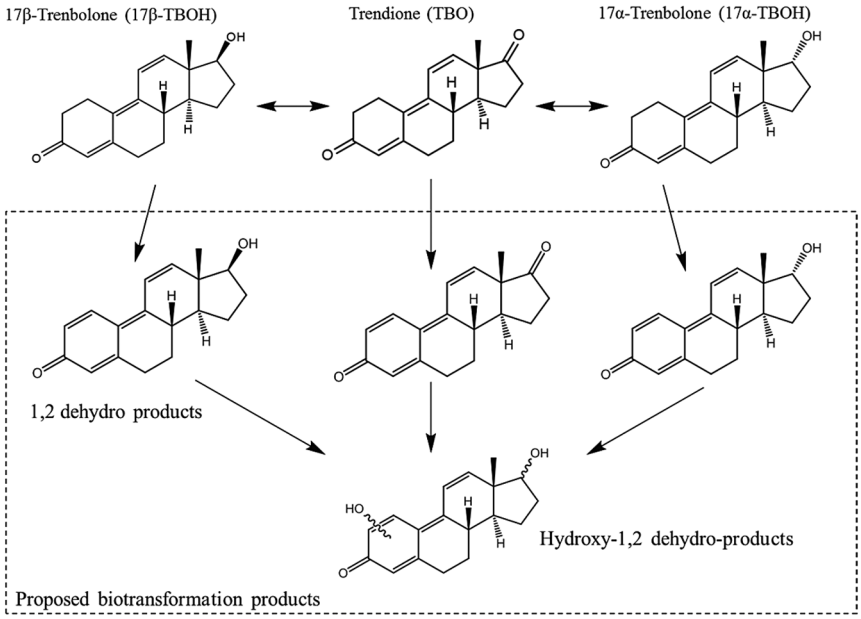

Figure 7. Structures of proposed microbial biotransformation products of trenbolone acetate metabolites. Although we propose 1,2-dehydrogenation products and A-ring hydroxylation in the present study, other configurations and isomers also are possible.

analysis [46]. Fractionation approaches such as toxicity identification evaluation used to identify bioactive constituents in complex mixtures can fail to identify causative agents [47] and are limited by the necessity for sensitive analytical methods developed around the availability of pure standards. Ecological risk and environmental hazard arise not only from contaminant persistence but also from the relationships between potency and yields in both parents and products, and we can expect that chemical analyses in isolation will often struggle to fully define these relationships. The best holistic approach to quantifying ecological risks would thus employ bioanalytical tools to improve the characterization of linkages between exposure, structure, and bioactivity in contaminants and their products.

SUPPLEMENTAL DATA

Figures S1–S3. (424 KB DOC).

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Data availability—For researchers interested in any data associated with the present study, please contact E. Kolodziej at kolodziej@uw.edu.

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