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Effect of Agricultural Antibiotics on the Persistence and Transformation of 17β-Estradiol in a Sequatchie Loam

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A laboratory incubation study was conducted to investigate the effect of agricultural antibiotics (sulfamethazine, tylosin, and chlortetracycline) on the persistence and transformation of 17β-estradiol in Sequatchie loam. We measured concentrations of 17β-estradiol and its primary metabolite (estrone) in soils spiked with antibiotics and 17β-estradiol. Dehydrogenase activity (DHA) was also measured as an indicator of the total microbial activity of the soils. The presence of antibiotics significantly decreased transformation of 17β-estradiol to estrone. There was a positive correlation between the DHA and the concentrations of estrone in soil spiked with 17β-estradiol only, implying that the reaction is mainly catalyzed by dehydrogenases. However, the positive correlation was weakened in soil spiked with 17β-estradiol and antibiotics together. We recommend that any study evaluating the fate and transport of estrogenic hormones in soil should include the effect of agricultural antibiotics because antibiotics and estrogenic hormones are commonly excreted together in environmental samples.

Key Words: 17β-Estradiol; Agricultural antibiotics; Persistence; Dehydrogenase activity.

INTRODUCTION

Endocrine-disrupting chemicals (EDCs), including steroid estrogens, can induce adverse health effects by disrupting the endocrine system of an organism

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or normal development in vivo[1] at extremely low concentrations, i.e., less than 1 ng L^{-1}.[2–4] This finding has recently intensified interest in their clinical measurement, primarily with respect to concerns regarding their roles in breast, ovarian, and possibly prostate cancer.[5,6] Because the potential risk posed by steroid estrogens is unclear, we need to better understand the environmental fate of these compounds.

The U.S. Geological Survey reported that steroid estrogens were frequently detected in 139 streams of 30 U.S. states in 1999 and 2000.[7] One of the sources of these environmental steroid estrogens is animal manure: these compounds are not only produced naturally by animals but also administered as growth promoters to increase the efficiency of feed use by livestock. The estimated concentrations of 17β-estradiol and estrone in manure ranged from below detectable limits to 1215 and 4728 μg kg^{-1}, respectively,[8] and cows and pigs are estimated to emit far more of these compounds than do humans.[9]

Colucci, Bork, and Topp[10] reported a rapid dissipation of 17β-estradiol in agricultural soils, and 17β-estradiol was transformed to its metabolites that have similar structures, such as estrone. Rapid transformation of 17β-estradiol (C-17 alcohol) to estrone (C-17 ketone) by oxidation has been frequently reported in environmental studies,[11–13] and it has been suggested that the reaction is mostly catalyzed by bacterial or fungal dehydrogenases.[14,15] However, mineralization of 17β-estradiol in soils was relatively slow:[10] only 12–17% of 1 mg kg^{-1} ^{14}C-17β-estradiol in loam, sandy loam, and silt loam was released as ^{14}C-CO_2 in three months. Previous studies have highlighted microbial effects on the persistence and transformation of 17β-estradiol in environmental samples.

In addition to concerns about steroid estrogens, the impact of agricultural antibiotics is also a major environmental issue because of the potential for the development of antibiotic resistance in exposed microbes.[16,17] Most livestock feedlots typically use large amounts of antibiotics for both disease prophylaxis and growth promotion. Producers in the United States are reported to administer the greatest amount of antibiotics to farm animals annually, 8×10^6 kg.[18] Therefore, antibiotics and steroid estrogens often occur as co-contaminants in ecosystems from animal production operations. Shore et al.[19] conducted an incubation study at different pH values, with and without added antibiotics (penicillin and streptomycin), to measure factors affecting persistence of testosterone in poultry litter. They found increased persistence when antibiotics were added and significant changes in the testosterone concentrations at pH 5 and 7, but no changes at pH 1 and 12. In our previous study, we found that 17β-estradiol changed total microbial biomass and their microbial community structure in soil, and the presence of antibiotics nullified the effect of 17β-estradiol on microbial community in soil.[20] Limited information is currently available regarding the effect of antibiotics on the persistence of estrogenic hormones in soil/water systems.

The main objective of this study was to investigate the effect of agricultural antibiotics on the persistence and transformation of 17β-estradiol in soil. We measured the concentrations of 17β-estradiol and its primary metabolite, estrone, in soils spiked with three different types of agricultural antibiotics and 17β-estradiol. We also measured the dehydrogenase activity (DHA) of the soils as an indicator of total microbial activity and evaluated the correlation between the microbial activity and the persistence and transformation of 17β-estradiol.

MATERIALS AND METHODS

A laboratory study was conducted using Sequatchie loam (fine-loamy, siliceous, semiactive, thermic humic Hapludults) obtained from the Agricultural Experiment Station at the University of Tennessee (Knoxville, TN). The chemical properties of the soil were pH, 6.9; total carbon, 7.9 mg kg^{-1}; total nitrate, 1.1 mg kg^{-1}. The soil was ground to pass through a 2-mm sieve and stored at -5°C.

Three different antibiotics (sulfamethazine, SFT; tylosin, TYS; chlortetracycline, CHTC), 17β-estradiol, and estrone were purchased from Sigma-Aldrich (St. Louis, MO) (Fig. 1). Stock solutions, 200 and 20,000 mg L^{-1} of each antibiotic and 200 mg L^{-1} of 17β-estradiol, were made in 70% methanol (70% methanol: 30% water, v/v).

The experimental treatments in this study were (1) 2 mg kg^{-1} 17β-estradiol (control), (2) 2 mg kg^{-1} SFT + 17β-estradiol, (3) 200 mg kg^{-1} SFT + 17β-estradiol, (4) 2 mg kg^{-1} TYS + 17β-estradiol, (5) 200 mg kg^{-1} TYS + 17β-estradiol, (6) 2 mg kg^{-1} CHTC + 17β-estradiol, and (7) 200 mg kg^{-1} CHTC + 17β-estradiol. For each treatment, 10 g of nonsterile soil was transferred to a 40-mL amber glass vial and supplemented with 100 μL of each antibiotic stock solution to make 2 and 200 mg kg^{-1} of antibiotic concentration. To the control soil, 100 μL of 70% methanol was added to account for the carbon energy value of other treatments. The moisture content of each soil sample was maintained at 70% of field capacity, 0.3 bar. Soil samples were pre-incubated for two days for equilibration. After the pre-incubation, 100 μL of 17β-estradiol stock solution was added to each sample and incubated for seven days at 37°C. The concentrations of 17β-estradiol and estrone were measured at days 1, 2, and 7 ($n = 3$).

For the analyses of 17β-estradiol and estrone, the soil sample was mixed with 25 mL of diethyl ether using a vortex for 5 min, and the mixture was centrifuged at 1000 g for 20 min. Ten mL of the extraction was collected from the ether layer and filtered through glass wool packed in the bottom of a pipette. The solution was dried under a stream of nitrogen gas at 37°C. All the extracted and dried samples for the analyses of 17β-estradiol and estrone were re-dissolved in 1 mL of a 1:1 (v/v) mixture of methanol and a mobile-phase (30% water: 70% acetonitrile, v/v). High-performance liquid chromatography (Hewlett-Packard 1100 system), coupled with electrospray and tandem mass spectrometry (PE Sciex API 365, Concord, ON, Canada), was used to measure the concentrations

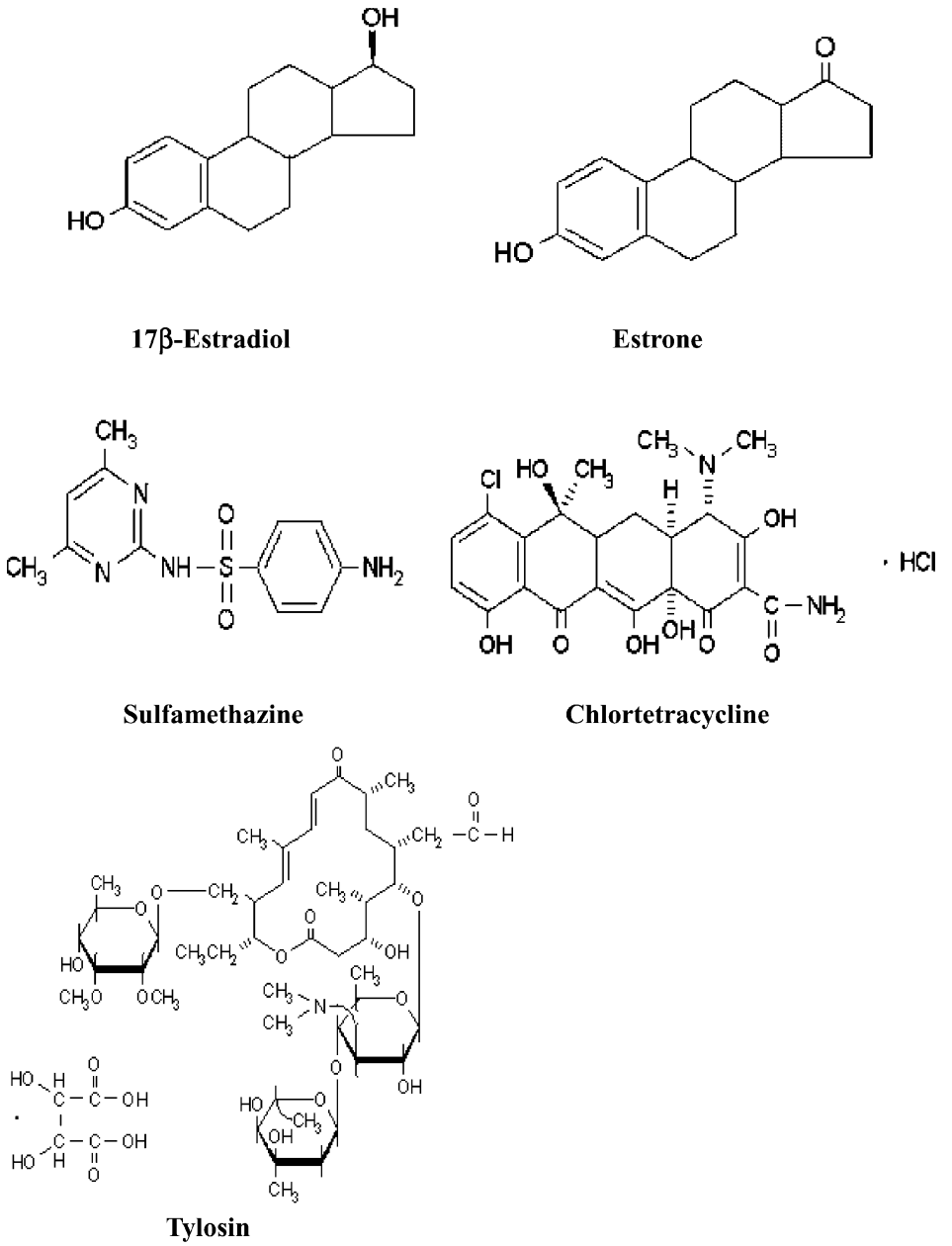

Figure 1: Structural formulas of 17β-estradiol, estrone, sulfamethazine, tylosin, and chlortetracycline.

of 17β-estradiol and estrone. The amounts of 17β-estradiol and estrone were determined based on the detection of their molecular ions (Q1) at *m/z* 271 and *m/z* 269, respectively, and their characteristic fragment ions (Q3) at *m/z* 183 and *m/z* 145, respectively. The method detection limits based on the ratio of

signal to noise (S/N $\geq$ 10/1) for the 200 μL of 5 mg L^{-1} standard solution of estrogens were 0.025 and 0.010 mg L^{-1} for 17β-estradiol and estrone, respectively. Good linearity of the standard calibration curves was obtained for concentrations from 0.025 to 2.0 mg L^{-1} for 17β-estradiol (R^2, 0.996) and estrone (R^2, 0.999).

Analysis of DHA is a sensitive method that responds quickly to induced changes in total microbial activity in soil[21,22] because it is linked to microbial oxidation of organic substances in the active microbial biomass.[23] In this study, the DHA was a particularly useful indicator of the effect of antibiotics on microbial activity and the microbial effect of the transformation of 17β-estradiol to estrone because transformation of 17β-estradiol occurs via the oxidation and reduction that are linked to DHA.[22] Dehydrogenase activity was measured by Tabatabai's method.[24] Briefly, the amount of DHA was determined using triphenyl tetrazolium chloride as an artificial electron acceptor; it was reduced to a red product, triphenyl formazan, by the microbial enzyme activity. The sample was extracted with ethanol, and the amount of triphenyl formazan produced was determined colorimetrically by absorbance at 485 nm.

Statistical significance ($Pr < 0.05$ probability level) among the treatments for each incubation day was evaluated using analysis of variance (ANOVA) and Tukey's multiple range test.[25] Pearson's correlation between the DHA and the concentration of estrone was determined[25] for each DHA value and the extractable estrone concentration (mean of three replicates), for the one-, two-, and seven-day incubations, in order to evaluate the effect of antibiotics on the correlation between the DHA and the transformation of 17β-estradiol to estrone in the soils.

RESULTS AND DISCUSSION

Table 1 shows the concentrations of 17β-estradiol and estrone in soil spiked with antibiotics and 17β-estradiol. In our preliminary study using both sterile and nonsterile soils (data not shown), mass recovery of 17β-estradiol during seven days of incubation ranged from 1 to 14%. The result is consistent with previous studies. Colucci, Bork, and Topp[10] reported that 17β-estradiol is transformed rapidly in soil and less than 30% of 17β-estradiol in loam was extracted following three days of incubation. The large octanol-water partition coefficient of 17β-estradiol, log K_{oc} 2.6–4.0, indicates a high sorption affinity to soil particles.[26,27] Casey et al.[28] reported less than 20% of extractable ^{14}C labeled 17β-estradiol in their batch and column experiments and found good correlation between 17β-estradiol sorption and soil surface area and cation exchange capacity. They also reported that the transport of steroid estrogens in soils is complicated due to simultaneous nonequilibrium transport and transformations.

Table 1: Concentrations of 17β-estradiol and estrone in Sequatchie loam spiked with antibiotics (sulfamethazine, SFT; tylosin, TYS; chlortetracycline, CHTC) and 17β-estradiol. The initial concentration of 17β-estradiol was 2 mg kg^{-1} ($n = 3$).

	Incubation day		
Treatment	**Day 1**	**Day 2**	**Day 7**
	17β-Estradiol (mg kg^{-1})		
17β-Estradiol (control)	0.172 ± 0.161^{a†}	0.137 ± 0.008^{a}	0.020 ± 0.017^{ab}
2 mg kg^{-1} SFT + 17β-estradiol	0.197 ± 0.005^{a}	0.083 ± 0.018^{bc}	0.011 ± 0.008^{ab}
200 mg kg^{-1} SFT + 17β-estradiol	0.203 ± 0.173^{a}	0.003 ± 0.001^{c}	0.002 ± 0.001^{b}
2 mg kg^{-1} TYS + 17β-estradiol	0.102 ± 0.050^{b}	0.107 ± 0.015^{b}	0.034 ± 0.002^{ab}
200 mg kg^{-1} TYS + 17β-estradiol	0.066 ± 0.025^{b}	0.038 ± 0.013^{bc}	0.023 ± 0.015^{ab}
2 mg kg^{-1} CHTC + 17β-estradiol	0.189 ± 0.109^{a}	0.101 ± 0.015^{b}	0.024 ± 0.007^{ab}
200 mg kg^{-1} CHTC + 17β-estradiol	0.220 ± 0.060^{a}	0.096 ± 0.080^{c}	0.045 ± 0.015^{a}
	Estrone (mg kg^{-1})		
17β-Estradiol (control)	0.286 ± 0.159^{a}	0.199 ± 0.046^{a}	0.099 ± 0.087^{a}
2 mg kg^{-1} SFT + 17β-estradiol	0.112 ± 0.019^{b}	0.104 ± 0.032^{ab}	0.065 ± 0.005^{b}
200 mg kg^{-1} SFT + 17β-estradiol	0.063 ± 0.050^{b}	0.006 ± 0.061^{b}	0.005 ± 0.004^{c}
2 mg kg^{-1} TYS + 17β-estradiol	0.148 ± 0.017^{ab}	0.075 ± 0.061^{ab}	0.073 ± 0.007^{b}
200 mg kg^{-1} TYS + 17β-estradiol	0.129 ± 0.048^{b}	0.112 ± 0.016^{b}	0.064 ± 0.019^{b}
2 mg kg^{-1} CHTC + 17β-estradiol	0.092 ± 0.046^{b}	0.140 ± 0.020^{ab}	0.065 ± 0.008^{b}
200 mg kg^{-1} CHTC + 17β-estradiol	0.077 ± 0.036^{b}	0.126 ± 0.016^{ab}	0.087 ± 0.003^{b}

†Within each column for the same incubation day, values with same letter are not significantly different at the 0.05 probability level by Tukey's multiple range test.

Rapid transformation of 17β-estradiol to estrone occurred in all the treatments, even with the addition of antibiotics. Concentrations of estrone were higher than concentrations of 17β-estradiol in many of the treatments for all three sampling days. For example, concentration of estrone in control was approximately 1.7 times higher than 17β-estradiol after one-day incubation. Note that total mass recovery of 17β-estradiol and estrone in the soil was 23% in this study.

It is noteworthy that the presence of sulfamethazine, tylosin, and chlortetracycline significantly reduced the transformation of 17β-estradiol in many of treatments. The reduced transformation is possibly due to the decreased oxidation of 17β-estradiol. The concentrations of estrone in the control (17β-estradiol only) and 200 mg kg^{-1} SFT + 17β-estradiol were 0.099 and 0.005 mg kg^{-1}, respectively.

Table 2 shows the DHA for Sequatchie loam spiked with antibiotics and 17β-estradiol. Antibiotics significantly altered DHA in most of the treatments. The DHA during pre-incubation (day^{-1}) and the total DHA were significantly reduced in the presence of all the antibiotics, except for the total DHA with TYS at 200 mg kg^{-1}. The peak values of DHA in each treatment during the seven days of incubation ranged from 71 to 553 mg triphenyl formazan kg^{-1}day^{-1}, and the peak values for antibiotics treatments were lower than that of the control.

Table 2: Analysis of dehydrogenase activity (DHA) in Sequatchie loam spiked with 2 and 200 mg kg^{-1} antibiotics (sulfamethazine, SFT; tylosin, TYS; chlortetracycline, CHTC) and 17β-estradiol. Antibiotic was first spiked to each soil sample, then 17β-estradiol was added after two days of incubation.

	DHA analysis (mg triphenyl formazan kg^{-1} day^{-1})					
Treatment	**Day −1**	**Day 0**†	**Day 1**	**Day 2**	**Day 7**	**Total**
17β-Estradiol (control)	668 ± 52^a	355 ± 33^b	209 ± 48^b	84 ± 9^c	14 ± 2^d	1329^b
2 mg kg^{-1} SFT + 17β-estradiol	130 ± 12^b	227 ± 56^b	123 ± 15^{bc}	66 ± 12^d	26 ± 7^c	527^c
200 mg kg^{-1} SFT + 17β-estradiol	43 ± 7^d	108 ± 16^c	153 ± 22^b	93 ± 12^c	39 ± 5^c	436^c
2 mg kg^{-1} TYS + 17β-estradiol	308 ± 43^b	244 ± 36^b	130 ± 48^{bc}	166 ± 18^b	72 ± 13^b	920^b
200 mg kg^{-1} TYS + 17β-estradiol	344 ± 20^b	553 ± 56^a	520 ± 28^a	273 ± 40^a	114 ± 12^a	1804^a
2 mg kg^{-1} CHTC + 17β-estradiol	75 ± 13^c	98 ± 18^c	114 ± 13^c	32 ± 2^d	28 ± 5^c	347^c
200 mg kg^{-1} CHTC + 17β-estradiol	36 ± 13^d	58 ± 24^d	71 ± 26^c	66 ± 22^d	48 ± 9^c	280^c

†The time at which 17β-estradiol was added.
‡Within each column, values with same letter are not significantly different at 0.05 probability level according to Tukey's multiple range test.

For example, peak value of DHA for 2 mg kg^{-1} SFT + 17β-estradiol was 227 mg triphenyl formazan kg^{-1} day^{-1}, whereas the peak DHA value for control was 668. The peak value is important because it is an indication of altered microbial activity in soil. In other words, the presence of antibiotics shifted the peak values to the right (later days), whereas peak values for control occurred at day^{-1}.

Figure 2 shows the correlation between DHA and concentrations of estrone. The DHA was significantly and positively correlated with the concentrations of estrone in the control ($R^2 = 0.96$). Again, the result indicates that transformation of 17β-estradiol to estrone in soil is mainly due to microbial oxidation in our study.[23] However, with the presence of antibiotics, the strong correlation disappeared, indicating that the antibiotics changed the microbial activity and thus degradation or oxidation of organic substances in the soil. Table 3 details the antibiotic-induced changes in the correlation between DHA and estrone. The presence of SFT and 2 mg kg^{-1} TYS + 17β-estradiol treatment maintained the strong correlation (R^2 ranged from 0.84 to 0.96), and the presence of the CHTC nullified the correlation with the DHA. The inconsistent results for the three antibiotics may be caused by differences in the microbes targeted and the metabolic effect of each antibiotic on the microbes.

Ternes et al.[13] reported that differences in the degradation of 17β-estradiol can also arise when different microbe populations are present in different treatment processes: in a Brazilian wastewater treatment plant over 99% of

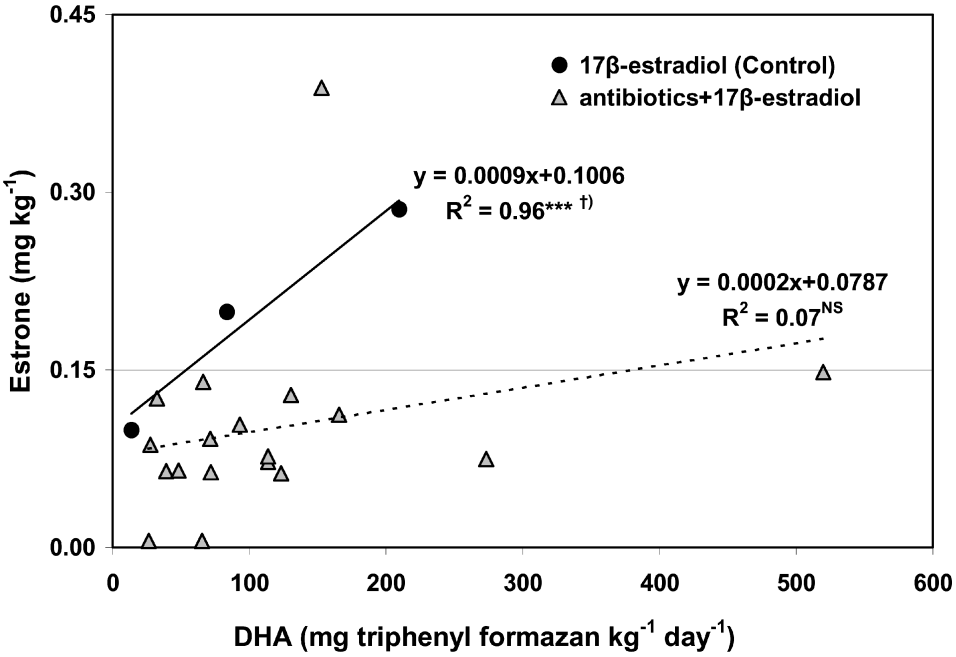

Figure 2: Correlation between dehydrogenase activity (DHA) and concentrations of estrone in Sequatchie loam with presence of antibiotics (sulfamethazine, SFT; tylosin, TYS; chlortetracycline, CHTC) and 17β-estradiol. †***is significant at $Pr < 0.001$ level and NS is not significant at $Pr < 0.05$.

the 17β-estradiol in its waste stream was degraded, whereas only 64% of the 17β-estradiol was removed in a German wastewater treatment plant. Moreover, estrogenic hormones are generally excreted in biologically inactive conjugated forms,[29] and these hormone conjugates are readily hydrolyzed back to the estrogenic hormone by bacteria products.[19,30] Again, Shore et al.[19] reported

Table 3: Correlations between dehydrogenase activity (DHA) analysis and the concentration of estrone in soils spiked with antibiotics (sulfamethazine, SFT; tylosin, TYS; chlortetracycline, CHTC) and 17β-estradiol ($n = 9$).

Treatment	Correlation (r^2) Estrone
17β-Estradiol (control)	0.96***†
2 mg kg^{-1} SFT + 17β-estradiol	0.85**
200 mg kg^{-1} SFT + 17β-estradiol	0.84**
2 mg kg^{-1} TYS + 17β-estradiol	0.96***
200 mg kg^{-1} TYS + 17β-estradiol	0.62*
2 mg kg^{-1} CHTC + 17β-estradiol	0.12NS
200 mg kg^{-1} CHTC + 17β-estradiol	0.38*

†*,**, and ***are significant at the 0.05, 001, and 0.001 levels, respectively; NS is not significant at $Pr < 0.05$.

a prolonged persistence of testosterone in poultry litter with the presence of antibiotics. However, our results did not show the prolonged persistence of 17β-estradiol with the addition of agricultural antibiotics. The presence of antibiotics significantly influenced DHA and decreased the transformation of 17β-estradiol to estrone in soil.

CONCLUSION

Agricultural antibiotics and steroid estrogens often coexist in animal manure and agricultural soils, and concern has arisen due to their potential adverse effect on the environment. We investigated the effect of three different agricultural antibiotics on the persistence and transformation of 17β-estradiol in a Sequatchie loam. The concentrations of estrone were lower in all the antibiotic treatments, implying that antibiotics reduced the transformation of 17β-estradiol. The presence of antibiotics significantly reduced the total DHA. The correlation between DHA and concentrations of estrone was weakened or eliminated, indicating that the microbial activity associated with the degradation and oxidation of 17β-estradiol in soil was complicated with the presence of antibiotics. Studies dealing with the fate and transport of steroid estrogens in soil should include the effect of antibiotics and other organic materials.

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