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Influence of Agricultural Antibiotics and 17 β-Estradiol on the Microbial Community of Soil

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Agricultural pharmaceuticals are a major environmental concern because of their hazardous effects on human and wildlife. This study analyzed phospholipid ester-linked fatty acids (PLFAs) and quinones to investigate the effects of a steroid (17β-estradiol) and agricultural antibiotics (chlortetracycline and tylosin) on soil microbes in the laboratory. Two different types of soil were used: Sequatchie loam (0.8% organic matter) and LaDelle silt loam (9.2% organic matter). The soils were spiked with 17β-estradiol and antibiotics, alone or in combination. In Sequatchie loam, 17β-estradiol significantly increased the microbial biomass, especially the biomarkers for beta proteobacteria (16:1ω7*c*, 18:1ω7*c*, *Cy*17:0, and UQ-8). The coexistence of antibiotics decreased the stimulatory effect of 17β-estradiol on the microbial community. In LaDelle silt loam, there were no significant differences in total microbial biomass and their microbial community structure among the treatments. Overall, 17β-estradiol changed the microbial community of soil and the presence of antibiotics nullified the effect of 17β-estradiol. However, the effects of 17β-estradiol and antibiotics on soil microbes were sensitive to the soil properties, as seen in the LaDelle silt loam.

Key Words: Microbial community; Phospholipid ester-linked fatty acids (PLFAs); Quinones; 17β-estradiol; Agricultural antibiotics.

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INTRODUCTION

Over the past decade, concerns about pharmaceuticals and other emerging organic contaminants in the environment have significantly increased. The United States Geological Survey reported that steroids were the compounds most frequently detected in 139 streams in 30 U.S. states from 1999 to 2000.[1] The report suggested that large numbers of people and wildlife might be exposed to steroids, a group of endocrine-disrupting chemicals (EDCs), through soil and water systems. One of the major sources of steroids released into the environment is animal manure. The concentrations of 17β-estradiol and estrone in manure range from below the detectable limit to 1,215 and 4,728 μg kg^{-1}, respectively.[2] Moreover, the 17β-estradiol concentrations in runoff from a field after manure application reached 0.15–2.3 μg L^{-1}.[3] Agricultural pharmaceuticals, such as steroids and antibiotics, are typically used at most livestock feedlots to promote growth, and these often occur as co-contaminants in soil at animal production operations. Each year 1.8 million metric tons of animal manure are applied to crop fields to supplement nutrients and condition soil in the USA.[4]

Since subculturing microbes on artificial media often reveals only 0.01–10% of the total microbes present in soil,[5] several biomarker analysis approaches that do not require artificial media have been developed to study the natural microbial community structure, *e.g.*, direct microscopic observation combined with gene probes, nucleic acid (DNA/RNA) analysis, and lipid analysis. Lipid analysis, such as ergosterols, phospholipid ester-linked fatty acids (PLFAs), and quinones, has become an important ecological tool for studying microbial biomass, community structure, metabolic status, and taxanomic classifications,[6–8] and has been widely used for several types of environmental research. In a field study, Schmidt et al.[9] reported that total PLFA levels increased when dissolved organic carbon was increased or when the addition of carbon was combined with fertilizer. However, the total amount of carbon did not have a direct relationship with the total amount of PLFAs in their study. Lundquist et al. [10] examined conventional, low-input, and organic farming systems, and reported that the PLFA composition in organic and conventional soils clearly differed for numerous fatty acids. The result indicates that soil environmental factors can influence microbial community structure. Bossio et al.[11] evaluated relative importance for changing PLFA composition in soils for the long term Sustainable Agriculture Farming Systems Project, and ranked the order: soil type > season > farming operation (*e.g.*, cover crop incorporation or sidedressing with mineral fertilizer) > management system (*e.g.*, conventional, low-input, or organic farming system) > spatial variation in a field. Although previous studies have reported that agricultural inputs are significantly associated with distinctive microbial community compositions,

the effects of agricultural pharmaceuticals on the microbial community in soil have not been studied.

In a previous study, we reported that antibiotics, sulfamethazine, tylosin, and chlortetracycline, can change the persistence and transformation of 17β-estradiol in soil and the presence of antibiotics nullifies the effect of 17β-estradiol.[12] However, limited information is currently available on the effect of antibiotics on the persistence of estrogenic hormones in soil/water systems. This study assessed the responses of the microbial community in soils exposed to 17β-estradiol and agricultural antibiotics, alone or in combination, using lipid analyses of PLFAs and quinones. Our study is important to determine the potential impact of pharmaceuticals on microbial community structure and function in soil and the ultimate fate of contaminants in the environment.

MATERIALS AND METHODS

Soil and Chemicals

A laboratory incubation study was conducted using two different types of soil: Sequatchie loam (fine-loamy, siliceous, semi-active, thermic humic Hapludults, 0.8% organic matter) obtained from the Agricultural Experiment Station at the University of Tennessee (Knoxville, TN) and LaDelle silt loam (fine-silty, mixed, super-active, frigid cumulic Hapludolls, 9.2% organic matter) obtained from North Dakota State University (Fargo, ND). The soils were air-dried and ground to pass through a 2-mm sieve. Fujie et al.[13] reported that the soil drying procedure did not affect the proportions of quinones as biomarker in soil, although the treatment decreased the amount of quinones.

Five grams of soil were placed into 40-mL amber glass vials and were spiked with 2 mg kg^{-1} antibiotics, 2 mg kg^{-1} 17β-estradiol, or combinations of both: the experiment treatments were (i) Control, (ii) 17β-estradiol, (iii) chlortetracycline, (iv) 17β-estradiol and chlortetracycline, (v) tylosin, and (vi) 17β-estradiol and tylosin. Chlortetracycline, tylosin, and 17β-estradiol were purchased from Sigma-Aldrich (St. Louis, MO). Because 17β-estradiol and antibiotics are hydrophobic compounds (*i.e.*, non-soluble in water), 200 mg L^{-1} stock solutions of each compound were made in 70% methanol (70% methanol: 30% water, v/v) at concentrations of 200 mg L^{-1}. Then, 100 μL of the stock solution was added for each treatment to make final concentration of 2 mg kg^{-1} of dry soil in each treatment. To the control soil, 100 μL of 70% methanol was added to account for the effect of methanol as a carbon energy source for microbe growth. Moisture content of each soil sample was adjusted to 70% of the field capacity in mass, and the sample was incubated for 7 d at 37°C. All glassware used in this experiment was thoroughly washed with deionized

water and heated for 4 h at 450°C to remove organic contaminants including lipids.

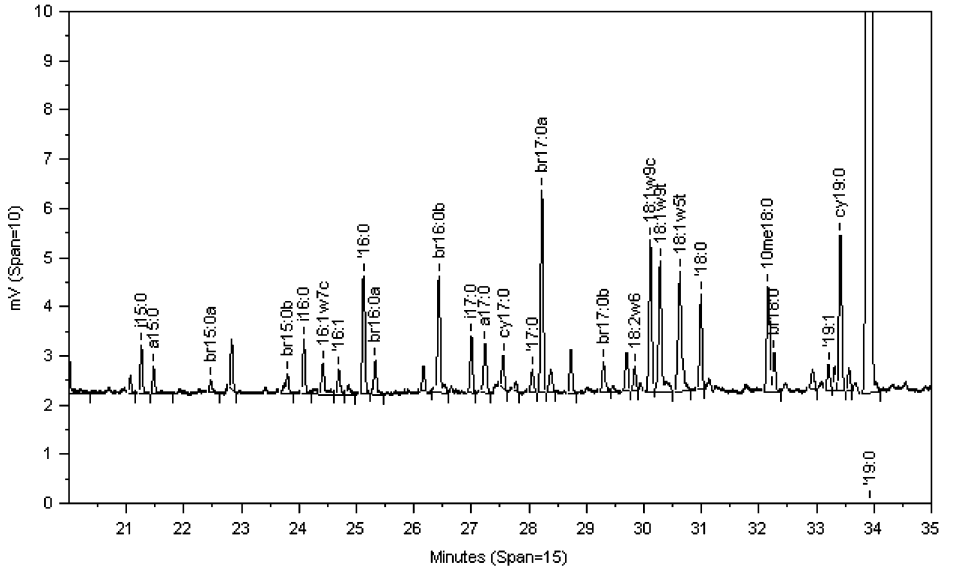
PLFA and Quinone Analyses

The samples for PLFAs and quinones were extracted with a single-phase solvent system of phosphate buffer (50 mM, pH 7.4), chloroform and methanol according to a modified Bligh and Dyer procedure.[14] The total-lipids extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography using three solvents of increasing polarity with bulk elution (chloroform < acetone < methanol). [15,16]

The polar lipid extract was trans-esterified to fatty acid methyl esters by a mild alkaline methanolysis prior to PLFA analysis.[15] The PLFA sample was analyzed by capillary gas chromatography with flame ionization detection on a Hewlett-Packard 5890 series II chromatograph with a 60-m non-polar column (ZB-1, 0.25 mm i.d., 0.25 μm film thickness). The definitive identification of peaks was accomplished by gas chromatography/mass spectroscopy of selected samples using a Hewlett-Packard 6890 series gas chromatograph interfaced with a Hewlett-Packard 5973 mass selective detector using a 60-m non-polar column (RTX-1, 0.25 mm i.d., 0.25 μm film thickness). Peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as internal standard and preliminary peak identification was performed by comparison of retention times with those of known standards (Fig. 1). Fatty acid nomenclature was used of the form A:BωC, where A designates the total number of carbon atoms,[17] B is the number of double bonds, and C is the distance of the closest unsaturation from the methyl end of the acyl chain. When different fatty acids had the same designation, they were distinguished by lower case letters suffixes: a, b, etc. The prefix *Cy* represents the cyclopropyl isomer of a fatty acid; the prefixes *i* and *a* refer to *iso* and *anti-iso* methyl branching, respectively. The suffixes *c* for *cis* and *t* for *trans* refer to conformational isomers. The number preceding the abbreviation Me indicates the position of a methyl group relative to the carbonyl carbon followed by the number of carbons in the fatty acid chain.

The neutral-lipid phase from silicic acid column chromatography was used for the quinones analysis. Ubiquinones (UQ) and menaquinones (MK) were analyzed using a liquid chromatography-tandem mass spectrometry system (PE Sciex API 365, Concord, ON, Canada) with an atmospheric pressure chemical ionization source. The MK-6 (*m/z* 581.3/187), MK-7 (*m/z* 649.9/187), MK-8 (*m/z* 718.0/187), MK-9 (*m/z* 786.1/187) and UQ-7 (*m/z* 659.6/197), UQ-8 (*m/z* 727.9/197), UQ-9 (*m/z* 795.7/197) and UQ-10 (*m/z* 863.8/197) were analyzed using the standard calibration curve for MK-4 (*m/z* 445.6/187) and UQ-6 (*m/z* 591.5/197), respectively (Fig. 2). Quinone homologs quantified by flow injection analysis were additionally verified by chromatographic separation of a sample

(a) Standard



(b) Soil Sample

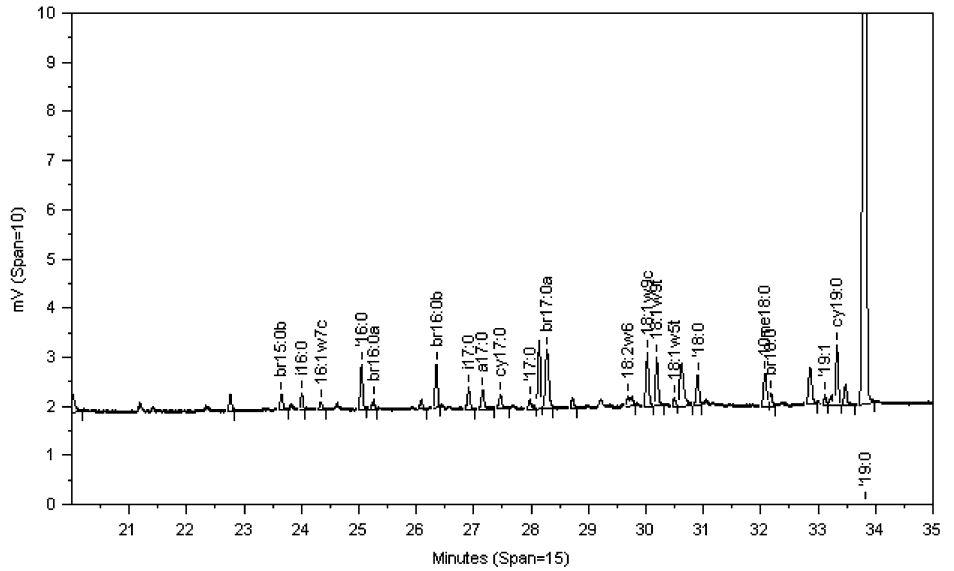


Figure 1: Chromatograms of a standard for phospholipid fatty acids mixture (a) and a soil sample (b). A 100 μL aliquot of a 1 mg L^{-1} standard solution of PLFAs mixture was used.

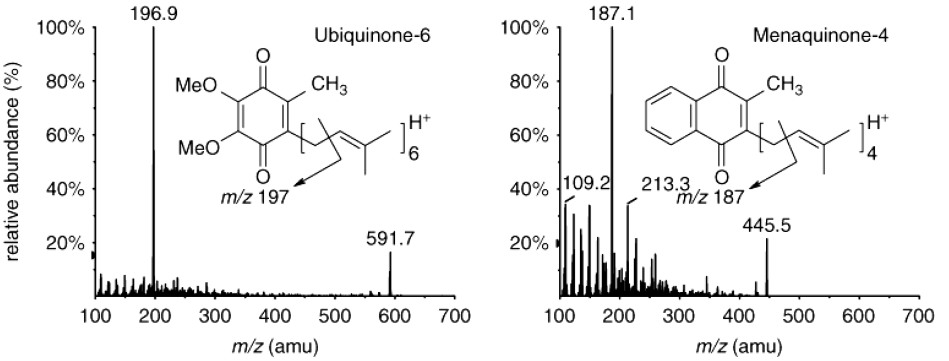


Figure 2: General structure of ubiquinones and menaquinones shown for UQ_6 and MK_4. The product ion spectra for *m/z* 591.7 (UQ_6) and *m/z* 445.5 (MK_4) revealed fragment ions, indicated with their *m/z* and structure, characteristic for the respective quinone class. The molecular ion $(M + H)^+$ and the respective diagnostic fragment ion, major ion in the mass spectra, were used.[18]

on a C_{18} LC column HAISIL 300 (30 $\times$ 1 mm, 5 μm, Higgins Analytical) and precursor ions that generated product ions at *m/z* 197 and *m/z* 187 were scanned, and multiple reaction monitoring transitions (*m/z* progenitor ion/fragment ion) were used for the detection of 10 major UQs and MKs (Fig. 3).

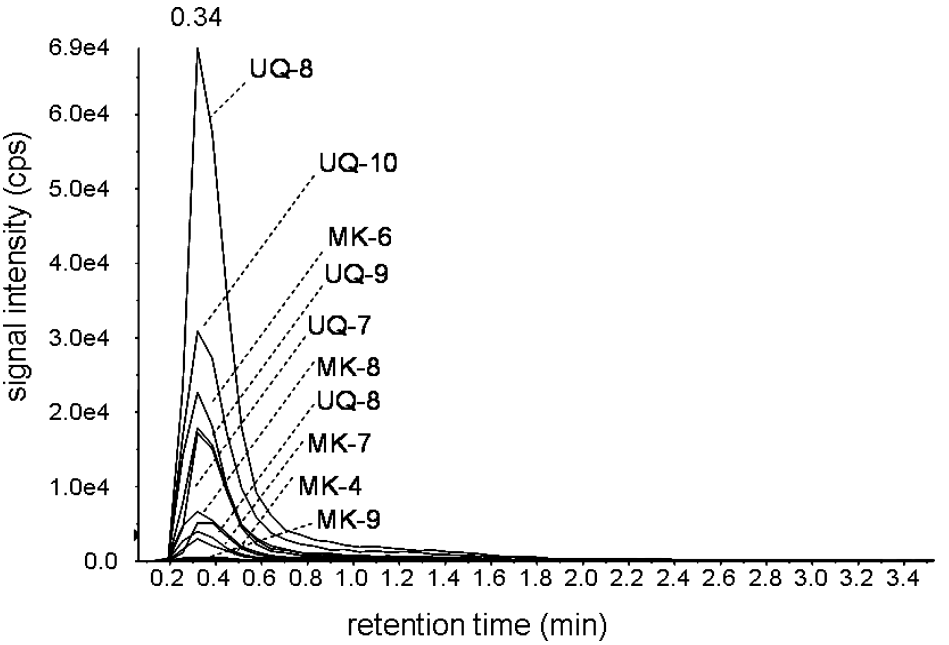


Figure 3: Flow injection analysis of 10 major ubiquinones and menaquinones in soil sample detected with APCI-MS/MS.

The determined lower limit of quantification at a calculated signal-to-noise ratio of 10 was 9.0, 6.8 and 23.3 fmol μL^{-1} for MK-4, UQ-6 and UQ-10, respectively.

Statistical Analysis

A completely randomized design with three replications was used. Cluster analysis was used to 40 different types of PLFA detected in this experiment to ensure the stability of the groups obtained (SAS Institute, Cary, NC). An analysis of variance (ANOVA) was performed on the factor scores, and Tukey's honest significant difference test (HSD) was used to identify significant differences among treatments ($\alpha = 0.05$).

RESULTS AND DISCUSSION

In Sequatchie loam, there were significant differences in the total amounts of PLFAs and quinones, *i.e.*, microbial biomass in soil, between some of the treatments (Fig. 3a and c). In the soil spiked with 17β-estradiol, the total amounts of PLFAs and quinones in the soil increased 30-fold and 33-fold, respectively. There were no significant differences in the amounts of PLFAs or quinones between control and the soil spiked with 17β-estradiol and antibiotics together. In LaDelle silt loam, there were no significant differences in the total amounts of PLFAs and quinones among the treatments (Fig. 3b and d). We believe that this was due to relatively high sorption and rapid degradation of the 17β-estradiol and antibiotics in the soil caused by high organic matter and microbe levels. Interestingly, a single treatment of antibiotics did not reduce the total amounts of PLFAs and quinones in both Sequatchie loam and LaDelle silt loam. The result did not comply with our hypothesis that antibiotics can reduce total microbial biomass. Among emerging contaminants in the environment, heavy metals are known to affect the growth, morphology, and metabolism of micro-organisms in soils.[19] Kelly et al.[20] reported that 47% reduction in total microbial biomass was observed in soils amended with 6,000 mg kg^{-1} of zinc after 15 days incubation. Overall, 17β-estradiol significantly increased total of PLFAs and quinones in LaDelle silt loam, and 17β-estradiol was not a factor to reduce total microbial biomass in both Sequatchie loam and LaDelle silt loam.

We performed a cluster analysis of the 40 different PLFAs in Sequatchie loam, which had significantly different total amounts of PLFAs and quinines (Fig. 4), and the clusters separated into two main groups (Fig. 5). The soil spiked with 17β-estradiol alone and the soils spiked with 17β-estradiol and an antibiotic together were in distinct clusters. The result indicates that the soil spiked with 17β-estradiol alone had different microbial communities compared to the soils spiked with 17β-estradiol and antibiotics. Table 1

Table 1: Phospholipid fatty acid (PLFA) and quinone contents (pmol kg^{-1}) as biomarkers in Sequatchie loam spiked with antibiotics and 17β-estradiol (n = 3). 17b, CHTC, UQ are 17β-estradiol, chlortetracycline, and ubiquinone, respectively.

	Species		ANOVA[†)]	Control	17b	CHTC
PLFA	Normal Saturates	16:0[‡)]	**	7 [B§]	630 [A]	376 [A]
		18:0	*	8 [B]	125 [A]	79 [B]
	Mid-chain branched saturates	*br*17:0	*	16 [B]	199 [A]	202 [A]
		10Me18:0	*	10 [B]	99 [A]	91 [A]
	Terminally branched saturates	*i*16:0	**	3 [B]	104 [A]	38 [B]
		*i*17:0	*	3 [B]	102 [A]	83 [A]
		*a*17:0	*	5 [B]	108 [A]	83 [A]
	Mono-unsaturates	16:1ω7*c*	**	1 [B]	638 [A]	107 [B]
		18:1ω7*c*	***	12 [B]	680 [A]	318 [B]
		*Cy*17:0	**	3 [B]	124 [A]	79 [A]
		*Cy*19:0	*	16 [B]	242 [A]	144 [B]
Quinone	UQ-8		**	0.1 [B]	11 [A]	0.6 [B]

[†)] *, **, and *** are significant at the 0.05, 001, and 0.001 levels, respectively.
[‡)] *br*, a branched fatty acid; *i*, iso methyl branching fatty acid; *a*, anti-iso methyl branching fatty acid; *Cy*, cyclopropyl isomer of a fatty acid; *c*, *cis* isomers of a fatty acid; number preceding the abbreviation Me, the position of a methyl group relative to the carbonyl carbon.
[§)] Within the same row, values with the same letter are not significantly different at the 0.05 probability level by the Tukey's Honest Significant Difference test.

summarizes the PLFAs and quinones used as biomarkers, indicating specific differences in the PLFA and quinone contents. Eleven of 40 PLFAs and 1 of 9 quinones in the soil exposed to 17β-estradiol differed significantly from those in the control soil. The amounts of 16:1ω7*c*, 18:1ω7*c*, and *Cy*17:0 increased significantly in the soil spiked with 17β-estradiol; these PLFAs characteristically occur in Gram-negative beta-subgroup proteobacteria. It has been reported that the monoenoic PLFAs 16:1ω7*c* and 18:1ω7*c* are usually increased in Gram-negative communities[21] when the Gram-negative bacteria move from the log phase to the stationary growth phase,[22] and that these are easily converted into cyclopropane PLFAs, such as *Cy*17:0 and *Cy*19:0. Some indicators of Gram-positive bacteria, *e.g.*, *br*17:0, also increased in the soil exposed to 17β-estradiol. Of the respiratory quinones, only UQ-8 differed significantly between soil spiked with 17β-estradiol and the control. Many Gram-negative bacteria contain UQ-8, QU-9, and Q-10,[7] and Gram-negative bacteria containing UQ-8 are classified primarily as beta proteobacteria.[23] The increase in UQ-8 suggests that microbes capable of aerobic respiration dominated the soil spiked with 17β-estradiol.[23,24] Therefore, we postulate that 17β-estradiol increases the Gram-negative microbial community of beta proteobacteria in Sequatchie loam under aerobic respiratory conditions.

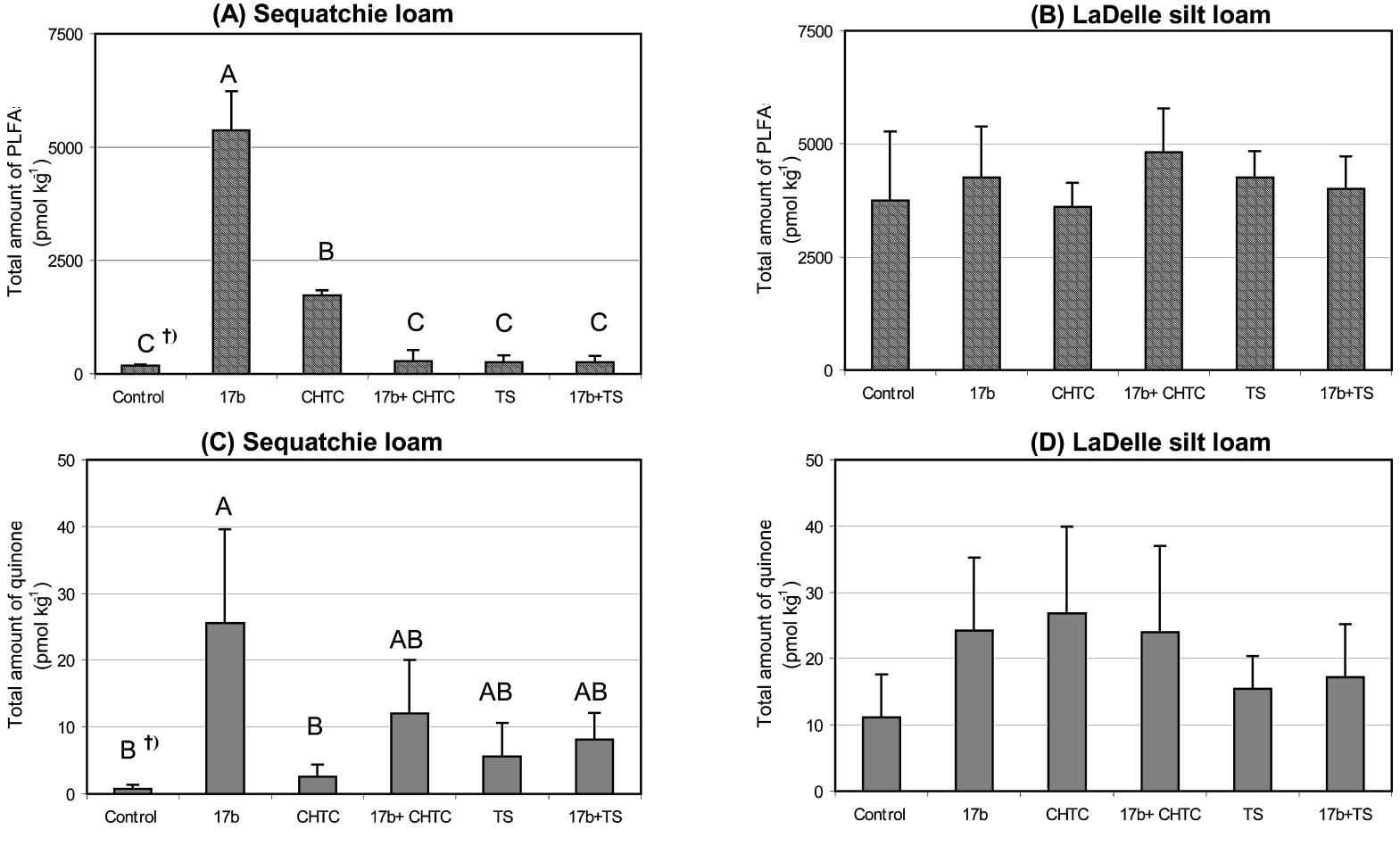


Figure 4: Total PLFAs and quinones as total of microbial biomass in Sequatchie loam (a and c) and LaDelle silt loam (b and d). 17b, CHTC, and TS represent 17β-estradiol, chlortetracycline, and tylosin, respectively.†) Values with the same letter are not significantly different at the 0.05 probability level according to the Tukey's Honest Significant Difference test (n = 3).

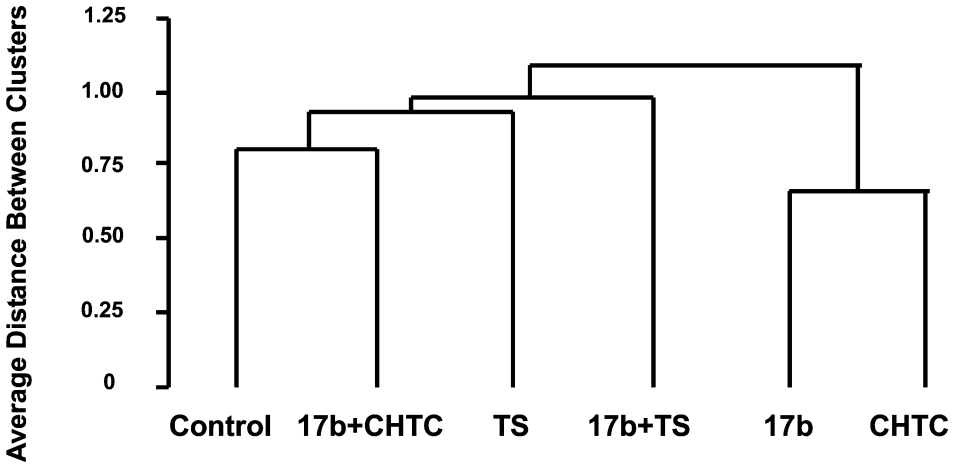


Figure 5: Cluster analysis based on 40 different types of phospholipid fatty acids in Sequatchie loam spiked with antibiotics and 17β-estradiol (n = 3). 17b, CHTC, and TS represent 17β-estradiol, chlortetracycline, and tylosin, respectively.

With respect to the physiological status of the microbial community,[25] the ratio of cyclopropyl to monoenoic precursors is usually less than 0.05 during unstressed log-phase growth, and the ratio increases to 2.5 or more as the stress of starvation and the stationary growth phase are prolonged.[26] The ratios of total cyclopropyl PLFAs to total monoenoic PLFAs changed significantly in the soil spiked with 17β-estradiol in this study, implying that 17β-estradiol influences the stress condition or stationary growth phase. Because we did not find a significant increase in *trans*-monounsaturated fatty acids that is a result of metabolic stress of toxicity,[27] we believe that 17β-estradiol was normally metabolized by microbes without metabolic stress of toxicity. On the other hand, indicators of the dikaryotic fungi *Ascomycota* and *Basidiomycota*, such as 18:2ω6 and 18:2ω9,[20,28] were unchanged in soils treated with 17β-estradiol and antibiotics, indicating that the change in the microbial community is not directly related to the fungi. To explain the persistence of 17β-estradiol, the oxidation of 17β-estradiol (C-17 alcohol) to estrone (C-17 ketone) is frequently cited.[12,29–31] Many studies have hypothesized that bacteria or fungi catalyze this reaction.[32,33] However, we did not find a significant increase in fungal indicators caused by 17β-estradiol in this study, and the PLFAs characteristic of beta proteobacteria (*i.e.*, 16:1ω7*c*, 18:1ω7*c*, *Cy*17:0, and UQ-8) showed the most significant increases in soil spiked with 17β-estradiol.

17β-Estradiol has been detected in ground and surface waters adjacent to land on which animal manure has been applied.[3] Estrogenic hormones such as 17β-estradiol are generally excreted in biologically inactive conjugated forms.[34] These hormone conjugates are readily hydrolyzed back to the estrogenic hormone by bacteria producing β-glucuronidase or sulfatase

enzymes.[35] Conjugates can reasonably be expected to be hydrolyzed in the environment rapidly and readily.[34,36] Overall, we found that 17β-estradiol can alter the microbial community in soil and that the presence of antibiotics as co-contaminants with 17β-estradiol nullifies the effect of 17β-estradiol on microbial community structure. We also found that the effects of 17β-estradiol and antibiotics can be influenced by specific soil properties.

CONCLUSION

PLFAs and quinones were analyzed to investigate the effect of agricultural pharmaceuticals on soil microbes. It is noteworthy that Gram-negative beta proteobacteria (16:1ω7*c*, 18:1ω7*c*, *Cy*17:0, and UQ-8) were significantly increased in 17β-estradiol spiked Sequatchie loam. However, the change was complicated when antibiotics were present as co-contaminants. We also found no significant differences in the effects of 17β-estradiol and antibiotics, alone or together, in LaDelle Silt loam. We postulate that this was due to their sorption in this organic rich soil. Overall, agricultural pharmaceuticals, including antibiotics and steroids, have a great influence on microbes in soil, and further study is needed for various soil and water systems.

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