Antibiotic Effects on Microbial Community Characteristics in Soils under Conservation Management Practices

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Veterinary antibiotics (VAs) administered to livestock are introduced to agroecosystems via land application of manure, posing a potential human and environmental health risk. Recent evidence suggests that agroforestry and grass vegetative filter strips (VFS) may act to mitigate VA transport or enhance VA degradation; however, VAs may adversely affect soil microbial communities within VFS and thus alter the primary functioning of the VFS. The objectives of this research were to investigate potential changes in microbial community structure and function and to quantify the development of antibiotic resistance in VFS and no-till soils exposed to various VA classes and concentrations. Laboratory mesocosms were established using soils collected from no-till cropland and two VFS (grass and agroforestry). Soils were treated with oxytetracycline or lincomycin (5–200 mg kg^{-1} soil). Individual mesocosms underwent destructive sampling at nine time points during 63 d, and the soils were tested for soil microbial function (C-utilization, dehydrogenase, and fluorescein diacetate hydrolysis assays), community structure (phospholipid fatty acid analysis), and antibiotic resistance. Functional assays associated with all VA treatments showed an initial inhibitory effect, but this trend was generally reversed by the seventh day. Shifts in microbial community structure and increased antibiotic resistance were not observed, suggesting that the soil microbial communities were robust to the effects of oxytetracycline and lincomycin at test concentrations. This work indicates that using VFS to mitigate VA loss from agroecosystems will not diminish important primary functions associated with VFS use in agriculture.

Abbreviations: FDA, fluorescein diacetate; LIN, lincomycin; MIC, minimum inhibitory concentration; OTC, oxytetracycline; PLFA, phospholipid fatty acid; VA, veterinary antibiotic; VFS, vegetative filter strips.

Veterinary antibiotics have been detected in surface water resources of the United States (Kolpin et al., 2002; Kim and Carlson, 2007), Europe (Christian et al., 2003), and Asia (Managaki et al., 2007). Surface water runoff from manured agroecosystems is considered to be a primary mechanism for VA transport to surface waters (Kay et al., 2005; Lin et al., 2011a), and surface runoff events are more problematic for soils with water-restrictive subsoil horizons (Needelman et al., 2004; Müller et al., 2006). The global distribution of such soils comprises an area of approximately 2.9 million km^2 (NRCS, 1998), which may exacerbate VA transport and presence in streams, rivers, and lakes. Recent studies have indicated, however, that VFS may have potential for mitigating VA loss from soils prone to surface water runoff events (Chu et al., 2010; Lin et al., 2010, 2011a).

Vegetative filter strips, land areas of either planted or indigenous vegetation situated between a pollutant source area and a surface water body that receives runoff, are a conservation practice implemented to reduce soil erosion and improve water quality (Schultz et al., 2000). Previous research has described the ability of

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VFS to minimize nutrient loss (Udawatta et al., 2002), reduce surface runoff and soil erosion (Coyne et al., 1995; Veum et al., 2009), and mitigate pesticide transport from agroecosystems (Krutz et al., 2005; Lin et al., 2011b). Other studies have cited increased biodiversity, wildlife habitat and corridors, and positive effects on stream habitat (Lovell and Sullivan, 2006) and water quality (Lerch et al., 2005).

Improved soil physical, chemical, and biological characteristics of VFS have been implicated in these ecosystem functions. For example, soils of VFS have been shown to have greater aggregate stability (Udawatta et al., 2008; Helgason et al., 2010), more complex and varied soil organic matter fractions (Mungai et al., 2005; Dornbush, 2007; Macdonald et al., 2009; Veum et al., 2011), and increased immobilization of CO_2 relative to cropped soils (Borin et al., 2010). Subsequently, VFS have been demonstrated to support more robust and diverse microbial communities (Martin et al., 1999).

The presence of VAs in manure is derived from the use of these drugs for therapeutic, prophylactic, and enhanced animal growth purposes (Wegener, 2003). A significant proportion of VAs ingested by animals is excreted unmetabolized (30–80%), however, and is subsequently land applied when manure is used to fertilize agricultural crops (Elmund et al., 1971; Levy, 1992; Halling-Sørensen et al., 2002). The three primary concerns surrounding the presence of VAs in the environment revolve around negative impacts to water quality, changes in soil microbial community structure and function, and the development of VA-resistant bacteria (Daughton and Ternes, 1999; Sarmah et al., 2006; Lee et al., 2007; Aga, 2008).

Although antibiotics have been observed in streams across the United States (e.g., Kolpin et al., 2002), the Safe Drinking Water Act lacks provisions for the regulation or monitoring of antibiotics (Bradford et al., 2008; Kemper, 2008). Recent updates to the USEPA's Drinking Water Strategy include the development of technologies to reduce contaminant chemicals in potable water supplies, but antibiotics are not included in the list of contaminants (USEPA, 2011). Yet, in 2000, a World Health Organization report focused on antibiotic resistance as one of the most critical human health challenges of the next century (World Health Organization, 2000), indicating that the spread of antibiotic resistance in environmental and clinical bacteria is of the upmost public health importance (Bradford et al., 2008; Kemper, 2008). Previous research has noted the presence of antibiotic-resistant genes in environmental samples collected near confined animal feed operations (Chee-Sanford et al., 2001; Koike et al., 2007) and adverse VA effects on soil microbial community structure and diversity (Hammesfahr et al., 2008; Gutiérrez et al., 2010).

Given the potential effects of VAs on soil microorganisms, there is concern that VAs intercepted and retained within VFS may alter soil microbial communities to an extent that other VFS ecosystem services are adversely impacted. Therefore, the objectives of this study were to investigate changes in (i) microbial community structure and (ii) microbial community function and (iii) to quantify the development of antibiotic resistance in VFS and non-VFS soils exposed to various VA classes and concentrations as a function of time.

We chose two common VAs for our investigations: oxytetracycline (OTC) and lincomycin (LIN). Oxytetracycline falls within the tetracycline class of VAs, which is one of the two heavily used antibiotic classes in animal agriculture (Bradford et al., 2008). Oxytetracycline is a broad-spectrum bacteriostatic antibiotic capable of treating a wide variety of infections; it is also widely used as a feed additive to stimulate growth in livestock (Boleas et al., 2005; Sarmah et al., 2006). Lincomycin, of the lincosamide class of VAs, is not as widely used or studied as OTC; however, LIN is used in disease prevention for swine and poultry (Sarmah et al., 2006) because it inhibits bacterial protein synthesis, particularly in Gram-positive bacteria.

METHODS

Study Site

Soils for this study were collected from the paired watershed study site at the University of Missouri's Greenley Memorial Research Center, Novelty, MO (40°1″ N, 92°11″ W) established in 1991 (Fig. 1). In 1997, grass VFS were established in the west watershed (3.16 ha) and agroforestry VFS were installed in the center watershed (4.44 ha); the east watershed (1.65 ha) contains no VFS and serves as a control. Vegetative filter strips, 4.5 m wide, follow the contour; they are separated by a distance of 36.5 m. Filter strips in the west and center experimental watersheds were planted with redtop (*Agrostis gigantea* Roth), bromegrass (*Bromus* spp.), and birdsfoot trefoil (*Lotus corniculatus* L.). Pin oak (*Quercus palustris* Münchh), swamp white oak (*Quercus bicolor* Willd.), and bur oak (*Quercus macrocarpa* Michaux) were planted 3 m apart in the center of the agroforestry VFS. Before the establishment of the filter strips, all three watersheds were under a corn (*Zea mays* L.)–soybean [*Glycine max* (L.) Merr.] rotation under no-till management. This corn–soybean rotation has been maintained in the control watershed and in between VFS of the west and center watersheds. For further study site information, see Veum et al. (2009) and references therein.

Soil Collection and Treatment

Bulk soil samples (three per watershed at the 0–10-cm depth) were collected in October 2009 from all watersheds. In the VFS watersheds, soils were collected at the shoulder landscape position, which equated to the second VFS from the top of the watershed (Fig. 1); soils were sampled at an equivalent position in the control watershed. Soils from this landscape position are characterized as Putnam silt loam (a fine, smectitic, mesic Vertic Albaqualf) (see Supplemental Table S1 for soil characterization data). Samples collected from the agroforestry VFS were located approximately 0.6 m from the base of the trees; this was to avoid root-wad soil from the nursery and the weed matting, which extended up to 0.5 m from the tree base.

Replicate soil samples were extracted and analyzed for initial LIN and OTC content before treatment. Soils were extracted by accelerated solvent extraction (ASE-300, Dionex Corp.). Dry soil and Hydromatrix (an inert diatomaceous earth sorbent, Agilent

Technologies) were mixed (15 g soil/2 g Hydromatrix) well and then poured onto 1 cm of sand in a 34-mL stainless steel extraction cell. The remainder of the extraction cell was filled with sand. Glass fiber filters were placed at both ends of the extraction cell and sealed with high-pressure end caps. The packed cells were then extracted using three static cycles with deionized water at 100°C and 10.340 MPa for LIN and three static cycles with 50% water (containing 5% NaOAc and 100 mmol L^{-1} ethylenediamine tetraacetic acid) and 50% methanol at 100°C and 10.340 MPa for OTC. Each static cycle was 5 min long and the final flush was 60% of the pore volume. Extracts were then concentrated using solid-phase extraction by diluting with 400 mL of deionized water. The resulting solutions had organic solvent contents <5% and could be passed through a conditioned Strata-X (Phenomenex) solid-phase extraction cartridge followed by three 20-mL rinses of nano-pure water. The cartridge was then dried for 2 min and eluted with 3 mL of 1:1 methanol/water. For OTC analysis, an additional cleanup was performed by adding 1 mL of 0.05 mol L^{-1} citric acid conditioning solution to the previous eluate and passing the solution through a Strata SAX cartridge. Oxytetracycline was eluted from the Strata SAX cartridge using 3 mL of methanol. Solutions containing OTC and LIN were evaporated to dryness under N_2 at 35°C, and the samples were reconstituted with 100 μL of methanol followed by 900 μL of nano-pure water. The samples were then transferred to high-performance liquid chromatography vials for liquid chromatography–mass spectrometry (LC-MS) analysis.

Lincomycin and OTC analysis was performed using LC-MS–MS electrospray (positive mode). Separation was performed using a Waters 2.1- by 30-mm XTerra MS C18 column with a 2.5-mm stationary phase. Operating conditions of the LC were: a mobile-phase flow rate of 0.25 mL min^{-1}, with a binary mobile phase of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Initial conditions were 10:90 acetonitrile/water, followed by isocratic flow for 1.5 min; at 1.5 min, a linear gradient from 10:90 acetonitrile/water to 90:10 acetonitrile/water was applied over 5 min, followed by 1.5 min of isocratic flow at 90:10 acetonitrile/water. Lincomycin eluted at 2.2 min and was quantified using the transition 407.2 → 126.2 m/z; OTC eluted at 5.7 min and was quantified using the transition 461.2 → 426.1 m/z.

Replicate soil samples collected in the field within each vegetative treatment were moist sieved and then divided into individual jars (60 g soil in a 150-mL magenta jar). Triplicate samples (one associated with each sampling location) from each vegetative treatment then received the following VA treatments: (i) no treatment (control); oxytetracycline hydrochloride (>95% purity; Sigma-Aldrich) at concentrations of (ii) 5, (iii) 50, or (iv) 200 mg kg^{-1} soil; or lincomycin hydrochloride (>95% purity; Sigma-Aldrich) at concentrations of (v) 5, (vi) 50, or (vii) 200 mg kg^{-1} soil. The antibiotic concentrations chosen for this study are quite extreme, but they were intentionally chosen to test the robustness of the systems. Antibiotic solutions were adjusted to pH 6.5 to 7.5; 6 mL of either VA solution or distilled water was pipetted into the appropriate sample jars, and the samples were shaken vigorously to help distribute the VA throughout the soil. The final soil moisture content was 35% (±2%). The jars were capped, wrapped in foil, and stored in the dark at room temperature (~25°C) during incubation to avoid VA photodegradation. Triplicate samples for each VFS–VA treatment were collected at 0, 3, 7, 14, 21, 28, 35, 49, and 63 d following the initial VA spike and were placed in cold storage (4°C) until analysis.

Soil Microbial Community Function

Dehydrogenase and fluorescein diacetate (FDA) hydrolysis enzyme assays along with C-utilization assays using Biolog ECO microplates were used to determine the soil microbial commu-

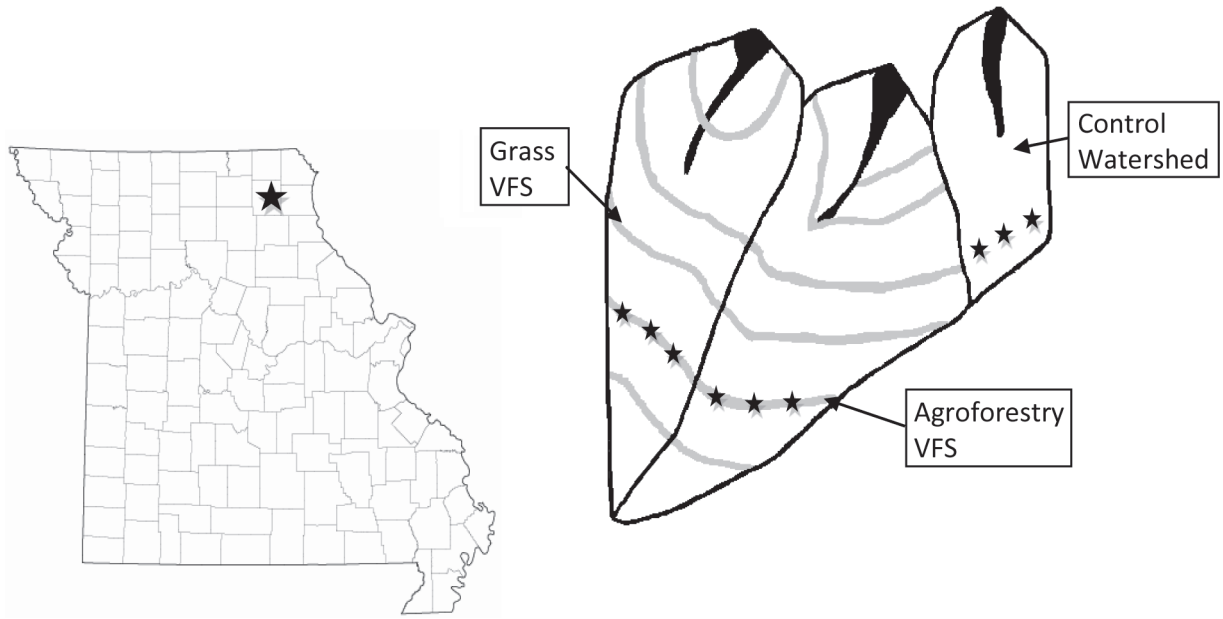

Fig. 1. Paired watershed study site at the University of Missouri's Greenley Memorial Research Center, Novelty, MO, and watershed map (courtesy of K. Veum). Gray bands indicate locations of grass or agroforestry vegetative filter strips (VFS); stars indicate sampling locations.

nity function. Dehydrogenase and FDA assays followed the procedures described by Kremer and Li (2003). The dehydrogenase enzyme assay was used to approximate the respiratory activity for soil microorganisms (Tabatabai, 1994). Soil (6 g fresh weight) was amended with 1.0 mL of 3% triphenyl-tetrazolium chloride and 3.0 mL of 0.2 mol L^{-1} $CaCO_3$ and incubated for 24 h at 37°C. Reactions were terminated by the addition of 50 mL of methanol, and the product, 2,3,4-triphenyl-tetrazolium formazan (TPF), was extracted for 30 min on a rotary shaker. The reaction mixtures were filtered, the concentration of TPF was determined spectrophotometrically at 485 nm, and the enzyme activity expressed as milligrams TPF released per gram dry soil per hour.

Soil microbial activity expressed as FDA hydrolysis was determined following the method of Schnuürer and Rosswall (1982) as modified by Kremer and Li (2003). Fluorescein diacetate is a general substrate for several hydrolytic enzymes including esterases, lipases, and certain proteases (Dick, 1997). For this assay, 1.0 g of soil was suspended in 20 mL of phosphate buffer (pH 7.6) in 50-mL Erlenmeyer flasks. After shaking for 15 min on a rotary shaker, 100 mL of FDA (4.8 mmol L^{-1}) was added and the flasks were returned to the rotary shaker at 100 rpm and incubated at 30°C for 105 min. The assay was terminated by extraction with acetone (10 mL) followed by filtration through filter paper (Whatman no. 2). Hydrolytic activity was detected by spectrophotometrically (490 nm) measuring the product of hydrolysis (fluorescein); enzyme activity is expressed as milligrams fluorescein released per gram dry soil per hour.

Biolog ECO microplates provided a C-utilization pattern of the soil microbial communities. This pattern is based on a battery of 31 substrates replicated three times in a 96-well microtiter plate and is visualized by color development after an incubation period. Soil samples (1 g) were extracted using 9 mL of 100 mmol L^{-1} phosphate buffer (phosphate-buffered saline at pH 7), serially diluted 10-fold to achieve a final concentration of 10^{-3}. Each microplate well was inoculated with 150 μL of extract, and the plates were incubated in the dark at 25°C for 72 h. Color development in the microplates was measured by an ELx808 absorbance microplate reader (Biotek Instruments) at 570 nm.

From ECO microplate absorbance readings, the following measures were calculated: average well color development (AWCD); substrate utilization richness; diversity; and evenness. The AWCD measures species activity and density as well as the ability of the microbial community to respond to a particular substrate (Zak et al., 1994). To determine the AWCD, initial readings were subtracted from final readings (72 h) for each well; any negative results were replaced with zeros before the totals for each plate replicate were divided by 31. The three replicates per plate were then averaged to achieve one reading per plate. Substrate utilization richness (S) was determined by the total number of positive responses (i.e., optical density > 0.10). Diversity was calculated using the Shannon diversity index ($H = -\Sigma p_i(\ln p_i)$, where p_i is the ratio of the activity on the ith substrate to the sum of activities on all substrates), and evenness was calculated as $H/\log S$ (Zak et al., 1994).

Soil Microbial Community Structure

Soil samples at 0, 3, 35, and 63 d were selected for PLFA analysis to determine the soil microbial community structure. Procedures were as described in Unger et al. (2009). Whole-soil PLFA procedures generally follow Bligh and Dyer (1959) as described by Petersen and Klug (1994). All reagents were high-performance liquid chromatography grade and purchased from Sigma-Aldrich, except where noted. Soil samples (2 g) were placed in Teflon-lined, screw-cap culture tubes (16 × 100 mm) and fatty acid methyl esters analysis was conducted based on saponification of soil at 100°C, acid methylation at 80°C, an alkaline wash, and an extraction of methyl esters of long-chain fatty acids and similar lipid compounds into hexane. Nonadecanoic acid methyl ester was included after the methylation step to enable quantification of the identified lipids on a molar basis. Samples for phospholipid analysis were separated by solid-phase extraction using 100-mg silica columns (Varian). The columns were conditioned with 3 mL of hexane, 1.5 mL of hexane/chloroform (1:1), and 100 mL of chloroform; a slight vacuum (2.5–5 cm of Hg) was applied to the columns after the addition of each solvent. The columns were rinsed by the sequential addition of 1.5 mL of chloroform/2-propanol (1:1) and 1.5 mL of 2% acetic acid in diethyl ether under vacuum. Finally, phospholipids were eluted from the columns with 2 mL of methanol, and evaporated under N_2 in preparation for PLFA extraction. The combined organic phase was evaporated to dryness under N_2 and redissolved in 75 mL of hexane/methyl tertiary butyl ether (1:1).

Fatty acid methyl esters were analyzed on a gas chromatograph (Agilent Technologies GC 6890) with a fused silica column and equipped with a flame ionization detector and integrator. ChemStation (Agilent Technologies) operated the sampling, analysis, and integration of the samples. Peak identification and integration of areas were performed under the Eukary method parameters by software supplied by Microbial Identification Systems. Peak chromatographic responses were translated into molar responses using the internal standard, and responses were recalculated as needed. Peaks that correspond to C chain lengths of 12 to 20 carbons are generally associated with microorganisms. Bacterial/fungi ratios were calculated for each sample. Stress indicators were calculated based on the ratios of the cyclopropyl fatty acids to monoenoic precursors and the total saturated to total monounsaturated fatty acids (Kieft et al., 1997; Bossio and Scow, 1998; Fierer et al., 2003). Biomass was calculated using the relationship determined by Bailey et al. (2002). Peaks used as markers for bacteria, fungi, Gram-positive bacteria, Gram-negative bacteria, mycorrhizae, and stress indicators are identified in Supplemental Table S2.

Quantification of Antibiotic Resistance

Soil samples at 0, 3, 21, 35, and 63 d were selected for quantification of antibiotic resistance. In the laboratory, approximate-

ly 1.0 g of moist soil was added to 5.0 mL of BBL Enterococcosel Bile Esculin broth (Becton, Dickinson and Co.) and placed onto a shaking incubator at 37°C to enrich *Enterococcus* (Gram-positive). An additional 1.0 g of moist soil was added to 5.0 mL of sterile BBL EC broth (Becton, Dickinson and Co.) and shaken at 37°C to selectively enrich total coliforms (Gram-negative). After 24 to 36 h, aliquots of each enrichment broth were spread onto plates prepared with Enterococcosel agar or MI agar (Becton, Dickinson and Co.) for isolation of enterococci and *Escherichia coli*, respectively. Following 24 to 36 h of incubation at 37°C, 16 isolates were collected from each plate. Following Gram staining to confirm identity as Gram-positive or Gram-negative, each isolate was assessed for antibiotic sensitivity as detailed below.

Sensitivity to LIN (Gram-positive isolates) and OTC (Gram-negative isolates) was determined by broth microdilution according to the Clinical and Laboratory Standards Institute (2009) standards for antimicrobial susceptibility testing. As such, each Gram-positive and Gram-negative plate included control (reference) isolates *Enterococcus fecalis* ATCC 29212 or *E. coli* ATCC 25922, respectively, to monitor the performance and reproducibility of the microdilution assay. All screening was performed utilizing 96-well plates containing dilution ranges of the target VAs from 0.5 to 32 mg L^{-1}. Following loading of the antibiotic solutions into the wells, 50 μL of the target isolate suspended in cation-adjusted Mueller–Hinton broth (initial target concentration of 5×10^8 colony-forming units L^{-1}) was added to each well. Each isolate was analyzed in duplicate; positive control wells with no VA added were included on each plate. The plates were sealed and placed into a 37°C incubator for 24 h.

Cell growth was determined using absorbance (600 nm) of individual wells using a BioTek microplate reader (BioTek Instruments). Absorbance readings from negative control wells (VA only, no isolate added) were subtracted from each isolate–antibiotic well before data analysis. The minimum inhibitory concentration (MIC), defined as the lowest concentration of VA that prevented growth, was calculated for each isolate.

Data Analysis

Analysis of variance (Proc Mixed) was used to evaluate the effects of the VA treatments on soil microbial community function and structure with time. Soils for this experiment were collected from three different locations within each VFS. For analyses, locations for each site were pooled; thus, the linear statistical model was location (i.e., agroforestry VFS, grass VFS, or cropland with no VFS), replicate within location, treatment (i.e., LIN 5, LIN 50, LIN 200, OXY 5, OXY 50, OXY 200, and no-VA control), time (i.e., 0, 3, 7, 14, 21, 28, 35, 49, and 63 d), and all possible interactions of location, treatment, and time. Replicate within location was used as the denominator of F to test location. All other effects in the model used the residual mean square for the denominator of F. Structuring the analysis in this manner allowed us to compare each VA concentration combination (e.g., LIN 5 vs. OXY 5, LIN 5 vs. OXY 50, LIN 5 vs. OXY 200, etc.) as opposed to the more limited comparisons that would result from lumping the VA treatments by type (e.g., LIN vs. OXY) or concentration (e.g., 5 vs. 50 vs. 200 mg kg^{-1} soil). Each dependent variable for microbial community function (i.e., dehydrogenase and FDA activity, AWCD, diversity, richness, and evenness) and microbial community structure (response variables for each microbial group as well as total biomass, bacteria/fungi ratio, and four stress indicators) was analyzed separately. Least square means were calculated and compared to determine significant differences among treatments. These analyses were conducted using SAS software (SAS Institute). For antibiotic resistance data, statistical significance of the results was assessed using repeated measures ANOVAs, with VFS treatment and VA concentration as fixed effects. These analyses were performed using Minitab 16 statistical software (Minitab Inc.).

RESULTS AND DISCUSSION

Initial Antibiotic Concentrations

Initial antibiotic concentrations were measured for each soil. In all cases, the VA concentrations were detectable but very low. The average concentration of three replicates for LIN before treatment was 1.0×10^{-2} μg kg^{-1} for the grass VFS soil, 7.0×10^{-3} μg kg^{-1} for the agroforestry VFS soil, and 4.2×10^{-3} μg kg^{-1} for the cropland soil. The initial OTC content was greater than that of LIN. The average OTC concentration of three replicates was 6.0×10^{-2} μg kg^{-1} for the grass VFS soil, 7.8×10^{-2} μg kg^{-1} for the agroforestry VFS soil, and 1.0×10^{-1} μg kg^{-1} for the cropland soil. In all cases, the initial antibiotic concentration was at least five orders of magnitude lower than the treatments. The highest pretreatment concentration of OTC was found in the cropland soil; however, it was 50,000 times lower than the lowest treatment concentration.

The presence of VAs in the pretreated soils may be due to small amounts of contamination during experimental establishment or residues remaining in the soil from prior land use. Farm records indicate that during the past 35 yr, no manure has been land applied on these watersheds. Before the establishment of the VFS in 1991, however, beef cows (*Bos taurus*) were allowed to graze the corn and soybean stubble in late fall and early winter (R. Smoot, personal communication, 2012). Since 1991, only inorganic fertilizers have been utilized in the watersheds while under corn rotation (Udawatta et al., 2002). Small-scale exposure to VAs via grazing may have occurred 20 yr ago.

Soil Microbial Community Function

The soil microbial community function was affected by the VA treatments (Table 1). Significant VA treatment × time interactions were observed for all ECO microplate variables except evenness; likewise, significant antibiotic treatment × time interactions were observed for both dehydrogenase and FDA enzyme assays (Table 1). Similar patterns were observed for the effects of both VAs on AWCD, richness, and diversity from the ECO microplate assay. This pattern showed an early decline in response (i.e., at 3 d), followed by a rapid recovery, with peak levels occurring around 35 d. Response dropped sharply by 49 d but then

recovered to near pretreatment levels by 63 d (Fig. 2a–2c and 3a–3c). While for the most part the VAs and the control treatments were not different, there were a few notable exceptions. For AWCD, both LIN and OTC at 50 mg kg^{-1} soil appeared to stimulate the microbial community because the AWCD for these treatments were significantly greater than those for the control at 7 d (Fig. 2a and 3a). Oxytetracycline at a concentration of 50 mg kg^{-1} soil also appeared to increase richness and diversity over control levels at 7 d (Fig. 3b and 3c). Also of interest is the significant decline in diversity compared with control treatments under LIN at 5 mg kg^{-1} soil at Day 7.

Enzyme assays also showed an early decline and recovery pattern (Fig. 2d–2e and 3d–3e). As with the ECO microplate assay, the VA treatments showed only a few significant differences from the control treatment. The greatest differences observed between the control and VA treatments in dehydrogenase activity occurred at 3 d (Fig. 2d and 3d). Dehydrogenase activity was significantly lower than the control treatment for both VAs at concentrations of 50 and 200 mg kg^{-1} soil; however, neither VA at 5 mg kg^{-1} was different than the control at 3 d. After 3 d, the dehydrogenase activity generally increased during the remainder of the experiment in soils amended with either VA. A decline in dehydrogenase activity was observed at 35 d; however, activity recovered by 63 d at greater than pretreatment levels. The greatest differences in FDA activity occurred at 7 d. In this case, FDA activity under LIN at concentrations of 50 and 200 mg kg^{-1} soil and under OTC at 200 mg kg^{-1} soil was significantly greater than FDA activity under the control treatment at 7 d (Fig. 2e and 3e). The FDA activity under both VAs recovered by 14 d and fluctuated within pretreatment levels for the remainder of the experiment (Fig. 2e and 3e).

Lincomycin and OTC had limited effects on the soil microbial community function. Veterinary antibiotics entering the soil environment face three primary fates: sorption, leaching, or degradation (Sarmah et al., 2006; Bradford et al., 2008). Both OTC and LIN are susceptible to strong sorption. Binding of OTC to soil has been related to ionic binding to divalent metal ions such as Ca^{2+} and Mg^{2+} (Boleas et al., 2005; Chander et al., 2005), the cation exchange capacity of organic matter (Kuchta et al., 2009), the soil clay content, and other chemical and physical properties associated with land management practices (Chander et al., 2005; Chu et al., 2010). Similarly, LIN sorbs to clay via cation exchange mechanisms (Wang et al., 2009; Watanabe et al., 2010). Sorption may occur quickly and may reduce the effectiveness of the VA (Kemper, 2008); however, soil-bound antibiotics have been shown to remain active (Kemper, 2008). For example, Chander et al. (2005) found that adsorbed OTC and tylosin were effective in inhibiting the growth of microorganisms; however, the effectiveness of these antibiotics was related to soil properties (clay content in particular). Given the clay content and cation exchange capacity of our soils, it is reasonable to assume that OTC and LIN were quickly adsorbed to the soils in both the experimental VFS and the cropland soils. A few declines in C utilization and enzyme activities were observed very early in our study (at 3 and 7 d), and this may correspond to the brief period during which a significant proportion of the VAs remained in soil solution.

Any VA effect on the soil microbial community function was short-lived. A number of abiotic and biotic processes may contribute to the degradation of antibiotics in the environment (Sarmah et al., 2006; Bradford et al., 2008). For example, a number of VAs are susceptible to photodegradation (Bradford et al., 2008), and microbial degradation via enzymatic reactions may also occur (Kemper, 2008). A half-life of 17 to 19 d has been reported for LIN (Kuchta et al., 2009) and a half-life of 30 d has been reported for OTC (Boleas et al., 2005). While care was taken to avoid photodegradation (e.g., sample jars were wrapped in foil and kept in closed boxes during incubation), microbial degradation may have occurred during the course of the experiment. Thus the VAs could be available as substrates for some members of the soil microbial community (Demoling et al., 2009); this is reflected by increased C utilization and enzyme activities between 10 and 35 d in our study (Fig. 2); however, the C-utilization and enzyme activity patterns for this time period do not differ between VA and control treatments. Therefore, these increases may simply be attributed to other changes in the soil microbial environment, in particular changes in soil moisture. Pretreatment soils had a moisture content of 25% (±2%); VA and control treatments raised the soil moisture content to 35% (±2%). This increase in soil moisture may have stimulated the soil microbial community. The declines in C uti-

Table 1. Analysis of variance results (*F* statistic) describing the effects of veterinary antibiotics (VAs) on soil microbial community function with and without vegetative filter strips (VFS). Significant results are in bold type.

Source of variation	Biolog ECO microplates				Enzyme assays	
	AWCD†	Richness	Diversity	Evenness	Dehydrogenase	Fluorescein diacetate
VFS	0.72	1.40	1.88	1.87	**11.44****	**13.22****
VA	1.11	1.10	**2.25***	0.36	**9.87‡**	1.45
VFS ´VA	1.04	0.89	**2.29****	0.87	**3.75‡**	0.82
Time	**72.18‡**	**67.65‡**	**53.25‡**	**6.55‡**	**111.46‡**	**52.35‡**
VFS × time	1.38	**2.15****	1.35	**3.38‡**	**4.89‡**	0.64
VA × time	**2.46‡**	**2.30‡**	**2.54‡**	0.65	**1.68****	**1.71****
VFS × VA × time	0.68	0.75	0.98	0.65	0.83	0.51

* Significant at the 0.05 level.

** Significant at the 0.01 level.

† Average well color development.

‡ Significant at the 0.0001 level.

lization between 35 and 49 d and FDA activity between 49 and 63 d suggest a density-dependent regulation mechanism. In other words, the microbial population increased due to environmental changes until the carrying capacity of the system was exceeded. At this point, competition and subsequent declines in microbial community function occurred. Members of the community lost due to competition became C and N substrates for those members of the community that survived and allowed microbial community function (i.e., C utilization) to recover to pretreatment levels by 63 d.

Soil Microbial Community Structure

In contrast to the results related to soil microbial function, analysis of soil microbial community structure data using ANOVA failed to reveal any significant VA treatment × time interactions (Table 2). Significant VFS treatment × time interactions were observed, however, for several variables (Table 2). In general, the soils of the agroforestry and grass VFS supported more robust populations of microorganisms than the cropland soil (Table 3). The grass VFS supported the greatest microbial biomass; likewise, populations of Gram-positive bacteria, fungi, mycorrhizae, and protozoa were greatest in the soils of the grass VFS treatment. The

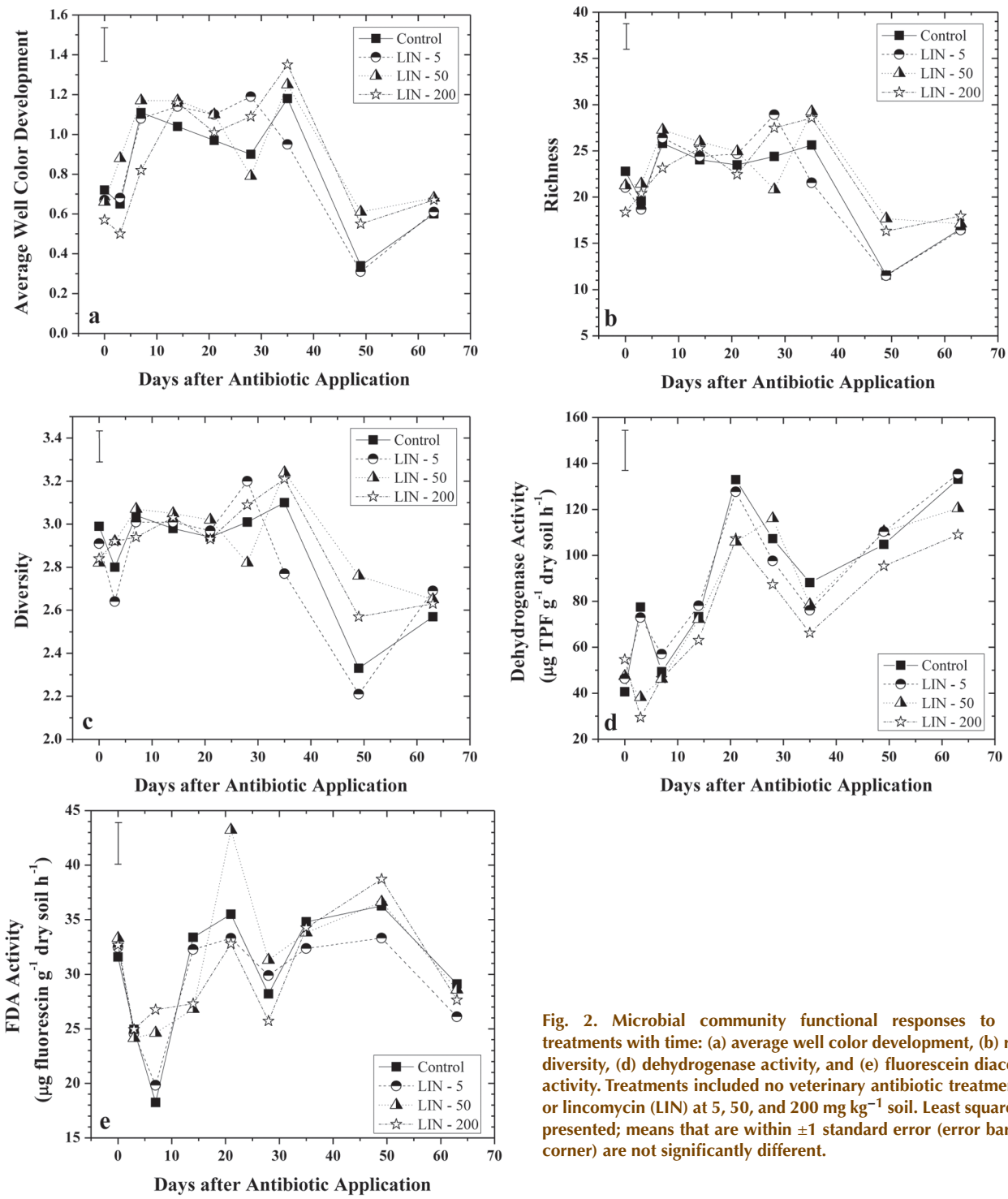

Fig. 2. Microbial community functional responses to lincomycin treatments with time: (a) average well color development, (b) richness, (c) diversity, (d) dehydrogenase activity, and (e) fluorescein diacetate (FDA) activity. Treatments included no veterinary antibiotic treatment (control) or lincomycin (LIN) at 5, 50, and 200 mg kg^{-1} soil. Least square means are presented; means that are within ±1 standard error (error bar, upper left corner) are not significantly different.

agroforestry VFS treatment supported the greatest total bacteria and had the highest responses for Gram-negative and anaerobic bacteria (Table 3). In contrast, the cropland soils supported the least microbial biomass and the lowest responses for total bacteria, Gram-positive bacteria, fungi, and mycorrhizae.

A general decline in PLFA marker response between 0 and 3 d roughly corresponds with the declines observed for the Biolog (substrate utilization) and enzyme assays (Table 4). All markers declined between 0 and 3 d; additional declines were observed in biomass, Gram-positive bacteria, Gram-negative bacteria, anaerobic bacteria, fungi, and mycorrhizae between 3 and 35 d. During this same time period, the bacteria/fungi ratio and protozoa response increased. The general decline in PLFA marker response during this time period does not appear to be reflected in soil microbial functional activity based on observed steady or increased C utilization and enzyme activity. Finally, between 35 and 63 d, Gram-positive bacteria, fungi, and mycorrhizae continued to decline, while biomass, the bacteria/fungi ratio, and the response of

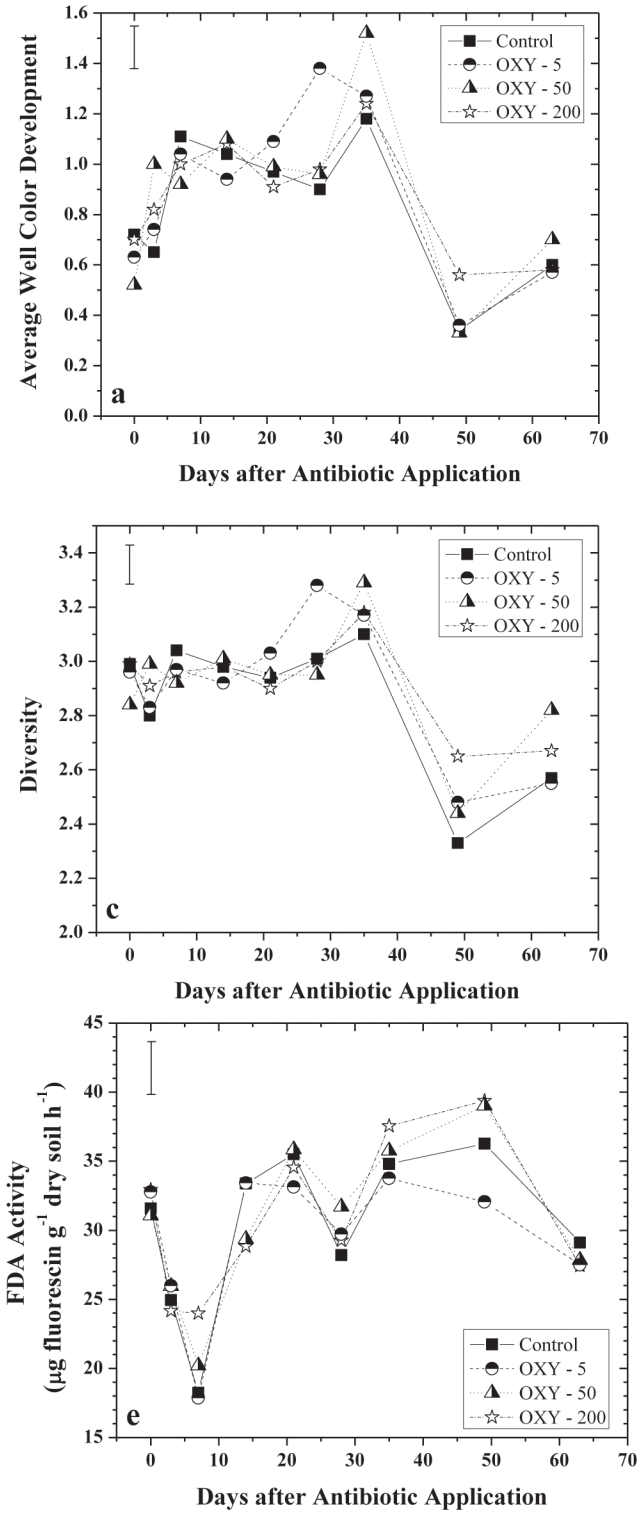

Fig. 3. Microbial community functional responses to oxytetracycline treatments with time: (a) average well color development, (b) richness, (c) diversity, (d) dehydrogenase activity, and (e) fluorescein diacetate (FDA) activity. Treatments included no veterinary antibiotic treatment (control) or oxytetracycline (OTC) at 5, 50, and 200 mg kg^{-1} soil. Least square means are presented; means that are within ±1 standard error (error bar, upper left corner) are not significantly different.

Table 2. Analysis of variance results (*F* statistic) describing the effects of veterinary antibiotics (VAs) on soil microbial community structure with and without vegetative filter strips (VFS). Significant results are in bold type.

Source of variation	Biomass	Bacteria/fungi ratio	Microbial response							Stress indicators†	
			Total bacteria	Gram − bacteria	Gram + bacteria	Anaerobic bacteria	Fungi	Mycorrhizae	Protozoa	MFA	Sat./MFA ratio
VFS	**26.08‡**	**42.69‡**	**79.51‡**	**55.02‡**	**69.58‡**	**56.86‡**	**97.41‡**	**98.36‡**	**95.22‡**	**178.13‡**	**55.95‡**
VA	1.50	1.43	1.08	0.99	1.49	0.78	1.76	1.44	0.49	**7.94‡**	**2.45***
VFS × VA	0.98	0.81	0.43	0.89	0.4	0.68	1.57	1.38	0.99	**15.1‡**	**5.94‡**
Time	**8.03‡**	**17.35‡**	**69.35‡**	**25.74‡**	**74.19‡**	**26.89‡**	**40.57‡**	**45.46‡**	**9.22‡**	1.14	1.8
VFS × time	1.00	**4.91‡**	**3.5*** **	**11.09‡**	1.75	**11.86‡**	**2.22***	**2.17***	**12.6‡**	2.38	0.95
VA × time	0.83	1.16	0.43	0.76	0.46	0.53	1.27	1.02	0.57	1.1	0.86
VFS × VA × time	0.71	**1.49***	0.68	0.55	0.85	0.3	1.34	1.2	0.77	**2.9‡**	1.37

* Significant at the 0.05 level.

*** Significant that 0.001 level.

† MFA, monounsaturated fatty acids; Sat./MFA, saturated/monounsaturated ratio.

‡ Significant at the 0.0001 level.

Gram-negative and anaerobic bacteria and protozoa continued to increase. Total bacteria remain unchanged for this final time period. Declines in Gram-positive bacteria, fungi, and mycorrhizae between 35 and 63 d may help explain the declines observed for both C utilization and enzyme activity at this time period.

The overall lack of response to VA treatment may be related to two factors: (i) the antibiotic application methods and (ii) initial differences in the soil microbial community structure. In this investigation, soils were collected from sites that lacked any previous exposure to VAs via land-applied manure. In addition, the antibiotics were applied to the soils only once during the course of the 63-d experiment and no manure or residue amendments were made. Working with sulfonamide antibiotics and a loamy sand soil from dairy farms in California, Gutiérrez et al. (2010) observed no significant effects of sulfonamides on dehydrogenase activity when the antibiotics were administered without a glucose amendment. When the glucose amendment was applied, however, significant inhibition was observed. Gutiérrez et al. (2010) speculated that a readily available C source was needed to promote microbial growth and subsequently observe effects due to the VAs. Similarly, Hammesfahr et al. (2008), working with sulfadiazine and manure-amended soils, observed a shift in the soil microbial structure following a single antibiotic application. The effects of this single antibiotic plus manure treatment were long lasting (2 mo) and the reseachers suggested that repeated applications of antibiotics, as are typically found in agricultural settings, are likely to intensify the observed effects (Hammesfahr et al., 2008). Finally, Demoling et al. (2009), working with sulfamethoxazole, found no significant difference in community structure in an unamended soil, even at the highest antibiotic concentrations. They suggested that a lag effect in the production of PLFAs might contribute to the lack of observed results. They speculated that PLFAs formed by microbial growth on added substrate are more likely to form than new PLFAs from tolerant species (Demoling et al., 2009). All of these examples are from experiments investigating sulfonamides, however, as opposed to OTC or LIN. Previous research at this site indicates significant differences in solid to solution partition coefficients (K_d values) between sulfonamides and tetracyclines. Chu et al. (2010) found OTC and sulfadimethoxine K_d values ranging from 670 to 1200 and 0.9 to 1.8 L kg^{-1}, respectively. Additionally, similar differences have been observed for sulfonamide and LIN added to smectites (Gao and Pedersen, 2005; Wang et al., 2009), the dominant mineral found in the clay fraction at this site (B. Chu, personal communication, 2012). Such differences in the sorption of sulfonamides, OTC, and LIN to soils inhibit our ability to readily state that the lack of shifts in the soil microbial community structure in our study was due to an insufficient supply of readily available C substrate or due to a single application of the chosen antibiotics. Nevertheless, future research investigating the effects of strongly sorbing VAs on soil microorganisms should consider these possibilities.

It should be further noted that the VFS and cropland soils differed in their initial soil microbial communities. Differences were most pronounced between the agroforestry VFS and the cropland soils, with the agroforestry VFS supporting larger populations of total bacteria, Gram-negative bacteria, anaerobic bacteria, and mycorrhizae than the cropland soil (data not shown). Likewise, previous studies of the soil microbial communities of the two VFS have demonstrated greater enzyme activities than the cropland system (Udawatta et al., 2008). Udawatta et al. (2008) measured FDA, β-glucosidase, β-glucosaminidase, and dehydrogenase; in every case, the agroforestry and grass VFS had greater activity levels than the cropland soils. For reasons noted above, it is possible that a single application of the antibiotics was not great enough to overcome these

Table 3. Differences in microbial community structure (as measured by phospholipid fatty acid analysis) due to vegetative filter strip treatment.

Microbial marker†	Agroforestry	Grass	Control
Biomass, g C kg^{-1} soil	318.38 b†	354.24 a	246.66 c
Bacteria/fungi ratio	3.100 b	2.659 c	3.594 a
Bacteria, mol%	0.190 a	0.183 b	0.160 c
Gram-negative bacteria, mol%	0.083 a	0.071 b	0.070 b
Gram-positive bacteria, mol%	0.106 b	0.110 a	0.091 c
Anaerobic bacteria, mol%	0.080 a	0.066 b	0.067 b
Fungi, mol%	0.065 b	0.072 a	0.046 c
Mycorrhizae, mol%	0.065 b	0.071 a	0.046 c
Protozoa, mol%	0.007 c	0.010 a	0.009 b
Monounsaturated fatty acids, mol%	0.309 c	0.452 a	0.367 b
Saturated/monounsaturated ratio	5.329 c	13.933 a	5.329 c

† Means followed by different letters are significantly different ($\alpha = 0.05$).

Table 4. Differences in microbial community structure (as measured by phospholipid fatty acid analysis) with time.

Microbial marker†	0 d	3 d	35 d	63 d
Biomass, g C kg^{-1} soil	353.41 a†	292.74 bc	270.47 c	309.09 b
Bacteria/fungi ratio	2.83 c	2.95 bc	3.07 b	3.61 a
Bacteria, mol%	0.200 a	0.181 b	0.164 c	0.166 c
Gram-negative bacteria, mol%	0.082 a	0.072 c	0.068 d	0.076 b
Gram-positive bacteria, mol%	0.116 a	0.108 b	0.095 c	0.090 d
Anaerobic bacteria, mol%	0.079 a	0.069 c	0.064 d	0.072 b
Fungi, mol%	0.073 a	0.065 b	0.057 c	0.050 d
Mycorrhizae, mol%	0.072 a	0.065 b	0.057 c	0.049 d
Protozoa, mol%	0.008 b	0.008 b	0.009 a	0.009 a
Monounsaturated fatty acids, mol%	0.376 a	0.382 a	0.379 a	0.367 a
Saturated/monounsaturated ratio	9.601 a	9.344 ab	8.801 ab	7.499 b

† Means followed by different letters are significantly different (a = 0.05).

initial differences. For significant changes in soil microbial structure to be observed, the VAs would have to have greatly altered the soil community structure of the VFS. Slight changes in microbial community structure due to VAs would fall within the overall community variation and not result in any significant changes.

Antibiotic Resistance

Minimum inhibitory concentrations (the lowest concentration of VA preventing growth) was determined for all Gram-negative (*E. coli*) and Gram-positive (*Enterococcus*) strains isolated from soils treated with OTC and LIN, respectively (Tables 5 and 6). It must be noted that MICs were calculated following selective enrichment of the bacterial community, methods that have been shown to bias the selection of isolates (Bruhn et al., 2005; Gorski, 2012). This bias, along with the knowledge that the two bacterial groups examined (*E. coli* and *Enterococcus*) represent but a small fraction of the total microbial community of the soil, may result in a failure to detect antibiotic resistance change occurring among other populations. The *E. coli* and enterococci were selected for resistance screening due to their clinical relevance, the propensity of these bacterial groups for the development of resistance (Hayes et al., 2003; Tadesse et al., 2012), and their reported ability to proliferate in soil environments (Byappanahalli and Fujioka, 2004).

Current clinical interpretive standards for *E. coli* state that an isolate showing MIC ≤4 mg L^{-1} is susceptible to tetracycline antibiotics (including OTC); isolates with MICs of 8 mg L^{-1} are classified as intermediate in resistance, and isolates with MICs ≥16 mg L^{-1} show high-level resistance (Clinical and Laboratory Standards Institute, 2010). Increased resistance resulting from a VA treatment might be revealed through a corresponding increase in the median MIC of bacterial isolates. Of the 12 VFS × VA treatments investigated, a total of eight (one agroforestry, three grass, and four cropland) showed a pattern of significantly ($P < 0.001$) increased median MIC from Days 0 to 63 (Table 5); however, the three sites with no OTC added were among those showing the most striking increases in mean MIC from the beginning to the end of the study. The microbial response to the applied concentration ranges of OTC were inconclusive; agroforestry VFS showed a significant (P

Table 5. Minimum inhibitory concentration (MIC) for Gram-negative bacteria isolated from soils treated with oxytetracycline at 0, 5, 50, or 200 mg kg^{-1}. Numbers represent median MICs of all isolates from each sample.

Site	Day of soil collection	Total isolates screened	Minimum inhibitory concentration			
			No OTC	OTC 5	OTC 50	OTC 200
		no.	mg L^{-1}			
Agroforestry	0	32	2	2	4	2
	3	28	2	1	2	1
	21	32	8†	4	1	2
	35	32	2	8†	2	1
	63	32	32‡	2	1	1
Grass	0	32	2	1	16‡	1
	3	32	2	2	1	16‡
	21	28	2	2	1	1
	35	32	8†	8†	8†	16‡
	63	32	16‡	8†	8†	32‡
Cropland	0	32	4	2	2	2
	3	32	2	1	1	2
	21	30	2	2	2	1
	35	32	8†	8†	4	4
	63	32	32‡	32‡	16‡	16‡

† Samples with isolate median MIC classified as intermediate in resistance (MIC = 8 mg L^{-1}).

‡ Samples with isolate median MIC classified as highly resistant (MIC ≥ 16 mg L^{-1}).

= 0.009) decrease in MIC from the control samples (no OTC) to the highest OTC concentration (i.e., 200 mg kg^{-1} soil) during the length of the study, while the grass VFS showed significant increases in MIC across the same treatments ($P = 0.044$). Only the cropland soils showed a strong trend of increased median MIC from Days 0 to 63 under all antibiotic applications, but the same trend was revealed in the cropland soils with no antibiotic added (Table 5).

The U.S. Clinical and Laboratory Standards Institute does not provide a published susceptibility range for Gram-positive bacteria exposed to LIN; however, the Public Health Agency of Canada classifies isolates with MICs ≥8 mg L^{-1} as highly resistant (Public Health Agency of Canada, 2004). All samples (including those with no added LIN) showed highly resistant median MICs at one or more times during this study (Table 6). Lincomycin resistance was significantly correlated with sampling date overall ($P = 0.002$), with the highest resistance levels found at 35 d, but decreasing in most sites by 63 d. Resistance levels were not significantly related to the applied VA concentration in agroforestry VFS, grass VFS, or cropland soils ($P = 0.385$, 0.945, and 0.270, respectively).

No increases in bacterial resistance were detected following antibiotic selective pressure by OTC or LIN, and high proportions of resistant Gram-positive and Gram-negative bacteria were detected in most study sites, including those with no added VAs. The prevalence of highly resistant organisms in the majority of sites studied was not surprising. Many studies have reported a diversity of antibiotic resistance genes in natural environments and have suggested that the primary ecological role for naturally produced antibiotics in soils is likely to be in inhibiting the growth of competing bacteria (Martínez, 2008). Båverud et al. (2004) reported OTC MICs averaging ≥32 mg L^{-1} in *Clostridium* spp. isolated from Swedish soils, and high levels of LIN resistance have also been reported in soil microbial communities (Gonzalez et al., 1999; Čermák et al., 2008).

Many studies have suggested a correlation between increased bacterial resistance levels in aquaculture and antibiotics used at the farms (DePaola et al., 1988; Guardabassi et al., 2000), yet studies in soils have largely failed to reveal clear-cut increases in microbial resistance following VA exposure (Hund-Rinke et al., 2004; Schmitt et al., 2006; Rodríguez-Sánchez et al., 2008). Exposure to anthropogenic metals rather than antibiotics is also thought to be important in selecting for antibiotic-resistant bacteria (Berg et al., 2005), suggesting that resistance acquisition can occur independently of antibiotic use.

Conclusions and Future Directions

The VAs OTC and LIN did not have a strong effect in this system. While a few changes in microbial function were observed, these changes were quickly mitigated. Community structural changes due to antibiotic application were not observed, nor were increased antibiotic resistance. It is probable that OTC and LIN were adsorbed quickly to the clay and organic matter fractions of the soil. This action may have resulted in reduced antimicrobial function and may have facilitated the biological degradation of these compounds. A single VA dose administered in the absence of any readily available C source (e.g., manure or residue) appears to be ineffective in altering the soil microbial community structure. Pretreatment differences in microbial community structure due to management imposed during the past 10+ yr appear to have superseded any effects of the VA treatments. Finally, the observed antibiotic resistance could not be linked to the OTC or LIN treatments. Vegetative filter strips may be effective in mitigating VAs; however, further investigations are warranted to determine whether multiple VA inputs to VFS during sporadic runoff events has a significant effect on soil microbial structure, function, and antibiotic resistance. Additional studies should examine the combined effects of VAs and soil amendments (i.e., manure), as well as the effect of multiple VA doses.

Table 6. Minimum inhibitory concentration (MIC) for Gram-positive bacteria isolated from soils treated with lincomycin at 0, 5, 50, or 200 mg kg^{-1}. Numbers represents median MICs of all isolates from each sample.

Site	Day of soil collection	Total isolates screened	Minimum inhibitory concentration			
			No LIN	LIN 5	LIN 50	LIN 200
		no.	mg L^{-1}			
Agroforestry	0	14	4	8†	4	4
	3	12	4	8†	8†	2
	21	20	8†	8†	2	8†
	35	28	8†	8†	4	8†
	63	32	4	1	2	1
Grass	0	14	4	4	4	4
	3	16	2	4	4	2
	21	12	8†	2	2	8†
	35	32	0.5	4	8†	8†
	63	32	4	8†	4	8†
Cropland	0	16	2	4	8†	2
	3	24	1	4	2	4
	21	16	2	1	4	1
	35	20	8†	8†	8†	8†
	63	28	2	2	8†	4

† Samples with isolate median MIC classified as highly resistant (MIC ≥ 8 mg L^{-1}).

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