



## Article

# Oxytetracycline and Monensin Uptake by Tifton 85 Bermudagrass from Dairy Manure-Applied Soil

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**Abstract:** To address concerns regarding the potential impact of antibiotic use in animal husbandry on antibiotic resistance in humans, we conducted a greenhouse-based study examining uptake of the veterinary antibiotics oxytetracycline (OTC) and monensin (MON) by Tifton 85 Bermudagrass (T85), the most commonly grown forage grass in the southeastern U.S.A. Since oxytetracycline is used in both veterinary and human medicine, its accumulation in animal products could impact human resistance to this antibiotic. Monensin is not used in human medicine but has a high potential for accumulating in the environment. Our research examined antibiotic uptake by forage grass T85, the effect of dairy manure application on its uptake, and antibiotic retention in soil. We compared unspiked, wet dairy manure to wet dairy manure spiked with MON or OTC that was soil surface applied to pots or incorporated into soil. After 6 wk, plant stem/leaf and root tissue, as well as soil samples, were assessed for antibiotic residues using enzyme-linked immunosorbent assay (ELISA). Results confirmed Tifton 85 MON and OTC uptake. Six weeks after adding the antibiotics, the greatest plant matter OTC and MON contents were  $157.9 \pm 70.6$  and  $234.4 \pm 19.6 \mu\text{g kg}^{-1}$ , respectively, and 17.6 and  $369.5 \mu\text{g kg}^{-1}$ , respectively, for soil. When spiked with OTC, manure incorporation led to decreased OTC uptake by T85 tissue. Bioaccumulation of these antimicrobials in livestock and in the environment is a potential concern for animal, environmental, and human health.

**Keywords:** antibiotics; monensin; oxytetracycline; Tifton 85; dairy manure; ELISA; surface application; incorporation; forage

## 1. Introduction

Antimicrobial use in animal production is a major contributor to the increase in antibiotic resistance [1]. A wide range of antimicrobial drugs are permitted for use in food and animal production in the U.S.A. as well as in other countries. Classes of antimicrobials used in cattle production include polypeptides, tetracyclines, macrolides, ionophores and sulfonamides [2]—almost all have some importance in human medicine, except for monensin (MON). It is not now, nor expected to be in the future, of importance to human medicine. Tetracycline is used to treat mastitis in cows and scours in calves, while the ionophore MON (commercially known as rumensin) can be used to treat coccidiosis [3], but is also commonly provided to cattle as a component of their feed to enhance growth and decrease methane release associated with rumination [4]. Of the 15,576,975 kg of various classes of antibiotics sold for domestic livestock use in 2015 [5], the ionophore class, including MON, accounted for 30%, while tetracycline accounted for 44% of sales.

Concentrated animal feeding operations (CAFOs) can be a significant source for antibiotic entry into the environment. Dairy operations in the U.S.A. produce 133 million Mg of manure per year (on a dry weight basis) [6]—most of which is land applied. This manure can be a significant source of antibiotic entry into the environment since these medications are not completely metabolized within treated animals. Approximately 23% of the oxytetracycline (OTC) and 40% of the MON administered to cattle ends up in their waste [7]. Antibiotics in land-applied manure can leach into the ground water, runoff into surface water, be degraded by the environment [8–10], or be taken up by plants. Manure–soil interactions are key factors affecting the environmental behaviors of manure-associated antimicrobial resistant elements such as antibiotics [11].

Potential environmental and human health impacts of land-applied manure contaminated with antimicrobials depends on their concentration in manure, method of land application, soil-based biological and chemical degradation processes, sorption of the antimicrobials to soil particles and plant uptake. When manure is soil surface applied, most manure aggregates are concentrated in the upper few centimeters, with minimum manure–soil mixing, resulting in immediate antimicrobial photodegradation, oxidation, sorption/desorption and leaching susceptibility [12]. Manure incorporation, typically through tillage, encourages the formation of manure–soil aggregates that can reduce the mobility of antimicrobials from manure that interact with soil organic and inorganic components to form less soluble complexes. Incorporation of manure below the soil surface also means that photodegradation becomes significantly reduced [13].

Most tetracyclines are sparingly water soluble and both tetracyclines and MON have strong binding affinities for clay minerals and soil organic matter [14]. Microbial degradation of these antimicrobials is affected by soil, type of manure, and climate conditions. Under field conditions, the reported half-life of tetracycline ranges from 18 to 79 days [15]. In two greenhouse-based studies [16] OTC was reported to have a 23-d [17] or a 33-d [16] half-life in manure-amended soil, compared to 31.5–43.3 d [17] or 56 d [16] in non-amended soils. Tetracyclines are also susceptible to photodegradation since they can effectively absorb light [18]. Soil sorption of MON is strongly influenced by soil organic matter and pH, with greatest sorption being observed on low pH soils with high levels of organic matter [10]. In studies of long-term surface applications of poultry litter to 1:1 silicate soils, MON sorption decreased with increased applications due to the subsequent increase in pH [19]. The half-life in soil for MON is considerable shorter than that of tetracyclines. Under greenhouse conditions, the half-life for MON was 13.5 d, while the MON half-life was 3.3 d with manure added and 3.8 d without manure under field conditions [20].

Tetracycline is readily taken up by vegetable crops. In an examination of vegetables fertilized with antimicrobial-containing manure, OTC uptake levels averaged  $76.8 \mu\text{g kg}^{-1}$  across all plants [21]. When  $1 \text{ mg kg}^{-1}$  of OTC was added to soil, uptake levels of  $7.2$  and  $<23 \mu\text{g kg}^{-1}$  OTC were assessed in lettuce and carrot, respectively [22]. In contrast, no detectable levels of OTC were found when 5 and  $10 \text{ mg kg}^{-1}$  of antibiotics were spiked into soil in a greenhouse study using a commercially available mixture of grass species seeds (75% English Ray Grass, *Lolium perenne*, 25% Field Meadow Grass, *Poa pratensis*, *Poa trivialis*) [23].

Only one study has examined plant MON uptake. Researchers [24] applied raw or composted hog or turkey manure to field plots where a variety of vegetable plants were grown. While both sulfamethazine and tylosin showed significant plant uptake, MON uptake levels in all plants were not significantly greater than the levels of quantification. Authors attributed limited plant uptake of MON to its rapid degradation rate in soil.

In the southeastern U.S.A., pastures and fields receiving farm manure applications are often planted to *Cynodon* spp. cv. Tifton 85 (T85) [25], which is among the most widely grown forage grasses in many tropical and subtropical regions. While studies on vegetable crops have shown plant uptake of tetracycline [26] and MON [24] when manure containing these antimicrobials are applied to soil, similar studies have not been conducted with T85. Previous plant uptake studies using T85 have been limited to nutrient uptake assessments [27]. Since T85 is often fed back to dairy

cows [28], it is possible that antibiotics in forage could be recycled back to farm animals and then back to the soil. Plant uptake and bioaccumulation of antibiotics could result in antibiotic introduction into the human food supply. Antibiotics have also been identified in dairy milk [29], while antibiotic resistant tetracycline genes have been identified in the meat [30] and milk [31] of cattle. Monensin from chicken manure might pose an environmental risk, under certain conditions, to soil invertebrates and microbial communities [32,33] while tetracyclines can also impact microbial activity and diversity in soil [34,35]. Although environmental antibiotic contents are typically below acute toxicity levels, very little is known about the risks associated with chronic low-level exposure or how the effects can be compounded by other natural or anthropogenic factors [36].

To address these concerns, we investigated antibiotic uptake by T85 and retention in soil with and without wet unspiked dairy cow manure, or dairy cow manure spiked with OTC or MON. In addition, we investigated the impact of varying application methods, surface or incorporated manure application, on plant uptake and soil retention of antibiotics. Our objective was to obtain insight into the potential for forage uptake and bioaccumulation of veterinary antibiotics which could impact human populations through ingestion of animal products with accumulated antimicrobial residues. We also investigated the effect of manure application method on the uptake and retention of antibiotics in order to identify potential farm management practices that could mitigate against their introduction into the food supply. Our hypothesis was that, whenever present, antibiotics would be taken up by T85 tissue and using the incorporation method for manure would lead to increased antimicrobial degradation and/or retention in soil.

## 2. Materials and Methods

### 2.1. Site Description, Treatments and Greenhouse Conditions

The experiment was conducted at the Texas A&M AgriLife Research and Extension Center in Stephenville, TX USA. It was performed in a greenhouse using Windthorst (fine, mixed, active, thermic Udic Paleustalfs) [37] topsoil that had not received manure applications for at least 5 y. Soil was collected 0 to 20 cm deep, homogenized, and analyzed for initial nutrient characteristics by the Texas A&M University Soil, Water and Forage Testing Laboratory (Table 1). Each pot received approximately 9.75 kg of soil which was watered to saturation (field capacity) once weekly and again daily whenever soil appeared dry. Greenhouse temperatures varied from 20 °C in the morning to 29 °C in the evening. Temperature in the center of the pots varied from 35 °C diurnal to 16 °C nocturnal.

**Table 1.** Nutrient concentration of soil and manure prior to planting.

	pH	Eh	NO <sub>3</sub> <sup>−</sup>	P	K	Ca	Mg	S	Na	Fe	Zn
	ppm										
Soil	5.3	120	7	16	92	403	91	6	4	12.24	0.15
DM	OTC	MON									
88.2%	1.0 μg kg <sup>−1</sup>	2.5 μg kg <sup>−1</sup>									
DM	OTC	MON				%				ppm	
Manure	84%	0.009 μg kg <sup>−1</sup>	3.2 μg kg <sup>−1</sup>	1.94	0.38	0.43	1.26	0.64	0.15	4345	107.3

Manure was collected from approximately 30 to −50 fresh cow piles from mature dairy cows in lactation fed a nutritionally complete ration at the Tarleton University Southwestern Dairy Center, Stephenville TX, USA. The cows were housed on concrete flooring, with feed and water were available for them in concrete feeders. Collection methods ensured that manure was not in contact with the concrete flooring or bedding materials. The dairy managers indicated to us that they administered neither of the antibiotics that we spiked into the collected manure. The manure was homogenized

prior to application and a subsample taken for testing at the Texas A&M University Soil, Water and Forage Testing Laboratory (Table 1).

The manure was either incorporated into the pot soil, applied to the surface or absent (control). Each of the manure application treatments had sub-treatments of unspiked (no antibiotics added), spiked with OTC, or spiked with MON. OTC was applied to the appropriate pots at  $11.3 \text{ mg kg}^{-1}$  and MON at  $5 \text{ mg kg}^{-1}$  [6,38–41]. Powdered MON was mixed into manure or soil as applicable prior to sprig transplant. Liquid OTC was pipetted into manure or soil as applicable and mixed for even distribution.

T85 stolons were collected from a field at the Texas A&M AgriLife Research Center, Stephenville TX USA that had been in T85 pasture for 12 y. The sprigs were washed to remove all soil from roots, trimmed to a uniform 30 cm length, planted three sprigs per pot at 6 cm depth. Any stolons that did not sprout after 2 weeks were replaced with fresh T85 sprigs, pruned and trimmed to meet original specifications.

## 2.2. Experimental Design

In total, 72 pots with  $23,000 \text{ cm}^3$  capacity (height: 29.2 cm, diameter: 35.6 cm), were washed with 10% bleach solution and then filled with 9.75 kg of soil (8.6 kg on a dry matter basis). Each pot was labelled and fitted with a tray to prevent uptake of leaked water by any of the adjacent pots on the tables. Each pot served as an experimental unit, with pots placed on two greenhouse benches in a randomized complete design four replications ( $n = 4$ ). Eighteen different treatment combinations were set up as described in Table 2. These included combinations of manure (+/−), antibiotic (OTC or MON or None), T85 (+/−), and application method (incorporated, surface applied) in a greenhouse.

**Table 2.** Treatment identification and specifications ( $n = 4$ ).

Treatment	Manure Application	Antibiotic Spike	Tifton 85	Manure
1	Incorporated	None	Present	Present
2	Surface applied	None	Present	Present
3	Incorporated	Oxytetracycline	Present	Present
4	Surface applied	Oxytetracycline	Present	Present
5	Incorporated	Monensin	Present	Present
6	Surface applied	Monensin	Present	Present
7	Incorporated	None	Absent	Present
8	Surface applied	None	Absent	Present
9	Incorporated	Oxytetracycline	Absent	Present
10	Surface applied	Oxytetracycline	Absent	Present
11	Incorporated	Monensin	Absent	Present
12	Surface applied	Monensin	Absent	Present
13	N/A	None	Present	Absent
14	N/A	Oxytetracycline	Present	Absent
15	N/A	Monensin	Present	Absent
16	N/A	None	Absent	Absent
17	N/A	Oxytetracycline	Absent	Absent
18	N/A	Monensin	Absent	Absent

Plants were harvested at 6 wk, and separated into stem, leaf, and root plant tissue, with an average total green weight of 110.8 g. Plant tissue samples were washed with sterile phosphate-buffered saline

to remove all soil and manure and stored in freezers at  $-20^{\circ}\text{C}$ . Soil samples were collected randomly within pots at 0 to 10 cm depth and ~5 cm from plant roots. For each applicable pot, all stem/leaf and root tissue from the three sprigs were combined, meaning that each treatment with Tifton 85 present had a total of three sprigs X four replications, or 12 samples. Each replicate, consisting of samples from three sprigs, was used for one composite treatment analysis (stem/leaf or root) and subsequent statistical analysis. Soil sampling taken from each pot was performed in triplicate and the composite sample was then used for analysis (three soil samples X four replications).

### 2.3. Monensin and Oxytetracycline Antibiotic Residue Analyses

Antibiotic uptake was quantified using the enzyme-linked immunosorbent assay (ELISA): a simple but sensitive and accurate technique for quantifying antigens based on a colorimetric analysis [42]. This test, commonly used in immunology to detect antibodies and antigens, uses an enzyme and substrate that reacts with antibodies to form colored products. Many researchers continue to use the ELISA technique due to its reliability and lack of clean up requirements [43]. A competitive ELISA test was used to quantify antibiotic residue contents in plant and soil samples. Commercially available kits were purchased from Abnova Corporation (Taipei, Taiwan) and Reagen LLC (Moorestown, NJ, USA) to perform the MON and OTC assays, respectively. More details can be found in the supplementary files (Supporting information S1).

### 2.4. Monensin Plant Tissue, Soil and Manure Extractions

MON extraction methods were adapted from previous research [24,44]. MON in plant tissues was extracted by adding 2 mL buffered peptone water (BPW, pH 7.0) to 1 g homogenized plant tissue. The mixture was vortexed for 1 min then put on a shaker for 1 h. It was then centrifuged at  $5000\times g$  for 15 min. The supernatant was extracted and stored in a freezer at  $-20^{\circ}\text{C}$  until ready for analysis.

MON was extracted from soil and manure by adding 4 mL BPW to 2 g of soil or manure then vortexing for 1 min. The suspensions were mixed on a shaker for 1 h then centrifuged at  $5000\times g$  for 15 min. The supernatant was extracted and stored at  $-20^{\circ}\text{C}$  until needed.

### 2.5. Oxytetracycline Plant Tissue, Soil and Manure Extractions

OTC extraction protocols were adapted from Reagen ELISA Test Kit RND99049 instructions, received on Nov 2016. To extract plant tissue, 3 mL 1X OXYTET extraction buffer was added to 1 g of the homogenized sample, vortexed for 10 min in a multitube vortexer and centrifuged for 10 min at  $4000\times g$ . Then, 200  $\mu\text{L}$  supernatant was transferred to a new tube containing 800  $\mu\text{L}$  1X OXYTET Feed Balance Buffer A and vortexed for 1 min. Each assay used 75  $\mu\text{L}$  per well (dilution factor 15). To extract soil for the assay, 4 mL of water was added to 2 g of each sample, vortexed and centrifuged at  $4000\times g$  for 5 min. A representative 100  $\mu\text{L}$  supernatant sample was added to a new tube with 300  $\mu\text{L}$  1X PBS MIX and 75  $\mu\text{L}$  was used per well for the assay (dilution factor 8).

### 2.6. ELISA Testing Protocol

The MON testing protocol was adapted from previous ELISA studies [24,43–45]. A standard curve was created and used to test extracts from the different sample types. The average absorbance (ODs) for each standard content of MON including 0 ng/mL (OD0) was then calculated. Percentage of Inhibition ( $100 - (\text{ODs}/\text{OD0}) \times 100$ ) was next and then the values of % of Inhibition were plotted against their corresponding contents. MON sample contents were then calculated by interpolation and multiplying by the sample's dilution factor to obtain the final content of MON. More details on conversion of antibiotic ELISA concentration to ELISA concentration samples can be found in supplementary information (Supporting information S2). The OTC testing protocol can be found in supplementary information (Supporting information S3). The  $R^2$  values and linear equations for all plates used can be found in the supplementary information (Table S1). The antibiotic concentrations in the sample extraction solutions can be found in the supplementary information (Tables S2 and S3).

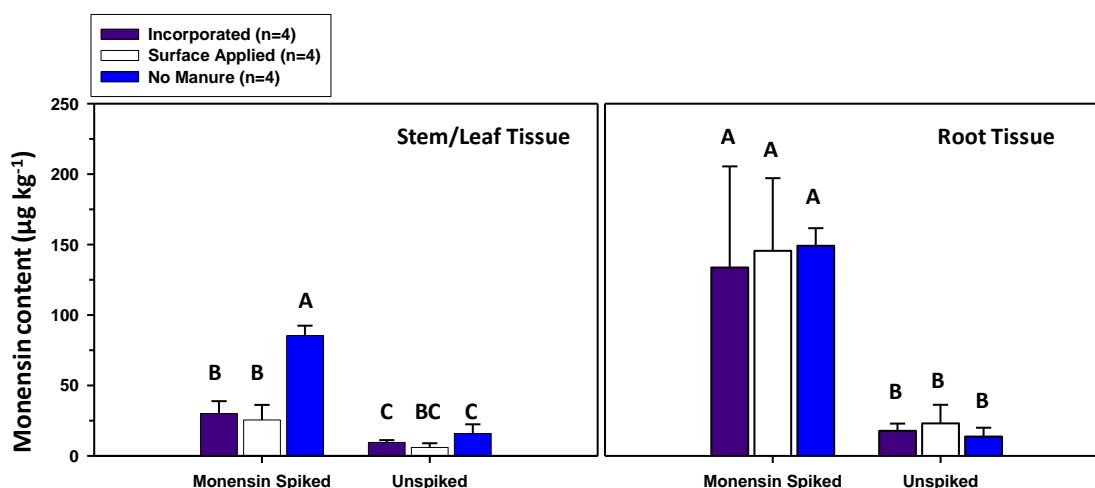
## 2.7. Statistical Analysis

Data were processed using JMP®Pro, Version 15 [46]. One-way ANOVA was performed with mean comparison for all pairs using Tukey–Kramer Honestly Significant Different (HSD) least significant multiple mean separation. Samples were separated by type (plant leaf/stem tissue and soil) and antibiotic (MON or OTC) initially, before applicable combinations of manure +/–, antibiotic +/–, T85 +/– and application method (where applicable) were compared and differences with  $p \leq 0.05$  were considered significant. Each subset of samples contained 24 samples in total, grouped based on treatment combination, that were all compared to each other.

## 3. Results and Discussion

### 3.1. Monensin Content in Herbage and Soils

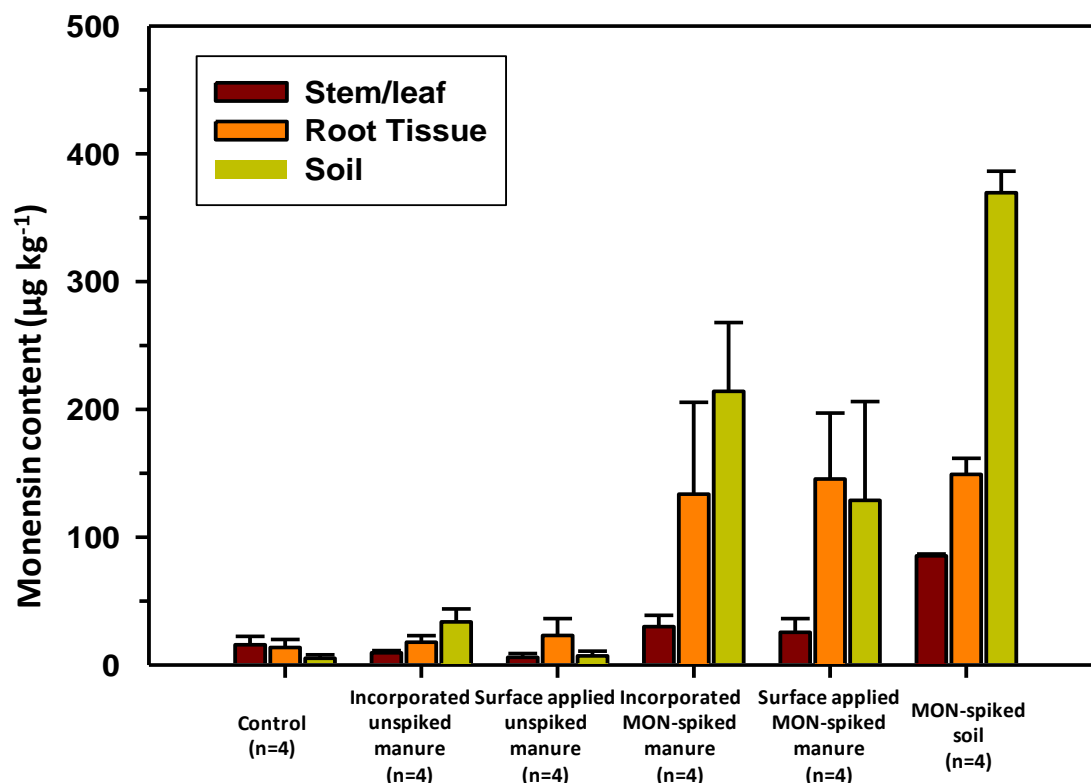
When MON was spiked, uptake by the root tissue occurred and was similar among treatments. Direct soil spiking of MON resulted in higher MON content in stem/leaf tissue ( $85.3 \pm 7.1 \mu\text{g kg}^{-1}$ ) when compared to plant tissue harvested from both MON spiked surface applied and soil incorporated manure treatments ( $p < 0.0001$ ) (Figure 1).



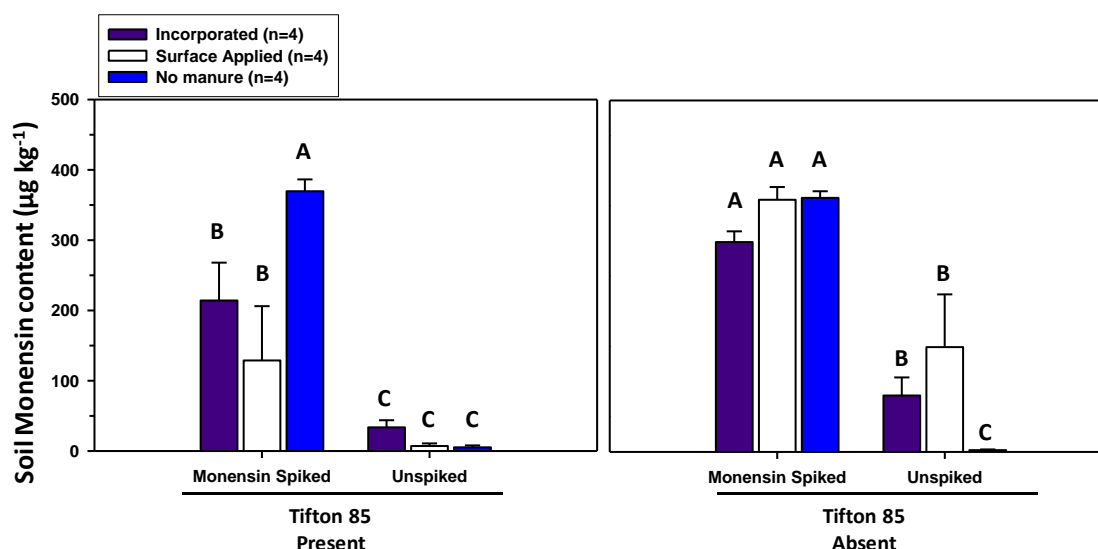
**Figure 1.** Tifton 85 plant tissue monensin content comparisons. The monensin content was measured using ELISA 6 weeks after treatment with direct soil monensin spiking ( $5 \text{ mg kg}^{-1}$ ), surface application of monensin-spiked manure ( $5 \text{ mg kg}^{-1}$ ), incorporation of monensin-spiked manure ( $5 \text{ mg kg}^{-1}$ ), unspiked manure, and no manure/no monensin treatments. Each error bar was constructed using standard deviation. Values with the same letter on top of columns do not differ according to Tukey–Kramer Honestly Significant Different (HSD) least significant multiple mean separation ( $p \leq 0.05$ ).

No MON uptake was assayed in either root or stem/leaf tissue when MON was not spiked. The lowest T85 stem/leaf tissue mean MON content ( $6.0 \pm 3.0 \mu\text{g kg}^{-1}$ ) was observed in plants from the pots with surface applied unspiked manure (Figure 2). When T85 was present, plant stem/leaf tissue MON uptake was consistent with MON levels in soil at the time of harvest. The MON-spiked, no-manure treatment, exhibited greater levels of both soil MON content ( $361.4 \mu\text{g kg}^{-1}$ ) (Figure 3), and MON stem/leaf tissue uptake ( $85.3 \pm 7.1 \mu\text{g kg}^{-1}$ ) (Figure 1) compared to either the MON-spiked manure, incorporated or surface-applied manure treatments for both of those sample types (soil and stem/leaf tissue respectively).





**Figure 2.** Monensin content in soil and herbage samples. The brown color represents T85 stem/leaf tissue, the orange color represents T85 root tissue and olive represents soil samples. Control samples have no MON or manure applied. A list of all concentrations can be found in Supplemental Tables S4 and S5. Each error bar was constructed using standard deviation. MON = Monensin.



**Figure 3.** Soil monensin content comparisons. The soil monensin content was measured using ELISA 6 weeks after treatment with direct soil monensin spiking ( $5 \text{ mg kg}^{-1}$ ), surface application of monensin-spiked manure ( $5 \text{ mg kg}^{-1}$ ), incorporation of monensin-spiked manure ( $5 \text{ mg kg}^{-1}$ ), unspiked manure, and no manure/no monensin treatments. Each error bar was constructed using standard deviation. Values with the same letter on top of columns do not differ according to Tukey–Kramer Honestly Significant Different (HSD) least significant multiple mean separation ( $p \leq 0.05$ ).

The lower MON plant stem/leaf tissue uptake when manure was added may be due to its rapid decomposition [47,48]. Monensin is highly soluble, which enhances its degradation rate [7] and

potential for leaching [49]. Its addition to soil or manure also increased uptake by T85 root tissue when compared to root tissue harvested from control soil pots ( $p \leq 0.003$ ). Varying the application method of MON-spiked manure did not impact the uptake by root tissue. Root uptake of MON in the MON-spiked plain soil treatment was greater than the surface applied or soil incorporated MON-spiked manure treatments ( $p \leq 0.002$ ) (Figure 1).

The greatest mean average for MON uptake by root tissue was almost double that seen in the greatest stem/leaf tissue (Figure 2). This is expected, as the plant roots are in initial contact with these antibiotics before translocation to aerial herbage. When plants absorb and abate antibiotics in soil, roots show the greatest absorption capacity [50].

In the three treatments where MON was applied with T85 present (5, 6 and 15), the MON stem/leaf contents were 30, 25.5 and 85.3  $\mu\text{g kg}^{-1}$ , respectively. In contrast, the greatest plant uptake contents obtained by [24] was 3.0  $\mu\text{g kg}^{-1}$ . Their uptake study involved growing spinach, lettuce, cabbage, carrot, radish, onion, garlic, tomato, green bell pepper, sweet corn, and potato in field plots harvested at peak maturity. The greatest content level of MON plant uptake was obtained in plots where the spiked turkey manure was applied at 227  $\text{g ha}^{-1}$  (0.34  $\text{mg kg}^{-1}$  compared to our application rate of 5  $\text{mg kg}^{-1}$ ). The approximately 0.88% MON uptake rate reported by [24] and 1.7 % MON uptake rate for our study's greatest stem/leaf tissue uptake content can be attributed to the rapid degradation rate of MON in soil [10], the short time allotted for the experiment, and potential environmental differences in greenhouse vs. field studies. The relatively low percentage of soil-available MON taken up by plants can be attributed to the strong tendency of MON to attach to soil cation exchange sites [10].

Monensin has a LD50 of 35  $\text{mg kg}^{-1}$ . In our experiment, the greatest mean combined stem, leaf and root tissue content of MON was 234.4  $\mu\text{g kg}^{-1}$  when MON was spiked at 5  $\text{mg kg}^{-1}$ . The antibiotic daily intake value (ADI), or the level that can be ingested daily by humans over a lifetime without any health risks, is 10  $\mu\text{g kg}^{-1}$  body weight  $\text{day}^{-1}$  for MON [26] while the maximum residue limit (MRL) in animal tissue is 2–100  $\mu\text{g kg}^{-1}$  raw weight of animal tissue [24]. Based on an average cow, weight of 753 kg, the greatest MON uptake content measured in this experiment, and assuming that all the antibiotic residue consumed from the forage grass deposits in the meat or milk, cows would need to consume roughly close to half of their body weight (321 kg) in fresh weight of T85 to create alarm for consumers ingesting their products.

MON content in soil was affected by MON +/-, manure application method and T85 +/- . When comparing MON treatments with T85 present, the plain soil-spiked MON treatment resulted in soil MON contents that were higher than the control soil, surface applied and incorporated MON-spiked manure ( $p < 0.0001$ ). The two latter treatments also resulted in soil MON contents that were higher than the background ( $p \leq 0.004$ ), but they were no different from each other (Figure 3). When comparing MON treatments with T85 absent, all three MON-spiked treatments (bare soil, incorporated and surface-applied manure) resulted in soil MON contents greater than the control soil ( $p < 0.0001$ ). Without T85 present, surface applied and incorporated unspiked manure resulted in higher levels of the antibiotic in soil when compared to the control soil ( $p \leq 0.05$ ) but there was no difference among treatments (Figure 3). The lower content of the antibiotic in soil when manure was present is consistent with previous research that showed manure and other organic matter enhances MON degradation [10,51].

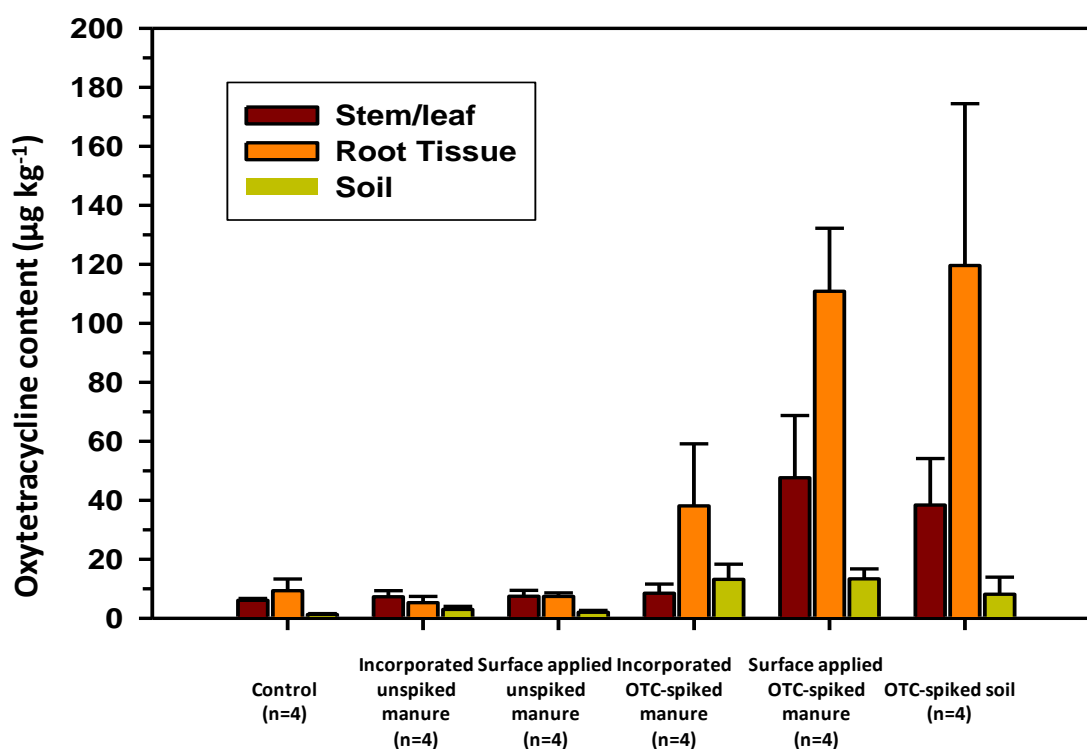
Manure application method had no effect on soil MON content regardless of whether MON was spiked or not. Since plant uptake of MON was greatest when MON was added without manure, the lower level of MON in manure-amended soils indicates that the manure enhanced MON degradation. However, there was no difference in soil MON content when the manure was incorporated compared to soil surface applied. When MON-spiked manure was surface applied to soil with T85 absent, soil MON content was greater than the same treatment with T85 present ( $p < 0.0001$ ). The same trend was seen when comparing MON-spiked manure that was incorporated with T85 absent to the same treatment with T85 present ( $p = 0.04$ ). These results again indicate that conditions provided by the manure cause MON breakdown and T85 bioaccumulation potential [10].



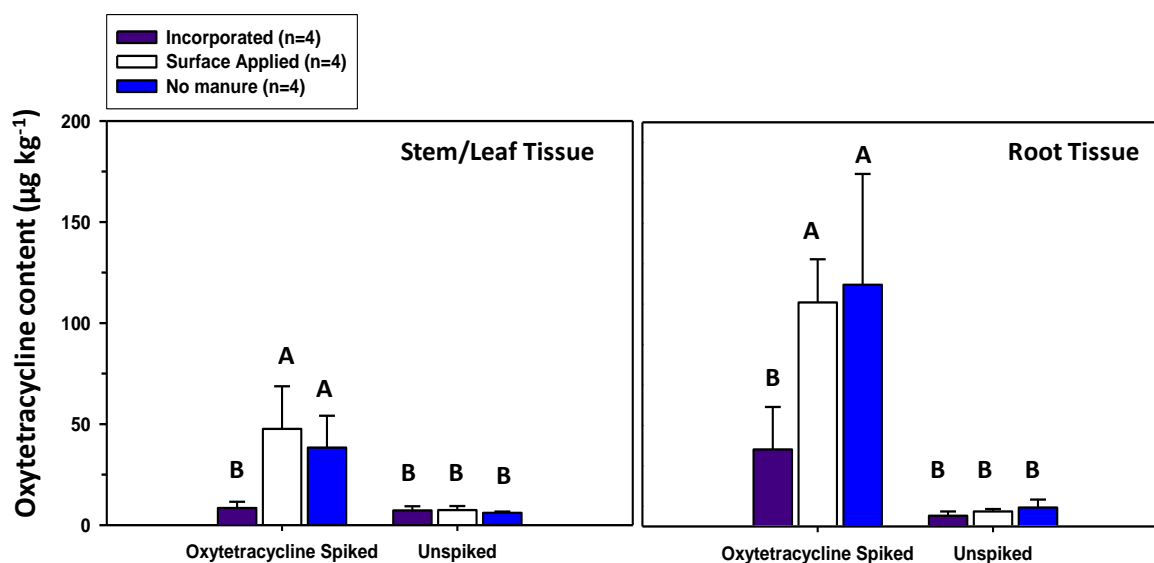
In the six soil treatments where MON was applied in our experiment, MON content ranged from  $128.8 \pm 77.3$  to  $369.5 \pm 16.8 \mu\text{g kg}^{-1}$  in soil. Other studies [10,24] confirmed the presence and persistence of MON in soil after manure application. In one study [24], soils treated with spiked turkey litter were assessed at  $18.0 \pm 25.2 \mu\text{g kg}^{-1}$  soil following harvest. While that study [24] obtained approximately 1.0 % recovery of applied MON, our study measured between 2.6% and 7.4%. This difference may have been due to differences in growing conditions and experiment timelines. Their experiment occurred over the course of a growing season and under field conditions compared to our 6 wk greenhouse-based experimental conditions. The relatively high levels of MON in our soil were likely due to the limited time provided for microbial degradation in the experimental design timeframe.

### 3.2. Oxytetracycline Contents in Soils and Herbage

OTC content in T85 stem/leaf tissue was affected by OTC +/- and manure application method when OTC was present. When T85 was present and OTC was added to the soil, levels of OTC remaining in the soil at the time of plant harvest were greater in all spiked treatments compared to the unspiked control (Figure 4). However, uptake of OTC by T85 root and stem/leaf tissue was greater than controls in the no manure OTC spiked and the OTC-spiked surface-applied manure treatments, but not in the OTC-spiked manure incorporated treatment. The greatest mean root OTC content ( $119.6 \mu\text{g kg}^{-1}$ ) was observed with direct soil OTC spiking while the lowest OTC content in root tissue ( $5.3 \mu\text{g kg}^{-1}$ ) was observed with plant tissue grown with unspiked incorporated manure. In the stem/leaf tissue, the greatest OTC content ( $47.6 \mu\text{g kg}^{-1}$ ) was found in the plants grown with surface applied OTC-spiked manure ( $p \leq 0.0003$ ) while the lowest OTC content ( $6.1 \mu\text{g kg}^{-1}$ ) was obtained from the plants grown in the no-OTC, no-manure control pots (Figure 5). In all OTC-spiked treatments, OTC uptake by roots was greater than in stem/leaf tissue. This supports prior findings [50] that when plants absorb and abate antibiotics in the soil, roots show greater absorption capacity than above-ground herbage.



**Figure 4.** Oxytetracycline content in soil and herbage samples. The brown color represents T85 stem/leaf tissue, and the orange color represents T85 root tissue and olive represents soil samples. Control samples have no MON or manure applied. A list of all concentrations can be found in Supplemental Tables S4 and S5. Each error bar was constructed using standard deviation.



**Figure 5.** Tifton 85 plant tissue oxytetracycline content comparisons. The oxytetracycline content was measured using ELISA 6 weeks after treatment with direct soil oxytetracycline spiking ( $11.3 \text{ mg kg}^{-1}$ ), surface application of oxytetracycline-spiked manure ( $11.3 \text{ mg kg}^{-1}$ ), incorporation of oxytetracycline-spiked manure ( $11.3 \text{ mg kg}^{-1}$ ), unspiked manure, and no manure/no monensin treatments. Each error bar was constructed using standard deviation. Values with the same letter on top of columns do not differ according to Tukey–Kramer Honestly Significant Different (HSD) least significant multiple mean separation ( $p \leq 0.05$ ).

Spiking OTC into soil or manure and then applying it to the soil surface resulted in increased uptake by T85 stem/leaf tissue ( $p \leq 0.006$ ) (Figure 5). OTC is naturally produced by soil bacteria [52] and thus trace levels are normally found in soil. Other research [53] found that OTC strongly interacted with natural organic matter, such as manure, resulting in poor extraction efficiency. Surface applying OTC-spiked manure and directly spiking OTC to soil caused the antibiotic to be taken into T85 stem/leaf tissue. When OTC-spiked manure was incorporated into soil extraction efficiency was possibly limited by OTC being strongly sorbed to organic matter and soil aggregates or it was more quickly broken down. Another researcher [16] reported a 33-d OTC half-life in manure-amended soil, compared to 56 d in non-amended soil. A third trial [54] reported more rapid degradation of OTC in unsterilized as compared to sterilized compost.

Uptake of OTC by plant stem/leaf tissue only occurred when soil was spiked with OTC. Surface application of OTC-spiked manure resulted in notably greater OTC uptake by the stem/leaf tissue compared to treatments where it was incorporated into soil. When OTC was not added, manure application method had no impact on OTC uptake into the stem/tissue. This is the first reported instance of manure application method affecting OTC uptake in plant tissue. OTC also exhibits strong binding potential to natural organic matter [55]. Less breakdown of the antibiotic can allow for greater uptake potential in the plant as well as persistence in the soil.

When plants absorb and abate antibiotics in the soil, roots show greater absorption capacity than above-ground herbage [50]. Our results support this tendency since root OTC content was greater than in stem/leaf tissue for both antibiotics. Both plant roots and stem/leaf tissue showed increased uptake in only two out of three spiked treatments (Figure 5). The results indicate that, as with MON, high soil OTC content increases T85 root uptake potential. The lack of significant OTC plant uptake in the OTC-spiked incorporated manure treatment despite similar soil OTC levels may be due to OTC sorption to soil particles combined with more rapid degradation of OTC within the rhizosphere. The soil used in our study was a very pale, slightly acid sand interbedded with light grey calcareous clay with a high illite content [37]. OTC adsorbs strongly to both soil components, clay and sand [56,57].

Recent studies [58] indicated that rhizosphere microbial communities can promote degradation of OTC. Growth of rhizobacteria may have been stimulated by the presence of incorporated manure.

In the three treatments where OTC was applied at  $11.3 \text{ mg kg}^{-1}$  (3, 4 and 14), T85 stem, leaf and root tissue contents were  $46.5$ ,  $158.4$  and  $157.9 \text{ } \mu\text{g kg}^{-1}$ , respectively, after 6 wk growth. The  $0.4\%$  to  $1.4\%$  plant OTC uptake is below plant uptake rates reported for other antibiotics ( $2\%$  to  $3\%$ ) [26,45], in prior plant uptake studies of veterinary medicines while some researchers [22,23] obtained non-detectable OTC uptake from spiked soils. The uptake of OTC is pH dependent, lowest at pH 5.0 and greatest at pH 7.0 [23], while soil sorption of OTC is most affected by percent clay and effective cation exchange capacity [59]. While our soil had a relatively low pH of 5.3, it had a higher clay content than the sandy soils used by other researchers [10,11]. It is possible that, due to the sandy nature of the soil used in their studies [22,23], leaching of OTC may have occurred. In prior research plant uptake of OTC was assessed in vegetable plants [22] but not in grasses [23]. The low but measurable plant uptake levels in this study may be due to the somewhat higher concentration of OTC spiking ( $11.3 \text{ mg kg}^{-1}$ ) used in this study combined with observations from several researchers [15,24] that plant uptake of antibiotics is highly dependent on the type of antibiotic, soil characteristics, and plant species.

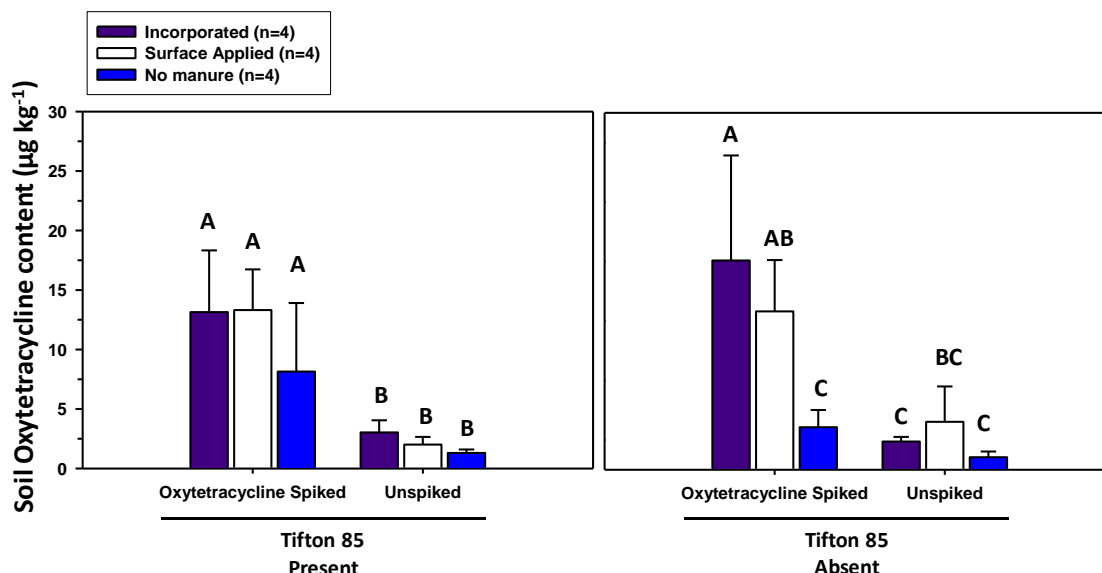
The ADI limit for human non-therapeutic tetracycline consumption is approximately  $30 \text{ } \mu\text{g kg}^{-1}$ , [24] while the MRL in animal tissue is  $1.2 \text{ mg kg}^{-1}$  raw weight of animal tissue [24]. Based on the greatest mean combined stem, leaf, and root tissue OTC content of  $158.4 \text{ } \mu\text{g kg}^{-1}$ , with the same assumptions as mentioned for MON, it would require cows to consume roughly 7.6 times their body weight ( $5,718 \text{ kg}$ ) of fresh weight T85 to raise concern for consumers ingesting their products. The actual potential for animal health risks from forage consumption following a single application of manure may be low but repeated applications of manure could potentially result in higher antibiotic contents in the forage.

As mentioned earlier, OTC contents detected in the samples were consistently low. These results could have been the result of the extraction protocol used for the soil and manure samples not being as efficient as expected or due to high levels of OTC sorption to soil and manure. However, higher OTC contents in soil were detected in soils spiked with OTC compared to unspiked treatments. The greatest mean OTC soil content ( $17.6 \text{ } \mu\text{g kg}^{-1}$ ) was observed when OTC-spiked manure was incorporated into the soil, while the lowest mean content ( $1.0 \text{ } \mu\text{g kg}^{-1}$ ) was seen when in soil without manure, T85 or OTC added. Our observations that OTC-spiked manure incorporated into the soil resulted in relatively high levels of residual soil OTC while allowing for low OTC uptake by both root and stem/leaf tissue appears to be consistent with incorporated OTC becoming bound to soil. Other research [60] reported that soils with high illite content (such as the Windthorst soil used in this experiment) have a tendency for OTC sorption.

When OTC was spiked into soil that had T85 present, both surface applying and incorporating the manure resulted in soil OTC contents that were higher than the background (no manure, no OTC control) ( $p \leq 0.002$ ) (Figure 6). For the treatments with T85 absent, addition of OTC-spiked manure, surface applied or incorporated into soil ( $p \leq 0.01$ ), caused increased OTC soil contents compared to the background soil contents (no manure, no OTC), but the two manure treatments were not different from each other in terms of soil OTC content (Figure 6).

The addition of OTC was the only factor that affected soil OTC content. T85 OTC uptake was confirmed, but its phytoremediative properties were not seen with changes in soil OTC content. In the six soil treatments where OTC was applied, its contents in soil ranged from  $3.6$  to  $17.6 \text{ } \mu\text{g kg}^{-1}$ , representing approximately  $0.03$  to  $0.16\%$  recovery of applied OTC. The OTC incorporated into the soil with manure could have adsorbed to the manure while the OTC in the treatment without plants or manure could have leached from the soil. In contrast to differences among manure application treatments when T85 was absent, no differences were observed when T85 was present and OTC spiked into the soil. Our observations that OTC-spiked manure incorporated into the soil resulted in relatively high levels of residual soil OTC while allowing for low OTC uptake by both root and stem/leaf tissue appears to be consistent with incorporated OTC becoming bound to soil minerals, manure, or organic

compounds produced in the rhizosphere. When another trial [56] examined OTC contents in sandy fields that had been continuously fertilized with liquid manure, they measured 0.15 mg OTC kg<sup>-1</sup> soil. Continued OTC application could account for the higher levels of soil OTC reported by Hamscher [56] despite their use of sandy soils as in the experiments reported by others [22,23].



**Figure 6.** Soil oxytetracycline content comparisons. The oxytetracycline content was measured using ELISA 6 weeks after treatment with direct soil oxytetracycline spiking (11.3 mg kg<sup>-1</sup>), surface application of oxytetracycline-spiked manure (11.3 mg kg<sup>-1</sup>), incorporation of oxytetracycline-spiked manure (11.3 mg kg<sup>-1</sup>), unspiked manure, and no manure/no monensin treatments. Each error bar was constructed using standard deviation. Values with the same letter on top of columns do not differ according to Tukey–Kramer Honestly Significant Different (HSD) least significant multiple mean separation ( $p \leq 0.05$ ).

#### 4. Conclusions

Our study demonstrates T85's capacity to take up MON and OTC from soils that have had antibiotic laden manure applied or effluent containing antibiotics in the mg kg<sup>-1</sup> range. If T85 is used to recycle nutrients from manure on CAFO farms, phytoaccumulation will result in these antibiotics being fed back to livestock. Based on reported liquid dairy manure OTC and MON spiking levels used in our study, these levels are not high enough to cause any immediate concern for these animals or human consumption of their products. While the antimicrobials tested may not be used or currently circulated in the environment at sufficient levels to impact human health, prior studies [32,33,35] indicate that these antimicrobials impact the health of soil ecosystems.

Manure application method had a negligible impact on plant MON uptake, but the presence of manure resulted in lower soil MON content compared to soils that were spiked with MON without manure addition. This indication of potential MON degradation in the presence of manure may serve to counteract the risks of MON buildup in soils. Stem/leaf tissue OTC content was greatest when this antibiotic was added to manure that was surface applied. This greater uptake was balanced against a lower OTC content in the soil for this treatment. These impacts will be affected by soil characteristics since OTC sorption potential increases with increasing organic matter percentage, cation-exchange capacity (CEC), and clay contents [59,60].

The most important and consistent factor was antibiotic spiking; whenever antibiotic contents were high, the potential for antimicrobial uptake and recycling through the system is also high. If manure antibiotic contents are low, our results indicate that application method is largely irrelevant. Oxytetracycline levels assessed in raw manure (0.009 mg kg<sup>-1</sup> dried manure) were lower than seen in

other studies while MON levels were consistent with those measured elsewhere ( $3.2 \text{ mg kg}^{-1}$  dried manure) [44].

Land application is the most common method for manure use and disposal. Our results make a case for manure soil incorporation instead of surface broadcast of manure to mitigate manure-borne OTC environmental impacts, when present at high levels. However, this may not be a practical method due to the associated issues with manure incorporation such as the impracticality of tilling perennial pastures, use of no-till practices, and stimulation of microbial activities that enhance carbon loss. Another way to reduce environmental impact from manure-associated antibiotics is to treat manure prior to land application by composting [51]. In situations where alternative application strategies and/or manure treatment is not practical, increasing hold times prior to manure application and applying manure following appropriate time and location practices can help reduce runoff of antibiotics into surface water and leaching into ground water as well as allowing for more antibiotic degradation before releasing into soil.

This research was carried out as a greenhouse study utilizing relatively low-cost ELISA analysis methods to provide an initial screening process. The findings can be used to inform the implementation of more detailed follow-up studies examining these antimicrobial uptake processes in the field using a broader range of antibiotics (other veterinary antibiotics used in livestock rearing that are also important for human medicine) and more precise analytical methods, such as Ultra High-Performance Liquid Chromatography (UHPLC MS/MS).

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/4/468/s1>, Supporting information S1, Monensin and oxytetracycline antibiotic residue analysis details; Supporting information S2, Conversion of antibiotic ELISA concentration to soil and plant tissue content; Supporting information S3, Oxytetracycline ELISA testing protocol; Table S1, Linear equations for ELISA kits; Table S2, Antibiotic concentration in plant tissue extraction solutions ( $n = 4$ ); Table S3, Antibiotic concentration in soil sample extraction solutions ( $n = 4$ ); Table S4, Antibiotic content per kg of plant tissue ( $n = 4$ ); Table S5: Antibiotic content per kg of soil ( $n = 4$ ).

**Author Contributions:** S.S.H. was involved with conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, visualization, writing the original draft and writing-review/editing. B.B. was involved with conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, visualization and writing-review/editing. J.A.B. was involved with conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization and writing-review/editing. J.P.M. was involved with conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, validation, and writing-review/editing. All authors have read and agreed to the published version of the manuscript.

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