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# Determination of the Persistence of Tetracycline Antibiotics and Their Degradates in Manure-Amended Soil Using Enzyme-Linked Immunosorbent Assay and Liquid Chromatography−Mass Spectrometry

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The persistence of manure-borne oxytetracycline in soil was investigated under field conditions. Soil cores were collected approximately once a month for over a period of two years and subsampled at depth increments of 0−5, 5−10, 10−15, 15−36, and 36−71 cm. Soil samples were analyzed by enzyme-linked immunosorbent assay (ELISA) and/or by liquid chromatography−mass spectrometry (LC-MS). Whereas LC-MS showed that oxytetracycline declined to <50% of its initial soil concentration after 3 weeks, ELISA showed that the total tetracyclines did not decline significantly 5 months after manure application. The differences between ELISA and LC-MS results are attributed to the broad cross-reactivity of the antibodies employed, which detect many structurally related tetracyclines, including their isomers and degradation products. Only trace amounts (≤1.0 μg/kg) of oxytetracycline were observed in the subsurface soil, and none was detected in water samples from field lysimeters, suggesting that oxytetracycline has low mobility in soil.

**KEYWORDS: Antibiotics; manure; ELISA; LC-MS; persistence; mobility**

## INTRODUCTION

The application of manure to cropland as a fertilizer source and soil conditioner is a common agricultural practice. Many animal confinement operations generate manure that contains antibiotics because animals receive antibiotics in feed rations, either as growth promoters or as therapeutic agents. The application of antibiotics to agricultural lands through repeated fertilization with animal manure potentially poses an ecological and environmental threat because the excreted antibiotics could contaminate soils, streams, and groundwater (*1*). Prolonged exposure of microorganisms to low doses of antibiotics could result in the selective pressure that favors the proliferation of resistant bacteria (*2*), including those that are pathogenic and have potential health risks to humans. Antibiotic resistance is a global threat because existing antibiotics are becoming increasingly ineffective in combating microbial infections in humans.

The tetracycline class is among the most widely used growth-promoting antibiotics in animal production. Depending on the animal species, up to 75% of a single dose of tetracycline is excreted in nonmetabolized form in urine or feces (*3*). A recent study conducted in northern Germany has shown that tetracycline and chlortetracycline residues can build up in soil from repeated application of liquid manure from livestock (*4*). A similar field study in the United Kingdom demonstrated the persistence of oxytetracycline (OTC) in soil during the first growing season, but not in the second growing season, when OTC was not detected in any of the samples taken (*5*). In a different study conducted to determine the persistence and distribution of OTC in soil after the application of 600 μg/mL OTC as drench, it was observed that low levels of OTC could be detected in soil 1.5 years after application (*6*). This latter study, however, did not represent the environmental conditions typical in agricultural fields receiving manure applications because OTC is normally present at much lower concentrations (∼10−25 μg/L) in liquid manure. Also, the latter study was not appropriate for predicting the fate of OTC in manure-amended soil because it did not take into account the effect of the high organic matter content of manure on the persistence and mobility of OTC in soil.

Despite the similarities in the chemistry and biological activities among the members of the tetracycline class of antibiotics, their individual environmental behaviors may differ significantly. The influence of soil conditions, climate, and application procedure (incorporation or surface application) and the frequency of manure applications are also important factors

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that could cause differences in the environmental fate of antibiotics. Therefore, the first objective of this study is to provide data on the persistence and mobility of OTC in a typical crop field in the midwestern United States and to determine the effect of repeated manure application on the fate of tetracyclines in soil.

In this study, an enzyme-linked immunosorbent assay (ELISA) was used in combination with liquid chromatography−mass spectrometry (LC-MS) to determine the antibiotic concentrations in soil. The conventional methods used to analyze tetracyclines in water and soil samples are high-performance liquid chromatography with ultraviolet detection (5, *7*) and LC-MS (*4*, *8*−*11*). LC-MS methods are sensitive and highly specific, but they require expensive instrumentation and time-consuming sample cleanup steps. Recently, the application of ELISA to determine the occurrence of tetracyclines in livestock manure (*12*) and soil (*13*) has been reported to circumvent the cost and practical limitations of conventional techniques. In addition, radioimmunoassay has been used previously to analyze for tetracyclines in water and wastewater (*14*, *15*). The antibodies used in these immunoassays have broad cross-reactivity toward several members of the tetracycline class of antibiotics and can detect transformation products that are only slightly modified, particularly when the general structure of the tetracycline rings is preserved. The broad cross-reactivity of antibodies used in the ELISA offers a unique advantage of detecting structurally related tetracyclines, such as unknown transformation products that would otherwise not be detected using LC-MS when operated under selected ion monitoring mode. It is important to consider the presence of tetracycline analogues and transformation products in soil because some of these compounds may have antibacterial activity that could change the structure of the natural soil microbial flora. Thus, the second objective of this study was to compare results obtained from ELISA with those from LC-MS to determine the effectiveness of ELISA as a screening tool to reduce the number of samples that need to be analyzed by LC-MS. This is the first study that shows the unique capability of ELISA in providing additional information on the persistence of tetracycline transformation products in soil. The data from ELISA combined with LC-MS results provide a more complete assessment of the environmental fate of tetracycline antibiotics in soil.

## MATERIALS AND METHODS

**Field Study Design and Manure Application.** The field study was conducted at the University of Nebraska West Central Research and Extension Center, located in North Platte, NE. The soil at this site is mapped as a Cozad silt loam (*Fluventic Haplustoll*). However, the surface texture within the experimental plots is predominantly loam. Average values for organic matter through the soil profile are 0−0.15 m, 1.14%; 0.15−0.30 m, 0.82%; 0.3−1.80 m, 0.54%; and 1.80−2.40 m, 1.03%. Soil pH averages 7.3 in the upper 0.15 m and 7.8 from 0.15 to 0.30 m and varies between 8.0 and 8.2 to a depth of 2.4 m. The experimental plots were 12 m by 6 m. Manure from feedlot cattle fed with 75 mg of OTC head$^{-1}$ day$^{-1}$ for ∼5 months was applied preplant as a nitrogen fertilizer source to corn plots. Manure was hand-applied using a grid system to ensure uniformity of application and then incorporated by disking. Manure application rates were based upon the University of Nebraska's nitrogen recommendation algorithm (*16*), which takes into account the nitrate-nitrogen content of the soil, the percent organic matter of the soil, the available nitrogen content of the manure, the expected corn grain yield, and corn nitrogen requirement. This algorithm also credits nitrogen derived from legumes, past manure applications, and irrigation water.

During the first year of the study (date of application, May 9, 2002), treatments consisted of manure applied at the recommended rate (1N) to supply the nitrogen needs of corn, manure at twice the recommended rate (2N) to simulate over-application of manure, and a control. The control received ammonium nitrate at the recommended rate to maintain uniform crop growth between treatments and triple superphosphate applied to match the phosphorus application rate from the 1N treatment. Each treatment was replicated four times in a randomized complete block design. During the second year of the study (date of application, May 21, 2003), each plot was split into two parts. The first half (6 m × 6 m plot) was treated with manure (1N and 2N rates) from feedlot cattle fed OTC (new), whereas the second half did not receive manure (residual). This design allowed for comparisons of antibiotic persistence and mobility between continued manure application and past manure application.

Plots were irrigated using a solid-set sprinkler system to match crop water needs and to minimize water losses through percolation and runoff. Soil moisture from each plot was measured weekly at 0.30 m depth increments to a depth of 2 m using a neutron probe. An automatic weather station located at the research site was used to calculate daily crop water use. Calculations were performed following procedures described by Allen et al. (*17*).

Leaching was measured using monolithic percolation lysimeters as described by Klocke et al. (*18*). The lysimeters installed adjacent to each of the plots received the same manure or chemical fertilizer application rates as the rest of the plot for both years. Twelve soil water extractors were inserted vertically upward through the lysimeter bottom. Extractors were open at one end to a drainage reservoir and closed at the other end. The extractors were porous tubes (13 mm o.d. × 150 mm long, 0.5-$\mu$m pores, 39 kPa air entry pressure), manufactured from sintered stainless steel (Mott Metallurgical Corp., Farmington, CT). Water from the extractors drained into a drainage reservoir (22 L) bolted to the bottom of the lysimeter and sealed with silicone. The reservoir was a shallow cylinder (0.6 m i.d. × 0.10 m deep) with a sloping bottom, fabricated from steel and coated with epoxy paint. The top of the reservoir was connected to a vacuum system through a copper tubing (9.5 mm o.d.). A water sampling line made of stainless steel tubing (9.5 mm o.d.) was connected to the bottom of the reservoir. Both the vacuum and sampling tubes were connected to a glass sampling container (3.78 L) installed in a manhole at the edge of the research plot. An electric vacuum pump was used to continuously apply vacuum to the extractors so that water in the lysimeters would drain even under unsaturated conditions.

**Sample Collection.** Water samples from the bottom of the lysimeters were collected approximately every 2 weeks during the growing season, to measure the water that leached from the crop root zone. Soil samples from the treated plots were collected using a soil auger with a 1.6-cm tip. Soil cores were collected and subsampled at depth increments of 0−5, 5−10, 10−15, 15−36, and 36−71 cm. Soil cores were collected prior to land application of manure and on a monthly basis for over a period of 2 years after manure application, except during the months of November−March when the soil was frozen. In addition, two surface soil samples were collected from the treated plots on the third year after application (sampling date, July 9, 2004). Samples were shipped frozen to either the University at Buffalo (Buffalo, NY) or the University of Nebraska−Lincoln (Lincoln, NE) for chemical analysis and were stored at −40 °C prior to analysis.

**Sample Analysis, Method 1.** During the first year of the study, the soil samples were sent to the Water Sciences Laboratory, University of Nebraska−Lincoln, Lincoln, for analysis by LC-MS. This method was modified from the water and wastewater method developed by Zhu et al. (*8*) and Snow et al. (*19*). Standards for oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), anhydrotetracycline, anhydrochlortetracycline, and $\beta$-apooxytetracycline were obtained from Acros Organics (Fisher Scientific, Houston, TX). Doxycycline (DOC) was obtained from Sigma-Aldrich (St. Louis, MO), and demeclocycline (DMCTC) was obtained from Fluka Chemical (Milwaukee, WI). HPLC or better grade methanol and acetonitrile, acetone, and reagent grade anhydrous citric acid and formic acid were obtained from Fisher Scientific. Stock, spiking, and calibration solutions were prepared in methanol in amber silane-treated vials.

Each soil sample was equilibrated four times, twice with citric acid and twice with acetone/formic acid, to maximize recovery of all

compounds. Briefly, a 10-g well-mixed soil sample was weighed into a Teflon centrifuge tube, spiked with a 200 ng/g of doxycycline surrogate, and mixed with 20 mL of 1 M citric acid. Each soil mixture was shaken for 30 min and centrifuged for 10 min, and the supernate was filtered into a 200-mL evaporation tube. The sample was equilibrated a second time with an additional 20 mL of 1 M citric acid followed by two 20-mL solutions of acetone and formic acid (pH 4). Each time, the mixtures were centrifuged and the supernatant from each sample was filtered and combined in evaporation tubes.

After the internal standard (demeclocycline) was added at 500 ng/g, the extract was evaporated under nitrogen to ~50% of its volume at 25 °C to remove most of the acetone and diluted to 100 mL with reagent water. The aqueous extract was then passed through a preconditioned Oasis HLB polymeric solid-phase extraction (SPE) cartridge (Waters, Milford, MA) to concentrate the tetracyclines and separate interferences. The cartridges were eluted with 0.5% formic acid in methanol prior to analysis by LC-MS. The extracts were analyzed using a 2695 HPLC with a triple-quadruple LC-MS (Waters Micromass Quattro) with electrospray ionization in positive ion mode. A well end-capped Kromasil BetaBasic C18 reverse phase HPLC column (250 × 2 mm) was used with a 25-$\mu$L injection volume. Analytes were separated isocratically using a mobile phase solution of 1% formic acid in water/acetonitrile/methanol (60:20:20, pH 2.5) at a constant temperature of 30 °C and a flow rate of 0.2 mL/min. A pseudo-molecular ion $[M + H]^+$ was selected as the parent ion for fragmentation, and corresponding fragment ions were selected for identification and quantitation. Ionization and collision energies are optimized on the basis of procedures described by the instrument manufacturer.

Recoveries of oxytetracycline, chlortetracycline, and tetracycline from eight uncontaminated soil samples fortified at 6.7 ng/g averaged 108 ± 3, 103 ± 4, and 99 ± 3%, respectively, whereas recoveries of the degradation products $\beta$-apooxytetracycline, anhydrochlortetracycline, and anhydrotetacycline averaged 45 ± 6, 58 ± 8, and 87 ± 4%, respectively. On the basis of the variability of the fortified sample, method detection limits ranged between 0.6 and 0.4 ng/g for the parent compounds and between 0.8 and 1.6 ng/g for the degradation products.

**Sample Analysis, Method 2.** During the second year of the study, soil and lysimeter water samples were sent to the Chemistry Department, University at Buffalo—State University of New York, for analysis. Soil samples were extracted using an automated accelerated solvent extraction (ASE) system (Dionex, Sunnyvale, CA). Approximately 5 g of soil was mixed with a sufficient amount of Hydromatrix (Dionex) to fill the 11-mL extraction cell. The extraction was performed in two steps. The first extraction step uses 100% aqueous McIlvaine buffer at pH 7.8, with 100 mM ethylenediaminetetraacetic acid (EDTA) additive. The second step of the extraction uses a 60:40 (v/v) water/methanol mixture as solvent. The first extraction was performed using two cycles at a pressure of 1500 psi and a temperature of 60 °C. The sample cell was filled with the solvent and allowed a static time of 5 min, after which the cell was purged with fresh solvent equivalent to 100% of the cell volume. The second extraction with methanol/water was performed using the same ASE conditions as above, except that the temperature was reduced to 30 °C as a caution to prevent possible degradation of the analytes from prolonged exposure to elevated temperature. A 100-$\mu$L aliquot was withdrawn from the first extract for screening by ELISA. The second extract was evaporated under a stream of nitrogen gas to reduce the percent methanol for effective SPE and the total volume of the extract to ~5 mL. The extracts from both steps were mixed, and the combined extracts were then subjected to SPE for cleanup and concentration. An internal standard, doxycycline, was added to the combined extract prior to SPE.

SPE was performed by passing the extracts through a 1-g strong anion exchange (SAX) Discovery cartridge (Supelco, Bellefonte, PA) in tandem with a 500-mg Oasis HLB cartridge. The cartridges were conditioned with 3 mL of methanol, followed by 3 mL of 0.04 M citric acid buffer at pH 4, similar to the method described by Jacobsen et al. (*10*). Because the extraction buffer used for ASE was at pH 7.8, the extracts were first adjusted to pH 4 with 1 M $H_2SO_4$ and diluted with distilled water to 200 mL prior to SPE. The samples were loaded onto the cartridges at ~5 mL $min^{-1}$. The SAX cartridge was then washed with 2 mL of citric acid buffer (pH 4) and then disconnected from the HLB cartridge. The HLB cartridge was dried for 15 min under vacuum and then eluted with 5 mL of methanol. The eluent volume was reduced to almost dryness under a gentle stream of nitrogen and reconstituted with 1 mL of 90:10 (v/v) McIlvaine buffer (pH 4)/acetonitrile. The sample extracts were vortexed for 5 min, sonicated for 10 min, and then transferred to the LC-MS autosampler vials.

LC-MS analysis was performed on an Agilent 1100 series LC-MSD (Agilent Technologies, Palo Alto, CA) using electrospray ionization in positive ion mode. The drying gas (nitrogen) flow rate was 10 L $min^{-1}$ at a temperature of 350 °C, and the capillary voltage was 4 kV. Analysis was performed using selected ion monitoring of the $[M + H]^+$ and two fragment ions produced at 140 V fragmentor voltage for oxytetracycline and at 130 V for the internal standard (doxycycline). A reversed-phase C18 column (Thermo Hypersil-Keystone, Bellefonte, PA) with dimensions of 100 × 2.1 mm and 3 $\mu$m particle size was used. Separation was performed using a gradient mobile phase at a flow rate of 250 $\mu$L $min^{-1}$ consisting of 100% acetonitrile (A) and 0.3% (v/v) formic acid/water (B). The gradient program was as follows: 10% A was held constant for 1 min, gradual increase in A such that at 12.6 min a composition of 55% A was reached, gradient was ramped to reach 95% A at 13 min, which was held for 4 min, and then the composition was returned to 10% A at 17 min.

The water samples collected from the lysimeters were concentrated by SPE using 500-mg Oasis HLB cartridges. The cartridges were conditioned with 3 mL of methanol, followed by 3 mL of reagent water. Then, 1-L water samples were passed through each cartridge at ~5 mL $min^{-1}$ using a vacuum manifold. The cartridges were dried under vacuum for 15 min. The analytes were then eluted with 5 mL of acetonitrile, and the eluates were evaporated to almost dryness under a stream of nitrogen gas. The extracts were reconstituted in 1 mL of 90:10 McIlvaine buffer (pH 4)/acetonitrile and transferred to autosampler vials for analysis by LC-MS method 1.

**ELISA Method for Tetracycline.** From the 100-$\mu$L aliquot of the extracts taken from method 2, three dilutions (1:1000, 1:5000, and 1:10,000) were prepared using the buffer provided in the ELISA kit (R-Biopharm GmbH, Darmstadt, Germany). All of the samples were analyzed using three dilutions. The concentrations obtained from these dilutions agreed with each other, indicating the absence of matrix effects. For highly concentrated samples more dilutions were prepared as needed until the concentration was within the dynamic range of the ELISA (0.1−5.0 ppb). All reagents except the wash buffer were provided in the kit. Samples or standards (50 $\mu$L) were added to individual microwells, followed by a solution of the anti-tetracycline antibodies (50 $\mu$L). The mixture was incubated for 1 h, and then the wells were washed with phosphate-buffered saline containing Tween 20 (Sigma-Aldrich, St. Louis, MO). A solution of a peroxidase-conjugated secondary antibody (100 $\mu$L) against the anti-tetracycline antibodies was added into each well and incubated for 1 h. The wells were then washed. A 100-$\mu$L mixture of urea peroxide and tetramethylbenzidine (1:1, v/v) was added into each well, and the plate was incubated for 30 min in the dark. Finally, the reaction was stopped by adding 100 $\mu$L of 1 M $H_2SO_4$ into each well. All reagents except the samples and standards were added into the wells using a multichannel pipet to minimize variability in reaction times between wells. The absorbances were measured at 450 nm using a Synergy-HR plate reader, and the concentrations were calculated on the basis of a four-parameter fit transformation performed on KC4 software (Bio-Tek Instruments, Winooski, VT).

## RESULTS AND DISCUSSION

**Persistence and Mobility of Oxytetracycline in Soil.** During the first year of the study, the initial OTC concentration in manure and the residues in soil cores were analyzed using LC-MS method 1, which had a limit of detection (LOD) of 0.4 $\mu$g/kg for OTC. The initial concentration of OTC in the cattle manure used to fertilize the soil was 2656 $\mu$g/kg. There were no residues of tetracycline in the surface soil layer from any of the plots prior to manure application (preapplication sampling date, April 11, 2002).

**Table 1.** Analysis of Surface Soil (0–5 cm) from the 2002 (Residual) and 2003 (New) Applications Using ELISA for the 1N and 2N Treatments (Concentrations in Micrograms per Kilogram)

| | plot 1 | | plot 2 | | plot 3 | |
|---|---|---|---|---|---|---|
| sampling date | new | res | new | res | new | res |
| | | | 1N | | | |
| April 21, 2003 | 140 | 140 | 351 | 351 | NS[a] | NS |
| June 4, 2003 | 194 | 216 | 145 | NS | 151 | 162 |
| Aug 18, 2003 | 205 | 1970 | 89 | 679 | 196 | NS |
| Sept 22, 2003 | 118 | 993 | 191 | NS | 113 | 5 |
| | | | 2N | | | |
| April 21, 2003 | 1250 | 1250 | 164 | 164 | 1943 | 1943 |
| June 4, 2003 | 1141 | 235 | 951 | 135 | 225 | 99 |
| Aug 18, 2003 | 261 | 129 | 2021 | 2251 | 1358 | 64 |
| Sept 22, 2003 | 167 | 148 | 84 | 138 | 133 | 241 |

[a] NS, no sample available for analysis.

The soil samples were first screened for the presence of tetracyclines using ELISA prior to LC-MS analysis. The ELISA method, which employs class-specific antibodies, has been previously characterized by Aga et al. (*12*) and was shown to detect several members of the tetracycline family of antibiotics, including OTC, and other structurally related transformation products. Therefore, the amount detected by ELISA is reported as "total tetracyclines" rather than OTC only. The LOD of the ELISA for tetracycline in soil was determined to be 1.0 ppb. Preliminary results from the ELISA analysis of soil cores collected from two selected plots showed no detectable tetracyclines in the subsurface soil layers. Therefore, for the remaining plots only the samples from the surface soil layer (0−5 cm) were analyzed by ELISA to reduce time and cost of analysis.

**Table 1** presents the results from the ELISA analysis of 0−5-cm soil samples from three plots that received manure to satisfy the corn nitrogen requirement (1N) and from three plots that received twice the corn nitrogen requirement (2N). In general, there is large spatial variability observed between replicate plots. This type of variability can be attributed to the difficulty in achieving homogeneous application of manure in the field and is typical of many field dissipation studies. A similar large spatial variability has also been observed in previous studies to investigate the fate of antibiotics in manure-amended soils (*4*, *5*). Despite the variability, the data presented provide evidence of tetracycline persistence over time. The wide variability in OTC concentration between plots and the sudden increase in concentrations between sampling periods observed in the field study by Hamscher et al. (*4*) were attributed to tetracyclines released from soil after a period of time due to changes in the composition of the organic material in soil, shifts in the microorganisms population, or variations of pH and redox potential.

Results from the ELISA analysis indicate no general trend in the concentrations of tetracyclines in soil over time. Overall, in 2003 no significant differences can be observed between the levels of tetracyclines in new plots that received a second treatment of manure and residual plots that had only residues from the 2002 application due to the large variability between replicate samples. However, it is apparent from **Table 1** that on the basis of the ELISA results, tetracycline and transformation products could persist in soil for at least 2 years. In addition, there was no significant difference in tetracycline concentrations between the 1N and 2N plots, indicating that the higher manure application rates did not influence the persistence of tetracycline in soil. To determine the presence of known tetracycline transformation products in the experimental plots, soil cores

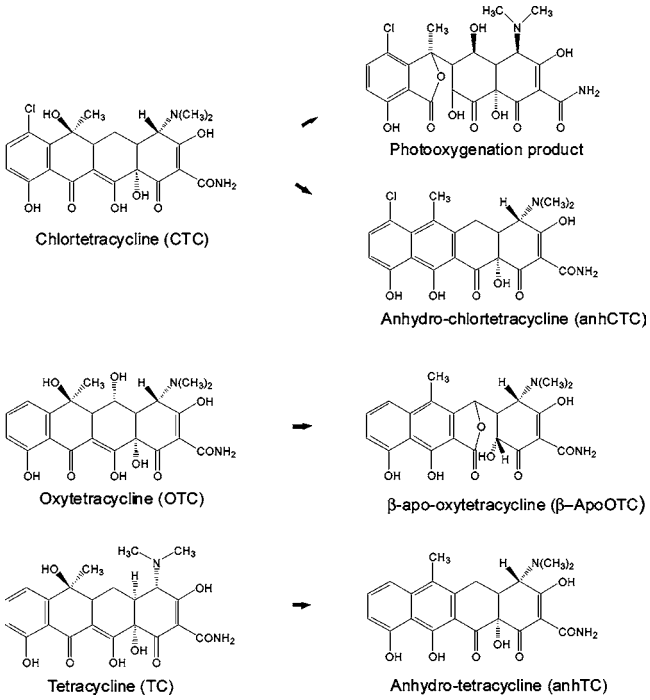


**Figure 1.** Chemicals structures of tetracycline antibiotics and their derivatives that were detected in the soil extracts.

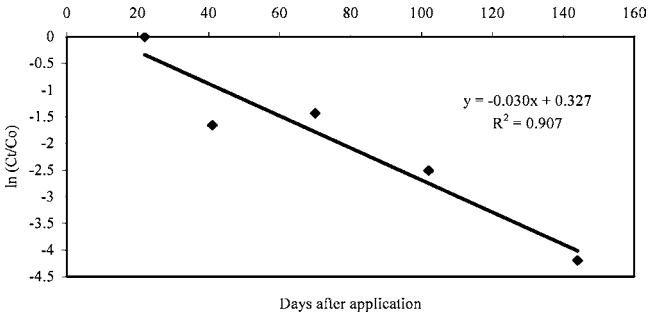


**Figure 2.** Estimation of dissipation half-life from plot 3 (2N).

collected from plot 3 (2N), which received manure at twice the crop nitrogen requirement in 2002, were analyzed using LC-MS method 1. Results of this analysis are shown in **Table 2**, and the structures of the tetracycline analogues detected are presented in **Figure 1**. The highest concentration of OTC observed was 270 $\mu$g/kg in the surface soil (0−5 cm) during the third week after application in 2002. The concentration decreased to 3.6 $\mu$g/kg after 4 months. These results were used to calculate the disappearance half-life of OTC in the surface soil, which was estimated to be ∼23 days following pseudo-first-order rate kinetics (**Figure 2**). To determine if tetracycline residues remained in soil 3 years after application, two surface soil samples collected on June 9, 2004, were analyzed by LC-MS and showed no detectable amounts of tetracyclines.

**Comparison of ELISA and LC-MS Results.** All extracts from the surface soil samples taken from plot 3 (2N) were analyzed using both ELISA and LC-MS to obtain a direct comparison between the two methods. The ELISA concentrations were generally several orders of magnitude higher than the LC-MS concentrations, and no correlation was observed between the two methods. Whereas LC-MS showed that OTC concentration decreased from 281 to 13 $\mu$g/kg in surface soil over a period of 16 months, ELISA results showed no decreasing trend (**Figure 3A**). Instead, results from ELISA showed a significantly higher concentration of total tetracycline residues

**Table 2.** Tetracycline Concentrations in Soil from Plot 3 (2N) during the 2002 Application (Results Obtained Using LC-MS Method 1) (Concentrations in Micrograms per Kilogram)[a]

| collection date | soil depth (cm) | TC | OTC | anhCTC | anhTC | β-apo-OTC | total TC |
|---|---|---|---|---|---|---|---|
| April 11, 2002 (28 days before application) | 0–5 | nd | nd | nd | nd | nd | nd |
| | 5–10 | nd | nd | nd | nd | nd | nd |
| | 10–15 | nd | nd | nd | nd | nd | nd |
| | 15–36 | nd | nd | nd | nd | nd | nd |
| | 36–71 | nd | nd | nd | nd | nd | nd |
| May 31, 2002 (22 days after application) | 0–5 | 11.29 | 270.05 | nd | nd | nd | 281.34 |
| | 5–10 | nd | nd | 1.44 | nd | 1.22 | 2.66 |
| | 10–15 | nd | nd | nd | nd | nd | nd |
| | 15–36 | nd | 0.55 | nd | nd | nd | 0.55 |
| | 36–71 | nd | nd | nd | nd | nd | nd |
| June 19, 2002 (41 days after application) | 0–5 | 1.69 | 51.89 | nd | nd | nd | 53.58 |
| | 5–10 | nd | 0.67 | nd | nd | nd | 0.67 |
| | 10–15 | nd | 0.77 | nd | nd | nd | 0.77 |
| | 15–36 | nd | 0.52 | nd | nd | nd | 0.52 |
| | 36–71 | 0.73 | 2.67 | nd | nd | nd | 3.40 |
| July 18, 2002 (70 days after application) | 0–5 | 2.27 | 64.98 | nd | nd | nd | 67.25 |
| | 5–10 | 0.6 | 0.81 | nd | nd | nd | 1.41 |
| | 10–15 | nd | nd | nd | nd | nd | nd |
| | 15–36 | nd | 0.9 | nd | nd | nd | 0.90 |
| | 36–71 | nd | nd | nd | nd | nd | nd |
| Aug 19, 2002 (102 days after application) | 0–5 | 0.48 | 22.08 | nd | nd | nd | 22.56 |
| | 5–10 | nd | nd | nd | nd | nd | nd |
| | 10–15 | nd | nd | nd | nd | nd | nd |
| | 15–36 | nd | nd | nd | nd | nd | nd |
| | 36–71 | nd | 1.1 | 0.97 | 0.49 | nd | 2.56 |
| Sept 30, 2002 (144 days after application) | 0–5 | nd | 3.6 | nd | nd | nd | 3.60 |
| | 5–10 | nd | nd | nd | nd | nd | nd |
| | 10–15 | nd | nd | 1.28 | 1.00 | nd | 2.28 |
| | 15–36 | nd | nd | 0.76 | nd | nd | 0.76 |
| | 36–71 | nd | 1.02 | 0.59 | nd | nd | 1.61 |

[a] TC, tetracycline; OTC, oxytetracycline; anhCTC, anhydrochlortetracycine; anhTC, anhydrotetracycline; β-apoOTC, apotetracycline.

even in soil samples collected 16 months after application (241 μg/kg). Analysis of surface soil from the plots that received a second manure application also showed the same magnitude of discrepancy between the LC-MS and ELISA results in 2003, as shown in **Figure 3B**.

The large differences between ELISA and LC-MS results suggest that there may be unidentified transformation products formed in soil, which are reacting to the ELISA. On the basis of the cross-reactivity profile of the ELISA reported by Aga et al. (*12*), many tetracycline derivatives containing the basic conjugated ring structure will produce a positive response to the assay. For example, epimers of OTC and other tetracycline derivatives will produce a positive response to ELISA. In addition, degradation products with only slight modifications on the tetracycline ring will most likely be recognized by the antibodies used in the ELISA and contribute to the response. Therefore, the information gained from ELISA is very important because it will indicate the presence of tetracycline-related residues in soil including unknown transformation products. The affinities of the anti-tetracycline antibodies toward different tetracycline compounds are of various degrees and cannot be easily deduced from the chemical structure of the compound without actual measurements. If an unknown compound has a much higher affinity toward the antibodies compared to OTC, a smaller concentration of the unknown could produce an ELISA response that is much higher than what is predicted if one is using OTC to construct a calibration curve.

The results presented in **Table 2** show that the highest concentrations of OTC were detected mainly in the top 0−5-cm soil layer, although there were traces of OTC detected in some of the deeper cores. The nondetection of tetracyclines in most of the subsurface soil by LC-MS is in agreement with the ELISA results. However, in addition to OTC, other derivatives of tetracyclines were detected by LC-MS, including tetracycline (TC), anhydrotetracycline (anhTC), anhydrochlortetracycline (anhCTC), and β-apooxytetracycline (β-apoOTC), at concentrations ranging from 0.48 to 11.29 μg/kg. The sources of these tetracycline derivatives may be from the degradation of OTC and from old manure that might have been mixed unintentionally with the newer manure from the OTC-fed cattle.

It is possible that there are many other unknown transformation products of tetracyclines in the soil extracts that are causing the ELISA response to be higher compared to the target-specific LC-MS method. There may also be side products of OTC production present in veterinary formulations of OTC, such as 2-acetyl-2-(decarboxamido)oxytetracycline reported by Lykkeberg et al. (*20*), that may be present in the manure and could be contributing to the ELISA response. Recently, a novel photooxygenation product of chortetracycline (CTC) has been identified in hog lagoons (*21*). This compound can be expected to produce a positive response to the tetracycline ELISA because it differs only from CTC by two additional hydroxyl groups. Unfortunately, a standard of this photooxygenate is not yet available to allow the determination of its cross-reactivity toward the tetracycline antibodies. Identification of trace amounts of unknown tetracycline degradates in soil can be challenging and is outside the scope of this study.

**Fate of Tetracyclines in Soil.** The high adsorption coefficient of OTC ($K_d$ values between 680 and 1030 L/kg in sandy loam) (*22*) suggests that OTC is immobile and is not expected to leach below the surface soil. Thus, the traces of OTC (generally <1 μg/kg) detected in the deeper layers of some soil cores may be attributed to preferential flow and/or potential cross-contamination of cores during sampling. LC-MS analysis of the water samples collected from all lysimeters did not show any detectable OTC, substantiating the hypothesis that OTC will

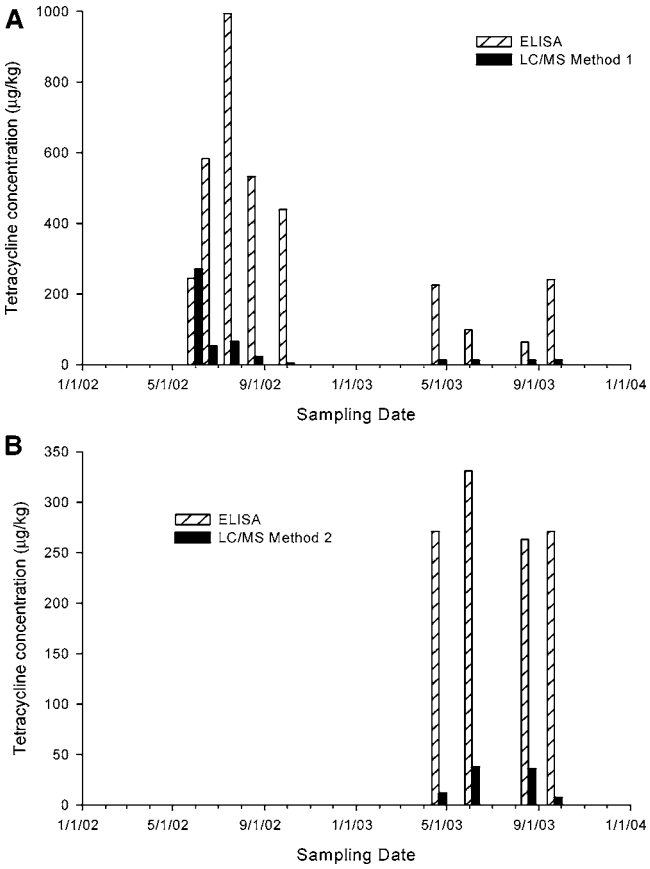


**Figure 3.** Comparison of LC-MS and ELISA results from the analysis of surface soil samples from a plot that received manure in 2002 (**A**) and a second manure application in 2003 (**B**).

have a very low leaching potential. It has been shown that OTC binds strongly to organic matter and to cations in soil (*6*, *23*). In addition, OTC is adsorbed in clay minerals by various mechanisms depending on the pH conditions (*24*, *25*). Therefore, adsorption is a significant factor contributing to the decrease in extractable OTC in soil, as has been indicated in previous studies (*26*, *27*). In the LC-MS analysis, additional derivatives of OTC were targeted to determine the presence of known transformation products, such as epi-OTC, iso-OTC, anhOTC, and $\beta$-apoOTC. None of these transformation products were detected except for $\beta$-apoOTC, which was detected in two samples at 0.57 and 1.22 $\mu$g/kg (**Table 2**). There has been no evidence indicating biodegradation of OTC in environmental samples, although abiotic transformations have been documented in soil (*28*). The lack of biotic degradation may be attributed to the strong sorption of tetracyclines to soil organic matter and clay content, which renders the antibiotics unavailable for microbial attack.

Another potential removal mechanism of OTC in the environment is photodegradation, because tetracyclines are known to be photosensitive (*29*, *30*). However, the relative importance of photodegradation on the environmental fate of tetracyclines has not been investigated. Results from the present study illustrate a unique feature of ELISA that could be useful in determining the presence of many tetracycline-related compounds in manure-amended soil, including novel degradation products of tetracyclines. Although it is not possible to quantify unknown degradates by ELISA, knowledge of their presence in soil several months after application indicates that the conventional methods may underestimate the antibiotic residues in soil. There is a need to investigate the identity and biological activity of these unknown degradates because it is possible that some of them may still have antimicrobial properties that could affect indigenous soil microbial populations.

## ABBREVIATIONS USED

CTC, chlortetracycline; DOC, doxycycline; OTC, oxytetracycline; TC, tetracycline; ELISA, enzyme-linked immunosorbent assay; LC-MS, liquid chromatography−mass spectrometry; ASE, accelerated solvent extraction; SPE, solid-phase extraction; EDTA, ethylenediaminetetraacetic acid; SAX, strong anion exchange.

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