RESEARCH REPORT
PORK SAFETY

Revised

Title: Detection of tetracyclines and tetracycline resistant bacteria in soils under long-term swine effluent application - **NPB #:** 10-103

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Industry Summary

These days, there are increasing concerns that the use of veterinary antibiotics could enhance persistence of antibiotic resistant bacteria in the environment, and consequently constitute a significant risk to the human health as well as animal well-being. Tetracyclines account for more than 50% of all antibiotics used in the swine industry. Unfortunately, only limited scientific evidence is available connecting the fate of antibiotics in the soil following animal waste application and the development of bacterial resistance. In the present study we quantify tetracyclines in 36 agricultural soils that have been continuously fertilized with swine effluent (50, 150, and 450 kg N ha^{-1}) for more than 15 years, and assess the level and occurrence of tetracycline resistant bacteria in these soils. Residue chlortetracycline concentrations were detected in the soils at all three swine effluent application rates. However, the presence of chlortetracycline had virtually no effect on the development of tetracycline resistance in bacteria isolated from these soils. Based on the testing of more than 3,000 soil bacteria isolates, we found no significant increase in the occurrence and level of chlortetracycline susceptible bacteria in the fertilized soil. To account for a possible transfer of tetracycline resistant bacteria from the swine effluent to soils, two commonly found tetracycline resistant genes were analyzed in the swine effluent and fertilized soils. While both genes were present in the swine effluent, they were not detectable in the swine effluent applied soils. Our findings suggest that while antibiotic resistant bacteria could potentially be added to the agricultural soils along with swine effluent, it would not necessarily lead to the development of antibiotic resistance of the bacteria originally present in the soil.

Keywords

Tetracycline, antibiotic, swine effluent, soil, antibiotic resistance

Scientific Abstract

The widespread use of veterinary antibiotics could potentially lead to persistence of antibiotic resistant bacteria in the environment, and consequently pose a significant risk to human health as well as animal well-being. Tetracyclines, including chlortetracycline and oxytetracycline, are one of the most broadly used classes of antibiotics in swine production. The main objective of the present study is to determine the presence of chlortetracycline and tetracycline resistant bacteria in the soils following over 15 years of annual swine effluent application. These soils were under continuous corn (*Zea mays* L.) cultivation since 1995; and swine effluent was applied using a center-pivot sprinkler system at rates of 62, 186, and 558 m^3 effluent/ha. Thirty-six soils collected at depths up to 45 cm were analyzed for chlortetracycline concentration by high performance liquid chromatography with a solid-phase extraction cleanup. Soil bacterial community was assessed by a culture-based method using tryptone soy agar plates. Susceptibility of soil bacterial isolates to tetracyclines was evaluated using the microbroth dilution method. Chlortetracycline was detected in the soils at all three application rates, even after 12 months of animal wastes application. Concentrations of chlortetracycline ranged from 0.02–0.26 mg/kg soil. The minimal chlortetracycline concentration needed to completely inhibit bacterial growth was less than 4 ppm for all the 3456 soil bacteria isolates, thus all of them were classified as non-resistant to chlortetracycline. However, 93% of bacteria isolated from the swine effluent was resistant to up to 16 ppm of chlortetracycline, and 3% was resistant to up to 32 ppm. Two common tetracycline resistant genes, tet(M) and tet(O), were also detected in the swine effluent, but not in the swine effluent fertilized soils. In conclusion, while bacteria resistant to antibiotics could potentially be added to the agricultural soils along with the swine effluent, it would not necessarily lead to the development of antibiotic resistant bacteria in the soil. At the same time, residue concentrations of chlortetracycline in the soils have virtually no effect on the development of bacterial tetracycline resistance in the fertilized soil.

Introduction

Antibiotics constitute a class of chemical compounds that kills bacteria or inhibit their growth. In 1940s antibiotics were introduced to agriculture to prevent, suppress, and treat bacterial infection in farm animals. Tetracyclines are one of the most broadly used antibiotic classes [1-3]. Their environmental fate is only poorly understood [4, 5]. It seems important to understand the role of dissipation of antibiotics in soils in the context of the spread of antibiotic resistance in the environment. The global emerge of antibiotic resistant bacteria has had serious consequences for human and animal health [6, 7]. Considerable controversy persists regarding the increase of antibiotic resistant bacteria as a result of antibiotics use in agricultural production [5, 8-10].

Development of antibiotic resistance is a complex phenomenon which can be due to the natural changes in the microbial response to the antibiotics, or could be due to human drug overuse. It is suggested that antibiotics released in the environment may contribute to contamination by antibiotic resistant pathogens, including *Campylobacter, Salmonella, Enterococcus* and *Escherichia coli*, and thereby increase risk of human infections by these and other resistant pathogens [8, 10, 11]. Limited direct scientific evidence is available connecting the fate of antibiotics in soils fertilized with animal manure and the development of bacterial resistance in these soils [4, 5]. The presence of antibiotic resistant bacteria in the soils close to animal production facilities and in some agricultural soils was reported by several research groups [12, 13]. At the same time, the detected concentrations of antibiotics in the corresponding soils were far below the minimal inhibitory concentration needed for the development of bacterial resistance [4, 14]. In one of the recent studies, it has been demonstrated that antibiotic-resistant strains of bacteria are already indigenous and highly prevalent in soils [15]. All 480 *Streptomyces* isolates from diverse soil locations (including

agricultural and native soils) were resistant to at least six antibiotics, and some to as many as 20 [15]. One thing which is commonly overlooked in studying antibiotic resistance in agricultural soils is the clearly defined control soil sample, making it extremely hard to confirm the development of the antibiotic bacterial resistance in agricultural soils as a result of agricultural activity. In addition, most studies were done on agricultural soils fertilized with swine effluent for rather short period of time (e.g., less than 10 years).

Objectives

The *general objective* of this project is to determine the concentrations of tetracyclines (which represent about 50% of all prophylactic antibiotics used) and the persistence of antibiotic resistant bacteria in the soils that have been continuously applied with swine effluent for more than 15 years. The *rationale* is to provide data that either sustain existing pork production practices or reinforce changes in animal management practices, or verify if bacteria resistant to antibiotics are not yet ubiquitous. Specifically, the following three specific objectives were addressed:

1. Develop a quantitative and reproducible method for analysis of tetracyclines in soil
2. Evaluate the mobility and persistence of tetracyclines in soil
3. Assess the level of antibiotic resistance in bacteria isolated from soil

Materials & Methods

Chemicals

The standards of tetracycline, chlorotetracycline, oxytetracycline, doxycycline, and demeclocycline (used as an internal standard) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and standards of 4-epitetracycline, anhydrotetracycline, 4-epi-anhydrotetracycline, 4-epichlorotetracycline, 4-epianhydrochlorotetracycline, epioxytetracycline, α-apo-oxytetracycline, β-apo-oxytetracycline were purchased from Acros Organics (Fair Lawn, NJ, USA). Acetonitrile, water, methanol and other solvents were of HPLC or LC/MS grades. Solvents and all other chemicals (at least of an analytical grade) were purchased from Sigma–Aldrich or Fisher (Pittsburgh, PA, USA).

Soil and swine effluent samples

Soil samples were obtained from the continuous corn (*Zea mays* L.) experiment located at the Oklahoma Panhandle Research and Extension Center near Goodwell, OK (36°35 N, 101°37 W, and elevation 992 m). Mean annual precipitation and temperature at the station are 30.3 mm and 13.4 °C, respectively. The predominant soil series at this site is a Gruver clay loam (fine, mixed, superactive, mesic Aridic Paleustolls). Experimental plots were arranged in a random block design. Dimensions of each plot were 4.572 m × 9.144 m. Six rows of corn were planted per each plot. Swine effluent was annually applied at 6-leaf stage using a center-pivot sprinkler system at application rates of 62, 186, and 558 m^3 effluent/ha/yr. The plots which were not fertilized with the swine effluent for 15 years were used as control plots. Samples of the swine effluent used for the fertilization were obtained from a commercial swine production facility located in Panhandle area, Oklahoma. Animals receive in-feed antibiotics including chlorotetracycline (100 g per ton of feed), tylosin, lincomycin, and carbadox (personal communication, 2010).

Soil sampling was performed four and twelve months after the most recent swine effluent application. Within each plot, five subsamples were taken from the center and four corners of the plot to form a composite sample. The soils were collected at three depths: 0–15, 15–30, and 30–45 cm. A total of 36 samples were obtained per sampling event. The field-moist soil samples were kept in amber plastic bags on

ice during transportation, then sieved (2-mm sieve) and mixed thoroughly within 48 h following sampling. The soils for the chemical analysis were stored at -80 °C, while the soils for the biological analysis were stored at 4 °C.

The soil pH value was measured in 0.01 M $CaCl_2$ at a ratio of 1:2.5 soil to solution (w/w). Particle-size distribution was determined by pipette analysis [16]. The total organic carbon and the total nitrogen content of the soil was determined using a LECO CN 2000 (LECO Corp., St. Joseph, MI, USA) by dry combustion [17]. The soil moisture content was determined gravimetrically after drying at 105 °C for 48 h. The total K, P, Na, Ca, and Mg of the soil samples were determined from the nitric acid digestions [18]. The total dissolved salts were calculated using the equation of *Chang et al.* [19] to provide the estimate amount of salt contributing to the soil electrical conductivity.

Analysis of tetracyclines in soils

Ultrasound-assisted solid-liquid extraction of tetracyclines from soil

For the analysis of four tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline), the analytical protocol based on the ultrasound-assisted extraction followed by the solid-phase extraction (SPE) cleanup and HPLC/UV quantification was developed based on the method described by *Kay et al.* [20] (Fig. 1).

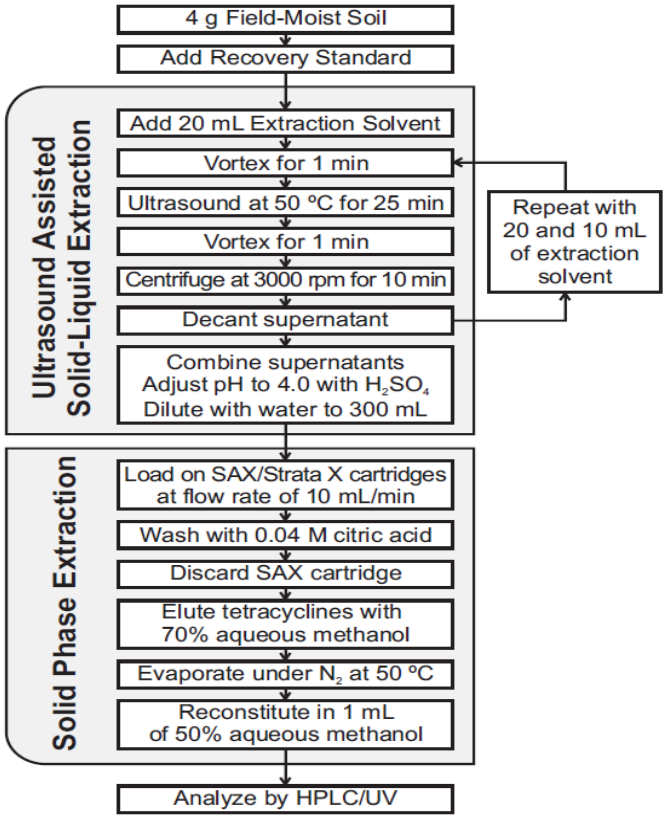

Figure 1. Scheme of analysis.

Conditions were optimized to maximize the recovery of tetracyclines and minimize interference from the soil matrix. The final procedure was as follows: Four grams of field-moist soil sample was accurately weighted into the 50 mL amber polypropelene centrifuge tubes, and 10 µL of 0.5 µg/mL of the recovery standard and 20 mL of extraction solvent (methanol:0.2 M EDTA-McIlvaine buffer pH 8, 1:1, v/v) were added. The samples were vortexed for 2 min, and then extracted in the ultrasound bath at 50 °C for 25 min. The soil suspension was vortexed again for two more min, and centrifuged for 10 min at 3000 rpm. The supernatant was decanted into the glass amber 500 mL bottles, and the extraction of the soil residues was repeated two more times with 20 and 10 mL of the extraction solvent. The supernatants were combined, diluted with water to 250 mL, and adjusted to pH 4.0 with 1 M H_2SO_4. The obtained diluted soil extracts were purified by SPE and submitted to HPLC/UV analysis. All the plasticware and glassware was pretreated with EDTA saturated methanol to minimize sorption of tetracyclines.

Accelerated solvent extraction (ASE) of tetracyclines from soil

The soils were extracted using a Dionex 150 (Sunnyvale, CA) accelerated solvent extraction (ASE) system. Five grams of the soil sample was mixed with Hydromatrix and loaded into the 66 mL stainless steel cell between Hydromatrix to occupy the dead volume in the tube. The stainless steel cell was lined with the EDTA washed cellulose filter. Extraction was carried out at 100 bar and room temperature. Static extraction time was 20 min, flush volume of 60%, and purge volume of 60%. Two extraction cycles were performed using water:methanol (1:3, v/v) containing 25 mM EDTA and 0.6 M sodium chloride (pH 8.0). The ASE extracts were diluted with enough water to reduce the organic strength to less than 5% by volume in the solution. The solution was adjusted to pH 4.0 with 1 M H_2SO_4. The obtained diluted soil extracts were purified by SPE and submitted to HPLC/MS/MS analysis.

Solid-phase extraction (SPE) of tetracyclines from soil extracts

Prior to HPLC/UV analysis, the soil extracts were purified by the solid-phase extraction (SPE). The Strata-X polymeric reverse phase and the Strata SAX SPE cartridges were set up in tandem. Both the Strata SAX (55µm, 70A) and Strata-X (33µm) cartridges contained 1 g of sorbent and were purchased from Phenomenex, Torrance, CA, USA.

The cartridges were activated with 2 × 3 mL of methanol and then conditioned with 2 × 3 mL of 0.04 M citric acid in water (pH 4.0). The soil extracts were loaded on the SPE cartridges at 5 mm Hg negative pressure. After loading, the SPE cartridges were washed with 2 × 3 mL of 10% methanol in 0.04 M citric acid (pH 4.0). Then the SAX cartridges were removed, and the Strata-X cartridges were allowed to dry for 15 min at 5 mm Hg negative pressure. Tetracyclines were eluted with 3 mL of 95% methanol in 0.04 M citric acid and 3 mL of 100% methanol. The methanolic fractions were combined and evaporated to dryness under the gentle steam of nitrogen in the water bath at 45 °C. The dry extracts were reconstituted in 1 mL of 50% aqueous methanol and submitted to HPLC/UV or HPLC/MS/MS analysis.

HPLC/UV analysis

HPLC analysis was performed using the Waters HPLC Breeze system equipped with the 1500 Series HPLC pump, 2487 dual wavelength absorbance detector, and 717 plus autosampler (Waters, Milford, MA, USA). The chromatographic separations of tetracyclines were carried out using XTerra RP 18, 150×4.6 mm, 5 micron column (Waters, Milford, MA, USA) at ambient temperature. The injection volume of 50 µL at the flow rate of 1.0 mL/min was used. The mobile phase consisted of 15 mM NaH_2PO_4 in water at pH 3.0 (solvent A), and acetonitrile (solvent B). The gradient elution program started with an isocratic elution at 5% solvent B for 2 min, and then was followed by a linear gradient to 60% solvent B from 2 to 12 min. Tetracyclines were quantified by their UV signal at 365 nm.

The calibration curves were obtained using the standard solutions of six different concentrations of tetracyclines (0, 0.625, 1.25, 2.5, 5, 10, 20 µg/mL). Internal standard method was employed for construction of the calibration curves using demeclocycline.

HPLC MS/MS analysis

HPLC-MS/MS analysis was performed using an Agilent series 1200 HPLC with the diode-array and Agilent 6320 Ion Trap mass spectrometer detector (Agilent Technologies, Palo Alto, CA). Chromatographic separation was carried out on the reverse-phase Agilent Zorbax Eclipse XDB-C18 (250×4.6 mm, 5 micron) analytical column, which was protected by a guard column with the same stationary phase (12.5×4.6 mm, 5 micron) (Agilent Technologies, Palo Alto, CA).

The column temperature was set at 40 °C, and the autosampler temperature was set at 4° C. The mobile phase consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in methanol (solvent B). The gradient elution program started with linear increase from 30% B to 90% B in 18 min, and then was followed by isocratic elution for 6 min at 90% B. For the first 7 min of the analysis the flow was diverted from the MS to prevent its contamination and ion suppression with salts and other polar species. The injection volume of 5 μL at the flow rate of 0.5 mL/min was used. MS data were collected in the positive ESI MS/MS mode. Nebulizer temperature was 350 °C, nebulizer pressure was 50 psi, and the drying gas glow rate was 10.0 L/min. The MS was manually tuned by infusing the 1 μg/mL tetracyclines mixture in methanol into the 50% aqueous methanol containing 0.1% formic acid at 30 μL/min. The parameters were optimized for a parent ion of each compounds (Table 1).

Table 1. Parameter of ion trap MS/MS for the analysis of tetracyclines.

Compound	**ETC**	**EOTC**	**OTC**	**ECTC**	**αAOTC**	**CTC**	**EATC**	**ATC**	**βAOTC**
Precursor ion, m/z	445	461	461	479	443	479	427	427	443
Target ion, m/z	410	426	426	444	408	444	365	365	408
Capillary, V	-4500	-4500	-4500	-4500	-4500	-4500	-4500	-4500	-4500
Skimmer, V	15.0	62.4	62.4	33.1	40.1	100.0	62.4	25.0	35.9
Capillary Exit, V	103.3	152.6	152.6	144.3	152.5	177.1	290.0	238.5	144.3
Octopole 1 DC, V	10.30	11.69	11.69	10.30	12.24	9.75	10.03	11.69	10.35
Octopole 2 DC, V	1.93	1.97	1.97	1.66	1.63	2.08	1.68	1.93	1.78
Octopole RF, Vpp	135.3	300.0	300.0	33.1	300.0	201.6	267.2	230.3	263.1
Lens 1, V	-6.2	-2.8	-2.8	-2.7	-5.7	-3.9	-6.7	-5.3	-4.2
Lens 2, V	-88.2	-52.8	-52.8	-43.9	-72.0	-54.3	-67.5	-72.0	-55.7
Trap Drive	43.0	53.0	53.0	48.9	50.5	49.3	45.4	50.5	49.7

Microbiological protocols

Isolation of soil bacteria

Bacteria were isolated from the soils by suspending soil samples (10 g) in the 0.85% saline solution and then plating them on the tryptone soya agar plates (tryptone, 10 g/L; soytone, 5 g/L; NaCl, 5 g/L; and agar, 15 g/L). Prior plating, the soils were pre-incubated for 10 days at 60% water holding capacity at room temperature in the dark. The soil bacteria population was enumerated by a culture based method. At least 18 replicate plates within three dilutions (10^{-6}, 10^{-5}, and 10^{-4}) were used for each soil sample. The viable cultures were counted on day 5 as it corresponded to the maximum growth of the 95% isolated bacteria. Additional counts were taken at day 10 to account for the population of slower growing microorganisms. For each soil sample, 104 random bacterial isolates were selected with a total of 3744 bacterial isolates for 36 soils. The obtained bacterial isolates were purified and preserved in glycerol: tryptone soy media (20:80, v/v) at -80 °C for further antibiotic susceptibility testing.

Antibiotic susceptibility test

Susceptibility of the bacteria isolates toward tetracyclines was examined by a standardized microdilution test following the National Committee for Clinical Laboratory Standards guidelines [21]. ATCC *Escherichia coli* 25922 strain was included in each experiment as quality control. Clorotetracycline was tested in two-

fold dilutions from 0.125 to 32 μg/mL. The minimal inhibitory concentration (MIC) was read as the lowest concentration without visible bacterial growth. All MIC determinations were performed in duplicate, and MIC_{50} (μg/mL) values were the concentrations at which 50% of isolates were inhibited. The breakpoint for tetracycline resistance is ≥ 16 μg/mL, and the resistance level for ATCC *Escherichia coli* 25922 strain is 0.5–2 μg/mL [21]. Briefly, about 0.4 μL of the bacteria with the turbidity adjusted to 0.5 McFarland standard was inoculated into the 20 μL of cation adjusted Mueller-Hinton broth containing 0–32 μg/mL of antibiotics. The microplates were sealed and incubated at 35 °C for 16–29 h. Bacteria growth was evaluated by the turbidity of suspensions at 625 nm as compared to the growth control with no antibiotic added.

Soil and swine effluent DNA extraction

Soil DNA was extracted from 0.7 g of soil using the Ultra Clean® Soil DNA Isolation Kit (Mo Bio Labs, Solana Beach, CA, USA), following the manufacturer's protocol for maximum yields. Briefly, the cells were lysed by a combination of mechanical and chemical methods. The soluble fraction was transferred to another tube and a protein precipitation reagent was added. The soluble fraction was then passed through a spin filter with a silica membrane to adsorb the DNA. The DNA was purified with the salt and ethanol washes and eluted with 50 μL of sterile elution buffer. The swine effluent DNA was extracted from 0.5 mL of the swine effluent using FastDNA® Sample Spin Kit for Feces (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol. The DNA samples were visualized on 1.5% (w/v) agarose gels containing ethidium bromide.

Tet(M) and tet(O) tetracycline resistance gene amplification

Amplification of tetracycline resistance genes tet(M) and tet(O) was performed in a total volume of 20 μL containing 2 μL of DNA template, 10 μL of GoTag Master Mix (Promega Corporation, Madison, WI, USA), 6 μL of nuclease-free water, 1 μL of 5 μM forward and reverse primers. Primer sequences were as follows: tet(M) forward, 5′-ACA GAA AGC TTA TTA TAT AAC-3′; tet(M) reverse, 5′-TGG CGT GTC TAT GAT GTT CAC-3′; tet (O) forward, 5′-ACG GAR AGT TTA TTG TAT ACC-3′; and tet(O) reverse, 5′-TGG CGT ATC TAT AAT GTT GAC-3′. Amplification was performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) according to *Aminov et al.* [22]: one step of denaturation (94 °C, 5 min), 25 cycles of denaturation-annealing-elongation steps [94 °C, 30 s; 55 °C (tet(M)) or 60 °C (Tet(O)), 30 s; 72 °C, 30 s], and a final elongation step (72 °C, 7 min). The amplified products were visualized on 2.5% (w/v) agarose gels containing ethidium bromide. Each PCR run contained a negative control (2 μL of nuclease-free water instead of template DNA) and a positive control (2 μL of ATCC *Escherichia coli* 25922 strain instead of template DNA).

Results

Method development for the analysis of tetracyclines in soil

Optimization of ultrasound-assisted extraction method

Ultrasound-assisted extraction of tetracyclines from soils was optimized using blank soil samples spiked with a mixture of tetracyclines at the three spike concentration levels: 1, 10, and 100 μg/g soil. Six combinations of McIlvaine buffer with EDTA and methanol at different pHs and methanol percentages, as well as two dichloromethane based solvent systems were evaluated in the study. The increase of pH from 4.0 to 8.0 resulted in a four- to six-fold increase in tetracyclines' recoveries. However, further increase of pH above 8.5 resulted in irreversible transformation of chlortetracycline into iso-chlortetracyline. With the increase in methanol percentage from 50 to 100%, the recoveries of tetracyclines did not increase. The use

of dichloromethane based solvents with three different compositions (CH_2Cl_2:MeOH:H_2O ratio was 2:1:1, 3:1:1, and 6:1:3, v/v/v) was not efficient for the extraction of tetracyclines from soils as extraction recoveries did not exceed 15%. The extraction solvent system containing methanol : 0.2 M EDTA : McIlvaine buffer (2:1:1, v/v/v) with pH 8.0 provided the highest recoveries across four tetracyclines (Table 2). The optimal ultrasound time of 25 min at 50 °C allowed for the quantitative recoveries with no significant decomposition and transformation of analytes. Similar recoveries were archived at room temperature with double extraction times. The use of ultrasound-assisted extraction resulted in the significant amount of suspended clay and organic matter. To separate soil from the soil debris, the soil extracts were centrifuged for 10 min at 3000 rpm. To assure quantitative recoveries, the extraction procedure was repeated three times.

Optimization of solid-phase extraction

For the selective removal of a co-extracted soil matrix and efficient pre-concentration of tetracyclines, several SPE modes were tested; which included: a neutral polymeric sorbent (Strata-X), a weak cation exchange polymeric sorbent (Strata-X-CW), a strong cation exchange polymeric sorbents (Strata-X-C), and a combination of a strong anion exchange and neutral sorbents (SAX/Strata-X). A mixture of four tetracyclines spiked with a blank soil extract was used for the evaluation of SPE sorbents (final concentration of each compound was 1 μg/g soil or 0.03 μg/mL of extract). Conditions of SPE such as pH, loading regime, wash and elution solvent systems were optimized for each SPE sorbent. Soil extract pH was adjusted to 4.0 before application to the SPE cartridge.

Table 2. Recoveries of tetracyclines from soils using the proposed method.

Added, μg/g soil / Analyte	Recovery (RSD), %		
	1	10	100
Oxytetracycline	104.5 (0.1)	90.9 (2.1)	112.2 (0.1)
Tetracycline	94.3 (3.7)	102.5 (3.5)	105.2 (0.4)
Chlortetracycline	69.3 (2.8)	90.2 (0.8)	88.1 (1.4)
Doxycycline	103.1 (5.0)	106.6 (2.8)	113.5 (4.0)

The performance of SPE was evaluated based on the tetracyclines' recoveries as well as the background levels in the following HPLC/UV analysis. The recoveries of four tested tetracyclines from Strata-X, Strata-X-CW, and SAX/Strata-X are presented in Figure 2. The use of Strata-X-C resulted in no detectable concentrations of tetracyclines (data not shown), possibly due to the strong retention of tetracyclines on the sorbent. For SAX/Strata-X, the recovery yields were almost quantitative for oxytetracycline, tetracycline, and doxycycline, while the recoveries for chlortetracycline were about 70%. The use of Strata-X and Stata-X-CW resulted in about 20% lower recoveries for oxytetracycline, tetracycline, and chlortetracycline. The Strata-X extracts contained a relatively high background due to the co-elution of soil matrix constituents. In comparison, the use of SAX/Strata-X or Strata-X-CW resulted in a lower background level. Both SAX/Strata-X and Strata-X-CW can be successively used for the cleanup of tetracyclines in soil extracts. Although Strata-X-CW provides lower recoveries, the use of a single cartridge SPE can be more economical. The final clean up procedure developed for the analysis of tetracyclines in soil samples used tandem SAX/Strata-X SPE.

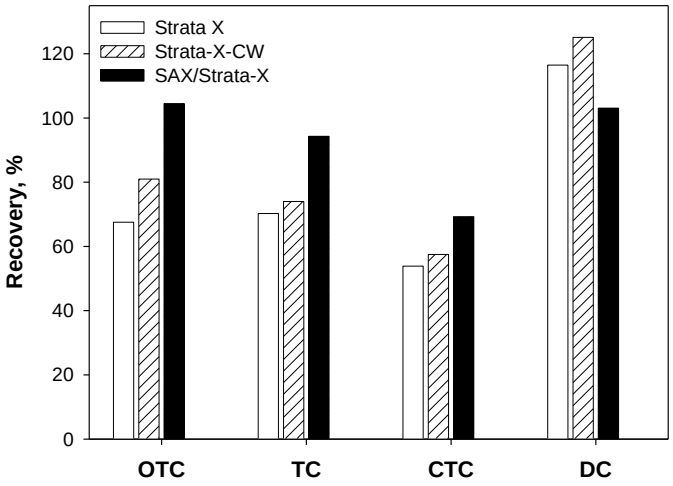

Figure 2. Recoveries of tetracyclines from solid-phase extraction using various sorbents. OTC - oxytetracycline, TC - tetracycline, CTC - chlortetracycline, and DC - doxycycline hyclate.

Optimization of HPLC/UV method

Three reverse phase HPLC columns were evaluated for the separation of tetracycline mixture: Waters Xterra RP 18 (150×4.6 mm, 5 micron), waters Symmetry RP Waters Nova-Pak (150×3.9 mm, 4 micron), and Phenomenex Kinetex PFP (50×4.6 mm, 2.6 micron). Xterra and Kinetex columns had somewhat similar performance, while Nova-Pak column was not selective enough to separate chlortetracycline and doxycycline. The use of Kinetex column was more time efficient and required significantly less solvent than the use of Xterra column. However, Xterra column was more stable over the time and was less susceptible to the changes in soil matrix components.

The mobile phase composition was optimized to provide baseline separation of tetracyclines and to have no affect on analytes' chemistry. Generally, the peak shape improved with the increase of NaH_2PO_4 content. However, increase of NaH_2PO_4 concentrations from 15 to 25 mM resulted in the increased system blockages.

The linearity of calibration curve for each analyte was determined by using series of standard solutions, and each standard solution was measured in triplicate. Linear relationships were obtained, and the correlation coefficients of all the calibration curves were found to be higher than 0.9996. Limits of detection (LOD) under the present chromatographic conditions were determined on the basis of response and slope of each regression equation at a signal-to-noise ratio (S/N) of 3.3. The LOD ranged from 7.2 to 9.3 ng.

Intra- and inter-day variations were chosen to determine the precision of the developed HPLC/UV method. The intra-day variation was determined by analyzing the same mixed standard solution for six times within one day, and accounted 0.12–0.18% for the retention times and 1.15–1.82% for the peak areas. The

inter-day variation was determined by analyzing the same mixed standard solution for consecutive 3 days, and accounted 0.12–0.34% for the retention times and 1.64–3.49% for the peak areas. The calibrations were conducted both before and after the analysis of sequence of samples to ensure that interferences did not affect the response of UV detector.

Using the developed analytical protocol, extraction recoveries of four studied tetracyclines from the soil samples were in the range of 69.3-114.5% with RDS between 0.1 and 5.0%. The limits of detection for the four studied tetracyclines in soil were as low as 0.03-0.4 µg/g soil.

2. Evaluation of the mobility of tetracyclines in soil

Four tetracyclines were analyzed in the soils collected four and twelve months after the most recent swine effluent application by the developed analytical protocol. The analysis of each soil was performed in triplicate to account for a spatial variability of each sample. Concentrations of tetracyclines were calculated against calibration curves developed on the day of analysis. The recovery standard was used to correct for extraction efficiency. In all the analyzed samples, no detectable concentrations of oxytetracycline, tetracycline, or doxycycline were found. Detected concentrations of chlortetracycline in all the analyzed soils did not exceed the maximum legal residue level, and ranged from 4 to 308 µg/kg soil (Fig. 3). The results are consistent with the antibiotic program of the farm which supplied swine effluent, and where chlortetracycline is used as a major tetracycline antibiotic.

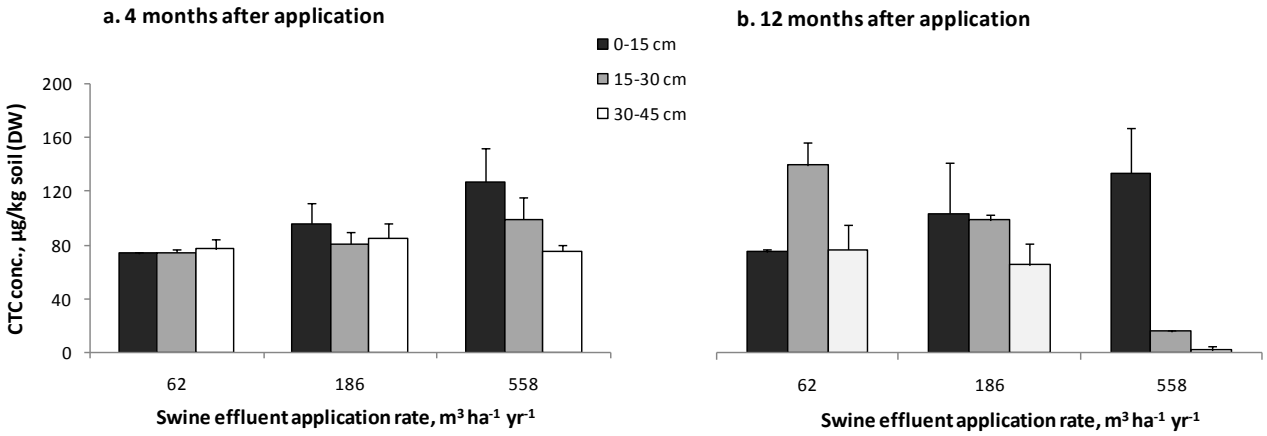

Figure 3. Detected concentrations of chlortetracycline in soils treated with swine effluent.

No statistically significant correlation between the swine effluent application rate and the residual concentration of chlortetracycline in soil sample was observed four months after the most recent swine effluent application. Chlortetracycline was equally distributed in the upper 45 cm layer of soil. In soil samples collected 12 months after the most recent swine effluent application, the concentration range of detected chlortetracycline was the same as eight months earlier, but the distribution within the depth of soil had changed. For the highest swine effluent application rate, chlortetracycline was concentrated in the upper 15 cm layer of soil. For the lower swine effluent application rates, the distribution of chlortetracycline within the soil depth did not change significantly.

3. Antibiotic resistance in bacteria isolated from soil

The viable culture counts of bacteria present in the studied soils were of the same order of magnitude for the studied sample (Table 3). No statistical difference in the number of bacteria isolates was observed as a function of the manure application rates. Also, no significant decline in the amount of aerobic bacteria cultured was observed with the increase of soil depth. Eleven major morphological types of bacterial isolates were observed for each soil. For most soils, about 80% of isolated bacteria were represented by only two major morphological types. These two types of bacteria were present in approximately the same amount in every soil sample regardless of the swine effluent application rate. Two distinct types of fungi and one type of actinomycetes were present on some of the cultured plates. Similar types and number of isolates were observed for soil sampled four and 12 months after the most recent swine effluent application (data not shown).

Table 3. Viable culture counts of bacteria present in soil (g^{-1}).

Soil Depth, cm	Swine effluent application rate, m^3 effluent ha^{-1}			
	0	62	186	558
0-15	6.4×10^6	4.8×10^6	9.3×10^6	6.6×10^6
15-30	5.7×10^6	5.6×10^6	6.8×10^6	4.0×10^6
30-45	3.9×10^6	5.9×10^6	7.3×10^6	3.7×10^6

Susceptibility testing of 3456 bacterial isolates from soils demonstrated that none of the isolates can be classified as resistant, based on the classification of National Committee for Clinical Laboratory Standards guidelines (Fig. 4) [21]. No detectable growth was observed in the media with concentrations of chlortetracycline above 4.0 ppm. At the same time, bacteria strains isolated from swine manure used for fertilization were resistant up to 16 ppm of chlortetracycline. There is a slight decrease in the level and number of chlortetracycline susceptible isolates with the increase in soil depth. No direct correlation was observed between the chlortetracycline susceptibility level and the swine effluent application rate.

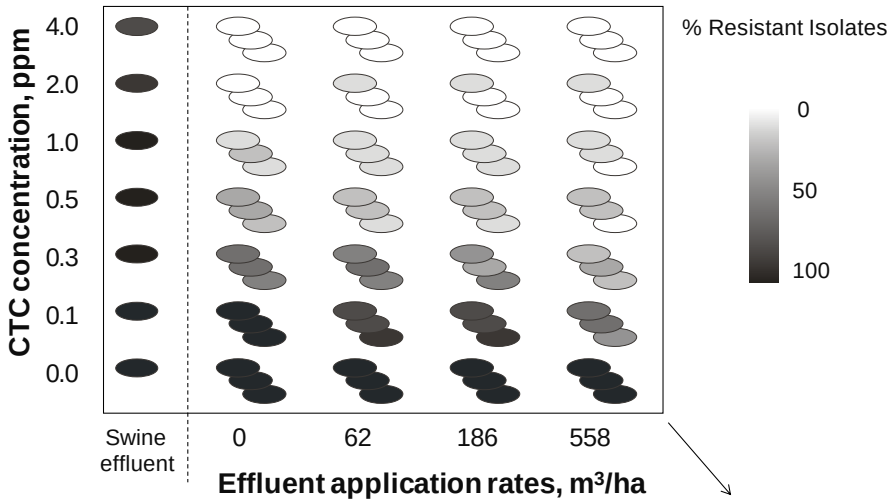

Figure 4. The occurrence of bacteria isolates resistant to chlorotetracycline at the specific concentrations. Bacteria were isolated from soil treated with swine effluent at three different application rates, and from soil never treated with swine effluent. The breakpoint for tetracycline resistance is ≥ 16 ppm.

The presence of two commonly found tetracycline resistance genes, tet(O) and tet(M), was analyzed in the studied soils as well as in the swine effluent. Based on the gel electrophoresis of PCR amplified products, it was revealed that no tet(O) or tet(M) were present in the soils, while these both genes were abundant in the swine effluent.

Discussion

Development of a method for the analysis of tetracyclines in soil

Analysis of tetracyclines in soils is a rather challenging task due to the strong interactions between tetracyclines and the soil matrix components. Tetracyclines are organic zwitter-ion molecules which can

interact ionically or hydrophobically with the soil's components such as clay minerals and soil organic matter. It is of note that the characteristics of the specific soil would almost always define the mechanism and the extent of tetracyclines sorption on that particular soil. For example, the range of partition coefficients for oxytetracycline can vary from 417 to 5259 L/kg in different soils [23, 24]. At lower pHs, tetracycline binding through the ionic interactions between the amine group of tetracycline and the negatively charged soil minerals is believed to be a major binding mechanism. However, based on the organic content of the soil, the contribution of hydrophobic interaction can also be significant [25]. In soils with high concentrations of metal ions, such as Ca^{2+} and Cu^{2+}, the coordination binding of tetracyclines on the metal ions is also significant [26].

To extract tetracyclines from the studied soils, an exhaustive desorption approach was employed (Fig. 1). To disturb ionic interactions of tetracyclines with the clay minerals, extractant pH was adjusted to 8.0. At this pH, the negative charge dominates on the tetracycline molecules, and adsorption of tetracyclines in the native and sodium forms of montmorillonite decreases with the increased pH [25]. The soils used in the study had relative high content of divalent metal ions due to the continuous application of swine effluent (See Supplemental Tables 1 and 2). Thus, relative high concentrations of EDTA were added to the extraction solvent to disturb complexation with the metal cations. While the procedure used provided high recoveries for all the four studied tetracyclines (Table 2), the use of basic conditions and the temperature resulted in the significant co-extraction of soil organic matter. It was shown that the use of anion exchange resin in the following SPE was efficient for removing most of the interferences (Figure 2). The amount of co-extractants was higher in the surface soils and up to two times lower in the deeper soils (data not shown).

The detected concentrations are within the range of previously reported values 0.7–41.8 μg/kg soil [14, 27]. In the present study, chlortetracycline was detected in 0 to 45 cm soil depth (Fig. 3). While in several studies most of the tetracyclines were detected in the upper 15 cm layer of soils and only trace amounts were detected at 30 cm depth; in the present study there was no significant decline in chlortetracycline concentration with the increase in soil depth (Fig. 3). The trend was consistent for the two lowest application rates, even after twelve months following the most recent swine effluent application. Apparently, tetracyclines are relatively immobile in the specific soil. In addition, continuous application of swine effluent can alter the soil properties such as pH and te content of soluble metal ions (See Supplemental Table 1). In fact, detected chlortetracycline concentrations correlate significantly with the sodium ($r=0.4288^{*}$, n=36) and potassium ($r=0.7395^{***}$, n=36) content in soil (Fig. 5). Because soluble salts affect the conductivity of soils, the ionic binding of tetracyclines can be promoted.

Interestingly, at the highest swine effluent application rate (558 m^3/ha), chlortetracycline concentrations in 15–30 and 30–45 cm of soil declined up to 90% in eight months (Fig. 3). To account for decomposition, the soil extracts were analyzed for possible degradation products by HPLC/MS/MS. Degradation products of tetracyclines can still carry biological properties, and sometimes are more toxic than the parent compound [28]. However, no common metabolites of chlortetracycline were detected in the soil (data not shown). Apparently, the higher swine effluent application rates had an effect on the mobility of chlortetracycline in the soil due to the increased amount of organic matter and/or high content of salts. Decreased sorption of oxytetracycline on soils with high dissolved organic matter content was also demonstrated by *Kulshrestha et al.* [25]. Unfortunately, monitoring of chlortetracycline concentrations in runoff and ground water was out of scope in this study, and no solid conclusions regarding potential leaching of antibiotics can be made. To rule out the possible bioaccumulation of chlortetracycline in corn grown on studied soils, the corn plants were analyzed for chlortetracycline. However, no detectable amount of chlortetracycline was present in the corn plants.

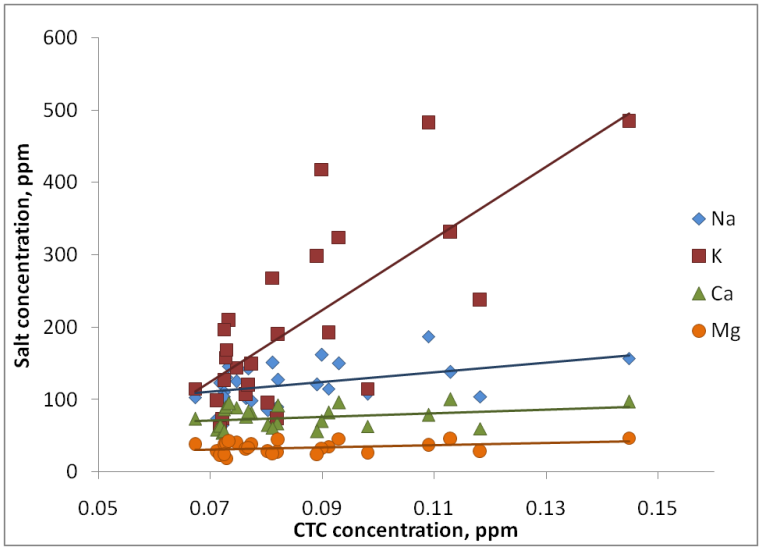

Figure 5. Correlation between selected metal ion content in soils and detected concentration of chlortetracycline in corresponding soils.

The presence of residue chlortetracycline concentrations in the studied soils had virtually no effect on the development of tetracycline resistance in soil bacteria (Figs. 3 and 4). In the present study, occurrence of chlortetracycline susceptible bacteria and the level of susceptibility in soils treated and never treated with the swine effluent were very similar (Fig. 3). Also, there was no significant increase in the number of tetracycline resistant bacteria after the swine effluent application. The presence of chlortetracycline susceptible bacteria in soils never treated with swine effluent is consistent with findings of *D'Costa et al.* [15], who demonstrated relatively high occurrence of antibiotic resistant bacteria in native soils. It should be noted, that many veterinary antibiotics were originally isolated from soil. Particularly, chlortetracycline was originally isolated from soil actimomycete *Streptomyces aureofaciens* [29, 30]. It is possible that soil bacteria already have a natural background of chlortetracycline resistance.

One of the culture based susceptibility testing limitations is that less of 1% of soil bacteria can be cultured under the laboratory conditions [31]. To account for this, the soils were directly analyzed for two common tetracycline resistant genes, tet(M) and tet(O). Tet(M) and tet(O) are ribosomal protection protein genes [32, 33]. Both tet(O) and tet(M) genes are described in a broad variety of gram-positive and gram-negative bacteria, as well as in plasmids [34-38]. Thus, these two genes could be used as indicators for the antibiotic resistance transfer from the swine effluent to soil.

In the present study, both analyzed genes were detected in the swine effluent used for the fertilization, but were not detected in the fertilized soil. Apparently, tetracycline resistance genes are diluted below the detection limit when the swine effluent is transferred to the soil. Also, if tetracycline resistance genes were

transferred by bacteria rather than by plasmids, the survival rate of bacteria in the soil should have been taken into account. For example, if the genes were carried by intestinal bacteria, they may not survive under environmental conditions. Several other research groups also reported the presence of antibiotic resistance genes in animal manure, but not in the soils fertilized with this manure [12, 39-41]. Low survival rate of antibiotic resistant bacteria originating from swine effluent was suggested.

In conclusion, while bacteria resistant to antibiotics could potentially be added to the agricultural soils along with the swine effluent, it would not necessarily lead to the development of antibiotic resistance in bacteria originally present in the soil. It is likely that bacteria introduced with the swine effluent could only survive for a limited time, and the level of antibiotic resistance in the swine effluent amended soils corresponds to the level in the soils never amended with the swine effluent. The residue concentrations of chlortetracycline in soils have virtually no effect on the selection of tetracycline resistance in the fertilized soil.

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Publications

The results of the present research were presented in a special session on Veterinary Pharmaceuticals in the Environment at 242nd American Chemical Society National Meeting & Exposition on August 28-September 1, 2011 in Denver, CO and in Soil Biology & Biochemistry session at ASA-CSSA-SSSA International Annual Meetings in on October 16-19, San Antonio, TX. The acknowledgment of the National Pork Board was cited.

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Supplemental Materials

Table 1. Description of soils used in the study. Data are presented as average of three field replicates (coefficient of variance).

Soil Depth, cm	Swine Effluent Application Rate, m^3/ha^{-1} yr^{-1}	pH ($CaCl_2$)	Na, ppm	K, ppm	Ca, ppm	Mg, ppm	Total Soluble Salts, ppm	Organic Carbon, %	Total Nitrogen, %	Sand, %	Silt, %	Clay, %
Gruver clay loam, Fine, mixed, superactive, mesic Aridic Paleustolls												
0-15	0	8.1 (1)	122 (9)	109 (6)	97 (15)	45 (12)	1436 (14)	2.2 (2)	0.14 (1)	32.5	42.5	25.0
15-30	0	7.9 (1)	101 (8)	85 (15)	95 (22)	42 (26)	1355 (24)	1.6 (8)	0.11 (10)	35.0	41.3	23.8
30-45	0	7.7 (3)	89 (1)	55 (7)	68 (3)	26 (6)	1205 (35)	1.3 (3)	0.10 (22)	40.0	35.0	25.0
0-15	62	7.9 (8)	120 (12)	139 (16)	84 (11)	39 (3)	1383 (13)	2.1 (4)	0.14 (4)	37.5	40.0	22.5
15-30	62	7.8 (8)	95 (20)	110 (13)	73 (19)	32 (16)	1156 (19)	1.5 (10)	0.10 (20)	35.0	42.5	22.5
30-45	62	7.9 (2)	93 (31)	69 (10)	62 (11)	25 (8)	959 (11)	1.2 (3)	0.09 (18)	35.0	42.5	22.5
0-15	186	8.1 (2)	138 (8)	282 (28)	96 (4)	45 (2)	1866 (17)	2.4 (3)	0.15 (4)	37.5	42.5	20.0
15-30	186	8.1 (1)	120 (20)	183 (18)	86 (9)	38 (9)	1531 (17)	1.7 (10)	0.11 (5)	37.5	38.8	23.8
30-45	186	7.9 (3)	112 (26)	110 (12)	70 (16)	29 (14)	1202 (20)	1.3 (4)	0.09 (6)	35.0	41.3	23.8
0-15	558	8.2 (1)	156 (21)	413 (30)	83 (16)	40 (14)	2123 (24)	2.3 (5)	0.15 (4)	35.0	41.3	23.8
15-30	558	8.0 (1)	129 (23)	318 (29)	62 (16)	28 (16)	1639 (26)	1.7 (10)	0.12 (8)	35.0	41.3	23.8
30-45	558	8.0 (1)	129 (18)	211 (24)	55 (12)	23 (14)	1316 (17)	1.3 (4)	0.09 (6)	36.3	40.0	23.8

Table 2. Description of swine effluent used in the study. Swine effluent samples were taken from the lagoon at three different times and presented as average.

Properties	Value
Moisture, %	99.3
Dry Matter, %	0.7
pH	8.0
EC, µS	$1.5 \cdot 10^5$
Soluble Salts, ppm	$1.3 \cdot 10^7$
P_2O_5, ppm	$0.6 \cdot 10^4$
Ca, ppm	$0.6 \cdot 10^4$
K_2O, ppm	$2.6 \cdot 10^5$
Mg, ppm	$2.8 \cdot 10^3$
Na, ppm	$1.1 \cdot 10^5$
S, ppm	$2.9 \cdot 10^3$
Fe, ppm	$3.2 \cdot 10^2$
Zn, ppm	$1.4 \cdot 10^2$
Cu, ppm	$2.2 \cdot 10^2$
Mn, ppm	1.5
Organic Carbon, %	29.3
Total Nitrogen, %	11.3