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Distribution, Sorption and Desorption of Tylosin, Chlortetracycline and their Metabolites in Pig Manure

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Abstract

Effects of pH on sorption, desorption and hysteresis of chlortetracycline (CTCs), tylosin (TYL) and their metabolites have been studied in pig manure samples collected from different pig farms. Approximately 36% of the samples analyzed tested positive (concentration > quantitative detection limit) for the antibiotics and metabolite residues. The degree of contamination depended upon the manure's pH. The acidic TYL-positive manure samples contained TYL-A and TYL-B, while the basic TYL-positive samples contained TYL-A, TYL-ALD and TYL-D. The CTC-positive manure samples contained mostly CTC, ECTC and ACTC at acidic pH, but mostly CTC, ICTC, EICTC and EACTC at basic pH. For acidic and neutral manure samples, the $\log K_D^{des}$ values were greater than the $\log K_D^{s}$ values for TYL-B, TYL-A, TYL-ALD, CTC, ACTC, ECTC and EACTC, indicating sorption-desorption hysteresis. TYL-D, ICTC and EICTC did not exhibit sorption-desorption hysteresis. At acidic and neutral pH, the residues remained solid-bound, but an increase in pH decreased hysteresis, resulting in greater leaching and contamination of the environment. Thus, environmental pH may determine the antibiotics and metabolite leaching in freshwater at or away from the site of production. In addition, analysis of the parent antibiotic residues may not provide an accurate assessment of the environmental health.

Keywords: Chlortetracycline, tylosin, manure, sorption, desorption, hysteresis

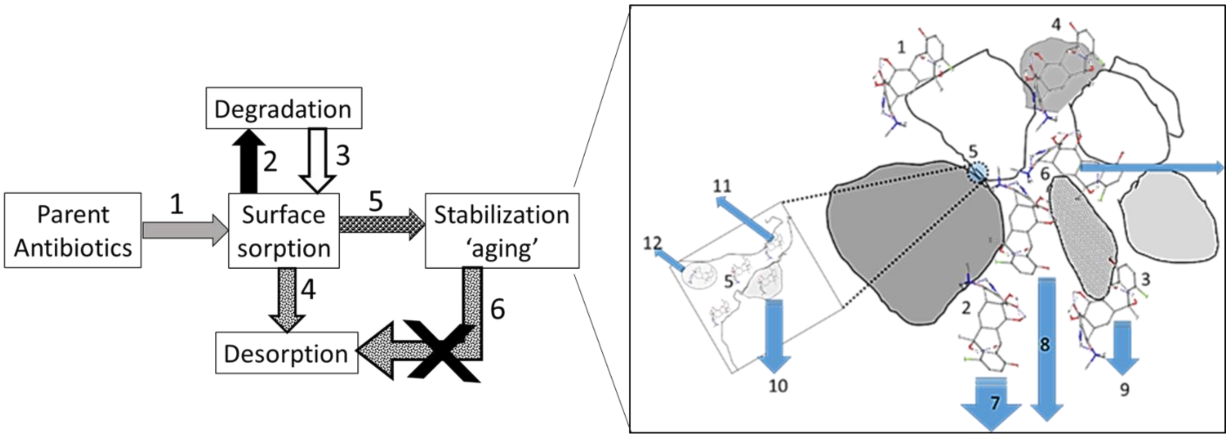
1. Introduction

CTC (broad-spectrum antibiotics) and TYL (a macrolide antibiotic isolated from a strain of

Streptomycets fradiae) are fed to the animals in feedlot operations for growth promotion and health maintenance (Pan et al. 2011, Hoese et al. 2009, APHIS 2006, Dewey et al. 1999, Powers 1999, Raman et al. 2004, Kerr et al. 2006). The antibiotics residues could contaminate the terrestrial compartment of the environment by either direct runoff or application of soil with contaminated manure. Pigs excrete a significant fraction of the ingested antibiotics in feces and urine (Elmund et al. 1971, Feinman and Matheson 1978, Alcock et al. 1999, Kolz et al. 2005). Earlier studies have reported the CTC and TYL concentrations in pig manure samples collected from different swine facilities in the United States, Europe and Asia ranged from 100 µg/Kg (solid) or L (liquid) to greater than 700,000 µg/Kg or L (Martinez- Carballo et al. 2007, Kumar et al. 2004, Hamscher et al. 2002, Jacobsen and Halling-Sørensen 2006, Karci and Balcioglu 2009, Zhang et al. 2005, Liu et al. 2009, Wei et al. 2009, Winckler et al. 2003, Hu et al 2008). The issue of antibiotic contamination is compounded by the fact that approximately 39% of all livestock in the U.S. are grown in confinement where they produce an estimated 175 million tons of manure (Kowalski et al. 2013, Lens et al. 2004) that are applied to the soil as an alternative to chemical fertilizer. Therefore, if manure is contaminated, it may also contaminate the applied fields due to leaching of the pollutants (Dolliver et al. 2007). The occurrence of antibiotics in the environment may be causally related to an increase in bacterial resistance in manure and waste effluent (Halling-Sorensen et al. 1998, Levy 1998, Goni-Urriza et al. 2000, Ogan and Nwiika 1993). Therefore, presence of antibiotic residues in the environment may pose a serious public health concern (Klaver and Matthews 1994, Halling-Sorensen 2001, Chee-Sanford et al. 2001, Boxall et al. 2003) that must be addressed. But, there is limited information on fate of antibiotics in animal manure since earlier studies have studied the fate of antibiotics in clays, organo-clays, humic materials and other soil types (Sassman and Lee 2005, Porubcan et al. 1978, Sithole and Guy 1987, Kay et al. 2004, 2005, Kin et al. 2011).

The main processes potentially affecting the distribution and fate of antibiotics in manure and soil are (i) their sorption to and desorption from manure or soil, (ii) chemical/biological transformations and (iii) transport through soil and water channels (Saltzman and Yaron 1986; van der Hoff and van Zoonen 1999). Sorption and desorption processes of a chemical depend upon the physicochemical properties of pollutants and chemical composition of manure determined by the differences in animal food sources and size of the operation (Kowalski et al. 2013). The solid fraction of manure contains (i) a mixture of soil, organic matter, enzymes that degrade chemicals, humus, and nutrients, (ii) anionic groups such as $-CO_2^-$ and $-O^-$ groups that attract cations such as Ca^{2+}, Mg^{2+}, and Al^{3+} and (iii) hydrophobic groups that retains hydrophobic chemicals (Brady 1974, Sanchez and Gonzalez 2005). Antibiotics may bind the soil particles by either replacing the cations, a process known as cation-exchange or binding via hydrophobic interaction (Sassman and Lee 2005). The observations that free antibiotics present in manure is taken up by plants that accumulate the antibiotics in stem, leaves and fruits (Kang et al. 2013, Dolliver et al. 2007, Kumar et al. 2005, Krebs et al. 1998) suggests that consumers may unknowingly be ingesting some of the antibiotics when they eat vegetables grown on manure-applied lands. However, substantial evidence support that, in addition to the parent antibiotics, their metabolites and some secondary metabolites exhibit may also exhibit biological activities (Habbu et al. 2016, Berdy 2005, 1995, 1989,1985, Donadio et al. 2002, Omura 1992, Demain 1999, Sassman and Lee 2005, Halling-Horensen et al. 2002, McCormick et al. 1957). Thus, in addition to CTC and TYL-A, their metabolites may also pose significant health risk to exposed humans and animals. Unfortunately, the fate of antibiotics metabolites in animal manure is not fully understood.

Therefore, the overall aim of the present investigation was to study the occurrence, sorption and desorption of TYL, CTC and their metabolites in manure samples obtained from pig farms. The hypotheses are that (i) the ionic and hydrophobic properties of CTC, TYL and their metabolites play a critical role in their binding to manure solids and (ii) TC and TYL metabolites are poorly retained by manure solids, exhibiting greater mobility (Figure-1).



A

B

Figure-1. The environmental fate of TYL, CTC and their metabolites in pig manure.

The Fate of antibiotics in manure is determined by their sorption to the manure-solid surface (Figure 1A-1) followed by their (i) desorption and migration (Figure 1A-4), (ii) 'aging' or stabilization (Figure 1A-6 and Figure 1B) and ensuing decrease in desorption, a process known as hysteresis (HYS), and (iii) biodegradation that results in formation of metabolites (Figure 1A-2) that may distribute between manure's liquid and solid (Figure 1A-3). Abbreviations for Figure 1B: 1 and 2 represent selective polar binding, 3 represents non-polar (hydrophobic) binding, 4 and 12 represents bacterial or root internalization and metabolism, 5 and 11 are chemical binding and stabilization (causing hysteresis), 6 is pore internalization, 7 to10 are desorption.

2. Materials and Methods

2.1 Chemicals and Supplies

TYL, CTC and metabolites were purchased from Sigma Chemicals Co. (St Louis, MO). The CTC metabolites were Iso-CTC hydrochloride (ICTC), anhydro-CTC hydrochloride (ACTC)), 4-epi-CTC hydrochloride (ECTC), and 4-epi-anhydro-CTC hydrochloride (EACTC). The column used for chromatographic separation of these antibiotics was 150 x 4.6 mm, Adsorbosphere-ODS, 5μ, C_{18} column (Alltech, CA). The manure samples were collected from farms (at least 1500 growing and finishing pigs) in Minnesota, Iowa, North

Dakota and North Carolina.

2.2 Physicochemical Properties of the Antibiotics and their Metabolites

The 2D structures of TYL-A, TYL-B, TYL-C, and TYL-D, OMT, DMT, CTC (+ - 0, 0 – 0 and + - - (Stephens et al., 1956), ECTC, ICTC, EICTC and EACTC (protonated and deprotonated) were drawn using ChemDraw. The 2D structures were imported to the ChemDraw 3D and MOE software and minimized to the minimum energy conformation before calculating their physicochemical, electronic and topological properties.

2.3 Processing and Properties of Manure Samples

2.3.1. Manure Processing

Whole manure samples were collected from pig farms and handled as described previously (Singh et al. 2013, Singh 2015, 2016). Immediately after collection, each sample was mixed with internal standards to track possible degradation and then split in two parts.

- *Part-1* (30% by weight) samples were thawed and autoclaved as described previously (Lesan and Bhandari 2003) to prevent antibiotic degradation during processing. The samples were screened for CTC, TYL and their and metabolites (using the HPLC method described below) to identify antibiotic-positive (antibiotic and metabolite concentrations greater than the method’s minimal detection limit (MDL)) and antibiotic-negative (antibiotic and metabolite concentrations less than the method’s MDL) manure samples. The screening showed that about 30% of the analyzed samples tested positive for CTC, TYL and their metabolite residues.
- *Part-2* (70% by weight) samples were thawed, sterilized and analyzed for antibiotic concentrations in liquid and solid fractions. The solid-water distribution and desorption of antibiotics were measured using isotherm equations described later.

All samples were analyzed for total organic carbon (OC), total wet manure solid (TS) and total Kjeldahl nitrogen (TKN) concentrations.

2.3.2 Manure Characterization

Total Organic Carbon was measured using a procedure described by Perrier and Kellogg (1960). Total Wet Manure Solid (TS) and Total Kjeldahl nitrogen (TKN) was determined using procedures described in a University of Wisconsin Madison Extension Publication A3769 (Peters 2003). Cation Exchange Capacity was measured using $BaCl_2$ and triethanolamine as described by Lax et al (1980). During the method validation phase, the $BaCl_2$ method was compared with the ammonium acetate method described by Sumner and Miller (1996).

2.3.3. Screening of Manure for CTC, TYL and their Metabolites

2.3.3.1. Extraction of antibiotics and metabolites from manure samples

Each manure sample was centrifuged and the solid and liquid phases were collected. The liquid samples were mixed with internal standards (demeclocycline (DMCTC) for CTC and roxithromycin (Ro) for tylosin) and concentrated to 10 ml using a freeze drier. The dried samples were stored at -80^{o}C for further analysis. The solid samples were washed with pure water (x10 w/v) followed by $CaCl_2$ solution (0.5M) (x 5 w/v) for elution of the antibiotics and the metabolites. Each pooled eluates were freeze dried and stored at -70^{o}C for further analysis.

.3.3.2. Extraction of CTC and Metabolites (Zhu et al 2001)

Manure samples (liquid or solid eluate) containing CTC and its metabolites were mixed with 10 ml of phosphate buffer (0.05M sodium phosphate mono-basic, 0.05M citric acid, pH 4, 0.05M EDTA). The samples were mixed thoroughly and centrifuged. Clear supernatant was mixed with 20 ml of reagent water that was poured onto a C_{18} column (500 mg, BOND ELUT, Varian, Harbor City, CA) conditioned with 10 ml methanol and 10 ml water. The column was prevented from drying completely after the entire samples had passed through it (the column efflux was retained for re-extraction). The bound-antibiotics were eluted with 2 ml of methanolic oxalic acid (0.01M) followed by acetonitrile. The eluates were pooled and concentrated to 0.1 ml, then analyzed directly.

2.3.3.3. Extraction of Tylosin and Metabolites (Nozal-Nalda et al 2006)

Polymeric sorbent SPE tubes (6 mL with 200 mg of sorbent, Strata X-33lm) conditioned with methanol and water (5 ml) were used. The pH of each concentrated extract was adjusted to 9 with carbonate buffer (100 mM, pH 9) and loaded on an conditioned SPE polymeric cartridge at about 1 mL/min using a suction system. Then, the columns were washed with 4 mL of 70:30 v/v water/ methanol (this rinse was stored for re-extraction is necessary). The analytes were eluted with 1 mL of methanol followed by 1 mL of acetonitrile.

2.3.3.4. Chromatography - CTC and Metabolites: The MS system used was Applied Biosystems API 3000 (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray source. Coupled to a Waters 2690 Alliance system (Milford, MA, USA) equipped with a tertiary gradient system. The columns and elution buffers used has been described previously (Soeborg et al. 2004). The separation was performed using the following mobile buffers: A - 200 ml methanol, 310 µl formic acid and milliQ water up to 1 L; and B- 950 ml methanol, 310 µl formic acid and milliQ water up to 1L. A procedure described by Tylova et al. 2013 and Soeborg et al. (2004) was used in this study. The MS analysis was performed in the positive ion mode using the settings described previously (Soeborg et al 2004). Product ions were m/z 461>444 for ACTC and EACTC, 479>444 for CTC, 479>462 for ICTC and 427>410 for EATC.

2.3.3.5. Chromatography - Tylosin and Metabolites

The procedure described by Nozal-Nalda et al. (2006) was modified to determine concentrations of TYL and its metabolites in manure liquid and solid samples. In brief, the analytes were separated a C18 column using formic acid 1% in water (solvent A), methanol (solvent B) and ACN (solvent C as mobile phase at a flow rate of 0.8 mL/min. A. The tertiary programming was modified for clear separation: 0 min 70/60/0 (A/B/C), 10 min 60/40/0, 20 min 70/5/25, 25 min 70/0/30 and 35 min 60/40/0 (equilibration for 5 min).

For quantification, a stock standard solutions for CTC, TYL-A and metabolites were prepared in water-methanol (70:30) or pure methanol at a concentration of 200 mg/L. A set of working standards (0 to 5 mg/ml) containing (1 mg/ml) roxithromycin (internal standard) were prepared using analyte-free manure liquid or solid eluates. All working standards were stored at 4°C and prepared fresh daily. The calibration samples were analyzed with each batch of analytes and a calibration curve was plotted using response (peak area or ion (m/z) intensity) on y-axis and concentration on x-axis. Manure analytes were determined using linear regression. At least three levels of the QAQC samples were used to ensure concentration accuracy.

2.4. Sorption, desorption and hysteresis of CTC, TYL-A and metabolites

2.4.1. Sorption coefficient (logK_D^s):

The antibiotic and metabolite concentration in solid and liquid fractions were fitted to equation-1 to calculate logK_D^s values.

$$LogK_D^S(L.Kg^{-1}) = log[\frac{A_e(\%)}{100-A_e\,(\%)}\ [\frac{V_0}{m_{solid}}]] \qquad \text{Equation 1}$$

In equation-1, A_e is % adsorption, V_0 is initial volume, and m_{solid} is total solid mass. An increase in logK_D^s is associated with a decrease in the leaching capacity of the compound (Sanchez-Camazano et al. 1994).

2.4.2 Desorption

Manure solids (pre-analyzed for TYL-A, CTC and metabolite concentrations) were centrifuged and the supernatant was completely aspirated. The liquid volume was recorded for each sample. The solid fraction was mixed with an antibiotic-free phosphate buffer, pH 6, 7, 8 or 9, mixed with 0.02M $CaCl_2$ solution (the volume of the buffer was equal to the sample's original liquid volume), mixed thoroughly, equilibrated for 24h at room temperature and centrifuged at 3000 ×*g* for 20 min. Then, 5 ml aliquot was removed from the supernatant and immediately replaced by equal volume of fresh desorption solution. Desorption step was repeated four times (Figure 2).

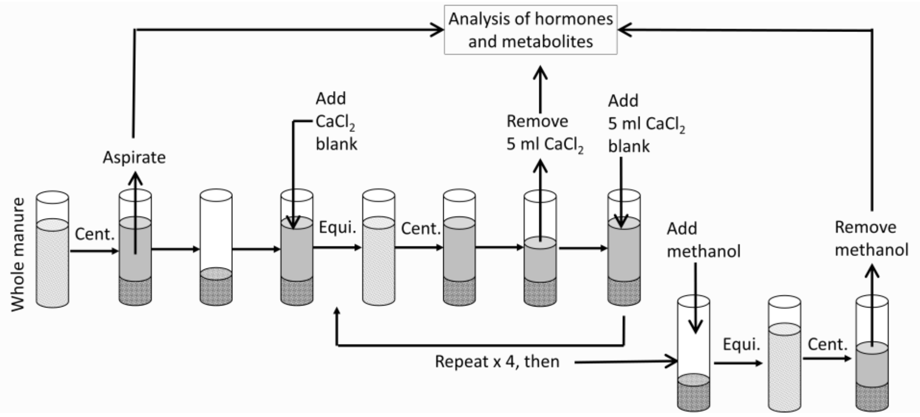


Figure2. The antibiotics desorption procedure used in this study.

Finally, the residues remaining in the solid were extracted with methanol. The supernatants were evaporated over nitrogen almost to dryness. A final volume of 1 mL was reconstituted in acetonitrile to be analyzed by HPLC/UV/MS-MS to determine the antibiotic concentrations in the equilibrium solution. Blank controls (solution + test substance without soil/sediment) were subject to the same test procedures to assess potential sorption to glass test vessels and potential of loss due to chemical hydrolysis. One ml of the blank buffer was removed and analyzed using HPLC/UV/MS-MS. The remaining solution was mixed with one ml of blank (antibiotic free $CaCl_2$) solution, incubated and analyzed. This was repeated four times, aqueous desorption steps 1 to 4. This was followed by a methanol desorption. The results were used to calculate $\log K_D^{des}$ using equation-2 (USEPA OECD/OCDE #106, 2004).

$$Log\ K_D^{des} = \log[\frac{m_s^{ad}\ (eq)}{m_{aq}^{des}\ (eq)}\ \frac{V_T}{m_{solid}}\ ]\ (\mathrm{L.Kg^{-1}}) \qquad \text{Equation-2}$$

In equation 2, m_{solid} is quantity of the soil phase (dry mass, (g)), m_{aq}^{des} (eq) is mass of the antibiotics or metabolites in the aqueous phase at desorption equilibrium (μg), m_s^{ad} is mass of the test substance remaining in the soil phase at desorption equilibrium (μg) and V_T is total volume of the aqueous phase in contact with the soil during the desorption (cm^3).

2.4.3 Hysteresis coefficient (HC)

HC was calculated using equation -3. An increase in HC values represents an increase in sorption-desorption hysteresis.

$$HC\ (\%) = \left[\frac{K_D^{des} - K_D^{s}}{K_D^{des}}\right] * 100 \qquad \text{Equation-3}$$

2.5 Statistics

The data were represented as mean ± standard deviation. SPSS (SPSS Inc. Chicago, IL) was used to determine significance of difference between two means at $\alpha = 0.05$.

3. Results

3.1 Manure Characteristics

Overall manure characteristics are listed in Table-1. The solids content of the manure samples collected in this study varied from <20% to 66%. TS, TOC and TKN (%) values for whole manure were insensitive to pH differences from 5.5 to >9. The CEC values decreased significantly as pH increased.

Table-1. Swine manure properties.

Indices	pH 5.5-6.5	pH >6.5 – 7.5	pH >7.5 – 8.5	pH ≥9
TS (%)	21±9	19±11	27±11	35±18
TOC (%)	35±21	31±13	23±10	21±9
TKN (g/kg)	23±11	26±9	29±7	26±8
CEC	111±22	79±17*	51±7*	17±10*

Please align Indices with pH values

TS: total solid, TOC: total organic carbon, TKN: Total kjeldahl nitrogen, and CEC: cation exchange capacity. * $p < 0.05$ significant when compared with acidic manure

3.2 Physicochemical Properties of Antibiotics

3.2.1. Tylosin-A and Metabolites

Figure-3 shows general structure of TYL and its metabolites. TYL-A, the parent compound contained, is a polyketide lactone substituted with three deoxyhexose sugars mycinose, mycaminose and mycarose. TYL metabolites are generated by loss of different substituted groups. Figure-4 shows the 3D structures of MM2 minimized 3D structures of TYL-A exhibiting distinct hydrophobic and hydrophilic surfaces with sugars in same plane, while the +ve charged NH_3^+ in an opposite plane. Although TYL-A and metabolites common pKa value, TYL-B is most hydrophobic and has least PSA. TYL-ALD was most hydrophilic metabolite. The molecular weight exhibited the following pattern: TYL-A > TYL-B, TYL-D > TYL-ALD.

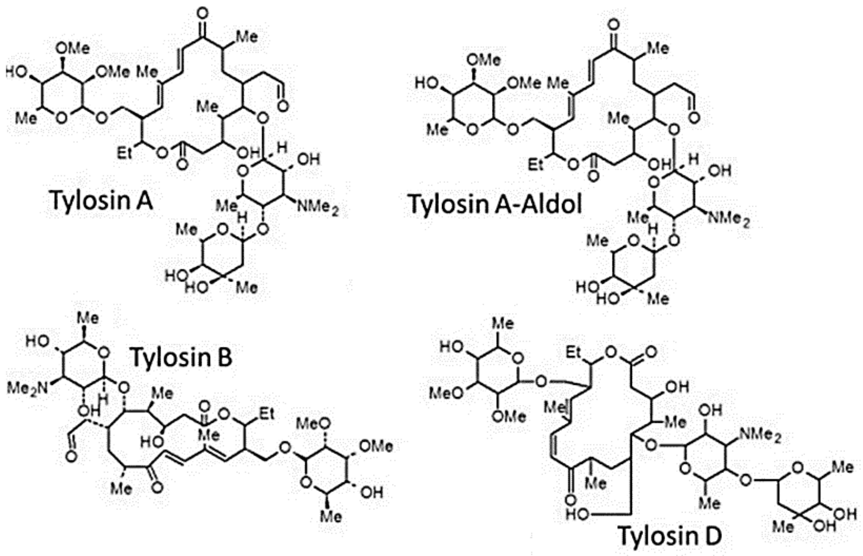


Figure 3. Chemical Structure of TYL-A and metabolites

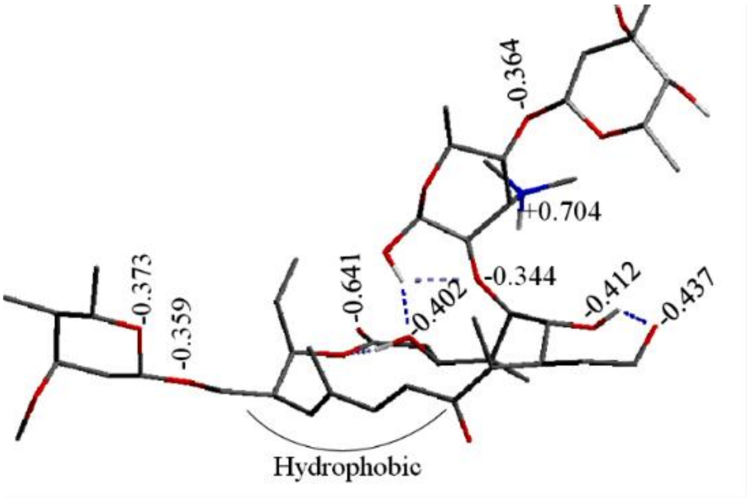


Figure-4: Three Dimensional Structure of TYL-A Showing Its Electronic Charges and Hydrophobic Site.

Table 2. Physicochemical Properties of TYL-A and Its Metabolites.

Indices	**TYL-A**	**TYL-B**	**TYL-D**	**TYL-ALD**
PSA	219	140	238	240
pKa	8.9	8.9	8.9	8.9
Lipinski rule				
Molecular weight (Mw) 180 - 500	900*	756*	756*	584*
# H bond acceptor (HBA) ≤ 10	15*	12*	12*	8
# H bond donor (HBD) ≤ 5	5	4	4	5
# rotatable bonds (RB) ≤10	13*	11*	11	7
LogP 0.4 0 - 5.6	1.66	2.3	1.7	1.3

PSA: polar surface area, pKa: dissociation constant, * Not conforming to the Lipinski rule.

3.2.2. Chlortetracycline and Metabolites

Figure-5 shows 2D structures of CTC metabolites 4-epi-CTC (ECTC), iso-CTC (ICTC), 4-epi-iso-CTC (EICTC), anhydro-CTC (ACTC) and epi-anhydro-CTC (EACTC). In general, CTC is based on the octahydro-naphthacene ring structure having (i) one aromatic (D) and three (ABC) alicyclic alkane rings (Figure 4B), (ii) six oxygen function groups in lower parts and C, (iii) a dimethylamine group at C4-ring A and (iv) a Cl substation at C7 in ring D (Figure 6).

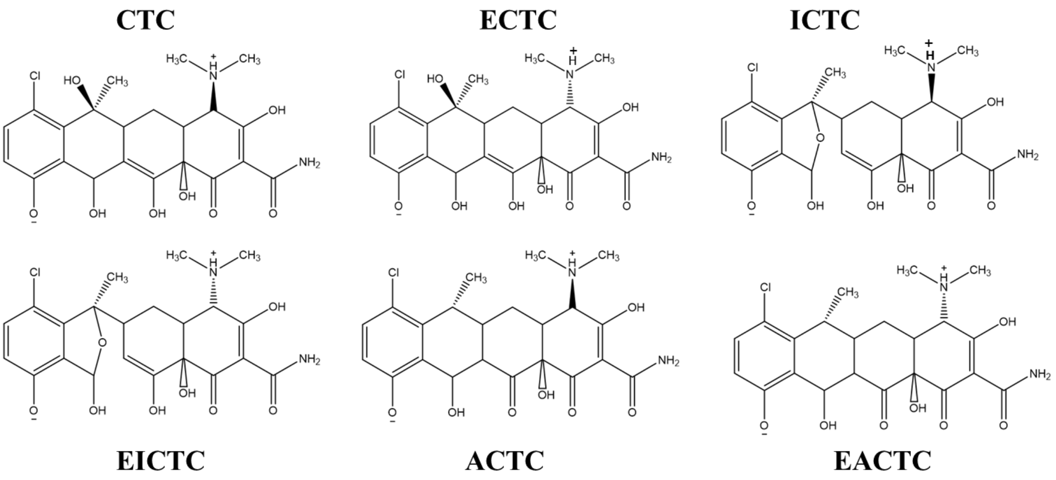


Figure 5. Chemical structures of chlortetracycline (CTC) and its metabolites.

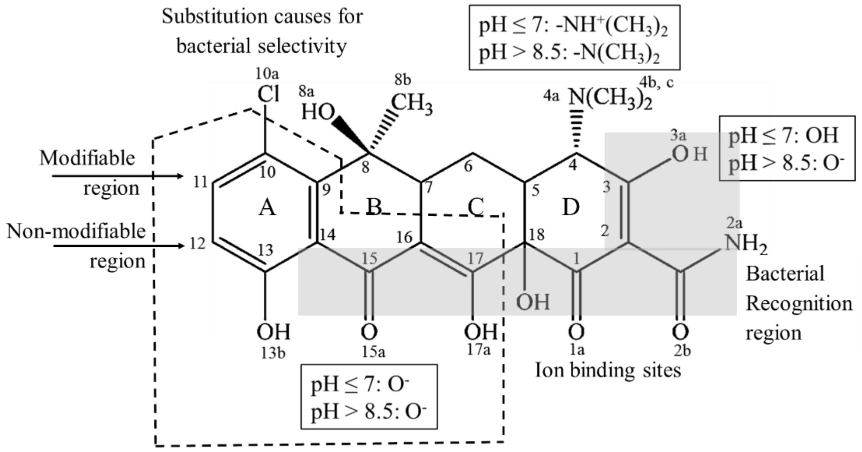


Figure-6. CTC structure-function relationship and ionization characteristics.

Abbreviations: ECTC is *epi*CTC, ACTC is anhydroCTC, EACTC is *epi*ACTC, ICTC is isoCTC and EICTC is *epi*ICTC.

Table 3 shows the physicochemical properties, while Table 4 lists the electronic properties of CTC and metabolites. The upper region of CTC is known as the modifiable region that can alter its biological properties and selectivity. The antibiotics also exhibits complex ionization patterns (Figure 6). At acidic pH, only the 4C nitrogen is protonated ($-NH(CH_3)_2^+$). At pH > 9, the two hydroxyl groups are sequentially deprotonated, resulting in formation of ionized O^- (O

3a and/or O 13b). At pH ranging from 7 to 8.5, CTC may exist as a zwitterion with net neutral (one O^-) or negative (two O^{-1}).

Table 3. Physicochemical properties of chlortetracycline and their metabolites.

Indices	CTC	ECTC	ICTC	EICTC	ACTC	EACTC
PSA	187	188	164	164	164	164
pKa1	9.3	9.3	8.4	8.4	9.4	9.4
pKa2	7.4	7.4	6.4	6.4	7.4	7.4
pKa3	4	4	3.6	3.6	4	4
Lipinski rule						
Molecular weight (Mw) 180 - 500	479	480	463	479	464	479
# H bond acceptor (HBA) ≤ 10	9	8	8	9	7	9
# H bond donor (HBD) ≤ 5	5	7	4	5	5	6
# rotatable bonds (RB) ≤10	3	2	2	2	2	2
LogP 0.4 0 - 5.6	-3.3	-3.3	-2.17	-2.17	-0.58	-0.58

The electronic charged in selected N and O atoms in different ionization forms of CTC is shown in Table 4. The ionized atoms exhibited expected positive and/or negative charge, respectively, for CTC^{+00} (+ve charge) and CTC^{0-0} (-ve charge) or CTC^{+--} (-ve charge). At higher pH, the deprotonated $–N(CH_3)_2$ at C4 also exhibited electron deficiency with positive charge > 1, possibly due to electron delocalization. Thus, CTC may behave like a zwitterion even if $-N(CH_3)_2$ remains deprotonated (Table 4).

Table 4. Electronic charge in O and N atoms in different CTC (Figure 6 for atom location).

	000	0 0 -	0 - 0	+ 0 0	+ - -
Atoms	-OH 3a Protonated $-N(CH_3)_2$ 4a Deprotonated	O^- 3a Deprotonated $-N(CH_3)_2$ 4a Deprotonated	O^- 13b Deprotonated $-N(CH_3)_2$ 4a Deprotonated	$N(CH_3)_2^+$ 4a Protonated OH 3a 13b Protonated	O^- 3a 13b Deprotonated $NH(CH_3)_2^+$ 4a Protonated
O 8a	-0.32	-0.291	**-1.025**	-0.31	**-1.069**
O 13b	-0.38	-0.38	-0.41	-0.373	-0.333
O 15a	-0.518	-0.528	-0.531	-0.513	-0.573
O 17a	-0.338	-0.341	-0.371	-0.329	-0.313
O 1a	-0.689	-0.619	-6.72	-0.632	-0.629
O 3a	-0.148	**-0.702**	-0.151	-0.121	**-0.732**
N 2a	0.09	0.05	0.07	0.05	0.07
O 2b	-0.825	-0.844	-0.867	-0.844	-0.813
N 4a	-0.143	1.141*	1.157*	0.716	0.923

3.3 Antibiotics Analysis and Quantification

The HPLC method used in this study provided clear separation of TYL, CTC and their metabolites from manure samples (Figure 7). The non-quantitative detection limit (nQDL) of the method was 1 μg/L, but the quantitative detection limit (QDL) of the method was 20 μg/L when the samples were extracted using C_{18}-column extraction procedure. The recovery of the antibiotics and their metabolites ranged from 80% to 97%. Approximately 45% of the analyzed samples tested positive for CTC, TYL-A and their metabolites, but only 36% of the positive samples contained the residues at concentrations ≥ QDL that were further subjected to quantitative analysis. As shown in Figure-8, the liquid antibiotics concentrations negatively correlated with the samples' pH values: acidic manure samples contained highest concentration of the antibiotic and metabolites that decreased gradually as pH increased. The acidic TYL-positive manure samples contained mostly TYL-A and TYL-B, while the basic TYL-positive samples contained TYL-A, TYL-ALD and TYL-D (Figure 8). The aqueous phase TYL-A and metabolite concentrations ranged from 1 to 200 μg/L. The CTC-positive manure samples contained mostly CTC, ECTC and ACTC at acidic pH, but mostly CTC, ICTC, EICTC and EACTC at basic pH. The aqueous phase CTC and metabolite concentrations ranged from < 1 to 700 μg/L (Figure 8).

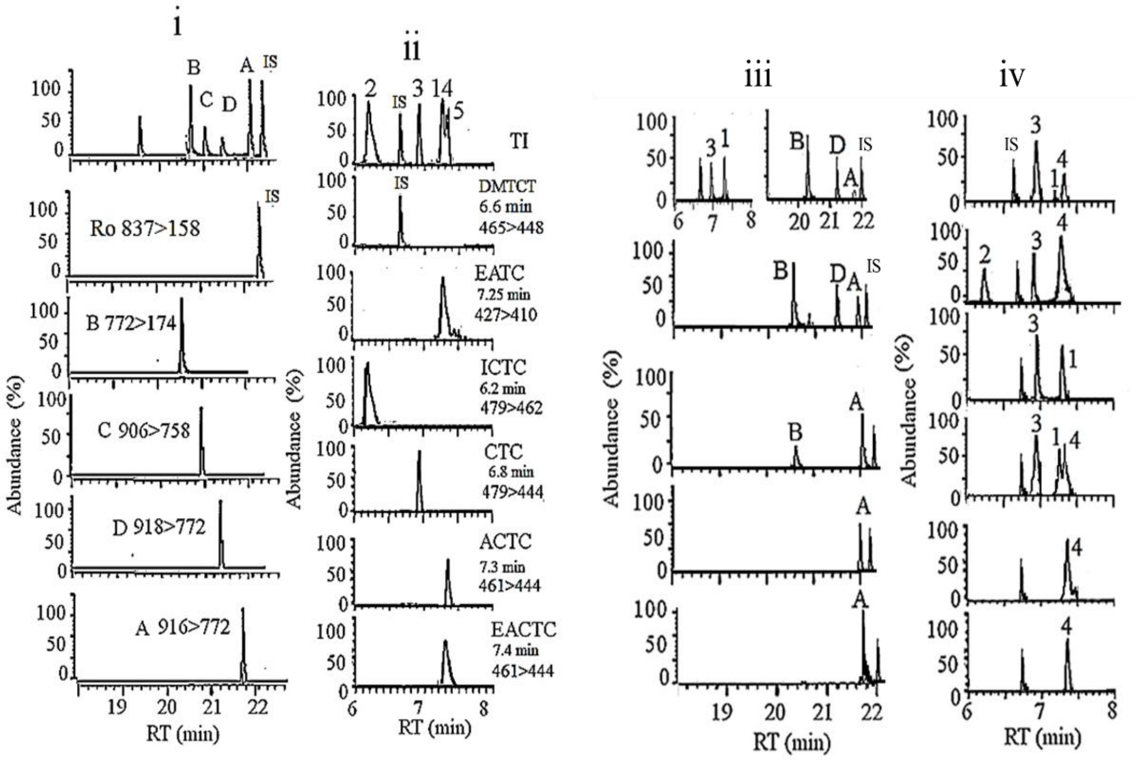


Figure 7. Typical chromatographic separation antibiotic residues.

(i) Separation of TYL-A and metabolites standards. The primary and secondary ions are shown for each analyte,

(ii) CTC and metabolites standard,

(iii) TYL-A and metabolites extracted from manure (iii top: this sample contained TYL and CTC both), and

(iv) (iv) CTC and metabolites extracted from manure.

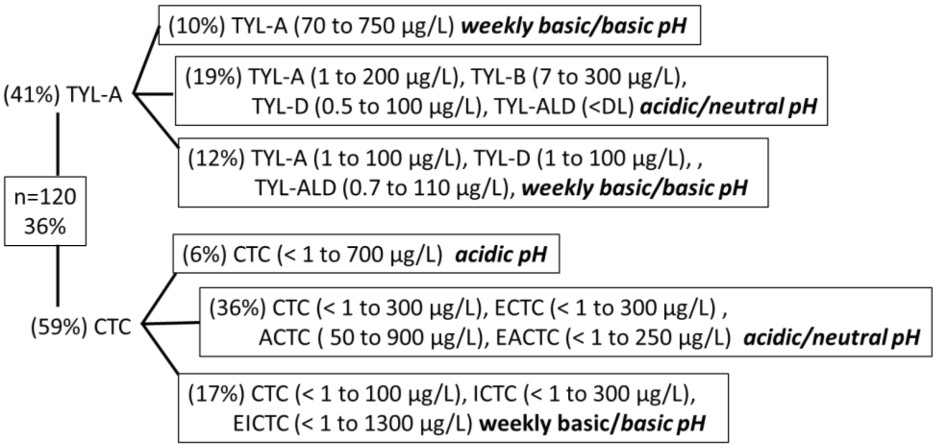


Figure 8. Concentration of TYL-A, CTC and their metabolites in manure liquid samples at different pH values.

3.4. Sorption and Desorption Coefficients

3.4.1. Chlortetracycline and Metabolites

Figures-9i show plots of pH versus $\log K_D^{des}$ and $\log K_D^{s}$ values and Figure 9ii shows the HYS values for CTC, ACTC and ICTC (values for epimers are not shown to avoid duplication), while the results of their statistical analysis (data from acidic (5.5 to 6.5), neutral (>6.5 to 7.5), slightly basic (>7.5 to 8.5) and basic (≥ 9) manure samples) are shown in Figure-10. As shown in Table-5, $\log K_D^{s}$, $\log K_D^{des}$ and HC values for CTC and metabolites in acidic, neutral, weakly basic and basic manure samples exhibited different patterns. For CTC, ECTC, ACTC and EACTC, the $\log K_D^{des}$ values were higher than the $\log K_D^{s}$ values in acidic and neutral manure samples, indicating sorption-desorption hysteresis. For ICTC and EICTC, $\log K_D^{s}$ and $\log K_D^{des}$ values were comparable, indicating lack of hysteresis. Epimerization did not affect the $\log K_D^{s}$, $\log K_D^{des}$ and HC values of CTC and metabolites in manure samples.

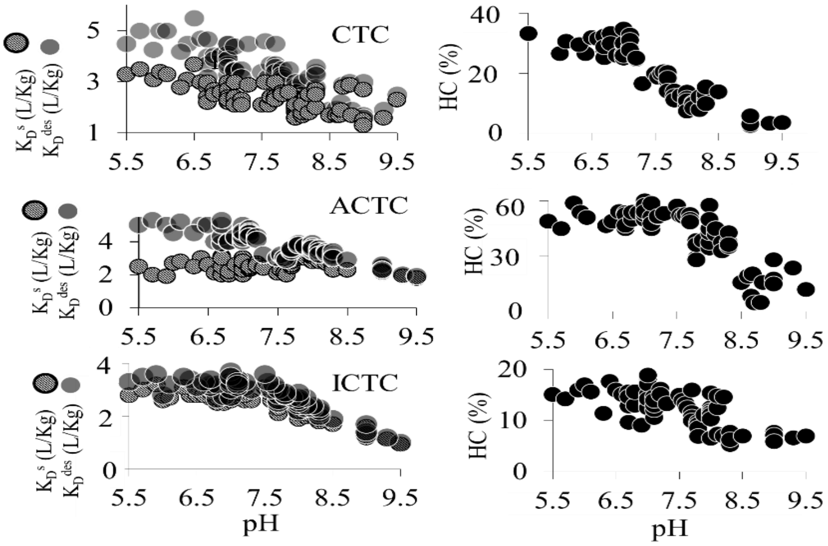


Figure 9. LogK_D^s or logK_D^{des} vs pH (i) and pH versus HYS (ii) plots for CTC, ACTC and ICTC in manure samples. The data for epimers were not shown since epimerization did not affect the respective values.

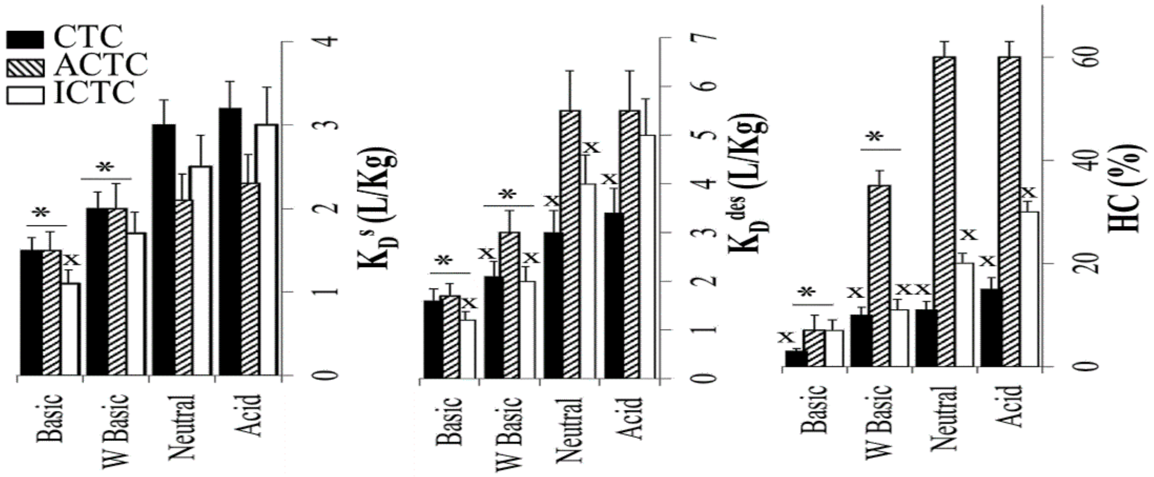


Figure-10. logK_D^s, logK_D^{des} and HC values for CTC, ACTC and ICTC in acidic, neutral, weakly (W) basic and basic manure samples.*: significant when compared with the acid-manure values and x: significant when compared with corresponding ACTC values.

Table5. Effect of manure pH on sorption, desorption and hysteresis of CTC and metabolites.

Sensitivity of logK_D^s, logK_D^{des} and HC of CTC and metabolites to manure pH	Effect of pH on relative distribution of logK_D^s, logK_D^{des} or HC values for CTC and metabolites in manure samples
CTC, ECTC K_D^s: Acidic, neutral > weakly-basic > basic manure K_D^{des}: Basic >> weakly-basic > acidic, neutral manure HC: Acidic, neutral > weakly-basice >> basic manure	*Acidic manure* K_D^s: CTC, ECTC > ACTC, EACTC > ICTC, EICTC K_D^{des}: ICTC, EICTC > CTC, ECTC > ACTC, EACTC HC: CTC, ECTC, ACTC, EACTC > ICTC, EICTC
ACTC, EACTC K_D^s: Acidic, neutral, weakly-basic > basic manure K_D^{des}: Basic >>acidic, neutral, weakly-basic manure HC: Acidic, neutral, weakly-basic > basic manure	*Neutral manure* K_D^s: ACTC, EACTC, CTC, ECTC, ICTC, EICTC K_D^{des}: ICTC, EICTC > ACTC, EACTC > CTC, ECTC HC: ACTC, EACTC > CTC, ECTC >> ICTC, EICTC
ICTC, EICTC K_D^s: Acidic, neutral, weakly-basic > basic manure K_D^{des}: Basic > weakly-basic, neutral, acidic manure HC: Acidic, neutral, weakly-basic > basic manure	*Weakly-basic manure* K_D^s: ACTC, EACTC > CTC, ECTC, ICTC, EICTC K_D^{des}: ICTC, EICTC > ACTC, EACTC, CTC, ECTC HC: CTC, EACTC > ACTC, EACTC > ICTC, EICTC
	Basic manure K_D^s: ACTC, EACTC > CTC, ECTC > ICTC, EICTC K_D^{des}: ICTC, EICTC > ACTC, EACTC, CTC, ECTC HC: CTC, ECTC > ICTC, EICTC, ACTC, EACTC

3.4.2. Tylosin and Metabolites

LogK_D^{des}, logK_D^s and HC values for TYL-A, TYL-B, TYL-D and TYL-ALD in manure are shown in Figure 11 and the statistical analyses of the data are shown in Figure 12. For acidic and neutral manure, individual logK_D^{des} values were greater than corresponding logK_D^s values, indicating sorption-desorption hysteresis. The HC values ranged from 6% to 15% for TYL-D, 25% to 30% for TYL-A and TYL-ALD and > 40% TYL-B. However, logK_D^{des} and logK_D^s

values were comparable in basic manure samples, indicating poor or lack of hysteresis. Comparative pattern of logK_D^s, logK_D^{des} and HC values for TYL-A and metabolites in acidic, neutral, weakly basic and basic manure samples are shown in Table-6. In general, except for TYL-D, the logK_D^s and HC values for TYL-A, TYL-B and TYL-ALD were lower than corresponding logK_D^{des} values in acidic, neutral and weakly-basic manure samples. As the manure pH increased to ≥ 9, the logK_D^s and HC values decreased.

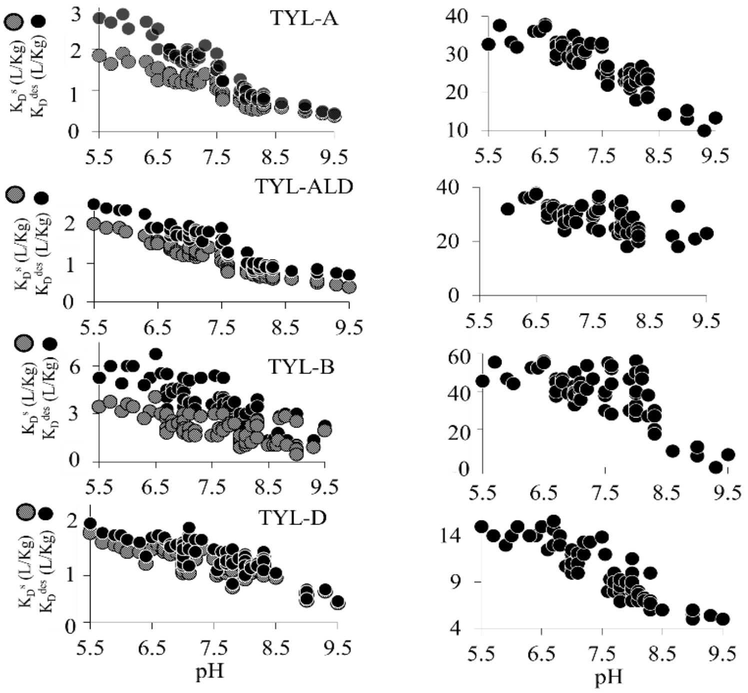


Figure-11. Typical pH versus logK_D^s or logK_D^{des} (11a) and pH versus HYS (11b) plots for TYL-A and metabolites in manure samples

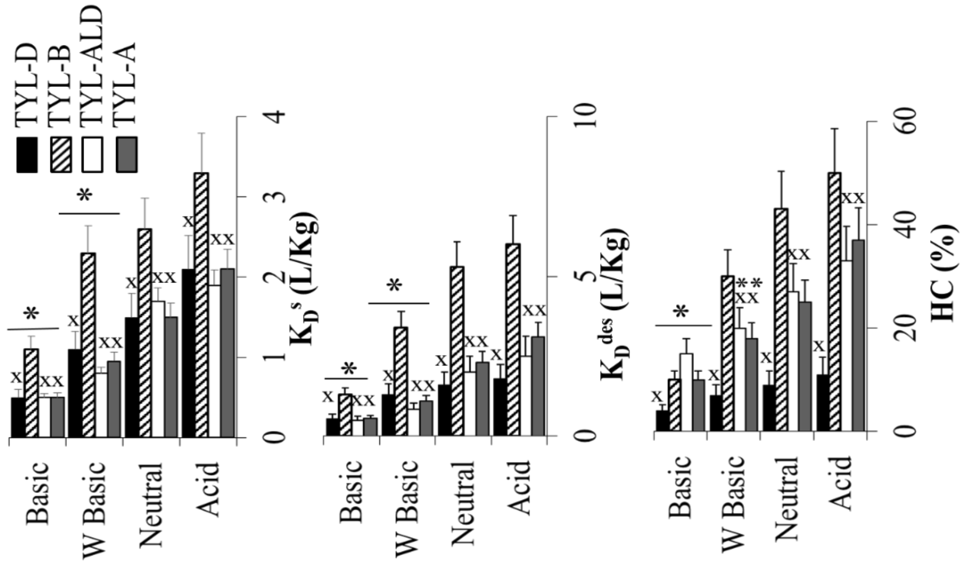


Figure-12. LogK_D^s, logK_D^{des} and HC values for TYL-A and metabolites in acidic, neutral, weakly-basic (W basic) and basic manure samples.

Table-6: Effect of manure pH on sorption, desorption and hysteresis of TYL-A and metabolites.

Sensitivity of $\log K_D^s$, $\log K_D^{des}$ and HC of TYL-A and metabolites to manure pH	Effect of pH on relative distribution of $\log K_D^s$, $\log K_D^{des}$ or HC values TYL-A and metabolites in manure samples
TYL-A, K_D^s: acidic, neutral < weakly basic, basic manure K_D^{des}: acidic, neutral < weakly basic, basic manure HC: acidic, neutral < weakly basic, basic manure	Acidic K_D^s: TYL-B > TYL-A, TYL-ALD > TYL-D K_D^{des}: TYL-D > TYL-A, TYL-ALD > TYL-B HC: TYL-A, TYL-ALD, TYL-B >> TYL-D
TYL-B K_D^s: acidic, neutral, weakly basic > basic manure K_D^{des}: acidic, neutral < weakly basic, basic manure HC: acidic, neutral, weakly basic, basic manure	Neutral K_D^s: TYL-B > TYL-A, TYL-ALD > TYL-D K_D^{des}: TYL-D > TYL-A, TYL-ALD > TYL-B HC: TYL-A, TYL-ALD, TYL-B >> TYL-D
TYL-ALD K_D^s: acidic, neutral V weakly basic, basic manure K_D^{des}: acidic, neutral < weakly basic > basic manure HC: acidic, neutral < weakly basic > basic manure	Weakly basic K_D^s: TYL-B > TYL-A, TYL-ALD > TYL-D K_D^{des}: TYL-D > TYL-A, TYL-ALD > TYL-B HC: TYL-B > TYL-A, TYL-ALD >> TYL-D
TYL-D K_D^s: acidic, neutral < weakly basic > basic manure K_D^{des}: acidic, neutral < weakly basic > basic manure HC: acidic, neutral < weakly basic > basic manure	Basic K_D^s: TYL-A, TYL-ALD, TYL-B, TYL-D K_D^{des}: TYL-D > TYL-A, TYL-ALD > TYL-B HC: TYL-A, TYL-ALD, TYL-B >> TYL-D

4. Discussion

4.1. Physicochemical Properties of Manure

Manure is a slurry of organic and inorganic matter containing animal feces, urine, soil, water, organic carbon, fatty acids, minerals and other nutrients, drugs and toxins. Manure solid contains particles of varying sizes with a series of negative-charged domains where the charge increases in the following order: organic matter > clay > slit > sand. Since only 10% to 40% of the soil's negative charges are pH dependent, we expect that soil may retain its negativity at pH ranging from 6 to > 9 (Christensen et al 2009, Gregory et al 1989). Manure solid would attract positively charged ions, but repel negatively charged ions present in the supernatant. An incomplete charge neutralization may yield net +ve and –ve charges at the surface (Gregory 1973, Vanotti and Hunt 1999, Hjorth et al 2010). Although the point of zero charge (PZC) for pig manure is not yet known, earlier study has proposed it to be at approximately pH 4.5 to 5.5 (Chen et al. 2013 and Trakal et al. 2014), thus manure may contain negative charge at pH > 6.0. Total CEC of manure ranges from 50 to 100 meq (Table-1, Jiang 2015) at pH 6 and the values decrease as the pH increases (Figueroa et al 2004). Based on the above and other relevant observations (Cashen 1959, Wiklander 1964, Barrow and Shaw 1979, Barrow 1985, Galleg et al 1976, Mulder and Cresser 1994, Apple et al 2003, Rengel 2003, Fontes and Alleoni 2006, Sposito 2008, Wegst-Uhrich et al. 2014), it is proposed that the following key ionic properties may determine sorption of an antibiotics on manure solid:

(i) Manure solid may exhibit net negative charge even at acidic pH that may directly bind the

$-NH^+(CH_3)_2$ group of TYL-A, CTC or the metabolites..

(ii) Cation exchange capacity (CEC) is highest in acidic manure, and the values decreased as the pH increased.

(iii) In acidic manure, the carboxylic groups remaind deionized (-COOH) and the basic groups or nutrients remained ionized (Al^{3+}, Na^+, Ca^{2+}, K^+, NH_4^+ etc). As pH increased, the ionized/deionized ratio of metals decreased, but the ratio of carboxylic acid increased. Cations such as Na^+, Ca^{2+} and NH_4^+ may replace the proton from –COOH and form $–COO^-Na^+$ that may be further replaced with cationic antibiotics, a process known as cation exchange.

(iv) Manure solid also contain hydrophobic sites that may interact with the hydrophobic sites of the antibiotics.

4.2. Physicochemical Properties of Antibiotics

CTC and its metabolites are polar compounds with logP ranging from -0.58 to -3.37 (logP pattern: ACTC, EACTC (-0.58) > ICTC, ETCTC (-2.17) > CTC, ECTC (-3.37)). They contain strong negative-charged and a weak positive-charged domains, separated by hydrophobic domains (Figure 6). TYL (log P pattern: TYL-B (2.3) > TYL-D (1.69), TYL-A (1.65) > TYL-ALD (1.3)) contains diffused positively charged sites with dispersed hydrophobic regions (Figure-4). CTC, depending on the solvent pH, exhibit complex ionization patterns because of multiple ionizing groups with different pKa values listed below (Cosentino et al 2005, Stephens et al 1956, Myers et al 1983, Nelson et al 2001 and Wang et al 2014):

- The pKa1for protonation of N_4 $(CH_3)_2$-C4 ranged from 9.1 to 9.7 for CTC, ECTC, ACTC and EACTC, while the values were 8.1- 8.4 for ICTC and EICTC. In this pH range, the following pattern may exist: cations (0-0, 0- -, +--) > zwitterion (+0-) >> anion (+00).
- The pKa2 for deprotonation of O_{10}-C_{10}, O_{11}-C_{11} and O_{12}-C_{12} ranged from 7.2 to 7.8 for CTC, ECTC, ACTC and EACTC, while 6.8 ICTC and EICTC. In this pH range, the following pattern may exist: zwitterion (+0-) > cations (0-0, 0- -, +--) > anions (+00).
- The pKa3 for O_3 and $–C_2(C_{2a}=O)N_{2a}H_2$ were <5 for CTC and its metabolites. In this pH range, anions (+00) may dominate.

CTC and metabolites, because of slight differences in their pKa values, may exhibit diverse ionic properties and ensuing interactions with manure. However, the pKa values for TYL-A and metabolites were 9.4, thus they may exist an anion (TYL^+) throughout the pH range of this study.

4.3. Concentration of CTC, TYL-A and their metabolites in pig manure liquid fraction

Antibiotics concentrations in manure and other environmental samples are generally determined by ELISA (for screening and determination of positive samples) and/or HPLC-MS (for quantitative determination of positive samples) (Aga et al 2003, Christiana et al 2003, Sorensen 2006, Ben et al. 2008, Hu et al. 2010, Shahbazi et al. 2015). An earlier study (Kumar et al. 2004) has shown that analysis of manure samples by ELISA and HPLC-MS yielded different results (concentrations of CTC and TYL were 0 to 7.93 mg/L and 0 to 5.23 mg/L, respectively, when analyzed by ELISA, but 0 to 5.23 and 0 to 3.8 mg/L, respectively, when analyzed by HPLC-MS), possibly be due to the presence metabolites that may have cross-reacted with the ELISA antibodies, as suggested by other investigators (Hu et al. 2008, Aga et al. 2005). Since many of the metabolites exhibit antibacterial activity and/or nonspecific toxicity (Henderson et al. 2008, Hu et al. 2008, Teeter and Meyerhoff 2003, Dos Santos et al. 2000), presence of active metabolites in manure samples may pose substantial risk of environmental contamination and ensuing risk of public health. Therefore, in the present study, a quantitative/confirmatory method using HPLC/MS-MS that provided clear separation of the antibiotics' and their metabolites was used. The method yielded the following results:

(i) Approximately 36% of pig manure samples tested positive (in quantitative terms) for CTC, TYL and/or their metabolites. Their concentrations ranged from 0.1 to >1 mg/L that were consistent with earlier values reported for the United States, Europe and China (Loke et al. 2002, Winckler and Grafe 2001, Chen et al. 2012, Zhao et al. 2010, Halling-Sorensen et al. 2005, Kumar et al. 2005).

(ii) The TYL-A metabolites were TYL-B, TYL-D and TYL-ALD, while CTC metabolites were ICTC, ACTC, ECTC, EICTC and EACTC.

(iii) The relative concentrations of the metabolites depended upon the manures' pH. The TYL-positive acidic and neutral samples contained mostly TYL-A and TYL-B, while the basic samples contained mostly TYL-D and TYL-ALD. CTC-positive acidic samples contained mostly CTC, ACTC and EACTC, while basic samples contained mostly ICTC and EICTC.

The pH-related changes in concentrations of TYL-A, CTC and metabolites depended on their ionization properties. TYL-A and metabolites are weekly basic in which the $–N(CH_3)_2$ group may remain protonated throughout the pH ranging from 5.5 to 9.5 in manure. CTC and metabolites may be a mixture of anions, cations and amphoteric whose proportion may depend upon the manures' pH. This suggests that pH may affect the fate (sorption, desorption and degradation) of the antibiotics and their metabolites in antibiotics-positive (antibiotics or metabolite concentration $\geq$ QDL) manure samples.

4.4 Solid-Liquid Partition Coefficient (K_D^s) of CTC, TYL-A and Metabolites

Antibiotics, upon entering manure, distribute between its solid and liquid fractions, a process determined by the parameter K_D^s (Tills 2001) that depends on (i) the manures' physicochemical characteristics such as particle size, pH, organic carbon, ionic charge, cation-exchange capacity, and (ii) the antibiotics' physicochemical properties such as lipophilicity, ionization status, dipole moment, ion exchange capacity, steric structure and topology (Thiele-Bruhn et al. 2004; Figueroa et al. 2004; Golet et al. 2003; Kulshrestha et al. 2004, Parolo et al. 2008). Manure solid may contain the following binding sites (Loke et al.

2002, Tolls 2001, Sithole and Guy 1987ab):

(i) hydrophobic sites that may interact with the hydrophobic sites of antibiotics and metabolites,

(ii) cation exchange (CE) sites on which a cationic ion is replaced by another cationic residue,

(iii) cationic (COO^-, SO_4^{2-}, Cl^-, etc.) sites that may bind the positively charged antibiotics such as (CTC^{+00} or TYL-A^+),

(iv) anionic (Ca^{2+}, Mg^{2+}, Na^+, NH_4^+) sites that may bind CTC^{0--} or CTC^{0-0},

(v) zwitterion (^-OOC-X- NH_3^+) sites that may bind CTC^{+0-}

Earlier studies have shown that sorption of antibiotics on manure solid may occur via diverse mechanisms including, but not limited to, Van der Waals and London dispersion forces, solvent partitioning-hydrophobic interactions, electrostatic attraction-repulsion, ion exchange and H-bonding (Chiou et al. 1989, Xia 1998, Evanko and Dzombak 1998, MacKay and Canterbury 2005, Lertpaitoonpan et al. 2009). TYL-A and its metabolites remain positively charged ($-NH^+(CH_3)_2$) at pH ranging from 5.5 to 9, while CTC and metabolites exist as a mixture different ions: (i) +00 > +0- at pH 5.5 to 6.5, (ii) +0- > +00 > 0-0 pH 6.5 to 7.5, (iii) 0-0 > +0- > 0- - at pH 7 and (iv) 0- - > 0-0 at pH greater than 9. In acidic manure or soils, sorption of CTC^{+00} and TYL-A^+ occur mostly via cation exchange (Gao and Pedersen 2005, Kahle and Stamm 2007). At pH range where the zwitterion CTC^{+0-} dominates, manure exerts a negative effect on antibiotics' sorption (Gao and Pedersen 2005). At pH > 9, CTC may exist as an anion and interact with manure's cationic sites via cationic bridging (Nowara et al. 1997; Otker and Akmehmet-Balcioglu, 2005).

$LogK_D^s$ values for TYL-A, -ALD, -B and -D ranged from <1.0 to 4.0 L/Kg (Table 4), while, the values for CTC and its metabolites ranged from <1 to 3.0 (Figure 10) that were comparable to the $logK_D^s$ values reported by Loke et al. 2002, Wegst-Uhrich et al. 2014, Ingerslev and Halling-Sorensen 2001 and Rabolle and Spliid 2000. This study showed that, in manure at pH 5.5 to 8.5, the $logK_D^s$ values for TYL-B were significantly greater than those for TYL-A, TYL-D and TYL-ALD, while the values were significantly lower in manure at pH ≥ 9. Sussman et al (2007) have shown that TYL-B is generated from the hydrolysis of the mycarose ring attached at position 4 of the 16-membered lactone ring of TYL-A, making the metabolite more hydrophobic. Thus, TYL-ALD, TYL-A and TYL-D may be more mobile than TYL-B. Similarly, Wegst-Uhrich et al. (2010) found significant differences in sorption behavior between TYL-A and its hydrolysis product TYL- B, but, contrarily, Sussman et al. (2007) found that (i) TYL-A and -D exhibited similar sorption characteristics and (ii) TYL-A's $logK_D$ values were greater than the TYL-B's $logK_D$ values. The cause for these differences is not yet understood. Studies have shown that the $logK_D^s$ values for CTC and TYL-A were inversely related to the manures' pH values: acidic manure exhibiting relatively higher $logK_D^s$ values than basic manure (Ter Laak et al. 2006, Spadotto et al. 2003, Westall et al. 1985, Ali et al. 2013, Sassman and Lee 2005, Loke et al. 2002). The present study yielded similar results for CTC, ECTC, ICTC, ETCTC, TYL-A, TYL-D and TYL-ALD, but not for

ACTC, EACTC and TYL-B whose K_D^s values were relatively resistant to pH changes.

4.5 Desorption Coefficient and Sorption-Desorption Hysteresis

Desorption (a process by which a chemical including antibiotics pre-adsorbed onto the manure solid is released into the environment) determines migration and bioavailability of chemicals in terrestrial and aquatic environment (Barriuso et al.1994). It is well established that, for manure, soil and other environmental samples, sorption and desorption of a chemical often do not follow the same path, a process known as sorption-desorption hysteresis (HYS). This occurs when an antibiotics (i) forms permanent bonds (such as covalent bond) with manure's organic matter (Bialk et al. 2005), (ii) competes with coexisting HOCs in soils and sediments (Rana et al. 2003), (iii) forms surface complex and hydrogen bonding (Xia and Pignatello, 2001; Sander et al. 2006, Tolls 2001, Thiele-Bruh 2003, Hamscher et al. 2004), (iv) is trapped within transient pores formed due to the complex nature of the liquid-phase configuration in unsaturated porous medium that presents hysteresis effects (Dane and Wier et al. 1975, Hillel 1980, O'Kane et al 2004, Poulovassilis 1961, Topp and Miller 1966) and (v) is entrapped in an air pocket which further reduces the water content of newly wetted soil that can accentuate the hysteresis effect (Hillel 1980, O'Kane et al. 2004). Hysteretic adsorption-desorption suggests that sorption of chemicals occur with a limited degree of reversibility depending upon both the physicochemical properties of the molecules and the soils involved (Gao et al. 1998a,b, Gramatica and Di Guardo 2002). An increase in hysteresis, therefore, indicates irreversible sorption and ensuing decrease in leaching and contamination (Gao et al. 1998a, Hong-Gian and Jiang 2010, Xu and Li 2010, Li et al. 2011, 2014). Chen (2012) and Kim et al. (2011) have suggested that the migration of desorbed antibiotics from manure to groundwater is one of the key aspects of the deterioration of groundwater quality and ensuing ecotoxicology. In the proceeding sections, possible significance of the $\log K_D^s$, $\log K_D^{des}$ and HC values in manure samples and associated health risks have been discussed.

4.5.1 Tylosin and Metabolites

$\text{Log}K_D^s$, $\log K_D^{des}$ or HC values for acidic, neutral and weakly-basic manure samples did not differ significantly, but they were significantly higher than corresponding values for basic manure samples. TYL-B (relatively most lipophilic) exhibited significantly higher HC values than TYL-A, TYL-ALD, TYL-D. Similarly to the present study, Sassman et al. (2007), Qian et al. (2011), Guo et al. (2014), Peng et al. (2014) have also reported sorption-desorption HC of TYL-A and other antibiotics in manure, soil and other environmental samples. A key observation of the present study was that TYL-A, TYL-D and TYL–ALD exhibited pH dependent hysteresis patterns in manures. At pH 5.5 to 6.5, the HC values were TYL-B >> TYL-A, -ALD > TYL-D. As pH increased the HC values decreased, but the decrease for TYL-B were several folds greater than that for TYL-A, -ALD and -D. At pH ≥ 9, $\log K_D^s$ and $\log K_D^{des}$ values were essentially comparable indicating absence of hysteresis. This suggests that, at acidic pH, TYL-B will be essentially immobile, TYL-A and –ALD will be moderately mobile and TYL-D will be freely mobile. At pH ≥ 9, all may be relatively freely mobile. Therefore, possibility of groundwater and/or surface waters contamination following field application of manure may range from 'highly likely' to 'not likely' depending upon the manure/soil physicochemical properties.

Possible mechanisms for pH-dependent sorption and desorption of TYL is shown in Figure-13. Since the –N$(CH_3)_2$ group in TYL remains mostly protonated (TYL^+) below pH 9, it will bind to manure solid via CE at broad pH range. At the manure pH > 9, the neutral species of TYL may appear, resulting in a moderate decrease in their sorption. Guo et al. (2014) have shown that hydrophobic interactions may become dominant at relatively higher pH, resulting in an increase in sorption of neutral TYL on manure at pH 9. The logK_D values at pH 9 may be attributed to the hydrophobic interactions between manure and TYL-A/metabolites.

4.5.2. CTC and Metabolites

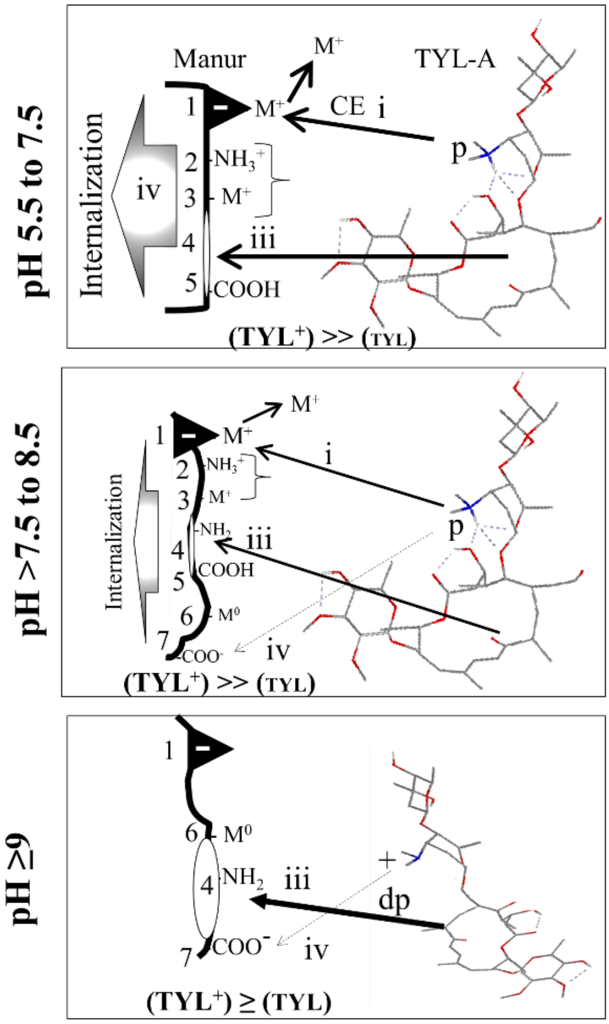
CTC and metabolites exhibits a complex ionization patterns, sorption, desorption and hysteresis as shown in Figure-14 and described below:

(i)*At pH 5.5 to 6.5*: CTC, ECTC, ACTC and EACTC may exist as anions (+00), while manure may exhibit a net –ve charge coupled to an exchangeable anion. At this pH range, CTC, ECTC, ACTC and EACTC exhibit sorption >> desorption, resulting in greater hysteresis. ICTC and EICTC exhibit comparable desorption and sorption, resulting in poor or no hysteresis. The present observations concur with earlier reports that CTC exhibits considerable sorption-desorption hysteresis in acidic soil (Sassman and Lee 2005, Zhao et al 2011 and Fernandez-Calvino et al 201).

(ii) *At pH >6.5 to 7.5:* ECTC, ACTC and EACTC may exist as anions > zwitterions, while ICTC and EICTC may exist mostly as zwitterions. Thus, desorption may increase and the HC values may decrease slightly at this pH range.

(iii) *At pH >7.5 to 8.5*: Cation 0-0 becomes prominent moiety followed by the zwitterions. ICTC and EICTC may exhibit more cations than CTC, ECTC, ACTC and EACTC.

(iv) *At pH ≥9*: Cations 0- - and 0-0 may be the predominant ions at this pH. CTC and the metabolites may exhibit comparable sorption and desorption, indicating lack of hysteresis.



Sorption
TYL-B >> TYL-A, TYL-ALD > TYL-D

Desorption
TYL-D > TYL-A, TYL-ALD > TYL-B

Sorption
TYL-B >> TYL-A, TYL-ALD > TYL-D

Desorption
TYL-D > TYL-A, TYL-ALD > TYL-B

Sorption
TYL-B, TYL-A, TYL-ALD > TYL-D

Desorption
TYL-D > TYL-A, TYL-ALD, TYL-B

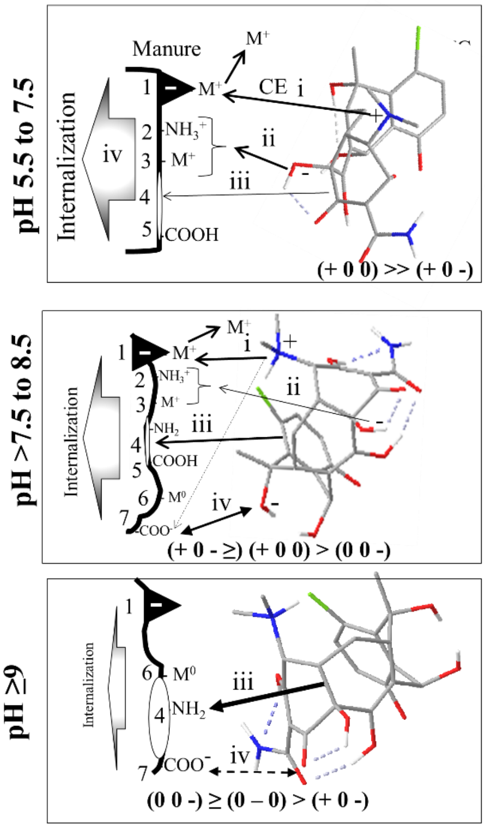
Figure 13. Proposed mechanisms for sorption and desorption of TYL-A and metabolites in manure.

Abbreviations: (1) Cation exchange sites, (2) ammonium ions, (3) metal ions, (4) hydrophobic sites, (5) protonated (non-ionized) carboxylic group, (6) non-ionized metals (0 charge), (7) deprotonated (ionized) carboxylic group, (i) cation exchange between M+ and TYL+ or metabolite+, resulting in sorption of TYL by manure solid at pH 5.5to 8.5, (ii) hydrophobic interaction between hydrophobic sites of TYL (or metabolites) and manure solid and (iii) a weal interaction between the antibiotic cation and the manure's COO- group.

To understand the complex effects of pH on desorption of CTC and metabolites from manure solid, it is important to understand the effects of pH of their physicochemical properties. As discussed above, manure contains (i) organic carbon mostly humic and long-chain fatty acids that accounts for its cationic negative charge, (ii) metal anions that bind the negative charges, (iii) ammonium ions that remains protonated (+ve charge) at acidic pH and (iv) hydrophilic and hydrophobic sites that interacts with hydrophilic and hydrophobic chemicals, respectively. As shown in Figure 12, at acidic pH, the –N(CH 3)$_2$ group of CTC, ECTC, ACTC and EACTC, because of their lower pKa1, may exhibit greater protonation(and positive charge) than ICTC and EICTC. Therefore, CTC, ECTC, ACTC and EACTC may exhibit lower sorption and greater desorption coefficients than ICTC and EICTC.

5. Conclusions and Environmental Consequences

As discussed earlier, the domestic livestock industry in the United States produces an estimated 175 million tons of manure each year. The most pragmatic means of disposing manure organic farming by amendment of arable soil with manure where its nutritive value can be utilized by growing plants. In swine intensive-farming, antibiotics are used to treat and prevent disease in swine, to protect swine welfare, and to improve growth rate and efficiency of feed utilization (Mackinnon 1993, Friendship 2000, Cromwell 2002, McEwen and Fedorka-Cray 2002). In the USA, approximately 534 tons of C and 166 tons of TYL are used annually in pig farming (Apley et al. 2012). Since pig manure has been shown to contain 3.5 mg/L TYL and 5.6 mg/L, the total load of these antibiotics in manure may be approximately 5.25 x 10^6 tons TYL and 8.75 x 10^6 tons, respectively.



Sorption
ACTC, EACTC > CTC, ECTC >> ICTC, EICTC

Desorption
EICTC, ICTC >> CTC, ECTC >> ACTC, EACTC

Sorption
ACTC, EACTC > CTC, ECTC >> ICTC, EICTC

Desorption
EICTC, ICTC >> CTC, ECTC >> ACTC, EACTC

Sorption
ACTC, EACTC, CTC, ECTC >> ICTC, EICTC

Desorption
EICTC, ICTC, CTC, ECTC >> ACTC, EACTC

Figure 14. Proposed mechanisms for sorption and desorption of CTC and metabolites in manure.

Abbreviations: (1 to 7) described in Figure 13, (i) cation exchange between M^+ and CTC^{+00} or $metabolite^{+00}$ at pH 5.5 to 6.5 and, as the pH increase sorption of CTC and metabolites to soil decrease, (ii) interaction between the anionic domain of CTC and metabolites and the cationic domain of manure, (iii) hydrophobic interaction between hydrophobic sites of CTC (or metabolites) and manure solid and (iv) interaction between the CTC and metabolite cation and the manure's COO^- group.

Therefore, during rain event or irrigation, antibiotics from contaminated manure leach to surface water and get carried away to distant sites, creating point and non-point contamination (Dragun 1988). Earlier studies have focused their attention mostly to the parent antibiotics and not to their metabolites that may also exhibit antibiotic properties or nonspecific toxicity. The aim of the present study was compares the scope, sorption and desorption of TYL, CTC and their metabolites in pig manure. The key observation of this study are following:

(1) Approximately 50% of liquid-phase of pig manure samples contained TYL, CTC and their metabolites, only 36% contained levels above the method's QDL. The antibiotics and metabolite residue present in the liquid fraction of manure will leach from the storage site directly into the environment. However, the scope of contamination may depend upon its pH since (i) the acidic TYL-positive manure samples contained TYL-A and TYL-B, while the basic TYL-positive samples contained TYL-A, TYL-ALD and TYL-D, and (ii) the CTC-positive manure samples contained mostly CTC, ECTC and ACTC at acidic pH, but mostly CTC, ICTC, EICTC and EACTC at basic pH. This suggests that the overall environmental impact of contaminated manure may depend upon the medium's pH.

(2) For acidic and neutral manure samples, the $\log K_D^{des}$ values for (i) TYL-B, TYL-A and TYL-ALD and (ii) CTC, ACTC, ECTC and EACTC were greater than corresponding $\log K_D^{s}$ values, indicating sorption-desorption hysteresis. At this pH, the residues remain manure-bound, thus will not leach and contaminate the environment. However, an increase in pH decreases hysteresis, resulting in greater leaching and contamination.

(3) TYL-D, ICTC and EICTC did not exhibit sorption-desorption hysteresis, thus the residues will leach on-site due to rain or irrigation.

Thus, once manure is applied to the agricultural field, TYL-A, CTC and their metabolites bound to manure-solid suspended in acidic environment would exhibit poor leaching. However, the rainwater would increase in residues' leaching if the soil is basic. Thus, environmental pH may determine the antibiotics and metabolite leaching in freshwater at or away from the site of production. In addition, analysis of the parent antibiotic residues may not provide an accurate assessment of the environmental health.

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