

# Degradation and Metabolite Production of Tylosin in Anaerobic and Aerobic Swine-Manure Lagoons

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**ABSTRACT:** Watershed contamination from antibiotics is becoming a critical issue because of increased numbers of confined animal-feeding operations and the use of antibiotics in animal production. To understand the fate of tylosin in manure before it is land-applied, degradation in manure lagoon slurries at 22°C was studied. Tylosin disappearance followed a biphasic pattern, where rapid initial loss was followed by a slow removal phase. The 90% disappearance times for tylosin, relomycin (tylosin D), and desmycosin (tylosin B) in anaerobically incubated slurries were 30 to 130 hours. Aerating the slurries reduced the 90% disappearance times to between 12 and 26 hours. Biodegradation and abiotic degradation occur, but strong sorption to slurry solids was probably the primary mechanism of tylosin disappearance. Dihydrodesmycosin and an unknown degradate with molecular mass of  $m/z$  934.5 were detected. Residual tylosin remained in slurry after eight months of incubation, indicating that degradation in lagoons is incomplete and that residues will enter agricultural fields. *Water Environ. Res.*, 77, 49 (2005).

**KEYWORDS:** tylosin, antibiotic degradation, anaerobic, aerobic, swine manure.

## Introduction

Antibiotic residues and increased numbers of antibiotic-resistant bacteria have been reported near confined animal feeding operations (CAFOs) and in agricultural watersheds (Campagnolo et al., 2002; Chee-Sanford et al., 2001; Haller et al., 2002; Kolpin et al., 2002). A major route for entry of veterinary pharmaceuticals into watersheds is through land application of animal biosolids and spills of animal waste at facilities using these drugs (Boxall et al., 2001; Daughton and Ternes, 1999). Swine CAFOs often use antibiotics for therapeutic or growth-promoting purposes. Manure generated at CAFOs, containing excreted residues, is commonly stored in earthen lagoons for several months before land application. Incomplete degradation of pharmaceuticals, in vivo and during manure storage before biosolids are land-applied, could be a contributing factor to the presence of these drugs in waterways.

In the year 2000, 92% of swine CAFOs reported using antibiotics in a nationwide survey, and the most commonly administered drug was tylosin, a macrolide antibiotic (USDA, 2002). Between 4 and 5 million pounds of tylosin and other macrolide antibiotics were sold annually in 2001 and 2000 (Vansickle, 2002). After oral administration of tylosin at a dose rate of 110 mg/kg, swine were found to excrete up to 40% of the tylosin in antibiotically potent forms (Sieck et al., 1978). These forms include (in order of prominence) relomycin (tylosin D), tylosin (tylosin A), dihydrodesmycosin, desmycosin (tylosin B), macrosin (tylosin C), and at least 10 other degradates in smaller quantities (Sieck et al., 1978; Teeter and Meyerhoff, 2003). Different forms of tylosin are shown in Figure 1.

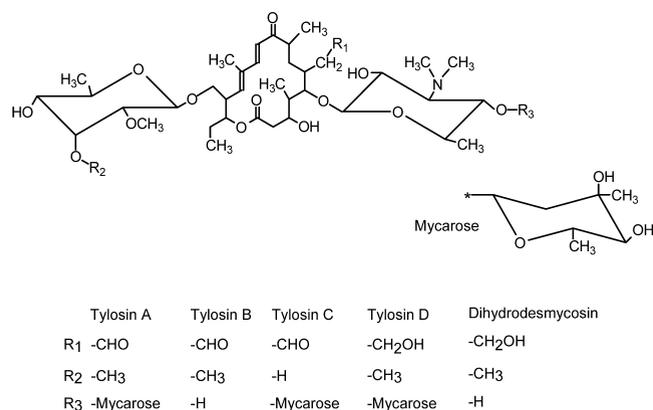
Degradation half-lives for tylosin reported in the literature average 4 to 8 days in swine, calf, and chicken manure; 2 to 8 days in aqueous manure and soil-manure mixtures; and 10 to 40 days in surface-water simulation systems (De Liguoro et al., 2003; Ingerslev and Halling-Sørensen, 2001; Ingerslev et al., 2001; Loke et al., 2000; Teeter and Meyerhoff, 2003). However, most of these studies focused on the degradation of tylosin A in aerobic systems and fresh manure. Tylosin degradation in an aqueous manure mixture, incubated under methanogenic conditions, produced several identified degradates; however, analysis was hampered by interference from components of the mixture (Loke et al., 2000). Degradation rates for tylosin A, in these systems, do not provide adequate information about the persistence of other forms of tylosin or the appearance of degradates in lagoon slurry, where waste may reside for six months before land application. Therefore, it is of interest to understand the fate of common veterinary drugs, such as tylosin, in lagoon storage, before the manure slurry is land-applied.

Typical anaerobic lagoons may have, as their design objectives, a reduction in solids, nutrients, odor, and sludge volume (Zhang, 2001). Retention times for the lagoons will vary with the production phase of the facility and season. In some cases, anaerobic lagoons may be modified to include mechanical aeration, or an aerated lagoon may follow as an additional stage for treatment of lagoon effluent. It is of interest to determine how aeration of lagoons, a common modification, may change degradation rates.

The objectives for the current research were to investigate the disappearance of tylosin and to determine the production of degradates in lagoon slurry. Lagoon slurries were collected from two swine CAFOs, amended with tylosin and incubated under aerobic and anaerobic conditions. The persistence of tylosin was also studied by incubating one of the spiked slurries anaerobically for eight months. Sodium-azide amended slurry and filtered lagoon liquid were included to distinguish biological from abiotic degradation or from sorption during the studies. Evaluation of solvent and solid-phase extraction systems was also done to optimize analyte recovery for liquid chromatography-tandem mass spectrometry analysis.

## Materials and Methods

**Slurry Collection and Characterization.** Manure slurries, identified as an open anaerobic lagoon (OL) and covered anaerobic lagoon (CL), were collected from two swine CAFO lagoons, an open lagoon, and a covered anaerobic lagoon, respectively. The OL slurry was a combination of settled solids and lagoon liquid collected from below the liquid surface of the lagoon, while CL was collected from a pump outlet of the covered lagoon. Collection took



**Figure 1—Structure of various tylosin forms.**

place in the summer, when the average temperature in the lagoons was approximately 25°C. The slurries were collected in 68-L (18-gal) rubber tubs, which were kept on ice during transport. The slurries were homogenized and stored in 1.9-L (2-qt) glass jars at 4°C until use.

Characterization tests were conducted to determine the pH, solids, mineral, and carbon content. Dissolved organic carbon in the supernatant was determined using UV-persulfate digestion with infrared detection. Organic and inorganic carbon solids content were determined by combustion and infrared spectrometry. Total solids were determined by weight difference after drying an aliquot of slurry at 105°C. The oxidation–reduction potential (ORP) and pH of the slurries were measured before and after incubation for the degradation experiments. All measurements were taken at 22 ± 1°C. The ORP was measured using a platinum-tipped ORP probe filled with a 3-M silver–silver chloride solution (Thermo Electron, Mississauga, Canada). A hydrogen standard +424 mV (mV E<sub>H</sub>) solution was used as a reference for the probe. The background concentrations of tylosin in each source material were analyzed by liquid chromatography with tandem mass spectrometry (LC–MS–MS).

**Tylosin Stability and Toxicity.** The stability of tylosin was monitored in several sterile matrices: (a) filtered lagoon liquids with 50 g/L sodium azide, (b) Milli-Q purified water (Millipore, Billerica, Massachusetts) with 50 g/L of sodium azide, and (c) Milli-Q water at pH 7 and 9.2. The filtered lagoon liquids were obtained by centrifuging the OL and CL slurries at 12 500 × g for 30 minutes. The lagoon liquids were decanted, and 50 g/L of sodium azide was added before filtration. Milli-Q water with 50 g/L of sodium azide and Milli-Q water without azide were adjusted with 0.1-M potassium hydroxide (KOH) to pH 9.2, for comparison with the filtered lagoon liquids. All matrices were filter-sterilized with sterile 0.2-µm pore-size cellulose-acetate filters and dispensed into autoclaved glassware, under a sterile laminar flow hood. Tylosin tartrate was spiked in all matrices to a concentration of 20 mg/L. The proportion of forms in aqueous tylosin tartrate stock solution was approximately 93% A, 5% D, 2% B, and 0.3% C. All containers were incubated in the dark at 22 ± 1°C, and aliquots were taken from the solutions periodically, under sterile conditions.

The toxicity of tylosin to slurry microbes was assessed by spiking CL slurry with 20 mg/L of tylosin. After 72 hours of incubation at 22°C, a 0.5-mL aliquot of slurry from the CL slurry assay was inoculated to 100 mL of a minimal salts broth. The broth was incubated at 25°C for seven days, and the growth of the slurry

microorganisms in the broth was observed after this period for signs of tylosin toxicity.

**Azide Effectiveness.** To assess the effectiveness of azide on microbial inhibition, 50 g/L of sodium azide was added to CL slurry and incubated for 96 hours. Aliquots of 0.5 mL were plated onto Luria Bertani agar to determine microbial growth.

**Anaerobic Studies.** Anaerobic degradation experiments were conducted by transferring 20 mL of either CL or OL slurries to amber glass vials with Teflon-lined caps. Before the slurries were transferred to the amber vials, the slurries were shaken for 10 minutes on a reciprocating shaker. For both OL and CL slurries, two sets of vials (15 to 18 vials per set) were prepared. For one set of vials, 400 µg of tylosin tartrate from aqueous stock solution were added to each vial giving a concentration of 20 mg/L. After addition of tylosin, the vials were capped, vortexed for 10 seconds, and the vial headspaces were immediately evacuated and filled with helium gas (99.99% pure). The second set of vials was spiked with 1 g of sodium azide (50 g/L) to inhibit biodegradation. After the addition of azide, the vials were vortexed and allowed to rest for 30 minutes at 22°C. This was followed by spiking the vials with 400 µg of tylosin and evacuating the headspaces of the vials. In addition, a set of vials were prepared with OL slurry, but spiked with 3900 µg of tylosin (195 mg/L) to observe differences in disappearance rates and products at this higher concentration.

A separate experiment using OL slurry was prepared under anaerobic conditions to study the persistence of residuals under long term incubation (eight months). In this study, 240 µg of tylosin tartrate was added (12 mg/L) to the vials. To inhibit biological activity, 0.08 g of azide per 20 mL of slurry (4 g/L) was added to another set of vials. All vials were incubated at 22 ± 1°C. Triplicate vials were sacrificed at each sampling event and measured for tylosin and degradates.

Tylosin was added to slurries rather than studying the disappearance rates of tylosin residuals already present in slurries, for several reasons. First, the detected tylosin residuals were too low to significantly determine disappearance rates. Second, tylosin B and D were the persistent forms of tylosin found in the lagoons, which were not consistent with the forms excreted by swine, which were primarily tylosin D and A.

**Aerobic Studies.** Aerated degradation experiments were prepared in a similar manner to the anaerobic experiments, except that immediately after spiking the manure slurry with tylosin the vials were capped and a 16-gauge Teflon tube inserted and compressed air (breathing-air grade) bubbled at a continuous rate. A 22-gauge needle was also inserted through the cap as a gas vent. Samples were incubated at room temperature (22 ± 1°C).

**Tylosin Extraction.** Three extraction solvent combinations were evaluated for tylosin recovery and stability in CL slurry 24 hours after spiking. The solvent combinations tested were methanol (Rabølle and Spliid, 2000), methanol–acetonitrile–0.1 M ascorbic acid (45:45:10, v:v:v) (Teeter and Meyerhoff, 2003), and acetonitrile–isopropyl alcohol (95:5, v:v) (Shang et al., 2001). Extraction recoveries, reported by the researchers, for the above extraction solvent combinations, from soil or manure-soil mixtures, ranged between 61% and 96%. Additional testing was conducted to determine if the acetonitrile–isopropyl alcohol combination amended with 5-M KOH (95:5:0.1, v:v:v) would be appropriate for use, because higher pH is often used to extract macrolides from tissues (Fedeniuk and Shand, 1998). Adjusting the solvent to above pH 9.4 was based on the assumption that tylosin's nonionized form may be easier to extract and occurs at pH above its pKa of 7.4 (O'Neil et al.,

2001). To evaluate each solvent combination, triplicate vials containing 20 mL of CL slurries were spiked with 400 µg tylosin tartrate (20 mg/L) and 1 g sodium azide (50 g/L) and incubated at 22°C for 24 hours. The extraction procedure consisted of two cycles of solvent extraction with sonication and shaking. To examine tylosin stability in the solvent, 400 µg tylosin tartrate and 1 g sodium azide were added to 20 mL of each solvent combination, without manure slurry, and were subjected to two cycles of sonication and shaking.

For the degradation studies, the slurry in each sacrificed vial was transferred to a 50-mL polypropylene centrifuge tube. The amber vial was rinsed with 10 mL of Milli-Q purified water, and the rinse water was transferred to the centrifuge tube. The slurry was centrifuged at  $12\,500 \times g$  for 30 minutes, resulting in a pellet weighing approximately 0.5 to 1 g and 20 to 30 mL of dark brown, translucent, aqueous supernatant. The supernatant, containing very small amounts of particulate material, was decanted, measured, and retained. Then, 10 mL of acetonitrile–isopropyl alcohol (95:5 v:v) was added to each tube. The tubes were vortexed for 1 minute, sonicated (Bransonic Model 5200, Branson Ultrasonics Corp., Danbury, Connecticut) for 30 minutes, shaken for 1 hour at 270 cycles per minute on a reciprocating shaker, and then centrifuged at  $12\,500 \times g$  for 15 minutes. The solvent was decanted and retained separately from the aqueous supernatant. The procedure was repeated by adding another 10 mL of fresh solvent to the pellet and the extract combined with the first 10 mL of solvent extract in an amber vial. The combined solvent extract was dried down to less than 5 mL by passing compressed air over the solvent at room temperature. The concentrated solvent extract was then transferred to a 250-mL glass beaker, including 20 mL of Milli-Q (deionized) water and 1 mL methanol rinse. The solvent extract was further diluted to 140 mL with Milli-Q water, and the pH was adjusted above pH 9.4 with 0.5-M KOH. Likewise, 2-mL of the decanted aqueous supernatant was transferred to a 100-mL glass beaker and diluted with 80 mL Milli-Q water. The pH of the dilution was raised above 9.4 with 5-M KOH.

Both the decanted aqueous supernatant and the diluted solvent extract were extracted separately using solid-phase extraction (SPE), under vacuum, at a flowrate less than 5 mL/min. Oasis Hydrophilic-Lipophilic Balance (HLB) cartridges (200 mg) (Waters, Milford, Massachusetts) were used and primed with 5 mL methanol, followed by 4 mL 0.5-M KOH. Glass fiber filters (Fisher Scientific, Hampton, New Hampshire) (1-µm pore-size) were inserted to prevent the SPE frit from clogging. The cartridges were rinsed with three 1-mL aliquots of methanol–water–ammonium hydroxide (60:38:2, v:v:v) to remove organic coextracted materials. Tylosin was eluted with four 0.5-mL aliquots of acetonitrile–glacial acetic acid (98:2, v:v). The tylosin-containing acetonitrile eluants were dried down completely, under nitrogen gas at 22°C, and reconstituted in 0.5 mL of 0.01-M ammonium acetate (pH 6.8) to improve tylosin stability before analysis. The tylosin recovered from the supernatant and the pellet was summed for each slurry sample.

**Tylosin Analysis.** Tylosin was analyzed using an Agilent 1100 Series high-pressure liquid chromatograph (HPLC) (Agilent Technologies, Palo Alto, California). Injection volume of samples was 25 µL, and UV detection wavelength for all tylosin forms was 284.8 nm. A ZORBAX SB-C18 4.6 × 250 mm column was used (Agilent Technologies). The eluants used were 0.01-M ammonium acetate–glacial acetic acid buffer (pH 4.6) and acetonitrile, at a constant flowrate of 0.5 mL per minute, starting with 10% acetonitrile increasing to 100% acetonitrile between 6 and 35 minutes for each

50-min run. Column temperature was maintained at 40°C. A calibration curve for tylosin tartrate was prepared, showing a strong linear area response ( $R^2 = 0.9997$ ). Calibrations for tylosin A, B, C, and D were made by assuming an equal-area-concentration response for all forms (Teeter and Meyerhoff, 2003). Retention times for tylosin forms B, D, C, and A were 21.2, 21.5, 21.8, and 22.3 min, respectively. The detection limit for tylosin, with UV, under these settings, was approximately 500 µg/L. For the degradation studies, HPLC analysis was not sufficient to determine tylosin C in the slurry assays. Liquid chromatograph-mass spectrometry was used to verify the presence of tylosin A, B, C, D, and dihydrodesmycosin, and to determine the molecular mass of degradates observed in some samples. The limit of detection for tylosin A, B, C, and D forms were each approximately 4 µg/L. Mass spectra of tylosin forms and degradates were acquired by positive and negative ion electrospray on an Agilent 1100 HPLC configured as above, with the addition of 0.1% acetic acid to acetonitrile, coupled to a 1100 mass selective detector (MSD) ion trap LC-MS-MS (Agilent, Palo Alto, California). The drying gas was operated at a flowrate of 12 mL/min at 350°C. The nebulizer pressure was 50 psig, scanning mass from 50 to 1200 m/z. Quantification was based on an external 15-point calibration from 8 to 800 mg/L of the base peak ion (protonated adduct of the molecular ion) of the analyte. For each compound, the protonated molecular ion,  $[M + H]^+$ , and at least one confirming ion was acquired, based on MS-MS of the base-peak and fragment ratios formed from the standards and confirmed by published spectra (Van Poucke et al., 2003 and 2004). Ion suppression tests for the elution of four typical samples showed no interference of tylosin A at the time window of interest.

**Disappearance Rate Modeling.** A two-compartment, first-order model was used to describe the total tylosin disappearance. This model assumes a pool of rapidly degrading tylosin ( $C_1$ ) and a pool of more persistent tylosin ( $C_2$ ).

$$C_t = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} \quad (1)$$

Where

- $C_t$  = tylosin concentration (% of added) at time  $t$ ;
- $C_1$  = initial tylosin concentration (% of added) in pool 1;
- $C_2$  = initial tylosin concentration (% of added) in pool 2 ( $C_1 + C_2 = 100\%$ );
- $k_1$  = first-order rate constant ( $\text{hour}^{-1}$ ) for pool 1;
- $k_2$  = first-order rate constant ( $\text{hour}^{-1}$ ) for pool 2; and
- $t$  = time (h).

The model was fit to the experiment data using nonlinear regression with SAS software (SAS, Cary, North Carolina). Note that  $C_2$  was set as equal to the total added (100%) minus  $C_1$ . If  $C_2$  or  $k_2$  is not significantly different from zero, the model becomes a simple first-order model.

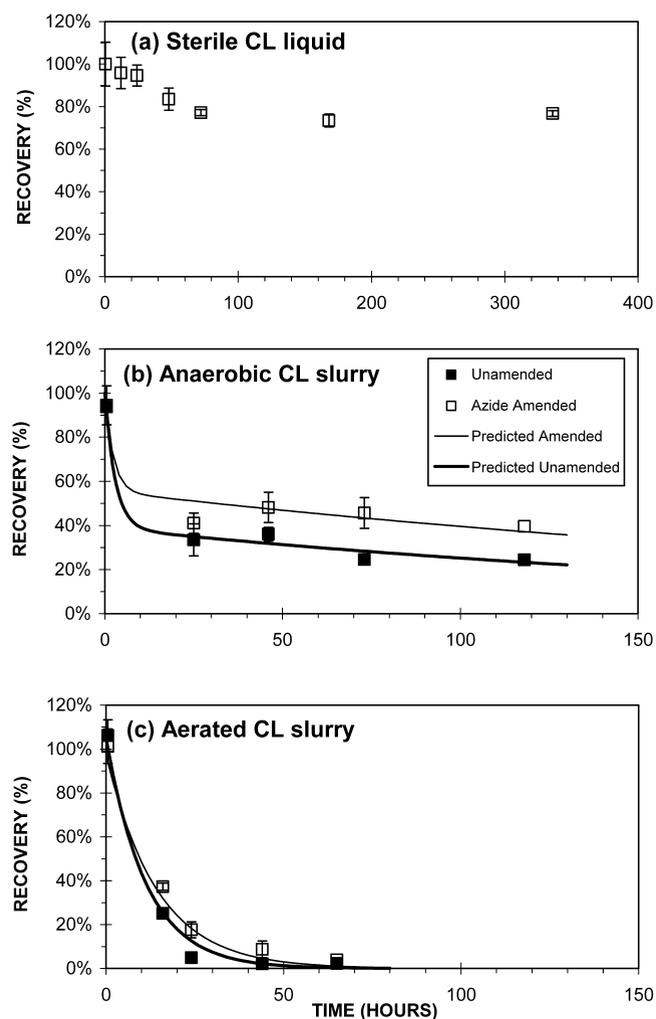
## Results and Discussion

**Slurry Characterization.** The characteristics of the two manure slurries are presented in Table 1. The pH for both manure slurries were similar, ranging between 8.5 to 9.1, before incubation. Background concentrations of tylosin B and D were approximately 50 and 15 µg/L in OL, respectively, and 1700 and 270 µg/L in the CL slurry, respectively. The OL slurries had higher total solids, organic carbon, and dissolved organic carbon as compared to CL slurry, but the total carbon values were similar.

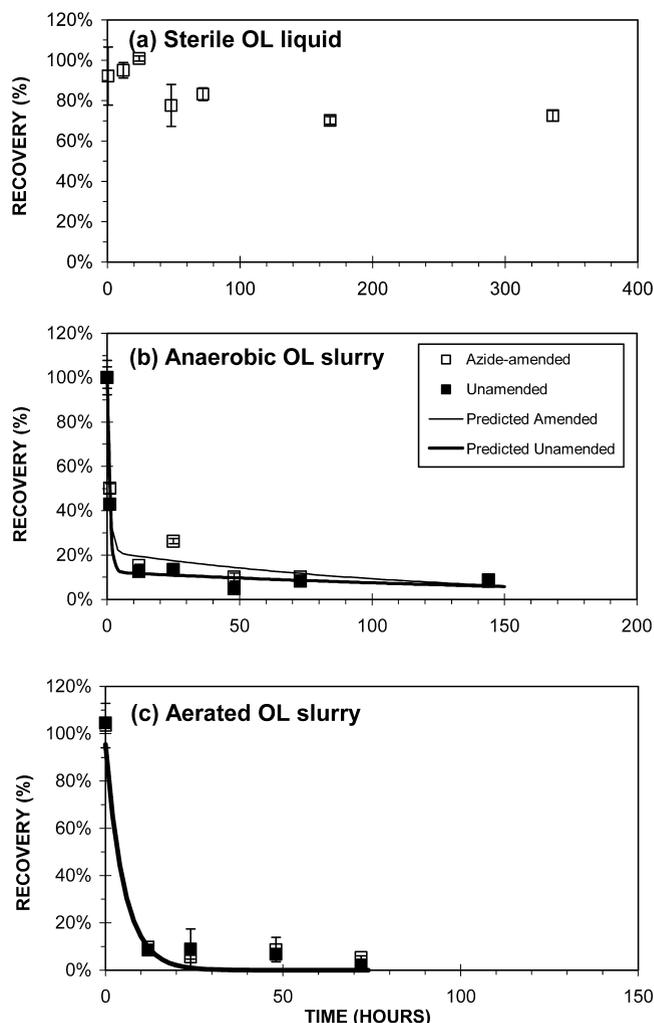
**Table 1—Swine manure characteristics from an open anaerobic lagoon (OL) and a covered anaerobic lagoon (CL).**

Analysis	Material	Open anaerobic lagoon (OL)	Covered anaerobic lagoon (CL)
pH	Slurry	8.5 to 9.1	8.7 to 9.0
Tylosin B, D (µg/L)	Slurry	50, 15	1700, 270
Total solids (g/kg)	Dried slurry	36	15
Nitrogen (g/kg)	Dried slurry	80	110
Organic carbon (g/kg)	Dried slurry	150	70
Phosphorus (g/kg)	Dried slurry	30	15

**Tylosin Stability and Toxicity.** Tylosin tartrate was stable for at least one month when stored in Milli-Q water at pH 5.7 to 6.7 at 22 ± 1°C. However, approximately 10% of added tylosin was degraded within the first 200 hours in Milli-Q water at pH 9.2.



**Figure 2—Tylosin recovered (% of added, sum of tylosin A, B, and D) from (a) sterile CL lagoon liquid, (b) anaerobic CL slurry, and (c) aerated CL slurry. Error bars are ± 1 standard deviation. Initial spiked concentration was 20 mg/L as tylosin tartrate.**



**Figure 3—Tylosin recovered (% of added, sum of tylosin A, B, and D) from (a) sterile OL lagoon liquid, (b) anaerobic OL slurry, and (c) aerated OL slurry. Error bars are ± 1 standard deviation. Initial spiked concentration was 20 mg/L as tylosin tartrate.**

In the case of azide-amended Milli-Q water at pH 9.3, the amount degraded was also approximately 10%.

Approximately 20 and 5% of added tylosin was degraded within the first 72 hours for the CL and OL 0.2-µm filtered lagoon liquids, respectively (see Figures 2a and 3a). In azide-amended and unamended Milli-Q water samples and both filtered lagoon liquids, an unknown degradate with molecular mass of m/z 934.5 appeared in an apparently base-catalyzed reaction (O’Neil et al., 2001; Paesen et al., 1995). The addition of sodium azide in the assays did not appear to influence tylosin stability.

The tylosin toxicity test showed that tylosin was not effective in suppressing microbial viability at 20 mg/L. The minimal salts broth became turbid after one week of incubation, and actinomycetes, amoebae, fungi, and bacteria were observed by light microscopy. This is consistent with previous studies, where tylosin was found to be active against gram-positive bacteria, but was only marginally active on gram-negative bacteria (Prescott, 2000).

**Azide Effectiveness.** Sodium azide was found to be effective at inhibiting microbial growth in the CL slurry, at a concentration of

**Table 2—Two-compartment model of tylosin disappearance in two manure slurries (CL and OL) under aerobic or anaerobic conditions, with or without sodium azide. Initial tylosin concentrations were 20 mg/L, except for OL anaerobic high treatment, which received 195 mg/L of tylosin. Rate constants are given in hours with 95% confidence intervals.**

Manure or treatment	$k_1$ ( $\text{h}^{-1}$ )	$k_2$ ( $\text{h}^{-1}$ )	$C_1$ (% of added)	$C_2$ (% of added)	90% Disappearance time (h)
CL aerated					
No azide	0.088 ± 0.024	0.029 ± 0.110 <sup>a</sup>	105.0 ± 0	NS <sup>b</sup>	26
Azide	0.070 ± 0.012	0	97.3 ± 5.1	NS <sup>b</sup>	32
OL aerated					
No azide	0.191 ± 0.132	0.001 ± 0.074 <sup>a</sup>	95.4 ± 18.1	NS <sup>b</sup>	12
Azide	0.195 ± 0.086	0.103 ± 0.572 <sup>a</sup>	94.8 ± 5.24	NS <sup>b</sup>	12
CL anaerobic					
No azide	0.344 ± 0.138	0.004 ± 0.002	61.1 ± 3.78	38.9	310
Azide	0.432 ± 0.183	0.003 ± 0.001	44.4 ± 6.25	55.6	500
OL anaerobic					
No azide	1.050 ± 0.172	0.005 ± 0.006 <sup>a</sup>	87.6 ± 4.22	12.4	40
Azide	1.005 ± 0.246	0.008 ± 0.006	78.5 ± 6.12	21.5	90
OL anaerobic high					
No azide	1.132 ± 0.238	0.045 ± 0.006	47.4 ± 6.74	52.6	30
Azide	1.123 ± 1.074	0.041 ± 0.012	26.8 ± 15.8	73.2	50

<sup>a</sup>  $k_2$  not significantly different from zero.

<sup>b</sup>  $C_2$  percentage tylosin not significantly different from zero.

50 g/L. Colonies or growth were absent from plates incubated for 96 hours with the azide-amended slurry.

**Extraction Recovery.** Tylosin concentrations are reported as the sum of the A, B, and D forms recovered from the separate analysis of slurry supernatant and slurry solid phase. Recoveries are expressed as a percentage of the tylosin added to the samples, after correction for tylosin concentrations present in the slurry at the time of collection. The average recoveries from CL and OL slurry using acetonitrile–isopropyl alcohol were 99% (8% RSD,  $n = 12$ ) and 93% (13% RSD,  $n = 12$ ), respectively, for vials sacrificed within 0.5 hours of spiking. Recoveries for acetonitrile–isopropyl alcohol averaged approximately 41% after 24 hours in CL. Methanol and acidified methanol each gave an average 38% recovery after 24 hours. Adding KOH to acetonitrile–isopropyl alcohol produced a higher proportion of tylosin B to tylosin A in the extracts. Because tylosin stability was desirable during extraction, the addition of KOH may not be appropriate. Of the three extraction solvents, acetonitrile–isopropyl alcohol (Shang et al., 2001) was chosen as the extractant also because of its use in the LC–MS–MS analysis. In addition, the SPE method outlined above for extracting tylosin forms and degradates was found to produce enhanced HPLC-baseline resolution as compared to other similar methods, which used acidic or non-pH modified rinses (Kolpin et al., 2002; Teeter and Meyerhoff, 2003). Oasis HLB cartridges provided recoveries of 96 to 105% from aqueous tylosin tartrate solutions and were resistant to pH changes up to pH 14, necessary to optimize the tylosin SPE procedure.

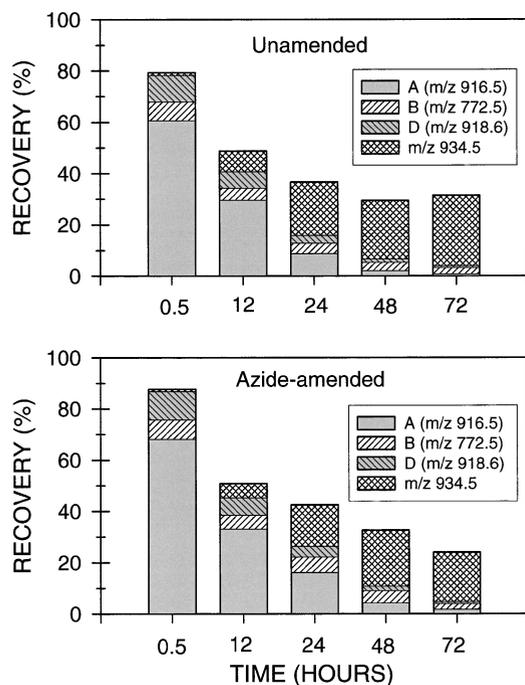
**Anaerobic Studies.** After 72 hours of incubation, the pH of the slurries changed from 8.5 to 9.1 to approximately 8.2 to 8.5 and dropped further to pH 7.5 to 7.8 after eight months of incubation. The addition of azide decreased pH of slurries by approximately 0.2 pH units. The ORP in slurries was between  $-10$  and  $-80$  mV  $E_H$  after 72 hours of incubation. The addition of azide resulted in a further decrease of the ORP to between  $-90$  and  $-160$  mV  $E_H$  after

72 hours incubation. Percent recoveries of tylosin (sum of forms A, B, and D) with time for CL and OL slurries spiked with 20 mg/L of tylosin are presented in Figures 2b and 3b, respectively. In both CL and OL slurries, there was a rapid disappearance of tylosin (60 to 85%) within the initial 24 hours, after which the disappearance of tylosin slowed in both the CL and OL slurries.

The two-compartment model (eq 1) was used to model the disappearance of tylosin in the OL and CL slurries. A first-order model was initially used, but was found to overestimate the amount of tylosin in anaerobic samples during the first 24 hours and underestimate the concentrations after 24 hours. The estimated tylosin disappearance rate constants,  $k_1$  and  $k_2$  and  $C_1$  and  $C_2$  for the anaerobic studies using OL and CL slurries, are summarized in Table 2. The  $k_1$  rate constants for the azide-amended and unamended OL and CL slurries were not significantly different, at a confidence interval of 95%. This implies that the rapid disappearance of tylosin within the first 24 hours may be because of sorption of tylosin to the solids. Substantial sorption of tylosin (90 to 99%) has been reported within 1 to 6 hours after spiking in soil and manure mixtures (Ingerslev and Halling-Sørensen, 2000).

The  $k_1$  rate constants for the OL slurries were two to three times larger than those for the CL slurries, indicating that tylosin disappearance was more rapid and residues were lower in OL slurry. Hydrophobic binding behavior has been related to sorption of tylosin (Loke et al., 2002; Tolls, 2001). The OL slurry had higher total solids and organic carbon content than CL, which supports sorption as a process contributing to tylosin disappearance. Teeter and Meyerhoff (2003) reported approximately 30% of the  $^{14}\text{C}$ -labeled tylosin accumulated as bound (nonextractable) residues in manure.

In the azide-amended CL slurry, the  $k_2$  rate constant was 0.003  $\text{h}^{-1}$ , and the estimated  $C_2$  value was 56%, showing that the tylosin continued to slowly disappear with time. For the unamended CL slurry, the  $k_2$  rate constant was 0.004  $\text{h}^{-1}$ , with a lower  $C_2$  value of



**Figure 4—Tylosin forms A, B, D, and unknown degradate (m/z 934.5) recovered at various times for OL lagoon slurry spiked with 195 mg/L tylosin and incubated anaerobically. Average recovery is expressed as the percent of tylosin added. Degradate recovery is based on the UV peak response for tylosin.**

39%. The difference in the estimated  $C_2$  values for the two CL slurries treatments indicated that there was less residual tylosin in the unamended CL slurry and may imply that biodegradation was occurring in the unamended CL slurry. The OL slurries behaved in a similar manner to the CL slurries, with respect to the azide treatment, but the estimates of  $C_2$  were less in the OL slurry than in the CL.

Half-lives may be used to compare the differences in the various treatments; however, because the disappearance of tylosin was very rapid, the time for 90% disappearance of tylosin was chosen as a comparison. The estimated time necessary for 90% tylosin disappearance was 40 and 310 hours for unamended OL and CL slurries, respectively. The 90% disappearance times for OL and CL azide-amended slurries were 90 and 500 hours, respectively, indicating that faster disappearance occurred in the unamended slurries.

For the anaerobic OL slurry treated with 195 mg/L tylosin, the  $k_1$  rate constant and the percent recovery were similar to that with 20 mg/L of tylosin. The estimated 90% disappearance time was also similar to that with 20 mg/L tylosin.

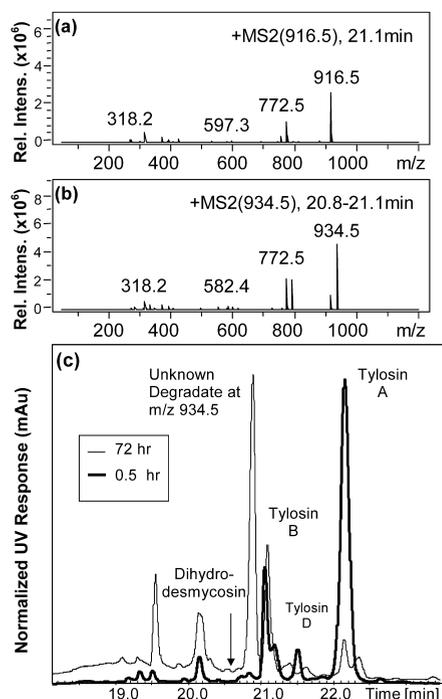
**Aerobic Studies.** The pH in the aerated slurries increased from 9.1 to 9.3 after 24 hours of aerobic incubation and remained above pH 9.0 after 12 days of aeration. Addition of sodium azide decreased the slurry pH by approximately 0.1 to 0.2 pH units. After aeration, the ORP was approximately +340 mV  $E_H$ . The ORP decreased by approximately 10 mV  $E_H$  in the aerated samples when azide was added.

As in the anaerobic studies, there was a rapid disappearance of tylosin. The disappearance of tylosin was almost completely described by the first term of the two-compartment model (eq 1),

making the disappearance follow a first-order model. The estimated  $k_1$  rate constants and  $C_1$  and  $C_2$  values are presented in Table 2. For the azide-amended and unamended OL slurries, the  $k_1$  rate constants were not significantly different. A similar assessment can be made for the  $k_1$  rate constants for the CL slurries. However, the  $k_1$  rate constants for OL were approximately 2 to 3 times larger than the CL slurries. Unlike the anaerobic studies, there was a lower amount of residual tylosin remaining at the end of the 72 hours. Less than 1% of the tylosin added remained after 12 days of aeration in OL slurry. The 90% disappearance time for the OL aerated slurries was 12 hours, as compared to 40 hours for the OL anaerobic slurries. For CL, the 90% disappearance times were 26 and 310 hours for the aerobic and anaerobic slurries, respectively.

Disappearance of tylosin in manure slurries can be attributed to biotic and abiotic degradation and to irreversible sorption (i.e., the formation of nonextractable bound residues). Assuming that the azide was effective in inhibiting microbial activity, the similar  $k_1$  rate constants for azide-amended and unamended slurries indicate that the portion of the initial tylosin disappearance attributed to biodegradation was quite small. In addition, the magnitude of abiotic degradation was small, with only 5 to 20% of the tylosin degraded in sterile filtered lagoon liquids. Therefore, much of the tylosin disappearance may be because of sorption, particularly in the anaerobically incubated slurries. Aeration increased slurry pH to 9.2 to 9.3, which may have accelerated a base-catalyzed reaction and resulted in lower residuals in aerated slurries. Faster disappearance rates in aerated samples correspond well to findings by other researchers in aerated and anaerobic surface water simulations and soil-manure slurries studies (Ingerslev and Halling-Sørensen, 2001; Ingerslev et al., 2001). In these systems, aeration decreased tylosin's half-life by 30 days when compared with anaerobic incubation and eliminated detectable residuals in soil-manure slurries within 12 to 15 days.

**Tylosin Degradates.** The proportions of the different forms of tylosin shifted through the tests, and some changes occurred immediately. The proportion of tylosin forms added to the lagoon slurries at the initiation of the experiments was approximately 93% A, 5% D, 2% B, and 0.3% C. Within 0.5 hours after spiking, the average proportion of forms A, D, B, and C in OL slurry spiked at 195 mg/L was 77% A, 13% D, 9% B, and 0.2% C (Figure 4). Similar changes in the proportions of tylosin forms were also noted in OL and CL samples after spiking 20 mg/L of tylosin. Tylosin A decreased more rapidly than D, and both decreased more rapidly than tylosin B. Tylosin B concentrations decreased in most slurries, except in anaerobically incubated CL slurry, where concentrations of B doubled during the first 72 hours. Tylosin B was the most predominant form in anaerobically incubated OL slurry, but this appears to be because of its increased relative persistence, as all forms decreased in concentration. The production of tylosin B was reported in aqueous anaerobically incubated manure mixtures and aerated soil manure slurries (Ingerslev and Halling-Sørensen, 2001; Loke et al., 2000). Tylosin D was also reported in these studies, but to a lesser extent than tylosin B. The highest relative proportion of tylosin D in the current research was in aerated CL slurry incubated for 72 hours, with 36% A, 36% D, and 27% B. Aerobically incubated OL slurry produced the smallest shifts in proportion of forms, with tylosin present as 93% A, 3% D, 4% B, and 0% C. After 72 hours, tylosin C was not detected in any of the samples. Dihydrodesmycosin was not detected in slurry-source materials, but increased to trace amounts during the first 72 hours of aerated and anaerobic incubation. Dihydrodesmycosin eluted at 20.2 minutes on



**Figure 5—Liquid chromatography with tandem mass spectrometry fragmentation patterns of (a) tylosin A ( $m/z$  916.5), (b) unknown degradate ( $m/z$  934.5), and (c) UV chromatograms of tylosin in CL slurry at 0.5 and 72 hours [in milliAbsorbance units (mAu)].**

chromatographs. Azide amendment did not significantly change the proportion of tylosin forms recovered in samples during short-term incubations.

In the long-term anaerobic tests using OL slurries, approximately 1% of the tylosin remained after eight months incubation. The tylosin forms detected after eight months were generally tylosin B and D. Comparison of the azide-amended and unamended slurries indicated that there was a greater amount of tylosin B, D, dihydrodesmycosin, and an unknown degradate (described below) in the azide-amended slurry than in the unamended slurry.

**Degradate Identification.** A degradate, eluting at 20.6 minutes, appeared within 12 hours after spiking in all unamended and azide-amended anaerobic and aerobically incubated assays. Neither aeration nor sodium azide affected the amount of degradate production. The degradate compound was not detected in slurry-source materials before tylosin addition or in tylosin tartrate standards at pH 7.0, but it did appear in sterile lagoon liquids and water at pH 9.2. Recovery of tylosin forms A, D, B, and the degradate are shown in Figure 4. Recovery of the degradate is based on an assumption of equal UV area response as tylosin. Typical UV chromatographs at 0.5 and 72 hours show the transformation of tylosin A, D, and B, and production of this degradate over time (Figure 5).

The base peak in the spectrum of the degradate was  $m/z$  934.5, and a major fragment ion was found at  $m/z$  772.5 when analyzed with LC-MS-MS, under positive ion mode. A low-abundance fragment ion, at  $m/z$  916.5, was also detected. Tylosin A's characteristic fragment ion of its 916.5  $[M + H]^+$  mass under positive ionization mode was 772.5  $[M + H - C_7H_{12}O_3]^+$ , indicating the loss of the mycarose sugar. The MS-MS mass

spectral fragmentation pattern for the degradate is compared with that of tylosin A in Figure 5. The degradate also responded under negative ionization mode, indicating that the compound had amphoteric properties. The mass spectral fragmentation pattern and peak UV absorbance of the degradate (285 to 290 nm) are strongly suggestive of a relation to tylosin A plus  $m/z$  18.

## Conclusions

Studies in manure-lagoon slurries indicated that the majority of tylosin was rapidly sorbed and degraded, with 90% disappearance occurring in less than five days. Both abiotic and biotic degradation were apparent. However, disappearance in anaerobic slurries slowed after 24 hours, and a residual pool of tylosin remained through the extended-anaerobic incubation. Aerating slurries significantly reduced tylosin residuals, leaving less than 1% of the added tylosin after 12 days. Tylosin B and D, which retain approximately 35 to 80% of the antibiotic activity of tylosin A (Teeter and Meyerhoff, 2003), were detected in slurry-source materials. Tylosin B and D were produced during the studies and remained after eight months of anaerobic incubation, in conventional-lagoon slurry. Dihydrodesmycosin and a degradate with undetermined antibiotic activity also formed during the experiments and persisted for eight months after tylosin addition. Based on these findings, residual tylosin degradates, with antibiotic activity, may be applied to agricultural fields with slurries and contribute to the detection of antibiotic residues near CAFOs.

## Acknowledgments

**Credits.** Funding for this work was provided by a grant from the Iowa State Water Resources Research Institute, Ames, Iowa. Mention of trade names or commercial products herein is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (Washington, D.C.).

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Submitted for publication May 4, 2004; revised manuscript submitted November 1, 2004; accepted for publication November 1, 2004.

The deadline to submit Discussions of this paper is May 15, 2005.

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