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## Abstract

Aerobic degradation, photolysis, and mobility of tylosin were investigated in the laboratory. Tylosin A is degraded with a half-life of 200 d in water, while it is stable in the dark. Tylosin C and D are relatively stable except in ultrapure water in the light. Slight increases of tylosin B and formation of two photoreaction products, isotylosin A alcohol (E,Z) and isotylosin A aldol (E,Z), were observed under exposure to light. In soil tylosin A and D has a dissipation half-life of about 1 wk. Sorption and abiotic degradation are the major factors influencing the loss of tylosin in the environment. No biotic degradation was observed at the test concentration of 50 µg/ml or µg/g either in pond water or in an agronomic soil, as determined by comparing dissipation profiles in sterilized and unsterilized conditions. At 7.5 ng/ml, biotransformation may play an important role in degradation of tylosin in water. Tylosin has strong sorption to various soils, and leachability is dependent on soil properties and manure amendment. Adsorbed tylosin in surface soil might run off to water bodies through soil erosion. In the end, pathways were proposed for tylosin degradation in the environment.

## Disciplines

Entomology | Veterinary Medicine | Veterinary Preventive Medicine, Epidemiology, and Public Health | Veterinary Toxicology and Pharmacology

## Comments

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## Chapter 7

# Environmental fate and chemistry of a veterinary antibiotic—tylosin

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Aerobic degradation, photolysis, and mobility of tylosin were investigated in the laboratory. Tylosin A is degraded with a half-life of 200 d in water, while it is stable in the dark. Tylosin C and D are relatively stable except in ultrapure water in the light. Slight increases of tylosin B and formation of two photoreaction products, isotylosin A alcohol (E,Z) and isotylosin A aldol (E,Z), were observed under exposure to light. In soil tylosin A and D has a dissipation half-life of about 1 wk. Sorption and abiotic degradation are the major factors influencing the loss of tylosin in the environment. No biotic degradation was observed at the test concentration of 50 µg/ml or µg/g either in pond water or in an agronomic soil, as determined by comparing dissipation profiles in sterilized and unsterilized conditions. At 7.5 ng/ml, biotransformation may play an important role in degradation of tylosin in water. Tylosin has strong sorption to various soils, and leachability is dependent on soil properties and manure amendment. Adsorbed tylosin in surface soil might run off to water bodies through soil erosion. In the end, pathways were proposed for tylosin degradation in the environment.

## Introduction

Over the last 30 years, intensive animal production has increased globally. As part of these intensive agricultural production practices, veterinary pharmaceuticals, primarily antibiotics, are widely being used as growth promoters or as therapeutic drugs. Many veterinary drugs are poorly adsorbed in

the gut of the animal, resulting in as much as 30-90% of the parent compound being excreted (1). Manure produced is usually stored in lagoons, and applied to agricultural fields as a fertilizer, which provides a pathway for veterinary pharmaceuticals and their metabolites to enter the environment. In addition, their metabolites can also be bioactive and can be transformed back to the parent compound after excretion (2). As a result, there is concern mounting over the over the fate and effects of low levels of veterinary antibiotics in the environment (3-7). The utilization of growth-promoting antibiotics in agriculture is one of the primary factors driving increased antibiotic resistance in humans (8).

Tylosin is a macrolide antibiotic used for the treatment of disease and growth promotion in cattle, swine, and poultry. Tylosin is also used to control American foulbrood, a wide-spread and devastating disease affecting honey bees (2). Tylosin consists of a mixture of tylosin A (Figure 1), tylosin B, tylosin C, and tylosin D. Approximately 80-90% of tylosin is composed of tylosin A (9). It has a broad anti-bacterial spectrum against gram positive bacteria and some gram negative bacteria, including vibrio, spirochete, and coccidia. It is also one of the first-choice drugs against infections caused by mycoplasma. Due to the wide-spread in agriculture, tylosin has been detected in 14% of 104 streams tested throughout the United States (10). There is concern that the presence of low levels of tylosin in the environment might promote bacterial resistance, which could decrease the effectiveness of erythromycin and other closely related antibiotics used to treat human diseases (11, 12).

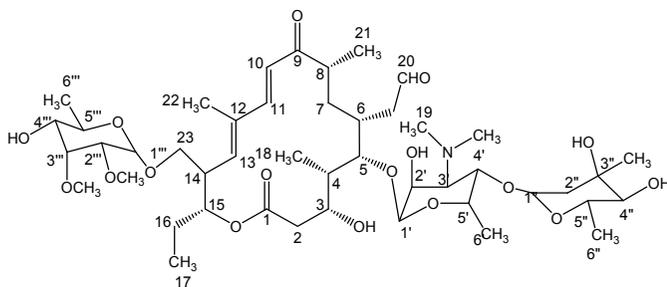


Figure 1. Molecular structure of tylosin A.

Understanding the environmental fate and chemistry of a chemical is a critical component of ecological and human risk assessments. The goal of this study was to characterize the environmental chemistry and fate of the veterinary antibiotic tylosin. Specifically we characterized the dissipation, movement and transformation of tylosin in water and soil. The studies here address: (1) dissipation in water, including the effects of light v. dark, ultrapure water v. pond water v. sterilized pond water, manure v. no manure, and submerged aquatic plant v. no plant; (2) dissipation in sterilized v. non-sterilized soil (one soil type); and (3) leaching through packed soil columns (three soil types).

## Materials and Methods

### Chemicals

Tylosin tartrate (95.0%, CAS NO. 74610-55-2) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All reagents used during extraction and analysis were analytical reagent grade or better.

### Water dissipation study

The methods for the water dissipation study have been previously reported (13). Briefly, pond water was collected from the Iowa State University Horticulture Farm Pond (Ames, IA, USA) and was stored in 250-ml French square bottles with perforated lids to maintain aerobic conditions. The pH of the pond water used (in the light and dark studies) was 8.1, the alkalinity is 103 mg/ml, and the total hardness is 190 mg/ml. Tylosin (50  $\mu\text{g/ml}$ ) was added to unsterilized pond water, sterilized pond water and ultrapure water, and the water microcosms were incubated. Water microcosms were sampled on days 0, 1, 3, 5, 7, 10, 14, 21, and 30, 60, 180 post-treatment. Tylosin samples were exposed to natural light at Ames, Iowa (42.01° N, 93.97° W) between April 15 and October 15 of 2005. All samples were maintained at a constant temperature of  $25 \pm 2$  °C with 12:12 natural light: dark cycle for 180 days. For the dark experiments, samples were kept in the same conditions but light was excluded by covering the bottles with aluminum foil.

A second water dissipation study (14) was conducted at the tylosin concentration of 7.5 ng/ml, which was selected to simulate the detected tylosin level in rivers (10). *Ceratophyllum demersum* (coontail) and pond water were collected from the ISU Horticulture Farm, and the manure used in the study was collected from swine on antibiotic-free diets at the ISU Swine Nutrition Farm. There were four microcosm treatments: no manure and no vegetation (pond water only), 0.1% manure (no vegetation), 2.3 g wet wt. coontail (no manure), 0.1% manure and 2.3 g wet wt. coontail. The mixture of Bristol's solution (nutrient-containing water for algal growth), pond water, and ultrapure water (at a ratio of 1:1:4) was placed in French square bottles for the test. The control was 33% Bristol's algal media. After a 3-day acclimation period, solutions were spiked to 7.5 ng/ml tylosin, to simulate tylosin concentrations in rivers. There were 5 replicates per treatment. The containers were maintained at constant temperature of  $25 \pm 2$  °C with 16:8 light (UV specific for plant growth): dark.

### Soil dissipation study (13)

Soil was collected from an agricultural field at the Iowa State University Agronomy and Agricultural Engineering Research Farm, near Ames, Iowa (from Field 55). The soil was classified as a sandy loam, a Nicollet-Webster

complex with 1.6% organic matter, 60% sand, 22% silt, 18% clay and pH 7.0. Soil samples (50 g), after passing through a No. 10 stainless steel sieve, were transferred to 250-ml French square glass bottles. Soil was sterilized by autoclaving at 121 °C for 30 min on three consecutive days. The soil moisture was adjusted to the field capacity water content (1/3 bar) using ultrapure water and was maintained throughout the study. The soil was spiked with 50 µg/g tylosin and incubated at 25°C in light/dark for 30 days. Samples were taken at days 0, 0.25, 0.5, 1, 3, 5, 7, 10, 14 and 21 and 30 post-treatment.

### Soil column study

Packed soil columns were used to evaluate the mobility of tylosin in soil. Soil columns consisted of PVC pipe (10-cm diameter and 30-cm length) fitted with a nylon mesh on the bottom. Columns were packed to a bulk density of 1.25 g/cm<sup>3</sup>. Columns were housed in upright position at 25°C for a one week prior to treatment and then for a 4-week testing period in an environmental chamber. Prior to treatment soil columns were saturated with 0.01M CaCl<sub>2</sub> solution and were allowed to drain overnight. After the treatment was applied, 3-cm rain events were simulated once a week for four weeks. Leachate was collected following each simulated rain event in 500 ml foil-covered glass bottles. At the end of the testing, columns were equally divided into three sections (called top, middle and bottom). Soil from each section was homogenized, and a 60-g subsample of soil was extracted. Three soil types were utilized: Canisteo clay loam, Nicollet-Webster sandy loam, and Hanlon sand; the properties of each soil is presented in Table II. There were three treatments for each soil type, including tylosin without manure, tylosin with manure, and only manure as control. The latter two treatment contained 30 g manure which was spiked with tylosin at 50 µg/g, and the manure was incorporated into about 2-cm soil surface. Four replications were applied for each treatment. Manure was attained from a swine farm feeding antibiotic-free diets, and was also confirmed to contain no tylosin using HPLC method.

### Analytical methods

Quantitative analysis of tylosin and its metabolites was achieved by reverse-phase HPLC (13). Analysis of the samples was performed using a Hewlett-Packard (Palo Alto, CA, USA) series 1100 HPLC system with a quaternary pump, an autosampler, a thermostatted column compartment, and a Spectroflow 757 absorbance detector (ABI Analytical, Kratos Division, Ramsey, NJ, USA). Data were collected and analyzed using HP Chemstation system software (REV. A.04.01). A Waters Atlantis<sup>TM</sup> (Milford, MA, USA) dC18 column (4.6×250 mm, 5-µm particle size) was used. Detection was conducted at 290nm, with a flow rate of 1.0 ml/min at room temperature. The mobile phases consisted of acetonitrile and 20 mM ammonium acetate (35:65, v/v, pH=6.0). The same ultraviolet-response capability of all tylosin-related compounds is assumed in this study because of lack of standards except for tylosin A. Soil samples (20g)

were extracted, using the mobile phase as the solvent, and the extracts were cleaned up with Oasis hydrophilic-lipophilic balance (HLB) cartridges (6 cc) (Waters, Milford, MA, USA) before HPLC analysis. Filtered water samples were directly injected (13). In order to prevent photodegradation, all extracts were stored in the dark and analyzed under minimum lighting.

## Results and Discussion

### Dissipation profile in water and soil

Tylosin-related compounds enter agricultural fields through application of tylosin-containing manure (15). Water dissipation study and soil dissipation study provided insights on persistence of tylosin A and its related compounds, which helps understand the risk posed by those tylosin residues in the environment. As a result, the dissipation profiles in water and soil are listed in Table I. Due to lack of analytical standards except for tylosin A, all tylosin-related compounds were identified using HPLC/MS (13).

Tylosin A had a dissipation time ( $DT_{50}$ ) of 200 d calculated from pooled data of three different treatments in the light, and tylosin A was stable, with less than 6% loss of initial spiked amount in the 6-month study. Tylosin C and D are relatively stable except in ultrapure water in the light. Slight increases of tylosin B after two months were observed in pond water, which is probably due to ionic strength or light exposure. Two photoreaction products were detected although the experimental conditions filtered out a majority of the UVA and UVB wavelengths which tylosin absorbs, and they were proved to be isotylosin alcohol and isotylosin aldol with application of HPLC-ESI-MS and  $^1\text{H}$  and  $^{13}\text{C}$  NMR. The structural elucidation of these two photoreaction products were conducted in our lab (16), and the structures of isotylosin alcohol and isotylosin aldol can be seen in Figure 5. Two photo-reaction products both have a (E, Z) configuration at the double-bond conjugated site, and one is isotylosin A alcohol (E, Z), and the other is isotylosin A aldol (E,Z) with two epimers.

Tylosin A and tylosin D had a  $DT_{50}$  of about 1 week in sterilized and unsterilized soil. The concentration of tylosin C increased slightly, and the mechanism of tylosin C formation is unknown, but it is probably facilitated by clay particles. The short  $DT_{50}$  of tylosin A in soil does not necessarily mean a rapid degradation. Tylosin might strongly adsorb to soil so that the recovery from the soil using the current extraction method is low. Although 80% of tylosin were able to be recovered right after the spike, the aging of tylosin in soil might remarkably reduce the final recovery from soil. It is evidenced that there are some bound tylosin residues in soils after solvent extraction by demonstrating biological activities of extracted soils (17).

**Table I. Dissipation profile of tylosin in water and soil in the laboratory**

Matrices and Conditions	Dissipation Profile <sup>a</sup>				
	Tylosin A	Tylosin B	Tylosin C	Tylosin D	
Ultrapure water	Light	227 d	Increase	200 d	280 d
	Dark	Stable	Stable	Stable	Stable
Pond water	Light	184 d	Stable	Stable	Stable
	Dark	Stable	Stable	Stable	Stable
	Sterilized + light	192 d	Increase	Stable	Stable
	Sterilized + dark	Stable	Increase	Stable	Stable
Soil	Sterilized	7 d	ND	Increase	8 d
	Unsterilized	7 d	ND	Increase	8 d

<sup>a</sup> Dissipation profile is described using DT<sub>50</sub> (Time to 50% dissipation, listed in days (d) where applicable); Stable: ≤6% loss; Increase: increase in concentration compared to study initiation; ND = not detected.

Although biodegradation of tylosin at concentration of 1 to 100 µg/L was found (18), no biodegradation was observed within the experimental periods by comparing the results from sterilized and unsterilized experimental conditions. The difference might be caused by the higher tylosin testing concentration and the lower microbial activity in our test system. The concentration of 30 µg/ml showed a complete inhibition of biodegradation (18), and in our studies the worst-case level of tylosin, 50 µg/ml in water or 50 µg/g in soil, was adopted for easy analysis of tylosin and its related compounds.

When the test concentration of tylosin was 7.5 ng/ml, the water dissipation study showed that there were significant differences after day 4 between treatments with manure and those without manure (Figure 2). At initial time point, tylosin concentrations between different treatments were not statistically different, and at later time points microbes from manure might play a role in dissipation or degradation of tylosin at this low level of tylosin. Coontail did not appear to have an effect on tylosin dissipation, indicating bioavailability of tylosin for coontail was low. In this separate water dissipation study, tylosin concentration was detected using a competitive direct enzyme-linked immunosorbent assay (ELISA) (19). The ELISA method was not totally specific for tylosin A, but showed cross-reactivity for other tylosin-related compounds. However, the ELISA detection results are in agreement with results from HPLC analysis within a 4-week experimental period, which is shown in another study done in our lab (16,19).

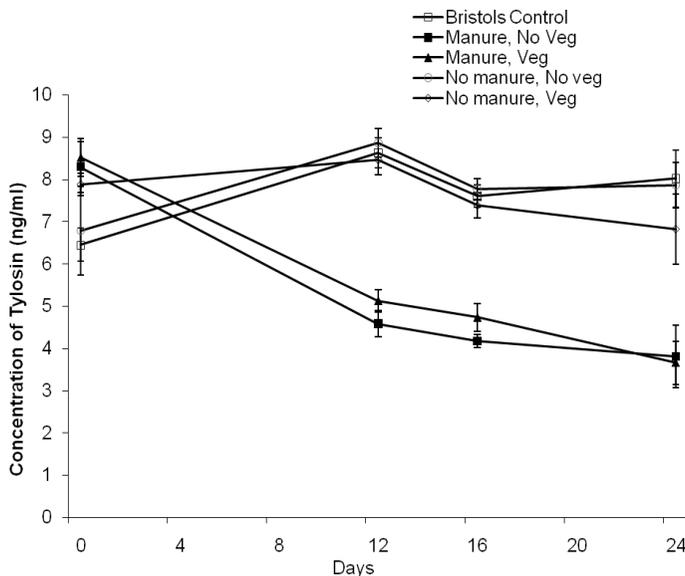


Figure 2. Dissipation of tylosin in surface water at 7.5 ng/ml.

### Mobility of tylosin in soil columns

Among three types of soil (Hanlon, Nicollet-Webster, and Canisteo) and two treatments (tylosin with or without manure) for each soil, tylosin A was detected at a range from 0 to 0.27 ng/ml in leachate from packed soil columns. An intact soil column study (20) showed that tylosin concentration was from 0.1 to 2.8 ng/ml in leachate. The average of 0.8 ng/ml from intact soil columns is at least 3 times higher than tylosin levels in leachate from packed soil columns, although the amount of spiked tylosin of 60  $\mu\text{g}$  (5  $\mu\text{g/g}$  in manure) on intact soil columns is 25 times lower than 1,500  $\mu\text{g}$  (50  $\mu\text{g/g}$  in manure) packed soil columns. Various factors might contribute to the difference such as soil properties, microbial bioactivity, etc. One important factor is the pore size of soils. All those intact soil columns were selected with a pre-leaching test in order to determine drainage, and columns with less than 24–48 h drainage were discarded. Packed soil columns would not have macropores inside which could drain water with chemicals quickly through soil columns with a low chance of adsorption to soil.

The distribution of recovered tylosin in packed soil columns is illustrated in Figure 3. Total recoveries of tylosin were below 4% of the initial spiked amount. Another study reported remarkably different recoveries of 61%–81% when soil column sections were extracted immediately after one rainfall event (21).

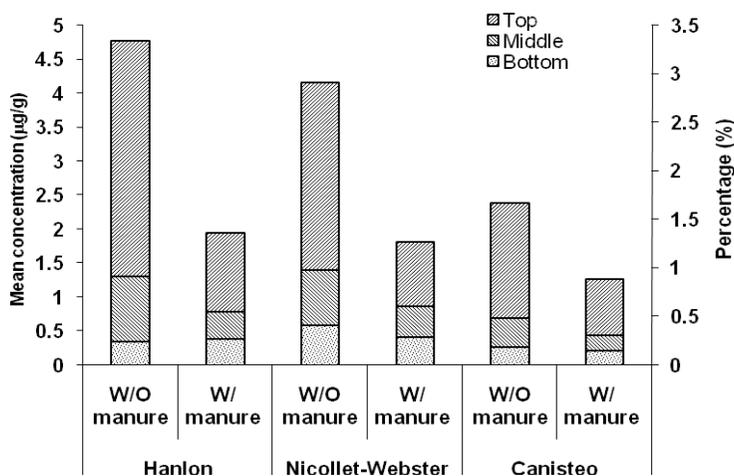


Figure 3. Distribution of recovered tylosin in packed soil columns

Table II. Soil properties and sorption coefficient (Kd) to tylosin

Soil types	texture	Sand%	Silt%	Clay%	%OM <sup>a</sup>	pH	CEC <sup>b</sup>	Kd (ml/g)
Canisteo	Clay loam	36	34	30	4.0	6.0	20.1	65
Nicollet-Webster	Sandy loam	47	36	17	2.6	6.8	14.9	42
Hanlon	Sand	90	6	4	0.5	8.1	6.9	24

<sup>a</sup> Organic matter content of soil

<sup>b</sup> Cation exchange capacity (Meq/100g)

In the one-month period of our soil column study, the majority of the tylosin might have dissipated before the soil extraction, since the  $DT_{50}$  in the soil was about 7 d. Decreased recovery from aged soils could be another factor, although the recovery of tylosin is more than 80% at the initial time point. It seems that there are more tylosin residues in the top section of soil columns than the middle and bottom sections. When tylosin is tightly bound to soil particles in highly adsorptive surface soil, soil adsorbed tylosin can be lost by erosion in runoff.

Soil properties such as organic matter, clay, C.E.C., etc. have been shown to influence the environmental behavior of a chemical (22). In this study, the three soils were selected to contain a wide range in composition to represent various soils in reality, and soil properties for each soil are listed in Table II. In Figure 3, the greatest leachability was observed in the Hanlon soil with no manure amendment. That soil contained highest sand content (90%), the lowest organic matter content (0.5%), and lowest C.E.C. (6.9 Meq/100 g) among the three soil types. Less or no tylosin was detected in the leachate from the other soil

columns. Correlation analysis (Figure 4) of soil properties with leachability indicates that soils high in organic matter, clay or C.E.C are the most adsorptive of tylosin, and manure amendment also decreased the leaching of tylosin through soil columns. In Hanlon and Nicollet-Webster soils amended with manure, tylosin concentrations in leachate were lower than in those without manure. This difference might be explained by higher sorption to manure which is evidenced by higher  $K_d$  value (285 ml/g) in manure compared to soils. Another possibility is that the swine manure contributed additional biodegraders to the test system.

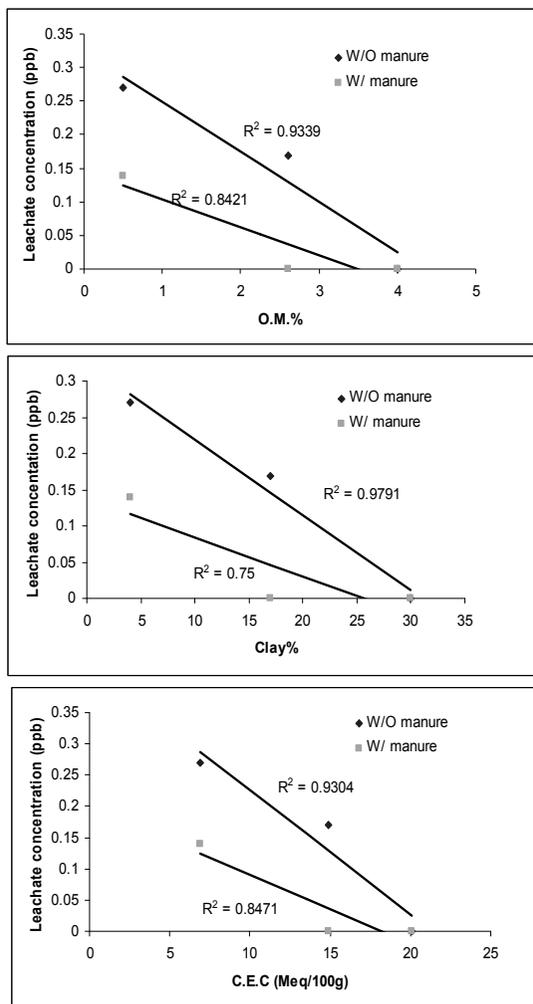


Figure 4. Correlation of leachability with OM%, clay%, and cation exchange capacity

## Proposed degradation pathways

Based on literature reports and our experimental findings, degradation pathways of tylosin in the environment are proposed and illustrated in Figure 5. Through cleavage of the mycarose sugar, tylosin B is formed from tylosin A in an acidic environment and light exposure (1,13,23). In neutral and alkaline conditions, tylosin aldol and some polar degradation products were detected (23). Tylosin D was found the major metabolite in swine and rat (technical sheet from Lilly Research Laboratories, Greenfield, IN, USA). Tylosin D was also detected in manure-containing test systems (1,15), which might be formed through microbial biotransformation, although it has yet to be determined. The soil dissipation study shows that tylosin C might form through abiotic transformation with facilitation by clay particles. Tylosin A associated with  $\text{NH}_4^+$  was found in our testing system, however, it is unknown if the formation appeared in the environmental matrices or in the HPLC mobile phase, which contained ammonium acetate. Under light exposure, isotylosin A aldol (E, Z) can be transformed from tylosin A or from the intermediate, tylosin A aldol. Isotylosin A alcohol (E, Z) can be formed from tylosin A or from tylosin D which is usually present with tylosin A, and it may possibly be biologically formed from tylosin A. Further degradation and mineralization of tylosin was observed (15,24).

Tylosin in the environment dissipates rapidly to very low concentrations which might not pose acute toxicity to living organisms in the ecosystem. However, persistence of low concentrations and their potential impact on the microbial community is not yet fully investigated and understood (14). For example, tylosin-resistant bacteria were isolated from cornfields (12). However, whether these tylosin-resistant bacteria were produced in the environment or brought from applied manure is still not clear. The impacts might include disturbance of soil respiration (25), soil enzyme activity (26), soil microbial communities (27,28), and soil nitrogen cycling (29), and analysis of effects on microbial community structure has been investigated using molecular genetic techniques (30-32). Recently, it has been shown that unextractable (bound) antibiotic residues, including bound tylosin residues, can still execute biological activity through unclear mechanisms (17), which may put selective pressure on bacteria in the terrestrial environment. Impact of low-level or bound residues of chemicals on the ecosystem, especially on the microbial community, should be a critical part of a complete risk assessment to ensure their safety to the environment and humans.

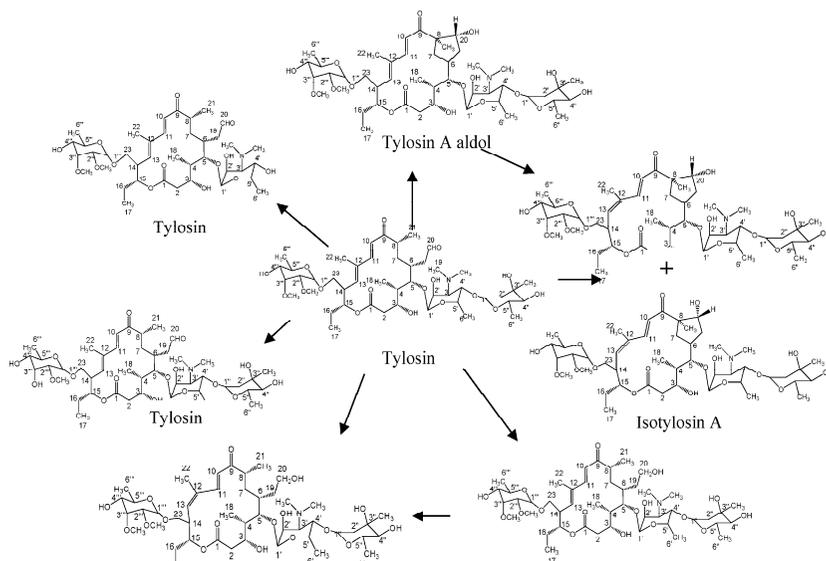


Figure 5. Possible transformation pathway of tylosin in the environment

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