Tylan® Soluble

Environmental Assessment for the Use of Tylosin Tartrate for Control of Necrotic Enteritis in Broiler Chickens

February 2012

Elanco Animal Health
A Division of Eli Lilly and Company
Lilly Corporate Center
Indianapolis, Indiana 46285

Table of Contents

List of Appendices

Tylan® Soluble

Environmental Assessment for the Use of Tylosin Tartrate for Control of Necrotic Enteritis in Broiler Chickens

1.0 Introduction

Tylosin tartrate is the active ingredient in Tylan® Soluble. Tylosin tartrate is already approved for oral use as Tylan Soluble (NADA 013-076) in chickens:

> As an aid in the treatment of chronic respiratory disease (CRD) associated with *Mycoplasma gallisepticum* sensitive to tylosin in broiler and replacement chickens. For the control of CRD associated with *Mycoplasma gallisepticum* sensitive to tylosin at the time of vaccination or other stress in chickens. For the control of CRD associated with *Mycoplasma synoviae* sensitive to tylosin in broiler chickens.

The following assessment is provided to support an application for the use of tylosin tartrate (as Tylan Soluble) at a targeted dose of up to 375 ppm in the drinking water of broiler chickens to reduce mortality caused by necrotic enteritis associated with *Clostridium perfringens*.

The environmental risk assessment has been conducted for the use of Tylan Soluble in broiler chickens based on the VICH guidelines for both Phase I (VICH GL6) and Phase II (VICH GL38) assessments and on normal use of chicken litter as fertilizer in the United States.

2.0 Pattern of Use and Relevant Exposure Routes

Tylosin tartrate will be administered to broiler chickens via the drinking water during an outbreak of necrotic enteritis associated with *Clostridium perfringens*. The treatment will be administered for a single 5 day period at a rate of 375 mg/L of tylosin in drinking water.

The use and exposure scenario is administration of tylosin-amended drinking water for 5 days during the production period of broiler chickens and subsequent application of chicken litter to agricultural land as this is the primary route for environmental exposure. Environmental exposure through spillage and breakage of containers is not expected since Tylan Soluble is marketed in small plastic jars.

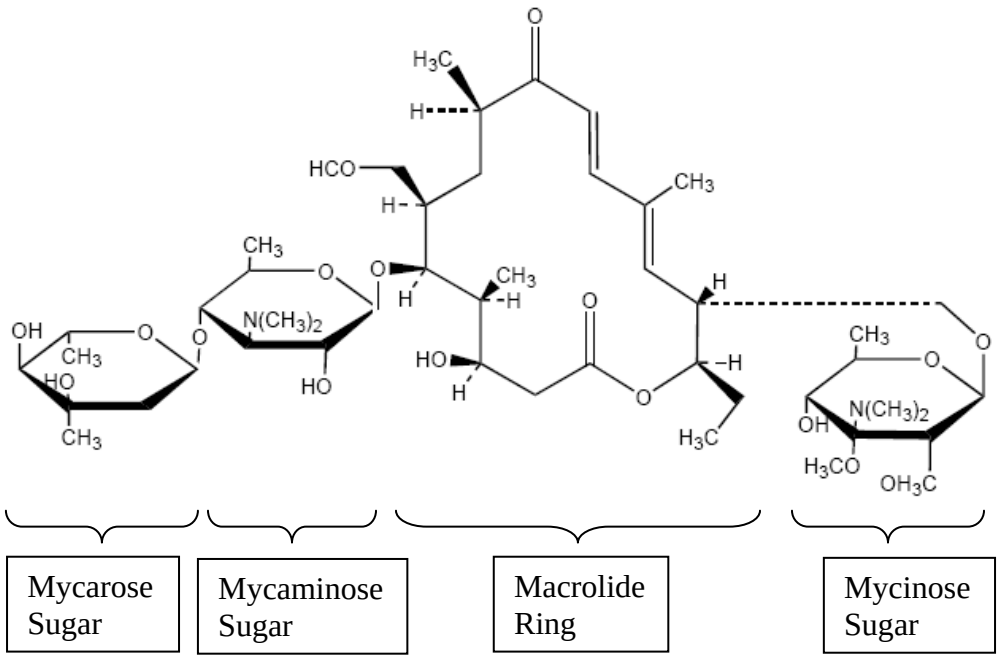
3.0 Description of the Product

Tylan Soluble is a formulation of tylosin for incorporation into drinking water. The active ingredient, tylosin, is prepared as a mixture of tylosin factors from fermentation and is predominantly tylosin factor A (not less than 74.5%), with tylosin factors B, C and D being minor components (Tylosin A+B+C+D not less than 91.3%). The tartrate salt is formed by the extraction of the tylosin bases with an aqueous solution of tartaric acid.

® Tylan (tylosin, Elanco Animal Health)

International Non-proprietary Name (INN):	Tylosin (factor A)
Chemical Name:	(4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[(6-deoxy-2,3-di-*O*-methyl-β-D allopyranosyl)oxy]methyl]-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-diene-2,10-dione
CAS Number (Tylosin base):	1401-69-0
Molecular Formula (Tylosin tartrate):	$(C_{46}H_{77}NO_{17})_2 \cdot C_4H_6O_6$
Molecular Weight:	Tylosin base: 916 Tylosin tartrate: 1981

Structural Formula for Tylosin A base:



There are different species of tylosin that are referred to as factors. Tylosin factor A, shown above, is the predominant factor in Tylan. Other factors include tylosin B (also known as desmycosin, in which the mycarose sugar is removed), tylosin C (also known as macrocin) is tylosin in which the mycinose sugar has been demethylated), and tylosin D (also known as relomycin in which the aldehyde on the macrolide ring has been reduced).

4.0 Phase I Environmental Impact Assessment

Final Guidance for Industry #89 (CVM, 2001) published by the FDA, Center for Veterinary Medicine, and the VICH GL6 Phase I guidance for Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) were consulted to

conduct the Phase I Environmental Impact Assessment for the use of Tylan Soluble in broiler chickens. In this Phase I assessment, the maximum concentration of tylosin in the litter and the soil has been calculated. No metabolism or degradation in litter is assumed and a total residue approach is taken for the Phase I assessment. The initiation of a Phase II assessment is dependent upon the trigger established in the VICH GL6 guidance: if the predicted environmental concentration of the total residue in soil is greater than 100 µg/kg, a Phase II assessment is warranted.

4.1 Concentration in Litter

The concentration of tylosin in chicken litter was estimated using the following values and calculations:

Concentration in Drinking Water	375 mg/L
Water Intake	0.2 L/day*
Duration of Dosing	5 days
Manure Production Period	50 days*
Amount of Diluent in Litter	15%*
Daily Manure	0.13 kg*

*These values are traditionally used values for environmental assessments and reflect typical agricultural practices such as those described by Midwest Plan Service (1985), National Research Council (1994), and Ensminger (1980).

$$Concentration\ in\ Litter = \frac{Total\ Tylosin\ Dosed}{Total\ Manure\ Produced \times Adjustment\ for\ Diluent\ in\ Litter}$$

$$= \frac{Concentration\ in\ Water \times Water\ Intake \times Duration}{Daily\ Manure \times Manure\ Production\ Period \times 1.15}$$

$$= \frac{375\frac{mg}{L} \times \frac{0.2L}{day} \times 5days}{0.13\frac{kg}{day} \times 50\ days \times 1.15} = \frac{375\ mg}{7.5\ kg} = 50\frac{mg}{kg}$$

4.2 Concentration in Soil

The maximum concentration of tylosin in the soil has been calculated using typical agronomy practices for application of poultry litter to land. Litter from poultry is applied to soil at a rate of 9200 kg/acre. Assuming a plow depth of 15 cm, the weight of the soil in an acre is approximately 910,500 kg. Assuming no degradation in manure or soil, the concentration of tylosin in soil after application of poultry litter could be as high as 505 µg/kg.

Application Rate of Litter to Soil	9200 kg/acre*
Plow Depth	15 cm*
Weight of Soil in 1 acre × 15 cm	910,500 kg soil/acre*

*These values are traditionally used values for environmental assessments and reflect typical agricultural practices such as those described by Simpson (1991) and Midwest Plan Services (1985).

$$Concentration\ in\ Soil = \frac{Concentration\ in\ Litter\ \times Application\ Rate\ of\ Litter\ to\ Soil}{Weight\ of\ Soil/acre}$$

$$= \frac{50\,\frac{mg}{kg} \times 9200\,\frac{kg}{acre}}{910{,}500\,\frac{kg}{acre}} = 0.505\,\frac{mg}{kg} = 505\ \frac{\mu g}{kg}$$

Since the initial concentration in the soil is more than 100 µg/kg, a Phase II environmental risk assessment was conducted, as per the VICH GL6 Final Guidance.

5.0 Phase II Environmental Impact Assessment

Since an initial soil concentration of tylosin of 505 µg/kg was calculated in the Phase I assessment, a Phase II environmental risk assessment has been conducted. Final Guidance for Industry #166 (CVM, 2006) published by the FDA, Center for Veterinary Medicine, and the VICH GL38 Phase II guidance for Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) were consulted to conduct the Phase II Environmental Impact Assessment for the use of Tylan Soluble in broiler chickens.

5.1 Tier A

5.1.1 Summary of Available Data

This section reviews environmental data that has been collected by the Sponsor and environmental data that has been reported in the published literature. Data collected by the Sponsor with tylosin include both older studies and more recent definitive studies that have been conducted according to current guidelines and Good Laboratory Practices. Only the studies with the data used in this risk assessment are included in the summaries appendix. Data from the literature is briefly described and is used to supplement data collected by the Sponsor.

5.1.1.1 Physical and Chemical Properties

The physical and chemical properties of tylosin indicate that the molecule is water soluble and exists as a solid at normal environmental temperatures.

Physical and Chemical Data (as Tylosin base)

Melting Point (Merck Index, 12th Edition (1996) p 1676)	128 - 132°C		
Aqueous Solubility (Merck Index, 12th Edition (1996) p 1676)	5000 mg/L at 25°C		
n-Octanol/Water Partition Coefficient (Study EC9907, 1999, Appendix A)	pH 5	pH 7	pH 9
	5	17	17
Vapor Pressure	As a high molecular weight solid, tylosin would be expected to have a negligible vapor pressure.		

5.1.1.2 Fate

5.1.1.2.1 Metabolism and Excretion

The metabolism and excretion of radiolabeled tylosin by broiler chickens has been described in Study T1Y720001 (2001, Appendix B). In this study, broiler chickens were administered ^{14}C-tylosin factor A in drinking water at a concentration of 528 mg/L.

A multi-stepped extraction procedure of the excreta was followed with the majority of the excreted radioactivity being recovered in the chloroform and aqueous fractions. Since tylosin predominantly partitions into the chloroform fraction, the chloroform extract was analyzed via HPLC coupled to tandem quadrupole mass spectroscopy with fraction collection and liquid scintillation counting. Tylosin A accounted for approximately half of the radioactivity in the chloroform fraction while tylosin D was about one fifth of the radioactivity. Other metabolites, including desmycosin (tylosin B) and 20-dihydrodesmycosin accounted for less than 10% in the chloroform fraction.

5.1.1.2.2 Degradation

5.1.1.2.2.1 Degradation in Excreta

The biodegradation of tylosin in chicken litter over 28 days has been examined in Study EC9908 (1999, Appendix E; Teeter and Meyerhoff, 2003). In that study, flasks containing chicken excreta collected from chickens fed ^{14}C-tylosin factor A were incubated under aerobic conditions at 20°C for 30 days. Negligible amounts of ^{14}C were measured in the volatile traps. Extracts of excreta were analyzed by an HPLC with ultraviolet detection using a method that was appropriate for detecting factors A, B, C and D of tylosin. HPLC/uv chromatograms of manure extracts from the beginning of the study contained two major peaks, one which corresponded to tylosin factor A and the other which had a retention time that was similar to that

expected for tylosin factor D. The presence of tylosin factors A and D is consistent with the chicken metabolism study, T1Y720001 (2001, Appendix B) in which the major metabolites in excreta are tylosin factors A and D. After 30 days of incubation of the manure under aerobic conditions, HPLC/uv chromatograms demonstrated that the manure extracts did not contain measurable levels of tylosin A. In fact, there were no peaks that matched the expected retention time of tylosin A, B, C or D. Therefore, if tylosin factors B, C, and/or D were formed as degradation products of tylosin factor A, those factors had also degraded by the end of the study. The first-order disappearance rate of tylosin A was estimated to be less than 7.5 days. Since very little ^{14}C was recovered as $^{14}CO_2$, it is likely that tylosin A, as well as the peak considered to be tylosin D, was degraded to one or several products. A radioprofile of the extracts was not generated, therefore, it is not known how many degradation products and their relative amounts were present. While the exact nature of the tylosin residues remaining in chicken manure is unknown, Study EC9908 (Appendix E) does demonstrate that the residues in aged manure do not contain tylosin factors A, B, C or D.

The disappearance of tylosin in poultry excreta is consistent with results reported by Dolliver, et al. (2008). In that study, turkey litter (excreta plus bedding material) was spiked with feed-grade tylosin and the litter stored indoors in one of three ways. Whether the litter was pushed into a pile without further manipulation, pushed into a pile and periodically turned and watered, or placed in a rotating steel drum with ventilation, the degradation rate of tylosin was similar and the reported half-life ranged from 16 to 23 days. The reported method used for measuring tylosin levels was an enzyme immunoassay which had cross-reactivity with tylosin factors A, B, C, and D (International Diagnostic Systems Elisa package insert). Therefore, the disappearance observed in this study included fate of tylosin factors other than A. Because broilers and turkeys are housed on similar bedding and have similar intestinal microflora (that is, lactobacilli and clostridium; Drasar and Barrow, 1985; Naqi et al, 1970), it is not unreasonable to assume that a similar amount of metabolic potential exists in poultry houses. Therefore, the study published by Dolliver et al. (2008) supports the assumption that tylosin and its factors will be subject to degradation within the litter of the chicken house as well as during any composting activities of the litter prior to application on land.

5.1.1.2.2.2 Degradation in Soil

The biodegradation of tylosin in four soils varying in pH and textural characteristics has been examined for 120 days at 20°C in aerobic conditions (Study 1982.6140, 2002, Appendix F). Soil was dosed with

approximately 1 mg/kg of ^{14}C-tylosin (as factor A). Over the course of the study some ultimate mineralization of tylosin occurred as evidenced by evolution of 1.6 to 9.3% of the applied radioactivity as $^{14}CO_2$. Tylosin factor A was also subject to primary degradation and was transformed to tylosin factors B, C, and D as well as to other metabolites. The half-life of tylosin A ranged from 50.3 to 105 days in the four soils. The sum of all tylosin factors decreased over the 120-day study (from 84.8 to 88% of applied radioactivity on Day 0 to 57.8 to 69.6% of applied radioactivity on Day 120). While tylosin A decreased over the course of the study, the sum of tylosin B, C, and D factors increased over the course of the study. However, the increase of these factors did not account for the entire disappearance of factor A. Therefore, the slower increase in these factors, as well as the evolution of $^{14}CO_2$, is likely due to the further degradation of factors B, C, and D. The bound residues increased over the duration of the study (ranging from 12.6 to 23.8% at the end of the study), suggesting continued degradation of tylosin to residues that are incorporated into the soil matrix. The results of this study demonstrate that tylosin A and its main factors do degrade in soil and are slowly degraded to CO_2.

The degradation of tylosin in soil described in Study 1982.6140 (Appendix F) is consistent with published studies on degradation of tylosin in soil. Gavalchin and Katz (1994) prepared a mixture of tylosin in chicken feces which was then used to fortify a sandy loam soil. The soil was incubated under aerobic conditions at 4, 20 and 30°C for 30 days. Periodically, samples were extracted and the extracts were evaluated for antimicrobial activity. After 30 days, 40% of activity remained in the 4°C condition. At both 20 and 30°C, no activity remained after 5 days. Using a soil-slurry test system, Ingerslev and Halling-Sorensen (2001) incubated tylosin in a slurry consisting of 1% or 10% swine manure in sandy and clay soils. The soils were incubated in columns which were aerated from the bottom. Tylosin A was quantified in filtered samples while tylosin B, C, and D were qualitatively identified by comparison to standards. The half-life of tylosin A ranged from 3.3 to 8.1 days. At time 0, tylosin A was the predominant peak in the samples. Degradation products including tylosin B, D and demycinosyl-tylosin were formed during the study, but disappeared shortly after tylosin A disappeared. Again, this report confirms that tylosin A and its related factors are degraded in soil. Halling-Sorensen et al. (2005) followed the concentrations of tylosin factors A, B, C and D in two field plots amended with manure from tylosin-treated swine. The calculated half-life values for tylosin A were 49 and 67 days. The half-life values for tylosin B and D were longer (84 to 140 days). The concentration of tylosin factor C, the factor with the lowest concentration at the beginning of the study, did not appear to decrease during the experimental phase (155 days). However, the concentrations of tylosin C in the field were below the

determined limit of quantitation and some were even below the limit of detection, therefore, the authors' conclusion that tylosin C does not degrade in the soils is not substantiated by their data. There is no structural reason to believe that tylosin C would be recalcitrant to degradation by microbes. In general, the published studies are in agreement with the Sponsor's data regarding the disappearance of tylosin from soil.

5.1.1.2.3 Soil Adsorption

The adsorption of tylosin to soil was evaluated in 3 different soils (Study EC9906, 1999, Appendix G). Freundlich binding isotherms were constructed for each soil at a range of concentrations and the resulting Freundlich Kd coefficients (6, 38, 67) were converted to Koc values on the basis of the organic carbon fraction in the soils. The Freundlich Koc values ranged from 200 to 2233 with a mean of 1362. The study data indicate that adsorption of ^{14}C-tylosin to the solids is limited and that subsequent desorption occurs readily.

The soil adsorption results from Study EC9906 (Appendix G) are consistent with published values. Tolls (2001) evaluated soil partitioning using a column displacement method with four soils and reported a range of Kd values for tylosin from 8.3 to 128 (the adjusted Koc values ranged from 550 to 7990). ter Laak et al. (2006) used a batch sorption method to determine the Kd values for tylosin in 11 soils; the Kd ranged from 12.4 to 387 with a mean value of 129.5 This group also reported that the sorption of tylosin was correlated to clay content and cation-exchange capacity but not to organic carbon content.

A soil leaching study (Appendix H) determined that tylosin factors A and D remained in the top 5 cm of soil after a total of 60 cm (23.5 inches) of water was applied over a period of six days. A similar conclusion, that tylosin is not subject to significant leaching in the soil, was reached by Rabølle and Spliid (2000) using four Danish soils in a column leaching study.

Thus, even though the soil adsorption for tylosin is limited, significant leaching into groundwater is not expected.

5.1.1.2.4 Hydrolysis and Photolysis

While formal hydrolysis and photolysis studies have not been conducted with tylosin, it has been observed that in static aquatic toxicity tests with daphnids and rainbow trout, tylosin concentrations remain relatively constant over 48- and 96-hour exposure periods. On the other hand, in a continuously illuminated algae study, the concentration of tylosin declined over the 72-hour exposure period in a vessel without algae. Therefore,

while there is evidence that tylosin is not subject to hydrolysis, it may have some potential for photolysis.

While not specifically measuring hydrolysis or photolysis, tylosin disappearance has been reported in an aquatic microcosm which had been repeatedly dosed with tylosin (Brain et al., 2005). When dosing ceased, the half-life of tylosin in the water (1 m deep with 50% of the bottom covered with trays of sediment) was determined to range from 9 to 10 days (Brain et al., 2005).

5.1.1.2.5 Bioconcentration

Tylosin has a very low n-octanol/water partition coefficient (Kow), so it is not likely to significantly bioconcentrate in aquatic organisms. Veith et al. (1979) generated a linear model to predict the bioconcentration factor for chemicals in fathead minnows:

$$\log BCF = 0.85 \times \log Kow - 0.70$$

Using this equation and the highest log Kow value (1.23), the estimated bioconcentration factor (BCF) for tylosin is 2.2. The rapid decline of tissue residues in chickens dosed with ^{14}C-tylosin supports the estimation that tylosin will not bioaccumulate in biological tissues (Study T1Y720001, 2001, Appendix B).

Fate Data

<table>
<tr><td>Degradation in Chicken Excreta
(Study EC9908, 1999, Appendix E)</td><td colspan="3">Tylosin A incubated aerobically for 30 days
No Tylosin A, B, C, or D was observed after 30 days</td></tr>
<tr><td rowspan="5">Soil Adsorption
(Study EC9906, 1999, Appendix G)</td><td>Soil</td><td>K_d</td><td>K_{oc}</td></tr>
<tr><td>Sandy Loam pH 4.6</td><td>6</td><td>200</td></tr>
<tr><td>Silt Loam pH 5.7</td><td>38</td><td>1652</td></tr>
<tr><td>Sandy Loam pH 7.6</td><td>67</td><td>2233</td></tr>
<tr><td>Mean</td><td>37</td><td>1362</td></tr>
<tr><td>Soil Leaching
(1978, Appendix H)</td><td colspan="3">Neither tylosin A nor D was found to leach from 30 cm tall columns of silt loam and sandy loam soil when 60 cm of artificial rainfall was applied over 6 or 7 days. Almost all of the tylosin activity remained in the top 5 cm of the columns.</td></tr>
</table>

Degradation in Soil (1982.6140, 2002, Appendix F)	Four soils were dosed with ^{14}C-tylosin and incubated aerobically for 120 days.	
	Half-life of tylosin A (days)	50.3 to 105
	Mineralization (% AR)	1.6 to 9.3
	^{14}C-Tylosin (∑A, B, C, D factors) Day 0 (% AR)	84.8 to 88
	^{14}C-Tylosin (∑A, B, C, D factors) Day 120 (% AR)	57.8 to 69.6
	Bound Residues Day 120 (% AR)	12.6 to 23.8

%AR: Percent of applied radioactivity

5.1.1.3 Toxicity

5.1.1.3.1 Soil Organisms

Definitive studies in soil microflora, plants, and earthworms have been conducted (Studies 1982.6222, 1982.6187, 6010-77, and 61741022, are described below, and are considered for the PNEC determination.

In a study following OECD guidelines 216 and 217 (Study 1982.6222, Appendix I), the highest concentration of tylosin tested, 5,000 µg/kg, resulted in changes in respiration and nitrogen fixation by soil microflora that were less than 25% different from the controls.

In a phytotoxicity test (Study 1982.6187, Appendix J) following OECD guideline 208, tomato, oat and soybean were exposed to tylosin incorporated in a sandy loam soil. There were no effects on emergence from seeds at the highest concentrations tested. Effects on growth were observed in all species. The lowest EC50 was 43,000 µg/kg in tomato, while the lowest no observed effect concentration (NOEC) was 3900 µg/kg in both soybean and oat.

The effects of tylosin on earthworms were evaluated in Studies 6010-77 (*Lumbricus terrestris*, Appendix K) and 61741022 (*Eisenia fetida*, Appendix L). In the first toxicity study, adult earthworms (*Lumbricus terrestris*) were exposed to tylosin-treated soil containing an initial nominal concentration of 102,600 µg/kg for 14 days. No mortality or change in growth rate was noted in the tylosin-exposed worms compared to the control. In the second chronic reproduction study, adult earthworms (*Eisenia fetida*) were exposed to tylosin-treated soil containing initial nominal concentrations up to 250,000 µg/kg for 28 days. Following 28 days, there was no mortality or inhibition of growth attributed to exposure to tylosin. The adult worms were removed from the soil and the

soil was maintained for an additional 28 days to allow cocoons to hatch. There were statistically significant decreases in the number of juveniles at the top two concentrations, 125,000 and 250,000 µg/kg. Therefore, the NOEC was 62,500 µg/kg.

A review of published reports on the effects of tylosin on soil organisms do not indicate that there are more sensitive endpoints or species in the terrestrial compartment than those studied and reported by the Sponsor. Baguer et al. (2000) exposed the springtail (*Folsomia fimetaria*), the enchytraeid (*Enchytraeus crypticus*), and the earthworm (*Aporrectodea caliginosa*) to various concentrations of tylosin in soil. Survival and reproduction were assessed for the springtail and enchytraeid exposures. For the earthworm, survival, reproduction, growth and hatchability of the cocoons were assessed. For all species and endpoints, 2,000,000 µg/kg was the lowest NOEC. Liu et al. (2009) evaluated the effect of tylosin plant growth of rice and cucumber; soil microbial respiration; and soil phosphatase activity. The NOEC for plant growth for tylosin was >500,000 µg/kg for rice and 50,000 µg/kg for cucumber, supporting the observation from the Sponsor's old phytotoxicity report that there is a difference in sensitivity to tylosin among plant species. However, the NOEC is still higher in this species than the NOEC in the Sponsor's definitive phytotoxicity study. Tylosin had no adverse effect on soil respiration up to 300,000 µg/kg. Tylosin did inhibit phosphatase activity in the soil, and the maximum inhibition compared to control was approximately 25% at 100,000 and 300,000 µg/kg.

5.1.1.3.2 Aquatic Organisms

Studies conducted to evaluate the toxicity of tylosin in algae, daphnia, and fish (Studies 1982.6139, 1982.6137, and 1982.6138) are described below.

The green alga, *Psuedokirchineriella subcapitata,* was exposed to tylosin under static conditions for 72 hours in Study 1982.6139 (Appendix M) that followed OECD guideline 201. Concentrations of tylosin in the treatment levels decreased from 96 to 109% of nominal concentrations at test initiation to 16 to 28% of nominal at the end of the study. The mean measured concentrations ranged from 25 to 390 µg/L. The decrease in tylosin concentration was probably due to photolysis since a similar drop in tylosin concentration also occurred in a vessel without algae. The results are based on mean measured concentrations. Concentration-dependent decreases in biomass and growth rate were observed. The EC50 values for biomass and growth rate were 220 and 310 µg/L, respectively. The NOEC value for both biomass and growth rate was 99 µg/L.

In Study 1982.6137 (Appendix N), *Daphnia magna* were exposed to tylosin in a static toxicity test following OECD guideline 202. The

exposure duration was 48 hours at a mean measured concentration of 87,000 µg/L. No immobilization or adverse effects were observed in daphnids exposed to tylosin. The EC50 was >87,000 µg/L and the NOEC was ≥87,000 µg/L.

Rainbow trout, *Oncorhynchus mykiss*, were exposed to tylosin in a static toxicity test following OECD guideline 203 (Study 1982.6138, Appendix O). The exposure duration was 96 hours at a mean measured concentration of 96,000 µg/L. There was no mortality or sublethal effects observed among fish exposed to tylosin. The LC50 was >96,000 µg/L and the NOEC was ≥96,000 µg/L.

Several studies have been published that describe the ecotoxicity of tylosin in aquatic species.

Wollenberger et al. (2000) tested tylosin in an acute daphnid study up to a nominal concentration of 1,600,000 µg/L and determined an EC50 of 680,000 µg/L and an EC10 of 483,000 µg/L. Wollenberger et al. (2000) also evaluated the effect of tylosin on *Daphnia magna* in a chronic exposure, monitoring survival and reproduction over 21 days over a nominal concentration range of 5600 to 180,000 µg/L. In that study, there was no effect observed on reproduction, but there was mortality at 90,000 and 180,000 µg/L. The NOEC was determined to be 45,000 µg/L.

Brain et al. (2004) evaluated the toxicity of tylosin to the duckweed *Lemna gibba* in a seven-day study over a nominal concentration range of 10 to 1000 µg/L. No phytotoxicity was observed at any concentration. In contrast, tylosin had a slight stimulatory effect on wet weight and chlorophyll *a* and *b*.

Yang et al. (2008) followed the OECD 201 guideline in testing tylosin with *Pseudokirchineriella subcapitata* for 72 hours. The reported EC50 from that study was 210 µg/L (based on nominal concentrations). Halling-Sørensen (2000) evaluated the comparative toxicity of tylosin to different algal species. Both a green algae and a blue-green algae (cyanobacteria) were exposed to a range of nominal concentrations of several antibiotics. The EC50 value for tylosin in *Selenastrum capricornutum* (green algae, now called *Pseudokirchineriella subcapitata*) and *Microcystis aeruginosa* (cyanobacteria) were determined to be 1380 and 34 µg/L, respectively. The exposure durations were three days and seven days for the green algae and cyanobacteria, respectively. However, the NOEC, EC10 and EC20 values were not reported for the two species. In general, the prokaryotic cyanobacteria were considerably more sensitive to compounds with antimicrobial activity than green algae.

Tylosin has also been evaluated using aquatic microcosms populated with macrophytes (Brain et al., 2005). The microcosms were dosed repeatedly

with nominal concentrations of tylosin ranging from 10 to 3000 µg/L. There were no inhibiting effects in macrophytes, but there was a stimulating effect at a measured concentration of 2415 µg/L.

Ecotoxicity Data

Terrestrial Effects Studies				
Respiration and Nitrogen Transformation Tests (28 days) (Study 1982.6222, 2004, Appendix I)	5,000 µg/kg results in <25% difference from control			
Terrestrial Plants – Seedling Growth (Study 1982.6187, 2003, Appendix J)		Emergence	Shoot Weight	
		NOEC µg/kg	EC50 µg/kg	NOEC µg/kg
	Tomato	≥ 63,000	43,000	16,000
	Soybean	≥ 250,000	53,000	3900
	Oat	≥ 1,000,000	140,000	3900
Earthworm 14-day Growth and Survival (*Lumbricus terrestris*) (Study 6010-77, 1978, Appendix K)	EC50 > 102,600 µg/kg NOEC ≥ 102,600 µg/kg			
Earthworm 56-day Reproduction (*Eisenia fetida)* (Study 61741022, 2011, Appendix L)	NOEC 62,500 µg/kg			
Aquatic Effects Studies				
Algal Growth Inhibition (Study 1982.6139, 2002, Appendix M)	Biomass EC_b50 220 µg/L $NOEC_b$ 99 µg/L Growth Rate EC_r50 310 µg/L $NOEC_r$ 99 µg/L			
Daphnia immobilization (Study 1982.6137, 2002, Appendix N)	EC50: >87,000 µg/L NOEC: ≥87,000 µg/L			
Fish Acute Toxicity (Study 1982.6138, 2002, Appendix O)	Rainbow Trout LC50 > 96,000 µg/L NOEC ≥ 96,000 µg/L			

5.2 PEC Calculations and Refinements (Exposure Assessment)

5.2.1 Soil

The $PEC_{soil\text{-}initial}$ for tylosin for treating necrotic enteritis in chickens was calculated in the Phase I assessment as 505 µg/kg using total tylosin residues in litter of 50 mg/kg. Per the VICH guideline (VICH GL38), this value can be refined using metabolism, degradation in manure and degradation in soil.

5.2.1.1 Refinement by metabolism

A very conservative refinement for metabolism by chickens is detailed in Appendix C. In this refinement, all identified metabolites that are factors of tylosin were corrected for their bioactivity relative to tylosin A. Additionally, all fractions that were not profiled were assumed to be tylosin A or something with activity equivalent to tylosin A. Using these assumptions, the amount of residues excreted from chickens following dosing with tylosin was decreased by 25%.

Therefore, when refined by metabolism, the concentration of tylosin in chicken litter from chickens treated for five days with narasin in drinking water at a concentration of 375 mg/L will be 37.5 mg/kg. The $PEC_{soil\text{-}refined\ for\ metabolism}$ is, therefore, 379 µg/kg.

5.2.1.2 Refinement by degradation in excreta:

Tylosin A and tylosin D degrade under aerobic conditions in chicken excreta (Study EC9908, Appendix E). Although present at Day 0, neither A nor D were detected after 30 days incubation. Using the limit of detection, a half-life was calculated for tylosin A of less than 7.5 days. Broilers are present in a barn for 50 days. Barns are typically not cleaned out between every set of broilers, instead fresh bedding is placed on top of the older bedding. Even when barns are cleaned out, litter is often piled into windrows or piles to allow composting to destroy pathogenic bacteria prior to application to land. Therefore, there is time for several half-lives of tylosin A to be degraded prior to application to fields. The data from the tylosin degradation in chicken excreta study also shows that tylosin D is degradable in 30 days and that after 30 days, factors A, B, C, and D are not present in chicken excreta. However, Study EC9908 (Appendix E) was not designed to track every degradation product and it is possible that major degradation products were formed. It is also possible, although unlikely, that some of the major degradation products have activities similar to that of tylosin. A very conservative assumption would be to make no refinement on the basis of degradation in chicken excreta. While the data indicate that none of the tylosin factors A, B, C, or D will be present in chicken litter, in this risk assessment, no refinement will be made for degradation in chicken litter prior to land application.

5.2.1.3 Refinement by degradation in soil:

Tylosin (sum of factors A, B, C, and D) degrades slowly in soil, but does eventually mineralize. Over 120 days, the sum of the factors decreased approximately 20 to 30%. Therefore, the soil concentration will not be refined for degradation in soil.

5.2.1.3.1 Calculation of $PEC_{soil\text{-}refined}$

Using the refined litter concentration, the $PEC_{soil\text{-}refined}$ can be calculated:

$$Concentration\ in\ Soil = \frac{Concentration\ in\ Litter\ \times Application\ Rate\ of\ Litter\ to\ Soil}{Weight\ of\ Soil/acre}$$

$$= \frac{37.5\frac{mg}{kg} \times 9200\frac{kg}{acre}}{910{,}500\frac{kg}{acre}} = 0.379\frac{mg}{kg} = 379\ \frac{\mu g}{kg}$$

5.2.1.3.2 Calculation of $PEC_{porewater}$

The partitioning coefficient of tylosin in soil was used to calculate the expected concentration of tylosin in pore water. The following calculation was used for deriving pore water concentration (ECB 2003):

$$PEC_{porewater} = \frac{PEC_{soil} \times Bulk\ Density_{wet\ soil}}{Kd\ \times 1000}$$

Using a wet soil bulk density value of 1700 kg/m^3, the lowest measured partition coefficient of 6, and the $PEC_{soil\text{-}refined}$ value of 379 µg/kg, the tylosin concentration in soil porewater was calculated to be 107 µg/L:

$$PEC_{porewater} = \frac{379\ \frac{\mu g}{kg} \times 1700\frac{kg}{m^3}}{6\ \times 1000} = 107\frac{\mu g}{L}$$

Given the minimal propensity of tylosin to leach through soil, this pore water concentration is not expected to be predictive of the groundwater concentration.

5.2.2 Surface Water

Movement of tylosin from soil to surface water may occur through runoff following rainfall events. A scenario of 1% runoff of compound from 10 acres of soil into a one-acre pond which is 2 m deep was considered. A one-acre pond that is 2 m deep has a volume of 8,100,000 L. Using the concentration of tylosin in litter and the application rate of litter per acre, the following calculation was performed to estimate the concentration of tylosin residues in the pond:

$$[Tylosin]_{pond} = \frac{[Tylosin]_{litter} \times kg\ litter\ per\ acre \times 10\ acres \times 0.01}{8{,}100{,}000\ L}$$

$$[Tylosin]_{pond} = \frac{37.5\frac{mg}{kg} \times 9200\frac{kg}{acre} \times 10\ acres \times 0.01}{8{,}100{,}000\ L} = 4.3\frac{\mu g}{L}$$

Therefore, the $PEC_{surface\ water}$ is 4.3 µg/L. Because tylosin has minimal propensity to adsorb to soil and sediment, all of the loss is assumed be present in the aqueous layer in the pond.

Regarding the assumption of 1% loss during a rainfall event, Davis et al. (2006) investigated the loss of tylosin during a simulated one hour rainfall event after surface application. In that evaluation, only 0.022% of tylosin was lost to runoff. Therefore, 1% is considered to be a worst-case assumption.

Reports of tylosin in surface water have been published. Kolpin et al (2002) reported in a reconnaissance study that out of 104 freshwater stream samples, tylosin was detected in 13.5% of them with a maximum concentration of 0.28 µg/L and a median concentration of 0.04 µg/L. Yang and Carlson (2004) report than a single sample taken from a river in Colorado at a site downstream of a wastewater treatment plant had a concentration of tylosin of 0.13 µg/L. Song et al. (2007) analyzed stagnant and drainage water from a livestock farm in which tylosin was used as a feed additive. Of the eleven samples, tylosin was detected in two samples, at a concentration of approximately 0.02 µg/L.

Reports from the published literature, therefore, support the use of 4.3 µg/L as a conservative estimate of the maximum possible surface water concentration of tylosin.

Summary of PEC Calculations for Total Residues of Tilmicosin

Compartment	Scenario	Concentration
Terrestrial	$PEC_{soil,\ total\ residues}$	505 µg/kg
	$PEC_{soil,\ refined\ for\ metabolism}$	379 µg/kg
Aquatic	$PEC_{surface\ water,\ refined\ for\ metabolism}$	4.3 µg/L

5.3 PNEC Calculations (Effect Assessment)

5.3.1 Terrestrial

The assessment factors applied to the toxicity values and the PNECs calculated for terrestrial species are tabulated below. The assessment factors are from the VICH GL38 Phase II guidance for Environmental Impact Assessments.

Terrestrial PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	<25% change from control at 5,000 µg/kg	1	5,000 µg/kg
Plants, Growth	EC50 = 43,000 µg/kg	100	430 µg/kg
Plants, Emergence	EC50 > 63,000 µg/kg	100	> 630 µg/kg
Earthworms	NOEC = 62,500 µg/kg	10	6250 µg/kg

Plants are the most sensitive species in the terrestrial compartment tested.

5.3.2 Aquatic

The assessment factors used and the PNECs calculated for aquatic species are tabulated below.

	Toxicity endpoint	Assessment Factor	PNEC
Algal Growth	EC50 = 220 µg/L	100	2.2 µg/L
	NOEC = 99 µg/L	10	9.9 µg/L
Daphnia Acute	EC50 > 87,000 µg/L	1000	> 87 µg/L
Fish Acute	LC50 > 96,000 µg/L	1000	> 96 µg/L

Algae are the most sensitive aquatic species tested. The EC50 and the NOEC used to calculate the PNEC for algae were from Study 1982.6139 (Appendix M), conducted according to OECD Guideline 201.

5.4 Risk Characterization

5.4.1 Soil

The predicted concentration of total residues of tylosin in soil (PEC_{soil}) after application of litter from broilers treated for necrotic enteritis with tylosin in drinking water is 505 µg/kg. When metabolism by chickens is considered, the $PEC_{soil\text{-}refined}$ can be calculated to be 379 µg/kg.

Of the terrestrial species tested, plant growth was observed to be affected by the lowest exposure to tylosin. The lowest EC50 for growth in plants was

43,000 µg/kg. Using an application factor of 100, the predicted no-effect concentration for plants is 430 µg/kg. The PEC/PNEC ratio for terrestrial organisms using the refined PEC_{soil} is less than 1.

5.4.2 Surface Water

The maximum predicted concentration of total residues of tylosin in surface water ($PEC_{refined\text{-}surface\ water}$) is 4.3 µg/L. Algae were the most sensitive aquatic species tested with tylosin. The PNEC calculated from the lowest EC50 (biomass) using an assessment factor of 100 is lower than the $PEC_{surfacewater}$. Therefore, the $PEC_{surfacewater}$ was compared to the PNEC value calculated from the NOEC divided by an assessment factor of 10. Using the resulting PNEC value of 9.9 µg/L, the PEC/PNEC ratio for aquatic organisms is less than 1. As previously described, Halling-Sørensen (2000) has reported a lower EC50 in a cyanobacteria algae species of 34 µg/L, based on nominal concentrations. The NOEC, EC10 and EC20 are not reported in that study. However, the effect concentration of 34 µg/L is eight times greater than the highest estimated PEC for surface water and over 120 times greater than the maximum concentration measured in surface waters.

Because the lowest PNEC for each environmental compartment is greater than the PEC in that compartment, there is no significant risk to organisms in that compartment.

PEC/PNEC Ratios

Compartment	Species	PEC*	PNEC	PEC/PNEC Ratio
Terrestrial	Plants	379 µg/kg	430 µg/kg	0.88
Surface Water	Algae	4.3 µg/L	9.9 µg/L	0.43

*For tylosin residues refined for metabolism

5.5 Conclusions Tier A

The environmental impact from the use of Tylan Soluble at a concentration of 375 mg/L (as tylosin) in drinking water in broiler chickens for 5 days to treat necrotic enteritis in high intensive rearing situations has been evaluated. The pathway for introduction of tylosin residues into the environment considered in this risk assessment was via the application of chicken litter as fertilizer to soil.

The predicted environmental concentrations were refined to consider metabolism by chickens. The predicted environmental concentration of tylosin in soil is 379 µg/kg after refinement. The highest calculated environmental concentration in surface water following rainfall events is 4.3 µg/L. This surface water level was over 15 times higher than any concentration of tylosin that has been measured in surface water. These predicted environmental concentrations of tylosin in soil and surface water are lower than the predicted no-effect concentrations for terrestrial and aquatic organisms.

Given its low octanol-water coefficient and extensive metabolism, tylosin is not expected to accumulate in environmental species. Given its degradation in excreta and soil, it is not expected to persist in the environment.

Treatment of broiler chickens with Tylan Soluble to treat necrotic enteritis is not expected to result in effects on terrestrial or aquatic organisms exposed to tylosin from chicken litter used to fertilize cropland soil.

6.0 Information on Environmental Assessment Expert

The following individual is responsible for the information in the Environmental Assessment Report for tylosin used as Tylan Soluble to treat necrotic enteritis in broiler chickens:

Name of the expert: Alison Nimrod Perkins
Author, Environmental Risk Assessment Group
Lilly Research Laboratories, Health, Safety, Environmental

Address: Eli Lilly and Company
Lilly Corporate Center
Indianapolis, IN 46285
USA

Signature: Alison Nimrod Perkins

Date: 10 February 2012

Brief information on the educational background, training and occupational experience:

Name: Alison Nimrod Perkins
Address: Lilly Research Laboratories
Lilly Corporate Center
Indianapolis, IN 46285

Degrees:

BS	Chemistry, Tulane University	1988
PhD	Pharmacology/Toxicology, University of Mississippi	1996

Current and Previous Appointments:

Principal Research Scientist, Environmental Risk Assessment, Lilly Research Laboratories (2010 to present)

Senior Research Scientist, Environmental Risk Assessment, Lilly Research Laboratories (2005 to 2010)

Senior Toxicologist, Senior Research Scientist, Environmental Science/Nonclinical Safety Assessment, Lilly Research Laboratories (2000 to 2005)

Editorial Board of Environmental Toxicology and Chemistry

Research Scientist, Research Institute of Pharmaceutical Sciences, U. Mississippi (1996 to 1999)

Publications:

Fifteen publications and numerous presentations and posters in the field of environmental toxicology.

7.0 References

Baguer AJ, Jensen J, Krogh PH. 2000. Effects of the antibiotics oxytetracycline and tylosin on soil fauna. Chemosphere 40:751-757.

Brain RA, Bestari K, Sanderson H, Hanson ML, Wilson CJ, Johnson DJ, Sibley PK, Solomon KR. 2005. Aquatic microcosm assessment of the effects of tylosin on *Lemna gibba* and *Myriophyllum spicatum*. Environ Pollut 133:389-401.

Brain RA, Johnson DJ, Richards SM, Sanderson H, Sibley PK, Solomon KR. 2004. Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven-day static-renewal test. Environ Toxicol Chem 23:371-382.

Davis JG, Truman CC, Kim SC, Ascough JC, Carlson K. 2006. Antibiotic transport via runoff and soil loss. J Environ Qual 35:2250-2260.

Dolliver H, Gupta S, Noll S. 2008. Antibiotic degradation during manure composting. J Environ Qual 37:1245-1253.

Drasar BS, Barrow PA. 1985. Intestinal Microbiology in Cole JA, Knowles CJ, Schlessinger D, eds. Aspects of Microbiology. American Society of Microbiology. 80 pp.

(ECB) European Commission - Joint Research Centre, Institute for Health and Consumer Protection, European Chemicals Bureau. 2003. Technical guidance document on risk assessment in support of Commission Directive 93/67/EEC on risk assessment for new notified substances, Commission Regulation (EC) No 1488/94 on risk assessment for existing substances, and Directive 98/8/EC of the European Parliament and of the council concerning the placing of biocidal products on the market. Luxembourg: Office for Official Publications of the European Communities. Part II. 328 p.

Ensminger, ME. 1980. Poultry Science, 2nd Edition. The Interstate, Danville, IL.

Gavalchin J, Katz SE. 1994. The persistence of fecal-borne antibiotics in soil. J AOAC Int 77:481-485.

Halling-Sørensen B. 2000. Algal toxicity of antibacterial agents used in intensive farming. Chemosphere 40:731-739.

Halling-Sørensen B, Jacobsen A-M, Jensen J, Sengelov G, Vaclavik E, Ingerslev F. 2005. Dissipation and effects of chlortetracycline and tylosin in two agricultural soils: A field-scale study in southern Denmark. Environ Toxicol Chem 24:802-810.

Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, Steitz TA. 2002. The structures of four macrolide antibiotics bound to the large ribosomal subunit. Mol Cell 10:117-128.

Ingerslev F, Halling-Sørensen. 2001. Biodegradability of metronidazole, olaquindox, and tylosin and formation of tylosin degradation products in aerobic soil-manure slurries. Ecotoxicol Environ Saf 48:311-320.

"Tylosin One-Step Elisa Procedure" package insert. International Diagnostic Systems, A Neogen® Company.

Kirst HA, Toth JE, Debono M, Willard KE, Truedell BA, Ott JL, Counter FT, Felty-Duckworth AM, Pekarek RS. 1988. Synthesis and evaluation of tylosin-related macrolides modified at the aldehyde function: A new series of orally effective antibiotics. J Med Chem 31:1631-1641.

Kirst HA, Wild GM, Baltz RH, Hamill RL, Ott JL, Counter FT, Ose EE. 1982. Structure-activity studies among 16-membered macrolide antibiotics related to tylosin. J Antibiotics 35:1675-1682.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streatms, 1999-2000: A national reconnaissance. Environ Sci Technol 36:1202-1211.

Liu F, Ying G-G, Tao R, Zhao J-L, Yang J-F, Zhao L-F. 2009. Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. Environ Pollut 157:1636-1642.

Mankin AS. 2008. Macrolide myths. Curr Opin Microbiol 11:414-421.

Merck Index, Twelfth Edition. 1996. Tylosin. p 1676.

Midwest Plan Service, 1985. Livestock Waste Facilities Handbook, Second Edition. MWPS-18.

Naqi SA, Lewis DH, Hall CF. 1970. The intestinal microflora of turkeys. Avian Dis 14:620-625.

National Research Council, 1994. Nutrient requirements of poultry, 9th revised edition. National Academy Press, Washington, D.C. pp. 26 and 37.

Omura S, Tischler M. 1972. Relationship of structures and microbiological activities of the 16-membered macrolides. J Med Chem 15:1011-1015.

Rabølle M, Spliid NH. 2000. Sorption and mobility of metronidazole, olaquindox, oxytetracycline and tylosin in soil. Chemosphere 40:715-722.

Simpson TW. 1991. Agronomic use of poultry industry waste. Poultry Sci 70:1126-1131.

Song W, Huang M, Rumbeiha W, Li H. 2007. Determination of amprolium, carbadox, monensin, and tylosin in surface water by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 21:1944-1950.

ter Laak TL, Gebbink WA, Tolls J. 2006. Estimation of soil sorption coefficients of veterinary pharmaceuticals from soil properties. Environ Toxicol Chem 25:933-941.

Teeter JS, Meyerhoff RD. 2003. Aerobic degradation of tylosin in cattle, chicken, and swine excreta. Environ Res 93:45-51.

Tolls J. 2001. Sorption of veterinary pharmaceuticals in soils: A review. Environ Sci Technol 35:3397-3406.

Veith GD, DeFoe DL, Bergstedt BV. 1979. Measuring and Estimating the Bioconcentration Factor of Chemicals in Fish. J Fish Res Board Can 36:1040-1048.

VICH 2000, Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) – Phase I, VICH GL6 Final Guidance.

VICH 2006, Environmental Impact Assessments (EIAs) for Veterinary Medicinal Products (VMPs) – Phase II, VICH GL38 Final Guidance.

Wollenberger L, Halling-Sørensen B, Kusk KO. 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. Chemosphere 40:723-730.

Yang L-H, Ying G-G, Su H-C, Stauber JL, Adams MS, Binet MT. 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures of the freshwater microalga *Pseudokirchneriella subcapitata*. Environ Toxicol Chem 27:1201-1208.

Yang S, Carlson KH. 2004 Solid-phase extraction – high-performance liquid chromatography-ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices. J Chromatography A 1038:141-155.

Zuzulova M, Kleinova D, Proksa B, Fuska J. 1995. In vitro activity of tylosin and its derivatives against *Ureaplasma urealyticum*. Arzneim Forsch/Drug Res 45:1222-1224.

Appendices

Appendix A - Study EC9907: A study to determine the octanol/water partition coefficient of tylosin (Compound 027892) in pH 5, 7, and 9 aqueous buffers. Report Date: 1999.

Methods:

A shake flask partition coefficient study following the requirements of OECD guideline 107 was performed with ^{14}C-tylosin at 25°C using pH 5, 7, and 9 aqueous buffers with octanol. After 21 hours of equilibration, triplicate aliquots of the octanol and aqueous phases were assayed by liquid scintillation counting for ^{14}C.

Results:

pH	Kow Mean (± standard deviation)
5	5 ± 0
7	17 ± 0
9	17 ± 0.7

Appendix B - Study T1Y720001: [^{14}C]Tylosin metabolism and residue decline in broiler chickens administered medicated drinking water. Report Date: 2001.

Methods:

Broiler chickens were dosed with ^{14}C-tylosin in drinking water (528 mg/L) for three consecutive days followed by 7 days of withdrawal. Excreta were collected daily from the animals. Tissue samples (liver, kidney, muscle, skin, fat and bile) were collected at intervals of 0, 2, 5 and 7 days after withdrawal.

Tissue aliquots were solubilized and quantified by liquid scintillation counting. Excreta aliquots were oxidized and quantified by liquid scintillation counting.

Liver and excreta samples were extracted using a multi-stepped fractionation scheme which extracted most of the radioactivity. The chloroform fraction in which majority of radioactivity was found was characterized by HPLC coupled to tandem quadrupole mass spectroscopy with fraction collection.

Results:

Decline of total radioactive residues after withdrawal:

Tissue	Zero days after withdrawal	Seven days after withdrawal
Liver	0.7 µg/g	< 0.1 µg/g
Kidney	0.368 µg/g	< 0.1 µg/g
Muscle, skin, fat	< 0.1 µg/g	< 0.1 µg/g

The above values represent the mean values from six animals. Although this study was not designed as a balance-excretion study, excreta were examined. At least 69% of the radioactive dose was eliminated via the excreta over a period of approximately 4 to 6 days.

The liver chloroform extract contained multiple radioactive components but the only residue able to be identified was tylosin D, due to the extensive sample matrix interference.

In the chloroform extract of the excreta collected during the final 24 hours of dosing, tylosin A and tylosin D were the most abundant residues at 29% and 12% of the total radioactivity residues in excreta, respectively. Other radioactive residues in the chloroform extract were each below 10% of the total radioactive residue. Two identified residues in this category were 20-dihydrodesmycosin and desmycosin.

Appendix C - Details of the Refinement of Excreted Residues Using Data from Study T1Y720001: [^{14}C]Tylosin metabolism and residue decline in broiler chickens administered medicated drinking water.

In Study T1Y720001, the amount of radioactivity recovered in the excreta was approximately 69% of the dose administered to the chickens via drinking water amendment.

The radioactivity from the excreta from the day prior to withdrawal and the day after withdrawal was extracted. On the first day, 99.2% of the radioactivity in the excreta was recovered by the extraction scheme, on the second day 102.9% radioactivity was recovered.

The fractionation procedure resulted in fractions of chloroform, carbon tetrachloride, aqueous, 1 N HCl, and acetone extracts, plus the pellet and a pool of rinses of the concentrating vessels (see Figure 2 in the report for study T1720001).

Table 1: Distribution of radioactivity among the fractions

Fraction	Day -1 to 0	Day 0 to 1
	% of radioactivity in excreta	
$CHCl_3$	61.7	56.8
CCl_4	0.2	0.2
Aqueous	18.6	15.3
1 N HCl	3.6	3.8
Acetone	0.5	0.6
Pellet	3.5	3.4
Miscellaneous (rinses)	11.1	22.8
Total	99.2	102.9

The miscellaneous fraction is the sum of radioactivity from rinses of the chloroform concentrating vessel and the aqueous vessel.

Table 2: Breakdown of miscellaneous fraction

Contribution	Day -1 to 0	Day 0 to 1
	% of radioactivity in excreta	
Aqueous flask	5.4	14.6
$CHCl_3$	5.7	8.2
Total in rinse	11.1	22.8

Therefore, the contribution from the rinse of the aqueous will be added to the aqueous and the contribution from the rinse of the chloroform will be added to the chloroform. It is assumed that these rinses have the same profile of residues as the aqueous and chloroform partitions.

Table 3: Revised distribution of radioactivity among the fractions, adding the radioactivity in the rinses to the aqueous and $CHCl_3$ fractions

Fraction	Day -1 to 0	Day 0 to 1
	% of radioactivity in excreta	
$CHCl_3$	67.3	65
CCl_4	0.2	0.2
Aqueous	24	29.9
1 N HCl	3.6	3.8
Acetone	0.5	0.6
Pellet	3.5	3.4

Using this fractionation procedure and based on tylosin's chemical characteristics, tylosin A and related factors are expected to be in the chloroform fraction and thus only that fraction was profiled in the metabolism study. The chloroform fraction was characterized by HPLC (with fractionation and LSC counting). Radioactive peaks were identified.

Table 4: Profile of radioactivity in the chloroform fraction

Peak ID	Day -1 to 0	Day 0 to 1
	% of recovered radioactivity in fraction	
Polar material	10.3	9.6
20-dihydrodesmycosin	6.4	9.2
Desmycosin (tylosin B)	7.5	6.8
Tylosin D	18.7	31.7
Tylosin A	47.0	32.0

The percentage of recovered radioactivity was then corrected for the percent that partitioned into chloroform and chloroform rinse:

Table 5: Components of chloroform fraction adjusted for % of excreta radioactivity

Peak ID	Day -1 to 0	Day 0 to 1
	% of excreta radioactivity	
	67.3%	65%
Polar material	6.9 (10.3*0.673)	6.2 (9.6*0.65)
20-dihydrodesmycosin	4.3 (6.4*0.673)	6.0 (9.2*0.65)
Desmycosin (tylosin B)	5.0 (7.5*0.673)	4.4 (6.8*0.65)
Tylosin D	12.6 (18.7*0.673)	20.6 (31.7*0.65)
Tylosin A	31.6 (47.0*0.673)	20.8 (32.0*0.65)

While dihydrodesmycosin (DHDM) and desmycosin (tylosin B) are less than 10% of the excreted radioactivity, these metabolites are included in the refinement because they are known to have some pharmacological activity. The metabolites with known activity are adjusted based on their relative bioactivity to tylosin A. Using data from published literature, the following relative bioactivities will be used in refinement (see Appendix D for more discussion on the structure activity relationship of tylosin analogs).

Table 6: Relative bioactivity of tylosin factors

A	B	C	D	DHDM
100*	100	100	25	50

*% relative to activity of tylosin A

The relative bioactivities are thus used to standardize the amount of activity in the fractions to tylosin factor A:

Table 7: Components of the chloroform fraction corrected for bioactivity relative to A

Peak ID	Day -1 to 0	Day 0 to 1
	% of Extracted Radioactivity	
20-dihydrodesmycosin	2.2 (50% * 4.3)	3.0 (50% * 6.0)
Desmycosin (tylosin B)	5 (100% * 5)	4.4 (100% * 4.4)
Tylosin D	3.2 (25% * 12.6)	5.2 (25% * 20.6)
Tylosin A	31.6 (100% * 31.6)	20.8 (100% * 20.8)
Total Equivalents as Tylosin A	**42.0**	**33.4**

While it is unlikely that tylosin and its factors partition into fractions other chloroform, it will be conservatively assumed that the small amounts of radioactivity in the CCl_4, 1 N HCl, acetone and pellet are also tylosin A. While it is even more unlikely that the radioactivity in the aqueous fraction is tylosin or even a single metabolite, it will be considered that all radioactivity in the aqueous fraction is at least as active as tylosin A. Therefore, the radioactivity in the non-profiled fractions will be added to the radioactivity due to parent and major metabolites.

Table 8: Radioactive residues considered to be tylosin A

Fraction	Day -1 to 0	Day 0 to 1
	% of radioactivity in excreta	
From $CHCl_3$	42.0	33.4
CCl_4	0.2	0.2
Aqueous	24	29.9
1 N HCl	3.6	3.8
Acetone	0.5	0.6
Pellet	3.5	3.4
TOTAL	**73.8**	**71.3**

The average of these numbers, 72.6%, is considered to be the maximum amount of excreted radioactivity that could be equivalent to tylosin A. Therefore, it will be assumed that no more than 75% of the dose will be excreted as residue equivalent to tylosin A.

Appendix D - Details of the structure activity relationship of tylosin factors.

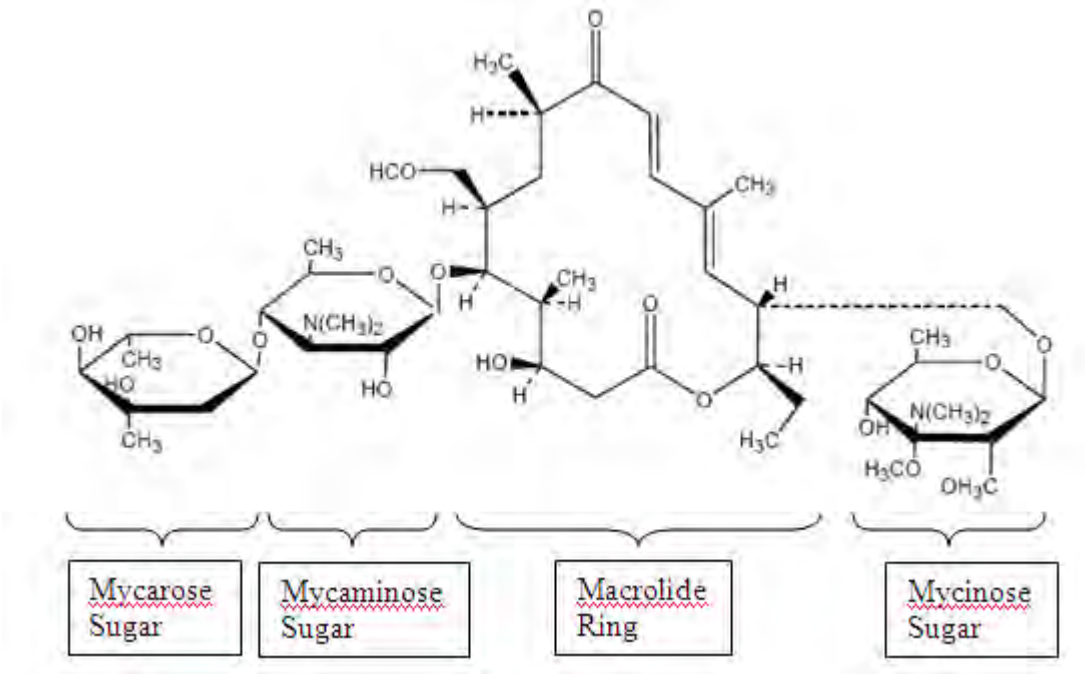
The relative bioactivity of the major tylosin factors used in the risk assessment is in the table below. In general, tylosins A, B, and C can be considered to be equipotent, while tylosin D and DHDM have less activity.

Relative bioactivity of tylosin factors

A	B	C	D	DHDM	Reference
100%	100%	100%	25%	50%	Kirst et al 1982 and 1988

*% relative to activity of tylosin A

To understand the differences in activity, it is helpful to refer to the structures of the tylosin factors and consider the mechanism of action of macrolide antibiotics. Below is a structure of tylosin factor A and a description of the major factors:



Tylosin Factors

Factor B	Desmycosin	Mycarose sugar is removed
Factor C	Macrocin	Demethylation of the mycinose sugar
Factor D	Relomycin	Reduction of the aldehyde on the macrolide ring
DHDM	dihydrodesmycosin	Mycarose sugar is removed and the aldehyde on the macrolide ring is reduced

Macrolides antibiotics act at the ribosome of bacteria. Specifically, they bind in the peptide exit tunnel through which the growing peptide leaves the site of polypeptide assembly. The position of the macrolide antibiotic in the tunnel appears to prevent the peptide chain from elongating. The binding is facilitated by interaction of the amino acids in the tunnel with the antibiotic via hydrophobic interactions with the ring itself and hydrogen binding with the substituent sugars on the ring (see review by Mankin 2008). Additionally, there is x-ray crystallographic evidence that a reversible covalent bond forms between the aldehyde on the macrolide ring and a nitrogen in the tunnel (Hansen et al 2002).

While providing some interaction with the binding site, the mycarose and mycinose sugars are not critical for antimicrobial activity. Thus, the changes in the mycarose and mycinose sugars that occur in tylosin factors B and C have some, but varying impact upon the antimicrobial activity. The data in Kirst et al (1982) and Kirst et al (1988) confirm that the antimicrobial activity of tylosins A, B, and C are roughly equal. On the other hand, removal of all the sugars to tylactone resulted in a complete loss of activity (Kirst et al 1982). The reduction of the aldehyde function of the macrolide ring removes the possibility of covalent binding of the antibiotic at its target. The importance of the aldehyde to the microbial activity is illustrated in two papers by Kirst et al (1982 and 1988) where the investigators tested several tylosin analogs against several species of gram-positive bacteria. The MIC values for tylosin D (reduced aldehyde of tylosin A) increased by 4- to 128-fold over the MIC values for tylosin. Therefore, tylosin D has at the most 25% activity of tylosin A. The MIC values for DHDM (reduced aldehyde of tylosin B) increased by 2- to 64-fold over the MIC values for tylosin. Therefore DHDM has at the most 50% activity of tylosin A. The decrease in antimicrobial activity with reduction of the aldehyde is confirmed by data published by Omura and Tischler (1972), Kirst et al (1982), and Zuzulova et al (1995).

Therefore, in this risk assessment, tylosin D and DHDM were considered to be only 25% and 50%, respectively, as active as tylosin A, while tylosins B and C were considered to have the same activity as tylosin A.

Appendix E - Study EC9908: A 30-day study to determine the aerobic degradation of tylosin (027892) in chicken excreta. Report Date: 1999.

Methods:

Foil covered flasks (n=6) containing ^{14}C-tylosin in chicken excreta were incubated under aerobic conditions in the dark at 20°C for 30 days. The excreta was obtained from chickens fed ^{14}C-tylosin A and stored frozen until study initiation. Prior to placement in flasks, the frozen excreta were thawed and mixed 1:1 with fresh excreta from non-treated chickens. On Days 0 and 30, samples were extracted and assayed by high performance liquid chromatography (HPLC) with ultraviolet detection to quantify the concentration of tylosin factor A in the excreta by comparison to a standard curve of tylosin A.

Results:

The vessels containing ^{14}C-tylosin evolved approximately 0.1% of the initial ^{14}C as collected volatiles. The mass balance of ^{14}C recovery for the system was 93.5%.

On Day 0, HPLC analytical values for tylosin factor A in the test material vessels averaged 35.01 mg/kg. Besides tylosin factor A there were two other predominant peaks noted that were not also in the control excreta chromatograms. Based on the retention times of standards for factors A, B, C, and D located in the data for a study to determine the aerobic biodegradation of tylosin in cattle excreta (Study EC9922)*, the larger of the two peaks could have been tylosin D. The third peak had a retention time that was earlier than these tylosin factors.

On Day 30, extracts of the test material samples did not contain measurable levels of tylosin factor A. Based on the analytical limit of detection and the dilution factor, the concentration of tylosin factor A was less than 2.23 mg/kg. Additionally, there were no peaks in the HPLC/uv chromatograms within the retention time window expected to contain tylosin factors A, B, C and D.

The first-order degradation half-life of tylosin factor A in chicken excreta was estimated as < 7.5 days.

Retention time of tylosin factor standards from study EC9922:

The relative retention times of peaks to that of tylosin A in EC9922 were compared to the retention times of peaks from study EC9908 to determine if these factors were present in the chromatograms of EC9908. The chromatographic conditions for the two studies were the same.

	*** Retention time (minutes)**
Tylosin Factor A	14.0 to 14.1
Tylosin Factor B	11.9
Tylosin Factor C	12.3 to 12.4
Tylosin Factor D	13.6

Appendix F - Study 1982.6140: [^{14}C]Tylosin – Conducting soil transformation studies under aerobic conditions following OECD guideline 307. Report Date: 2002.

Methods:
^{14}C-Tylosin (approximately 1 mg/kg, as factor A) was incubated in 4 soils at 20°C for 120 days under aerobic conditions. Test vessels were connected to volatile traps. Duplicate vessels were sacrificed on Days 0, 4, 14, 30, 60 and 120 and soils were analyzed for parent compound and extractable metabolites using high performance liquid chromatography with radiochemical detection.

Results:

	Sandy Loam pH 7.3 8.9% OM	**Loamy Sand pH 5.6 2.2% OM**	**Clay Loam pH 6.5 5.7% OM**	**Sandy Loam pH 6.4 3.2% OM**
Half-life of tylosin factor A (days)	105	50.3	92.7	70.1
Mineralization (% AR)	9.3	2.0	1.6	2.0
^{14}C-Tylosin (∑A,B,C,D) - Day 0 (% AR)	87.1	84.8	86	88
^{14}C-Tylosin (∑A,B,C,D) - Day 120 (% AR)	69.6	66.8	57.8	60.7
Bound Residues - Day 120 (% AR)	12.6	17.9	23.8	22.3

%AR = Percent of applied radioactivity

Tylosin factor A was subject to primary degradation and transformed to tylosin factors B, C, and D as well as to other metabolites. The sum of tylosin factors decreased over the 120 day study. The bound residues increased over the duration of the study, suggesting that degradation products were incorporated into the soil matrices. Tylosin was slowly degraded to CO_2.

Appendix G - Study EC9906: A study to determine the sorption of tylosin (027892) in three soils. Report Date: 1999.

Methods:

The adsorption of tylosin to soil was estimated according to principles of OECD guideline 106 and FDA guideline 3.08. In the study, duplicate containers of four concentrations of ^{14}C-tylosin (as factor A) were incubated with three different soils for approximately 2 days at 20°C. Two subsequent desorption steps were performed to determine the extent to which tylosin was removed from soils. Samples of the equilibrium solution after centrifugation were assayed by liquid scintillation counting and Freundlich isotherms were constructed from the sorption data.

Results:

	Sandy Loam pH 7.6 OC* 3%	**Sandy Loam pH 4.6 OC 3%**	**Silt Loam pH 5.7 OC 2.3%**
Adsorption			
Kd	67	6	38
Koc	2233	200	1652
First Desorption			
Kd	94	8	48
Koc	3133	267	2087
Second Desorption			
Kd	106	12	52
Koc	3533	400	2261

*OC = Organic Carbon

The results indicate that adsorption of ^{14}C-tylosin from soil solution to the soil solid is limited and that subsequent desorption occurs readily.

Appendix H - Laboratory soil leaching of tylosin. Report Date: 1978.

Methods:
The leaching characteristics of tylosin factors A and D were determined using two different soil textures (silt loam and sandy loam) in a soil column leaching study using a study design described in accordance to EPA guidelines published in 1975. Tylosin (factor A or D) was applied to the top of each of six 30 cm x 6.35 cm i.d. soil columns (3 columns for factor A and 3 columns for factor D). The columns were leached with the equivalent of 60 cm rainfall over 6 or 7 days (a total of 1.872 L of water) in six 312 mL increments and the leachate from each increment was collected separately for analysis. At the end of the leaching process, each column was dismantled and divided into six 5 cm sections for analysis. The leachate and the soil were evaluated for tylosin activity using a microbiological method.

Results:
The data indicated that minimal leaching occurred with most of the tylosin (factor A or D) remaining in the top 5.0 cm soil segment. No tylosin was observed in the leachates (detection limit of 0.5 mg/L).

Analysis results in soil

	% Total[a]			
Sample (cm)	**Silt Loam Soil**		**Sandy Loam Soil**	
	Factor A	**Factor D**	**Factor A**	**Factor D**
0-5	83.4-94.2%[b]	76.1-84.3%	106.4-122.1%	84.0-103.7%
5-10	0.3-0.6%	0-8.3%	ND	0-2.0%
10-15	ND[c]	ND	ND	ND
15-20	ND	ND	ND	ND
20-25	ND	ND	ND	ND
25-30	ND	ND	ND	ND

[a]Percent recovered based on each column being initially fortified with 25 mg factor A or 19.36 mg factor D.

[b]Range of 3 values from 3 columns

[c]ND none detected at detection limit of 0.5 mg/kg

Appendix I - Study 1982.6222: Tylosin (Compound 027892) – Determination of the effects on soil microflora activity following OECD guideline 216 and 217. Report Date: 2004.

Methods:
Tylosin was incorporated into sandy loam soil at nominal concentrations of 1 and 5 mg/kg (dry weight). Soils were incubated under aerobic conditions for 28 days in the dark at 20°C. On days 0, 7, 14, and 28, vessels were removed for evaluation of carbon respiration and nitrogen transformation. Carbon respiration was evaluated by adding glucose and monitoring CO2 production. Nitrogen transformation was evaluated by analyzing concentrations of ammonium, nitrite and nitrate. Dinoseb, an inhibitor of microflora activity, was used in the study as a positive control.

Results:

Carbon transformation

	Mean calculated carbon respiration rates (mg CO_2/kg/hr, standard deviation)	**Deviation from Control (%)**
Control	14.1 (0.642)	
Tylosin 1 mg/kg	13.0 (0)	-7.63
Tylosin 5 mg/kg	12.4 (0.185)	-11.7

Nitrate transformation

	Mean nitrate transformation rates (mg NO_3^-/kg/hr, standard deviation)	**Deviation from Control (%)**
Control	5.20 (0.110)	
Tylosin 1 mg/kg	5.76 (0.627)	10.9
Tylosin 5 mg/kg	6.20 (0.236)	19.3

After 28 days, tylosin had no effect on carbon respiration and nitrogen transformation that was equal to or greater than 25% deviation from the untreated soil controls at either 1 or 5 mg/kg.

Dinoseb inhibited carbon transformation throughout the study, but only inhibited microbial nitrification in the first half of the study. Nitrification recovered in the second half of the study.

Appendix J - Study 1982.6187: Tylosin – Determination of effects on seedling emergence and seedling growth of three plant species. Report Date: 2003.

Methods:
A seedling emergence and growth study following OECD Guideline 208 was conducted to determine the effects of tylosin on tomato (*Lycopersicon esculentum*), soybean (*Glycine max*), and oat (*Avena sativa*). Seeds of each species were planted in loamy sand (85% sand, 12% silt, 3% clay) that had been amended with tylosin. For each species 4 seeds were planted per replicate pot and there were 10 replicates per treatment level. HPLC analysis of the treatment solutions was performed to insure the soil and sand substrates were dosed with the appropriate amount of tylosin. The replicate pots were incubated in a greenhouse in which the temperature was maintained between 15 and 32°C, with a mean temperature of 23°C. The seeds and seedlings were observed for 25 (soybean and oat) or 26 (tomato) days to determine percent emergence, mortality and morphological abnormalities. At test termination, shoot portions of each plant were severed at the substrate surface, dried for a minimum of three days, and individually weighed to assess growth.

Results:

Tomato

	Mean Emergence, % (standard deviation)	**% of plants with chlorosis or necrosis**	**Mean Weight, g (standard deviation)**	**% Inhibition of Growth**
Control	98 (8)	0	0.2839 (0.0474)	
Solvent Control	98 (8)	0	0.2650 (0.0391)	
Pooled Control	98 (8)		0.2744 (0.0434)	
0.24 mg/kg	98 (8)	0	0.2003 (0.0584)	27*
0.98 mg/kg	98 (8)	0	0.2906 (0.0641)	-6
3.9 mg/kg	100 (0)	0	0.2496 (0.0366)	9
16 mg/kg	100 (0)	0	0.2403 (0.0373)	12
63 mg/kg	98 (8)	65	0.0126 (0.0075)	95*

*Significantly different from pooled control, effect at 0.24 mg/kg is not considered treatment related due to lack of concentration response.

Soybean

	Mean Emergence, % (standard deviation)	% of plants with chlorosis or necrosis	Mean Weight, g (standard deviation)	% Inhibition of Growth
Control	100 (0)	3	1.0157 (0.1513)	
Solvent Control	100 (0)	0	1.0429 (0.0956)	
Pooled Control	100 (0)		1.0293 (0.1240)	
0.98 mg/kg	100 (0)	3	0.9629 (0.1356)	6
3.9 mg/kg	95 (11)	0	0.9938 (0.1540)	3
16 mg/kg	90 (24)	3	0.8449 (0.1603)	18*
63 mg/kg	98 (8)	29	0.4170 (0.1330)	59*
250 mg/kg	95 (11)	83	0.0853 (0.0911)	92*

*Significantly different from pooled control.

Oat

	Mean Emergence, % (standard deviation)	% of plants with chlorosis or necrosis	Mean Weight, g (standard deviation)	% Inhibition of Growth
Control	98 (8)	0	0.3012 (0.0439)	
Solvent Control	98 (8)	0	0.3729 (0.0403)	
Pooled Control	98 (8)			
3.9 mg/kg	98 (8)	0	0.3487 (0.0433)	6
16 mg/kg	95 (11)	0	0.3229 (0.0478)	13*
63 mg/kg	98 (8)	0	0.2514 (0.0295)	33*
250 mg/kg	98 (8)	17	0.0956 (0.0128)	74*
1000 mg/kg	98 (8)	98	0.0006 (0.0012)	100*

*Significantly different from solvent control.

	Percent Emergence			Shoot Dry Weight		
	EC25	EC50	NOEC	EC25	EC50	NOEC
Tomato	> 63	> 63	≥ 63	27	43	16
Soybean	> 250	> 250	≥ 250	24	53	3.9
Oat	> 1000	> 1000	≥ 1000	44	140	3.9

Appendix K - Study 6010-77: The toxicity of compound 27892 (tylosin) to earthworms in a 14 day soil incorporated study. Report Date: 1978.

Methods:

Tylosin (100 mg) was blended with 25 g wet rabbit feces, 850 g dry potting soil, and water to achieve a nominal concentration of 102.6 mg/kg based on total media weight. Two replicates with 5 worms (*Lumbricus terrestris*) per replicate were established in glass jars. The jars were covered with gauze and held at 10 ± 1°C. Worms were separated from soil, weighed and their appearance noted on days 0, 7, and 14.

Results:

There were no mortalities or physical signs of toxicity observed in worms exposed to tylosin. There were no changes in growth rate.

	Weight (g)		
	Day 0	**Day 7**	**Day 14**
Control	16.6	20.4	22.0
Tylosin (102.6 mg/kg)	25.0	28.7	31.0
Tylosin (102.6 mg/kg)	25.0	27.4	30.0

Therefore, the NOEC is equal to or greater than the concentration tested and will be considered to be ≥ 102.6 mg/kg dry weight.

Appendix L - Study 61741022: Effects of tylosin on reproduction and growth of *Eisenia fetida* in artificial soil. Report Date: 2011.

Methods:
Following OECD Guideline 222, artificial soil was spiked with nominal concentrations of tylosin A (as tylosin tartrate) of 7.81, 15.63, 31.25, 62.5, 125, and 250 mg/kg. Adult worms (10 per replicate, 8 replicates for the control and 4 replicates per treatment level) were incubated for 4 weeks under fed conditions. After 4 weeks adults were removed from soil, assessed for health and weighed. Test vessels were incubated for an additional 4 weeks. After 4 weeks, reproduction was assessed by enumerating offspring. Offspring were removed from the soil by placing vessels in a warm water bath to drive worms to the surface of the soil where they could easily be removed. Additionally, the soil was carefully sorted through by hand to gather any additional worms.

Results:
The pH in the soil (measured at initiation and at the end of the study) ranged from 6.0 to 6.3; the water content was 28.9% to 34.6%, the temperature was maintained within the range of 18 to 22°C, and the photoperiod was 16 hours light:8 hours dark.

Survival and growth in adult worms in the control treatment was 100% and +59.1%, respectively. Survival in all treatment groups was 100%. Growth of the adult worms in the treatment groups ranged from +61.3% to +70.7% and there were no trends or statistical differences from control. The mean number of offspring per replicate in the control group was 478 ± 16. The mean number of offspring in the 7.81, 15.63, 31.25, 62.5, 125, and 250 mg tylosin/kg treatment groups was 512, 517, 472, 445, 376, and 365 offspring per replicate, respectively. There were no statistical differences in the treatment levels up to and including 62.5 mg/kg. The numbers of offspring in the 125 and 250 mg/kg treatment levels were statistically lower than the control value.

Therefore, the NOEC for mortality and growth of adult earthworms is 250 mg tylosin A/kg while the NOEC for reproduction is 62.5 mg tylosin A/kg.

Appendix M - Study 1982.6139: Tylosin – Acute toxicity to the freshwater green alga *Pseudokirchneriella subcapitata*, following OECD guideline #201. Report Date: 2002.

Methods:

A static toxicity test was conducted to evaluate the effects of tylosin on green algae. Algal cells were cultured for 72 hours in a liquid nutrient medium that contained tylosin at nominal concentrations of 0.0, 0.04, 0.08, 0.16, 0.32, 0.64 mg/L. Concentrations of tylosin were measured at the beginning and end of the exposure. Each treatment consisted of three replicate 250-ml sterile Erlenmeyer flasks containing 100 ml of nutrient medium with an initial algal density of 10,000 cells/ml. Flasks were incubated in continuous light (3200 to 5400 lux), at a shaking rate of 100 rpm, and in an environmental chamber that maintained temperature at 24°C. The number of cells was determined at 24, 48 and 72 hours using a hemacytometer.

Results:

Measured concentrations:

	Measured Concentration (mg/L)		
Nominal	0 hour	72 hour	Mean
Control	<0.0086	<0.0064	NA
0.04	0.04	0.01	0.025
0.08	0.077	0.018	0.047
0.16	0.17	0.032	0.099
0.32	0.35	0.088 0.050*	0.22
0.64	0.61	0.17	0.39

*At 0.32 mg/L an abiotic control was included to assess whether algae influenced degradation

Measured concentrations decreased over the duration of the study by 75% or more. The concurrent decrease in the abiotic control suggests that the decrease is due to adsorption to the vessels, photolysis or hydrolysis. In other static toxicity studies, there is no evidence of hydrolysis or adsorption; therefore, it is likely that the disappearance in the continuously illuminated algae study is due to photolysis of tylosin.

Biomass and Growth Rate:

Treatment	Biomass (× 10^4 cells·day/mL)		Growth Rate (day^{-1})	
	Mean (SD)	% Inhibition	Mean (SD)	% Inhibition
Control	26.8 (2.06)		1.13 (0.04)	
0.025	24.9 (0.81)	7	1.1 (0.02)	3
0.047	23.3 (7.71)	13	1.06 (0.11)	6
0.099	23.6 (7.1)	12	1.04 (0.1)	8
0.22	8.2 (3.82)*	69	0.66 (0.1)*	42
0.39	5.8 (3.35)*	78	0.44 (0.16)*	61

*Significantly different from control

The EC50 values for biomass and growth rate were 0.22 and 0.31 mg/L, respectively. The NOEC for both parameters was 0.099 mg/L.

Appendix N - Study 1982.6137: Tylosin – Acute toxicity to water fleas, (*Daphnia magna*) under static conditions, following OECD guideline #202. Report Date: 2002.

Methods:

Daphnids, ≤24 hours old, were exposed for 48 hours to control water or to 87 mg/L (mean measured) tylosin for 48 hours under static conditions. Both the control and the tylosin treatment had four replicate beakers with five daphnids in each beaker. Each replicate beaker contained 200 ml of test solution. Temperature, dissolved oxygen, and pH of the test solutions were measured daily. Total alkalinity, total hardness, and conductivity were measured in the dilution water and the test solutions. Daphnids were assessed for immobilization.

Results:

The measured concentration at test initiation was 94% of nominal (100 mg/L) and after 48 hours it was 80% of nominal. The mean measured concentration was defined as 87 mg/L. The mechanism of the decrease in concentration of tylosin is unknown.

The water quality characteristics were as follows: pH, 8.0 to 8.2; dissolved oxygen concentration, at least 98% of saturation; temperature, 20˚C to 21°C; total alkalinity, 120 mg/L (as $CaCO_3$); total hardness, 170 mg/L (as $CaCO_3$); and conductivity, 500 µmhos/cm.

No immobilization or adverse effects were observed in daphnids exposed to tylosin. The EC50 was considered to be > 87 mg/L and the NOEC was considered to be greater than or equal to 87 mg/L, the highest concentration tested.

Appendix O - Study 1982.6138: Tylosin – Acute toxicity to rainbow trout (*Oncorhynchus mykiss*), under static conditions following OECD guideline #203. Report Date: 2002.

Methods:

Juvenile rainbow trout (mean individual weight, 1.1 g; mean length 47 mm) were exposed to control water or to 96 mg/L (mean measured) tylosin for 96 hours under static conditions. Both the control and the tylosin treatment level had three replicate aquaria with 10 rainbow trout in 15 L in each aquarium. Dissolved oxygen concentrations, pH, and temperature of the solutions were recorded daily. Total alkalinity, total hardness, and conductivity of the dilution water were determined. Behavioral signs of toxicity (sluggishness, hypoactivity, minimal swimming behavior, labored respiration, and prostration) and mortality were monitored for fish in each aquarium on a daily basis.

Results:

At test initiation the measured concentration of tylosin was 97% of nominal (100 mg/L). At test termination the concentration averaged 96% of nominal. The mean measured concentration for the study was 96 mg/L.

Water quality characteristics were as follows: pH, 6.7 to 7.4; dissolved oxygen, 6.9 to 10.2 mg/L; temperature, 13˚C to 14°C; total hardness, 42 to 54 mg/L (as $CaCO_3$); alkalinity, 31 to 37 mg/L (as $CaCO_3$); and conductivity, 140 to 150 µmhos/cm.

No mortality or adverse effects were noted in fish exposed to tylosin. The 96-hour median lethal concentration was considered to be > 96 mg/L and the NOEC was considered to be greater than or equal to 96 mg/L, the highest concentration tested.