Pulmotil AC
(aqueous tilmicosin)

Environmental Assessment for the Use of Pulmotil AC to Control Swine Respiratory Disease

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1.0 Introduction

Tilmicosin is the active ingredient in Pulmotil AC. Tilmicosin is already approved for oral use as Pulmotil 90 Type A Medicated Article for use in the feed of pigs and cattle for control of swine and bovine respiratory disease (NADA 141-064). Additionally, tilmicosin is approved for use in cattle for treatment of bovine respiratory disease as an injectable, Micotil 300 Injection (NADA 140-929).

The following assessment is provided to support an application for the use of tilmicosin at a targeted dose of 200 mg/L in the drinking water of swine for the control of swine respiratory disease associated with *Mycoplasma hyopneumoniae* and/or *Pasteurella multocida*, and/or *Haemophilus parasuis*, and/or *Actinobacillus pleuropneumoniae* and/or *Actinobacillus suis* in herds infected with Porcine Respiratory and Reproductive Syndrome Virus (PRRSV).

This environmental risk assessment has been conducted based on the VICH guidelines for both phase I (VICH GL6) and phase II (VICH GL38) assessments and on normal use of swine manure as fertilizer in the United States.

2.0 Pattern of Use and Relevant Exposure Routes

Pulmotil AC will be administered to swine via drinking water for 5 days at any time during the production cycle. Administration will be by veterinary prescription only.

Pulmotil AC will be added to the drinking water to provide a targeted daily dose of tilmicosin of 10 to 20 mg/kg of bodyweight. Once diluted in drinking water, the concentration of tilmicosin will be 200 mg/L. It is possible that only a select group of animals within a barn will be administered tilmicosin, however, 100% barn treatment does represent the most conservative scenario for the purposes of this assessment.

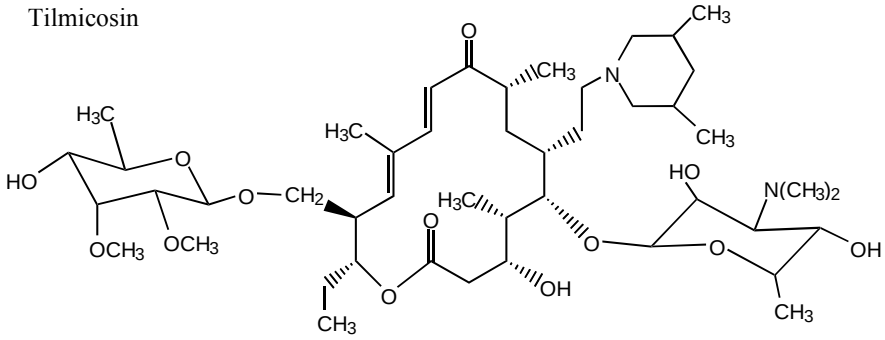
The primary route of environmental exposure to tilmicosin will be from swine manure applied to agricultural land. Concentrated animal feeding operations will be the use pattern evaluated in the assessment since this is the most prevalent production environment for swine in the United States. In addition, the amount of tilmicosin introduced into the environment from use of Pulmotil AC in concentrated animal feeding operations is expected to be much greater than that introduced from use in pastured swine. Therefore, concentrated animal feeding operations represent the most appropriate exposure scenario for evaluation. Runoff from swine facilities as a route of environmental exposure to tilmicosin will not be considered because swine facilities are designed such that manure-contaminated runoff is not expected. Manure storage is either covered such that rain does not enter or, in the case of uncovered storage facilities, the storage capacity can accommodate waste as well as rainfall events. State government regulations, including those for states which are primary swine producers, dictate the

need to contain manure (e.g. Iowa IAC [567] 65.2; North Carolina 15A NCAC 02T.1307).

3.0 Description of the Product

Pulmotil AC is an aqueous formulation of tilmicosin containing 250 mg/ml in the final formulation. The active ingredient, tilmicosin, is prepared from desmycosin which itself is derived from tylosin phosphate concentrate by mild acid hydrolysis.

International Non-proprietary Name (INN):	Tilmicosin
Chemical Name:	20-deoxy-20-(3,5-dimethylpiperidin-1-yl)-desmycosin
CAS Number (Tilmicosin):	108050-54-0
Molecular Formula:	$C_{46}H_{80}N_2O_{13}$
Molecular Weight:	869.15
Structural Formula:	Tilmicosin



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 Pulmotil AC in swine. In this Phase I assessment, the maximum concentration of tilmicosin in the manure and the soil has been calculated. No metabolism or degradation in manure 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 Calculation of Predicted Environmental Concentration

4.1.1 Calculation of concentration in manure

The concentration of tilmicosin in the manure of swine was estimated using the following equation and assumptions in Table 1:

$$[Tilmicosin]_{manure} = \frac{Dose\ per\ day\ \times Fraction\ of\ animals\ treated\ \times Dosing\ duration}{Total\ Manure\ Production}$$

Table 1 Assumptions used to calculate tilmicosin concentration in swine manure

Fraction of animals treated:	1.0
Duration: 5	days
Concentration in Drinking Water:	200 mg/L
Water Intake:	5 L/day*
Manure Production:	4.1 kg/day*
Manure Production Period:	90 days[$]
Total manure produced in production period:	369 kg

* The water intake and manure production values are traditionally used values for environmental assessments and reflect typical agricultural practices such as those described by ASAE, 2003; Ohio Livestock Manure Management Guide 2006; and NRC 1998.

[$]The US EPA states that the storage capacity of liquid swine manure varies from 3 to 12 months (http://www.epa.gov/agriculture/ag101/porkmanure.html).

Note: The duration of dosing, 5 days, is shorter than the manure production period. Therefore, the concentration in manure is essentially diluted by the period of the manure production during which no dosing is occurring. The shortest manure production periods of 90 days was used to minimize dilution.

Thus, the concentration of total tilmicosin residues (e.g. tilmicosin plus any metabolites) in manure was calculated as:

$$[Tilmicosin]_{manure} = \frac{\frac{200\ mg}{L}\ X\ 5\ L\ \times 1.0\ \times 5\ days}{369\ kg} = 13.6\ mg/kg$$

4.1.2 Calculation of concentration in soil

The maximum concentration of tilmicosin in the soil has been calculated using industry accepted and commonly used agronomic and manure management practices for application of swine manure to agricultural land.

In current intensive pork production systems, swine are reared in total confinement facilities that collect swine manure and wastewater from washing and drinking water into large structures and the resulting liquid manure slurry is applied to land. To obtain the maximum benefit from manure as a fertilizer it is injected or incorporated to minimize ammonia volatilization, conserve nutrients, control odor emissions and reduce runoff potential. The application rate of

manure to soil is based on its nutrient content and, for this assessment, an upper rate of 22,700 kg/acre will be used to calculate the soil concentration. Assuming an incorporation depth of 15 cm and an average bulk density of soil of 1500 kg/m^3, the weight of the soil in an acre is approximately 910,500 kg:

$$Weight\ of\ Soil = 1\ acre\ \times 4046.85 \frac{m^2}{acre} \times 0.15\ m \times 1500\ kg/m^3$$

Assuming no degradation in manure or soil, the concentration of tilmicosin in soil after application of swine manure could be as high as 339 µg/kg.

$$[Tilmicosin]_{soil} = \frac{[Tilmicosin]_{manure} \times Application\ Rate}{Weight\ of\ Soil}$$

Table 2 Assumptions used to calculate tilmicosin concentration in soil

Application Rate of Swine Manure to Soil*:	22,700 kg/acre*
Average Bulk Density of Soil*:	1500 kg/m^3*
Incorporation Depth:	15 cm*
Weight of soil 15 cm deep:	910,500 kg

*These are traditionally used values in environmental assessments and reflect typical agricultural practices.

$$[Tilmicosin]_{soil} = \frac{13.6\ \frac{mg}{kg} \times \frac{22{,}700kg}{acre} \times 1000 \frac{\mu g}{mg}}{910{,}500 \frac{kg}{acre}} = 339\ \mu g/kg$$

The initial estimated predicted environmental concentration in the soil is greater than 100 µg/kg, which is the trigger for conduct of a Phase II environmental risk assessment, as per the VICH GL6 Final Guidance.

5.0 Phase II Environmental Impact Assessment

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 Pulmotil AC in swine. In the Phase II assessment, data describing the physical/chemical properties, environmental fate and environmental effects of tilmicosin are used to assess the environmental risk of the use of tilmicosin. Phase II progresses as two tiers: a basic set of studies is evaluated in Tier A and is used to prepare a conservative risk assessment. If that risk assessment cannot rule out the possibility of a risk to the environment, then a Tier B assessment is conducted.

5.1 Tier A

5.1.1 Summary of Available Data

5.1.1.1 Physical and Chemical Properties

The physical and chemical properties of tilmicosin (Table 3) indicate that the molecule is water soluble and exists as a solid at normal environmental temperatures.

Table 3. Physical and Chemical Properties of Tilmicosin

Dissociation Constants (McFarland et al., 1997)	8.18, 9.56		
Aqueous Solubility (Study RMK8701, 1988, Appendix A)		5°C	25°C
	pH 5	NT	Extremely viscous due to high solubility
	pH 7	NT	566 mg/mL
	pH 9	72.5 mg/mL	7.7 mg/mL
Thermogravimetric Analysis (Study JJL8910, 1985, Appendix B)	At about 167°C, decomposition of tilmicosin begins.		
n-Octanol/Water Partition Coefficient (Study AAC8728, 1988, Appendix C)	pH 5	pH 7	pH 9
	< 10	< 10	376

NT: Not tested

5.1.1.2 Fate

The fate of tilmicosin in pigs and in the environment is described in detail below. The environmental fate data collected with tilmicosin is summarized in Table 4.

5.1.1.2.1 Metabolism and Excretion

The metabolism and excretion of radiolabeled tilmicosin by swine has been evaluated in Study T5C759201 (1992, Appendix D). In this study, pigs were administered ^{14}C-tilmicosin in feed for 5 days. The total radioactivity excreted was measured by combustion of feces and liquid scintillation counting of urine. Approximately 70% of the radioactivity of the total dose was excreted and recovered within 14 days following cessation of dosing. The excreted radioactivity was primarily in the feces (91.8% of the total excreted radioactivity) with only 8.2% found in the urine. The feces were extracted and the extract and the urine were profiled by HPLC with radiometric detection. In both the urine and the feces extract, tilmicosin was the predominant component. A major metabolite was identified in both urine and feces extract, T-4. The T-4 metabolite structure is tilmicosin with a reduced double bond in the macrolide ring and a sulfate moiety on the macrolide ring. The activity of the T4 metabolite is less than that of tilmicosin (T-4 has less than 5% of the

activity as tilmicosin against *Azotobacter*; T-4 Study V00397, 1997, Appendix Q; tilmicosin Study Z00493, 1993, Appendix K).

5.1.1.2.2 Degradation

At pH values of 5 and 7, tilmicosin is hydrolytically stable in water, with a calculated half-life of 1 year or more at 25°C (Study RMK8702, 1988, Appendix E). At pH 9, the average hydrolysis rate constant at 25°C was calculated to be 18.53 x 10^{-5} hours. This corresponds to a half-life of 156 days and indicates a moderate degree of hydrolytic instability.

The degradation of tilmicosin in swine manure was investigated in Study 1982.1094.6111.727 (1995, Appendix F). In this study ^{14}C-tilmicosin was incubated in a slurry of fresh swine manure under anaerobic conditions for 73 days. Evolved gasses were assessed for ^{14}C (as ^{14}C-CO_2 and ^{14}C-CH_4) and extracts of the contents of the incubation vessels were assayed by HPLC for degradation products. In the study, sodium benzoate was also evaluated as a positive control. While respiration indicated that the microbial population was active, neither tilmicosin nor sodium benzoate was degraded in the swine manure suggesting that the metabolic activity of the microbial population in this study may have been impaired.

The biodegradation of tilmicosin in soil has been examined (Studies ABC-0404, 1988, Appendix G; T5C749301, 1993, Appendix H). Both of these studies investigated the degradation of ^{14}C-tilmicosin in clay loam, loam and sandy loam soils after incubation for either 64 (Study ABC-0404) or 56 days (Study T5C749301).

A small amount of degradation of tilmicosin occurred. A minimal amount of $^{14}CO_2$ (<1% to 6.75%) evolved during the incubations. The majority of the radioactivity in soil extracts at the end of the incubation period was identified as tilmicosin (either by qualitative thin-layer chromatography or by quantitative HPLC). Thin-layer chromatography of the extracts in ABC-0404 showed that there were at least two degradation products other than tilmicosin, but tilmicosin was the predominant component. HPLC analysis of the extracts in T5C749301 showed that 10 to 12% of the extracted radioactivity in the three soils was not tilmicosin. The quantification of radioactivity by HPLC demonstrates that these degradation products are minor in nature (e.g. <10% of the applied radioactivity).

In both soil degradation studies, significant amounts of radioactivity were not extractable at the end of the incubation. The extraction of soil was conducted using methanol with ammonium hydroxide. Only 62% to 80% was recovered by extraction in Study ABC-0404 and 53% to 68% in Study T5C749301. It is unlikely that the unextractable radioactivity is bioavailable.

Using the quantitative data in Study T5C749301, that 10% of the extractable radioactivity was not tilmicosin at the end of the study, and adjusting for the extraction recovery, about 6% of tilmicosin degraded over 56 days. Assuming first order degradation kinetics, a half-life of 1.7 years can be estimated with an associated rate of degradation of 0.404 $year^{-1}$ (Appendix H).

The two soil degradation studies agree that within the 56 to 64 day duration of these studies, there is minimal degradation of tilmicosin to transformation products. Tilmicosin is slowly degraded in soil.

5.1.1.2.3 Soil Adsorption

The adsorption of tilmicosin to soil was evaluated in 4 different soils (Studies ABC-0396, 1988; ABC-0450, 1990, Appendix I). Freundlich binding isotherms were constructed for each soil at a range of concentrations and the resulting Freundlich K_d coefficients were converted to K_{oc} values on the basis of the organic carbon fraction in the soils. The Freundlich K_d coefficients ranged from 86 to 318 while the K_{oc} values ranged from 4244 to 36150. The magnitude of these values as well as the difficult extraction of tilmicosin from soil during the soil degradation studies indicate that tilmicosin will be strongly absorbed to the soil and will be unlikely to move extensively through soil.

5.1.1.2.4 Photolysis

Tilmicosin undergoes very rapid photolysis in water exposed to summer sunlight at 40 degrees north latitude, with a half-life of about 0.82 hours (Study JJL8704, 1987, Appendix J). Further, chromatographic analysis demonstrated that even the initial degradation products disappeared within one day.

5.1.1.2.5 Bioconcentration

Tilmicosin has a low n-octanol/water partition coefficient (K_{ow}), 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:

$$\log BCF = (0.85 \times \log K_{ow}) - 0.70$$

Using this equation and the highest log K_{ow} value for tilmicosin (2.575), the estimated bioconcentration factor (BCF) for tilmicosin is 31.

Table 4. Environmental Fate of Tilmicosin

Soil Adsorption/Desorption (ABC-0396, 1988 and ABC-0450, 1990, Appendix I)		Freundlich Coefficient	K_{oc}
	Clay Loam pH 6.9	318	36150
	Sandy Loam pH 5.7	129	8214
	Loam pH 8.9	86	4244
	Loam pH 6.5	181	16667
Hydrolysis (RMK8702, 1988, Appendix E)	Hydrolytically stable at pH values 5 and 7. Half-life of 156 days at pH 9 at 25°C.		
Degradation in Soil (ABC-0404, 1988, Appendix G)	Biodegradation to $^{14}CO_2$ was less than 1% for the three soils incubated for 64 days. 62 to 80% of the radioactivity was extracted from soil. Tilmicosin was the predominant component of the extract based on TLC. Two or more minor transformation products were observed.		
Degradation in Soil (T5C749301, 1993 Appendix H)	Biodegradation to $^{14}CO_2$ ranged from <1% to about 7% over 56 days in loam, clay loam and sandy loam soils. 53 to 68% of the radioactivity was extracted from the soil. The majority of the ^{14}C in the extract was identified as tilmicosin by HPLC/RAM, 10% of the extractable radioactivity was not tilmicosin. Using 6% degradation in 56 days; the half-life for tilmicosin in soil of is approximately 1.7 years, with a degradation rate constant of 0.404 $year^{-1}$.		
Degradation in Swine Manure under Anaerobic Conditions (1982.1094.6111.727, 1995, Appendix F)	No significant degradation of tilmicosin or the positive control (sodium benzoate) over 73 days.		
Photolysis – DT50 values, hours (JJL8704, 1987, Appendix J)	pH 5	pH 7	pH 9
	0.84	0.82	0.82

5.1.1.3 Toxicity

The environmental effects of tilmicosin in the terrestrial and aquatic compartments are described below and summarized in Tables 5 and 6.

5.1.1.3.1 Terrestrial Organisms

Definitive studies in soil microflora, plants, and earthworms have been conducted, are described below and considered for predicted no effect concentration determination.

Tilmicosin has been tested for inhibitory activity on the nitrogen-fixing soil microbe, *Azotobacter chroococcum* (Study Z00493, 1993, Appendix K). When tilmicosin was incorporated in agar, the minimum inhibitory concentration (MIC) for tilmicosin on *Azotobacter* was 5000 µg/L. The sulfated metabolite of tilmicosin, T4, was found to be at least 20 times less active than tilmicosin to *Azotobacter* as no inhibitory concentration was found up to 100,000 µg/L (Study V00397, 1997, Appendix Q).

When tilmicosin was incorporated into soil, no effects on respiration or nitrogen fixation by a natural population of soil microflora were observed with tilmicosin at the highest concentration tested, 20,000 µg/kg (Study 389681, 1997, Appendix L).

In a seedling germination test with four crop species, only cucumber seeds were affected by exposure to tilmicosin via filter paper saturated with aqueous tilmicosin solutions (Study ABC-0399, Appendix M). There were no effects on germination for any of the four species. However, at the highest concentration, 100,000 µg/L, the cucumber radicle length was reduced 45.5%. Thus the NOEC for cucumbers was 10,000 µg/L.

In a seedling growth test, six crop species seedlings were transplanted into sandy loam soil or sand-only substrate treated with tilmicosin (Study 42631, Appendix N). The endpoints for the study were shoot lengths and weights and root weights.

In the sandy loam soil test, no effects were observed in ryegrass, soybeans, tomatoes and wheat up to the highest concentration tested, 300,000 µg/kg. For these four species, the EC50 values were all >300,000 µg/kg, and the NOEC values were 300,000 µg/kg. In corn, a significant, treatment-related decrease (43% decrease compared to control) in root weight was observed at 300,000 µg/kg, but not at 100,000 µg/kg; therefore, the EC50 for corn was >300,000 µg/kg and the NOEC was 100,000 µg/kg. As in the germination test, cucumber was sensitive to tilmicosin exposure. Shoot lengths of cucumber were decreased by 45% at 300,000 µg/kg and shoot weights were decreased by 22% and 69% at 100,000 and 300,000 µg/kg, respectively. Root weights were significantly decreased by 63% and 76% at 100,000 µg/kg and 300,000 µg/kg, respectively. The EC50 values for cucumber shoot lengths, weights, and root weights are >300,000, 205,000 and 90,800 µg/kg, respectively. The NOEC values for cucumber shoot

lengths, weights, and root weights are 100,000; 30,000; and 30,000 µg/kg, respectively.

In the sand-only test, all species showed reduced growth in a dose-response fashion. Ryegrass, soybean, cucumber and tomato all had the lowest NOEC value of 3,000 µg/kg.

Taken together, the two plant studies with tilmicosin indicate that cucumbers have a particular sensitivity to tilmicosin; the mechanism for this sensitivity is unknown. Liu et al (2009) found that cucumbers were more sensitive than rice to tylosin, another macrolide with antimicrobial activity. Comparison of the NOEC in the seed germination study, in which the exposure medium was tilmicosin-saturated filter paper, with the NOEC in the seedling growth study, in which the exposure medium was sand, indicates that seedling growth is a slightly more sensitive endpoint than radicle development (NOEC of 10,000 µg/L for radical development versus 3,000 µg/kg for growth). Additionally, binding to soil reduces bioavailability since phytotoxicity occurs at lower concentrations when the plants are grown in sand compared to soil. Soil more accurately reflects the exposure scenario of manure applied to agricultural fields, therefore, the toxicity endpoints determined in the soil media will be used for the environmental assessment. It is also noted that with its high sand content (70%), the soil media used in Study 42361 is appropriately conservative for evaluating toxicity.

The effects of tilmicosin on earthworms were evaluated in two studies. In Study W00788 (1988, Appendix O), *Lumbricus terrestris* were exposed to tilmicosin for 28 days with no adverse effects on survival, growth, or behavior observed at concentrations up to 918,000 µg/kg in the test media. In a chronic study including a reproduction endpoint (Study 1982.6336, 2008, Appendix P), *Eisenia fetida* were exposed to tilmicosin in soil up to a concentration of 1,000,000 µg/kg. After 4 weeks, adults were removed, leaving cocoons and any juveniles. After a second 4-week period, juvenile worms were counted to assess reproduction. No effects were observed on survival and growth of the adult worms. Slight decreases in the number of juveniles per replicate of approximately 25% compared to control were observed at the top two concentrations. Based on these decreases, the NOEC was conservatively estimated to be 310,000 µg/kg.

5.1.1.3.2 Aquatic Organisms

Definitive studies in microorganisms, algae, daphnia, and fish have been conducted, are described below and are considered for determination of the predicted no effect concentrations.

The inhibitory effects of tilmicosin on *Nostoc sp*. and *Anabaena flos-aquae*, photosynthetic cyanobacteria, have been evaluated (Study Z00493,

1993, Appendix K; Study 1982.6397, 2012, Appendix R). The minimum inhibitory concentration (MIC) for *Nostoc sp.* was determined to be 500 µg/L while the MIC for *A. flos-aquae* was 177 µg/L. In these studies, the stock solutions used to prepare the agar were analyzed to confirm the dosing levels of tilmicosin, however, the stability of tilmicosin in the test system (i.e. the agar under the lighting conditions required to grow the cyanobacteria) was not evaluated.

The green alga, *Pseudokirchneriella subcapitata*, was exposed to tilmicosin in a static toxicity test for 14 days at initial assayed concentrations ranging from 12 to 1173 µg/L (Study J00693, 1993, Appendix S). The concentrations of tilmicosin in the treatments decreased to non-detectable levels by the end of the study in most of the treatment solutions. The calculated average concentrations over the first five days of the study ranged from 9.1 to 891 µg/L. The specific growth rate over the first four days of the exposure and the algal cell counts after 5 days were the endpoints of the study used in the risk assessment. The EC50 values for the specific growth rate and algal cell counts were 221 and 84 µg/L, respectively, while the NOEC values were 85 and 41 µg/L, respectively. The decline in tilmicosin concentrations was likely due to photolysis. Since light is required for algae to grow, an algal toxicity test cannot be conducted without the possibility of photolysis. Accurate calculation of the median effective concentration and the no-observed effect concentration at the end of the 14-day study was not possible since the concentration of tilmicosin in most of the exposure solutions could not be quantified. After 5 days, tilmicosin concentrations decreased by approximately 50%. Therefore, results from this study confirm that tilmicosin degrades fairly rapidly in aqueous solutions exposed to light.

Daphnia magna were exposed to tilmicosin in a static toxicity test for 48 hours at average concentrations ranging from 2,600 to 95,000 µg/L (Study C00189, 1989, Appendix T). Hypoactivity and immobilization were observed at concentrations of 9,000 µg/L and higher. The EC50 was 57,300 µg/L and the NOEC was 2,600 µg/L.

In Studies F00189 (1989, Appendix U) and F00289 (1989, Appendix V), bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) were exposed to tilmicosin in static toxicity tests for 96 hours at mean measured concentrations ranging from 214,000 to 679,000 µg/L (bluegill) and 98,000 to 875,000 µg/L (rainbow trout). For bluegill, the LC50 of tilmicosin was 716,000 µg/L and the NOEC was 214,000 µg/L. For rainbow trout, the LC50 of tilmicosin was 851,000 µg/L and the NOEC was 534,000 µg/L.

Table 5. Terrestrial Effects of Tilmicosin

Microbial Inhibition Test with *Azotobacter chroococcum* (Z00493, 1993, Appendix K)	MIC = 5000 µg/L			
Respiration and Nitrogen Transformation Tests (28 days) (Inveresk 389681, 1997, Appendix L)	Deviation from control was less than 25% at 20,000 µg/kg			
Terrestrial Plants – Seedling Germination (ABC-0399, 1988, Appendix M)		NOEC µg/L		
	Corn	100,000		
	Cucumber	10,000		
	Soybean	100,000		
	Wheat	100,000		
Terrestrial Plants – Seedling Growth (ABC-42631, 1995, Appendix N) (in soil)		EC50 µg/kg		
		Shoot Length	Shoot Weight	Root Weight
	Ryegrass	>300,000	>300,000	>300,000
	Wheat	>300,000	>300,000	>300,000
	Corn	>300,000	>300,000	>300,000
	Soybean	>300,000	>300,000	>300,000
	Cucumber	>300,000	205,000	90,800
	Tomato	>300,000	>300,000	>300,000
		NOEC µg/kg		
		Shoot Length	Shoot Weight	Root Weight
	Ryegrass	300,000	300,000	300,000
	Wheat	300,000	300,000	300,000
	Corn	300,000	300,000	100,000
	Soybean	300,000	300,000	300,000
	Cucumber	100,000	30,000	30,000
	Tomato	300,000	300,000	300,000
Earthworm 28-day Growth and Survival (Study W00788, 1988, Appendix O)	NOEC = 918,000 µg/kg			
Earthworm Reproduction (Study 1982.6336, 2008, Appendix P)	NOEC = 310,000 µg/kg			

Table 6. Aquatic Effects of Tilmicosin

Microbial Inhibition Test with *Nostoc sp.* (Z00493, 1993, Appendix K)	MIC = 500 µg/L The effects of photolysis on test concentrations during the study could not be evaluated		
Microbial Inhibition Test with *Anabaena flos-aquae* (1982.6397, 2012, Appendix R)	MIC = 177 µg/L The effects of photolysis on the test concentrations during the study could not be evaluated		
Algal Growth Inhibition (J00693, 1993, Appendix S)		EC50 µg/L	NOEC µg/L
	Average Growth Rate (4 days)	221	85
	Algal Cell Counts (5 days)	84	41
Daphnia immobilization (C00189, 1989, Appendix T)	EC50 = 57,300 µg/L NOEC = 2,600 µg/L		
Fish Acute Toxicity (F00189 and F00289, 1989, Appendix U and Appendix V)	Bluegill LC50 = 716,000 µg/L NOEC = 214,000 µg/L Rainbow Trout LC50 = 851,000 µg/L NOEC = 534,000 µg/L		

5.1.2 PEC Calculations and Refinements (Exposure Assessment)

5.1.2.1 Terrestrial

The initial PEC_{soil} was calculated in the Phase I assessment as 339 µg/kg. This concentration is from the worst case scenario, i.e. all pigs in a barn will be treated, all residue eliminated is tilmicosin or metabolite as active as tilmicosin, manure storage is 90 days, and that all pigs will be treated with Pulmotil AC for the maximum duration of five days.

Per the VICH guideline (GL38), this total residue value will be refined based on the actual composition of the dose excreted by the treated animal by adding the active substance and the active metabolites (those that are 10% or more of the administered dose). The refinement is described in detail in Appendix W. Due to metabolism to minor and inactive components, the percentage of excreted radioactivity considered to be as active as tilmicosin is 81.8%. This is a conservative estimate of refinement since all non-profiled radioactivity was considered to be tilmicosin. Therefore, the concentration of tilmicosin in manure is revised to 11.1 mg/kg (13.6 X 0.818) and the concentration of tilmicosin in soil is revised to 277 µg/kg.

Tilmicosin slowly degrades in soil. For slowly degrading compounds, the possibility of accumulation in soil from repeated yearly application of manure containing tilmicosin residues can be evaluated. To understand the potential impact of yearly application, a half-life for tilmicosin was estimated based on the degradation observed in soil in Study T5C759201. HPLC profiles of soil extracts after 56 days of aerobic incubation showed that approximately 90% of the extracted radioactivity was tilmicosin. However, at the end of the study, only 53% to 68% of the radioactivity could be extracted from the soil. By multiplying the amount extracted by the percentage of tilmicosin in the HPLC profile, 5.1%, 6%, and 6.9% of the tilmicosin was degraded to minor residues in the three soils. If 6% degrades in 56 days and first order degradation kinetics are assumed ($C = C_0 * e^{-kt}$), then the half-life of tilmicosin in soil would be about 1.7 years with a degradation rate of 0.404 $year^{-1}$ (see calculations in Appendix H). If the half-life is rounded up to 2 years, the degradation rate would be 0.3465 $year^{-1}$. The values for half-life and degradation rate constant are only estimates. They ignore any mineralization that could occur in some soils and assume that all unextractable residues from the soil are tilmicosin and are bioavailable. These estimates also assume that degradation will continue at the same rate as observed in Study T5C759201.

Assuming that swine manure with tilmicosin is applied annually to the same field and tilmicosin degrades in soil at a rate of 0.3465 $year^{-1}$, then each year 277 µg/kg is added and 70.7% (from e^{-kt} or $e^{-0.3465*1}$ or 0.707) remains from the previous year. At the beginning of the 10th year, the new addition of manure results in a soil concentration of 916 µg/kg (about 3.3 times the concentration after a single application). Given the uncertainty of the half-life estimate, if the half-life was twice as long, e.g. 4 years (which would have a degradation rate of 0.173 $year^{-1}$), repeated application for 10 years would result in an environmental concentration of 1434 µg/kg (about 5.2 times the concentration after a single application).

5.1.2.2 Aquatic

Movement of tilmicosin 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. Inserting the concentration of total tilmicosin residues in manure and the application rate of manure per acre, the following calculation was performed to estimate the concentration of tilmicosin residues in the pond:

$$[Tilmicosin\ residues]_{pond} = \frac{[Tilmicosin\ residues]_{manure} \times Application\ Rate\ \times 10\ acres\ \times 0.01}{8{,}100{,}000\ L}$$

The concentration of total tilmicosin residues in swine manure is 13.6 mg/kg. If a total of 22,700 kg of fresh swine manure is applied per acre, then 3,087,200 mg of tilmicosin residues will be applied per 10 acres. After a rainfall event 30,872 mg (1%) would enter the pond. A one-acre pond that is 2 m deep has a volume of 8,100,000 L. Therefore, the concentration of tilmicosin in the pond, $PEC_{surface\ water}$, would be 3.8 µg/L. Refining the residues in manure for metabolism results in $PEC_{surface\ water\text{-}metabolism\ refined}$ of 3.1 µg/L.

The concentration in the water can also be refined by the propensity of tilmicosin to adsorb to soil and sediment. Since the tilmicosin that runs off soil is bound to soil particles, the Kd values of tilmicosin for soil were used. The measured Kd values for tilmicosin to soil ranged from 86 to 318. For purposes of the risk assessment, the lowest Kd value of 86 was used. The PEC refined for adsorption is calculated by the following equation:

$$PEC_{surface\ water-adsorption\ refined} = \frac{mass_{tilmicosin}}{mass_{water} + (mass_{sediment} \times K_d)}$$

The mass of tilmicosin that enters the pond is 25,253 mg (refined for metabolism), the mass of water in the pond is 8,100,000 kg, and the mass of the sediment, assuming mixing into the top 5 cm of sediment, is 300,000 kg. Therefore the PEC for surface water refined for adsorption to soil and sediment is 0.7 µg/L.

Assuming a half-life of 2 years in soil and repeated annual application for 10 years, the amount of tilmicosin and relevant metabolites available from runoff is about 3.3 times that after a single application. Therefore, after repeated application, the $PEC_{surfacewater\text{-}adsorption\text{-}refined}$ is 2.3 µg/L (0.7 µg/L x 3.3 for accumulation) for total active residues.

Assuming a half-life of 4 years in soil and repeated annual application for 10 years, the amount of tilmicosin and relevant metabolites available from runoff is about 5.2 times that after a single application. Therefore, after repeated application, the $PEC_{surfacewater\text{-}adsorption\text{-}refined}$ is 3.6 µg/L (0.7 µg/L x 5.2 for accumulation) for total active residues.

The surface water concentration would decline over time due to photolysis.

Table 7 summarizes the predicted environmental concentrations for tilmicosin in the terrestrial and aquatic compartments.

Table 7. Summary of PEC Calculations for Tilmicosin (Total Residues and Refinements)

Compartment	Scenario	Concentration
Terrestrial	$PEC_{soil,\ total\ residues}$	339 µg/kg
	$PEC_{soil,\ metabolism}$	277 µg/kg
	$PEC_{soil,\ metabolism,\ accumulation\ over\ 10\ years,\ 2\ year\ half\ life}$	916 µg/kg
	$PEC_{soil,\ metabolism,\ accumulation\ over\ 10\ years,\ 4\ year\ half\ life}$	1434 µg/kg
Aquatic	$PEC_{surface\ water,\ total\ residues}$	3.8 µg/L
	$PEC_{surface\ water,\ metabolism}$	3.1 µg/L
	$PEC_{surface\ water,\ metabolism,\ adsorption}$	0.7 µg/L
	$PEC_{surface\ water,\ metabolism,\ adsorption,\ accumulation\ over\ 10\ years,\ 2\ year\ half\ life}$	2.3 µg/L
	$PEC_{surface\ water,\ metabolism,\ adsorption,\ accumulation\ over\ 10\ years,\ 4\ year\ half\ life}$	3.6 µg/L

5.1.3 PNEC Calculations (Effect Assessment)

In accordance with VICH GL38 Phase II guidance for Environmental Impact Assessments (EIA's), predicted no-effect concentrations (PNECs) were calculated using the recommended data set and the appropriate assessment factors.

5.1.3.1 Terrestrial

The assessment factors applied to the toxicity values and the PNECs calculated for terrestrial species are included in Table 8. The assessment factors are from the VICH GL38 Phase II guidance for Environmental Impact Assessments.

Table 8. Terrestrial PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	≤25% change from control = 20,000 µg/kg	1	20,000 µg/kg
Plants, growth – soil	$EC50_{cucumbers}$ = 90,800 µg/kg $EC50_{other\ plants}$ = >300,000 µg/kg	100	908 µg/kg >3,000 µg/kg
Earthworms	NOEC = 310,000 µg/kg	10	31,000 µg/kg

Plants were the most sensitive terrestrial species. Since seedlings germinated on filter paper and plants grown in sand provided unrealistic exposure conditions, the PNEC for plants was based on results from plant growth studies with sandy loam soil. The lowest predicted no effect concentration in

the terrestrial compartment is 908 µg/kg based on plants, specifically on inhibition of the root growth of cucumbers.

5.1.3.2 Aquatic

The assessment factors used and the PNECs calculated for aquatic species are included in Table 9.

Table 9. Aquatic PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Algal Growth	$EC50_{growth\ rate}$ = 221 µg/L $EC50_{algal\ cell\ count}$ = 84 µg/L	100	0.84 µg/L
Daphnia acute	EC50 = 57,300 µg/L	1000	57.3 µg/L
Fish Acute	LC50 = 716,000 µg/L	1000	716 µg/L

The lowest aquatic PNEC is that calculated for the endpoint of algal cell counts for algae, 0.84 µg/L. This PNEC is less than the effective concentrations for inhibition of cyanobacteria by tilmicosin, therefore, this PNEC is protective of cyanobacteria.

5.1.4 Risk Characterization

5.1.4.1 Terrestrial

The predicted maximum concentration of total residues of tilmicosin in soil (PEC_{soil}) after a single application of manure from swine treated with Pulmotil AC for 5 days is 339 µg/kg. After consideration of swine metabolism of tilmicosin the maximum concentration is predicted to be 277 µg/kg. After repeated annual applications of manure to the same soil, the highest concentration in soil could be in the range of 916 to 1434 µg/kg (assuming that the degradation half-life in soil is between 2 and 4 years).

The PEC values in soil were compared to the PNEC value for plants grown in sandy-loam soil (Table 10). The PNEC value for plants was 908 µg/kg, which is based on the EC50 for decreased root weight in cucumbers. The PEC/PNEC ratios indicate that, for single applications of tilmicosin-containing manure, there is no significant risk to plants or other terrestrial species. When considering repeated annual applications, the range of predicted soil concentration range after 10 years is greater than the PNEC. The terrestrial PEC range for repeated application is lower than the PNEC values determined for soil microflora (20,000 µg/kg), earthworms (31,000 µg/kg), and plant species (3000 µg/kg) other than cucumber.

5.1.4.2 Aquatic

The predicted concentration of tilmicosin residues in surface water after a single application refined for metabolism and adsorption to soil and sediment is 0.7 µg/L. Assuming repeated annual applications for 10 years and a half-life of 4 years, the predicted concentration in surface water could in the range of 2.3 to 3.6 µg/L (assuming that the degradation half-life in soil is between 2 and 4 years).

The PEC values were compared to the PNEC values for aquatic organisms (Table 10). The PNEC calculated for tilmicosin was 0.84 µg/L based on the EC50 for algal cell counts. The PEC/PNEC ratio for aquatic organisms is 0.8. The PEC/PNEC ratios indicate that, for single applications of tilmicosin-containing manure, there is no significant risk to algae or other aquatic species. When considering repeated annual applications for 10 years and a half-life between 2 and 4 years, the predicted concentration range of tilmicosin (2.3 to 3.6 µg/L) is greater than the PNEC of 0.84 µg/L. The aquatic PEC value for repeated soil application is lower than the PNEC values for aquatic species other than algae, namely daphnia (57.3 µg/L) and fish (716 µg/L).

Table 10. Tier A PEC/PNEC Ratios for Tilmicosin

Compart-ment	Species	PEC*	PNEC	PEC/ PNEC Ratio
Terrestrial	Plants (cucumbers)	$PEC_{soil,\ metabolism}$: 277 µg/kg	908 µg/kg	0.3
		$PEC_{soil,\ metabolism,\ accumulation\ over\ 10\ years,\ half\ life\ of\ 2\ years}$: 916 µg/kg		1.0
		$PEC_{soil,\ metabolism,\ accumulation\ over\ 10\ years,\ half\text{-}life\ of\ 4\ years}$: 1434 µg/kg		1.6
Aquatic	Green algae	$PEC_{surface\ water,\ metabolism,\ adsorption}$: 0.7 µg/L	0.84 µg/L	0.8
		$PEC_{surface\ water,\ metabolism,\ adsorption,\ accumulation\ over\ 10\ years,\ half\text{-}life\ of\ 2\ years}$: 2.3 µg/L		2.7
		$PEC_{surface\ water,\ metabolism,\ adsorption,\ accumulation\ over\ 10\ years,\ half\text{-}life\ of\ 4\ years}$: 3.6 µg/L		4.3

*for tilmicosin residues refined for metabolism

5.1.5. Summary of Tier A

The terrestrial PEC/PNEC ratios calculated in Tier A do not preclude a risk to cucumbers in the terrestrial environment when considering the scenario of repeated application of manure from swine treated with Pulmotil AC to land. The aquatic PEC/PNEC ratios calculated in Tier A do not preclude a risk to algae

following runoff into surface water from soil to which manure from swine treated with Pulmotil AC has been repeatedly applied. Therefore, a Tier B assessment is conducted below to further refine the calculations for repeated annual applications of tilmicosin residues in swine manure to cropland soil.

5.2 Tier B

The PEC/PNEC ratios from the Tier A assessment above are only of concern for terrestrial plants and aquatic algae exposed to tilmicosin following repeated annual applications of manure from swine treated with Pulmotil AC. This Tier B assessment is completed to further evaluate PEC/PNEC ratios for repeated annual applications of tilmicosin residues.

5.2.1. Terrestrial Plants

Because 1) six plant species (rather than just three) were tested in Study 42631; 2) the most sensitive species, cucumber, was confirmed in two different phytotoxicity studies (Studies 42631 and ABC-0399); and 3) there is evidence in the literature that cucumber is sensitive to macrolides such as tilmicosin (Liu et al 2009); for the refinement of the risk assessment for cucumbers, a factor of 10 will be applied to the NOEC values for cucumber as per VICH GL38 Phase II guidance for Environmental Impact Assessments to recalculate the terrestrial PNEC value (Table 11).

Table 11. Tier B Terrestrial PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Cucumbers, growth - soil	NOEC – shoot length: 100,000 µg/kg	10	10,000 µg/kg
	NOEC – shoot weight: 30,000 µg/kg		3,000 µg/kg
	NOEC - root weight: 30,000 µg/kg		3,000 µg/kg

The lowest PNEC value, 3000 µg/kg, was compared to the PEC values in the terrestrial compartment (Table 12).

Table 12. Tier B Terrestrial PEC/PNEC Ratios

PEC_{soil}	PNEC	PEC/PNEC Ratio
$PEC_{soil, metabolism}$: 277 µg/kg	3000 µg/kg	0.1
$PEC_{soil, metabolism, accumulation over 10 years: half-life of 2 years}$: 916 µg/kg		0.3
$PEC_{soil, metabolism, accumulation over 10 years: half-life of 4 years}$: 1434 µg/kg		0.5

The PEC values in the soil are all lower than the PNEC based on the NOEC for cucumber even when repeated annual application for 10 years is considered.

5.2.2. Algae

To refine the risk assessment for algae, an assessment factor of 10 is applied to the NOEC values from Study J00693 as per VICH GL38 Phase II guidance for Environmental Impact Assessments to recalculate the aquatic PNEC values (Table 13).

Table 13. Tier B Aquatic PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Algae	NOEC – growth rate: 85 µg/L	10	8.5 µg/kg
	NOEC – algal cell counts: 41 µg/L		4.1 µg/kg

The lowest PNEC value, 4.1 µg/L, is compared to the PEC values in the aquatic compartment (Table 14).

Table 14. Tier B Aquatic PEC/PNEC Ratios

$PEC_{surface\ water\ –\ adsorption\ refined}$	PNEC	PEC/PNEC Ratio
$PEC_{surface\ water,\ metabolism,\ adsorption}$: 0.7 µg/L	4.1 µg/L	0.2
$PEC_{surface\ water,\ metabolism,\ adsorption,\ accumulation\ over\ 10\ years,\ 2\ year\ half\text{-}life}$: 2.3 µg/L		0.6
$PEC_{surface\ water,\ metabolism,\ adsorption,\ accumulation\ over\ 10\ years,\ 4\ year\ half\text{-}life}$: 3.6 µg/L		0.9

The PEC values in the surface water are all lower than the PNEC based on the NOEC for algae even when repeated annual application for 10 years is considered.

5.2.3 Summary of Tier B

The risk assessments for plants and algae were refined using the NOEC values from the studies reviewed in Tier A. The PEC/PNEC ratios were less than one indicating that there is no significant risk to the terrestrial or aquatic compartments from the administration of tilmicosin as Pulmotil AC to swine via drinking water even when accumulation in the soil following 10 years of repeated manure applications was considered.

5.3 Summary and Conclusion

The environmental impact from the use of Pulmotil AC for 5 days in swine drinking water at a concentration of 200 mg tilmicosin/L to control swine respiratory disease in concentrated animal feeding operations has been evaluated using a base set of data collected on the physical/chemical properties, environmental fate, and environmental effects of tilmicosin. The pathway for introduction of tilmicosin into the environment considered in this risk assessment was via the application of swine manure as fertilizer to soil. Runoff to surface water from soil fertilized with swine excreta containing tilmicosin residues was also considered.

Tilmicosin is fairly water soluble and is not expected to bioconcentrate in tissues. While stable in aqueous solutions under dark conditions, tilmicosin is subject to rapid photolysis in aqueous media under light conditions. Tilmicosin has a propensity to bind to soil which will limit its movement into runoff and groundwater.

The predicted environmental concentrations were calculated using the administration rate of tilmicosin and typical animal husbandry and agronomy practices. The concentrations were refined by considering the metabolism of tilmicosin by swine to minor or inactive metabolites. The predicted environmental concentration of tilmicosin in soil is 277 µg/kg after refinement for metabolism of tilmicosin by pigs. Given the slow degradation of tilmicosin in soil, the potential maximum soil concentration of tilmicosin in soil following 10 years of repeated annual application of manure was calculated to range from 916 to 1434 µg/kg (assuming a degradation half-life between 2 and 4 years in soil). The maximum predicted environmental concentration in surface water following rainfall runoff from field soil where tilmicosin could accumulate is calculated to range from 2.3 to 3.6 µg/L.

The most sensitive tested species to tilmicosin were cucumbers and green algae. Predicted no-effect concentrations for cucumbers and algae were determined to be 3000 µg/kg and 4.1 µg/L, respectively, using the no-observed effect concentration and an appropriate safety factor. The predicted no-effect concentrations were higher than the predicted environmental concentrations, even considering possible accumulation in soil. Therefore, the treatment of swine with tilmicosin as Pulmotil AC for control of swine respiratory disease in high intensive rearing situations is not expected to result in any substantial environmental impact through the application of swine manure to cropland soil.

6.0 Information on Environmental Assessment Expert

The following individual is responsible for the information in the Environmental Assessment Report for tilmicosin used as Pulmotil 90 Type A Medicated Article to control swine respiratory disease:

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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

American Society of Agricultural Engineers 2003. Manure Production and Characteristics. ASAE D384.1 FEB03.

(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.

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 Pollution 157:1636-1642.

McFarland JW, Berger CM, Froshauer SA, Hayashi SF, Hecker SJ, Jaynes BH, Jefson MR, Kamicker BJ, Lipinski CA, Lundy KM, Reese CP, Vu CB. 1997. Quantitative structure-activity relationships among macrolide antibacterial agents: In vitro and in vivo potency against *Pasteurella multocida*. J Med Chem 40:1340-1346.

National Research Council (NRC). 1998. Nutrient Requirements of Swine. 10th rev. ed. Natl. Acad. Press, Washington, DC.

The Ohio State University Extension. 2006. Ohio Livestock Manure Management Guide. Bulletin 604.

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.

Appendices

Appendix A - Study RMK8701: Water solubility of tilmicosin. Study Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Excess tilmicosin was added to water solutions maintained at pH levels of 5, 7, and 9 by addition of phosphoric acid. The test temperature was 25°C for all three pH levels, and also 5°C for pH 9. The samples were filtered to remove undissolved tilmicosin and assayed by HPLC.

Results:
The test results show that the water solubility of tilmicosin is very dependent on temperature and pH. At pH 9, solubilities of 7.7 and 72.5 mg/ml were obtained at temperatures of 25°C and 5°C, respectively. Tilmicosin is considerably more soluble as the pH is lowered, having a solubility of 566 mg/mL at pH 7 and 25°C. At pH 5, the solubility is so great that a sticky paste is formed.

Appendix B - Study JJL8910: Thermogravimetric of tilmicosin. Study Date: 1985.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Approximately 10 mg of tilmicosin was weighed onto a sample pan of a thermogravimetric analyzer. The initial temperature of the analyzer was 20˚C. The heating rate was set at 5˚C/min under a nitrogen flow of 40 cc/min. A thermogram representing percent weight loss versus temperature was recorded.

Results:
The thermogram indicated a weight loss of only 1.6% from 23˚C to about 129˚C. This loss represents a loss of water and other minor volatile impurities. No losses were observed around the tilmicosin melting point range, 107˚C to 112˚C. A continuous loss through decomposition of tilmicosin was observed to begin at 167˚C. These results indicate that tilmicosin is a non-volatile solid.

Appendix C - Study AAC8728: N-Octanol/water partition coefficients of tilmicosin. Study Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{14}C-Tilmicosin

Methods:
Solutions of ^{14}C-radiolabeled tilmicosin in n-octanol were equilibrated with aqueous buffers having pH levels of 5, 7, and 9 at a temperature of 25°C. The concentration of tilmicosin in each phase was determined by radiochemical analysis.

Results:
The n-octanol/water partition coefficients (K_{OW}) were determined to be <10 at pH levels of 5 and 7 and 376 at pH 9. These low values indicate that tilmicosin would not bioaccumulate in lipoid tissue.

Appendix D - Study T5C759201: Tilmicosin metabolism study in tissues and excreta of pigs fed 400 ppm ^{14}C tilmicosin. Study Date: 1992.

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{14}C Tilmicosin phosphate

Methods:

Six crossbred swine (3 males and 3 females) weighing approximately 22 kg were fed ^{14}C tilmicosin phosphate equivalent to 400 ppm tilmicosin base in the feed for 5 days. Tissues from a male and a female were assayed at 0, 7 and 14 days withdrawal. Muscle, liver, kidney, fat, and bile were assayed for total radioactivity. Selected tissues were profiled for parent and metabolites by HPLC. Urine and feces were assayed for ^{14}C tilmicosin.

Urine and feces were collected from a period of 1 day predose to 7 days after the last dose from the 14-day withdrawal group. Urine and feces were collected from the beginning of dose to slaughter (0-day withdrawal group) or until 2 days after the last dose (7-day withdrawal group). Feces were assayed by total radioactivity by combustion followed by LSC. Urine was assayed for total radioactivity by LSC.

Feces from Day 5 of the study from one pig were extracted using methanol then subjected to a partitioning scheme which resulted in several fractions. The $CHCL_3$:hexane and the $CHCL_3$ fractions were combined, concentrated and assayed by HPLC to identify parent and metabolites.

Urine from Day 4 from the same pig as the profile feces was subject to HPLC analysis directly.

Results:

The total recovery of dosed radioactivity from urine and feces was 70.4 and 69.9%, respectively, for the male and female of the 14 day withdrawal group. Only 5.7 to 5.8% of dosed radioactivity (8.2% of the excreted radioactivity) was found in urine while 64.1 to 64.7% was found in feces (91.8% of the excreted radioactivity).

From the radioprofiles the feces extracts, approximately 54% and 10% of the fecal radioactivity was identified as tilmicosin and the T4 metabolite, respectively. Of the

urinary radioactivity, approximately 75% and 25% were identified as tilmicosin and the T4 metabolite, respectively.

Structural elucidation experiments including accurate mass measurement, fast atom bombardment, mass spectrometry and nuclear magnetic resonance suggested a reduction of a carbon-carbon double bond on the macrolide ring and addition of sulfate to the ring of tilmicosin to form the T4 metabolite.

Appendix E - Study RMK8702: Hydrolysis of tilmicosin in aqueous buffer solutions. Study Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Sterile, aqueous buffer solutions of pH 5, 7, and 9 were fortified with 250 mg/L tilmicosin and maintained in the dark at 50°C. The solutions were assayed for tilmicosin 5 days after initiation of the study. To further define the extent of base promoted hydrolysis, sterile aqueous buffer solutions of pH 9 were fortified with 250 mg/L tilmicosin and maintained in the dark at 25°C. Samples were periodically removed and assayed by HPLC during the 28-day test period.

Results:
At pH 5 and 7, tilmicosin was hydrolytically stable in water, with a calculated half-life of 1 year or more at 25°C. At pH 9, the average hydrolysis rate constant at 25°C was calculated to be 18.53×10^{-5} hours. This corresponds to a half-life of 156 days and indicates a moderate degree of hydrolytic instability.

Appendix F - Study 1982.1094.6111.727: Determination of the biodegradation potential of tilmicosin (EL-870) under anaerobic conditions. Report Date: 1995.

Performing Laboratory: Springborn Laboratories

Test Article: ^{14}C Tilmicosin

Methods:
The biodegradability of tilmicosin was evaluated in an anaerobic test system using a slurry of fresh swine manure as the source of the microbial inoculums. Test procedures were adapted from TSCA guidelines (U.S. EPA, 1992) for studying anaerobic biodegradation in domestic sewage. Tilmicosin was tested at concentrations of 1 mg/L using radiolabeled test material and 78.6 mg/L using nonradiolabeled test material. ^{14}C-Sodium benzoate was used as the reference material. Control, reference, and test material vessels were incubated at approximately 35°C for 73 days. Vessels were amended with an additional source of carbon (glucose and ethanol) on Day 59. Gas production (CO_2 plus CH_4) was measured periodically in control, reference, and test material vessels. Concentrations of sodium benzoate and tilmicosin were determined by HPLC of extracts of reaction vessel contents.

Results:
There was no mineralization of tilmicosin or sodium benzoate observed in the study based on evolved $^{14}CO_2$. At the same time, total volumes of gas produced in the sodium benzoate-treated and tilmicosin-treated vessels were not significantly different from control vessels at study determination.

HPLC analysis of the reference compound vessels for sodium benzoate showed that all of the sodium benzoate added at study initiation was present after 73 days of incubation, indicating that sodium benzoate did not degrade under these test conditions. The distribution of radioactivity in extracts from test vessels containing 1 mg/L ^{14}C-tilmicosin showed that the extractable radioactivity was accounted for as tilmicosin after 73 days of incubation. These data indicate that tilmicosin did not degrade significantly under these test conditions.

Active microbial counts and production of gas in control, reference, and test material vessels indicated that a viable microbial population was present in all vessels. Under the conditions of this study, tilmicosin was not degraded by swine waste maintained under anaerobic conditions for 73 days.

Appendix G - Study ABC-0404: Biodegradation of ^{14}C tilmicosin in soil. Study Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{14}C Tilmicosin

Methods:
The biodegradation study was conducted according to procedures described in the Environmental Assessment Technical Assistance Handbook, FDA, CVM. Clay loam, loam, and sandy loam soils were fortified with ^{14}C glucose (positive controls) or with unlabeled glucose plus 10 ppm ^{14}C tilmicosin. The samples were adjusted to moisture levels of 75% of field capacity and incubated at room temperature in the dark for 64 days. The flasks were fitted with traps to collect organic volatiles and $^{14}CO_2$ and the sample trains were aerated twice each day. Radioactivity in the traps was determined by liquid scintillation counting (LSC) using samples from a negative control for backgrounds. At the end of the study, radioactivity remaining in the soils was determined by extraction and then by combustion of the spent soil, coupled with LSC. Radioactivity in the soil extracts was profiled using thin-layer chromatography (TLC).

Results:
Results are summarized in Table 1. Recovery of $^{14}CO_2$ from the ^{14}C glucose positive controls ranged from 31% to 62%, indicating that the soils were viable. Recovery of $^{14}CO_2$ in the ^{14}C tilmicosin treatment samples was <1%, indicating a low degree of biodegradation. Neutral solvent extraction of the soils recovered <10% of the radioactivity. Extraction with methanol containing 1% ammonium hydroxide recovered approximately 62% to 80% of the radioactivity and most of this fraction appeared to be parent tilmicosin. The spent soils contained 14% to 24% of the original ^{14}C tilmicosin radioactivity. These results indicate that the half-life for degradation of tilmicosin in the soils was longer than 64 days, since approximately two-thirds to three-fourths of the tilmicosin remained as parent compound as observed using TLC. Two minor degradation products were also observed with TLC.

Table 1. Radioactivity Distribution (%) Among Various Fractions from ^{14}C Tilmicosin and ^{14}C Glucose Treated Soils[a/]

	Tilmicosin-treated			Glucose-treated		
Fraction	**Loam**	**C Loam**	**S Loam**	**Loam**	**C Loam**	**S Loam**
Volatiles	0	0	0	2	1	<1
$^{14}CO_2$	0	<1	<1	31	43	62
Neut. Solv.	8	9	4	2	1	2
Meth./NH_4OH	78	79	62	<1	<1	<1
Spent Soil	19	14	24	36	31	27

a/ Values are given as % of the total added to the sample.

Appendix H - Study T5C749301: Biodegradation of ^{14}C tilmicosin in soil. Study Date: 1993.

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{14}C Tilmicosin

Methods:
The biodegradation study was generally conducted according to procedures described in the Environmental Assessment Technical Assistance Handbook, FDA, CVM. Clay loam, loam, and sandy loam soils were fortified with ^{14}C glucose (positive controls), unlabeled glucose (negative control), or 1 ppm ^{14}C tilmicosin plus unlabeled glucose. The samples were adjusted to moisture levels of 75% of field capacity and incubated at room temperature in the dark for 8 weeks. The flasks were fitted with traps to collect organic volatiles and $^{14}CO_2$. Radioactivity in the traps was determined by liquid scintillation counting (LSC). At the end of the study, radioactivity remaining in the soils was determined by extraction (with methanol containing 1% ammonium hydroxide) with LSC and by combustion of the spent soil. The soil extracts were profiled by HPLC.

Results:
Biodegradation of the ^{14}C glucose to $^{14}CO_2$ ranged from 50 to 68 percent in the three soils. Minimal biodegradation of ^{14}C tilmicosin to $^{14}CO_2$ occurred during the experimental period. Organic volatiles and $^{14}CO_2$ traps accounted for less than 1 percent of the total ^{14}C tilmicosin radioactivity in clay loam and sandy loam soils. In loam soil, about 7 percent of the total ^{14}C tilmicosin radioactivity was recovered as $^{14}CO_2$. Extraction of subsamples of tilmicosin-treated soils recovered 53% to 68% of the radioactivity. HPLC analysis showed that the radioactivity extracted from the soils by ammoniac methanol was predominantly unchanged tilmicosin (88% -90%).

Assuming that 90% of the radioactivity in the extracts was tilmicosin and the remainder was degraded, approximately 6% of the tilmicosin degraded during the 56 day study (10% adjusted for the extraction recovery). Assuming first order degradation kinetics ($C = C_0 * e^{-kt}$), a rate constant of 0.404 $year^{-1}$, can be calculated:

$$94\% = 100\% * e^{-k*56days}$$

$$\ln\left(\frac{94}{100}\right) = -k * 56\ days$$

$$k = 0.0011\ days^{-1} = 0.404\ years^{-1}$$

Such a rate constant would result in a half-life of 1.7 years:

$$t_{½} = [\ln (0.5)] \div (-0.404) = 1.7 \text{ years}$$

Appendix I - Studies ABC-0396 and ABC-0450: Tilmicosin soil sorption/desorption and ^{14}C tilmicosin supplementary sorption study. Report Dates: 1988 and 1990

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{14}C Tilmicosin

Methods:

Study ABC-0396

Eight grams of sandy loam (pH 5.7), loam (pH 6.5), and clay loam (pH 6.9) were equilibrated in glass centrifuge tubes with 40 ml of 0.01 M $CaCl_2$ solution containing various concentrations of ^{14}C tilmicosin. Equilibration was done by mixing on a mixing wheel at 25 ± 1°C. The ^{14}C tilmicosin, Lot 702-SZ0-23, had equimolar distribution of radioactivity in the macrolide ring and piperidine ring. The specific activity was 1.29 mCi/mg and the purity was approximately 95%. Samples were run in triplicate with appropriate blanks and controls. After mixing for the appropriate interval, samples were centrifuged at 2230 x g and aliquots of solution were assayed for radioactivity by liquid scintillation counting.

Study ABC-0450

The same methodology was used as in Study ABC-0396 with eight gram samples of silica gel (for dry column chromatography, Activity III/30 mm, Woelm 04530) and with samples of loam soil adjusted to pH 8.9 using $Ca(OH)_2$.

Results:

A preliminary experiment was conducted using a concentration of 1 mg/ml ^{14}C tilmicosin equilibrated as described above for 24, 48, and 72 hours, to determine the time required for equilibration. After equilibration, two desorption steps were performed with fresh $CaCl_2$ solution to determine the degree of desorption. A 24-hour mixing time was sufficient to achieve equilibration. Almost all (>95%) of the radioactivity was adsorbed to the soils. Very little (<3%) was desorbed by mixing with fresh $CaCl_2$ solution.

A second set of samples was run for 24 hours at concentrations of 0.2 to 25 mg/ml to determine the Freundlich sorption coefficients (K) for the three soils. The sorption coefficients were 318, 181, and 129 for clay loam, loam, and sandy loam soils, respectively. Thus, tilmicosin was tightly sorbed to all three soil types.

In Study ABC-0450, a Freundlich sorption coefficient (K) was determined for loam soil adjusted to pH 8.9 using three concentrations of ^{14}C tilmicosin ranging from 1 to 25 ppm. This study demonstrated that even at a high soil pH, tilmicosin is strongly sorbed to soil, with a K value of 86. When a 1-ppm solution of ^{14}C tilmicosin was mixed with silica gel, only 2% was recovered from the supernate. The silica gel adsorbed 98% of the tilmicosin from the solution.

Appendix J - Study JJL8704: Aqueous photodegradation study of tilmicosin. Study Date: 1987.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Buffer solutions were prepared at pH 5, 7, and 9 with sterile, air-saturated, HPLC-grade water. Reaction solutions were prepared by dissolving tilmicosin reference standard material in each buffer solution to a final concentration of 8.7 µg/mL (10^{-5} M). Aliquots of these sterile test solutions were poured into sterile quartz tubes, sealed, and exposed to summer sunlight at approximately 30° from the vertical.

Based on initial data on samples exposed to sunlight for 0, 1, 3, and 7 days, triplicate sample sets for each pH were exposed to sunlight at shorter intervals of 0, 1, 2, and 4 hours. At each pH, identical positive control solutions contained in quartz tubes were wrapped in aluminum foil to exclude sunlight and were sampled at the same time intervals as the exposed samples. Blank buffer solutions were also exposed to sunlight to check for any interferences. The concentration of tilmicosin in the samples was determined by high-performance liquid chromatography with UV detection at 280 nm.

Results:
No degradation of tilmicosin was observed in the positive control solutions and no interferences were observed in the blank buffer solutions. Tilmicosin was determined to undergo rapid, aqueous photodegradation under sunlight conditions at all three pH levels tested. Degradation products observed in the 1-, 2-, and 4-hour chromatograms essentially disappear within 1 day as evidence by the 1-day chromatograms. The aqueous photodegradation rate constants (k) for tilmicosin at pH 5, 7, and 9 were 0.83 ± 0.11, 0.84 ± 0.09, and 0.84 ± 0.12 hrs^{-1}, respectively. Using these calculated rate constants, the corresponding half-life values for tilmicosin at pH 5, 7, and 9 were 0.84 ± 0.11, 0.82 ± 0.10, and 0.82 ± 0.12 hours, respectively. These results are quantitatively accurate for the test conditions and should qualitatively reflect photodegradation rates at other latitudes. Based on these data, tilmicosin and its degradation products should not accumulate in the aquatic environment.

Appendix K - Study Z00493: Microbial (*Nostoc*, *Azotobacter*) growth inhibition from exposure to tilmicosin (Compound 177370). Report Date: 1993.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Tilmicosin was evaluated for potential inhibitory effects on the growth of pure cultures of the free-living nitrogen-fixing bacteria, *Azotobacter chroococcum*, and blue-green alga, *Nostoc sp*. Tilmicosin was incorporated into an agar-based media in a series of decreasing concentrations. Duplicate series of the test plates were inoculated with the pure cultures. Test plates with *Azotobacter* were incubated at 24.9 to 25.5°C with no direct illumination. Test plates with *Nostoc* were incubated between 25.7 and 27.2°C at a light intensity of approximately 150 $\mu E/m^{-2}sec^{-1}$. Growth observed visually was used as an indication of inhibitory effects and a minimum inhibitory concentration (MIC) value was reported for each organism. The MIC was defined as the lowest concentration of tilmicosin that inhibited the growth of the test microorganism. Agar containing tilmicosin at nominal concentrations of 0 (control), 1.0, 2.5, 5.0, and 10.0 mg/L were used for *Azotobacter chroococcum*. Agar flasks containing tilmicosin at nominal concentrations of 0 (control), 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/L were used for *Nostoc sp.* The study protocol was based on FDA's 4.02 guideline.

Results:
HPLC/uv analysis of the stock solutions used to prepare the agar confirmed that the stock concentrations ranged from 85 to 105% of the target concentrations.

An MIC value of 0.5 mg tilmicosin/L was observed for *Nostoc sp*.

An MIC value of 5.0 mg tilmicosin/L was observed for *Azotobacter chroococcum*.

Appendix L - Study 389681: Effect of tilmicosin (177370) on soil microflora. Study Date: 1997.

Performing Laboratory: Inveresk Research

Test Article: Tilmicosin Aqueous N.I.

Methods:
The study design complied with the BBA Guidelines for the Official Testing of Plant Protection Products, Part VI, Section 1-1 and was consistent with the OECD Guidelines 216 and 217. Tilmicosin was incorporated into sandy loam and sandy silt loam soils at nominal concentrations of 2 and 20 mg/kg. Soils were incubated under aerobic conditions for 28 days. Throughout the test microbial respiration rates were determined. At 28 days post-treatment, concentrations of ammonia, nitrite and nitrate were measured.

Results:
Rates of microbial respiration in treated soil of both types deviated from those in the untreated soil controls by less than 15%. Concentrations of ammonia, nitrite and nitrate in treated soil of both types deviated from those in untreated soil controls by less than 15%.

Tilmicosin did not adversely affect respiration, mineralization of organic nitrogen, or nitrification activity of soil microflora at concentrations up to 20 mg/kg. The NOEC for soil microflora exposed to tilmicosin is ≥ 20 mg/kg.

Appendix M - Study ABC-0399: Determination of the effect of tilmicosin on seed germination. Report Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Seeds of corn (*Zea mays*), cucumber (*Cucumis sativus*), soybean (*Glycine max*), and wheat (*Triticum aestivum*) were soaked for 1 hour in distilled water. They were then germinated in Petri dishes in filter paper saturated with solutions of 1, 10, or 100 ppm of tilmicosin. After germination, the percent germination and the radicle length of seedlings were determined.

Results:
The results show that tilmicosin did not have an effect on seed germination in any of the four cultivars or on radicle development of corn, soybean, or wheat. The development of the cucumber radicle was not affected at tilmicosin concentrations of 1 or 10 ppm, but there was a 45.5% reduction in radicle length at 100 ppm.

Appendix N - Study ABC-42631: Determining the effects of tilmicosin on the seedling growth of terrestrial plants. Study Date: 1995.

Performing Laboratory: ABC Laboratories

Test Article: Tilmicosin

Methods:
A 21-day seedling growth study was conducted to determine the effects of tilmicosin on corn (*Zea mays*), cucumber (*Cucumis sativus*), perennial ryegrass (*Lolium perenne*), soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), and wheat (*Triticum aestivum*). Seedlings of each species were transplanted into sand and sandy loam that was then treated with nutrient media containing tilmicosin. Treatment levels in the sandy loam soil were 0, 1, 3, 10, 30, 100, and 300 mg/kg (mg tilmicosin per kg of dry soil). These same treatment levels and an additional treatment of 0.3 mg/kg were used to the test the effects of tilmicosin in sand as a substrate. HPLC analysis of the treatment solutions was performed to insure the soil and sand substrates were dosed with the appropriate amount of tilmicosin. Five plants were used for each replicate and five replicates were used for each treatment level. The seedlings were cultured in an environmentally controlled room for 21 days. Seedlings were sub-irrigated on an as-needed basis with half strength Hoagland's nutrient solution. Shoot lengths were measured for all seedlings on days 0, 7, and 14. Shoot lengths, shoot weights, and root weights were measured for all plants at the end of the study.

Results:

Well-defined dose-response relationships were evident for all species when exposed to high tilmicosin levels in sand. The no-observed effect concentrations are tabulated below for the six species in sand.

Plants Grown in Sand

	NOEC mg/kg		
Species	**Shoot Length**	**Shoot Weight**	**Root Weight**
Corn	100	30	100
Cucumber	1	3	3
Ryegrass	10	10	3
Soybeans	10	3	3
Tomatoes	30	3	10
Wheat	300	100	100

The effects of tilmicosin on seedling growth were significantly reduced when tilmicosin was introduced into sandy loam soil. Tilmicosin strongly sorbs to soil, but least of all to sandy loam. Even so, sorption to the sandy loam soil was apparently strong enough to significantly reduce the effects of tilmicosin on the seedlings. The EC50s and NOECs for the six species are tabulated below. Only cucumbers and corn were significantly affected by tilmicosin in sandy loam soil with cucumbers being more sensitive than corn. In sandy loam soil, no significant adverse effects were found for the other four species tested at a tilmicosin level of 300 mg/kg. Thus, the no-observed effect concentration for the study is 30 mg/kg.

Plants Grown in Sandy Loam Soil

	EC50 mg/kg		
Species	**Shoot Length**	**Shoot Weight**	**Root Weight**
Corn	>300	>300	>300
Cucumber	>300	205	90.8
Ryegrass	>300	>300	>300
Soybeans	>300	>300	>300
Tomatoes	>300	>300	>300
Wheat	>300	>300	>300

Plants Grown in Sandy Loam Soil

	NOEC mg/kg		
	Shoot Length	**Shoot Weight**	**Root Weight**
Corn	300	300	100
Cucumber	100	30	30
Ryegrass	300	300	300
Soybeans	300	300	300
Tomatoes	300	300	300
Wheat	300	300	300

Appendix O - Study W00788: The toxicity of soil-incorporated tilmicosin to the earthworm in a 28-day test. Study Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Tilmicosin was blended with pulverized rabbit feces, sandy loam soil, and water to achieve average measured tilmicosin concentrations of 0.0, 74, 423, and 918 ppm. Four replicates, each containing 2.0 kg of test media and 10 earthworms, were used for a control and at each treatment level. Every 7 days the earthworms were observed (normal, flaccid, prostrate or dead). Worms were weighed at the beginning and end of the study. Earthworms were exposed to the test media for 28 days.

Results:
No mortality or physical signs of toxicity were observed in earthworms at any tilmicosin concentration tested. The mean body weight gain (35.6%) by earthworms at the tilmicosin concentration of 918 ppm was significantly higher than the mean body weight gain (28.6%) by control worms after 28 days of exposure but there were no differences in body weight gain in the other concentrations.

It was concluded that no behavioral effects or reductions in body weight gain resulted when earthworms were exposed for 28 days to soil containing concentrations of tilmicosin as high as 918 ppm.

Appendix P - Study 1982.6336: Chronic toxicity and reproduction test exposing the earthworm *Eisenia fetida* in artificial soil, based on the OECD guideline 222. Report Date: 2008.

Performing Laboratory: Springborn Smithers Laboratories

Test Article: Tilmicosin premix (formulated with corncobs)

Methods:
Artificial soil was spiked with nominal concentrations of tilmicosin of 95, 170, 310, 560, and 1000 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. Vessels were incubated for an additional 4 weeks. After 4 weeks, reproduction was assessed by carefully sifting through the soil in each vessel and removing and counting offspring.

Results:
Survival and growth in adult worms in the control treatment was 100% and +27%, respectively. Survival and growth of the adult worms in the treatment groups ranged from 98 to 100% and +27 to +32%, respectively, and there were no statistical differences from control. The mean number of offspring per replicate in the control group was 98 ± 16. The mean number of offspring in the 95, 170, 310, 560, and 1000 mg tilmicosin/kg treatment groups was 91, 108, 92, 78, and 73 offspring per replicate. There were no statistical differences between the number of offspring in the treatment groups and that in the control. However, a conservative estimate of the biological significance is that the slightly lower reproduction at the two highest concentration indicated the beginning of an effect of tilmicosin on earthworms.

The LC50 and EC50 values were estimated to be > 1000 mg tilmicosin/kg. The NOEC for earthworm survival, growth, and reproduction was conservatively estimated to be 310 mg/kg.

Appendix Q - Study V00397: Acute toxicity of metabolite T4 of tilmicosin to *Azotobacter chroococcum* in an agar diffusion test system. Report Date: 1997.

Performing Laboratory: Lilly Research Laboratories

Test Article: T4 Metabolite of Tilmicosin

Methods:
The metabolite T4 of tilmicosin was evaluated for potential inhibitory effects on the growth of a pure culture of the free-living nitrogen-fixing bacteria, *Azotobacter chroococcum*. Metabolite T4, a sulfated form of tilmicosin, was incorporated into an agar-based media and triplicate test wells were inoculated with a pure culture of *Azotobacter*. The nominal test concentrations were 0 (control), 1.0, 2.5, 5.0, 10, 25, 50, and 100 mg/L. Wells were incubated in the dark between 26 and 27°C. Growth observed visually was used as an indication of inhibitory effects. The study protocol was based on FDA's 4.02 guideline.

Results:
Analysis of the stock solution (nominal 1 mg/mL) by HPLC/ISP-MS confirmed that test wells were dosed with the T4 metabolite. The measured concentration of the T4 in the stock solution was 1.4 mg/mL.

Azotobacter chroococcum was not inhibited by exposure to nominal metabolite T4 concentrations up to 100 mg/L. The nominal MIC of metabolite T4 to *Azotobacter chroococcum* was >100 mg/L.

Appendix R - Study 1982.6397: Tilmicosin – Determination of growth inhibition of *Anabaena flos-aquae* following FDA Environmental Assessment Technical Assistance Handbook, Document 4.02. Date: 2012.

Performing Laboratory: Smithers Viscient

Test Article: Tilmicosin

Methods:
Tilmicosin was evaluated for potential inhibitory effects on the growth of a pure culture of the cyanobacterium, *Anabaena flos-aquae*. Tilmicosin in aqueous stocks was incorporated into an agar-based media in a series of decreasing concentrations with triplicate plates per concentration. The target concentrations were 0.05, 0.075, 0.10, 0.125, 0.15, and 0.30 mg/L in the agar. The aqueous stocks were analyzed for tilmicosin by liquid chromatography/mass spectrometry to confirm dosing. The prepared plates were inoculated with culture using a micropipette and streaking with a sterile loop. Test plates with *Anabaena flos-aquae* were incubated at 22 to 24°C under a continuous light intensity of 160 to 240 footcandles for 13 days. Growth observed visually was used as an indication of inhibitory effects and a minimum inhibitory concentration (MIC) value was reported. The MIC was defined as the lowest concentration of tilmicosin that inhibited the growth of the test microorganism. The study protocol was based on FDA's 4.02 guideline.

Results:
Based on the results of the LC/MS analysis of the stock solutions, the test concentrations were 0.058, 0.084, 0.119, 0.143, 0.177, and 0.432 mg/L.

Complete growth inhibition occurred at the 0.177 and 0.432 mg/L concentrations. Therefore, the MIC was determined to be 0.177 mg/L

Appendix S - Study J00693: The 14-day acute toxicity of tilmicosin to the freshwater green alga (*Selenastrum capricornutum*) in a static test system. Study Date: 1993.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
A static toxicity test was conducted to evaluate the effects of tilmicosin on the green alga, *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*). Algal cells were cultured for 14 days in a liquid nutrient medium that contained tilmicosin at initial assayed concentrations of 0.0, 12, 25, 54, 112, 240, 468, and 1173 µg/L. Tilmicosin concentrations in the test solutions were measured again at the end of the test at each treatment level. Each treatment consisted of three replicate 500-ml Erlenmeyer flasks containing 100 ml of nutrient medium with an initial algal density of 1000 cells/ml. The algal population of each flask was quantified on Days 2, 3, 4, 5, 7, 10, and 14.

Results:
Tilmicosin rapidly degraded in the test systems, likely due to photolysis. At Day 14, tilmicosin concentrations were below detectable levels in all but the highest two treatments. Large declines were also found at these highest two treatment levels. Three analytical controls that did not contain algae but were incubated under the same conditions were measured on Day 5. Using the average decrease in tilmicosin concentration in the analytical controls, concentrations of tilmicosin at Day 5 were estimated for all test solutions. An average tilmicosin concentration over the first five days was determined from the initial measured concentration and calculated Day 5 concentration.

Initial Measured Concentration µg/L	Measured Concentration in Analytical Controls at Day 5 µg/L	Calculated Concentrations at Day 5, adjusted for decrease in Analytical Controls µg/L	Calculated Average Concentration for First Five Days µg/L	Measured Concentration after 14 Days µg/L
12		6.2	9.1	ND
25	ND	13	19	ND
54		28	41	ND
112	44	58	85	ND
240		125	183	ND
468		243	356	51
1173	756	609	891	250

ND = not detected, the limit of quantitation was 25 µg/L, the limit of detection was 10 µg/L.
On Day 5, the mid and high analytical controls were 39.3% and 64.5% of their initial concentrations, respectively, or an average of 51.9% of their initial. This average factor was used to calculate levels for all concentrations on Day 5.

The average specific growth rate, calculated over the first 4 days of the study, was significantly reduced at initial tilmicosin concentrations ≥ 240 µg/L or an average concentration over the first five days of ≥ 183 µg/L. The EC50* for average growth rate was approximately 221 µg/L and the NOEC was 85 µg/L, based on the calculated average concentrations. The algal cell counts were also decreased after 5 days exposure to tilmicosin. The EC50* and NOEC values for the cell counts were 84 and 41 µg/L, respectively, based on the calculated average concentration. After 14 days, the algal biomass was decreased at treatments with an initial concentration of 112 µg/L and greater, the EC50 was approximately 112 µg/L (initial concentration).

Initial Measured Concentration µg/L	Calculated Average Concentration for First Five Days µg/L	Average Growth Rate (Day 0-4) day^{-1}	Average Growth Rate % of control	Algal Cell Counts Day 5 (100 cells/mL)	Algal Cell Counts Day 5 % of Control	Algal Cell Counts Day 14 (100 cells/mL)	Algal Cell Counts Day 14 % of Control	Algal Biomass on Day 14 mg/mL	Algal Biomass on Day 14 % of control
Control		1.32		502		8583		0.094	
12	9.1	1.31	99	471	94	7333	85	0.094	100
25	19	1.30	98	442	88	7458	87	0.095	101
54	41	1.31	99	392	78	7375	86	0.087	93
112	85	1.23	93	254#	51	7583	88	0.045#	49
240	183	0.64#	48	16#	3	6542#	76	0.037#	39
468	356	0.39#	30	6#	1	21#	0	0.002#	2
1173	891	0.41#	31	9#	2	15#	0	0.000#	0

Significantly different from control. Statistical significance ($p < 0.01$) was determined using Dunnett's Multiple Comparison Test (Algal Cell Counts Day 5 and Average Growth Rates for Days 0-4) and Tukey's multiple comparison test (Algal Cell Counts Day 14 and Algal Biomass Day 14).

	EC50* µg/L	NOEC µg/L
Average Growth Rate	221	85
Algal Cell Counts Day 5	84	41
Biomass Day 14	112 (initial)	54 (initial)
*The EC50 values were determined with CETIS software using a three-parameter log-logistic model in June 2012. The values are based on the calculated average concentrations.		

Appendix T - Study C00189: The acute toxicity of tilmicosin to *Daphnia magna* in a static test system. Study Date: 1989.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
A group of 20 *Daphnia*, <24 hours old, were exposed for 48 hours to control water and to solutions of tilmicosin with average measured concentrations of 0.0 (water control), 2.6, 9.0, 26.4, 38.5, 58.6, and 95.0 mg/L. 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 diluent water and the test solutions. *Daphnia* were assessed for hypoactivity, prostration, and immobility.

Results:
The water quality characteristics were as follows: pH, 8.0 to 8.5; dissolved oxygen concentration, at least 90% of saturation; temperature, 19.4˚C to 21.6°C; total alkalinity, 142 to 152 mg/L (as $CaCO_3$); total hardness, 137 mg/L (as $CaCO_3$); and conductivity, 282 to 301 mS/cm. At tilmicosin concentrations ≥9.0 ppm, exposure-related signs of toxicity ranged from hypoactivity to immobility. The 48-hour median effective concentration, the 95% confidence limits, and the slope of the concentration-response curve were 57.3 ppm, 51.5 to 64.8 ppm, and 10.5, respectively. No immobilization or physical signs of toxicity were observed in animals exposed to a tilmicosin concentration of 2.6 mg/L.

Appendix U - Study F00189: The acute toxicity of tilmicosin to bluegill (*Lepomis macrochirus*) in a static system. Study Date: 1989.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Groups of 20 juvenile bluegill (mean individual weight, 0.87 g) were exposed to average measured tilmicosin concentrations of 0.0 (water control), 214, 524, 528, 604, and 679 ppm for 96 hours. Aquaria with 30 L of test or control solution were used to contain each group of 20 fish. 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:
Water quality characteristics were as follows: pH, 8.1 to 9.3; dissolved oxygen at least 87% saturation; temperature, 21.0˚C to 21.8°C; total hardness, 137 mg/L (as $CaCO_3$); alkalinity, 138 to 230 mg/L (as $CaCO_3$); and conductivity, 306 to 360 mS/cm. Fish exposed to tilmicosin concentrations ≥ 524 ppm exhibited sluggishness, hypoactivity, or prostration. The 96-hour median lethal concentration, its 95% confidence limits, and the slope of the concentration-response curve were 716 ppm, 635 to 807 ppm, and 12.6, respectively. No mortalities and no behavioral signs of toxicity were found for fish exposed to a tilmicosin concentration of 214 ppm.

Appendix V - Study F00289: The acute toxicity of tilmicosin to rainbow trout (*Salmo gairdneri*) in a static test system. Study Date: 1989.

Performing Laboratory: Lilly Research Laboratories

Test Article: Tilmicosin

Methods:
Groups of 20 juvenile rainbow trout (mean individual weight, 0.53 g) were exposed to average measured tilmicosin concentrations of 0.0 (water control), 98, 196, 424, 534, 659, and 875 ppm for 96 hours. Aquaria with 30 L of test or control solution were used to contain each group of 20 fish. 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:
Water quality characteristics were as follows: pH, 8 to 9.2; dissolved oxygen, at least 78% saturation; temperature, 12.0˚C to 13.4°C; total hardness, 120 to 136 mg/L (as $CaCO_3$); alkalinity, 124 to 258 mg/L (as $CaCO_3$); and conductivity, 181 to 231 mS/cm.

Fish exposed to tilmicosin concentrations ≥659 ppm exhibited sluggishness, hypoactivity, or prostration. The 96-hour median lethal concentration, its 95% confidence limits, and the slope of the concentration-response curve were 851 ppm, 784 to 988 ppm, and 12.3, respectively. No mortalities and no behavioral signs of toxicity were found for fish exposed to tilmicosin concentrations of 534 ppm or lower.

Appendix W – Details of the refinement of excreted residues using data from Study T5C759201: Tilmicosin metabolism study in tissues and excreta of pigs fed 400 ppm ^{14}C tilmicosin.

In Study T5C759201, the total recovery of dosed radioactivity in the excreta (urine and feces) was 70.4 and 69.9% in the male and female pigs, respectively. Most of the radioactivity was recovered in the feces:

Table 1: Distribution of excreted radioactivity

	% of dosed radioactivity female	% of dosed radioactivity male	Average % of dosed radioactivity	% of excreted radioactivity
Urine	5.7	5.8	5.75	8.2
Feces	64.7	64.1	64.4	91.8
Total excreta	70.4	69.9	70.15	100

The distribution of radioactivity in the Day 5 feces and urine from one pig were characterized.

Characterization of radioactivity in feces:
Feces were extracted using methanol and the extract was subjected to a multistep fractionation procedure resulting in several fractions.

Table 2 Distribution of radioactivity among the fractions of the feces extract

	% of radioactivity in the feces	% of total excreted activity
CCl_4	0.7	0.6
2:1 $CHCl_3$:hexane	65.3	59.9
$CHCl_3$	10.9	10
80:20 methanol:water	5.7	5.2
Spent aqueous methanol	5.3	4.9
Spent feces residue	12	11.0
Total Recovery	**99.5%**	
Percent profiled by HPLC (pooled chloroform fractions)	76.2	69.9
Percent not profiled by HPLC (all other fractions)	23.7	21.8

The $CHCl_3$:hexane and $CHCl_3$ fractions were pooled and the radioactivity profiled by gradient elution HPLC with liquid scintillation counting of the resulting fractions. Since the fecal radioactivity was 91.8% of the total excreted radioactivity, 69.9% of the excreted radioactivity was in the pooled fractions that were profiled ([65.3% + 10.9%] * 91.8%). Tilmicosin was the predominant component identified while T-4 was also present.

Table 3 Profile of radioactivity in the pooled chloroform fractions

	% of extract radioactivity	% of excreted radioactivity
Tilmicosin	77	53.8
T-4	14	9.8
Remaining activity	9	6.3

The other fractions of the feces extract were not profiled. While it is likely that the majority of tilmicosin is extracted in the chloroform-containing fractions, the components of the other fractions are unknown.

Characterization of radioactivity in urine

The urine was profiled by directly injecting the urine onto gradient elution HPLC with liquid scintillation counting of the resulting fractions. Again, tilmicosin was the predominant component identified while T-4 was also present.

Table 4 Profile of radioactivity in the urine

	% of urine radioactivity	% of excreted radioactivity
Tilmicosin	75	6.15
T-4	25	2.05

Excreted radioactive residues considered to be tilmicosin
While it is unlikely that much tilmicosin is in fractions other than the chloroform-containing fractions, it will be conservatively assumed that the small amounts of radioactivity in the unprofiled fractions of the feces extract are equivalent to tilmicosin. Because T-4 is known to be significantly less active that tilmicosin, it will be excluded.

Table 5 Excreted radioactive residues considered to be tilmicosin

	% of excreted radioactivity
Unknown radioactivity in other fractions of feces extract	21.8
Tilmicosin identified in feces	53.8
Tilmicosin identified in urine	6.15
TOTAL	81.8

Therefore, 81.8% of the tilmicosin residues excreted are considered to be equivalent to tilmicosin. The remaining residues are either metabolites with no activity (T-4) or minor metabolites. The value of 81.8% will be used to adjust the amount of tilmicosin residues that are excreted from swine.