Avilamycin
(Type A Medicated Article/Avilamycin Premix)

Environmental Assessment for the Use of Avilamycin to Prevent/Reduce Incidence of Mortality Caused by Necrotic Enteritis in Broiler Chickens

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1.0 Introduction

Avilamycin is the active ingredient in a Type A medicated article-containing feed premix. The following assessment is provided to support an application for the use of this premix to give a maximum concentration of avilamycin of 45 ppm (the range is 15 to 45 ppm) in the final feed of broiler chickens for the prevention and/or reduction in mortality caused by necrotic enteritis associated with *Clostridium perfringens*.

This environmental assessment was prepared to support a supplemental approval for the use of avilamycin in broiler chickens and reflects the proposed dose, duration, and treatment conditions requested.

This environmental risk assessment has been conducted based on the VICH guidelines for both phase I (VICH GL6) and phase II (VICH GL38).

2.0 Pattern of Use and Relevant Exposure Routes

Avilamycin will be administered to broiler chickens for a maximum of 21 days. The use of avilamycin in broiler chickens must be initiated by animal age day 18. A dose range of 15 to 45 ppm in the feed will be used (e.g. up to 45 mg avilamycin activity/kg feed).

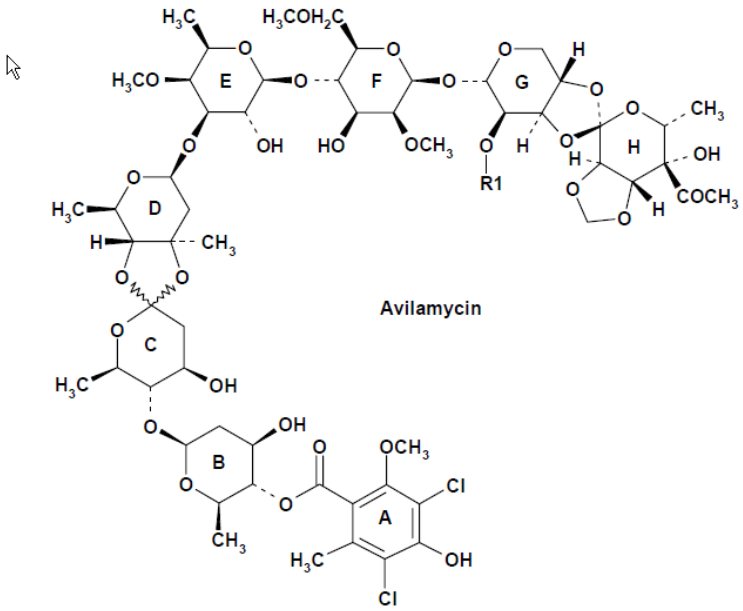
The primary route of environmental exposure to avilamycin will be from application of chicken litter to agricultural land. Chicken litter will contain manure from treated chickens. Any contribution from spillage and breakage of the primary container of Type A medicated article will be minor and occur at a separate site (feed mill). These spills will be insignificant and handled through municipal waste systems.

Runoff from chicken facilities as a route of environmental exposure to avilamycin will not be considered because chicken facilities and stored litter are covered such that contaminated runoff is not expected.

3.0 Description of the Product

The active ingredient is avilamycin. Avilamycin is an antibiotic of the orthosomycin family consisting of a six-member oligosaccharide with dichloroiosoeverninic acid at one end and methyl eurekanate at the other. Avilamycin is produced by fermentation of *Streptomyces viridochromogenes* and is composed of a mixture of avilamycin A (>60%), avilamycin B (<18%) and 14 minor factors, with none of the minor avilamycins contributing more than 6% of the total avilamycin content.

Avilamycin is primarily active against gram positive bacteria through inhibition of protein synthesis by binding to the 50S ribosomal subunit and preventing the correct positioning of tRNA and initiation factor 2 (Kofoed and Vester, 2002).

International Non-proprietary Name (INN):	Avilamycin
Chemical Name:	O-(1R)-4-C-acetyl-6-deoxy-2,3-O-methylene-D-galactopyranosylidene-(1-3-4)-2-O-(2-methyl-1-oxopropyl)-α-L-lyxopyranosyl O-2,6-dideoxy-4-O-(3,5-dichloro-4-hydroxy-2-methoxy-6-methylbenzoyl)-β-D-arabino-hexopyranosyl-(1-4)-O-2,6-dideoxy-D-arabino-hexopyranosylidene-(1-3-4)-O-2,6-dideoxy-3-C-methyl-β-D-arabino-hexopyranosyl-(1-3)-O-6-deoxy-4-O-methyl-β-D-galactopyranosyl-(1-4)-2,6-di-O-methyl-β-D-mannopyranoside
CAS Number:	11051-71-1 (Avilamycin) 69787-79-7 (Avilamycin A)
Molecular Formula:	$C_{61}H_{88}Cl_2O_{32}$ (Avilamycin A)
Molecular Weight:	1404.2 (Avilamycin A)
Structural Formula:	

Avilamycin

Factor	R1	Other Modifications
A	$-COCH(CH_3)_2$	
A'	$-COCH_2CH_3$	–H replaces $-COCH_3$ on Ring H
B	$-COCH_3$	
E	–H	$-CHOHCH_3$ replaces $-COCH_3$ on Ring H
F	$-COCH(CH_3)_2$	–H replaces –Cl adjacent to $-OCH_3$ and –OH replaces $-OCH_3$ on Ring A
G	$-COC_4H_9$	
H	$-COCH(CH_3)_2$	–H replaces –Cl adjacent to $-OCH_3$ on Ring A
I	$-COCH_2CH_3$	
J	$-COCH(CH_3)_2$	–OH replaces $-OCH_3$ on Ring F

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 a Type A feed premix containing Avilamycin in broiler chickens. In this Phase I assessment, the maximum concentration of avilamycin in the litter (manure plus bedding material) and the soil has been calculated. No metabolism or degradation in litter is assumed and a total residue approach is taken for the Phase I assessment. The initiation of a Phase II assessment is dependent upon the trigger value 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 Litter

To estimate the concentration of avilamycin in litter, it was assumed that all broilers in the barn were fed avilamycin for 21 days during an approximately 42 day growing cycle. The other assumptions used to calculate a litter concentration are in Table 1. The maximum concentration of avilamycin in litter is therefore, 19.5 mg/kg.

Table 1. Assumptions used to calculate avilamycin concentration in chicken litter

Maximum Concentration in Final Feed	45 mg/kg
Daily Feed Intake	0.13 kg/bird[a]
Daily Manure Produced	0.13 kg/bird[a]
Dilution of Manure by Bedding	15%[a]
Daily Litter Produced	0.15 kg/bird[b]
Treatment Duration	21 days
Growing Cycle	42 days[a]

[a] These are traditionally used values in environmental assessments and reflect typical agricultural practices.

[b] The daily litter produced is calculated by multiplying the manure produced by 115%.

$$Concentration\ in\ Litter = \frac{Daily\ Avilamycin\ Dosed}{Daily\ Litter\ Produced} \times \frac{Treatment\ Duration}{Growing\ Cycle}$$

$$= \frac{Concentration\ in\ Feed \times Daily\ Feed\ Intake}{Daily\ Litter\ Produced} \times \frac{Treatment\ Duration}{Growing\ Cycle}$$

$$= \frac{45 \frac{mg_{avilamycin}}{kg_{feed}} \times 0.13\ kg_{feed}}{0.15\ kg_{litter}} \times \frac{21d}{42d} = 19.5\ mg/kg$$

4.1.2 Calculation of concentration in soil

The maximum concentration of avilamycin in the soil has been calculated using typical agronomy practices for application of poultry litter to land. Litter from chickens is applied to soil at an upper rate of 9200 kg/acre and is incorporated into soil. 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 into which the manure is mixed is approximately 910,500 kg:

$$Weight\ of\ Soil = 1\ acre\ \times 4046.856 \frac{m^2}{acre} \times 0.15\ m \times 1500\ kg/m^3$$
$$= 910{,}500\ kg$$

The assumptions used to calculate the soil concentration are in Table 2. Assuming no degradation in litter or soil, the concentration of avilamycin in soil after application of poultry litter could be as high as 197 µg/kg.

Table 2. Assumptions used to calculate avilamycin concentration in soil

Application Rate of Litter to Soil	9200 kg/acre[a]
Plow Depth	15 cm[a]
Weight of Soil in 1 acre x 15 cm	910,500 kg soil

[a]These are traditionally used values in environmental assessments and reflect typical agricultural practices.

$$[Avilamycin]_{Soil} = \frac{Concentration\ in\ Litter\ \times Application\ Rate\ of\ Litter\ to\ Soil}{Weight\ of\ Soil}$$

$$\frac{19.5 \frac{mg}{kg} \times 9200\ kg/acre}{910{,}500 kg/acre} = 0.197 \frac{mg}{kg} = 197\ \mu g/kg$$

Assuming no degradation in litter or soil, the concentration of avilamycin in soil after application of broiler litter could be as high as 197 µg/kg. Since the initial concentration in the soil is more than 100 µg/kg, a Phase II environmental risk assessment was conducted, as per the VICH GL6 Final Guidance.

5.0 Phase II Environmental Impact Assessment

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 avilamycin in broiler chickens. In the Phase II assessment, data with regard to the physical/chemical properties, environmental fate and environmental effects of avilamycin are used to assess the environmental risk of the use of avilamycin. Phase II progresses as two tiers; in the first (Tier A), a basic set of less complex studies is evaluated and is used to prepare a conservative risk assessment. If that risk assessment cannot rule out the possibility of a risk to the environment, more complex studies are then conducted and evaluated in Tier B. In the current assessment of the risk for the use of avilamycin in broiler chickens, only a Tier A assessment has been conducted.

5.1 Summary of Available Data

This section reviews environmental data that has been collected with avilamycin. The Sponsor has collected data with avilamycin since the early 1980's.

5.1.1 Physical and Chemical Properties

The physical and chemical properties of avilamycin are listed in Table 3.

The aqueous solubility of avilamycin is pH-dependent with higher solubility at higher pH values. The solubility values for avilamycin A were determined to be < 0.125, 41 and 113 mg/L at pH 5, 7, and 9, respectively (Study I-EWD-82-07, 1982, Appendix A). The solubility values were also determined for a mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', and 0.9% D1+D2). The aqueous solubility of the avilamycin mixture was slightly higher than avilamycin A alone: < 0.125, 75, and 222 mg/L at pH 5, 7, and 9, respectively.

The logarithm of the octanol-water partition coefficient at pH 7.0 was determined to be 2.09 for avilamycin A and 1.94 for a mixture of avilamycin containing 28.7% avilamycin B (Study I-EWD-82-16, 1982, Appendix B). Avilamycin A appears to be slightly more lipophilic than the other avilamycin factors. In Study 66306 (2011, Appendix C), the octanol-water partition coefficient of avilamycin A was found to be related to pH and inversely proportional to its aqueous solubility. The log Pow of avilamycin A was determined to be 3.97, 2.55 and 0.681 at pH 4.5, 7, and 9, respectively.

The solubility and octanol-water partition data are consistent for a compound with an acidic dissociation constant. Additionally, the available data are in agreement that avilamycin A is more nonpolar than the other factors.

Table 3. Physical and Chemical Properties of Avilamycin

Melting Point (Merck Index, 1996)		Avilamycin A	Mixture of Avilamycin Factors
		181-182°C	188-189.5
Aqueous Solubility (I-EWD-82-07, 1982, Appendix A)		Avilamycin A	Mixture of Avilamycin Factors
	pH 5	< 0.125 mg/L	< 0.125 mg/L
	pH 7	41 mg/L	75 mg/L
	pH 9	113 mg/L	222 mg/L
n-Octanol/Water Partition Coefficient (Study 66306, 2011, Appendix C)	pH 4.5	pH 7	pH 9
	3.97	2.55	0.681
Dissociation Constant, pKa (internal data measured by potentiometric titration)	5.71, acidic		

5.1.2 Fate

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

5.1.2.1 Metabolism and Excretion

When chickens (predosed for 7 days with non-radiolabeled avilamycin at 20 ppm in feed) were orally dosed with a single capsule of ^{14}C-avilamycin, 50 to 78% of the dose was recovered in excreta in the first 24 hours (Study ABC-0230, 1984, Appendix D). In the first 4 days, 78 to 96% of the radioactive dose was recovered in excreta.

The metabolism of avilamycin by rats and swine has been described by Magnussen et al (1991). In those species, avilamycin is extensively transformed to a flambic acid, which interconverts with flambalactone. Flambic acid has been shown to have less pharmacological activity than the parent avilamycin (Study MR11MS-ELA, 2011, Appendix E). However, the metabolites of avilamycin in chickens have not been identified, primarily because avilamycin residues in tissues are very low. Additionally, excreted avilamycin residues from chickens have not been characterized.

5.1.2.2 Degradation

5.1.2.2.1 Hydrolysis

Avilamycin is not stable in water and undergoes hydrolysis more rapidly in acidic and basic media as compared to neutral conditions. The half-lives of avilamycin incubated in the dark at pH 5, 7, and 9 are 12, 230, and 52 hours, respectively (Study S-AAC-82-04, 1983, Appendix F). In this study, concentration was measured by a microbiological assay (with *Micrococcus flavus)*; therefore, the hydrolysis products were not microbiologically active.

5.1.2.2.2 Photolysis

Avilamycin is rapidly photodegraded in aqueous solutions. In Study S-AAC-82-04 (1983, Appendix G), solutions of avilamycin in pH 7 buffer were irradiated under fluorescent lamps which replicate an ultraviolet spectral energy distribution similar to natural sunlight. The first order degradation rate was calculated to be -0.59 hr^{-1} and the half-life was 1.2 hours.

In Study EWD8429 (1984, Appendix H), the degradation rate of avilamycin was calculated to be 7.24 $days^{-1}$ in natural sunlight at 40°N latitude in the summer (partly cloudy on the day that this data was gathered). Using the light intensity in the study and the light intensity at various northerly latitudes, the half-lives under clear skies was calculated. The half-lives ranged from 0.086 days at 20°N in summer to 1.33 days at 50°N in winter.

In both photolysis studies above, the concentration of avilamycin was measured by a microbiological assay (with *Micrococcus flavus)*; therefore, the photolysis products of avilamycin are not microbiologically active. Avilamycin was rapidly degraded under experimental conditions in which the aqueous media was clear and shallow allowing maximal light penetration. In the surface waters impacted by agricultural runoff, degradation of avilamycin will likely be slower due to the presence of particles and dissolved materials as well as depth which limit light penetration.

Based on its hydrolytic and photolytic potential, avilamycin is not expected to persist in aqueous environments. However, because degradation rates are likely slower in real-world aqueous compartments, the environmental concentrations in the surface water will not be refined for photolysis and hydrolysis.

5.1.2.2.3 Degradation in Soil

The field dissipation of avilamycin residues was evaluated in Study ABC-0236 (1984, Appendix I). In this study, manure from chickens fed 30 ppm ^{14}C-avilamycin in feed was applied to the top 5 cm of a silty loam soil. The concentration of avilamycin residues measured in the soil in this study (0.38 mg/kg) was slightly higher but relatively comparable with expected concentration following application of chicken manure to soil (PEC_{soil} = 0.197

mg/kg). Core samples up to 30 cm deep were evaluated for total radioactivity over the course of a year. The majority of the recovered radioactivity was always found in the top 7.5 cm. After 4 weeks only about 50% of the applied radioactivity was found in the core samples.

A more recent degradation study of avilamycin in soils was conducted to fully evaluate the kinetics and identify major degradation products in four different soils (Study 66679, 2012, Appendix J). In this study, the soils were amended with ^{14}C-avilamycin at a rate of 1 mg/kg and incubated at 20°C under aerobic conditions in the dark. Evolved $^{14}CO_2$ was trapped using KOH and soils were extracted and extracts profiled using HPLC with radiometric detection. Major degradation products were identified using LC/MS/MS. Avilamycin was rapidly transformed in the soils: the maximum DT50 and DT90 values were 1.5 and 28.6 days, respectively. Additionally, there was significant mineralization; after 120 days, 15.1 to 42.7% of the applied radioactivity (AR) evolved as $^{14}CO_2$ in three of the soils, and in the fourth soil, 58.0% of the AR was evolved as $^{14}CO_2$ after just 51 days. A large amount of nonextractable residue was observed in the study. Since the three soils with the most $^{14}CO_2$ evolution had the highest nonextractable residue, it is likely that the residue is composed of small degradation products that were incorporated into the microbial-soil matrix. Six transformation products were observed that were greater than 10% of the applied radioactivity. The predominant degradation pathway was hydrolysis leading to the same cleavage of the orthoester linkage between the C and D rings in avilamycin similar to what is observed in metabolism studies. In the soil metabolism study, both flambic acid and flambalactone were observed as well as the corresponding remaining moiety from the other side of the molecule. All three of these products experienced further degradation over the course of the study. Another minor pathway of degradation resulted in hydrolysis of the avilamycin at a different location and loss of the methyl eurekanate entity. This degradation product was also observed to further degrade. The only degradation product that did not clearly show further degradation during the study was found at levels greater than 10% in only one of the soils and only at the last three time points of the study, Days 73, 98 and 120. The structure of this degradation product was not determined, but the molecular weight was only 230 g/mole (versus 1404 g/mole for avilamycin A), therefore, this degradation product is unlikely to have the same bioactivity as avilamycin.

5.1.2.2.4 Degradation in Excreta

No studies have been conducted to evaluate the degradation of avilamycin in chicken manure or in litter.

5.1.2.3 Soil Adsorption

The adsorption of avilamycin to soil and its potential soil mobility have been evaluated.

In Study EWD8609 (1986, Appendix K), a batch sorption study was conducted in which ^{14}C-avilamycin in 0.01 M $CaCl_2$ was equilibrated with three different soils (sandy loam, loam, and clay loam) for 16 hours. To reduce degradation during the study, the 0.01 M $CaCl_2$ was boiled to drive off CO_2 which thereby raised the pH. In addition, the adsorption of avilamycin to glass was corrected by subtracting the amount that bound to glass in a soil-less control. The amount of radioactivity bound to soil was determined by subtraction of the supernatant from the amount added to the equilibration mixtures. The resulting Kd values were 51, 23, and 109 for the sandy loam, loam and clay loam soils, respectively.

In a more recent study, Study 66678 (2012, Appendix L) following OECD guideline 106, ^{14}C-avilamycin was equilibrated with five different soils for up to 20 hours. The 0.01 M $CaCl_2$ was boiled to drive off dissolved CO_2 and the pH of the solution was adjusted to 7 to minimize degradation and increase solubility of avilamycin. Additionally, the test tubes were pre-conditioned with excess non-radiolabeled avilamycin to reduce adsorption to glass. The soil and aqueous phases were separated by centrifugation and Kd values for total radioactivity were calculated from the total radioactivity in the supernatant and the calculated amount in the soil by subtracting the supernatant radioactivity from what was originally added. For the five soils, the mean Kd values for total radioactivity ranged from 2.05 to 143 with an average of 54 and the average Koc value over the 5 soils was 3643. Additionally, the soil pellets were extracted and the extracts and the aqueous supernatants were profiled by HPLC fractionation followed by liquid scintillation counting. Using the profile data, Kd values specific for avilamycin were calculated. For avilamycin, the mean Kd values ranged from 2.01 to 29.3 with an average of 15 while the average Koc value was 1060. While the properties of avilamycin (instability especially at low pH values, low water solubility especially at low pH values, adsorption to glass) make it difficult to conduct a robust batch sorption study, the data from both of these studies can be taken as estimations of the binding of avilamycin to soil. Given the issues with the conduct of a batch sorption study with a compound possessing these characteristics, simulation of leaching may provide an alternative way to understand of the potential soil mobility of avilamycin.

In Study ABC-0337 (1986, Appendix M), the potential soil mobility of avilamycin and flambalactone were investigated using soil thin-layer chromatography. The distance that the compounds travelled in three different soils (coarse, medium, and fine) was compared to mobile and immobile reference standards. Avilamycin was classified as a low mobility compound. Flambalactone, which can be easily hydrolyzed to the more polar carboxylic acid (flambic acid), was found to be more mobile than avilamycin in the soils.

In the two studies in Study S-AAC-82-04 (1983, Appendix N), the mobility of avilamycin and its soil degradation products were investigated using soil columns. In the first study, four columns were prepared each with a different soil. ^{36}Cl-Avilamycin was applied to the top of the columns and then the columns were leached with water to simulate 60 cm of rainfall over approximately six days. Total amounts of radioactivity found in the leachates were 22.1, 53.3, 26.7, and 22.7% for

the sand, sandy loam, loam and clay loam soils, respectively. In another part of this study, ^{36}Cl-avilamycin was aged for 30 days in the sandy loam soil, prior to placing that soil on the top of a soil column prepared with untreated sandy loam soil. Over 45 days, 40-mL of water per day, equivalent to 1.25 cm, was added to the tops of the columns and leachate collected. At the end of the study, 84.4% of the applied radioactivity was found in the leachate.

The studies together suggest that avilamycin can sorb to soil, but its rapidly forming degradation products are likely to be more mobile in soil.

5.1.2.3.1 Bioconcentration

One way to estimate bioconcentration potential of a chemical is to consider its lipophilicity. The log P_{ow} values for avilamycin across an environmental range of pH ranges from 3.97 (at pH 4.5) to 0.681 (at pH 9). Veith and Kosian (1983) generated a linear model using a training set of 122 molecules to predict the bioconcentration factor for chemicals in fathead minnows:

$$\log BCF = (0.79 \times \log K_{ow}) - 0.40$$

Using this equation and the highest log P_{ow} value for avilamycin (3.97), the estimated bioconcentration factor (BCF) for avilamycin is only 545. Bioaccumulation that affects the food chain typically becomes a concern with bioconcentration factors greater than 1000 or more. Additionally, the potential for avilamycin to bioaccumulate is very low given its limited half-life in water (due to hydrolysis and photolysis, especially in more acidic environments) and its propensity for metabolism across species evaluated (swine and rat).

Table 4. Environmental Fate of Avilamycin

Soil Adsorption/Desorption (Study 66678, 2012, Appendix L)		Total Radioactivity Koc		Avilamycin Koc
	Sand pH 5.2	559		605
	Loam pH 6.0	3321		1325
	Silty Clay Loam pH 6.3	1987		537
	Sandy Clay Loam pH 8.0	397		388
	Sandy Clay Loam pH 6.8	11,952		2444
Hydrolysis (Study S-AAC-82-04, 1983, Appendix F)	Hydrolytically unstable			
		pH 5	pH 7	pH 9
	Half-life (hours)	12	230	52
Photolysis (Study EWD8429, 1984, Appendix H)	Half-lives range from 0.086 to 1.33 days from 20 to 50 degrees north latitude over summer and winter			
Degradation in Soil for up to 120 days (Study 66679, 2012, Appendix J)	Biodegradation to $^{14}CO_2$ ranged from 15.1 to 58.0% of applied radioactivity. After 7 days, less than 11% AR in all soils was identified as avilamycin. The DT50 and DT90 for avilamycin ranged from 0.2 to 1.5 days and 0.6 to 28.6 days, respectively. Six major degradation products were observed, including flambic acid and flambalactone which further degraded over the course of the study.			

5.1.2.4 Toxicity

The environmental effects of avilamycin in the terrestrial and aquatic compartments are described below and summarized in Table 5.

5.1.2.4.1 Terrestrial Organisms

The toxicity of avilamycin to soil microflora, plants, and earthworms has been evaluated.

The effects of avilamycin on soil microflora, specifically carbon transformation (respiration) and nitrate formation (nitrification), were evaluated in Study 70541080 (2012, Appendix O). The initial soil concentrations of avilamycin (applied as crystalline avilamycin) were 1, 5, and 15 mg/kg soil (dry weight). There were no effects on respiration throughout the study. After 28 days, the carbon transformation rate in the highest treated group differed from the control rate by only 4%. There were transient effects on nitrification by avilamycin treatment. The data suggested that avilamycin treatment stimulated the formation of nitrate nitrogen early in the study. By Day 28, the nitrate formation rate in the

controls had caught up to that in 1 and 5 mg avilamycin/kg treatments but not the 15 mg avilamycin/kg which was increased over the control by 34%. By Day 56, the difference in nitrate formation rate between the 15 mg avilamycin/kg and the control was only 11%. Therefore, no long-term impacts from avilamycin are expected on the soil microflora population.

An older study was conducted on the effects of avilamycin on the nitrification in soil (S-AAC-82-19, 1983, Appendix P). However, in the older study, the test substance was actually avilamycin residues applied as manure from pigs fed avilamycin. Based on avilamycin activity measured at the initiation of the test, the tested concentrations were 0.83 and 1.66 mg avilamycin/kg soil. After 28 days, the amount of nitrate-nitrogen in the soil was the same as that in the feces-free soil control and only slightly higher than the soil control (≤ 18% difference) amended with the same amount of feces from pigs not treated with avilamycin.

Both the recent study (Study 70541080, 2012, Appendix O) and the older study (Study S-AAC-82-19, 1983, Appendix P) are in agreement that avilamycin and its residues are not expected to have a significant effect on soil microflora. This environmental risk assessment will be conducted using the data from the more recent study (Study 70541080, 2012) because it was conducted to current guidelines and utilizes crystalline avilamycin.

In a seedling germination test with six species, no seeds were affected by direct exposure to avilamycin via filter paper saturated with aqueous avilamycin solutions (Study ABC-0263, 1984, Appendix Q). The highest concentration of avilamycin in this study was equivalent to the exposure to avilamycin residues which would result from the application of approximately 14,000 kg litter/acre, that is, about 150% of the maximum exposure expected in the soil.

The phytotoxicity of manure from chickens fed avilamycin (at 100 mg/kg in feed) was evaluated in Study ABC-0262 (1984, Appendix R). Seeds of corn, wheat, soybean, and tomato plants were grown in the amended soil. Control pots were amended with manure from chickens that were not fed avilamycin. The calculated exposure concentration was 0.365 mg avilamycin residues/kg soil based on the measured residues in the manure. At 21 days after planting the seeds, there were no statistical differences for corn or wheat when compared to the controls. There was a 6% decrease from controls in soybean shoot weight which was statistically significant at day 21; however, this decrease is not considered biologically significant. There was also a 34% decrease in root weight of treated wheat compared to the control which was statistically significant (t-test, $p<0.05$). Given the difficulty in removing the root mass completely and cleanly from soil, it is unclear if the decrease in root weight is meaningful, especially considering that the decrease in shoot weight (17.9% compared to control) was not as pronounced and was not statistically significant. In addition, there was a decrease in size of tomato plants grown in soil amended with avilamycin manure in both mean weight (shoots decreased by 45% and roots decreased by 43%) and mean height (decreased by 21%). These differences were statistically significant.

The concentration of avilamycin residues in the exposure pots in this older phytotoxicity study (0.365 mg/kg) was similar to that expected in the soil (PEC_{soil} = 0.197 mg/kg). However, another more recent phytotoxicity study has been conducted following the OECD 208 guideline to evaluate seed emergence and growth (Study 66369, 2012, Appendix S). In the new study, six crop species (corn, oat, radish, soybean, sugar beet, and tomato) were planted in avilamycin-fortified sandy loam soil. Avilamycin was added to soil as crystalline avilamycin at much higher concentrations than were tested in the previous phytotoxicity study and at higher concentrations than what is expected in soil. No effects were observed on percent emergence of the seeds, survival of seedlings after emergence, individual shoot length, and replicate shoot weight for any species up to the highest concentration tested, 500 mg avilamycin/kg soil (dry weight).

All three of the phytotoxicity studies are consistent with the conclusion that in general plants are not particularly sensitive to exposure to avilamycin. The study in which soil was amended with manure from chickens treated with avilamycin suggests that tomatoes may be more sensitive to avilamycin residues, however, in the more recent study, concentrations of avilamycin that were orders of magnitude greater did not cause stunted growth even in tomatoes. Since the concentrations tested in the older phytotoxicity study are not as high as the predicted environmental concentration in soil, this environmental risk assessment will be conducted using the data from the more recent study (Study 66369, 2012).

The effects of avilamycin on earthworms were evaluated in a subchronic growth and survival study and in a reproduction study. In Study W01882 (1988, Appendix T), *Lumbricus terrestris* were exposed to avilamycin for 14 days with no adverse effects on survival, growth, or behavior observed at concentrations up to 100 mg avilamycin/kg in soil (dry weight). In a chronic study including a reproduction endpoint (Study 66370, 2011, Appendix U), *Eisenia fetida* were exposed to avilamycin in soil up to a concentration of 1,300 mg avilamycin/kg soil (dry weight). 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. While there were no statistically significant differences in the number of juveniles found in the soil treated with avilamycin, there were 18 to 20% fewer earthworms in the two highest doses (670 and 1,300 mg/kg) compared with the pooled control. Based on these decreases, the NOEC was conservatively estimated to be 330 mg/kg. The results of the subchronic and reproduction studies are consistent. Therefore, this environmental risk assessment will be conducted using the data from the more recent study (Study 66370, 2011, Appendix U).

Given the rate of degradation of avilamycin in soil observed in Study 66679 (2012, Appendix J) in which DT50s ranged from 0.2 to 1.5 days for four soils, organisms in these terrestrial toxicity studies were likely exposed to significant concentrations of avilamycin degradation products, including flambic acid and/or flambalactone. No terrestrial organism tested appears to have a particular sensitivity to avilamycin or its residues.

5.1.2.4.2 Aquatic Organisms

The toxicity of avilamycin to microorganisms, cyanobacteria, daphnia, and fish has been evaluated.

In Study S-AAC-82-08 (1983, Appendix V), the toxicity of avilamycin to sewage microorganisms was investigated by repeatedly treating an activated sludge inoculum with increasing concentrations of avilamycin, up to 102.6 mg/L (theoretical), under aerobic conditions. The highest measured concentration of avilamycin activity in the test systems was 87.6 mg/L. Measurements of BOD, colony-forming units, pH, and solids content did not indicate that avilamycin would have a deleterious impact upon the digestive process of a diverse microbial population. The design of the study, increasing the theoretical concentration from 0.1 to 102.6 mg/L could have resulted in acclimation of the system to effects of avilamycin.

The inhibitory effects of avilamycin on *Synechococcus leopoliensis*, a photosynthetic cyanobacterium, have been evaluated in a 72 hour static exposure (Study T4EFR0701, 2007, Appendix W). The nominal concentrations were 0.625, 1.25, 2.5, 5.0 and 10 mg/L and the actual concentrations were not measured. Avilamycin caused a decrease in the growth rate which was more pronounced after 24 hours than at the end of the study (72 hours). This may reflect a decreasing concentration of bioactivity as avilamycin is degraded due to photolysis and/or hydrolysis. The EC50 values for growth rate and yield were determined to be >10 mg/L and 6.85 mg/L, respectively, based on nominal concentrations. The NOEC values for growth rate and yield were considered to be 2.5 and 1.25 mg/L, respectively. It is likely that avilamycin was degraded by hydrolysis and photolysis during this study in which the aqueous cultures were exposed to 72 hours of constant illumination of 3700 lux. In Studies S-AAC-82-04 (Appendix G) and EWD8429 (Appendix H), the half-life of avilamycin was determined to be as low as 1.2 hours in aqueous solutions exposed to simulated or actual sunlight. Hydrolysis occurs at a slower rate when solutions of avilamycin are incubated in the dark with half-lives of 230 and 52 hours at pH values of 7 and 9, respectively (in the cyanobacteria study the pH ranged from 8.07 to 8.25), in Study S-AAC-82-04. If the faster hydrolysis rate is considered, approximately 60% of the avilamycin would hydrolyze over the 72-hour study. The amount that would photolyze is more difficult to estimate. Therefore, the average true exposure concentrations during the cyanobacteria study are likely much lower than the nominal concentrations upon which the EC50 and NOEC values are based. Adding to the uncertainty in the relationship of toxicity to exposure is that,

in considering a realistic aqueous environment in which avilamycin residues run off into surface water, hydrolysis and photolysis will also occur, although photolysis will likely be attenuated due to lower light penetration.

To evaluate the toxicity of avilamycin to aquatic invertebrates, two acute toxicity studies with *Daphnia magna* have been conducted. In Study C03382 (1983, Appendix X), daphnia were exposed to avilamycin (as dried fermentation product) in a static toxicity test for 48 hours at a single concentration. The nominal concentration was 100 mg/L but the measured concentrations ranged from 18.6 to 29.0 mg/L over the test with a mean measured concentration of 23.8 mg/L (concentrations were measured as antimicrobial activity). The disparity between the nominal and measured concentrations was likely due to a combination of the susceptibility of avilamycin to hydrolysis and photolysis (especially during the prolonged pre-study stirring period) and the limited solubility of the dried fermentation product. No mortality, immobilization or sublethal effects were observed. The EC50 was >23.8 mg/L and the NOEC was 23.8 mg/L. Recently, another acute toxicity study with daphnia exposed to avilamycin was conducted in order to confirm that the material was not toxic to daphnids at higher concentrations by using crystalline avilamycin (Study 66272, 2011, Appendix Y). Daphnids were exposed to nominal concentrations up to 160 mg/L and again no toxicity was noted in any daphnid at any concentration. The mean measured concentrations were slightly lower than nominal and the highest treatment level was measured to be 138 mg/L (concentrations were measured using an HPLC/uv method). Therefore, the EC50 was >138 mg/L and the NOEC was 138 mg/L. In Study 66272, there was little evidence of degradation under the relatively low light conditions of 604 lux with a 16 h:8 h light:dark cycle. This environmental risk assessment will be conducted using the data from the more recent study (Study 66272, 2011) because higher concentrations were tested using crystalline avilamycin and because the nominal and measured concentrations were similar and, therefore, there is less uncertainty in the results.

In Studies F12782 (1983, Appendix Z) and F12682 (1983, Appendix AA), bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) were exposed to avilamycin (as dried fermentation product) in static toxicity tests for 96 hours at a mean measured concentration of 35.4 mg/L (bluegill) and up to 47.8 mg/L (rainbow trout). Concentrations were measured as antimicrobial activity. For both of these studies, the nominal maximum concentration tested was 100 mg/L. As with Study C03382 (Appendix X), the disparity between the nominal and measured concentrations was likely due to a combination of instability of avilamycin in water and in light as well as low water solubility of the dried fermentation product. The pH values measured during the tests ranged from 7.9 to 8.7. Therefore, it is likely that the bluegill and rainbow trout would have been exposed to some of the degradation products of avilamycin. No mortality or signs of toxicity were noted in any fish of either species. Therefore, for both species, the LC50 is greater than the highest concentration tested and the NOEC is equal to the highest concentration tested.

The photolysis and hydrolysis products of avilamycin have not been identified. However, it is likely that some of them are the same as the transformation products observed in the soil degradation study since hydrolysis was a component of the degradation pathway in soil. Therefore, it cannot be ruled out that the aquatic species were exposed to some degree to the degradation products of avilamycin.

Table 5. Environmental Effects of Avilamycin

Terrestrial Effects Studies			
Soil Microflora Respiration and Nitrogen Transformation Tests (56 days) (Study 70541080, 2012, Appendix O)	Concentration as high as 15,000 µg/kg resulted in less than 25% difference from control after 56 days		
Terrestrial Plants – Emergence and Seedling Growth (Study 66369, 2012, Appendix S)		µg/kg	
		EC50/LC50	NOEC
	Corn	>500,000	500,000
	Oat	>500,000	500,000
	Radish	>500,000	500,000
	Soybean	>500,000	500,000
	Sugar beet	>500,000	500,000
	Tomato	>500,000	500,000
Earthworm Reproduction (Study 66370, 2011, Appendix U)	NOEC 330,000 µg/kg		
Aquatic Effects Studies			
Inhibition of Sewage Microorganisms (Study S-AAC-82-08, 1983, Appendix V)	Concentrations of avilamycin up to 87,600 µg/L had no significant effect on sewage microorganisms.		
Blue-green Algae (Cyanobacterial) Growth Inhibition (Study T4EFR0701, 2007, Appendix W)		µg/L	
		Yield	Growth Rate
	EC50	6,850	>10,000
	NOEC	1,250	2,500
Daphnia immobilization (Study 66272, 2011, Appendix Y)	EC50: >138,000 µg/L NOEC: 138,000 µg/L		
Fish Acute Toxicity (Studies F12782 and F12682, 1983, Appendix Z and Appendix AA)	Bluegill LC50: >35,400 µg/L NOEC: 35,400 µg/L Rainbow Trout LC50: >47,800 µg/L NOEC: 47,800 µg/L		

5.2 PEC Calculations and Refinements (Exposure Assessment)

5.2.1 Soil

The initial PEC_{soil} was calculated in the Phase I assessment as 197 µg/kg.

The PEC_{litter} concentration was first calculated using the following assumptions: all chickens in the barn are treated with the maximum dose of avilamycin for 21 days of a 42-day growing period and all residues eliminated are avilamycin and/or metabolites as active as avilamycin. The PEC_{litter} of total avilamycin residues using these assumptions was 19.5 mg/kg.

Since the composition of metabolites that are excreted by chickens has not been characterized, the PEC_{litter} will not be refined based on metabolism. It is noted that in other species, avilamycin is extensively metabolized to nonactive transformation products. Additionally, as avilamycin is rapidly degraded in soil and there is evidence that the breakdown products are also degraded in soil, it is likely that avilamycin residues are also unstable in the litter environment in the barn. However, no data has been collected on the degradation of avilamycin residues in litter. Therefore, for the purposes of this risk assessment, no metabolism or degradation in the litter will be assumed. The PEC_{litter} and PEC_{soil} will be considered to be 19.5 mg/kg and 197 µg/kg, respectively.

Avilamycin degrades rapidly in soil to several degradation products which in turn break down, eventually to CO_2, with observed half-lives of avilamycin ranging from 0.2 to 1.5 days. Therefore, there is no concern that avilamycin residues will persist and build up in the terrestrial environment.

5.2.2 Groundwater

Avilamycin and its residues are moderately sorbed to soil, with mean K_{oc} values ranging from 399 to 11952 in various soils (Study 66678, 2012; Appendix L). Substantial proportions of avilamycin are degraded to CO_2 in soil, with most of the remaining residues undergoing primary metabolism. It is very unlikely that significant levels of avilamycin or active residues would be found in groundwater.

5.2.3 Surface Water

Movement of avilamycin 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 avilamycin residues in chicken litter (19.5 mg/kg) and the application rate of litter per acre (9200 kg/acre), the following calculation was performed to estimate the maximum concentration of avilamycin residues in the pond:

$$[Avilamycin\ residues]_{pond}$$

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

$$[Avilamycin\ residues]_{pond} = \frac{19.5\frac{mg}{kg} \times 9200\frac{kg}{acre}\ \times 10\ acres\ \times 0.01}{8{,}100{,}000\ L} = 2.2\ \mu g/L$$

The concentration of total residues of avilamycin in litter manure is 19.5 mg/kg. A total of 9200 kg of poultry litter is applied per acre, such that 1,794,000 mg of avilamycin residues will be applied per 10 acres. Therefore, 17,940 mg 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 total avilamycin residues in the pond, $PEC_{surface\ water}$, would be 2.2 µg/L.

The measured Koc values for avilamycin residues to soil ranged from 399 to 11952. While, the $PEC_{surface\ water}$ might be somewhat lower due to binding to sediments suspended in the pond, for purposes of this risk assessment, no consideration for adsorption to soil is used to refine the $PEC_{surface\ water}$.

Table 6 summarizes the maximum predicted environmental concentrations for avilamycin residues in the terrestrial and aquatic compartments.

Table 6. Summary of PEC Calculations for Total Residues of Avilamycin

Compartment	Scenario	Concentration
Terrestrial	$PEC_{soil,\ total\ residues}$	197 µg/kg
Aquatic	$PEC_{surface\ water,\ total\ residues}$	2.2 µg/L

5.3 PNEC Calculations (Effect Assessment)

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

5.3.1 Terrestrial

The assessment factors used and the calculated PNECs for terrestrial species are included in Table 7. Terrestrial species were not particularly sensitive to avilamycin, which may reflect the rapid degradation of avilamycin in soil. The lowest PNEC value was in plants: 5,000 µg/kg.

Table 7. Terrestrial PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	≤25% change from control = 15,000 µg/kg	1	15,000 µg/kg
Plants, growth – soil	EC50 > 500,000 µg/kg	100	5,000 µg/kg
Earthworms	NOEC = 330,000 µg/kg	10	33,000 µg/kg

5.3.2 Aquatic

The assessment factors used and the PNECs calculated for aquatic species are included in Table 8. Cyanobacteria (i.e. blue-green algae) appear to be more susceptible to toxicity from avilamycin than fish or daphnids based on the median effective concentration. Additionally, while the EC50 and NOEC for cyanobacteria are reported to be 6850 and 1250 µg/L, respectively, these values are based on the initial nominal concentrations, which likely declined under the conditions of the study. Because the average exposure concentrations in the cyanobacteria study were likely lower than the initial nominal concentrations, an additional assessment factor has been used in the calculation of the PNEC value to account for this difference. An assessment factor of 3 will be used to account for the difference between nominal and actual concentrations in addition to the typical default assessment factor of 100. Therefore, a total assessment factor of 300 was applied to the EC50 for cyanobacteria. The lowest PNEC value for aquatic species is in cyanobacteria: 22.8 µg/L.

Table 8. Aquatic PNEC Values

	Toxicity endpoint	Assessment Factor	PNEC
Blue-green Algae (Cyanobacterial) Growth	EC50 = 6,850 µg/L NOEC = 1,250 µg/L	300	22.8 µg/L
Daphnia acute	EC50 > 138,000 µg/L	1000	138 µg/L
Fish Acute (bluegill)	LC50 > 35,400 µg/L	1000	35.4 µg/L

5.4 Risk Characterization

5.4.1 Terrestrial Compartment

The predicted maximum concentration of total residues of avilamycin in soil (PEC_{soil}) after a single application is 197 µg/kg. The lowest terrestrial PNEC value for avilamycin was 5,000 µg/kg.

The PEC/PNEC ratio for the terrestrial compartment is 0.04 (Table 9). The ratio is less than one indicating that there is no significant risk to plants or other soil-dwelling species. Given the rapid degradation of avilamycin in soil, there is no concern for significant risk following repeated applications of litter from chickens fed avilamycin.

Table 9. PEC/PNEC Ratio for Terrestrial Compartment

Species	PEC_{soil}	PNEC	PEC/PNEC Ratio
Plants	Total avilamycin residues: 197 µg/kg	>5000 µg/kg	0.04

5.4.2 Aquatic Compartment

The maximum predicted concentration of avilamycin residues in surface water is 2.2 µg/L. The lowest aquatic PNEC value calculated for avilamycin was 22.8 µg/L.

The PEC/PNEC ratio for the aquatic compartment is 0.10 (Table 10). The ratio is less than one, indicating that there is no significant risk to blue-green algae or other aquatic-dwelling species. Given the potential for photolysis and hydrolysis, there is no significant risk for accumulation of avilamycin in the aquatic compartment.

Table 10. PEC/PNEC Ratios for Surface water Compartment

Species	$PEC_{surface\ water}$	PNEC	PEC/PNEC Ratio
Blue-green algae (Cyanobacteria)	Total avilamycin residues: 2.2 µg/L	22.8 µg/L	0.10

5.5 Summary and Conclusions

The environmental impact from the administration of avilamycin via feed for 21 days at a targeted maximum concentration of 45 ppm in feed to prevent and/or reduce incidence of mortality caused by necrotic enteritis in broiler chickens has been evaluated. The evaluation included review of a base set of data collected on the physical/chemical properties, environmental fate, and environmental effects of avilamycin and its degradation products. The pathway for introduction of avilamycin into the environment was via the application of chicken manure as litter as fertilizer to soil. Runoff to surface water from soil fertilized with chicken litter containing avilamycin residues was also considered. The risk assessment utilized worst case assumptions of the maximum number of days of administration, the maximum dose, and the maximum application rate of litter based on nitrogen as well as a total residue approach.

The maximum predicted concentration of total avilamycin residues in the soil is 197 µg/kg and the maximum predicted concentration in surface water following runoff is 2.2 µg/L. The lowest predicted no effect concentration in the terrestrial compartment is calculated to be 5,000 µg/kg in soil (based on plants) and the lowest predicted no effect concentration in surface water is 22.8 µg/L (based on blue-green algae). In both compartments the predicted environmental concentrations are lower than the predicted no effect concentration. The PEC/PNEC ratios are 0.04 and 0.10 for terrestrial and aquatic compartments, respectively. Since avilamycin is extensively metabolized by animals and extensively degraded in soil and water, it is not expected to persist in the environment or accumulate in environmental species.

The avilamycin PEC/PNEC ratios are less than one and avilamycin will not persist or accumulate in the environment. Therefore, the use of avilamycin in broiler chickens treated with a maximum concentration in feed of 45 ppm is not expected to result in environmental impact through the application of chicken litter to cropland soil.

Because this conservative risk assessment has concluded that there is no expected harm to the environment, further data collection (e.g. a Tier B assessment) is not required.

6.0 Information on Environmental Assessment Expert

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Appendices

Appendix A - Study I-EWD-82-07: The solubility of avilamycin in aqueous solutions. Report Date: 1982.

Performing Laboratory: Lilly Research Laboratories

Test Articles:

Crystalline Avilamycin A
Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Excess test article was added to pH 5.0, 7.0 and 9.0 buffers. The solutions were shaken at 25°C and aliquots were removed for analysis periodically. The samples were filtered through a 20 micron filter and the filtrate was assayed in triplicate using a micro-biological agar well plate method.

Results:

	Maximum solubility mg/L (timepoint achieved)	
	Avilamycin A	**Mixture of Factors**
pH 5.0	<0.125 mg/L	<0.125 mg/L
pH 7.0	41 mg/L (3 hrs)	75 mg/L (24 hrs)
pH 9.0	113 mg/L (3 hrs)	222 mg/L (3 hrs)

At pH 5.0, the solubilities of avilamycin A and the mixture were both less than 0.125 mg/L which was the limit of detection for the assay. At pH values of 7.0 and 9.0, after reaching maximum solubility, the avilamycin concentrations decreased, apparently due to hydrolysis. The results suggest that avilamycin A is slightly less water soluble than the other avilamycin factors.

Appendix B- Study I-EWD-82-16: n-Octanol/Water Partition Coefficient of Avilamycin (EL-750). Report Date: 1982.

Performing Laboratory: Lilly Research Laboratories

Test Article:

Crystalline Avilamycin A
Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Solutions of avilamycin in n-octanol at two different concentrations were equilibrated with pH 7.0 buffer at a temperature of 25°C for one hour. The phases were separated by centrifugation for 20 minutes and the concentration of avilamycin in each phase was analyzed using a microbiological agar well plate assay.

Results:

	Mean Log Kow at pH 7.0
Avilamycin A	2.09
Mixture of Avilamycin Factors	1.94

The results indicate that avilamycin A is likely more nonpolar than avilamycin B.

Appendix C - Study 66306: Avilamycin: Determination of n-octanol/water partition coefficient (shake flask method). Report Date: 2011.

Performing Laboratory: ABC Laboratories, Inc.

Study Design: GLP, OECD 107

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Solutions of avilamycin in n-octanol were equilibrated with aqueous buffers having pH levels of 4.5, 7, and 9 at a temperature of 20°C. The concentration of avilamycin A in each phase was determined by LC/MS/MS.

Results:

	Log Pow* (standard deviation)
pH 4.5	3.97 (0.12)
pH 7	2.55 (0.05)
pH 9	0.681 (0.023)

While the test article was a mixture of avilamycin factors, the LC/MS/MS method specifically measured avilamycin A. Therefore, the log Pow values reported are for avilamycin A.

*The partition coefficient values are not necessarily for the neutral species. Therefore, instead of Kow values which implies the partition coefficient of the neutral species, they have been reported as Pow values.

Appendix D - Study ABC-0230: ^{14}C Avilamycin balance-excretion study in chickens. Report Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Study Design: GLP

Test Article: ^{14}C Avilamycin fermented from uniformly labeled glucose

Methods:

Two female and two male broiler chickens were fed ad libitum a ration containing unlaveled avilamycin at 20 ppm of microbiological activity for seven days. At the end of the pre-dosing period, each chicken was dosed once with a capsule containing 4.0 mg of ^{14}C avilamycin.

Results:

During the 13-day collection period, the two females and the two males excreted 92.8, 99.2, 96.6, and 84.4%, respectively, of the total dose administered. The bulk of the dosed ^{14}C in the four birds was excreted in the first four days (78.33 to 96.41%) with 50 to 78% during the first 24 hour collection period.

Appendix E - Study MR11MS-ELA: Determination of the minimal inhibitory concentration of avilamycin and flambic acid against various bacteria. Study Date: 2011.

Performing Laboratory: Microbial Research, Inc.

Study Design: Susceptibility testing conducted in the spirit of Standards M11-A7 and M31-A3 from the Clinical and Laboratory Standards Institute

Test Articles: Avilamycin and Sodium Flambate

Methods:
The bacteria tested included several strains of each of *Costridium perfringens*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus aureus*. Ninety-six well plates for MIC testing were prepared with either SBB (*Clostridium perfringens*) or MHB (Enterococci and Staphylococci) media. Avilamycin and flambic acid (as sodium flambate) were dissolved in methanol and added to the wells at various concentrations. The concentrations of each test item ranged from 0.06 to 128 µg/mL. The wells were inoculated with the appropriate bacteria and incubated for 46 to 48 hours under anaerobic (for *Clostridium perfringens*) conditions or 16 to 20 hours under aerobic (for Enterococci and Staphylococci) conditions. At the end of incubation, growth in the wells was assessed.

Results:

	Avilamycin	**Flambic Acid**
***Clostridium perfringens* (10)**		
MIC range	0.25 to 2 µg/mL	>128 µg/mL
***Enterococcus faecalis* (10)** ***Enterococcus faecium* (10)**		
MIC range	1 to 8 µg/mL	>128 µg/mL
***Staphylococcus aureus* (10)**		
MIC range	4 to 8 µg/mL	>128 µg/mL

No microbiological activity was observed with flambic acid at even the highest concentration tested, 128 µg/mL. The molecular weight of flambic acid is approximately 40% that of avilamycin (527 versus 1404). If the MIC concentrations are expressed as molar concentration, then the highest concentration of flambic acid tested (128 µg/mL or 0.24 mM) is approximately 40 times greater than the highest MIC for avilamycin (8 µg/mL or 0.006 mM). That is, the pharmacological activity of flambic acid is at least 40 times less than that of avilamycin.

Appendix F - Study S-AAC-82-04: Hydrolysis of avilamycin in buffer solution. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Sterile, aqueous buffer solutions of pH 5, 7, and 9 were fortified with 1.25 mg/L avilamycin and maintained in the dark at 24°C. Periodically samples were taken for analysis using an agar plate method and the organism *Micrococcus flavus* to measure bioactivity.

Results:
Avilamycin degraded in all solutions studied.

	pH 5	pH 7	pH 9
Half-life (hours)	12	230	52

Appendix G - Study S-AAC-82-04: An aqueous photolysis rate study with avilamycin. Study Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline mixture of avilamycin factors (63.8% A, 28.7% B, 6.7% A', 0.9% D_1+D_2)

Methods:
Sterile buffer solutions of avilamycin (1.25 mg/L) were prepared in pH 7 buffer and placed in 20-mL ampoules. The ampoules were irradiated using fluorescent sunlamps and black lights with an ultraviolet spectral energy distribution similar to natural sunlight. Samples were withdrawn for analysis after 1, 2, 4, 5, and 7 hours using an agar plate method and the organism *Micrococcus flavus*. Two ampoules were wrapped in aluminum foil and placed in the irradiation apparatus for seven hours as positive controls.

Results:
No significant degradation was observed in the control samples. In the irradiated samples, least squares analysis of the data obtained resulted in a first-order rate constant of -0.59 hr^{-1} and a half-life of 1.2 hours. Photolysis is likely to be a significant mode of dissipation in the environment.

Appendix H - Study EWD8429: Photodegradation of avilamycin in sunlight. Study Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Test Article: Avilamycin

Methods:
Solutions of avilamycin at 2 to 10 mg/L in 0.02 M pH 7 buffer, contained in quartz tubes, were exposed to summer sunlight. Avilamycin concentrations were determined at initiation and at 2, approximately 7 (0.5 days), and approximately 14 hours (1 day) using an agar plate method and the organism *Micrococcus flavus*. The sunlight intensity during the studies was monitored using the chemical actinometer p-nitroacetophenone.

Results:
Avilamycin degraded rapidly during the experimentation. In most samples exposed to sunlight for approximately 7 and 14 hours, no avilamycin activity was detected. Control samples of avilamycin incubated in the dark did not demonstrate degradation.

In one sample set, the concentration of avilamycin decreased from 10.0 mg/L at initiation to 0.258 mg/L after 7 hours (0.5 days). The first order degradation rate in this sample was 7.24 $days^{-1}$ corresponding to a half-life of 0.0958 days. Using the PNAP concentration to determine the sunlight intensity, avilamycin half-lives for other latitudes in summer and winter were calculated:

Latitude (Degree North)	Half-Life (Days)	
	Summer	Winter
20	0.086	0.150
30	0.091	0.236
40	0.096	0.475
50	0.110	1.33

Appendix I - Study ABC-0236: Field Dissipation of ^{14}C avilamycin chicken manure metabolites. Study Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Study Design: GLP

Test Article: Manure from chickens fed 30 ppm ^{14}C avilamycin

Methods:

Manure from chickens fed 30 ppm of ^{14}C avilamycin was mixed with the top 5 cm of soil contained in a 0.65 m^2 galvanized steel ring buried in soil (silty loam, pH = 6.6) to approximately 0.75 m. The amount of dried manure mixed into the soil was 0.252 kg, which theoretically contained 42.8 mg of avilamycin residues. At each sampling time, six core samples were taken at random and divided into 7.5 cm segments. Each corresponding 7.5 cm segment from the six cores was pooled and treated as a composite sample. The depth of the cores varied between 7.5 and 30 cm. Total radioactivity in the composite samples was measured by combustion.

Calculation of avilamycin residues in the soil:

Assuming the bulk density of soil is 1500 kg/m^3, the total weight of soil to which the manure was added was 48.75 kg (0.65 m^2 × 0.05 m × 1500 kg/m^3). Therefore, the calculated concentration of avilamycin residues in the top 5 cm of the ring is 0.88 mg/kg (42.8 mg ÷ 48.75 kg). Because measurements were taken for 7.5 cm segments, the mean concentration in the top 7.5 cm segment would be expected to be approximately 0.587 mg/kg (0.88 mg/kg ÷ 1.5) due to the inclusion of 2.5 cm of "clean" soil. However, the initial measured concentration of radioactive residues (as measured by combustion of the core soil sample taken from 0 to 7.5 cm depth) was 0.38 mg/kg. The amount of avilamycin residues measured in the soil at the initiation of this experiment (0.38 mg/kg) is comparable to expected concentrations in the soil (PEC_{soil} = 0.197 mg/kg) as calculated in Section 4.1.2).

Results:

Avilamycin-related substances dissipated quickly during the first 2 weeks of the study (only 77.9% was found at the 2 week sample). During this time there was very little precipitation (less than 1 cm) indicating that the loss was not due to leaching. Within 4 weeks only 46.6% of the initial radioactivity was accounted for in the soil with the majority of the upper 15 cm (34.4% in the 0 to 7.5 cm core and 6.6% in the 7.5 to 15 cm core). Throughout the yearlong study, the 0 to 7.5 cm core sample contained most of the radioactivity. The radioactivity present in the first 7.5 cm of soil continued to decline in subsequent sampling periods. Samples from the 0 to 7.5 cm

depth still contained the majority of radioactivity even 36 and 52 weeks after application. However a small amount of leaching did occur as evidenced by counts in the 15 to 30 cm depths.

Time (weeks)	% Initial Radioactivity			
	Core Segment (cm)			
	0-7.5	7.5-15	15-22.5	22.5-30
0	100	NT	NT	NT
1	63.2	6.6	NT	NT
2	57.1	20.8	NT	NT
3	54.7	13.7	18.4*	4.2
4	34.4	6.6	4.2	1.4
6	23.1	5.7	5.7	2.4
8	25.9	3.8	NT	NT
15	17.9	4.7	1.9	9.4
36	19.8	4.7	1.4	3.8
52	16	3.8	1.9	2.4

"NT" = not tested
*=sample considered to be contaminated

Appendix J - Study 66679: Avilamycin: Aerobic transformation in four soils. Report Date: 2012.

Performing Laboratory: ABC Laboratories Inc.

Study Design: GLP, OECD Guideline 307

Test Article: ^{14}C Avilamycin, the radiolabel is distributed uniformly throughout the molecule

Methods:
Four soils were fortified with 1 mg/kg ^{14}C-avilamycin and incubated under aerobic conditions at 20°C in the dark for up to 120 days with trapping of effluent gases in KOH. At sampling timepoints, evolved $^{14}CO_2$ was measured by liquid scintillation counting of the KOH traps, soils were extracted, and post-extracted soils were combusted. The radioactivity in the soil extracts was profiled by HPLC with radiometric detection. Major degradation products were isolated by fractionation and identified by LC/MS/MS.

The following summarizes the soil characteristics:

Name	USDA Textural Class	pH	% Organic Matter
Audrain	Loam	5.9	5.1
Tift	Loamy Sand	5.1	1.3
MSL-PF	Sandy Clay Loam	6.8	2.0
Raymondville	Sandy Clay Loam	8.0	0.83

Results:
Avilamycin showed rapid degradation in the four soils evaluated. After 7 days only 11% or less of the dosed avilamycin was detected in all four soils. A varying amount of avilamycin was mineralized to CO_2. At the end of incubation the amount of radioactivity in the KOH traps ranged from 15.1 to 58.0% of the applied radioactivity (AR).

There was a large amount of nonextractable residue observed in the study. Since the three soils with the highest amount of mineralization (27% to 58.0% AR) were also the soils with the largest amount of AR as nonextractable residue (31.7 to 37.1% AR), it is likely that the non-extractable residue was composed of small degradation products that had been incorporated in to the microbial-soil matrix.

Mass Balance and Transformation Kinetics:

	Audrain		Tift		MSL-PF		Raymondville	
Day	**7**	**120**	**7**	**120**	**7**	**120**	**7**	**51**
Mass Balance*	100.7	99.5	99.6	95.6	101.2	93.8	101.2	102.1
$^{14}CO_2$*	2.6	15.1	2.4	27.7	1.5	42.7	2.2	58.0
Nonextractable Residue*#	7.9	15.3	7.4	31.7	9.9	27.8	13.0	37.1
Extractable Radioactivity*	90.2	69.1	89.8	36.1	89.7	23.2	85.9	7.1
Avilamycin*$	1.8	0.3	10.0	0.4	7.5	0.1	10.9	1.1
DT50 (days)	0.2		1.5		0.4		1.0	
DT90 (days)	0.6		5.1		1.2		28.6	

*All values are percent of applied radioactivity
#By combustion
$In extract
DT50 and DT90 values are for avilamycin

Six major degradation products were observed over the course of the study. The degradation products suggest that there are two degradation pathways of avilamycin. In the primary pathway, hydrolysis leads to a cleavage of the avilamycin to the smaller flambic acid (M4 in the table below) which is can convert to flambalactone (M3) and the remainder of the avilamycin molecule (M2). In the other degradation pathway, which was observed in the Audrain soil more than in the other three soils, the eurekanate portion of avilamycin is removed to form M5.

Summary of the observed transformation products, with the maximum amount reached and the day of the maximum amount observed:

	Maximum %Applied Radioactivity observed in various soils			
Product	**Audrain**	**Tift**	**MSL-PF**	**Raymondville**
Avilamycin	75.7 (Day 0)	86.0 (Day 0)	87.4 (Day 0)	88.7 (Day 0)
M1	62.4 (Day 1)	19.2 (Day 10)	20.0 (Day 14)	26.4 (Day 3)
M2	51.1 (Day 2)	45.5 (Day 7)	58.2 (Day 3)	39.4 (Day 7)
M3	15.4 (Day 73)	15.7 (Day 10)	< 10	< 10
M4	13.8 (Day 2)	16.4 (Day 3)	23.7 (Day 3)	< 10
M5	22.9 (Day 51)	< 10	< 10	< 10
M6	27.9 (Day 120)	< 10	< 10	< 10

In general, the major degradation products of avilamycin were further degraded in the soil.

Appendix K - Study EWD8609: Soil adsorption of avilamycin. Report Date: 1986.

Performing Laboratory: Lilly Research Laboratories

Study Design: Similar to OECD 106

Test Article: ^{14}C Avilamycin obtained by fermentation starting with ^{14}C-diethylmalonate as a precursor; more than 85% of the label is in the dichloroisoeverninic moiety.

Methods:
A soil adsorption study was conducted with ^{14}C avilamycin in sandy loam, loam, and clay loam soils. Ten gram portions of the soils were equilibrated for 16 hours with 40 mL portions of 0.01 M $CaCl_2$ and ^{14}C avilamycin in 50 mL centrifuge tubes. After centrifuging, the avilamycin content of the aqueous layer was determined radiochemically. The amount adsorbed to the soil was determined by difference.

The pH value of 0.01 M $CaCl_2$ was brought to 7.3 by autoclaving for 20 minutes and then boiling for an additional 10 minutes to remove dissolved CO_2. Soilless controls were included to monitor adsorption to the glass vessels. Approximately 5% was found to bind to the vessels without soil. It was assumed that this amount also bound to the vessels when soil was added, thus, the amount assumed to adsorb to glass was subtracted from the amount bound to soil.

Results:

Soil	pH	% Organic Matter	% Organic Carbon[1]	Kd	Koc[2]
Sandy loam	4.9	2.6	1.5	51	3400
Loam	6.7	2.2	1.3	23	1769
Clay loam	7.0	5.1	3.0	109	3633

[1]Soil organic matter contains approximately 58% organic carbon, %OC was calculated by multiplying %OM by 0.58.
[2]Koc was calculated by dividing Kd by the fraction of organic carbon.

The Kds were based on total radioactivity. Given the pH values of the soil, it is likely that there was some degradation of avilamycin over the 16 hours of the equilibration study.

Appendix L - Study 66678: Avilamycin: Determination of Adsorption/Desorption Using the Batch Equilibrium Method. Report Date: 2012 .

Performing Laboratory: ABC Laboratories Inc.

Study Design: GLP, OECD Guideline 106

Test Article: ^{14}C Avilamycin, the radiolabel is distributed uniformly throughout the molecule

Methods:
Tier 1: Preliminary method development: The stability, sorption to filters and vessels, and solubility of avilamycin were investigated along with various soil:solution ratios. During the preliminary work, changes to the protocol were made to overcome the instability of avilamycin in acidic soils, the low solubility of avilamycin in acidic media, and the tendency of avilamycin to adsorb to filters and vessels. These changes were implemented in Tier 2 and included boiling and adjusting the 0.01 M $CaCl_2$ to pH 7 and preconditioning the vessels to reduce adsorption of avilamycin to the vessels.

Tier 2 Kinetic adsorption: ^{14}C-avilamycin was equilibrated with 5 soils suspended in 0.01 M $CaCl_2$ in pre-conditioned glass tubes at an initial aqueous concentration of 1 µg/mL. At 0.25, 1, 2, 4, and 20 hours, duplicate tubes were sacrificed and the phases separated by centrifugation. Partitioning between soil and water was calculated by measuring total radioactivity in the aqueous supernatant and calculating the amount in the soil by subtracting the supernatant radioactivity from what was originally added. Additionally, the soils were extracted and both the soil extract and the aqueous phase were analyzed via fractionation with radiometric detection. Based on the fractionation data, the partition coefficient specific for avilamycin was determined.

Tier 3: Isotherm test with the Raymondville soil (in which avilamycin was stable): Varying concentrations of ^{14}C-avilamycin (0.01 to 1 µg/mL in the aqueous phase) were equilibrated with Raymondville soil suspended in 0.01 M $CaCl_2$ (1:5 soil:solution ratio). Partitioning between phases was evaluated after a 2-hour adsorption step followed by a 2-hour desorption step.

Soils Used:

Name	USDA Textural Class	pH[a]	% Organic[b] Matter
Tift	Sand	5.2	1.1
Tehama	Loam	6.0	2.9
Audrain	Silty Clay Loam	6.3	5.4
Raymondville	Sandy Clay Loam	8.0	0.89
MSL-PF[c]	Sandy Clay Loam	6.8	2.0

[a] pH in 1:1 soil:water ratio

[b] Determined by Walkley-Black method

[c] Mutcher Sandy Loam-Pesticide Free

Results:

Avilamycin was found to degrade rapidly in all soils except the most alkaline sandy clay loam (Raymondville), thus, equilibrium could not be established. Partitioning coefficients were determined for total radioactivity and also for avilamycin. The Kd and Koc values presented below for each soil were determined using all timepoints for which a value was calculated.

Soil	pH	%OM	%OC	Total Radioactivity		Avilamycin	
				Kd	Koc	Kd	Koc
Sand	5.2	1.1		3.57	559	3.87	605
Loam	6.0	2.9		56.0	3321	22.3	1325
Silty clay loam	6.3	5.4		66.5	1987	16.9	537
Sandy clay loam	8.0	0.89		2.05	397	2.01	388
Sandy clay loam	6.8	2.0		143	11952	29.3	2444
Average over all five soils				**54**	**3643**	**15**	**1060**

Appendix M - Study ABC-0337: Evaluation of the soil mobility of avilamycin and its major fecal metabolite by soil thin-layer chromatography. Report Date: 1986.

Performing Laboratory: Lilly Research Laboratories

Study Design: GLP

Test Article: ^{14}C Avilamycin obtained by fermentation starting with ^{14}C-diethylmalonate as a precursor

Methods:
Soil TLC plates were prepared using coarse, medium and fine-textured soils. Radiolabeled herbicides trifluralin, atrazine, and dicamba were used as reference compounds representative of immobile, intermediately mobile, and highly mobile chemicals, respectively. ^{14}C Avilamycin and ^{14}C Metabolite A (flambalactone) were applied to to soil TLC plates along with the reference compounds. Plates were developed in water and radioautographs were prepared. Frontal R_f values of test and reference compounds were determined.

Results:
Avilamycin exhibited low mobility on all three soils. It was more mobile than triflualin but considerably less mobile that atrazine or dicamba. Using the soil mobility classification system described by Helling and Turner (1968), avilamycin would be considered a class 2 (low mobility) compound.

On the medium and fine-textured soils, flambalactone was much more mobile than avilamycin. Compared to the reference standards on these two soils, flambalactone was more mobile than atrazine but less mobile than dicamba. On the coarse soil, flambalactone exhibited low mobility of Metabolite A which could be due to the low pH of the soil (pH 4.9) and to the chemical nature of the metabolite itself (a lactone which can be readily hydrolyzed to the corresponding carboxylic acid). Using Helling and Turner's classification system, flambalactone would be considered a mobile compound (class 4 or class 5) on nonacidic soils and a compound of low mobility (class 2) on acidic soils (pH less than 5.0).

Helling CS, Turner BC. 1968. Pesticide Mobility: Determination by Soil Thin-Layer Chromatography. Science 162:562-563.

Soil thin-layer R_f values for test and reference compounds:

	R_f Value				
Soil Type	**Trifluralin**	**Avilamycin**	**Atrazine**	**Flambalactone**	**Dicamba**
Coarse	0.07	0.09	0.48	0.27	0.88
Medium	0.05	0.26	0.58	0.84	0.98
Fine	0.07	0.12	0.68	0.95	1.00

Appendix N - Study S-AAC-82-04: A four-soil laboratory leaching study with avilamycin and an aged soil leaching study with avilamycin. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: ^{36}Cl Avilamycin

Methods:
Radiolabeled ^{36}Cl avilamycin was applied to the top of soil columns prepared with sand, sandy loam, loam, and clay loam soils. Each column was 30 cm by 1.0 cm i.d. The columns were leached with the equivalent with 60 cm water. Leachate was collected in 10-cm fractions and was analyzed radiochemically. At the end of the leaching process, the columns were broken into 5-cm sections, the sections were extracted, and the extracts and leachates were analyzed radiochemically.

In a second study to determine the mobility of soil degradation products, a sandy loam soil fortified with 7.6 µg/g ^{36}Cl avilamycin was aged 30 days. After 30 days, the soil was applied to the top of a 30 cm by 6.35 cm (i.d.) soil column. The column was leached with the equivalent of 1.25 cm rainfall per day for 45 days. At the end of the experiment, the column was broken into 5-cm sections, and the soil sections and leachate fractions were analyzed radiochemically.

Results:
Radioactivity leached in all four soils employed in the first part of the study. Total amounts of applied radioactivity found in the leachate were 22.1, 53.3, 26.7 and 22.7 % found for the sand, sandy loam, loam, and clay loam soils, respectively. Significant radioactivity was found in all soil sections of the columns. The results of the study indicate that avilamycin or its degradation products leached under the conditions employed and suggest that avilamycin or its hydrolysis products has potential for leaching in the environment.

The results of the part of the study that looked at radioactivity after incubation in soil, indicate that avilamycin and/or soil degradation products are susceptible to leaching. A total of 84.4 % of the radioactivity was found in the leachate. Only 11.8% of the applied radioactivity remained on the column and the majority of it was in the top section. A total of 96.2% of the applied radioactivity was accounted for at the end of the experiment.

Appendix O - Study 70541080: Effects of crystalline avilamycin on the activity of the soil microflora in the laboratory. Report Date: 2012.

Performing Laboratory: IBACON GmbH

Study Design: GLP, OECD Guidelines 216 and 217

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Three concentrations of avilamycin in a loamy soil (1, 5, and 15 mg avilamycin/kg soil (dry weight)* were evaluated for effects on carbon and nitrogen transformation compared to untreated controls. Soil for the nitrogen transformation test was amended with lucerne meal prior to dosing with avilamycin. The concentration of avilamycin in the dosing solution (acetone) was confirmed by HPLC with uv detection. The soils were divided into three replicates per concentration for each test (carbon and nitrogen) and were incubated in plastic boxes at 20 to 23°C.

For carbon transformation, at 0, 7, 14, and 28 days after treatment with avilamycin, soil samples were removed in triplicate and supplemented with glucose. CO_2 production was measured for 24 hours following glucose addition.

For nitrogen transformation, at 0, 7, 14, 28, 42, and 56 days, the soil was sampled and analyzed for nitrate. The nitrogen transformation rate was calculated from nitrate levels at the time point compared to the nitrate levels at Day 0.

Results:

Carbon Transformation:

		Treatment Level		
	Control	1 mg/kg*	5 mg/kg	15 mg/kg
DAY 28 Respiration Rate mg CO_2/kg dry soil per hour	11.9	11.9	11.5	11.4
% Deviation from Control		-1	-4	-4

Nitrogen Transformation:

		Treatment Level		
	Control	1 mg/kg	5 mg/kg	15 mg/kg
DAY 28 Nitrate Content	32.9	33.4	36.4	38.6
DAY 28 Nitrogen Transformation Rate mg/kg dry soil per day	0.58	0.60	0.68	0.78
% Difference from Control		3	17	34
DAY 56 Nitrate Content	63.8	---	---	69.0
DAY 56 Nitrogen Transformation Rate mg/kg dry soil per day	0.84	---	---	0.93
% Difference from Control		---	---	11

The respiration rates in the soil treated with avilamycin up to 15 mg/kg were within 25% of the control soil.

Therefore, exposure to avilamycin has no impact on respiration activities of soil microorganisms up to 15 mg avilamycin/kg.

During the early weeks of the study, the soils treated with avilamycin had an increased amount of nitrate content compared to the control soil. The increase was concentration-dependent. On Day 28, the nitrogen transformation rate in the highest avilamycin treatment (15 mg/kg) was increased over the control by 34%. At 1 and 5 mg/kg, the nitrogen transformation rates were less than 25% different from the control. Therefore, the control and the highest avilamycin-treated soil were continued in incubation and evaluated at Day 42 and Day 56. By Day 56, the nitrogen transformation rate in the highest avilamycin treatment was increased over the control by only 11%.

Exposure to avilamycin results in transient increases in nitrogen transformation over the control. By Day 28, those increases are less than 25% that of the control in the 1 and 5 mg/kg. By Day 56, the increase in nitrogen transformation rate in the highest concentration is less than 25% that of the control. Therefore, avilamycin has no lasting impact on nitrogen transformation activities of soil microorganisms.

*Test concentrations are given as mg avilamycin/kg soil (dry weight)

Appendix P - Study S-AAC-82-19: The effect of avilamycin on nitrification in soil. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Manure from swine fed a diet containing 80 mg/kg avilamycin

Methods:
Swine feces were collected from pigs on a basal ration with and without 80 mg/kg avilamycin as the dried fermentation product. A soil classified as a loamy sand was fortified with 400 mg/kg ammonium nitrogen by means of spraying with an aqueous solution of ammonium sulfate. Dried feces were mixed into the soil at two rates equivalent to 15 and 30 grams wet weight manure/kg dry soil. Two controls were included, soil without feces and soil amended with 30 grams wet weight manure/kg soil from pigs not treated with avilamycin. The soil-manure mixtures were added to pots and watered to a moisture level of 75% of saturation.

The avilamycin activity in the treated manure was determined at initiation of the study using a microbiological agar plate method using *Micrococcus flavus* as the indicator organism. Based on microbiological activity in the feces, when the manure was applied to soil, the soil-manure mixtures contained 0.83 and 1.66 mg avilamycin per kilogram dry soil.

At initiation and at 1, 2, 3, 4, and 5 week intervals, soil samples were removed and analyzed for ammonium and nitrate to determine if nitrification, the process of ammonium oxidation to nitrate, was affected by avilamycin.

Results:
During the early weeks of this study, avilamycin demonstrated a concentration-response relationship with regard to the inhibition of nitrification in soil. The presence of manure alone in the soil also inhibited nitrification. The effect of avilamycin and manure appears to be transient. By 4 weeks, the amount of ammonia remaining in the samples is less than 10% of the initial values in all treatments. Also, at 4 weeks, all treatments have reached an approximate plateau in nitrate levels and neither avilamycin treatment is more than 25% different from either control.

Treatment		Ammonium Nitrogen (ppm)					
	Soil Rep	0 wk	1 wk	2 wk	3 wk	4 wk	5 wk
Manure-free soil	1	287	238	98	4	6	1
	2	280	255	121	10	7	1
30 grams/kg* control manure	1	364	236	170	34	18	4
	2	374	234	127	24	12	3
15 grams/kg* avilamycin manure	1	330	263	119	20	10	2
	2	328	272	138	12	12	3
30 grams/kg* avilamycin manure	1	338	316	230	46	12	3
	2	357	326	258	51	12	3

*kg of dry soil

Treatment		Nitrate Nitrogen (ppm)					
	Soil Rep	0 wk	1 wk	2 wk	3 wk	4 wk	5 wk
Manure-free soil	1	32	54	259	363	362	375
	2	32	65	244	384	397	394
30 grams/kg* control manure	1	18	10	101	263	315	310
	2	13	16	104	248	339	389
15 grams/kg* avilamycin manure	1	29	30	226	407	406	406
	2	22	42	210	389	331	388
30 grams/kg* avilamycin manure	1	13	11	68	316	365	395
	2	25	12	65	346	408	425

*kg of dry soil

The following table shows further post-study analysis of the nitrate data included in the report to calculate the effects as percent differences from the controls.

Treatment	Average Nitrate Nitrogen at 4 weeks	% Difference from Manure-Free Soil	% Difference from Manure Control Soil
Manure-free soil	379.5	--	--
30 g/kg control manure	327	-13	--
15 g/kg avilamycin manure	368.5	-3	+13
30 g/kg avilamycin manure	386.5	2	+18

Appendix Q - Study ABC-0263: Determination of the effect of avilamycin on seed germination. Report Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Study Design: GLP

Test Article: Crystalline mixture of avilamycin factors (72.8% A, 9.9% B, 3.2% A', 2.1% C, 1.1% D1, 0.5% D2, 0.1% E and 10.3% unidentified components)

Methods:
Aliquots of 20 mL of distilled water containing avilamycin were added to two layers of filter paper in a petri dish. There were four replicates of each avilamycin treatment: 0.274 mg/petri dish, 0.548 mg/petri dish, and 1.096 mg/petri dish. There were also 4 replicates of a control treatment with just distilled water. Seeds of corn (*Zea mays*), wheat (*Triticum aestivum*), soybean (*Glycine max*), pinto bean (*Phaseolus vulgaris*), sweet pepper (*Capsicum annuum*), and tomato (*Lycopersicon esculentum*) were placed on the filter paper and germinated in the dark for 4 to 7 days at 25°C. Seeds were monitored for germination.

Results:
Seeds of all six plant species treated with avilamycin at all treatment levels had germination rates similar to that of the control seeds.

	% Germination					
mg/dish	Pinto Bean	Soybean	Corn	Wheat	Sweet Pepper	Tomato
0	62.0	72.0	90.0	68.3	66.7	91.7
0.274	68.0	72.0	94.7	72.7	64.0	82.7
0.548	75.0	78.0	92.8	65.0	65.0	88.3
1.096	62.0	75.0	85.3	79.3	62.7	87.3

To put the treatments in this study in perspective for the current risk assessment for broiler chickens, the amount of avilamycin per petri dish can be used to calculate the equivalent amount of avilamycin applied per acre as litter. Using the surface area of the petri dish (0.0081 m^2), the amount of mg avilamycin applied to an acre (4046.856 m^2) is calculated. Then, assuming that there are 19.5 mg avilamycin per kg of litter (Section 4.1.1), the amount of litter applied to an acre:

mg/petri dish	mg avilamycin/acre	kg litter/acre
0.274	136,893.6	3,510
0.548	273,787.3	7,020
1.096	547,574.6	14,040

Appendix R - Study ABC-0262: Phytotoxicity study with manure from chickens treated with avilamycin. Report Date: 1984.

Performing Laboratory: Lilly Research Laboratories

Study Design: GLP

Test Article: Manure from chickens fed a diet containing 100 ppm avilamycin

Methods:
Corn (*Zea mays*), tomato (*Lycopersicon esculentum*), soybean (*Glycine max*), and wheat (*Triticum aestivum*) plants were grown from seed in pots containing soil dosed with manure from chickens maintained on a control ration or a ration containing 100 ppm avilamycin. Each experimental pot was dosed with 1.8 g of dried manure (7.4 g wet manure) which theoretically contained 1.46 mg of avilamycin residues. The pots were six inches in diameter (0.1524 m; thus the surface area was 0.01824 m^2) containing soil 6 inches deep. Assuming that the soil had a mass of approximately 1500 kg/m^3, the weight of soil in the pot was approximately 4 kg (0.01824 $m^2 \times 0.1524$ m $\times$ 1500 kg/m^3). Therefore, the approximate concentration in the pots of avilamycin residues was 0.365 mg/kg (1.46 mg ÷ 4 kg).

Measurements of shoot height were made 14 and 21 days after planting. At the termination of the test, 21 days after seeding, the shoots were cut at the soil line and the fresh weight of both the shoots and the roots (as composite samples) were measured.

Results:

Plant Type	Height of Plants (cm)			
	14 Days		21 Days	
	Control	Avilamycin	Control	Avilamycin
Corn	38.5	39.4	64.7	65.4
Wheat	25.4	24.8	32.1	29.6
Soybean	11.1	12.0	16.8	17.0
Tomato	3.0	3.0	7.3	5.8*

Plant Type	Mean Weight/Plant (g)			
	Shoots		Roots	
	Control	Avilamycin	Control	Avilamycin
Corn	8.82	10.21	4.69	4.04
Wheat	1.12	0.92	0.47	0.31*
Soybean	3.61	3.39*	2.29	2.29
Tomato	1.48	0.81*	0.14	0.08*

*Significantly different from control using t-test (statistical analysis using replicate data was conducted after the final report was complete)

At 21 days, tomato plants grown in soil treated with avilamycin-manure were significantly smaller than the controls both in height and in shoot and root weight (t-test, $p<0.05$). The treated tomato plants were greener than control plants and morphologically normal.

In wheat, there was a 34% decrease in the root weight of the treatment group compared to the control which was statistically significant (t-test, $p<0.05$). Given the difficulty in removing the root mass completely and cleanly from soil, it is unclear if the decrease in root weight is meaningful, especially considering that the decrease in shoot weight (17.9% compared to control) was not as pronounced and was not statistically significant.

In soybean, there was a 6% decrease in shoot weight in the treatment group compared to control. This decrease is statistically significant (t-test, $p<0.05$), but is not considered biologically meaningful given the small size of the change and the lack of effect in other parameters.

Manure from avilamycin-treated chickens had no effect on the growth of corn.

Appendix S - Study 66369: Effects of avilamycin on the seedling emergence and early seedling growth of terrestrial plants following OECD guideline 208. Report Date: 2012.

Performing Laboratory: ABC Laboratories Inc.

Study Design: OECD 208, GLP

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
A 21-day seed emergence and growth study was conducted to determine the effects of avilamycin on corn (*Zea mays*), soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), oat (*Avena sativa*), radish (*Raphanus sativus*), and sugar beet (*Beta vulgaris*). A sandy loam soil was fortified with avilamycin at concentrations of 7.79 (tomato only), 15.6, 31.2, 62.5, 125, 250, and 500 mg avilamycin activity/kg soil (dry weight). Soil fortification was achieved by dissolving avilamycin in acetone, applying the acetone dosing solution to sand, and then mixing the sand with the soil. The concentrations of avilamycin in the acetone dosing solutions were confirmed analytically by HPLC/uv. A blank control and a solvent control were included in the study. There were six replicate pots per treatment level with five seeds planted in each pot. The study was conducted in a greenhouse and over the course of the study, the average temperature was 28.4°C, the average relative humidity was 81%, and the average daily light was 497 $\mu E\ m^{-2}s^{-1}$. Emergence, survival, visual injury, shoot length and replicate shoot weight were evaluated as endpoints.

Results:
There were no significant trends in emergence, survival, shoot length or shoot weight following treatment with avilamycin. Therefore, LC50 for survival was >500 mg/kg soil (dry weight) for all species. The EC50 values for emergence, shoot length, and shoot weight were >500 mg/kg soil (dry weight) for all species tested. The no-observed-effect concentrations for all endpoints in all species were equal to 500 mg/kg soil (dry weight).

Emergence and Survival				Avilamycin mg/kg in soil (dry weight)						
	Blank	**Vehicle**	**Pooled**	**7.79**	**15.6**	**31.2**	**62.5**	**125**	**250**	**500**
Corn										
% Emergence	97	97	97	--	100	100	100	97	100	97
% Survival	100	100	100	--	100	100	100	100	100	100
Oat										
% Emergence	87	100	93	--	100	97	87	100	93	100
% Survival	100	100	100	--	100	100	100	100	100	100
Radish										
% Emergence	100	97	98	--	87	97	97	97	93	97
% Survival	100	97	98	--	100	100	100	100	100	100
Soybean										
% Emergence	100	90	95	--	100	97	90	93	97	93
% Survival	100	96	98	--	100	100	100	96	100	100
Sugar Beet										
% Emergence	90	97	93	--	93	97	93	100	90	100
% Survival	100	100	100	--	100	100	100	90*	96	100
Tomato										
% Emergence	90	97	93	90	84*	97	97	97	86	97
% Survival	100	97	98	100	100	100	97	100	100	100

*These were significantly lower than the pooled control. However, given the lack of dose response these decreases were not considered to be related to avilamycin.

Individual Shoot Length and Replicate Shoot Weight				Avilamycin mg/kg in soil (dry weight)						
	Blank	**Vehicle**	**Pooled**	**7.79**	**15.6**	**31.2**	**62.5**	**125**	**250**	**500**
Corn										
Shoot length mm	752 (43.9)	739 (68.1)	746 (21.8)	--	785 (57.1)	745 (44.1)	750 (53.0)	742 (48.0)	765 (41.9)	734 (60.5)
Shoot weight g	3.445 (0.560)	3.517 (0.441)	3.481 (0.441)	--	4.116 (0.288)	3.320 (0.334)	3.628 (0.331)	3.610 (0.300)	3.461 (0.339)	3.141 (0.427)
Oat										
Shoot length mm	310 (20.4)	329 (12.1)	320 (8.28)	--	327 (37.7)	319 (25.0)	345 (20.0)	316 (15.3)	306 (21.6)	310 (12.4)
Shoot weight g	0.2439 (0.0626)	0.3304 (0.0645)	0.2872 (0.0577)	--	0.2752 (0.0240)	0.2901 (0.0281)	0.2704 (0.0766)	0.2871 (0.0391)	0.2580 (0.0467)	0.2711 (0.0374)
Radish										
Shoot length mm	134 (26.7)	149 (14.9)	142 (17.4)	--	150 (9.25)	142 (11.4)	142 (15.3)	132 (24.6)	142 (18.4)	143 (10.1)
Shoot weight g	0.4032 (0.122)	0.5256 (0.144)	0.4644 (0.0723)	--	0.4956 (0.0954)	0.5052 (0.0969)	0.5460 (0.110)	0.3907 (0.0671)	0.4631 (0.0769)	0.4328 (0.0698)
Soybean										
Shoot length mm	489 (59.9)	495 (97.7)	493 (70.0)	--	510 (58.5)	488 (58.0)	484 (112)	488 (103)	515 (50.2)	507 (64.6)
Shoot weight g	2.975 (0.202)	2.616 (0.417)	2.795 (0.185)	--	2.906 (0.275)	2.703 (0.342)	2.562 (0.283)	2.586 (0.397)	2.768 (0.157)	2.496 (0.323)
Sugar Beet										
Shoot length mm	114 (22.3)	132 (12.1)	123 (11.3)	--	129 (19.7)	138 (14.5)	150 (15.3)	109 (23.6)	124 (16.8)	130 (9.93)
Shoot weight g	0.2629 (0.105)	0.3318 (0.0440)	0.2973 (0.0644)	--	0.3028 (0.0454)	0.3337 (0.0612)	0.3863 (0.0643)	0.2194 (0.106)	0.2635 (0.0768)	0.3158 (0.0468)
Tomato										
Shoot length mm	94.6 (11.9)	93.3 (9.11)	93.9 (8.03)	86.6 (6.84)	88.1 (7.17)	90.9 (5.95)	88.8 (9.25)	95.5 (6.76)	88.2 (7.17)	85.3 (7.13)
Shoot weight g	0.1523 (0.0278)	0.1528 (0.0236)	0.1525 (0.0232)	0.1377 (0.0403)	0.1666 (0.0517)	0.1497 (0.237)	0.1544 (0.0240)	0.1727 (0.0165)	0.1303 (0.0294)	0.1403 (0.0168)
There were no significant differences in treatment groups compared to the pooled control.										

Appendix T - Study W01882: The toxicity of soil-incorporated avilamycin (EL-750, Compound 48740) to earthworms (*Lumbricus terrestris*) in a 14-day test. Revised Report Date: 1988.

Performing Laboratory: Lilly Research Laboratories

Study Design: GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
Test media (rabbit feces, loamy sand soil, and water) was placed in 2-L glass jars. Three replicate jars were used for each of the following: control media; media containing 10 mg/kg soil (dry weight)* of nominal avilamycin activity; and media containing 100 mg/kg of nominal avilamycin activity. These media levels are equivalent to 0.0, 67.1, and 671 mg of dried fermentation product/kg. Five worms, about 4 g each, were placed into each of the nine jars at the beginning of the study. The test was conducted at 12°C. On day 7 and day 14 of the study, the worms were weighed, examined and described as follows: normal, flaccid, soft and flaccid, moribund, or dead.

Results:
All worms were found to be normal throughout the study. No flaccid, soft and flaccid, moribund or dead earthworms were found in any control or treatment group. The average body weights of worms exposed to 10 and 100 mg/kg were 4.530 and 4.397 g, respectively, and these were not significantly different from the average weight of control worms, 4.448 g. The average body weight increased during the study. Percent average body weight increases for the worms were: control, 11.5%; 10 mg/kg treatment, 11.4%; 100 mg/kg treatment, 10.7%. The average weight gains of worms in the two treatment levels were not statistically different from the average weight gain of control worms.

The 14-day no-observed-effect concentration (NOEC) for avilamycin in earthworms was 100 mg/kg and the EC50 was >100 mg/kg.

*Test concentrations are given as mg avilamycin activity/kg soil (dry weight)

Appendix U - Study 66370: Avilamycin: Survival and reproduction test with the earthworm, *Eisenia fetida*. Report Date: 2011.

Performing Laboratory: ABC Laboratories Inc.

Study Design: OECD 222, GLP

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Artificial soil was fortified with nominal concentrations of avilamycin of 87, 170, 330, 670, and 1300 mg/kg soil (dry weight). Fortification was conducted using acetone to deliver avilamycin to sand and then mixing the sand into the artificial soil. A blank control and a vehicle control were included. The concentration of avilamycin in the acetone dosing stock solutions was confirmed using an HPLC/uv method. Adult worms (10 per replicate, 8 replicates each for the blank and solvent controls and 4 replicates per avilamycin 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:
After 28 days of exposure, the percent mortality of adult worms was 3, 3, 0, 3, 0, 0, and 0% in the control, vehicle control, 87, 170, 330, 670, and 1300 mg/kg treatments. All of the live earthworms were observed to be normal.

The control worms lost an average of 17% in replicate mass during the 28-day exposure which the vehicle control worms lost an average of 13%. The percent change in replicate mass of worms in the avilamycin treatments ranged from a loss of 3% to a gain of 8%.

The average reproduction (% coefficient of variation) for the blank and vehicle control groups were 65 (27%) and 81 (27%). The mean number of juveniles per replicate in the pooled control (blank + vehicle) was 73 (% CV of 29%). The average number of juveniles per replicates was 78, 73, 66, 58, and 60 in the 87, 170, 330, 670, and 1300 mg/kg treatment levels which were 107%, 100%, 90%, 79%, and 82% of the pooled control value. While there was a trend towards reduced offspring with increasing concentration of avilamycin in the soil, there was no statistically significant reduction in the reproductive output of worms exposed to the test substance as compared to the pooled control reproduction.

The LC50 and EC50 values were estimated to be > 1300 mg avilamycin/kg. The highest concentration in which there was no statistically significant effect on earthworm survival, growth, and reproduction was 1300 mg/kg. Given the trend to decreased reproduction and the fact that the reproduction the highest two concentrations was decreased by more than 15%, the conservative NOEC will be considered to be 330 mg avilamycin/kg soil (dry weight).

*Test concentrations are given as mg avilamycin activity/kg soil (dry weight)

Appendix V - Study S-AAC-82-08: Avilamycin: Interaction with Sewage Microorganisms Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Test Article: Avilamycin dried fermentation product, containing 16.7% avilamycin activity

Methods:
Inoculum from a sewage plant's aerated lagoon was treated with avilamycin as the dried fermentation product. Treatments were made daily by adding increasing concentrations of avilamycin up to a maximum of 102.6 mg/L. Total volume in the aeration vessels was held constant by removing a portion of the supernate before replacing it with nutrient solution. Aeration was accomplished with laboratory air flowing at 3 ft^3/hr.

The effect of avilamycin on sewage microorganisms was determined by measuring biochemical oxygen demand (BOD, 5 day), viable cell counts, pH and dry weights.

The concentration of avilamycin activity in the test system was measured using a microbiological assay.

Results:
Analyses made on treated systems were compared to non-treated controls.

After daily treatments, initial BODs were naturally high in all systems, but subsequent analyses showed the expected reduction of nutrients. Even at high avilamycin concentrations, the microbiological digestive activity was not inhibited.

Colony-forming units were lower in the avilamycin-treated samples in the first eleven days. These findings indicate that avilamycin may adversely affect the microbial population in the sewage system for the first two weeks, but it is clear that the effect is transient. pH and solids in treated samples were not different from untreated controls.

Confirmatory dissolved oxygen utilization tests with acclimated inocula indicated no inhibition of respiration of sewage microorganisms.

The highest concentration of avilamycin activity measured in the test systems was 87.6 mg/L.

Appendix W - Study T4EFR0701: Blue-green algae (cyanobacterial) growth inhibition test. Report Date: 2007.

Performing Laboratory: CIT

Study Design: GLP, OECD Guideline 201

Test Article: Crystalline avilamycin

Methods:
A static toxicity test was conducted to evaluate the effects of avilamycin on the cyanobacterial strain, *Synechococcus leopoliensis*, over a 72 hour exposure. Cell suspensions were exposed to nominal concentrations of 0.625, 1.25, 2.5, 5 and 10 mg avilamycin activity/L. Three replicates were included for each test concentration and six replicates for a dilution water control. Cell growth data was recorded at 24, 48, and 72 hours by means of a Malassez cell counter. Data was expressed as yield and average specific growth rate.

Results:
During the test, the pH ranged from 8.07 to 8.25 and the temperature ranged from 22.6 to 23.7°C. The test was conducted under continuous lighting at 3700 to 3880 lux. There was some evidence of precipitation in 2.5, 5, and 10 mg/L test solutions.

Exposure to avilamycin caused a decrease in growth rate at higher concentrations. The magnitude of the decrease was greatest at 24 hours when the percent inhibition compared to control was 16% and 41% in the 5 and 10 mg/L treatment levels, respectively. After 72 hours, the inhibition was 0, 0, 2, 7, and 20% compared to control in the 0.625, 1.25, 2.5, 5 and 10 mg/L, respectively. The growth rates at 5 and 10 mg/L was statistically significantly different from that of the control. The EC50 for growth rate was >10 mg/L and the no observed effect level was 2.5 mg/L.

Yield at 72 hours was inhibited by 0, 0, 11, 33, and 69% compared to the control at 0.625, 1.25, 2.5, 5, and 10 mg/L, respectively. The yields at 5 and 10 mg/L were statistically different from that of the control, but since the inhibition at 2.5 was 11%, the NOEC was conservatively considered to be 1.25 mg/L. The EC50 for yield was determined to be 6.85 mg/L.

Appendix X - Study C03382: The acute toxicity of avilamycin (EL-750, Compound 48740) to *Daphnia magna* in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Study Design: EPA, ASTM, GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
A group of 30 and a group of 29 *Daphnia*, ≤24 hours old, were exposed to control water and to a nominal avilamycin activity concentration of 100 mg/L, respectively, for 48 hours. Each of the three replicate beakers contained 200 ml of test solution and were used to contain 9 or 10 daphnia for the treatment and control. Water samples were taken at 0 and 48 hours for analysis of the avilamycin concentration using an agar well method with *Micrococcus flavus*. Temperature, dissolved oxygen, and pH of the test solutions were measured at least at the beginning and end of the study. Total alkalinity, total hardness, and conductivity were measured for the dilution water. *Daphnia* were assessed for hypoactivity, prostration, and immobility.

Results:
The mean measured concentration of avilamycin in the test solution was 23.8 mg/L. No avilamycin was detected in the control solutions.

The water quality characteristics were as follows: pH, 7.3 to 8.0; dissolved oxygen concentration, at least 83% of saturation; temperature, 20.0˚C; total alkalinity, 148 mg/L (as $CaCO_3$); total hardness, 137 mg/L (as $CaCO_3$); and conductivity, 250 µmhos/cm.

No mortalities were found. No daphnia were hypoactive, prostrate or immobile in this study. The 48-hour no-observed-effect concentration (NOEC) for avilamycin in daphnia was 23.8 mg/L and the EC50 was >23.8 mg/L.

Appendix Y - Study 66272: Avilamycin: Acute toxicity to the water flea, Daphnia magna, determined under static test conditions following OECD guideline 202. Report Date: 2011.

Performing Laboratory: ABC Laboratories Inc.

Study Design: OECD 202, GLP

Test Article: Crystalline mixture of avilamycin factors (69.8% A, 8.02% B, 4.14% I+A', 2.95% L+M+N, 2.70% unknown avilamycin)

Methods:
Daphnids, <24 hours old, were exposed to each nominal treatment level of control, 9.98, 20.1, 40.1, 80.3, and 160 mg avilamycin activity/L (nominal) for 48 hours. Each of the four replicate beakers at each treatment level contained 200 ml of test solution and was used to contain 5 daphnids. Water samples were taken at 0 and 48 hours for analysis of the avilamycin concentration using an HPLC/uv method. Temperature, dissolved oxygen, and pH were measured at test initiation and termination. Total alkalinity, total hardness, and conductivity were measured in the dilution water at test initiation. *Daphnia* were assessed for immobility and sublethal effects.

Results:
Given evidence of insolubility (cloudiness) at the two highest nominal concentrations, the analytical samples were centrifuged prior to analysis. The mean measured concentrations of avilamycin in the test solutions were 0, 9.67, 19.8, 39.1, 68.8, and 138 mg/L.

The water quality characteristics were as follows: pH, 8.2 to 8.6; dissolved oxygen concentration, at least 93% of saturation; temperature, 20.2 to 21.0˚C; total alkalinity, 156 mg/L (as $CaCO_3$); total hardness, 148 mg/L (as $CaCO_3$); and conductivity, 337 µS.

No mortalities or sublethal effects were observed. The 48-hour no-observed-effect concentration (NOEC) for avilamycin in daphnia was 138 mg/L and the EC50 was >138 mg/L.

In the analysis, avilamycin had a short retention time under the chromatography conditions used. Therefore, it is possible that some degradation products may have been present but were integrated under the peak of avilamycin. For C03382 the pH ranged from 7.3 to 8.0, while in Study 66272 the pH ranged from 8.2 to 8.6. Avilamycin is more stable at pH 7 than at pH 9, so there was more hydrolytic potential in Study 66272. Therefore, it is possible that the invertebrates could have been exposed to some concentrations of the degradation products of avilamycin.

Appendix Z - Study F12782: The acute toxicity of avilamycin (EL-750, Compound48740) to bluegill (*Lepomis macrochirus*) in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Study Design: ASTM, EPA, GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
Three groups of 10 juvenile bluegill (mean individual weight, 1.0 g) were exposed for 96 hours to nominal avilamycin concentrations of 0.0 and 100 mg/L (671 mg/L of dried fermentation product). Samples of test solutions were taken at 0, 24, 48, 72, and 96 hours for analysis for avilamycin concentration using an agar well method with *Micrococcus flavus*. Dissolved oxygen concentration, pH and temperature were recorded daily for each jar. Total hardness, total alkalinity, and conductivity were recorded once for the dilution water. Behavioral signs of toxicity and mortalities were noted for fish in each jar on a daily basis.

Results:
Analyzed concentrations of avilamycin throughout the test ranged from 30.1 to 41.8 mg/L for the three replicate 15 L treatment jars and the overall avilamycin level was 35.4 mg/L. Avilamycin was not detected in any of the three 15L control jars. Water quality characteristics were as follows: pH, 8.0 to 8.7; dissolved oxygen at least 93% saturation; temperature, 20.0˚C; total hardness, 120 mg/L (as $CaCO_3$); alkalinity, 140 mg/L (as $CaCO_3$); and conductivity, 225 µmhos/cm.

No mortalities occurred in this study. No behavioral signs of toxicity were noted for any fish in this study. The 96-hour no-observed-effect concentration (NOEC) for avilamycin in bluegill was 35.4 mg/L and the LC50 was >35.4 mg/L.

Appendix AA - Study F12682: The acute toxicity of avilamycin (EL-750, Compound 48740) to rainbow trout in a static test system. Report Date: 1983.

Performing Laboratory: Lilly Research Laboratories

Study Design: ASTM, EPA, GLP

Test Article: Avilamycin dried fermentation product, containing 14.9% avilamycin activity

Methods:
Groups of 10 juvenile rainbow trout (mean individual weight, 1.09 g) were exposed to nominal concentrations of 0.0 (water control), 25, 50, 75, and 100 mg avilamycin activity/L for 96 hours. Jars with 15 L of test or control solution were used to contain each group of ten fish. Samples of test solutions were taken at 0, 24, 48, 72, and 96 hours for analysis of avilamycin concentration using an agar well method with *Micrococcus flavus*. 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 and mortalities were monitored for fish in each jar on a daily basis.

Results:
The mean measured concentrations of avilamycin for the five treatment levels were 0, 20.5, 39.8, 44.7, and 47.8 mg/L. Water quality characteristics were as follows: pH, 7.9 to 8.4; dissolved oxygen, at least 96% saturation in all test solutions; temperature, 12.0˚C; total hardness, 120 mg/L (as $CaCO_3$); total alkalinity, 140 mg/L (as $CaCO_3$); and conductivity, 225 µmhos/cm.

No mortalities occurred in this study. No behavioral signs of toxicity were noted for any fish in this study. The 96-hour no-observed-effect concentration (NOEC) for avilamycin in rainbow trout was 47.8 mg/L and the LC50 was >47.8 mg/L.