Elanco Animal Health
A Division of Eli Lilly and Company
2500 Innovation Way
Greenfield, IN 46140
USA

ENVIRONMENTAL ASSESSMENT REPORT

Environmental Assessment for the Use of Maxiban™ (narasin and nicarbazin) in Feed for Prevention of Coccidiosis in Broiler Chickens

September 2017

Table of Contents

List of Tables

List of Appendices

Environmental Assessment for the Use of Maxiban™ in Feed for Prevention of Coccidiosis in Broiler Chickens

1.0 Introduction

Maxiban™ (narasin and nicarbazin) is a Type A Medicated Article that is incorporated into chicken feed. Maxiban™ is approved (NADA 138-952) for the prevention of coccidiosis in broiler chickens caused by *Eimeria tenella*, *E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti*, and *E. mivati*. The current approval is for continuous feeding with a withdrawal time of 5 days before slaughter. The maximum concentrations of active ingredients of Maxiban™ in feed are 45 grams narasin/ton (50 mg/kg) and 45 grams nicarbazin/ton (50 mg/kg).

The following assessment is provided to support an application for the same use of Maxiban™ at the same maximum dosing but without a withdrawal period.

The environmental risk assessment has been conducted based on the VICH guidelines for both phase I (CVM 2001, VICH 2000) and phase II (CVM 2006, VICH 2004) assessments and on typical use of chicken litter as fertilizer in the United States.

2.0 Pattern of Use and Relevant Exposure Routes

Narasin and nicarbazin will be continuously administered to broiler chickens via the feed for the prevention of coccidiosis.

In this risk assessment, the use and exposure scenario is continuous administration of feed amended with narasin and nicarbazin (as Maxiban™) during the production period for broiler chickens and subsequent application of chicken litter to agriculture land. This is the primary route for environmental exposure.

3.0 Description of the Product

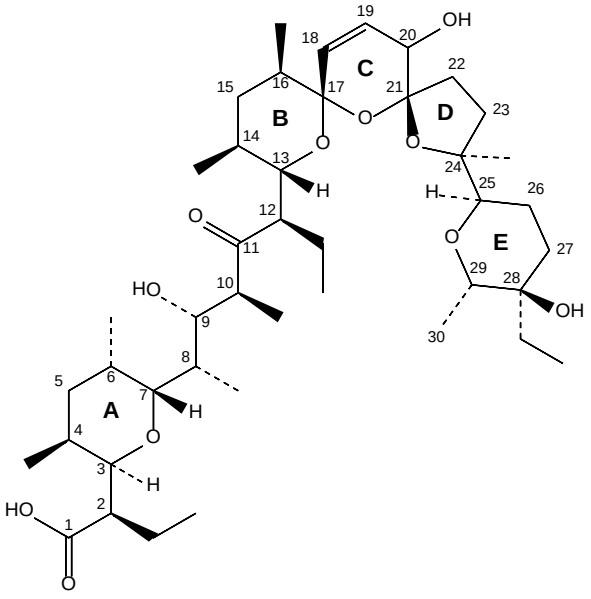
Maxiban™ is a Type A premix for incorporation into complete feed for broiler chickens. Narasin and nicarbazin are the active ingredients in Maxiban™.

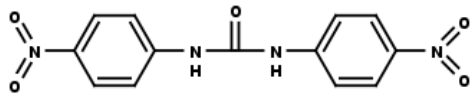
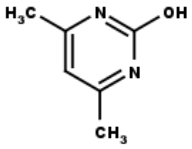
Narasin is produced by fermentation using a strain of *Streptomyces aureofaciens*. The fermentation culture is harvested such that the narasin is obtained mixed with the mycelial cells of the producing organism and unused components of the feed-stock used in the fermentation process. Thus, the dried mycelia or biomass form of narasin contains nutrients which are suitable for use in broiler feed. Maxiban™ contains sufficient quantities of this dried fermentation product to achieve a narasin concentration of 36 grams/pound of premix.

Narasin is a monocarboxylic polyether ionophore which complexes with monovalent alkali cations and has antimicrobial and anticoccidial activity.

Nicarbazin is a 1:1 molar complex of 4,4'-dintrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HDP). On a weight basis, DNC makes up about 70% while HDP is 30% of the nicarbazin. DNC and HDP form the nicarbazin complex when stirred in a methanol solution at room temperature (Rogers et al. 1983). The complexation of DNC, the biologically active component, with HDP serves to significantly increase its bioavailability from the gut compared to when DNC is administered alone or as a simple mixture of DNC and HDP (Rogers et al. 1983). The concentration of nicarbazin in the premix is 36 gram/pound.

Identification of Active Ingredients

Narasin	
Chemical Name:	(αβ, 2β, 3α, 5α, 6α)-α-ethyl-6-[5-[5-(5α-ethyltetrahydro-5β-hydroxy-6α-methyl-2H-pyran-2β-yl)-3"α,4,4",5,5"α,6"-hexahydro-3'β-hydroxy-3"β,5α,5"β-trimethylspiro]furan-2(3H),2'-[2H]pyan-6'(3'H),2"-[2H]pyran]-6"α-yl]-2α-hydroxy-1α,3β-dimethyl-oxoheptyl]-tetrahydro-3,5-di-methyl-2H-pyran-2-acetic acid or (4*S*)-4-Methylsalinomycin
CAS Number (Narasin):	55134-13-9
Molecular Formula (acid form):	$C_{43}H_{72}O_{11}$
Molecular Weight:	765.03 g/mol
Structural Formula for Narasin:	

Nicarbazin	
Chemical Name:	Equimolar complex of DNC and HDP
CAS Number:	330-95-0
Molecular Formula:	$C_{19}H_{18}N_6O_6$
Molecular Weight:	426 g/mol
DNC	
Chemical Name:	1,3-bis(4-nitrophenyl)urea; 4,4'-dinitrophenylurea; 4,4'-dinitrocarbanilide
CAS Number:	587-90-6
Molecular Formula:	$C_{13}H_{10}N_4O_5$
Molecular Weight:	302 g/mol
Structural Formula for DNC:	
HDP	
Chemical Name:	2-hydroxy-4,6-dimethylpyrimidine ; 4,6-dimethylpyrimidin-2-ol
CAS Number:	108-79-2
Molecular Formula:	$C_6H_8N_2O$
Molecular Weight:	124 g/mol
Structural Formula for HDP:	

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) (VICH 2000) were consulted to conduct the Phase I Environmental Impact Assessment for the use of Maxiban™ in broiler chickens. In this Phase I assessment, the maximum concentrations of narasin and the components of nicarbazin (DNC and HDP) in the excreta and the soil have been considered. No metabolism or degradation in excreta is assumed such that a total residue approach is taken for the Phase I assessment. The initiation of a Phase II assessment is dependent upon the trigger established in the VICH GL6 guidance: if the predicted environmental concentration of the total residue in soil is greater than 100 µg/kg, then a Phase II assessment is warranted.

4.1 Concentration in Litter

The concentrations of the components of Maxiban™ in chicken litter (Table 2) were estimated using the assumptions in Table 1 and the following calculations:

Table 1. Assumptions for calculating Maxiban™ components concentrations in litter

Concentration in Feed	narasin	50 mg/kg
	DNC	35 mg/kg
	HDP	15 mg/kg
Daily Feed Intake	0.13 kg/day	
Duration of Dosing	Continuous	
Excreta Production Period	50 days	
Amount of Diluent in Litter	15%	
Daily Excreta	0.13 kg	

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

$$= \frac{Concentration\ in\ Feed \times Feed\ Intake\ per\ Day}{Daily\ Manure\ Production \times 1.15}$$

$$= \frac{50\frac{mg\ narasin}{kg\ feed} \times \frac{0.13\ kg\ feed}{day}}{0.13\frac{kg\ manure}{day} \times 1.15} = 43.5\frac{mg\ narasin}{kg\ litter}$$

Table 2. Concentration of Maxiban™ components in chicken litter

Compound	Narasin	DNC	HDP
mg/kg in chicken litter	43.5	30.4	13.0

4.2 Concentration in Soil

The maximum concentrations of active ingredients in the soil have been calculated using typical agronomy practices for application of poultry litter to land.

In order to understand how poultry litter is applied to soil, nutrient management experts and extension service agents from several states which are leading producers of broilers in the United States were contacted. These experts and agents work with farmers on the nutrient management plans for their agricultural land. The rate of land application of chicken litter (e.g., tons applied per acre) varies depending on nutrient needs of the crops to be grown, potential yield of the crop, soil types, competing sources of organic fertilizer available, availability of nitrogen in the litter, the frequency of split fertilizer application (organic plus inorganic nitrogen), presence of

watersheds such that phosphorus limits apply, cost of transportation of litter to site of application and limited availability of litter. The actual rate of land application of chicken litter ranges from 2 to 4 tons per acre a single time per year. Although there are some situations of multiple applications of a lower rate or rare specific conditions where a maximum rate of 5 tons per acre once per year may be recommended (e.g., if a previously unused piece of land was cleared for agricultural use or if sufficient irrigation is available such that a higher crop yield can be achieved). Therefore, for this risk assessment, a maximum rate of 5 tons per acre will be assumed since this value should cover extreme situations. While tillage practices are also geographically specific, there are areas in which no-till practices predominate. In a no-till scenario, even without mechanical incorporation, a mixing of the litter into the top 5 cm of the soil is assumed due to wind and soil structure. Therefore, to calculate the maximum concentrations in soil, an application rate of 5 tons of litter per acre (4536 kg litter/acre) will be considered with mixing of the litter into the top 5 cm of the soil surface. The weight of 5 cm of soil in an acre (4047 m^2) is approximately 303525 kg (assuming a bulk density of 1.5 g/cm^3). The maximum concentrations of the components of Maxiban™ in soil (Table 4) were calculated assuming no degradation in excreta or soil and the assumptions in Table 3 as follows:

Table 3. Assumptions for calculating Maxiban™ component soil concentrations

Application Rate of Litter to Soil	4536 kg/acre
Mixing Depth into Soil	5 cm
Weight of Soil in 1 acre × 5 cm	303525 kg soil/acre

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

$$= \frac{43.5\frac{mg\ narasin}{kg\ litter} \times 4536\frac{kg\ litter}{acre}}{303525\frac{kg\ soil}{acre}} = 0.650\frac{mg}{kg} = 650\ \frac{\mu g\ narasin}{kg\ soil}$$

Table 4. Phase I Maxiban™ component soil concentrations

Compound	Narasin	DNC	HDP
µg/kg in soil	650	454	194

Since the initial soil concentration of each component of Maxiban™ is greater than 100 µg/kg, a Phase II environmental risk assessment was conducted for each component, as indicated in the FDA CVM Guidance for Industry #89 (CVM 2001) and the VICH GL6 Guidance (VICH 2000).

5.0 Phase II Environmental Impact Assessment

Since the initial soil concentrations of narasin, DNC and HDP were calculated to be 650, 454, and 194 µg/kg, respectively, in the Phase I assessment, a Phase II environmental risk assessment has been conducted. The 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) (VICH 2004) were consulted to conduct the Phase II assessment of the use of Maxiban™ in broiler chickens.

5.1 Tier A

5.1.1 Summary of Available Data

This section reviews environmental data that has been collected by the Sponsor and the Sponsor's partners as well as data that has been reported in the published literature. While the Sponsor and partners have collected environmental data since the late 1970's, only the definitive data used in the risk assessment have been summarized in the appendices and/or submitted in full study reports with this assessment. Information from the published literature is used for comparison when available.

5.1.1.1 Physical and Chemical Properties

The physical and chemical properties of narasin and components of nicarbazin are presented in Table 5. Neither narasin nor the nicarbazin components are expected to volatilize. Narasin and HDP are water soluble (Poole et al., Appendix A; Study 206378, Appendix B), while DNC has low solubility in water (based on weight of evidence from aquatic toxicity studies).

The hydrophobicity of narasin and the nicarbazin components has been measured as log Kow in studies that used a shake flask design. HDP has a very low log Kow reflective of its high water solubility (Study ADM-56, Appendix D). Narasin has the highest measured log Kow (Study 151C-120, Appendix C) of the three components.

For the DNC log Kow determination, ^{14}C-labeled DNC with a radiopurity of 99 to 100.1% was used (Study ADM-56, Appendix D) and the reported log Kow was 3.6. The amount of radioactivity found in the aqueous phase was very small compared to the amount of radioactivity in the octanol phase. Therefore, it is possible that some of the aqueous radioactivity could have been impurities and not DNC, which would underestimate the log Kow. The reported value of 3.6 is considered the minimum log Kow for DNC. As perspective, calculated log Kow values for DNC are similar to 3.6, indicating that it is unlikely that the log Kow is much higher than 3.6. For example, the Environmental Protection Agency's KOWWIN™ model (version 1.68; run using EpiWeb 4.1) predicts a value for DNC of 3.76 (using SMILES notation for DNC of O=C(NC1=CC=C(C=C1)N(=O)=O)NC1=CC=C(C=C1)N(=O)=O as input).

Table 5. Physical and Chemical Data for Narasin and Nicarbazin Components

	Narasin	DNC	HDP
Melting Point	98 to 100°C (Merck Index 2017)	312°C (Merck Index 2017)	201 to 205°C (Sigma-Aldrich 2014)
Aqueous Solubility (mg/L)	pH 7.0 102 pH 9.0 681 (Study Poole et al., Appendix A)	Expected to be less than 0.1 (weight of evidence from aquatic toxicity tests conducted between 12 and 22.3°C)	66000 to 71000 (pH 4 to 9) at 20°C (Study 206378, Appendix B)
Log Kow	pH 5 4.79 pH 7 4.85 pH 9 5.06 (Study 151C-120, Appendix C)	pH 5 ≥3.6 pH 7 ≥3.6 pH 9 ≥3.6 (Study ADM-56, Appendix D)	pH 5 -1.0 pH 7 -0.9 pH 9 -0.9 (Study ADM-56, Appendix D)

5.1.1.2 Fate

5.1.1.2.1 Metabolism and Excretion of Narasin

The metabolism and excretion of radiolabeled narasin by broiler chickens has been described in Study ABC-0260 (Appendix E) and Study T4H969301 (Appendix F).

In Study ABC-0260 (Appendix E), broiler chickens were administered ^{14}C-narasin in feed at a concentration of 80 mg/kg for 7 days. The excreta from days 4 through 7 was pooled, air-dried, homogenized and then assayed for total radioactivity and for parent using HPLC and microbiological assay. Relative quantities of metabolites were estimated from column and thin-layer chromatography. The concentration of narasin in excreta as measured by HPLC was 12.1 mg/kg excreta (on an air-dried basis) and 6.5 mg/kg excreta on a wet weight basis. The concentration of narasin activity was 11.5 mg/kg (on an air-dried basis) by microbiological assay. Parent narasin accounted for 5% of the total radioactivity in the excreta and accounted for all of the microbiological activity. The remaining radioactivity was characterized by fractionation followed by thin layer chromatography. Several previously identified metabolites were present, two trihydroxylated narasins and four dihydroxylated narasins.

In Study T4H969301 (Appendix F), broiler chickens were administered ^{14}C-narasin in feed at a concentration of 50 mg/kg for five days. A second group was administered ^{14}C-narasin and nicarbazin in feed, each at a concentration of 50 mg/kg for five days. Residues in the excreta were extracted and subjected to liquid chromatography (LC) to isolate narasin and its metabolites. Identification of residues was conducted using high performance liquid chromatography/ion spray/mass spectrometry. Using fractionation on a preparative silica column, fifteen separate metabolite peaks were identified and quantified using the amount of radioactivity in the fraction. The metabolites included a tetrahydroxylated narasin, several di- and trihydroxylated narasins and the corresponding derivatives of narasin factor B. Narasin B is narasin with the hydroxyl group on ring C oxidized to the ketone. About 50% of the total radioactivity in the excreta was characterized as hydroxylated metabolites and about 3% as parent narasin. The remaining radioactivity included 4% that was non-extractable, 6 to 8% that

was more polar and left in the aqueous methanol, and minor fractions in the LC in concentrations too low to characterize by HPLC/ISP/MS/LSC. The data showed that nicarbazin has no effect on the metabolism of narasin by chickens.

5.1.1.2.1.1. Biological Activity of Chicken Metabolites of Narasin

As discussed above, microbiological characterization in Study ABC-0260 (Appendix E) shows that the narasin metabolites (of which the majority are di- and trihydroxylated narasin) are not biologically active, because the amount of microbiological activity in the excreta expressed as narasin equivalents was equal only to the actual amount of narasin itself in the excreta. Therefore, the excreted metabolites do not contribute to the microbiological activity in the excreta.

Wong et al. (1977) demonstrated that the ionophoric activity of narasin could be characterized in isolated rat liver mitochondria. In this assay, ATPase activity, or ATP hydrolysis, induced by the addition of either valinomycin or monazomycin and alkali metal cation, was reduced by narasin. The effects of narasin metabolites on ATPase and oxygen uptake in rat liver mitochondria were then further characterized by Wong (Appendix G). In this study, four narasin metabolites were tested: metabolite F (a dihydroxy narasin), NM-3 (a dihydroxy narasin), NM-2 (a trihydroxy narasin), and the fourth was a mixture of NM-6 (a dihydroxy narasin) and NM-3. These four were individually tested in terms of their effects on ATPase activity and oxygen uptake upon oxidation of malate and glutamate in rat liver mitochondria. The results indicated that the four narasin metabolites exhibited relatively weak effects on ATPase activity and oxygen uptake rates of rat liver mitochondria. The relative ionophoric activity in rat liver mitochondria of the four metabolite preparations was only 0.47% or less compared to narasin.

Manthey and Goebel (Appendix H) also characterized the biological activity of di- and tri-hydroxylated metabolites of narasin isolated from excreta from chickens fed ^{14}C-narasin. Six metabolites were evaluated by a standard narasin thin-layer chromatography bioautographic assay system against *Bacillus subtilis,* a microbial species that is susceptible to narasin. Metabolites (equivalent to 500 ng of narasin based on radioactivity) were applied to TLC plates alongside 25 ng of narasin. None of the metabolites exhibited zones of antimicrobial activity while narasin had a zone of inhibition demonstrating its activity. The metabolites were, therefore, at least 20 times less active than narasin.

In summary, narasin is extensively metabolized by chickens to numerous minor metabolites. Narasin makes up 3% to 5% of the excreted residue. The metabolized residue including several di-and tri-hydroxylated narasin structures is essentially inactive. Therefore, for the purposes of this risk assessment, the measured amount of narasin in chicken excreta as measured in Study ABC-0260 (Appendix E) will be used to calculate the predicted environmental concentrations. In that study, the narasin concentration in the feed was 80 mg/kg and the amount of narasin in the chicken excreta was 6.5 mg/kg on a wet weight basis. For the risk assessment, that number will be adjusted to 4.1 mg/kg to represent a concentration of 50 mg narasin/kg in the feed.

5.1.1.2.2 Metabolism and Excretion of Nicarbazin

The metabolism and excretion of the components of nicarbazin by chickens in the presence and absence of narasin have been evaluated.

5.1.1.2.2.1 Metabolism and Excretion of HDP

In Study T4H749304 (Appendix K), two groups of broilers were fed ^{14}C-labeled nicarbazin (the radiolabel was located in the HDP component) for five consecutive days. The concentration of nicarbazin in the feed of both groups was 50 mg/kg. To evaluate the effect of narasin on the metabolism and excretion of HDP, the feed for one group also contained 50 mg/kg narasin (unlabeled). All excreta was collected daily beginning one day prior to initiation of study until end of treatment. Extraction and characterization of the extracts showed that the HDP accounted for more than 84% of the total radioactivity in the excreta and, therefore, it was concluded that ^{14}C HDP was not significantly metabolized in either test group and that the presence of narasin did not alter the metabolic pattern.

In Study 805286 (Appendix I), chickens were administered nicarbazin twice daily by gavage for 7 days. The nicarbazin contained ^{14}C-HDP. The amount of nicarbazin dosed daily divided by the amount of food that each chicken consumed daily was approximately 130 mg/kg feed (or 92.2 mg/kg DNC and 37.8 mg/kg HDP). Excreta were collected from the birds at 24-hour intervals throughout the dosing period until sacrifice, which was 240 hours after the last morning dose was administered. Elimination of total radioactivity was rapid with a mean of 96.71% of the total dose recovered within 16 hours of the last dose administration. Extraction and processing of excreta resulted in recovery of 71% to 77% of the total radioactive residues. Several minor metabolites were observed using HPLC with radiometric detection, but all were less than 7% of the dose, and most were less than 5%. The minor metabolites were not identified and the majority of the radioactivity in the excreta was identified as HDP (~65% of total radioactive residues). Therefore, this more recent study confirms the conclusion in Study T4H749304 that there is minimal metabolism of HDP by broiler chickens.

5.1.1.2.2.2 Metabolism and Excretion of DNC

In Study ABC-0293 (Appendix L), two groups of broilers were fed ^{14}C-labeled nicarbazin (the radiolabel was located in the DNC component) for five consecutive days. The concentration of nicarbazin in the feed of both groups was 50 mg/kg. To evaluate the effect of narasin on the metabolism and excretion of DNC, the feed for one group also contained 50 mg/kg narasin (unlabeled). Portions of the excreta were collected during the dosing period. Extraction and characterization of the extracts showed that DNC accounted for the majority of the radioactivity in the excreta with a minor metabolite resulting from cleavage of the DNC molecule. Therefore, DNC was not significantly metabolized by chickens and the presence of narasin did not alter the metabolic pattern.

In Study 805129 (Appendix J), chickens were administered nicarbazin twice daily by gavage for 7 days. The nicarbazin contained ^{14}C-DNC. The amount of nicarbazin dosed daily divided by the amount of food that each chicken ate daily was approximately 100 mg/kg feed (or 70.9 mg/kg DNC and 29.1 mg/kg HDP). Excreta were collected from birds at 24 hour intervals

throughout the dosing period until sacrifice, which was 240 hours after the last morning dose was administered. Twenty-four (24) hours after the last morning dose, the concentration of total radioactive residues in excreta expressed as mg DNC equivalents/kg was 53.66 mg DNC equivalents/kg, and the cumulative amount of radioactivity that had been excreted was ~85% of the dose. At 48 hours after the last morning dose, 95% of the dose had been excreted. Extraction and processing of excreta resulted in 41% to 50% of the total radioactive residues being characterized. Most of the radioactivity in the excreta was identified as DNC. Therefore, this more contemporary study confirms the conclusion in Study ABC-0293 (Appendix L) that there is minimal metabolism of DNC by broiler chickens.

In summary, the components of nicarbazin (HDP and DNC) are minimally metabolized by chickens and the excretion of HDP is rapid. Additionally, when dosed to chickens, the components of the nicarbazin complex dissociate such that the HDP and DNC are excreted separately. The dissociation of the two components is evident in their different pharmacokinetics, tissue distribution, and excretion in the chicken (Study 805286, Appendix I; Study 805129, Appendix J). Since formation of the nicarbazin complex requires the components to be in a methanolic solution (Rogers et al. 1983), the complex will not reform in the excreta (or the litter or the environment). Therefore, this environmental risk assessment is conducted for DNC and HDP separately, not on the nicarbazin complex.

5.1.1.2.3 Degradation of Narasin

5.1.1.2.3.1 Degradation of Narasin in Chicken Excreta

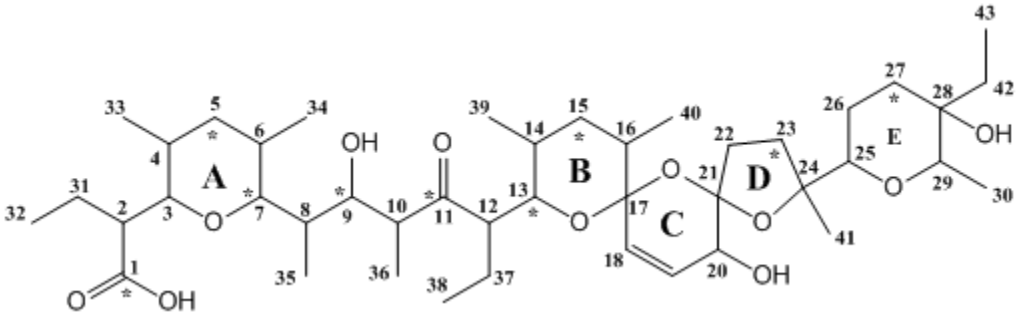
The biodegradation of narasin in fresh chicken excreta and in a mixture of fresh chicken excreta and litter over 35 days has been examined in Study 151E-125 (Appendix M). In that study, flasks containing ^{14}C-narasin (0.5 mg/kg) were incubated with the chicken excreta and with a mixture of chicken excreta and litter under aerobic conditions at 20°C for 35 days. At the end of 35 days, only 1.45 to 2.76% of the applied radioactivity was evolved as $^{14}CO_2$ in the excreta and excreta/litter test systems. By day 7, the amount of narasin in the test systems was 47 to 56% of the applied radioactivity, and at the end of the study on Day 35, the amount of narasin was 37 to 50% of applied radioactivity (data includes both test systems). There were similar amounts of degradation in the excreta and excreta/litter test systems. Using fractionation, only two major metabolites (> 10% of applied radioactivity) were observed. HPLC-MS/MS techniques were used to identify these two major metabolites as a trihydroxylated narasin and narasin with the A ring removed. The other degradation products were less than 5% of applied radioactivity.

The disappearance of narasin reached approximately 50% by Day 7, and appeared to plateau for the remainder of the study. While it is unknown as to whether this plateau was due to an artifact of the test system, the parameters used to characterize the viability of the test systems do indicate that there was some decrease in microbial viability over the duration of the study. This degradation study is considered to be a conservative estimate of the potential degradation of narasin in excreta because, in a chicken barn, fresh excreta would be continuously added, replacing the older microbial population in the litter. Additionally, this study was conducted at lower temperatures than those that can exist in the litter matrix of a chicken house or in a composting pile or windrow.

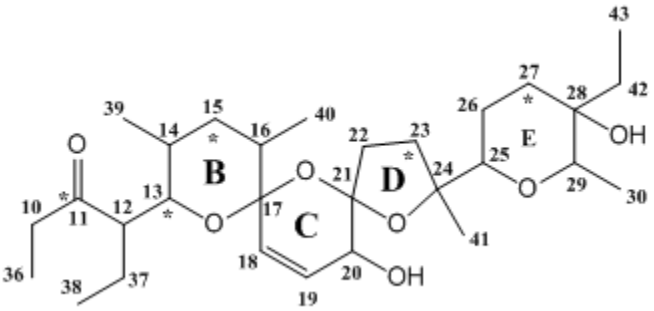
5.1.1.2.3.2 Biological Activity of Narasin Transformation Products formed in Chicken Excreta

The major metabolites identified were a trihydroxy narasin and narasin with the "A" ring removed. Trihydroxy narasin has already been discussed as having no microbiological activity. Based on the requirement for ionophoric activity for microbiological efficacy and the structure activity relationship of narasin, the degradation product without the “A” ring is also considered to have no biological activity.

Structure of ^{14}C narasin used in the chicken excreta degradation study (asterisks indicate potential sites of ^{14}C incorporation):



Structure of Degradant without “A” ring:



This same structure has been identified as a microbial enzymatic degradation product of the narasin-related ionophore, salinomycin (Vértesy et al. 1987). Narasin differs from salinomycin in only the presence of an additional methyl group on the "A" ring. In this report, salinomycin was incubated with an enzymatic preparation from *Pseudomonas stutzeri*, a soil bacterium which degrades salinomycin. This soil bacterium is naturally resistant to salinomycin. After incubation with the enzymatic preparation, there was a decrease in both the amount of salinomycin (as observed on thin layer chromatography, TLC) and the microbiological activity. Additionally, a new substance was observed on TLC. This degradation product was isolated and evaluated with NMR and mass spectrometry. The structure was determined to be the same as that found for the narasin degradant without the “A” ring in the chicken excreta degradation study. The results of this study indicate that the microbiological activity of the degradation product is at least less than that of salinomycin.

Miyazaki et al. (1976) demonstrated the importance of the carboxylic acid associated with the "A" ring in a structure activity relationship investigation of salinomycin. When Miyazaki et al. substituted a methyl ester or a methyl alcohol for the carboxylic acid group on the "A" ring, the binding affinity of salinomycin for cations decreased at least two orders of magnitude and the minimum inhibitory concentrations (MICs) for *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, and *Mycobaterium phlei* increased from a range of 1.56 to 12.5 mg/L to more than 100 mg/L for all tested species. Esterification or reduction of the carboxylic acid associated with the "A" ring in salinomycin results in extensive or complete loss of biological activity against susceptible bacteria. Since the "A" ring contains the carboxylic acid in both salinomycin and narasin, loss of the "A" ring would result in extensive or complete loss of biological activity. Therefore, the narasin transformation product found in chicken excreta with loss of the "A" ring and its associated carboxylic acid is very likely to lack biological activity.

Martinek et al. (2000) used NMR techniques to investigate the three dimensional structure of narasin and how the molecule complexes cations. Six oxygens of narasin were involved in the cation complexation process, including two from the carboxylic acid and the hydroxyl group closest to the "A" ring. In the identified degradation product, these three oxygens have been removed from the molecule. Without three of the six oxygens involved in complexation of cations, the ionophoric activity in this narasin transformation product is expected to be very low or completely lost.

Therefore, based on the results of Study 151E-125 (Appendix M), at least 50% of narasin, and narasin activity, excreted from chickens will be quickly eliminated by microbial degradation in the excreta.

5.1.1.2.3.3 Degradation of Narasin in Soil

The biodegradation of narasin in three soils varying in pH and textural characteristics has been examined for 84 days at 20°C in aerobic conditions (Study 802374, Appendix N). Soil was treated with approximately 1.6 mg/kg of ^{14}C-narasin. Over the course of the study, narasin was mineralized, as evidenced by evolution of 64%, 19% and 54% of the applied radioactivity as $^{14}CO_2$ in the sandy loam, silt loam, and clay loam soils, respectively. Several unidentified transformation products were observed, none of which accounted for more than 16% of the applied radioactivity using HPLC with radiometric detection. At the end of the study, the sum of transformation products observed in the extracts made up 9.19%, 34.39%, and 15.56% of the applied radioactivity in the sandy loam, silt loam and clay loam soils, respectively, while 6.91%, 26.22%, and 14.94% of the applied radioactivity was identified as narasin. The half-life of narasin ranged from 21 to 49 days in the three soils. The DT90 values were 69 to 162 days. The results of this study demonstrate that narasin degrades in soil and is degraded to CO_2.

In an older study, Study 276A-3480-22 (Appendix O), greenhouse soil fortified with 10 mg/kg narasin (air-dried weight) was incubated under field moisture conditions at temperatures ranging from 21° to 30°C. In this study, the concentration of narasin was determined by microbiological assay. Microbiological activity declined rapidly such that less than 10% of the original level was present after 26 days. The degradation rate was somewhat faster in this greenhouse soil degradation study than in Study 802374 (Appendix N). The soil transformation products of narasin that formed in soil did not retain any significant biological activity.

Therefore, narasin degrades in soil and is not expected to accumulate with successive yearly applications of chicken litter.

5.1.1.2.4 Degradation of Nicarbazin

The degradation of nicarbazin or its components in chicken litter has not been evaluated.

5.1.1.2.4.1 Degradation of Nicarbazin in Soil

5.1.1.2.4.1.1 Degradation of DNC in Soil

The degradation of DNC in three soils varying in pH and textural characteristics has been examined for 120 days at 20°C in aerobic conditions (Study 804853, Appendix P). Soil was treated with approximately 0.86 mg/kg of ^{14}C-DNC. Over the study, the bound (unextractable) radioactivity increased to 25% to 37% of the applied radioactivity on Day 120. The amount of radioactivity evolved as $^{14}CO_2$ was 2% in one soil and less than 1% in the other two soils, suggesting minimal mineralization of DNC. The radioactivity that could be extracted was characterized by HPLC with radiometric detection. Minor degradation products were observed after 64 and 120 days, but none of these was more than 3% of the applied radioactivity (mean). Therefore, the major route of disappearance of DNC was irreversible binding; it is unknown whether the entity bound is DNC or some degradation product(s). The calculated DT50 values for disappearance of extractable (and presumably bioavailable) DNC for the three soils were 193, 239 and 257 days (average 230 days).

5.1.1.2.4.1.2 Degradation of HDP in Soil

The degradation of HDP in three soils varying in pH and textural characteristics has been examined for 120 days at 20°C in aerobic conditions (Study 804869, Appendix Q). Soil was treated with approximately 0.354 mg/kg of ^{14}C-HDP. The bound (unextractable) radioactivity ranged from 59% to 71% of applied radioactivity on Day 4 and did not increase significantly by Day 120 when it ranged from 66% to 74%. There was evidence of degradation based on the presence (in small amounts) of degradation products observed using HPLC analysis of soil extracts and significant radioactivity evolved as gas over the duration of the study (ranging from 27% to 32%; $^{14}CO_2$ was 22% or more). Therefore, the route of disappearance of HDP included irreversible binding and degradation. It is unknown whether the bound radioactivity is HDP or some degradation product(s). However, given the amount of mineralization observed as evidenced by $^{14}CO_2$, it is likely that the radiolabel was incorporated into the biomass and therefore is not HDP. The calculated DT50 values for disappearance of HDP for the three soils ranged from 3 to 7 days.

5.1.1.2.4.1.3 Degradation of a mixture of Nicarbazin and Narasin in Soil

In Study ABC-0209 (Appendix R) the soil degradation of ^{14}C-nicarbazin (DNC was the radiolabeled component) was evaluated singly or in the presence narasin in a greenhouse study. Even in the presence of nicarbazin, narasin degraded rapidly as evidenced by bioactivity quantification. The radioactivity in the soil remained the same throughout the study, while the

amount of DNC detected in extracts declined to approximately 75% after 18 weeks but there was no further decline.

In Study ABC-0284 (Appendix S) the soil degradation of ^{14}C-nicarbazin (DNC was the radiolabeled component) was evaluated singly or in the presence of narasin in a field plot. Following incorporation into the top 15 cm of soil, core samples to a depth of 15 cm were collected and evaluated for total radioactivity, and extracted and evaluated for narasin and nicarbazin. The total radioactivity did not decline in the plot indicating that there was no leaching of the DNC nor was there any degradation to volatile degradation products. The levels of DNC in the extracts declined with a DT50 of 48.6 weeks. The analysis of narasin was variable, but narasin did decline to levels less than 10% of starting values within 6 weeks.

These older studies (Study ABC-0209, Appendix R; Study ABC-0284, Appendix S) demonstrate that the presence of nicarbazin does not impact the soil degradation of narasin. They also confirm that the degradation of DNC is slow and proceeds by irreversible binding to soil. DNC is not expected to leach from soil.

5.1.1.2.5 Soil Adsorption

5.1.1.2.5.1 Soil Adsorption of Narasin

The adsorption of narasin to soil was evaluated in five different soils (Study 151E-107, Appendix T) following OECD Guideline 106. The resulting adsorption Kd values ranged from 5.4 to 150 L/kg and the desorption Kd values ranged from 7.1 to 108 L/kg. The adsorption Koc ranged from 507 to 3670 L/kg, with a mean value of 1619 L/kg (log Koc = 3.2). Even though narasin only moderately adsorbs to soil, significant leaching into groundwater is not expected due to the rapid degradation of narasin in soil.

5.1.1.2.5.2 Soil Adsorption of DNC

The adsorption of DNC to soil was evaluated in three different soils at two different concentrations of DNC (Study 804848, Appendix U) following OECD Guideline 106. The resulting adsorption Kd values ranged from 286 to 2066 L/kg. The adsorption Koc values ranged from 16137 to 123923 L/kg, with a mean value of 48012 L/kg (log Koc = 4.7). DNC strongly adsorbs to all soils tested with higher adsorption at higher concentrations. DNC is expected to be immobile in soil.

5.1.1.2.5.3 Soil Adsorption of HDP

The adsorption of HDP to soil was evaluated in three different soils (Study 804832, Appendix V) following OECD Guideline 106. The resulting adsorption Kd values ranged from 1.1 to 3.6 L/kg. The adsorption Koc ranged from 33 to 154 L/kg, with a mean value of 102 L/kg (log Koc = 2). HDP is expected to be mobile in soil.

5.1.1.2.6 Hydrolysis and Photolysis

5.1.1.2.6.1 Hydrolysis and Photolysis of Narasin

Narasin is stable in water at pH 7 and 9, but undergoes hydrolysis at pH 5 (Poole et al., Appendix A). The stability of narasin in neutral water is corroborated by analytical results in the static aquatic toxicity tests with daphnids and fish over 48- and 96-hour exposure periods (Study C01883, Appendix GG; Study F05283, Appendix HH; Study F05183, Appendix II). Photolysis of narasin was evaluated when exposed to simulated sunlight (Poole et al.Appendix A). In that study, the half-life was approximately 1.5 days. However, narasin concentrations were stable over the 72-hour algae growth inhibition test (Study 802573, Appendix FF) under constant light conditions. While hydrolysis and photolysis might contribute to the dissipation of narasin in the environment under certain conditions, these routes of dissipation will not be used to calculate predicted environmental concentrations of narasin for the Phase II assessment.

5.1.1.2.6.2 Hydrolysis and Photolysis of DNC and HDP

Neither DNC nor HDP is susceptible to hydrolysis (Study P0000693, Appendix W). Definitive data on the susceptibility of DNC and HDP to photolysis are not available.

5.1.1.2.7 Bioconcentration

5.1.1.2.7.1 Potential for Bioconcentration of Narasin

The empirically-determined log n-octanol/water partition coefficient (log Kow) of narasin ranges from 4.79 to 5.06 (shake-flask design, Study 151C-120, Appendix C). Based on the water solubility of narasin, the measured range of log Kow in Study 151C-120 (Appendix C) is substantially higher than expected. The value derived from a comparative HPLC analysis (Study 341587, Appendix X) is even higher than the value determined in the shake flask study. Therefore, estimation of Kow for the ionophorous narasin appears to be influenced by the conditions under which it is evaluated.

Considered alone, the magnitude of the Kow value indicates that there is some potential for narasin to bioconcentrate in tissues. However, narasin is water soluble and extensively metabolized and degraded, indicating that significant bioaccumulation is unlikely to occur. Little tissue accumulation was observed in Study T4H969301 (Appendix F), in which chickens were fed a diet with 50 mg/kg ^{14}C-narasin in their feed for five days. At the end of five days, the highest amount of radioactive residues was in the liver with a mean concentration of 0.319 mg/kg. Fat was the next highest with 0.116 mg/kg. In fat, narasin was the predominant residue representing approximately 60% of the total radioactivity in that tissue. In the excreta only 3% of the total radioactivity was identified as narasin, while a total of 50% was identified as di-, tri-, and tetrahydroxy derivatives of narasin. The remaining radioactivity was spread out among several minor peaks. Tissue residue amounts were similar in cattle as in chickens (Study ABC-0137, Appendix Y). Extensive metabolism to several minor hydroxylated narasin metabolites was also observed in cattle, dogs and rats (Studies ABC-0126 and ABC-0127, Appendix Z). Therefore, despite its measured octanol-water partitioning coefficients, narasin is

not expected to bioaccumulate given its susceptibility to extensive oxidative metabolism and limited accumulation in tissues of several species.

5.1.1.2.7.2 Potential for Bioconcentration of DNC and HDP

The log Kow value of HDP is very low (Study ADM-56, Appendix D) indicating that the potential for bioconcentration in biological tissues is also low.

While the minimum log Kow of DNC of 3.6 (Study ADM-56, Appendix D) also indicates a low potential for bioconcentration, it is possible that the log Kow is greater than 3.6. As discussed, predictive Kow modeling supports the conclusion that the log Kow is unlikely to be much higher than 3.6.

Additional evidence for the low bioaccumulation potential of DNC in biological tissues can be found in the chicken metabolism and residue studies. In Study 805129 (Appendix J), after 7 days of dosing nicarbazin (125 mg nicarbazin/kg feed) containing ^{14}C-DNC, the levels of radioactivity in liver, kidney, skin with fat, and muscle tissues were 27.797, 16.776, 5.122, and 4.431 mg equivalents/kg. Four days after cessation of dosing, the concentration of radioactivity in those tissues decreased by at least 97%. In the pivotal residue Study T4HAUK0703 (Appendix AA), chickens were dosed with narasin and nicarbazin for 35 days via feed containing 50 mg narasin/kg feed and 50 mg nicarbazin/kg feed. The calculated daily doses of nicarbazin ranged from 4.65 to 8.91 mg/kg body weight. On day 35, one group of chickens were sacrificed and the DNC tissue levels ranged from 1.610 (muscle) to 9.190 (liver) mg DNC/kg with levels in kidney and skin with fat falling within the range. The same tissues evaluated after 3 days of withdrawal from fortified food reflected significant clearance from tissues of 73 to 93%. After 5 days of withdrawal, the concentrations in tissues were even lower, reflecting at least 96 to 98% clearance from tissues. Both of these tissue residue studies are supportive of a low potential for bioaccumulation in biological tissues based on the clearance of the DNC from tissues.

5.1.1.2.8 Summary of Environmental Fate Data

Table 6. Narasin: Fate Data

Abiotic Degradation (Poole et al., Appendix A)	Hydrolysis Stable at pH 7.0 and 9.0 Half-life of ~3.5 days at pH 5.0 Photolysis At pH 7.0 half-life is ~1.5 days		
Degradation in Chicken Excreta (Study 151E-125, Appendix M)	Incubated aerobically for 35 days Approximately 50% degraded after 7 days		
Soil Adsorption (Study 151E-107, Appendix T)	Soil	Kd L/kg	Koc L/kg
	Clay Loam pH 7.2, OC 5%	25	507
	Sandy Clay Loam pH 6.2, OC 1.9%	22	1171
	Clay Loam pH 5.2, OC 4.1%	150	3670
	Loamy Sand pH 5.7, OC 1.3%	26	1971
	Clay pH 7.7, OC 0.7%	5.4	778
	Mean	*46*	*1619*
Degradation in Soil (Study 802374, Appendix N)	Three soils were dosed with ^{14}C-narasin and incubated aerobically for 84 days.		
	Half-life of narasin	21 to 49 days	
	Mineralization to $^{14}CO_2$	19 to 64 % AR	
	Bound Residues Day 84	18 to 25 % AR	
	Narasin Day 84	6.91 to 26.22 % AR	

%AR: Percent of applied radioactivity

Table 7. DNC: Fate Data

Abiotic Degradation (P0000693, Appendix W)	Hydrolysis: Stable at pH 5, 7, and 9			
Soil Adsorption (Report 804848, Appendix U)	Soil	Initial Concentration mg/L	Kd L/kg	Koc L/kg
	Sandy Loam (pH 7.5, 1.3% OC)	0.13	1611	123923
		0.02	286	21962
	Clay Loam (pH 7.3, 3.1% OC)	0.13	2033	62591
		0.02	533	16137
	Silt Loam (pH 6.1, 2.5% OC)	0.13	1664	66560
		0.02	423	16900
	Mean	*0.13*	*1769*	*84358*
		0.02	*414*	*18333*
Degradation in Soil (Report 804853, Appendix P)	Three soils were dosed with ^{14}C-DNC and incubated aerobically for 120 days.			
	Half-life of DNC	193 to 257 days		
	Mineralization to $^{14}CO_2$	0.81 to 1.96 %AR		
	Bound Residues Day 120	26 %AR		
	DNC Day 120	60 to 70 %AR		

%AR: Percent of applied radioactivity

Table 8. HDP: Fate Data

Abiotic Degradation (P0000693, Appendix W)	Hydrolysis: Stable at pH 5, 7, and 9			
Soil Adsorption (Report 804832, Appendix V)	Soil	Initial Concentration mg/L	Kd L/kg	Koc L/kg
	Sandy Loam (pH 7.5, 1.3% OC)	5.00	1.6	119
		0.05	2.0	154
	Clay Loam (pH 7.3, 3.3% OC)	5.00	1.1	33
		0.05	1.5	45
	Silt Loam (pH 6.1, 2.5% OC)	5.00	2.9	114
		0.05	3.6	144
	Mean		*2.1*	*101.5*
Degradation in Soil (Report 804869, Appendix Q)	Three soils were dosed with ^{14}C-HDP and incubated aerobically for 120 days.			
	Half-life of HDP	3 to 7 days		
	Mineralization to $^{14}CO_2$ Day 8	4 to 9 %AR		
	Mineralization to $^{14}CO_2$ Day 120	22 to 31 %AR		
	Bound Residues Day 120	74 %AR		
	HDP Day 8	15 to 28 %AR		

%AR: Percent of applied radioactivity

5.1.1.3 Ecotoxicity

This risk assessment of Maxiban™ will be conducted using pivotal ecotoxicity data collected with narasin, HDP and DNC under Good Laboratory Practices. The pivotal data are described below for each component and summarized in the tables that follow. Supplementary data are also discussed. Supplementary data includes older studies as well as studies that were conducted on mixtures of the components.

5.1.1.3.1 Ecotoxicity: Narasin

5.1.1.3.1.1 Ecotoxicity: Narasin: Soil Organisms

Definitive studies in soil microflora, plants, and earthworms have been conducted with narasin.

5.1.1.3.1.1.1 Ecotoxicity: Narasin: Soil Organisms: Soil Microflora

In a study following OECD guidelines 216 and 217 (Study 802458, Appendix BB), soil was amended with narasin at two concentrations, 3784 and 17430 µg/kg. There were no biologically important effects (i.e., greater than 25% change from control) on carbon or nitrogen transformation by soil microflora at either concentration.

5.1.1.3.1.1.2 Ecotoxicity: Narasin: Soil Organisms: Terrestrial Plants

In a phytotoxicity test (Study 802442, Appendix CC) following OECD guideline 208, winter oat, mung bean and radish were exposed to narasin incorporated in a sandy loam soil at concentrations of 375, 3381, and 29260 µg/kg. Endpoints were number of seedlings that emerged and the fresh weight of seedlings (shoot only). In winter oat, there was no effect on emergence at any concentration. The growth of seedlings in the 29260 µg/kg treatment was significantly decreased compared to control by 38%. Thus, for winter oat, both the LC50 based on emergence and the EC50 based on growth were greater than the highest concentration tested, 29260 µg/kg. In mung beans, there was a concentration-dependent decrease in the percent emergence compared to control, the decrease in emergence was 40% at the highest concentration. Likewise, there was a concentration-dependent decrease in shoot weight of mung beans, the decreases were 15%, 27%, and 67% compared to control in the 375, 3381, 29260 µg/kg treatments, respectively. Thus, for mung beans, the LC50 based on emergence and the EC50 based on growth were >29260 µg/kg and 8990 µg/kg, respectively. In radish, there was no effect on emergence at the two lower concentrations while no seedlings emerged at the highest concentration, 29260 µg/kg. In the report for this study, an LC50 of 5.07 µg/kg was calculated using the probit method. However, given the lack of partial responses, the probit method is not appropriate. Instead, a binomial method, taking the mean of the 3381 and 29260 µg/kg treatments, estimates an LC50 of 16300 µg/kg. For radish seedling weight, concentrations of 375 and 3381 µg/kg resulted in 13% and 25% decrease in weight compared to control (no seedlings emerged at 29260 µg/kg). The EC50 for growth is, therefore, greater than 3381 and less than 29260 µg/kg. Using 100% growth inhibition for seedlings that did not emerge, including those at the highest concentration, a more precise EC50 can be calculated using the ICp approach (Norberg-King, 1993). The resulting EC50 is 6183 µg/kg (see

discussion in Appendix CC). The lowest EC50 for plant toxicity is, therefore, 6183 µg/kg for growth in radish.

Supplementary information from older studies provides additional context for the potential of narasin to have phytotoxic effects. In 14 test species, narasin had no effects on 14 plant species at 150 µg/kg, slight effects at 1500 µg/kg and severe phytotoxicity at 10000 and 40000 µg/kg (Lilly 1977a). In another older study (Lilly 1977b), litter from the pens of broilers fed with ration containing 100 mg narasin/kg was applied to a field prior to cultivation. When the seeds were planted, the narasin concentration was estimated to be 50 to 100 µg/kg (as measured by microbiological assay) and there was no phytotoxicity observed in the seven plant species evaluated.

5.1.1.3.1.1.3 Ecotoxicity: Narasin: Soil Organisms: Earthworms

The effects of narasin on earthworms in subchronic and chronic exposures were evaluated in Study 802568 (Appendix DD) and Study 1982.6391 (Appendix EE).

In Study 802568 (Appendix DD), earthworms (*Eisenia fetida*) were exposed to soil fortified with narasin at concentrations ranging from 4300 to 270900 µg/kg for 14 days following OECD guideline 207. The endpoints were survival and body weight. The percent mortality in the solvent and blank controls was 5% and 7.5%, respectively. The percent mortality in the narasin treatment levels was 12.5%, 22.5%, 87.5%, 100%, and 100% for the 4300, 34300, 67700, 137700, and 270900 µg/kg, respectively. The LC50 was determined to be 46400 µg/kg and the no observed effect concentration for survival was 4300 µg/kg. The mean weight change in the solvent and blank controls was -9.7 and -8.7%, respectively. The mean weight change in the 4300, 34300, and 67700 was -8.1, -4.7 and -30.7%, respectively. The EC50 for weight change was greater than 67700 µg/kg and the NOEC was 34300 µg/kg.

In an earthworm reproduction study following OECD guideline 222, mature *Eisenia fetida* were exposed to narasin incorporated in soil for 28 days (Study 1982.6391, Appendix EE). Soil concentrations of narasin were 3100, 6300, 13000, 25000, and 50000 µg/kg. At the end the 28-day exposure, the adult worms were removed, assessed for survival and weighed. For another 28 days, the soil was incubated under the same conditions allowing any cocoons deposited by the adult worms to hatch. At the end of the second 28 days, the young worms were separated from the soil by means of a Berlese funnel and enumerated. Endpoints of the study include survival and body weight change in the adults and number of young worms produced per surviving female. There was a significant effect on survival at 50000 µg/kg, with only 25% of adult worms surviving this exposure. The LC50 was calculated to be 41000 µg/kg. There was no significant decrease in body weight or offspring. Therefore, the NOEC for the study was 25000 µg/kg based on survival in the adult worms.

Supplementary subchronic data from older studies with *Lumbricus terrestris* (Lilly 1979; Study W00783 1985), indicate that *L. terrestris* are slightly more sensitive than *E. fetida* with LC50 values between 17900 and 40000 µg/kg.

5.1.1.3.1.2 Ecotoxicity: Narasin: Aquatic Organisms

The toxicity of narasin has been assessed in invertebrates, fish, and algae.

5.1.1.3.1.2.1 Ecotoxicity: Narasin: Aquatic Organisms: Algae

The green alga, *Pseudokirchneriella subcapitata (*formerly *Selenastrum capricornutum),* was exposed to narasin under static conditions for 72 hours in Study 802573 (Appendix FF) that followed OECD guideline 201. Concentrations of narasin in the treatment levels were fairly stable over the study: 100% to 108% of theoretical concentrations at test initiation and 89% to 103% of theoretical at the end of the study. The mean measured concentrations ranged from 35 to 4170 µg/L (the lowest treatment levels, 35 and 230 µg/L, were below the quantification limit and the theoretical concentrations were used). Concentration-dependent decreases in biomass and growth rate were observed. The EC50 values for biomass (area under the curve) and growth rate were 770 and 2920 µg/L, respectively. The NOEC value for both biomass and growth rate was estimated to be 230 µg/L.

5.1.1.3.1.2.2 Ecotoxicity: Narasin: Aquatic Organisms: Daphnids

In Study C01883 (Appendix GG), *Daphnia magna* were exposed to narasin in a 48-hour static toxicity test using procedures that generally followed guidelines available at the time (ASTM, 1980). Procedures were similar to those in the current OECD guideline 202. The treatment levels of narasin ranged from mean measured concentrations of 4690 to 42180 µg/L. The 48-hour median effective concentration for immobilization was determined to be 20560 µg/L.

5.1.1.3.1.2.3 Ecotoxicity: Narasin: Aquatic Organisms: Fish

In Study F05283 (Appendix HH) rainbow trout, *Oncorhynchus mykiss*, were exposed to narasin in a static toxicity test using procedures that generally followed guidelines available at the time (ASTM, 1980). Procedures were similar to those in the current OECD guideline 203. The exposure duration was 96 hours to a mean measured concentration range of 103 to 5260 µg/L. Sublethal effects and mortalities were noted for fish exposed to 316 µg/L and higher. The LC50 was determined to be 2230 µg/L.

In Study F05183 (Appendix II) bluegill, *Lepomis macrochirus*, were exposed to narasin in a static test toxicity test for 96 hours using procedures that generally followed guidelines available at the time (ASTM, 1980). Procedures were similar to those in the current OECD guideline 203. The mean measured concentrations, which were stable over the duration of the test, ranged from 880 to 9550 µg/L. Mortality and sublethal effects including hypoactivity and labored respiration were observed in fish at concentrations of 2800 µg/L and higher. The LC50 was determined to be 5020 µg/L.

The fish and daphnid studies described above for narasin are used as pivotal data in the risk assessment because they were conducted using standard guidelines and in compliance with Good Laboratory Practices. The pivotal studies were conducted using mycelial narasin and are included in the table of narasin ecotoxicity data. There are older, supplementary studies that

were conducted with crystalline narasin that give results that are consistent with the studies described above.

5.1.1.3.1.3 Summary of Narasin Ecotoxicity Data

Table 9. Narasin: Pivotal Ecotoxicity Data

Terrestrial Effects Studies				
Respiration and Nitrogen Transformation Tests (28 days) (Study 802458, Appendix BB)	Exposure to concentrations up to 17,430 µg/kg had results that varied less than 25% from controls for carbon or nitrogen transformation			
Terrestrial Plants – Seedling Growth (14 days after control emergence) (Study 802442, Appendix CC)		Emergence	Growth (Shoot Weight)	
		LC50 µg/kg	EC50 µg/kg	NOEC or LOEC µg/kg
	Winter Oats	>29,260	>29,260	NOEC 3,381
	Mung Beans	>29,260	8,990	LOEC 375
	Radish	16,300	6,183	NOEC 375
Earthworm Growth and Survival (14 days) (Study 802568, Appendix DD)	*Eisenia fetida* LC50 46,400 µg/kg NOEC 4,300 µg/kg			
Chronic Earthworm Reproduction (56 days) (Study 1982.6391, Appendix EE)	*Eisenia fetida* LC50 41,000 µg/kg NOEC 25,000 µg/kg			
Aquatic Effects Studies				
Algal Growth Inhibition (72 hours) (Study 802573, Appendix FF)	Biomass EC_b50 770 µg/L $NOEC_b$ 230 µg/L Growth Rate EC_r50 2,920 µg/L $NOEC_r$ 230 µg/L			
Daphnia immobilization (48 hours) (Study C01883, Appendix GG)	*Daphnia magna* EC50 20,560 µg/L NOEC < 4,690 µg/L			
Fish Acute Toxicity (96 hours) (Study F05283, Appendix HH)	Rainbow Trout LC50 2,230 µg/L NOEC 190 µg/L			
Fish Acute Toxicity (96 hours) (Study F05183, Appendix II)	Bluegill LC50 5,020 µg/L NOEC 1,660 µg/L			

5.1.1.3.2 Ecotoxicity: DNC

5.1.1.3.2.1 Ecotoxicity: DNC: Soil Organisms

Definitive studies in soil microflora, plants, and earthworms have been conducted with DNC.

5.1.1.3.2.1.1 Ecotoxicity: DNC: Soil Organisms: Soil Microflora

In a study following OECD guideline 216 (Report 804984, Appendix JJ), soil was amended with DNC at two concentrations, 800 and 8,000 µg/kg. After 28 days, the amount of nitrate in the treated soils did not differ from that in the control soil by more than 25%. Therefore, there were no biologically important effects on nitrogen transformation by soil microflora at either concentration.

5.1.1.3.2.1.2 Ecotoxicity: DNC: Soil Organisms: Terrestrial Plants

In a phytotoxicity study (Report 805024, Appendix KK) following OECD guideline 208, ryegrass, oats, mung bean, lettuce, radish, and turnip were exposed to DNC incorporated in a loamy sand soil at concentrations of 800, 4000, and 8000 µg/kg. Endpoints were number of seedlings that emerged and the fresh and dry weight of seedlings (shoot only). There were no effects on emergence of any species tested. Comparing means, there were no decreases in growth (on either a per replicate or a per plant basis) that were greater than 22% compared to control for the oats, mung bean, lettuce, radish or turnip. Based on statistical analysis by Dunnett's two-tailed test, there were no significant differences from control at any concentration. For ryegrass grown in 8000 µg/kg treated soil, the fresh and dry shoot weights were 47% and 38%, respectively, lower than the control on a per plant basis. Based on statistical analysis by Dunnett's two-tailed test, these decreases were not significantly different from control. The dry shoot weight per plant and the fresh shoot weight (per replicate and per plant) were not statistically significant from control at any concentration for any species. Therefore, all of the EC50 values for growth are greater than 8000 µg /kg.

A second phytotoxicity study (Study 151P-104, Appendix LL) following OECD guideline 208, was conducted with ryegrass, wheat and corn to evaluate whether the effects in the ryegrass were reproducible. In this second study, seeds were planted in an artificial soil amended with DNC at concentrations of 2900, 4300, 6500, 9700, 14600 and 21900 µg/kg. There were no significant changes in seedling emergence or survival or dry shoot weight or height for any treatment level in any species. The largest changes from (pooled) control were observed in weight for the ryegrass. However, considering all differences from the pooled control (11% decrease, 2% increase, 6% increase, 23% decrease, 17% decrease, and 26% increase for the 2900, 4300, 6500, 9700, 14600 and 21900 µg DNC/kg treatments, respectively), none of which were significant, leads to the conclusion that these differences reflect only biological variability and not treatment with DNC. Therefore, the EC50 is greater than 21900 µg /kg and the NOEC is 21900 µg /kg for the monocots tested in Study 151P-104.

While the first phytotoxicity study tested monocots and dicots at concentrations up to 8000 µg DNC/kg, the only observed effects were with the monocot ryegrass. The second study evaluated whether the effects in monocots were reproducible. There is greater confidence in the second

study, since the study was conducted using higher replication, more test concentrations, higher test concentrations and the test concentrations were verified. Therefore, for this risk assessment the overall NOEC for plants is considered to be 21900 µg/kg.

5.1.1.3.2.1.3 Ecotoxicity: DNC: Soil Organisms: Earthworms

The effects of DNC on earthworms in a subchronic exposure have been evaluated. In Study CYT 012/014574 (Appendix MM), earthworms (*Eisenia fetida*) were exposed to soil fortified with DNC at concentrations ranging from 93000 to 982000 µg/kg for 14 days. Methods followed OECD guideline 207. The endpoints were survival and body weight. There were no mortalities in the test and there were no significant effects on body weight compared to the controls. The LC50 and NOEC values for the study were >982000 µg/kg and 982000 µg/kg, respectively.

5.1.1.3.2.2 Ecotoxicity: DNC: Aquatic Organisms

The toxicity of DNC has been assessed in invertebrates, fish, and algae.

5.1.1.3.2.2.1 Ecotoxicity: DNC: Aquatic Organisms: Algae

The green alga, *Pseudokirchneriella subcapitata (*formerly *Selenastrum capricornutum*), was exposed to DNC under static conditions for 72 hours in Study 811794 (Appendix NN). Methods followed OECD guideline 201. Concentrations of DNC in the treatment levels were not stable over the study with losses ranging from 47% to 83% of the nominal concentrations. Nominal concentrations ranged from 13 to 100 µg/L and geometric mean measured concentrations ranged from 8.29 to 42.25 µg/L. There were no concentration-dependent decreases in yield or growth rate and the EC50 and NOEC values were >42.25 and 42.25 µg/L, respectively.

5.1.1.3.2.2.2 Ecotoxicity: DNC: Aquatic Organisms: Daphnids

In Study 573A-104A (Appendix OO), *Daphnia magna* were exposed to DNC in a 48-hour static acute toxicity test using procedures that followed OECD guideline 202. The treatment levels of DNC ranged from mean measured concentrations of 17 to 93 µg/L. The 48-hour median effective concentration for immobilization was > 93 µg/L. There were some observations of lethargy at concentrations of 40, 64 and 93 µg/L. In addition, there was 5%, 25%, and 5% immobility at 40, 64, and 93 µg/L, respectively. No signs of toxicity were observed at 27 µg/L.

Study 151A-150 (Appendix PP) evaluated the effect of chronic exposure to DNC in *Daphnia magna* in a full life-cycle test following OECD 211. Daphnia were exposed to five concentrations of DNC. The test was conducted with daphnids less than 24 hours old and continued for 21 days during which survival, growth and reproductive output were evaluated. The mean measured DNC concentrations were 2.7, 5.9, 14, 35, and 85 µg/L. Survival was 0% in the highest concentration of 85 µg /L and there were no significant effects on survival at the lower concentrations. Reproduction and weight were statistically decreased (decreases of 44% and 16%, respectively, compared to control) in the 35 µg /L treatment. Length was also significantly decreased at 35 and 14 µg /L, by 13.5% and a 1.8%, respectively, compared to control. While the 1.8% decrease in length at 14 µg/L was statistically significant, the magnitude

suggests that it is at the very beginning of the length response. A decrease of 1.8% is not considered to have ecological significance and its lack of biological significance is supported by absence of toxicity in the other apical endpoints. Therefore, the NOEC is considered to be 14 µg/L based on statistically and biologically significant effects at 35 µg/L.

5.1.1.3.2.2.3 Ecotoxicity: DNC: Aquatic Organisms: Fish

In Study 573A-106 (Appendix QQ) rainbow trout, *Oncorhynchus mykiss*, were exposed to DNC in a static toxicity test using procedures that followed OECD guideline 203. The exposure duration was 96 hours to a mean measured concentration of 69 µg/L. No sublethal effects or mortalities were noted for rainbow trout exposed to DNC. The LC50 was greater than 69 µg/L.

In Study 573A-105 (Appendix RR) bluegill, *Lepomis macrochirus*, were exposed to DNC in a static toxicity test using procedures that followed OECD guideline 203. The exposure duration was 96 hours to a mean measured concentration of 72 µg/L. No sublethal effects or mortalities were noted for bluegill exposed to DNC. The LC50 was greater than 72 µg/L.

Since DNC is known to cause reproductive toxicity in birds, a reproductive study was conducted with DNC in fathead minnows using a protocol similar to OECD 229 (Study 151A-151, Appendix SS). Groups of sexually mature, "proven spawners" (2 males, 4 females per group) were exposed to each of five concentrations of DNC for 4 weeks. Once each week during exposure, eggs were incubated at each concentration to evaluate hatching success. The exposures were conducted under flow-through conditions at mean measured concentrations of 0.80, 2.6, 8.9, 28, and 91 µg/L. There were no significant effects on survival, fecundity (eggs per female per day), egg fertility, or egg hatchability. The NOEC in this study was 91 µg/L.

5.1.1.3.2.3 Summary of DNC Ecotoxicity Data

Table 10. DNC: Pivotal Ecotoxicity Data

Terrestrial Effects Studies			
Soil Microflora Nitrogen Transformation Tests (28 days) (Study 804984, Appendix JJ)	Exposure to concentrations up to 8000 µg/kg had results that varied less than 25% from controls for nitrogen transformation		
Terrestrial Plants – Seedling Growth (14 days after emergence) (Study 805024, Appendix KK)		Emergence	Growth (Shoot Weight)
		LC50 µg/kg	EC50 µg/kg
	Ryegrass	>8000	>8000
	Winter Oats	>8000	>8000
	Mung Beans	>8000	>8000
	Lettuce	>8000	>8000
	Radish	>8000	>8000
	Turnip	>8000	>8000
Terrestrial Plants – Seedling Growth (14 days after emergence) (Study 151P-104, Appendix LL)		Emergence	Growth (Shoot Weight & Height)
		NOEC µg/kg	NOEC µg/kg
	Ryegrass	21900	21900
	Wheat	21900	21900
	Corn	21900	21900
Earthworm Growth and Survival (14 days) (Study CYT 011/014574, Appendix MM)	*Eisenia fetida* LC50 >982000 µg/kg NOEC 982000 µg/kg		
Aquatic Effects Studies			
Algal Growth Inhibition (72 hours) (Study 811794, Appendix NN)	Yield EC_b50 > 42.25 µg/L NOEC$_b$ 42.25 µg/L Growth Rate EC_r50 > 42.25 µg/L NOEC$_r$ 42.25 µg/L		
Daphnia Acute Toxicity (48 hours) (Study 573A-104A, Appendix OO)	*Daphnia magna* EC50 >93 µg/L NOEC 27 µg/L		
Daphnia Chronic Toxicity (21 days) (Study 151A-150, Appendix PP)	*Daphnia magna* NOEC 14 µg/L		
Fish Acute Toxicity (96 hours) (Study 573A-106, Appendix QQ)	Rainbow Trout LC50 >69 µg/L NOEC 69 µg/L		
Fish Acute Toxicity (96 hours) (Study 573A-105, Appendix RR)	Bluegill LC50 >72 µg/L NOEC 72 µg/L		
Fish Reproductive Toxicity (28 days) (Study 151A-151, Appendix SS)	Fathead minnow NOEC 91 µg/L		

5.1.1.3.3 Ecotoxicity: HDP

5.1.1.3.3.1 Ecotoxicity: HDP: Soil Organisms

Definitive studies in soil microflora, plants, and earthworms have been conducted with HDP.

5.1.1.3.3.1.1 Ecotoxicity: HDP: Soil Organisms: Soil Microflora

In a study following OECD guideline 216 (Report 805003, Appendix TT), soil was amended with HDP at two concentrations, 350 and 3500 µg/kg. After 28 days, the amount of nitrate in the treated soils did not differ from that in the control soil by more than 25%. Therefore, there were no biologically important effects on nitrogen transformation by soil microflora at either concentration.

5.1.1.3.3.1.2 Ecotoxicity: HDP: Soil Organisms: Terrestrial Plants

In a phytotoxicity test (Report 805019, Appendix UU) following OECD guideline 208, ryegrass, oats, mung bean, lettuce, radish, and turnip were exposed to HDP amended in a loamy sand soil at concentrations of 350, 1750, and 3500 µg/kg. Endpoints were number of seedlings that emerged and the fresh and dry weight of seedlings (shoot only). There were no effects on emergence of lettuce, oats, ryegrass and turnip seedlings. There were decreases in emergence on radish and mung bean and LC50 values were determined to be 2780 and 2890 µg/kg, respectively. Comparing means, there were no significant changes in growth (on either a per replicate or a per plant basis) in ryegrass, oats, lettuce, or turnip based on statistical analysis by Dunnett's two-tailed test. Significant effects were detected for mung bean for the mean replicate dry weight which was decreased by 67% compared to control in the 3500 µg/kg treatment. The mean replicate fresh weight was also decreased by 61% compared to control in the 3500 µg/kg, but it was not statistically significant. These means were calculated from all the replicates, including the replicate in the high group in which had no plants emerge; the replicate weight for this replicate was 0 g. When the growth was expressed as a per plant weight, the decreases were less severe (22% and 30% for fresh and dry weight, respectively). If the replicate which had no emergence was omitted, the treatment mean per plant weights were essentially the same as control. However, there is wide variability in the replicate per plant weights suggesting that the reduced number of remaining plants in the replicate could have influenced weight of the remaining plants. The mung bean results suggest that there could be a detrimental effect on growth at the highest concentration, 3500 µg/kg. Significant effects were also detected for radish growth. The mean per plant fresh weight was significantly increased (141% of the control) in the high treatment. Again the mean calculation included the replicate in which no seedlings emerged, which had a per plant fresh weight of 0 g. Excluding this group, the mean per plant fresh weight was even greater, 187% of the control at 3500 µg/kg. There is some evidence that the reduced number of remaining plants in the replicates could have contributed to the increase in weight. However, there was one replicate that had all 5 seedling emerged, and even the per plant weight for this replicate was 150% of the control mean. The radish results suggest that the highest concentration, 3500 µg/kg, could have a detrimental effect on radishes, if it is assumed that the significant increased plant size is a detrimental effect, which is debatable. Because there was no decrease in per plant weight which was greater than 50%, all of the EC50 values for growth are >3500 µg/kg.

A second phytotoxicity study (Study 151P-103, Appendix VV) following OECD guideline 208, was conducted to further investigate the effect of HDP on radish and mung beans and two other dicots (soybean and peas) from the mung bean family. In the second study, seeds were planted in soil with concentrations of HDP ranging from 1100 to 5900 µg/kg. For radish, soybeans and peas, there were no statistically significant changes in emergence, survival, dry shoot weight or height with any treatment compared to the control. In mung bean, there were no significant changes in emergence, survival, or dry shoot weight compared with control. However, there was a statistically significant reduction in plant height at the two highest concentrations, 3900 and 5900 µg/kg, compared to the control. The detrimental effect of these differences is debatable given that the magnitude of height reduction for both concentrations was only 6 to 7% and the absence of detrimental effects in condition or the other apical endpoints. For the study, the statistical NOEC is 2630 µg/kg. Given the lack of effects in emergence, the lowest EC50 for emergence in the dicots tested in this study is >5900 µg/kg.

For this risk assessment the overall EC50 for plants is considered to be >3500 µg/kg based on the highest concentration tested in monocots and the NOEC is considered to be 2630 µg/kg based on the statistical results with mung bean.

5.1.1.3.3.1.3 Ecotoxicity: HDP: Soil Organisms: Earthworms

The effects of HDP on earthworms in a subchronic exposure have been evaluated. In Study CYT 012/014575 (Appendix WW), earthworms (*Eisenia fetida*) were exposed to soil fortified with HDP at concentrations ranging from 94 to 989000 µg/kg for 14 days following OECD guideline 207. The endpoints were survival and body weight. There were no moralities and no significant effects on body weight observed when compared to the control. The LC50 and NOEC values for the study were >989000 µg/kg and 989000 µg/kg, respectively.

5.1.1.3.3.2 Ecotoxicity: HDP: Aquatic Organisms

The toxicity of HDP has been assessed in invertebrates, fish, and algae.

5.1.1.3.3.2.1 Ecotoxicity: HDP: Aquatic Organisms: Algae

The green alga, *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), was exposed to HDP under static conditions for 72 hours in Study 811810 (Appendix XX). Methods followed OECD guideline 201. Concentrations of HDP were stable over the study and approximated nominal concentrations. Mean measured concentrations of HDP ranged from 5084 to 46362 µg/L. No treatment level resulted in significant differences from the control in yield or growth rate and the EC50 and NOEC values were >46362 and 46362 µg/L, respectively.

5.1.1.3.3.2.2 Ecotoxicity: HDP: Aquatic Organisms: Daphnids

In Study 573S-107C (Appendix YY), *Daphnia magna* were exposed to HDP in a 48-hour static toxicity test using procedures that followed OECD guideline 202. The treatment levels of HDP ranged from mean measured concentrations of 15000 to 107000 µg/L. There were no observations of immobility or toxicity, therefore, the 48-hour median effective concentration > 107000 µg/L and the NOEC was 107000 µg/L.

5.1.1.3.3.2.3 Ecotoxicity: HDP: Aquatic Organisms: Fish

In Study 573A-109 (Appendix ZZ) rainbow trout, *Oncorhynchus mykiss*, were exposed to HDP in a static toxicity test using procedures that followed OECD guideline 203. The exposure was for 96 hours to a mean measured concentration of 110000 µg/L. No sublethal effects or mortalities were noted for rainbow trout exposed to HDP. The LC50 was determined to be >110000 µg/L.

In Study 573A-108 (Appendix AAA) bluegill, *Lepomis macrochirus*, were exposed to HDP in a static toxicity test using procedures that followed OECD guideline 203. The exposure was for 96 hours to a mean measured concentration of 122000 µg/L. No sublethal effects or mortalities were noted for bluegill exposed to HDP. The LC50 was determined to be >122000 µg/L.

5.1.1.3.3.3 Summary of HDP Ecotoxicity Data

Table 11. HDP: Pivotal Ecotoxicity Data

Terrestrial Effects Studies			
Soil Microflora Nitrogen Transformation Tests (28 days) (Study 805003, Appendix TT)	Exposure to concentrations up to 3,500 µg/kg had results that varied less than 25% from controls for nitrogen transformation		
Terrestrial Plants – Seedling Growth (14 days after emergence) (Study 805019, Appendix UU)		Emergence	Growth (Shoot Weight)
		LC50 µg/kg	EC50 µg/kg
	Ryegrass	>3500	>3500
	Winter Oats	>3500	>3500
	Mung Beans	2890	>3500
	Lettuce	>3500	>3500
	Radish	2780	>3500
	Turnip	>3500	>3500
Terrestrial Plants – Seedling Growth (14 days after emergence) (Study 151P-103, Appendix VV)		Emergence	Growth (Shoot Weight & Height)
		NOEC µg/kg	NOEC µg/kg
	Mung Beans	5900	2630
	Radish	5900	5900
	Soybean	5900	5900
	Peas	5900	5900
Earthworm Growth and Survival (14 days) (Study CYT 012/014575, Appendix WW)	*Eisenia fetida* LC50 >989,000 µg/kg NOEC 989,000 µg/kg		
Aquatic Effects Studies			
Algal Growth Inhibition (72 hours) (Study 811810, Appendix XX)	Biomass EC_b50 >46,362 µg/L $NOEC_b$ 46,362 µg/L Growth Rate EC_r50 >46,362 µg/L $NOEC_r$ 46,362 µg/L		
Daphnia immobilization (48 hours) (Study 573A-107C, Appendix YY)	*Daphnia magna* EC50 >107,000 µg/L NOEC 107,000 µg/L		
Fish Acute Toxicity (96 hours) (Study 573A-109, Appendix ZZ)	Rainbow Trout LC50 >110,000 µg/L NOEC 110,000 µg/L		
Fish Acute Toxicity (96 hours) (Study 573A-108, Appendix AAA)	Bluegill LC50 >122,000 µg/L NOEC 122,000 µg/L		

5.1.1.3.4 Ecotoxicity: Nicarbazin (mixture of DNC and HDP)

Nicarbazin (a complex of DNC and HDP in a 1:1 molar ratio) has been tested in ecotoxicity studies with earthworms, daphnids and fish.

5.1.1.3.4.1 Ecotoxicity: Nicarbazin: Soil Organisms: Earthworms

Earthworms (*Eisenia fetida*) were exposed to soil fortified with nicarbazin at five concentrations ranging from 95,000 to 1,000,000 µg/kg for 14 days (Study CYT 010/014573, 2002). Methods followed those described in OECD guideline 207 and EEC Directive 87/302/EEC, Part C. Endpoints were survival and body weight. Concentrations of nicarbazin (as DNC or HDP) were not verified. There were no treatment related effects on survival or body weight. The LC50 was determined to be greater than 1,000,000 µg/kg.

In an older study, earthworms (*Lumbricus terrestris*) were exposed to soil fortified with nicarbazin at concentrations of 10,000 and 100,000 µg/kg for 14 days (Study W01382, Appendix BBB). Methods followed those described by Karnak and Hamelink (1982). Endpoints were physical signs of toxicity, changes in body weight, and mortality. Concentrations of nicarbazin (as DNC or HDP) were not verified. There were no signs of toxicity or mortalities due to nicarbazin and there was no apparent effect on body weight.

5.1.1.3.4.2 Ecotoxicity: Nicarbazin: Aquatic Organisms: Daphnids

Daphnids (*Daphnia magna*) were exposed to nicarbazin at a nominal concentration of 100,000 µg/L under static conditions for 48 hours (Study C02782, Appendix CCC). Methods generally followed those described in ASTM E729-80 (ASTM 1980). Endpoints were physical signs of toxicity and immobility. Concentrations were verified by measuring concentrations of HDP only. DNC was not measured due to its low water solubility. HDP represented 26.8% of nicarbazin by assay. The measured concentrations of HDP were consistent during the study and the mean measured concentration was 24,200 µg/L. There were no observations of immobility or toxicity.

5.1.1.3.4.3 Ecotoxicity: Nicarbazin: Aquatic Organisms: Fish

Bluegill (*Lepomis macrochirus*) were exposed to nicarbazin at a nominal concentration of 100,000 µg/L under static conditions for 96 hours (Study F08982, Appendix DDD). Methods generally followed those described in ASTM E729-80 (ASTM 1980). Concentrations were verified by measuring HDP only. DNC was not measured due to its low water solubility. HDP represented 26.82% of nicarbazin. The measured concentrations of HDP were consistent during the study and mean measured concentration was 28,700 µg/L. There were no observations of mortality or toxicity.

Rainbow trout (*Oncorhynchus mykiss*) were exposed to nicarbazin at a nominal concentration of 100,000 µg/L under static conditions for 96 hours (Study F09082, Appendix EEE). Methods generally followed those described in ASTM E729-80 (ASTM 1980). Concentrations were verified by measuring HDP only. DNC was not measured due to its low water solubility. HDP represented 26.82% of nicarbazin. The measured concentrations of HDP were consistent during

the study and mean measured concentrations was 26,700 µg/L. There were no observations of mortality or toxicity.

5.1.1.3.4.4 Summary of Ecotoxicity of Nicarbazin (complex of DNC and HDP)

From ecotoxicity studies with nicarbazin, there is no evidence that there is more toxicity when earthworms, daphnids and fish are exposure to the nicarbazin (the complex of DNC and HDP) compared to when they are exposed to DNC and HDP individually.

The ecotoxicity data with nicarbazin is considered supplementary to the pivotal data collected with the individual components of nicarbazin and is not used to calculate risk.

5.1.1.3.5 Ecotoxicity: Mixture of Narasin and Nicarbazin

The ecotoxicity of a 1:1 mixture (by weight) of narasin and nicarbazin has been evaluated in fish, daphnids and earthworms. In these studies, both DNC and HDP were fortified at the nominal concentrations listed, constituting the 1:1 mixture (by weight). In the fish and daphnid studies, the concentrations of narasin and HDP were confirmed by analysis. The median effect concentration and the NOEC are expressed as the nominal concentration of narasin and the measured concentration of narasin.

5.1.1.3.5.1 Ecotoxicity: Narasin-Nicarbazin Mixture: Soil Organisms: Earthworms

Earthworms (*Lumbricus terrestris*) were exposed to soil fortified with narasin and nicarbazin at five nominal concentrations ranging from 700 to 25000 µg/kg for 14 days (Study W01083, 1983). Methods followed those described by Karnak and Hamelink (1982). Endpoints were physical signs of toxicity and body weight. Concentrations of nicarbazin (as DNC or HDP) were not verified. Significant decreases in growth and sublethal signs of toxicity were observed at 16000 µg/kg and higher. No treatment-related decreases in body weight or other physical signs of toxicity were observed at 2750 µg/kg or below. Based on nominal concentrations, the NOEC was established as 2750 µg/kg

5.1.1.3.5.2 Ecotoxicity: Narasin-Nicarbazin Mixture: Aquatic Organisms: Daphnia

Daphnia magna were exposed to eight nominal narasin and nicarbazin concentrations ranging from 500 to 25000 µg/L for 48 hours under static conditions (Study C02883, 1983). Methods followed those described in ASTM E729-80 (ASTM 1980). Endpoints were immobilization and sublethal signs of toxicity. Concentrations were verified by measuring narasin and HDP; DNC was not measured due to its low water solubility. HDP represented 26.72% of nicarbazin, by assay. The measured concentrations of narasin and HDP were consistent during the study. Mean measured concentrations ranged from 160 to 7800 µg/L for HDP and 459 to 24000 µg/L for narasin. No physical signs of toxicity observed at 500 µg/L (nominal) or below. Based on nominal concentrations, the NOEC was 500 µg/L (459 µg/L based on mean measured narasin concentrations) and the LC50 was 21260 µg/L (20650 µg/L based on mean measured narasin concentrations).

5.1.1.3.5.3 Ecotoxicity: Narasin-Nicarbazin Mixture: Aquatic Organisms: Fish

Bluegill (*Lepomis macrochirus*) were exposed to nine nominal narasin and nicarbazin concentrations ranging from 1800 to 10000 µg/L for 96 hours under static conditions (Study F07383, 1983). Methods followed those described in ASTM E729-80 (ASTM 1980). Endpoints were survival and sublethal signs of toxicity. Concentrations were verified by measuring narasin and HDP; DNC was not measured due to its low water solubility. HDP represented 26.72% of the nicarbazin, by assay. The measured concentrations of narasin and HDP were consistent during the study. Mean measured concentrations ranged from 540 to 3500 µg/L for HDP and 1610 to 9700 µg/L for narasin. At 1800 µg/L (nominal) and below there were no physical signs of toxicity observed. Based on nominal concentrations the NOEC was established as 1800 µg/L (1610 µg/L based on mean measured narasin concentrations) and the LC50 was 5050 µg/L (4970 µg/L based on mean measured narasin concentrations).

Rainbow trout (*Oncorhynchus mykiss*) were exposed to ten nominal narasin and nicarbazin concentrations ranging from 450 to 5600 µg/L for 96 hours under static conditions (Study F07483, 1983). Methods followed those described in ASTM E729-80 (ASTM 1980). Endpoints were survival and sublethal signs of toxicity. Concentrations were verified by measuring narasin and HDP; DNC was not measured due to its low water solubility. HDP represented 26.72% of the nicarbazin, by assay. The measured concentrations of narasin and HDP were consistent during the study. Mean measured concentrations ranged from 140 to 1810 µg/L for HDP and 390 to 5380 µg/L for narasin. Physical signs of toxicity were observed at all concentrations, therefore, a NOEC was not established. Mortality ranged from 20 to 100% at concentrations 0.80 to 5.6 µg/L (nominal). Based on nominal concentrations, the LC50 was determined to be 1610 µg/L (1480 µg/L based on mean measured narasin concentrations).

A second study with rainbow trout (*Oncorhynchus mykiss*) was conducted to establish a NOEC (Study F08683, 1983). Trout were exposed to four nominal narasin and nicarbazin concentrations ranging from 160 to 800 µg/L for 96 hours under static conditions. Endpoints were survival and sublethal signs of toxicity. Concentrations were verified by measuring narasin and HDP; DNC was not measured due to its low water solubility. HDP represented 26.72% of the nicarbazin, by assay. The measured concentrations of narasin and HDP were consistent during the study. Mean measured concentrations ranged from 43 to 250 µg/L for HDP and 133 to 732 µg/L for narasin. The NOEC was established as 160 µg/L (nominal), where no physical signs of toxicity were observed, or 133 µg/L based on mean measured narasin concentrations.

5.1.1.3.6 Summary of Ecotoxicity of a Mixture of Narasin and Nicarbazin

In ecotoxicity studies in which fish, daphnids and earthworms were exposed to the active ingredients individually, narasin is more toxic than nicarbazin. In the studies in which fish and daphnids were exposed to a 1:1 (by weight) mixture of narasin and nicarbazin the EC50 results (expressed as measured narasin concentration) are very similar to the results with narasin alone. There is no evidence that HDP and DNC have a synergistic effect on the toxicity of narasin to these environmental species.

5.1.2 PEC Calculations and Refinements (Exposure Assessment)

5.1.2.1 Soil PEC

The calculated $PEC_{soil\text{-}initial}$ values following continuous administration to broiler chickens of 50 mg narasin/kg feed and 50 mg nicarbazin/kg feed were calculated in the Phase I assessment (Section 4.2) as 650, 454, and 194 µg/kg of narasin, DNC, and HDP using the total residues in litter of 43.5, 30.4, and 13.0 mg/kg, respectively, and assuming an application rate of 5 tons/acre with mixing into the top 5 cm of the soil. Per the VICH Phase II guidance (CVM 2006, VICH 2004), this value can be refined using metabolism, degradation in excreta and degradation in soil.

5.1.2.1.1 Soil PEC: Narasin

5.1.2.1.1.1 Soil PEC: Narasin: Refinement by metabolism

As described in Section 5.1.1.2.1, narasin is extensively metabolized by broiler chickens to many minor metabolites which do not have biological activity. In Study ABC-0260 (Appendix E), excreta from broiler chickens fed 80 mg narasin/kg feed for 7 days contained 6.5 mg narasin/kg excreta (wet weight basis). Adjusting for a feed concentration of 50 mg narasin/kg feed, a concentration of 4.1 mg narasin/kg excreta (on a wet weight basis) will be used as a refined concentration in excreta.

5.1.2.1.1.2 Soil PEC: Narasin: Refinement by degradation in excreta

As described in Section 5.1.1.2.3, narasin is degraded in chicken excreta under aerobic conditions such that after 7 days approximately 50% of narasin and narasin activity are gone. Two major degradation products are formed, but since their biological activity is expected to be far lower than that of narasin, they will not be considered in the risk assessment. Additionally, because excreted narasin is such a small percent of dosed narasin, these degradation products are a small part of the total inactive residue. A cycle of broiler chickens is typically in the barn for 50 days. It will be assumed that at least 50% of the remaining narasin in the excreta is degraded after excretion and in the barn.

The concentration of narasin in litter at the end of the broiler cycle can be calculated to be 1.8 mg/kg as shown below:

$$Litter\ concentration_{end\ of\ 50\text{-}day\ cycle} = \frac{Excreta\ Concentration\ \times M}{Litter\ Dilution}$$

Where:

Excreta Concentration: 4.1 mg/kg
M: Excreta Degradation Removal: 0.5
Litter Dilution: 1.15

$$Litter\ concentration_{end\ of\ 50\text{-}day\ cycle} = \frac{4.1\ \frac{mg}{kg} \times 0.5}{1.15} = 1.8\ \frac{mg}{kg}$$

5.1.2.1.1.3 Soil PEC: Narasin: Refinement by degradation in soil

Narasin degrades rapidly in soil. However, a conservative approach will be taken assuming that crops are sown immediately after litter containing narasin residues is applied to soil. This approach is also appropriate for assessing risk to earthworms and soil microflora, species which will be present in soil at the time of application. Therefore, the soil concentration will not be refined for degradation in soil.

5.1.2.1.1.4 Soil PEC: Narasin: Calculation of $PEC_{soil\text{-}refined}$

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

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

$$= \frac{1.8\frac{mg}{kg} \times 4536\frac{kg}{acre}}{303525\frac{kg}{acre}} = 0.027\frac{mg}{kg} = 27\ \frac{\mu g}{kg}$$

5.1.2.1.2 Soil PEC: DNC

As described in Section 5.1.1.2.4.1.1, there was minimal metabolism by chickens, no refinement based on metabolism will be used in the calculation of the PEC_{soil}. In soil, the disappearance half-life of DNC is less than a year (257 days was the longest half-life, equivalent to a rate of 0.984 $year^{-1}$). If the maximal soil concentration after application is 454 µg/kg, then, theoretically, a year after application the amount of DNC that can be detected in soil is 37.4% (from e^{-kt} or $e^{-0.984*1}$) of the initial concentration or 170 µg/kg. However, the disappearance is due, in part, to formation of non-extractable residue. While there is some evidence of actual degradation of DNC (detected degradation products including volatiles ranged from 1.7% to 3.95% of the applied radioactivity after 120 days), no refinement due to degradation in soil will be applied. For this risk assessment, the maximum PEC of DNC in soil after a single application is considered to be 454 µg/kg.

For slowly degrading compounds like DNC, the possibility of accumulation in soil from repeated yearly application should be considered. To understand the potential impact of yearly application, it was assumed that chicken litter with DNC is applied annually to the same field and DNC degrades in soil at a rate of 0.984 $year^{-1}$. Each year 454 µg/kg is added to 37.4% of the maximum concentration from the previous year. After 10 years, the maximum concentration in soil reaches a plateau of 725 µg/kg, or about 1.6 times the concentration after a single application.

5.1.2.1.3 Soil PEC: HDP

As described in Section 5.1.1.2.4.1.1, there was minimal metabolism by chickens, no refinement based on metabolism will be used in the calculation of the PEC_{soil}. In soil, the disappearance half-life of HDP ranged from 3 to 7 days. There is considerable evidence of actual degradation

of HDP based on the mineralization observed during the study. However, for this risk assessment, it is assumed that plants, earthworms and soil microflora will be exposed to the maximum concentration of HDP in soil, 194 µg/kg.

5.1.2.2 Groundwater PEC

5.1.2.2.1 Groundwater PEC:Narasin

Narasin is substantially degraded to carbon dioxide in soil, with most of the remaining residues undergoing primary degradation and/or sorption (Section 5.1.1.2.3.3). Narasin itself is moderately sorbed to soil, with a mean Kd value of 46 in various soils (Section 5.1.1.2.5.1). Given these characteristics and the very low initial concentration of narasin in soil, it is very unlikely that significant levels of narasin would be found in groundwater.

5.1.2.2.2 Groundwater PEC:DNC

DNC is strongly adsorbed to soil (Section 5.1.1.2.5.2) and has a very low water solubility (Section 5.1.1.1). These characteristics indicate that DNC will have low mobility in soil and is unlikely to be found in significant quantities in ground water.

5.1.2.2.3 Groundwater PEC:HDP

Given its water solubility (Section 5.1.1.1) and low Kd (Section 5.1.1.2.5.3), HDP is likely to be mobile in soil. However, it is also rapidly degraded in soil with a half-life of 3 to 7 days (Section 5.1.1.2.4.1.2). Therefore, it is unlikely that HDP will persist long enough in soil to contaminate ground water.

5.1.2.3 Surface Water PEC

5.1.2.3.1 Surface Water PEC: Narasin

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

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

$$[Narasin]_{pond} = \frac{1.8\frac{mg}{kg} \times 4536\frac{kg}{acre} \times 10\ acres \times 0.01}{8{,}100{,}000\ L} = 0.1\ \frac{\mu g}{L}$$

Therefore, the narasin $PEC_{surface\ water}$ refined for metabolism and degradation in excreta is 0.1 µg/L. Because narasin has only a moderate propensity to adsorb to soil and sediment, all of the run-off loss is assumed be present in the aqueous layer in the pond.

Reports of narasin in surface water have been published. In a survey of several sites of the Cache la Poudre River in Colorado, including ones located in agricultural areas, Kim and Carlson (2006, 2007) report the highest narasin concentration in surface water as 0.038 µg/L. Thompson et al (2009) looked for narasin in surface waters collected from twenty-three agricultural watersheds in Canada. In a total of 237 samples, only four contained narasin with the highest concentration measured at 0.019 µg/L.

Reports from the published literature, therefore, support the use of 0.1 µg/L as an appropriately conservative estimate of the maximum possible surface water concentration of narasin.

5.1.2.3.2 Surface Water PEC: DNC

The estimation of DNC in surface water of a pond was calculated using a similar scenario as that for narasin as above.

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

$$[DNC]_{pond} = \frac{30.4\frac{mg}{kg} \times 4536\frac{kg}{acre} \times 10\ acres \times 0.01}{8{,}100{,}000\ L} = 1.7\ \frac{\mu g}{L}$$

The concentration in the water can also be refined by the propensity of DNC to adsorb to soil and sediment. Since the DNC that runs off soil is bound to soil particles, the Koc values of DNC for soil were used. For the purpose of this risk assessment, the lowest measured Koc value for DNC to soil (16137 L/kg, Table 7) was conservatively used to refine the surface water concentration. The Koc value was transformed to a Kd value using the organic content of a standard sediment (16137 L/kg × 2.9%) or 468 L/kg. The PEC refined for adsorption is calculated by the following equation:

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

$$PEC_{surface\ water-adsorption\ refined} = \frac{13789\ mg}{8{,}100{,}000\ kg + (300{,}000\ kg \times 468\frac{L}{kg})} = 0.09\ \frac{\mu g}{L}$$

The mass of the sediment used assumes mixing into the top 5 cm of sediment and is 300,000 kg. Therefore, the PEC for surface water refined for adsorption to soil and sediment is 0.09 µg/L. Assuming a half-life of 257 days in soil and repeated annual application, the maximum amount of DNC available from runoff is about 1.6 times that after a single application. Therefore, after repeated application, the $PEC_{surfacewater\text{-}adsorption\text{-}refined}$ is 0.14 µg/L (0.09 µg/L x 1.6) for total residues.

5.1.2.3.3 Surface Water PEC: HDP

The estimation of HDP in surface water of a pond was calculated using the same runoff scenario:

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

$$[HDP]_{pond} = \frac{13.0\frac{mg}{kg} \times 4536\frac{kg}{acre} \times 10\ acres \times 0.01}{8{,}100{,}000\ L} = 0.7\ \frac{\mu g}{L}$$

Because HDP has only a moderate propensity to adsorb to soil and sediment, all of the loss is assumed be present in the aqueous layer in the pond.

5.1.2.4 Summary of Refined Predicted Environmental Concentrations

Table 12. Predicted Environmental Concentrations

Compartment	Narasin	DNC	HDP
Litter µg/kg – no refinements	43500	30400	13000
Litter, refined for metabolism, degradation in manure, µg/kg	1800	30400	13000
Soil, µg/kg – no refinements	650	454	194
Soil, refined for metabolism, degradation in manure µg/kg	27	454	194
Soil, refined for metabolism, degradation in manure after multiple annual applications µg/kg	27	725	194
Surface water, refined for metabolism, degradation in manure µg/L	0.1	1.7	0.7
Surface water, refined for metabolism, degradation in manure, adsorption to soil µg/L	0.1	0.09	0.7
Surface water, refined for metabolism, degradation in manure, adsorption to soil after multiple annual applications µg/L	0.1	0.14	0.7

5.1.3 Tier A PNEC Calculations (Effect Assessment)

The assessment factors applied to the toxicity values and the Tier A PNEC values for narasin, DNC, and HDP for terrestrial and aquatic species are tabulated below. The assessment factors are from the VICH GL38 Phase II guidance for Environmental Impact Assessments (CVM 2006, VICH 2004).

5.1.3.1 PNECs: Narasin

Table 13. Tier A Terrestrial PNEC Values: Narasin

Test	Species	Toxicity endpoint (Affected endpoint)	Assessment Factor	PNEC
Soil Microflora	--	28-day NOEC = 17430 µg/kg (<25% change from control)	1	17430 µg/kg
Plants	Mung Bean	14-day EC50 = 6183 µg/kg (growth)	100	61.8 µg/kg
Earthworm	*Eisenia fetida*	56-day NOEC = 25000 µg/kg (survival)	10	2500 µg/kg

Table 14. Tier A Aquatic PNEC Values: Narasin

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Algal Growth	*Pseudokirchneriella subcapitata*	72-hour EC50 = 770 µg/L (biomass)	100	7.7 µg/L
Daphnia Acute	*Daphnia magna*	48 hour EC50 = 20560 µg/L (immobilization)	1000	20.6 µg/L
Fish Acute	*Oncorhynchus mykiss*	96 hour LC50 = 2230 µg/L (mortality)	1000	2.2 µg/L

5.1.3.2 PNECs: DNC

Table 15. Tier A Terrestrial PNEC Values: DNC

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	--	28-day NOEC = 8000 µg/kg (<25% change from control)	1	8000 µg/kg
Plants	Ryegrass, Winter Oat, Mung Bean, Lettuce, Radish, Turnip	14-day EC50 > 8000 µg/kg (emergence and growth)	100	>80 µg/kg
Earthworms	*Eisenia fetida*	14-day NOEC = 982000 µg/kg (survival and body weight)	1000	982 µg/kg

The earthworm study was a 14-day subchronic exposure with endpoints of mortality and growth. The VICH GL38 Phase II guidance (CVM 2006, VICH 2004) recommends using an assessment factor of 10 on the NOEC from a study that includes earthworm reproduction as an endpoint. Because reproduction was not included, an assessment factor of 1000 applied to the concentration at which there were no effects on mortality or growth to protect for earthworm reproduction.

Table 16. Tier A Aquatic PNEC Values: DNC

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Algal Growth	*Pseudokirchneriella subcapitata*	72-hour EC50 > 42.25 µg/L (yield and growth rate)	100	>0.42 µg/L
Daphnia Acute	*Daphnia magna*	48 hour EC50 > 93 µg/L (immobilization)	1000	>0.093 µg/L
Fish Acute	*Oncorhynchus mykiss*	96 hour LC50 > 69 µg/L (mortality)	1000	>0.069 µg/L

5.1.3.3 PNECs: HDP

Table 17. Tier A Terrestrial PNEC Values: HDP

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Soil Microflora	--	28-day NOEC= 3500 µg/kg (<25% change from control)	1	3500 µg/kg
Plants	Radish	14-day LC50 = 2780 µg/kg (emergence)	100	27.8 µg/kg
Earthworm	*Eisenia fetida*	14-day NOEC = 989000 µg/kg (survival and body weight)	1000	989 µg/kg

The earthworm study was a 14-day subchronic exposure with endpoints of mortality and growth. The VICH GL38 Phase II guidance (CVM 2006, VICH 2004) recommends using an assessment factor of 10 on the NOEC from a study that includes earthworm reproduction as an endpoint. Because reproduction was not included, an assessment factor of 1000 applied to the concentration at which there were no effects on mortality or growth to protect for earthworm reproduction.

Table 18. Tier A Aquatic PNEC Values: HDP

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Algal Growth	*Pseudokirchneriella subcapitata*	72-hour EC50 > 46,362 µg/L (yield and growth rate)	100	>463 µg/L
Daphnia Acute	*Daphnia magna*	48-hour EC50 > 107,000 µg/L (immobilization)	1000	>107 µg/L
Fish Acute	*Oncorhynchus mykiss*	96-hour LC50 > 110,000 µg/L (mortality)	1000	>110 µg/L

5.1.3.4 Summary of Tier A PNEC Values

Table 19. Tier A PNEC Values

	Narasin	DNC	HDP
Soil microflora, µg/kg	17430	8000	3500
Plants, µg/kg	61.8	>80	27.8
Earthworms, µg/kg	2500	982	989
Algae, µg/L	7.7	>0.42	>463
Daphnia, µg/L	20.6	>0.093	>107
Fish, µg/L	2.2	>0.069	>110

5.1.4 Risk Characterization

5.1.4.1 Risk Characterization: Narasin

5.1.4.1.1 Risk Characterization: Narasin: Soil

The predicted concentration of total residues of narasin in soil (PEC_{soil}) after application of litter from broilers treated with Maxiban™ is 650 µg/kg (Section 4.2). When metabolism and degradation in chicken excreta is considered, the $PEC_{soil\text{-}refined}$ can be calculated to be 27 µg/kg (Section 5.1.2.1.1.4) and this PEC is used for risk characterization.

The PEC/PNEC ratios for terrestrial organisms using the refined PEC_{soil} (27 µg/kg) are all less than 1 (Table 20).

5.1.4.1.2 Risk Characterization: Narasin: Surface Water

The maximum predicted concentration of narasin in surface water ($PEC_{refined\text{-}surface\ water}$) is 0.1 µg/L (Section 5.1.2.3.1). The PEC/PNEC ratios for aquatic organisms using the refined $PEC_{surfacewater}$ are all less than 1 (Table 20).

Because the PNEC values (Section 5.1.3.1) in each compartment are all higher than the PEC in that compartment, the PEC/PNEC ratios are all less than 1 (Table 20) and there is no significant risk to organisms in that compartment.

Table 20. PEC/PNEC Ratios: Narasin

Compartment	Species	PEC*	PNEC	PEC/PNEC Ratio
Terrestrial	Microflora	27 µg/kg	17430 µg/kg	0.002
	Plants		61.8 µg/kg	0.4
	Earthworms		2500 µg/kg	0.01
Surface Water	Algae	0.1 µg/L	7.7 µg/L	0.01
	Daphnia		20.6 µg/L	0.005
	Fish		2.2 µg/L	0.05

* refined for metabolism and degradation in excreta

5.1.4.2 Risk Characterization: DNC

5.1.4.2.1 Risk Characterization: DNC: Soil

The maximum predicted concentration of total residues of DNC in soil (PEC_{soil}) after application of litter from broilers treated with Maxiban™ is 454 µg/kg (Section 4.2). DNC is not significantly metabolized by chickens and while there is some evidence of degradation in soil, it is not extensive. Therefore, the risk assessment is conducted on the maximum possible residue concentration of 454 µg/kg. Additionally, the potential for DNC to accumulate after repeated annual application of chicken litter to soil was evaluated. The maximum soil concentration after repeated annual applications is 725 µg/kg (Section 5.1.2.1.2).

The PEC/PNEC values for microflora and earthworms are less than 1 (Table 21). Therefore, there is no significant risk to those organisms for DNC.

The Tier A PNEC value for plants, >80 µg/kg (Section 5.1.3.2), is less than the maximum PEC_{soil} values of 454 and 725 µg/kg for a single and repeated applications, respectively, and the PEC/PNEC values are greater than 1 for plants (Table 21).

A Tier B assessment will be conducted for DNC in the terrestrial compartment, specifically for protection of plants.

5.1.4.2.2 Risk Characterization: DNC: Surface Water

As described in Section 5.1.2.3.2, the predicted concentration of total residues of DNC in surface water is 1.7 µg/L. When adsorption to soil is considered, the concentration in water can be refined to 0.09 µg/L ($PEC_{refined\text{-}surface\ water}$). Additionally, considering accumulation after annual application, the maximum surface water concentration (refining for adsorption to soil) is 0.14 µg/L. The refined PEC values in surface water considering single and annual application of chicken litter to soil are both evaluated in the risk characterization.

The PEC values for DNC in surface water for single and annual application are both lower than the PNEC for algae, therefore, the PEC/PNEC ratios are less than 1 and there is no risk to algae from DNC (Table 21).

For daphnids, the PEC/PNEC ratio is less than 1 following a single application, but with repeated annual applications of chicken litter to soil, the PEC/PNEC ratio is greater than 1 (Table 21).

For fish, the PEC/PNEC ratios are greater than 1 for both single and repeated annual application (Table 21).

A Tier B assessment will be conducted for DNC in the aquatic compartment, specifically for protection of daphnids and fish. Additionally, since DNC is known to affect reproduction in birds, the Tier B assessment in fish will evaluate reproduction.

Table 21. PEC/PNEC Ratios: DNC

Compartment	Species	PEC	PNEC	PEC/PNEC Ratio
Terrestrial				
Single application	Microflora	454 µg/kg	8000 µg/kg	0.06
	Plants		>80 µg/kg	< 5.7
	Earthworms		982 µg/kg	0.5
Annual application	Microflora	725 µg/kg	8000 µg/kg	0.09
	Plants		>80 µg/kg	< 9.1
	Earthworms		982 µg/kg	0.7
Surface Water				
Single application	Algae	0.09 µg/L*	>0.42 µg/L	<0.2
	Daphnia		>0.093 µg/L	< 1.0
	Fish		>0.069 µg/L	<1.3
Annual application	Algae	0.14 µg/L*	>0.42 µg/L	<0.3
	Daphnia		>0.093 µg/L	< 1.5
	Fish		>0.069 µg/L	<2.0

* refined for adsorption

5.1.4.3 Risk Characterization: HDP

5.1.4.3.1 Risk Characterization: HDP: Soil

The maximum predicted concentration of total residues of HDP in soil (PEC_{soil}) after application of litter from broilers treated with Maxiban™ is 194 µg/kg (Section 5.1.2.1.3). HDP is not significantly metabolized by chickens and but does degrade in soil with a half-life that ranges from 3 to 7 days (Section 5.1.1.2.4.1.2). To protect terrestrial species that are present in soil immediately following land application, the Tier A risk assessment is conducted on the maximum possible residue concentration of 194 µg/kg.

The PEC/PNEC values for microflora and earthworms are less than 1 (Table 22) and, therefore, there is no significant risk to those organisms. The Tier A PNEC value for plants, 27.8 µg/kg (Section 5.1.3.3), is less than the maximum PEC_{soil} value of 194 µg/kg and the PEC/PNEC value is greater than 1 for plants (Table 22).

A Tier B assessment will be conducted for HDP in the terrestrial compartment, specifically for protection of plants.

5.1.4.3.2 Risk Characterization: HDP: Water

The maximum predicted concentration of total residues of HDP in surface water ($PEC_{surface\ water}$) is 0.7 µg/L (Section 5.1.2.3.3). The PNEC values in surface water are all greater than the PEC in surface water (Table 22).

Because the PEC/PNEC values in surface water are all less than 1, there is no significant risk to organisms in that compartment for HDP.

Table 22. PEC/PNEC Ratios: HDP

Compartment	Species	PEC	PNEC	PEC/PNEC Ratio
Terrestrial	Microflora	194 µg/kg	3500 µg/kg	0.06
	Plants		27.8 µg/kg	7.0
	Earthworms		989 µg/kg	0.2
Surface Water	Algae	0.7 µg/L	>463 µg/L	<0.002
	Daphnia		>107 µg/L	<0.007
	Fish		>110 µg/L	<0.006

5.1.5 Summary of Tier A

The environmental impact from the continuous use of Maxiban™ Type A premix in feed for broiler chickens to prevent coccidiosis in high intensive rearing situations has been evaluated. The specific concentrations of narasin and nicarbazin in the feed that were evaluated were 50 mg/kg feed (each), the highest recommended concentration. The pathway for introduction of narasin and nicarbazin into the environment considered in this risk assessment was via the application of chicken litter as fertilizer to soil. Runoff to surface water from that soil was also considered. The risk of narasin and the two components of nicarbazin (DNC, and HDP) were considered separately.

5.1.5.1 Summary of Tier A - Narasin

For narasin, the predicted environmental concentrations were refined to consider metabolism and degradation in excreta during the litter holding period. The predicted environmental concentration of narasin in soil is 27 µg/kg after refinement (Section 5.1.2.1.1). The predicted environmental concentration in surface water following run-off events is 0.1 µg/L after refinement (Section 5.1.2.3.1). These predicted environmental concentrations of narasin in soil and surface water are lower than the predicted no-effect concentrations for terrestrial and aquatic organisms calculated from endpoints determined in ecotoxicity studies (Table 20). Since narasin is extensively metabolized by animals, and degraded in excreta and soil, it is not expected to persist in the environment or accumulate in environmental species. Therefore, a Tier B risk assessment for narasin was not necessary.

5.1.5.2 Summary of Tier A - DNC

DNC has very low water solubility and is not extensively metabolized by chickens. Additionally, it adsorbs extensively and degrades slowly in soil. Therefore, the risk characterization considered the potential accumulation in soil that could occur if chicken litter is repeatedly applied to soil. The predicted maximum concentration of DNC in soil following repeated application is 725 µg/kg, assuming no degradation (Section 5.1.2.1.2). The predicted maximum concentration in surface water due to run-off events is 0.14 µg/L (Section 5.1.2.3.2). While no toxicity was observed in the terrestrial and aquatic organisms that have been conducted with DNC, the resulting PNECs from those studies are lower than the predicted environmental concentrations (Table 21). Additionally, there were concerns regarding potential reproductive effects in fish. Therefore, DNC is considered in a Tier B risk assessment below.

5.1.5.3 Summary of Tier A - HDP

HDP is not significantly metabolized by chickens. However, HDP is much more soluble than DNC and is degraded rapidly in soil with evidence of mineralization and incorporation into the soil biomass. The predicted maximum concentration of HDP in soil is 194 µg/kg and the half-life is only 7 days (Section 5.1.2.1.3). Following a run-off event, the maximum surface water concentration is calculated to be 0.7 µg/L (Section 5.1.2.3.3), which is more than 100 times lower than the predicted no effect concentration in aquatic species (Table 22). However, the lowest PNEC for terrestrial organisms (in plants) was 27.8 µg/kg, less than the maximum predicted concentration of HDP in soil, therefore HDP in the terrestrial compartment is considered in a Tier B risk assessment below.

5.2 Tier B

The PEC/PNEC ratios from the Tier A assessment above are only of concern for terrestrial plants and aquatic organisms exposed to DNC in soil and surface water, respectively, and for plants exposed to HDP in soil. These scenarios are considered below.

5.2.1. DNC - Terrestrial Plants

In two phytotoxicity studies, eight plant species representing crops were evaluated (Study 805024, Appendix KK; Study 151P-104, Appendix LL). Study 151P-104 was conducted using more replicates, higher test concentrations and more test concentrations, therefore, the NOEC from that study (21900 µg/kg) will be used to derive the PNEC in Tier B. For the refinement of the risk assessment for DNC in plants, a factor of 10 will be applied to the NOEC value.

Table 23. Tier B Terrestrial PNEC Values: DNC

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Plants	Ryegrass, wheat and corn	14-day NOEC 21900 µg/kg (emergence and growth)	10	2190 µg/kg

This lowest PNEC value, 2190 µg/kg, was compared to the PEC values in the terrestrial compartment.

Table 24. Tier B Terrestrial PEC/PNEC Ratios: DNC

	PEC_{soil}	PNEC	PEC/PNEC Ratio
PEC_{soil}:	454 µg/kg	2190 µg/kg	0.2
$PEC_{soil,\ accumulation\ over\ repeated\ application}$:	725 µg/kg		0.3

The PEC values in the soil are lower than the PNEC based on the NOEC for plants even when repeated annual applications are considered. Therefore, there is no significant risk to terrestrial plants.

5.2.2 DNC – aquatic organisms

Chronic studies were conducted in fish and daphnia. In a fish reproduction study (28 days, Study 151A-151, Appendix SS), no toxicity was observed at 91 µg/L, the highest concentration that could be tested due to limited aqueous solubility of DNC. A life-cycle study with *Daphnia magna* (21 days, Study 151A-150, Appendix PP) resulted in a NOEC of 14 µg/L. An assessment factor of 10 was applied to these NOECs to calculate Tier B PNEC values for the fish and daphnid species (Table 25). During the daphnia life-cycle study, the daphnia were fed at a rate 1.5 times greater than that recommended in the OECD 211 test guideline. Since there were no available data to demonstrate that the increased feed did not affect the outcome of the study, either in respect to the physiology of the daphnia or the dynamics of the chemical in solution, an additional assessment factor of 2 was applied to the PNEC for the daphnia species (Table 25).

Table 25. Tier B DNC Aquatic PNEC Values

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Daphnia Chronic	*Daphnia magna*	21-day NOEC 14 µg/L (survival, growth, reproduction)	20	0.7 µg/L
Fish Reproduction	*Pimephales promelas*	28-day NOEC 91 µg/L (survival, fecundity, embryo fertility and hatchability)	10	9.1 µg/L

Table 26. Tier B DNC Aquatic PEC/PNEC Ratios

Compartment	Species	PEC	PNEC	PEC/PNEC Ratio
Single application	Daphnia	0.09 µg/L	0.7 µg/L	0.1
	Fish		9.1 µg/L	0.01
Annual application	Daphnia	0.14 µg/L	0.7 µg/L	0.2
	Fish		9.1 µg/L	0.02

* refined for adsorption

The PEC values in the surface water are all lower than the PNEC values for daphnia and fish based on the NOEC even when repeated annual application is considered (Table 26). Therefore, there is no significant risk to daphnids or fish.

5.2.3 HDP - Terrestrial Plants

A total of eight plant species were tested in two phytotoxicity studies (Study 805019, Appendix UU; Study 151P-103, Appendix VV). Only mung bean showed signs of toxicity in both the first and the repeat study. In the repeat study (Study 151P-103, Appendix VV) more concentrations were tested and the effects on mung bean were either less in magnitude or absent compared to the first study. Additionally, the magnitude of the only effect in the repeat study (a decrease of 6 to 7% in height compared to control) was of questionable biological significance. The statistical NOEC from the repeat study (2630 µg/kg) was used to derive the PNEC value by dividing by an assessment factor of 10 (Table 27).

Table 27. Tier B Terrestrial PNEC Values: HDP

Test	Species	Toxicity endpoint	Assessment Factor	PNEC
Plants	Mung bean	14-day NOEC 2630 µg/kg (growth)	10	263 µg/kg

Table 28. Tier B HDP PEC/PNEC Ratios

Compartment	Species	PEC	PNEC	PEC/PNEC Ratio
Terrestrial	Plants	194 µg/kg	263 µg/kg	0.7

The PEC value in the soil is lower than the PNEC based on the NOEC for plants (Table 28). Therefore, there is no significant risk to terrestrial plants.

5.2.4 Summary of Tier B

The PNEC values for terrestrial and aquatic organisms were estimated in Tier B by using the NOEC values from chronic studies with fish and daphnids and additional phytotoxicity data with terrestrial plants (Table 27). The Tier B PEC/PNEC ratios were less than one (Table 28) indicating that there is no significant risk to organisms in the terrestrial or aquatic compartments.

5.2.5 Additional Risk Consideration: Nicarbazin in Non-target Avian Species

As already noted in Section 5.1.1.3.2.2.3, nicarbazin has a known reproductive toxicity to birds. The potential for adverse effects on non-target birds in the environment was evaluated.

5.2.5.1 Toxicity of nicarbazin to birds

5.2.5.1.1 Acute dietary studies

Nicarbazin exhibited low toxicity to birds in acute (5 day) dietary studies in which mallards and bobwhite quail were administered nicarbazin in feed (Study A01182, 1985; Study A01482, 1985). In both species, the LC50 values are greater than 5000 mg nicarbazin/kg feed. Body weight gain was reduced in birds fed diets containing nicarbazin, The lowest no observed effect concentration in these 5 day studies was in mallards at 62 mg nicarbazin/kg feed (Study A01182, 1985).

5.2.5.1.2 Reproductive studies

There are several reports in the published literature on changes in reproductive endpoints in birds following exposure to nicarbazin. The reproductive endpoints include pigmentation of eggs, egg production, egg weight, egg fertility, and hatchability. Most of these studies have been conducted in chickens. Some of these studies are summarized below.

Jones et al (1990a) administered nicarbazin to Hubbard female layers and Vantress male breeders by incorporating it into feed at concentrations of 0, 20, 50 and 100 ppm (mg

nicarbazin/kg feed). The feeding regimen was a 3-day pretreatment phase with control diet, 10 days of feeding nicarbazin-fortified feed, followed by 20 days of withdrawal with control diet. Egg concentrations of DNC were measured and were found to be correlated with nicarbazin feed level. Egg production, egg weight and fertility were not impacted. Depigmentation of the egg shell was observed at the 50 and 100 ppm level with the duration of the observation being higher in the 100 ppm level. Hatchability of fertile eggs was decreased at all test levels, but the duration of this effect and the severity was related to dose. At 100 ppm hatchability was decreased from Treatment day 5 to Withdrawal day 6 and was the most depressed hatchability was 14%. At 50 ppm, hatchability was decreased from Treatment day 7 to Withdrawal day 4 and the most depressed hatchability was 49%. At 20 ppm, the hatchability was only decreased on Withdrawal days 1 to 2 and the hatchability at this time period was 60%. All other time points were not significantly different from the pre-treatment hatchability of 84%.

In a similar study conducted in White Leghorn layers, Jones et al (1990b) administered nicarbazin in feed at concentrations of 0, 20, 50, and 100 ppm. All hens were fed control diet for three days, then nicarbazin-fortified diet for 10 days followed by control diet for 14 days. In this study, egg weights were decreased compared to controls at 50 and 100 ppm, with a higher incidence of observation for the 100 ppm level. A similar pattern was observed with egg production, but there was more variability that confounded some results. There was also an increased incidence of egg mottling which was related to dose with 9 and 11% of eggs with mottling observed on 4 days at 20 ppm compared to 16 days at 100 ppm with incidence observations ranging from 4 to 81%.

Hughes et al (1991) administered nicarbazin in feed to broiler chickens at 25, 50 and 100 ppm for three different durations (2, 4, 6 days followed by a withdrawal period) to evaluate scenarios of accidental feed contamination with nicarbazin. There were no effects on egg production at 25 and 50 ppm when administered for 2 days, but there was a decrease in production noted at 100 ppm only on days 7 and 8 of the study. When administered contaminated feed for 4 days, all levels had decreased egg production with severity and duration to recovery related to dose. The decreased egg production in all treatment levels after feeding for 6 days was increased in duration and severity compared to 2 and 4 days but was dose responsive. Hatchability also followed a similar pattern. At 25 ppm, decreased hatchability of fertile eggs was observed only after 4 day of exposure on days 5 to 8 of the study and after 6 days of exposure on days 7 and 8 of the study. On day 7 and 8 of the study, the decreased hatchability was 38.9% and 33.3% of control after 4 and 6 days of exposure, respectively. Decreased hatchability was dose-dependent and observed for more study days at 100 ppm than the lower doses.

Jones et al (1990c) administered nicarbazin in feed at levels of 0, 10, 20, 50 and 100 ppm to brown egg layers. Birds were treated for 10 days with a withdrawal period of 12 days. Egg production and egg weight was decreased at 100 ppm only. Fertility was not affected at any level, but hatchability of fertile eggs was decreased at levels 20, 50 and 100 ppm with severity, duration of effect, and time to recovery all dependent on the treatment level. The authors concluded that there were no adverse effects at 10 ppm nicarbazin in feed.

Leeson et al. (1989) exposed White leghorn layer chickens to 5, 10, 20, 40, 80, 125 and 200 ppm nicarbazin in feed. Birds were fed treated diet for 4 weeks, followed by a withdrawal period of 4 weeks. Egg production was significantly lower than controls at 125 and 200 ppm, and was lower

but not significant at 40 and 80 ppm. Egg weight was also significantly decreased compared to controls at 125 and 200 ppm, but these treatment levels also showed decreased food intake by the layers. The eggs at 125 and 200 ppm also had decreased hatchability. The authors concluded that significant loss in egg production occurred at 125 and 200 ppm while treatments of 40 and 80 ppm resulted in depressed egg production.

As nicarbazin is used as a contraceptive for nuisance avian populations, the effect of nicarbazin on reproduction of other species (mallards, Canada geese, Pekin ducks, and pigeons) has been investigated. Results for mallards and Canada geese indicate that some species are less sensitive than chickens to reproductive inhibition (Yoder et al. 2006, Bynum 2005) which may be due in part to differential adsorption of DNC (Yoder et al. 2005). Reinoso (2008) fed a commercial diet fortified with nicarbazin to Pekin ducks for two weeks followed by a 16-day recovery period. The concentration of nicarbazin in the diet ranged from 31 to 500 ppm. During the two-week treatment, egg production and fertility declined slowly in a dose responsive way. Within approximately one week into the recovery period, the lowest dose returned to control levels almost immediately and the higher doses took longer to return to control levels. Pigeons are also susceptible to reproductive effects (Avery et al. 2008), although the only concentration of nicarbazin in the bait used was very high, 5000 ppm. Of the species that have been evaluated, no other species appears to be more sensitive to the reproductive effects of nicarbazin than chickens.

The EPA (2005) concluded that 10 ppm (10 mg nicarbazin/kg feed) was the level in feed that that does not have reproductive effects in chickens, based on a review available data. The 10 ppm no effect level is consistent with the published data reviewed above.

5.2.5.1.3 No effect levels of DNC in birds

Since DNC is the biologically active ingredient in nicarbazin, because DNC plasma levels are much higher in birds than HDP levels, and since HDP is not persistent in soil, the risk evaluation for non-target birds was performed based on exposure to DNC. In the acute toxicity studies, the no effect level for reduced weight gain is 62 mg nicarbazin/kg feed, or 43.4 mg DNC/kg feed (as DNC is 70% of the nicarbazin feed concentration on a weight basis, e.g. 0.7 x 62 mg/kg). The no effect level for reproductive effects in chickens is 10 mg nicarbazin/kg feed, or 7 mg DNC/kg feed. Since no bird species tested appears to be more sensitive to reproductive effects of nicarbazin than chickens, the no effect level of 7 mg DNC/kg feed will be used to evaluate the potential for adverse effects in all birds.

5.2.5.1.4 Exposure of birds to DNC

It was assumed that non-target avian species could experience the highest exposure to DNC by either directly eating soil to which chicken litter had been applied or eating terrestrial species that may have accumulated DNC from the soil. The maximum concentration of DNC in soil is 725 µg/kg after land application of chicken litter to soil (after multiple annual applications). Since DNC is hydrophobic, there may be some accumulation in terrestrial species such as earthworms. Assuming that earthworms are living in soil that have a DNC concentration of 725 µg/kg, the DNC concentration in earthworms can be calculated to be 184 µg/kg based in uptake from pore

water and soil in the gut (calculations are based on ECHA (2016) and described in Appendix FFF). Therefore, the estimated concentrations of DNC in food sources are 184 µg/kg in earthworms and 725 µg/kg in soil.

5.2.5.2 Risk analysis

5.2.5.2.1 Earthworms as food source

Assuming that worms were the sole food source of birds, the concentration of DNC in the food would be 236 times lower than the no effect level in food for reduced weight gain in mallards and bobwhite quail (43400 µg/kg ÷ 184 µg/kg). The concentration of DNC in the food would be 38 times lower than the no effect level for reproductive effects in chickens (7000 µg/kg ÷ 184 µg/kg).

5.2.5.2.2 Soil as food source

Considering the very conservative assumption that birds eat soil as a sole food source, the concentration of DNC in that "food" would still be 60 times lower than the no effect level for reduced weight gain (43400 µg/kg ÷ 725 µg/kg) and approximately 10 times lower than the no effect level for reproductive effects in chickens (7000 µg/kg ÷ 725 µg/kg).

5.2.5.2.3 Implications of non-complexation

Additionally, for the risk analyses above, the margins of safety are actually larger because the DNC that is in the soil is not complexed with HDP (Study 805286, Appendix I and Study 805129, Appendix J). The bioavailability (oral absorption) of DNC is higher when DNC is complexed with HDP. The difference in bioavailability between a complex of DNC and HDP and a simple mixture of the two components has been demonstrated in chickens on the basis of the difference in anticoccidial activity by Cuckler et al (1955) and on the basis of plasma levels of DNC in chickens by Porter and Gilfillan (1955). Cuckler et al (1955) reported that the anticoccidial activity of DNC in chickens when dosed in the feed as a complex with HDP was increased at least tenfold compared to when dosed alone. Chickens administered a single oral dose of 1000 mg nicarbazin/kg body weight (bw) had a plasma concentration of 8.9 µM DNC /L after 4 hours, while it was only 1.1 µM DNC/L when chickens were dosed with both (uncomplexed) 650 mg DNC/kg bw and 350 mg HDP/kg bw (Porter and Gilfillan, 1955). The increased bioavailability of the complex is also demonstrated in Study 130-136 (2009). The study determined the pharmacokinetic profiles of DNC in rats dosed via oral gavage as a complex with HDP and dosed as a simple mixture with HDP. The exposure to DNC (as Area under the Curve) when dosed as a simple mixture is only about 5% of that when dosed as a complex with HDP.

Therefore, the risk to non-target avian species that could be exposed to the components of nicarbazin following application of chicken litter to agricultural land is minimal.

5.3 Summary and Conclusion

The environmental impact from the continuous use of Maxiban™ Type A premix in the feed of broiler chickens to prevent coccidiosis in concentrated animal feeding operations has been evaluated. The specific concentrations of narasin and nicarbazin in the feed evaluated were 50 mg/kg feed (each), the highest recommended concentration. The risks posed by narasin, DNC, and HDP (DNC and HDP are the two components of nicarbazin) were considered separately. The risk assessment considered data collected on the physical/chemical properties, environmental fate, and environmental effects of narasin and HDP and DNC.

The pathway for introduction of narasin, DNC and HDP into the environment considered in this risk assessment was via the application of chicken litter to agricultural land. Runoff to surface water from that land was also considered. The predicted environmental concentrations of narasin, DNC, and HDP were calculated using the maximum administration rate of Maxiban™ and typical animal husbandry and agronomy practices for land application of chicken litter. The concentrations of narasin were refined by considering the metabolism of narasin by chickens to minor or inactive metabolites and the degradation of narasin in litter during the holding period. While narasin and HDP are degraded in soil, DNC is degraded slowly. Therefore, the predicted environmental concentration of DNC was refined considering the possibility of accumulation in the soil due to repeated annual applications of litter. Surface water concentrations were calculated considering potential runoff from fields during rainfall events. DNC is not very soluble in water and adsorbs to soil; therefore, calculation of the surface water concentration of DNC was refined considering the adsorption to soil and sediment. The Tier A assessment was sufficient for risk characterization for narasin, since all PEC/PNEC ratios were less than one (Table 29). For DNC, the Tier A PEC/PNEC ratios were above one for terrestrial plants, daphnia and fish (Table 30). Additionally, there were concerns regarding potential reproductive effects in egg laying species (i.e., fish) due to the mechanism of action of DNC. To further characterize the risk, a Tier B assessment was conducted to assess the toxicity to plants and fish and daphnia reproduction. A Tier B assessment, was also conducted with HDP in the terrestrial compartment, specifically for the protection of plants. The Tier A PEC/PNEC ratios for HDP were all less than one with the exception of plants (Table 31). Following the Tier B assessment, the PEC/PNEC ratios for DNC in the terrestrial and surface water compartments and for HDP in the terrestrial compartment were all less than one (Tables 30 and 31) indicating that there is no significant risk to organisms in the terrestrial or aquatic compartments.

Table 29. PEC/PNEC Ratios: Narasin

Compartment	Species	PEC*	PNEC	PEC/PNEC Ratio
Tier A Assessment				
Terrestrial	Microflora	27 µg/kg	17430 µg/kg	0.002
	Plants		61.8 µg/kg	0.4
	Earthworms		2500 µg/kg	0.01
Surface Water	Algae	0.1 µg/L	7.7 µg/L	0.01
	Daphnia		20.6 µg/L	0.005
	Fish		2.2 µg/L	0.05

* refined for metabolism and degradation in excreta

Table 30. PEC/PNEC Ratios: DNC

Compartment	Species	PEC	PNEC	PEC/PNEC Ratio
Tier A Assessment				
Single Application				
Terrestrial	Microflora	454 µg/kg	8000 µg/kg	0.06
	Earthworms		982 µg/kg	0.5
Surface Water	Algae	0.09 µg/L*	>0.42 µg/L	<0.2
Annual Application#				
Terrestrial	Microflora	725 µg/kg	8000 µg/kg	0.09
	Earthworms		982 µg/kg	0.7
Surface Water	Algae	0.14 µg/L*	>0.42 µg/L	<0.3
Tier B Assessment				
Single Application				
Terrestrial	Plants	454 µg/kg	2190 µg/kg	0.2
Surface Water	Daphnia	0.09 µg/L*	0.7 µg/L	0.1
	Fish		9.1 µg/L	0.01
Annual Application#				
Terrestrial	Plants	725 µg/kg	2190 µg/kg	0.3
Surface Water	Daphnia	0.14 µg/L*	0.7 µg/L	0.2
	Fish		9.1 µg/L	0.02

refined for accumulation in soil with yearly application

* refined for adsorption

Table 31. PEC/PNEC Ratios: HDP

Compartment	Species	PEC	PNEC	PEC/PNEC Ratio
Tier A Assessment				
Terrestrial	Microflora	194 µg/kg	3500 µg/kg	0.06
	Earthworms		989 µg/kg	0.2
Surface Water	Algae	0.7 µg/L	>463 µg/L	<0.002
	Daphnia		>107 µg/L	<0.007
	Fish		>110 µg/L	< 0.006
Tier B Assessment				
Terrestrial	Plants	194 µg/kg	263 µg/kg	0.7

In all cases, the predicted environmental concentration is less than the predicted no effect concentration and the PEC/PNEC ratio is less than one. Therefore, the treatment of broiler chickens with narasin and nicarbazin as Maxiban™ for control of coccidiosis without a withdrawal period is not expected to result in any significant environmental impact through the application of chicken litter to agricultural land.

5.4 Cumulative Impacts Assessment

5.4.1 Narasin

Since other marketed products contain narasin, the potential cumulative impacts of narasin were considered. Monteban™ is approved as a feed additive for broilers (NADA 118-980) and Skycis™ (NADA 141-340) is approved as a feed additive for swine. The maximum narasin PEC_{soil} values following use of Maxiban™, Monteban™, and Skycis™ are 27 µg/kg, 54 µg/kg, and 45 µg/kg, respectively. The PEC_{soil} for Monteban™ uses the same assumptions for land application and refinement as in the current Maxiban™ environmental assessment (EA) along with a narasin feed concentration that is twice that of Maxiban™. The PEC_{soil} for Skycis™ uses assumptions particular for land application of liquid swine manure and refinement. The soil concentration is greatest for the Monteban™ usage in broilers and the refined $PEC_{surface\ water}$ in this scenario would be 0.2 µg/L. The PEC/PNEC ratios or risk quotients (RQ) for Monteban™ usage are greater than those for Maxiban™: 0.8 for the most sensitive organism in the terrestrial compartment (plants) and 0.1 for the most sensitive organism in surface water (algae).

Considering the use of narasin for multiple indications in the same animals on the same farm, both Monteban™ and Maxiban™ are labeled for use as the sole ration, therefore they could not be used in the same broilers. However, it is possible that different flocks (or houses) of chickens on the same farm might be treated with different feed additives. The litter from those flocks could be stored together prior to being applied to land. The worst case scenario would be that all of the chicken litter is from chickens treated with Monteban™. Since the RQs for Monteban™ are less than 1, no substantial risk is posed to terrestrial or surface water organisms.

Considering the use of narasin for different species on the same farm, it is unlikely that swine and chickens would both be treated with narasin on the same farm, since concentrated animal feeding operations, where use of feed additives primarily occurs, are typically single species facilities. Nonetheless, land application of swine manure from swine treated with Skycis™, results in a slightly lower soil concentration than from Monteban™. Therefore, the RQs will not be higher than those estimated for Monteban™ above.

Considering the use of narasin at different farms in the same watershed, for feed additives used primarily in concentrated animal feed operations, the route of entry to the environment is considered to be land application of litter or manure to crops or pasture for fertilization purposes and run off from that land into surface waters. Runoff directly from locales of husbandry is not expected for these operations. Additionally, land application may not occur at the same geographic location as where the animal husbandry occurs. The highest PEC_{soil} and $PEC_{surface\ water}$ for narasin will still be that resulting from treatment of chickens with Monteban™. Use of Maxiban™ or Skycis™ will result in lower environmental concentrations and RQs. Since the RQs for Monteban™ are lower than 1, it is concluded that these potential scenarios of cumulative effects of narasin would not result in a risk to terrestrial or aquatic species.

5.4.2 Nicarbazin

The following potential scenarios of cumulative impacts were considered: (1) use of nicarbazin for multiple indications in the same animals on the same farm, (2) use of nicarbazin in different species on the same farm, and (3) use of nicarbazin in the same or different species on different farms in the same watershed. The only approved indication for nicarbazin in food animal species is for the prevention of coccidiosis in broiler chickens, as evaluated in the current assessment. The approved use requires continuous feeding as the sole ration and does not allow multiple products containing nicarbazin to be fed simultaneously. Therefore, there is no potential for Maxiban™ to contribute to levels of DNC or HDP in the environment above those that could be contributed by other approved products containing nicarbazin. Based on the RQs derived in this EA, the potential scenarios of cumulative impacts from the use of nicarbazin in Maxiban™ would not result in a substantial risk to the environment.

5.5 Alternatives to the Proposed Action

The only alternative to the proposed action is the "no action" alternative, which would be the failure to approve the supplemental new animal drug application (NADA) for Maxiban™ (narasin, nicarbazin) Type A Medicated Article. However, based on our analysis in this environmental assessment, we do not believe that significant environmental impacts will occur from this action. Therefore, the "no action" alternative was eliminated from consideration.

5.6 Agencies and Persons Consulted

No other agencies or persons were consulted in the preparation of this environmental assessment.

6.0 Information on Environmental Assessment Expert

The following individuals were responsible for the information in the Environmental Assessment Report for narasin and nicarbazin used as Maxiban for prevention of coccidiosis in broiler chickens:

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

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

Signature: Emiko S. Freklau on behalf of A. Perkins

Date: 09/27/2017

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

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

Degrees:

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

Current and Previous Appointments:

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

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

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

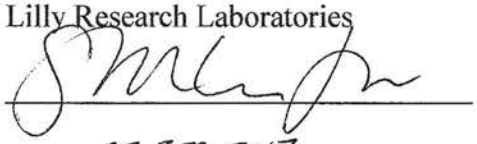
Editorial Board of Environmental Toxicology and Chemistry

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

Publications:

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

Name of the expert: Michael Robert Lee
Environmental Risk Assessment Group
Lilly Research Laboratories

Signature: 

Date: 27 SEP 2017

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

Name: Michael Robert Lee
Address: Lilly Corporate Center
Indianapolis, IN 46285
USA

Degrees:

BA	Biology, Rhode Island College	2005
MS	Biology, University of Massachusetts, Dartmouth	2015

Current and Previous Appointments:
Associate Consultant, Environmental Risk Assessment, Lilly Research Laboratories (2016 to present)
Senior Associate, Environmental Risk Assessment, Lilly Research Laboratories (2013 to 2016)
Biologist II, Ecotoxicology, Smithers Viscient (2006 to 2013)
Biological Laboratory Technician, Commonwealth Sciences (2005 to 2006)
Technical Field Intern, Rhode Island Department of Environmental Management (2005)

Publications:
One publication and several presentations and posters in the field of environmental toxicology

7.0 References

1. ASTM. 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. Publication E729-80, American Society for Testing and Materials, Philadelphia, PA.

2. Avery ML, Keacher KL, Tillman EA. 2008. Nicarbazin bait reduces reproduction by Pigeons (*Columba livia*). Wildlife Research 35:80-85.

3. Bynum K, Yoder C, Eisemann J, Johnston J, Miller L. 2005. Development of nicarbazin as a reproductive inhibitor for resident Canada geese. Wildlife Damage Management Conferences – Proceedings. Paper 101. http://digitalcommons.unl.edu/icwdm_wdmconfproc/101

4. CVM. 2001. Guidance for Industry #89, Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) – Phase I, VICH GL 6, Final Guidance. Docket No. 99D-2975. Center for Veterinary Medicine, US Food and Drug Administration. 10 pp.

5. CVM. 2006. Guidance for Industry #166, Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) – Phase II, VICH GL 38, Final Guidance. Docket No. 2004D-0156. Center for Veterinary Medicine, US Food and Drug Administration. 37 pp.

6. Cuckler AC, Malanga CM, Basso AJ, O'Neill RC. 1955. Antiparasitic activity of substituted carbanilide complexes. Science 122:244-245.

7. ECHA. 2016. European Chemicals Agency. Guidance on information requirements and chemical safety assessment Chapter R.16: Environmental Exposure Assessment Version 3.0. ECHA-16-G-03-EN. https://echa.europa.eu/documents/10162/13632/information_requirements_r16_en.pdf

8. EPA. 2005. Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances. Pesticide Fact Sheet Nicarbazin. 22 pp.

9. Hughes BL, Jones JE, Toler JE. 1991. Effects of exposing broiler breeders to nicarbazin contaminated feed. Poultry Science 70: 476-482.

10. Jones JE, Solis J, Hughes BL, Castaldo DJ, Toler JE. 1990a. Reproduction responses of broiler-breeders to anticoccidial agents. Poultry Science 69: 27-36.

11. Jones JE, Solis J, Hughes BL, Castaldo DJ, Toler JE. 1990b. Production and egg-quality responses of white leghorn layers to anticoccidial agents. Poultry Science 69: 378-387.

12. Jones JE, Solis J, Hughes BL, Castaldo DJ, Toler JE. 1990c. Effect of nicarbazin on brown-egg layer-breeders. Applied Agricultural Research 5:149-152.

13. Karnak RE, Hamelink JL. 1982. A standardized method for determining the acute toxicity of chemicals to earthworms. Ecotoxicology and Environmental Safety 6: 216-222.

14. Kim S-C, Carlson K. 2006. Occurrence of ionophore antibiotics in water and sediments of a mixed-landscape watershed. Water Research 40:2549-2560.

15. Kim S-C, Carlson K. 2007. Quantification of human and veterinary antibiotics in water and sediment using SPE/LC/MS/MS. Analytical and Bioanalytical Chemistry 387:1301-1315.

16. Leeson S, Caston LJ, Summers JD. 1989. The effect of graded levels of nicarbazin on reproductive performance of laying hens. Canadian Journal of Animal Science 69: 757-764.

17. Lilly. 1977a. Greenhouse test for narasin phytotoxicity – experiments Q61-3474-287, 276A-3482-29, and B79-3328-66. Eli Lilly and Company, Greenfield Laboratories, Plant Research Science, Greenfield, Indiana.

18. Lilly. 1977b. Phytotoxicity test in field plots manured with floor pen litter from narasin-treated broilers – experiment 276A-3477-8. Eli Lilly and Company, Greenfield Laboratories, Plant Research Science, Greenfield, Indiana.

19. Lilly. 1979. The toxicity of compound 79891 (narasin) to earthworms in a 14-day soil incorporated study, study nos. 6018-78 and 6026-78. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

20. Martinek T, Riddell FG, Wilson CF. 2000. The conformations of narasin-metal complexes in solution determined by NMR spectroscopy. Journal of Chemical Society, Perkin Trans 2:2192-2198.

21. The Merck Index Online, Royal Society of Chemistry, 2017; M7771 (CAS No. 55134-13-9,) M4574 (CAS No. 587-90-6). https://www.rsc.org/merck-index (accessed Sept. 18, 2017)

22. Miyazaki Y, Kinashi H, Otake N, Mitani M, Yamanishi T. 1976. Chemical modification and structure-activity correlation of salinomycin. Agricultural Biological Chemistry 40:1633-1640.

23. Norberg-King, T.J. 1993. Linear interpolation method for sublethal toxicity: The inhibition concentration (ICp) approach (Version 2.0). National Effluent Toxicity Assessment Center Technical Report 03-93. Duluth, MN.

24. Porter CC, Gilfillan JL. 1955. The absorption and excretion of orally administered nicarbazin by chickens. Poultry Science 34:995-1001

25. Reinoso V. 2008. Contraceptive action of nicarbazin in white pekin ducks (Masters Thesis).

26. Rogers EF, Brown RD, Brown JE, Kazazis DM, Leanza WJ, Nichols JR, Ostlind DA, Rodino TM. 1983. Nicarbazin complex yields dinitrocarbanilide as ultrafine crystals with improved anticoccidial activity. Science 222:630-632.

27. Sigma-Aldrich. 2014. Material Safety Data Sheet, 2-Hydroxy-4,6-dimethylpyrimidine, Product 225886. Version 5.2, Revision Date: 31 July 2014.

28. Study 130-136. 2009. Pilot laboratory study: Relative bioavailability of DNC in rats administered alone, mixed with HDP and as nicarbazin. Sponsor: Elanco Animal Health, Performing Laboratory MPI Research, Inc.

29. Study A01182. 1985. The toxicity of nicarbazin (compound 93760) to mallards in a five-day dietary study. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

30. Study A01482. 1985. The toxicity of nicarbazin (compound 93760) to bobwhite in a five-day dietary study. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

31. Study C02883. 1983. The acute toxicity to *Daphnia magna* of narasin and nicarbazin (EL-9193, compound 79891 and 93760) tested as a 1:1 premix. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

32. Study CYT 011/014573. 2002. Nicarbazin Technical – Acute Toxicity (LC50) to the Earthworm. Sponsor: Koffolk Ltd., Performing Laboratory: Huntingdon Life Sciences Ltd.

33. Study F07383. 1983. The acute toxicity to bluegill (*Lepomis macrochirus*) of narasin and nicarbazin (EL-9193, compound 79891 and 93760) tested as a 1:1 premix. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

34. Study F07483. 1983. The acute toxicity to rainbow trout (*Salmo gairdneri*) of narasin and nicarbazin (EL-9193, compound 79891 and 93760) tested as a 1:1 premix – I. Determination of the LC50. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

35. Study F08683. 1983. The acute toxicity to rainbow trout (*Salmo gairdneri*) of narasin and nicarbazin (EL-9193, compound 79891 and 93760) tested as a 1:1 premix – II. Determination of the NOEC. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

36. Study W01083. 1983. The toxicity to earthworms (*Lumbricus terrestris*) of soil-incorporated narasin and nicarbazin (EL-9193, compound 79891 and 93760) tested as a 1:1 premix. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

37. Study W00783. 1985. The toxicity of soil-incorporated narasin (compound 79891) to earthworms *(Lumbricus terrestris*) in a 14-day test. Eli Lilly and Company, Lilly Research Laboratories, Greenfield, Indiana.

38. Thompson TS, Noot DK, Forrest F, van den Heever JP, Kendall J, Keenliside J. 2009. Large volume injection for the direct analysis of ionophores and avermectins in surface water by liquid chromatography-electrospray ionization tandem mass spectrometry. Analytica Chimica Acta 633:127-135.

39. Vértesy L, Heil K, Fehlhaber H-W, Ziegler W. 1987. Microbial decomposition of salinomycin. Journal of Antibiotics 40:388-390.

40. VICH. 2000. Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) – Phase I, VICH GL6. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products. June 2000. 9 pp.

41. VICH. 2004. Environmental Impact Assessments for Veterinary Medicinal Products – Phase II Guidance, VICH GL38. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products. June 2000. 38 pp.

42. Wong DT, Berg DH, Hamill RH & Wilkinson JR. 1977. Ionophorous properties of narasin, a new polyether monocarboxylic acid antibiotic, in rat liver mitochondria. Biochemical Pharmacology 26: 1373-1376.

43. Yoder CA, Graham JK, Miller LA, Bynum KS, Johnston JJ, Goodall MJ. 2006. Effect of method of delivering nicarbazin to mallards on plasma 4,4'-dinitrocarbanilide levels and reproduction. Poultry Science 85:1442-1448.

44. Yoder CA, Miller LA, Bynum KS. 2005. Comparison of nicarbazin absorption in chickens, mallards, and Canada geese. Poultry Science 84:1491-1494.

Appendices

Please note that some the study summaries included in the appendices are from studies that were conducted by and are owned by companies other than Elanco. As indicated in a footer to the summaries, permission has been granted by the owner of the data to Elanco Animal Health to use the data contained in those studies.

Appendix A – Poole, et al. The Solubility, Hydrolysis, and Photolysis of Narasin in Aqueous Solutions. 1981.

	Report Title:	***The Solubility, Hydrolysis, and Photolysis of Narasin in Aqueous Solutions***				
	Performing Laboratory:	***Eli Lilly and Company***				
	Authors:	***Poole, GM; West, SD; Donoho, AL***				
	Report Date:	***1981***				
STUDY SUMMARY						
Materials & Methods						
Buffers	***pH 5.0 Buffer***	0.01 M sodium acetate (acetic acid)				
	pH 7.0 Buffer	0.01 M boric acid (sodium hydroxide)				
	pH 9.0 Buffer	0.01 M sodium bicarbonate (sodium carbonate)				
Materials & Methods - Solubility						
Test Article	***Name:***	Narasin				
Solution Preparation	***Amount of test article:***	Narasin added to visible excess				
Environmental Conditions	***Temperature:***	25°C				
	Agitation:	Wrist-action shaker (continuous)				
	Test duration:	72 hours				
Sample Measurement	***Sample volume:***	50 mL				
	Filtration:	0.2-µm Nalgene Filter Unit				
	Analysis:	Turbidimetric method (microbiological activity)				
Results - Solubility						
Water Solubility		***Narasin concentration (mg/L)***				
	Buffer	**24 hr**	**48 hr**	**72 hr**	**Average**	
	pH 5.0	Not stable	Not stable	Not stable	Not stable	
	pH 7.0	103	101	Not tested	102	
	pH 9.0	>552	676	687	681	
Materials & Methods - Hydrolysis						
Test Article	***Name:***	^{14}C Narasin				
Solution Preparation	***Concentrations:***	pH 5: 0.5 mg/L pH 7: 2.0 mg/L pH 9: 1.0 mg/L				
Environmental Conditions	***Temperature:***	25°C in the dark				
	Test duration:	pH 5: 7 days				
	Test duration:	pH 7 & 9: 30 days				
Sample Measurement	***Sample analysis:***	Turbidometric method (microbiological activity), TLC Total radioactivity (extraction followed by LSC)				
Results - Hydrolysis						
	Narasin concentration (mg/L) (by turbidometric assay)					
Buffer	***Day 1***	***Day 2***	***Day 4***	***Day 7***	***Day 15***	***Day 30***
pH 5	0.170	0.120	0.034	0.011	Not tested	Not tested
pH 7	1.04	Not tested	Not tested	0.927	0.948	0.88
pH 9	0.706	Not tested	Not tested	0.698	0.764	0.730

Materials & Methods - Photolysis		
Test Article	***Name:***	^{14}C Narasin
Solution Preparation	***Concentrations:***	pH 7: 2.0 mg/L
	Test vessel:	sterile glass ampule
Environmental Conditions	***Temperature:***	28°C
	Irradiation:	Fluorescent sunlamps (to mimic natural summer sunlight)
	Light intensity:	4.5 x 10^4 uW/cm^2
Sample Measurement	***Sample analysis:***	Turbidometric method (microbiological activity)

Results - Photolysis							
	Narasin concentration (mg/L)						
Trial	***Day 0***	***Day 1***	***Day 2***	***Day 3***	***Day 7***	***Day 15***	***Day 30***
Initial	Not tested	0.875	Not tested	Not tested	0.0	0.045	Not tested
Second	1.60	0.70	0.28	0.14	Not tested	Not tested	Not tested
Dark Control	Not tested	1.04	Not tested	Not tested	0.927	0.948	0.880

Summary			
Buffer	***Solubility***	***Hydrolysis (DT50)***	***Photolysis (DT50)***
pH 5	Not stable	3.5 days	NA
pH 7	102 mg/L	Stable	~1.5 days
pH 9	681 mg/L	Stable	NA

Appendix B – Study 206378. 1,3-Bis (4-nitrophenyl)urea and 4,6-Dimethyl pyrimidine-2-ol Determination of the Water Solubility of 1,3-Bis (4-nitrophenyl)urea and 4,6-Dimethyl pyrimidine-2-ol. Report Date: March 2005.

	Report Title:	***1,3-Bis(4-nitrophenyl)urea and 4,6-Dimethyl pyrimidine-2-ol Determination of the Water Solubility of 1,3-Bis(4-nitrophenyl)urea and 4,6-Dimethyl pyrimidine-2-ol***
	Project Number:	***206378***
	Guidance Document:	***OECD 105***
	GLP Compliance:	***OECD***
	Report Number:	***23964***
	Report Date:	***24 March 2005***
STUDY SUMMARY		
Materials & Methods – 1,3-Bis (4-nitrophenyl) urea		
Test Article	***Name:***	1,3-Bis(4-nitrophenyl)urea (DNC)
	Name:	4,6-Dimethyl pyrimidine-2-ol (HDP)
Analytical Method		HPLC/uv
Buffers	***Reverse Osmosis water***	
	pH 4.0 Buffer:	*0.1 M citrate (adjusted with sodium hydroxide)*
	pH 7.0 Buffer:	*0.1 M phosphate (adjusted to sodium hydroxide)*
	pH 9.0 Buffer:	*0.1 M boric acid*
Sample Preparation	***DNC:***	~ 10 mg DNC and 50 mL of buffer added to 50 mL amber glass jar
	HDP:	~ 1.2 g of HDP and 5 mL of buffer added to 15 mL amber glass jar
	Replication :	3 replicates for each time point
Environmental Conditions	***Preincubation:***	30°C for 24, 48 and 72 hours
	Incubation:	20 ± 0.5°C for 24 hours
Sample Measurement	***Sampling time points:***	24, 48 and 72 hour
Results – 1,3-Bis (4-nitrophenyl) urea (DNC)		
Water Solubility	***MilliRO Water:***	< 0.02 mg/L
	pH 4.0 Buffer	< 0.02 mg/L
	pH 7.0 Buffer	< 0.02 mg/L
	pH 9.0 Buffer	< 0.02 mg/L
Results – 4,6-Dimethyl pyrimidine-2-ol (HDP)		
Water Solubility	***MilliRO Water:***	69.23 g/L
	pH 4.0 Buffer	70.72 g/L
	pH 7.0 Buffer	66.32 g/L
	pH 9.0 Buffer	71.45 g/L

Study 206378 was conducted by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 206378.

Appendix C – Study 151C-120. Narasin – Determination of the n-Octanol/Water Partition Coefficient by the Shake Flask Method Following OECD Guideline #107. January 2008.

	Report Title:	***NARASIN – Determination of the n-Octanol/Water Partition Coefficient by the Shake Flask Method Following OECD Guideline #107***
	Project Number:	***151C-120***
	Guidance Document:	***OECD 107***
	GLP Compliance:	***FDA & OECD***
	Report Date:	***30 January 2008***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Narasin
Buffers	***pH 5.0 Buffer:***	0.01 M sodium acetate & acetic acid
	pH 7.0 Buffer:	0.01 M mono-potassium phosphate & sodium hydroxide
	pH 9.0 Buffer:	0.01 M sodium hydroxide, boric acid & potassium chloride
Methods	***Method:***	Shake-flask
	Water : octanol ratios:	1:7, 1:3 & 1:1 at each pH
	Test vessel:	Teflon centrifuge tube
	Replication:	2 vessels per solvent ratio
	Temperature:	25°C
	Agitation:	5 minutes in agitating waterbath
	Phase separation:	Centrifugation
Analytical Method		HPLC/MS

Results

	Mean Log Pow		
Solvent Ratio	***pH 5***	***pH 7***	***pH 9***
1:7	4.79	5.01	4.86
1:3	4.92	4.85	5.01
1:1	4.56	4.69	5.31
Mean ± SD	**4.79 ± 0.175**	**4.85 ± 0.167**	**5.06 ± 0.262**

Appendix D – Study ADM-56. The Determination of the Distribution Coefficients of the Components of Nicarbazin between 1-octanol and Aqueous Buffers. March 1986.

	Report Title:	***The determination of the distribution coefficients of the components of nicarbazin between 1-octanol and aqueous buffers (ADM-56)***
	Project Number:	***ADM-56***
	Report Date:	***24 March 1986***
STUDY SUMMARY		
Materials & Methods		
Test Articles	***Names:***	14C-HDP (radiopurity 99.4%) 14C-DNC (radiopurity 99 to 100.1%)
Buffers	***pH 5 Buffer:***	0.05 M sodium acetate adjusted with acetic acid
	pH 7 Buffer:	0.05 M potassium phosphate adjusted with sodium hydroxide
	pH 9 Buffer:	0.05 M sodium borate adjusted with acetic acid
Solvents	***Buffers and 1-octanol:***	Mutually pre-saturated
Methods	***Method:***	Shake-flask
	Buffer: octanol ratios:	10 mL:1 mL for DNC 5 mL:5 mL for HDP
	Test vessel:	15-mL centrifuge tube
	Replication:	2 vessels per pH per test article
	Temperature:	25°C
	Agitation:	3 minutes by vortexing, then in a shaking water bath
	Equilibration duration:	1 vessel for 1 hour and 1 vessel for 24 hours
	Phase separation:	Centrifugation
Analytical Method		LSC of duplicate samples of octanol and buffer layers

Results

Equilibration Times (hr)	*pH*	*Mean DNC Kow**	*DNC Kow at pH (log Kow)*	*Mean HDP Kow*	*HDP Kow at pH (log Kow)*
1	*5*	*4106*	*pH 5*	*0.112*	*pH 5*
24	*5*	*4241*	*4174 (3.6)*	*0.113*	*0.113 (-0.95)*
1	*7*	*3865*	*pH 7*	*0.122*	*pH 7*
24	*7*	*4320*	*4093 (3.6)*	*0.122*	*0.122 (-0.91)*
1	*9*	*3301*	*pH 9*	*0.114*	*pH 9*
24	*9*	*3950*	*3626 (3.6)*	*0.115*	*0.115 (-0.94)*
	Overall Mean Kow	**3964**		**0.116**	
	Std Dev	**367**		**0.005**	
	Log Kow	**3.60**		**-0.93**	

*As described in the report, the radioactivity in the buffer layers in the DNC test was less than the radioactivity that may be impurities in the test substance. Therefore, the reported Kow and log Kow are the minimum possible values.

Study ADM-56 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study ADM-56.

Appendix E – Study ABC-0260. Chemical and Radiochemical Characterization of ^{14}C Residues in Excreta from Chickens Dosed with Ration Containing 80 ppm ^{14}C Narasin. June 1984.

	Report Title:	***Chemical and Radiochemical Characterization of ^{14}C Residues in Excreta from Chickens Dosed with Ration Containing 80 ppm ^{14}C Narasin***
	Performing Laboratory:	***Eli Lilly and Company***
	Study Number:	***ABC-0260***
	GLP Compliance:	***FDA & OECD***
	Report Date:	***28 June 1984***
STUDY SUMMARY		
Materials & Methods		
Radiolabelled Test Article	***Name:***	^{14}C-narasin
	Radiopurity:	> 94%
Non-Radiolabelled Test Article	***Name:***	Narasin
Dosing Preparation	***Preparation of treated ration:***	Isotopically-diluted ^{14}C-narasin was mixed with chicken ration to obtain 80 ppm in feed
	Dose confirmation:	Dose confirmed by liquid scintillation counting of methanol extracts
Test Species		Male and female broiler chickens, 8 weeks old at initiation of dosing
Exposure Design	***Dose Groups:***	Test group – ^{14}C-narasin-dosed ration Control group - non-dosed ration
	Duration:	7 days
	Route:	Oral, via feed
	Feed and Water:	Ad libitum, food consumption measured daily
Collection of Samples	***Excreta:***	Excreta was collected between days 4 and 7 as a pooled test sample from treated and control chickens
Evaluation of Residues	***Total radioactivity in excreta:***	Combustion followed by liquid scintillation counting
	Characterization of metabolites:	Excreta was extracted with methanol, extracts were fractionated, and fractions subjected to thin layer chromatography
	Measurement of Narasin in Excreta:	Excreta was extracted with methanol and extracts fractionated by HPLC. Narasin was quantified in fractions by uv following derivatization. Methanol extracts were also measured for narasin content by a microbiological potency assay against *Streptococcus faecalis*.
Results		
Dose	***Total daily narasin intake:***	12.5 mg/broiler
Excreta Residues	***Narasin content by HPLC:***	12.1 mg/kg
	Narasin content by microbiological activity:	11.5 mg/kg
	Total radioactivity in excreta:	237 mg/kg (narasin equivalents)
	% of radioactive residue that is narasin:	4.9 to 5.1%
	Metabolite characterization:	Narasin was converted to many metabolites, no single metabolite exceeded 11.3% of the total radioactivity.
	Identified metabolites:	NM-1, NM-2, NM3, NM-4, NM-5, NM-6 and NM-7 were approximately 25% of the excreta radioactivity.

Appendix F – Study T4H969301. A Comparative Metabolism Study in Tissues and Excreta of Chickens Dosed with 14C-Narasin with and without Nicarbazin. April 1994.

	Report Title:	***A Comparative Metabolism Study in Tissues and Excreta of Chickens Dosed with 14C-Narasin with and without Nicarbazin***
	Performing Laboratory:	***Eli Lilly and Company***
	Study Number:	***T4H969301***
	Test Dates:	***May and June 1993***
	GLP Compliance:	***FDA & OECD***
	Report Date:	***4 April 1994***
STUDY SUMMARY		
Materials & Methods		
Radiolabelled Test Article	***Name:***	^{14}C-narasin (multiple positions)
	Radiopurity:	97.4%
Non-Radiolabelled Test Articles	***Name:***	Narasin
	Name:	Nicarbazin premix
Dosing preparation	***^{14}C-Narasin ration, Treatment 01:***	Isotopically diluted ^{14}C-narasin was mixed with chicken ration to give 50 ppm narasin in feed
	Narasin + Nicarbazin ration, Treatment 02:	Nicarbazin and isotopically diluted ^{14}C-narasin mixed with chicken ration to give concentration in feed of 50 ppm narasin and 50 ppm nicarbazin.
Test Species		Broiler chickens, male and female approximately 6 weeks old at initiation of dosing
Exposure Design	***Dose Groups:***	Control – basal ration Treatment 01 – 50 ppm ^{14}C-narasin ration Treatment 02 –ration containing 50 ppm ^{14}C-narasin and 50 ppm nicarbazin ration 3 males and 2 females per group
	Duration	5 days
	Route	Oral, via feed
	Feed and Water	ad libitum, food consumption measured daily
Exposure Assessment	***Endpoints:***	Liver, kidneys, muscle, fat, and skin; collected at slaughter Excreta collected daily beginning one day before initiation and continuing until end of treatment
	Methods:	Excreta combusted and analyzed by LSC for total radioactivity Tissues solubilized and analyzed by LSC for total radioactivity Excreta and tissues extracted, fractionated and analyzed by HPLC/ISP/MS/LC and thin layer chromatography

Results				
Total Tissue Residues	***Mean ppm narasin equivalents at slaughter***		***Treatment 01***	***Treatment 02***
		Liver	0.32[‡]	0.27[‡]
		Kidney	0.04	0.04
		Muscle	<0.04	<0.02
		Fat	0.12[‡]	0.07[‡]
		Skin/Fat	0.08	0.05
		[‡]only tissues with sufficient residues for metabolite characterization		
Characterization of Metabolites	***Liver***	61% and 75% radioactivity extracted from Treatments 01 and 02. Radiochromatograms showed similar wide distribution of radioactivity.		
	Fat	Parent narasin represented 61% and 56% of the total radioactivity in fat for Treatments 01 and 02.		
	Excreta	90% of the radioactivity in excreta was extracted and characterized. Almost 50% was characterized as hydroxylated metabolites, 3% as parent narasin, and the remaining radioactivity was in minor fractions and was too low to characterize.		
Impact of nicarbazin	Nicarbazin had no effect on the metabolism of narasin			

Appendix G – Wong. Effect of Narasin Metabolites on ATPase and Oxygen Uptake in Rat Liver Mitochondria. 1978

Performing Laboratory: Lilly Research Laboratories

Test Article: Narasin metabolite preparations from chicken and cattle excreta

Methods:
Narasin and four narasin metabolite preparations were evaluated for determination of ionophorous properties by measurement of their effects on ATPase activity in the presence of a alkali metal cation and oxygen uptake upon oxidation of malate and glutamate in rat liver mitochondria. Mitochondria were isolated from the livers of male Sprague-Dawley rats (110 – 115 g). The four narasin metabolites tested were: metabolite F (a dihydroxy narasin), NM-3 (a dihydroxy narasin), NM-2 (a trihydroxy narasin) and the fourth was a mixture of NM-3 and NM-6 (a dihydroxy narasin). To determine effects on ATPase activity and oxygen uptake, the metabolites were individually tested at concentrations up to approximately 20 µg/mL. For comparison, the IC50 for ATPase activity and oxygen uptake was determined for narasin.

Results:
All four of the metabolite preparations were at least 200 times less active as ionophores than narasin.

Appendix H – Manthey and Goebel. Isolation and Characterization of Narasin Metabolites Derived from Excreta of Orally Dosed Chickens. 1982.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline ^{14}C Narasin

Methods:
Excreta from broiler chickens which were dosed for five days with 100 ppm ^{14}C narasin was extracted with methanol to recover ^{14}C narasin and metabolites. The extract was subjected to fractionation by liquid-liquid partitioning, silica gel column chromatography, thin-layer chromatography and reversed phase HPLC to characterize the distribution of narasin metabolites. Specific metabolite fractions were purified and subjected to analysis by mass spectrometry.

Additionally, the purified metabolites were assayed for relative antimicrobial activity against *Bacillus subtilis* in a standard narasin TLC bioautographic assay. Radioactivity equivalent to 500 ng of each metabolite was applied to TLC plates, along with 25 ng of narasin.

Results:
Seven individual metabolites were isolated, labeled as NM-1 through 7. Six metabolites were identified by mass spectometry. The seventh metabolite (NM-5) was not isolated in sufficient quantity for mass spectral analysis. None of the metabolites constituted a large percentage of the total radioactivity.

The metabolites were found to be di- or trihydroxylated narasin in which the hydroxy groups were substituted for hydrogen in various positions on the rings of the narasin molecule. Thus, in chickens a primary mode of narasin metabolism is hydroxylation of the narasin molecule.

None of the metabolites exhibited zones of antimicrobial activity, while narasin did provide an antimicrobial response. In this assay, the metabolites were 20 times less active than parent narasin against *Bacillus subtilis*.

Appendix I – Study 805286. The Absorption, Distribution, Metabolism and Excretion of [^{14}C]-HDP Following Multiple Administrations of Nicarbazin Containing [^{14}C]-HDP to Broiler Chickens. 2007.

	Report Title:	***The Absorption, Distribution, Metabolism and Excretion of [^{14}C]-HDP Following Multiple Administrations of Nicarbazin Containing [^{14}C]-HDP to Broiler Chickens***
	Study Number:	***805286***
	GLP Compliance:	***OECD***
	Report Number:	***24715***
	Report Date:	***15 February 2007***
STUDY SUMMARY		
Materials & Methods		
Radiolabelled Test Article	***Name:***	4,6-dimethyl pyrimidine-2-ol, [2-^{14}C]; [^{14}C]-HDP
	Radiochemical purity:	99.78%
Non-Radiolabelled Test Articles	***Name:***	2-hydroxy-4,6-dimethyl pyrimidine (HDP)
	Name:	4,4' dinitrocarbanilide (DNC)
Dose Preparation	***Dose preparation:***	Gelatin capsules loaded with ^{14}C-nicarbazin (mixture of 14C-HDP and DNC)
Test Species		Broiler chickens approximately 3 weeks old at initiation of dosing
Exposure Design	***Target dose:***	125 mg nicarbazin/kg food/day
	Duration:	7 days
	Dosing:	Twice daily (am and pm) administered in gelatin capsules by oral gavage
	Treatment Groups:	Group 1: 3 Males, 3 Females sacrificed 24 h after last am dose Group 2: 3 Males, 3 Females sacrificed 72 h after last am dose Group 3: 3 Males, 3 Females sacrificed 120 h after last am dose Group 4: 3 Males, 3 Females sacrificed 240 h after last am dose
	Environmental conditions:	16 h light/8 h dark cycle Temperature range 19 to 22 °C Humidity range 33 to 75%
	Feed/Water:	ad libitum with daily food consumption recorded
Exposure Assessment	***Endpoints:***	Tissue residues in liver, kidneys, skin with fat and muscle Excreta samples collected from Group 4 at 24 hour intervals throughout dosing period until sacrifice Body weights prior to dosing and prior to sacrifice
		Excreta and tissues analyzed by combustion/LSC Excreta analyzed by HPLC w/ radiodetection Tissues analyzed by HPLC with fraction collection and LSC Extracts analyzed by LC/MS for metabolite identification

Results					
Dosing	***Mean dose levels***	126.289 to 130.241 mg/kg food (based on food consumption)			
Excreta Residues	***Total Radioactive Residues in Excreta:***	***Time***		***% Administered Dose***	
		168 h	(24 h after last am dose)	96.71	
		192 h	(48 h after last am dose)	98.22	
		216 h	(72 h after last am dose)	98.89	
		240 h	(96 h after last am dose)	99.27	
		264 h	(120 h after last am dose)	99.44	
		288 h	(144 h after last am dose)	99.66	
		312 h	(168 h after last am dose)	99.79	
		336 h	(192 h after last am dose)	99.94	
		360 h	(216 h after last am dose)	100.04	
		384 h	(240 h after last am dose)	100.23	
	Characterization of Excreta Residues:	***Excreta***	***% of TRR extracted and characterized***	***%TRR identified as HDP***	
		2 males	71.46% and 73.85%	58 to 66% of TRR depending on method used	
		2 females	77.14% and 73.96%		
		Other polar peaks were observed that were less than 10% of the TRR in excreta			
Tissue Residues	***Total Radioactive Residues in Tissues:***	***Tissue***	***Mean Concentration µg equiv/g***			
			24 hrs after last am dose	72 hrs after last am dose	120 hrs after last am dose	240 hrs after last am dose
		Kidney	0.134	<0.005	<0.002	<0.002
		Skin with fat	0.106	0.027	<0.017	<0.006
		Liver	0.095	0.008	<0.006	<0.002
		Muscle	0.084	<0.003	<0.002	<0.001
	Characterization of Tissue Residues:	The primary component of extractable radioactivity in liver, muscle and skin with fat was HDP; the primary component in kidney was an unidentified, more polar peak, this unidentified peak was also a major component in the other three tissues			

Study 805286 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 805286.

Appendix J – Study 805129. The Residue Depletion and Metabolic Identification of [^{14}C]-DNC in Chickens Following Repeated Administrations of Nicarbazin-Containing [^{14}C]-DNC. 2007.

	Report Title:	***The Residue Depletion and Metabolic Identification of [^{14}C]-DNC in Chickens Following Repeated Administrations of Nicarbazin-Containing [^{14}C]-DNC***
	Study Number:	***805129***
	GLP Compliance:	***OECD***
	Report Number:	***24697***
	Report Date:	***16 March 2007***
STUDY SUMMARY		
Materials & Methods		
Radiolabelled Test Article	***Name:***	1,3-bis(4-nitrophenyl)urea, [phenyl-^{14}C(U)], [14C]-DNC
	Radiochemical purity:	99%
Non-Radiolabelled Test Articles	***Name:***	4, 4-dinitrocarbanilide (DNC)
	Name:	2-hydroxy-4,6-dimethyl-pyrimidine (HDP)
Dose Preparation		Isotopically diluted [^{14}C]-DNC was mixed with HDP to prepare ^{14}C-nicarbazin (confirmed by LSC).
Test Species		Broiler chickens, male and female, approximately 3 weeks old at initiation of dosing
Exposure Design	***Dose:***	125 mg nicarbazin/kg food consumed/day
	Duration:	7 days
	Dosing:	Two equal portions of 62.5 mg/kg administered in gelatin capsules by oral gavage (in am and in pm)
	Treatment Groups:	Group 1: 3 Males, 3 Females Sacrificed 24 hr after last am dose Group 2: 3 Males, 3 Females Sacrificed 120 hr after last am dose Group 3: 3 Males, 3 Females Sacrificed 240 hr after last am dose
Exposure Assessment	***Endpoints:***	Tissue residues in liver, kidneys, skin with fat and muscle Excreta samples collected from Group 3 at 24 hour intervals throughout dosing period until sacrifice
	Methods:	Excreta and tissues analyzed by combustion/LSC Excreta and tissues extracted and analyzed by LSC and HPLC w/ radiodetection Excreta and tissue extracts analyzed by LC/MS for metabolite identification

Results					
Dosing	***Actual dose on a per kg food consumed:***	Group 1		104.590 mg/kg	
		Group 2		105.331 mg/kg	
		Group 3		101.185 mg/kg	
Excreta Residues	***Total Radioactive Residues in Excreta:***	***Time***	***Hours after last am dose***	***Mean or Range µg equiv/g***	***% Administered Dose***
		168 h	24 h	53.660	85.04
		192 h	48 h	22.944	91.16
		216 h	72 h	12.494	94.72
		240 h	96 h	8.273	96.97
		264 h	120 h	3.569	97.91
		288 h	144 h	2.705	98.74
		312 h	168 h	1.076	99.02
		336 h	192 h	1.051	99.28
		360 h	216 h	0.663	99.46
		384 h	240 h	0.413	99.58
	Characterization of Excreta Residues:	***Excreta***	***% of TRR extracted and characterized***	***%TRR identified as DNC***	
		Pooled male	50.17	45.15	
		Pooled female	41.82	36.05	
		Several other peaks were observed that were individually less than 3% of the TRR in excreta			
Tissue Residues	***Total Radioactive Residues in Tissues***	***Tissue***	***Mean Concentration µg equiv/g***		
			24 hr after last am dose	120 hr after last am dose	240 hr after last am dose
		Liver	27.797	0.608	0.050
		Kidney	16.776	0.369	0.033
		Skin with fat	5.122	0.151	0.024
		Muscle	4.431	0.069	0.002
	Characterization	The primary component of extractable radioactivity was DNC. An acetyl derivative of DNC was observed and several components less than 10% of the total radioactive residues.			

Study 805129 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 805129.

Appendix K – T4H749304. ^{14}C Nicarbazin Tissue Residues and Metabolism in Chickens Fed ^{14}C Nicarbazin With and Without Unlabeled Narasin. 1994.

	Report Title:	***^{14}C Nicarbazin Tissue Residues and Metabolism in Chickens Fed ^{14}C Nicarbazin With and Without Unlabelled Narasin***
	Performing Laboratory:	***Eli Lilly and Company***
	Study Number:	***T4H749304***
	GLP Compliance:	***OECD & FDA***
	Report Date:	***24 May 1994***
STUDY SUMMARY		
Materials & Methods		
Radiolabelled Test Article	***Name:***	^{14}C-4,6-dimethyl-2-pyrimidinol (HDP)
	Lot number:	553-88C-103
Non-Radiolabelled Test Articles	***Name:***	N,N'-bis-(4-nitrophenyl)urea (DNC)
	Name:	Nicarbazin
	Name:	Narasin
Dosing preparation	***Treatment 01 Ration Preparation:***	^{14}C nicarbazin (a mixture of 14C-HDP and DNC) was mixed with chicken feed to give a ration with 50 ppm nicarbazin
	Treatment 02 Ration Preparation:	^{14}C nicarbazin (a mixture of 14C-HDP and DNC) and narasin was mixed with chicken feed to give a ration with 50 ppm nicarbazin and 50 ppm narasin
Test Species	***Broiler chickens:***	Broiler chickens, male and female approximately 6 weeks old at initiation of dosing
Exposure Design	***Dose Groups:***	Control – fed basal CK-22 ration Treatment 01 – ration containing 50 ppm 14C-nicarbazin Treatment 02 – ration containing 50 ppm 14C-nicarbazin and 50 ppm narasin
	Duration:	5 days
	Route:	oral, via feed
	Lighting:	continuous light
	Withdrawal Period:	No withdrawal
	Feed:	ad libitum, food consumption measured daily
Exposure Assessment	***Endpoints:***	Liver, kidneys, muscle, fat, and skin; collected at slaughter and pooled by sex and treatment Excreta collected daily from two treatment groups beginning one day before initiation and continuing until end of treatment
	Methods:	Excreta combusted and analyzed by LSC for total radioactivity Tissues solubilized and analyzed by LSC for total radioactivity Excreta and tissues extracted, fractionated and analyzed by HPLC/ISP/MS/LC and thin layer chromatography

Results				
Total Tissue Residues	***Mean ppm ^{14}C-nicarbazin equivalents:***		***Treatment 01***[‡]	***Treatment 02***[‡]
		Liver	0.44	0.47
		Kidney	0.73	0.83
		Muscle	0.36	0.38
		Fat	0.04	0.04
		Skin/Fat	0.12	0.12
		[‡]Radioactivity from all tissues was >90% extractable		
Characterization of Metabolites	***Liver, Kidney, Muscle:***	HDP accounted for 67% to 84% of total radioactivity in these tissues		
	Excreta:	HDP accounted for greater than 84% of the total radioactivity in excreta		
	Influence of narasin:	There were no significant differences in metabolism between the two treatment groups.		

Appendix L – Study ABC-0293. A ^{14}C Nicarbazin-Narasin Metabolism Study in Broiler Chickens. 1985.

	Report Title:	***A ^{14}C Nicarbazin-Narasin Metabolism Study in Broiler Chickens***
	Performing Laboratory:	***Eli Lilly and Company***
	Study Number:	***ABC-0293***
	GLP Compliance:	***FDA & OECD***
	Report Date:	***5 December 1985***
STUDY SUMMARY		
Materials & Methods		
Radiolabelled Test Article	***Name:***	^{14}C-N,N'-bis-(4-nitrophenyl) urea (^{14}C-BNPU aka ^{14}C-DNC)
Non-Radiolabelled Test Articles	***Name:***	N,N'-bis-(4-nitrophenyl) urea (BNPU aka DNC)
	Name:	4,6-dimethyl-2-pyrimidinol HCl (HDP)
	Name:	Narasin (mycelial)
Dosing preparation	***Preparation of ^{14}C-Nicarbazin ration:***	^{14}C-nicarbazin (a mixture of isotopically diluted ^{14}C-DNC and HDP) was mixed with ration such that the concentration of nicarbazin was 50 ppm in feed
	Preparation of ^{14}C-Nicarbazin + Narasin ration:	^{14}C-Nicarbazin and narasin were added to chicken ration such that the concentration of nicarbazin was 50 ppm in feed and the concentration of narasin was 50 ppm in feed
Test Species	***Broiler chickens:***	male and female, 8 weeks old at dosing initiation
Exposure Design	***Dose Groups:***	1. 14 Chickens fed ration with 50 ppm ^{14}C nicarbazin 2. 8 Chickens fed ration with 50 ppm ^{14}C nicarbazin and 50 ppm narasin
	Duration:	5 days
	Route:	oral, via feed
	Withdrawal Period:	No withdrawal
	Feed/Water:	ad libitum until time of sacrifice; food consumption recorded
Evaluation of Residues	***Methods:***	Total radioactivity in tissues: LSC following solubilization Excreta and tissues extracted and analyzed by LPLC and TLC chromatography Mass spectrometry to identify isolated metabolites
Results		
Residues	***Characterization of Residues***	TLC of tissue and excreta extracts presented identical patterns of metabolite distribution for the ^{14}C-nicarbazin-treated chickens and the ^{14}C-nicarbazin+narasin-treated chickens.
		Tissue and excreta contained primarily parent ^{14}C DNC with small quantities of 3 other metabolites.
		Metabolism proceeded primarily through reduction and acetylation of one nitro group or both nitro groups. There was also evidence of cleavage of the DNC molecule

Appendix M – Study 151E-125. Narasin: Aerobic mineralization & transformation in chicken manure. Report Date: March 2011.

Test Article: Crystalline ^{14}C Narasin

Methods:
The degradation of ^{14}C narasin was evaluated in two test systems: fresh chicken manure and fresh chicken manure mixed with used litter in a 4:1 ratio (based on dry weight). The test systems were placed in the test chambers, dosed with 0.5 mg [^{14}C]-narasin/kg (oven-dry weight), and incubated in air-flow-through systems under aerobic conditions at approximately 20°C for 35 days. Laboratory air was pulled through the chamber by means of a vacuum to provide aerobic conditions and air exiting the test chambers was passed through a sorbent material and then a KOH solution to trap organic volatiles and $^{14}CO_2$, respectively.

Duplicate test chambers were sacrificed on Days 3, 7, 14, 28 and 35 and contents were extracted with methanol. The methanol extracts were concentrated by partitioning into chloroform. The concentrated extracts were analyzed with liquid scintillation counting (LSC) and High Performance Liquid Chromatography (HPLC/β-RAM).

Matrix controls and viability controls (test systems dosed with ^{14}C glucose) were incubated under the same test conditions as the test chambers. Evolved $^{14}CO_2$ was trapped in KOH traps. Microbial viability was also assessed by measurement of respiration (total CO_2 evolved from the test systems), microbial biomass and enumeration of colony-forming units.

Concentrated samples of the methanol extract collected on Days 7 and 14 were profiled for identification and quantification of parent material and degradation products using LSC, HPLC/β-RAM, HPLC/MS/MS, HPLC Ion-trap MS/MS, and UPLC Time of flight/MS.

Results:
Microbial biomass, glucose mineralization rate, and enumeration of colony-forming units provided evidence that microbial population was viable and metabolically active at the beginning of the test and trended down at the end. Viability and metabolic activity of two test systems were similar.

The amount of narasin in the test systems declined to about 50% of the initial dose levels by test Day 7 and remained around that level for the rest of the study. About 2% of the dosed radioactivity was captured as carbon dioxide or volatile organics by the end of the study. Between about 10 and 15% of the radioactivity could not be extracted from solids throughout the study. The distribution of radioactivity in the test systems is included in the table:

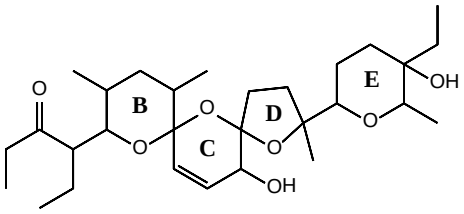
Test Day Sampled	Test System	Mean Recovery of applied radioactivity (%)				
		Total recovered	Total captured as CO_2 plus Volatiles	Non-extractable residues in solid	Total extracted in methanol	Total in extract identified as narasin
0	Manure	113	na	14.3	99	91.6
	Manure+Litter	120	na	12.5	107	102
3	Manure	105*	0.033	9.7	96	85.5
	Manure+Litter	112	0.017	11.7	100	72.2
7	Manure	103	0.065	14.3	89	48.1
	Manure+Litter	107	0.113	15.5	91	55.4
14	Manure	110	0.111	13.8	96	52.6
	Manure+Litter	111	0.150	14.3	97	51.8
28	Manure	106	0.872	12.8	92	52.9
	Manure+Litter	110	0.678	15.3	94	47.6
35	Manure	107	2.196	13.5	91	49.0
	Manure+Litter	108*	1.800	14.6	91	36.9

*Rounded mean values in table do not exactly add up to the rounded mean total recovery values

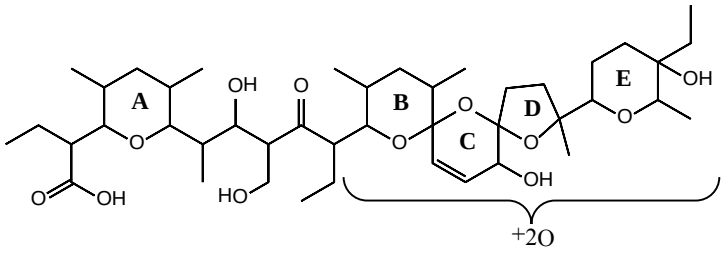
Degradation profiling analysis was performed by ABC Laboratories, Inc. A total of nine transformation product peaks were resolved in a concentrated sample of the methanol extract, along with narasin. These peaks were quantified in the Day 7 and Day 14 chicken manure and manure/litter extracts by fractionation and liquid scintillation counting. The other peaks of radioactivity in the extracts were very minor.

Among the nine degradation products, Peak 3 and Peak 9 were the largest and accounted for approximately 27-29% and 19-24% of the radioactivity in the concentrated sample of extract, respectively; while the other seven quantified peaks were each less than 6%. Parent narasin accounted for approximately 38 to 41% of the radioactivity in the concentrated methanol extract. This profile was consistent in both Day 7 and Day 14 samples.

The isolated components in Peaks 3 and 9 were investigated using LC-MS/MS techniques. The data for Peak 3 are consistent with narasin without the A ring. A proposed structure for degradation product Peak 3 is shown below:



The data for Peak 9 are consistent with a trihydroxylated narasin. A proposed structure for degradation product Peak 9 is shown below with an indication that two additional oxygen atoms exist in hydroxyl groups somewhere in the indicated portion of the molecule:



Appendix N – Study 802374. The Degradation of [^{14}C]-Narasin in Soil Under Aerobic Conditions. 2002.

	Report Title:	**The Degradation of [^{14}C]-Narasin in Soil Under Aerobic Conditions**			
	Guidance Document:	***Procedures for Assessing the Environmental Fate and Ecotoxicology of Pesticides , SETAC 1995***			
	GLP Compliance:	***OECD, US FDA, US EPA, Japan MHLW, Japan MAFF, Japan METI***			
	Project Number:	***802374***			
	Report Number:	***21463***			
	Report Date:	***26 June 2002***			
STUDY SUMMARY					
Materials & Methods					
Radiolabelled Test Article	***Name:***	[^{14}C(G)]-Narasin			
	Radiochemical purity:	97.00%			
Non-Radiolabelled Test Article	***Name:***	Narasin			
Test Soils	***Characteristics of soils:***	*Classification*	*Sandy Loam*	*Clay Loam*	*Silt Loam*
		pH (0.01M KCl)	7.4	7.3	7.5
		Organic carbon	1.5%	1.6%	2.4%
		CEC (mEq/100g)	18.4	34.3	27.5
Exposure Design	***Pre-incubation:***	12 days at 20 ± 2°C			
	Test duration:	84 days			
	Test chambers:	50 g soil in 250 mL Erlenmeyer flask			
	Microbial biomass:	Measured on day 0 and 84 using Anderson Domsch Method			
	Incubation conditions:	In the dark at 20 ± 2°C			
	Ventilation:	Moist CO_2-free air (5-15 mL/min)			
	Volatile Traps:	Ethanediol (trap non-specific [^{14}C]-organic volatiles); 1M sodium hydroxide (trap liberated $^{14}CO2$)			
Sample Analysis	***Sampling intervals:***	Days 0, 7, 14, 21, 28, 42, 56, 70, and 84			
	Radiochemical analysis:	Soil extracts, traps, and test chamber washes analyzed via LSC Soil residue analyzed via combustion followed by LSC			
	Chromatographic analysis:	Radiolabel narasin and degradation products from soil extracts characterized and quantified by HPLC and TLC			

Results					
Sample Analysis			***Sandy Loam***	***Clay Loam***	***Silt Loam***
	Microbial biomass (mgC/100g):	*Initial (day 0)*	38	79	29
		Final (day 84)	33	66	31
	Radiochemical analysis at day 84 (% AR):	*Soil extract*	16.10%	30.50%	60.60%
		^{14}C volatiles	0.06%	0.02%	Not detected
		$^{14}CO_2$	64.20%	54.37%	18.62%
		Non-extractable residue	17.70%	25.33%	19.73%
		Test chamber wash	0.02%	0.04%	0.02%
		Total at day 120	98.08%	110.26%	98.97%
	HPLC characterization of soil extracts at day 84 (%AR):	*Narasin*	6.91%	14.94%	26.22%
		Additionally, there were 5 unidentified peaks, all less than 10% AR except one in one soil that was 14.25% AR			
	TLC characterization of soil extracts at day 84:	*Narasin*	4.43%	11.90%	22.54%
Rate of Degradation	***DT Values (assuming first order kinetics)***	***Soil Type***	***DT50 (days)***	***DT90 (days)***	***Rate ($days^{-1}$)***
		Sandy Loam	21	69	-0.03324
		Clay Loam	29	96	-0.02392
		Silt Loam	49	162	-0.01424

Appendix O – Study 276A-3480-22 - Decline of Narasin in Greenhouse Soil. 1977.

	Report Title:	***Decline of Narasin in Greenhouse Soil***	
	Performing Laboratory:	***Eli Lilly and Company***	
	Project Number:	***276A-3480-22***	
	Report Date:	***1977***	
STUDY SUMMARY			
Materials & Methods			
Test Article	***Name:***	Narasin (crystalline)	
Test Soil	***Soil:***	1:1 mixture of sand and Brookston loam	
Exposure Design	***Addition of Narasin to Soil:***	50 mg narasin in methanol added dropwise to 5 kg aliquot of soil and blended	
	Test duration:	41 days	
	Test chambers:	metal flat (31.5 x 21.5 x 8.0 cm^3) lined with plastic, placed in a large plastic bag	
	Application rate:	10 ppm	
	Incubation conditions:	Ambient greenhouse conditions	
	Temperature range:	21°C to 30°C	
	Soil moisture:	Brought to field capacity	
Sample Analysis	***Sampling:***	subsamples taken to include soil through entire soil layer	
	Analysis:	Samples air-dried and analyzed for narasin using a microbiological assay.	
Results			
Narasin remaining in soil		Days	ppm
		0	9.83
		11	4.7
		26	0.62
		41	0.26
		Sensitivity of microbiological assay: 0.25 ppm	
Kinetics of disappearance	***first order model:***	$C_t = C_0 \times e^{-kt}$	
	rate constant:	0.079 day^{-1}	
	R2:	0.99	
	half-life:	8.8 days	

Appendix P – Study 804853 - The Degradation of [Phenyl-^{14}C(U)]-1,3-Bis-(4-nitrophenyl) Urea in Soil Under Aerobic Conditions. May 2007.

	Report Title:	***The Degradation of [Phenyl-14C(U)-1,3-Bis-(4-nitrophenyl) Urea in Soil Under Aerobic Conditions***			
	Study Number:	***804853***			
	Guidance Document:	***OECD 307***			
	GLP Compliance:	***OECD***			
	Report Number:	***24325***			
	Report Date:	***07 May 2007***			
STUDY SUMMARY					
Materials & Methods					
Radiolabelled Test Article	***Name:***	[phenyl ^{14}C(U)] 1,3-bis-(4-nitrophenyl) urea, also known as [^{14}C]-DNC			
	Radiochemical purity:	99%			
Non-Radiolabelled Test Article	***Name:***	1,3-bis-(4-nitrophenyl) urea (DNC)			
Test Soils	***Characteristics of soils:***	*USDA classification*	*Sandy Loam*	*Sandy Clay Loam*	*Silt Loam*
		pH (0.01M $CaCl_2$)	6.3	7.0	5.6
		Organic carbon	2.2%	2.7%	3.8%
		CEC (cmol+/kg)	13.7	17.5	16.8
		Microbial biomass (mgC/100g)	37.21	47.62	42.81
Exposure Design	***Pre-incubation duration:***	12 days			
	Test duration:	120 days			
	Test chambers:	250 mL Erlenmeyer flask with 50 g soil			
	Sample replication:	2 per soil per sampling interval			
	Test article application:	43 µg (in 200 µL acetone) added dropwise 200 µL acetone added to biomass samples			
	Incubation conditions:	In the dark at 20 ± 2°C Moisture content of the soils maintained at 40-60% of water holding capacity (WHC)			
	Ventilation:	Moist CO_2-free air			
	Traps:	ethanediol (trap volatile 14C-organics); 1M sodium hydroxide (trap liberated $14CO_2$)			
Sample Analysis	***Sampling intervals:***	Days 0, 2, 4, 8, 16, 32, 64, 120			
	Radiochemical analysis:	Soil extracts analyzed via LSC Nonextractable residues analyzed via combustion then LSC Trap samples analyzed via LSC Test chamber washes analyzed via LSC			
	Chromatographic analysis:	HPLC/RAM and TLC of extracts			

Results					
Sample Analysis	***Radiochemical analysis at day 120 (% Applied Radioactivity):*** ***(based on replicate mean)***	*USDA classification*	*Sandy Loam*	*Sandy Clay Loam*	*Silt Loam*
		Soil extract	70.83%	63.24%	66.85%
		$14CO_2$	0.92%	0.81%	1.96%
		14C volatiles	0.07%	0.07%	0.10%
		Non-extractable residue	25.94%	Not quantified	Not quantified
		Test chamber wash	0.02%	0.10%	0.02%
		Total at day 120	96.87%	64.22%	68.93%
	HPLC characterization of soil extracts at day 120 (%AR) ***(based on replicate mean)***	*DNC component*	70.14%	60.17%	65.98%
		Three unidentified peaks present at 8%AR or less			
	TLC characterization of soil extracts at day 120 (%AR):	*DNC*	66.97%	58.19%	63.42%
		5 unidentifed spots of radioactivity all less than 2% AR			
Rate of Degradation	***DT50 Values***	***Soil Type***	***Coefficient of Determination (R2)***		***DT50 (days)***
		Sandy Loam	0.9508		239
		Sandy Clay Loam	0.9665		193
		Silt Loam	0.8952		257

Study 804853 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 804853.

Appendix Q – Study 804869 - The Degradation of [2-^{14}C]-4, 6-Dimethyl Pyrimidine-2-ol in Soil under Aerobic Conditions. 2006.

Report Title:	***The Degradation of [2-14C]-4, 6-Dimethyl Pyrimidine-2-ol in Soil under Aerobic Conditions***				
Study Number:	***804869***				
Guidance Document:	***OECD 307***				
GLP Compliance:	***OECD***				
Report Number:	***24329***				
Report Date:	***28 April 2006***				
STUDY SUMMARY					
Materials & Methods					
Radiolabelled Test Article	***Name:***	[2-14C]-4,6-dimethyl-pyrimidine-2-ol, also known as [14C]-HDP			
	Radiochemical purity:	99.78%			
Non-Radiolabelled Test Article	***Name:***	2-hydroxy-4,6-dimethyl pyrimidine (HDP)			
Test Soils	***Characteristics of soils:***	*USDA classification*	*Sandy Loam*	*Sandy Clay Loam*	*Silt Loam*
		pH (0.01M $CaCl_2$)	6.3	7.0	5.6
		Organic carbon	2.2%	2.7%	3.8%
		CEC (cmol+/kg)	13.7	17.5	16.8
		Microbial biomass (mgC/100g)	37.21	47.62	42.81
Exposure Design	***Pre-incubation duration:***	24 days			
	Test duration:	120 days			
	Test chambers:	250 mL Erlenmeyer flask with 50 g soil			
	Sample replication:	2 per soil per sampling interval			
	Test article application:	17.7 µg in water added dropwise			
	Incubation conditions:	In the dark at 20 ± 2°C Moisture content of the soils maintained at 40-60% of water holding capacity (WHC)			
	Ventilation:	Moist CO_2-free air			
	Traps:	ethanediol (trap volatile 14C-organics); 1M sodium hydroxide (trap liberated $14CO_2$)			
Sample Analysis	***Sampling intervals:***	Days 0, 2, 4, 8, 16, 32, 64, 120			
	Radiochemical analysis:	Soil extracts analyzed via LSC Nonextractable residues analyzed via combustion then LSC Trap samples analyzed via LSC Test chamber washes analyzed via LSC			
	Chromatographic analysis:	HPLC/RAM and TLC of extracts			

Results					
Sample Analysis	***Radiochemical analysis at day 120 (% Applied Radioactivity): (based on replicate mean)***	*USDA classification*	*Sandy Loam*	*Sandy Clay Loam*	*Silt Loam*
		Soil extract	1.29%	1.36%	0.83%
		$14CO_2$	22.05%	28.00%	31.19%
		14C volatiles	5.24%	1.68%	1.82%
		Non-extractable residue	74.03%	Not analyzed	Not analyzed
		Test chamber wash	0.01%	0.02%	Not detected
		Total at day 120	102.61%	31.06%	33.84%
	HPLC characterization of soil extracts at day 16 (for Sandy Loam and Sandy Clay Loam) and day 8 (for Silt Loam) (%AR): (based on replicate mean)	*HDP component*	11.55%	16.82%	15.32%
		Several unidentified peaks all less than 1.0% AR			
	TLC characterization of soil extracts at day 16 (for Sandy Loam and Sandy Clay Loam) and day 8 (for Silt Loam) (%AR) :	*HDP component*	10.69%	17.61%	14.49%
		4 unidentified spots of radioactivity all less than 1.5% AR			
Data Analysis	***DT Values***	***Soil Type***	***R^2***	***DT50 (days)***	***DT90 (days)***
		Sandy Loam	0.8809	6	20
		Sandy Clay Loam	0.9149	7	23
		Silt Loam	0.8902	3	11

Study 804869 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 804869.

Appendix R – Study ABC-0209 - A Greenhouse Study to Determine the Rate of Decline of Soil Incorporated Narasin and ^{14}C Nicarbazin Singly and in Combination. April 1985.

	Report Title:	***A Greenhouse Study to Determine the Rate of Decline of Soil Incorporated Narasin and ^{14}C Nicarbazin Singly and in Combination***
	Performing Laboratory:	***Eli Lilly and Company***
	Study Number:	***ABC-0209***
	GLP Compliance:	***OECD & FDA***
	Report Number:	***24329***
	Report Date:	***12 April 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article - Nicarbazin	***Radiolabeled article:***	^{14}C-Nicarbazin (radiolabel located in the DNC component)
	Radiochemical purity:	99%
	Non-radiolabelled article:	Nicarbazin
Test Article - Narasin	***Non-radiolabelled article:***	Narasin acid
Test Soil	***Components:***	1:1 v/v local silt loam and coarse sand mixture
Exposure Design	***Test concentrations:***	Control 10 mg/kg ^{14}C-nicarbazin 10 mg/kg narasin mixture of 10 mg/kg ^{14}C-nicarbazin & 10 mg/kg narasin
	Test article carrier:	Narasin: ethyl acetate Nicarbazin: ethylene glycol monoethyl ether
	Test article application:	Dosing solution added dropwise to soil then evaporated
	Test chambers:	Galvanized metal flat (20-cm x 30-cm x 9-cm)
	Incubation conditions:	Ambient greenhouse conditions Soil moisture content adjusted to 75% Flat placed inside plastic bag to prevent water evaporation
	Test duration:	52 weeks
Sample Analysis	***Sampling intervals:***	0, 1, 2, 4, 6, 8, 12, 18, 24, 35, 42 and 52 weeks after application
	High levels of narasin:	Extracts analyzed using a turbidimetric method with *Streptococcus faecalis*
	Low levels of narasin:	Extracts analyzed by a thin-layer chromatographic bioautographic method with *Bacillus subtilis*
	Nicarbazin assay (non-radiolabel)	Extracts analyzed by HPLC for DNC
	Nicarbazin assay (radiolabel)	Soil samples combusted and analyzed via LSC

Results		
Conclusion	***Narasin***	Under greenhouse conditions, soil incorporated narasin at 10 mg/kg degrades rapidly; by week 4 the concentration was less than 10% of initial.
	Nicarbazin	Soil incorporated nicarbazin at 10 mg/kg declined by about 72 to 80% of initial levels after 18 weeks and remained unchanged thereafter. No appreciable terminal oxidation of ^{14}C-nicarbazin by soil organisms
	Narasin/Nicarbazin mixture	Neither narasin or nicarbazin affected the degradation rate of the other

Appendix S – Study ABC-0284 - A Study to Determine the Rate of Depletion of Narasin and ^{14}C-Nicarbazin in a Field Soil Plot. 1986.

	Report Title:	***A Study to Determine the Rate of Depletion of Narasin and ^{14}C-Nicarbazin in a Field Soil Plot***
	Performing Laboratory:	***Eli Lilly and Company***
	Study Number:	***ABC-0284***
	GLP Compliance:	***OECD & FDA***
	Report Date:	***21 April 1986***
STUDY SUMMARY		
Materials & Methods		
Test Article - Nicarbazin	***Radiolabeled article:***	^{14}C-Nicarbazin (labeled in the DNC component)
	Radiochemical purity:	≥99%
Test Article - Narasin	***Non-radiolabelled article:***	Mycelial narasin
Exposure Design	***Test concentrations:***	Control, mixture of ^{14}C-nicarbazin & narasin each added to the soil at a rate of 2.47 kg/ha
	Replication:	1
	Incubation conditions:	Ambient field conditions
	Test duration:	53 weeks
Field Soil	***Location:***	Greenfield, Indiana
	Top soil:	Silt loam (fortified with control chicken excreta) Plot confined to a 0.914 m dia. section of galvanized metal culvert
Sample Analysis	***Sampling intervals:***	0, 1, 2, 3, 4, 6, 8, 12, 16, 41, 44, 49 and 53 weeks after application
	Soil Sampling:	Composite of 4 core samples (sample depth ~15 cm)
	Replicates per sample:	2
	Non-radiolabel analysis:	Assayed for narasin via HPLC-derivatization Assayed for nicarbazin (DNC) via HPLC
	Radiolabel analysis:	Soil samples combusted and analyzed via LSC
Results		
Conclusion	***Nicarbazin***	Slowly declined with a half-life of 48.6 weeks
	Narasin	Rapidly declined to <10% initial levels in 6 weeks

Appendix T – Study 151E-107 - Narasin – Adsorption/Desorption Characteristics in Five Representative Soils Following OECD Guideline 106. 2008.

Report Title:	***NARASIN – Adsorption/Desorption Characteristics in Five Representative Soils Following OECD Guideline 106***						
Guidance Document:	***OECD 106***						
GLP Compliance:	***OECD, FDA, MAFF***						
Study Number:	***151E-107***						
Report Date:	***'24 January 2008***						
STUDY SUMMARY							
Materials & Methods							
Test Article	***Name:***	Narasin (Compound 079891)					
Test Soils	***Characteristics of soils:***	*Texture class*	*Clay Loam*	*Sandy Clay Loam*	*Clay Loam*	*Loamy Sand*	Clay
		% organic carbon	5.0%	1.9%	4.1%	1.3%	0.7%
		CEC (mEq/100g)	25.9	17.8	23.2	12.4	30.8
		pH (0.01M CaCl2)	7.2	6.2	5.2	5.7	7.7
Analytical Method	***Instrument:***	LC/MS					
Tier 2 Assessment	***Adsorption Kinetics***	*Test vessel:* *Solution:* *Soil/solution ratio:* *Analysis*	Pyrex glass tubes 0.01 M CaCl2 1:5 Aqueous phase measured by LC/MS over 48 hours				
	Desorption Kinetics	*Set up:* *Analysis:*	Replace CaCl2 from Adsorption Kinetics with fresh CaCl2 Aqueous phase measured by LC/MS over 48 hours				
Tier 3 Assessment	***Adsorption Isotherm***	*Maximum concentration adsorbed in soil (M_{ad}):* *Concentrations:* *Soil/solution ratio:* *Analysis:*	10 mg/kg 0.05, 0.1, 0.2, 0.5 and 1 M_{ad} 1:5 Aqueous phase measured by LC/MS after 4 hours				
	Desorption Kinetics	*Set up:* *Analysis:*	Replace CaCl2 from Adsorption Isotherm with fresh CaCl2 Aqueous phase measured by LC/MS after 4 hours				
Results							
Tier 2 Assessment		***Texture class***	***Clay Loam***	***Sandy Clay Loam***	***Clay Loam***	***Loamy Sand***	**Clay**
	Adsorption Kinetics	K_d^{ads}	25	22	150	26	5.4
		K_{OC}^{ads}	507	1171	3670	1971	778
	Desorption Kinetics	K_d^{des}	42	27	108	24	7.1
		K_{OC}^{des}	840	1423	2628	1841	1016
Tier 3 Assessment	***Adsorption Isotherm***	K_d^{ads}	44	18	106	15	8.8
		K_{OC}^{ads}	873	927	2575	1149	1263
	Freundlich Isotherm	$log(K_F^{ads})$	1.7074	1.2833	2.0606	1.2310	0.9625
	Desorption Isotherm	K_d^{des}	49	39	151	24	20
		K_{OC}^{des}	983	2060	3677	1878	2844
	Freundlich Isotherm	$log(K_F^{des})$	1.667	1.2848	1.9718	1.3605	1.2861

Appendix U – Study 804848 – Adsorption /Desorption of [Phynyl-^{14}C(U)]-1,3-Bis-(4-nitrophenyl) Urea in Soil. 2006.

Report Title:	***Adsorption/Desorption of [Phenyl-14C(U)]-1,3-Bis-(4-nitrophenyl) Urea in Soil***				
Study Number:	***804848***				
Guidance Document:	***OECD 106***				
GLP Compliance:	***OECD***				
Report Number:	***25127***				
Report Date:	***05 June 2006***				
STUDY SUMMARY					
Materials & Methods					
Radiolabelled Test Article	***Name:*** ***Radiochemical purity:***	[phenyl-14C(U)]-1,3-bis-(4-nitrophenyl) urea, also known as [14C]-DNC 99%			
Non-Radiolabelled Test Article	***Name:***	1,3-bis-(4-nitrophenyl) urea (DNC)			
Test Soils	***Characteristics of soils***	*USDA classification*	*Sandy Loam*	*Clay Loam*	*Silt Loam*
		pH (0.01M $CaCl_2$)	4.7	7.3	6.1
		Organic carbon	1.3%	3.1%	2.5%
		CEC (mEq/100g)	9.9	24.6	19.8
Tier 2 Assessment	***Adsorption***	*Test vessel:*	Nalgene centrifuge vessels		
		Solution:	0.01 M CaCl2		
		Soil/solution ratio:	1:100		
		Adsorption time	2 hours		
		Analysis	Aqueous phase measured by LSC		
	Desorption	*Set up:*	Replace CaCl2 from Adsorption with fresh CaCl2		
		Desorption time:	24 hours		
		Analysis:	Aqueous phase measured by LSC		
Results					
Tier 2 Assessment	*Soil Type*	*Initial Concentration (mg/L)*	*Adsorption*		*Desorption*	
			Kd	*Koc*	*Kd*	*Koc*
	Sandy Loam	*0.13*	1611	123923	1641	126193
		0.02	286	21962	766	58885
	Sandy Clay Loam	*0.13*	2066	62591	2564	77682
		0.02	533	16137	1608	48712
	Silt Loam	*0.13*	1664	66560	2414	96560
		0.02	423	16900	2167	86680

Study 804848 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 804848.

Appendix V – Study 804832 – Adsorption / Desorption of HDP. 2006.

Report Title:	***Adsorption/Desorption of [2-14C]-4,6-Dimethyl Pyrimidine-2-ol in Soil***				
Guidance Document:	***OECD 106***				
GLP Compliance:	***OECD***				
Study Number:	***804832***				
Report Number:	***25128***				
Report Date:	***05 June 2006***				
STUDY SUMMARY					
Materials & Methods					
Radiolabeled Test Article	***Name:***	[2-14C]-4,6-dimethyl pyrimidine-2-ol, also known as [14C] HDP			
	Radiochemical purity:	99.78%			
Non-Radiolabeled Test Article	***Name:***	2-hydroxy-4,6-dimethyl pyrimidine (HDP)			
Test Soils	***Characteristics of soils***	*Texture*	*Sandy Loam*	*Clay Loam*	*Silty Clay Loam*
		pH (0.01M $CaCl_2$)	4.7	7.3	6.1
		Organic carbon	1.3%	3.1%	2.5%
		CEC (mEq/100g)	9.9	24.6	19.8
Tier 2 Assessment	***Adsorption***	*Test vessel:*	Nalgene centrifuge vessels		
		Solution:	0.01 M CaCl2		
		Soil/solution ratio:	1:1		
		Adsorption time	168 hours		
		Analysis	Aqueous phase measured by LSC		
	Desorption	*Set up:*	Replace CaCl2 from Adsorption with fresh CaCl2		
		Desorption time:	48 hours		
		Analysis:	Aqueous phase measured by LSC		
Results					
Tier 2 Assessment	*Soil Type*	*Initial Concentration (µg/mL)*	*Adsorption*		*Desorption*	
			Kd	*Koc*	*Kd*	*Koc*
	Sandy Loam	*5.00*	1.6	119	2.0	150
		0.05	2.0	154	2.4	185
	Sandy Clay Loam	*5.00*	1.1	33	1.6	47
		0.05	1.5	45	2.0	61
	Silt Loam	*5.00*	2.9	114	3.6	144
		0.05	3.6	144	4.4	176

Study 804832 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 804832.

Appendix W – Study P0000693 – Aqueous Hydrolysis of Nicarbazin Under Laboratory Conditions. 2004.

Report Title:	***Aqueous Hydrolysis of Nicarbazin Under Laboratory Conditions***						
Guidance Document:	***EPA 540/9-82-021***						
GLP Compliance:	***US EPA 40 CFR 160***						
Project Number:	***P0000693***						
Report Date:	***9 November 2004***						
STUDY SUMMARY							
Materials & Methods							
Test Articles	***Name:***	4,4'-Dinitrocarbanilide (DNC)					
	Purity:	98.0%					
	Name:	4,6-Dimethyl-2-pyrimidinol (HDP)					
	Purity:	97%; 99.4%					
Sterile Buffers	***pH 4.0 Buffer:***	0.02M ammonium acetate					
	pH 7.0 Buffer:	0.02M ammonium acetate					
	pH 9.0 Buffer:	0.02M ammonium hydroxide					
Incubation Conditions	***Concentration:***	DNC: 0.5 µg/mL HDP: 250 µg/mL					
	Volume:	4 mL					
	Test vessels:	4-mL sterile, silylated amber glass vials					
	Replication:	2 replicates per sampling interval					
	Temperature:	25±1°C in the dark					
	Duration:	30 days					
Analysis	***Sampling:***	Sampling intervals for analysis of DNC and HDP Days 0, 1, 2, 4, 8, 14, 21, 30 Additional aliquots removed at Days 0 and 30 for microbial sterility					
	Methods:	LC/MS/MS					
Results							
		DNC			***HDP***		
		pH 5	***pH 7***	***pH 9***	***pH 5***	***pH 7***	***pH 9***
Recovery from Time 0	***Day 0***	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
	Day 30	106.3%	115.4%	99.6%	98.7%	100.2%	111.8%
Mass Balance	***Day 0***	84.2%	79.8%	88.9%	92.1%	95.5%	94.1%
	Day 30	89.5%	92.2%	88.6%	90.9%	95.7%	105.2%
Summary							
DNC and HDP hydrolytically stable at pH 5, 7 and 9 at 25°C for up to 30 days							

Study P0000693 was conducted by Innolytics, LLC. Innolytics, LLC has granted permission to Elanco Animal Health to use the data in Study P0000693.

Appendix X – Study 341587. Physico-Chemical Testing with Narasin: Partition Coefficient. 2002.

	Report Title:	***Physico-Chemical Testing with Narasin: Partition Coefficient***
	Guidance Document:	***OECD 117***
	GLP Compliance:	***OECD***
	Project Number:	***341587***
	Report Number:	***21362***
	Report Date:	***26 June 2002***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Narasin
Methods	***Column:***	Partisil ODS3
	Standards:	Thiourea (unretained) Bromobenzene Acetanilide Naphthalene Methyl benzoate Fluoranthene Ethyl benzoate DDT
	Dilution matrix and mobile phase:	75:25 (v/v) methanol:0.1M citrate buffer (pH 4.0)
Results		
Narasin peak:	Narasin eluted as a split peak after the last standard peak. Therefore, the Pow of narasin was considered to be greater than the known Pow of the last standard.	
Pow:	>6.2	

Appendix Y – Study ABC-0137. A ^{14}C Narasin Tissue Residue and Comparative Metabolism Study in Cattle. 1982.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline ^{14}C Narasin

Methods:
Hereford cattle, six steers and three heifers, ranging in weight between 218 to 319 kg were dosed with ^{14}C narasin equivalent to a feeding level of approximately 18 g/ton of feed. ^{14}C Narasin was administered orally in gelatin capsules morning and evening for three, five or seven days. The experiment was conducted in three stages using three groups of three animals. Twelve hours after the last dose, representative specimens of muscle, liver, kidney and back fat were collected from 2 steers and 1 heifer and then assayed for radioactivity. Parent narasin in liver tissue of the three cattle dosed for seven days was determined by microbiological assay.

Results:
Radioactivity in liver was approximately 0.8 ppm calculated as narasin equivalents and there was no significant difference between animals in the three dosing groups. Therefore, steady state concentrations were approximated within three days' dosing. Muscle, kidney and fat residues were all less than 0.033 ppm (based on group means). Narasin concentrations in livers of the cattle dosed for seven days were approximately 8% of the total radioactivity. Chromatographic profiles of liver radioactivity from the three, five and seven-day animals were similar.

Appendix Z – Studies ABC-0126 and ABC-0127. Comparative Metabolism of ^{14}C Narasin in Orally Dosed Cattle, Dog and Rats. 1986.

Performing Laboratory: Lilly Research Laboratories

Test Article: Crystalline ^{14}C Narasin

Methods:
Cattle, a dog, and rats were dosed orally for up to 7 days with ^{14}C narasin. The radiochemical residues extracted from the liver and feces were subjected to fractionation by solvent partitioning, silica gel column chromatography and thin-layer chromatography (TLC). Cattle liver metabolites were visualized by TLC autoradiography and relative quantities of individual metabolites were estimated by liquid scintillation counting of zones scraped from TLC plates.

Results:
All three species produced multiple narasin metabolites. Fecal extracts contained more than twenty radioactivity metabolites and no single metabolite constituted a significant proportion of the total radioactivity. Comparison of column elution profiles and TLC autoradiograms indicated that metabolite patterns were qualitatively similar among cattle, rats, and dogs. There were, however, some quantitative differences among these three species.

Eight of the di- and trihydroxy derivatives of narasin have been previously identified in chickens. Two novel metabolites, a monohydroxylated narsin (NM-12) and a dihydroxylated narasin (NM-13), were identified in this study. These two were produced by all three species but were most prevalent in cattle.

Quantitative fractionation of radioactivity in cattle liver indicated that metabolite NM-12 was the most abundant (approximately 16%) and that metabolites NM-3 and NM-6, which are dihydroxy narasin derivatives, each accounted for approximately 4% of the total. The remainder of the liver radioactivity consisted of several metabolites with low relative abundance, approximately 10% nonextractable radioactivity, and 10 to 15% intractable polar residues. Very little (<3%) of the liver residue was parent narasin.

Appendix AA – Study T4HAUK0703. Residue Depletion of Nicarbazin and Narasin in Edible Tissues from Chickens Following Administration of Maxiban™ G160 via Feed. 2008.

	Report Title:	***Residue Depletion of Nicarbazin and Narasin in Edible Tissues from Chickens Following Administration of Maxiban™ G160 via Feed***
	Study Number:	***T4HAUK0703***
	GLP Compliance:	***OECD***
	Report Number:	***28890***
	Report Date:	***22 May 2008***
STUDY SUMMARY		
Materials & Methods		
Test Item	***Name:***	Maxiban™ G160
Test Species		Broiler chickens, male and female, one day old at test initiation
Exposure Design	***Dose:***	658.8 mg Maxiban™ G160/kg food (50 mg narasin/kg, 50 mg nicarbazin/kg)
	Dose Preparation:	Maxiban™ G160 was incorporated into appropriate commercially available non-medicated broiler chicken diets
	Duration:	42 days (35 days fed Maxiban™ incorporated diet, 7 days withdrawal)
	Dosing:	Fed *ad libitum* and food consumption for the group was measured and recorded approximately 24 hours later
	Treatment Groups:	Group 1: 3 males, 3 females sacrificed at 0 days withdrawal Group 2: 3 males, 3 females sacrificed at 3 days withdrawal Group 3: 3 males, 3 females sacrificed at 5 days withdrawal Group 4: 3 males, 3 females sacrificed at 7 days withdrawal
Exposure Assessment	***Endpoints:***	Tissue residues in kidneys, liver, muscle (composite of breast and thigh), skin with fat, and abdominal fat pad
	Methods:	Tissue residues extracted and analyzed by LC/MS/MS Four aliquots were analyzed from each of the processed tissue samples for each bird

Results					
Dosing	***Average dose as mg/kg body weight based on food consumed:***	***Day***	***Narasin (mg/kg)***		***Nicarbazin (mg/kg)***
		7	8.50		8.91
		14	5.60		5.87
		21	6.14		6.39
		28	4.46		4.65
Tissue Residues	***Withdrawal Day***	***Mean Tissue Residues (µg/kg)***			
		Liver	***Kidney***	***Muscle***	***Skin with Fat***
		Narasin			
	0	< LOQ	< LOQ	< LOQ	< 27.2
	3	ND	ND	ND	ND
	5	ND	ND	ND	ND
	7	ND	ND	ND	ND
		Nicarbazin (as DNC)			
	0	9190	4290	1610	2040
	3	2450	295	187	313
	5	355	LOQ	< 27.8	59.6
	7	< 87.8	< LOQ	< LOQ	< 25.9
	For narasin LOQ = 25 ug/kg For DNC LOQ = 50 ug/kg, 100 ug/kg, 25 ug/kg and 25 ug/kg for liver, kidney, muscle and skin with fat, respectively				

Appendix BB – Study 802458. Soil Microorganisms: Carbon and Nitrogen Transformation Tests with Narasin. 2002.

	Report Title:	***Soil Microorganisms: Carbon and Nitrogen Transformation Tests with Narasin***		
	Guidance Document:	***OECD 216 & OECD 217***		
	GLP Compliance:	***OECD***		
	Project Number:	***802458***		
	Report Number:	***21529***		
	Report Date:	***16 July 2002***		
STUDY SUMMARY				
Materials & Methods				
Test Article	***Name:***	Narasin		
Organic substrate	***Type:***	Lucerne (Alfalfa, *Medicago sativa*)		
Test Soil:	***Characteristics***	Sandy loam topsoil collected from the upper 20 cm horizon.		
		Microbial biomass (mgC/kg)	283.98	
Exposure Design	***Test concentrations:***	0 (control), 3.5 and 17.5 mg/kg		
	Treatment solution carrier:	Acetone		
	Test chambers:	1.2 kg soil in 2.5L plastic containers		
	Replication:	3 test chambers per test and per concentration		
	Test article application:	Treatment solution added to quartz sand and mixed into soil		
	Control sample:	Acetone added to quartz sand and mixed into soil		
	Organic substrate application:	0.5% (w/w) lucerne (for nitrogen transformation test)		
	Incubation conditions:	In darkness at 20 ± 2°C		
Endpoint measurements:	***Determination of respiration:***	50 g sub-sample amended D-glucose and evolved CO_2 over 12 hours measured with infra-red gas analyzer		
	Determination of nitrate:	50 g sub-sample extracted with KCl and nitrate ions in soil extracts measured photometrically		
Results				
Respiration Rate	***On Day 28***	***Test concentration***	***Respiration Rate (mean ± SD; mL CO_2/(kg soil hour))***	***% Deviation from Control***
		Control	5.02 ± 0.17	--
		3.5 mg/kg	4.43 ± 0.14	-11.8%
		17.5 mg/kg	4.86 ± 0.48	-3.2%
Nitrification	***On Day 28***	***Test concentration***	***Nitrate (mean ± SD; mg N/kg soil)***	***% Deviation from Control***
		Control	68.3 ± 0.4	--
		3.5 mg/kg	71.9 ± 0.3*	5.3%
		17.5 mg/kg	72.6 ± 1.3*	6.3%
	*Measured values are significantly different from control			
Conclusions				
Respiration:	No treatment effect at 3.5 or 17.5 mg/kg (< 25% deviation from control)			
Nitrification:	No treatment effect at 3.5 or 17.5 mg/kg (< 25% deviation from control)			

Appendix CC – Study 802442. Terrestrial Plant Growth Test with Narasin. 2002.

	Report Title:	***Terrestrial Plant Growth Test with Narasin***	
	Guidance Document:	***OECD 208***	
	GLP Compliance:	***OECD***	
	Project Number:	***802442***	
	Report Number:	***21317***	
	Report Date:	***27 June 2002***	
STUDY SUMMARY			
Materials & Methods			
Test Article	***Name:***	Narasin	
Test Soil/Sand	***Characteristics of soil***	*Loamy sand topsoil collected from the upper 20 cm horizon*	
		Organic carbon	0.4%
Test Species	***Common name (species):***	Winter oat (*Avena sativa* cv Jalna) Radish (*Raphanus sativus* cv French Breakfast 3) Mung bean (*Phaseolus aureus*)	
Exposure Design	***Test concentrations:***	0 (control), 0.35, 3.5 and 35 mg/kg	
	Treatment solution carrier:	Acetone	
	Test soil composition:	25% test sand and 75% test soil	
	Test article application:	Treatment solution added to sand and mixed into soil	
	Control preparation:	Acetone added to sand and mixed into soil	
	Test chambers:	1.1L plastic pots	
	Replication per species:	4 replicates per concentration , 5 seeds per replicate	
	Replicate arrangement:	Random block design; separate blocks for each species	
	Environmental conditions:	Maintained in glasshouse Temperature ranged from 16 to 23 °C Water administered via saucers below pots	
	Exposure duration:	14 days after at least 50% control emergence	
Treatment Solution Analysis		validated HPLC/uv method to confirm narasin concentration	
Exposure Assessment	***Endpoints:***	Emergence (survival): LC50 Growth (based on fresh weight of above-ground seedling): EC50	
	Statistical Analysis:	LC50 & EC50: probit method NOEC: ANOVA ($\alpha = 0.05$)	

<table>
<tr><th colspan="5">Results</th></tr>
<tr><td>Treatment Solution Analysis</td><td colspan="4">Soil concentrations were determined to be 0.0 (control, 0.375, 3.381, and 29.260 mg/kg</td></tr>
<tr><td rowspan="8">Emergence Rate</td><td rowspan="2">Treatment rate (mg/kg)</td><td colspan="3">Emergence (seedlings emerged/total seeds)</td></tr>
<tr><td>Winter oat</td><td>Radish</td><td>Mung bean</td></tr>
<tr><td>0</td><td>20/20</td><td>19/20</td><td>20/20</td></tr>
<tr><td>0.375</td><td>19/20</td><td>19/20</td><td>20/20</td></tr>
<tr><td>3.381</td><td>20/20</td><td>18/20</td><td>16/20</td></tr>
<tr><td>29.26</td><td>19/20</td><td>0/20</td><td>12/20</td></tr>
<tr><td>LC50</td><td>> 29.26 mg/kg</td><td>16.3 mg/kg[a]</td><td>> 29.26 mg/kg</td></tr>
<tr><td colspan="4">[a] Report calculated LC50 using probit method (5.07 mg/kg); probit method inappropriate due to lack of partial effects. LC50 estimated by applying binomial method to treatment means (3.381 & 29.26 mg/kg)</td></tr>
<tr><td rowspan="9">Growth</td><td rowspan="2">Treatment rate (mg/kg)</td><td colspan="3">Mean fresh weight per seedling ± SD (g)</td></tr>
<tr><td>Winter oat</td><td>Radish</td><td>Mung bean</td></tr>
<tr><td>0</td><td>0.399 ± 0.056</td><td>0.696 ± 0.057</td><td>0.580 ± 0.059</td></tr>
<tr><td>0.375</td><td>0.447 ± 0.022</td><td>0.603 ± 0.058</td><td>0.494 ± 0.084*</td></tr>
<tr><td>3.381</td><td>0.530 ± 0.076*</td><td>0.522 ± 0.097*</td><td>0.422 ± 0.018*</td></tr>
<tr><td>29.26</td><td>0.248 ± 0.041*</td><td>NA</td><td>0.194 ± 0.033*</td></tr>
<tr><td>EC50</td><td>> 29.26 mg/kg</td><td>> 3.381 mg/kg</td><td>8.99 mg/kg</td></tr>
<tr><td>NOEC/NOAEC</td><td>3.381 mg/kg</td><td>0.375 mg/kg</td><td>< 0.375 mg/kg</td></tr>
<tr><td colspan="4">* statistically significant effects determined by ANOVA</td></tr>
<tr><td rowspan="7">Re-calculated Radish Growth</td><td>Treatment rate (mg/kg)</td><td colspan="3">Mean weight per seedling ± SD (g)[b]</td></tr>
<tr><td>0</td><td colspan="3">0.661 ± 0.087</td></tr>
<tr><td>0.375</td><td colspan="3">0.571 ± 0.071</td></tr>
<tr><td>3.381</td><td colspan="3">0.459 ± 0.083</td></tr>
<tr><td>29.26</td><td colspan="3">0.000 ± 0.000</td></tr>
<tr><td>EC50</td><td colspan="3">6.183 mg/kg</td></tr>
<tr><td colspan="4">[b] Report did not calculate an EC50. EC50 estimated using ICp method (Norberg-King, 1993). Replicate weights re-calculated using 0 g for seeds that did not emerge.</td></tr>
</table>

Appendix DD – Study 802568 – Narasin – Determination of Acute Toxicity (LC_{50}) to Earthworms. 2002.

	Report Title:	***Narasin – Determination of Acute Toxicity (LC50) to Earthworms***
	Guidance Document:	***OECD 207***
	GLP Compliance:	***OECD***
	Project Number:	***802568***
	Study Number:	***21585***
	Report Date:	***08 July 2002***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Narasin
Test Organism	***Common name:***	Earthworm
	Species:	*Eisenia foetida foetida*
	Age at initiation:	at least 2 months
	Weight at initiation:	300 - 600 mg
Exposure Design	***Test medium:***	Artificial soil
	Dosing solution solvent:	Acetone
	Route of administration:	Sand
	Soil dosing:	Sand dosed with narasin stock solution; then mixed with soil
	Solvent control dosing:	Sand dosed with acetone; then mixed with soil
	Duration:	14 days
	Nominal test concentrations:	5, 40, 80, 160 & 320 mg/kg as test article
	Controls:	Solvent control & untreated control
	Replication:	4 chambers per treatment; 10 organisms per replicate
	Test chambers:	1 L glass jar
	Mass of test medium:	750 g per replicate
Environmental Conditions:	***Room Temperature:***	20 to 22°C (extremes recorded daily)
	Soil pH:	6.84 to 7.16 (measured on day 0 & 14)
	Soil moisture content:	29 to 33% (measured on day 0 & 14)
Analytical Measurement		Narasin concentration in dosing solutions measured prior to dosing using a validated HPLC/uv method
Exposure Assessment	***Organism observations:***	Mortality at day 7 and 14 Individual body weights at day 0 and 14

Results				
Stock Solution Analysis	Soil concentrations were determined to be 0.0 (control), 4.3, 34.3, 67.7, 137.7, 270.9 mg/kg			
Organism Observations	***Treatment rate (mg/kg)***	**Mortality on Day 14**	**Bodyweight change**	
	0 (untreated)	7.5% (3/40)	-8.70%	
	0 (solvent control)	5% (2/40)	-9.70%	
	4.3	12.5% (5/40)	-8.10%	
	34.3	22.5% (9/40)	-4.70%	
	67.7	87.5% (35/40)	-30.70%	
	137.7	100% (40/40)	NA	
	270.9	100% (40/40)	NA	
Data Analysis			*Day 7*	*Day 14*
	Mortality	*LC50*	51.1 mg/kg	46.4 mg/kg
		NOEC	34.3 mg/kg	4.3 mg/kg
	Bodyweight	*NOEC*	34.3 mg/kg	4.3 mg/kg

Appendix EE – Study 1982.6391. Narasin – Chronic Toxicity and Reproduction Test Exposing the Earthworm *Eisenia fetida* in Artificial Soil, Based on OECD Guideline 222. 2011.

	Report Title:	***Narasin – Chronic Toxicity and Reproduction Test Exposing the Earthworm Eisenia fetida in Artificial Soil, Based on OECD Guideline 222***
	Guidance Document:	***OECD 222***
	GLP Compliance:	***OECD & FDA***
	Study Number:	***1982.6391***
	Report Date:	***08 February 2011***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Narasin
Test Organism	***Common name:***	Earthworm
	Species:	*Eisenia fetida*
	Age at initiation:	at least 2 months old; mature with clitellum
	Weight at initiation:	250 - 600 mg
Exposure Design	***Test medium:***	Artificial soil
	Dosing solution solvent:	Acetone
	Test article administration:	Sand dosed with narasin dosing solution; acetone evaporated; sand incorporated into soil
	Solvent control dosing:	Sand dosed with acetone; acetone evaporated; sand incorporated into soil
	Duration:	56 days
	Nominal test concentrations:	3.1, 6.3, 13, 25 & 50 mg/kg
	Controls:	Solvent control & untreated control
	Replication:	4 per treatment levels and solvent control, 10 organisms per replicate 8 for the untreated control, 10 organisms per replicate
	Test chambers:	1000 mL glass beakers
	Mass of test media:	600 g (dry weight) per replicate
	Feeding:	5 g of moist cattle manure per feeding per chamber Fed on days 1, 7, 14, 21 and 28
Environmental Conditions	***Soil Temperature:***	17 to 21°C (extremes recorded daily)
	Soil Moisture content:	18 to 38% (measured day 0 and 28)
Analytical Measurement	Narasin concentration in dosing solutions measured using a validated LC/MS/MS method	
Exposure Assessment	***F0 organism observations:***	Mortality and health assessment (day 28) Group body weight (day 0 and 28) F0 organisms removed from soil on Day 28
	F1 organism observations:	Number of juveniles (day 56)
	Endpoints:	F0 survival F0 body weight change number of juveniles (F1)

Results				
Test Solution Analysis	Measured concentrations of narasin in dosing solutions ranged from 93 to 110% of nominal. Therefore, the biological results are reported using nominal concentrations.			
Organism Observations	***Treatment rate (mg/kg)***	***F0***		***F1***
		Survival (%)	***Bodyweight Change (%)***	***Juveniles per adult***
	0 (untreated)	100%	21 ± 3.9%	7.4 ± 1.4
	0 (solvent control)	100%	22 ± 4.5%	6.8 ± 1.8
	3.1	100%	20 ± 9.0%	7.7 ± 1.8
	6.3	100%	11 ± 7.6%	9.0 ± 2.1
	13	100%	18 ± 10%	8.3 ± 1.6
	25	95%	31 ± 11%	5.6 ± 1.4
	50	25%	37 ± 42%	9.2 ± 9.5
Data Analysis	***LC50/EC50***	**41 mg/kg**	**>50 mg/kg**	**>50 mg/kg**
	NOEC	**25 mg/kg**	**50 mg/kg**	**50 mg/kg**

Appendix FF – Study 802573. Narasin – Alga, Growth Inhibition Test (72 h, EC_{50}). 2002.

	Report Title:	***Narasin – Alga, Growth Inhibition Test (72 h, EC50)***	
	Guidance Document:	***OECD 201 EC Guideline C3***	
	GLP Compliance:	***OECD***	
	Project Number:	***802573***	
	Report Number:	***21559***	
	Report Date:	***16 July 2002***	
STUDY SUMMARY			
Materials & Methods			
Test Article	***Name:***	Narasin	
Test Organism	***Common name:***	Freshwater green alga	
	Species:	*Selenastrum capricornutum*	
	Source:	Laboratory culture	
Exposure Design	***Test medium:***	ISO freshwater algal growth medium	
	Duration:	72 hours	
	Nominal test concentrations:	0.0 (control), 0.05, 0.32, 0.75, 1.5, 3, 6 mg/L	
	Abiotic Controls:	0.32 and 3.6 mg/L (no algae added)	
	Number of replicates:	3 replicates for narasin treatments 6 replicates for control	
	Test chambers:	250 mL Erlenmeyer flasks with foil lids	
	Solution volume:	100 mL	
	Inoculation concentration:	10^4 cells/mL	
Environmental Conditions	***Photoperiod:***	Continuous at 6,000 to 10,000 lux	
	Temperature:	21 to 23°C	
	Agitation:	Orbital shaking device set at 100 rpm	
	pH:	7.92 to 10.42	
	Analytical confirmation of test article:	Measured at time 0 and 72 hr, using a validated HPLC/uv method	
Biological Data	***Cell counts:***	24, 48 and 72 hr	
	Endpoints:	72 hr EC50 & NOEC for biomass (AUC) & growth rate	
	Statistical analysis:	EC50 - log-probit method NOEC - one-tailed Dunnett's ($\alpha = 0.05$)	
Results			
Test Solution Analysis		Narasin concentrations were stable over the test duration. Biological data is reported as mean measured concentrations.	
Biological Data	***Mean Measured Concentration (mg/L)***	***72 hr Growth Parameters***	
		Biomass (AUC, 10^5 cells/hr/ml)	***Growth Rate (μ_{ave}/day)***
	<LOQ	3.23	1.54
	0.0352	2.94	1.53
	0.23	2.47	1.45
	0.54	1.85*	1.37*
	1.06	1.45*	1.32*
	2.16	0.69*	1.06*
	4.17	0.11*	0.47*
	*Significantly different from control, Dunnett's		
Statistical Analysis		***Biomass (10^5 cells/hr/ml)***	***Growth Rate (μ_{ave}/day)***
	NOEC	0.23	0.23
	EC50	0.77	2.92

Appendix GG – Study C01883. The Acute Toxicity of Narasin (Compound 79891) to *Daphnia magna* in a Static Test System. 1985.

	Report Title:	***The Acute Toxicity of Narasin (Compound 79891) to Daphnia magna in a Static Test System***		
	Performing Laboratory:	***Eli Lilly and Company***		
	Guidance Document:	***ASTM E729-80***		
	GLP Compliance:	***US FDA, OECD***		
	Study Identification:	***C01883***		
	Report Date:	***September 1985***		
STUDY SUMMARY				
Materials & Methods				
Test Article	***Name:***	Narasin		
Test Organism	***Common name:***	Water flea		
	Species:	*Daphnia magna*		
	Age at initiation:	≤ 24 hours		
Exposure Design	***Test medium:***	Conditioned well water		
	Duration:	48 hours		
	Nominal test concentrations:	0.0 (control), 5.0, 8.0, 12.5, 20.0, 30.0 and 45.0 mg/L		
	Replication:	3 replicates/treatment, 10 organisms/replicate		
	Test chambers:	250 mL borosilicate glass beakers		
	Solution volume:	200 mL		
	Solution renewal:	None, static		
	Analytical confirmation of test article:	Turbidometric assay using *Streptococcus faecalis*		
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark		
	Dissolved oxygen	7.2 mg/L (average, ≥49% of saturation throughout test)		
	pH	7.7 to 8.5		
	Total hardness:	120 mg/L (as $CaCO_3$)		
	Total alkalinity:	147 mg/L (as $CaCO_3$)		
	Temperature:	Averaged 20.4°C		
Biological Data	***Organism observation:***	At initiation and 24 and 48 hr		
	Organism observations:	Physical condition (normal, hypoactive, prostrate, immobile)		
	Endpoints:	48 hour EC50 based on immobility		
	Statistical Analysis:	Log-probit method		
Results				
Biological Data:	***Average Measured Concentration (mg/L):***	# Hypoactive 48 hr	# Immobile 48 hr	Cumulative Immobilization (%) 48 hr
	< 0.10 (control)	0	0	0
	4.69	2	1	3
	7.86	4	4	13
	12.45	21	0	0
	18.96	14	9	30
	35.08	4	26	87
	42.18	0	30	100
Endpoint Analysis	***48 hr EC50:***	20.56 mg/L		

Appendix HH – Study F05283. The Acute Toxicity to Rainbow Trout (*Salmo gairdneri*) of Narasin (Compound 79891). 1985.

	Report Title:	***The Acute Toxicity to Rainbow Trout (Salmo gairdneri) of Narasin (Compound 79891)***
	Performing Laboratory:	***Eli Lilly and Company***
	Guidance Document:	***ASTM E729-80***
	GLP Compliance:	***US FDA, OECD***
	Study Identification:	***F05283***
	Report Date:	***September 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Narasin
Test Organism	***Common name:***	Rainbow Trout
	Species:	*Salmo gairdneri* (currently *Oncorhynchus mykiss*)
	Age class:	Juvenile
Exposure Design	***Test medium:***	Conditioned well water
	Route of administration:	Water (whole body exposure)
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control), 0.125, 0.225, 0.365, 0.62, 1.1, 2.0, 3.3 and 5.6 mg/L
	Replication:	1 replicate/treatment, 10 organisms/replicate
	Feeding:	Not fed during test
	Test chambers:	glass jars
	Solution volume:	15 L
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark
	Dissolved oxygen	Averaged 10.2 mg/L (≥ 92% of saturation throughout test)
	pH	8.2 to 8.6
	Temperature	13°C
	Total hardness:	120 mg/L (as $CaCO_3$)
	Total alkalinity:	148 mg/L (as $CaCO_3$)
	Conductivity:	225 µmhos/cm
	Analytical confirmation of test article:	Turbidimetric assay using *Streptococcus faecalis*
Biological Data	***Organism observation:***	At initiation and 24, 48, 72 and 96 hr
	Organism observations:	Condition/behavior pattern
	Endpoints:	96 hour LC50

Results			
Biological Data:	***Average Measured Concentration (mg/L):***	Cumulative Mortality (%)	Observations
		96 hr	At concentrations ≥0.316 mg/L, fish exhibited signs of toxicity including hypoactivity, impaired swimming behavior, labored respiration, and death.
	< 0.10 (control)	0	
	0.103	0	
	0.190	0	
	0.316	0	
	0.561	0	
	1.00	0	
	1.82	30	
	3.04	80	
	5.26	100	
Endpoint Analysis	***96 hr LC50:***	2.23 mg/L	
	NOEC:	0.19 mg/L	

Appendix II – Study F05183. The Acute Toxicity to Bluegill (*Lepomis macrochirus*) of Narasin (Compound 79891). 1985.

	Report Title:	***The Acute Toxicity to Bluegill (Lepomis macrochirus) of Narasin (Compound 79891)***
	Performing Laboratory:	***Eli Lilly and Company***
	Guidance Document:	***ASTM E729-80***
	GLP Compliance:	***US FDA & OECD***
	Study Identification:	***F05183***
	Report Date:	***September 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Narasin
Test Organism	***Common name:***	Bluegill
	Species:	*Lepomis machrochirus*
	Age class:	Juvenile
Exposure Design	***Test medium:***	Condition well water
	Route of administration:	Water (whole body exposure)
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control), 1.0, 1.8, 3.0, 5.0, 6.2, 7.0, 8.0, 9.0 and 10.0 mg/L
	Replication:	1 replicate/treatment, 10 organisms/replicate
	Feeding:	Not fed during test
	Solution volume:	15 L
	Solution renewal:	None, static
Exposure Conditions	***Photoperiod:***	16 hours light, 8 hours dark
	Dissolved oxygen:	Averaged 8.75 mg/L (≥ 83% of saturation throughout test)
	pH:	8.05 to 8.6
	Temperature:	20.1°C
	Total hardness:	120 mg/L (as $CaCO_3$)
	Total alkalinity:	152 mg/L (as $CaCO_3$)
	Conductivity:	250 µmhos/cm
	Analytical confirmation of test article:	Turbidimetric assay using *Streptococcus faecalis*
Biological Data	***Organism observation:***	At initiation and 24, 48, 72 and 96 hr
	Organism observations:	Condition/behavior pattern
	Endpoints:	96 hour LC50

Results			
Biological Data:	***Average Measured Concentration (mg/L):***	Cumulative Mortality (%)	
		96 hr	At concentrations ≥2.80 mg/L, fish exhibited signs of toxicity including hypoactivity, impaired swimming behavior, labored respiration, and death.
	< 0.10 (control)	0	
	0.88	0	
	1.66	0	
	2.80	0	
	4.68	30	
	6.00	90	
	6.40	100	
	7.80	100	
	8.70	100	
	9.55	100	
Endpoint Analysis	***96 hr LC50 (Upper, Lower CI):***	5.02 mg/L (4.61, 5.46 mg/L)	
	NOEC:	1.66 mg/L	

Appendix JJ – Study 804984 – 4,4'-Dinitrocarbanilide (DNC) Soil Microorganisms: Nitrogen Transformation Test (OECD Guideline for the Testing of Chemicals, Document 216). 2005.

	Report Title:	***4,4'-Dinitrocarbanilide (DNC) Soil Microorganisms: Nitrogen Transformation Test (OECD Guideline for the Testing of Chemicals, Document 216)***				
	Study Number:	***804984***				
	Guidance Document:	***OECD 216***				
	GLP Compliance:	***OECD***				
	Report Number:	***25353***				
	Report Date:	***26 May 2005***				
STUDY SUMMARY						
Materials & Methods						
Test Article	***Name:***	4,4'-dinitrocarbanilide (DNC)				
Organic substrate	***Type:***	Lucerne (Alfalfa, *Medicago sativa*)				
Test Soil	***Characteristics of soil:***	*USDA classification*	*Sandy Loam*			
		pH	6.4			
		Organic carbon	1.3%			
		Microbial biomass (mgC/kg)	145			
Exposure Design	***Test concentrations:***	0 (control), 0.8 mg/kg soil, 8.0 mg/kg soil				
	Test article carrier:	Acetone				
	Test chambers:	5L plastic container with 1.2 kg soil (dry weight)				
	Soil sample replication:	3 per concentration				
	Test article application:	DNC solution added to quartz sand and mixed into soil				
	Control sample:	Acetone added to quartz sand and mixed into soil				
	Organic substrate application:	0.5% (w/w) lucerne mixed into each soil sample				
	Incubation conditions:	In darkness at 20 ± 2°C Moisture content maintained at 40% WHC				
Sample Analysis	***Sampling intervals:***	0-3 hrs, 7, 14 and 28 days post application				
	Determination of nitrate:	50 g subsamples extracted with KCl and nitrate measured photometrically.				
	Endpoint:	mg nitrate/kg of dry soil				
	Statistical analysis:	2-tailed multiple comparison Dunnett's test (α=0.05)				
Results						
Results	***Nitrogen transformation:***		*Concentration of Nitrate (mean ± SD; mg N/kg soil)*			
	% Inhibition:		*% Deviation from Control*			
		Test concentration	0 -3 hr	7 days	14 days	28 days
		0.8 mg/kg	-1.9%	10.2%	7.2%	5.1%
		8 mg/kg	-3.7%	0.0%	8.3%	-10.9%
	Endpoint Analysis:	No impact on nitrogen transformation				

Study 804984 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 804984.

Appendix KK – Study 805024. Evaluation of the Potential Impact of 4,4'-Dinitrocarbanilide (DNC) on Seedling Plant Species at 1x, 5x and 10x the Maximum Predicted Environmental Concentration in Soil. 2006.

	Report Title:	***Evaluation of the Potential Impact of 4, 4' - Dinitrocarbanilide (DNC) on Seedling Plant Species at 1x, 5x and 10x the Maximum Predicted Environmental Concentration in Soil***	
	Study Number:	***805024***	
	Guidance Document:	***OECD 208***	
	GLP Compliance:	***OECD***	
	Report Number:	***24866***	
	Report Date:	***21 July 2006***	
STUDY SUMMARY			
Materials & Methods			
Test Article	***Name:***	4,4'-dinitrocarbanilide (DNC)	
Test Soils	***Characteristics of soil:***	*Sandy Loam topsoil collected from the upper 20 cm horizon.*	
		pH	5.6
		Organic carbon	0.4%
Test Species	***Common name (species):***	Mung Beans (*Phaseolus aureus*) Oats (*Avena sativa*) Perennial Ryegrass (*Lolium perenne*) Lettuce (*Lactuca sativa*) Turnip (*Brassica rapa* cv Golden Ball) Radish (*Raphanus sativu*s cv French Breakfast 3)	
Exposure Design	***Test concentrations:***	0 (control), 0.8, 4.0 and 8.0 mg DNC/kg soil	
	Test article carrier:	Acetone	
	Test chambers:	1.1L plastic pots	
	Test article application:	DNC solution added to sand and sand mixed into soil to give approximately a 25%:75% sand:soil ratio	
	Control samples:	Acetone added to sand and mixed into soil to give approximately a 25%:75% sand:soil ratio	
	Replication:	4 replicates per concentration, 5 seeds per chamber	
	Replicate arrangement:	Random block design; separate blocks for each species	
	Environmental conditions:	Maintained in glasshouse Temperature (12 to 24°C), Humidity (48% to 90%) Water administered via saucers below pots	
	Exposure duration:	14 days after 50% control emergence	
Exposure Assessment	***Endpoints:***	Phytotoxic effects Emergence (survival) Fresh shoot weight (cut at soil surface) Dry shoot weight	
	Statistical evaluation:	Emergence (survival): LC50 Shoot weight: EC50 and NOEC	

Results				
Endpoint Analysis	***Species***	***Emergence***	***Fresh Shoot Weight***	***Dry Shoot Weight***
		LC50	***EC50***	***EC50***
	Mung bean	>8.0 mg/kg	>8.0 mg/kg	>8.0 mg/kg
	Oats	>8.0 mg/kg	>8.0 mg/kg	>8.0 mg/kg
	Ryegrass	>8.0 mg/kg	>8.0 mg/kg	>8.0 mg/kg
	Lettuce	>8.0 mg/kg	>8.0 mg/kg	>8.0 mg/kg
	Turnip	>8.0 mg/kg	>8.0 mg/kg	>8.0 mg/kg
	Radish	>8.0 mg/kg	>8.0 mg/kg	>8.0 mg/kg
Conclusion	DNC did not impact emergence or shoot weight for any species at any concentration			

Study 805024 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 805024.

Appendix LL – Study 151P-104. 4,4'-dinitrocarbanilide (DNC) – A Toxicity Study to Determine the Effects on the Seedling Emergence and Growth of Three Species of Plants Following OECD Guideline 208. 2016.

	Report Title:	***4,4'-dinitrocarbanilide (DNC) – A Toxicity Study to Determine the Effects on the Seedling Emergence and Growth of Three Species of Plants Following OECD Guideline 208***	
	Study Number:	***151P-104***	
	Guidance Document:	***OECD 208***	
	GLP Compliance:	***OECD, FDA***	
	Report Date:	***December 2, 2016***	
STUDY SUMMARY			
Materials & Methods			
Test Article	***Name:***	4,4'-dinitrocarbanilide (DNC); purity 98.8%	
Test Soils	***Characteristics of soil:***	*Artificial soil: 88% sand, 6% silt, 6% clay*	
		pH (water method)	6.9
		Organic matter	1.9%
		Organic carbon	1.1%
Test Species	***Common name (species):***	Perennial Ryegrass (*Lolium perenne*) Wheat (*Triticum aestivum*) Corn (*Zea mays*)	
Exposure Design	***Test concentrations:***	0 (control), 0 (solvent control), 2.9, 4.3, 6.5, 9.7, 14.6, and 21.9 mg DNC/kg soil (dry weight)	
	Test article vehicle:	Acetone	
	Test chambers:	6 ½" standard plastic pots	
	Test article application:	DNC stock in acetone added to sand and acetone allowed to evaporate. Spiked sand mixed into soil.	
	Replication:	8 replicates per control and concentration, 5 seeds per replicate	
	Replicate arrangement:	Randomized complete block design	
	Environmental conditions:	Maintained in greenhouse Temperature (18.70 to 34.33°C; mean 25.71°C), Humidity (46% to 91%), PAR (8.5 to 12.1 moles) Initial watering was from above, subsequent watering was sub-irrigation	
	Exposure duration:	14 days after 50% control emergence	
Exposure Assessment	***Endpoints:***	Condition (qualitative only) Emergence Survival of emerged seedlings Dry shoot weight Shoot height	
	Statistical evaluation:	NOECs determined for Emergence, Survival of emerged seedlings, Shoot height, Dry shoot weight per replicate; evaluated for differences from pooled control using William's or Jonckheere-Terpstra Trend tests	

Results					
Endpoint Analysis	***Species***	***Emergence***	***Survival of Emerged Seedlings***	***Fresh Shoot Height***	***Dry Shoot Weight***
		NOEC	***NOEC***	***NOEC***	***NOEC***
	Ryegrass	21.9 mg DNC /kg	21.9 mg DNC/kg	21.9 mg DNC/kg	21.9 mg DNC/kg
	Wheat	21.9 mg DNC/kg	21.9 mg DNC/kg	21.9 mg DNC/kg	21.9 mg DNC/kg
	Corn	21.9 mg DNC/kg	21.9 mg DNC/kg	21.9 mg DNC/kg	21.9 mg DNC/kg
Conclusion	DNC did not impact emergence, survival, height or weight for any species at any concentration tested				

Appendix MM – Study CYT 011/014574 – DNC (4,4'-dinitrocarbanilide) Acute toxicity (LC_{50}) to the Earthworm. 2002.

	Report Title:	***DNC (4,4'-dinitrocarbanilide) Acute Toxicity (LC50) to the Earthworm***
	Guidance Document:	***OECD 207***
	GLP Compliance:	***OECD, UK***
	Study Identification:	***CYT 011/014574***
	Report Date:	***23 January 2002***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	DNC (4,4'-Dinitrocarbanilide)
Test Organism	***Common name:***	Earthworm
	Species:	*Eisenia foetida*
	Size at initiation:	300 to 600 mg
Exposure Design	***Test medium:***	Artificial soil
	Duration:	14 days
	Nominal test concentrations:	0.0 (control), 95, 171, 309, 556, 1000 mg/kg (as test article)
	Nominal test concentrations:	0.0 (control), 93, 168, 303, 546, 982 mg/kg (as DNC, corrected for purity)
	Replication:	4 replicates/treatment, 10 organisms/replicate
	Test chambers:	1 L glass containers with 737g soil
	Temperature:	21 to 22°C
	Soil moisture:	Initiation: 34 to 35% Termination: 31 to 32%
Biological Data	***Organism observation frequency:***	Initiation, day 7 and day 14
	Biological data:	Body weight on Days 0 and 14 Health (behavioral, pathological observations) Mortality
	Endpoint:	LC50, Body weight change
Results		
Organism Observations	***Physical condition:***	No mortalities and all worms appeared normal
	Body Weight:	% increase in weight ranged from +3 to +7% with no concentration-response trend
Endpoint Analysis	***LC50:***	> 1000 mg/kg (> 982 mg/kg, corrected for purity)
	NOEC:	1000 mg/kg (982 mg/kg, corrected for purity)

Study CYT 011/014574 is owned by Koffolk Ltd. Koffolk Ltd. has granted permission to Elanco Animal Health to use the data in Study CYT 011/014574.

Appendix NN – Study 811794. Freshwater Alga, Growth Inhibition Test with DNC. 2014.

	Report Title:	***Freshwater Alga, Growth Inhibition Test with DNC***
	Guidance Document:	***OECD 201***
	GLP Compliance:	***OECD***
	Project Number:	***811794***
	Report Number:	***35247***
	Report Date:	***07 August 2014***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	4,4-dinitrocarbanilide (DNC)
Test Organism	***Common name:***	Freshwater green alga
	Species:	*Pseudokirchneriella subcapitata*
Exposure Design	***Test medium:***	ISO freshwater algal growth medium
	Carrier solvent:	Dimethyl formamide (DMF) at 0.10 mL/L
	Duration:	72 hours
	Nominal test concentrations:	0.0 (control), 0.0 (solvent control), 13, 22, 36, 60, 100 µg/L
	Abiotic Controls:	13 and 100 µg/L (no algae added)
	Number of replicates:	3 replicates for DNC treatments 6 replicates for control and solvent control 1 replicate for each abiotic control
	Test chambers:	250 mL silanised glass Erlenmeyer flasks with foil lids
	Solution volume:	100 mL
	Inoculation concentration:	0.96 x 10^4 cells/mL
Environmental Conditions	***Photoperiod:***	Continuous light at 6297 lux
	Temperature:	22.6 to 23.4°C
	Agitation:	Orbital shaking device set at 100 rpm
	pH:	7.96 to 9.21
	Analytical confirmation of test article:	Measured at time 0 and 72 hr, using a validated LC-MS/MS method
Biological Data	***Cell counts:***	0, 24, 48 and 72 hr
	Endpoints:	72 hr EC50 & NOEC for yield & average specific growth rate

Results				
Test Article Analysis	***Target concentration (µg/L):***	***Measured concentration (µg/L)***		***Geometric Mean Measured (µg/L)***
		0 hr	***72 hr***	
	0 (control)	<LOD‡	<LOD	<LOD
	0 (solvent control)	<LOD	<LOD	<LOD
	13	11.9	5.77	8.29
	13 (abiotic)	--	7.44	--
	22	20.1	8.49	13.06
	36	32.9	17.5	23.99
	60	58.9	13.7	28.41
	100	102	17.5	42.25
	100 (abiotic)	--	35.7	--
	‡ *LOD = 0.0306 µg/L*			
Biological Data	***Geometric Mean Measured (µg/L)***	***72 hr Growth Parameters***		
		Yield (µg/mL)		***Growth Rate (μ_{ave}/day)***
	0 (control)	29.47		1.59
	0 (solvent control)	27.27		1.57
	8.29	27.03		1.56
	13.06	28.43		1.58
	23.99	23.03		1.51
	28.41	26.76		1.56
	42.25	26.47		1.56
Statistical Analysis		***Yield***		***Growth Rate***
	NOEC	42.25 µg/L		42.25 µg/L
	EC50	>42.25 µg/L		>42.25 µg/L

Study 811794 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 811794.

Appendix OO – Study 573A-104A. 4,4'-Dinitrocarbanilide (DNC): A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). 2004.

	Report Title:	***4,4'-Dinitrocarbanilide (DNC): A 48-Hour Static Acute Toxicity Test with the Cladoceran (Daphnia magna)***
	Guidance Document:	***US EPA OPPTS 850.1010; OECD 202; EPA FIFRA 72-2; ASTM E729-88a***
	GLP Compliance:	***US EPA, OECD and Japan MAFF***
	Study Identification:	***573A-104A***
	Report Date:	***23 November 2004***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	4,4'-Dinitrocarbanilide (DNC)
Test Organism	***Common name:***	Waterflea
	Species:	*Daphnia magna*
	Age at initiation:	< 24 hours
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Carrier solvent:	Dimethylformamide (DMF)
	Solvent concentration:	500 µL DMF/L
	Duration:	48 hours
	Nominal test concentrations:	0.0 (control), 0.0 (solvent control), 13, 22, 36, 60, 100 µg DNC/L
	Replication:	2 replicates/treatment, 10 organisms/replicate
	Test chambers:	glass beakers
	Solution volume:	200 mL
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark with 30 minute transition period Light intensity: 426 lux
	Dissolved oxygen:	≥ 89% of saturation throughout test
	pH:	Ranged from 8.3 to 8.5
	Temperature:	20.2 to 20.5°C
	Hardness:	130 mg/L (as $CaCO_3$, measured at initiation)
	Alkalinity:	180 mg/L (as $CaCO_3$, measured at initiation)
	Conductivity:	315 µmhos/cm (measured at initiation)
Analytical confirmation of test article:		Measured at initiation and termination using a validated LC/MS/MS method.
Biological Data	***Organism observations:***	Mortality, immobilization, clinical signs of toxicity or abnormal behavior
	Endpoints:	24 and 48 hour EC50 and NOEC based on mortality/immobility

Results				
Biological Data:	***Organism Observations:***	Mean Measured Concentration (µg DNC/L)	Toxicity*	Mortality
			48 hr	48 hr
		Control	0%	0%
		Solvent Control	0%	5%
		17	0%	5%
		27	0%	0%
		40	0%	5%
		64	55%	25%
		93	20%	5%
		*Toxicity observations were lethargy		
Endpoint Analysis:	***48 hr EC50:***	> 93 µg DNC/L		
	NOEC:	27 µg DNC/L		

Study 573A-104A is owned by Innolytics LLC. Innolytics LLC has granted permission to Elanco Animal Health to use the data in Study 573A-104A.

Appendix PP – Study 151A-150. 4,4'-dinitrocarbanilide (DNC) – A Semi-Static Life-Cycle Toxicity Test with the Cladoceran (Daphnia magna) following OECD Guideline 211. 2016.

	Study Title:	***4,4'-dinitrocarbanilide (DNC) – A Semi-Static Life-Cycle Toxicity Test with the Cladoceran (Daphnia magna) following OECD Guideline 211***
	Guidance Document:	***OECD 211***
	GLP Compliance:	***US FDA & OECD***
	EAG Laboratories Study Number:	***151A-150***
	Report Date:	***December 2, 2016***
STUDY SUMMARY		
Materials & Methods		
Test Substance	***Name:***	4,4'-dinitrocarbanilide (DNC)
	Standard number:	1110-90-10
	Purity:	98.8%
Test Organism	***Species:***	*Daphnia magna* (cladoceran)
	Source:	EAG Laboratories – Easton cultures
	Age at initiation:	< 24 hours
Exposure Design	***Dilution water:***	Well water (filtered and UV sterilized)
	Carrier solvent:	Triethylene glycol (0.1 mL/L)
	Test chambers:	Test chambers: 250 mL glass beakers
	Test solution volume:	Approximately 200 mL
	Test solution renewal:	daily
	Photoperiod:	16 hours light: 8 hours darkness (30-min transition)
	Duration:	21 days
	Controls:	Negative control and solvent control
	Nominal treatment levels:	2.6, 6.3, 16, 40 and 99 µg DNC/L
	Replication:	10 replicates, 1 daphnid per replicate
	Feeding:	YCT, vitamin, algae once daily†
Analytical	***Method:***	LC/MS/MS
	Limit of Quantitation:	0.750 µg DNC/L (LOQ)
Biological Data	***Measured Endpoints:***	Survival (lack of immobilization) Reproduction (live neonates per surviving parent) Growth (length and dry weight)

Results				
Environmental Conditions	***pH:***		8.0 to 8.6	
	Dissolved oxygen:		8.0 to 9.1 mg/L (≥89% saturation)	
	Temperature:		19.0 to 20.6ºC	
	Specific Conductivity:		330 – 395 µS/cm	
	Hardness as $CaCO_3$:		140 – 148 mg/L	
	Alkalinity as $CaCO_3$:		176 – 180 mg/L	
	Illumination:		548 to 724 lux	
Mean Measured Concentration (µg DNC/L)	***Percent Survival***	***Mean No. Live Neonates Per Surviving Parent ± Std. Dev.***	***Mean Length ± Std. Dev. (mm)***	***Mean Dry Weight ± Std. Dev. (mg)***
<LOQ (Negative Control)	90	271 ± 9.2	5.04 ± 0.073	1.1 ± 0.20
<LOQ (Solvent Control)	90	257 ± 15.5	5.04 ± 0.053	1.1 ± 0.10
Pooled Controls	90	--[1]	5.04 ± 0.062	1.1 ± 0.16
2.7	80	256 ± 31.0	5.04 ± 0.052	1.4 ± 0.18
5.9	60	275 ± 14.5	5.02 ± 0.075	1.3 ± 0.24
14	80	266 ± 19.2	4.95 ± 0.076*	1.5 ± 0.17
35	90	143 ± 35.9*	4.36 ± 0.22*	0.92 ± 0.17*
85	0*	--[2]	--[2]	--[2]
EC_{10} (µg DNC/L) (95% CI)	--	32 (32 – 33)	--	--
EC_{50} (µg DNC/L) (95% CI)	56 (46 – 60)	--	--	--

* Indicates a statistically significant decrease in comparison to the control ($p \leq 0.05$); the reduction in length at 14 µg/L is not considered biologically significant.

[1] there was a statistically significant difference between the negative and solvent control groups, treatment data were compared to the solvent control.

[2] 100% immobility

CI = Confidence Interval.

Conclusions		
Most Sensitive Endpoint	***LOEC (µg DNC/L)***	***NOEC (µg DNC/L)***
Reproduction (mean neonates per surviving parental daphnid):	35	14

[†] daphnia fed at rate 1.5 times (0.6 mg C/daphnid/day in 200 mL solution) recommended level in OECD 211 guideline (0.1 to 0.2 mg C/daphnid/day in 50 mL to 100 mL solution)

Appendix QQ – Study 573A-106. 4,4'-Dinitrocarbanilide (DNC): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). 2004.

	Report Title:	***4,4'-Dinitrocarbanilide (DNC): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (Oncorhynchus mykiss)***
	Guidance Document:	***US EPA OPPTS 850.1075; ASTM E729-88a; EPA FIFRA 72-1; OECD 203***
	GLP Compliance:	***US EPA, OECD and Japan MAFF***
	Study Identification:	***573A-106***
	Report Date:	***23 November 2004***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	4,4'-Dinitrocarbanilide (DNC)
Test Organism	***Age at initiation:***	Rainbow Trout, *Oncorhynchus mykiss* Juvenile
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Carrier solvent:	Dimethylformamide (DMF)
	Solvent concentration:	500 µL DMF/L
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control) 0.0 (solvent control) and 100 µg/L
	Replication:	3 replicate/treatment, 10 organisms/replicate
	Test Solution volume:	30 L
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark with 30 minute transition period Light intensity - 130 lux
	Dissolved oxygen:	≥ 74% of saturation throughout test
	pH:	8.3 to 8.6
	Temperature:	12.0 to 12.5°C
	Hardness:	132 mg/L (as $CaCO_3$)
	Alkalinity:	180 mg/L (as $CaCO_3$)
	Conductivity:	280 µmhos/cm
Analytical confirmation of test article:		DNC concentrations in test solutions measured on days 0, 2, and 4 using a validated LC/MS/MS method.
Biological Data	***Organism observations:***	Mortality and clinical signs of toxicity or abnormal behavior
	Endpoints:	LC50 and NOEC based on mortality and toxicity
Results		
Test Article Analysis	***Mean measured concentrations (µg/L):***	<LOQ (control), <LOQ (solvent control) and 69 µg /L LOQ 2.5 µg DNC/L
Biological Data:		No mortality or toxicity was observed in any treatment during study
Endpoint Analysis	***24, 48, 72 and 96 hr EC50:***	> 69 µg DNC/L
	NOEC:	69 µg DNC/L

Study 573A-106 is owned by Innolytics LLC. Innolytics LLC has granted permission to Elanco Animal Health to use the data in Study 573A-106.

Appendix RR – Study 573A-105 – 4,4'-Dinitrocarbanilide (DNC): A 96-Hour Static Acute Toxicity Test with the Bluegill (*Lepomis macrochirus*). 2004.

	Report Title:	***4,4'-Dinitrocarbanilide (DNC): A 96-Hour Static Acute Toxicity Test with the Bluegill (Lepomis macrochirus)***
	Guidance Document:	***US EPA OPPTS 850.1075; OECD 203; ASTM E729-88a; EPA FIFRA 72-1***
	GLP Compliance:	***US EPA, OECD and Japan MAFF***
	Study Identification:	***573A-105***
	Report Date:	***23 November 2004***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	4,4'-Dinitrocarbanilide (DNC)
Test Organism		Bluegill, *Lepomis macrochirus*
	Age at initiation:	Juvenile
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Carrier solvent:	Dimethylformamide (DMF)
	Solvent concentration:	500 µL/L
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control) and 100 µg/L
	Replication:	3 replicates/treatment, 10 organisms/replicate
	Solution volume:	30 L
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark with 30 minute transition period Light intensity 578 lux (measured at initiation)
	Dissolved oxygen:	≥ 77% of saturation throughout test
	pH:	8.5 to 8.7
	Temperature:	21.2 to 22.3°C
	Hardness:	130 mg/L, as $CaCO_3$ (measured at initiation)
	Alkalinity:	181 mg/L, as $CaCO_3$ (measured at initiation)
	Conductivity:	320 µmhos/cm (measured at initiation)
Analytical confirmation of test article:		DNC in test solutions measured on days 0, 2 and 4 with a validated LC/MS/MS method
Biological Data	***Organism observations:***	Mortality and clinical signs of toxicity or abnormal behavior
	Endpoints:	LC50 and NOEC based on mortality and toxicity
Results		
Test Article Analysis	***Mean measured concentrations (mg/L):***	<LOQ (control), 72 µg/L
Biological Data:	***Organism observations:***	No mortality or toxicity was observed in any treatment during the study
Endpoint Analysis	***LC50:*** > 72 µg/L	**NOEC:** 72 µg/L

Study 573A-105 is owned by Innolytics LLC. Innolytics LLC has granted permission to Elanco Animal Health to use the data in Study 573A-105.

Appendix SS – Study 151A-151 – 4,4'-Dinitrocarbanilide (DNC) – Fish Short-Term Reproduction Assay with the Fathead Minnow (*Pimephales promelas*). 2016.

	Study Title:	***4,4'-Dinitrocarbanilide (DNC) – Fish Short-Term Reproduction Assay with the Fathead Minnow (Pimephales promelas)***
	Guidance Document:	***OECD 229***
	GLP Compliance:	***US FDA & OECD***
	Study Number:	***151A-151***
	Report Date:	***December 2, 2016***
STUDY SUMMARY		
Materials & Methods		
Test Substance	***Name:***	4,4'-Dinitrocarbanilide (DNC)
	Reference number:	1110-90-10
	Purity:	98.8%
Test Organism	***Species:***	*Pimephales promelas* (fathead minnow)
	Source:	Osage Catfisheries, Inc. Osage Beach, Missouri 65065
	Age of adults at pre-exposure initiation:	approximately 6 months
Exposure Design	***Dilution Water:***	EAG Laboratories - Easton well water, UV sterilized and filtered
	Carrier solvent:	Triethylene glycol (100 µL/L)
	Exposure System:	Continuous-flow diluter Temperature-controlled room Photoperiod: 6 hours light: 8 hours darkness with a 30-minute transition period Turnover rate: 10 volume additions per day Two separate exposure systems for adult and egg exposures Instantaneous adult biomass loading: 1.4 g fish/L
	Duration:	Adult exposure: 28 days Egg exposure: embryo through 2 days post-hatch
	Test Groups:	5 treatment levels with a negative control and solvent control
	Nominal treatment levels:	5.1, 13, 32, 80, 200 and 500 µg LY2835219/L
	Replication:	4 replicates for each test group Adult exposure: 6 fish per replication (2 males, 4 females) Egg exposure: 50 eggs per replicate per week, when available
	Feeding:	Adult exposure: commercial flake food and live brine shrimp nauplii (twice daily) Egg exposure: not fed
	Test chambers:	Adult exposure: 12-L glass aquaria with 10 L of water Egg exposure: 9-L glass aquaria with 7 L of water
Analytical Measurements	***Method:***	Liquid chromatography with mass spectrometry (LC/MS/MS)
	Limit of Quantitation:	0.475 µg DNC/L (LOQ)

Results			
Test Conditions	***Exposure period:***	Adult Exposure	Egg Exposure
	pH:	7.9 – 8.4	8.0 – 8.3
	Dissolved oxygen:	4.7 – 8.2 mg/L	7.8 – 8.2 mg/L
	Temperature:	24.0 – 25.6°C	24.4 – 25.9°C
	Conductivity:	347 – 377 µS/cm	350 – 367 µS/cm
	Hardness (as $CaCO_3$):	140 – 148 mg/L	140 – 148 mg/L
	Alkalinity (as $CaCO_3$):	176 – 180 mg/L	174 – 180 mg/L
	Illumination:	548 to 724 lux	503 to 702 lux
Analytical Measurements	***Mean Measured Concentrations:***	Adult exposure: 0.80, 2.6, 8.9, 28 and 91 µg DNC/L Egg exposure: 0.77, 2.6, 8.4, 27 and 85 µg DNC/L	

Biological Measurements

Adult Exposure

Mean Measured Concentration (µg DNC/L)	Percent Survival to Termination (Mean ± Std. Dev.)	Eggs per Female per Reproductive Day (Mean ± Std. Dev.)	Percent Fertility (Mean ± Std. Dev.)
Negative Control	100 ± 0.0	27.6 ± 5.15	97.7 ± 1.2
Solvent Control	95.8 ± 8.4	18.4 ± 3.94	96.9 ± 1.9
Pooled Control	97.9 ± 5.9	--	97.3 ± 1.5
0.80	100 ± 0.0	23.3 ± 4.07	98.2 ± 1.1
2.6	100 ± 0.0	25.7 ± 9.24	97.2 ± 1.8
8.9	100 ± 0.0	19.7 ± 7.12	96.7 ± 1.6
28	95.8 ± 8.4	24.3 ± 9.04	97.4 ± 1.4
91	95.8 ± 8.4	21.6 ± 6.45	96.8 ± 1.9

Egg Exposure

Mean Measured Concentration (µg DNC/L)	Percent Hatching Success (Week #)				Percent Post-Hatch Survival (Week #)			
	1	2	3	4	1	2	3	4
Negative Control	99.5	100	--	99.5	100	99.5	--	100
Solvent Control	99.0	99.0	95.5	98.0	100	99.0	100	100
Pooled Control	99.3	99.5	--	98.9	100	99.3	--	100
0.77	97.5	99.0	100	100	99.5	99.5	99.3	99.3
2.6	99.3	99.3	100	99.0	99.3	100	92.0	97.5
8.4	100	98.5	96.0	98.7	100	100	97.1	100
27	99.5	96.7†	100	100	99.0	98.0	100	98.0
85	98.7	98.0†	94.0	99.3	99.3	99.0	97.2†	99.3

†Statistically significant effect according to the Jonckheere-Terpstra trend test ($p < 0.05$). Effect not considered biologically relevant, since similar effects were not observed in the weeks preceding and following.

Conclusions

Most Sensitive Endpoint	NOEC (µg DNC/L)	LOEC (µg DNC/L)
Not Applicable	91	>91

Appendix TT – Study 805003 - 2-Hydroxy-4,6-dimethylpyrimidine (HDP) Soil Microorganisms: Nitrogen Transformation Test (OECD Guideline for the Testing of Chemicals, Document 216). 2005.

	Report Title:	***2-Hydroxy-4,6-dimethylpyrimidine (HDP) Soil Microorganisms: Nitrogen Transformation Test (OECD Guideline for the Testing of Chemicals, Document 216)***				
	Study Number:	***805003***				
	Guidance Document:	***OECD 216***				
	GLP Compliance:	***OECD***				
	Report Number:	***25354***				
	Report Date:	***26 May 2005***				
STUDY SUMMARY						
Materials & Methods						
Test Article	***Name:***	2 hydroxy 4,6 dimethylpyrimidine (HDP)				
Organic substrate	***Type:***	Lucerne (Alfalfa, *Medicago sativa*)				
Test Soils	***Characteristics of soils***	*USDA classification*	*Sandy Loam*			
		Microbial biomass (mgC/kg)	145			
Exposure Design	***Test concentrations:***	0 (control), 0.35 mg/kg soil, 3.5 mg/kg soil				
	Test article carrier:	Milli-Q water				
	Test chambers:	5L plastic container with 1.2 kg soil (dry weight)				
	Replication:	3 per treatment group				
	Test article application:	Aqueous HDP solution added to soil				
	Control sample:	Milli-Q water added to soil				
	Organic substrate application:	0.5% (w/w) lucerne mixed into each soil sample				
	Incubation conditions:	In darkness at 20 ± 2°C Moisture content maintained at 40-60% of water holding capacity (WHC)				
Sample Analysis	***Sampling intervals:***	3.25 hrs, 7, 14 and 28 days post application				
	Determination of nitrate:	50 g subsamples extracted with KCl and nitrate measured photometrically				
	Endpoint:	mg nitrate/kg of dry soil				
Results						
Results	***Nitrogen transformation***		*Concentration of Nitrate (mean ± SD; mg N/kg soil)*			
	% Inhibition		*% Deviation from Control*			
		Test concentration	0 -3 hr	7 days	14 days	28 days
		0.35 mg/kg	10.9%	-10.5%	6.6%	1.8%
		3.5 mg/kg	0.0%	9.5%	7.2%	-4.0%
	Endpoint Analysis:	No impact on nitrogen transformation				

Study 804003 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 805003.

Appendix UU – Study 805019 - Evaluation of the Potential Impact of 2-Hydroxy-4,6-dimethyl pyrimidine (HDP) on Seedling Plant Species at 1x, 5x and 10x the Maximum Predicted Environmental Concentration in Soil. 2006.

	Report Title:	***Evaluation of the Potential Impact of 2-Hydroxy-4,6-dimethyl pyrimidine (HDP) on seedling Plant Species at 1x, 5x and 10x the Maximum Predicted Environmental Concentration in Soil***
	Study Number:	***805019***
	Guidance Document:	***OECD 208***
	GLP Compliance:	***OECD***
	Report Number:	***24883***
	Report Date:	***21 July 2006***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	2-hydroxy-4,6-dimethyl pyrimidine (HDP)
Test Soils	***Characteristics of soil***	*Sandy Loam topsoil collected from the upper 20 cm horizon*
		pH 5.6
		Organic carbon 0.4%
Test Species	***Common name (species):***	Mung Beans (*Phaseolus aureus*) Oats (*Avena sativa*) Perennial Ryegrass (*Lolium perenne*) Lettuce (*Lactuca sativa*) Turnip (*Brassica rapa* cv Golden Ball) Radish (*Raphanus sativu*s cv French Breakfast 3)
Exposure Design	***Test concentrations:***	0 (control), 0.35, 1.75 and 3.5 mg HDP/kg soil (predicted environmental concentration, 0.35 mg HDP/kg)
	Test article carrier:	Milli-Q water
	Test chambers:	1.1L plastic pots
	Test article application:	HDP solution added to quartz sand and the sand mixed into soil at a ratio of approximately 25%:75% sand:soil Milli-Q added to sand and mixed into soil
	Replication per species:	4 replicates per concentration, 5 seeds per chamber
	Environmental conditions:	Maintained in glasshouse Temperature (12 to 24°C), Humidity (49% to 90%) Water administered via saucers below pots
	Exposure duration:	14 days after 50% control emergence
Exposure Assessment	***Endpoints***	Phytotoxic effects Emergence (survival) Fresh shoot weight Dry shoot weight
	Statistical evaluation	Emergence (survival): LC50 Shoot weight: EC50 and NOEC

Results				
Endpoint Analysis	***Species***	***Emergence***	***Fresh Shoot Weight***	***Dry Shoot Weight***
		LC50	***EC50***	***EC50***
	Mung bean	2.89 mg/kg	>3.5 mg/kg	>3.5mg/kg
	Oats	>3.5mg/kg	>3.5mg/kg	>3.5mg/kg
	Ryegrass	>3.5mg/kg	>3.5mg/kg	>3.5mg/kg
	Lettuce	>3.5mg/kg	>3.5mg/kg	>3.5mg/kg
	Turnip	>3.5mg/kg	>3.5mg/kg	>3.5mg/kg
	Radish	2.78 mg/kg	>3.5 mg/kg	>3.5mg/kg
Conclusion	Phytotoxic effects were determined in mung bean and radish at 3.5 mg HDP/kg			

Study 805019 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 805019.

Appendix VV – Study 151P-103 - 4,6-dimethyl-2-pyrimidinol (HDP) – A Toxicity Study to Determine the Effects on the Seedling Emergence and Growth of Four Species of Plants Following OECD Guideline 208. 2016.

	Report Title:	***4,6-dimethyl-2-pyrimidinol (HDP) – A Toxicity Study to Determine the Effects on the Seedling Emergence and Growth of Four Species of Plants Following OECD Guideline 208***	
	Study Number:	***151P-103***	
	Guidance Document:	***OECD 208***	
	GLP Compliance:	***OECD, FDA***	
	Report Date:	***December 2, 2016***	
STUDY SUMMARY			
Materials & Methods			
Test Article	***Name:***	4,6-dimethyl-2-pyrimidinol (HDP). 99.8%	
Test Soils	***Characteristics of soil:***	*Artificial soil: 88% sand, 6% silt, 6% clay*	
		pH (water method)	6.9
		Organic matter	1.9%
		Organic carbon	1.1%
Test Species	***Common name (species):***	Soybean (*Glycine max)* Pea *(Pisum sativum*) Radish *(Raphanus sativus*) Mung bean *(Vigna radiata)*	
Exposure Design	***Test concentrations:***	0 (negative control), 1.1, 1.75, 2.63, 3.9, and 5.9 mg HDP/kg dry soil	
	Test chambers:	6 ½" standard plastic pots	
	Test article application:	HDP stock in RO water added directly to the test soil.	
	Replication:	8 replicates per control and concentration, 5 seeds per replicate	
	Replicate arrangement:	Randomized complete block design	
	Environmental conditions:	Maintained in greenhouse Mung bean, radish and soybean: Temperature (18.70 to 34.33°C; mean 25.94°C), Humidity (46.00% to 91.00%), PAR (8.5 to 12.1 moles) Pea: Temperature (14.77 to 29.50°C; mean 21.16°C), Humidity (26.35% to 98.90%), PAR (11.3 to 18.2 moles) Initial watering was from above, subsequent watering was sub-irrigation	
	Exposure duration:	14 days after 50% control emergence	
Exposure Assessment	***Endpoints***	Condition (qualitative only) Emergence Survival of emerged seedlings Shoot height Dry shoot weight	

Results					
Endpoint Analysis	***Species***	***Emergence***	***Survival of Emerged Seedlings***	***Fresh Shoot Height***	***Dry Shoot Weight***
		NOEC mg HDP/kg	***NOEC*** mg HDP/kg	***NOEC*** mg HDP/kg	***NOEC*** mg HDP/kg
	Soybean	5.9	5.9	5.9	5.9
	Pea	5.9	5.9	5.9	5.9
	Radish	5.9	5.9	5.9	5.9
	Mung bean	5.9	5.9	2.63	5.9

Appendix WW – Study CYT 012/014575 - HDP (2-Hydroxy-4, 6-Dimethylpyrimidine) Acute Toxicity (LC_{50}) to the Earthworm. 2004.

	Report Title:	***HDP (2-Hydroxy-4, 6-Dimethylpyrimidine) Acute Toxicity (LC_{50}) to the Earthworm***
	Guidance Document:	***OECD 207***
	GLP Compliance:	***OECD, UK***
	Study Identification:	***CYT 012/014575***
	Report Date:	***21 January 2002***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	HDP (2-Hydroxy-4, 6-Dimethylpyrimidine)
Test Organism	***Common name:***	Earthworm
	Species:	*Eisenia foetida*
	Size at initiation:	300 to 600 mg
Exposure Design	***Test medium:***	Artificial soil
	Duration:	14 days
	Nominal test concentrations:	0.0 (control), 95, 171, 309, 556, 1000 mg/kg (as test article)
	Nominal test concentrations:	0.0 (control), 94, 169, 306, 550, 989 mg/kg (as HDP, corrected for purity)
	Replication:	4 replicates per treatment, 10 organisms per replicate
	Number of organisms per replicate:	10
	Test chambers:	1 L glass containers with 736 g soil
	Temperature:	21°C
	Soil moisture:	Day 0: 34% Day 14: 31 to 32%
Biological Data	***Biological data:***	Body weight on Days 0 and 14 Health (behavioral, pathological observations) Mortality
	Endpoint:	LC50, Body weight change
Results		
Organism Observations:	***Physical condition:***	No mortalities and all worms appeared normal
	Body Weight:	% change in weight ranged from -2 to +8% with no concentration-response trend
Endpoint Analysis	***LC50:***	> 1000 mg/kg (> 989 mg/kg, corrected for purity)
	NOEC:	1000 mg/kg (989 mg/kg, corrected for purity)

Study CYT 011/014575 is owned by Koffolk Ltd. Koffolk Ltd. has granted permission to Elanco Animal Health to use the data in Study CYT 011/014575.

Appendix XX – Study 811810. Freshwater Alga, Growth Inhibition Test with HDP. 2014.

	Report Title:	***Freshwater Alga, Growth Inhibition Test with HDP***
	Guidance Document:	***OECD 201***
	GLP Compliance:	***OECD***
	Project Number:	***811810***
	Report Number:	***35248***
	Report Date:	***07 August 2014***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Dimethyl-2-pyrimidinol (HDP)
Test Organism	***Common name:***	Freshwater green alga
	Species:	*Pseudokirchneriella subcapitata*
Exposure Design	***Test medium:***	ISO freshwater algal growth medium
	Duration:	72 hours
	Nominal test concentrations:	0.0 (control), 5, 12.5, 25, 37.5, 50 mg/L
	Abiotic Controls:	5 and 50 mg/L (no algae added)
	Number of replicates:	3 replicates for HDP treatments 6 replicates for control 1 replicate for each abiotic controls
	Test chambers:	250 mL silanised glass Erlenmeyer flasks with foil lids
	Solution volume:	100 mL
	Inoculation concentration:	0.99 x 10^4 cells/mL
Environmental Conditions:	***Photoperiod:***	Continuous light at 6147 lux
	Temperature:	22.5 to 23.2°C
	Agitation:	Orbital shaking device set at 100 rpm
	pH:	7.51 to 9.14
	Analytical confirmation of test article:	Measured at time 0 and 72 hr, using a validated LC-MS/MS method
Biological Data	***Cell counts:***	0, 24, 48 and 72 hr
	Endpoints:	72 hr EC50 & NOEC for yield & average specific growth rate

Results			
Test Article Analysis	***Measured concentrations were consistent over the duration of the study in all treatment levels and abiotic controls.***		
Biological Data	***Mean Measured Concentration (mg/L)***	***72 hr Growth Parameters***	
		Yield	***Growth Rate (μ_{ave}/day)***
	0 (control)	33.93	1.64
	5.08	32.83	1.63
	12.03	29.17	1.58
	24.46	34.51	1.64
	37.03	33.53	1.63
	46.36	29.55	1.59
Toxicity Endpoints		***Yield***	***Growth Rate***
	NOEC	46.36 mg/L	46.36 mg/L
	EC50	>46.36 mg/L	>46.36 mg/L

Study 811810 is owned by Phibro Animal Health. Phibro Animal Health has granted permission to Elanco Animal Health to use the data in Study 811810.

Appendix YY – Study 573A-107C. 2-Hydroxy-4,6-dimethylpyrimidine (HDP): A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). 2004.

	Report Title:	***2-Hydroxy-4,6-dimethylpyrimidine (HDP): A 48-Hour Static Acute Toxicity Test with the Cladoceran (Daphnia magna)***
	Guidance Document:	***US EPA OPPTS 850.1010; OECD 202; EPA FIFRA 72-2; ASTM E729-88a***
	GLP Compliance:	***US EPA, OECD and Japan MAFF***
	Study Identification:	***573A-107C***
	Report Date:	***23 November 2004***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	2-Hydroxy-4,6-dimethylpyrimidine (HDP)
Test Organism	***Common name:***	Waterflea
	Species:	*Daphnia magna*
	Age at initiation:	< 24 hours
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Duration:	48 hours
	Nominal test concentrations:	0.0 (control), 16, 26, 43, 72 and 120 mg/L
	Replication:	2 chambers/replicate, 10 organismsreplicate
	Test chambers:	250 mL glass beakers
	Solution volume:	200 mL
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark with 30 minute transition period Light intensity 506 lux
	Dissolved oxygen:	≥ 86% of saturation throughout test
	pH:	8.2 to 8.6
	Temperature:	20.3 to 20.8°C
	Hardness:	128 mg/L (as $CaCO_3$, measured at initiation)
	Alkalinity:	174 mg/L (as $CaCO_3$, measured at initiation)
	Conductivity:	330 µmhos/cm (measured at initiation)
Analytical confirmation of test article:		Measured HDP concentrations at initiation and termination using a validated LC/MS/MS method.
Biological Data	***Organism observations:***	Mortality, immobilization, clinical signs of toxicity or abnormal behavior
	Endpoints:	24 and 48 hour EC50 and NOEC based on mortality/immobility

Results		
Test Article Analysis	***Mean measured concentrations (mg/L):***	<LOQ (control), 15, 24, 39, 66, 107 mg/L (LOQ 12.5 µg HDP/L)
Biological Data:	***Observations:***	1 mortality at 15 mg/L and 1 lethargic at 66 mg/L All other organisms in treatment and control groups remained in normal physical condition
Endpoint Analysis	***48 hr EC50:*** ***NOEC:***	> 107 mg HDP/L 107 mg HDP/L

Study 573A-107C is owned by Innolytics LLC. Innolytics LLC has granted permission to Elanco Animal Health to use the data in Study 573A-107C.

Appendix ZZ – Study 573A-109 - 2-Hydroxy-4,6-dimethylpyrimidine (HDP): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*). 2004.

	Report Title:	***2-Hydroxy-4,6-dimethylpyrimidine (HDP): A 96-Hour Static Acute Toxicity Test with the Rainbow Trout (Oncorhynchus mykiss)***
	Guidance Document:	***US EPA OPPTS 850.1075; ASTM E729-88a; EPA FIFRA 72-1; OECD 203***
	GLP Compliance:	***US EPA, OECD and Japan MAFF***
	Study Identification:	***573A-109***
	Report Date:	***23 November 2004***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	2-Hydroxy-4,6-dimethylpyrimidine (HDP)
Test Organism	***Species:***	Rainbow Trout, *Oncorhynchus mykiss*
	Age at initiation:	Juvenile
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control) and 120 mg/L
	Replication:	3 replicates/treatment, 10 organisms/replicate
	Solution volume:	30 L (19.1 cm depth)
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark with 30 minute transition period Light intensity - 120 lux
	Dissolved oxygen:	≥ 76% of saturation throughout test
	pH:	8.3 to 8.6
	Temperature:	12.2 to 12.5°C
	Hardness:	128 mg/L (as $CaCO_3$)
	Alkalinity:	180 mg/L (as $CaCO_3$)
	Conductivity:	280 µmhos/cm
Analytical confirmation of test article:		HDP concentrations in test solutions measured on days 0, 2, and 4 using a validated LC/MS/MS method.
Biological Data	***Organism observations:***	Mortality and clinical signs of toxicity or abnormal behavior
	Endpoints:	LC50 and NOEC based on mortality and toxicity
Results		
Test Article Analysis	***Mean measured concentrations:***	<LOQ (control), 110 mg/L LOQ 12.5 µg HDP/L
Biological Data:		No mortality or toxicity was observed in any treatment during study
Endpoint Analysis:	***24, 48, 72 and 96 hr EC50:***	> 110 mg/L
	NOEC:	110 mg/L

Study 573A-109 is owned by Innolytics LLC. Innolytics LLC has granted permission to Elanco Animal Health to use the data in Study 573A-109.

Appendix AAA – Study 573A-108 - 2-Hydroxy-4,6-dimethylpyrimidine (HDP): A 96-Hour Static Acute Toxicity Test with the Bluegill (*Lepomis macrochirus*). 2004.

	Report Title:	***2-Hydroxy-4,6-dimethylpyrimidine (HDP): A 96-Hour Static Acute Toxicity Test with the Bluegill (Lepomis macrochirus)***
	Guidance Document:	***US EPA OPPTS 850.1075; OECD 203; ASTM E729-88a; EPA FIFRA 72-1***
	GLP Compliance:	***US EPA, OECD and Japan MAFF***
	Study Identification:	***573A-108***
	Report Date:	***23 November 2004***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	2-Hydroxy-4,6-dimethylpyrimidine (HDP)
Test Organism	***Species:***	Bluegill, *Lepomis macrochirus*
	Age at initiation:	Juvenile
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control) and 120 mg/L
	Replication:	3 replicates/treatment, 10 organisms/replicate
	Number of organisms per replicate:	10
	Solution volume:	30 L
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark with 30 minute transition period Light intensity 743 lux
	Dissolved oxygen:	≥ 84% of saturation throughout test
	pH:	8.5 to 8.7
	Temperature:	21.4 to 22.4°C
	Hardness:	134 mg/L (as $CaCO_3$)
	Alkalinity:	182 mg/L (as $CaCO_3$)
	Conductivity:	320 µmhos/cm
Analytical confirmation of test article:		HDP in test solution measured on days 0, 2, and 4 with a validated LC/MS/MS method
Biological Data	***Organism observations:***	Mortality and clinical signs of toxicity or abnormal behavior
	Endpoints:	LC50 and NOEC based on mortality/immobility
Results		
Test Article Analysis	***Mean measured concentrations (mg/L):***	<LOQ (control), 122 mg/L
Biological Data:	***Organism Observations:***	All organisms in treatment and control groups appeared healthy and normal throughout test
Endpoint Analysis	***LC50: >122 mg/L***	**NOEC**: 122 mg/L

Study 573A-108 is owned by Innolytics LLC. Innolytics LLC has granted permission to Elanco Animal Health to use the data in Study 573A-108.

Appendix BBB – Study W01382 - The Acute Toxicity of Soil-Incorporated Nicarbazin (Compound 93760) to Earthworms (*Lumbricus terrestris*) in a 14-Day Test. 1985.

	Report Title:	***The Acute Toxicity of Soil-Incorporated Nicarbazin (Compound 93760) to Earthworms (Lumbricus terrestris) in a 14-Day Test***
	Performing Laboratory:	***Eli Lilly and Company***
	Guidance Document:	***Karnak, 1982***
	GLP Compliance:	***OECD and FDA***
	Study Identification:	***W01382***
	Report Date:	***September 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Nicarbazin
	Components:	71.83% 1,3-bis(4-nitrophenyl)urea (DNC) 26.82% 4,6-dimethylpyrimidine-2-ol (HDP)
Test Organism	***Common name:***	Earthworm
	Species:	*Lumbricus terrestris*
Exposure Design	***Test medium:***	Loamy sand soil (850g loamy sand soil, 50g rabbit feces, 100 mL deionized water)
	Duration:	14 days
	Nominal test concentrations:	0.0 (control), 10 and 100 mg/kg soil
	Replication:	3 replicates/treatment, 5 organisms/replicate
	Test chambers:	2 L glass jar with 1000 g soil
	Temperature:	13°C
Biological Data	***Organism observations:***	Physical condition, Body weight gain
	Endpoint:	LC50, NOEC
Results		
Organism Observations:		No signs of toxicity were observed No significant decrease in body weight gain with treatment
Endpoint Analysis	***NOEC:***	100 mg/kg (nominal, as nicarbazin)
	EC50:	>100 mg/kg (nominal, as nicarbazin)

Appendix CCC – Study C02782 - The Acute Toxicity of Nicarbazin (Compound 93760) to *Daphnia magna* in a Static Test System. 1985.

	Report Title:	***The Acute Toxicity of Nicarbazin (Compound 93760) to Daphnia magna in a Static Test System***
	Performing Laboratory:	***Eli Lilly and Company***
	Guidance Document:	***ASTM E729-80 (1980)***
	GLP Compliance:	***OECD and FDA***
	Study Identification:	***C02782***
	Report Date:	***September 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Nicarbazin
	Components:	71.83% 1,3-bis(4-nitrophenyl)urea (DNC) 26.82% 4,6-dimethylpyrimidine-2-ol (HDP)
Test Organism	***Common name:***	Water flea, *Daphnia magna*
	Age at initiation:	≤ 24 hours
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Duration:	48 hours
	Nominal test concentrations:	0.0 (control) and 100 mg/L
	Replication:	3 replicates/treatment, 10 organisms/replicate
	Test chambers:	250 mL borosilicate glass beakers
	Solution volume:	200 mL
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark
	Dissolved Oxygen:	all chambers at least 100% of saturation
	pH:	8.5 to 8.6
	Temperature:	19.5°C
	Total hardness:	103 mg/L (as $CaCO_3$)
	Total alkalinity:	120 mg/L (as $CaCO_3$)
	Conductivity:	230 µmhos/cm
Analytical confirmation of test article:		HPLC-UV analysis of HDP in test solutions
Biological Data	***Organism observations:***	Physical condition
	Endpoints:	Mortality (immobility)
Results		
Test Article Analysis	***Measured concentration of HDP (mg/L):***	<LOQ (control), 24.2 mg/L
Biological Data:	***Observations:***	No physical signs of toxicity or immobility were observed
Endpoint Analysis	***EC50:***	>100 mg/L (nominal, as nicarbazin) >24.2 mg/L (measured as HDP)
	NOEC:	100 mg/L (nominal, as nicarbazin) 24.2 mg/L (measured as HDP)

Appendix DDD – Study F08982 - The Acute Toxicity to Bluegill (*Lepomis macrochirus*) of Nicarbazin (Compound 93760). 1985.

	Report Title:	***The Acute Toxicity to Bluegill (Lepomis macrochirus) of Nicarbazin (Compound 93760)***
	Performing Laboratory:	***Eli Lilly and Company***
	Guidance Document:	***ASTM E729-80 (1980)***
	GLP Compliance:	***OECD and FDA***
	Study Identification:	***F08982***
	Report Date:	***September 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Nicarbazin
	Components:	71.83% 1,3-bis(4-nitrophenyl)urea (DNC) 26.82% 4,6-dimethylpyrimidine-2-ol (HDP)
Test Organism	***Species:***	Bluegill, *Lepomis macrochirus*
	Age at initiation:	Juvenile
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control) and 100 mg/L
	Replications:	3 replicates/treatment, 10 organisms/replicate
	Number of organisms per replicate:	10
	Solution volume:	200 mL
	Solution renewal:	None, static
Environmental Conditions	***Photoperiod:***	16 hours light, 8 hours dark
	Dissolved oxygen:	all chambers at least 100% of saturation
	pH:	8.25 to 8.6
	Temperature:	22 to 23°C
	Total hardness:	103 mg/L (as $CaCO_3$)
	Total alkalinity:	120 mg/L (as $CaCO_3$)
	Conductivity:	240 µmhos/cm
Analytical confirmation of test article:		HPLC-UV analysis of HDP component
Biological Data	***Organism observations:***	Physical condition
	Endpoints:	Mortality
Results		
Test Article Analysis	***Average measured concentration of HDP (mg/L):***	<LOQ (control), 28.68 mg/L
Organism Observations:	***Physical condition:***	No physical symptoms of toxicity or mortality observed
Endpoint Analysis	***NOEC:***	100 mg/L (nominal, as nicarbazin) 28.68 mg/L (average measured as HDP)
	EC50:	>100 mg/L (nominal, as nicarbazin) >28.68 mg/L (average measured as HDP)

Appendix EEE – Study F09082 - The Acute Toxicity to Rainbow Trout (*Salmo gairderni*) of Nicarbazin (Compound 93760). 1985.

	Report Title:	***The Acute Toxicity to Rainbow Trout (Salmo gairderni) of Nicarbazin (Compound 93760)***
	Performing Laboratory:	***Eli Lilly and Company***
	Guidance Document:	***ASTM E729-80 (1980)***
	GLP Compliance:	***OECD and FDA***
	Study Identification:	***F09082***
	Report Date:	***September 1985***
STUDY SUMMARY		
Materials & Methods		
Test Article	***Name:***	Nicarbazin
	Components:	71.83% 1,3-bis(4-nitrophenyl)urea (DNC) 26.82% 4,6-dimethylpyrimidine-2-ol (HDP)
Test Organism		Rainbow Trout, *Salmo gairdneri*
	Age at initiation:	Juvenile
Exposure Design	***Test medium:***	Laboratory well water (freshwater)
	Duration:	96 hours
	Nominal test concentrations:	0.0 (control) and 100 mg/L
	Replication:	3 replicates/treatment, 10 organisms/replicate
	Solution volume:	15 L
	Solution renewal:	None, static
Environmental Conditions	***Dissolved oxygen:***	all chambers at least 88% of saturation
	pH:	Ranged from 8.0 to 8.5
	Temperature:	Ranged from 12 to 13°C
	Total hardness:	102 mg/L (as $CaCO_3$)
	Total alkalinity:	120 mg/L (as $CaCO_3$)
	Conductivity:	220 µmhos/cm
Analytical confirmation of test article		HPLC-UV analysis of HDP component
Biological Data	***Organism observations:***	Physical condition
	Endpoints:	Mortality
Results		
Test Article Analysis	***Average measured concentration of HDP (mg/L):***	<LOQ (control), 26.68 mg/L
Organism Observations:	***Physical condition:***	No physical symptoms of toxicity or mortality observed
Endpoint Analysis	***NOEC:***	100 mg/L (nominal, as nicarbazin) 28.68 mg/L (average measured as HDP)
	EC50:	>100 mg/L (nominal, as nicarbazin) >28.68 mg/L (average measured as HDP)

Appendix FFF – Calculations for Concentration in Earthworm

In Section 5.2.5, exposure to DNC via worm consumption was considered. The contents of worm gut contain soil and, therefore, non-target avian species may be affected by the amount of DNC in the soil. To derive a concentration of DNC in earthworms inhabiting soil containing DNC residues, REACH (ECHA 2016) was consulted.

To derive the concentration of DNC in earthworms, first the earthworm BCF ($BCF_{earthworm}$) was determined. There is no measured value, therefore, the BCF was estimated using the measured log Kow (>3.6, Table 5). The earthworm BCF can be calculated using the Kow of DNC ($10^{3.6}$), the default density of an earthworm (1 $kg_{wwt} \cdot L^{-1}$) and equation R.16-66 of REACH (ECHA 2016).

$$BCF_{earthworm} = \frac{(0.84 + 0.012 \times Kow)}{RHO_{earthworm}}$$

$$BCF_{earthworm} = \frac{(0.84 + 0.012 \times 10^{3.6})}{1\ kg_{wwt} \cdot L^{-1}} = 49\ \frac{L}{kg_{wwt}}$$

The concentration in the earthworm ($C_{earthworm}$) is the total concentration of DNC in the worm as a result of bioaccumulation and adsorption of DNC through pore water and soil in the gut. Prior to deriving $C_{earthworm}$, the concentration in pore water ($PEC_{porewater}$) must be calculated.

In order to estimate the concentration in pore water, the partitioning to pore water is calculated. First, the partitioning coefficient to soil (Kp_{soil}) is calculated to be 322.74 $L \cdot kg^{-1}$. The value is derived assuming that soil has a percent organic carbon content of 2% (Foc_{soil}) and using the lowest measured K_{oc} value measured in soil (16137 L/kg, Table 7) and equation R.16-6 of REACH (ECHA 2016):

$$Kp_{soil} = Foc_{soil} \times Koc$$

$$Kp_{soil} = 0.02\ \frac{kg_{oc}}{kg_{soil}} \times 16137\ \frac{L_{water}}{kg_{oc}} = 322.74\ \frac{L_{water}}{kg_{soil}}$$

The bulk density of soil (RHO_{soil}) is calculated to be 1700 $kg \cdot m^{-3}$. The value is derived using equation R.16-16 and default values in table R.16-8 of REACH (ECHA 2016). Default values used are for the fraction of solids in soil ($Fsolid_{soil}$), the density of the solid phase (RHOsolid), the fraction of water in soil ($Fwater_{soil}$), the density of the water phase (RHOwater), the fraction of air in soil ($Fair_{soil}$), and the density of the air phase (RHOair).

$$RHO_{soil} = Fsolid_{soil} \times RHOsolid + Fwater_{soil} \times RHOwater + Fair_{soil} \times RHOair$$

$$RHO_{soil} = 0.6\frac{m^3_{solid}}{m^3_{soil}} \times 2500\frac{kg_{solid}}{m^3_{solid}} + 0.2\frac{m^3_{water}}{m^3_{soil}} \times 1000\frac{kg_{water}}{m^3_{water}} + 0\frac{m^3_{air}}{m^3_{soil}} \times 1.3\frac{kg_{air}}{m^3_{air}}$$

$$= 1700\frac{kg_{soil}}{m^3_{soil}}$$

The dimensionless partitioning coefficient to the soil compartment ($K_{soil\text{-}water}$) is calculated to be 484.31 $m^3 \cdot m^{-3}$. The value is derived using Kp_{soil} (322.74 $L \cdot kg^{-1}$) and default values in table 16-8 and equation R.16-7 of REACH (ECHA 2016):

$$K_{soil-water} = Fwater_{soil} + Fsolid_{soil} \times \frac{Kp_{soil}}{1000} \times RHOsolid$$

$$K_{soil-water} = 0.2\frac{m^3_{water}}{m^3_{soil}} + 0.6\frac{m^3_{solid}}{m^3_{soil}} \times \frac{322.74\ \frac{L_{water}}{kg_{soil}}}{1000\frac{L_{water}}{m^3_{water}}} \times 2500\ \frac{kg_{soild}}{m^3_{solid}} = 484.31\frac{m^3_{soil}}{m^3_{water}}$$

The predicted environmental concentration in pore water ($PEC_{pore\ water}$) is calculated to be 2.5 $\mu g \cdot L^{-1}$. The value is derived using the PEC_{soil} (725 $\mu g \cdot kg^{-1}$, Section 5.1.2.1.2), $K_{soil\text{-}water}$ (484.31 $m^3 \cdot m^{-3}$), the bulk density of soil (1700 $kg \cdot m^{-3}$) and equations R.16-55 and R.16-56 of REACH (ECHA 2016):

$$PEC_{pore\ water} = \frac{PEC_{soil} \times RHO_{soil}}{K_{soil-water} \times 1000}$$

$$PEC_{pore\ water} = \frac{725\frac{\mu g}{kg_{soil}} \times 1700\frac{kg_{soil}}{m^3_{soil}}}{484.31\frac{m^3_{soil}}{m^3_{water}} \times 1000\frac{L_{water}}{m^3_{water}}} = 2.5\frac{\mu g}{L}$$

A factor to convert soil concentration from dry weight to wet weight ($CONV_{soil}$) is needed to determine $C_{earthworm}$. $CONV_{soil}$ is derived using RHO_{soil} (1700 $kg \cdot m^{-3}$), RHO_{solid} (2500 $kg \cdot m^{-3}$), $Fsolid_{soil}$ (0.6 $m^3 \cdot m^{-3}$), and equation R.16-64 of REACH (ECHA 2016).

$$CONV_{soil} = \frac{RHO_{soil}}{F_{solid} \times RHO_{solid}}$$

$$CONV_{soil} = \frac{1700\frac{kg_{wwt,soil}}{m^3{}_{soil}}}{0.6\frac{m^3{}_{solid}}{m^3{}_{soil}} \times 2500\ \frac{kg_{dwt,solid}}{m^3_{solid}}} = 1.13\frac{kg_{wwt,soil}}{kg_{dwt,solid}}$$

The concentration in earthworms ($C_{earthworm}$) is calculated to be 184 $\mu g \cdot kg$. $C_{earthworm}$ is derived using the $BCF_{earthworm}$, $PEC_{porewater}$, PEC_{soil}, $CONV_{soil}$ (1.13 $kg_{wwt} \cdot kg_{dwt}^{-1}$), a default value for the fraction of gut loading (dry weight to wet weight) in the worm (F_{gut}, 0.1 $kg_{dwt} \cdot kg_{wwt}^{-1}$)and equation R16.65 of REACH (ECHA 2016). The characteristics of DNC (Sections 5.1.1.1 and 5.1.1.2.5.2) indicate that it will have low mobility in soil. Therefore, $C_{earthworm}$ was conservatively calculated assuming earthworms only inhabited soil around the point of litter application.

$$C_{earthworm} = \frac{BCF_{earthworm} \times PEC_{porewater} + PEC_{soil} \times F_{gut} \times CONV_{soil}}{1 + F_{gut} \times CONV_{soil}}$$

$$C_{earthworm} = \frac{49\ \frac{L}{kg_{wwt}} \times 2.5\frac{\mu g}{L} + 725\frac{\mu g}{kg_{soil}} \times 0.1\frac{kg_{dwt}}{kg_{wwt}} \times 1.13\frac{kg_{wwt}}{kg_{dwt}}}{1 + 0.1\frac{kg_{dwt}}{kg_{wwt}} \times 1.13\frac{kg_{wwt}}{kg_{dwt}}}$$

$$= 184\frac{\mu g}{kg_{wwt,earthworm}}$$