## SCIENTIFIC OPINION

# Scientific Opinion on the safety and efficacy of Koffogran (nicarbazin) as a feed additive for chickens for fattening[1]

**EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)**[2,3]

European Food Safety Authority (EFSA), Parma, Italy

## ABSTRACT

Koffogran (containing 25 % nicarbazin) is intended to be used as a coccidiostat for chickens for fattening up to an age of 28 days. Feeding 125 mg nicarbazin/kg feed was safe for chickens for fattening (margin of safety about 1.5) and effective in controlling coccidiosis. Ingested nicarbazin is rapidly split into its components 2-hydroxy-4,6-dimethylpyrimidine (HDP) and 4,4'-dinitrocarbanilide (DNC). DNC is the marker residue, liver the target tissue. HDP-related residues are much lower than those derived from DNC. Nicarbazin is not genotoxic. In sub-chronic rat studies, no NOAEL could be derived for nicarbazin, but a NOAEL of 709 mg/kg bw/day was identified for DNC. Those differences may be related to the higher systemic exposure of rats to DNC when administered as nicarbazin instead of DNC alone. No major concern appeared in multigeneration reproduction studies in rats at 300 mg DNC + 100 mg HDP/kg bw/day and in developmental studies with rats and rabbits at 70 and 60 mg nicarbazin/kg bw, respectively. As the consumer is exposed to DNC rather than nicarbazin, an ADI was set for DNC with 0.77 mg/kg bw/day, derived from the NOAEL for elevated serum ALT in a two-year dog study (154 mg DNC/kg bw/day) applying a safety factor of 200. MRLs of 15, 6, 4 and 4 mg DNC/kg liver, kidney, muscle and skin/fat, respectively, were proposed. Applying those MRLs consumer exposure would not exceed 24 % of the ADI. A one-day withdrawal period was considered as adequate. Nicarbazin was not an irritant or sensitiser to skin, but a slight irritant to eyes. Inhalatory exposure of users to nicarbazin was negligibly small. No safety concern for the soil compartment, groundwater or by secondary poisoning was identified, the risk for surface water could however not be assessed.

## KEY WORDS

Coccidiostat, nicarbazin, DNC, HDP, chickens for fattening, safety, ADI, MRLs, efficacy

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## SUMMARY

Following a request from the European Commission, the European Food Safety Authority (EFSA) was asked to deliver an opinion on the safety and efficacy of Koffogran (containing 25 % nicarbazin as active substance), intended for use as a coccidiostat in chickens for fattening up to an age of 28 days.

Nicarbazin from Koffogran at a maximum use level of 125 mg/kg complete feed was considered safe for chickens for fattening (margin of safety about 1.5) under normal climate conditions and as far as the microbiological risk is concerned.

Ingested nicarbazin was rapidly split into its two components, 2-hydroxy-4,6-dimethylpyrimidine (HDP) and 4,4'-dinitrocarbanilide (DNC), which behaved independently with respect to their pharmacokinetics and metabolism. DNC from nicarbazin was considerably more available for the animal than DNC given alone or administered simultaneously with HDP in similar proportions as in nicarbazin. DNC appeared as the marker residue, and liver was the target tissue. DNC residues declined rapidly from tissues following nicarbazin withdrawal. HDP-related residues are much lower than those derived from DNC.

Following the submission of further mutagenicity studies, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that nicarbazin is not genotoxic. No NOAEL for nicarbazin in a sub-chronic rat study could be derived since nicarbazin exerted adverse effects at all the dose levels tested (lowest dose 181 mg/kg bw/day). However, a NOAEL of 709 mg/kg bw/day could be derived for DNC from a sub-chronic rat study using DNC alone. These apparently conflicting results may be related to the higher systemic exposure of rats to DNC when administered as nicarbazin instead of as DNC. 154 mg DNC (+ 51 mg HDP)/kg bw/day from a two-year dog study was considered as the lowest NOAEL. Despite the shortcomings of the rat multigeneration reproduction studies, no major concern appeared at the highest doses tested (300 mg DNC + 100 mg HDP/kg bw/day). The FEEDAP Panel concluded that there are no concerns on developmental toxicity in rats up to 70 mg nicarbazin/kg bw/day, based on fetotoxic effects at 200 mg/kg bw/day. The NOAEL for maternal toxicity in the rabbit study could be conservatively established as 60 mg nicarbazin/kg bw/day, whereas the NOAEL for developmental toxicity was 120 mg nicarbazin/kg bw/day (the highest dose given).

p-Nitroaniline (PNA), a nicarbazin associated impurity, is a suspected carcinogen. Considering the PNA disposition data in rats and the maximum PNA level in nicarbazin recommended by the FEEDAP Panel, an exposure of the consumer to PNA resulting from the consumption of tissues from chickens for fattening fed diets supplemented with Koffogran at the maximum proposed level would be negligibly low.

As the consumer is only exposed to DNC, an ADI of 0.77 mg/kg bw for DNC was set, derived from the NOAEL of the two-year dog study (154 mg DNC/kg bw/day) and applying a safety factor of 200. MRLs for DNC in liver (15 mg/kg), kidney (6 mg/kg), muscle (4 mg/kg) and skin/fat (4 mg/kg) were proposed. A worst case calculation by dietary exposure following application of these MRLs showed that only 24 % of the ADI is used up by these MRLs. A one-day withdrawal period was considered as adequate.

Nicarbazin showed no potential for skin irritation or sensitisation, it was slightly irritating to the eyes (transient effects). Inhalatory exposure to nicarbazin from handling Koffogran was considered negligibly small.

The FEEDAP Panel does not expect that the use of nicarbazin at the recommended dose will pose a foreseeable risk for the soil compartment, groundwater or secondary poisoning. In the absence of raw toxicity data for algae, the risk for surface water could, however, not be assessed.

The FEEDAP Panel considered nicarbazin at the maximum dose proposed (125 mg/kg complete feed) to be effective in controlling coccidiosis in chickens for fattening.

The FEEDAP Panel made some recommendations concerning the reduction of PNA in nicarbazin and further monitoring of potential *Eimeria* resistance in chickens for fattening.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 1831/2003 establishes rules governing the Community authorisation of additives for animal nutrition and in particular defines, by Article 25, the transitional measures that have to be met for authorisation of the additive under conditions of Council Directive 70/524/EEC.

The Commission received a supplementary dossier from the applicant company, Phibro Animal Health, to complete the missing data on the product "Koffogran", based on nicarbazin, when it is used as a feed additive for chickens for fattening.

## TERMS OF REFERENCE BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority to issue an opinion on the safety and efficacy of "Koffogran", based on nicarbazin, when used for chickens for fattening, evaluating the supplementary data provided by the applicant.

**Table 1:** Description and conditions of use of the additive as proposed by the applicant

| | |
|---|---|
| **Additive** | Nicarbazin (Koffogran) |
| **Registration number/EC No/No (if appropriate)** | |
| **Category of additive** | Coccidiostats and histomonostats |
| **Functional group of additive** | n/a |

| **Description** | | | |
|---|---|---|---|
| **Composition, description** | **Chemical formula** | **Purity criteria (if appropriate)** | **Method of analysis (if appropriate)** |
| Nicarbazin 250.00 g/kg<br>Stearic acid 126.00 ± 5%<br>Polysorbate 20 13.90 ± 10%<br>Wheat middlings to 100 %<br><br>Active Substance:<br>Nicarbazin | **Nicarbazin**<br>$C_{19}H_{18}N_6O_6$<br>CAS number: 330-95-0<br>Equimolecular complex of 1,3-bis(4-nitrophenyl)urea and 4,6-dimethylpyrimidin-2-ol, in granular form<br>IUPAC Nomenclature:<br>4,4' Dinitrocarbanilid and 2-hydroxy-4,6-dimethyl pyrimidine Complex (equimolar 1:1) | **Nicarbazin**<br>Related impurities:<br>≤ 1%<br>p-nitroaniline<br>max. 0.5% | **Nicarbazin** is a 1:1 molar mixture of 4,4[1]-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HDP). It is assayed using a reverse-phase isocratic method, which measures the DNC moiety at a wavelength of 340nm |

| | |
|---|---|
| **Trade name (if appropriate)** | Koffogran |
| **Name of the holder of authorisation (if appropriate)** | Phibro Animal Health s.a. Belgium BE 4033 |

| **Conditions of use** | | | | |
|---|---|---|---|---|
| **Species or category of animal** | **Maximum Age** | **Minimum content** | **Maximum content** | **Withdrawal period (if appropriate)** |
| | | **mg/kg of complete feedingstuffs** | | |
| Chickens for fattening | 28 days | 100 | 125 | 5 days |

| **Other provisions and additional requirements for the labelling** | |
|---|---|
| **Specific conditions or restrictions for use** (if appropriate) | |
| **Specific conditions or restrictions for handling** (if appropriate) | Use prohibited at least 5 days before slaughter |
| **Post market monitoring** (if appropriate) | |
| **Specific conditions for use in complementary feedingstuffs** (if appropriate) | - |

| **Maximum Residue Limit (MRL) (if appropriate)** | | | |
|---|---|---|---|
| **Marker residue** | **Species or category of animal** | **Target tissue(s) or food products** | **Maximum content in tissues** |
| 4,4' dinitrocarbanilide (DNC) | Chickens for fattening | Liver | 750 µg/kg |

## ASSESSMENT

### 1. Introduction

Koffogran contains 25 % nicarbazin as active substance. It is applied for use in chickens for fattening as a coccidiostat at a dose range of 100–125 mg nicarbazin/kg complete feedingstuffs. The FEEDAP Panel made an assessment of the data submitted by the applicant in 2003 (EFSA, 2003).

The toxicological part of the dossier was considered to be far from satisfactory. Conclusions on the safety for the consumer and user could not be drawn. In addition, the risk for the terrestrial and aquatic environment could not be assessed due to insufficient information provided. Moreover, a full assessment of the efficacy based on recent data was not possible as an insufficient number of up-to-date trials were provided.

The applicant submitted in 2008 a supplementary dossier to compensate the inadequacies identified previously which is the basis of the present assessment.[4]

EFSA received a cross-reference letter from the applicant of Maxiban® G160 (containing narasin and nicarbazin) allowing the sharing of the safety and technical data in the Maxiban® G160 dossier in the context of this opinion.

### 2. Characterisation of the additive

In its former assessment (EFSA, 2003), the FEEDAP Panel did not make any comments on this section of the dossier. However, many questions were raised by the Member States.

A series of questions were related to the specifications of various compounds and to analytical issues. Specifications were provided in the current dossier for nicarbazin,[5] for polysorbate 20,[6] for stearic acid,[7] for wheat middlings,[8] for packaging of Koffogran,[9] for packaging of nicarbazin,[10] and for Koffogran.[11] The Koffogran product data sheet was provided.[12] Following the approach normally adopted by the FEEDAP Panel, those data are not commented upon unless there is a safety concern.

Information on the analytical methods was given for free HDP in Koffogran,[13] for the Israeli Standard on microbiological analysis including a certificate of microbiological analysis of wheat middlings,[14] for the analytical method for DNC,[15] on the determination of nicarbazin in premixtures and feeds,[16] for the validation of the method for residual solvents[17] (and chromatograms for solvents),[18] and for the validation of analytical methods.[19] Those data are not commented upon either by the FEEDAP Panel. A certificate of analysis of the reference standard was attached.[20]

The following chapters are based on the relevant information in the current dossier and, if appropriate, on the information already provided in the former dossier.

[4] EFSA Dossier reference: FAD-2008-0062
[5] Technical dossier/Quality folder/Appendix 1
[6] Technical dossier/Quality folder/Appendix 3
[7] Technical dossier/Quality folder/Appendix 4
[8] Technical dossier/Quality folder/Appendix 5
[9] Technical dossier/Quality folder/Appendix 6
[10] Technical dossier/Quality folder/Appendix 12
[11] Technical dossier/Quality folder/Appendix 15
[12] Technical dossier/Quality folder/Appendix 38
[13] Technical dossier/Quality folder/Appendix 2
[14] Technical dossier/Quality folder/Appendix 5-2
[15] Technical dossier/Quality folder/Appendix 9
[16] Technical dossier/Quality folder/Appendix 13-1
[17] Technical dossier/Quality folder/Appendix 14
[18] Technical dossier/Quality folder/Appendix 11
[19] Technical dossier/Quality folder/Appendix 17
[20] Technical dossier/Quality folder/Appendix 10

## 2.1. Identity of the additive

Koffogran is a feed additive to be used for the control of coccidiosis in chickens fattening. It is a straw to brown coloured granular solid composed of (% w/w): nicarbazin: 25.00 (Specification 23.7–27.5 %),[21] stearic acid: 12.60, polysorbate 20: 1.39 and wheat middlings: 61.01. The nicarbazin content of Koffogran was analysed in four batches,[22] it varied between 24.3 and 25.1 % (average 24.8 ±0.3 %).

More than 90 % of the particles in the final formulation have a diameter of 850 μm, and less than 7 % of the particles have diameters < 125 μm. The bulk density of the product is about 0.5 g/mL.

Particle size data obtained by laser diffraction analysis were used to estimate the inhalatory exposure of users/workers.

Heavy metals were analysed in three batches.[23] The content, expressed as lead, was < 10 mg/kg. The dioxin content of Koffogran was determined in three batches.[24] The values were consistently low and ranged between 0.040 and 0.047 ng WHO-PCDD/F-TEQ/kg.

## 2.2. Characterisation of the active substance

Nicarbazin, a chemically synthesised product, is an equimolecular complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) with the formula $C_{19}H_{18}N_6O_6$ and the CAS No. 330-95-0 (MW=426.38). The structural formula is given in Figure 1.

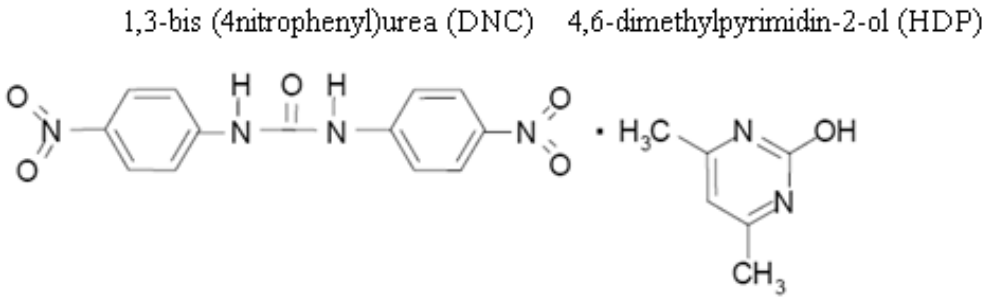


**Figure 1:** Structural formula of nicarbazin

Nicarbazin purity, as specified by the petitioner, should be above 95.1 %.[25] Potential impurities are free HDP (max. 3.0 %), p-nitroaniline (PNA, max. 0.5 %), ammonia (max. 0.5 %), chloride (max 0.3 %), sulphate (max. 1.5 %), methanol (max.0.02 %), diethylbenzene (max. 0.02 %) and other unspecified impurities (max. 0.2 %). Specifications, analytical methods and data were provided.[26,27]

Content uniformity of nicarbazin (specification 28.0–30.0 % HDP, 67.7–73.0 DNC) has been analytically confirmed by analysis of six batches (HDP 28.9–29.4 %, DNC 67.7–69.1 %).[28,29] PNA content was analysed in six batches and resulted between 0.1 and 0.2 %.[30] It did not increase either during a storage period of 36 months.[31] Free HDP (not involved in the complex) represented 1.5 % of

[21] Technical dossier/Quality folder/Appendix 15
[22] Technical dossier/Quality folder/Appendix 19
[23] Technical dossier/Quality folder/Appendix 18
[24] Technical dossier/Quality folder/Appendix 19
[25] Technical dossier. Monograph section
[26] Technical dossier. Monograph section
[27] Technical dossier/Quality folder/Appendix 8
[28] Technical dossier/Quality folder/Appendix 21
[29] Technical dossier/Quality folder/Appendix 27
[30] Technical dossier/Quality folder/Appendix 21
[31] Technical dossier/Quality folder/Appendix 22

nicarbazin (three batches).[32] The specifications for residual solvents are below the VICH[33] thresholds; confirmed by analysis of six batches[34] (below 0.01 %). Control methods are in place.

The physical and chemical properties of DNC and HDP are summarised in Table 2 (EFSA, 2003). The vapour pressure of DNC and HDP is not provided, however, for the environmental risk assessment (ERA) partitioning to air is considered negligible.

**Table 2:** Relevant properties of 4,4'-dinitrocarbanilide (DNC) and 2- hydroxy-4-6-dimethylpyrimidine (HDP)

| | DNC | HDP |
|---|---|---|
| **Molecular Weight** | 302 g/mole | 124 g/mole |
| **Water solubility** | < 0.02 mg/L | appr. 70 g/L |
| **Log $K_{ow}$** | 3.6 at pH 5-9 | -0.94 at pH 5–9 |

## 2.3. Manufacturing processes

### 2.3.1. Active substance

The production process of nicarbazin is fully described in the dossier. Nicarbazin is synthesised using a chemical process from DNC and HDP. The company also provided additional information on the manufacturing process of DNC and HDP.

### 2.3.2. Additive

Wheat middlings and the polysorbate 20 are mixed for 15 minutes. Nicarbazin is added and the compounds are mixed for a further 15 minutes. The stearic acid is added and the mix is mixed for a further 15 minutes. The preparation is then heated to 75 to 85°C and further mixed for 30 minutes. The product is cooled during pneumatic transfer. [35]

## 2.4. Physico-chemical and technological properties of the additive

### 2.4.1. Stability

It should be noted that the nicarbazin complex, stabilised by hydrogen bounds, dissociates easily in solvents but not in water.[36] The assays performed after solvent extraction are therefore carried out by analysing each of the two moieties (DNC and HDP) separately.

#### 2.4.1.1. Shelf life of the additive

The shelf life of the product was already demonstrated in the previous dossier.[37] In summary, two Koffogran batches (stored at 25 °C/60 % relative humidity (RH)) were analysed for nicarbazin (measured as DNC and HDP separately) up to 24 months. Initial values were 24.7 and 25.0 %. After 24 months, the values were 23.8 and 25.1 %, respectively. A third batch was followed for nine months (initial: 24.5 %, final: 24.7 % nicarbazin). Two more batches were stored at 40 °C/75 % RH, one for 12 and the other for nine months. Initial values were 24.7 % and 24.5 % nicarbazin, 23.9 % after nine months and 24.0 % after 12 months, respectively. Two batches were stored in a non air-conditioned room (5–35 °C and 25–60 % RH). DNC and HDP were determined for 43 and 32 months, respectively, and found to be stable (initial and final HDP: 28 %; initial DNC 69 %, final 70 %). A third batch was stored at 25 °C/60 % RH for up to 36 months and also found to be stable (initial and final HDP: 29 %, initial DNC; 69 %, final 68 %). As no change of the respective contents of both

[32] Technical dossier/Quality folder/Appendix 27
[33] http://www.vichsec.org/en/guidelines.htm
[34] Technical dossier/Quality folder/Appendix 21
[35] Technical dossier. Monograph section
[36] Technical dossier/Quality folder/Appendix 31
[37] Technical dossier/Quality folder/Appendix 30

components was observed, it can be concluded that each nicarbazin component was stable. This is supported by the absence of potential decomposition products (PNA and paranitrophenylurea) during a storage period of 36 months.[38] In a forced degradation study, no decomposition (no decrease of HDP and DNC) could be observed after treatment with $H_2O_2$ or heat (four hours at 100 °C), but a limited significant decomposition occurred under acidic or basic conditions.

The data support the shelf life proposed by the applicant[39] of 24 months when stored at not more than 25 °C.

2.4.1.2. Stability of the additive used in premixtures and feedingstuffs

Three recent studies on the stability of nicarbazin from Koffogran in premixtures for chickens for fattening (with trace elements) were provided.[40] The target concentration was 1.04 % nicarbazin. Nicarbazin (measured as DNC) was determined after four, eight and 12 weeks of storage at 30 °C/65 % RH and 40 °C/75 % RH, respectively. In the first study, the analysed initial value was 0.94 % nicarbazin; after three months, 0.95 % was measured at 30 °C/65 % RH and 0.86 % at 40°C/75 % RH. Studies 2 and 3 showed a similar outcome. The applicant concludes from the nicarbazin concentrations measured after three months under accelerated conditions on a six-month stability of nicarbazin in premixtures under normal storage conditions.

Three recent studies[41] on the stability of nicarbazin (125 mg nicarbazin/kg complete feed) from Koffogran in mash and pelleted feed (typical starter diet for chickens for fattening) were submitted. In the first study, nicarbazin (measured as DNC) averaged in three samples 118 mg/kg mash feed and 111 mg/kg pelleted feed. DNC was determined after four, eight and 12 weeks of storage at 30 °C/65 % RH and 40 °C/75 % RH, respectively. In the first study, after 12 weeks at 30 °C/ 65 % RH, nicarbazin in mash feed amounted to 106 mg/kg, in the pelleted diet to 103 mg/kg. The corresponding values at 40 °C/75 % RH were 124 and 101 mg/kg, respectively. Studies 2 and 3 showed a similar outcome.

### 2.4.2. Homogeneity

Koffogran was added to a complete premixture at a level of 1.78 % nicarbazin and six random samples were taken for assay. Homogeneity was demonstrated by a mean value of 1.72 % and a coefficient of variation (CV) of 1.1 %.[42]

The recent nicarbazin stability studies in premixtures for chickens for fattening and in mash and pelleted feeds (see Section 2.4.1.2)[43] allow also conclusions on the distribution of the additive. Three samples each were analysed in the three studies. In premixtures (target concentration: 1.04 % nicarbazin), 1.00 (CV 3.9 %); 1.02 (CV: 4.7 %) and 0.97 % (CV: 5.8 %) were measured. In mash feed (target concentration 125 mg/kg), 118 (CV: 4.1 %); 117 (CV: 4.1 %) and 124 mg/kg (CV: 6.9 %) were analysed, in pelleted feed 111 (CV: 7.3 %); 103 (CV: 4.2 %) and 109 mg nicarbazin/kg (CV: 2.5 %).

The results confirm the homogenous distribution of nicarbazin from Koffogran in premixtures and final feedingstuffs for chickens for fattening.

### 2.4.3. Cross contamination

A study on the likely cross contamination when preparing feedingstuffs was provided.[44] A typical commercial poultry diet containing 125 mg nicarbazin/kg was formulated, then mixed and pelleted. Thereafter the entire equipment was flushed and a non-supplemented diet was mixed and pelleted. This procedure was repeated three times. The target content of nicarbazin was analytically confirmed

[38] Technical dossier/Quality folder/Appendix 22
[39] Technical dossier. Monograph section
[40] Technical dossier/Quality folder/Appendix 40
[41] Technical dossier/Quality folder/Appendix 39
[42] Technical dossier/Quality folder/Appendix 32
[43] Technical dossier/Quality folder/Appendix 39 and 40
[44] Technical dossier/Quality folder/Appendix 13-0

in the supplemented diets within acceptable variance. The results of the assays performed on mash samples (from the mixer) and finished pellets of the non-supplemented diets after flush showed no detectable nicarbazin (LOD of the method: 1 mg nicarbazin/kg feed) in all of the three replicates. The results indicate that nicarbazin has no tendency to adhere to the mixing equipments.

### 2.5. Conditions of use

Koffogran is a feed additive to be used for the control of coccidiosis in chickens for fattening of not more than 28 days of age at a concentration of 100–125 mg nicarbazin/kg complete feed. The applicant proposes a five–day withdrawal period.

### 2.6. Analytical methods

#### 2.6.1. Analytical methods for routine control of the active substance in premixtures and feedingstuffs

The applicant makes reference to an analytical method validated and inter-laboratory studied, applied to premixtures and feedingstuffs for chickens for fattening originating from different European countries.[45] It consists of a reverse phase liquid chromatography coupled to UV detection of DNC. The relative standard deviation was 5.7 % for the premixtures. The recovery was 91–108 %, the limit of detection < 20 mg DNC/kg and the relative standard deviation 2.6–10.2 % for feedingstuffs.

#### 2.6.2. Analytical method for the determination of the residues of the active substance in target tissues

The Koffogran opinion (EFSA, 2003) reported an HPLC/UV analytical method which allowed the determination of DNC in chicken tissues with a LOQ of 0.1 mg/kg.

The applicant has submitted a new and validated method[46] which consists of the LC-MS/MS determination of DNC in chicken tissues. The mean recoveries of spiked samples were all between 70–110 % with coefficients of variation of ≤ 15% for concentrations ≥ 100 µg/kg. LOQs are 0.050, 0.100, 0.025 and 0.025 mg DNC/kg liver, kidney, muscle and skin/fat, respectively.

## 3. Safety

### 3.1. Safety for the target species

#### 3.1.1. Tolerance studies in the target species

No new data were provided by the applicant. In its opinion on Koffogran (EFSA, 2003), the FEEDAP Panel concluded that:

> 'The safety margin is around 1.5 based on body weight gain. Because of the adverse effects observed on the reproductive system contamination of feed for laying and breeding stock should particularly be avoided.'

To the knowledge of the FEEDAP Panel there are no new data available that requires reconsideration of the above conclusions.

#### 3.1.2. Microbiological safety of the additive

No new data were provided by the applicant. In its opinion on Koffogran (EFSA, 2003), the FEEDAP Panel concluded on the microbiological findings [recent insertion]:

> 'At feed level concentration nicarbazin, DNC and HDP had no antimicrobial activity against pathogenic and non-pathogenic, endogenous and exogenous bacteria [five strains each of

[45] Technical dossier/Quality folder/Appendix 13-1
[46] Technical dossier/Monograph folder/Appendix 25

> *Salmonella, Staphylococcus, Enterococcus, Escherichia coli, Proteus, Lactobacillus, Campylobacter, Clostridium, Bacteroides*] in a standardised *in vitro* test [agar dilution test: MICs all >256 mg/L]. At 490 mg/kg nicarbazin had no antimicrobial activity on *Salmonella enterica* (serovar Enteritidis). Nicarbazin had no effect on colonisation or excretion of *Salmonella enterica* (serovar Enteritidis).'

### 3.1.3. Interactions/incompatibilities

No new data were provided by the applicant. In its opinion on Koffogran (EFSA, 2003), the FEEDAP Panel concluded that:

> 'Results of compatibility studies are not presented. Incompatibilities or interactions with feedingstuffs, carriers, other approved additives or medicinal products are not to be expected by means of the so far known history of the product because no incidents from practical use have been materialised.'

### 3.1.4. Heat stress

Nicarbazin at the recommended use level is known to aggravate symptoms of heat stress in chickens for fattening. It increases body temperature and heart rate (Beers et al., 1989) as well as heat production (Wiernusz and Teeter, 1995) under the condition of heat stress.

### 3.1.5. Conclusions on the safety for the target species

Nicarbazin at the recommended maximum use level (125 mg/kg complete feed) is considered safe for chickens for fattening (margin of safety about 1.5) under established temperature regimes.

Nicarbazin is considered to be safe as far as the microbiological risk is concerned.

## 3.2. Safety for the consumer

### 3.2.1. Metabolism and residue studies

#### 3.2.1.1. Metabolism

In its former assessment of Koffogran (EFSA, 2003), the FEEDAP Panel has drawn the following conclusions:

i) metabolic steady state in chickens is reached after six days,

ii) metabolic fate of nicarbazin in chickens shows that the DNC and HDP components of the complex are split and behave independently, the DNC moiety (and metabolites) being mainly excreted in the faeces (46 % unchanged DNC) while the HDP moiety mainly appears in the urine (90 % unchanged HDP),

iii) DNC metabolic fate in chickens has been established and its main metabolites identified in the excreta, bile and tissues: monoacetylamino-DNC resulting from the reduction and acetylation of one nitro group, diacetylamino-DNC corresponding to the reduction and acetylation of both nitro groups, and N,N'-1,4-phenylenebis(acetamide) resulting from the split, reduction and acetylation of the molecule,

iv) unchanged HDP represents about 85 % of total HDP residues in the excreta, liver and kidney, the other metabolites each representing less than 10 %,

v) liver is the target tissue and DNC can be considered as the marker residue,

vi) metabolic fate of nicarbazin is qualitatively similar in the chicken and the rat.

In a new study,[47] six-week old chickens (five animals of each sex) received 50 mg [$^{14}$C]-DNC-nicarbazin/kg feed for five consecutive days. The animals were killed at zero-day withdrawal and excreta and bile were sampled. The identification of DNC-derived metabolites confirmed the metabolic pathway of nicarbazin formerly described (EFSA, 2003).

In another new study,[48] 21-day old chickens received twice daily (capsule) and for seven consecutive days, a dose equivalent to 125 mg [$^{14}$C]-DNC-nicarbazin/kg feed. Steady state in plasma was reached after five days. The metabolic profiling (radio-HPLC) of the cumulative excreta (day 1 to day 7) of four animals confirmed that unchanged DNC was the main component excreted (about 90 % of the extractable radioactivity), followed by the monoacetyl-derivative of DNC (5 %) then thirteen minor metabolites representing each less than 2 % of the total radioactivity.

A third study[49] has been carried out following a similar protocol, but using 125 mg [$^{14}$C]-HDP-nicarbazin/kg feed. Steady state in plasma was reached after six days. About 97 % of the administered radioactivity was recovered in the excreta after 16 hours, which indicates that HDP from nicarbazin is processed rapidly. Unchanged HDP represented about 65 % of the total radioactivity in the excreta, whereas minor metabolites represented each less than 6 %. After a zero-day withdrawal, HDP was also a major component of the residual radioactivity (77 %, 22 %, 57 % and 46 % in the liver, kidney, muscle and skin/fat, respectively) found in tissues.

*Interaction of nicarbazin constituents*

The relative oral bioavailability of DNC from nicarbazin in rats vs. DNC alone or in combination with HDP was studied in rats.[50] Nine groups of five rats each, jugular vein-canulated, received single oral doses of 50, 150 and 450 mg nicarbazin/kg bw; 50, 150 and 450 mg of a mixture of DNC plus HDP (in the same molecular ratio as in nicarbazin)/kg bw and 150, 450 and 900 mg DNC/kg bw, respectively. Blood samples were collected at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 hours after receiving the dose, DNC concentration was determined and pharmacokinetic analysis was performed. The results indicate that when administered as nicarbazin, DNC shows a bioavailability which is 33 to 65 times higher than when administered alone or as a mixture with HDP.

Those results confirm a previous observation made in chickens concerning superior coccidiostatic action of nicarbazin compared to a mixture of DNC+HDP (Cuckler et al., 1955). The data support also a previous hypothesis (Rogers et al., 1983) of a dramatic increase (expected 80 to 100 fold) of the bioavailability of DNC from nicarbazin, based on the observation that ultrafine crystals were formed in water, which would result in an increased absorption of DNC.

3.2.1.2. Residues

*Total residues*

Two studies concerning the kinetics of tissue residues derived from [$^{14}$C]-HDP and [$^{14}$C]-DNC-nicarbazin administered at the dose proposed for use have been submitted.

In the first one,[51] four groups of six chickens (three males and three females, 21-day old) received twice daily (capsule) and for seven consecutive days a total dose corresponding to 125 mg [$^{14}$C]-HDP-nicarbazin/kg feed. Groups of birds were sacrificed 24 hours after the last morning dose administration, i.e. a 16-hour withdrawal considered conservatively as a one-day withdrawal, then after three, five and ten days. Total residues were measured and metabolite profiling established based on radio-HPLC methods. Total residues resulting from the HDP moiety of nicarbazin are presented in Table 3.

[47] Cross reference/Maxiban® G160 Technical dossier/Section III/Annex 13
[48] Technical dossier/Human safety section/Appendix 7
[49] Technical dossier/Human safety section/Appendix 8
[50] Cross reference/Maxiban® G160 Technical dossier/Supplementary information/ September 2009/Annex 7
[51] Technical dossier/Human safety section/Appendix 8

**Table 3:** Kinetics of total residues in tissues of chickens (six animals per time point) administered 125 mg [$^{14}$C]-HDP-nicarbazin/kg feed for seven consecutive days followed by a withdrawal period (expressed as mg equivalent HDP/kg wet tissue)[a]

| | Withdrawal period (days) | | | |
|---|---|---|---|---|
| | **1** | **3** | **5** | **10** |
| **Liver** | 0.095 ± 0.041 | 0.008 ± 0.003 | 0.006 ± 0.002 | 0.0022 ± 0.002 |
| **Kidney** | 0.134 ± 0.061 | 0.0051 ± 0.003 | 0.0022 ± 0.001 | 0.0022 ± 0.001 |
| **Muscle** | 0.084 ± 0.037 | 0.0032 ± 0.001 | 0.0022± 0.001 | - |
| **Skin/fat** | 0.106 ± 0.036 | 0.027 ± 0.010 | 0.0172 ± 0.010 | 0.0062 ± 0.002 |

[a]: average values including LOQ values (liver, kidneys and muscle: 0.004 mg/kg; skin/fat: 0.011 mg/kg) for samples below these values.

The low resolution of the methodology used to separate tissue metabolites allows the FEEDAP Panel to conclude only on the prevalence of HDP as the major metabolite in all tissues (45 % to 64 % in the liver, 26 % in the kidneys) and the presence of a low proportion (less than 10 % each) of metabolites.

A second study[52] was carried out following a similar protocol, but using 125 mg [$^{14}$C]-DNC-nicarbazin/kg feed and retaining three withdrawal times only. As in the former study, the animals were sacrificed 24 hours after the last morning dose administration, i.e. a 16-hour withdrawal considered to correspond to a one-day withdrawal, then after five and ten days. An improved analytical approach (LC/MS/MS) was used to establish metabolic profiles.

Radioactivity steady state in plasma was reached after a four-day withdrawal. Total residues resulting from the DNC moiety of nicarbazin are presented in Table 4. At one-day withdrawal the figures are orders of magnitude higher than those obtained with [$^{14}$C]-HDP-nicarbazin, i.e. from 50 (skin/fat) to 300 (liver) times. A very fast decline of the radioactivity was observed in all tissues over the first four days of withdrawal. Liver was the target tissue.

**Table 4:** Kinetics of total residues in tissues of chickens (six animals per time point) administered 125 mg [$^{14}$C]-DNC-nicarbazin/kg feed for seven consecutive days followed by a withdrawal period (expressed as mg equivalent DNC/kg wet tissue)[a]

| | Withdrawal period (days) | | |
|---|---|---|---|
| | 1 | 5 | 10 |
| **Liver** | 27.797 ± 1.445 | 0.608 ± 0.198 | 0.050 ± 0.014 |
| **Kidney** | 16.776 ± 1.254 | 0.369 ± 0.121 | 0.033 ± 0.011 |
| **Muscle** | 4.431 ± 0.571 | 0.069 ± 0.016 | 0.002 ± 0.001 |
| **Skin/fat** | 5.122 ± 0.320 | 0.151 ± 0.039 | 0.024 ± 0.010 |

[a] average value including LOQ value (0.004 mg/kg) for samples below this value

Total residue extractability from tissues was high after a one-day withdrawal (94 %, 83 % and 90 % in the liver, muscle and skin/fat, respectively) but decreased with increasing withdrawal periods, namely in the liver (75 % after five days and 27 % after ten days). DNC and acetyl-DNC (by decreasing order) appeared as the major residual compounds in all tissues at one-day withdrawal, accompanied by a great number of minor metabolites (13 in the liver, four in the muscle and skin/fat) representing each less than 10 %. The ratio of DNC vs. total residues in tissues is presented in Table 5.

[52] Technical dossier/Human safety section/Appendix 7

**Table 5:** Ratio of DNC vs. total radioactivity in chicken tissues according to the withdrawal period (DNC as % of total DNC-related residues)

| | Withdrawal period (days) | |
|---|---|---|
| | 1 | 5 |
| **Liver** | 38–49 | 23–48 |
| **Kidney** | 31–42 | 0–5 |
| **Muscle** | 21–27 | 0–18 |
| **Skin/fat** | 40–55 | - |

*Marker residue*

A study[53] of the residue depletion of nicarbazin measured as DNC in chicken tissues in field conditions has been performed. Groups of six birds (one-day old, three of each sex) were administered feed supplemented with 125 mg nicarbazin from Koffogran/kg (analytically controlled) for 28 days. Groups of animals were slaughtered after 1, 5, 7, 9, 11 and 14 days of withdrawal and tissues sampled.

DNC residues were quantified using an analytical method with LOQs of 0.050, 0.100, 0.025 and 0.025 mg/kg for the liver, kidney, muscle and skin/fat, respectively. The results are presented in Table 6.

**Table 6:** DNC residue depletion in tissues of chicken fed for 28 days a diet supplemented with 125 mg nicarbazin from Koffogran/kg followed by a withdrawal period (mg DNC/kg tissue)

| | Withdrawal period (days) | | |
|---|---|---|---|
| | 1 | 5 | 7 |
| **Liver** | 9.249 ± 1.804 | 0.453 ± 0.047 | <LOQ |
| **Kidney** | 3.007 ± 1.095 | <LOQ[a] | <LOQ |
| **Muscle** | 2.110 ± 0.506 | 0.045 ± 0.008 | <LOQ |
| **Skin/fat** | 2.327 ± 0.372 | 0.131 ± 0.033 | <0.027 ± 0.004 |

[a] LOQ = 0.05, 0.1, 0.025 and 0.025 mg/kg for the liver, kidney, muscle and skin/fat

All the results for withdrawal periods longer than seven days were below the LOQs.

3.2.1.3. Conclusion on the metabolism and residue studies

The complementary studies and data previously supplied confirm (i) the former conclusions of the FEEDAP Panel concerning the metabolism of nicarbazin from Koffogran in chickens (EFSA, 2003), and (ii) that DNC from nicarbazin is considerably more available for the animal than DNC given alone or administered simultaneously with HDP in similar proportions as in nicarbazin.

Moreover, total residues resulting from either the HDP or the DNC moieties of nicarbazin (administered at the highest recommended dose) indicate that (i) HDP-related residues are much lower than those derived from DNC, (ii) after five days all tissues are essentially devoid of HDP-derived residues, and (iii) DNC appears to be the marker residue. Following nicarbazin withdrawal, DNC residues decline rapidly from tissues, liver being the target tissue.

### 3.2.2. Toxicological studies

3.2.2.1. Acute toxicity

The applicant did not provide new studies. It should be noted that the two acute studies assessed in the previous opinion (EFSA, 2003) were both pre-GLP. The results showed that nicarbazin was of low acute toxicity.

[53] Technical dossier/Human safety section/Appendix 9

3.2.2.2. Genotoxicity (mutagenicity and clastogenicity)

The FEEDAP Panel assessed (EFSA, 2003) two bacterial mutagenicity studies with nicarbazin.

Nicarbazin, HDP and DNC were tested in the Ames mutagenicity test using *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 with and without metabolic activation by liver enzymes in rats pre-treated with Aroclor 1254 (S9 mix). The concentrations (in DMSO) of nicarbazin, HDP and DNC were 100–2 000 µg/plate, 200–2 000 µg/plate and 100–2 000 µg/plate, respectively. It was noted that nicarbazin and DNC formed precipitates at concentrations greater than 500 and 300 µg/plate, respectively. The substances did not produce a mutagenic response in this test.

Ohta et al. (1980) showed that nicarbazin (up to 10 000 µg/plate in 10% DMSO) was mutagenic for *Salmonella typhimurium* strains TA98 and TA1538 with and without metabolic activation by S9 mix from livers of Aroclor 1254-treated male rats. It was not mutagenic for the strains TA100, TA1535 and TA1537 or *Escherichia coli* Wp2 *hcr trp*. In a separate rec-assay for DNA repair, *Bacillus subtilis* H17 (rec+) and M45 (rec-) strains showed no differential zones of inhibition when exposed to nicarbazin indicating a negative result for mutagenicity in this test.

The applicant has provided in the recent submission a third report of Ames tests.[54] Nicarbazin was tested for mutagenic activity in *Salmonella typhimurium*, TA1537, TA98 and TA100 and *Escherichia coli* WP2*uvr*A at concentrations (in DMSO) ranging from 3 to 1 000 µg per plate. Precipitation of the test material was observed at 1 000 µg per plate in both activation conditions, but not at up to 333 µg per plate. Two independent tests were conducted at each concentration in the presence and absence of S9 mix from Aroclor 1254-induced livers of male Fischer rats. Mutagenic activity was confirmed in the frame shift tester strain TA98, in the presence and absence of S9 mix, in both independent tests. These findings confirm the mutagenicity of nicarbazin in the Ames test.

In the recent submission of new data, nicarbazin was assayed[55] for mutagenic potential in a mouse lymphoma assay (L5178Y cell line, clone 3.7.2.C) that was optimised for detecting both point mutations (tk-locus) and chromosomal effects by separately scoring 'small' and 'large' colonies. Four independent experiments were conducted: two in the absence and two in the presence of S9 mix from livers of Aroclor 1254-treated male Fischer rats, exposing the cells for four and 24 hours to concentrations of nicarbazin of up to 100 µg/mL. Duplicate cultures were used at each concentration for each treatment. The results of the experiments showed no evidence of any mutagenicity or clastogenicity of nicarbazin.

To evaluate the *in vivo* genotoxic potential of nicarbazin, a micronucleus test in bone marrow erythrocytes of male and female CD-1 mice was submitted in the early application (EFSA 2003).[56] Nicarbazin did not cause any increase in the number of micronucleated polychromatic erythrocytes in mice given two daily oral doses of 2 000 mg/kg bw. The absorption of nicarbazin was confirmed by analysis showing the presence of DNC in plasma following dosing. Those results indicated an absence of genotoxicity of nicarbazin and DNC in this test.

Another *in vivo* test has been provided in the recent submission.

The genotoxic potential of nicarbazin was tested[57] using the *ex vivo* unscheduled DNA synthesis (UDS) assay in hepatocytes of male Fischer rats treated by the oral route with 1 000 mg/kg bw and 2 000 mg/kg bw. Expression times of 12 –16 hours and 2–4 hours were used. No UDS was observed at either nicarbazin concentration using either expression time. It can be concluded that, in this test, nicarbazin was not genotoxic to the hepatocytes from rats exposed *in vivo*.

[54] Technical dossier/Human safety section/Appendix 1
[55] Technical dossier/Human safety section/Appendix 2
[56] Technical dossier/Human safety section/Appendix 15
[57] Technical dossier/Human safety section/Appendix 3

Since genotoxicity of nicarbazin was absent in mammalian systems, including *in vivo* exposure to somatic cells at two different organ sites, the FEEDAP Panel concludes that nicarbazin does not present a genotoxic risk. As nicarbazin readily metabolises to DNC and HDP it is reasonable to conclude that DNC and HDP are also non-genotoxic.

3.2.2.3. Sub-chronic oral toxicity

The applicant provided on request of EFSA two 90-day studies in rats. One study was conducted with nicarbazin and the other with DNC alone.

The first study[58] was conducted to evaluate the sub-chronic toxicity of nicarbazin (specifications analytically confirmed) and was based on OECD guideline 408. Three treatment groups of 15 male and 15 female Sprague Dawley-derived rats were given intended doses of 200, 600/400, and 1 000/600 mg/kg bw/day in the diet for 13 weeks. One additional group of 15 animals/sex served as the control. Dose levels were maintained by adjusting the concentration weekly based on the mean body weight and food consumption. Due to decreased food consumption and body weights after the first week by nicarbazin administration, the mid and high doses were adjusted to 400 mg/kg/day and 600 mg/kg/day, respectively. The intended dietary concentrations were analytically confirmed. The calculated mean test article consumption over the treatment period was 181, 384 and 599 mg/kg bw/day for the males and 189, 400, and 619 mg/kg bw/day for the females.

One male (1/15) at 1 000/600 mg/kg/day was found dead on day 11. Two males (2/15) at 600/400 mg/kg/day were found dead on day 88 and day 90. Two females (2/15) at 1 000/600 mg/kg/day were both found dead on day 85. Due to the limited number of tissues examined microscopically, the cause of death in those animals could not be determined. A clear nicarbazin-related effect on body weight gain was observed in a dose-dependent manner in all treated groups. This decrease in body weight is assumed to be directly related to the decrease in food consumption. Food consumption was distinctively decreased in each treated group. There were dose dependent decreases in erythrocyte counts, haemoglobin and hematocrit in both sexes and all treated groups. In both sexes and all treated groups there were dose-dependent increases in blood urea nitrogen and plasma creatinine. There also were dose-dependent increases in plasma phosphorus in all treated groups and both sexes. All plasma protein parameters (total protein, albumin and globulin) were generally decreased in a dose-dependent manner in all treated groups. There were increases in plasma triglyceride in both sexes at the mid- and high-dose levels and in plasma cholesterol in both sexes at all three dose levels that generally followed dose dependencies.

In almost all the dose groups an increase of relative organ weight including kidney, spleen, brain, adrenal glands, epididymides, heart, liver, ovaries, pituitary gland, testes, thymus, thyroid/parathyroid glands and uterus with cervix accompanied with macroscopic changes could be observed. Microscopic examinations were limited to kidneys, testes and epididymides. Increased kidney weights correlated microscopically with tubular degeneration/regeneration, chronic inflammation and fibrosis. Crystals were found within renal tubules, and occasionally in the glomeruli, the pelvis and the interstitium.

The testes had minimal to severe degeneration/atrophy of the seminiferous tubules in males at the highest dose. A NOAEL could not be derived (<181 mg/kg bw/day).

In the second study (conducted according OECD guideline 408),[59] DNC was administered by oral gavage (10 mL/kg) once a day for 91 consecutive days to three treatment groups of 15 male and 15 female Sprague Dawley-derived rats at dose levels of 106, 284 and 709 mg/kg bw. The DNC doses are equivalent to 150, 400 and 1000 mg nicarbazin/kg bw/day. One additional group of 15 animals/sex served as the control and received the vehicle only (0.5 % methylcellulose). Additionally, three groups of four animals/sex/group served as toxicokinetic (TK) animals and received DNC at the same dosages

[58] Cross reference/Maxiban® G160 technical dossier/Supplementary information/September 2009/Appendix 1
[59] Cross reference/Maxiban® G160 Technical dossier/Supplementary information/November 2009

Observations for morbidity, mortality, injury and the availability of food and water were conducted twice daily for all animals. Detailed clinical and mass observations were made. Water and food consumption were measured and recorded daily and reported weekly for main study animals. Ophthalmoscopic examinations were conducted pretest and prior to terminal necropsy.

Blood and urine samples for clinical pathology evaluations were collected from designated animals during week 6 and prior to terminal necropsy. Blood samples for determination of the plasma concentrations of the test substance were collected from designated TK animals prior to dosing once per week during the study and at designated time points on day 91. The TK parameters were determined for the test article.

At study termination, necropsy examinations were performed in all relevant organs, organ weights were recorded and tissues were microscopically examined.

All animals survived to their scheduled terminal necropsy on day 92. No adverse DNC-related effects on clinical signs, functional observational battery evaluations, body weight, food consumption, water consumption, ophthalmological examination, clinical pathology, organ weights, macroscopic and microscopy pathology, were observed during the study.

Male and female $AUC_{0-\tau}$ and $C_{max}$ increased in an approximately dose-proportional manner between 106 and 284 mg/kg/day, but there was no increase in either $AUC_{0-\tau}$ or $C_{max}$ between 284 and 709 mg/kg/day on day 91.

Under the conditions of this study, the NOAEL of 709 mg DNC/kg/day is established.

3.2.2.4. Two-year oral toxicity study

Pre-GLP two-year oral studies in rats[60] and dogs[61] with a mixture of DNC and HDP (weight ratio 3:1) have been assessed previously (EFSA, 2003). No major potential concern appeared from the available data, even if both studies showed shortcomings in design and reporting.

The NOAEL of the rat study was judged to be the highest dose administered (300 and 100 mg/kg bw per day of DNC and HDP, respectively).

In the dog study, a statistically significant elevation of serum ALT was observed in the highest-dose group (600 mg DNC and 200 mg HDP/kg bw given six times a week). Based on this finding, the NOAEL of the dog study was 180 mg DNC and 60 mg HDP/kg bw per day, calculated on the basis of administration on six days per week. Calculated on the basis of the total weekly dosage, the NOAELs would be 154 mg DNC and 51 mg HDP/kg bw per day.

3.2.2.5. Reproduction toxicity including developmental toxicity

In its former opinion on Koffogran (EFSA, 2003) the FEEDAP Panel assessed two pre-GLP multigeneration studies in rats, both performed with DNC+HDP at a weight ratio of 3:1.

The first study was judged to be unclear with regard to study design and the relationships of the satellite study to the main study. The second study,[62] presented as a sequence of interim reports, was poorly described and not adequate for current standards, particularly in relation to the apparent selection of each generation of animals without reference to their prior *in utero* exposure. Despite the shortcomings of both studies no major concern appeared at the highest doses tested (300 mg DNC + 100 mg HDP/kg bw/day).

[60] Technical dossier/Human safety section/Appendix 17
[61] Technical dossier/Human safety section/Appendix 18
[62] Technical dossier/ Human safety section/Appendix 19

A developmental study with nicarbazin in rats[63] was already assessed by the FEEDAP Panel in 2003 (EFSA, 2003). In experiment III of this study, a total of 24 pregnant female rats received orally from (day 7–17) 0, 70, 200 and 600 mg nicarbazin/kg bw, respectively. The following abnormalities were observed: decreased ossifications of vertebral bodies of the tail with doses of 200 and 600 mg/kg ($p < 0.01$) and extra lumbar ribs at 200 not at 600 mg/kg ($p < 0.05$). Significantly higher water intake was observed in five out of 21 rats in the mid- and high-dose groups. At the top dose level there was an increased maternal mortality, decreased feed intake and body weight of dams and live fetuses. Despite some deficiencies in the reporting of data, the FEEDAP Panel concludes there are no concerns on developmental toxicity up to 70 mg nicarbazin/kg bw.

A recent developmental toxicity study in rabbits with nicarbazin has been provided.[64] Groups of 24 mated female rabbits were dosed by gavage with 0, 30, 60 or 120 mg nicarbazin/kg bw/day once daily from days 6 to 28 of gestation. The pregnant females were monitored twice daily during the study. Body weight was measured every three days and feed intake recorded daily. All animals were killed at day 29 of gestation and gross necropsy was performed on adult animals. At necropsy, all tissues were examined and special attention was given to the uterus and contents. Number and assessment of corpora lutea, implantations, and fetuses was recorded. Fetuses were inspected by dissection prior to fixation and processing for skeletal examination.

There were no treatment-related effects on clinical observations or behaviour; body weight and feed intake were similar in all groups. There was no indication of any treatment-related effects on the reproductive parameters and fetal toxicity. At 120 mg/kg bw/day, two out of 24 animals had prominent lobulation of the liver; normally, this incidence would be considered too small to be attributed to treatment but in a dose range finding study,[65] two out of six females each at 200 and 400 mg/kg/day showed this finding. Consequently, the effect on the liver observed at 120 mg/kg bw/day should be considered as treatment-related. However, the toxicological relevance remains unclear. The NOAEL for maternal toxicity can be conservatively established as 60 mg nicarbazin/kg bw, whereas the NOAEL for developmental toxicity was 120 mg nicarbazin/kg bw (the highest dose given).

3.2.2.6. Genotoxicity and carcinogenicity of p-Nitroaniline

p-Nitroaniline (PNA) is one of the starting materials in the DNC synthesis and residual amounts remain as impurity in nicarbazin.

The Health Council of The Netherlands (GR, 2008) concluded in its review that PNA is a suspected carcinogen on the basis of the observed increase of haemangiosarcomas in male mice in a study performed in the frame of NTP (1993). Hepatic haemangiosarcomas in males showed a tendency for a dose-related incidence from 0/50 for controls to 1/50 by 3 mg PNA/kg bw to 2/50 by 30 mg PNA and to 4/50 by 100 mg PNA/kg bw (historical control: 2.1 %; range 0–6 %). Female mice did not show noticeable results. Two other carcinogenicity studies showed negative results, but the doses used were considered by the Council either too low (0.25–9 mg/kg bw of rats for two years) or the information on the study was insufficient (2–70 mg/kg bw of mouse for two years). How far the findings on potential carcinogenicity are related to a genotoxic effect remains unclear because of the inconsistent results of the reviewed studies on genotoxicity. *In vitro* bacterial mutagenicity assays showed that PNA caused frame shift mutations in presence and in absence of an exogenous metabolic system. Other mutagenicity assays were negative. In mammalian cells PNA also induced chromosomal aberrations in presence and in absence of a metabolic system; however, negative outcomes were reported as well. In one assay, PNA induced sister chromatid exchanges in the presence of an activation system, but negative or equivocal results were found by other investigators using the same assay. Negative results were reported from *in vivo* studies (sex linked recessive lethal mutation assay using *Drosophila melanogaster*, unscheduled DNA synthesis in liver cells of male rats).

[63] Technical dossier/ Human safety section/Appendix 20
[64] Technical dossier/ Human safety section/Appendix 5
[65] Technical dossier/ Human safety section/Appendix 4

The Health Council of The Netherlands concluded that PNA has been insufficiently investigated and that there is a cause for concern.

Chopade and Matthews (1984) studied the disposition of *p*-[$^{14}$C]-nitroaniline (PNA) in male F-344 rats following oral and intravenous administration. The gastrointestinal absorption of PNA was near complete and was not affected by dose in the range studied (0.276 and 13.8 mg/kg bw orally). PNA was rapidly distributed and showed no marked affinity for any particular tissue. The clearance of [$^{14}$C]-PNA-derived radioactivity from various tissues was rapid, the whole body half-life of PNA was approximately one hour. Within three days clearance of PNA-derived radioactivity from the body was almost complete. [$^{14}$C]-PNA was rapidly cleared by metabolism to nine metabolites which were excreted primarily in the urine and to a lesser extent in faeces. Most (56 %) of the urinary radioactivity was in the form of sulfate conjugates of two metabolites of PNA; the excretion of un-metabolised PNA was minimal (less than 3 %). Biliary excretion of [$^{14}$C]-PNA was significant; however, much of this PNA-derived radioactivity underwent enterohepatic circulation and was subsequently excreted in urine. Those data are considered to indicate that an exposure of the consumer to PNA resulting from the consumption of tissues from chickens for fattening fed Koffogran-supplemented diets (0.006 mg PNA/kg chicken bw, based on the FEEDAP Panel recommendation for maximum 0.1% PNA in nicarbazin) would be negligibly low.

3.2.2.7. Conclusions on toxicological studies

Nicarbazin gave positive results for mutagenicity in two bacterial studies in some *Salmonella* strains. Since genotoxicity of nicarbazin was absent in mammalian systems (micronucleus and unscheduled DNA synthesis and mouse lymphoma assay, including *in vivo* studies of effects in two somatic tissues), the FEEDAP Panel concludes that nicarbazin is not genotoxic.

In a sub-chronic rat study, nicarbazin exerted adverse effects at all the dose levels tested (lowest dose 181 mg/kg bw/day). A NOAEL could not be established. A sub-chronic rat study using DNC alone was performed. No DNC-related effects were observed at the highest dose tested and a NOAEL of 709 mg DNC/kg bw was derived. These apparently conflicting results may be related to the higher systemic exposure of rats to DNC when administered as nicarbazin instead of DNC alone (or as a simple mixture with HDP).

Two-year toxicity studies performed in rat and dog resulted in a lowest NOAEL of 154 mg DNC (+ 51 mg HDP)/kg bw derived from the dog study.

Despite the shortcomings of the rat multigeneration studies no major concern appeared at the highest doses tested (300 mg DNC + 100 mg HDP/kg bw/day).

Despite some deficiencies in the reporting of data, the FEEDAP Panel concludes there are no concerns on developmental toxicity in rats up to 70 mg nicarbazin/kg bw, based on fetotoxic effects at 200 mg/kg bw. The NOAEL for maternal toxicity in the rabbit study can be conservatively established as 60 mg nicarbazin/kg bw, whereas the NOAEL for developmental toxicity was 120 mg nicarbazin/kg bw (the highest dose given).

p-Nitroaniline (PNA), a nicarbazin associated impurity, is a suspected carcinogen. Considering the PNA disposition data in rats and the maximum PNA level in nicarbazin recommended by the FEEDAP Panel, an exposure of the consumer to PNA resulting from the consumption of tissues from chickens for fattening fed diets supplemented with Koffogran at the maximum proposed level would be negligibly low.

### 3.2.3. Consumer safety

#### 3.2.3.1. Proposal for an acceptable daily intake (ADI)

In establishing the ADI for nicarbazin, the JECFA (WHO, 1998) 'noted the absence of certain toxicological studies in support of nicarbazin; however, the other data available provided sufficient information to overcome most of these deficiencies. It was noted that nicarbazin has been used in veterinary medicine in many countries for over 40 years. On the basis of this long history of use and the fact that use is restricted to starter rations in broiler chickens, the Committee considered that an ADI could be supported. The Committee established an ADI of 0-400 µg nicarbazin/kg bw on the basis of the NOEL of 200 mg/kg bw per day in the study of developmental toxicity in rats and using a safety factor of 500, chosen to account for the limitations in the database.'

Limitations leading to the high safety factor of 500 consisted mainly of (i) the presented equivocal results on genotoxicity of nicarbazin (studies on mammalian systems were not available yet) and (ii) the deficiencies in design and reporting of the developmental study with nicarbazin in rats.

The FEEDAP Panel notes that the results of a recent sub-chronic study in rats and of the developmental studies in rats and rabbits, all performed with nicarbazin, indicate a lower NOAEL for nicarbazin than that considered by JECFA. Indeed, no NOAEL could be identified in the sub-chronic rat study.

As the consumer will not be exposed to nicarbazin but to DNC (HDP residues are negligible, the level of consumer exposure is expected to be several orders of magnitude less than the levels showing toxicity in animals), a nicarbazin-based ADI would not reflect consumer safety. Only DNC should be considered for the consumer safety assessment.

The lowest NOAEL derived from the two-year dog toxicity following the administration of DNC (together with HDP in a weight ratio of 3:1) was 154 mg DNC/kg bw/day. The validity of the results of the pre-GLP study is indirectly supported by the results of the recent sub-chronic study with DNC on rats. An uncertainty factor of 200 is applied in deriving an ADI from the NOAEL. The additional factor of two, applied to the conventional safety factor of 100, takes into account the shortcomings of the dog study with regard to design and protocol. This corresponds to an ADI of 770 µg DNC/kg bw (corresponding to 46 mg DNC per 60 kg person).

For the assessment of user safety, an ADI for nicarbazin may be helpful. The lowest NOAEL was 60 mg nicarbazin/kg bw observed in a developmental study with rabbits. Since a NOAEL in a 90-day study in rats with nicarbazin could not be established, only a provisional ADI can be derived from the developmental study. Applying the same uncertainty factor of 200 as above, a provisional ADI of 300 µg nicarbazin/kg bw (18 mg/day for a 60 kg person) is proposed.

#### 3.2.3.2. Consumer exposure and proposal for maximum residue limits (MRLs)

Considering the similarity of the metabolic fate of nicarbazin in chickens and rats, the FEEDAP Panel concludes that the ADI for DNC based on laboratory animals is a suitable basis for MRL calculation.

As the two moieties of nicarbazin behave separately, the whole residues resulting from the metabolic fate of both entities should be in principle considered together to evaluate the exposure of the consumer to the toxicologically relevant metabolites. However, considering that residues resulting from the DNC moiety of nicarbazin are orders of magnitude higher than those arising from HDP, the former were considered only.

Taking a conservative approach, the FEEDAP Panel considers that the total metabolites derived from DNC represent a risk which is at most equal to an equivalent quantity of DNC.

The exposure of the consumer to DNC-related residues has been calculated according to daily consumption values of animal products set in Regulation (EC) No 429/2008, and the calculated DNC

total residues plus 2SD (95% confidence limit) measured in the different tissues and at different withdrawal times. The results are presented in Table 7, with the percentage of the ADI taken up.

**Table 7:** Human consumer daily exposure to whole DNC derived residues from relevant chicken tissues after one and five-day withdrawals and corresponding percentage of the acceptable daily intake (ADI)

| | Liver | Kidney | Muscle | Skin/fat | Sum |
|---|---|---|---|---|---|
| **Total DNC residues** | | | | | |
| **(mg DNC equivalent/kg tissue), average + 2 SD** | | | | | |
| **1-day** | 30.687 | 19.284 | 5.573 | 5.762 | |
| **5-day** | 1.004 | 0.611 | 0.101 | 0.229 | |
| **Exposure (mg DNC/person/day)** | | | | | |
| **1-day** | 3.069 | 0.193 | 1.672 | 0.519 | 5.453 |
| **5-day** | 0.100 | 0.006 | 0.030 | 0.021 | 0.157 |
| **% of the ADI (46 mg/person/day)** | | | | | |
| **1-day** | - | - | - | - | 12 |
| **5-day** | - | - | - | - | 0.3 |

The results indicate that consumer exposure to the whole DNC derived residues present in chicken tissues at one-day withdrawal complies with the ADI.

Considering a one-day withdrawal, the following MRLs are proposed: 15, 6, 4 and 4 mg DNC/kg for the liver, kidney, muscle and skin/fat, respectively. The applied tissue specific ratios marker vs. total residue are derived from the data in Table 5.

**Table 8:** Safety of the proposed MRLs for edible tissues from chickens fed Koffogran

| | Liver | Kidney | Muscle | Skin/fat | Sum |
|---|---|---|---|---|---|
| **Proposed MRLs (mg/kg tissue)** | 15 | 6 | 4 | 4 | |
| **Ratio marker vs total residue** | 0.4 | 0.3 | 0.2 | 0.4 | |
| **Consumption (kg/day)** | 0.1 | 0.01 | 0.3 | 0.09 | 0.5 |
| **DITR[a] (mg/day)** | 3.75 | 0.20 | 6.00 | 0.90 | 10.85 |
| **Consumption (% ADI)** | | | | | 24 |

[a] Daily intake of total residues

The consumer exposure resulting from the consumption of tissues containing the corresponding MRLs complies with the ADI.

3.2.3.3. Withdrawal period

The FEEDAP Panel considers a withdrawal period of one day as adequate.

### 3.3. Safety for the user

With regard to the risk assessment, the FEEDAP Panel concluded in 2003 (EFSA, 2003) that:

> 'Nicarbazin shows no skin irritation potential, no skin sensitisation potential and only transient conjunctiva irritation. It is likely to be poorly absorbed through the skin, due to its poor solubility in organic solvents. Because of the effects during short term exposure in rats and the chronic exposure of workers adverse effects on the respiratory system of workers can not be excluded.'

Concerning particle size and dusting potential, new data has been provided.

### 3.3.1. Dusting potential of Koffogran

A Stauber-Heubach test was performed with three batches of Koffogran. Dustiness was in 100 L air: 7.9; 3.3 and 3.1 mg/100 g, corresponding to 0.079, 0.033 and 0.031 g Koffogran/m³.[66]

The particle size distribution in nicarbazin (six batches) and Koffogran (six batches) was determined by Laser diffraction.[67] The results are given in Table 9. About 50 % of the nicarbazin particles are of respirable size (≤ 8.3–12.5 µm), the different pattern of Koffogran reflects the effects of granulation and the larger particles of the diluents (wheat middlings). All samples contain particulate matter with particle sizes < 10 µm but limit values concerning alveolar dust (100 % < 7.1 µm, 50 % < 5.0 µm), which are assumed to be health hazardous,[68] are not obtained.

**Table 9:** Particle size (µm) of nicarbazin and Koffogran

| Nicarbazin | | | | Koffogran | | | |
|---|---|---|---|---|---|---|---|
| Batch [a] | 10 percentile | 50 percentile | 90 percentile | Batch [a] | 10 percentile | 50 percentile | 90 percentile |
| 031184 | 1.28 | 9.47 | 31.15 | 5_01_37 | 19.39 | 278.6 | 477.6 |
| 031185 | 1.75 | 12.51 | 68.81 | 5_01_38 | 20.91 | 284.7 | 484.6 |
| 031186 | 1.35 | 11.14 | 47.45 | 5_01_39 | 24.92 | 301.4 | 510.2 |
| 0301869 | 3.3 | 8.3 | 18.3 | | | | |
| 0307880 | 3.1 | 8.3 | 20.0 | | | | |
| 0306851 | 3.1 | 8.9 | 20.3 | | | | |

[a] Batches nicarbazin 031184, 031185, and 031186 and Koffogran 5_01_37, 5_01_38, and 5_01_39 from recent production, nicarbazin batches 0301869, 0307880 and 0306851 from years 1999, 2000, and 2001

Nicarbazin in dust was not analysed. For further calculations it is assumed that the nicarbazin content in dust is equal to that in Koffogran.

### 3.3.2. Worker/user inhalatory exposure estimate

There are different operations in a premixture factory during which the worker could be exposed to dust from Koffogran:

- taking Koffogran from its bag for weighing in the dispensary,
- emptying bags of previously weighed material in the hopper or mixers,
- packing the final premixture.

The factors to be considered in a worst case scenario as well as the estimation of that scenario are listed in Appendix A-1.

The nicarbazin uptake of persons handling Koffogran in a premixture factory via the respiratory route during an eight-hour working day is estimated to 250 µg. However, this total inhalable amount would not quantitatively reach the lower respiratory tract (trachea, bronchi, bronchioles, alveoli). Particles in the nasopharynx, trachea, bronchi and broncheoles may adhere to the mucus and be absorbed only at a very low level. It can be assumed that the mucus is expectorated, swallowed and absorbed in the gastrointestinal tract in a similar way to dietary exposure, with involvement of the gut flora and first-pass metabolism by the liver.

The Koffogran consists to about 10 % of particles which are assumed to reach the absorptive surface of the alveoli (≤ 20 µm, DIN EN 481). But these particles show a different alveolar availability (see Appendix A-2, DIN EN 481). The amount of nicarbazin in fine dust particles is calculated on the basis of the table reported in Appendix A-2 and the laser diffraction analysis[69] of sample 5_01_37.

[66] Technical dossier/Quality section/Appendix 25-1
[67] Supplementary information. December 2009
[68] TA Luft 2002 in: Feldhaus/Hansel Bundesimmissionsschutzgesetz, 15. Auflage 2002, C.F. Müller Verlag, Heidelberg
[69] Supplementary information. December 2009

Because laser diffraction data on particle size were only provided for Koffogran and not for the dust of Koffogran, it is assumed that dust would not contain particles >100 µm. The sum of particles up to 100µm in Koffogran was 25.5 %; the data for the fraction with alveolar availability from Koffogran were therefore multiplied with 100/25.5 to simulate Koffogran dust.

Whereas the corrected sum of particles ≤16 µm in sample 5_01_37 is 26.5 %, its amount reaching the alveoli is only 7.3 %. Consequently, only 18 µg nicarbazin (0.3 µg/kg bw) would reach the alveoli during an eight-hour working day (six minutes direct exposure).

Data for inhalatory toxicity of nicarbazin in laboratory animals are not available; the provisional ADI for nicarbazin proposed by the FEEDAP Panel (300 µg/kg bw) is 1 000 times higher than the worst case inhalatory exposure of users.

The applicant provided information that in two production factories no adverse reactions of workers to nicarbazin have been observed at least during the last ten years.[70]

### 3.3.3. Conclusions on user safety

In view of this assessment and the extensive experience of handling Koffogran/nicarbazin in the workplace and the lack of evidence of any adverse consequence in users, the FEEDAP Panel concludes that no special safety measures are required for users above those normally employed for handling dust-generating products.

## 3.4. Safety for the environment

The active ingredient is not a physiological/natural substance of established safety for the environment. The additive is not intended for companion animals. Consequently, the Phase I assessment has to be continued to determine the predicted environmental concentration (PEC).

In Phase I and II a total residues approach will initially be taken to estimate a worst case $PEC_{initial}$. It will be assumed that nicarbazin is excreted 100 % as parent compound. Since nicarbazin is a complex of the components DNC and HDP, the environmental risk assessment will be based on both of these compounds separately at the recorded maximum dose in a ratio 70:30 w/w. Distribution to other compartments is also based on the properties of both components as long as no data on relevant metabolites are submitted.

### 3.4.1. Exposure assessment

#### 3.4.1.1. Fate and behaviour

**Fate in manure**

No data on the fate of nicarbazin in manure of the target animals have been submitted.

**Fate in soil**

*Adsorption*

The adsorption/desorption behaviour of [$^{14}$C]-DNC was investigated in three soil types: sandy loam, clay loam and silty clay loam.[71] The pH of the soils ranged from 6.1 to 7.5,and the soil organic carbon content ranged from 1.3 % to 3.1 %. As adsorption was rapid and complete the procedures outlined in the OECD Guideline 106 could not be followed completely. An important deviation is that the test was performed with two instead of five concentrations, which hampers the interpretation of the test results and prevents obtaining accurate $K_d/K_{oc}$. At the lowest concentration (0.02 µg/L) the $K_{oc}$ values

[70] Supplemetary information. May 2009
[71] Technical dossier/Environment section/Appendix 1

were much lower, ranging from 16137–21962, than at the highest concentration tested (0.15 µg/L), ranging from 62591–123923. As the highest test concentration was close to the water solubility, the high $K_d/K_{oc}$ is considered to be an over prediction of the adsorption to soil. Therefore, the lowest values are considered to be more reliable. As only three soils are tested the lowest $K_{oc}$ value is used for the risk assessment.

The adsorption/desorption behaviour of [$^{14}$C]-HDP was investigated in three soil types: sandy loam, clay loam and silty clay loam.[72] The pH of the soils ranged from 6.1 to 7.5 and the soil organic carbon content ranged from 1.3% to 3.1 %. As no adsorption equilibrium could be established, the procedures outlined in the OECD Guideline 106 could not be followed completely. Another important deviation is that the test was performed with two instead of five concentrations. Both test concentrations did however resulted in comparable the $K_{oc}$ values ranging from 33–114. As only three soils are tested the lowest $K_{oc}$ value is used for the risk assessment.

*Degradation*

The aerobic degradation of DNC in accordance with OECD 307 was evaluated in sandy loam, sandy clay loam and silt loam soils[73] using [$^{14}$C]-DNC and included the estimation of the fate and behaviour in soil. Evolved [$^{14}$C]-$O_2$ was low throughout the study period, for all soils, accounting for 1 to 2 % of applied radioactivity after 120 days. Chromatographic analyses indicated that DNC was the only significant component present in all soil types. At 64 and 120 days, up to four minor components ($\leq$ 3 % each) were detected but not identified. The dissipation was mainly attributed to the formation of bound residues accounting for 27 % in the sandy loam. In the other soil types the non-extractable residues were not determined. The $DT_{50}$, based on first order kinetics, was 239, 193 and 257 days in sandy loam, sandy clay loam and silt loam soil types, respectively.

The aerobic degradation of [$^{14}$C]-HDP was studied as described above for DNC, using the same soil types.[74] Formation of [$^{14}$C]-$O_2$ was relatively high throughout the study, accounting for 22 to 31 % of applied radioactivity after 120 days. HDP was the only significant component present in all samples from all soil types; no metabolites representing more than 10 % of the total radioactivity applied were found. Dissipation was strongly attributed to the fast formation of non-extractable residues as demonstrated in sandy loam soil (not determined in the other soil types). Based on linear regression and first order kinetics, the $DT_{50}$ was calculated as 6, 7 and 3 days, the $DT_{90}$ as 20, 23 and 11 days, in sandy loam, sandy clay loam and silt loam, respectively.

**Fate in water**

No data have been submitted on (photo-) biodegradation of DNC and HDP in water.

3.4.1.2. Predicted environmental concentrations (PECs)

The methods to estimate PEC in manure, soil, groundwater and surface water are described in the technical guidance for assessing the safety of feed additives for the environment (EFSA, 2008). The calculated values are shown in Table 10.

[72] Technical dossier/Environment section/Appendix 2
[73] Technical Dossier/Environment section/Appendix 3
[74] Technical dossier/Environment section/Appendix 4

**Table 10:** Initial predicted environmental concentrations (PECs) of DNC, HDP in soil (µg/kg), groundwater and surface water (µg/L)

| | Arable land | |
|---|---|---|
| | **DNC[a]** | **HDP** |
| Soil | 690 | 189 |
| Ground water | 2.42 | 270 |
| Surface water | 0.81 | 90 |

(a) The $DT_{50}$ of DNC is > 60 days, therefore a PEC plateau is calculated to take the potential of accumulation in soil into account

All Phase I PEC trigger values for soil and groundwater are exceeded. Therefore, a Phase II assessment is considered necessary.

### 3.4.2. Effect assessment

3.4.2.1. Toxicity to soil organisms

*Effects on soil micro-organisms*

The effects of DNC and HDP on soil nitrification were determined in two separate tests using sandy loam soil following the OECD guidelines 216.[75,76] No effects above the 25 % trigger selected for those studies were observed for concentrations ≤ 8.0 mg DNC and 3.5 mg HDP/kg soil.

*Effects on plants*

The toxicity of HDP[77] on the following terrestrial plants has been tested in loamy sand soils: Oats (*Avena sativa*), lettuce (*Lactuca sativa*), turnip (*Brassica rapa*), perennial ryegrass (*Lolium perenne*), mung bean (*Phaseolus aureus*) and radish (*R. sativus*). The OECD guideline 208 was not completely followed as instead of using a concentration range, the effects were measured at one, five and ten times the estimated PEC of 0.35 mg/kg. No effect of HDP on the emergence and growth of lettuce, oats, ryegrass and turnip seedlings. HDP did have a phytotoxic effect on the emergence of both radish and mung bean, resulting in $LC_{50}$ values of 2.8 and 2.9 mg/kg, respectively. The mean radish shoot fresh weight was higher in the x10 rate group compared with the controls, although this was not confirmed with the dry weight analysis. For mung bean the total dry weight at the 3.5 mg/kg (x10) was significantly lower than the controls. However, fewer plants emerged in the 3.5 mg/kg group than in the controls. The NOEC for both plants was 1.75 mg/kg.

The toxicity of DNC[78] to terrestrial plants has been tested using the same species and test design as used for HDP (see above). The effects were measured at one, five and ten times the estimated PEC of 0.8 mg/kg. No effects were observed on emergence of any of the crop species tested. DNC did not show any effect on the growth of lettuce, mung bean, radish, ryegrass and turnip seedlings. The mean fresh weight of oats decreased slightly but not significantly as the concentration of DNC increased. The NOEC was 8 mg/ kg.

*Effect on earthworms*

The effect of DNC[79] and HDP[80] on *E. foetida* was tested in two separate studies conducted in an artificial soil (70 % sand, 20 % clay and 10 % organic matter) under laboratory conditions, using six

[75] Technical dossier/Environment section/Appendix 6
[76] Technical dossier/Environment section/Appendix 7
[77] Technical dossier/Environment section/Appendix 9
[78] Technical dossier/Environment section/Appendix 8
[79] Technical dossier/Environment section/Appendix 12
[80] Cross reference/Maxiban® G160 technical dossier/Section III/Appendix 55

test concentrations of 0, 95, 171, 309, 556 and 1 000 mg/kg soil (each treatment in four fold), for an exposure period of 14 days at 21–22 °C, according OECD guideline 207. No mortality occurred at any test concentration. The $LC_{50}$ for both DNC and HDP is > 1000 mg/kg soil.

3.4.2.2. Toxicity to aquatic organisms

The acute toxicity of nicarbazin to *D. magna* and rainbow trout (*O. mykiss)* was determined following the US EPA and ASTM guidelines, which are similar to the OECD guideline 202.[81] Toxicity was performed as a limit test at a concentration of 100 mg nicarbazin/L. Because DNC is considered by the applicant insoluble in water, only HDP was measured. The measured concentration in the *D. magna* study was 24.2 mg HDP/L, in the trout study 26.7 mg HDP/L, both concentrations remaining stable up to the end of the test. No acute toxicity was observed in *D. magna*, indicating that the $LC_{50}$ for HDP is > 24.2 mg/L. No acute toxicity in trout was observed indicating that the $LC_{50}$ for HDP is > 26.7 mg/L.

As DNC was not analysed in either of the studies, it is unclear to which DNC concentration the animals were exposed.

In a more recent study, the toxicity of DNC to *D. magna*,[82] rainbow trout (*O. mykiss)*[83] and Blue Gill *(Lepomis macrochirus*)[84] were determined following OECD guidelines 202 and 203. All test conditions were within the acceptable limits. In those studies the exposure concentrations were also measured. The toxicity to *D. magna* was tested in a dose range, whereas the toxicity to the two fish species was tested in a limit test at a nominal concentration of 100 µg/L, resulting in a measured concentration around the reported maximum water solubility (i.e. 69 and 72 µg/L, respectively). The 48-h $EC_{50}$ for *D. magna* and the 96-h $LC_{50}$ for *O. mykiss* and *L. macrochirus* could not be determined because $E(L)C_{50}$ was not reached up to the maximum water solubility.

In an older study, not reported in detail, both DNC and HDP were tested for acute toxicity to the algae *Chlorella pyrenoidosa, D. magna* and the fishes *Poecilia reticulata* (guppy) and *O. mykiss* (rainbow trout).[85] The study has already been assessed by the FEEDAP Panel in its former opinion on Koffogran (EFSA, 2003). Because of the deficiencies in analysing essential parameters and in reporting data, the FEEDAP Panel does not consider this study valid, as it did not in 2003.

The applicant was informed that a full assessment of the algae study[86] would not be possible because information on the chemical analysis and detailed experimental results are lacking. The applicant informed EFSA that he was unable to obtain more details about this study. The applicant submitted instead for consideration that there should be no reason for doubts about the reliability of the study performed by a well-reputed research institute. The FEEDAP Panel could not follow those arguments and reiterates its position that in the absence of raw data, the toxicity of DNC and HDP to algae cannot be evaluated.

No data on the toxicity of DNC and HDP to sediment dwelling organisms has been submitted.

3.4.2.3. Conclusion

*PNEC for the terrestrial compartment*

The most sensitive endpoint observed for HDP is seed emergence with a NOEC of 1.75 mg/kg. This results in a PNEC of 0.18 mg/kg using an assessment factor of 10. For DNC the lowest endpoint is a NOEC of 8 mg/kg for plant and micro-organism, resulting in a PNEC of 0.8 mg/kg using an assessment factor of 10.

[81] Technical dossier/Environment section/Appendix 10
[82] Cross reference/Maxiban® G160 technical dossier/Supplemetary information/July 2009/ERA attachments/Annex 7
[83] Cross reference/Maxiban® G160 technical dossier/Supplemetary information/July 2009/ ERA attachments/Annex 8
[84] Cross reference/Maxiban® G160 technical dossier/Supplemetary information/July 2009/ ERA attachments/Annex 9
[85] Cross reference/Maxiban® G160 technical dossier/Section III/Appendix 69
[86] Cross reference/Maxiban® G160 technical dossier//Section III/Appendix 69

*PNEC for the aquatic compartment*

Although the toxicity for *D. magna* and fish has sufficiently been investigated, for both DNC and HDP, the toxicity data for algae could not be evaluated. Consequently, a PNEC for the aquatic environment for both DNC and HDP cannot be determined.

3.4.2.4. Bioaccumulation

No experimentally determined bioconcentration factors (BCF) for earthworm and fish have been submitted. Since the log $K_{ow}$ DNC is 3.6, the compound has a potential for bioaccumulation.

### 3.4.3. Risk characterisation

3.4.3.1. Risk for soil

Based on the assumption that 100 % of the highest use dose is excreted as parent compound, the PEC/PNEC for DNC is < 1 (Table 11). The PEC/PNEC for HDP is slightly above 1. However, based on the excretion data provided, showing that approximately 65 % of the dose is excreted as parent compound and no metabolites are > 10 %, the PEC can be refined resulting in a PEC/PNEC < 1.

It should be noted that the PNEC for DNC is based on an unbounded NOEC. Furthermore, in a plant study with nicarbazin no effects were observed up to 1 000 mg/kg (EFSA, 2003). An unacceptable risk as a result of a combined effect of DNC and HDP is therefore not expected.

**Table 11:** The PEC/PNEC comparison based on 100 % of the proposed recommended dose and refined based on metabolism data

| | Compound | |
|---|---|---|
| | **DNC** | **HDP** |
| $PEC_{soil,\ initial}$ (mg/kg) | 0.69 | 0.19 |
| $PEC_{soil,\ refined}$ (mg/kg) | - | 0,12 |
| PNEC (mg/kg) | 0.8 | 0.18 |
| $PEC/PNEC_{initial}$ | 0.9 | 1.1 |
| $PEC/PNEC_{refined}$ | - | 0.7 |

3.4.3.2. Risk for surface water

Since the toxicity data on algae submitted for DNC and HDP could not be evaluated, the risk for the aquatic environment cannot fully be assessed.

It should be noted that for crustaceans and fish no acute toxicity was observed up to water solubility. An absence of short-term toxicity does, however, not necessarily mean that a substance has no long-term toxicity. As a general rule used for industrial chemicals and biocides, long-term toxicity tests are required for substances with log $K_{ow}$ > 3 (or BCF > 100) and a PEC > 1/100th of the water solubility. In the case of HDP these criteria are clearly not met. The initial PEC for DNC (log $K_{ow}$ > 3) is close to 1/100th of the water solubility. Using the FOCUS models, only one scenario (R3) gives a comparable initial concentration, whereas the time weighted average of this scenario and the initial PEC of other scenario are lower than that. In addition, QSAR calculations for amides and substituted urea, using EcoSAR v.1.0, show that the chronic toxicity value of DNC is ≥ 10 than the initial PEC. The FEEDAP Panel therefore assumes there is a sufficient margin of safety and does not consider chronic toxicity studies for crustaceans and fish necessary. For the same reason it is not expected that the initial PEC for sediment based on equilibrium partitioning will cause an unacceptable risk for sediment dwelling organisms. No further tests are considered necessary.

3.4.3.3. Risk for groundwater

Based on the screening model described in the technical guidance for assessing the safety of feed additives for the environment (EFSA, 2008), the PEC ground water for DNC and HDP is > 0.1 μg/L. However, taking the reported degradation rates in soil into account and using the FOCUS PEARL model, the calculated concentrations did not exceed the groundwater trigger value of 0.1 μg/L.

3.4.3.4. Risk for secondary poisoning

DNC has a log $K_{ow}$ of 3.6 and therefore has a potential for bioaccumulation. As no Bioconcentration Factor (BCF) values for earthworms and fish were provided, these were estimated using the QSARs described in the REACH guidance for existing chemicals, resulting in values of 48.6 and 229, respectively. The calculated $PEC_{oral}$ for fish eating bird/mammal and worm eating birds/mammals based on the initial concentration in surface water and soil, assuming that 50 % of the diet is taken from exposed soil/water, are presented in Table 12.

The lowest NOEL for DNC in birds/mammals is 154 mg/kg feed. Using an Assessment Factor of 30, in accordance with the REACH guidance for existing chemicals, the $PNEC_{oral}$ is 5 mg/kg feed. When compared to the calculated $PEC_{oral}$, both PEC/PNEC ratios are < 1 (see Table 12). The FEEDAP Panel therefore concludes that there are no safety concerns with regard to secondary poisoning.

**Table 12:** Risk assessment for secondary poisoning for DNC based on 100 % of the proposed recommended dose

| | $PEC_{oral, sw}$ (mg/kg) | $PEC_{oral,soil}$ (mg/kg) | $PNEC_{oral}$ (mg/kg) | $PEC/PNEC_{sw}$ | $PEC/PNEC_{soil}$ |
|---|---|---|---|---|---|
| **DNC** | 0.093 | 0.15 | 5 | 0.019 | 0.03 |

### 3.4.3. Conclusion

Considering the condition of use of Koffogran and based on the data provided, a safety concern for the soil compartment, groundwater or by secondary poisoning could not be identified. In the absence of raw toxicity data for algae the risk for surface water can, however, not be assessed.

## 4. Efficacy

Nicarbazin causes the destruction of second generation schizonts suggesting a coccidiocidal mode of action. However, there were also findings indicating a coccidiostatic action or both (Chapman, 1993).

No recent dose titration studies under experimental conditions are required for the re-evaluation of a coccidiostat. The applicant consequently did not submit new data for this purpose. To assess the efficacy of a coccidiostat that has already been in use for several years, three recent floor pen studies and three recent field trials are considered necessary, conducted under current use conditions (and using recently isolated *Eimeria* strains in floor pen studies).

### 4.1. Efficacy in chickens for fattening

#### 4.1.1. Dose titration and dose confirmation

The FEEDAP Panel summarised dose-titration and confirmation studies in its previous opinion on Koffogran (EFSA, 2003):

> ˙Four battery trials (1955/56) showed in controlled conditions using nicarbazin in a dose range from 50 to 1000 mg/kg feed that the optimum dose regarding efficacy against *E. necatrix*, *E. acervulina* and *E. tenella* and body weight (bw) gain was 125 mg nicarbazin/kg complete feed.

Dose confirmation studies were performed in 1959, 1970 and 1992. The efficacy of 125 mg nicarbazin/kg feed was confirmed including other species like *E. mivati*, *E. hagani* and *E. praecox*.'

### 4.1.2. Battery cage trials

A series of 11 recent battery trials conducted between 2006 and 2008 was provided.[87] In each trial, nicarbazin (125 mg/kg feed, confirmed by analyses ± 2 0 %) was compared to a polyether coccidiostat (60 mg/kg, confirmed by analyses) and to an uninfected untreated control group (UU) as well as an infected untreated control group (IU). In each trial a total of 224 male Ross chickens were randomised to four groups (seven birds/cage, eight replicates/group). The chickens were treated from 12 days of age and infected at 14 days of age with *E. tenella* (3 trials), *E. acervulina* (3 trials), *E. maxima* (3 trials), *E.brunetti* (1 trial) and *E. necatrix* (1 trial), which were field isolated in different countries: France, UK, Germany and the Netherlands.

The parameters measured were: coccidiosis induced mortality, body weight, weight gain, feed intake and feed to gain ratio from day 12 to 21, intestinal lesions were scored on day 21 and oocyst excretion was determined from day 19 to 22. Details on the respective inoculum doses as well as the results are given in Appendix B.

The overall results for the 11 trials are summarised in Table 13. No mortality occurred in trials 8, 9 and 11. When not considering those three trials, the mortality decreased from 12.7 % in the IU group to 7.1 % in the nicarbazin-fed group. In trial 1, 5 and 6 the mortality in the nicarbazin group ranged from 10.7 to 23.2 %. However, no coccidial lesions were observed in those chickens; it can therefore be assumed that the high mortality rate was not related to coccidiosis. If those three trials were excluded from the mean values presented in Table 13, the mortality rates would be 0, 14.6, 0.4 and 10.0 % for the groups UU, IU, nicarbazin and the polyether coccidiostat, respectively.

The lesion scores were significantly reduced by nicarbazin in all trials. In all trials, except trial 2, nicarbazin significantly reduced oocyst excretion while the polyether coccidiostat did not.

In all trials nicarbazin significantly improved body weight gain and feed to gain ratio (except trial 2) compared to the IU group and similar to the polyether coccidiostat. However, nicarbazin (and the polyether coccidiostat) could not fully compensate for the depressive effect of coccidiosis on body weight gain and feed to gain ratio.

**Table 13:** Summary on the mortality, lesion score, oocyst excretion, body weight gain and feed to gain ratio of the 11 battery trials performed with chickens for fattening infected with *Eimeria* field isolates[a]

| Group | Mortality (%) [b] | Lesion score | Daily oocyst excretion per bird (x $10^3$) | Body weight gain (g) | Feed to gain ratio (g/g) |
|---|---|---|---|---|---|
| **Un-infected Untreated (UU)** | 1.6 | 0 | 0 | 563 | 1.40 |
| **Infected Untreated (IU)** | 12.7 | 3.3 | 92,522 | 374 | 1.84 |
| **Nicarbazin (125 mg /kg)** | 7.1 | 0.7 | 15,596 | 471 | 1.57 |
| **Polyether (60 mg/kg)** | 8.7 | 2.8 | 84,950 | 466 | 1.58 |

[a] Values for mortality, body weight gain and feed to gain ratio were obtained during days 12 to 21 of the experimental period, lesion score was measured on day 21, and daily oocyst excretion was determined over the period 19 to 22.
[b] Values do not consider trials 8, 9 and 11, trials in which mortality did not occur.

### 4.1.3. Floor pen trials

Three floor pen trials are considered in this evaluation. The first study (Fitz-Coy and Edgar, 1992) was already considered in the former evaluation (EFSA, 2003). The second and third are recent floor pen trials conducted in France in 2007/2008.

[87] Technical dossier/Efficacy section/Appendices 1-11

The first study (Fitz-Coy and Edgar, 1992) was conducted to compare the efficacy of 125 mg nicarbazin/kg complete feed (analyses of the dose proposed not provided in the publication) to an un-infected untreated control and an infected untreated control group in controlling a virulent challenge infection of *E. mitis* in chickens for fattening. The trial was performed with 21-day-old chickens which were randomised to five groups (six birds/cage, five replicates/group). Infection took place at day 21 by crop incubation (B4 and C2 strains, challenge dose, approximate number of oocysts/bird: *E. acervulina*: 51 000; *E. maxima*: 9 300; *E. tenella*: 19 300; *E. mitis*: 8 260; *E. praecox*: 1 520). Body weight was recorded on day 2, 0 and 7 post-inoculation and feed to gain ratio was calculated between day 0 and 7 post-inoculation.

No mortalities were recorded during the trial. Treatment of *E. mitis* strain B4-infected chickens with 125 mg nicarbazin/kg feed resulted in a growth that was comparable to uninfected untreated controls, but in chickens infected with strain C2 a significant growth reduction was observed. Feed conversion was significantly higher (Table 14).

**Table 14:** Effect of nicarbazin on body weight gain and feed to gain ratio of infected chickens for fattening from day 0 to day 7 post inoculation

| ***E. mitis* strain** | **Treatment** | **Body weight gain (g, %)[1]** | **Feed conversion (g/g)** |
|---|---|---|---|
| **None** | None | 306, 100[a] | 2.06[a] |
| **B4** | None | 214, 70[c] | 2.35[b] |
| **B4** | Nicarbazin | 284, 93[a] | 2.33[b] |
| **C2** | None | 184, 60[d] | 2.41[b] |
| **C2** | Nicarbazin | 265, 87[b] | 2.46[b] |

[1] body weight gain expressed in total grams and percentage considering the result of the uninfected untreated group as the 100 %

[a,b,c,d]: Means within columns with different superscripts are significantly different ($P < 0.05$)

The two recent studies[88,89] were conducted as a randomised complete block design. In each trial, the efficacy of 125 mg nicarbazin/kg feed (confirmed by analyses) was compared to an infected untreated control (IU) and a group of chickens that received a polyether coccidiostat (intended dose 60 mg/kg; analytical values 47 to 48 mg/kg). In each trial, a total of 960 male chickens for fattening were randomised to the three groups (40 birds/cage, eight replicates/group). The duration of the treatment period was 30 and 31 days for trial 1 and 2, respectively, followed by four days of withdrawal. Infection was performed on day 16/17 (trial 1/2) by oral administration (via feed) of infectious material (220 000 sporulated *E. acervulina*, 40 000 *E. maxima*, 220 000 *E. tenella* oocysts).

Mortality, body weight and feed intake were monitored and feed to gain ratio calculated. On days 22/23 and 30/31 in trial 1 and 2, respectively, intestinal lesions were scored and faecal samples were obtained for the determination of oocysts (expressed as nb/g faeces).

The results of both trials are summarised in Table 15. No significant differences were observed on the mortality but it was numerically lower in the treated groups (nicarbazin and polyether) compared to the IU birds. Body weight was higher in the nicarbazin group compared to the IU group and the polyether-fed group.

The lesion scores were the lowest in the nicarbazin group on day 22/23 and on day 30/31. The lesion scores in duodenum, jejunum and caeca on day 22/23 and in jejunum and caeca on day 30/31 were significantly lower in the nicarbazin group compared to control groups (IU, polyether).

The nicarbazin treatment decreased significantly the oocyst excretion compared to the IU in trial 1 and compared to the IU and the polyether-fed group in trial 2. On day 30/31, *E. maxima* oocyst excretion

[88] Technical dossier/ Efficacy section/ Appendix 12
[89] Technical dossier/ Efficacy section/ Appendix 13

was significantly reduced in the nicarbazin group compared to the IU group and the polyether-fed group.

**Table 15:** Effect of nicarbazin on the mortality, lesion score, oocyst excretion, body weight gain and feed to gain ratio in infected chickens for fattening[1]

| Trial | Group | Mortality (%) | Lesion score[2] | | | Oocyst excretion (nb/g of feces) | Final Body weight (g) | Feed conversion ratio (g/g) |
|---|---|---|---|---|---|---|---|---|
| | | | (Du) | (JI) | (Ca) | | | |
| | **IU** | 19.9 | $3.3^{b}$ | $2.5^{b}$ | $3.1^{b}$ | $1{,}504{,}750^{a}$ | $1656^{c}$ | 1.527 |
| **1** | **Nicarbazin** | 1.1 | $1.6^{a}$ | $1.3^{a}$ | $2.2^{a}$ | $550{,}500^{b}$ | $1937^{a}$ | 1.503 |
| | **Polyether** | 13.4 | $3.2^{b}$ | $2.3^{b}$ | $3.5^{b}$ | $774{,}250^{ab}$ | $1712^{b}$ | 1.526 |
| | **IU** | 19.4 | $3.5^{b}$ | $2.9^{b}$ | $3.6^{b}$ | $2{,}345{,}250^{a}$ | $1767^{c}$ | 1.554 |
| **2** | **Nicarbazin** | 1.1 | $1.9^{a}$ | $0.8^{a}$ | $1.8^{a}$ | $459{,}750^{b}$ | $2078^{a}$ | 1.530 |
| | **Polyether** | 12.9 | $3.4^{b}$ | $3.0^{b}$ | $3.8^{b}$ | $2{,}026{,}750^{a}$ | $1843^{b}$ | 1.541 |

[1] Mortality results during days 16/17 to 22/23 (trial 1/2, respectively), lesion score measured on day 22/23, total oocyst excretion was determined on day 22/23.
[2] Du, duodenum; JI, jejunum and ileum; Ca, caecum.
[a,b,c] Means within columns with different superscripts are significantly different.

### 4.1.4. Field trials with shuttle program

A total of four field trials were conducted in 2008, three in France and one in the UK. In each of these comparisons 125 mg nicarbazin/kg feed was fed during the initial phase of a shuttle program (until 28 days of age) and then followed by an ionophore anticoccidial. The intended dosage of coccidiostats in each trial was analytically determined; dosage was confirmed in most cases except some values of the fourth trial. The endpoints (body weight, lesion score and oocyst excretion) were measured at different time points, mainly on a reduced number during the trial and rough body weight by the end of the trial. Building averages and applying statistical analysis is therefore not possible. The intended coccidiostats concentrations in each trial were analytically determined. They could be confirmed in most cases except some values of the fourth trial.

The first trial[90] compared a shuttle program of nicarbazin (125 mg/kg) followed by a polyether coccidiostat (70 mg/kg) with a similar program involving a combination (47.2 mg of the same polyether and 47.2 mg nicarbazin/kg) followed by the same polyether (70 mg/kg) as a control. About 30 600 male and female mixed chickens for fattening were raised in two similar houses (one per treatment). Nicarbazin and the combination, first phase, were fed until day 28 and the polyether, second phase, until day 33. Females were slaughtered on day 37 and males on day 49. Lesion scores (20 birds per treatment) and litter oocysts counts from each program were collected on day 23 and day 30. Microscopic oocysts evaluation of intestines was carried out on day 30. Total broiler body weight was recorded at slaughter. Mean body weight at slaughter was not different between the two groups (nicarbazin 1.58 kg for the females and 2.94 kg for the males; control 1.66 kg and 3.01 kg, respectively). Feed to gain ratio (1.9 for both groups) and mortality (3.43% dead and culled for nicarbazin, 2.88 % for the control) also showed very small differences.

The second trial[91] compared a shuttle program of nicarbazin (125 mg/kg) fed for 28 days followed by a polyether coccidiostat (25 mg/kg) for nine days with another polyether coccidiostat (60 mg/kg) fed for 28 days as control. About 25 300 chickens for fattening of both sexes were raised in four sections (two replicates per treatment). After cessation of the coccidiostatic treatments birds were fed an untreated control diet until slaughter (day 49). On days 21 and 28, lesion scores were examined and litter oocyst counts determined (ten chicks per replicate). On day 35, 100 birds per pen (200 birds/treatment) were weighed. Total chicken body mass was recorded at slaughter (by difference of truck weights). Final body weight was 1.78 kg for the nicarbazin group and 1.84 for the control, feed

[90] Technical dossier. Efficacy section. Appendix 14
[91] Technical dossier. Efficacy section. Appendix 15

to gain ratio was 2.1 for both groups. Mortality in the nicarbazin group was 3.7 and the control 4.5. Oocysts excretion was reduced in nicarbazin compared to control, however this was not reflected in the lesion score.

In the third trial,[92] the same experimental design was applied as in the second trial to a total of 24 240 chickens for fattening with the exception that birds were slaughtered on day 42. Lesion score and oocyst excretion were recorded on day 21 and 25. On days 9, 21, 28 and 35, the body weight of 100 birds per pen (200 birds/treatment) was measured. Total chicken body mass was recorded at slaughter (by difference of truck weights). Final body weight was 2.11 kg for the nicarbazin group and 2.14 for the control, and mortality in the nicarbazin group was 4.7 and in the control group 5.9. Oocysts excretion was reduced in nicarbazin compared to control, however this was not reflected in the lesion score.

The fourth trial[93] compared nicarbazin (125 mg/kg) fed for the first 28 days with a combination (50 mg of a polyether and 50 mg nicarbazin/kg) fed for the first 23 days. In both cases the treatment was followed by another polyether coccidiostat (100 mg/kg) until day 32. A withdrawal feed was offered until slaughter (average age at slaughter: about 40 days for female chicks and for 'as hatched' chicks, 44 days for males chicks). About 165 000 chickens for fattening of both sexes were raised in six broiler houses (three houses per treatment). Ten chicks per house were scored for the intestinal lesion on day 21 and oocyst counts were determined in litter on day 22 and 29. On days 7, 14, 21, 28, 35 and 42, 150 chickens per house (300 chickens/treatment) were weighed. Total chicken body mass was recorded at slaughter (by difference of truck weights). The differences among the houses of one treatment group appeared greater than that between the treatments groups concerning body weight (average 2.16 kg), feed to gain ratio (average 1.9), mortality (range 1.4–6.8 % in the six houses) and oocyst excretion. Intestinal lesions were not detected.

### 4.1.5. Resistance of *Eimeria* to nicarbazin

Although the development of resistance of *Eimeria* to coccidiostats is a common problem in poultry, induction of resistance to nicarbazin under laboratory conditions was difficult and mostly not successful. *Eimeria* showing cross resistance to several coccidiostats were still sensitive to nicarbazin (Chapman, 1993).

### 4.1.6. Product quality

Data on the influence of nicarbazin on product quality were not provided. However, no adverse effects on product quality were reported or became known during the long period of nicarbazin use.

### 4.1.7. Conclusion on efficacy

The principal effect of nicarbazin in preventing coccidiosis in chickens for fattening from several *Eimeria* strains has been demonstrated over a long period of years (1955-1992); 125 mg/kg feed was established as optimum dose. A series of 11 battery trials recently performed in different countries with different *Eimeria* field strains confirm this conclusion.

In three floor pen trials, nicarbazin at a dose of 125 mg/kg feed could be shown to be effective in controlling coccidiosis by improving weight gain, reducing mortality, lesion score and oocyst excretion after artificial infection with three different *Eimeria* strains.

Four field trials with a total of 245 000 birds under a shuttle program with nicarbazin treatment (125 mg/kg feed) for the first four weeks support the conclusion that nicarbazin is as effective as the other coccidiostats used for comparison. The inherent variation of field studies data does not allow a more detailed comparison.

[92] Technical dossier. Efficacy section. Appendix 16
[93] Technical dossier. Efficacy section. Appendix 17

The FEEDAP Panel considers nicarbazin at the maximum dose proposed (125 mg/kg complete feed) to be effective in controlling coccidiosis in chickens for fattening.

## 5. Post-market monitoring

No specific risks associated with the use of Koffogran could be identified. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[94] and Good Manufacturing Practice.

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

Nicarbazin at the maximum use level (125 mg/kg feed) is considered safe for chickens for fattening (margin of safety about 1.5) under normal climate conditions. Nicarbazin is considered to be safe as far as the microbiological risk is concerned.

Nicarbazin, when ingested, is rapidly split in its two components HDP and DNC which behave independently. DNC from nicarbazin is considerably more available for the animal than DNC given alone or administered simultaneously with HDP in similar proportions as in nicarbazin.

DNC appears as the marker residue. DNC residues decline rapidly from tissues following nicarbazin withdrawal. Liver is the target tissue. HDP-related residues are much lower than those derived from DNC.

Since genotoxicity of nicarbazin was absent in mammalian systems (micronucleus and unscheduled DNA synthesis and mouse lymphoma assay, including *in vivo* studies of effects in two somatic tissues), the FEEDAP Panel concludes that nicarbazin is not genotoxic.

No NOAEL for nicarbazin in a recent sub-chronic rat study could be derived since nicarbazin exerted adverse effects at all the dose levels tested (lowest dose 181 mg/kg bw/day). However, a NOAEL of 709 mg/kg bw/day for DNC could be derived from a recent sub-chronic rat study using DNC alone. These apparently conflicting results may be related to the higher systemic exposure of rats to DNC when administered as nicarbazin instead of DNC alone.

154 mg DNC (+ 51 mg HDP)/kg bw/day from a two-year dog study is considered as the lowest NOAEL.

Despite the shortcomings of the rat multigeneration reproduction studies, no major concern appeared at the highest doses tested (300 mg DNC + 100 mg HDP/kg bw/day). In spite of some deficiencies in the reporting of data, the FEEDAP Panel concludes that there are no concerns on developmental toxicity in rats up to 70 mg nicarbazin/kg bw/day, based on fetotoxic effects at 200 mg/kg bw/day. The NOAEL for maternal toxicity in the rabbit study can be conservatively established as 60 mg nicarbazin/kg bw, whereas the NOAEL for developmental toxicity was 120 mg nicarbazin/kg bw/day (the highest dose given).

p-Nitroaniline (PNA), a nicarbazin associated impurity, is a suspected carcinogen. Considering the PNA disposition data in rats and the maximum PNA level in nicarbazin recommended by the FEEDAP Panel, an exposure of the consumer to PNA resulting from the consumption of tissues from chickens for fattening fed diets supplemented with Koffogran at the maximum proposed level would be negligibly low.

As the consumer is only exposed to DNC, an ADI of 0.77 mg/kg bw for DNC is set, derived from the NOAEL of the two-year dog study (154 mg DNC/kg bw/day), applying a safety factor of 200.

[94] OJ L 35, 8.2.2005, p.1

MRLs for DNC in liver (15 mg/kg), kidney (6 mg/kg), muscle (4 mg/kg) and skin/fat (4 mg/kg) are proposed. A worst case calculation by dietary exposure following the application of those MRLs shows that only 24 % of the ADI is met. A one-day withdrawal period is considered as adequate.

Nicarbazin shows no potential for skin irritation or sensitisation, it is slightly irritating to eyes (transient effects). Inhalatory exposure to nicarbazin from handling Koffogran is considered negligibly small.

The FEEDAP Panel does not expect that the use of nicarbazin at the recommended dose will pose a foreseeable risk for the soil compartment, groundwater or secondary poisoning. In the absence of raw toxicity data for algae, however, the risk for surface water cannot be assessed.

The FEEDAP Panel considers nicarbazin at the maximum dose proposed (125 mg/kg complete feed) to be effective in controlling coccidiosis in chickens for fattening.

### RECOMMENDATIONS

p-Nitroaniline in nicarbazin should be minimised to the lowest concentration possible. The FEEDAP Panel recommends reducing the maximum content in nicarbazin from 0.5% to 0.1%.

Field monitoring of *Eimeria spp*. resistance to nicarbazin shall be undertaken, preferably during the latter part of the period of authorisation.

As the consumer is not exposed to nicarbazin (only to DNC), the maximum levels in animal tissues and products resulting from the unavoidable carry over of cross contamination of non-target feeds, set by Regulation (EC) No 124/2009 for nicarbazin, should be reconsidered.

## DOCUMENTATION PROVIDED TO EFSA

1. Response files for the dossier Koffogran. December 2008. Submitted by Member State Rapporteur (Belgium).
2. Cross-reference letter (90-day study in rat with nicarbazin) from Eli Lilly and Company Limited (Elanco Animal Health). March 2009.
3. Supplementary information on manufacturing process of nicarbazin. May 2009. Submitted by Member State Rapporteur (Belgium).
4. Supplementary information on worker safety reports. May 2009. Submitted by Member State Rapporteur (Belgium).
5. Cross-reference letter (safety and technical data) from Eli Lilly and Company Limited (Elanco Animal Health). June 2009.
6. Access letter for DNC aquatic toxicity studies and water solubility data from Innolytics, LLC. August 2009.
7. Phibro Animal Health. Supplementary information. December 2009.
8. Dutch opinion on the October 2008 response file for the dossier Koffogran. March 2009. Medicines Evaluation Board Veterinary Medicinal Products Unit. The Netherlands.

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## APPENDICES

## APPENDIX A-1

- A factory with a large throughput can prepare 40 premixture batches per day (8 hours per shift)
- The maximum time for weighing/emptying is 20 seconds
- In the same factory about 20 % of premixtures[95] may contain Koffogran
- All dust comes from Maxiban
- Total air volume available for inspiration is saturated with Koffogran dust
- The maximum dust concentration is 0.08 $g/m^3$
- The nicarbazin concentration in dust is 25 % (as in Koffogran)
- The total breathed air per worker is 10 $m^3$ per 8 hours = 1.25 $m^3$ per hour
- The use of personal protection equipment (coverall, goggles, gloves and mask of the type P2, that reduces the inhalation exposure to 10 %)

The scenario:

| | |
|---|---|
| Batches with potential exposure | 40 (total) x 0.2 (percentage of Koffogran containing premixtures) = 8 batches |
| Time of exposure | 8 (batches) x 20 sec = 160 seconds ~ 3 minutes, for safety reasons 6 minutes of contact with Koffogran will be considered |
| Inhaled air during exposure | 1.25 $m^3$ per hour x 0.1 hours (6 minutes) = 0.125 $m^3$ |
| Nicarbazin in air | 0.08 $g/m^3$ (Koffogran dust) x 25 (% nicarbazin in dust) = 0.02 $g/m^3$ |
| Nicarbazin in inhaled air | 0.02 $g/m^3$ x 0.125 $m^3$ = 0.0025 g (2.5 mg) |
| Reduction by filter mask | 2.5 mg x 0.1 (reduction to 10 %) = 0.25 mg |

[95] In 2008, the total feed production volume in the EU is estimated to be 150,570,000 t, 32.5 % of the total is poultry feed. From the data of the member states it can be extracted that not more than 50 % would be broiler feed, the animal category, for which Maxiban is applied for authorisation. 16.25 % is rounded to 20 %.

## APPENDIX A-2

Numerical values of the separation curve following DIN EN 481 to compile the aerosol fraction relevant for occupational health, related to the whole air transported Aerosol.

| Aerodynamic Diameter (mm) | Breathable fraction (%) | Thoracic fraction (%) | Alveolar fraction (%) |
|---|---|---|---|
| 0 | 100.0 | 100,0 | 100,0 |
| 1 | 97.1 | 97.1 | 97.1 |
| 2 | 94.3 | 94.3 | 91.4 |
| 3 | 91.7 | 91.7 | 73.9 |
| 4 | 89.3 | 89.0 | 50.0 |
| 5 | 87.0 | 85.4 | 30.0 |
| 6 | 84.9 | 80.5 | 16.8 |
| 7 | 82.9 | 74.2 | 9.0 |
| 8 | 80.9 | 66.6 | 4.8 |
| 9 | 79.1 | 58.3 | 2.5 |
| 10 | 77.4 | 50.0 | 1.3 |
| 11 | 75.8 | 42.1 | 0.7 |
| 12 | 74.3 | 34.9 | 0.4 |
| 13 | 72.9 | 28.6 | 0.2 |
| 14 | 71.6 | 23.2 | 0.2 |
| 15 | 70.3 | 18.7 | 0.1 |
| 16 | 69.1 | 15.0 | 0 |
| 18 | 67.0 | 9.5 | |
| 20 | 65.1 | 5.9 | |
| 25 | 61.2 | 1.8 | |
| 30 | 58.3 | 0.6 | |
| 35 | 56.1 | 0.2 | |
| 40 | 54.5 | 0.1 | |
| 50 | 52.5 | 0 | |
| 60 | 51.4 | | |
| 80 | 50.4 | | |
| 100 | 50.1 | | |

1. Inhalable fraction: the separation curve corresponds to the average probability of inhalation

2. Thoracic fraction: the separation curve corresponds to the average probability for particles entering the tracheo-bronchial-tree and the alveolar area

3. Alveolar (respirable) fraction: That fraction is part of a thoracic fraction. The separation curve corresponds to the average probability for particles entering the alveolar area

4. Extrathoracic fraction: This fraction results from the difference between the inhalable fraction and the thoracic fraction

5. Trachebronchial fraction: This fraction results from the difference between the thoracic fraction and the aveolar fraction. The separation curve is not numerically defined

## APPENDIX B

### The 11 battery trials with field Eimeria isolates
### Experimental design, Mortality, Lesion Score, Oocyst Excretion, Body Weight Gain and Feed to Gain Ratio of the Chickens for fattening

| Trial No.<br>Eimeria sp.<br>Oocyst-dosage<br>Country | Group | Mortality, %<br>D12 to D21 | Lesion score<br>D21 | Daily oocyst excretion per bird x $10^3$ | Body weight gain, g<br>D12 to D21 | g feed/ g gain<br>D12 to D21 |
|---|---|---|---|---|---|---|
| **1** | NU | 3.6 | 0 c | 0 | 624 a | 1.36 a |
| *E.tenella* | IU | 19.6 | 3.8 a | 52,562 | 443 c | 1.71 c |
| 25,000 | Nicarbazin | 10.7 | 0.8 b | 23 | 528 b | 1.56 b |
| France | Polyether | 17.9 | 3.7 a | 41,119 | 538 b | 1.59 b |
| **4** | NU | 0 b | 0 d | 0 | 628 a | 1.35 a |
| *E.tenella* | IU | 25.0 a | 3.9 a | 88,160 | 460 d | 1.70 c |
| 25,000 | Nicarbazin | 1.8 b | 1.1 c | 8 | 536 c | 1.49 b |
| UK | Polyether | 10.7 a | 3.2 b | 83,720 | 569 b | 1.46 b |
| **7** | NU | 0 b | 0 c | 0 | 590 a | 1.44 a |
| *E.tenella* | IU | 23.2 a | 3.3 a | 57,061 | 415 c | 1.89 b |
| 25,000 | Nicarbazin | 0 b | 0.5 b | 0 | 483 b | 1.60 a |
| Germany | Polyether | 32.1 a | 3.3 a | 53,920 | 478 b | 1.63 a |
| **2** | NU | 0 | 0 a | 0 | 627 a | 1.32 b |
| *E.acervulina* | IU | 7.1 | 3.4 d | 154,126 | 479 c | 1.52 a |
| 250,000 | Nicarbazin | 0 | 1.4 b | 158,960 | 527 b | 1.58 a |
| France | Polyether | 5.4 | 3.0 c | 164,630 | 524 b | 1.45 ab |
| **5** | NU | 3.6 b | 0 b | 0 | 556 a | 1.40 c |
| *E.acervulina* | IU | 1.8 b | 3.4 a | 369,466 | 380 d | 1.81 a |
| 250,000 | Nicarbazin | 23.2 a | 0.1 b | 2,741 | 461 c | 1.57 b |
| UK | Polyether | 0 b | 3.3 a | 236,872 | 488 b | 1.45 c |
| **8** | NU | 0 | 0 b | 0 | 549 a | 1.51 d |
| *E.acervulina* | IU | 0 | 3.2 a | 233,652 | 391 d | 1.93 a |
| 250,000 | Nicarbazin | 0 | 0 b | 9,276 | 477 b | 1.66 c |
| Germany | Polyether | 0 | 3.3 a | 251,661 | 451 c | 1.74 b |
| **3** | NU | 0 | 0 c | 0 | 601 a | 1.36 d |
| *E.maxima* | IU | 1.8 | 3.3 a | 18,559 | 333 d | 2.02 a |
| 50,000 | Nicarbazin | 0 | 1.6 b | 156 | 502 b | 1.51 c |
| France | Polyether | 1.8 | 3.4 a | 22,825 | 418 c | 1.75 b |
| **6** | NU | 5.4 b | 0 c | 0 | 447 a | 1.44 c |
| *E.maxima* | IU | 7.1 b | 2.3 a | 8,305 | 300 d | 1.91 a |
| 50,000 | Nicarbazin | 21.4 a | 0.02 c | 7 | 360 c | 1.63 b |
| UK | Polyether | 1.8 b | 1.6 b | 9,156 | 392 b | 1.58 b |
| **9** | NU | 0 | 0 d | 0 | 469 a | 1.46 c |
| *E.maxima* | IU | 0 | 3.3 a | 11,947 | 340 c | 1.80 a |
| 50,000 | Nicarbazin | 0 | 1.4 c | 389 | 381 b | 1.66 b |
| The Netherlands | Polyether | 0 | 2.7 b | 44,546 | 347 c | 1.74 ab |
| **10** | NU | 0 | 0 b | 0 b | 554 a | 1.35 a |
| *E.necatrix* | IU | 16.0 | 3.41 a | 449 a | 336 c | 1.73 c |
| 50,000 | Nicarbazin | 0 | 0.13 b | 0 b | 476 b | 1.47 b |
| France | Polyether | 0 | 0.20 b | 636 a | 547 a | 1.34 a |
| **11** | NU | 0 | 0 d | 0 | 547 a | 1.36 a |
| *E.brunetti* | IU | 0 | 3.0 a | 23,450 | 242 d | 2.23 d |
| 80,000 | Nicarbazin | 0 | 0.87 c | 359 | 453 b | 1.52 b |
| France | Polyether | 0 | 2.56 b | 25,369 | 377 c | 1.69 c |