Azadirachtin
Evaluation of Classification and Labelling Proposal with regard to Developmental Toxicity

Date: 17.11.2014

Report Number: 234379-A2-050601-01

Author:

Prof. Dr. Wolfgang Pfau

GAB Consulting GmbH
Hinter den Höfen 24
21769 Lamstedt
Germany

Phone: +49 4773 8889 141
Fax: +49 4773 8889 20
Email: wolfgang.pfau@gabconsulting.de

AUTHENTICATION

Report No.:	234379-A2-050601-01
Report Title:	Azadirachtin: Evaluation of Classification and Labelling Proposal with regard to Skin Sensitisation

I, the undersigned, hereby declare that the present report has been prepared by GAB Consulting GmbH, Hinter den Höfen 24, 21769 Lamstedt, Germany.

Signature

Date

17 November 2014

Prof. Dr. Wolfgang Pfau
certified Toxicologist (DGPT, EUROTOX)

Table of Contents

1 Executive Summary 4
2 Introduction 4
3 CLH-Proposal - Reproduction Toxicity 5
4 Available Data 5
4.1 Developmental Toxicity Study NeemAzal 5
4.2 Ventricular Septal Defects 8
4.2.1 Spontaneous Incidences /Historical Control Data 8
4.2.2 Chemically Induced VSD 9
4.3 Developmental Toxicity study in the Rat with Fortune Aza 9
4.4 Other Data 10
4.4.1 Human Evidence 10
4.4.2 Reproduction Toxicity Studies 10
4.4.3 Developmental Toxicity Studies 11
5 Opinion of PRAPeR Meeting 13
6 EFSA Conclusion on Pesticide Peer review 14
7 Conclusion 14
8 References 14
8.1 Proprietary studies 14
8.2 Published data 15

1 Executive Summary

Azadirachtin is a refined medium polarity extract from the kernels of the Neem tree. It is approved in the EU as active substance for plant protection products.

During the plant protection approval process opinions were divided concerning the developmental toxicity of the active substance. In fact a majority of experts agreed not to propose any classification based mainly on the low incidences observed in the developmental toxicity study in rats.

RMS Germany prepared a CLH-Dossier to define an appropriate harmonized classification for Azadirachtin and, regarding developmental toxicity, proposed classification in Category 2 (H361d).

With regard to developmental toxicity, the attention is focused on a teratogenicity study in the rat, especially on visceral malformations, namely the incidences of small and severe ventricular septal defect (VSD). Because incidences of VSD were very close to historical control data for the laboratory and well within the published historical control data of MARTA (1996), it is appropriate to re-investigate this issue taking into account related relevant experimental data in order to assess these findings in a weight-of-evidence approach. The aim is to define a hazard classification that is both protective of human health but avoids an over-classification.

Low incidences of malformations were noted in a rat teratogenicity toxicity study with one case of ventricular septal defect in the mid-dose treatment group (0.66%, within the historical control range of the laboratory). In the high dose group, two pups were affected with VSD (1.33%, within the published historical control range for this rat strain). Given the low incidence level of this rather common observation in rats, it is appropriate to consider further data that are available: Treatment caused maternal toxicity in the dams, at both the mid-dose and high-dose group, noted as reduced body weight gain. Although this effect was only transient, it occurred around days 6 to 8 of pregnancy, which has been identified as critical time in the development of the foetal heart. It is, therefore, reasonable to assume that the observed low incidence of VSD is a high dose effect secondary to maternal toxicity rather than a direct effect of Azadirachtin.

The low incidences of visceral malformations in one developmental toxicity study were very close to the laboratory historical control incidence level and within the published historical control data. It is appropriate to consider a weight of evidence approach taking into account the likely influence of maternal toxicity and the lack of similar observations in numerous other experimental studies on reproductive toxicity. It is concluded that a classification of Azadirachtin for reproductive toxicity is not warranted supported by the following experimental findings:

- no indication of adverse developmental effects was noted in a 2-generation study,
- no adverse effects were reported in another teratogenicity study in the rat with Azadirachtin extract,
- no effects on the developing heart were reported in a teratogenicity study in rabbits,
- no adverse effects were reported in supplemental studies including a segment I reproductive toxicity study, a segment II study and two 2-generation studies,
- no adverse effects in pups were reported in a published 2-generation study which included detailed investigations on the developmental toxicity of Azadirachtin extract
- no increased incidence of visceral malformations were reported in two published developmental toxicity studies with Azadirachtin extract

. Based on the experimental evidence it is appropriate to not classify Azadirachtin with regard to reproductive toxicity.

Therefore, the classification and labelling according to Regulation (EC) No 1272/2008 with regard to developmental toxicity should be that Azadirachtin has not to be classified.

2 Introduction

Azadirachtin is a refined medium polarity extract from the kernels of the Neem tree. Azadirachtin was included into Annex I of Directive 91/414/EEC by Commission Directive 2011/44/EU (13. April 2011) for use as insecticidal pesticide in the EU. Following entry into force of Regulation (EC) No 1107/2009, Azadirachtin is now included in the Annex to Commission Implementing Regulation (EU) No 540/2011[1].

[1] (OJ L 153/77, 01 January 2009 and OJL 153/18, 01 June 2004, p. 1-186).

Azadirachtin is one of very few insecticides permitted in organic farming[2]. It has been used safely since 20 years as an active ingredient in several plant protection products (e.g. NeemAzal®-T/S).

3 CLH-Proposal - Reproduction Toxicity

In the CLH report (Proposal for Harmonised Classification and Labelling) the RMS Germany concluded regarding classification and labelling that

- Regarding effects on fertility, the data are considered conclusive but not sufficient to trigger classification for such effects.
- Regarding developmental toxicity, classification in Category 3 (R63, DSD criteria) and Category 2 (H361d, CLP criteria) is considered appropriate.
- No data are available to judge whether there are specific effects on or via lactation (R64, H362).

In contradiction to the classification in Category 2 (H361d, CLP criteria) the RMS noted in the CLH report:(4.11.5) *"Considering the findings seen in the developmental toxicity study in rats performed with NeemAzal (interventricular septal defects, malrotated heart, supernumerary ribs), the effects were seen at or around doses, where maternal toxicity could be observed. Additionally, the incidences in the rat study were increased only slightly and the possibility of non-specific causes such as general toxicity could not be excluded"*.

4 Available Data

4.1 Developmental Toxicity Study NeemAzal

The discussed effects were noted in the following developmental toxicity study:

Report: Myers, D. P., Dawe, I. S. (1997a)
NeemAzal technical – A Study of the Developmental Toxicity in Rats (Gavage administration)
Huntingdon Life Sciences Ltd., Huntingdon, England
unpublished report No. EIP 2/952493; TOX9700514

4.1.1 Executive Study Summary

In a GLP-conform study according to OECD TG 414 mated Charles River CD® female rats, assigned to one control and three treatment groups of 25 animals each, were used to determine the teratogenic potential of Neem extract. Dosage levels of 50, 225 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19 of gestation at a volume of 10 mL/kg in 1 % methylcellulose in this study. Observations on mortality, clinical signs of toxicity and body weights were recorded. On gestation day 20, all females were sacrificed, the number and location of viable and nonviable foetuses, early and late resorptions and *corpora lutea* were recorded. Uterus weights were determined. Gross lesions were recorded. Half of the foetuses from each dam were examined for skeletal deformations and the remaining were sectioned for identifying visceral anomalies.

Based on the initially reduced bodyweight and food consumption in the high and mid-dose groups the no observable adverse effect level (NOAEL) was 50 mg NeemAzal /kg bw/d for maternal effects corresponding to 18.3 mg Azadirachtin A/kg bw/d.

One litter was affected by the mottled foetus syndrome and one by the squat foetus syndrome; both were discounted from further evaluation as these are considered spontaneous.

No effects on foetal number were observed.

A statistically not significant increase in the number of foetuses with supernumerary ribs was noted at the highest dose level but was not statistically significant. There were observations of visceral anomalies such as small ventricular defects.

[2] Commission Regulation (EC) No 889/2008 of 5 September 2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control

Incidences of malformations affecting the heart of the foetuses were within or just outside the historical control data. Therefore, we conclude that there was no clear indication of an adverse developmental effect. The NOAEL was 1000 mg/kg bw/d.

Conclusions (Cited from the CLH report, 9.10.2 Developmental toxicity)

"Based on the initial reduced bodyweight gain, food consumption and the increased water consumption in high dose animals, the no observable adverse effect level was 225 mg/kg bw/day for maternal effects. The post dose salivation observed for dams at 225 and 1000 mg/kg bw/day is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance. Increased incidences of malformations were noted in the foetuses of the high and mid dose treatment groups affecting the heart (ventricular septal defect, malrotation of heart) and an increased incidence of supernumerary ribs occurred in the high dose group. Even though maternal toxicity was not observed in this study, liver toxicity in dams can be expected, which had a LOAEL of 123 mg NeemAzal/kg bw/d (1600 ppm) in the 90-d study in rats (NOAEL: 32 mg/kg bw/d (400 ppm)). Additionally, incidences were increased only slightly."

4.1.2 Maternal Toxicity

Undisputedly, maternal toxicity was observed at the highest dose level of 1000 mg/kg bw/d (Table 1).

In the mid dose group (225 mg/kg bw/d) a significantly reduced feed intake (days 6-7) and a reduced body weight gain (days 6-8; 8.5 g vs 10.5 g (-19%), p=0.18) was observed when all pregnant dams are considered. This reduction in body weight gain by 19% is only a transient and not-statistically significant sign of maternal toxicity. However, it is just below the value of 20% decreased body weight gain mentioned in a recent review on the effects of maternal toxicity in developmental toxicity studies (Beyer et al, 2011). Thus, in conclusion, maternal toxicity has to be considered at mid dose level.

Table 1: Maternal body weight changes and feed intake

	Dose Level			
NeemAzal technical [mg/kg bw/d]	**0**	**50**	**225**	**1000**
Azadirachtin A [mg/kg bw/d]	**0**	**18.3**	**82.4**	**366**
Number of animals§	23	23	23	23
Body weight gain				
Weight gain Day 6 – Day 8 [g/rat]	10.4	10.5	8.5#	6.1**
Weight gain Day 8 – Day 20 [g/rat]	133.1	143.8	138.7	143.0
Feed intake				
Number of animals§	25	25	25	25
Feed intake Days 6 – 7 [g/rat/d]	26	26	24**	23**
Feed intake Days 8 – 9 [g/rat/d]	28	29	28	27
Feed intake Days 10 – 11 [g/rat/d]	30	31	29	28*
Feed intake Days 12 – 13 [g/rat/d]	30	29	29	27

#p = 0.18
**p < 0.01, * p < 0.05
§ excluding non-pregnant animals for body weight (two per dose group)

Interventricular septal defect (VSD) is a relatively common anomaly and malformation in laboratory animals and is noted also in untreated control groups (see below Point 4.3). It has been reported to may have a genetic basis as evidenced by the observation that the rate of VSD is high in inbred rat strains (Solomon, 1997). Also chemical inducers have been identified such as the anticonvulsant trimethadione or phenobarbital. These have been reported to induce rates of up to 93% incidence of VSD and are most effective in the induction of these defects when administered around gestation day 8 (Fleeman 2004, Purssell 2012). Thus, the observed transient toxicity in the mid dose group (manifest as reduced feed intake and reduced body weight gain) was during that very period of the foetal development that is important for the organogenesis also of the heart.

4.1.2.1 Toxicity of Azadirachtin in the Rat upon Repeated Oral Administration

Table 2: Lowest observed adverse effect levels in repeated dose studies on oral toxicity in the rat for determination of maternal toxicity (as summarised in the DAR)

Study type	Test item	LOAEL Azadirachtin (a.i.) (mg/kg bw/d)	LOAEL Azadirachtin A (mg/kg bw/d)	Report
28-day toxicity	NeemAzal	301*	83.1	Waterson, (1997a)
28-day toxicity	Fortune Aza	400	40.8	Waterson, Dawe, (1997a)
90-day toxicity	NeemAzal	123	33.9	Waterson, (1997b)
90-day toxicity	Fortune Aza	137	14.0	Waterson, Dawe (1997b)
90-day toxicity	ATI-720	178	13.8	Johnson, (1994)
Prelim teratogenicity	Fortune Aza	>1000	100	Waterson (1997d)
Teratogenicity	Fortune Aza	>1000	100	Waterson (1997c)
Prelim teratogenicity	NeemAzal	300	108	Myers, Dawe (1997b)
Teratogenicity	NeemAzal	1000	360	Myers, Dawe (1997a)#

*lowest dose level tested, no NOAEL in this study
study discussed here

Comparing the lowest adverse effect levels observed in rats upon repeated oral administration (Table 2) the range is between 123 and >1000 mg a.i./kg bw/d for Azadirachtin (corresponding to 33.9-360 mg Azadirachtin A). The values of 1000 mg a.i./kg bw/d were observed in teratogenicity studies of dose range finders with no thorough examination such as blood or organ analyses.

The lowest limit values (LOAEL 123 – 178 mg a.i./kg bw/d) were recorded for administration with feed whereas bolus administration by gavage, as in the teratogenicity study, is known to generally lead to more pronounced effects at lower levels.

In the subchronic repeat dose toxicity studies (90-day-toxicity) performed with the technical products, signs of toxicity were seen. Thus mentioned in the DAR. The NOAELs of the different extracts in the subchronic studies were 32 and 35 mg a.i./kg bw/d. Based on these, an overall NOAEL of 32 mg a.i. /kg bw/d (corresponding to 32 mg NeemAzal technical) was defined in the plant protection approval process. This was the base for the limit values AOEL = ADI = 0.1 mg a.i./kg bw/d including a safety factor of 300.

Thus, the lowest LOAEL observed was 123 mg a.i./kg bw/d. This value corresponds to about half of the mid dose level of 225 mg a.i./kg bw/d in the teratogenicity study with NeemAzal, which we consider to be the LOAEL for maternal toxicity.

Based on these considerations it appears reasonable to consider the mid-dose level of 225 mg a.i./kg bw/d as LOAEL for maternal toxicity in the Myers & Dawe (1997a) study.

4.1.3 Malformations - Low Number of Incidences

An important point for considerations on developmental toxicity is the number of malformations in dosage groups, compared to control data. For a proper judgement, malformations, which are considered as spontaneous (such as squat or mottled foetus syndrome) should be excluded from examination.

After exclusion of mottled foetus syndrome and squat foetus syndrome, only two malformations remain at the mid dose level and four malformations at the high dose level affecting one or three litters, respectively.

Upon close re-examination it was only one foetus of 306 affected with malformations associated with the heart (one foetus in litter 63 was diagnosed with both, VSD and malrotated heart) in the mid dose group (Table 3).

Three foetuses of 308 in the high dose group were affected with malformations associated with the heart: one foetus (litter 88) was diagnosed with VSD, atrial septal defect and malrotated heart. One foetus (litter 80) was diagnosed with duplicated inferior vena cava and one foetus (litter 84) was diagnosed with duplicated inferior vena cava and VSD (Table 3).

Other visceral findings occurred only once in the mid-dose or high dose group, and in most cases in combination with VSD, and are considered incidental observations. The observed increase of the number of foetuses showing

supernumerary ribs was not statistically significant. This is in line with the statement of the study director on both the mid (225 mg a.i./kg bw) and high (1000 mg a.i./kg bw) dosage group: "*Neither the type, or incidence of skeletal anomalies, or the incidence of foetuses with sternebrae indicated any obvious adverse effects of treatment*."

Table 3: Foetal (litter) incidences of visceral malformations

Observation	Dose level (mg/kg bw/d)			
	0	**50**	**225**	**1000**
Number of foetus (litters) examined:	305 (23)	323 (23)	306 (23)	308 (23)
Interventricular septal defect + malrotated heart	0 (0)	0 (0)	1 (1)	0 (0)
Interventricular septal defect + duplicated inferior vena cava	0 (0)	0 (0)	0 (0)	1 (1)
Interventricular septal defect + malrotated heart + atrial septal defect with narrow pulmonary vein	0 (0)	0 (0)	0 (0)	1 (1)
Duplicated inferior vena cava	0 (0)	0 (0)	0 (0)	1 (1)

4.2 Ventricular Septal Defects

Ventricular septal defects are rather commonly observed defects in the rat foetus (Solomon, 1997). Generally, these are categorised as "small" VSD which are considered to be a variation, a delay in cardiac development; these close postnatally without any adverse effect. The more severe VSD, which are considered as malformations are less common and would result in mortality (Fleeman, 2004).

4.2.1 Spontaneous Incidences /Historical Control Data

Both, small and severe VSD, occur spontaneously with apparently a genetic causal component as judged by the observation that in certain inbred strains of rats the incidence may be as high as 25% in untreated control animals in the Wistar Kyoto rat (Solomon, 1997; Slama, 2002).

For the Sprague-Dawley rat (Crl:CD®BR) the historical control data at Huntingdon Life Sciences Ltd, the laboratory conducting the Myers & Dawe (1997a) study, were provided with the study report for 11 recent developmental studies. This overview covered only the 7-month period preceding Myers & Dawe (1997a) study-report, the developmental toxicity studies conducted from July 1994 until February 1995. Concerning severe VSD resulting in malformation, a maximum incidence of one affected pup was noted per study (0.63%; 144 -176 pups examined per study) in the untreated control animals. Accordingly, only one litter was affected at maximum, corresponding to 4.2% of litters affected. (Table 4)

As pointed out in Table 4 incidences of VSD were 1/152 = 0.66% in the mid-dose group and 2/151 = 1.33% in the high dose group. This was within (or only one affected pup above) the incidence of 1/158 = 0.63% from historical control data for the laboratory. These control data cover eleven studies conducted between July 1994 and February 1995 at Huntingdon Life Sciences Ltd., UK. The incidences in both groups (225 and 1000 mg a.i./kg bw) were within the historical control data (1.34%) published for Crl:CD®(SD)BR rats observed in a high number of laboratories (MARTA, 1996; Solomon, 1997).

Considering small VSD (which is judged as anomaly) the incidence levels were at one, three and two affected pups for the mid dose and high dose, respectively. This was within the range of the historical laboratory incidence and was well within the historical control range reported in MARTA (1996).

Table 4 Ventricular septal defects (VSD) incidences and historical control maximum data

		Fetal data				Litter data	
		VSD malformation		VSD small anomaly		VSD mal-formation	VSD small anomaly
		No/no examined per study	%	No/no examined per study	%	%	%
Myers & Dawe, 1997a	Control	0/152	0	0/152	0	0	0
	50 mg/kg bw/d	0/159	0	1/159	0	0	0
	225 mg/kg bw/d	1/152	0.66	3/152	1.97	4.3(1/23)	13.0 (3/23)
	1000 mg/kg bw/d	2/151	1.33	2/151	1.32	8.7 (2/23)	8.7 (2/23)
Historical control VSD (11 studies 07/1994 – 02/1995 at Huntingdon, UK)		1/158	0.63*	3/144	2.08*	4.2*(1/24)	13.6 (3/22)*
Historical control VSD (MARTA, 1996)		unknown	1.34*	unknown	10.3*	10.0*	40.9*

*value calculated based on the worst control out of all control studies

4.2.2 Chemically Induced VSD

Increased incidences of VSD have been reported for rats following treatment of pregnant animals with certain chemical substances, such as the anti-convulsant trimethadione, dimethadione or trichloroethylene. These have been reported to induce rates of up to 93% incidence of VSD. Purssell (2012) noted that although these chemicals are structurally diverse a common denominator appears to be the induction of toxicity affecting the heart and cardiovascular effects. No such effects, however, were noted for Azadirachtin.

Ventricular septal defects are observed rather commonly as spontaneous anomalies regarded as delayed development or visceral malformation. Also, certain chemical substances including anti-epileptic drugs and organic solvents have been reported to induce an increased incidence of VSD. The low incidence of VSD and other observations reported in the Myers & Dawe (1997a) study are within the historical control background incidences or at worst 0.02% above the spontaneous level. Thus, it appears not appropriate to translate this finding into a classification of Azadirachtin with regard to a developmental toxicological hazard.

4.3 Developmental Toxicity study in the Rat with Fortune Aza

Based on the data submitted, it was concluded in the process for approval of Azadirachtin as active substance for plant protection that three Azadirachtin extracts (NeemAzal, Fortune Aza and Mitsui extract) are equivalent. Thus, the study on teratogenicity in the rat conducted with Fortune Aza is to be regarded as additional study on an equivalent test item.

This GLP-conform study according to OECD TG 414 was conducted at the same laboratory (Huntingdon, UK) by the same personnel (foetal pathologist), in the same year (1997) with the same animal strain (Crl:CD®BR VAF/Plus) from the same source (Charles River UK) as the study with NeemAzal (Myers and Dawe, 1997a).

Report: Waterson, L.A. (1997b)
Fortune Aza technical - A Study of the Developmental Toxicity in Rats, Huntingdon Life Sciences Ltd., Huntingdon, England
unpublished report No. FBT 2/960340

This study demonstrated a lack of adverse effects on foetuses following administration of Azadirachtin during pregnancy at dose levels of 100, 300 or 1000 mg/kg bw/d as concluded by the RMS. In fact the number of interventricular septal defects was highest in the control group with three animals from three litters showing small VSD while one foetus was affected in the mid-dose group. No malformations affecting the heart or blood vessels or any other kind of malformations were noted.

4.4 Other Data

4.4.1 Human Evidence

No epidemiological studies have been reported indicating an adverse effect of Azadirachtin on development. Furthermore, there are no casuistic reports on teratogenic or other adverse developmental effects in humans despite the common use of Azadirachtin products.
As pointed out in the CLH report (Point 9.12.3) there are numerous published reports on the use of extracts from other parts of the Neem tree (leaves, bark, roots) or Neem oil in Indian folk medicine. Because of a marked different composition, these have no relevance for the refined Neem extract Azadirachtin that is considered here.

4.4.2 Reproduction Toxicity Studies

Additional information is available that was disregarded in the process for approval of Azadirachtin as active substance for plant protection (PPP process), but can very well serve at least as supportive and supplemental information for the classification of Azadirachtin according to Regulation (EC) No. 1272/2008.

Malformations such as severe VSD lead to the death of affected offspring. Thus, reduced survival in reproductive studies would be indicative of increased incidences of severe malformations. However, no increase in mortality in pups was reported in a two generation study (Ramamoorthy, 2000a) at dose levels of up to 750 ppm NeemAzal technical in the diet. This study, was accepted in the DAR and the CLH report.

Report:	Ramamoorthy (2000a) Evaluation of Toxicity of NeemAzal Technical to Reproductive Process in Wistar Rats - Segment IV - Toxicity to two Generation Reproductive Process, Report-no. 4826,

Additionally, reports on three further generational studies are available that were disregarded in the PPP process and judged “not acceptable” in the CLH report. However, these can serve at least as supportive and supplemental information for the classification of Azadirachtin despite considerable deficiencies in reporting and/or study design or the test substance information.

These include a two generation reproduction study with the plant protection product NeemAzal F 5% (Mani 1996). No adverse effects induced by this treatment were observed in the offspring regarding clinical signs, body weight, sex ratio and litter size in F1a and F1b pups or F2a and F2b pups.
No lesions were noted in F2b pups that were subjected to necropsy, neither with regard to gross pathology nor to histopathological examinations performed on control animals and in the group with the highest dose.
Reproductive performance and other litter parameters were not affected by the treated diet at any dose.

In a segment I study (Ramamoorthy 2000b) rats received NeemAzal at 100 and 1000 mg/kg bw/d by gavage. There were no treatment related developmental effects reported regarding litter size, fertility, pup weight or any other signs in the offspring. The NOAEL was 1000 mg NeemAzal /kg bw/d. Although this study was not accepted in the CLH report it points out that even at a dosage of 1000 mg NeemAzal/kg bw/d no effects on general reproduction parameters were determined.

In a published two generation study the absence of developmental toxicity of Azadirachtin (technical Azadirachtin 12%) was demonstrated (Srivastava & Raizada 2007):
Rats were fed 100, 500 and 1000 ppm technical Azadirachtin through diet which is equivalent to 5, 25 and 50 mg/kg bw of rats. There were no toxicological effects in parent rats as evidenced by clinical signs of toxicity. The litters of F(1B) and F(2B) generations were devoid of any morphological, visceral and teratogenic changes. There were no major malformations in foetuses, while some insignificant minor skeletal variations like missing 5th sternebrae and bipartite thoracic centre were found. These were not compound or dose related. No significant pathomorphological changes were observed in liver, kidney, brain and gonads of F(2B) pups.
In conclusion, rats fed technical Azadirachtin showed no evidence of cumulative effects on postnatal development and reproductive performance over two generations.
The lack of teratogenic potential is judged by the observation that offspring mortality was unaffected and also no teratogenic effects were observed in a subgroup of foetuses investigated for skeletal or visceral malformations.

The latter report was disregarded in the DAR (based on non-equivalence of the test item) and not mentioned in the CLH report. However, this published study adds to the database on Azadirachtin/Neem kernel extract supporting the notion that the malformations reported in the Myers & Dawe (1997a) study are a spontaneous and incidental finding.

4.4.3 Developmental Toxicity Studies

Further evidence for the observation of maternal toxicity of technical extract at, or around 200-300 mg a.i./kg bw/d and the lack of any developmental toxicity is given in the dose range finding studies for the full studies summarised in 4.1 and 4.2 and a developmental toxicity study (Segment II study):

Study 1) A GLP-conform dose finding teratogenicity study was performed in mated Charles River CD® rats according to OECD guideline 414 (Myers and Dawe, 1997b). One control and three treatment groups of 10 animals, each, were used to determine the teratogenic potential of NeemAzal technical. Dosage levels of 0, 100, 300 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19.
Observations on mortality, clinical signs of toxicity and body weights were recorded. On gestation day 20, all females were sacrificed and the number and location of viable and nonviable foetuses, early and late resorptions and *corpora lutea* were recorded. Uterus weights were determined. Gross lesions were recorded.
Maternal toxicity: The post dose salivation observed for dams at 300 and 1000 mg/kg bw/d is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance.
Based on the initially reduced bodyweight gain (Table 5) and food consumption and the increased water consumption in the high dose group, the no observable adverse effect level for maternal toxicity in this preliminary study was set at 100 mg/kg bw/d corresponding to 36.6 mg Azadirachtin A/kg bw/d.
Developmental toxicity: No effects on foetal number and development or incidences of malformations were observed at any treatment levels. Thus, the NOAEL for developmental toxicity was >1000 mg/kg bw/d.

Table 5: Maternal body weight changes in dose finding teratogenicity study (excerpt from Myers & Dawe, 1997b)

	Dose level [mg/kg bw/d]			
NeemAzal® technical	**0**	**100**	**300**	**1000**
Azadirachtin A	**0**	**36.6**	**109.8**	**366.0**
Number of animals	9	8	8	8
Weight gain Day 2-Day 6 (g/rat)	42.6	39.6	29.6	42.6
Weight gain Day 6-Day 8 (g/rat)	14.2	13.8	8.8*	8.6*
Weight gain Day 8-Day 10 (g/rat)	14.7	15.1	17.9	19.0
Weight gain Day 10-Day 20 (g/rat	116.0	114.3	118.0	117.6
Final bodyweight (g/rat)	410.1	406.5	409.3	413.0

* $p<0.01$

Study 2) In a GLP-conform dose range finding study (Waterson, 1997c) according to OECD guideline 414 (1981) mated Charles River CD® female rats, assigned to one control and three treatment groups of 10 animals, each, were used to determine the teratogenic potential of Fortune Aza technical. Dosage levels of 0, 100, 300 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19 of gestation at a volume of 10 mL/kg in 1% methylcellulose in this study. Observations on mortality, clinical signs of toxicity and body weights were recorded. On gestation day 20, all females were sacrificed and the number and location of viable and nonviable foetuses, early and late resorptions and *corpora lutea* were recorded. Uterus weights were determined. Gross lesions were recorded.
Based on the initially reduced bodyweight and food consumption, and the increased water consumption in the high dose group the no observable adverse effect level was 300 mg/kg bw/d for maternal toxicity (19.8 mg Azadirachtin A/kg bw/d.
The post dose salivation observed for dams at 300 and 1000 mg/kg bw/d is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance.

No effects on foetal number and development or incidences of malformations were observed at any treatment levels. Thus, the NOEL for developmental toxicity was 1000 mg/kg bw/d.

Study 3) In a segment II study (Pugazhenthi, 1998) groups of 20 pregnant female Wistar rats were used to determine the teratogenic potential of NeemAzal. This study was not performed according to GLP but adhered closely to a similar quality system.
Dosage levels of 0, 250, 500 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 15 of gestation at a volume of 10 mL/kg in distilled water in this study.
Based on reduced uterus weight and reduced food consumption in the high dose group the no observable adverse effect level was 500 mg/kg bw/d for maternal effects.
Only increased incidences of moles were noted in the foetuses of the high treatment groups. No other effects on foetal number and development were observed. No VSD were reported.
The latter report was not evaluated in the CLH report due to reporting deficits but adds further to the evidence that malformations reported in the Myers & Dawe (1997a) study are a spontaneous and incidental finding.

Study 4) In a GLP-conform teratogenicity study in the rabbit (Ryan, 1994) according to OECD guideline 414 (1981) four groups of pregnant New Zealand White rabbits were treated daily on gestation days 6 to 18 by gavage. The animals received suspensions of ATI-720 in 0.5% aqueous carboxymethyl cellulose at 20, 100 and 500 mg/kg bw/d, and a control group was treated with vehicle alone (5 mL/kg bw).

Based on bloody urine in the high dose group, and reduced bodyweight and food consumption in the high dose and mid dose group the no observable adverse effect level was 20 mg ATI-720/kg bw/d for maternal effects.

Significant signs of developmental toxicity were observed in the high dose group only and were related to maternal toxicity. No effects on foetal number and development were observed in the mid dose and low dose group. Thus, the NOAEL for developmental toxicity was 100 mg/kg bw/d. No visceral malformations affecting the heart were observed. This study was conducted in rabbits but may be considered to add further to the evidence that malformations affecting the heart reported in the Myers & Dawe (1997a) study are a spontaneous and incidental finding.

Study 5) Most recently Dallaqua et al. (2013) compared Azadirachtin (extract of Neem seed kernels with 70% aqueous ethanol) and Neem seed kernel oil in a developmental toxicity study in Sprague-Dawley rats. The study was not conducted under GLP, and deviations from the OECD guideline 414 included that only one dose level (1.4 g/L) and a control group was tested, only 11 dams in each group were included and thus only 39 pups were tested for visceral malformations and 43 for skeletal malformations. In conclusion, while Neem seed kernel oil treatment administered during pregnancy caused abnormalities in rat foetuses, showing teratogenic effect, the Azadirachtin (ethanolic extract) presented no impairment in the foetuses.

Considering the deficiencies and deviations from the relevant guideline, this study may only be considered as supplemental but it adds to the evidence that Neem seed kernel extract does not possess a teratogenic potential.

Study 6) In a published teratogenic study (Srivastava & Raizada, 2001) the absence of developmental toxicity of Azadirachtin (technical Azadirachtin 12%) was demonstrated. Rats were fed with 500, 1000 and 1500 mg/kg bw/d technical Azadirachtin through diet. Technical Azadirachtin at different doses did not produce any significant embryo/foetotoxic effects. There were no major malformations, while some minor variants found in animals at the high doses were not compound or dose related effects. The absence of anomalies in foetal gross, visceral morphology and skeleton suggests that technical Azadirachtin is not teratogenic in rats at the doses tested. Even if the equivalence of the extracts is not fulfilled, this published study adds to the database on Azadirachtin/ Neem seed kernel extract supporting the notion that the malformations reported in the Myers & Dawe (1997a) study are a spontaneous and incidental finding.

Table 6 Summary of the weight of evidence regarding developmental toxicity of Azadirachtin

Study type exposure route species	**Guideline followed Test item**	**Developmental toxicity effects**	**Reference**
Developmental toxicity; oral gavage, rat	OECD TG 414 NeemAzal	Adverse effects only marginally above historical laboratory controls but within the published historical control range	Myers and Dawe, 1997a
Developmental toxicity; oral gavage, rat	OECD TG 414 Fortune Aza	No adverse effect	Waterson, 1997b
2-generation oral (with feed), rat	Gaitonde Guideline NeemAzal	No adverse effects	Ramamoorthy, 2000a
2-generation & developmental toxicity oral (with feed), rat	Published No guideline mentioned	No adverse effects	Srivastava & Raizada 2007
2-generation oral (with feed), rat	Gaitonde Guideline NeemAzal F 5%	No adverse effects	Mani 1996
Segment I study oral gavage, rat	Gaitonde Guideline NeemAzal	No adverse effects	Ramamoorthy, 2000b
Developmental toxicity; oral gavage, rat	Published No guideline mentioned	No adverse effects	Srivastava & Raizada, 2001
Developmental toxicity; oral gavage, rat	OECD TG 414 NeemAzal	No adverse effect	Myers and Dawe, 1997b
Developmental toxicity; oral gavage, rat	OECD TG 414 Fortune Aza	No adverse effect	Waterson, 1997c
Segment II study oral gavage, rat	Gaitonde Guideline NeemAzal	No adverse effect	Pugazhenthi, 1998
Developmental toxicity; oral gavage, rabbit	OECD TG 414 ATI 720	No visceral findings Effects only at severe maternal toxic dose levels	Ryan, 1994
Developmental toxicity; oral gavage, rat	Non guideline 70% ethanolic extract	No adverse effect	Dallaqua, 2013

5 Opinion of PRAPeR Meeting

EFSA organised a peer review of the initial DAR-evaluation. During the PRAPeR expert meeting 79 (section mammalian toxicity) the developmental toxicity was discussed explicitly. The most important conclusion of this expert meeting is cited in the draft CLH report (Chapter 4.11.5):

"During an expert consultation in the PPP procedure, it was discussed, whether classification with R63 should be proposed: "There was a feeling that R63 was not appropriate based on the dataset available and incidences seen in the rat studies. […] Experts voted on the classification issue and a majority agreed to not propose any classification" (cited from the meeting minutes). This recommendation was based mainly on the low incidences observed in the developmental toxicity study in rats with NeemAzal."

6 EFSA Conclusion on Pesticide Peer review

After the PRAPeR expert meetings, EFSA published its conclusion on pesticide peer review with the following statement on developmental effects (EFSA2011):

„No developmental effects were observed in rats with Trifolio-M and Sipcam sources….."

7 Conclusion

Low incidences of malformations were noted in a rat teratogenicity toxicity study with one case of ventricular septal defect in the mid-dose treatment group (within the historical control range of the laboratory). In the high dose group two pups were affected (within the published historical control range for this rat strain) suggestive of a dose-related effect. However, this malformation is a rather common spontaneous observation in rats. Also, the treatment caused maternal toxicity in the dams both at mid-dose and high-dose level noted as reduced body weight gain. Although this effect was only transient, it occurred around days 6 to 8 of pregnancy, which has been identified as critical time in the development of the foetal heart. Furthermore, toxicity noted upon repeated dosing was reported at 123 mg/kg bw/d corresponding to half the mid-dose level of 225 mg/kg bw/d.

No indication of adverse developmental effects were noted in a two-generation study, also no adverse effects were reported in other teratogenicity studies with Azadirachtin extract with rats, no effects on the developing heart were reported in a teratogenicity study in rabbits. No adverse effects were reported in supplemental studies including a phase I reproductive toxicity study and a two-generation study. No adverse effects in pups were reported in a published two-generation study, which included detailed investigations on teratogenicity of Azadirachtin extract.

It is considered appropriate to <u>not classify</u> Azadirachtin with regard to reproductive toxicity because the malformation incidences were only marginally (0.02%) outside the laboratory historical control level, the occurrence of these malformations is likely influenced by maternal toxicity and similar observations are lacking in numerous other experimental studies.

The proposed classification and labelling according to Regulation (EC) No 1272/2008 with regard to human health is therefore only: H317 May cause an allergic skin reaction.

8 References

8.1 Proprietary studies

Johnson, W.D. (1994) 90-day oral (diet) toxicity study of ATI-720 in rats, Report-no. L 08424 Study No 4, GLP: yes, Published: no

Kumar, T. (2000) Long-term carcinogenicity study of NeemAzal technical in Wistar Rats, Report-no. 7291, GLP: yes, Published: no

Mani, B. (1996) Reproduction Toxicity Study (Segment-IV) of NeemAzal-F 5% in Charles Foster Rat, Report-no. 1542/JRF/TOX/96, GLP: yes,

Moorthy, M.V. (1996) Carcinogenicity study of Neemazal-F 5% in mice, Report-no. 1544/JRF/TOX/96, GLP/GEP: no,

Myers, D.P., Dawe, I.S. (1997a) A study of developmental toxicity in rats (gavage administration) Report-no. 05-293-97, EIP 2/952493, GLP: yes,

Myers, D.P., Dawe, I.S. (1997b) NeemAzal technical - A preliminary study of developmental toxicity in rats (gavage administration) Report-no. EIP 2/952493, GLP: yes,

Pugazhenthi, R. (1998) Effect of NeemAzal technical 27.3% on reproductive processes: Segment II teratological study in wistar rats, Report-no. 4824,GLP/GEP: no,

Ramamoorthy, S. (2000a) Evaluation of Toxicity of NeemAzal Technical to Reproductive Process in Wistar Rats - Segment IV - Toxicity to two Generation Reproductive Process, Report-no. 4826, GLP: yes,

Ramamoorthy, S. (2000b) Evaluation of toxicity of NeemAzal technical to general reproductive process and fertility in Wistar rats - Segment I, Report-no. 4823, GLP/GEP: no

Ryan, B. (1994) A developmental toxicity study of orally administered ATI-720 in rabbits, Report-no. 129601, L 08424 Study

No2b, GLP: yes,

Waterson, L.A. (1997a) NeemAzal technical toxicity study in rats by dietary administration for 4 weeks, Report-no. EIP 3/960397, GLP: yes,

Waterson, L.A. (1997b) NeemAzal technical toxicity study in rats by dietary administration for 13 weeks, Report-no. EIP 4/963100, GLP: yes,

Waterson, L.A. (1997c) Fortune Aza technical - A Study of the Developmental Toxicity in Rats, Report-no. FBT 2/960340, GLP: yes,

Waterson, L.A. (1997d) Fortune Aza technical - A Preliminary Study of the Developmental Toxicity in Rats, Report-no. FBT 1/952837, GLP: yes,

Waterson, L.A., Dawe, I.S. (1997a): Fortune Aza technical Toxicity study in rats by dietary administration for 4 weeks, Report-no. FBT 3/961630, GLP: yes,

Waterson, L.A., Dawe, I.S. (1997b) Fortune Aza technical - Toxicity Study in Rats by Dietary Administration for 13 Weeks, Report-no. FBT 4/962744, GLP: yes,

8.2 Published data

Beyer, B. K., Chernoff, N., Danielsson, B. R., Davis-Bruno, K., Harrouk, W., Hood, R. D., Scialli, A. R. (2011). ILSI/HESI maternal toxicity workshop summary: Maternal toxicity and its impact on study design and data interpretation. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, 92(1), 36-51.

Dallaqua, B., Saito, F. H., Rodrigues, T., Calderon, I. M. P., Rudge, M. V. C., Volpato, G. T., & Damasceno, D. C. (2013). *Azadirachta indica* treatment on the congenital malformations of fetuses from rats. *Journal of ethnopharmacology*, *150*(3), 1109-1113.

Fleeman, T.L., Cappon, G.D and Hurtt , M.E. Postnatal Closure of Membranous Ventricular Septal Defects in Sprague-Dawley Rat Pups After Maternal Exposure With Trimethadione Birth Defects Res B 71:185–190, 2004

MARTA (Middle Atlantic Reproduction and Teratogenicity Association)(1996) Historical Control Data (1992 - 1994) for Developmental and Reproductive Toxicity Studies using the Crl:CD®(SD)BR Rat, Charles River Laboratories.

Purssell E, Weston AD, Thomson JJ, Swanson TA, Brown NA, Ozolinš TR. (2012). Noninvasive high-resolution ultrasound reveals structural and functional deficits in dimethadione-exposed fetal rat hearts in utero.*Birth Defects Res B Dev Reprod Toxicol.* 95(1):35-46

Raizada, R.B. et al. (2001) Azadirachtin, a neem biopesticide: subchronic toxicity assessment in rats, *Food Chem Toxicol*. 39, 477-483.

Rufer, E. S., Hacker, T. A., Flentke, G. R., Drake, V. J., Brody, M. J., Lough, J., & Smith, S. M. (2010). Altered Cardiac Function and Ventricular Septal Defect in Avian Embryos Exposed to Low-Dose Trichloroethylene. *Toxicological Sciences*, 113(2), 444-452.

Slama, M., Susic, D., Varagic, J., & Frohlich, E. D. (2002). High rate of ventricular septal defects in WKY rats. *Hypertension*, 40(2), 175-178.

Solomon, H.M. et al. (1997) Spontaneous and induced alterations in the cardiac membraneous ventricular septum of fetal, weanling and adult rats, *Teratology*, 55, 185-194.

Srivastava, M. K., & Raizada, R. B. (2001). Assessment of embryo/fetotoxicity and teratogenicity of azadirachtin in rats. *Food and chemical toxicology*, 39, 1023-1027.

Srivastava, M. K., & Raizada, R. B. (2007). Lack of toxic effect of technical azadirachtin during postnatal development of rats. *Food and chemical toxicology*, 45(3), 465-471.