# Committee for Risk Assessment

# RAC

## Opinion

proposing harmonised classification and labelling
at EU level of

## Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents]

## EC Number: 283-644-7
## CAS Number: 84696-25-3

CLH-O-0000006926-62-01/F

## Adopted

## 10 December 2020

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# OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

**Chemical name:** **Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents]**

**EC Number:** **283-644-7**

**CAS Number:** **84696-25-3**

The proposal was submitted by **Germany** and received by RAC on **31 October 2019.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

# PROCESS FOR ADOPTION OF THE OPINION

**Germany** has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **25 November 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **24 January 2020**.

### ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Annemarie Losert**

Co-Rapporteur, appointed by RAC: **Pietro Paris**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **10 December 2020** by **consensus**.

**Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)**

| | Index No | Chemical name | EC No | CAS No | Classification | | Labelling | | | Specific Conc. Limits, M-factors and ATE | Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Hazard Class and Category Code(s) | Hazard statement Code(s) | Pictogram, Signal Word Code(s) | Hazard statement Code(s) | Suppl. Hazard statement Code(s) | | |
| Current Annex VI entry | No current Annex VI entry | | | | | | | | | | |
| Dossier submitters proposal | TBD | Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] | 283-644-7 | 84696-25-3 | Repr. 2<br>Skin Sens. 1<br>Aquatic Chronic 1 | H361d<br>H317<br>H410 | GHS07<br>GHS08<br>GHS09<br>Wng | H361d<br>H317<br>H410 | | M=10 | |
| RAC opinion | TBD | Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] | 283-644-7 | 84696-25-3 | Repr. 2<br>Skin Sens. 1<br>Aquatic Chronic 1 | H361d<br>H317<br>H410 | GHS07<br>GHS08<br>GHS09<br>Wng | H361d<br>H317<br>H410 | | M=10 | |
| Resulting Annex VI entry if agreed by COM | TBD | Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] | 283-644-7 | 84696-25-3 | Repr. 2<br>Skin Sens. 1<br>Aquatic Chronic 1 | H361d<br>H317<br>H410 | GHS07<br>GHS08<br>GHS09<br>Wng | H361d<br>H317<br>H410 | | M=10 | |

# GROUNDS FOR ADOPTION OF THE OPINION

## RAC general comment

"Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents]" (hereinafter "*Margosa Extract with water*") is an active (UVCB) substance in the meaning of Regulation (EU) No 528/2012 (approved under Directive 98/8/EC) and therefore subject to harmonised classification and labelling (Regulation (EC) No 1272/2008 Article 36.2).

The EINECS entry (EC No. 283-644-7, CAS No. 84696-25-3) is a general entry covering all extracts from *Azadirachta indica,* irrespective of the extraction conditions. According to the Guidance for identification and naming of substances under REACH and CLP, the different extracts receive different names, depending on the origin of the plant material in combination with the extraction/manufacturing method. However, the EC name and number is valid for all extracts from *Azadirachta indica*.

This CLH dossier was prepared for *Margosa Extract with water*. This extract is approved as a biocidal active substance in product type 18 (Insecticides, Acaricides and Products to control other Arthropods) since 2014 and is included in the Union list in the Biocide Regulation with an expiration date of 30/04/2024.

Currently it is known that three other margosa extracts (all covered by the same EINECS entry) are on the market:

- Margosa, extract, cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide. At the BPC 19 (March 2017) the approval as a biocidal active substance was concluded, a CLH dossier was submitted in 2017 and the RAC opinion adopted in 2018.[1]

- Margosa, extract from the kernels of *Azadirachta indica* extracted with organic solvents at elevated temperatures.

- Margosa, extract from press-cake of kernels of *Azadirachta indica* after removal of the "Neem Oil", extracted with organic solvents at elevated temperatures.

The substance "*Margosa Extract with water*" formally differs from the active substance "Azadirachtin", which has been evaluated and authorised under the PPP Regulation in 2007. The PPP active substance "Azadirachtin" covers:

(i) Margosa extract from the kernels of *Azadirachta indica* extracted with organic solvents at elevated temperatures;

(ii) Margosa extract from presscake of kernels of *Azadirachta indica* after removal of the "Neem Oil", extracted with organic solvents at elevated temperatures; and

[1] https://echa.europa.eu/de/registry-of-clh-intentions-until-outcome/-/dislist/details/0b0236e180a7225e

(iii) Margosa extract from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents.

The CLH Dossier for "*Margosa Extract with water*" considers only the data for the latter ((iii), above) of the three extracts covered by the PPP "Azadirachtin" active substance approval.

"*Margosa Extract with water*" is a UVCB substance and only a few constituents are identified, e.g. Azadirachtin A (the most abundant), Azadirachtin B, Nimbin and Salannin.

Under the PPP and BPR procedures, the whole extract was considered to be the toxicologically relevant substance, as no toxicological data were available to demonstrate that particular components were responsible for the observed toxicological effects.

Aflatoxins might be present in the extract, with defined maximum residue levels, since they are relevant impurities in the meaning of the PPP regulation.

All of the toxicological studies were performed with *Margosa Extract with water*. However, the content of Azadirachtin A varies.

- The vast majority of studies were performed with *Margosa Extract with water* containing 36.6 % Azadirachtin A.
- Some studies were performed with extracts with a lower content of Azadirachtin A, which is indicated in the study descriptions. This concerns the following studies: acute toxicity studies in Wistar rats and Swiss albino mice (Anonymous, 1993a and 1993b), 14-day study in CD rats (Anonymous, 1995), micronucleus assay *in vivo* (Azadirachtin A content of 27 %), carcinogenicity study in Swiss albino mice (Anonymous, 1996e, NeemAzal-F 5 % (formulation, 5 % Azadirachtin A content), 2-generation study in Charles Foster rats (Anonymous, 1996d; NeemAzal-F 5 % formulation, 5 % Azadirachtin A content).

In addition, two other technical extracts were submitted for the evaluation as the pesticide active ingredient "azadirachtin" which are not included in this dossier. The notifiers named their extracts "FortuneAza" or "NPI720"/"ATI 720" which are also technical extracts of seed kernels of the Neem tree obtained by a different extraction procedure. Where applicable, it is indicated whether data on those extracts are in agreement with observations for *Margosa Extract with water*.

## RAC evaluation of physical hazards

### Summary of the Dossier Submitter's proposal

The dossier submitter (DS) presented studies or justifications for non-testing for all relevant physical hazards. *Margosa Extract with water* was tested in the following hazard classes.

Testing of flammability according to A.10 resulted in a negative result in the preliminary test. EC test A.14 gave a negative result on explosive properties. No self-ignition was observed up to the melting point in a study conducted according to EC test A.16. A test for oxidising solids was conducted according to EC test A.17, which showed negative result. Based on this, the DS concluded that classification as explosive, flammable solid, oxidising solid and as self-igniting solid is not justified.

Flammability in contact with water and pyrophoric solids were not tested because experience in production and handling had shown that the substance does not react with water and is stable in air for several days. Testing for self-reactive properties can be omitted if the decomposition

energy is below 300 J/g. Differential scanning calorimetry (EC test A.14) showed low decomposition energies of about 177 J/g. Based on this, the DS concluded that no further testing is necessary and no classification as flammable in contact with water, as pyrophoric solid and as a self-reactive substance is justified.

The hazard class self-heating properties was not open during the consultation of the CLH report.

As *Margosa Extract with water* is a solid, the following hazard classes are not relevant: flammable gases and liquids, oxidising gases and liquids, gases under pressure, flammable aerosols, pyrophoric liquids and no organic peroxides are present.

Overall, no classification was proposed by the dossier submitter for physical hazards.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

In line with the DS, RAC considers the presented studies to be relevant for assessing the physical hazards. It is noted that explosive and oxidising properties have been tested according to EC methods A.14 and A.17, respectively, and not according to the recommended UN RTDG test methods. The relevant chemical structures for the aforementioned hazard classes of *Margosa Extract with water* are unsaturated C-C bonds, O-C bonds and O-H bonds, which are exempted from testing for oxidising properties according to the CLP Regulation. Explosive properties can be excluded, as the decomposition energy is below 500 J/g as stated in result of EC test A.14. Self-heating properties were not open for comment during consultation.

Corrosive to metals: the justification provided by the DS was not fully in line with the CLP regulation, however RAC notes that the substance has a melting point above 55°C, hence no existing test method is applicable.

Overall RAC considers the available test results and information sufficient to support the DS's proposal for **no classification for physical hazards**.

## HUMAN HEALTH HAZARD EVALUATION

## RAC evaluation of acute toxicity

### Summary of the Dossier Submitter's proposal

*Margosa Extract with water* was tested in three oral acute toxicity studies (Anonymous, 1997c, rat; Anonymous, 1993a, rat; Anonymous, 1993b, mouse), in one dermal acute toxicity study (Anonymous, 1997d, rat) and one inhalation acute toxicity study (Anonymous, 1997b, rat). The observations after acute oral and dermal exposure indicate $LD_{50}$ values above the relevant upper limits for classification according to the CLP Regulation.

In one study, 20 % mortality was seen after oral exposure to 4760 mg/kg bw. Clinical signs and reduced locomotor activity were seen at oral doses ≥ 3365 mg/kg bw.

***Table**: Overview on the available acute oral toxicity studies (from the CLH report)*

| **Animal species & strain / Test material** | **Number of animals per dose level** | **Doses, route of administration, vehicle** | **$LD_{50}$ (mg/kg bw) Test compound** | **Reference, year, Method** |
|---|---|---|---|---|
| Rat, Hsd/Ola:Sprague -Dawley (CD) / *Margosa Extract with water* (37 % Azadirachtin A) | 5 M & 5 F | 5000 mg/kg bw, gavage, distilled water (10 mL/kg bw) | > 5000 Clinical signs: piloerection, pallors of the extremities, reduced bw gain in some rats | Anonymous, 1997c, EPA FIFRA Guideline 152-15 (equivalent to OECD TG 401,no deviation), GLP: yes |
| Rat, Wistar / *Margosa Extract with water* (≥ 25 % Azadirachtin A*) | 5 M & 5 F | 0, 1190, 2380, 4760 mg/kg bw gavage DMSO (20 mL/kg bw) | > 4760 (at 4760 mg/kg bw: 20 % mortality, dullness and reduced activity) | Anonymous, 1993a TG and GLP-status unknown |
| Mouse, Swiss albino / *Margosa Extract with water* (≥ 25 % Azadirachtin A*) | 5 M & 5 F | 0, 1190, 2380, 3365 mg/kg bw gavage DMSO (15 mL/kg bw) | > 3365 (at 3365 mg/kg bw: reduced locomotor activity) | Anonymous, 1993b TG and GLP-status unknown |

* No certificate of analysis provided in study report

No mortalities and no abnormal macroscopic pathological findings were observed. Slightly lower body weight gain was observed in all male rats and one female rat on day 8, and one male and four females on day 15.

***Table**: Overview on the available acute dermal toxicity studies (from the CLH report)*

| **Animal species & strain / Test material** | **Number of animals per dose level** | **Doses, route of administration, vehicle** | **LD50 (mg/kg bw) Test compound** | **Reference, year, Method** |
|---|---|---|---|---|
| Rat, Hsd/Ola:Sprague -Dawley (CD) / *Margosa Extract with water* (37 % Azadirachtin A) | 5 M & 5 F | 2000 mg/kg bw, dermal (24 h), water moistened | > 2000 | Anonymous, 1997d EPA Pesticide Assessment Guideline 152-14 (1984) (equivalent to OECD TG 403, limit, no deviation), GLP: yes |

In the inhalation study, the maximum attainable concentration was 0.72 mg/L (4h, whole body), which is within the concentration limits for acute inhalation toxicity, category 3 (dusts and mists). During the exposure period hunched posture, partially closed eyes and test material on fur were reported, but no signs of toxicity were reported during the observation period. It was concluded that the $LC_{50}$ is > 0.72 mg/L. A short statement on two studies with two other technical extracts ("Fortune Aza" & "NPI 720) was presented, also indicating $LC_{50}$ values > 2.4 mg/L and reporting that one death of a female animal occurred at that dose ("Fortune Aza").

***Table**: Overview on the available acute inhalation toxicity studies (from the CLH report)*

| Animal species & strain / Test material | Number of animals per dose level | Doses, route of administration, vehicle | $LC_{50}$ (mg/L) Test compound | Reference year Method |
|---|---|---|---|---|
| Rat, Sprague-Dawley /<br>*Margosa Extract with water* (37 % Azadirachtin A). No signs of toxicity were observed. | 5 M & 5 F | 0.72 mg/L air (4 h), whole body | > 0.72 (highest attainable conc.) | Anonymous, 1997b;<br>EPA FIFRA Guideline 152-14 (1984)<br>(equivalent to OECD TG 402, limit, no deviation),<br>GLP: yes |

On the basis of the presented results the DS concluded that no classification for acute toxicity is warranted.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

In addition to the analysis presented above, the CLH dossiers also contained (limited) human data. While routine medical observation of workers exposed to Neem tree extracts did not show adverse health effects (Anonymous, 2003, 2004, 2005a,b), reports from the open literature described intoxications (including fatal cases), mainly from the use of "Neem Oil" and other "Neem tree extracts" as medication. However, as the composition of these extracts is unknown these data are not considered relevant for the evaluation of *Margosa Extract with water*.

RAC concurs with the DS and supports **no classification for acute toxicity via the oral, dermal and inhalation routes.**

## RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

### Summary of the Dossier Submitter's proposal

The DS did not propose to classify *Margosa Extract with water* as STOT SE 1 or 2, considering that the non-lethal effects reported after acute exposure were transient and not of considerably adverse nature, as there was no significant impact on health or the effects were only seen at high doses, clearly exceeding those required for classification for STOT SE. In addition, as no narcotic effects or irritation of the respiratory tract were observed following oral, dermal or inhalation exposure, the DS concluded that *Margosa Extract with water* does not meet the criteria to be classified as STOT SE 3 for respiratory tract irritant or narcotic effects.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

No signs of organ toxic effects were observed in the acute oral, dermal or inhalation toxicity studies in rats and mice exposed to *Margosa Extract with water*. The clinical signs observed in the acute toxicity studies were transient and not severe or were only seen at doses clearly exceeding the respective guidance values for classification in the CLP regulation. The animal data submitted did not provide evidence for respiratory tract irritation or narcotic effects.

Information on human poisoning incidents following exposure to "Neem Oil" and other "Neem tree extract" are considered by RAC to be of limited relevance, as explained in the section on acute toxicity (above). In addition, routine medical observation of workers exposed to Neem tree extracts did not show adverse health effects (Anonymous, 2003, 2004, 2005a,b)

RAC concurs with the DS that **no classification for STOT SE is warranted.**

## RAC evaluation of skin corrosion/irritation

### Summary of the Dossier Submitter's proposal

The DS provided a study in which *Margosa Extract with water* was tested according to EPA FIFRA Guidelines 152-12 (1984), which is equivalent to OECD 404 (no deviations; GLP; Anonymous, 1996f). Very slight erythema (score 1) was seen in 3 of 6 exposed male New Zealand albino rabbits (scored only on the first day of exposure). No signs of systemic toxicity were reported.

The DS also mentioned that for two other technical extracts ("Fortune Aza", "NPI 720", which are different from *Margosa Extract with water*), no skin irritating properties were reported.

***Table**: Overview on the available skin irritation study (from the CLH report)*

| **Animal species & strain / Test material** | **Number of animals** | **Doses** | **Result** | **Reference Method** |
|---|---|---|---|---|
| Rabbit, New Zealand albino / *Margosa Extract with water* (37 % Azadirachtin A) | 6 M | 0.5 g (4 h) | Not irritating (highest erythema score: 1), resolved by day 2 | Anonymous, 1996f (TG equivalent to OECD 404, no deviations GLP: yes) |

The DS concluded that the criteria for classification (in 2/3 animals, a mean value of ≥ 2.3 - ≤ 4.0 for erythema / eschar or oedema) were not fulfilled.

On that basis no classification for skin irritation was proposed.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

RAC considers the presented study reliable and adequate to demonstrate the absence of skin irritating properties of *Margosa Extract with water.* RAC further notes that also in the acute

dermal toxicity study no signs of irritation were reported. On that basis RAC concurs with the DS and supports **no classification for skin irritation.**

## RAC evaluation of serious eye damage/irritation

### Summary of the Dossier Submitter's proposal

The DS provided a study in which *Margosa Extract with water* was tested according to EPE FIFRA Guideline 152-13 (1984), which is equivalent to OECD 405 (no deviations; GLP; Anonymous, 1996f).

Dulling of the cornea in one animal and discharge and redness of the conjunctiva were seen in all animals 1h after instillation of test compound. Effects declined and were absent within one or two days after instillation.

The DS also mentioned that for two other technical extracts ("Fortune Aza", "NPI 720", which are different from *Margosa Extract with water*) no eye irritating properties were reported.

***Table**: Overview on the available eye irritation study studies (from the CLH report)*

| **Animal species & strain / Test material** | **Number of animals** | **Doses** | **Result*** | **Reference, Method** |
|---|---|---|---|---|
| Rabbit, New Zealand albino /<br><br>*Margosa Extract with water* (37 % Azadirachtin A) | 5 M & 1 F | 70 mg | Mean scores:<br>Not irritating Cornea opacity: 0.0 / 0.0 / 0.0<br>Iris: 0.0 / 0.0 / 0.0<br>Redness of conjunctivae: 1.0 / 0.3 / 0.2<br>Chemosis: 0.7 / 0.3 / 0.0 | Anonymous, 1996g,<br>(TG equivalent to OECD 405, no deviations,<br>GLP: yes) |

*mean scores at the reading times (24 h / 48 h / 72 h)

The CLP criteria, which state that for classification at least in 2/3 animals a score of ≥ 1 for corneal opacity and / or ≥ 1 for iritis and/or ≥ 2 for conjunctival redness and/or ≥ 2 conjunctival oedema (chemosis) must be achieved, were not fulfilled.

On that basis no classification for eye irritation was proposed.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

RAC considers the presented study reliable and adequate to demonstrate the absence of eye irritating properties of *Margosa Extract with water.* On that basis RAC concurs with the DS and supports **no classification for skin corrosion/irritation.**

## RAC evaluation of respiratory sensitisation

### Summary of the Dossier Submitter's proposal

There were no specific studies performed with *Margosa Extract with water*. The DS commented that there was no evidence from single or repeated dose animal studies that *Margosa Extract with water* had any potential to cause respiratory sensitisation.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

There is no evidence from the available single or repeated dose toxicity studies that *Margosa Extract with water* has a potential to cause respiratory sensitisation, and as stated in previous sections, the available human data from routine medical observation of workers exposed to Neem tree extracts did not show any adverse health effects (Anonymous, 2003, 2004, 2005a,b).

On that basis **RAC supports the DS's proposal for no classification for respiratory sensitization.**

## RAC evaluation of skin sensitisation

### Summary of the Dossier Submitter's proposal

The DS presented a guinea-pig maximisation test conducted according to the method of Magnusson & Kligman investigating the skin sensitising properties of *Margosa Extract with water* (Anonymous, 1997a). The study was conducted according to EPA FIFRA Guideline 152-15, which is equivalent to OECD 406, with no deviations and according to GLP.

Slight irritation was observed in all animals after intradermal application of *Margosa Extract with water* with solvent. Necrosis was recorded at sites receiving the test material in combination with Freund's complete adjuvant. One day before dermal application, the skin was treated with a 10 % solution of SDS in petrolatum. Slight erythema was observed after topical application of the test compound or vehicle in treated or control animals, respectively. On challenge, no skin reactions were observed in control animals. In contrast, all animals of the treatment group (40 or 80 % in acetone) showed slight to well defined oedema and erythema upon challenge with *Margosa Extract with water* solutions (results of the single animals are listed in the CLH report, table 21).

The DS mentioned two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) which are also skin sensitising.

Regarding human data, the DS reported that no case reports on hypersensitivity to *Margosa Extract with water* were available. Only single cases of contact dermatitis following dermal application of "Neem Oil" are reported in the open literature (Greenblatt *et al*. 2012, Reutemann and Ehrlich 2008).

Based on the results from Anonymous (1997a) the dossier submitter concluded that *Margosa Extract with water* has skin sensitising properties. However, as only relatively high concentrations were tested it was not possible to assess whether the substance fulfils the criteria for classification in category 1A. Hence, a classification in category 1 without sub-category was proposed.

## Comments received during consultation

No comments were received during consultation.

## Assessment and comparison with the classification criteria

*Margosa Extract with water* was tested in a study equivalent to OECD 406 (Anonymous, 1997a). The details of the study are presented in the table below.

***Table**: Guinea pig maximisation test (Anonymous, 1997a), adapted from the CLH report*

| **Animal species & strain / Test material** | **Number of animals** | **Doses** | **Result** | **Reference Method** |
|---|---|---|---|---|
| Guinea pig, Dunkin Hartley albino / *Margosa Extract with water* (37 % Azadirachtin A) | 20 M treated<br>10 control | Intradermal:<br>5 % test material in acetone/alembicol<br>5% teat material in Freund's Complete Adjuvant 1:1 with water<br>Dermal:<br>10 % SDS in petrolatum to induce irritation<br>80 % test material in acetone for topical induction<br>40 % and 80 % test material in acetone for topical challenge (after 3 weeks) | Sensitising (M&K) [all animals sensitised]<br>Challenge after 3 weeks at 40% and 80%<br>Scored after 48 h and 72 h, respectively: 20/20; 20/20<br>negative control: 0/10, 0/10<br>positive control: 66/70 * | Anonymous, 1997a<br>EPA FIFRA Guideline 152-15<br>(equivalent to OECD 406, no deviation)<br>GLP: yes |

* Seven earlier tests with alpha-hexylcinnamic aldehyde as positive reference substance (performed in 1992-1995) resulted in allergic reactions and have shown the sensitivity of the guinea pig strain used.

Based on the positive result in all animals exposed to 40% and 80% test material in acetone via dermal application and 5% test material intradermally (with and without Freund's complete adjuvant) it can be concluded that *Margosa Extract with water* is a skin sensitiser.

While the results of the dermal application part of the study were presented in the CLH report (Table 21 of the CLH report), the results of the intradermal part were not presented on an individual animal basis.

Skin sensitisation was observed in all exposed animals, however, as no concentration ≤ 1% was tested it cannot be concluded whether the test material would be sufficiently potent to justify a classification in sub-category 1A.

Without any details available, the information on skin sensitising properties of two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) and on single cases of contact dermatitis following dermal application of "Neem Oil" to human skin (Greenblatt *et al.* 2012, Reutemann and Ehrlich 2008) are considered marginally supportive.

RAC supports the DS's proposal to **classify *Margosa Extract with water* as Skin Sens 1, without sub-categorisation.**

## RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

### Summary of the Dossier Submitter's proposal

The DS presented three repeated dose toxicity studies in rats with dietary exposure to *Margosa Extract with water*, including a 14-day study (Anonymous, 1995), a 28-day study (Anonymous, 1997h) and a 90-day study (Anonymous, 1997i).

While no detailed information except body weight, food consumption and daily observations were available for the 14-day study, the 28 day and the 90-day study demonstrated liver- and thyroid-related effects. The DS considered these effects as not severe enough to support a classification as STOT RE.

In addition, the DS reported on feeding studies from farm animals (cows, calves, bulls, buffalo calves, growing pigs and sheep) exposed to water-washed Neem seed kernel cake via the diet (Anonymous, 2002, Anonymous, 2005c). For more details on the composition of the administered test material see the CLH report (section 4.7.2.6). These feeding studies were conducted for up to twelve months and investigated a diverse spectrum of parameters, but no adverse effects were reported.

Overall, the DS concluded that no STOT RE classification for *Margosa Extract with water* is required.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

The dietary repeated dose toxicity studies with *Margosa Extract with water* in rats are presented in the table below.

***Table**: Summary of the repeated dose dietary toxicity studies in rats (from the CLH report, slightly modified).*

| **Animal species & strain / Test material** | **Number of animals** | **Doses, vehicle, duration** | **Result** | **Reference** |
|---|---|---|---|---|
| Rat, CD<br><br>/ *Margosa Extract with water* (Azadirachtin content not stated) | 5 M & 5 F | 20000, 50000 ppm (equivalent to 2000, 5000 mg/kg bw/d)<br>**Feed**<br>**2-wk** | LOAEL: 20000 ppm (2000 mg/kg bw/d)<br>bw ↓; feed intake (50000 ppm) ↓ | Anonymous, 1995<br>(only data on bodyweight, food consumption, daily observations) |

| Animal species & strain / Test material | Number of animals | Doses, vehicle, duration | Result | Reference |
|---|---|---|---|---|
| Rat, Crt: CD (SD) BR<br><br>/ *Margosa Extract with water* (37 % Azadirachtin) | 5 M & 5 F | 0, 3200, 8000, 20000 ppm (0, 320, 770, 1850 mg/kg bw/d in males; 0, 300, 790, 1750 mg/kg bw/d in females)<br>**Feed**<br>**4-wk** | LOAEL: 300 mg/kg bw/d (3200 ppm)<br>All dose levels: hepato-toxicity (periportal hepatocyte eosinophilia with clumping), thyroid toxicity (follicular epithelial hypertrophy)<br>**Liver weights** (g): (0-3200-8000-20,000 ppm)<br>M: 19-19.2-21.3*-20.6**<br>F: 11.2-12.6-13.6*-16.6**<br>**Thyroid weights** (mg): (0-3200-8000-20,000 ppm)<br>M: 17.9-20.1-24.7-22.9<br>F: 16.2-18.7-23.3*-24.2*<br>Adrenal weights (mg): (0-3200-8000-20,000 ppm):<br>M: 62.3-51.4-52.5-49.3*<br>F: 69.0-69.8-70.5-63.0<br>20000 ppm: hepatocyte hypertrophy; **lower bw gain (% control)**: M: 67 %; days 8-29; F: days 1-4: -25 % (bw loss); days 4-8: 67 %; days 8-29: 70 %<br>8000 ppm: **lower bw gain in females** (% control): days 1-4/4-8/8-29: 42 %/78 %/93 %, resp. | Anonymous, 1997h |
| Rat, Crt: CD BR<br><br>/ *Margosa Extract with water* (26.8 – 28.4 % Azadirachtin content) | 10 M & 10 F | 0, 100, 400, 1600, 6400 ppm (0, 8, 32, 123, 490 mg/kg bw/d in males; 0, 9, 36, 135, 525 mg/kg bw/d in females)<br>**Feed**<br>**90-d** | NOAEL: 32 mg/kg bw/d (400 ppm)<br>Haematological parameters: 0-100-400-1600-6400 ppm<br>**APTT (s)**:<br>M: 19.2-20.4-21.0-22.1-24.1<br>F: 16.4-16.8-16.2-15-8-15.6<br>**TT (s)**<br>M:25-26-26-27-30**)<br>F: 20-20-32-20-19*<br>**MCV (fL)**<br>M: 53.8-53.6-52.6-52.2*-52.2*<br>F: 56.3-55.4-55.2-55.1-53.1**<br>**PCV (%)**<br>M: 48.1-18.2-49.4-48.5-48.1<br>F: 46.8-46.5-45.7-45.7-44.8**<br>**Liver weights (g)**<br>0-100-400-1600-6400 ppm<br>M: 20.6-18.3-20.6-20.0-23.0*<br>F: 11.1-10.1-11.1-11.9-14.5*<br>6400 ppm: liver (wt ↑: approx. 11%; hepatocyte hypertrophy, periportal fat deposition, blood protein levels ↑), thyroid (rel. wt↑(F) : approx. 17 %; follicular epithelial hypertrophy)<br>1600 ppm: liver (periportal fat deposition in females), haematology: prolonged APTT in males (+15 % *vs*. control) | Anonymous, 1997i |

*p < 0.05; **p < 0.01

APTT: activated partial thromboplastin time, TT: thrombin time, MCV: mean corpuscular volume (erythrocytes), PCV: packed cell volume (erythrocytes)

In the rat 28-day study, general toxicity (i.e. lower body weight gain) was seen in the mid- and top–dose group. Liver weights were statistically significantly increased in males and females of the mid- and top-dose groups, while thyroid weight was only increased in females in these two dose groups. These observations occurred at doses above the upper guidance value for STOT RE 2 (300 mg/kg bw/day for 28-day studies). Periportal hepatocyte eosinophilia with clumping and follicular cell hypertrophy were seen in all dose groups but were not considered severe enough to support a classification as STOT RE 2.

Also in the 90 day study, the main target organs of toxicity were liver and thyroid. At the top dose (490 mg/kg bw/day in males, 525 mg/kg bw/day in females) liver weights were increased in both sexes (by approx. 11% relative to controls) and hepatocyte hypertrophy and periportal fat deposition were observed. In addition, blood parameters related to liver toxicity were affected: increases in blood protein levels, the TT value was increased in males, but decreased in females and the APTT was also increased in males (indicating prolonged blood coagulation time).

Thyroid weight was also increased in females (by approx. 17%) and follicular epithelial hypertrophy was described, but no other related parameters were affected. No studies were available that investigated whether the observed thyroid effects were secondary to liver enzyme induction, however, the increased liver weight might be an indication of a link.

At the next lower dose (123 mg/kg bw/day in males, 135 mg/kg bw/day in females) an increase in the incidence and severity of periportal fat deposition was only seen in females, slightly increased blood protein levels were seen in both sexes and prolonged APTT occurred in males only. No thyroid effects were seen at that dose. This dose is clearly above the relevant guidance value of 100 mg/kg bw/day for STOT RE 2. Although the gap to the next lower dose is rather large (32 and 36 mg/kg bw/day in males and females, respectively), the observed fat deposition in females only is not considered supportive for a classification as STOT RE 2, considering further that a decrease in the effect is assumed for a dose of 100 mg/kg bw/day and lower.

At the two top doses slight effects on red blood cells, MCV and PCV were reported, however, these effects were not severe and occurred at dose levels above the relevant guidance values.

In the carcinogenicity section of the CLH report two carcinogenicity studies in the rat (Anonymous, 2000a) and mouse (Anonymous, 1996e) are presented. The mouse study tested a formulation (NeemAzal-F 5%). No indication of toxicity was seen in either study, except some indications for prolonged blood coagulation time in male rats at the top dose of 448 mg/kg bw/day, after 190 and 360 days (not statistically significant). In the rat study, comparable doses to those in the 90 day study were tested. The lack of any relevant toxicity might be explained by the use of a different rat strain (Rat, Crt: CD (SD) BR in the 90-day study vs. Wistar rat in the carcinogenicity study). For further details see the section on carcinogenicity. In the absence of any relevant toxicity findings in these two studies they do not support a STOT RE classification.

The presented data on repeated dose studies in farm animals exposed to different plant extracts of the Neem tree are not considered to have a strong impact on the conclusion, but also indicate that there is not remarkable target organ toxicity.

Overall RAC concurs with the DS's proposal and supports **no classification for STOT RE.**

## RAC evaluation of germ cell mutagenicity

### Summary of the Dossier Submitter's proposal

#### *In vitro studies*

The DS presented three *in vitro* studies with *Margosa Extract with water*, one AMES test (Jones & Gant, 1997), one HPRT gene mutation study in CHO cells (Admans & Kirkpatrick, 1997) and a chromosomal aberration test in human lymphocytes (Stien, 2006).

While the two gene mutation studies were negative, the chromosomal aberration (CA) test in human lymphocytes was positive at cytotoxic concentrations (lower mitotic index at concentrations ≥ 2500 µg/mL, at these concentrations the test compound precipitated) without enzymatic activation (-S9) and negative with enzymatic activation (+S9) (for details see the table below).

***Table**: In vitro mutagenicity studies with Margosa Extract with water (table from CLH report)*

| **Test system / Test material** | **Test object** | **Concentration** | **Results**<br>**Test compound** | **Reference**<br>**Method** |
|---|---|---|---|---|
| Ames test /<br>***Margosa Extract with water* (37 % Azadirachtin A)** | *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538 | 50-5000 µg/plate | Non mutagenic (+/- S9) | Jones & Gant, 1997 TOX9700511<br>OECD 471 |
| CA /<br>***Margosa Extract with water* (37 % Azadirachtin A)** | Cultured human lymphocytes | 312.5-5000 µg/mL | Clastogenic (- S9) at cytotoxic concentrations, non-clastogenic (+ S9) | Stien, 2006 TOX2006-739<br>OECD 473 |
| HPRT gene mutation /<br>***Margosa Extract with water* (37 % Azadirachtin A)** | CHO cells | (25)200-1250 µg/mL | Non mutagenic (+/- S9) | Adams & Kirkpatrick, 1997 TOX9700512<br>OECD 476 |

#### *In vivo* studies

*Margosa Extract with water* was also tested in an *in vivo* bone marrow mouse micronucleus study. No increase in micronucleated erythrocytes was observed, despite the slight effect on the ratio of polychromatic to normochromatic erythrocytes (which indicated that the bone marrow was exposed to the test substance).

***Table**: In vivo mutagenicity study with Margosa Extract with water (table from CLH report)*

| **Test system / Test material** | **Method** | **Route of administration** | **Dose levels** | **Result** | **Reference** |
|---|---|---|---|---|---|
| Mice, CD-1 /<br>***Margosa Extract with water* (azadirachtin A: 27 %)** | Micronucleus test, bone marrow | Gavage (1 % methyl cellulose) | 0, 1250, 2500, 5000 mg/kg bw | Non genotoxic | Anonymous, 1997g |

Two further studies with other technical extracts also did not show mutagenic potential in respective bone marrow micronucleus studies in mice ("Fortune Aza", "NPI 720"). No further information presented in the CLH report.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

Based on the overall negative test results from *in vitro* and *in vivo* studies there was no evidence for a mutagenic potential of *Margosa Extract with water*.

The slight indication for clastogenicity at cytotoxic concentrations *in vitro* (chromosomal aberration test) could not be confirmed in the *in vivo* bone marrow micronucleus study. Although the test material in the latter had a slightly lower Azadirachtin A content, this is not considered relevant, as a specific relevance of this specific constituent for the investigated effect is not known or demonstrated.

On that basis RAC agrees with the DS's conclusion that **classification for germ cell mutagenicity is not warranted**.

## RAC evaluation of carcinogenicity

### Summary of the Dossier Submitter's proposal

The DS presented two studies, one two-year carcinogenicity study in rats (Anonymous, 2000a) with *Margosa Extract with water* and a mouse carcinogenicity study (Anonymous, 1996e) with the formulation NeemAzal-F 5%.

Although the top doses applied in the rat carcinogenicity (448 mg/kg bw/day in males, 635 mg/kg bw/day in females) were comparable to those used in the 90 day rat study (490 mg/kg bw/day in males, 525 mg/kg bw/day in females), no comparable toxicity was seen in the carcinogenicity study. No increase in tumour incidence or related findings (hypertrophy) was observed, with the only finding being a slightly prolonged coagulation time in males at the top dose (not statistically significant). A slight decrease in survival in all the dosed groups was not considered treatment related (See table below).

The DS also referred to the OECD Guidance Document 116 (Guidance on the conduct and design of chronic toxicity and carcinogenicity studies, supporting OECD 451, 452 and 453, OECD 2014) and noted that the top dose of the rat carcinogenicity study (Anonymous, 2000a) did not fulfil the criteria for an MTD (maximum tolerable dose) described in that document.

In addition, an expert consultation within the framework of the PPP process resulted in the conclusion that the study quality was questionable, especially as no effects were seen including at the highest dose tested. There were uncertainties with regard to the specification of test material and no tumours or hypertrophy was seen in any of the control animals over 2 years. They concluded that only limited information on long-term toxicity and carcinogenicity can be drawn from the study.

In contrast, in the biocides framework (Dir 98/8/EC) the study was considered reliable and it was considered that the difference between the 90 day and the carcinogenicity study in observed toxicity could be explained by the different strains of rat used in these studies. As up to half the limit dose was tested, it was concluded in the biocides framework that the top dose was sufficiently high. They classified the study as Klimisch 2 based on minor deficiencies (see above) and because the conducting laboratory had no GLP status.

The mouse carcinogenicity study (Anonymous, 1996e) was carried out with the formulation NeemAzal-F 5% (contains approx. 20% *Margosa Extract with water* and 80% polyethylene oxide) and this did not demonstrate any carcinogenic or histopathological findings up to the top dose (63 mg/kg bw/day in males, 72 mg/kg bw/day in females). No other effects were described and the top dose was considered to be the NOAEL. As the content of *Margosa Extract with water* was only 20%, the notifier under the Biocidal Products Regulation proposed to use a correction factor of 5, resulting in a NOAEL of 12.6 mg/kg bw/day.

No studies were available for any other formulation.

***Table**: Overview of the available carcinogenicity studies (from the CLH report)*

| **Animal species & strain / Test material** | **Number of animals** | **Doses, vehicle, duration** | **Results** | **Reference**<br>**Method** |
|---|---|---|---|---|
| Rat, Wistar /<br>*Margosa Extract with water* (37 % Azadirachtin A) | 50 M & 50 F | 0, 400, 1600, 6400 ppm (0, 29, 114, 448 mg/kg bw/d in males; 0, 38, 167, 635 mg/kg bw/d in females)<br>**Feed**<br>**7 d/wk; 105-wks** | NOAEL: 448 mg/kg bw/d (6400 ppm) No toxic effects reported.<br>Slightly increased (not significant) coagulation time observed in medium and high dose in male rats.<br>Gross Pathology:<br>Rounded or irregular growths in the teat region in females: (at 0-400-1600-6400ppm, respectively) 2-1-3-3.<br>Males: 2 tumours in the lower abdomen (6400, 400 ppm), 1 tumour in the prostate (6400 ppm). No carcinogenic effects reported (observed tumours were considered incidental):<br>Tumour rates: (at 0-400-1600-6400ppm, respectively):<br>Mammary tumours:<br>F: 2-1-3-3<br>Lymphosarcoma:<br>M: 0-1-0-1<br>Prostatic carcinoma:<br>M: 0-0-0-1<br>Death rates were increased in all treatment groups but were considered not treatment related.<br>**Number of Deaths**: (at 0-400-1600-6400ppm, respectively):<br>M: 4-6-3-10<br>F: 1-5-5-5 | Anonymous, 2000a (clinical chemistry performed)<br>Gaitonde Committee Guideline 6.3.0.C.iv. – corresponds to OECD TG 452<br>GLP |
| Mouse, Swiss albino /<br>NeemAzal-F 5 % (formulation, 5 % Azadirachtin A content) | 50 M & 50 F | 0, 100, 300, 1000 ppm (0, 6.6, 18.4, 63 mg/kg bw/d in males; 0, 7.0, 21, 72 mg/kg bw/d in females)<br>**Feed**<br>**18-mo** | NOAEL: 63 mg/kg bw/d (1000 ppm)<br>No toxic effects reported. No carcinogenic effects reported | Anonymous, 1996e (feed analysis not performed, clinical signs not reported)<br>Gaitonde Committee Guideline 6.3.0.C.iv. – corresponds to OECD TG 452<br>GLP |

Based on the available results the DS did not propose a classification of *Margosa Extract with water* as carcinogenic, however, they concluded that the studies had limitations and did not enable a firm conclusion to be drawn.

### Comments received during consultation

No comments were received during consultation.

### Assessment and comparison with the classification criteria

RAC agrees with the DS's analysis of the available data. In the rat carcinogenicity study the MTD was not achieved and other limitations (uncertainties with regard to the specification of test material, no evidence of tumours or hypertrophy in any of the control animals in 2 years, discrepancy with the results observed in the 90 day study – despite the different rat strains used in these studies) were also described by the DS. Although the top dose made up for half the limit dose, the study was not conducted in line with the OECD guidance document 116 (conduct of carcinogenicity studies). On that basis RAC is of the opinion that no firm conclusion can be drawn from the rat carcinogenicity study.

In the mouse study only a formulation was tested. The applied doses were very low and the formulation only had a concentration of 20% *Margosa Extract with water*. No signs of toxicity or carcinogenicity were observed, but the applied doses were clearly below those recommended for a carcinogenicity study (MTD not reached).

RAC notes that the available studies do not indicate any carcinogenic potential, but the available data are limited and have several deficiencies. Consequently, RAC proposes **no classification of *Margosa Extract with water* for carcinogenicity due to inconclusive data.**

## RAC evaluation of reproductive toxicity

### Summary of the Dossier Submitter's proposal

#### *Adverse effects on sexual function and fertility*

The DS presented a dietary rat two-generation study with *Margosa Extract with water* (Anonymous, 2000b), testing doses up to 50.7 mg/kg bw/day in males and 59.6 mg/kg bw/day in females. No effects on sexual function and fertility were observed (for effects on offspring see the section on developmental toxicity). Some organ weights were affected and the number of live pups was reduced in the P1 generation. However, as these observations were either not dose related or were not repeated in subsequent generations, the DS did not consider them as adverse.

In addition there were some cases of tubular hypoplasia and hyperaemia in the testes in the P0 generation and tubular atrophy and focal interstitial oedema in the testis in the P1 generation and hyperaemia of the uterus in P1 females of the mid and top dose, but incidences of these findings in the P0 and P1 generation were low and single cases of these observations were also seen in the respective controls (see Assessment and Comparison with Classification Criteria).

The DS concluded that there were no treatment related effects and a NOAEL at the top dose of 750 ppm (51 mg/kg bw/day in males, 60 mg/kg bw/day in females) was derived.

Another rat two-generation reproduction study (Anonymous, 1996d) in which the formulation NeemAzal-F 5% (containing 20% *Margosa Extract with water* in 80% polyethylene oxide, resulting in a concentration of approx. 5% w/w Azadirachtin A) was tested, was judged as "not

acceptable" by the DS (no data on feed analysis, time to fertilisation or duration of gestation was reported). In this study relative weights of ovaries and spleen were increased in maternal animals at all doses (approx. 13 – 333 mg/kg bw/day). Bodyweights of the mid- and top-dose animals were reduced, but no effects on sexual function and fertility were reported (for effects on offspring see section on developmental toxicity).

A third study (Anonymous, 2000c), a one-generation study, was mentioned and judged as "not acceptable", but no information on this study was presented.

The DS also reported various findings with respect to fertility or reproduction from the open literature. However, the DS noted that these reports cover different compounds (other extraction methods, other starting material, etc.) and are therefore not relevant for the *Margosa Extract with water* in focus of the present evaluation.

Based on the absence of effects on reproductive organs in repeated dose studies (rat 28 day and 90 day studies, section specific target organ toxicity, repeated exposure) and no effects on reproduction and fertility in a two generation study of acceptable quality (Anonymous, 2000a), supported by the absence of effects in a two-generation and a one-generation study of low quality (not acceptable), the DS concluded that no classification of *Margosa Extract with water* for effects on sexual function and fertility was necessary.

***Developmental toxicity***

The DS presented a developmental toxicity study in rats conducted according to OECD TG 414 (Anonymous, 1997f) as well as the respective dose-range finding study (Anonymous, 1997e). In addition, the DS considered the relevant results for the assessment of developmental toxicity from the two-generation studies (Anonymous, 2000b and Anonymous 1996d, with Neem Azal F 5%).

The DS also presented developmental toxicity studies with other Neem tree extracts, including a study in rabbits with the extract "ATI 720", which was described as toxic to dams and foetuses and a study in rats, which tested the extract "Fortune Aza" which gave similar results as the rat study with *Margosa Extract with water* (Anonymous, 2000b). No further information was provided on these studies.

The DS considered the study by Anonymous (1997e, f) to be the most relevant for the assessment of developmental toxicity. In this study slight maternal toxicity was observed at the mid and top dose, which included minor effects on body weight gain, feed intake and water consumption. While in the preliminary study (Anonymous, 1997e) no effects on foetuses were seen up to the dose of 1000 mg/kg bw/day (though there were only 10 F per dose group and only external morphology examinations were conducted), in the main study an increased incidence of malformations (among other findings: interventricular septal defects, malrotated heart in the mid- and top-dose groups and increased incidence of supernumerary ribs in the top dose group, see table "Visceral malformations and anomalies") was observed.

The DS reported that an expert consultation within the framework of the PPP process concluded that the maternal (reduced body weight) and supernumerary ribs in foetuses of the top dose group were relevant findings and set the maternal and foetal NOAELs at the mid dose. However, as these findings were considered to be of low incidence the majority of the experts voted against classification for developmental toxicity.

The DS considered the observed developmental effects as dose related and adverse. Although only one litter was affected by heart associated malformations (interventricular septal effects and malrotated heart were classified as malformations, and in addition haemorrhagic thyroid and subcutaneous oedema was described in this litter) at the mid dose, where no adverse effects on

the dams were observed, this was not considered an isolated finding. The same and further heart-related malformations were seen at the top dose, where slight maternal toxicity was evident (for details on maternal toxicity see table "Maternal body weight / body weight changes, Anonymous (1997f)" and related text). Therefore the DS proposed to classify *Margosa Extract with water* as Repr. 2; H361d.

***Lactation***

The DS summarised that there were no data available to assess whether there are specific effects on or via lactation (H362). Under the conditions of the two-generation study (Anonymous, 2000b), no effects on any of the investigated parameters were reported up to the highest dose tested. On that basis the DS did not propose a classification for lactation.

## Comments received during consultation

During the consultation, three general comments were received by two Companies/Manufacturers and an individual. Their comments mainly concerned the substance identity and that substances that also cover the presently evaluated *Margosa Extract with water* are currently approved under different regulatory frameworks and that the classification process should be aligned with other regulatory processes.

The DS clarified that the present CLH report covers a clearly defined extract of the neem tree (both regarding material used and extraction method) and the CLH process is independent from the other cited processes. RAC agrees with this response.

Six companies, a trade organisation and a non-governmental organisation commented on the proposed classification as Repr. 2, H361d. In their comments they argued against the classification proposal. The main arguments were that several organisations, including EFSA and US EPA, had conducted risk assessments and had concluded that certain Neem tree extracts did not pose a risk regarding reproductive toxicity.

The DS responded that hazard and risk assessment are not the same and that the present CLH proposal covers a specific Neem tree extract, and assesses the studies relevant for this specific extract. In this regard the present CLH proposal only considered those studies that are relevant for this extract.

The commenters also referred to additional studies, e.g. a developmental toxicity study in rabbits via the dermal route, but this study was not submitted, hence the relevance to the present CLH proposal could therefore not be assessed. It is further noted that the classification proposal for Category 2 is based on developmental toxicity observed in rats, after oral application, hence a negative study in rabbits via the dermal route would not overrule the findings in a different species with a different route of application.

In addition, the commenters did not agree with the analysis of the available animal study. They were of the view that the effects were only marginally increased and occurred in the presence of maternal toxicity only.

The DS considered the mid dose to be a dose without maternal toxicity and the observed heart related malformations at this dose as relevant findings, mainly because the same and further heart related effects were also seen at the top dose. In addition, the DS was of the view that there was no evidence that would demonstrate that the observed developmental effects seen at the top dose were secondary non-specific consequence of the slight maternal toxicity.

One company manufacturer further commented, that if a classification as Repr. 2; H361d was agreed, a specific concentration limit above the generic concentration limit should be set, as they were of the view that the ED10 value was above 400 mg/kg bw/day (low potency group).

The DS responded that the available data set was of insufficient quality to enable a reliable derivation of an ED10 value and referred to in the Guidance on the application of the CLP criteria, version 5.0, July 2017 (hereafter "CLP Guidance") ), which states (section 3.7.2.6.2) that "*if the classification of a substance in Category 2 is done on the basis of 'limited evidence', the quality of the available data will in such cases determine whether a potency assessment is possible. In cases where no further evaluation is possible, the generic concentration limits* [GCL] *of CLP apply.*"

In addition, some of the comments received pointed out that Neem tree extracts are highly popular, traditional botanicals and used for multiple purposes over hundreds of years, without any evidence that the use could lead to damage to the unborn child. RAC notes that no reliable epidemiological study was provided that would allow a thorough assessment of developmental effects of these extracts in humans. The fact that Neem tree extracts are considered to be rather diverse regarding their composition (depending on source material as well as extraction method applied) further complicates an assessment of potential effects of these extracts in humans.

One company provided further historical control data (HCD) from 24 developmental toxicity studies, conducted in the same laboratory that had carried out the developmental toxicity study Anonymous (1997e, f). These data were used for the current assessment.

## Assessment and comparison with the classification criteria

### *Adverse effects on sexual function and fertility*

The DS presented a two-generation study with acceptable quality (Anonymous, 2000b), as well as a two-generation (Anonymous, 1996d) and a one-generation study (Anonymous, 2000c), both judged as "not acceptable" by the DS.

Details of Anonymous, 2000b and 1996d are presented in the table below (no details on Anonymous, 2000c were presented in the CLH report).

***Table**: Summary of Anonymous 2000b and 1996d (adapted from the CLH report)*

| **Animal species & strain / Test material** | **Number of animals** | **Doses, vehicle, duration, guideline** | **Results** | **Reference** |
|---|---|---|---|---|
| Rat, Wistar / ***Margosa Extract with water* (37.3 % Azadirachtin A)** | 10 M & 20 F | 0, 250, 500, 750 ppm (0, 16.8, 34, 50.7 mg/kg bw/d in males; 0, 19.9, 38.9, 59.6 mg/kg bw/d in females)<br>Feed<br>Equivalent to OECD 416<br>GLP | Parental: No effects on parents. NOAEL: 50 mg/kg bw/d (750 ppm)<br>- statistically significant reduction in relative organ weights (testis, brain, heart), no dose-response, only P0)<br>Offspring:<br>- low incidence histopathological findings in the testis & uterus in first litters of P0 only (also seen in controls)<br>- reduced number of live pups only in the first litter of P1<br>Reproductive: No effects on reproduction NOAEL: 50 mg/kg bw/d (750 ppm) | Anonymous, 2000b<br>(no data on feed analysis, time to fertilisation not reported)<br>for more details, see table "Overview on organs weight, rat two-generation study" below. |

| | | | | |
|---|---|---|---|---|
| Rat, Charles Foster /<br>**NeemAzal F 5 %** | 10 M & 20 F | 0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d)<br>Feed<br>2-gen. study<br>Similar to OECD TG 416<br>GLP status unknown | Parental: spleen, ovary wt ↑, bw ↓ LOAEL: appr. 13 mg/kg bw/d (200 ppm)<br>Reproductive: No effects on reproduction NOAEL: appr. 333 mg/kg bw/d (5000 ppm) | Anonymous, 1996d<br>(no data on feed analysis, time to fertilisation and duration of gestation not reported) |

***Table**: Overview on organs weight, rat two-generation study (Anonymous, 2000b) (from the CLH report); F0 males*

| ***Absolute values*** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Dose level (ppm)** | **Fasted body-weight (g)** | **Liver (g)** | **Brain (g)** | **Kidney§ (g)** | | **Heart (g)** | **Adrenal§ (mg)** | | **Testis § (g)** | |
| 0 | 273.8 | 10.59 | 1.79 | 0.99 | 0.99 | 0.93 | 31 | 33 | 1.48 | 1.47 |
| 250 | 300.0 | 11.20 | 1.82 | 1.02 | 1.02 | 0.91 | 32 | 33 | 1.46 | 1.47 |
| 500 | 287.3 | 10.77 | 1.79 | 1.04 | 1.04 | 0.93 | 33 | 34 | 1.46 | 1.45 |
| 750 | 310.4 | 11.61 | 1.84* | 1.05 | 1.02 | 0.92 | 34* | 33 | 1.48 | 1.49 |
| ***Relative values*** | | | | | | | | | | |
| **Dose level (ppm)** | | **Liver (%)** | **Brain (%)** | **Kidney§ (%)** | | **Heart (%)** | **Adrenal§ (%)** | | **Testis § (%)** | |
| 0 | | 3.86 | 0.66 | 0.36 | 0.36 | 0.34 | 0.011 | 0.012 | 0.54 | 0.54 |
| 250 | | 3.74 | 0.62 | 0.35 | 0.35 | 0.31 | 0.011 | 0.011 | 0.49 | 0.50 |
| 500 | | 3.75 | 0.62 | 0.36 | 0.36 | 0.32 | 0.012 | 0.012 | 0.51* | 0.51* |
| 750 | | 3.73 | 0.59** | 0.34 | 0.34 | 0.30** | 0.011 | 0.011* | 0.48** | 0.48** |

*, $p < 0.05$; **, $p < 0.01$; §, left and right organs

In Anonymous (2000b) some effects on organ weights, some low incidence histopathological changes which were also seen in respective controls and reduced number of live pups (in the litters from the first mating of the P0 generation), were reported.

These effects were seen at low incidence (histopathological findings, see table "Overview on histopathological findings in animals of different generations") and as they did not show a dose-response relationship (organ weight effects, see table "Overview on organs weight, rat two-generation study (Anonymous, 2000b)") and/or were not repeated in subsequent litters of the same generation or subsequent generations (histopathological findings, effects on organ weights, reduced number of live pups), RAC agrees with the DS's conclusion that the study does not demonstrate adverse effects on reproductive function and fertility.

In addition, no effects on reproductive organs were seen in the repeated dose toxicity studies (see the STOT RE section).

***Table**: Overview on histopathological findings in animals of different generations*

| **Dose (ppm)** | **0** | **250** | **500** | **750** |
|---|---|---|---|---|
| **P0 generation** | | | | |
| Tubular hypoplasia | 1 | - | - | 2 |
| Hyperaemia in testes | - | - | - | 3 |
| **P1 generation** | | | | |
| Tubular atrophy & focal interstitial oedema | 1 | 1 | 2 | 2 |
| Hyperaemia of the uterus | 1 | - | 2 | 3 |
| **F2b generation** | | | | |
| No gross pathology or histopathological findings | | | | |

It is noted that the study has some drawbacks, including that time to fertilisation was not determined and that feed analysis was not performed. No information on the stability of the *Margosa Extract with water* in feed was found in the CLH report. In addition RAC considers the

top dose of 50.7 mg/kg bw/day in males and 59.6 mg/kg bw/day in females, which was the parental NOAEL, rather low and concludes that higher doses could have been tested.

The second two generation study (Anonymous, 1996d) is considered less relevant for the assessment as it tested the formulation Neem Azal-F 5%. Although this formulation contains only 20% *Margosa Extract with water*, higher toxicity was observed, even at the lowest dose of 13 mg/kg bw/day (equivalent to 2.6mg/kg bw/day *Margosa Extract with water*). The DS judged this study as "not acceptable" and mentioned that no data on feed analysis, time to fertilisation and duration of gestation were presented. Overall the study is considered of minor relevance for the assessment of reproductive toxicity.

RAC notes that studies from the open literature indicate adverse effects on fertility and on reproduction, however, as pointed out by the DS, supported by the PPP expert group, they were conducted with Neem tree extracts different from the one presently under investigation. RAC agrees with the DS that these results have no influence on the assessment of *Margosa Extract with water*.

In line with the DS, RAC is of the view that the observed effects do not warrant classification for sexual function and fertility, but the available data are limited and have several deficiencies. Consequently, RAC proposes **no classification of *Margosa Extract with water* for classification for sexual function and fertility due to inconclusive data**.

### *Developmental toxicity*

In the table below, the relevant studies for the assessment of developmental toxicity are described.

***Table**: Studies relevant to assess developmental toxicity (adapted from the CLH report).*

| **Reference / Test material** | **Protocol Species** | **Doses** | **Maternal effects Test compound** | **Developmental effects** |
|---|---|---|---|---|
| Anonymous, 1997e / ***Margosa Extract with water* (36.7 % Azadirachtin A)** | OECD 414, pre-study (only 10 F per dose group, only external morphology examination)<br>Rat, Crl:CD BR VAF/plus | 0, 100 ,300, 1000 mg/kg bw/d | 300, 1000 mg/kg bw/d: Bw ↓, feed intake (only 1000) ↓, post-dosage salivation<br>NOAEL: 100 mg/kg bw/d | No effects on foetuses<br>NOAEL: 1000 mg/kg bw/d |
| Anonymous, 1997f / ***Margosa Extract with water* (36.7 % Azadirachtin A)** | OECD 414, main study<br>Rat, Crl:CD BR VAF/plus<br>- Gavage (vehicle: 1% methylcellulose)<br>- Exposure: GD 6-19 | 0, 50, 225, 1000 mg/kg bw/d | 1000 mg/kg bw/d: Bw ↓, feed intake ↓, post-dosage salivation<br>NOAEL: 225 mg/kg bw/d | 255 mg/kg bw/d: Malformations (cf. Table 36), supernumerary ribs (only 1000)<br>NOAEL: 50 mg/kg bw/d |
| Anonymous, 2000b / ***Margosa Extract with water* (37.3 % Azadirachtin A)** | Similar OECD TG 416 (no data on feed analysis, time to fertilisation not reported)<br>for more details, see section 4.10.1.1<br>OECD 416<br>Rat | 0, 250, 500, 750 ppm (0, 16.8, 34, 50.7 mg/kg bw/d in males; 0, 19.9, 38.9, 59.6 mg/kg bw/d in females)<br>Feed | Parental: No effects on parents NOAEL: 50 mg/kg bw/d (750 ppm) | Developmental: No effects on offspring NOAEL: 50 mg/kg bw/d (750 ppm)<br>- low incidence histopathological findings in testis & uterus in first litters of P0 only (also seen in control)<br>- reduced number of live pups only in the first litter of P1 |

| | | | | |
|---|---|---|---|---|
| Anonymous, 1996d /<br>**NeemAzal F 5 %** | Similar OECD TG 416 (no data on feed analysis, time to fertilisation and duration of gestation not reported)<br>2-gen. study<br>Rat | 0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d)<br>Feed | Parental: spleen, ovary wt ↑, bw ↓ LOAEL: appr. 13 mg/kg bw/d (200 ppm) | Developmental: No effects on offspring NOAEL: appr. 333 mg/kg bw/d (5000 ppm) |

This table does not include information on the developmental toxicity study in rabbits which tested "ATI 720" or on the rat developmental study with "FortuneAza". It is noted that extracts "FortuneAza" or "NPI720"/"ATI 720" which are also technical extracts of seed kernels of Neem tree are obtained by a different extraction procedure and therefore are not directly relevant to the present evaluation.

- The information on the developmental toxicity study in rabbits on "ATI 720" (equivalent to OECD 414) presented in the CLH report is quite limited (CLH report, p47, 48). No reference is given in the CLH report, however, based on the study description and the formulation tested (i.e. "ATI 720") it appears that Anonymous (1994) described in the CAR (2006) is the respective study.
  New Zealand White rabbits (16-17 animals per group) were gavage dosed (0, 20, 100 & 500 mg/kg bw/day) from GD 6 - 18. Considerable effects on maternal weight / weight gain were seen at the top dose, but also at the mid dose (NOAEL maternal = 20 mg/kg bw/day), while developmental toxicity was only seen at the top dose and consisted of significantly reduced foetal weight, number of live foetuses, number of viable litters and significantly elevated number of *in utero* deaths.
  RAC concludes that this study gives no support for a classification for developmental toxicity, as the applied test material differs considerably from *Margosa Extract with water* and it is noted that the Azadirachtin A concentration of ATI 720 is only about a quarter of that in *Margosa Extract with water* (i.e. ~ 9%).
- Another developmental toxicity study in rats is mentioned in the CLH report which tested "Fortune Aza" (CLH report, p47). It was concluded that the results were comparable to *Margosa Extract with water* with respect to maternal toxicity and no developmental effects on foetuses were observed. No further information was presented or could be located in the CAR report.

No relevant findings on the offspring were reported in the two two-generation studies presented in the table above, one of them (Anonymous, 1996d) was considered not acceptable for the evaluation of *Margosa Extract with water*, the other study (Anonymous, 2000b) tested much lower doses than Anonymous (1997e,f) (for details, see the section on fertility and reproductive performance).

Several studies from the open literature also investigated developmental toxicity of different Neem tree extracts in rat. While some of them did not observe any adverse effects on development, Dallaqua *et al.* (2013) described an increase in visceral malformations in rat foetuses upon *in utero* exposure to Neem seed oil, which was not seen with an azadirachtin solution. As these studies cover different Neem tree extracts they are not considered relevant for the present opinion.

Consequently RAC focussed on the assessment of the developmental toxicity study in rats (Anonymous, 1997e,f).

Maternal toxicity

In line with the DS, RAC is of the opinion that adverse effects on dams were seen at the top dose, but the mid dose can be considered the maternal NOAEL. In the following table the maternal body weight and body weight changes are listed.

While final body weights were comparable among all groups, some decrease in body weight gain was seen in the mid and top dose groups, which was statistically significant in the top dose between days 6 - 8. The reduced weight gain was accompanied by reduced food consumption on days 6 and 7.

***Table**: Maternal body weight / body weight changes, Anonymous (1997f) (table from the CLH report)*

| **Dose level (mg/kg bw/d)** | **0** | **50** | **225** | **1000** |
|---|---|---|---|---|
| Number of animals § | 23 | 23 | 23 | 23 |
| Weight gain (g) on days 2 - 6 | 40.1 | 39.9 | 36.9 | 34.3 |
| Weight gain (g) on days 6 - 8 | 10.4 | 10.5 | 8.5 | 6.1** |
| Weight gain (g) on days 8 - 20 | 133.1 | 143.8 | 138.7 | 143.0 |
| Final bodyweight | 408.7 | 420.3 | 409.7 | 408.1 |

**, p < 0.01; §, excluding non-pregnant animals

Other signs of maternal toxicity were increased salivation 1 hour after dosing in all top dose animals and 4 animals of the mid dose group. Top dose animals further showed increased water consumption. No other clinical signs were described.

***Development***

***Table**: Visceral malformations and anomalies (adapted from the CLH report)*

| **Visceral findings** | | **Dose level (mg/kg bw/day)** | | | | **HCD [1]** | **HCD [2]** |
|---|---|---|---|---|---|---|---|
| | | **0** | **50** | **225** | **1000** | | |
| Number of fetuses examined viscerally | | 152 | 159 | 152 | 151 | 1690* | 3542 |
| Number of fetuses examined, total | | 305 | 323 | 306 | 308 | - | 7112 |
| Number of (litters) examined | | (23) | (23) | (23) | (23) | (257) | (553) |
| **Thoracic malformations** | Malformed systemic / pulmonary arteries | 0 (0) | 0 (0) | 0 (0) | 1 (1)[a] | - | - |
| | Atrial septal defect with narrow pulmonary vein | 0 (0) | 0 (0) | 0 (0) | 1 (1)[a] | - | - |
| | Interventricular septal defect | 0 (0) | 0 (0) | 1 (1)[f]<br>0.7%<br>0.33%<br>(4.4%) | 2(2)[a,b]<br>1.3%<br>0.65%<br>(8.7%) § | 0 – 0.6%<br>(0 – 4.1%) | 0 – 1.5%*<br>0 – 0.74%**<br>(0 – 8.3%) *** |
| | Malrotated heart | 0 (0) | 0 (0) | 1 (1)[f, §] | 1 (1)[a,§] | - | 0 (0) |
| | Duplicated inferior vena cava | 0 (0) | 0 (0) | 0 (0) | 2 (2)[b,c]<br>1.3%§<br>0.65%§<br>(8.7%) § | - | 0 – 0.6%*<br>0 – 0.3%**<br>(0 – 4.4%) *** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Thoracic anomalies** | Anomalous cervico-thoracic arteries | 1 (1) | 0 (0) | 0 (0) | 0 (0) | - | - |
| | Interventricular septal defect, small | 0 (0) | 1 (1)<br>0.63%<br>0.3%<br>(4.4%) | 3 (3)[g,h,i]<br>2%<br>0.98%<br>(13%) | 2 (2)[d,e, ≠]<br>1.3%<br>0.7%<br>(8.7%) | 0 – 2,1%<br>(0 – 13.6%) | 0 – 2.3%*<br>0 – 1.1%**<br>(0 – 13.6%) *** |
| **Thoracic malformations & anomalies together** | Interventricular septal defect & Interventricular septal defect, small | 0 (0) | 1 (1)<br>0.6%<br>0.3%<br>(4.4%) | 4 (4)[f,g,h,i]<br>2.6%<br>1.3%<br>(17.4%) § | 4 (4)[a,b,d,e, ≠]<br>2.7%<br>1.3%<br>(17.4%) § | - | 0 – 3.1%*<br>0 – 1.5%**<br>(0 – 16.7%) *** |

a: litter 88; b: litter 84, c: litter 80, d: litter 85, e: litter 98, ≠ an additional litter (litter 98) with small interventricular septal defect was discounted here because mottled foetus syndrome occurred, f: litter 63, g: litter 65, h: litter 68, i: litter 74

* Based on foetuses examined, visceral; ** Based on foetuses / litters examined (total); *** in all litters about half the foetuses examined viscerally

1 Huntington (1994 – 1995), 2 Huntington (1994 – 1997)

§ … indicates where HCDs are exceeded based on HCD 2

The historical control data (HCD) presented in the CLH report were from 11 studies conducted between July 1994 and February 1995 at the conducting laboratory (Huntington, 1994 – 1995). In this laboratory an interventricular septal defect (small) was classified as a visceral anomaly and an interventricular septal defect was classified as a visceral malformation. Interventricular septal defect (malformation) was recorded in only two studies of the eleven presented, in one foetus each, while interventricular septal defect (small, anomaly) was seen in 7 of the 11 studies.

During consultation of the CLH report, industry provided further HCD from the conducting laboratory. These HCD were provided by Envigo, the successor institute of the performing laboratory (Huntington). They consisted of 24 studies (including the 11 studies part of Huntington, 1994 – 1995) conducted between July 1994 and February 1997.

In these 24 studies interventricular septal defect (malformation) was seen in 4 studies, in 3 of which a single foetus showed the effect and in 1 study 2 foetuses of 2 litters had the effect. Based on all 24 studies, only the top dose incidences on a litter basis exceeded the HCDs, while based on Huntington, 1994 – 1995, the top dose incidences exceeded the HCDs on a foetus and on a litter basis.

In addition, Huntington (1994 – 1997) also covered incidences for malrotated heart and duplicated vena cava. No incidence of malrotated heart was seen in any of the 24 studies, therefore the observed cases in the mid- and top-dose (one case each) are above background incidence levels.

Duplicated vena cava was seen in 1 foetus of the 24 studies. The observed incidences in the top dose therefore exceed the HCDs on litter and foetus basis.

No data were presented for the other heart related malformations observed in the study (i.e. malformed systemic / pulmonary arteries, atrial septal defect with narrow pulmonary vein).

***Table**: Incidence of supernumerary rib 14 (from CAR 2006, described as skeletal variants in this document)*

| **Dose** | **Foetuses examined** | **Foetuses with** | | | |
|---|---|---|---|---|---|
| | | **13 ribs** | | **14 ribs** | |
| **mg/kg be/day** | **n** | **n** | **%** | **n** | **%** |
| 0 | 152 | 137 | 90.6 | 15 | 9.4 |
| 50 | 159 | 145 | 91.4 | 14 | 8.6 |
| 225 | 149 | 138 | 93.3 | 11 | 6.7 |
| 1000 | 149 | 114 | 75.7 | 35 | 24.3 |

Though not statistically significant, there was a clear increase in supernumerary rib 14 in the top dose (~ 2.5-fold increase compared to controls).

The HCD from Huntington (1994 – 1997), also provided incidences for supernumerary rib 14. In these data it was differentiated between full and short rib 14. In only 1 of the 24 studies full supernumerary rib 14 was seen in 2 foetuses from 1 litter (foetuses: 0 – 1.2%, litters: 4%). Short supernumerary rib was seen in all studies with incidences ranging from 4.5% - 20% in foetuses and 25 - 48% in litters. The full study report did not clearly state whether the incidences listed in the table "Incidence of supernumerary rib 14" were for full or short rib or for both effects together. Regarding the relative rareness of full additional rib 14 it might be concluded that the numbers presented in the table "Incidence of supernumerary rib 14" consider either both, incidences of short and full rib 14 together, or only short rib 14 incidences. Based on the available information no direct comparison with the provided HCD is possible.

***Table**: Skeletal and visceral malformations – incidence summary (from CLH report)*

**Skeletal and visceral malformations - incidence summary**

| | Group/dosage (mg/kg/day) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Foetuses | | | | Litters | | | |
| | 1 Control | 2 50 | 3 225 | 4 1000 | 1 Control | 2 50 | 3 225 | 4 1000 |
| No. examined | 305 | 323 | 306 | 308 | 23 | 23 | 23 | 23 |
| No. affected | 1 | 5 | 5 | 8 | 1 | 3 | 3 | 5 |
| REGION/Description | Incidence | | | | | | | |
| **CRANIAL** | | | | | | | | |
| Cleft palate | - | - | 1[c] | - | - | - | 1 | - |
| Brachygnathia with bridge of ossification mandibles | - | - | 1[c] | - | - | - | 1 | - |
| Misshapen basisphenoid | - | - | 1[c] | - | - | - | 1 | - |
| Partially fused occipital condyle to cervical vertebral arch | - | 1[a] | - | - | - | 1 | - | - |
| **CERVICAL** | | | | | | | | |
| Lordosis | - | - | - | 1[f] | - | - | - | 1 |
| Scoliosis, minimal | - | 1[a] | - | - | - | 1 | - | - |
| Fused/partially fused vertebral elements | 1 | 1[a] | - | - | 1 | 1 | - | - |
| **THORACIC** | | | | | | | | |
| Malformed systemic/ pulmonary arteries | - | - | - | 1[e] | - | - | - | 1 |
| Atrial septal defect with narrow pulmonary vein | - | - | - | 1[e] | - | - | - | 1 |
| Interventicular septal defect | - | - | 1[b] | 2[de] | - | - | 1 | 2 |
| Malrotated heart | - | - | 1[b] | 1[e] | - | - | 1 | 1 |
| Duplicated inferior vena cava | - | - | - | 2[d] | - | - | - | 2 |
| Diaphragmatic hernia | - | 4 | - | - | - | 2 | - | - |
| Distorted ribcage with thickened ribs | - | - | - | 1[f] | - | - | - | 1 |
| **LUMBAR/ABDOMINAL** | | | | | | | | |
| Umbilical hernia | - | 1[a] | - | - | - | 1 | - | - |
| **APPENDICULAR** | | | | | | | | |
| Forelimb flexure | - | - | - | 1[f] | - | - | - | 1 |
| Brachymelia with curved ulnae and radii | - | - | - | 1[f] | - | - | - | 1 |
| **OTHER** | | | | | | | | |
| Squat foetus syndrome | - | - | 3 | - | - | - | 1 | - |
| Mottled foetus syndrome | - | - | - | 4 | - | - | - | 1 |

Superscripts indicate findings common to one foetus

In line with the analysis carried out by the DS, RAC considers the observed visceral malformations and anomalies related to the heart as evidence for developmental toxicity. Though only one litter and foetus was affected at the mid dose (interventricular septal effects and malrotated heart were classified malformations, in addition haemorrhagic thyroid and subcutaneous oedema were described in this litter), where no maternal toxicity was observed, the same and further heart related malformations and anomalies were seen at the top dose (duplicated inferior vena cava 2 (2), atrial septal defect with narrow pulmonary vein 1 (1), malformed systemic/pulmonary artery 1 (1)) in 3 foetuses of 3 litters. As such the effects cannot be disregarded and this was also

supported by the study authors. For two of the findings (interventricular septal defect (small), interventricular septal defect) HCD from the conducting laboratory were considered by the DS (Huntington, 1994 – 1995). These HCD incidences were exceeded for interventricular septal defect in the mid dose on a litter, but not on a foetus basis. At the top the dose historical control incidences were exceeded on both litter and foetus basis. The incidence of interventricular septal defect (small) did not exceed historical controls, but further indicated that the heart was a target organ.

Considering the HCD provided during the consultation (Huntington, 1994 – 1997) the historical incidences for interventricular septal defect were only exceeded for litters inat the top dose. For duplicated inferior vena cava the historical incidences were exceeded in the top dose for foetuses and litters. Also the observed cases of malrotated heart in the mid- and top-dose group (one case each) exceeded the historical controls, as this effect was not seen in any of the 24 studies.

Taking all observed alterations in this organ system in the foetuses together, an increased incidence of heart related effects with dose and a dose related trend in severity can be observed.

In addition, there was a clear increase in supernumerary rib 14 in the top dose. Though this effect is not considered a malformation but a variant and the incidence was only seen concomitant with slight maternal toxicity, the increase was judged to be a relevant finding by the PPP expert group. RAC agrees with this conclusion and considers the effect as supportive evidence for classification.

RAC further considers the observed maternal toxicity, evidenced by reduced body weight gain between GD 6 – 8 of gestation (the time when test material administration started, see table "Maternal body weight / body weight changes, Anonymous (1997f)") is insignificant and there is no information available that would indicate that the observed effects in rat offspring were a secondary non-specific consequence of maternal toxicity.

Comparison with the classification criteria

No appropriate human data are available that could support a classification of *Margosa Extract with water* in Category 1A.

Studies considered relevant for this hazard class are the developmental toxicity study in rats (Anonymous, 1997e,f) and the two-generation study in rats (Anonymous, 2000b).

Other studies are not considered relevant for the assessment of developmental toxicity of *Margosa Extract with water*, for various reasons explained in the previous sections.

No developmental toxicity was seen in the two-generation study, though it should be noted that relatively low doses were applied in this study (for details, see section on adverse effects on sexual function and fertility) and the design of the two-generation study does not cover all aspects of development in a way comparable to a TG-compliant developmental toxicity study (such as OECD 414).

In the rat developmental toxicity study an increase in visceral malformations and anomalies of the heart at doses without or only insignificant maternal toxicity (limited to slight reductions in maternal weight gain between GD 6 – 8 in the top dose) was observed.

The increase was only slight (1 foetus at the mid dose and 3 foetuses of 3 litters at the top dose), but some of the effects exceeded historical controls (see table "Visceral malformations and anomalies" and section on HCD). The foetuses were affected by several types of malformations, the number of which was clearly higher at the top dose, indicating increased severity. Although the heart related anomalies observed at the low, mid and top dose were not increased above historical control levels, they are still considered supportive findings, as they further support the conclusion that the heart is a target organ.

The increase in supernumerary rib 14 at the top dose is also considered supportive evidence for a classification.

Though the increase in the observed findings was not very strong, it was above historical controls for some of the observed malformations. An increase in the severity of the effects with dose was observed and although the heart related anomalies did not exceed historical control incidences, they further support that the heart was a target organ. Also the increase in the incidence of supernumerary rib 14 in both foetuses and litters at the top dose is considered supportive evidence for a classification. There is no evidence that would indicate that the effects were a secondary consequence of the (insignificant) maternal toxicity in top dose dams.

In conclusion RAC considers the observed findings **warrant classification as Repr. 2, H361d.**

***Specific concentration limits (SCL)***

During the consultation, one company pointed out, that the observed low incidences of malformations and anomalies would indicate that *Margosa Extract with water* belongs to the low potency group, defined by an ED 10 ≥ 400 mg/kg bw/day.

The incidences of malformations or malformations and anomalies together on a foetus basis would indicate low potency, with ED 10 values > 1000 mg/kg bw/day. However, on a litter basis an ED 10 value close to 400 mg/kg bw/day can be derived based on malformations alone. When considering both malformations and anomalies together, the resulting ED 10 is below 225 mg/kg bw/day, indicating medium potency. As the classification proposal for category 2 is based on all heart related effects that were seen in Anonymous (1997e, f), including malformations as well as anomalies, it appears relevant to also consider both sets of effects for deriving an ED 10 value, indicating that the medium potency group would be more appropriate for *Margosa Extract with water*.

Section 3.7.2.6.2 of the CLP Guidance further specifies that "*if the classification of a substance in Category 2 is done on the basis of 'limited evidence', the quality of the available data will in such cases determine whether a potency assessment is possible. In cases where no further evaluation is possible, the generic concentration limits* [GCL] *of CLP apply."* In the present case the available study appears sufficiently reliable for assessing the potency of the test material in this study. The low incidences of malformations observed are considered to represent the limited evidence supporting classification in category 2. In section 3.7.2.6.5 The CLP guidance several modifying factors are listed which should be considered when deciding whether SCLs should be applied in specific cases. These modifying factors are discussed for their relevance for *Margosa Extract with water* in the following section.

- Type and severity of the effect:

The observed heart related malformations are considered severe effects, relevant for humans. In contrast the observed heart anomalies are not considered to be severe effects, but they support the conclusion that the developing heart is a target organ. Overall, the severity of the effect supports retaining *Margosa Extract with water* in the medium potency group.

- Data availability:

There is only a single relevant study available for *Margosa Extract with water*. No information from a second species is available. The limited information available counts against moving *Margosa Extract with water* to the low potency group.

- Dose-response relationship:

A slight increase in malformations was seen at the mid and top doses. The relevance of these findings cannot be excluded. ED10 values above the cut—off for low potency (malformation and

malformations & anomalies together, per foetus) as well as below the cut-off for low potency (malformations & anomalies together, per litter) can be derived (see above).

- Mode or mechanism of action:

As no information on a possible underlying mode or mechanism of action is available, the relevance of the observed malformations for humans cannot be excluded. This information does not indicate the need for adapting the potency group.

- Toxicokinetics:

There is no information on the toxicokinetics of *Margosa Extract with water*. It is not known whether a single component of this UVCB substance or the extract as a whole is responsible for the observed effects on development. It is not known whether the extract or components of the extract have the potential to accumulate. This information does not indicate the need for adapting the potency group.

- Conclusion on modifying factors and potency group:

Overall, the assessment of modifying factors indicates that *Margosa Extract with water* should remain in the medium potency group and the general concentration limit of 3% should be applied.

In this respect, it is also relevant to note Section 3.7.2.6.5 of the CLP Guidance: *"In general, more conclusive evidence is required when moving a substance to a lower potency group than to a higher potency group."* In conclusion, **RAC recommends not to deviate from the generic concentration limit for category 2 (i.e. 3%)**.

***Lactation***

No respective findings were observed in the two-generation study in rats (Anonymous, 2000b) that would support a classification, however, it is noted that the doses applied in that study were rather low. In the absence of relevant data on effects on or via lactation RAC concurs with the DS's proposal for **no classification for effects on or via lactation.**

# ENVIRONMENTAL HAZARD EVALUATION

## RAC evaluation of aquatic hazards (acute and chronic)

### Summary of the Dossier Submitter's proposal

For environmental hazards, the DS proposed a classification as Aquatic Chronic 1 (H410) with an M-Factor of 10, based on the findings in the relevant ecotoxicological studies on Chironomids, described below.

***Dossier Submitter Remarks on data used for environmental classification***

The technical active substance consists of a complex mixture of related triterpenoids extracted from the seed kernels of the neem tree Azadirachta indica A. JUSS. Since it is not possible to synthesize *Margosa Extract with water* chemically, the major individual component, **Azadirachtin A**, was chosen as the lead substance for describing the behaviour of *Margosa Extract with water* in the environment.

Only ecotoxicological test data for exactly this water extract further processed with organic solvent was considered as relevant, due to a fundamental difference with other extracts, concerning the content of the ecotoxicological relevant components Azadirachtin A (and B): 34 % Azadirachtin A for *Margosa Extract with water* (approved as insecticide) versus < 0.2 % in total

in another biocidal Margosa Extract (approved as repellent). The other contained limonoids (Salannin and Nimbin) are only minor constituents for the extracts with a mainly insecticidal mode of action, whereas they are exceeding the concentration of Azadirachtin for the Margosa Extract approved as repellent.

The table below reports the definitions used for the environmental section of the CLH report:

| | **CLH dossier for Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvent]** | **Characterisation / Components (average)** | **Used synonyms (e.g. study reports, other dossiers)** |
|---|---|---|---|
| **Lead component (measured in all studies)** | Azadirachtin A | Azadirachtin exists in the different isomeric forms A, B, H, J. Azadirachtin A is the most frequent and continuously measured form. It is also considered as the ecotoxicological most relevant component. | Sometimes no differentiation between Azadirachtin A and B reported in the studies |
| **Active substance** | Margosa Extract with water | 34 % Azadirachtin A | NeemAzalTechnical |
| **Formulated product** | Neem Azal-T/S (as plant protection product) | 1 % Azadirachtin A | NeemProtect (as biocidal product) |

***Degradation***

A hydrolysis study (Tross, 1996), performed according to OECD TG 111, was run on the active substance (a.s.) *Margosa Extract with water* at pH 4, 7 and 8 and at 30 and 40 °C. Azadirachtin A was used as lead substance since it is the major component of *Margosa Extract with water*. The hydrolysis of Azadirachtin A is pH-dependent as indicated by a significant increase in the rate of degradation with increasing pH. At high temperatures of 30 to 40 °C, Azadirachtin A has a half-life of 5 to 23 hours in slightly alkaline conditions at pH 8. In acidic conditions at pH 4 half-lives ranged from 56 (at 40 °C) to 256 hours (at 30 °C). The extrapolation of the test results to the average outdoor temperature in the EU of 12 °C using the Arrhenius equation yields a half-life of 112.7, 40.9 and 8.2 days at pH 4, 7 and 8, respectively. Hydrolysis products are not detectable due to the technical limitations with regard to radiolabelling of the test substance and synthesis of reference substances. Further information is available from the DAR of Azadirachtin, providing hydrolysis half-lives for Azadirachtin A of 18.1 d, 9.6 d, and >1d at pH values of 4, 7, and 10, respectively, determined at 25 °C in buffered solution. For Azadirachtin B, half-lives of 24.0 d, 12.3 d, and >1 d were reported in the same study (Molinari, 2002).

In conclusion, Azadirachtin A and B undergo hydrolytic degradation. The rate of degradation is pH and temperature dependant, increasing at higher pH and temperature.

Aqueous photolytic half-lives for *Margosa Extract with water* were calculated based on the quantum yield and UV/VIS data from the direct phototransformation study in water of *Margosa Extract with water* (Werle, 1995) and parameters included in the computer model "ABIWAS". The half-life times ranged from 26.5 days to 7.2 months for January and from 3.8 to 19.2 days for July.

Regarding biotic degradation, the key study based on which DS concluded on degradability is a ready biodegradability test on the lead component Azadirachtin A, performed according to OECD TG 301F. A mixture of fresh non-adapted activated sludge and aqueous soil extract containing soil micro-organisms was used as inoculum. The incubation was conducted at 22±1°C and pH

7.4-7.6. Toxicity controls were set out, demonstrating no inhibitory effect of Azadirachtin A on the inoculum at tested concentration of 100 mg/L. Biological degradation of Azadirachtin A at the end of the 28-day incubation was 21.6%, leading to the conclusion, that the component Azadirachtin A is not readily biodegradable.

This result is supported by other three tests on ready biodegradability performed with the a.s. *Margosa Extract with water*. A summary of the relevant information is provided in the following table.

| Method/ Guideline | Inoculum | | | Test substance conc. | Degradation | | Reference |
|---|---|---|---|---|---|---|---|
| | Type | Concen-tration | Adaptation | | Incub. period | Degree [%] | |
| OECD TG 301 F<br><br>**Key study** | activated sludge & aqueous soil extract with soil micro-organisms | 1.8 x $10^4$ CFU/mL corresponding to 30 mg/L dry matter | no | 100 mg Azadirachtin A/L | 28 days | 21.6 | Hund, K. (1999b) |
| OECD TG 301 D | activated sludge | not specified | no | 1.8, 3.6 & 5.4 mg *Margosa Extract* (a.s.)/L, 33.4 % Azadirachtin A | 28 days | 5.6 | Werle (1998) |
| OECD TG 301 F | activated sludge | 9.3 x $10^4$ CFU/mL corresponding to 30 mg/L dry matter | no | 100 mg *Margosa Extract* (a.s.)./L, 34 % Azadirachtin A | 35 days | 36.8 | Hund, K. (1998a) |
| | activated sludge & aqueous soil extract with soil micro-organisms | 1.2 x $10^5$ CFU/mL corresponding to 30 mg/L dry matter | no | 100 mg *Margosa Extract* (a.s.)/L, 34 % Azadirachtin A | 35 days | 48.2 | |
| OECD TG 301 F | activated sludge & aqueous soil extract with soil micro-organisms | 2.4 x $10^4$ CFU/mL corresponding to 30 mg/L dry matter | no | 100 mg *Margosa Extract* (a.s.)/L (dissolved in DMSO), 34% Azadirachtin A | 47 days | 49.1 | Hund, K. (1999a) |
| OECD TG 301 D | activated sludge | not specified | no | 1 & 2 mg NeemAzal-T/S/L, 1% Azadirachtin A | 28 days | 65.7 | Lenz, G. (1995) |

In all studies, the incubations were conducted at 20±2°C and pH 7. Toxicity controls were set out, demonstrating no inhibitory effect of *Margosa Extract with water* on the inoculum. The results of these tests confirmed *Margosa Extract with water* as not being readily biodegradable.

Furthermore, one study using the formulated product (NeemAzal-T/S) as test substance is available. The product NeemAzal-T/S contains only ~1% Azadirachtin A in total. The test showed > 60 % degradation within 10 days and thus the criteria of classification as 'readily biodegradable' was formally met. However, the 'ready biodegradability' of the product NeemAzal-T/S is probably attributable to the properties of the formulation additives, representing the bulk of the product (96%).

Azadirachtin A and B were found to dissipate from water with half-lives between 2.4-66.4 days (12 °C) in several non-guideline studies on freshwater and water-sediment. Neither information regarding the degree of ultimate degradation, nor on degradation products, is available from these studies.

*Margosa Extract with water* is a complex substance of natural origin. According to the Guidance on the Application of the CLP criteria, a complex substance of natural origin has to be regarded as not rapidly degradable if it contains a not rapidly degradable constituent with a proportion of ≥ 20% or in case the constituent is hazardous, of even lower proportions. *Margosa Extract with water* contains ~ 34% Azadirachtin A, which is considered as the compound mainly responsible for the ecotoxicological effect on the target organisms and does not meet the criteria for ready biodegradability.

Based on the abovementioned data, the DS concluded that *Margosa Extract with water* cannot be considered rapidly degradable.

***Bioaccumulation***

Determination of n-octanol/water partition coefficient values for *Margosa extract with water* is technically not feasible. However, log $K_{ow}$ was determined for some selected azadirachtins (Troß 1996). The authors reported log $K_{ow}$ values of 0.99 for Azadirachtin A, 1.29 for Azadirachtin B and 0.68 for Azadirachtin H.

Based on the reported log Kow values, the bioconcentration factors ($BCF_{fish}$) for Azadirachtin A and Azadirachtin B were estimated using the standard equation

$$\log BCF = 0.85 \times \log K_{ow} - 0.7$$

resulting in a $BCF_{fish}$ of 1.38 L/kg for Azadirachtin A and a $BCF_{fish}$ of 2.5 L/kg for Azadirachtin B.

The DS concluded that the calculated $BCF_{fish}$ values indicate a low potential for aquatic bioaccumulation of the main components of *Margosa Extract with water*.

***Aquatic toxicity***

Short-term and long-term aquatic toxicity data are available for all three trophic levels. A summary of the relevant information is provided in the following table (the key endpoint used by DS in hazard classification is highlighted in bold). All studies were performed under (semi-)static conditions with results expressed in terms of mean measured concentrations (mmc). Studies available were either performed with the active substance *Margosa extract with water* (equivalent to NeemAzal or NeemAzal technical) or with the product NeemAzal-T/S. In all studies, Azadirachtin A was used as analytical lead component and the content of Azadirachtin A in *Margosa extract with water* or NeemAzal-T/S is always given.

| Method | Results | Remarks | Reference |
|---|---|---|---|
| OECD TG 203: Oncorhynchus mykiss, mortality | 96h-$LC_{50}$ = 4.14 mg a.s./L | Study performed with the product NeemAzal-T/S containing 1 % Azadirachtin A; effect values related to active substance *Margosa Extract with water* | Anonymous (1996b) |
| OECD TG 202: Daphnia magna, immobilisation | 48h-$EC_{50}$ = 9.69 mg a.s/L | Study performed with the a.s. M*argosa extract with water* | Anonymous (1999b) |
| OECD TG 201: Scenedesmus subspicatus; growth rate inhibition | 72h-$E_rC_{50}$ = 1041 mg/L<br>72h-$E_rC_{10}$ = 332 mg/L | Study performed with the a.s. M*argosa extract with water;* No exponential growth during the whole test duration | Wenzel, A. (2002) report no. TRF-001/4-30 |
| OECD TG 204: Oncorhynchus mykiss, mortality and growth; study design comparable to OECD TG 215 with validity criteria fulfilled | 28d-NOEC = 1.9 mg/L | Study performed with product NeemAzal-T/S, containing 1 % Azadirachtin A; effect values related to active substance *Margosa Extract with water* | Anonymous (1999a) |
| OECD TG 202, Pt. II: Daphnia magna Reproduction & mortality | 21d-NOEC=1.84 mg/L | Study performed with the a.s. Margosa extract with water;<br>Effect values based on mean measured concentrations | Anonymous (1999b) |
| OECD TG 202 Pt II: Daphnia magna, reproduction | 21d-NOEC = 0.1 mg/L | Study performed with product NeemAzal-T/S, containing 1 % Azadirachtin A, effect values related to active substance *Margosa Extract with water* | Schmitz A. (1999) Report no. TRF-002/4-21 |
| OECD TG 219: Chironomus riparius emergence and development test | 28d-NOEC = 0.0075 mg a.s/L | Study performed with the a.s. M*argosa extract with water* | Gonsior, G. (2008a) report no. 2007/1356/01-ASCr |
| OECD TG 219: Chironomus riparius emergence and development test | **28d-NOEC = 0.006 mg a.s./L** | **Study performed with the product NeemAzal-T/S containing 1 % Azadirachtin A, effect values related to active substance *Margosa Extract with water*** | **Gonsior, G. (2008b) report no. 2007/1355/01-ASCr** |

Short-term toxicity

*Fish*

One reliable acute toxicity study to fish is provided in the CLH Report for purpose of classification. In this study acute toxicity of *Margosa Extract with water* to rainbow trout (*O. mykiss*) was determined from a semi-static test with the formulated product NeemAzal-T/S (containing 1% Azadirachtin A) as test substance and performed according to OECD TG 203 (1992). Five test substance concentrations (between 50 and 800 mg/L of NeemAzal-T/S) and a control were established and the effect values were based on geometric mean of the measured concentrations at test start (t=0) and after 48 h (before renewal of test solution). A 96h-$LC_{50}$ of 4.14 mg/L ($LC_{50}$)

was calculated based on the mean measured concentrations for the leading compound Azadirachtin A and the mean Azadirachtin A content in *Margosa extract with water* of 34%, being this component regarded as the ecotoxicological most relevant. This study is considered acceptable and useful for the effects assessment of *Margosa extract with water*.

A further fish short-term toxicity study performed on *Cyprinus carpio* with the product NeemAzal-T/S as a limit test (100 mg/L NeemAzal-T/S containing 1.1% Azadirachtin A) is reported in the CLH report as supportive information of low acute toxicity to. However, as no analytical monitoring of the test substance concentration was performed, the study is considered as not valid for purpose of acute classification and therefore not included in the Table above.

*Aquatic invertebrates*

One acceptable and reliable short-term toxicity study with aquatic invertebrates (*Daphnia magna*) is available for *Margosa extract with water* (purity 33.4% Azadirachtin A), according to OECD TG 202 (Pt. I). Immobilisation was assessed at six concentrations tested between 20.5 and 80 mg a.s./L (nominal). The 48-h $EC_{50}$ was determined to be 9.6 mg/L (value based on initial measured concentration).

*Algae and aquatic plants*

Only one 72-h growth inhibition study (static test) with the green algae *Scenedesmus subspicatus* was performed with *Margosa extract with water* (purity 35% Azadirachtin A) according to OECD TG 201 (1984).

Azadirachtin A and Azadirachtin B were measured at test start and end. The effect values are based on nominal concentration (0, 10, 50, 100, 500 and 1000 mg a.s./L.) because Azadirachtin A was not stable in the test system (degradation by 96%) and the concentration measured at test start was in the range 85-113%. Azadirachtin B, used as leading compound, results stable in the test system, but its concentration was above 120% of nominal concentration. Therefore, it is unclear which of the components is responsible for the effects observed.

Although a 72 h-$E_rC_{50}$ of 1041 mg/L and a 72 h-$E_rC_{10}$ of 332 mg/L were calculated (based on nominal concentration of Azadirachtin B), in the control cultures no exponential growth during the whole test duration was observed; as exponential growth is a prerequisite for growth rate evaluation, the test should be considered acceptable just as supporting study to confirm that algae are not the most sensitive group (see Comment section).

Long-term toxicity

*Fish*

Two chronic toxicity studies to fish are available and included in the CLH Report, although only one is used for purpose of chronic classification. The reliable long-term toxicity study was carried out on *Oncorhynchus mykiss* with the formulated product NeemAzal-T/S (containing 1 % Azadirachtin A). Although this test was performed according to OECD TG 204, however the study design was rather comparable and conform to OECD TG 215, with regards to the test duration and the evaluated endpoints (exposure period of 28 d; growth as sub-lethal endpoint); as also reported by the DS, validity criteria for fish tests according to OECD TG 215 were fulfilled and, therefore, this test can be considered as an acceptable long term toxicity study for classification purposes. Six test substance concentrations (between 4.7 and 150 mg/L of NeemAzal-T/S) as well as a control were examined in a flow-through system over 28 days. The effect values related to active substance were calculated based on the mean measured concentrations for the leading compound Azadirachtin A and presuming a mean Azadirachtin A content *in Margosa extract with water* of 34%. A 28d-NOEC (for mortality) of 63.6 mg/L NeemAzal-T/S was found (based on

mean measured concentrations), corresponding to a NOEC value related to the active substance *Margosa Extract with water* of 1.9 mg/L. No significant effects on growth rate or on other sublethal parameters were found. Although the study was performed with the formulated product instead of the active substance as such, it is considered as valid and useful for addressing the effects assessment of the active substance as well as for purpose of chronic classification.

A further chronic toxicity study conducted on zebra fish, *Danio rerio*, with a.s. *Margosa Extract with water* (purity 29.9 % Azadirachtin A) according to OECD TG 210 (1992) is provided in the CLH report. No statistically significant difference between any test substance treatment and the control was found. A NOEC value was established at 2.0 mg a.s./L. However, as the average survival of fertilized eggs in the control was < 70% after 37 d, the study is considered by the DS as not valid and therefore cannot be used for the effects assessment.

*Aquatic invertebrates*

Two long-term toxicity studies on *Daphnia magna* according to OECD TG 202 (Pt. II) are available in the CLH report.

In the first reproduction study, the chronic toxicity *of Margosa extract with water* (purity 33.4% Azadirachtin A) was determined in a semi-static test, where a 21 d-NOEC was established as 1.84 mg a.s./L. The toxicity value is based on mean measured concentrations of 0.1, 0.21, 0.42, 0.90 and 1.84 mg a.s./L.

In the second reproduction study, the toxicity of the formulated product NeemAzal-T/S, containing 1% Azadirachtin A, was tested. This is a semi-static test and the mean measured concentration were in the range of 1.7 to 62.5 mg/L. A 21 d-NOEC = 3.4 mg/L of NeemAzal-T/S was estimated, that corresponds to a NOEC related to active substance *Margosa extract with water* of 0.102 mg/L.

*Other aquatic organisms (including sediment)*

Two long-term toxicity studies on *Chironomus riparius* according to OECD TG 219 were provided by DS.

One Study (Gonsior, G., 2008a) was performed with the a.s. *Margosa extract with water* of (purity 34 % Azadirachtin A).

Samples taken from the water phase, the pore water and the sediment were analysed at day 0, 7 and 28. The analytical measurements after 7 and 28 days showed a degradation of the test substance below the limit of quantification (LOQ) of 0.00625 mg/L for water and pore water and 0.0156 mg/kg for sediment. In the sediment, the *Margosa Extract with water* concentrations did not exceed the LOQ during the whole study. Consequently, the chironomids were not exposed to the nominal concentrations over the whole time. Therefore the mean of the NOEC based on nominal concentrations and the ½ LOQ (for water and pore water, because no test substance was found in the sediment) was calculated. The NOEC based on the geometric mean concentration was calculated to be 0.0075 mg/L.

In a further study (Gonsior, G., 2008b) the toxicity of the formulated product NeemAzal-T/S (purity 1 % Azadirachtin A) to *Chironomus riparius* was studied. Chironomid larvae were exposed to nominal concentrations of 0.0717, 0.143, 0.287, 0.573, 1.15, 2.29, 4.59 and 9.17 mg/L of NeemAzal-T/S. The overall NOEC was estimated to be 0.573 mg/L NeemAzal T/S.

Samples of the overlying water, pore water and the sediment were taken 1 hour, 7 days and 28 days. The analytical measurements after 7 and 28 days showed a degradation of the test substance below the limit of quantification (LOQ) of 0.183 mg NeemAzal-T/S/L for water and pore water and 0.475 mg/kg for sediment. In the sediment the NeemAzal-T/S concentrations did not exceed the LOQ during the study (measured on day 0, 7 and 28). Consequently the

chironomids were not exposed to the nominal concentrations over the whole time. Therefore the mean of the NOEC based on measured concentration at test start and the ½ LOQ (for water and pore water, because no test substance was found in the sediment) was calculated.

The NOEC based on the geometric mean concentration was calculated to be 0.2 mg/L of NeemAzal-T/S. This corresponds to a NOEC related to the active substance *Margosa Extract with water* of 0.006 mg/L.

This effect value was calculated based on the mean measured concentrations for the leading compound Azadirachtin A and the mean Azadirachtin A content in *Margosa extract with water* of 34 %.

## Comments received during consultation

For the environmental aspects, two comments were provided: one by a Company-Manufacturer and one by a Member State.

The Company agreed to the Chronic classification but it does not share the M factor proposed by DS. In particular, they complained that the values used for the NOEC derivation for the two long-term studies with *Chironomus riparius*, calculated as geometric mean of measured concentrations, should not be considered due to the poor recovery rates of the lead component (below the limit of quantification). Based on the life cycle of chironomids and the intention of the test system to represent a single exposure event (drift, drainage), they considered most reasonable to use the nominal or initially measured concentrations instead of geometric mean.

Therefore in their opinion, endpoint for chronic toxicity classification should be the nominal NOEC (28d-NOEC= 0,0184mg Margosa, ext./l) for the midge larvae *Chironomus riparius*. Consequently, they not agree with the M-factor = 10 proposed in the CLH report, suggesting M = 1.

The DS clarified that the NOEC based on mean measured concentrations using LOQ/2 was already agreed in 2012 by EU MS for the assessment of Margosa extract in the BP and PPP assessment. Moreover, as no measured test substance concentrations in the sediment are available, the only reliable solution is to calculate a mean concentration based on LOQ/2, as recommended in OECD TG 23.

RAC agrees with DS response. Moreover the ECHA guidance on CLP foresees that the L(E)$C_{50}$ and NOEC may be calculated based on the geometric mean concentration of the start and end of test. "*Where concentrations at the end of test are below the analytical detection limit, such concentrations shall be considered to be half that detection limit*". In conclusion, although RAC notes some minor uncertainties in the substance behaviour in the experiment media, the calculated values are acceptable to obtain a valid NOEC.

The commenting MS supported the Chronic classification proposed by DS. Moreover commented some specific endpoints.

Regarding studies on Chironomids, MS suggested RAC to be aware of the composition of the formulations. DS clarified that the composition of the biocidal product NeemAzal-T/S is contained in the confidential Appendix to the CAR. The identity of the other components does not indicate that they would increase the toxicity of the active substance. However they noted the consistency of the NOEC values from the *Chironomus* studies performed with the formulation product, compared with the substance (*Margosa Extract with water*) that are in the same concentration range when based on the concentration of *Margosa extract with water*.

Regarding the toxicity to algae, MS suggested to derive the mean measured concentration based on Azadirachtin A as for other endpoints.

The DS clarified that, the calculation of a mean concentration based on Azadirchatin A is not necessary, mainly considering that the study is acceptable just to support that algae are clearly the least sensitive of the tested aquatic organisms.

## Assessment and comparison with the classification criteria

### *Degradation*

*Margosa Extract with water* is a complex substance of natural origin. According to the Guidance on the Application of the CLP criteria (version 5, July 2017) a complex substance, such as UVCBs, should be regarded as not rapidly degradable if the constituents that are not rapidly degradable constitute a significant part of the substance, e.g. more than 20%, or for a hazardous constituent an even lower content.

*Margosa Extract with water* contains ~34% Azadirachtin A, which is considered as the compound mainly responsible for the ecotoxicological effect on the target organism. Azadirachtin A itself does not meet the criteria for ready biodegradability, showing only 21.6% degradation within 28 days. Extrapolation of the hydrolysis test results for Azadirachtin A to the average outdoor temperature in the EU (12 °C) yields half-lives of 112.7, 40.9 and 8.2 days at pH 4, 7 and 8, respectively. According to the Guidance on the Application of the CLP criteria (version 5, July 2017), data on hydrolysis might be considered for classification purposes only when the longest half-life determined within the pH range 4-9 is shorter than 16 days. Thus, hydrolysis cannot be considered for classification purposes, since the longest half-life determined within the pH range 4-9 is longer than 16 days. Azadirachtin A and B were found to dissipate from water with half-lives between 2.4-66.4 days (12 °C) in several non-guideline studies on freshwater and water-sediment. Neither information regarding the degree of ultimate degradation, nor on degradation products, is available from these studies.

Based on the abovementioned data, Azadirachtin A cannot be considered rapidly degradable. Consequently, *Margosa Extract with water* with a content of ~34% Azadirachtin A has to be considered not rapidly degradable as well.

### *Bioaccumulation*

No measured $BCF_{fish}$ data is available. The measured log $K_{OW}$ for Azadirachtin A and Azadirachtin B is below the CLP trigger value of ≥ 4. Therefore, RAC agrees with the DS's conclusion that the substance has a low bioaccumulation potential.

### *Aquatic toxicity*

Adequate **acute toxicity data** are available for all three trophic levels (fish, crustacean, algae/aquatic plants).

The lowest acute value is the 96h-$LC_{50}$ of 4.14 mg a.s./L from an acute toxicity test with rainbow trout. All acute effect data exceed acute classification trigger value ($LC_{50}$ ≤ 1 mg/l) therefore **no aquatic acute classification is warranted** *for Margosa Extract with water*.

Adequate **chronic toxicity data** are available for all three trophic levels (fish, crustacean, algae/aquatic plants). Invertebrates represent the most sensitive trophic level for chronic toxicity in the aquatic compartment. RAC agrees with the DS that the test for fish performed according to OECD TG 204 can be considered as an acceptable long term toxicity study for classification purposes, because it conforms to OECD TG 215 with regards to the test duration, the evaluated endpoints and test validity criteria.

The lowest long-term effect values were found for the midge larvae *Chironomus riparius* in two water-sediment studies according to OECD TG 219 (spiked water). The substance tested was *Margosa extract with water* in Gonsior, 2008(a) and NeemAzal-T/S in Gonsior, 2008 (b). The Azadirachtin A was the lead component in both studies. The corresponding values, calculated for the active substance *Margosa Extract with water* are 28d-NOEC = 0.0075 mg/L in Gonsior, 2008(a) and 28d-NOEC =0.006 mg a.s./L in Gonsior, 2008(b).

RAC agrees that although these are not standard test systems for classification, the use of *Chironomus riparius* values is justified by the insecticidal mode of action of the substance, as well as by the fact that exposure of the test organisms was predominantly via the water phase. This is supported by the measured concentrations below the LOQ in the sediment throughout the duration of the study. Moreover, Chironomids were already considered by RAC as key organisms to classify a number of active substances with the same insecticidal mode of action (e.g. Spirotetramat, Sulfoxaflor, Thiacloprid, Thiamethoxam). In another recent case (Imidacloprid), a key study with no guideline and performed with non-standard invertebrate species was considered by RAC relevant as well as reliable for use in classification due to the substance's insecticidal mode of action.

Despite the lowest value is a NOEC = 0.006 mg a.s./L by Gonsior, 2008 (b), RAC considers more appropriate the results obtained on the test substance as such i.e. *Margosa Extract with water* with a NOEC = 0.0075 mg/L. However, the results from the two chironomus studies are in good agreement and this does not affect the classification proposed by DS: **Aquatic Chronic 1, H410, with M = 10** (considering 0.001 mg/L < NOEC < 0.01 mg/L for non-rapidly degradable substances).

**ANNEXES:**

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).