Committee for Risk Assessment

RAC

Annex 1

Background document

to the 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

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

10 December 2020

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

Substance 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**

Index Number: **-**

Contact details for dossier submitter:

BAuA
Federal Institute for Occupational Safety and Health
Federal Office for Chemicals
Friedrich-Henkel-Weg 1-25
44149 Dortmund, Germany

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance 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
Annex VI Index number:	-
Degree of purity:	100 %
Impurities:	UVCB substance, thus no impurities are assigned

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	-
Current proposal for consideration by RAC	Repr. 2; H361d Skin Sens. 1; H317 Aquatic Chronic 1; H 410 M-Factor 10
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Repr. 2; H361d Skin Sens. 1; H317 Aquatic Chronic 1; H 410 M-Factor 10

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON MARGOSA, EXT. [FROM THE KERNELS OF AZADIRACHTA INDICA EXTRACTED WITH WATER AND FURTHER PROCESSED WITH ORGANIC SOLVENTS]

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None		None	conclusive but not sufficient for classification
2.2.	Flammable gases	-		-	conclusive but not sufficient for classification
2.3.	Flammable aerosols	-		-	conclusive but not sufficient for classification
2.4.	Oxidising gases	-		-	conclusive but not sufficient for classification
2.5.	Gases under pressure	-		-	conclusive but not sufficient for classification
2.6.	Flammable liquids	-		-	conclusive but not sufficient for classification
2.7.	Flammable solids	None		None	conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures	None		None	conclusive but not sufficient for classification
2.9.	Pyrophoric liquids	-		-	conclusive but not sufficient for classification
2.10.	Pyrophoric solids	None		None	conclusive but not sufficient for classification
2.11.	Self-heating substances and mixtures	-		-	Data lacking
2.12.	Substances and mixtures which in contact with water emit flammable gases	None		None	conclusive but not sufficient for classification
2.13.	Oxidising liquids	-		-	conclusive but not sufficient for classification
2.14.	Oxidising solids	None		None	conclusive but not sufficient for classification
2.15.	Organic peroxides	None		None	conclusive but not sufficient for classification
2.16.	Substance and mixtures corrosive to metals	-		-	conclusive but not sufficient for classification

3.1.	Acute toxicity - oral	None		-	conclusive but not sufficient for classification
	Acute toxicity - dermal	None		-	conclusive but not sufficient for classification
	Acute toxicity - inhalation	None		-	conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	None		-	conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	None		-	conclusive but not sufficient for classification
3.4.	Respiratory sensitisation	-		-	data lacking
3.4.	Skin sensitisation	Skin Sens 1, H317		-	
3.5.	Germ cell mutagenicity	None		-	conclusive but not sufficient for classification
3.6.	Carcinogenicity	None			conclusive but not sufficient for classification
3.7.	Reproductive toxicity	Repr. 2 H361d		-	
3.8.	Specific target organ toxicity –single exposure	None		-	conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	None		-	conclusive but not sufficient for classification
3.10.	Aspiration hazard	-		-	data lacking
4.1.	Hazardous to the aquatic environment	Aquatic Chronic 1, H410	M=10	-	
5.1.	Hazardous to the ozone layer	-		-	

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Table 4: Proposed labelling based according to the CLP Regulation

	Labelling	Wording
Pictograms	GHS07 GHS08 GHS09	
Signal Word	Warning	
Hazard statements	H361d H317 H410	Suspected of damaging the unborn child May cause an allergic skin reaction Very toxic to aquatic life with long lasting effects
Suppl. Hazard statements	-	-

Proposed notes assigned to an entry: -

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

No previous classification and labelling available.

In 2014 CLH dossiers for two different margosa extracts were submitted by the German CA, namely one for the biocidal active substance "Margosa Extract" (approved for the use as insecticide in PT 18) and another for "Azadirachtin", the active substance approved for the use in plant protection products.[1]

However, both CLH dossiers have been withdrawn in 2015 after it was decided that the substance identity had to be redefined based on the ECHA "Guidance for identification and naming of substances under REACH and CLP" and the guidance "Botanical Active Substances Used in PPP". Currently four different "margosa substances" are formally identified based on the origin of the plant material in combination with the extraction / manufacturing method (see section 1.1).

2.2 Short summary of the scientific justification for the CLH proposal

Considering the reported findings in the relevant toxicological studies, a classification of the technical material as skin sensitiser (Skin Sens. 1; H317) and as developmental toxicant (Repr. 2; H361d) is proposed. For the other toxicological hazards, either the data were conclusive but not sufficient for classification or the relevant data were lacking.

Considering the reported findings in the ecotoxicological studies, a classification as Aquatic Chronic 1 (H 410) with an M-Factor = 10 is proposed.

2.3 Current harmonised classification and labelling

No entry in Annex VI.

2.4 Current self-classification and labelling

No entry in C&L inventory.

[1] https://echa.europa.eu/harmonised-classification-and-labelling-previous-consultations/-/substance-rev/3393/term?_viewsubstances_WAR_echarevsubstanceportlet_SEARCH_CRITERIA_EC_NUMBER=601-089-4&_viewsubstances_WAR_echarevsubstanceportlet_DISS=true

https://echa.europa.eu/harmonised-classification-and-labelling-previous-consultations/-/substance-rev/3392/term?_viewsubstances_WAR_echarevsubstanceportlet_SEARCH_CRITERIA_EC_NUMBER=283-644-7&_viewsubstances_WAR_echarevsubstanceportlet_DISS=true

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.[2]

- 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

[2] 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*.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

"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 substance in the meaning of Regulation (EU) No 528/2012 (approved under Directive 98/8/EC)[3] and therefore subject to harmonised classification and labelling (Regulation (EC) No 1272/2008 Article 36.2).

[3] http://dissemination.echa.europa.eu/Biocides/factsheet?id=0043-18

Part B.

SCIENTIFIC EVALUATION OF THE DATA

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

The EINECS entry (EC No. 283-644-7, CAS No. 84696-25-3) is a general entry covering all kinds of extracts from *Azadirachta indica, Meliaceae* irrespective of the extraction conditions:

Extractives and their physically modified derivatives such as tinctures, concretes, absolutes, essential oils, oleoresins, terpenes, terpene-free fractions, distillates, residues, etc., obtained from Azadirachta indica, Meliaceae.

According to the guidance for identification and naming of substances under REACH and CLP the different extracts get different names, based on the origin of the plant material in combination with the extraction / manufacturing method. However, the EC name and number is valid for all kinds of extracts from *Azadirachta indica, Meliaceae*.

This current CLH dossier was prepared for the following extract:

- Margosa, extract from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents (hereafter *"Margosa Extract with water"*). This extract is already approved as biocidal active substance and is included in the Union list in the Biocide Regulation.[3]

Currently there is knowledge of three other margosa extracts (all covered by the same EINECS entry) being 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 biocidal active substance was concluded, a CLH dossier was submitted in 2017.[4]
- Margosa, extract from the kernels of *Azadirachta indica* extracted with organic solvents at elevated temperatures (CLH proposal expected to be submitted in the framework of the PPP renewal process).
- Margosa, extract from presscake of kernels of *Azadirachta indica* after removal of the Neem oil, extracted with organic solvents at elevated temperatures (CLH proposal expected to be submitted in the framework of the PPP renewal process).

[4] https://echa.europa.eu/harmonised-classification-and-labelling-previous-consultations/-/substance-rev/16111/term?_viewsubstances_WAR_echarevsubstanceportlet_SEARCH_CRITERIA_EC_NUMBER=283-644-7&_viewsubstances_WAR_echarevsubstanceportlet_DISS=true

Concluding, since now in total four margosa extracts (all covered by the EINECS entry) are known to be on the market. This dossier was prepared for one of these extracts (*Margosa Extract with water*).

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

The 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 finally

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

The approval of the active substance Azadirachtin will expire in 2021.[5]

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

Table 5: Substance identity

EC number:	283-644-7
EC name:	Margosa, ext.
CAS number (EC inventory):	84696-25-3
CAS number:	84696-25-3
CAS name:	Margosa, ext.
Name	Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents]
IUPAC name:	Not available.
CLP Annex VI Index number:	-
Molecular formula:	Not available since substance is an UVCB substance.
Molecular weight range:	Not available since substance is an UVCB substance.

Structural formula:

Not available since substance is an UVCB substance.

[5] http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.detail&language=EN&selectedID=976

1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Please refer to confidential Annex.			

Table 7: Impurities (non-confidential information)

Even though the substance is an UVCB substance, for which per definition no impurities are assigned, some toxicologically relevant constituents are given here to highlight their presence in the extract.

Impurity	Typical concentration	Concentration range	Remarks
Aflatoxines B1 (main compound), B2, G1, G2	Sum < 100 µg/kg		

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
-				

1.2.1 Composition of test material

Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] (hereinafter "*Margosa Extract with water*").

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON MARGOSA, EXT. [FROM THE KERNELS OF AZADIRACHTA INDICA EXTRACTED WITH WATER AND FURTHER PROCESSED WITH ORGANIC SOLVENTS]

Property	**Value**	**Reference**	**Comment (e.g. measured or estimated)**
State of the substance at 20°C and 101,3 kPa	*Margosa Extract with water* technical is a pale yellow to light brownish powder with garlic like odour (purity 100 % *Margosa Extract with water*) Azadirachtin A is a white odourless powder.	Kleeberg, 1994a/b (Assessment report for biocidal active substance)	experimental result
Melting/freezing point	*Margosa Extract with water* partially liquefies above 120 °C and decomposes above 200 °C (purity 100 % *Margosa Extract with water*)	Werle, 1995a (Assessment report for biocidal active substance)	experimental result
Boiling point	The boiling point of *Margosa Extract with water* cannot be observed since decomposition occurs already during melting.	-	-
Relative density	D^{20}_4 = 1.340 at 20 °C (purity 100 % *Margosa Extract with water*)	Thom, 2007 (Assessment report for biocidal active substance)	experimental result
Vapour pressure	No test conducted (extraction mixture). Based on the calculated vapour pressure of 3.6·10-13 Pa for Azadirachtin A the vapour pressure of the extraction mixture should be << 10-5 Pa.	-	estimated
Surface tension	Test not applicable because no saturated test solution with the same ratio of components as in *Margosa Extract with water* could be produced.	-	-
Water solubility	Test not conducted (extraction mixture) solubility of Azadirachtin A: 2.9 g/L at 20 °C	Troß, 1995b (Assessment report for biocidal active substance)	experimental result
Partition coefficient n-octanol/water	Test not applicable (extraction mixture)	-	[*Margosa Extract with water* was used in this study, but only the partition coefficients for Azadirachtin A, B, and H could be determined based on the analytical quantitation of the three solutes in either phase.]
Flash point	The flash point is only relevant to liquids		

Flammability Flammability upon ignition (solids, gases) Flammability in contact with water Pyrophoric properties	 Preliminary test: The burning time for the distance of 200 mm was 5 minutes and 47 seconds (347 s). The test item is not a flammable solid sense of REGULATION (EC) No 1272/2008. The study does not need to be conducted because the experience in production or handling shows that the substance does not react with water, e.g. the substance is manufactured with water or washed with water. The classification procedure needs not to be applied because the substance is known to be stable into contact with air at room temperature for prolonged periods of time (days)	 Franke, 2005a Report No. 20050679.02 BAM 2.2 (2012) BAM 2.2 (2012)	 92/69/EEC, A.10
Explosive properties	maximum exothermic decomposition energy: 177 J/g The heat of decomposition was below 500 J/g. (DSC) The test substance has no explosive properties.	Smeykal, 2002 Report No. 20020457.01	92/69/EEC, A.14 (DSC)
Self-ignition temperature for solids -	No self-ignition temperature was observed up to the melting point.	Franke, 2005b Report No. 20050679.03	92/69/EEC, A.16
Oxidising properties	The maximum burning rate of the mixture of the test item and cellulose (0.82 mm/s) was lower than the maximum burning rate of the reference mixture of cellulose and barium nitrate (1.05 mm/s). Due to this, the test item has no oxidizing properties.	Franke, 2005d Report No. 20050679.04	92/69/EEC, A.17
Stability in organic solvents and identity of relevant degradation products	Solubility tests suggest the active substance to be acceptably stable	Troß, 1995c (Assessment report for biocidal active substance)	experimental result
Dissociation constant	Test not required (extraction mixture)	-	-

Data waiving

Information requirement: Flammable gases (including chemically unstable gases)
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is a solid.

Information requirement: Aerosols
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is no aerosol.

Information requirement: Oxidising gases
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is a solid.

Information requirement: Gases under pressure
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is a solid.

Information requirement: Flammable liquid
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is a solid.

Information requirement: Self-reactive substances and mixtures
Reason: study scientifically not necessary
Justification: The study does not need to be conducted because the exothermic decomposition energy is less than 300 J/g and hence, the classification procedure does not need to be applied.

Information requirement: Pyrophoric liquids
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is a solid.

Information requirement: Pyrophoric solids

Reason: study scientifically not necessary

Justification: The study does not need to be conducted because the substance is known to be stable in contact with air at room temperature for prolonged periods of time (days) and hence, the classification procedure does not need to be applied.

Information requirement: Substances and mixtures which in contact with water emit flammable gases
Reason: study scientifically not necessary
Justification: The study does not need to be conducted because the experience in production or handling shows that the substance does not react with water, e.g. the substance is manufactured with water or washed with water.

Information requirement: Oxidising liquids
Reason: study technically not feasible
Justification: The study does not need to be conducted because *Margosa Extract with water* is a solid.

Information requirement: Organic peroxides
Reason: study scientifically not necessary
Justification: The study does not need to be conducted because the substance does not fall under the definition of organic peroxides according to GHS and the relevant UN Manual of tests and criteria.

Information requirement: Corrosive to metals
Reason: study technically not feasible
Justification: The study does not need to be conducted because there is no established suitable test method for solid substances.

2 MANUFACTURE

The active substance *Margosa Extract with water* is an extract derived from ground seed kernels of the tropical neem tree *Azadirachta indica* using the manufacturing method developed by the applicant in the biocidal approval process (hereinafter *"Margosa Extract with water"*).

2.1 Identified uses

The substance is used as a biocide and pesticide.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 10: Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference
Refer to Table 9			

3.1 Summary and discussion

The preliminary test according to method 92/69/EEC, A.10 was performed. Taking into account the results obtained during the preliminary test, no main test was performed. The test item was not considered as highly flammable solid under the experimental conditions. Experience in handling and use indicates *Margosa Extract with water* is not pyrophoric and does not react with water to liberate flammable gases.

Further, it was also tested in a standard self-ignition temperature study (92/69/EEC, A.16) and no self-ignition temperature was observed up to the melting point.

For the evaluation of explosive properties the screening method differential scanning calorimetry (DSC) was used. The two DSC-measurements showed exothermal effects in the temperature range 280 – 440 °C with low decomposition energies of 177 J/g and 168 J/g, respectively. Therefore, explosive properties are excluded and the classification procedure for the hazard class "Self-reactive substances and mixtures" does not need to be applied.

A test according to method 92/69/EEC, A.17 was performed. The test item didn’t show oxidising properties.

3.2 Comparison with criteria

Due to the lack of data, it is not possible to assess the hazard class Self-heating substances and mixtures.

However, on the basis of the available data, it can be concluded that *Margosa Extract with water* does not pose other physical hazards.

3.3 Conclusions on classification and labelling

No classification and labelling with regard to the physical hazards are proposed.

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 the physical hazards.

Comments received during consultation

No comments were received.

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**.

4 HUMAN HEALTH HAZARD ASSESSMENT

Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] (*Margosa Extract with water*) is an UVCB substance.

Constituents of kernels can differ from the constituents of other parts of neem tree (e.g., leaves, flowers, stem bark) qualitatively and quantitatively. Additionally, the extraction process (e.g., pre-processing, solvent, temperature, clean up) has a great impact on the composition of the technical extract. Therefore, it is difficult to compare the results of published literature studies with the results of the studies that were submitted for the PPP/BP evaluation, as they were most often conducted with different test substances. Furthermore, only few constituents of neem tree extracts are identified.

Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] (*Margosa Extract with water*) consist of several constituents, e.g., Azadirachtin A, Azadirachtin B, Nimbin or Salannin, of which Azadirachtin A has the highest abundance. Finally, both in the PPP and the BP procedure, the whole extract was considered the toxicologically relevant substance because no toxicological data were available to demonstrate that certain components were responsible for the observed toxicological effects.

Aflatoxins might be present in the extract; being relevant impurities in the meaning of the PPP regulation, maximum levels were defined for them.

All of the toxicological studies were performed with *Margosa Extract with water*. However, *Margosa Extract with water* varies in the content of Azadirachtin A. 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 tables. This concerns studies as follows: acute toxicity 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 all (except micronucleus assay *in vivo*), results from studies with *Margosa Extract with water* with 36.6 % Azadirachtin A are available.

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 "Fortune Aza" or "NPI720"/"ATI 720" which are also technical extracts of seed kernels of 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*.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

No studies were available on absorption, distribution, metabolism and excretion in animals.

4.1.2 Human information

No studies submitted by the applicants

4.1.3 Summary and discussion on toxicokinetics

No studies were available on absorption, distribution, metabolism and excretion. Such studies require radioactive labelled compounds to allow the sensitive detection and identification of parent compound and metabolites. *Margosa Extract with water* is a mixture of several different limonoids and other compounds extracted from the seed kernels of the Neem tree. It is therefore not feasible to perform a kinetic study with *Margosa Extract with water*. It is furthermore also not possible to perform such a study for its analytically leading compound Azadirachtin A due to the unavailability of chemically synthesised and radioactively labelled Azadirachtin A, since it can be obtained by extraction and clean-up of the seed kernels of the Neem tree only. [Note: in open literature a total synthesis of Azadirachtin A was described (reviewed in Jauch, 2008). However, having an overall recovery of 0.00015 %, it is considered of no practical use.] Therefore, it is not possible to obtain radioactive labelled material and it was accepted, that no studies on metabolism and toxicokinetics were submitted.

No information was available on the products of mammalian metabolism. From *in vitro* experiments it was evident that mammalian metabolism resulted in reduced cytotoxicity.

In vitro studies indicated that azadirachtin was hydrolysed in aqueous media also at neutral pH values. Therefore, it was conceivable that ester groups were hydrolysed in the mammalian body.

4.2 Acute toxicity

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

No mortalities were observed in all studies but that of Anonymous (1993a) with 20 % dead rats in the high dose group. Clinical signs of toxicity (such as piloerection, pallor of the extremities, dullness and reduced activity) were seen, but resolved within a few days.

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to their LD_{50} values (both > 5000 mg/kg bw).

Table 11: Summary of acute oral toxicity

Animal species & strain	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)	5 M & 5 F	5000 mg/kg bw, gavage, distilled water (10 mL/kg bw)	> 5000 *Margosa Extract with water* (37 % Azadirachtin A) Clin. signs: piloerection, pallors of the extremities, reduced bw gain in some rats	Anonymous, 1997c
Rat, Wistar	5 M & 5 F	0, 1190, 2380, 4760 mg/kg bw gavage DMSO (20 mL/kg bw)	> 4760 *Margosa Extract with water* (≥ 25% Azadirachtin A*) (at 4760 mg/kg bw: 20 % mortality, dullness and reduced activity)	Anonymous, 1993a
Mouse, Swiss albino	5 M & 5 F	0, 1190, 2380, 3365 mg/kg bw gavage DMSO (15 mL/kg bw)	> 3365 *Margosa Extract with water* (≥ 25 % Azadirachtin A*) (at 3365 mg/kg bw: reduced locomotor activity)	Anonymous, 1993b

* No certificate of analysis provided in study report

4.2.1.2 Acute toxicity: inhalation

No mortalities and no abnormal macroscopic pathological findings were observed. Clinical signs of toxicity were seen during exposure (hunched posture, partial closed eyes and test material on fur) in all animals but not during the observation period (no more details reported in the study report).

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to their LC_{50} values (both > 2.4 mg/L, highest attainable dose). One female animal died ("Fortune Aza").

Table 12: Summary of acute inhalation toxicity

Animal species & strain	Number of animals per dose level	Doses, route of administration, vehicle	LC_{50} (mg/L) Test compound	Reference year Method
Rat, Sprague-Dawley	5 M & 5 F	0.72 mg/L air (4 h), whole body	> 0.72 (highest attainable conc.) *Margosa Extract with water* (37 % Azadirachtin A). No signs of toxicity were observed.	Anonymous, 1997b

4.2.1.3 Acute toxicity: dermal

No mortalities were observed in all studies. No clinical signs of toxicity were seen.

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to their LD_{50} values (both > 2000 mg/kg bw).

Table 13: Summary of acute dermal toxicity

Animal species & strain	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)	5 M & 5 F	2000 mg/kg bw, dermal (24 h), water moistened	> 2000 *Margosa Extract with water* (37 % Azadirachtin A)	Anonymous, 1997d

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

4.2.1.4 Acute toxicity: other routes

No studies with application via other routes were available.

4.2.2 Human information

No studies were available.

4.2.3 Summary and discussion of acute toxicity

Margosa Extract with water was of low acute toxicity following oral, dermal or inhalation exposure. No further mortalities or signs of toxicity were observed in rats upon treatment with single doses via either route.

4.2.4 Comparison with criteria

Table 14 presents the relevant CLP criteria for the highest category that would require classification. LD_{50} values after oral, dermal or inhalation administration of Margosa, ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] were above the threshold levels leading to a classification. The highest achievable dose following inhalation was within the concentration limits which are required for classification as Acute Tox 3, but no signs of toxicity were observed. LC_{50} value following inhalation exposure: > 0.72 mg/L air.

Table 14: CLP criteria for classification for acute toxicity

CLP criteria
Cat 4 (H302): $300 < LD_{50} \leq 2000$ mg/kg (oral) Cat. 3 (H301): $50 < LD_{50} \leq 300$ mg/kg (oral) Cat. 2 (H300): $5 < LD_{50} \leq 50$ mg/kg (oral) Cat. 1 (H300): $LD_{50} \leq 5$ mg/kg (oral)
Cat. 4 (H332): $1.0 < LC_{50} \leq 5.0$ (dusts and mists) Cat. 3 (H331): $0.5 < LC_{50} \leq 1.0$ (dusts and mists) Cat. 2 (H330): $0.05 < LC_{50} \leq 0.5$ (dusts and mists) Cat. 1 (H330): $LC_{50} \leq 0.05$ (dusts and mists)
Cat. 4 (H312): $1000 < LD_{50} \leq 2000$ mg/kg (dermal) Cat. 3 (H311): $200 < LD_{50} \leq 1000$ mg/kg (dermal)

CLP criteria
Cat. 2 (H310): 50 < LD_{50} ≤ 200 mg/kg (dermal)
Cat. 1 (H310): LD_{50} ≤ 50 mg/kg (dermal)

4.2.5 Conclusions on classification and labelling

In summary and based on the submitted data, *Margosa Extract with water* does not meet the criteria to be classified for oral, dermal or inhalation toxicity according to the criteria of the CLP regulation.

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 / *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; EPA FIFRA Guideline 152-14 (1984) (equivalent to OECD TG 402, limit, no deviation), 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.**

4.3 Specific target organ toxicity – single exposure (STOT SE)

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

Transient and mild clinical signs of toxicity (piloerection and pallor of the extremities, dullness and reduced locomotor activity were seen in animals treated with single oral high doses (above 3300 mg/kg bw) of *Margosa Extract with water*. No narcotic effects or irritation of the respiratory tract were observed following, oral, inhalation and dermal exposure.

4.3.2 Comparison with criteria

Table 15: Classification criteria for Categories 1 and 2 of specific target organ toxicity-single exposure (C: guidance value)

CLP criteria	
Category 1 (H370) Oral (rat): C ≤ 300 mg/kg bw Dermal (rat or rabbit): C ≤ 1000 mg/kg bw Inhalation (rat, dust/mist/fume): ≤ 1 mg/L/4 h	Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure - reliable and good quality evidence from human cases or epidemiological studies; or - observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations.
Category 2 (H371) Oral (rat): 2000 ≥ C > 300 mg/kg bw Dermal (rat or rabbit): 2000 ≥ C > 1000 mg/kg bw Inhalation (rat, dust/mist/fume): 5 ≥ C > 1 mg/L/4 h	Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following single exposure - observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.
Category 3 (H335/H336) Guidance values do not apply (mainly based on human data)	Transient target organ effects This category only includes narcotic effects and respiratory tract irritation. These are target organ effects for which a substance does not meet the criteria to be classified in Categories 1 or 2 indicated above. These are effects which adversely alter human function for a short duration after exposure and from which humans may recover in a reasonable period without leaving significant alteration of structure or function.

4.3.3 Conclusions on classification and labelling

Considering that the observed non-lethal effects reported after acute exposure were transient and were not of considerably adverse nature with no significant impact on health or which were only seen in high doses clearly exceeding those required for classification as STOT SE, no classification as STOT SE is proposed.

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.**

4.4 Irritation

4.4.1 Skin irritation

4.4.1.1 Non-human information

Very slight erythema (score: 1) was seen in animals treated with *Margosa Extract with water* which resolved within one day. No signs of systemic toxicity were reported.

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to irritating properties (not irritating).

Table 16: Summary of skin irritation

<table>
<tr><th>Animal species & strain</th><th>Number of animals</th><th>Doses</th><th>Result</th><th>Reference Method</th></tr>
<tr><td>Rabbit, New Zealand albino</td><td>6 M</td><td>0.5 g (4 h)</td><td>Not irritating (highest erythema score: 1), resolved by day 2
Margosa Extract with water (37 % Azadirachtin A)
<table>
<tr><th rowspan="2">Rabbit number and sex</th><th rowspan="2">E = Erythema
O = Oedema</th><th colspan="4">Day</th></tr>
<tr><th>1*</th><th>2</th><th>3</th><th>4</th></tr>
<tr><td>568 ♂</td><td>E
O</td><td>1
0</td><td>0
0</td><td>0
0</td><td>0
0</td></tr>
<tr><td>570 ♂</td><td>E
O</td><td>1
0</td><td>0
0</td><td>0
0</td><td>0
0</td></tr>
<tr><td>583 ♂</td><td>E
O</td><td>0
0</td><td>0
0</td><td>0
0</td><td>0
0</td></tr>
<tr><td>584 ♂</td><td>E
O</td><td>1
0</td><td>0
0</td><td>0
0</td><td>0
0</td></tr>
<tr><td>585 ♂</td><td>E
O</td><td>0
0</td><td>0
0</td><td>0
0</td><td>0
0</td></tr>
<tr><td>586 ♂</td><td>E
O</td><td>0
0</td><td>0
0</td><td>0
0</td><td>0
0</td></tr>
</table>
* Approximately 60 minutes after removal of the dressing</td><td>Anonymous, 1996f</td></tr>
</table>

4.4.1.2 Human information

No studies submitted by the applicants.

4.4.1.3 Summary and discussion of skin irritation

Margosa Extract with water exhibited no irritating potential to skin.

4.4.1.4 Comparison with criteria

Table 17: CLP criteria

CLP criteria
Irritating to skin (Category 2, H315): at least in 2/3 tested animal a positive response of: Mean value of ≥ 2.3 - ≤ 4.0 for erythema/eschar or for oedema

Highest score observed in skin irritation studies was 1 for erythema.

As the results do not meet the criteria laid down in the CLP regulation, classification and labelling for skin irritation is not needed.

4.4.1.5 Conclusions on classification and labelling

In summary and based on the submitted data, *Margosa Extract with water* does not meet the criteria to be classified for skin irritation/corrosion according to the criteria in the CLP regulation.

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.**

4.4.2 Eye irritation

4.4.2.1 Non-human information

Dulling of cornea in one animal, discharge and redness of conjunctiva in all animals were seen 1 h after instillation of test compounds. Effects declined with time and were absent within one or two days. Signs of eye irritation were less severe than the criteria for classification would require.

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to its eye irritating properties (not irritating).

Table 18: Summary of eye irritation

Animal species & strain	Number of animals	Doses	Result*	Reference Method
Rabbit, New Zealand albino	5 M & 1 F	70 mg	Not irritating Cornea opacity: 0.0 / 0.0 / 0.0 Iris: 0.0 / 0.0 / 0.0 Redness of conjunctivae: 1.0 / 0.3 / 0.2 Chemosis: 0.7 / 0.3 / 0.0 *Margosa Extract with water* (37 % Azadirachtin A)	Anonymous, 1996g

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

4.4.2.2 Human information

No studies submitted by the applicant.

4.4.2.3 Summary and discussion of eye irritation

Margosa Extract with water exhibited very slight and reversible irritating potential to the eye.

4.4.2.4 Comparison with criteria

Margosa Extract with water exhibited very slight and reversible irritating potential to the eye. The severity of findings did not reach the critical thresholds to be classified as eye irritant.

Table 19: CLP criteria

CLP criteria
Irritating to eyes (Category 2, H319): at least in 2/3 tested animal a positive response of: corneal opacity: ≥ 1 and/or iritis: ≥ 1 and/or conjunctival redness: ≥ 2 and/or conjunctival oedema (chemosis): ≥ 2

4.4.2.5 Conclusions on classification and labelling

In summary and based on the submitted data, *Margosa Extract with water* did not meet the criteria to be classified for eye irritation/corrosion according to the criteria in CLP regulation.

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 / *Margosa Extract with water* (37 % Azadirachtin A)	5 M & 1 F	70 mg	Mean scores: Not irritating Cornea opacity: 0.0 / 0.0 / 0.0 Iris: 0.0 / 0.0 / 0.0 Redness of conjunctivae: 1.0 / 0.3 / 0.2 Chemosis: 0.7 / 0.3 / 0.0	Anonymous, 1996g, (TG equivalent to OECD 405, no deviations, 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 irritation.**

4.4.3 Respiratory tract irritation

No specific studies (conducted in non-humans or humans) concerning respiratory tract irritation were available. In the acute inhalation studies in rats, no irritation or other respiratory effects were observed. Neither histopathological findings nor practical observations in humans are available. In summary and based on the submitted data, *Margosa Extract with water* does not meet the criteria to be classified as a respiratory tract irritant.

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.**

4.5 Corrosivity

No specific studies regarding corrosion were submitted. Corrosion was not seen in the studies for dermal or eye irritation. Hence, no classification for corrosion of skin or eye is proposed. Please compare also section 4.4 (Irritation).

4.6 Sensitisation

4.6.1 Skin sensititsation

4.6.1.1 Non-human information

Margosa Extract with water was tested according to the protocol of Magnusson & Kligman, *Margosa Extract with water* showed sensitising potential upon skin contact.

Table 20: Summary of skin sensitisation

Animal species & strain	Number of animals	Doses	Result	Reference Method
Guinea pig, Dunkin Hartley albino	20 M treated 10 control	Intradermal: 5 % (w/v) in acetone/alembicol Dermal: 80 % in acetone	Sensitising (M&K) [all animals sensitised] *Margosa Extract with water* (37 %) Scored after 48 h and 72 h, resp.: 20/20; 20/20, negative control: 0/10, 0/10, positive control: 20/20, 20/20, respectively.	Anonymous, 1997a

Slight irritation was observed in all animals after intradermal application of *Margosa Extract with water* with solvent (Anonymous, 1997a). Necrosis was recorded in sites receiving 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 showed slight to well defined oedema and erythema upon challenge with *Margosa Extract with water* solutions (40 and 80 % in acetone). Hence, *Margosa Extract with water* showed sensitising properties by skin contact. Individual data after challenge are depicted in Table 21.

Two other technical extract ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to their sensitising properties (sensitising).

Table 21: Individual erythema and oedema scores after challenge

Freund's treated control animals:

Guinea-pig number	E = Erythema O = Oedema	Score					
		24 Hours		48 Hours		72 Hours	
		A	P	A	P	A	P
795	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
796	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
797	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
798	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
799	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
800	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
801	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
802	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
803	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
804	E	0	0	0	0	0	0
	O	0	0	0	0	0	0

A Anterior site, exposed to NeemAzal Technical, 80 % w/v in acetone
P Posterior site, exposed to NeemAzal Technical, 40 % w/v in acetone

Test animals:

Guinea-pig number	E = Erythema O = Oedema	Score						Results Positive (+) Negative (-) Inconclusive (±)
		24 Hours		48 Hours		72 Hours		
		A	P	A	P	A	P	
805	E	2	L2	2	2	2	2	+
	O	1*	0	2*	1*	2*	1*	
806	E	L2	0	L2	0	L1	0	+
	O	0	0	0*	0	0*	0	
807	E	L2	2	2	2	Ø2	Ø2	+
	O	0	0	1*	0*	2	2	
808	E	2	2	2	2	Ø2	Ø2	+
	O	0	0	0*	0*	2	2	
809	E	L2	0	L2	0	L2	0	+
	O	0	0	0*	0	1*	0	
810	E	2	L2	Ø2	L2	Ø2	L1	+
	O	1	0	2	0	2	0*	
811	E	1	2	2	1	2	2	+
	O	0*	0	2*	1*	2*	1*	
812	E	2	1	2	2	Ø2	Ø2	+
	O	1	0	2*	1*	2	2	
813	E	2	L2	Ø2	ØL2	Ø2	Ø2	+
	O	0	0	2	2	2	1	
814	E	2	2	Ø2	Ø2	ØL2	ØL2	+
	O	1*	1	2	1	1	1	
815	E	L2	L2	ØL1	L1	1	1	+
	O	0	0	0	0	0*	0	
816	E	2	2	Ø2	Ø2	ØNP2	L1	+
	O	1	0	2	1	3	1*	
817	E	L2	0	2	0	2	L2	+
	O	0	0	0*	0	2*	1*	
818	E	2	L2	2	L2	Ø2	Ø2	+
	O	0	0	1*	0	1	1	
819	E	2	2	Ø2	Ø2	Ø2	Ø2	+
	O	2	1	2	2	2	2	
820	E	L2	L2	L2	L2	2	2	+
	O	0	0	0*	0*	2*	2*	
821	E	2	2	Ø2	Ø2	Ø2	Ø2	+
	O	1	1	2	2	2	2	
822	E	2	2	Ø2	Ø2	ØNP2	Ø2	+
	O	1	1	1	2	2	2	
823	E	L2	L2	ØL2	ØL2	Ø2	Ø2	+
	O	0	0	1	1	2	2	
824	E	2	2	2	2	Ø2	Ø2	+
	O	1	0	1*	1*	2	1	

L Localised dermal reaction (restricted to a small area of the challenge site)
NP Necrotic patch
* Dryness and sloughing of the epidermis
Ø Thickening, dryness and sloughing of the epidermis
A Anterior site, exposed to NeemAzal Technical, 80% w/v in acetone
P Posterior site, exposed to NeemAzal Technical, 40% w/v in acetone

Six tests with hexyl cinnamic aldehyde as positive reference substance (performed in December 1992 to January 1999) resulted in allergic reactions and have shown the sensitivity of the guinea pig strain used.

4.6.1.2 Human information

No studies submitted by the applicant. No case reports on hypersensitivity to *Margosa Extract with water* are 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). No more case reports were retrieved.

4.6.1.3 Summary and discussion of skin sensitisation

Margosa Extract with water showed sensitising potential by skin contact.

4.6.1.4 Comparison with criteria

Table 22 presents the toxicological results in comparison with the CLP criteria.

Table 22: Results of skin sensitisation tests in comparison with CLP criteria

Toxicological result	**CLP criteria**
Margosa Extract with water: 20/20 animals positive 5 % intra dermal induction concentration	Guinea pig maximisation test Category 1A (H317): ≥ 30 % responding at ≤ 0.1 % intradermal induction dose or ≥ 60 % responding at > 0.1 % to ≤ 1 % intradermal induction dose Category 1B (H317): ≥ 30 % to < 60 % responding at > 0,1 % to ≤ 1 % intradermal induction dose or ≥ 30 % responding at > 1 % intradermal induction dose

Results with *Margosa Extract with water* in the concentration tested lead to a classification in category 1B. However, as all animals responded and information on lower concentration is not available, subcategory 1A cannot be excluded. Therefore, classification in category 1 (without subcategorisation) is proposed.

4.6.1.5 Conclusions on classification and labelling

In summary and based on the submitted data, *Margosa Extract with water* meets the criteria laid down in the CLP regulation (as amended) to be classified as Skin sensitisation category 1 (H317 - May cause an allergic skin reaction).

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 10 control	Intradermal: 5 % test material in acetone/alembicol 5% teat material in Freund's Complete Adjuvant 1:1 with water Dermal: 10 % SDS in petrolatum to induce irritation 80 % test material in acetone for topical induction 40 % and 80 % test material in acetone for topical challenge (after 3 weeks)	Sensitising (M&K) [all animals sensitised] Challenge after 3 weeks at 40% and 80% Scored after 48 h and 72 h, respectively: 20/20; 20/20 negative control: 0/10, 0/10 positive control: 66/70 *	Anonymous, 1997a EPA FIFRA Guideline 152-15 (equivalent to OECD 406, no deviation) 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.**

4.7 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.7.1 Respiratory sensitisation

No data/information (from non-humans or humans) was submitted that would allow an evaluation of sensitising properties for the respiratory tract.

4.7.2 Non-human information

Studies in rats with repeated oral administration of test compound were available. Neither studies with other species, nor studies with other routes of administration were submitted.

4.7.2.1 Repeated dose toxicity: oral

Rats were treated with repeated doses of *Margosa Extract with water* in a range of 14 to 90 daily doses.

Clear evidence of toxicity was observed in the 28-d study with *Margosa Extract with water* (Anonymous, 1997h) in rats receiving dose levels of 3200, 8000 or 20000 ppm. Upon histopathological examination, all treated animals showed signs of substance effects in the thyroid (follicular epithelial hypertrophy) and the liver (periportal hepatocyte eosinophilia with clumping). Bodyweight gain was reduced in animals with dietary dose levels of 20000 and 8000 ppm. In animals receiving 20000 ppm, hepatocyte hypertrophy was noted. A NOAEL could not be established, the LOAEL was the lowest dose tested of 300 mg/kg bw/d (3200 ppm).

After treatment of rats for 90 d with 6400 ppm of *Margosa Extract with water* in feed (achieved dose 490 and 525 mg/kg bw/d for males and females, respectively), evidence of hepatotoxicity (in both sexes: organ weight increase, hepatocyte hypertrophy; in females only: periportal fat deposition, (minimally) increased blood protein levels) was observed (Anonymous, 1997i). Furthermore, effects on haematology (females: higher mean platelet values, (slightly) reduced thrombotest-values; males: prolonged blood coagulation (APTT), prolonged thrombotest-values) and thyroid (increased relative weight, slight increase of incidence of follicular epithelial hypertrophy) were seen. At 1600 ppm (achieved dose 123 and 135 mg *Margosa Extract with water*/kg bw/d for males and females, respectively) increased incidence and severity of periportal fat deposition was noted in females only, while slightly increased total protein levels were noted for both sexes and prolonged APTT values for males only.

For male rats, statistically significant elevated red blood cell counts for the 400 ppm, 1600 ppm and 6400 ppm and lower mean corpuscular values (MCV) were noted for the 1600 ppm and 6400 ppm dose groups. Females of the 6400 ppm treatment group had significantly reduced packed cell volume (PCV), MCV and reduced platelet count values. MCHC values were elevated for the 1600 ppm and 6400 ppm dose groups. The coagulation parameter TT was prolonged for males but reduced for females of the highest dose group, while APTT was dose-related prolonged for 400, 1600 and 6400 ppm males. These effects were statistically significant but marginal at 400 ppm. The effects seen at 400 ppm were considered to be toxicologically not relevant, as they were only marginal. It was concluded that at 400 ppm (achieved dose 32 and 36 mg/kg bw/d for males and females, respectively) and 100 ppm (achieved dose 8 and 9 mg/kg bw/d for males and females, respectively) no signs of toxicity were observed. The NOAEL in this study was 32 mg/kg bw/d (400 ppm).

Table 23: Summary of oral RDT

Animal species & strain	**Number of animals**	**Doses, vehicle, duration**	**Result**	**Reference Test compound Method**
Rat, CD	5 M & 5 F	20000, 50000 ppm (equivalent to 2000, 5000 mg/kg bw/d) **Feed** **2-wk**	LOAEL: 20000 ppm (2000 mg/kg bw/d) bw ↓; feed intake (50000 ppm) ↓ *Margosa Extract with water* (Azadirachtin content not stated)	Anonymous, 1995. (only data on bodyweight, food consumption, daily observations)
Rat, Crt: CD (SD) BR	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) **Feed** **4-wk**	LOAEL: 300 mg/kg bw/d (3200 ppm) <u>All dose levels:</u> hepato-toxicity (periportal hepatocyte eosinophilia with clumping), thyroid toxicity (follicular epithelial hypertrophy) **Liver weights** (g): (0-3200-8000-20,000 ppm) M: 19-19.2-21.3*-20.6** F: 11.2-12.6-13.6*-16.6** **Thyroid weights** (mg): (0-3200-8000-20,000 ppm) M: 17.9-20.1-24.7-22.9 F: 16.2-18.7-23.3*-24.2* Adrenal weights (mg): (0-3200-8000-20,000 ppm): M: 62.3-51.4-52.5-49.3* F: 69.0-69.8-70.5-63.0 <u>20000 ppm:</u> 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 % <u>8000 ppm:</u> **lower bw gain in females** (% control): days 1-4/4-8/8-29: 42%/78%/93%, resp.	Anonymous, 1997h *Margosa Extract with water* (37 % Azadirachtin content)

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference Test compound Method
Rat, Crt: CD BR	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) **Feed** **90-d**	NOAEL: 32 mg/kg bw/d (400 ppm) Haematological parameters: 0-100-400-1600-6400 ppm **APTT (s)**: M: 19.2-20.4-21.0-22.1-24.1 F: 16.4-16.8-16.2-15-8-15.6 **TT (s)** M:25-26-26-27-30**) F: 20-20-32-20-19* **MCV (fL)** M: 53.8-53.6-52.6-52.2*-52.2* F: 56.3-55.4-55.2-55.1-53.1** **PCV (%)** M: 48.1-18.2-49.4-48.5-48.1 F: 46.8-46.5-45.7-45.7-44.8** **Liver weights (g)** 0-100-400-1600-6400 ppm M: 20.6-18.3-20.6-20.0-23.0* F: 11.1-10.1-11.1-11.9-14.5*<u>6400 ppm:</u> liver (wt ↑: approx. 11%; hepatocyte hypertrophy, periportal fat deposition, blood protein levels ↑), thyroid (rel. wt↑(F) : approx.. 17 %; follicular epithelial hypertrophy) <u>1600 ppm:</u> liver (periportal fat deposition in females), haematology: prolonged APTT in males (+15 % *vs.* control)	Anonymous, 1997i *Margosa Extract with water* (26.8 – 28.4 % Azadirachtin content)

*p < 0.05; ** p < 0.01

4.7.2.2 Repeated dose toxicity: inhalation

No studies with repeated dose inhalation administration were available.

4.7.2.3 Repeated dose toxicity: dermal

No studies with repeated dose dermal administration were available.

4.7.2.4 Repeated dose toxicity: other routes

No studies with repeated dose administration via other routes were available.

4.7.2.5 Human information

No studies submitted by the applicants

4.7.2.6 Other relevant information

No studies with other mammalian species were submitted. There was no indication for toxic effects from feeding studies published in open literature conducted in various farm animals (cows, calves, and bulls, buffalo calves, growing pigs, sheep) with water-washed Neem seed kernel cake (typical contents were between 0.1 and 1 g Azadirachtin A/kg) (studies summarised by the notifier: Anonymous, 2002; Anonymous, 2005c). No signs of toxicity regarding a diverse spectrum of parameters tested were reported upon admixing up to 45 % water-washed Neem seed kernel cake to the regular concentrate mixture. Such feeding studies in farm animals were conducted for up to twelve months and no adverse effects were noted. Parameters were milk production in cows, sperm quality in bulls, growth rate in piglets, and cattle, meat characteristics. Also red and white cell counts as well as haemoglobin and liver enzymes were unaffected.

Unfortunately, the available data allow only a very rough estimate of the amount of azadirachtin to which the farm animals were exposed. According to the applicant, the highest concentration of *Margosa extract* in the diet of goats receiving 25 % "water washed neem seed kernel cake" (WWNSKC) as protein concentrate mixture was 375 ppm. Growing calves were fed a concentrate mixture containing 45 % water-washed Neem seed kernel cake, based on the Azadirachtin A content, this was equivalent of a dietary dose of approx. 675 ppm *Margosa Extract with water*. Using standard conversion factors for goats and cattle to adjust dietary concentrations to a mean daily intake per kg bodyweight, assuming a fraction of one third of the protein concentrate mixture in the total diet and taking into account the variability in Azadirachtin A content in the extracts and other neem products, a mean daily dose of Azadirachtin A in the range of 3-9 mg/kg bw (equivalent to 9-27 mg *Margosa Extract with water*/kg bw) may be calculated. This would be in the same order of magnitude as the NOAEL in the subchronic study in rats and is much lower than doses that produced adverse effects in those experiments.

4.7.2.7 Summary and discussion of repeated dose toxicity

Effects seen in the repeated-dose 90-d study with *Margosa Extract with water* in rats revealed a NOAEL of 32 mg/kg bw/d with a LOAEL of 123 mg/kg bw/d. Effects were seen predominantly in the liver. Thyroid follicular epithelium hypertrophy was seen in the study with *Margosa Extract with water* (Anonymous, 1997h) at a dose level of 6400 ppm (achieved dose 490 and 525 mg/kg bw/d for males and females, respectively); no studies were submitted to explore if this effect was secondary to liver enzyme induction, which might be indicated by liver weight increase.

4.7.2.8 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

No severe effects were seen in the 28-d and 90-d study in rats with *Margosa Extract with water*.

4.7.2.9 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

Table 24: presents the CLP criteria for classification.

CLP criteria
Category 1 (H372): Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 30 mg/kg bw/d 90-day: ≤ 10 mg/kg bw/d
Category 2 (H373): Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 300 mg/kg bw/d 90-day: ≤ 100 mg/kg bw/d

No severe or significant findings were observed in rats at dose levels below the respective guidance values. Hence, it is proposed not to classify for STOT-RE.

4.7.2.10 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

Classification for effects seen in repeated-dose studies was considered not necessary.

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 / *Margosa Extract with water* (Azadirachtin content not stated)	5 M & 5 F	20000, 50000 ppm (equivalent to 2000, 5000 mg/kg bw/d) **Feed** **2-wk**	LOAEL: 20000 ppm (2000 mg/kg bw/d) bw ↓; feed intake (50000 ppm) ↓	Anonymous, 1995 (only data on bodyweight, food consumption, daily observations)
Rat, Crt: CD (SD) 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) **Feed** **4-wk**	LOAEL: 300 mg/kg bw/d (3200 ppm) All dose levels: hepato-toxicity (periportal hepatocyte eosinophilia with clumping), thyroid toxicity (follicular epithelial hypertrophy) **Liver weights** (g): (0-3200-8000-20,000 ppm) M: 19-19.2-21.3*-20.6** F: 11.2-12.6-13.6*-16.6** **Thyroid weights** (mg): (0-3200-8000-20,000 ppm) M: 17.9-20.1-24.7-22.9 F: 16.2-18.7-23.3*-24.2*	Anonymous, 1997h

			Adrenal weights (mg): (0-3200-8000-20,000 ppm): M: 62.3-51.4-52.5-49.3* F: 69.0-69.8-70.5-63.0 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 % 8000 ppm: **lower bw gain in females** (% control): days 1-4/4-8/8-29: 42 %/78 %/93 %, resp.	
Rat, Crt: CD 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) **Feed** **90-d**	NOAEL: 32 mg/kg bw/d (400 ppm) Haematological parameters: 0-100-400-1600-6400 ppm **APTT (s)**: M: 19.2-20.4-21.0-22.1-24.1 F: 16.4-16.8-16.2-15-8-15.6 **TT (s)** M:25-26-26-27-30**) F: 20-20-32-20-19* **MCV (fL)** M: 53.8-53.6-52.6-52.2*-52.2* F: 56.3-55.4-55.2-55.1-53.1** **PCV (%)** M: 48.1-18.2-49.4-48.5-48.1 F: 46.8-46.5-45.7-45.7-44.8** **Liver weights (g)** 0-100-400-1600-6400 ppm M: 20.6-18.3-20.6-20.0-23.0* F: 11.1-10.1-11.1-11.9-14.5* 6400 ppm: liver (wt ↑: approx. 11%; hepatocyte hypertrophy, periportal fat deposition, blood protein levels ↑), thyroid (rel. wt↑(F) : approx. 17 %; follicular epithelial hypertrophy) 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.**

4.8 Germ cell mutagenicity (Mutagenicity)

4.8.1 Non-human information

4.8.1.1 In vitro data

The results of the submitted tests did not show a potential to induce gene mutations under the test conditions used. However, NeemAzal showed clastogenic activity in cytotoxic concentrations in chromosomal aberration test in cultured human lymphocytes.

In the chromosomal aberration study with *Margosa Extract with water* (Stien, 2006, TOX2006-739), cytotoxicity (lower mitotic index) was observed in concentrations of 2500 µg/mL and above; in these concentrations, test compound was observed to precipitate. Significantly increased CA rate was observed at 5000 µg/mL without metabolic activation (4 h exposure). The aberration rates in the other incubations were within the range of incubations with solvent or within the range of historical control incubations.

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to their *in vitro* mutagenic properties (non mutagenic in AMES test and HPRT gene mutation assay, no results of a clastogenic assay *in vitro* available).

Table 25: Summary of in vitro mutagenicity

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

4.8.1.2 In vivo data

The tested extract *Margosa Extract with water* (content Azadirachtin A: 27 %) did not induce micronucleated polychromatic erythrocytes, when tested in mouse micronucleus assay. Ratio of polychromatic to normochromatic erythrocytes was slightly decreased in mice treated with *Margosa Extract with water* (significant at highest dose and at 24 h only).

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were comparable with respect to their *in vivo* genotoxic properties (non genotoxic *in vivo* in the micronucleus assay in mice).

Table 26: Summary of in vivo mutagenicity

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

4.8.2 Human information

No studies submitted by the applicants

4.8.3 Other relevant information

No other relevant information available.

4.8.4 Summary and discussion of mutagenicity

Neem Azal technical (content Azadirachtin A: 37 % in *in vitro* studies, 27 % in the *in vivo* study) was tested in a three *in vitro* and one *in vivo* genotoxicity assays, measuring different mutagenicity endpoints such as gene mutations in bacterial and mammalian cells, and chromosomal mutations *in vitro* and *in vivo*.

The results of all the tests did not show a potential to induce gene mutations of the azadirachtin technical extract under the test conditions used. However, clastogenic activity was observed in cytotoxic concentrations in chromosomal aberration test in cultured human lymphocytes. The tested extract with a slightly lower content of Azadirachtin A (27 % vs. 37 % *in vitro*) did not show genotoxic potential in an *in vivo* micronucleus test in mice.

4.8.5 Comparison with criteria

Table 27: Following criteria for classification for gem cell mutagens are given in CLP regulation:

CLP regulation
The classification in Category 1A is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans. The classification in Category 1B is based on: — positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or — positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or — positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people. The classification in Category 2 is based on: — positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from: — somatic cell mutagenicity tests in vivo, in mammals; or — other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays. Note: Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.

No human data are available; hence a classification in category 1A is not possible. Neither *in vivo* heritable germ cell mutagenicity tests nor positive results from *in vivo* somatic cell mutagenicity tests in mammals are available; hence a classification in 1B is not possible. Results of one *in vitro* study (clastogenicity) were positive in cytotoxic concentrations, others (Ames, HPRT) and the respective

in vivo studies showed a negative outcome, hence a classification in category 2 is considered not necessary.

4.8.6 Conclusions on classification and labelling

No classification for mutagenicity was considered necessary, as the criteria laid down in CLP regulation were not met.

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** **Test compound**	**Reference** **Method**
Ames test / ***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 OECD 471
CA / ***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 OECD 473
HPRT gene mutation / ***Margosa Extract with water* (37 % Azadirachtin A)**	CHO cells	(25)200-1250 µg/mL	Non mutagenic (+/- S9)	Adams & Kirkpatrick, 1997 TOX9700512 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 / ***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 **the criteria for germ cell mutagenicity are not fulfilled**.

4.9 Carcinogenicity

4.9.1 Non-human information

4.9.1.1 Carcinogenicity: oral

In a two year carcinogenicity study in rats (Anonymous, 2000a), *Margosa Extract with water* was dosed up to 448 mg/kg bw in males or 635 mg/kg bw/d in females (6400 ppm in feed). No test substance related carcinogenic effect was seen in this study. Gross and histopathologic findings were considered incidental and typical of the rat strain employed. No effects were found, thus the high dose level was considered the NOAEL. Deficiencies in the study design of this study concerning requirements for chronic toxicity studies (urinalysis not performed; haematology and clinical chemistry performed only at study initiation, after 6 and 12 months of treatment and at necropsy with limited parameters assessed) can be put aside with information of subchronic and carcinogenicity

studies (*urinalysis:* histopathological investigation of kidneys and blood urea nitrogen concentration in this long-term study and urinalysis in a 90-d study did not indicate nephrotoxicity; *haematology/clinical chemistry:* full macro- and microscopic pathological investigation showed no adverse findings (all findings were considered incidental and typical for the rat strain employed) and full clinical chemistry analysis was performed in a 90-d study and showed only few modified parameters which were not investigated in this long-term study [MCV, MCHC, globulin]). In conclusion and considering the information requirements for pesticides and biocides, the list of parameters examined in this study was not complete as compared to requirements of OECD guidelines 452 and 453. It however appears unlikely that toxicologically relevant adverse changes with respect to these parameters have been overlooked by these omissions.

The results of this study are not in agreement with the results of the 90-d feeding studies in rats. In the subchronic study's findings were hepatotoxicity, follicular epithelial hypertrophy, and prolonged coagulation time in male rats. One explanation for these differences might be the use of different rat strains (Wistar rats in carcinogenicity and reproductive study, Crl: CD BR rats in subchronic studies). However, there were some indications for prolonged coagulation time in male rats in the highest dose group at days 190 and 360 compared to day 0 but values were not statistically significant. Dose selection for carcinogenicity testing was based on results of the 90-d study (Anonymous, 1997i). According to OECD Guidance Document 116 (Guidance document 116 on the conduct and design of chronic toxicity and carcinogenicity studies, supporting test guidelines 451, 452 and 453, OECD 2014), the highest dose group would not have been accepted as maximum tolerated dose (MTD).

This study was discussed during an expert consultation of the PPP procedure: "The validity of the study was questioned, especially as no effects were seen at the highest dose tested (approx. 400 and 500 mg/kg bw/day in males and 560 and 700 mg/kg bw/day in females). In the 90-d-study effects were observed at 32 mg/kg bw/day. […] Strong doubts were raised about the validity of the long term study: - Uncertainties over the specification of material tested; - No control animals developed tumours (and no hypertrophy) after two years. The doubts raised for this study mean that there is no reliable long term information on long term toxicity for Azadirachtin (the mouse study was deemed unacceptable because only a 5 % Azadirachtin formulation was used). It was questioned whether the effects seen in the 90-d study be adaptive? No conclusion on long term toxicity and/or carcinogenicity can be drawn due to the limited information available" (cited from the meeting minutes).

In contrast, the carcinogenicity study was accepted within the framework of Dir 98/8/EC. In the peer review process according to biocide active substance approval, the more severe results in the 90-d study compared to the chronic study were addressed. The possible explanation, differences may be caused by the use of different rat strains (90-day study: SD rats; chronic study: Wistar rats) obtained from different breeders was accepted. This point was not part of TM discussion. The "Technical Meeting" (TM III/2010) recommended Annex I inclusion for *Margosa extract with water*.

Due to minor deviations and the lack of GLP status for the laboratory at that time, the carcinogenicity study was accepted with restrictions (reliability 2). Treatment related tumours were not observed in the rat study up to doses approximately half the limit dose. Thus, endpoints of carcinogenicity are considered adequately addressed in the study.

We were informed by UK GLP authority that the testing facility was not part of its GLP monitoring program.

The mouse carcinogenicity study (Anonymous, 1996e) with the formulation NeemAzal-F 5 % (contains approx. 20 % *Margosa Extract with water* and 80 % polyethylene oxide) showed no carcinogenic potential and also no treatment related histopathological findings were noted (highest

dose tested: 63 mg/kg bw/d in males, 72 mg/kg bw/d in females (1000 ppm)). Gross and histopathologic findings were considered incidental and typical of the mouse strain employed. No effects were found, thus the high dose level was considered the NOAEL. The notifier proposed a correction factor of 5 to calculate *Margosa Extract with water* dose levels from NeemAzal-F5 % dose levels, leading to an estimated NOAEL of 12.6 mg/kg bw/d.

No studies on carcinogenicity were submitted for two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) for the evaluation as the pesticide active ingredient "azadirachtin".

Table 28: Summary of oral carcinogenicity

Animal species & strain	Number of animals	Doses, vehicle, duration	Result Test compound	Reference Method
Rat, Wistar	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) **Feed** **7 d/wk; 105-wks**	NOAEL: 448 mg/kg bw/d (6400 ppm) No toxic effects reported. Slightly increased (not significant) coagulation time observed in medium and high dose in male rats. Gross Pathology: Rounded or irregular growths in teat region in females 0-400-1600-6400ppm, respectively): 2-1-3-3. Males: 2 in lower abdomen (6400, 400 ppm), 1x prostate (6400 ppm) No carcinogenic effects reported (observed tumours considered incidental): Tumour rates: 0-400-1600-6400ppm, respectively): Mammary tumours: F: 2-1-3-3 Lymphosarcoma: M: 0-1-0-1 Prostatic carcinoma: M: 0-0-0-1 *Margosa Extract with water* (37 % Azadirachtin A) Death rates were increased in all treatment groups but were considered not treatment related. **Number of Deaths**: 0-400-1600-6400ppm, respectively): M: 4-6-3-10 F: 1-5-5-5	Anonymous, 2000a (clinical chemistry performed)
Mouse, Swiss albino	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) **Feed** **18-mo**	NOAEL: 63 mg/kg bw/d (1000 ppm) No toxic effects reported No carcinogenic effects reported NeemAzal-F 5 % (formulation, 5 % Azadirachtin A content)	Anonymous, 1996e (feed analysis not performed, clinical signs not reported)

4.9.1.2 Carcinogenicity: inhalation

No information concerning carcinogenicity after inhalation administration available.

4.9.1.3 Carcinogenicity: dermal

No information concerning carcinogenicity after dermal administration available.

4.9.2 Human information

No information concerning carcinogenicity in humans available.

4.9.3 Other relevant information

No other relevant information available.

4.9.4 Summary and discussion of carcinogenicity

Based on this information, *Margosa Extract with water* did not induce tumours in rats. However, the limitations of the available studies need to be taken into account.

4.9.5 Comparison with criteria

Table 29 presents CLP criteria.

Table 29: Criteria for classification

CLP regulation
A substance is classified in Category 1 (known or presumed human carcinogens) for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as: Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from: — human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or — animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). — In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals. The placing of a substance in Category 2 (suspected human carcinogens) is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies. […] 3.6.2.2.3. Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance. Sufficient human evidence demonstrates causality between human exposure and the development of cancer, whereas sufficient evidence in animals shows a causal relationship between the substance and an increased incidence of tumours. Limited evidence in humans is demonstrated by a positive association between exposure and cancer, but a causal relationship cannot be stated. Limited evidence in animals is provided when data suggest a carcinogenic effect, but are less than sufficient. The terms ‘sufficient’ and ‘limited’ have been used here as they have been defined by the International Agency for Research on Cancer (IARC) and read as follows: **(a) Carcinogenicity in humans** The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

- sufficient evidence of carcinogenicity: a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence;
- limited evidence of carcinogenicity: a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

(b) Carcinogenicity in experimental animals

Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals. The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

- sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;
- limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

3.6.2.2.4. Additional considerations (as part of the weight of evidence approach (see 1.1.1)). Beyond the determination of the strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans. The full list of factors that influence this determination would be very lengthy, but some of the more important ones are considered here.

3.6.2.2.5. The factors can be viewed as either increasing or decreasing the level of concern for human carcinogenicity. The relative emphasis accorded to each factor depends upon the amount and coherence of evidence bearing on each. Generally there is a requirement for more complete information to decrease than to increase the level of concern. Additional considerations should be used in evaluating the tumour findings and the other factors in a case-by-case manner.

3.6.2.2.6. Some important factors which may be taken into consideration, when assessing the overall level of concern are:

a) tumour type and background incidence;
b) multi-site responses;
c) progression of lesions to malignancy;
d) reduced tumour latency;
e) whether responses are in single or both sexes;
f) whether responses are in a single species or several species;
g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity;
h) routes of exposure;
i) comparison of absorption, distribution, metabolism and excretion between test animals and humans;
j) the possibility of a confounding effect of excessive toxicity at test doses;
k) mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity.

Mutagenicity: it is recognised that genetic events are central in the overall process of cancer development. Therefore evidence of mutagenic activity in vivo may indicate that a substance has a potential for carcinogenic effects.

There are no relevant data from epidemiological studies submitted by the notifier, hence no classification with Cat 1A according to the CLP regulation is proposed.

Considering the limitations of the studies regarding carcinogenicity with *Margosa Extract with water* (as discussed during an expert consultation of the PPP procedure), no sufficient data seem to be available to allow a robust evaluation.

4.9.6 Conclusions on classification and labelling

On the basis of the rat study, no classification for carcinogenicity was considered necessary, as the criteria laid down in the CLP regulation are not met. However, as a mice study was only performed with the formulation, data is lacking to allow a firm conclusion.

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** **Method**
Rat, Wistar / *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) **Feed** **7 d/wk; 105-wks**	NOAEL: 448 mg/kg bw/d (6400 ppm) No toxic effects reported. Slightly increased (not significant) coagulation time observed in medium and high dose in male rats. Gross Pathology: Rounded or irregular growths in the teat region in females: (at 0-400-1600-6400ppm, respectively) 2-1-3-3. 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): Tumour rates: (at 0-400-1600-6400ppm, respectively): Mammary tumours: F: 2-1-3-3 Lymphosarcoma: M: 0-1-0-1 Prostatic carcinoma: M: 0-0-0-1 Death rates were increased in all treatment groups but were considered not treatment related. **Number of Deaths**: (at 0-400-1600-6400ppm, respectively): M: 4-6-3-10 F: 1-5-5-5	Anonymous, 2000a (clinical chemistry performed) Gaitonde Committee Guideline 6.3.0.C.iv. – corresponds to OECD TG 452 GLP
Mouse, Swiss albino / NeemAzal-F 5 % (formulation, 5	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,	NOAEL: 63 mg/kg bw/d (1000 ppm) No toxic effects reported. No carcinogenic effects reported	Anonymous, 1996e (feed analysis not performed, clinical

% Azadirachtin A content)		72 mg/kg bw/d in females) **Feed** **18-mo**		signs not reported) Gaitonde Committee Guideline 6.3.0.C.iv. – corresponds to OECD TG 452 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.**

4.10 Toxicity for reproduction

4.10.1 Effects on fertility

4.10.1.1 Non-human information

In the two generation reproduction study *Margosa Extract with water* (Anonymous, 2000b) had no impact on clinical signs, bodyweight, feed consumption and gross (and microscopic) pathology of

parental animals (highest dose tested: 50.7 mg/kg bw/d in males, 59.6 mg/kg bw/d in females (750 ppm)). Treatment with *Margosa Extract with water* had no influence on reproduction. Information on the observations in offspring is provided in section 4.10.2.1.

In another (not acceptable) two generation reproduction study (Anonymous, 1996d) with the formulation NeemAzal-F 5 % (containing 20% *Margosa Extract with water* in 80% polyethylene oxide, equivalent to approx. 5 % w/w Azadirachtin A), increased relative weights of ovaries and spleen in maternal rats were noted in all treatment groups (approx. 13-333 mg/kg bw/d or 200-5000 ppm). Additionally, mean bodyweights in intermediate and high dose animals were reduced. The formulation had no effect on reproduction. Information on the observations in offspring is provided in section 4.10.2.1.

A third (not acceptable) one generation reproductive toxicity study (Anonymous, 2000c) could not be taken into account due to deficiencies in the study design and the study report.

No studies on fertility were submitted for two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) for the evaluation as the pesticide active ingredient "azadirachtin".

Table 30: Summary of effects on fertility

Animal species & strain	Number of animals	Doses, vehicle, duration	Result Test compound	Reference
Rat, Wistar	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) Feed 2-gen. study	Parental: No effects on parents NOAEL: 50 mg/kg bw/d (750 ppm) Reproductive: No effects on reproduction NOAEL: 50 mg/kg bw/d (750 ppm) *Margosa Extract with water* (37.3 % Azadirachtin A)	Anonymous, 2000b (no data on feed analysis, time to fertilisation not reported) for more details, see table below.
Rat, Charles Foster	10 M & 20 F	0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d) Feed 2-gen. study	Parental: spleen, ovary wt ↑, bw ↓ LOAEL: appr. 13 mg/kg bw/d (200 ppm) Reproductive: No effects on reproduction NOAEL: appr. 333 mg/kg bw/d (5000 ppm) NeemAzal F 5 % (formulation of 5% azadirachtin in 95% polyethyleneoxide)	Anonymous, 1996d (no data on feed analysis, time to fertilisation and duration of gestation not reported)

Table 31: Bodyweights and organ weights of males P0 animals (absolute and relative values)

Absolute values										
Dose level (ppm)	**Fasted body-weight (g)**	**Liver (g)**	**Brain (g)**	**Kidney[§] (g)**		**Heart (g)**	**Adrenal[§] (mg)**		**Gonads[§] (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

Absolute values										
Dose level (ppm)	**Fasted body-weight (g)**	**Liver (g)**	**Brain (g)**	**Kidney§ (g)**		**Heart (g)**	**Adrenal§ (mg)**		**Gonads§ (g)**	
Relative values										
Dose level (ppm)		**Liver (%)**	**Brain (%)**	**Kidney§ (%)**		**Heart (%)**	**Adrenal§ (%)**		**Gonads§ (%)**	
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 male rats of the P1 generation a reduced relative mean brain weight noted at the lowest dose was considered incidental. Also reduced relative testes weights were observed in the 250 and 500 ppm treatment group. However, these effects were marginal and only confined to one side and, thus, considered no signs of toxicity. No significant changes in relative or absolute means of organ weights were observed in females of the P1 generation.

Table 32: Bodyweights, absolute and relative organ weights in male P1 animals – means

Dose level (ppm)	**Fasted bodyweight (g)**	**Brain (g)**	**Brain (%)**	**Heart (g)**	**Heart (%)**	**Gonads§ (mg)**		**Gonads§ (%)**	
0	344.1	1.81	0.52	0.93	0.27	1.42	1.46	0.41	0.42
250	348.5	1.79	0.51*	0.90	0.26	1.42	1.41	0.41	0.40*
500	349.5	1.81	0.52	0.93	0.27	1.44	1.41	0.42	0.41*
750	347.9	1.81	0.53	0.93	0.27	1.44	1.44	0.42	0.42

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

Administration of *Margosa Extract with water* did not influence pup bodyweights for the male and female offspring for all matings of both generations. Total number of live pups was reduced in the litter from the first mating of the P1 generation, both, number of male and female pups were reduced in the 500 and 750 ppm dose groups. However, in the subsequent matings number of pups (F2b and F2c) was not different from control animals and thus this effect is considered not treatment related. The proportion of male pups was reduced in the F1a litter in the highest dose group. However, since sex ratio was normal (48.1 % male) in the litters of the subsequent mating (F1b), this observation was not considered treatment related. Reproductive performance and the other litter parameters assessed, e.g. bodyweight and sex ratio were not affected by ingestion of test diets at any level tested.

Table 33: Effect of treatment on mean bodyweights (g) for the offspring from all matings of both generations

Litter	Dose level (ppm)	Total number of live pups		Sex ratio (% male)	Mean bodyweight at lactation day					
					0		4		21	
		M	f		m	f	m	f	m	f
F1a	0	69	81	46.0	5.10	5.06	9.26	9.12	25.25	25.76
	250	74	77	49.0	5.14	5.06	9.31	9.16	25.78	25.93
	500	73	97	42.9	5.14	5.16	9.26	9.23	24.71	24.77
	750	62	97	39.0	5.08	4.93	9.00	9.12	24.34	24.43
F1b	0	78	78	50.0	5.24	5.32	8.38	8.35	33.92	33.86
	250	70	67	51.1	5.33	5.40	8.08	8.00	33.76	34.00
	500	73	71	50.7	5.44	5.44	8.16	7.96	34.96	35.14
	750	74	80	48.1	5.47	5.40	8.11	8.01	35.23	34.70
F2a	0	72	75	49.0	4.22	4.25	8.73	8.83	30.03	29.05
	250	68	66	50.7	4.44	4.42	8.54	8.40	30.53	30.43
	500	63	58	52.1	4.54	4.55	8.19	8.59	29.54	30.24
	750	61	51	54.5	4.75	4.76	8.77	8.76	31.44	30.98
F2b	0	79	66	54.5	4.71	4.41	8.72	8.41	29.80	29.64
	250	74	57	56.5	4.59	4.32	8.47	8.16	29.12	29.32
	500	64	64	50.4	4.89	4.84	8.45	8.39	31.45	30.81
	750	78	64	54.9	4.50	4.25	8.29	8.15	29.37	28.72
F2c	0	67	62	51.9	4.49	4.34	8.48	8.42	28.03	29.42
	250	71	79	47.3	4.49	4.46	8.18	8.20	27.73	29.15
	500	75	63	54.4	4.64	4.70	8.44	8.35	29.23	29.76
	750	69	70	49.6	4.48	4.38	8.29	8.37	28.98	29.98

P0 generation: In the testes of two animals of the high dose group tubular hypoplasia was noted. This was not observed in any other dose group and only in one male of the control group. In three cases of the high dose group hyperaemia of substance was reported in the testes of the high dose group. This was not observed in any other dose or control group.

P1 generation: Tubular atrophy and focal interstitial oedema were noted in two males each of the high dose and the intermediate dose level, while this observation was reported in one male of the low dose and control group of the P1 parental generation. Hyperaemia of the uterus was noted in three and two females of the high and mid dose respectively, while this was noted only in one case of the control group. Several other sporadic effects were noted but there was no substance related effects since similar observations were made in control animals. No lesions were noted in F2b that were subjected to necropsy neither with regard to gross pathology nor histopathological examinations.

Conclusions:

There were no treatment related reproductive effects reported regarding litter size or fertility. The NOEL/NOAEL was 750 ppm with regard to reproductive parameters, corresponding to 51 mg and 60 mg *Margosa Extract with water*/kg bw/day for males and female, respectively. No dose related effects were noted in parental animals, the NOAEL is, thus, equivalent to the maximal dose tested, 750 ppm corresponding to 51 or 60 mg *Margosa Extract with water*/kg bw/d for males or females respectively.

4.10.1.2 Human information

No studies submitted by the applicants

4.10.2 Developmental toxicity

4.10.2.1 Non-human information

The results of the available studies are summarised in Table 34.

Table 34: Summary for developmental toxicity

Reference	Protocol Species	Doses	Maternal effects Test compound	Developmental effects
Anonymous, 1997e	OECD 414 (only 10 F per dose group, only external morphology examination) 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 NOAEL: 100 mg/kg bw/d *Margosa Extract with water* (36.7 % Azadirachtin A)	No effects on foetuses NOAEL: 1000 mg/kg bw/d
Anonymous, 1997f	OECD 414 Rat, Crl:CD BR VAF/plus	0, 50, 225, 1000 mg/kg bw/d	1000 mg/kg bw/d: Bw ↓, feed intake ↓, post-dosage salivation NOAEL: 225 mg/kg bw/d *Margosa Extract with water* (36.7 % Azadirachtin A)	255 mg/kg bw/d: Malformations (cf. Table 36), supernumerary ribs (only 1000) NOAEL: 50 mg/kg bw/d
Anonymous, 2000b .	Similar OECD TG 416 (no data on feed analysis, time to fertilisation not reported) for more details, see section 4.10.1.1 2-gen. study 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) Feed	Parental: No effects on parents NOAEL: 50 mg/kg bw/d (750 ppm) *Margosa Extract with water* (37.3 % Azadirachtin A)	Developmental: No effects on offspring NOAEL: 50 mg/kg bw/d (750 ppm) at 750 ppm: mild but stat. significant lower relative testes, brain, and heart weights (only in F_0), not considered adverse
Anonymous, 1996d	Similar OECD TG 416 (no data on feed analysis, time to fertilisation and duration of gestation not reported) 2-gen. study Rat	0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d) Feed	Parental: spleen, ovary wt ↑, bw ↓ LOAEL: appr. 13 mg/kg bw/d (200 ppm) NeemAzal F 5 % (formulation of 5% azadirachtin in 95% polyethyleneoxide)	Developmental: No effects on offspring NOAEL: appr. 333 mg/kg bw/d (5000 ppm)

Maternal body weight changes are depicted in Table 35.

Table 35: Maternal bodyweights and bodyweight changes (Anonymous, 1997f)

	Dose level (mg/kg bw/d)			
	0	**50**	**225**	**1000**
Number of animals §	23	23	23	23
Weight gain Day 2-Day 6	40.1	39.9	36.9	34.3
Weight gain Day 6-Day 8	10.4	10.5	8.5	6.1**
Weight gain Day 8-Day 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

Treatment of pregnant rats with high (and intermediate) doses of *Margosa Extract with water* (≥ 300 mg/kg bw/d) induced signs of toxicity (reduced bodyweight gain (Table 35), lower feed intake and higher water consumption). In a preliminary study (Anonymous, 1997e) no effects on foetuses were observed (up to 1000 mg/kg bw/d), whereas in the main study (Anonymous, 1997f) an increase of the incidence of malformations (interventricular septal defects, malrotated heart; *c.f.* Table 36) were observed in litters of high and intermediate dose groups (1000 and 225 mg/kg bw/d) and an increase of the incidence of supernumerary ribs in litters of high dose groups.

The developmental toxicity studies were discussed during an expert consultation of the PPP procedure. For the main study with *Margosa Extract with water*, it was agreed to set the NOAELs for maternal and developmental effects at 225 mg/kg bw/d based on bodyweight effects or 14th ribs, respectively.

In the rat developmental study with *Margosa Extract with water*, litter 63 (of mid dose group) and litters 80, 84, 88 (of high dose group) showed malformations associated with heart. Variations associated with the heart were seen in litter 33 (of low dose group: interventricular septal defect, small) litters 65, 68, 74 (of mid dose group) and litters 85, 98 (of high dose group).

The manufacturer argued that malformations were seen only at maternally toxic doses and were not relevant because they were induced by high maternal toxicity. In the mid dose group, initial (GD 6-8) bodyweight gain (8.5 g vs. 10.4 g in controls) was slightly reduced and the initial (GD 6-7) feed intake (24 g vs. 26 g in controls) was significantly reduced. However, bodyweight was comparable to the control group and later on, bodyweight gain and feed intake were comparable to controls. Hence, the DS did not consider the findings observed in the mid dose group as adverse (and established the NOAEL at the mid dose level). In high dose dams, initial (GD 6-8) bodyweight gain (6.1 g vs. 10.4 g in controls) and the initial (GD 6-7) feed intake (23 g vs. 26 g in controls) were significantly reduced and water intake was significantly increased.

In the mid dose group only one litter was affected with heart-associated malformations. In this litter interventricular septal defects and malrotated heart were classified as malformation, haemorrhagic thyroid and subcutaneous oedema were also observed. Indeed (as argued by the manufacturer), in case this finding had been observed in isolation it probably would have been dismissed as incidental, however, in the high dose group the same and further heart-associated malformations were detected. Therefore, the findings observed in the mid dose group were considered as dose-related and adverse. This evaluation is in line with the evaluation by the study director (study report, page 23): "*Of the remaining 2 malformed foetuses, it was noted that one showed interventricular septal defect. A further 3 foetuses (3 further litters affected) showed small interventricular septal defect (classified as a visceral anomaly). The overall combined incidence of interventricular septal defect (4 foetuses (4 litters affected)) was comparable to that observed at 1000 mg/kg/day and, as such, the possibility that this isolated finding may be attributable to treatment cannot be discounted.*"

Historical control data of the performing laboratory (Huntingdon Life Sciences) summarised data of 11 studies with a total of 191 litters and 1690 foetuses. Interventricular septal defect (classified as malformation) were seen in two studies each with one foetus and one litter affected, whereas small interventricular septal defects (classified as visceral anomaly) were found in 7 studies (12 animals in 12 affected litters, see Table 38.
In comparison, the total number of thoracic malformations in the highest dose group (1000 mg/kg bw/d) was 7 (3 litters), and interventricular septal defects (malformations) were observed in (2 foetuses in 2 litters) in the highest dose group and in 1 foetus in the mid dose group, the latter also had a malrotated heart.

Two other technical extracts ("Fortune Aza", "NPI 720" - different from *Margosa Extract with water*) were submitted for the evaluation as the pesticide active ingredient "azadirachtin".
The developmental toxicity study in rats ("Fortune Aza") is comparable to *Margosa Extract with water* with respect to maternal toxicity and no developmental effects on foetuses were observed.
"ATI 720" was highly toxic in rabbits to dams and foetuses (maternal NOAEL 20 mg/kg bw/d, developmental NOAEL: 100 mg/kg bw/d). Due to the high level of toxicity observed in the top dose group, the low number of available litters and the low mean litter size of 0.9 live foetuses per litter (compared to 8.4 in the control group), the dose level of 500 mg/kg bw/d was considered too high (compared to test guideline requirements), when taking into account the extent of foetotoxicity.

Table 36: Foetal (litter) incidences of selected findings (Anonymous, 1997f)

Observation		**Dose level (mg/kg bw/d)**			
		0	**50**	**225**	**1000**
Number of foetus (litters) examined:		305 (23)	323 (23)	306 (23)	308 (23)
Visceral findings					
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]	2 (2)[a,b]
	Malrotated heart	0 (0)	0 (0)	1 (1)[f]	1 (1)[a]
	Duplicated inferior vena cava	0 (0)	0 (0)	0 (0)	2 (2)[b,c]
Thoracic (anomalies)	Anomalous cervicothoracic arteries	1 (1)	0 (0)	0 (0)	0 (0)
	Interventricular septal defect (small)	0 (0)	1 (1)	3 (3)[g,h,i]	2 (2)*,[d,e]

a: litter 88; b: litter 84, c: litter 80, d: litter 85, e: litter 74, * an additional litter (litter 98) with small interventricular septal defect was discounted here because mottled foetus syndrom occurred, f: litter 63, g: litter 65, h: litter 68, i: litter 74 (see also Table 37 below)

Table 37: Skeletal and visceral malformations – incidence summary

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[c]	-	-	-	1
Atrial septal defect with narrow pulmonary vein	-	-	-	1[c]	-	-	-	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

Table 38: Control incidence of interventricular septal defects of the performing laboratory

Control incidence of interventricular septal defects

Study	1	2	3	4	5	6	7	8	9	10	11
Animal source	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK
Date of sacrifice	07.94	07.94	09.94	09.94	10.94	11.94	11.94	01.95	01.95	02.95	02.95
No. foetuses examined	144	146	144	161	158	164	171	139	147	160	156
No. litters examined	22	22	22	24	24	24	25	23	24	23	24
Description	Incidence (Foetuses (litters))										
Interventricular septal defect A	-	-	-	-	1(1)	-	1(1)	-	-	-	-
Interventricular septal defect (small) B	3(3)	-	-	1(1)	-	-	2(2)	2(2)	1(1)	2(2)	1(1)
Total (anomalous and malformed)	3(3)	-	-	1(1)	1(1)	-	3(3)	2(2)	1(1)	2(2)	1(1)

A Classified as malformation
B Classified as visceral anomaly
CR/UK Charles River UK rats

In the two generation reproduction study *Margosa Extract with water* (Anonymous, 2000b) had no impact on clinical signs, bodyweight, feed consumption and gross (and microscopic) pathology of parental animals (highest dose tested: 50.7 mg/kg bw/d in males, 59.6 mg/kg bw/d in females (750 ppm)). Treatment with *Margosa Extract with water* had no influence on the development of the offspring.

In another (not acceptable) two generation reproduction study (Anonymous, 1996d) with the formulation NeemAzal-F 5 % (containing 20% *Margosa Extract with water* in 80% polyethylene oxide, equivalent to approx. 5 % w/w Azadirachtin A), increased relative weights of ovaries and spleen in maternal rats were noted in all treatment groups (approx. 13-333 mg/kg bw/d or 200-5000 ppm). Additionally, mean bodyweights in intermediate and high dose animals were reduced. The formulation had no effect on developmental parameters.

4.10.2.2 Human information

Purified neem oil was used in first clinical trials as intravaginal/-uterineal used contraceptive (Talwar *et al.*, 1995, TOX2006-3053, 1997, TOX2006-3054). No information on the Neem seed extract used (composition, content of azadirachtin, purity, extraction method etc.) was given in the publication by Tawar et al. (1997). In a publication cited by Talwar (1997: Mukherjee et al. 1996), the free fatty acid composition was described as follows: Palmitic acid (19.6 %, stearic acid (17.2 %), oleic acid (41.2 %), linoleic acid (0.82 %; and other undetected minor acids (1.65 %). For the bitter principles (constituents responsible for the bitter taste) another publication was cited (Siddiqui et al. 1988) in which the composition of the dichloromethane extract of the fresh, undried, uncrushed neem twigs was described. As extraction method and solvent as well as parts of the plants are different and no information on limonoids were reported, information from the study cannot be used for the evaluation of *Margosa Extract with water*. A clinical trial – as mentioned by Talwar et al. (1995 cited from Talwar et al. 2002 IN: Schmutterer H. (ed.): The neem tree and other meliaceous plants. Sources of

unique natural products for integrated pest management, medicine, industry and other purposes, 2nd ed, Neem Foundation Mumbai 2002, 893 pp) was conducted in 18 healthy tubectomized women with administration of purified neem oil. Three milliliter of purified neem oil (Praneem Vilci) were administered by an intrauterine cathether under aseptic conditions. The composition is also considered different from *Margosa Extract with water* and should not be used for the evaluation of *Margosa Extract with water*. Overall, all studies mentioned here were listed for the sake of completeness. Information on the neem extracts/preparations used is generally sparse. Constituents of kernels differ from the constituents of other parts of the neem tree (e.g., leaves, flowers, stem bark). Additionally, the extraction process (e.g., pre-processing, solvent, temperature, clean up) has a great impact on the constitution of the technical extract. It is difficult to compare the results of published literature studies with the results of the studies that were submitted for this evaluation, as they were most often conducted with different test compounds. Furthermore, only few constituents of neem trees are identified. All studies listed above from the published literature are considered not relevant for the evaluation of *Margosa Extract with water*.

4.10.3 Other relevant information

Various extracts or oil of different parts of neem tree were reported in literature to induce reproductive toxic effect. An aqueous leave extract was reported to reduce fertility in male mice (Deshpande et al., 1980, TOX2006-3046; Sadre et al., 1984, TOX2006-3049, both extracts not comparable to *Margosa Extract with water*, no information on limonoid content), whereas a methanolic seed kernel extract had no impact on fertility (Krause & Adami, 1984, TOX2006-3047, 0.1 mL of 10 % methanolic extract dissolved in ethanol and diluted with water to a 1 % solution, no information on limonoid content). *In vitro* treatment of spermatozoe with neem seed kernel oil had spermatocidal effects (Sinha et al., 1984, TOX2006-3051, no further information available). Intrauterine application of the oil in various species prevented gravity (Tewari et al., 1986, TOX2006-3055; Lal et al., 1986, TOX2006-3048 no further information available; Talwar et al., 1997, TOX2006-3054). Furthermore, female rats showed reduced implantation rates and increased resorption rates after intravaginal, oral, or subcutaneous application (Sinha, Riar, Tiwary et al., 1984, TOX2006-3052; Tewari et al., 1986, TOX2006-3055neem oil from crushed seeds. administered dose: 0.2 ml s.c., no information on limonoids reported; Lal et al., 1986, TOX2006-3048). Abortus was seen in female baboons after oral intake of neem oil (Talwar et al., 1997, TOX2006-3054, no details on the extract).

Overall, all studies mentioned here were listed for the sake of completeness. Information on the neem extracts/preparations used is generally sparse. Constituents of kernels differ from the constituents of other parts of the neem tree (e.g., leaves, flowers, stem bark). Additionally, the extraction process (e.g., pre-processing, solvent, temperature, clean up) has a great impact on the constitution of the technical extract. It is difficult to compare the results of published literature studies with the results of the studies that were submitted for this evaluation, as they were most often conducted with different test compounds. Furthermore, only few constituents of neem trees are identified. All studies listed above from the published literature are considered not relevant for the evaluation of *Margosa Extract with water*.

4.10.4 Summary and discussion of reproductive toxicity

For the evaluation of **effects on fertility or reproduction**, findings in single-dose (e.g. histopathology of testes, short-term, long-term, multi-generation and one-generation studies can be used. *Margosa Extract with water* was evaluated in short-term studies in rats as well as in a long-term, a 2-generation, and a 1-generation study.

In the 28-d, 90-d and long-term studies in rats with *Margosa Extract with water*, no findings on sex organs were reported in the study reports. No effects on fertility or reproduction were observed in the submitted 1-generation (considered not acceptable) or 2-generation (considered acceptable) toxicity studies with *Margosa Extract with water*. Dose levels in the 2-generation study were calculated as mean of the compound intake in weeks 0, 5, 10 and 15 (Anonymous, 2009). Therefore, compound intake was based only on the intake during the pre-mating period.

In reports from open literature, various findings with respect to fertility or reproduction are described. However, in the literature reports different test compounds (other extraction methods, other starting materials, etc.) were used when compared to the technical extracts used for PPP and biocidal products. There seem to be some differences in properties, when comparing different preparations of different parts of neem tree (e.g., flower, leaves, seed kernel). In the available reproductive toxicity study, no effects on fertility were observed.

This argumentation was supported by the participants of an expert consultation in the PPP procedure.

Considering the findings seen in the **developmental toxicity** study in rats performed with *Margosa Extract with water* (interventricular septal defects, malrotated heart, supernumerary ribs), the effects were seen at or around doses where maternal toxicity was observed. Additionally, the incidences were increased only slightly and the possibility of non-specific causes such as general toxicity could not be excluded.

Considering that the effects described in section 4.10.2.2 and 4.10.3 were seen after administration of extracts prepared from neem seed kernels or neem leaves which were not identical to the *Margosa Extract with water* evaluated here, it is considered appropriate that these effects are not used for classification and labelling of *Margosa Extract with water*.

This argumentation was supported by the participants of an expert consultation in the PPP procedure.

4.10.5 Comparison with criteria

Table 39 and Table 40 present the CLP criteria.

Adverse effects on sexual function and fertility:

Table 39: Classification criteria concerning adverse effects on sexual function and fertility

CLP criteria
Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on sexual function and fertility in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility and - where the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

In the submitted 2-generation studies, under the conditions of the studies, no findings with relevance for a classification for adverse effects on sexual function and fertility were reported up to the highest dose tested.

There are no epidemiological data to evaluate effects on fertility, hence *Margosa Extract with water* cannot be placed in category 1A (CLP).

Therefore, no classification for effects on fertility/reproduction is proposed.

Adverse effects on development:

Table 40: Classification criteria concerning adverse effects on development

CLP criteria
Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on development in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development and - the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

There are no appropriate epidemiological studies available on developmental effects in humans. Hence, classification with Category 1A according CLP regulation is not possible.

The prenatal developmental toxicity was investigated in rats and rabbits complying with international test guidelines and GLP.

Considering the findings seen in the developmental toxicity study in rats performed with *Margosa Extract with water* (interventricular septal defects, malrotated heart, supernumerary ribs), the effects were seen at or around doses, where maternal toxicity could be observed. Additionally, the incidences in the rat study were increased only slightly and the possibility of non-specific causes such as general toxicity could not be excluded.

Considering that the effects described in sections 4.10.2.2 and 4.10.3 were seen after administration of extracts prepared from neem seed kernels or neem leaves which were not identical to the technical extract evaluated here, it is considered appropriate that these effects are not used for classification and labelling of *Margosa Extract with water*.

This argumentation was supported by the participants of an expert consultation in the PPP procedure.

According to regulation (EC) No 1272/2008 major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency.

ECHA's Guidance on the application of the CLP criteria (Version 5.0 July 2017, Section 3.7.2.2.1.1, p. 400-401) cites the CLP regulation: "Annex I: 3.7.2.4.3 Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1. However, when a substance is so toxic that maternal death or severe inanition results, or the dams are prostrate and incapable of nursing the pups, it is reasonable to assume that developmental toxicity is produced solely as a secondary consequence of maternal toxicity and discount the developmental effects. Classification is not necessarily the outcome in the case of minor developmental changes, when there

is only a small reduction in foetal/pup body weight or retardation of ossification when seen in association with maternal toxicity."

No information is available to judge whether the observed effects on (rat) offspring have to be regarded as secondary non-specific consequences of maternal toxicity. There were proposals to correlate the maternal and offspring findings in the developmental toxicity study. However, due to the few maternal parameters determined in developmental toxicity studies and taking into account the toxicological profile of the present compound, this exercise is not expected to provide meaningful insights into the question whether the offspring findings are secondary non-specific consequences of maternal toxicity.

In summary, classification in Category 2 (H361d, CLP criteria) is considered appropriate especially taking into account the low incidences of the malformations and the possible impact of maternal toxicity.

The manufacturer considered a classification as a developmental toxicant as not necessary, because in their opinion, effects on foetuses occurred in the presence of maternal toxicity only. Hence, the effects were deemed as secondary non-specific consequences of maternal toxicity which would not warrant classification.

During an expert consultation in the PPP procedure, it was discussed whether classification with R63 (corresponding to H361d according to CLP criteria) should be proposed: "There was a feeling that R63 was not appropriate based on the dataset available and incidences seen in the rat studies. […] Experts voted on the classification issue and a majority agreed to not propose any classification" (cited from the meeting minutes). This recommendation was based mainly on the low incidences observed in the developmental toxicity study in rats with *Margosa Extract with water*.

Adverse effects on lactation:

No data are available to judge whether there are specific effects on or via lactation (H362). Under the conditions of the 2-generation study, no effects on any investigated parameter were reported up to the highest dose tested.

4.10.6 Conclusions on classification and labelling

Regarding effects on fertility, the data are considered conclusive but not sufficient to trigger classification for such effects.

Regarding developmental toxicity, classification in Category 2 (H361d, CLP criteria) is considered appropriate.

No data are available to judge whether there are specific effects on or via lactation (H362).

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) Feed Equivalent to OECD 416 GLP	Parental: No effects on parents. NOAEL: 50 mg/kg bw/d (750 ppm) - statistically significant reduction in relative organ weights (testis, brain, heart), no dose-response, only P0) Offspring: - low incidence histopathological findings in the testis & uterus in first litters of P0 only (also seen in controls) - reduced number of live pups only in the first litter of P1 Reproductive: No effects on reproduction NOAEL: 50 mg/kg bw/d (750 ppm)	Anonymous, 2000b (no data on feed analysis, time to fertilisation not reported) for more details, see table "Overview on organs weight, rat two-generation study" below.
Rat, Charles Foster / **NeemAzal F 5 %**	10 M & 20 F	0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d) Feed	Parental: spleen, ovary wt ↑, bw ↓ LOAEL: appr. 13 mg/kg bw/d (200 ppm)	Anonymous, 1996d (no data on feed analysis, time to fertilisation and

		2-gen. study Similar to OECD TG 416 GLP status unknown	Reproductive: No effects on reproduction NOAEL: appr. 333 mg/kg bw/d (5000 ppm)	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) 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 NOAEL: 100 mg/kg bw/d	No effects on foetuses NOAEL: 1000 mg/kg bw/d
Anonymous, 1997f / ***Margosa Extract with water* (36.7 % Azadirachtin A)**	OECD 414, main study Rat, Crl:CD BR VAF/plus - Gavage (vehicle: 1% methylcellulose) - Exposure: GD 6-19	0, 50, 225, 1000 mg/kg bw/d	1000 mg/kg bw/d: Bw ↓, feed intake ↓, post-dosage salivation NOAEL: 225 mg/kg bw/d	255 mg/kg bw/d: Malformations (cf. Table 36), supernumerary ribs (only 1000) 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) for more details, see section 4.10.1.1 OECD 416 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) 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) - low incidence histopathological findings in testis & uterus in first litters of P0 only (also seen in control) - reduced number of live pups only in the first litter of P1
Anonymous, 1996d / **NeemAzal F 5 %**	Similar OECD TG 416 (no data on feed analysis, time to fertilisation and duration of gestation not reported) 2-gen. study Rat	0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d) 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] 0.7% 0.33% (4.4%)	2(2)[a,b] 1.3% 0.65% (8.7%) [§]	0 – 0.6% (0 – 4.1%)	0 – 1.5%* 0 – 0.74%** (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] 1.3%[§] 0.65%[§] (8.7%) [§]	-	0 – 0.6%* 0 – 0.3%** (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) 0.63% 0.3% (4.4%)	3 (3)[g,h,i] 2% 0.98% (13%)	2 (2)[d,e, ≠] 1.3% 0.7% (8.7%)	0 – 2,1% (0 – 13.6%)	0 – 2.3%* 0 – 1.1%** (0 – 13.6%) ***
Thoracic malformations & anomalies together	Interventricular septal defect & Interventricular septal defect, small	0 (0)	1 (1) 0.6% 0.3% (4.4%)	4 (4)[f,g,h,i] 2.6% 1.3% (17.4%) [§]	4 (4)[a,b,d,e, ≠] 2.7% 1.3% (17.4%) [§]	-	0 – 3.1%* 0 – 1.5%** (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[c]	-	-	-	1
Atrial septal defect with narrow pulmonary vein	-	-	-	1[c]	-	-	-	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.**

4.11 Other effects

4.11.1 Non-human information

4.11.1.1 Neurotoxicity

A 21-d study on repeated-dose delayed neurotoxicity in chicken was conducted (Anonymous, 1998) with a 21-d post-dosing recovery period. After gavage of *Margosa Extract with water* (up to 1000 mg/kg bw/d, Neem Azal technical; 27.3 % azadirachtin), neither neurotoxicological nor other effects were observed. Deficiencies in the study design were that neuropathy target esterase was not measured and that only 3 animals per dose group were used.

Margosa Extract with water is not known to contain organophosphorous structures; therefore, no additional studies on delayed neurotoxicity were necessary.

No neurotoxicity studies in rats were submitted.

4.11.1.2 Immunotoxicity

No studies were submitted.

4.11.1.3 Specific investigations: other studies

No studies were submitted.

4.11.1.4 Human information

Routine medical observation (general [e.g., fever, weakness, sweating] and special signs [*gastro intestinal:* e.g., nausea, vomiting; *neuromuscular:* e.g., headache, dizziness; *cardio respiratory:* e.g., nasal discharge, cough, tachycardia; *eye:* e.g., ophthalmic examination, double vision; *psychological:* e.g., temperament, nervousness] of toxicity, vital signs [e.g., blood pressure, pulse, respiratory rate], blood chemistry, haematology) of workers exposed to neem extracts did not show adverse health effect (Anonymous, 2002b, Anonymous, 2003, Anonymous, 2004, Anonymous, 2005a, Anonymous, 2005b).

There were reports in open literature about intoxications (and deaths) of infants after intake of neem oil as medication (estimated intake: 5-50 mL). Initial clinical signs included vomiting, convulsion, and at later stages metabolic acidosis with coma. Post-mortem examination revealed histological liver damage, such as lipid infiltration in hepatocytes, damage of mitochondria, and sometimes encephalopathy (Sundaravalli et al., 1982, TOX2006-3064; Sinniah et al., 1981, TOX2006-3062; Sinniah et al., 1982, TOX2006-3061). In some reports relatively high case numbers are given, e.g. more than 60 (supposed or verified) intoxications of children with neem oil within 5 years in one hospital in Madras/India (Sinniah et al., 1981, TOX2006-3062). Neem oil is a common treatment in southern Asia, therefore, the incidence of cases with such severe adverse effects cannot be judged. Clinical signs, occurrence in children following often an infection, and pathology results are similar to Reye-syndrome, which occurs rarely, but most times after virus infections (influenza, chicken pox) and subsequent treatment with certain drugs (e.g., acetyl salicylic acid) (Sinniah & Baskaran, 1981, TOX2006-3060; Beers & Berkow, 1999, TOX2006-3056; Gerok, 1996, TOX2006-3058). A Reye-like syndrome was induced by treatment of rats and mice with neem oil. In contrast to humans, however, microsomal liver enzymes were not decreased, and brain oedema did not occur (Sinniah et al., 1985, TOX2006-3063).

The toxic substance and the mode of action were unknown. Therefore, the observed effects could not be attributed to any single constituent of neem oil.

Neem oil and *Margosa Extract with water* are both generated from neem seed (kernels). Neem oil is generated out of crushed kernels by pressing or by extraction with hexane. *Margosa Extract with water* is generated by extraction with polar protic and aprotic solvents and precipitation with a non-polar solvent.

Chemical composition of the extracts was described by the manufacturer, but the composition of neem oil is unknown up to a great extent. Lipids/fatty acids (total fatty acid content: 10-90 % (wt/wt)), azadirachtin (between "not detectable" up to 2323 ppm), nimbin (between "not detectable" up to 18132 ppm) and salannin (between "not detectable" up to 47150 ppm) have been described in neem oil (Kumar & Parmar, 1996). Therefore, even though neem oil, extracts prepared with organic

solvents and *Margosa Extract with water* have – in part – the same constituents, it is unknown if the observed effects on human and rat livers were caused by these known compounds. Hence, it is proposed not to use the results derived from other extracts than *Margosa Extract with water* for classification and labelling.

4.11.2 Summary and discussion

No relevant information on *Margosa Extract with water* available.

4.11.3 Comparison with criteria

No data available to allow a comparison

4.11.4 Conclusions on classification and labelling

Data lacking.

5 ENVIRONMENTAL HAZARD ASSESSMENT

General remark on data used for classification

All data used for classification in this dossier has already been submitted and accepted in 2006 in the framework of biocidal active substance approval. Hence, the **quality of data and reported information in the studies** does not always reflect the actual scientific standards. However, as currently no better data is available the presented CLH proposal is based on the best available information.

Concerning the analysis of the **environmental behaviour** of *Margosa Extract with water* it has to be kept in mind, that 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.. Taking into consideration the origin of the extract from higher plants and the biosynthetic pathway leading to these triterpenoids, radiolabelling of the main components of the active substance is not feasible, since it is not possible to synthesize *Margosa Extract with water* chemically. In view of this dilemma, the major individual component of *Margosa Extract with water*, i.e., Azadirachtin A, which accounts for about one third of the total mass of the extract, was chosen as the lead substance for describing the behaviour of *Margosa Extract with water* in the environment.

A way to synthesize the individual component Azadirachtin A has only been available since 2007 (S. Ley et al., (2007): Angewandte Chemie, 119, 40, 7773-7776) and therefore a considerable time after the acceptance of the dossier as complete for the process of approval as biocidal active substance. Hence, the synthesis of the lead substances was technically not feasible for the applicant at the time of dossier submission in 2006.

As far as the **effect assessment** is concerned, only ecotoxicological test data for exactly this water extract further processed with organic solvent was considered as relevant, because compared to the other known Margosa extracts there is a fundamental difference 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). With regard to the other contained limonoids Salannin and Nimbin they are only minor constituents for the extracts with a mainly insecticidal mode of action, whereas Salannin and Nimbin are exceeding the concentration of Azadirachtin for the Margosa Extract approved as repellent. Hence, the data for the other *Margosa Extracts* (e.g. repellent) are not considered to be relevant for the current CLH proposal and consequently the respective data are not included in the CLH dossier.

Based on the above explanations, the following definitions have been used for the environmental section:

	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)

5.1 Degradation

Table 41: Summary of relevant information on degradation

Method	Results	Remarks	Reference
OECD 301 F	21.6 % after 28 d	Test substance Azadirachtin A Not readily biodegradable	Hund, K. (1999b), report no. TRF-003/3-15
OECD 301 D	5.6 % after 28 d	Test substance *Margosa Extract with water* (34% Azadirachtin A) Not readily biodegradable	Werle (1998), report no. 97 50 40 787
OECD 301 F	36.8 – 48.2 % after 35 d	Test substance *Margosa Extract with water* (33.4 % Azadirachtin A) Not readily biodegradable	Hund, K. (1998a), report no. TRF-001/3-15
OECD 301 F	49.1 % after 47 d	Test substance *Margosa Extract with water* (34 % Azadirachtin A) Not readily biodegradable	Hund, K. (1999a), report no. TRF-001/3-15/1
OECD 301 D	65.7% after 28 d	Test substance NeemAzal T/S (1 % Azadirachtin A) Ready biodegradable	Lenz, G. (1995), report no. 94 50 41 389 D
OECD 111	Half life at 12 °C: pH 4 = 112.7 d pH 7 = 40.9 d pH 8 = 8.2 d	hydrolytic degradation, increasing with temperature and pH Test substance Azadirachtin A	Troβ, R. (1996a), report no. TM 1195.15 and Troß, R. (1997), report no. LP 97.04

5.1.1 Stability

It has to be noted that for the available stability studies the a.s. *Margosa Extract with water* was the test substance and Azadirachtin A was used as lead substance since it is the major component (34 ± 9 %) of *Margosa Extract with water*.

Table 42: Hydrolytic degradation

Method / Guideline	pH	Temperature [°C]	Initial TS concentration, C_0 & [mol/L x 10^{-4}]	Reaction rate constant, Kh [1/h]	Half-life, DT_{50} [h]	Coefficient of correlation, r^2	Reference
OECD 111	4	30	0.82	0.00271	256	0.9174	Troß, R. (1996a), report no. TM 1195.15 A7.1.1.1.1-01
	7		0.78	0.00610	114	0.9927	
	8		1.12	0.03027	23	0.9987	
	4	40	1.24	0.01061	65	0.9604	
	7		1.24	0.02376	29	0.9986	
	8		1.22	0.12891	5	0.9963	
	4	40	1.16	0.01244	56	0.9749	
	7		1.13	0.02201	31	0.9945	
	8		1.09	0.12636	5	0.9993	
Method / Guideline	**pH**	**Temperature [°C]**	**Initial TS concentration, C_0 & [mol/L x 10^{-4}]**	**Reaction rate constant, Kh [1/h]**	**Half-life, DT_{50} [d]**	**Coefficient of correlation, r^2**	**Reference**
OECD 111 (Mathematical Calculation)	4	18		0.00042	68.8		Troß, R. (1997), report no. LP 97.04 A7.1.1.1.1-02
	7			0.00111	26.1		
	8			0.00472	6.1		
	4	20		0.00058	49.9		
	7			0.00148	19.5		
	8			0.00651	4.4		
	4	22		0.00079	36.4		
	7			0.00198	14.6		
	8			0.0892	3.2		
	4	12		$2.563 \cdot 10^{-4}$	112.7		
	7			$7.056 \cdot 10^{-4}$	40.9		
	8			$3.503 \cdot 10^{-3}$	8.2		

& concentrations refer to Azadirachtin A, i.e. the major component (ca. 30 % of TS) of the test substance *Margosa Extract with water*

In the first study the hydrolysis of Azadirachtin A as function of the pH was tested at two temperatures, 30 °C and 40 °C. The hydrolytic stability of Azadirachtin A is strongly pH-dependent as indicated by a significant increase in the rate of degradation with increasing pH. At high water temperatures of 30 to 40 °C, Azadirachtin A has a rapid half-life of 5 to 23 hours in slightly alkaline conditions at pH 8 to ca. 2 ¼ to 10 days in acidic conditions at pH 4.

In the second study no materials were used, the study involves a mathematical calculation. The experimental determination of the reaction rate for the hydrolysis of Azadirachtin A has been conducted at two temperatures (30 and 40 °C, refer to the first study). These reaction rate values were extrapolated for other temperatures (18, 20 and 22 °C) with the help of the "Arrhenius equation": Ln $k = \ln A - E_a/RT$.

The extrapolation of the test results to the average outdoor temperature in the EU of 285.15 K 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; submitted under DAR: IIA 7.8.3/01).

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

Table 43: Photolysis in water

Method / Guideline	**Initial TS concentration, C_0 [&] [mol/L x 10^{-6}]**	**Total recovery of test substance [% of applied a.s.]**	**Photolysis rate constant (k^c_p)**	**Direct photolysis sunlight rate constant (k_{pE})**	**Reaction quantum yield (Φ^c_E)**	**Half-life ($t_{1/2E}$)**	**Reference**
OECD Draft (part A) "Direct Phototrans-formation", 1990	9.1	test conducted with unlabelled TS, therefore no balance established	not given	not given	5.55 x 10^{-4}	not determined	Werle, H. (1995), report no. 95 50 40 827 B A7.1.1.1.2-01 Werle, H. (1999), report no. 99 50 40 819 (calculation) A7.1.1.1.2-02

[&] concentration refer to azadirachtin A, i.e. the major component (ca. 30% of TS) of the test substance *Margosa Extract with water*

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* and parameters included in the computer model "ABIWAS" (initial Azadirachtin A concentration: 10^{-5} mol/L; water body: 100 m^2 surface, 0.1 m depth; degradation only via direct photolysis; spectral photon irradiance latitude 55°N; January scenario: 2 °C, 8.0-hour day; July scenario: 20 °C, 16.1-hour day).

The half-life times for January were estimated to be:
Minimum: 26.5 days; Normal: 1.8 months; Maximum: 7.2 months.

The half-life times for July were estimated to be:
Minimum: 3.8 days; Normal: 5.5 days; Maximum: 19.2 days.

Table 44: Phototransformation in air

Method / Guideline	**Time-dependent OH-radical concentration [OH radicals cm^{-3}]**	**Overall reaction rate constant k [cm^3 x $molecule^{-1}$ x s^{-1}]**	**Half-life [h]**	**Reference**
Model calculation using estimation method by AOPWIN version 1.88	24-h average 5.0 x 10^5	227.03 x 10^{-12}	1.696	Müller, M. (1999), report no. not given A7.3.1-01

Degradation of organic compounds in the atmosphere is mainly based on the reaction with hydroxyl radicals. For this reaction the rate constant can be determined by AOP. Together with an assumed hydroxyl radical concentration in the atmosphere an estimate of the atmospheric half-life is possible. The calculated half-life for Azadirachtin A is 1.696 h (equivalent to 0.071 d).

With regard to this estimated value for Azadirachtin A, long-term transport and accumulation in air are not to be expected.

Furthermore, the tendency of azadirachtins, the major components of *Margosa Extract with water*, to enter the atmosphere is considered to be low taking into account both the vapour pressure of these compounds (3.6 x 10^{-13} Pa) and the Henry's Law Constant (2.4 x 10^{-14} Pa m^3/mol).

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

No estimation of biodegradation was conducted.

5.1.2.2 Screening tests

Table 45: Ready biodegradability

Method/ Guideline	Test type	Test parameter	Inoculum			Additional substrate	Test substance conc.	Degradation		Reference
			Type	Concentration	Adaptation			Incub. period	Degree [%]	
OECD 301 F **Key study**	ready	oxygen consumption	activated sludge & aqueous soil extract with soil micro-organisms	1.8 x 10^4 CFU/mL correspon-ding to 30 mg/L dry matter	no	no	100 mg Azadi-rachtin A/L	28 days	21.6	Hund, K. (1999b), report no. TRF-003/3-15 A7.1.1.2.1-05
OECD 301 D	ready	oxygen consumption	activated sludge	not specified	no	no	1.8, 3.6 & 5.4 mg *Margosa Extract* (a.s.)/L, 33.4 % Aza-dirachtin A	28 days	5.6	Werle (1998), report no. 975040787 A7.1.1.2.1-02
OECD 301 F	ready	oxygen consumption	activated sludge	9.3 x 10^4 CFU/mL correspon-ding to 30 mg/L dry matter	no	no	100 mg *Margosa Extract* (a.s.)./L, 34 % Aza-dirachtin A	35 days	36.8	Hund, K. (1998a), report no. TRF-001/3-15 A7.1.1.2.1-03
			activated sludge & aqueous soil extract with soil micro-organisms	1.2 x 10^5 CFU/mL correspon-ding to 30 mg/L dry matter	no	no	100 mg *Margosa Extract* (a.s.)/L, 34 % Aza-dirachtin A	35 days	48.2	
OECD 301 F	ready	oxygen consumption	activated sludge & aqueous soil extract with soil micro-organisms	2.4 x 10^4 CFU/mL correspon-ding to 30 mg/L dry matter	no	no	100 mg *Margosa Extract* (a.s.)/L (dissolved in DMSO), 34 % Aza-dirachtin A	47 days	49.1	Hund, K. (1999a), report no. TRF-001/3-15/1 A7.1.1.2.1-04

OECD 301 D	ready	oxygen con-sumption	activated sludge	not specified	no	no	1 & 2 mg NeemAzal-T/S /L, 1 % Azadi-rachtin A	28 days	65.7	Lenz, G. (1995), report no. 94 50 41 389 D A7.1.1.2.1-01

It has to be noted, that in general screening tests on ready biodegradability are intended for pure chemicals and may be extended on mixtures only in exceptional cases, such as mixtures of structurally similar chemicals like oils and surface-active substances (surfactants). Consequently, screening tests are not suitable for complex mixtures, such as natural extracts, consisting of structurally different components, with each component possessing individual degradation behaviours.

In case of *Margosa Extract with water*, an OECD 301F study (Hund, 1999b) is available for the lead component Azadirachtin A, i.e., the major component of the a.s. *Margosa Extract with water* in regards to both amount (~34% w/w) and biological activity. In this study, the test substance Azadirachtin A was degraded to 21.6% only within 28 days, leading to the conclusion, that the component Azadirachtin A is not readily biodegradable.

This result is supported by three tests on ready biodegradability performed with the a.s. *Margosa Extract with water*.

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 sludge microorganisms.

The first test performed with *Margosa Extract with water* was conducted according to OECD guideline 301 D using activated sludge as inoculum. In this test, *Margosa Extract with water* was shown to be not readily biodegradable with 5.6 % degradation within 28 days.

In the second test, conducted according to OECD guideline 301 F, ready biodegradability was investigated using two different kinds of inoculum, activated sludge and a mixture of activated sludge and aqueous soil extract containing soil microorganisms. The results of this test confirmed *Margosa Extract with water* as not being readily biodegradable with 36.8 % and 48.2 % degradation within 35 days, respectively. At the end of the 10-day window the *Margosa Extract with water* was degraded to 23.7 % and 36 %, respectively.

The third test was also conducted according to OECD guideline 301 F and investigated ready biodegradability of *Margosa Extract with water* using a mixture of activated sludge and aqueous soil extract containing soil microorganisms. The result of 49.1 % degradation within 47 days (28.1 % at the end of the 10-day window) is in line with the two other tests, demonstrating *Margosa Extract with water* to be not 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 %).

Simulation tests

Biodegradation in freshwater

Guideline	Test substance	DT_{50}	Remarks	Reference
No guideline	40µg/L Azadirachtin A	11.9 d (20°C)	Pond water	Sundaram et al., 1995; Formulation Selection and Investigation of Azadirachtin –A Persistence in Some Terrestrial and Aquatic Compenents of a Forest Environment; Journal of Liquid Chromatography, 18 (2) (1995), PP. 363-376
No guideline	Neem-EC nominal conc. 0.2 and 0.4 mg Azadirachtin A/L	8-13 d (no temp. determined)	glass aquaria placed in forest ground	Sundaram et al., 1997; Hydrolysis of Azadirachtin in Buffered and Natural Waters; Pestic. Sci. 0031-613X , 1997, pp. 74 – 90
No guideline	Neemix 4.5 nominal conc. 0.026 – 0.690 mg Azadirachtin A/L	24.7-29.2 d (no temp. determined)	enclosures set up in a small forest lake	Thompson et al. (2002); Fate and persistence of Azadirachtin A following applications to mesocosms in a small forest lake; Canadian Forest Service, Great lakes Forestry Centre, Canada; Bulletin of Environmental Contamination and Toxicology
No guideline	19 µg/mL Azadirachtin A	0.4-10.7 d (35°C) 2.4-66.4 d (12°C)	creek and lake water samples in the dark, pH 6.2-8.1	Szeto & Wan, 1996; Hydrolysis of Azadirachtin in Buffered and Natural Waters; J. Agr. Fd.Chem. 44 (1996), pp. 1160-1163
No guideline	42.70 mg/L Azadirachtin A, 13.05 mg/L Azadirachtin B	8.8-12.6 d (25°C) 16.7-23.9 d (12°C)	incubation in the dark at 25 °C in river water samples up to 60 days	Molinari, 2002; submitted under DAR: IIA 7.8.3/01

No standard water/sediment study is available and information is gained mostly from published literature. The only water sediment system analysed was established under outdoor conditions. Due to this and based on the reasons mentioned above, mass balances are incomplete, providing only dissipation instead of degradation half-lives and neither information regarding the degree of ultimate degradation nor on degradation products.

5.1.3 Summary and discussion of degradation

Margosa Extract with water is a complex substance of natural origin. According to the Guidance on the Application of the CLP Criteria, Version 4.0 (2013) a complex substance should be regarded as not rapidly degradable if it contains not-rapidly-degradable constituents 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.

Azadirachtin A itself does not meet the criteria for ready biodegradability, showing only 21.6 % degradation within 28 days.

Extrapolation of the hydrolysis stabilisation test results for Azadirachtin A to the average outdoor temperature in the EU (285.15 K) yields half-lives of 112.7, 40.9 and 8.2 days at pH 4, 7 and 8, respectively. 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. Additionally, hydrolysis products are not detectable due to the technical limitations with regard to radiolabelling of the test substance and synthesis of reference substances.

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 above mentioned 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.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

The adsorption/desorption study was conducted with *Margosa Extract with water* (30 % azadirachtin A) as test substance and Azadirachtin A was used as lead substance since it is the major component (34 ± 9 %) of *Margosa Extract with water*.

Table 46: Adsorption/desorption screening test

Method / Guideline	Tested soils/ Classifi-cation	Adsorbed a.s. & [%]	K_a[1]	K_{aOC}[2]	K_d [3]	K_{dOC}[4]	K_a/K_d[5]	Degradation products		Reference
								Name	[%] of a.s.	
OECD 106	Speyer 2.1/ sand	7.55	0.405	65.4	n.d.	--	n.a.	none	--	Troβ, R. (1996b), report no. TM 995.12 A7.1.3-01
	Speyer 2.2/ loamy sand	8.70	0.479	20.6	n.d.	--	n.a.	none	--	
	Speyer 2.3/ loamy sand	6.95	0.373	30.6	n.d.	--	n.a.	none	--	

[1] K_a = Adsorption coefficient; [2] K_{aOC} = Adsorption coefficient based on organic carbon content; [3] K_d = Desorption coefficient; [4] K_{dOC} = Desorption coefficient based on organic carbon content; [5] K_a / K_d = Adsorption / Desorption distribution coefficient

[&] concentration refer to azadirachtin A, i.e. the major component (ca. 30 % of TS) of the test substance *Margosa Extract with water*; n.d. = not determined due to the low adsorption (< 10 %); n.a. = not applicable

The adsorption properties of Azadirachtin A were investigated in three soils of two different soil types (sand, sandy loam) in the study of Troß (1996b). The resulting K_{OC} values were in the range of 20.6 mL/g in loamy sand to 65.4 mL/g in sand. With regard to the low K_{OC} values in the tested soils, Azadirachtin A is slightly adsorbed to soil, indicating a high to moderate potential mobility in soil.

5.2.2 Volatilisation

The tendency of azadirachtins, the major components of *Margosa Extract with water*, to enter the atmosphere is considered to be low taking into account the low vapour pressure of these compounds (3.6×10^{-13} Pa) and the Henry's Law Constant (2.4×10^{-14} Pa m^3/mol).

5.2.3 Mobility

The column leaching study was conducted with *Margosa Extract with water* as test substance and azadirachtin A was used as lead substance since it is the major component of *Margosa Extract with water*.

Table 47: Column leaching study

Method/ Guideline	Soils / Classifi-cation	OC	pH	Design	Application rate	Residues in leachate [% of applied Aza A]	Reference
BBA Part IV, 4-2	Speyer 2.1/ sand	0.62	5.9	glass columns, 65 mm i.d.; 30 cm soil depth of water-saturated soil; 200 mm rain within 2 d	33 mL of 10 % aq. solution of NeemAzal-T/S eqv. to 32.8 mg azadirachtin A	90.4	Troβ, R. (1995), report no. TM 995.11 A7.2.3.2-01
	Speyer 2.2/ loamy sand	2.32	5.6			55.1	
	Speyer 2.3/ loamy sand	1.22	6.4			42.1	

i.d. = inner diameter

The high mobility of Azadirachtin A in soil as already indicated by the low K_{OC} is confirmed under the stringent conditions of the laboratory column leaching test, i.e., highly exaggerated concentration of substance applied to soil, maximum water saturation of soils at test start, watering with 200 mm rain within two days following test substance application. However, contamination of groundwater by Azadirachtin A under actual use conditions seems to be unlikely taking into account its short degradation half-life in soil.

5.3 Aquatic Bioaccumulation

Table 48: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
Calculation	BCF: 2.5 (Azadirachtin B) BCF: 1.38 (Azadirachtin A)	Low potential for bioaccumulation	

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

Table 49: Determination of aquatic bioaccumulation

Basis for estimation	log Kow (measured)	Estimated BCF for fish (freshwater) on wet weight basis	Estimated BCF for fish eating bird/predator	Reference
Standard equation (74), TGD on Risk Assessment (2003), Part II, chapter 3.8.3.2	1.29 (Azadirachtin B)[1]	2.5 L/kg	- -	- -
	0.99 (Azadirachtin A)[2]	1.38 L/kg	-	-

[1]content of Azadirachtin B in *Margosa extract with water:* 5.6 %
[2] content of Azadirachtin A in *Margosa extract with water*: 34 %

Determination of log K_{ow} values for *Margosa extract with water* is technically not feasible.

However, the n-octanol/water partition coefficient (log K_{ow}) was determined for some selected azadirachtins (Troß 1996). The authors reported log Kow 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.

5.3.1.2 Measured bioaccumulation data

No data available.

5.3.2 Summary and discussion of aquatic bioaccumulation

The calculated BCF_{fish} values of 2.5 L/kg (Azadirachtin B) and 1.38 L/kg (Azadirachtin A) indicate a low potential for aquatic bioaccumulation of the main components of *Margosa Extract with water*.

Furthermore, no other indicators point to an intrinsic potential for bioconcentration; the surface tension, for instance, is 56.4 mN/m and thus lies above the trigger value of ≤ 50 mN/m.

5.4 Aquatic toxicity

Table 50: Summary of relevant information on aquatic toxicity

Method	**Results**	**Remarks**	**Reference**
OECD 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 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 201: *Scenedesmus subspicatus*; growth rate inhibition	72h-ErC50 = 1041 mg/L 72h-ErC10 = 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 204: Oncorh*ynchus mykiss*, mortality and growth	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 211: Daphnia manga, 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 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 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

For the following effects assessment studies were available that 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.

In addition, for some studies performed with product also the content of *Margosa extract with water* in the product, either as 4 % or as maximum 4 %. However, as the content of *Margosa extract with water* in the product was not proven by further data, for those the measured concentration of Azadirachtin A as well as a mean content of Azadirachtin A in *Margosa extract with water* of 34 % was used for the derivation of the effect value related to *Margosa extract with water*.

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Table 51: Short-term toxicity to fish

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	LC_0	LC_{50}	LC_{100}		
OECD 203 (1992)	Rainbow trout (*Onco-rhynchus mykiss*)	Mortality	Semi-static (48-hour intervals)	96 hours	0.9	4.14	8.5	effect values based on geometric mean of the measured concentrations at t=0 and t=48 h test substance: Neem/Azal-T/S, containing 1 % Azadirachtin A , effect values related to active substance *Margosa Extract with water*	Anonymous. (1996b)

The acute toxicity of *Margosa Extract with water* to rainbow trout was extrapolated from a semi-static test with the product NeemAzal-T/S. The test was conducted according to OECD No. 203 (1992). Each test system comprised ten fish in a volume of 30 L tap water. Five test substance concentrations (50/100/200/400/800 mg/L NeemAzal-T/S) and a control were established. The test organisms were transferred to fresh medium after 48 h. Analytical determination of the leading component Azadirachtin A was performed at test start and after 48 h (before renewal of test solution). It is assumed that the mean measured concentration for the first phase of the test is also representative for the second phase (48-96 h). Therefore, the effect values are based on the geometric mean of the measured concentrations at test start and after 48 h. The effect value for *Margosa extract with water* 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 %.

A further fish short-term study with *Cyprinus carpio* was performed with the product NeemAzal-T/S as a limit test (100 mg/L NeemAzal-T/S containing 1.1 % Azadirachtin A) (Anonymous, 1996c). No mortality was found. However, as no analytical monitoring of the test substance concentration was performed, the study was considered as not valid and is therefore not used for the effects assessment of *Margosa extract with water*.

5.4.1.2 Long-term toxicity to fish

Table 52: Long-term toxicity to fish

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]		Remarks	Reference
			design	duration	NOEC	LOEC		
OECD 204	Rainbow trout, *Oncorhynchus mykiss*	Mortality, growth	Flow-through	28 d	1.9	4.4	Study performed with product NeemAzal-T/S containing 1 % Azadirachtin A, effect values related to active substance *Margosa extrac with watert*	Anonymous (1999a)
OECD 210	Zebra fish, *Danio rerio*	Hatching and survival rate, length and weight (FI-, FII-generation); daily egg production and fertilisation rate (FI-generation)	Flow-through	174 days	2.0	6.4	Study performed with the a.s. M*argosa extract with water*; Not valid, as survival of fertilized eggs in contol was < 70 %	Anonymous (2000c)

A long-term fish test is available for the product NeemAzal-T/S. The study was performed according to OECD 204, however the study design is rather comparable to OECD 215 (exposure period of 28 d; growth as sublethal endpoint) and therefore acceptable as long-term study. Test species was *Oncorhynchus mykiss*. Six test substance concentrations (4.7/9.4/18.8/37.5/75/150 mg/L NeemAzal-T/S) as well as a control were prepared. 10 fish per concentration were exposed in a flow-through system over 28 days. Analytical monitoring of the test substance concentration was performed two times per week using Azadirachtin A as leading compound (1 % content in NeemAzal-T/S). The mean measured concentrations were in the range of 3.9 to 147.5 mg NeemAzal-T/S/L. A 28d-NOEC for mortaliy of 63.6 mg NeemAzal-T/S/L was found (based on mean measured concentrations). This corresponds to a NOEC related to the active substance *Margosa Extract with water* of 1.9 mg/L. This effect value was 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 %.

No significant effects on growth rate or 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 adequate for the effects assessment of the active substance. According to the available data on the two formulation additives, the ecotoxicity of the biocidal product is expected to be associated with the a.s. rather than to any of those additives.

In a further study the chronic toxicity of *Margosa Extract with water* (purity 29.9 % Azadirachtin A) to zebra fish, *Danio rerio*, was investigated under flow-through conditions according to OECD No. 210 (1992). Four test substance treatments (nominal 0.20, 0.63, 2.00 and 6.40 mg a.s./L) and one blank control were set up at test start with each two replicates containing each 100 fertilized eggs in 12 L test medium. Survival and growth (body weight, length) of larvae was recorded on day 37. On day 38, juvenile fish were transferred to chambers with 25 L volume. On day 50, the number of fish per replicate was impartially equated to 50 and on day 84, when sexual development was finished, number of fish was further reduced to 24 per replicate (sex ratio 2:1 male:female). Reproduction of F1 generation was evaluated between days 91 and 118. On day 135, 100 fertilised eggs of each replicate were transferred to 12 L test medium, and survival and growth of fry (F2) was determined after another 38 days. Nominal concentrations were satisfactorily maintained up to and including the reproduction phase, but significantly lower than nominal during the second (F2) early life stage phase. No statistically significant difference between any test substance treatment and the control was found during the entire test period for any test parameter using average values of both replicates for the statistics. In one replicate of the 6.4 mg a.s./L treatment group, however, survival of fry of F1 was clearly decreased indicating a threshold for survival of fry at this concentration level. Although there was no similar finding with the F2 generation, this is not considered to disqualify the indication of a toxic effect in the F1 due to the significant decrease in the test substance concentrations during the second ELS phase. Therefore, the NOEC is established at 2.0 mg a.s./L. However, as the average survival of the control was only 56.6 % after 37 d, the study is not valid and cannot be used for the further effects assessment.

No further long-term fish studies are available for *Margosa extract with water*.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

Table 53: Short-term toxicity to invertebrates

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	EC_0	EC_{50}	EC_{100}		
OECD 202, Pt. I	*Daphnia magna*	Immobility	static	48 hours	2.00	9.69	>26.34	Study performed with the a.s. *Margosa extract with water;* effect values based on initial measured conc.	Anonymous (1999b)

The acute toxicity of *Margosa Extract with water* (purity 33.4 % Azadirachtin A) to *Daphnia magna* was determined in a static test according to OECD No. 202 (1984). Five neonates (< 24 h) were held in 60 mL glass beakers containing 25 mL test medium and four replicate test systems were set up per treatment group. Six test substance concentrations (nominal: 2.5, 5.0, 10, 20, 40 and 80 mg a.s./L) were prepared adding the same volume of appropriate stock solutions in acetone to the test medium (≤ 0.01 %). A blank and a vehicle control were tested in addition. Concentrations of the test substance were measured at 0 and 48 h using azadirachtin A as lead substance. The measured concentrations were lower than nominal at 0 hours and increasing by 48 hours in the medium and higher treatments (probably due to inhomogeneous mixing at start of the test). Therefore, as a worst-case approach, the toxicity values are calculated based on measured initial concentrations. Immobility of test organisms, determined at 24 and 48 hours, was increasing with time showing a concentration-effect relationship (90 % at the highest treatment level). Despite the analytical peculiarities, the test is considered acceptable and the toxicity data are reliable.

5.4.2.2 Long-term toxicity to aquatic invertebrates

Table 54: Long-term toxicity to invertebrates

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]		Remarks	Reference
			design	duration	NOEC	LOEC		
OECD 202, Pt. II	*Daphnia magna*	Reproduction & mortality	semi-static	21 days	1.84	>1.84	Study performed with the a.s. M*argosa extract with water*; Effect values based on mean measured conc.	Anonymous (1999b)
OECD 202, Pt. II	*Daphnia magna*	Reproduction & mortality	semi-static	21 days	0.1	0.22	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 A 7.4.3.4/02

The chronic toxicity of *Margosa Extract with water* (purity 33.4 % Azadirachtin A) to *Daphnia magna* was determined in a semi-static test according to OECD No. 202, Pt. II (1984). Ten daphnids per treatment level were individually confined in 60 mL glass beakers containing 50 mL test medium. The concentration of the test substance in the medium varied more than ± 20 %, therefore, the toxicity values were based on mean measured concentrations of 0.10, 0.21, 0.42, 0.90 and 1.84 mg a.s./L. Mortality of adults daphnids, appearance of first young and number of young daphnids were regularly checked. There was no statistically significant difference for any test parameter between any treatment level and the blank control. Accordingly, the NOEC was established as 1.84 mg a.s./L. The test is considered acceptable and the toxicity data are reliable.

In a second reproduction study with *Daphnia magna* the chronic toxicity of the formulated product NeemAzal-T/S was examined. 10 daphnids per concentration were individually exposed in a semi-static system to 6 test substance concentrations (3.125/6.25/12.5/25/50/100 mg NeemAzal-T/S/L). Analytical monitoring of the test substance concentration was performed in fresh and old medium at each medium change using Azadirachtin A as leading compound (1 % content in NeemAzal-T/S). The mean measured concentrations were in the range of 1.7 to 62.5 mg NeemAzal-T/S/L. A 21 d-NOEC for reproduction of 3.4 mg NeemAzal-T/S/L was found (based on mean measured concentrations). This corresponds to a NOEC related to the active substance *Margosa Extract with water* of 0.102 mg/L. This effect value was 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 %.

Although the study was performed with the formulated product instead of the active substance, it is considered as adequate for the effects assessment of the active substance. According to the available data on the two formulation additives, the ecotoxicity of the b.p. is expected to be associated with the a.s. rather than to any of those additives.

5.4.3 Algae and aquatic plants

Table 55: Toxicity to algae

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	E_rC_{10}	E_bC_{50}[1]	E_rC_{50}[2]		
OECD 201	*Scenedesmus subspicatus* (green alga)	Cell density, biomass, growth rate	static	72 hours	332	482	1041	Study performed with the a.s. M*argosa extract with water* ;Effect values based on nominal concentration; no exponential growth during the whole test duration	Wenzel, A. (2002) report no. TRF-001/4-30 A 7.4.1.3

The toxicity of *Margosa Extract with water* (purity 35 % Azadirachtin A) to the green alga *Scenedesmus subspicatus* was determined in a static test according to OECD No. 201 (1984). At the start of the test, alga inoculum of 10^4 cells/mL was introduced in a volume of 100 mL test medium in a 250 mL glass flask. Three replicate flasks were set up per treatment group and maintained under continuous light and shaking. The nominal test concentrations were 0, 10, 50, 100, 500 and 1000 mg a.s./L. Both azadirachtin A and azadirachtin B were measured at test start and end. As azadirachtin A was not stable in the test system (degradation by 96 %), azadirachtin B was used as leading compound and was found to be stable also after 72 h. The concentration of azadirachtin B was > 120 % of nominal and the concentration of Azadirachtin A at test start was in the range of 85-113 %. As it is unclear which azadirachtin is responsible for the effects, the effect values are based on nominal concentrations.

Clear adverse effects on the growth of algae were found at the two highest treatment levels in comparison with the control. The 72h-E_rC_{50} was calculated as 1041 and the respective E_bC_{50} was 482 mg a.s./L., The 72h-E_rC_{10} was calculated as 332 mg a.s./L. The control cultures did not follow exponential growth during the whole test duration. Instead, a lag phase was observed for the first 24 h. As exponential growth is a prerequisite for growth rate evaluation, the test is formally not valid. However, as algae are clearly the least sensitive of the tested aquatic organisms, the test is regarded as acceptable for the effects assessment.

No further algae studies are available for *Margosa extract with water.*

5.4.4 Other aquatic organisms (including sediment)

Table 56: Long-term toxicity to Chironomid larvae

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]		Remarks	Reference
			design	duration	NOEC	LOEC		
OECD 219	*Chironomus riparius*	Emergence, development rate	static	28 days	0.0184 (nominal conc.) 0.0075 (mean measured conc.)	0.0368 (nominal)	Study performed with the a.s. M*argosa extract with water*	Gonsior, G. (2008a) report no. 2007/1356/01-ASCr A 7.4.3.5.1
OECD 219	*Chironomus riparius*	Emergence, development rate	static	28 days	0.018 (nominal conc.) 0.006 (mean measured conc.)	0.036 (nominal)	Study performed with 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 B 7.7.1.1

The long-term toxicity of *Margosa Extract with water* (purity 34 % Azadirachtin A) to *Chironomus riparius* was examined according to OECD 219. Chironomid larvae were exposed to 0.0023, 0.0046, 0.00919, 0.0184, 0.0368, 0.0735, 0.147 and 0.294 mg a.i./L in a static water-sediment system for a period of 28 days. Four replicate test vessels were prepared for each test substance treatment group and for a blank control group. Additional 18 vessels were prepared for chemical analyses of the test item. During the experimental phase, the larvae were fed daily with 1 mg fish food per larvae.

Based on the nominal concentrations, the 28-day EC_{50} for emergence was determined to be 0.0329 mg/L. The number of emerged midges in the test item treatments did not show a significant difference to the control at the nominal concentration up to and including 0.0184 mg/L. The time course of emergence, represented by the development rate, did not show a significant difference to the control at the nominal concentration up to and including 0.0368 mg/L. The overall NOEC was estimated to be 0.0184 mg/L and the overall LOEC was estimated to be 0.0368 mg/L.

Samples taken from the water phase, the pore water and the sediment of 0.0184 and 0.294 mg/L test vessels and of the control vessels 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 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 NeemAzal-T/S/L and an untreated control in using the same test design as described above.

Based on the nominal concentrations, the 28-day EC_{50} for emergence was determined to be 1.15 mg NeemAzal-T/S/L. The number of emerged midges in the test item treatments did not show a significant difference to the control at the nominal concentration up to and including 0.573 mg NeemAzal T/S/L. The time course of emergence, represented by the development rate, did not show a significant difference to the control at the nominal concentration up to and including 1.15 mg/L. The overall NOEC was estimated to be 0.573 mg NeemAzal T/S/L and the overall LOEC was estimated to be 1.15 mg NeemAzal T/S/L.

Samples of the overlying water, pore water and the sediment were taken 1 hour, 7 days and 28 days after application for the concentrations 0.573 and 9.17 mg NeemAzal T/S/L and for the control. 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 NeemAzal-T/S/L. 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 %.

The results from both studies related to *Marogsa extract* are in good agreement. For both studies it can be expected that the exposure of the test organism occurred predominantly via the water phase as the sorption potential of the analytical lead component Azadirachtin A is low. This conclusion is also supported by the measured concentrations in the sediment which were below the LOQ in both studies. Therefore, the studies should be considered for the classification of the active substance *Margosa extract with water*.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

Degradation: not rapidly degradable;

As *Margosa Extract with water* is a complex substance of natural origin, it has to be regarded as not rapidly degradable since it contains a not-rapidly-degradable constituent (i.e. Azadirachtin) with a proportion of ≥ 20 % (i.e. ~ 34 %). Azadirachtin is also considered as the compound mainly responsible for the ecotoxicological effect on the target organisms.

5.1 → Biodegradation: not readily biodegradable ´
Azadirachtin A is not readily biodegradable, since it was degraded to only 21.6 % within 28 days in an OECD 301F test.

→ Hydrolysis: hydrolytically degradable
According to the "Guidance on the application of the CLP criteria" hydrolysis might be considered for classification only when the longest half-life determined with the pH-range 4-

9 is shorter than 16 days and if the hydrolysis products do not fulfil the criteria for classification as hazardous to the aquatic environment. Because the longest half-life for Azadirachtin A is 112.7 days, hydrolysis will not be considered.

→ Biodegradation in freshwater
A substance is demonstrated to be ultimately degraded in a surface water simulation test with a half-life of < 16 days (corresponding to a degradation of > 70 % within 28 days); or primarily degraded biotically or abiotically e.g. via hydrolysis, in the aquatic environment with a half-life < 16 days (corresponding to a degradation of > 70 % within 28 days), and it can be demonstrated that the degradation products do not fulfil the criteria for classification as hazardous to the aquatic environment. Azadirachtin does not meet these criteria, since it was 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, whereas neither information regarding the degree of ultimate degradation nor on degradation products are available from these studies.

5.2 →Adsorption/desorption: not relevant for classification and labelling

Volatilisation: not relevant for classification and labelling
According to "Guidance on the application of the CLP criteria", volatilization only represents removal of a chemical from the water phase, and not degradation, the Henry's Law constant cannot be used for assessment of degradation in relation to aquatic hazard classification of substances.

Mobility: not relevant for classification and labelling

5.3 → Aquatic Bioaccumulation: log K_{ow}< 4 (low bioaccumulation potential)

5.4 → Aquatic Toxicity: not acutely toxic (EC/LC_{50}> 1 mg/L), but toxic to aquatic life with long lasting effects (NOEC < 0.1 mg/L)

Adequate **acute toxicity data** is available for all three trophic levels (fish, crustacean, algae/aquatic plants). The criterion for classification as H400 "Very toxic to aquatic life" is a $LC_{50} \leq 1$ mg/l. As 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 the trigger value. Therefore *Margosa Extract with water* does not fulfil the classification criterion and **no classification as Aquatic Acute 1, H400** is necessary.

Adequate **chronic toxicity data** is available for all three trophic levels (fish, crustacean, algae/aquatic plants). Hence, according to Regulation (EC) 286/2011 (2nd ATP) the classification of the long-term aquatic hazards has to be based on the available chronic data. Invertebrates represent the most sensitive trophic level for chronic toxicity in the aquatic compartment.

The lowest long-term effect value (28d-NOEC = 0.006 mg a.s./L) was found for the midge larvae *Chironomus riparius* in a water-sediment study according to OECD 219 (spiked water). Although this is not a standard test system for classification, the use of this value 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.

For substances not fulfilling criteria for rapid degradation, the criterion for classification as H410 "Very toxic to aquatic life with long lasting effects" is EC_{10}/NOEC ≤ 0.1 mg/L. *Margosa Extract with water* fulfils this criterion and should be classified as **Aquatic Chronic 1, H410**, with a chronic

multiplication factor **$M_{chronic}$ = 10** (considering 0.001 mg/L < NOEC < 0.01 mg/L for non-rapidly degradable substances).

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Considering the availability of adequate acute data for all three trophic levels (→ classification criteria not fulfilled) and adequate chronic toxicity data for all three trophic levels and the fact that *Margosa Extract with water* represents a non-rapidly degradable substance, the following classification for the environment can be concluded:

Category Chronic 1 with multiplying factor $M_{chronic}$ = 10

With regard to the environment and in accordance to Regulation of European Parliament (EC) No 1272/2008, the substance *Margosa Extract with water* has therefore to be classified with H410, Category Chronic 1, $M_{chronic}$ = 10.

For the labelling the GHS pictogram GHS09 and the hazard statement "Very toxic to aquatic life with long lasting effects" has to be applied with the signal word 'Warning'.

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 **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 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; 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).

<u>Long-term toxicity</u>

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).

6 OTHER INFORMATION

None

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8 ANNEXES

Confidential Annex