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: **-**

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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

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

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)[2] and therefore subject to harmonised classification and labelling (Regulation (EC) No 1272/2008 Article 36.2).

[2] 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.[2]

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.[3]
- 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).

[3] 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.[4]

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.

[4] 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

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)	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.	Franke, 2005a Report No. 20050679.02	92/69/EEC, A.10
Flammability in contact with water	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.	BAM 2.2 (2012)	
Pyrophoric properties	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)	BAM 2.2 (2012)	
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.

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) Cat. 2 (H310): $50 < LD_{50} \leq 200$ mg/kg (dermal) Cat. 1 (H310): $LD_{50} \leq 50$ 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.

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.

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

Animal species & strain	Number of animals	Doses	Result	Reference Method
Rabbit, New Zealand albino	6 M	0.5 g (4 h)	Not irritating (highest erythema score: 1), resolved by day 2 *Margosa Extract with water* (37 % Azadirachtin A)	Anonymous, 1996f

Rabbit number and sex	E = Erythema O = Oedema	Day 1*	2	3	4
568 ♂	E	1	0	0	0
	O	0	0	0	0
570 ♂	E	1	0	0	0
	O	0	0	0	0
583 ♂	E	0	0	0	0
	O	0	0	0	0
584 ♂	E	1	0	0	0
	O	0	0	0	0
585 ♂	E	0	0	0	0
	O	0	0	0	0
586 ♂	E	0	0	0	0
	O	0	0	0	0

* Approximately 60 minutes after removal of the dressing

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.

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.

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.

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).

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.

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.

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.

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
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[e]	-	-	-	1
Atrial septal defect with narrow pulmonary vein	-	-	-	1[e]	-	-	-	1
Interventicular septal defect	-	-	1[b]	2[de]	-	-	1	2
Malrotated heart	-	-	1[b]	1[e]	-	-	1	1
Duplicated inferior vena cava	-	-	-	2[d]	-	-	-	2
Diaphragmatic hernia	-	4	-	-	-	2	-	-
Distorted ribcage with thickened ribs	-	-	-	1[f]	-	-	-	1
LUMBAR/ABDOMINAL								
Umbilical hernia	-	1[a]	-	-	-	1	-	-
APPENDICULAR								
Forelimb flexure	-	-	-	1[f]	-	-	-	1
Brachymelia with curved ulnae and radii	-	-	-	1[f]	-	-	-	1
OTHER								
Squat foetus syndrome	-	-	3	-	-	-	1	-
Mottled foetus syndrome	-	-	-	4	-	-	-	1

Superscripts indicate findings common to one foetus

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).

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 corresponding to 30 mg/L dry matter	no	no	100 mg Azadirachtin 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 % Azadirachtin 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 corresponding to 30 mg/L dry matter	no	no	100 mg *Margosa Extract* (a.s.)./L, 34 % Azadirachtin 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 corresponding to 30 mg/L dry matter	no	no	100 mg *Margosa Extract* (a.s.)/L, 34 % Azadirachtin 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 corresponding to 30 mg/L dry matter	no	no	100 mg *Margosa Extract* (a.s.)/L (dissolved in DMSO), 34 % Azadirachtin 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 consumption	activated sludge	not specified	no	no	1 & 2 mg NeemAzal-T/S /L, 1 % Azadirachtin 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 K_{OW} (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),	1.29 (Azadirachtin B)[1]	2.5 L/kg	-	-

Basis for estimation	log Kow (measured)	Estimated BCF for fish (freshwater) on wet weight basis	Estimated BCF for fish eating bird/predator	Reference
Part II, chapter 3.8.3.2			-	-
	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. *Margosa 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 $\mathbf{M_{chronic} = 10}$ (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’.

6 OTHER INFORMATION

None

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8 ANNEXES

Confidential Annex