Committee for Risk Assessment

RAC

Annex 1
Background document
to the Opinion proposing harmonised classification and labelling at EU level of

N,N'-methylenediacrylamide

EC Number: 203-750-9
CAS Number: 110-26-9

CLH-O-0000007157-72-01/F

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

Adopted

15 September 2022

CLH report

Proposal for Harmonised Classification and Labelling

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

Chemical name: *N,N'*-methylenediacrylamide

EC Number: 203-750-9

CAS Number: 110-26-9

Index Number: -

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CONTENTS

1 IDENTITY OF THE SUBSTANCE 1

1.1 NAME AND OTHER IDENTIFIERS OF THE SUBSTANCE 1
1.2 COMPOSITION OF THE SUBSTANCE 2

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING 3

2.1 PROPOSED HARMONISED CLASSIFICATION AND LABELLING ACCORDING TO THE CLP CRITERIA 3

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING 4

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL 5

5 IDENTIFIED USES 5

6 DATA SOURCES 5

7 PHYSICOCHEMICAL PROPERTIES 5

8 EVALUATION OF PHYSICAL HAZARDS 7

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION) 7

9.1 SHORT SUMMARY AND OVERALL RELEVANCE OF THE PROVIDED TOXICOKINETIC INFORMATION ON THE PROPOSED CLASSIFICATION 8

10 EVALUATION OF HEALTH HAZARDS 8

10.1 ACUTE TOXICITY - ORAL ROUTE 9
10.2 ACUTE TOXICITY - DERMAL ROUTE 9
10.3 ACUTE TOXICITY - INHALATION ROUTE 9
10.4 SKIN CORROSION/IRRITATION 9
10.5 SERIOUS EYE DAMAGE/EYE IRRITATION 9
10.6 RESPIRATORY SENSITISATION 9
10.7 SKIN SENSITISATION 9
10.8 GERM CELL MUTAGENICITY 9
10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity 15
10.8.2 Comparison with the CLP criteria 17
10.8.3 Conclusion on classification and labelling for germ cell mutagenicity 17
10.9 CARCINOGENICITY 21
10.10 REPRODUCTIVE TOXICITY 21
10.11 SPECIFIC TARGET ORGAN TOXICITY-SINGLE EXPOSURE 21
10.12 SPECIFIC TARGET ORGAN TOXICITY-REPEATED EXPOSURE 21
10.13 ASPIRATION HAZARD 22

11 EVALUATION OF ENVIRONMENTAL HAZARDS 22

12 EVALUATION OF ADDITIONAL HAZARDS 22

13 ADDITIONAL LABELLING 22

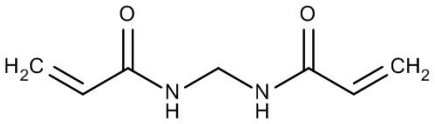
14 REFERENCES 22

15 ANNEXES 23

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Names in the IUPAC nomenclature or other international chemical names	Bis(acrylamido)methane Bisacrylamide Methylene-bis-acrylamide N,N'-Methylendiacrylamid N,N'-methylenebis-2-propenamide N,N'-Methylenebisacrylamide N,N-Methylenbisacrylamide N-[(prop-2-enamido)methyl]prop-2-enamide N-[(Prop-2-enoylamino)methyl]prop-2-enamide
Other names (usual name, trade name, abbreviation)	MBA
ISO common name	*n.a.*
EC number	203-750-9
EC name	*N,N'*-methylenediacrylamide
CAS number	110-26-9
Other identity code	*n.a.*
Molecular formula	C7H10N2O2
Structural formula	
SMILES notation	
Molecular weight or molecular weight range	154.17 g/mol
Information on optical activity and typical ratio of (stereo) isomers	*n.a.*
Description of the manufacturing process and identity of the source (for UVCB substances only)	*n.a.*
Degree of purity (%) (if relevant for the entry in Annex VI)	*n.a.*

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)
N,N'-methylenediacrylamide EC 203-750-9	-	None	Acute Tox. 3, H301 Actue Tox. 4, H312, H332 Muta. 1B, H340 (Oral) Carc. 1B, H350 (Oral) Repr. 2, H361 (Oral) STOT RE 1, H372 (Peripheral nervous system) (Oral)

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
-				

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
-					

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 5: Proposed harmonised classification and labelling according to the CLP criteria.

	Index No	**Chemical name**	**EC No**	**CAS No**	**Classification**		**Labelling**			**Specific Conc. Limits, M-factors and ATEs**	**Notes**
					Hazard Class and Category Code(s)	**Hazard statement Code(s)**	**Pictogram, Signal Word Code(s)**	**Hazard statement Code(s)**	**Suppl. Hazard statement Code(s)**		
Current Annex VI entry	No current Annex VI entry										
Dossier submitter's proposal	TBD	*N,N'*-methylenediacrylamide	203-750-9	110-26-9	Muta. 1B, H340	H340	GHS06 Dgr	H340			
Resulting Annex VI entry if agreed by RAC and COM	TBD	*N,N'*-methylenediacrylamide	203-750-9	110-26-9	Muta. 1B, H340	H340	GHS06 Dgr	H340			

Table 6: Reason for not proposing harmonised classification and status under consultation

Hazard class	Reason for no classification	Within the scope of consultation
Explosives	Hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	Hazard class not assessed in this dossier	No
Oxidising gases	Hazard class not assessed in this dossier	No
Gases under pressure	Hazard class not assessed in this dossier	No
Flammable liquids	Hazard class not assessed in this dossier	No
Flammable solids	Hazard class not assessed in this dossier	No
Self-reactive substances	Hazard class not assessed in this dossier	No
Pyrophoric liquids	Hazard class not assessed in this dossier	No
Pyrophoric solids	Hazard class not assessed in this dossier	No
Self-heating substances	Hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	Hazard class not assessed in this dossier	No
Oxidising liquids	Hazard class not assessed in this dossier	No
Oxidising solids	Hazard class not assessed in this dossier	No
Organic peroxides	Hazard class not assessed in this dossier	No
Corrosive to metals	Hazard class not assessed in this dossier	No
Acute toxicity via oral route	Hazard class not assessed in this dossier	No
Acute toxicity via dermal route	Hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	Hazard class not assessed in this dossier	No
Skin corrosion/irritation	Hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	Hazard class not assessed in this dossier	No
Respiratory sensitisation	Hazard class not assessed in this dossier	No
Skin sensitisation	Hazard class not assessed in this dossier	No
Germ cell mutagenicity	Harmonised classification proposed	Yes
Carcinogenicity	Hazard class not assessed in this dossier	No
Reproductive toxicity	Hazard class not assessed in this dossier	No
Specific target organ toxicity-single exposure	Hazard class not assessed in this dossier	No
Specific target organ toxicity-repeated exposure	Hazard class not assessed in this dossier	No
Aspiration hazard	Hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	Hazard class not assessed in this dossier	No
Hazardous to the ozone layer	Hazard class not assessed in this dossier	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

No previous harmonised classification and labelling.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at Community level.

5 IDENTIFIED USES

The substance (N,N'-methylenediacrylamide; MBA) is used as crosslinking agent and as monomer in polymerisation. It is used by professional workers for the production of electrophoresis gels.

6 DATA SOURCES

Scientific publications were gathered from Search in PubMed open literature using search terms like "N,N'-methylenediacrylamide", "Methylene-bis-acrylamide", "acrylamide analogues" and similar.

The registration dossier in ECHA dissemination site (https://echa.europa.eu/sv/registration-dossier/-/registered-dossier/21036) mainly contains studies conducted on the structurally similar substance acrylamide (using read across). As the mutagenicity data on MBA itself is considered strong enough for classification purposes, and the data on acrylamide does not justify classification in a different (sub-) category, the data on acrylamide has only been summarised and used as supporting evidence.

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g., measured or estimated)
Physical state at 20°C and 98.2 kPa	Solid	REACH registration (ECHA dissemination, 2021)	Measured
Melting/freezing point	173.7 °C and 185.9 °C (probably polymerisation)	REACH registration (ECHA dissemination, 2021)	Measured. Two thin endothermic peaks were recorded at 173.7 °C and 185.9 °C which probably correspond to phase transition of the test item due to polymerisation.
Boiling point	333.8 °C	REACH registration (ECHA dissemination, 2021)	Measured
Relative density	1.216 ± 0.006 at 20.0 °C	REACH registration (ECHA dissemination, 2021)	Measured
Vapour pressure	3.0 x 10-6 Pa	REACH registration (ECHA dissemination, 2021)	Measured
Surface tension	70.3 ± 0.1 mN/m	REACH registration (ECHA dissemination, 2021)	Measured
Water solubility	34.1 g/L	REACH registration (ECHA dissemination,	Measured

Property	Value	Reference	Comment (e.g., measured or estimated)
		2021)	
Partition coefficient n-octanol/water	-0.08	REACH registration (ECHA dissemination, 2021)	Measured
Flash point	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant
Flammability	Not flammable	REACH registration (ECHA dissemination, 2021)	Measured
Explosive properties	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant
Self-ignition temperature	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant
Oxidising properties	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant
Granulometry	The average particle size is 99.92 µm.	REACH registration (ECHA dissemination, 2021)	Measured
Stability in organic solvents and identity of relevant degradation products	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant
Dissociation constant	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant
Viscosity	-	REACH registration (ECHA dissemination, 2021)	Waived by the registrant

8 EVALUATION OF PHYSICAL HAZARDS

Not evaluated in this CLH proposal.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8: Summary table of toxicokinetic studies for acrylamide.

Method	Results	Remarks	Reference
Basic toxicokinetics in vivo. GLP Reliability according to Registrant: 1. The objective was to compare the metabolism of acrylamide administered orally (p.o.), dermally, intraperitoneally (i.p.), or by inhalation, and to measure the haemoglobin adducts produced. Rats and mice were exposed to 2.9 ppm [1,2,3-13C] and [2,3-14C]acrylamide for 6 h. [2,3-14C]acrylamide (162 mg/kg) or [1,2,3-13C]acrylamide (138 mg/kg) in water was administered dermally to rats for 24 h, and [1,2,3-13C]acrylamide was administered i.p. (47 mg/kg). Urine and faeces were collected for 24 h.	Uptake due to dermal and inhalation exposure is lower than for ip. Urine was the major elimination route in rats and mice. Signals in the 13C-NMR spectra of urine were assigned to previously identified metabolites derived from acrylamide glutathione conjugation and conversion to glycidamide (GA). Acrylamide-GSH was a major metabolic route in rats accounting for 69% (i.p.), 71% (p.o.), 52% (dermal), and 64% (inhalation). In mice, acrylamide-GSH accounted for only 27% (inhalation) of the total urinary metabolites. The remaining urinary metabolites were derived from GA. Valine haemoglobin adducts of acrylamide and GA were characterized using liquid chromatography-mass spectrometry. The ratio of acrylamide to GA adducts paralleled the flux through pathways based on urinary metabolites. This study demonstrates marked species differences in the metabolism and internal dose (Hb-adducts) of acrylamide following inhalation exposure and marked differences in uptake comparing dermal with p.o. and i.p. administration.	Test substance: acrylamide.	Unnamed 2001, summarised in registration dossier, ECHA's dissemination site, 2021.
In vivo basic toxicokinetics. GLP Reliability according to Registrant: 1.	No bioaccumulation potential based on study results This study demonstrates that acrylamide is rapidly distributed throughout the body where it is readily metabolized prior to excretion. The biotransformation of acrylamide was mainly mediated through glutathione conjugation followed by excretion in the urine of the	Test substance: acrylamide	Unnamed 2005, summarised in registration dossier, ECHA's dissemination site, 2021.

Method	Results	Remarks	Reference
	mercapturic acid, N-acetyl-S-(3-amino-3oxypropyl)cysteine. Other metabolites are also excreted but their significance is unknown. The half-life of parent acrylamide in the body is extremely short; however, a small percentage of radiolabel remains in tissues for several weeks.		
Dermal absorption in vivo. GLP. Reliability according to Registrant: 1	The study demonstrated that dermal absorption in humans is slow. Only 4.5% of the applied dose was absorbed from an occluded patch on the forearm over a 24-hour period which equates to less than 0.2% absorption per hour.	Test substance: acrylamide	Unnamed 2006. summarised in registration dossier, ECHA's dissemination site, 2021.

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification

No experimental toxicokinetics studies on MBA are available. In the registration dossier, three toxicokinetics studies on the structurally similar substance acrylamide are summarised (see table 8 above). Based on several different types of information, including structure similarity, physical-chemical properties, and genotoxicity profile, the dossier submitter considers that biological and toxicological similarities between the two substances are likely. Although the molecular weight of MBA is twice as high as acrylamide's (154.17 g/mol vs. 71.8 g/mol, respectively), both substances are hydrophilic and water soluble (log Pow -0.9/-0.08, WS 2155/34.1 g/L for acrylamide and MBA, respectively), and share the identical organic functional group (the acrylamide moiety) which indicate that the two substances may behave similarly in the body. Both substances are solids in room temperature. The data demonstrating similar genotoxic properties of MBA and acrylamide are discussed in more detail in section 10.8. Below is a brief summary of the toxicokinetic behaviour of acrylamide (summarised from EFSA's report, 2015).

Both animal and human studies have shown that acrylamide is rapidly and extensively absorbed from the gastrointestinal tract and the parent compound and/or its metabolites are then widely distributed throughout the body (including liver, kidney, lung, muscles, brain, testes, sciatic nerve, spinal cord, skin, fat and small intestine). Acrylamide is also transferred into the fetus and to a low extent to milk. Acrylamide is extensively metabolised, mostly by conjugation with GSH but also by epoxidation to form the more reactive metabolite glycidamide (GA), an epoxide. The formation of GA represents a metabolic activation pathway preferentially mediated by CYP2E1. Metabolic inactivation reactions comprise the hydrolysis of GA to 2,3-dihydroxypropionamide, as well as the GST-driven formation of GSH adducts of acrylamide and GA, which are further processed to the respective mercapturic acids and excreted in urine. Conjugation of acrylamide and GA with GSH appear to be the predominant detoxification pathway, while GA hydrolysis plays a minor role. The GST isoforms involved in the conjugation of acrylamide and GA with GSH in animals and humans are not known. The acrylamide metabolites are rapidly and almost completely excreted with the urine, mostly as mercapturic acids of the GSH conjugates of acrylamide and GA, and there is no indication of tissue accumulation, except for residual protein adducts (EFSA, 2015).

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Not evaluated in this CLH proposal.

10.2 Acute toxicity - dermal route

Not evaluated in this CLH proposal.

10.3 Acute toxicity - inhalation route

Not evaluated in this CLH proposal.

10.4 Skin corrosion/irritation

Not evaluated in this CLH proposal.

10.5 Serious eye damage/eye irritation

Not evaluated in this CLH proposal.

10.6 Respiratory sensitisation

Not evaluated in this CLH proposal.

10.7 Skin sensitisation

Not evaluated in this CLH proposal.

10.8 Germ cell mutagenicity

Table 5: Summary table of mutagenicity/genotoxicity tests in vitro

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Bacterial Reverse Mutation Assay, OECD TG 471. Not GLP. Reliability according to Registrant: 2	MBA (purity >95%) dissolved in water	Strains: S. typhimurium TA 1535, TA 1537, TA 1538, TA 98 and TA 100 With and without metabolic activation (Aroclor S9) Concentrations: 0, 5, 50, 500, 1000 and 5000 µg/plate. Positive controls included.	Not mutagenic (with or without metabolic activation). Cytotoxicity was not specified.	Study by Hashimoto and Tanii 1985, summarised in registration dossier, ECHA's dissemination site, 2021.
In vitro gene mutation study in bacteria, similar to OECD TG 471.	MBA (purity not known) dissolved	S. typhimurium TA 1535, 97, 98, 100. With and without metabolic activation	Mutagenic in Strain TA1535 with 10% Hamster S9 and Strain TA100 with 30% Hamster S9). The mutagenic response increased dose-	Study from NTP (Zeiger et al. 1988), summarised in registration dossier, ECHA's

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
GLP not specified. Reliability according to Registrant: 2	in DMSO.	(S9) Doses: 0, 100, 333, 1000, 3333, 10000 µg/plate Positive controls included.	dependently. Cytotoxicity was not specified.	dissemination site, 2021.

Table 10: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo.

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
In vivo mammalian somatic cell study: cytogenicity / erythrocyte micronucleus, OECD TG 474. GLP not specified. Reliability according to Registrant: 2	MBA (purity not specified)	Male B6C3F1 mice (5 per group) dosed by i.p. injection of 0, 25, 50 and 100 mg/kg. Doses given: 2 Duration: 2 days. Sampling time: 24 hrs. Positive control included.	Mutagenic (statistically significant effects seen at 25, 50 and 100 mg/kg).	Study from NTP 1988, summarised in registration dossier, ECHA dissemination site, 2021.
Comet assay on testicular samples from exposed mice. Not GLP.	MBA (purity not stated).	Male CD-1 mice (5 per group) dosed orally by 0, 50, 100 and 190 mg/kg bw. Doses given: 2 Duration: 2 days. Sampling time: 2-4 hrs after second dosing.	MBA exposure cause statistically significant increased DNA damage (comets) in testicular cells at 190 mg/kg (reported as average % tail DNA).	Hansen et al. 2014
Dominant lethal and heritable translocation study, similar to OECD TG 478. GLP not specified.	MBA (purity 99.9%)	Study I: Male $(C3Hx101)F_1$ hybrid stock mice (36 per group) dosed by i.p injections at 0 and 225 mg/kg bw. Matings occurred 1-50 days post-treatment with untreated female mice (T-stock). All post stem-cell stages were sampled. Study II: Male $(C3Hx101)F_1$ hybrid stock mice (30 per group) dosed by i.p injections at 0 and	Study I: Dominant lethal effects were observed in matings during the first 4 days post-treatment. The number of implantations/pregnant females exposed to MBA was statistically significant decreased compared to controls (7.4 vs. 9.7). Living embryos per pregnant female was 5.1 in MBA-treated group vs. 8.1 in controls (statistically significant). Reduced number of pregnant females, implantations per pregnant females and of living embryos was also seen in matings at 36.5-43.5 days post-treatment. Study authors suggest involvement of cytotoxic	Rutledge et al. 1990 (NTP study).

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		225 mg/kg bw. Matings occurred 1-9 days post-treatment with untreated female mice (SECxC57BL)F1. Late spermatids and mature spermatozoa stages were sampled. Study III: Male mice (C3Hx101)F_1 hybrid stock (60 and 65 mice per group, respectively) dosed by i.p. injections at 0 and 90 mg/kg bw given daily for 5 days. Matings occurred 1-12 days post-treatment with untreated female mice, both (C3Hx101)F_1 and (SECxC57BL)F1. Midspermatids to mature spermatozoa stages were sampled. Study IV: Male mice (C3Hx101)F_1 hybrid stock (25 and 97 mice per group, respectively) dosed by i.p. injections at 0 and 90 mg/kg bw given daily for 5 days. Matings occurred 1-5 days post-treatment with female mice (SECxC57BL)F1. Only male offspring were kept for further study to identify translocation carriers. Male offspring (F1 generation) were further mated with untreated control females to identify sterile and semisterile offspring for cytogenetic analysis, as well as anatomical analysis of fetuses. Prior to mutagenicity test a 30-day acute toxicity study for single and multiple doses was performed to determine the maximum tolerated dose (<250 mg/kg bw). Positive controls were not included. Data on historical controls were available. No general clinical	effects in differentiating spermatogonia to explain the latter. Study II: Dominant lethal effects were seen in matings 0.5-3.5 days post-treatment. MBA exposure resulted in 14 percent dead implants (vs. 2 in controls, statistically significant) and 10 percent dominant lethals. Study III: Dominant lethals were seen in maturing sperm (matings between 0.5-3.5 days post-treatment). Matings of the two different females' stocks resulted in 22-26 and 34-46 percent dominant lethals. Study IV: MBA induced an increased incidence of semisterile offspring (10 percent vs. 0.8 in controls). The frequency of translocations was 13.4% (47/350) in offspring of MBA-treated mice, vs 0.8% in controls (1/127). No information on general toxicity of parental generation is available.	

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		observations were reported. Only one dose was tested. Percent dominant lethals = (1 – (living embryos/pregnant female (treated)/living embryos/pregnant female (control))) x 100.		

Table 11: Summary table of supporting studies.

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
NTP's Reproductive Assessment by Continuous Breeding (RACB). GLP.	MBA (97-99%)	Swiss CD-1 mice were dosed with MBA in drinking water at doses 10, 30 and 60 ppm (doses corresponded to 1.6, 4.7 and 9.3 mg/kg bw/day in Task 2/3 and 2.6, 9.6 and 16.3 mg/kg bw/day in Task 4). Task 1. 28 days dose range finding study. Task 2. Control group (n=40 mating pairs) and 3 dose groups (n=20 mating pairs). 7 days of dosing (housed separately), followed by 98 days of dosing (as mating pairs), followed by 6 weeks dosing (housed separately). Any litter delivered during last dosing period were dosed until Day 74 of age. Dominant lethal test: Control- and high dose-treated F1 males were cohabited with 3 untreated females for up to 4 nights. Pregnant females were killed on Gestation Day 16 to evaluate implants and dominant lethal effects in the males. Task 3. A crossover mating trial was conducted after the 6 weeks holding	Task 1: Dose range finding study. Task 2 (continuous breeding): No difference in the proportion of fertile pairs, average number of litters per pair, proportion of pups born alive, and proportion male pups per litter between groups. In mid- and high dose groups the mean number of live pups/litter was statistically significantly decreased (12.9* ± 0.5 and 12.4* ± 0.7 for mid- and high dose groups, respectively, vs. 14.4 ± 0.3 in controls). In high dose group, pups' weights were significantly decreased (1.49* ± 0.02 vs. 1.59 ± 0.02 in controls. Dominant lethal potential: Pregnancy rates in high-dose males mated with naive females did not differ between the groups. A slight but statistically significant increase in early resorptions were seen in the high dose group (1.34* ± 0.13 vs. 0.83 ± 0.17 in controls), resulting in increased total post implantation loss (1.52* ± 0.12 vs. 1.06 ± 0.19 in controls). Task 3 (cross mating trial): No difference among groups regarding number of offspring, however pup weight was significantly lower for pups from treated dams (adjusted live pup weight 1.46* g ± 0.04 in control + treated female vs. 1.66 ± 0.04 g in controls). Task 2/3 necropsy results (F0): a slight dose-related but statistically significant decrease in right testis weight were seen	Chapin et al. 1995

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		period, where high dose and control groups were mated with partners in the other dose groups (control males x control females, high-dose males x control females, and control males x high-dose females). Task 4. Fertility assessment. F1 mice dosed in task 2 until Day 74 were mated with partner from the same treatment group (nonsiblings). Fertility and reproductive endpoints were assessed.	in mid- and high dose groups (134.0 ± 5.4, 131.7 ± 5.1, 118.8* ± 4.9, 113.7* ± 4.2 mg, from controls to high dose, respectively). In addition, there was a statistically significant dose-related decrease in total number of spermatids/testis in mid- and high dose groups (13.6 ± 0.5, 13.7 ± 0.9, 11.1* ± 0.6, 9.1*± 0.7). MBA did not alter estrous cycle length or progression. Testicular degeneration was seen in one control male and three high dose males in F0 animals. Task 4 (fertility assessment): No difference in percent of fertile pairs, pregnant females, or pregnant females that delivered litters. Fewer live pups were delivered in the high dose group (9.1* ± 1.0 vs. 13.6 ± 0.4 in controls) and adjusted pup weights were statistically significantly decreased in both mid- and high dose groups (1.53* ± 0.03 and 1.39* ± 0.04 g for mid- and high dose groups, respectively, vs. 1.67 ± 0.03 g in controls). Dam body weights were statistically significantly decreased in all treated groups (92, 91 and 84% of controls). Necropsy data Task 4: all treated F1 males weighed less than controls (mean terminal body weight 34.9* ± 0.7 g vs. 39.2 ± 1.0 g, in high dose group and controls, respectively). In males from the high dose group, right cauda epididymis weights were significantly decreased (12.4* ± 0.6 mg vs. 14.5 ± 0.4 mg in controls), and testis weights were decreased in males of mid- and high dose groups (102.3* ± 3.3 mg and 83.8* ± 3.8 mg in mid- and high dose groups, respectively, vs. 129.7 ± 3.0 mg in controls). A slight but statistically significant decrease in total spermatids per testis was seen in mid- and high dose groups, compared to controls (total spermatid heads x 10^6/testis: 12.5* ± 0.7 and 14.0 *± 0.5 in mid- and high dose groups, respectively, vs. 15.0 ± 0.4 in controls). Degeneration of testes were seen in two control mice, one at mid-dose and in two in high dose group. In females, right ovary weight was statistically significantly decreased in the high dose group (8.7* ± 0.8 mg vs. 13.0 ± 0.8 mg in controls, a dose	

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
			related trend). *Significant p<0.05	
Study on sperm count and morphology, and testicular histopathology in mice. Not GLP.	MBA (of "specific reagent grade")	Male ddY strain mice dosed orally once at 0, 50, 100 or 200 mg/kg bw. Sperm count and morphology were evaluated in caput epididymis 3 to 75 days after treatment. Testicular histopathological changes were studied 45 days post-treatment.	Statistically significant testicular weight loss was seen at 25- and 30-days post-treatment at 200 mg/kg bw. Weights recovered to control levels within 45 days. At 100 mg/kg MBA, testicular weight reached a minimum at 30 and 35 days, although not statistically significant compared to control. At 50 mg/kg bw, a slight decrease in testicular weight and testis to body weight ratio was seen at 35 days. Sperm count and morphology were dose-dependently affected. Exposure to 200 mg/kg MBA caused a statistically significant decrease in sperm count 30-60 days post-treatment. A statistically significant increase in abnormal sperm occurred from 7 to 45 days. High abnormality remained 75 days after treatment. Exposure to 100 mg/kg bw dose caused sperm count decrease at 30 and 35 days, and a significant increase in sperm abnormality was seen at 30 days. After treatment with 50 mg/kg, no significant decrease in sperm count was seen, but a high rate of sperm abnormality appeared 30-35 days after treatment. After exposure to 200 mg/kg MBA sperm abnormality appeared in diphase, which corresponded with early histopathological changes in testis 1-3 days after treatment (including reduction in number of resting, leptotene, and zygotene spermatocytes, and a degeneration of nuclei of round spermatids in stages I-III) . The marked loss of spermatids in maturation phase at 30 days coincided with the decrease in sperm count in the caput epididymis seen 35 days post-treatment with 100 and 200 mg/kg bw of MBA.	Sakamoto and Hashimoto 1988
A modified reproductive assessment by continuous breeding (RACB) study. GLP.	MBA (purity 98%) dissolved in water	CD-1 Swiss mice were given 0, 3, 10 and 30 mg/kg bw orally on GD 6 through 17.	Maternal body weights were statistically significantly decreased at GD17 at 10 and 30 mg/kg bw (by 9%). Gravid uterine weights of the high dose group were statistically significantly lower than controls (by 18%). Average fetal body weight per litter was statistically significantly reduced (by 25%) in the high dose group compared	George et al. 1998

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			to controls. The percentage of live fetuses with variations per litter was statistically significantly increased in mid- and high doses (57 and 78%, respectively, compared to 27% in controls). This was due to an increase in the incidence of extra ribs on the first lumbar vertebra. MBA did not increase the incidence of fetuses per litter with malformations.	
Sex-linked recessive lethal test in Drosophila. OECD TG 477 (TG deleted in 2014). GLP not specified.	MBA (purity >99%) dissolved in water.	Male drosophila melanogaster was exposed to 0 or 600 ppm. Exposure duration: 72 h	MBA exposure caused 26% mortality and 34% sterility. Percent lethals was 49 (vs. 14 in controls) and the substance was considered mutagenic in the SLRL test. Reciprocal translocation experiment was negative for MBA.	Foureman et al. 1994 (NTP study)

Table 12: Summary table of human data relevant for germ cell mutagenicity

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
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10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

There are two mutagenicity tests in bacteria available on MBA, one of which showed positive results and one study was negative. In the positive study, the mutagenic effect appeared to require metabolic activation and the response increased dose-dependently (see study details in Annex I). No information on cytotoxicity is available.

An in vivo study (OECD TG 474, by NTP 1988) in mice via i.p. administration demonstrated micronuclei formation in bone marrow, statistically significant at 25, 50 and 100 mg/kg bw MBA, which indicates cytogenetic damage of the substance. Hansen et al. (2014) used an in vivo comet assay to study DNA damage by MBA in testicles from mice administered twice at 24 hours apart with oral doses of 50, 100 and 190 mg/kg bw. The test showed statistically significant increased amount of DNA damage in animals treated at the highest dose (190 mg/kg bw) compared to controls. The result demonstrates that MBA given by oral route can reach the gonads and induce DNA damage in testicular cells.

In the study by Rutledge et al.1990, MBA caused dominant lethal effects (i.e., post-implantation loss, expressed in the study as periimplantation embryonic death) at 225 mg/kg bw in male mice. Only one dose, given by i.p. injection, was tested in the study. The effect was statistically significant at matings 0.5 - 3.5 days post-treatment, compared to controls, indicating genetic damage in sperm during the later stages of spermatogenesis. A reduced number of pregnant females and of living embryos was seen at matings at 36.5 - 43.5 days post-treatment, possibly an effect caused by cytotoxicity in differentiating spermatogonia. In

addition, the study showed that MBA exposure caused an increased incidence of semisterile offspring, and heritable reciprocal translocations were demonstrated in these animals.
Interestingly, the effects observed for matings during days 0.5-3.5 and 36.5-43.5 post-treatment with MBA at 225 mg/kg bw (Rutledge et al. 1990) coincided in time after treatment with effects seen on sperm count and morphology in the study of Sakamoto and Hashimoto (1988). Sperm effects (count and morphology) and testicular histopathology in mice was studied after exposure to a single oral dose of MBA at 50, 100, and 200 mg/kg. Both sperm count and morphology were dose-dependently affected. Testicular histopathological changes showed that resting spermatocytes were either absent or reduced 1-3 days after treatment with 200 mg/kg MBA. At 35 days post-treatment with 100 and 200 mg/kg a decrease in the number of sperm was seen in the caput epididymis.

The continuous breeding study by Chapin et al (1995) showed that MBA had an effect on male fertility (reductions in reproductive organ weights and in sperm indices) and caused dominant lethal reproductive toxicity (a slight increased number of early resorptions and increased post-implantation loss). Developmental toxicity included decreased number of live pups and reductions in pup weight in the absence of neurotoxicity or other marked general toxicity of the parental generation. The highest dose used in the experiment was relatively low at approximately 9-16 mg/kg bw per day.

A few other supporting studies are also available, e.g., Foureman et al. (1994), which showed that MBA is able to induce sex-linked recessive lethal mutations in post meiotic and meiotic germ cells in male Drosophila melanogaster. George et al. (1998) investigated the developmental toxicity of MBA in mice. No teratogenic effects were seen, however slight perturbation of the developmental process, as indicated by reduced fetal weight and increased incidence of fetuses with anatomical variations at mid- and high doses (10 and 30 mg/kg bw/d, respectively).

Taken together, the available evidence clearly shows that MBA can reach the germ cells and interact with DNA in the germ cells and cause genetic damage. The mutagenic effects are demonstrated to be passed on to the offspring. In vitro results support a mutagenic mechanism, whereas several in vivo studies indicate a clastogenic effect by MBA.

Comparison with acrylamide

MBA is structurally similar to acrylamide, as its structure is composed of two acrylamide moieties. Acrylamide has harmonised classifications including Muta. 1B, Carc. 1B and Repr. 2. The mutagenic property of acrylamide has been extensively studied.

Acrylamide is a reactive electrophilic compound that can react with nucleophilic targets, such as amino acids and proteins. Reactivity towards certain proteins is proposed to be the predominant mechanism of action underlying the neurotoxic property of acrylamide. Acrylamide appears to react poorly with DNA. Both in vivo and in vitro studies indicate that the mutagenic and carcinogenic effects of acrylamide are mediated via the formation of the more reactive epoxide glycidamide, which readily forms covalent adducts with DNA (especially N7-GA-Gua) (EFSA, 2015).

There are other hypothesized mechanisms for acrylamide-induced DNA damage, irrespective of glycidamide formation. At very high doses, there are indications of intracellular generation of reactive oxygen species resulting in oxidative DNA damage. In addition, a mechanism involving disruption of hormone levels or hormone signalling has been suggested to cause tumors in hormonally sensitive tissues (EFSA, 2015). No such data are available for MBA.

Studies indicate that, in general, acrylamide does not cause gene mutations in bacteria. *In vitro* studies on mammalian cells have shown that acrylamide appears to be a weak gene mutagen but an effective clastogen, whereas glycidamide is a strong gene mutagen *in vitro,* as well as a strong clastogen (EFSA, 2015). Based on results from one of the two bacterial gene mutation tests available for MBA, which was positive, the gene mutagenic profile appears to differ between acrylamide and MBA. Other data, however, point towards a clastogenic effect by MBA.

The genotoxic and mutagenic potential of acrylamide has been demonstrated by positive results in *in vivo* mammalian tests, such as the comet assay and the micronucleus test. In addition, acrylamide has been shown to induce heritable genetic damage in *in vivo* germ cell studies, such as the dominant lethal assay, specific locus mutation and heritable translocation assays. Specific locus mutations in offspring of male mice exposed to 50-125 mg/kg bw have been reported (reviewed in EFSA, 2015). Transmitted mutations appear in offspring of female mice mated with males 8-14 days and 15-21 days post-treatment (suggesting effects on spermatids and spermatozoa in late stage of spermatogenesis). Wildtype mice and CYP2E1-null mice exposed to 0-50 mg/kg acrylamide/bw on 5 consecutive days demonstrated the importance of CYP2E1 in the dominant lethality, as only wild-types showed dose-related increased in resorptions and decreased number of pregnant females (study reviewed in EFSA, 2015). Glycidamide, the reactive metabolite of acrylamide has also been tested in a rodent dominant lethal test (Generoso et al. 1996). Exposure to glycidamide (125 mg/kg) resulted in a marked reduction in the number of living implants and increases in the proportion of resorption moles in females mated during the interval 2.5-11.5 days post-treatment. The maximum dominant lethal response occurred during the 4.5-9.5 days post-treatment interval (corresponding to treated late spermatids and early spermatozoa). A heritable translocation test demonstrated a frequency of 20.18%, about two orders of magnitude higher than the spontaneous frequency for heritable translocations observed in that laboratory (Generoso et al.1996).

Acrylamide is listed as one of the positive controls to be used in the rodent dominant lethal test (at dose 50 mg/kg bw) (OECD TG 478). MBA appears to behave similar to acrylamide, as dominant lethal effects are induced at similar exposure levels, and the two substances both affect later stages of the spermatogenesis.

Long-term carcinogenicity studies of acrylamide in animals have demonstrated multiple-site carcinogenicity (NTP 2012, NTP 2014, EFSA review 2015). No information on carcinogenic effects by MBA is available.

10.8.2 Comparison with the CLP criteria

Classification in Muta. 1A is not appropriate as it should be based on human data and no human data are available.

Classification in Muta. 2 is not appropriate as the experimental evidence available clearly demonstrates a mutagenic effect of MBA in germ cells in *in vivo* heritable germ cell mutagenicity tests in mammals.

The criteria for classification in Muta. 1B for germ cell mutagenicity are considered fulfilled since two studies demonstrate positive results from in vivo heritable germ cell mutagenicity tests in mammals. In addition, WoE supporting evidence of the mutagenic effect is available, including increased micronuclei in bone marrow after i.p. injection in vivo and positive in vivo comets in testicular cells after oral exposure. The available data provide clear evidence that the substance reach the germ cells and interact with germ cell DNA. The mutagenic effect of MBA can be passed on to the progeny. Classification of MBA as Muta. 1B, H340 is warranted.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Classification of MBA for germ cell mutagenicity in category 1B is warranted: Muta. 1B H340.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The substance *N,N*'-methylenediacrylamide (MBA) is used as a crosslinking agent and as a monomer for polymerisation. It is used by professional workers in the production of electrophoresis gels.

The scope of the current CLH report and ODD is focussed on germ cell mutagenicity.

The DS proposed Muta. 1B for MBA since:

- two studies with MBA demonstrated positive results from heritable germ cell mutagenicity tests in mammals (one study after intraperitoneal (i.p). exposure, the other after oral exposure);
- supporting evidence of the mutagenic effect of MBA is available from *in vivo* studies, including increased micronuclei in bone marrow after i.p. injection and positive comet assays in testicular cells after oral exposure;
- the available data provided clear evidence that MBA reaches the germ cells and interacts with germ cell DNA;
- the mutagenic effect of MBA can be passed on to the progeny.

The DS also considered MBA to be structurally similar to acrylamide, a chemical which has a harmonised classification as Muta. 1B and whose mutagenic effects are well known. For the assessment of germ cell mutagenicity the DS considers the information on MBA as sufficient for classification and the information on acrylamide is only used as supporting evidence.

Comments received during consultation

Comments were received from two MSCAs.

Both MSCAs supported Muta. 1B based on positive results from *in vivo* heritable germ cell mutagenicity tests in mammals, supporting positive results seen in other genotoxicity studies and the similarity with acrylamide.

In response to a comment from one MSCA concerning the summary of one of the *in vitro* studies (Hashimoto and Tanii, 1985), the DS confirmed that considering the context, the text should be read as "No cytotoxicity" instead of "Cytotoxicity was not specified".

Assessment and comparison with the classification criteria

The following table provides a summary of the available data on MBA for the endpoint germ cell mutagenicity:

Study	Reference	Result	Remarks
In vitro			
Bacterial reverse mutation assay *S. typhimurium* TA 1535, 1537, 1538, 98 and 100 0, 5, 50, 500, 1000 and 5000 µg/plate MBA purity >95%, in water	Hashimoto and Tanii, 1985	Negative (+/- S9)	Details on experimental design not provided. *S. typhimurium* TA 102 or *E. coli* WP2 uvrA not tested. No cytotoxicity.

Bacterial reverse mutation assay *S. typhimurium* TA 1535, 97, 98 and 100 0, 100, 333, 1000, 3333, 10000 µg/plate MBA purity not known, in DMSO	Zeiger *et al*., 1988 (NTP)	Positive in TA1535 (+S9) and TA100 (+S9). Negative in TA1535 (-S9), TA100 (-S9), TA97 (+/-S9) and TA98 (+/-S9).	*S. typhimurium* TA 102 or *E. coli* WP2 uvrA not tested. Cytotoxicity was not specified.
In vivo			
Dominant lethal and heritable reciprocal translocation assay, mouse, i.p. 0 and 225 mg/kg bw, single exposure 0 and 90 mg/kg bw, 5 consecutive days MBA purity 99.9%	Rutledge *et al*., 1990 (NTP)	Positive for dominant lethal effects and heritable reciprocal translocations	Information on general toxicity of parental generation not reported.
Micronucleus assay, mouse, bone marrow (OECD TG 474), i.p. 0, 25, 50 and 100 mg/kg bw, 2 consecutive days MBA purity not specified	NTP, 1988	Positive Statistically significant increased number of MN in PCE at all dose levels	
Comet assay, mouse, testis, oral (gavage) 0, 50, 100 and 190 mg/kg bw, 2 consecutive days MBA purity not specified	Hansen *et al*., 2014	Positive Statistically significant increased mean % tail DNA in high dose group	Animals were observed during study period twice daily for abnormalities in clinical appearance. Information on adverse effects not reported.
Additional			
Reproductive Assessment by Continuous Breeding (RACB) study, mouse, oral (drinking water) 0, 1.6, 4.7 and 9.3 mg/kg bw/d MBA purity 97-99%	Chapin *et al*., 1995 (NTP)	Slightly, statistically significant, increased number of early resorptions and increased post-implantation loss noticed in high dose group	This study included a dominant lethal segment with pre-mating exposure of the male animals (control and high dose only). Only the results of the dominant lethal segment are reported in this table.
Sperm count, morphology and	Sakamato and	Dose-dependent effect on sperm count and	

testicular histopathology; mouse; oral 0, 50, 100 and 200 mg/kg bw, single exposure MBA of "specific reagent grade"	Hashimoto, 1988	histopathology	
Sex-linked recessive lethal test, Drosophila melanogaster (OECD TG 477) 0 and 600 ppm MBA purity >99%	Foureman *et al*., 1994	Positive for sex-linked recessive lethal mutations. Negative for reciprocal translocations.	

Since classification in category 1A requires human evidence, this category is not applicable in this case as no human data are available for MBA. Classification in category 2 is also not appropriate as the experimental results clearly point towards a mutagenic effect of MBA in the germ cells as pointed out below.

One *in vivo* heritable germ cell mutagenicity test in mammals is presented in the dossier and provides clear positive results. The dominant lethal assay of Rutledge *et al*. (1990), involved four experiments, three focussing on dominant lethal effects and one on heritable translocation effects. In the first experiment, focusing on the effect of MBA at various stages of spermatogenesis, the number of implantations/pregnant females and the number of live embryos per pregnant females was statistically significantly reduced compared to controls, and the percent dead implants was subsequently increased after single i.p. pre-mating treatment (225 mg/kg bw) of male mice with MBA. The dominant lethal effects were observed during the first 4 days post-treatment. Also, a reduced number of pregnant females and of living embryos were observed in females mated 36.5-43.5 days post-treatment to exposed males, which the study authors considered an effect caused by cytotoxicity in differentiating spermatogonia. The occurrence of dominant lethal effects as observed during the first 4 days post-treatment was verified in a second experiment using single exposure (225 mg/kg bw) with matings during 1-9 days post-treatment and in a third experiment after 5× repeated i.p. treatment (90 mg/kg bw per day) with matings during 1-12 days post-treatment and using two different strains of untreated female animals. The results of these second and third experiments confirmed the findings of the first experiment with dominant lethal effects during 0.5-3.5 days post-treatment. The fourth experiment of Rutledge *et al*. (1990) was a heritable translocation assay upon 5× repeated i.p. treatment. The results of this experiment demonstrated an increased incidence of male offspring with reduced fertility and heritable reciprocal translocations in these animals after treatment of the male parental generation with MBA.

The DS indicated that the effects observed from matings during the first four days and during days 36.5-43.5 post-treatment in the dominant lethal study of Rutledge *et al*. (1990) matched in time with effects seen in sperm count and sperm morphology in the mouse study of Sakamoto and Hashimoto (1988). After single oral treatment with MBA, resting spermatocytes were either absent or reduced 1-3 days post-treatment, whereas

at 35 days post-treatment a decrease in the number of sperm was observed in the caput epididymis (Sakamoto and Hashimoto, 1988).

Overall, the results of the dominant lethal study of Rutledge *et al*. (1990) provide clear evidence of an effect of MBA on the germ cells. This is supported by the Reproductive Assessment by Continuous Breeding (RACB) study of Chapin *et al*. (1995) which included a dominant lethal segment. In this specific part of the study, dominant lethal reproductive effects were observed, i.e. a slightly increased number of early resorptions resulting in an increased post-implantation loss, after oral (drinking water) pre-mating treatment of males with MBA.

Further support is provided by positive results from *in vivo* somatic cell mutagenicity and genotoxicity testing. An *in vivo* mouse micronucleus test with i.p. administration of MBA demonstrated formation of micronuclei in the bone marrow (NTP, 1988). RAC notes that the i.p. administration, as applied in this *in vivo* mouse micronucleus study as well as in the dominant lethal study of Rutledge *et al*. (1990), is not a regular route of human exposure. However, the *in vivo* comet assay by Hansen *et al*. (2014) demonstrated DNA damage in testicular cells after oral (gavage) treatment of mice, indicating that MBA can also reach the gonads upon oral exposure. RAC further notes that in the above-described dominant lethal segment of the RACB study of Chapin *et al*. (1995), treatment with MBA was via the oral route.

Finally, two bacterial mutagenicity tests were presented in the dossier of which the study of Hashimoto and Tanii (1985) showed negative results with and without metabolic activation, while in the study of Zeiger *et al*. (1988), MBA was found to be mutagenic with metabolic activation in two (TA100 and TA1535) out of four included strains. MBA was further shown to be mutagenic in a sex-linked recessive lethal test in Drosophila melanogaster, though negative for reciprocal translocation in this test (Foureman *et al*., 1994). RAC notes the structural similarity with acrylamide, a known mutagenic chemical, and considers that the data on acrylamide gives additional support for the proposed classification.

Overall, these data provide clear evidence of germ cell mutagenicity. Therefore, for *N,N'*-methylenediacrylamide (MBA), RAC concurs with the DS that **Muta. 1B (H340) is warranted**.

10.9 Carcinogenicity

Not evaluated in this CLH proposal.

10.10 Reproductive toxicity

Not evaluated in this CLH proposal.

10.11 Specific target organ toxicity-single exposure

Not evaluated in this CLH proposal.

10.12 Specific target organ toxicity-repeated exposure

Not evaluated in this CLH proposal.

10.13 Aspiration hazard

Not evaluated in this CLH proposal.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Not evaluated in this CLH proposal.

12 EVALUATION OF ADDITIONAL HAZARDS

Not evaluated in this CLH proposal.

13 ADDITIONAL LABELLING

Not relevant.

14 REFERENCES

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15 ANNEXES

Annex I to the CLH report