Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

dinitrogen oxide

EC Number: 233-032-0
CAS Number: 10024-97-2

CLH-O-0000007281-79-01/F

Adopted

16 March 2023

P.O. Box 400, FI-00121 Helsinki, Finland | Tel. +358 9 686180 | Fax +358 9 68618210 | echa.europa.eu

16 March 2023

CLH-O-0000007281-79-01/F

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

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

Chemical name: **dinitrogen oxide**

EC Number: **233-032-0**

CAS Number: **10024-97-2**

The proposal was submitted by **France** and received by RAC on **25 April 2022.**

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

PROCESS FOR ADOPTION OF THE OPINION

France has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **16 May 2022**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **15 July 2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Betty Hakkert**

Co-Rapporteur, appointed by RAC: **Gordana Mendaš Starčević**

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

The RAC opinion on the proposed harmonised classification and labelling was adopted on **16 March 2023** by **consensus**.

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

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT SE 3 STOT RE 1 Ozone 1	H360Df H336 H372 (nervous system) H420	GHS08 GHS07 Dgr	H360Df H336 H372 (nervous system) H420			
RAC opinion	TBD	dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT SE 3 STOT RE 1 Ozone 1	H360Df H336 H372 (nervous system) H420	GHS08 GHS07 Dgr	H360Df H336 H372 (nervous system) H420			
Resulting Annex VI entry if agreed by COM	TBD	dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT SE 3 STOT RE 1 Ozone 1	H360Df H336 H372 (nervous system) H420	GHS08 GHS07 Dgr	H360Df H336 H372 (nervous system) H420			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

The scope of the CLH dossier and current RAC opinion is focussed on the hazard classes STOT SE, STOT RE, reproductive toxicity and hazardous to the ozone layer.

Use and application

Dinitrogen oxide (N_2O) has many uses and applications:

- N_2O is used in surgery as an adjuvant in inhalational anaesthesia, for pain relief during delivery, or for short analgesia during minor medical procedures (e.g., dentistry). The dossier submitter (DS) provided an indication for the concentration used for anaesthesia (50% N_2O and 50% O_2).
- N_2O is used as a food additive (E942).
- N_2O is an in-canister propellant used in many preparations and uses (e.g., aerate whipping cream, inflate balloons).

"Recreational" misuse of the gas has been identified as strongly increasing in recent years.

RAC notes that it has the task to evaluate the information presented to it for the hazard classes included in the CLH dossier and which were open for consultation; this may include human data from clinical case studies. However, this evaluation is independent of the uses of the substance.

Toxicokinetics and proposed mechanism of action

N_2O is rapidly absorbed through inhalation. The alveolar concentration is similar to the inhaled concentration within 5 minutes and the blood/gas partition coefficient is 0.47 (ANSM, 2014). N_2O is rapidly distributed throughout the body in dissolved form, especially to richly vascularised tissues. It easily penetrates into the brain and is able to cross the placental barrier (INRS, 2018). N_2O is poorly metabolised (0.004%). However, N_2O is reduced to nitrogen in the reaction with the cobalt (Co^{2+}) of vitamin B12 (MAK, 1993). N_2O is mostly eliminated unchanged through the lungs within a few minutes, and only small amounts are eliminated via the urine.

As indicated by the DS, N_2O probably acts by directly inhibiting vitamin B12 formation and inactivating methionine synthase through oxidation of the Co^{2+}. Methionine synthase inhibition leads to the impairment of the generation of methyl groups for DNA methylation. This results in the decrease in the synthesis of deoxythymidine, thymidine and DNA among other products. Stewart *et al.* (2019) highlighted that it is still unknown whether deficiency of methyl substituents, necessary for synthesis of myelin, DNA, other essential reactions, or accumulation of homocysteine to toxic levels, accounts for the pathophysiology alone or in concert. The specific impacts of the proposed mechanism of action are further discussed under the relevant hazard classes in question. However, it is noted that the CLP Regulation does not require a mode of action to be conclusively demonstrated for classification in these hazard classes.

Test concentrations in animal studies

For the human health endpoints, the DS presented animal data derived from inhalation studies using whole body exposure, in addition to human data. Regarding the level of applied test concentration in these animal studies, RAC notes the following:

- OECD test guidelines, such as TG 412/413 (section 13) state that '*The maximum concentration tested should consider: 1) the maximum attainable concentration, 2) the need to maintain an adequate oxygen supply, and/or 3) animal welfare considerations. In the absence of data-based limits, the acute limits of the United Nations Globally*

Harmonized System of Classification and Labelling of Chemicals [GHS] may be used (i.e., up to a maximum concentration of 5 mg/L for aerosols, 20 mg/L for vapours, and 20000 ppm for gases). Justification should be provided if it is necessary to exceed these limits when testing gases or highly volatile test chemicals (e.g. refrigerants).'

- OECD TG 414 (section 17) states that '*Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test chemical often may indicate the maximum attainable level of exposure (for example, dermal application should not cause severe localised toxicity).'*
- Specific information on reproductive toxicity testing is also stated in the CLP Regulation (EC) 1272/2008 Annex I (part 3: Health Hazards, chapter 3.7: Reproductive toxicity, section 3.7.2.5.7) as '*There is general agreement about the concept of a limit dose, above which the production of an adverse effect is considered to be outside the criteria which lead to classification, but not regarding the inclusion within the criteria of a specific dose as a limit dose. However, some guidelines for test methods, specify a limit dose, others qualify the limit dose with a statement that higher doses may be necessary if anticipated human exposure is sufficiently high that an adequate margin of exposure is not achieved. Also, due to species differences in toxicokinetics, establishing a specific limit dose may not be adequate for situations where humans are more sensitive than the animal model.'*

RAC has taken note of the various exposure concentrations applied in the available animal studies. These can reach concentrations far above the indicated GHS level of 20000 ppm. In line with the OECD TGs/GD (guidance document) and CLP Regulation (Annex I, 3.7.2.5.7), the adverse effects observed at high test concentrations (i.e., above 20000 ppm) are considered relevant for classification by RAC because the anticipated human exposure is high, as indicated by the use as an anaesthetic and foreseeable recreational use. When following the 20000 ppm guidance value, an adequate margin of exposure is not likely to be reached. However, RAC further notes that OECD GD 39 (section 61) states that '*An oxygen concentration of at least 19%, a carbon dioxide concentration not exceeding 1%, and an evenly distributed exposure atmosphere should be ensured*[1]*.'*, leaving a maximum of 80% (800000 ppm) for the test item.

HUMAN HEALTH HAZARD EVALUATION

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

Summary of the Dossier Submitter's proposal

The DS proposed to classify N_2O as STOT SE 3 (H336).

The DS noted the following when concluding on the classification:

- In several studies in rats (Jevtovic-Todorovic *et al.,* 2000; 2001; 2003; 2005), significant and severe changes in the nervous system were observed >300000 ppm.
- In mice significant behavioural changes were observed >250000 ppm
- In a human volunteer study, Bruce and Bach (1976) reported a slight impairment of audio-visual capacity at 50 ppm, and lower performance in visual acuity, audio-visual capacity,

[1] OECD GD 39 (section 60) contains a similar statement for nose only exposure: '*Each exposure port should have similar exposure conditions with an oxygen concentration of at least 19% and a carbon dioxide concentration not exceeding 1%*.'

immediate memory and vigilance response following 4h exposure to N_2O at 500 ppm. However, the volunteers may not have been representative for the general population, as indicated by a letter published in 1991 by the same authors. Several other acute studies which were performed at higher concentrations reported effects on the nervous system. A LOAEC of 50-500 ppm can be identified for these effects.

Although effects in some studies were considered by the DS to be sufficiently severe to fulfil the criteria for STOT SE 1 or 2, no experimental studies were found at concentration levels and exposure duration that would fulfil the criteria for classification for STOT SE (Cat 2. C ≤20000 ppmV/4h).

Based on the effects observed on cognitive function (e.g., lower performance in audio-visual capacity, memory and vigilance, reaction time) in human volunteer studies using controlled exposure levels, classification of the substance in category 3 (narcotic effects) for STOT SE was considered relevant by the DS. The effects are expected to be reversible. Although not assessed, it was reported by Fagan *et al.* (1994) that dizziness, paraesthesia and euphoria observed in volunteers exposed to N_2O were rapidly reversible. In addition, reversible ataxia was also noted in animals (Singh *et al.,* 2015, detailed in STOT RE section), supporting a classification as STOT SE 3 for narcotic effects.

Comments received during consultation

Two Member State Competent Authorities (MSCAs) commented and agreed with the proposed classification.

Six industry representatives disagreed with the proposal based on several arguments which questioned the quality and validity of the data, including:

- lack of substance characterisation
- inadequate methodology and acceptance/evaluation criteria
- limited reporting of methodology
- data search only in a single database (PubMed); however, RAC notes that secondary literature and the REACH database were consulted as well

The industry representatives all considered that the reliability of the human volunteer studies cannot be assessed when considering their limitations.

Assessment and comparison with the classification criteria

The DS discussed eight non-guideline neurotoxicity animal studies with exposure via inhalation, five in rats and three in mice. All of these had limitations which included lack of information on purity, substance origin, GLP status, housing conditions and sometimes animal age, exposure duration, analytical control and information on general toxicity. RAC notes that no information was provided on the exposure method (whole body or nose only).

Three studies by the same author (Jevtovic-Todorovic *et al*., 2000; 2001; 2003; 2005) exposed rats to concentrations above 800000 ppm N_2O under hyperbaric conditions (higher pressure allows higher concentrations). The artificial nature of these studies and unclear oxygen levels makes it difficult to assess the relevance of the toxic effect of N_2O for humans. The carbon dioxide levels may also have exceeded the recommended 1% in these studies (1-3%). The effects reported in the studies included (reversible) neuron vacuolation (>500000 ppm) and neuronal cell death at very high concentrations (>1000000 ppm). Overall, the results of these studies by

Jevtovic-Todorovic *et al.* are only used as supportive evidence for classification, as they add information to the general picture of neuronal effects caused by N_2O.

A summary of the animal studies relevant for classification is presented in the table below.

***Table**. Summary of the relevant animal studies for STOT SE*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
Rats			
Non-guideline neurotoxicity study Klimisch score: 2 Courtière *et al.,* 1997	Male Long-Evans rats N=10/group	Inhalation, single continuous exposure 300000; 400000; 500000; 600000; 700000 ppm N_2O	No information on general toxicity ≥300000 ppm: Dose-related dec. performance (stat. sign.) in visual vigilance performance task ≥400000 ppm: Dose-related dec. (stat. sign.) in locomotor activity
Non-guideline neurotoxicity study Klimisch score: 2 Dzoljic *et al.,* 1994	Male rats (strain not specified) N=8-10/group	Single continuous exposure (24h) 700000 ppm mixed in O_2	Transient dec. in visual evoked potential amplitude. Dec. in nocturnal locomotion. Tolerance observed during the following light-dark cycle.
Mice			
Non-guideline neurotoxicity study Klimisch score: 3 Li *et al.,* 2001	Male NIH Swiss mice N=12-15/group	0; 250000; 500000; 750000 ppm N_2O mixed in O_2 Unknown exposure duration	No data on general toxicity. At 500000 ppm: Inc. (stat. sign.) in the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner.
Non-guideline neurotoxicity study Klimisch score: 2 Caton *et al.,* 1994	Male Swiss-Webster mice N=15-20/group	35-60 min single exposure 500000 ppm N_2O	No data on general toxicity. Inc. behavioural anxiolytic effects.
Non-guideline neurotoxicity study Klimisch score: 3 Dorris *et al.,* 1993	Male mice, strain not specified	1h single exposure 500000 ppm N_2O	No data on general toxicity. Inc. locomotor activity.

Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant

In rats, neurological effects observed included:

- ≥300000 ppm (exposure duration not reported): Dose-related decrease in locomotor activity, alteration of visual detection task (Courtière *et al.,* 1997).
- 700000 ppm: Transient decrease in visual evoked potential amplitude. Decrease in nocturnal locomotion. Effect on tolerance observed during the following light-dark cycle (Dzoljic *et al.,* 1994).
- Supportive: >500000 ppm: reversible neuron vacuolation (studies by Jevtovic-Todorovic *et al.,* 2000; 2001; 2003; 2005) conducted under hyperbaric conditions.

In mice, neurological effects observed included:

- 500000 ppm: Increased behavioural anxiolytic effects (Caton *et al.,* 1994), increased locomotor activity (Dorris *et al.,* 1993, but disregarded by the DS because of poor reporting/many limitations).
- >500000 ppm (no information on exposure duration): Increased time spent in the light compartment and in the number of intercompartmental transitions. (Li *et al.*, 2001).

In addition to the animal studies, nine studies with human volunteers were presented by the DS. These studies had limitations including a limited number of volunteers per study and non-standardised study designs. However, RAC notes that such limitations are usually present with human volunteer studies and the number of participants in some of the studies and the total number of human volunteers cannot be considered limited.

Table: *Summary of the human volunteer studies for STOT SE*

Type of study/report	**Test substance, Route of exposure, relevant information about the study**	**Observations**	**Reference**
Human volunteer study	5 ♂ + 6 ♀ Chamber (nasal mask) 5 sessions (~190 min): air (control session); 100000; 200000; 300000; 400000 ppm N_2O	Significant impairment on auditory reaction time and eye-hand coordination. No acute tolerance to N_2O LOAEC=300000 ppm Rapid recovery (5 min)	Yajnik *et al.,* 1996
Human volunteer study	8 ♂ + 4 ♀ Chamber (nasal mask) 5 sessions (~ 60 min): air; 50000; 100000; 200000; 400000 ppm N_2O	Significant differences: impairment of reaction time and attention LOAEC=100000 ppm Rapid recovery (few minutes)	Fagan *et al.,* 1994
Human volunteer study	15 ♂ Chamber (nasal mask) 4 sessions (duration not specified): air (training session & control session); 200000; 400000 ppm N_2O	Impairment on psychomotor tests: - Symbol digit - Finger tapping - Test response latency LOAEC=200000 ppm	Mahoney *et al.,* 1988
Human volunteer study	6 (sex not specified) Chamber (nasal mask) 4 sessions (10 + 20 min): air; 100000; 200000; 400000 ppm N_2O in O_2	Impairment on psychomotor tests: - Continuous performance test - Finger tapping LOAEC=100000 ppm	Estrin *et al.,* 1988
Human volunteer study	24 ♂ Chamber (nasal mask) 4h exposure: placebo, 50 ppm N_2O	Psychomotor performance: No effect Mood: effects observed but no statistical difference NOAEC=50 ppm	Venables *et al.,* 1983
Human volunteer study	20 ♂/group Chamber (nasal mask) 4h exposure, twice:	Impairment on psychomotor tests: - Memory - Visual acuity - Audio-visual capacity	Bruce and Bach, 1976

	- 25 ppm N_2O + 0.5 ppm of halothane, - 50 ppm of N_2O, - 50 ppm + 1 ppm halothane, - 500 ppm N_2O, - 500 ppm N_2O + 10 ppm halothane	NOAEC=50 ppm N_2O	
Human volunteer study	30 ♂ Chamber 4h exposure: air, 500 ppm N_2O	Impairment on psychomotor test: - digit-span test LOAEC=500 ppm	Bruce and Bach, 1975
Human volunteer study	5 ♀ + 3 ♂ Facial mask 2 sessions (15-min exposure) air (control session), 200000 ppm N_2O + 30% O_2 + room air	Significant activation in the anterior cingulate cortex and deactivation in the posterior cingulate hippocampus LOAEC=200000 ppm	Gyulai *et al.,* 1996
Human volunteer study	N=15 (sex not specified) Facial mask, 15-min exposure: 100000; 300000; 500000 ppm N_2O mixed in O_2	Significant decrease in cerebral function analysing monitor LOAEC=300000 ppm	William *et al.,* 1984

In summary, the neurological effects observed in nine studies with human volunteers included:

- 500 ppm: small reversible changes in audio visual acuity/capacity, digit span, memory and vigilance response (Bruce and Bach, 1974; 1975; 1976). Studies may have been affected with sampling bias of sensitive subjects as noted in a letter by the authors a few years after publication.
- ≥100000 ppm for 1h: impairment of reaction time and attention (Fagan *et al.,* 1994).
- ≥100000 ppm, several sessions, exposure duration not specified: impairment on psychomotor tests including test response latency, finger tapping (Mahoney *et al.,* 1988, Estrin *et al.,* 1988).
- 200000 ppm: significant activation in the anterior cingulate cortex and deactivation in the posterior cingulate hippocampus (Gyulai *et al.,* 1996).
- >300000 ppm for 190 min: significant impairment on auditory reaction time and eye-hand coordination. (Yajnik *et al.,* 1996). Rapid reversibility (<5min after exposure).
- ≥300000 ppm: significant decrease in cerebral function analysing monitor (William *et al.,* 1984).

RAC notes that N_2O is used for inhalational anaesthesia in human medicine, implying it has known sedative properties on the nervous system. In addition, similar effects on the nervous system have also been observed after repeated exposure (see section on STOT RE).

Applicability of STOT SE 1 or 2

The CLP criteria allow classification for STOT SE 1 or 2 based on animal studies if adverse non-lethal effects are observed at concentrations below 20000 ppm for 4h (CLP Regulation, Annex I, 3.8.1 and Table 3.8.2). In the available studies, animals were not exposed at concentrations below this level. Therefore, a classification for STOT SE 1 or 2 is not warranted based on the information from animal studies.

There are no guidance values (GVs) for classification for STOT SE 1 or 2 based on human studies.

According to the CLP Regulation, substances may be classified for STOT SE 1 based on human data *"if substances that have produced significant toxicity in humans after single exposure."* Further the CLP Regulation Annex I 3.8.2.1.7.1. states that *"Classification is*

supported by evidence associating single exposure to the substance with a consistent and identifiable toxic effect."

The neurological effects observed in human volunteers (and supported by findings in animals), i.e., mostly reductions in psychomotor activity, reaction times, attention and coordination, are signs of nervous system depression, which is considered as a consistent finding between studies. RAC considers these findings to be transient and reversible in nature, rather than "significant" and "adverse". The reversibility was fast in the human volunteer studies where this information was reported, which is supported by the fast toxicokinetic of N_2O after single exposure.

Overall, the effects after single exposure in animal studies occur only at concentrations above the guidance values and the neuronal effects in humans are considered transient and reversible in nature. The toxic effects cannot be considered "significant" or sufficiently adverse to warrant a classification as STOT SE 1 or 2. In conclusion, RAC agrees with the DS that a classification as STOT SE 1 or 2 is not warranted.

Applicability of STOT SE 3

The CLP criteria (Annex I, Section 3.8.2.2.2) indicate that for classification as STOT SE 3 is based on: `

(a) central nervous system depression including narcotic effects in humans such as drowsiness, narcosis, reduced alertness, loss of reflexes, lack of coordination, and vertigo are included. These effects can also be manifested as severe headache or nausea, and can lead to reduced judgment, dizziness, irritability, fatigue, impaired memory function, deficits in perception and coordination, reaction time, or sleepiness;

(b) *narcotic effects observed in animal studies may include lethargy, lack of coordination, loss of righting reflex and ataxia. If these are not transient in nature, they shall be considered to support classification for Category 1 or 2 specific target organ toxicity single exposure*.'

All the studies with human volunteers presented by the DS have limitations. Most notably, the study quality and reliability are difficult to assess for various reasons including non-standardised study designs and limited number of volunteers in the individual studies. This hampers the interpretation of the individual results. However, since many of these studies consistently indicate (narcotic) neurological effects, and that overall, the total number of human volunteers in these studies is rather high, it is unlikely an effect of chance or study design. In further support, the substance is used in human medicine as an anaesthetic and the observed effects are of similar nature, although less severe, as the reported effects from recreational use (presented with STOT RE). Therefore, RAC considers that the narcotic effects observed in the human volunteer studies are relevant for classification.

Narcotic effects following a single inhalation exposure were noted in human volunteers supported with similar findings in studies with rats and mice. These narcotic effects included impaired locomotor/psychomotor activity, reaction time, attention and coordination. They are considered the be transient in nature starting at concentrations of 100000 ppm in the presented human volunteer studies.

In conclusion, RAC agrees with the DS that **classification as STOT SE 3; H336 (May cause drowsiness or dizziness) is warranted**.

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

Summary of the Dossier Submitter's proposal

The DS proposed to classify N_2O as STOT RE 1; H372 (nervous system) based on neurological effects after repeated exposure in humans.

The DS considered the following arguments when concluding on the classification:

- In rats, severe adverse effects in the meninges, brain and the spinal cord were noted in several studies. Since only high concentrations of N_2O were tested, these effects are not relevant for classification as STOT RE 2.
- In mice, no histopathological findings in brain were noted up to 500000 ppm in Rice *et al.* (1985). Nevertheless, the behavioural changes observed in Fung *et al.* (1993) after 8-d exposure to 1000 ppm could support a classification of N_2O as STOT RE 2.
- In humans, there are numerous cases of reported subacute combined degeneration of the spinal cord. Although some of the cases were due to recreational use, severe effects were also seen under controlled conditions of N_2O exposure (e.g., pain management). The full reversibility of the effect is questionable and longer follow-up would be needed to conclude on this point with more certainty.
- Results from occupational exposure studies support that these neurobehavioral effects may also occur at low concentration levels.
- According to the ECHA guidance on the application of the CLP criteria (CLP guidance, 2017), "*where the same target organ toxicity of similar severity is observed after single and repeated exposure to a similar dose, it may be concluded that the toxicity is essentially an acute (i.e. single exposure) effect with no accumulation or exacerbation of the toxicity with repeated exposure. In such a case classification with STOT SE only would be appropriate*". Human recreational abuse cases support that repeated exposure to N_2O can lead to exacerbation of toxicity as shown by the severe effects such as myelopathy with potential irreversible consequences. The mode of action (irreversible inactivation of vitamin B12 through oxidation of the cobalt ion) proposed by the DS also supports that STOT RE may be more relevant than STOT SE as the accumulation of homocysteine levels and/or deficiency of methyl constituent will be responsible for the observed effects.

Based on significant adverse health effects in the nervous system of humans exposed to N_2O, classification of N_2O for the nervous system as primary target organ system is considered relevant. As the most severe effects on the nervous system are noted following repeated exposure in human, classification as STOT RE 1 was considered appropriate by the DS.

A specific concentration limit (SCL) was not proposed by the DS as N_2O did not induce target organ toxicity at a concentration level clearly below the guidance values according to the CLP Regulation.

Comments received during consultation

Two MSCAs agreed with the proposed classification.

Comments from six industry representatives argued against the proposed classification based on effect concentrations found in animals above the guidance values for STOT RE. Regarding the studies, the industry representatives considered there were several issues:

- All of the available animal data had limitations. These studies were not conducted according to GLP or any relevant test guideline.

- Many studies included a single concentration and as a result, no concentration-response relationship can be established.
- The number of animals per group were generally lower than recommended in test guidelines, thereby making a statistical analysis (if undertaken) difficult to interpret.
- Where adversity was observed this occurred at concentrations that exceeded the maximum recommended concentration for repeated dose inhalation studies, without consideration of hypoxic effects.
- Similar to the human volunteer studies under STOT SE, limitations hamper a reliability assessment for the human data. These limitations include no standardised studies, unclear exposure, potential co-exposure and other possible confounders.
- The effects observed in the case studies may have been confounded with factors such as co-exposure to other drugs. These confounders have not been discussed or considered.
- No reliability assessments were or can be undertaken for the human data.

Assessment and comparison with the classification criteria

Animal data

The DS discussed seven non-guideline inhalation neurotoxicity studies in animals, four in rats, two in mice and one with monkeys. All of these had limitations including lack of information on GLP status, unclear substance purity and origin and lack of information on general toxicity. The rats were exposed (whole body) for >400000 ppm and exposure duration varied from 1.5h/d to 8h/d from 7 days to six months. As the exposure levels were clearly above the GV for STOT RE, only the main findings are presented. These studies provide supportive information on possible effects after repeated exposure to N_2O.

At 500000 ppm (whole body, for 1.5 - 2h/d for 60 days), clinical signs (ataxia), structural changes in brain and spinal cord (vacuolation of neurons, demyelination) were observed as well as neurochemical effects (hyperhomocysteinemia, decrease in methionine synthase activity, decreased glutamate and vitamin B12 levels) (Singh *et al.,* 2015; Mistra *et al.,* 2020). Both Singh *et al.* (2015) and Mistra *et al.* (2020) reported behavioural changes, including reductions in locomotor activity and grip strength, following repeated high concentration exposure to N_2O. Dyck *et al.* (1980) found no changes after exposing rats to 700000 ppm for 4h/d, 5d/week for 6 months, but the investigation was limited to recording nerve conduction, electromyographic abnormalities in caudal nerve and morphometric and teased fibre abnormalities. Hayden *et al.* (1974) found cortical cell count changes in the brain at 400000 ppm after exposure 8h/d for 7-14 days. However, this information could only be obtained from an abstract and is therefore of limited value.

Mice were exposed to lower concentrations (i.e., below GV for STOT RE) by Fung *et al.* (1993), at 1000-2000 ppm (whole body) N_2O for 8h/d for 8 days. Reduced locomotor activity and neural cell counts were noted at the end of the period, but the changes were not statistically significant. Rice *et al.* (1985) exposed mice to 500000 ppm for 4h/d, 5 d/week for 14 weeks but did not find any statistically significant changes apart from lower body weight gain.

In monkeys, continuously exposed to 150000 ppm for 56 days, severe ataxia, peripheral nerve degeneration, spinal cord degeneration and demyelination was observed (Dinn *et al.,* 1980). However, only a single monkey/group was used in this study which means it has a low reliability.

Human data

The DS summarised numerous case studies and retrospective case series which reported on recreational use of N_2O. In addition, a poison centre analysis and three occupational exposure studies in the form of observational studies were included.

Poison control centre data

The French poison control centres analysed 66 cases, including 39 men and 27 women, recorded between 1 January 2017 and 31 December 2019. The median age was 21 years, ranging from 14 to 49 years, and 54.5% of the cases concerned people between 20 and 25 years of age. The type of N_2O consumed was almost exclusively N_2O contained in cartridges for food use, available over the counter and inhaled via balloons. The duration and history of consumption varied widely, ranging from occasional consumption to several times a day for months. The individuals reported taking quantities ranging from just a few cartridges to several hundred a day, and with great variety in the total quantities. Fifty-nine people reported adverse symptoms following inhalation of N_2O. Neurological and neuromuscular problems were the most common signs. Forty were mild, 14 were moderate and 5 were severe. For three of them, the symptoms were due to consumption with concurrent use of one or more psychoactive substances (alcohol with or without drugs) at a party. For one person suffering from cardiorespiratory arrest, heart disease was later discovered in the hospital. The two others experienced convulsive episodes, with one person falling into a coma and suffering myoclonus. The two other severe cases involved chronic consumption of N_2O – around ten cartridges a day for one person, and around forty a day for the other – at home and without taking any other psychoactive substances. Both had neurological symptoms. At least one neurological and neuromuscular symptom was reported in 42 cases (71.2%). Among them, 73.8% had at least one motor or sensory symptom such as paraesthesia, tremor in the extremities, or muscle pain. Four people reported symptoms suggestive of peripheral neuropathy following chronic use of N_2O. Half of the 42 cases suffered from at least one symptom such as headaches, dizziness or balance disorders.

Case report series

Oussalah *et al*. (2019) reviewed 100 cases published between 1966 and 2018 where individuals were exposed to at least one cartridge/month between 1966 and 2018. 76% had regular exposure. The three main diagnoses were subacute combined degeneration of the spinal cord (28%), myelopathy (26%) and generalised demyelinating polyneuropathy (23%). In patients that underwent Magnetic Resonance Imaging (MRI), changes in the spinal cord (T2 signal hyperintensity) were noted in 68% of the patients.

Neurological symptoms were reported in 96% of the patients, including paraesthesia in extremities (80%), walking impairment or unsteady gait (58%), weaknesses (43%), fallings or equilibrium disorders (24%), Lhermitte's signs (15%) and ataxia (12%).

Clinical chemistry analysis revealed that at least 72% of the patients had haematological abnormalities (e.g., low haemoglobin level). Vitamin B12 deficiency was noted in 71% of the patients and elevated methylmalonic acid and homocysteine levels in 90 and 94% of the patients, respectively.

The neurological disorders reported in other case reports were similar to the ones included in this systematic review study. Almost all the case reports presented by the DS were included in the systematic review by Oussalah *et al.* (2019) except some published papers not including data on biological parameters or where preventive treatment with vitamin B12 before N_2O exposure was used.

Several follow-up studies revealed persistent symptoms. The duration of follow-up was in most of the cases only a few months. Nevertheless, in Sleeman *et al.* (2016), full recovery was not observed after 2-year follow-up. However, this was based on a single case report of a 29-year-old woman. The reported rates for persistent numbness and accidental injury were 4.3% and 1.2%, respectively (Oussalah *et al.,* 2019). Garakani *et al.* (2016) also focussed on neurological sequelae and psychiatric disorders following N_2O abuse. In the 59 cases for which follow-up information was available, neurological symptoms improved in 46 cases and persisted in 3 cases.

Symptoms fully resolved in 10 cases. It is noted that there may be uncertainties surrounding the full stop of exposure in these follow-up studies. Overall, it seems most symptoms resolved after a while, but there are exceptions where some of the symptoms persisted.

Occupational exposure

Dreyfus *et al.* (2008) reported the cases of two anaesthetists who developed a chronic toxic encephalopathy (CTE) after many years of exposure to anaesthetic gases in operating rooms (where ventilation was deficient for three years). The authors reported high levels of anaesthetics gases such as N_2O (mean 311 ppm, peaks 1600 ppm) and halogenated gases (mean 16 ppm, peaks 1600 ppm). A direct relationship between N_2O exposure and CTE is uncertain in these cases, because of anaesthetists are also exposed to many other neurotoxic agents including halogenated anaesthetic gases.

Lucchini *et al.* (1996) conducted a study in an Italian hospital, examining 30 operating room workers. The workers were exposed to N_2O for 1 week with gaseous anaesthetics and given a personal sampling device for N_2O for 3 hours. On the last day of the gaseous anaesthesia week, mean N_2O air concentration was 54.2 (SD=22.8) ppm and mean urine N_2O concentration was 25.6 (SD=22.1) µg/L. The study showed a prolonged reaction time and increased serum prolactin levels in exposed workers only when they worked with gaseous anaesthesia. Notably, no information on potential co-exposure was provided and therefore the effects measured may not be solely caused by N_2O.

Lucchini *et al.* (1997) also conducted a multi-centre study in Italy with a group of 112 workers from 10 Italian hospitals who were exposed to anaesthetic gases (N_2O and isoflurane). For atmospheric N_2O, the geometric mean and 95^{th} percentile were 23.2 ppm and 127 ppm, respectively, on the 1^{st} day before the shift and 20.6 ppm and 114 ppm, respectively, on the last day of the shift; the corresponding values for isoflurane were 0.4 ppm and 3.8 ppm, and 0.3 ppm and 2.7 ppm. No statistical difference was observed between exposed and control subjects for neurobehavioral effects, stress and arousal levels.

Scapellato *et al.* (2008) investigated effects of N_2O exposure on behavioural functions in workers of an Italian hospital for one year. In subjects with urinary concentrations of N_2O below 27 µg/L, there was a linear decrease in reaction times in the colour word vigilance test results from Monday morning to Friday evening, indicating an effect on learning. In subjects with N_2O urinary concentrations above 27 µg/L, the mean reaction time results were essentially steady across a work week, indicating that learning performances may have been impaired. A similar effect was observed for 'arousal' in the mood scale measures suggesting a cumulative effect of anaesthetic gases over a week of exposure.

In summary, neurobehavioral changes could occur following repeated low exposure levels to N_2O. However, there was possible co-exposure with other anaesthetic gases. In addition, the cumulative effects during a working week were reversible following the weekend.

RAC notes that human data is often hampered by unclear exposure levels and potential confounders. In some but not all of the available studies, confounding factors were identified. Although the value of individual data is limited, the high number of case reports can be considered sufficient evidence to support effects after repeated exposure in a weight of evidence assessment.

Proposed mechanism of action

The neurological effects are proposed to be the result of the irreversible inactivation of methionine synthase function by oxidation of the Co^+ ion of vitamin B12. This results in the decrease in the synthesis of deoxythymidine and thymidine and DNA synthesis and myelin among other products. Irreversible inactivation of methionine synthase by N_2O is identical between species.

RAC recognises that studies in both animals and humans indicate that exposure to N_2O is associated with deficiency in vitamin B12. Vitamin B12 deficiency relates amongst others to neuro(developmental) effects. Therefore, it is plausible that the resulting neurological effects seen after N_2O exposure are at least partly a consequence of the vitamin B12 deficiency. It is also noted there is no clear evidence that the effects could be attributed to hypoxia in the presented studies. RAC notes that it not necessary to demonstrate a mode of action for classification.

Applicability of classification as STOT RE 1 or 2

According to the CLP Regulation, *'Substances are to be classified for target organ toxicity after repeated exposure in category 1 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.'*

Guidance values are not applicable for effects observed after repeated exposure in humans. According to the CLP Regulation, Annex I, section 3.9.2.7.3, the following effects shall be taken into consideration for classification (only relevant criteria cited):

'*(b) significant functional changes in the central or peripheral nervous systems or other organ systems, including signs of central nervous system depression and effects on special senses (e.g. sight, hearing and sense of smell);'*

-> After N_2O exposure, neurological effects are consistently observed in humans including subacute combined degeneration of the spinal cord, myelopathy and generalised demyelinating polyneuropathy. A significant number also suffered from changes in the spinal cord observed with MRI techniques.

Neurological symptoms included paraesthesia in extremities, locomotor activity impairment such as walking impairment or unsteady gait, weaknesses, fallings or equilibrium disorders, Lhermitte's signs and ataxia. In some cases, effects were irreversible or only partly reversible.

-> In support of these findings, changes (mostly suppression of) in locomotor activity were noted such as lower grip strength. Nerve and spinal cord degeneration were also observed in animals (rats and a monkey).

'*(c) any consistent and significant adverse change in clinical biochemistry, haematology, or urinalysis parameters;'*

-> The most notable and consistent effects in both humans and animals are vitamin B12 deficiency and increased homocysteine levels. Vitamin B12 deficiency can be related to neurological findings.

Such effects observed in humans can normally be considered sufficiently clear and adverse for classification. However, RAC has also considered some additional factors in their assessment.

In relation to whether effects arising from the recreational use of a substance should be considered for classification, RAC notes the provisions in CLP Article 9(5): '*When evaluating the available information for the purposes of classification, the manufacturers, importers and downstream users shall consider the forms or physical states in which the substance or mixture is placed on the market and in which it can reasonably be expected to be used'.*

Although there were some comments received during consultation of the CLH report indicating that non-intentional use should not be taken into account, Article 9(5) indicates that the *form* for its intended use must be considered in the evaluation for classification. Clearly the form of N_2O

as placed on the market is the same as in the provided studies and recreational cases. Therefore, the studies with this form of the substance, whether it is from recreational use or not, can be used to support classification.

Considering the limitations of the case reports, such as unknow exposure levels, evidence from a single case report would be considered insufficient to support classification. This is also mentioned in the CLP guidance: *'A single case report from deliberate exposure (i.e., abuse) is unlikely to provide sufficiently robust evidence to support classification without other evidence'*. In this case, the number of case reports is large. The French poison centres summarised over 66 cases and the reports series review by Oussalah *et al.* (2019) covered about 100 cases. RAC considers such a large number of case reports indicating similar, neurological effects to provide sufficiently strong evidence for classification of the intrinsic properties of N_2O.

Apart from the recreational use, there is no information on the exposure duration and the frequency. RAC notes the effects after recreational use/repeated exposure are more severe compared to a single high exposure (STOT SE). After a short/single medical or recreational inhalation exposure, there is a neurological effect that quickly (seconds to minutes) disappears after the end of the exposure (see the toxicokinetic section). These neurological effects might be more related to mechanisms like reversible receptor binding. However, the neurological effects after prolonged inhalation exposure do not reverse quickly after the end of exposure. It is a slower process in the range of days – months rather than minutes. This is likely more related to the decrease in vitamin B12 and its slow recovery and aligns with the proposed mechanism of action.

Overall, the neuronal effects observed in humans after repeated exposure are clear and sometimes irreversible. There is no indication that the severity of these effects could be obtained after a single high exposure in humans. Therefore, RAC considers it most appropriate to evaluate the findings relevant in the presented context, i.e., effects after prolonged and repeated exposure.

The available animal data indicate adverse biological and behavioural neuronal effects after repeated N_2O exposure at high concentrations, most notably in rats. These effects occurred only after exposure levels above the guidance values for classification as STOT RE in the CLP Regulation, Annex I, Table 3.9.2 and Table 3.9.3. The non-statistically significant reduced locomotor activity and neural cell counts observed in mice after exposure to low levels of N_2O (below the GV of 2500 ppm) is not considered sufficiently significant and severe for classification for STOT RE. Overall, the animal studies provide further support for the effects on the neuronal system observed in human case reports and occupational exposure data.

In summary, RAC considers that there is sufficient evidence from the large number of human case studies, reporting severe, sometimes irreversible neurological effects including subacute combined degeneration of the spinal cord and suppression of locomotor activity. These effects are supported with similar findings in animal studies. Therefore, RAC agrees with the DS and concludes that **classification as STOT RE 1; H372 (Causes damage to the nervous system through prolonged or repeated exposure) is warranted**.

Since effects were either above the guidance values or the exposure was not clear, no SCL can be derived.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The DS proposed classification as Repr. 1B (H360Df) for N_2O.

Regarding **adverse effects on sexual function and fertility**, the DS considered that:

- None of the animal studies were performed in compliance with OECD TG. All animal studies had limitations, e.g., single concentration level, low number of animals, few parameters investigated, and were either performed on male or female animals.
- Decreased fertility and oestrous cycle changes observed consistently in female rats in two studies (Kugel *et al.*, 1990: 1989) leads to a clear concern for female fertility.
- The effects observed on testis and spermatogenesis in a rat study (Kripke *et al*., 1976) and the decrease in litter size upon exposure of male animals in another rat study (Vieira *et al.*, 1983a) support concern for potential male fertility effects.
- Adverse effects were only observed in rats and not in mice. The DS had no explanation for the absence of effects in mice compared to rats, leading to some uncertainties. However, the DS noted that fertility and oestrus cycle were not investigated in female mice. Therefore, the DS considered it not possible to disregard the effect observed in rats based on the negative results observed in mice.
- Although fertility effects were seen in the two human studies (Ahlborg *et al*., 1996; Rowland *et al.*, 1992), limited characterisation of N_2O exposure levels and co-exposure impaired a firm conclusion on the observed effects in humans.

Overall, the DS considered Category 2 for adverse effects on sexual function and fertility as more appropriate than Category 1B due to the limited number of parameters investigated in the animal studies and the absence of effects in mice.

Whether an SCL or generic concentration limit (GCL) should be applied to the data, was not specifically discussed by the DS.

Regarding the mode of action, the DS hypothesised that the effect of N_2O on fertility may be related to disturbance of folate metabolism via inactivation of methionine synthase induced by N_2O. However, the DS considered that data investigating this are not available.

Regarding **adverse effects on development**, the DS considered the following:

- Developmental findings were observed in numerous available developmental toxicity studies in rats, mice or hamsters. The main findings were the increase in resorptions and in malformations, and further, delayed development and decreased foetal weight.
- N_2O caused embryotoxicity and teratogenicity in rats during gestation day (GD)8 to GD10 after single 24h exposure with a NOAEC for both effects of 350000 ppm (Mazze *et al.*, 1987). Following continuous exposure during the whole gestation period, a NOAEC for malformations of 500 ppm was identified (Vieira *et al.*, 1980). Following intermittent exposure to N_2O (4-8h/d exposure during critical window or whole gestation period), embryofoetal toxicity (decrease foetal weight, resorption or dose related decrease in litter size) was observed. No significant increase in malformations upon intermittent exposure was noted, though in one study an increase in major malformations and external malformations were observed (without statistical significance) at 750000 ppm (Mazze *et al.*, 1986).
- Maternal toxicity was not described in all the studies. Reported maternal toxicity at up to 750000 ppm included mostly decreased body weight gain and mild sedation, not indicative of excessive toxicity.

- The teratogenic effects in animals were considered not to be secondary to maternal toxicity including the mild sedation.
- Inconsistent findings were noted regarding abortion in humans. Although some human studies indicated that N_2O may induce congenital abnormalities or reduced birth weight, interpretation of the human data is difficult due to co-exposure and bias.

Overall, the DS proposed classification in Category 1B given the clear embryo-lethality observed in animals following N_2O exposure, which was considered not to be secondary to unspecific maternal toxicity.

Whether an SCL or GCL should be applied to the data, was not specifically discussed by the DS.

Regarding the mode of action, the DS hypothesised that the effect of N_2O on teratogenicity may be related to inhibition of vitamin B12 formation and methionine synthase and involvement of folate. However, the DS considered that the exact mechanism by which N_2O acts as a teratogen is still not fully understood.

Adverse effects on or via lactation was not assessed by the DS due to lack of data.

Comments received during consultation

A total of ten comments were received, with one comment from an individual, six comments from industry representatives and three comments from MSCAs.

Two MSCAs supported the proposed overall classification as Repr. 1B (H360Df). A third MSCA supported the proposed Category 2 for adverse effects on sexual function and fertility. With regards to adverse effects on development this MSCA indicated to have a slight preference for Category 2 rather than Category 1B, given the omission of data on maternal toxicity in some of the available studies. This issue was also mentioned by one of the other two MSCA (though supporting Category 1B).

The individual considered the wording in section 4 of the CLH report ('Justification that action is needed at community level') to be misleading as they considered it untrue that there is evidence of reproductive harm in humans.

All industry representatives disagreed with the proposed classification, both for adverse effects on sexual function and fertility and for adverse effects on development. Their comments focussed on:

- Quality of the animal studies, i.e., studies being non-guideline/non-GLP, low number of animals and lack of statistical analysis, single concentration group (so no assessment of concentration-response relationship), lack of details in study methodology, few parameters included.
- (Ir)relevance of high concentration levels.
  - Concentration levels being above a limit concentration.
  - Considering the role of hypoxia.
- Lack of information on general toxicity or maternal toxicity in the animal studies.
- Adverse effects being the consequence of the anaesthetised state.
- Quality of the human data, i.e., limited quantitative characterisation of N_2O exposure, lack of information on co-exposures and confounding factors.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

Table: *Summary of the available data on* ***female rats*** *for adverse effects on sexual function and fertility*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
FEMALE RAT			
Study examining effects on brain (1), ovulatory cycle (2) and fertility (3) Non-guideline; GLP not stated; Klimisch 2 Kugel *et al.*, 1990 *Limitations:* No information on general toxicity in dams; Only one concentration tested; Low number of animals used in each group; No statistical analysis performed	Sprague-Dawley rat Experiment 1: 8 females/exposure group, divided as 4/group in proestrus and 4/group in metestrus Experiment 2: 12 females/exposure group** Experiment 3: 12 females/exposure group, divided as 6 animals/group in proestrus and 6 animals/group in random stage of ovulatory cycle. Mated with non-exposed males.	N_2O (purity not stated) 0; 300000 ppm N_2O in (compressed) air* Inhalation, whole body 8h/d for 4 days or one ovulatory cycle	<u>General toxicity</u> No information provided. <u>Reproductive toxicity</u> Experiment 1 (brain): increase in LHRH (luteinising hormone-releasing hormone) cell counts in hypothalamus in animals exposed on proestrus. No effects in animals exposed in metestrus. Experiment 2 (ovulatory cycle): disrupted cycles following the first day of exposure and 11 out of 12 exposed rats went into constant proestrus. Effects resolved within 3 weeks. Normal cycle in controls. Experiment 3 (fertility): mating occurred in all animals. Only 6 out of 12 treated animals (3 in group treated in proestrus and 3 in random phase of the cycle) gave birth vs. all animals in control. No effects on litter size and weight***

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

** based on the original publication

*** in the original publications this is literally presented as "No significant difference was noted in litter size and weight (between exposed and control animals)." RAC assumes that this refers to those animals that gave birth.

Table: *Summary of the available data on* ***male rats*** *for adverse effects on sexual function and fertility.*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
MALE RAT			
Dominant lethal test & paternal study (including behavioural examination) Non-guideline; GLP not stated; Klimisch 2	Sprague-Dawley rat 12 (paternal study) and 24 (dominant lethal test) males per exposure group Mated with non-exposed females	N_2O (purity checked though not stated) 0, 1000; 5000; 10000 ppm N_2O in air* Inhalation, whole body	<u>General toxicity</u> No effect on bw <u>Reproductive toxicity</u> *Dominant lethal test*: no effect on conception rate, total number of implants/litter or live foetuses/litter. Concentration-related trend (not stat.

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
Holson *et al.*, 1995 (sub-study: fertility) *Limitations*: Few parameters investigated in the study; No information on survival or clinical signs in the sires or dams in the study	(dominant lethal test and paternal study)	6h/d, 5d/week for 9 weeks	sign.) to an inc. in the number of resorptions at the highest concentration. *Paternal study*: no effect on litter size, no behavioural effect on offspring. Slight tendency (not stat. sign.) towards fewer pups per litter in the N_2O-exposed groups.
Fertility study Non-guideline; non-GLP; Klimisch 2 Vieira *et al.*, 1983a *Main limitations:* Single concentration; Exposure period too short to cover whole spermatogenic cycle; Few parameters examined; No information on general toxicity	Wistar rat 12 males/exposure group Mated with non-exposed females, directly upon exposure and after a 6-month recovery period.	N_2O (purity not stated) 0; 5000 ppm N_2O in air* Inhalation, whole body 6h/d, 5d/week for 30 days	<u>General toxicity</u> No information provided. <u>Reproductive toxicity</u> Dec. in litter size and developmental delay in offspring (i.e., bw, tail length and body length) upon mating directly at the end of exposure period. No effect on offspring upon mating after a 6-month recovery period.
Study focusing on testis toxicity Non-guideline; non-GLP; Klimisch 2 Kripke *et al.*, 1976 *Limitations:* Low number of animals per group; Only one concentration level; Too short exposure period (<10 week necessary to cover the whole spermatogenic cycle); No detailed results (incidence, grade of the lesions); Although several organs were sampled, only findings in testes were published.	LEW/f Mai rat 4-6 males/exposure group	N_2O (purity not stated) 0; 200000 ppm N_2O in air* Inhalation, whole body Experiment 1: 8h/d for 1 to 35 days Experiment 2: 24h/d for 32 days; sacrifice after 3, 6 or 10 days recovery	<u>General toxicity</u> No information provided. <u>Reproductive toxicity</u> Dec. absolute testis weight (experiment 1 and 2). Effect reversed after 6d recovery (experiment 2). Dec. in the number of spermatogenic cells with some disorganisation of the normal architecture (experiment 1). Damage and destruction of spermatogenic cells with increasing severity and frequency with exposure duration (experiment 2). No effect in Leydig cells and supporting cells within the tubules, no effect on serum testosterone levels.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant; bw = body weight

Table: *Summary of the available data on* ***mice*** *for adverse effects on sexual function and fertility*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
MOUSE			
Subchronic inhalation toxicity study Non-guideline, non-GLP; Klimisch 2 Rice *et al.*, 1985 *Limitations:* Few organs and parameters examined; Short exposure duration (4h/d)	Swiss Webster mouse 15/sex/exposure group	N_2O (purity not stated) 0; 5000; 50000; 500000 ppm N_2O in air* Inhalation, whole body 4h/d, 5d/week for 14 weeks	<u>General toxicity</u> All animals survived. Dec. bw gain (M: 77%, F: 63%). <u>Reproductive toxicity</u> No histopathological changes in testes and ovaries.
Study focusing on germ cell toxicity Non-guideline, non-GLP; Klimisch 2 Mazze *et al.*, 1983 *Limitations:* Short exposure duration (4h/d); No information on the source of test material; Low number of animals per group for oocyte examination (n=6); Only few organs and parameters examined; Low level of information on general toxicity; No information if experimenters were blind to treatment	Swiss Webster mouse 15/sex/exposure group	N_2O (medical grade; purity not stated) 0; 5000; 50000; 500000 ppm N_2O in air* Inhalation, whole body 4h/d, 5d/week for 14 weeks Positive controls used for male germ cell toxicity (methyl methanesulfonate) and oocyte count (methylchloranthrene)	<u>General toxicity</u> Normal behaviour. No excitement or general anaesthesia. <u>Reproductive toxicity</u> No effect on testis weight, percentage abnormal sperm, sperm count or histopathological appearance of testis. Number of oocytes unaffected. Positive response obtained for positive controls.
Fertility study Non-guideline, non-GLP; Klimisch 2 Mazze *et al.*, 1982 *Limitations*: Few reproductive parameters investigated; No information on general toxicity	Swiss/ICR mouse 18-21 males/exposure group Mated with non-exposed females	N_2O (medical grade; purity not stated) 0; 5000; 50000; 500000 ppm N_2O in air* Inhalation, whole body 4h/d, 5d/week for 9 weeks	<u>General toxicity</u> No information <u>Reproductive toxicity</u> No effect on abilities of males to impregnate females. No effect on litter size, foetal wastage (resorption, death), foetal growth.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

bw = body weight

***Table**: Summary of the available human data for adverse effects on sexual function and fertility*

Study, reference	Characteristics	Results
Retrospective cohort study Ahlborg *et al.*, 1996 *Limitations:* No information on co-exposure; Quantitative characterisation of N_2O exposure limited	Questionnaire sent to 3985 women midwifes born in 1940 and thereafter; 84% responded (n=3358). N_2O exposure based on average number of deliveries per month at which the midwife assisted where N_2O was used, and the type of work and work schedule. Detailed information on number of menstrual cycles required to achieve pregnancy and working conditions during that period were obtained concerning the most recent planned pregnancy. Probability of becoming pregnant (per cycle) calculated. Fecundability ratio (as relation to the unexposed) determined.	No effect of N_2O exposure on fecundability noted, except for the small group of midwifes that assisted >30 deliveries with N_2O per month showing a reduced fecundability ratio (0.64; 95% CI: 0.44-0.95)
Retrospective cohort study Rowland *et al.*, 1992 *Limitations:* No information on co-exposure; Quantitative characterisation of N_2O exposure limited	Screening questionnaires sent to 7000 women dental assistants aged 18 to 39; questionnaire completed by 4856. 459 women met criteria set by study authors and were followed up by detailed telephone interviews, resulting in a total of 418 women. N_2O exposure based on number of hours of exposure per weeks in room where N_2O was being used and the presence or absence of scavenging systems. Fertility measured by the number of menstrual cycles without contraception that the women required to become pregnant.	No relation between scavenged N_2O exposure and fertility. Reduced fertility only noticed in women with >5 h per week exposure to unscavenged N_2O.

The available human data (Ahlborg *et al.*, 1996; Rowland *et al.*, 1992) do not provide clear evidence for classification on its own, given the limitations regarding characterisation of the N_2O exposure and co-exposure. Therefore, classification in Category 1A is not appropriate.

All animal studies included inhalation exposure using whole body application. Studies with Klimisch score 2 are used in the RAC assessment and included in the table above. RAC notes that studies with Klimisch score 3 or 4 were included in the CLH report (table 11). The available animal studies were not performed in compliance with OECD TG and GLP and have limitations such as application of a single (high) concentration level, small animal group size and limited number of parameters investigated. Furthermore, either male or female animals were exposed. Nevertheless, RAC considers that in case observations are noted in studies with limited parameters or applying a single exposure concentration, they can in a weight of evidence approach, still point towards serious substance-specific adverse effects relevant for classification.

Evidence for an adverse effect on sexual function and fertility is primarily obtained from rat studies. Regarding female fertility, the results of the rat study by Kugel *et al.* (1990) point towards decreased fertility. Following inhalation exposure to 300000 ppm N_2O 8h/d for 4 days or one ovulatory cycle, only half of the exposed females (6/12) gave birth after mating with non-exposed males compared to 12/12 in controls. In addition, disrupted oestrus cycles immediately following day 1 of exposure were observed with 11/12 exposed female rats being into constant

proestrus. Though the adverse effects on oestrus cyclicity resolved, it required 3 weeks for normalisation.

Regarding male fertility, results of the rat studies of Kripke *et al.* (1976) and Vieira *et al.* (1983a) provide supportive evidence. Inhalation exposure of male rats to 200000 ppm N_2O in air, being intermittent for 8h/d for 1-35 days or continuous for a total of 32 days, resulted in reduced absolute testis weight (data on relative testis weight not presented). Also, damage and destruction of the spermatogenic cells was shown, with both incidence and severity being more pronounced in continuously exposed rats compared to intermittently exposed rats (Kripke *et al.*, 1976). It is noted that the exposure duration is too short to cover the full period of spermatogenesis; nevertheless effects on some reproductive parameters were observed.

In the rat study of Vieira *et al.* (1983a), a statistically significant reduction in litter size was seen after inhalation exposure of male animals to 5000 ppm N_2O 6h/d, 5 d/week for 30 days and subsequent mating with non-exposed females. This effect on litter size was not observed after a 6-month recovery of the exposed males followed by mating with non-exposed females. Nevertheless, the reduced litter size itself is considered an irreversible effect. In the control group one litter comprised of nine offspring while the remaining 35 litters included 11-15 offspring (mean number of offspring per litter: 12). A similar pattern was observed in the recovery group with one litter with eight offspring and the remaining 35 litters ranging from 10-14 offspring (mean number of offspring per litter: 11). In the group mated directly upon exposure to N_2O one litter comprised of 14 offspring but the remaining 35 litters ranged between two and six offspring (mean number of offspring per litter: 7 ($p<0.001$)). In addition, a significant decrease in body weight, tail length and body length were noticed in offspring of this latter group compared to control. Such effects were not observed in the recovery group.

In the rat study of Holson *et al.* (1995), no effect on conception rate, total number of implants/litter or live foetuses/litter (dominant lethal test), no effect on litter size and no behavioural effect on offspring (paternal test) was observed upon inhalation exposure of male animals up to 10000 ppm N_2O 6h/d, 5/week for 9 weeks and subsequent mating with non-exposed females. A concentration-related trend to an increase in the number of resorptions at the highest concentration (dominant lethal test) and a slight tendency towards fewer pups per litter in the N_2O-exposed groups (paternal test) were noted, though these trends were not statistically significant.

RAC notes an apparent inconsistency between the rat studies that investigated male fertility. Kripke *et al.* (1976) applied a high exposure concentration and observed clear adverse effects on spermatogenesis, though they did not include mating in the study design. While in the study of Vieira *et al.* (1983a) a significantly reduced litter size was observed upon mating of exposed males with non-exposed females, this was not the case for the study of Holson *et al.* (1995) despite that in this latter study a 2× higher exposure concentration and 1.5× longer exposure duration was applied when compared to the study of Veira *et al.* (1983a). It is noted that different rat strains were used in both studies, i.e., Wistar in Vieira *et al.* (1983a) and Sprague-Dawley in Holson *et al.* (1995), which might explain the observed difference.

The CLP guidance states that '*Adverse effects on fertility and reproductive performance seen only at dose levels causing marked systemic toxicity (e.g. lethality, dramatic reduction in absolute body weight, coma) are not relevant for classification purposes.*' Information on general toxicity was not provided for the rat studies that demonstrated adverse effects on sexual function and fertility. Concentration levels pointing towards adverse effects on sexual function and fertility in rats were up to 300000 ppm. However, adverse effects on fertility were also observed after repeated exposure of rats to lower concentrations (e.g., 5000 ppm in Vieira *et al.*, 1983a). In the developmental toxicity studies (see below), maternal toxicity did not occur <350000 ppm for 24h in rats (Mazze *et al.*, 1984; 1987). Maternal toxicity was limited, when exposed up to 600000

N_2O ppm for 24h, to reduced body weight and mild sedation. However, at 600000 ppm lethality was observed. In the repeated dose studies (see section STOT RE), serious general toxicity was not reported between 400000 and 700000 ppm in rats (Hayden *et al.*, 1974; Singh *et al.*, 2015; Mistra *et al.*, 2020; Dyck *et al.*, 1980). Lower concentration levels were not tested. Overall, RAC agrees with the DS that the presence of marked systemic toxicity can reasonably be excluded.

The studies performed in mice do not provide evidence of an adverse effect on sexual function or fertility. Upon repeated inhalation exposure up to 500000 ppm, 4h/d, 5d/week for 9-14 weeks, no adverse effect on testes, spermatogenesis and fertility were noted in male mice nor was an adverse effect on ovaries and oocytes noticed in female mice (Rice *et al.*, 1985; Mazze *et al.*, 1982; Mazze *et al.*, 1983). As already noted by the DS, fertility and oestrus cyclicity were not investigated in female mice.

In summary, adverse effects on sexual function and fertility were noted in studies where rats were exposed by inhalation to N_2O. However, the available studies are not OECD TG- or GLP-compliant and included only limited parameters. Upon exposure of female animals, serious effects included oestrus cycle disturbances and a marked decreased (50%) fertility upon exposure to 300000 ppm N_2O, 8h/d for 4 days (Kugel *et al.*, 1990). Upon exposure of male animals, adverse effects on spermatogenesis were noted at 200000 ppm N_2O, being intermittent for 8h/d for 1-35 days or continuous for a total of 32 days (Kripke *et al*., 1976). In addition, marked reduced litter size was observed upon 5000 ppm N_2O 6h/d, 5d/week for 30 days and subsequent mating to non-exposed females (Vieira et al., 1983a). RAC further noted an inconsistency as in the third study investigating male rat fertility and applying higher exposure concentration and longer exposure duration no such significant effects were noted (Holson *et al*., 1995). This could be due to strain differences or other effects and introduces an uncertainty.

RAC considers the evidence for adverse effect on fertility and sexual function to be stronger for female than for male rats. Further, RAC considers that the negative findings in the mouse studies do not negate the positive findings in rat. The diverse findings in these two species might point towards species differences in sensitivity but is considered not to reduce the concern. The CLP guidance does indicate that in the absence of mechanistic information showing otherwise, humans are generally assumed to be the most sensitive species. RAC notes that there is no information available to disregard the positive findings in rat. Further, the absence of adverse effects in mice is not considered to reduce the concern and the human data are considered supportive.

Overall, these data provide evidence for an adverse effect on fertility and sexual function. RAC considers the observed adverse effects on fertility and sexual function relevant for humans and not to be a consequence of other unspecific toxic effects. However, considering that the available data are limited, and the apparent inconsistent findings in rats introduces uncertainties, RAC agrees with the DS and concludes that **classification for adverse effects on sexual function and fertility in Category 2 (Repr. 2; H361f Suspected of damaging fertility) is warranted**.

Given the effective concentration levels, and also taking into account that in most studies a single concentration level was applied (which hampers assessment of concentration-response relationship, derivation of ED_{10} values and establishing the potency), the available data on sexual function and fertility do not support the calculation of an SCL.

Adverse effects on development

Human data

The DS presented a number of studies investigating the developmental toxicity of N_2O in humans.

Several studies investigated the potential risk for spontaneous abortion related to N_2O exposure. However, the interpretation of the results of these studies were hampered by poorly defined

exposure concentrations and lack of measurements, and the results being inconsistent. No effect was observed in the two most recent cross-sectional studies, though a high risk of bias was identified, with either no control groups or missing information on exposure and potential confounding factors hampering the reliability of the studies (Eftimova *et al.*, 2017; Uzun *et al.*, 2014). In the retrospective cohort study of Axelsson *et al.* (1996), no increased risk for abortion was observed in midwives. In contrast, in the retrospective cohort study of Rowland *et al.* (1995) an increased risk of abortion was observed among female dental assistants exposed at least 3 hours per week to unscavenged N_2O, though no clear concentration-response was noticed in this study. Furthermore, Heidam *et al.* (1984) identified an effect of N_2O on abortion among dental assistants, though the characterisation of the exposure was limited. Finally, Cohen *et al.* (1980) observed a significant increase in spontaneous abortions in dental assistants exposed to N_2O versus controls. However, exposure evaluation was based on questionnaires rather than measurements.

Regarding developmental abnormalities, Teschke *et al.* (2011) identified an increased risk of congenital abnormalities in offspring among female nurses exposed to N_2O. Exposure probability was estimated based on employment information retrieved during a telephone survey. Cohen *et al.* (1980) found an increase in congenital anomalies in offspring to dental assistants exposed to N_2O. A 1.5-fold increase in the rate of musculoskeletal defects was observed in the exposed group. However, adjustment for potential other risk factors was limited. A reduced birth weight and an increase in odds of being small for gestational age was noticed in offspring of midwives exposed to N_2O (Bodin *et al.*, 1999). Exposure was not quantified, and co-exposure was not considered in this study.

Overall, the available human data do not provide clear evidence on its own for classification, given the inconsistent findings, the limitations regarding characterisation of the N_2O exposure, co-exposure and other confounding factors.

Animal data

The DS presented multiple animal studies for the endpoint adverse effect on development:

- Rat prenatal developmental toxicity studies with a repeated intermittent inhalation exposure, i.e., 6-8 hours per day during specific parts of/or the whole gestation period (see table below).
- Rat prenatal developmental toxicity studies with a single 24h inhalation exposure during a specific day of the gestation period as well as studies with inhalation exposure of 23-24h/d during multiple days of the gestation period or during the whole period of gestation (see table below).
- Rat studies investigating the pre- and/or postnatal (neuro)development upon intermittent exposure (see Background document for details).
- Prenatal developmental toxicity studies in other species (see Background document for details).

Table**: Detailed summary of the available animal data on N_2O for the endpoint adverse effects on development: **rat prenatal developmental toxicity studies with intermittent exposure

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
Prenatal developmental toxicity study in rats Non-guideline; Non-GLP; Klimisch 2 (DS)/3 (RAC)	Sprague-Dawley rats N=19-50/group	N_2O (medical grade; purity not stated)	Maternal toxicity: dec. in body weight gain (stat. sign. in dams exposed on GD13-15). Developmental toxicity:

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
Mazze *et al.,* 1986 *Limitations:* Single concentration level		0; 750000 ppm N_2O in oxygen and air Inhalation, whole body 6h/d on GD13-15, GD10-12 or GD7-9	- Dec. foetal weight (GD13-15) - Inc. resorptions and foetal wastage (dead and resorbed) in dams exposed during GD13-15 window - Inc. major malformations and external abnormalities in dams exposed on GD7-9 and skeletal malformations in dams exposed during GD13-15 (not stat. sign.).
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Vieira *et al*., 1983b *Limitations:* Low number of animals per groups; Size of the inhalation chamber not specified; No information on maternal toxicity; Potential effects of high latitude (1700m) and pressure is unknown; Few details on study method and results	Female Wistar rats N=12/group	N_2O (purity not stated) 0; 250; 500; 1000; 5000 ppm N_2O in air Inhalation, whole body 6h/d, 5d/week, whole gestation period (3 weeks)	Maternal toxicity: No information Developmental toxicity: Dose-related dec. in litter size (stat. sign. at 5000 ppm). No effect on foetal weight or crown-rump length of foetuses. No malformations reported.
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 (DS)/3 (RAC) Pope *et al.,* 1978 *Limitations:* Low number of animals per treatment group; Animals were exposed simultaneously in the chambers; No information on how animals were sacrificed; Results for maternal liver and kidney weight not provided, representative maternal tissues fixed for microscopic examination not specified; Detailed results of skeletal examination not provided; No analysis of visceral abnormalities; Unknown if the 5-6 foetuses selected for skeletal examination was randomly done	Sprague-Dawley rats N=8-10 per groups	N_2O (purity not stated) 0; 10000; 100000; 500000 ppm N_2O in air, additional stress group as control Inhalation, whole body 8h/d, whole gestation (GD0-20)	Maternal toxicity: no effect on body weight or on food consumption Developmental toxicity: - Delayed development (foetal weight, crown-rump length, delayed ossification), stat. sign. at ≥100000 ppm - Dec. placental weight, stat. sign. at ≥10000 ppm - Inc. foetal loss at the low and mid dose but not stat. sign. and inside spontaneous range of the laboratory. No increase in foetal loss at 500000 ppm.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

** Plug day=day 0 of gestation.

Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant; bw = body weight

Table: *Summary of the available animal data on N_2O for the endpoint adverse effects on development:* ***rat prenatal developmental toxicity studies with continuous exposure (23-24h/d)***

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Fujinaga *et al.,* 1991 *Limitations*: Only one concentration level; Single day of exposure; No information if experimenters were blind to treatment and control groups; Few details on maternal toxicity (only bw before and after exposure and on GD20 was provided); No information on clinical signs; It is not specified if the death observed in the treated group was treatment related.	Sprague-Dawley rats N=25 in exposed group and 30 in control group	N_2O (medical grade; purity not stated) 0; 600000 ppm N_2O mixed with O_2 and air Inhalation, whole body 24h exposure at GD8 Sacrifice: GD20	<u>Maternal toxicity</u> Exposed dams appeared mildly sedated and rested quietly during exposure period. No stat. sign. difference in mean bw on GD20 (334 g vs 368 g in controls). Mortality in one out of 25 exposed dams. <u>Developmental effects</u> - Stat. sign. inc. foetal resorptions/litter (48% vs 5% in controls) - Stat. sign. dec. live foetuses/litter (mean 6.5±4.1 vs 12±2.1 in controls) - Stat. sign. dec. mean number of foetuses/litters - Stat. sign. inc. major visceral malformations - Stat. sign. inc. minor visceral anomalies - Stat. sign. inc. minor skeletal anomalies and variants.
Prenatal developmental toxicity study Non-guideline, Non-GLP; Klimisch 2 Fujinaga *et al.,* 1990 Only survival and *situs inversus* in foetuses examined *Limitations:* No information on the age and body weight of the animals at the start of the study; Body weight of dams analysed but results were not reported; General toxicity of dam not provided; No information on foetuses weight; Single dose level, single exposure; No information if experimenters were blind to treatment	Sprague-Dawley rats N=35/group	N_2O (medical grade; purity not stated) 0; 700000-750000 ppm N_2O mixed in air and oxygen Inhalation, whole-body 24h exposure at GD8 Sacrifice: 4-6 rats on GD11, 12, 13, 14, 15, 16, 18 or 20 Only survival and situs inversus in foetuses examined	<u>Maternal toxicity</u> No information <u>Developmental toxicity</u> Stat. sign. inc. in embryo-foetal mortality rate on GD14 and onward compared to control. Stat. sign. inc. in altered laterality at all stages of development compared to controls.
Prenatal developmental toxicity study in rat Non-guideline; Non-GLP; Klimisch 2 Fujinaga *et al.,* 1989 *Limitations*: Low number of litters examined; Lack of details on maternal toxicity (food consumption, clinical signs, weight); Only one concentration level; No	Sprague-Dawley rats 20 females/N_2O exposure group, 30 controls	N_2O (medical grade; purity not stated) 0; 600000 ppm N_2O mixed in air and oxygen Inhalation, whole-body Exposure: 24h at GD6, 7, 8, 9, 10, 11 or 12	<u>Maternal toxicity</u> Mortality in 2 to 4 dams per group except in control and GD10 exposed group. Mild sedation during exposure. Dec. (stat. sig.) mean maternal bw at caesarean section in all treated groups (Table 8 in confidential Annex I, no further information). <u>Developmental toxicity</u>

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
information if experimenters were blind to treatment		Sacrifice: GD20	- No effects on the number of implantations, live foetuses, mean foetal weight, sex ratio - Inc. % of resorptions per litter on GD8 and 11 (stat. sign.) - Inc. skeletal malformations following exposure on GD9 (stat. sign.) - Inc. skeletal variations following exposure on GD8 (stat. sign.) - Inc. visceral malformations and minor visceral anomalies when dams exposed on GD8 or GD9 (stat. sign.).
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Mazze *et al.,* 1988 *Limitations:* Few details on maternal toxicity (survival, food consumption); Only one concentration reported (results at 50% and 75% N_2O were pooled); No information if experimenters were blind to treatment; No justification of differences in the number of animals per groups; Unclear why only 5 out of 20 exposed animals for methionine synthase activity were selected; Controls may not have been exposed to air in inhalation chamber as the exposed animals (unclear in the paper)	Sprague-Dawley rats Experiment 1: 90 rats: - 20 animals exposed to N_2O or folinic acid or folinic acid + N_2O - 30 controls (air) Experiment 2: 116 rats - 37 controls (air) - 26 animals exposed to N_2O - 27 animals exposed to folinic acid - 26 animals exposed to folinic acid + N_2O Methionine synthase activity: 65 non-pregnant rats - 5 controls (air) - 20 exposed to N_2O- 20 exposed to N_2O + halothane - 20 exposed to folinic acid + N_2O - only 5 of each of these groups were killed for the assay.	N_2O (medical grade; purity not stated) Experiment 1 and 2: Control; 500000 ppm (experiment 1); 750000 ppm N_2O (experiment 2); folinic acid; 750000 ppm N_2O + folinic acid Methionine synthase activity: Control; 500000 ppm N_2O; 500000 ppm N_2O + halothane; 500000 ppm N_2O + folinic acid Inhalation, whole body, 24h at GD8 Sacrifice at 24, 48 and 72h post-treatment	<u>Maternal toxicity</u> Mild sedation in both experiments at 500000 and 750000 ppm Stat. sign. dec. bw due to lower number of live foetuses <u>Developmental toxicity</u> - No effect on weight of pups - Inc. early and late resorptions (stat. sign.) - Inc. visceral malformations (stat. sign.) - Inc. minor skeletal anomalies and variants (stat. sign.) Teratogenic effect still observed with co-administration of folinic acid. No correlation with methionine synthase activity.
Developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Fujinaga *et al.,* 1987 *Limitations:* Single concentration tested; Single day of exposure	Sprague-Dawley rats N=40 females in controls and 30 in treated groups Sacrifice: GD20	N_2O (medical grade; purity not stated) 0; 500000 ppm N_2O mixed in oxygen and air	<u>Maternal toxicity</u> No mortality Mild sedation during exposure Dec. body weight gain on GD12 and 20 compared to controls (stat. sign.). No significant effect on GD6, 8, 9, 14, 16.

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
		Inhalation, whole body 24h on GD8	Developmental toxicity Stat. sign. effects: increased early and late resorption, increased foetal wastage (dead and resorbed) and increase major visceral malformations (right side aortic arch, in 5/26 litters).
Developmental toxicity study in rats Non-guideline; Non-GLP; Klimisch 2 Mazze *et al.,* 1987 *Limitations*: Two concentration levels; Single day of exposure	Sprague-Dawley rats N=34-40 in controls and 24-30 in treated groups	N_2O (medical grade) 0; 350000; 500000 ppm N_2O mixed in O_2 and air Inhalation, whole body 24h on GD8 Sacrifice: GD20	Maternal toxicity - Mild sedation of dams (350000 and 500000 ppm) - Sign. dec. bw gain on GD6-21 compared to controls (135g in controls compared to 106g in exposed group) and to mean body weight at caesarean section compared to controls (321g vs 351g in controls). Mean weight at caesarean section and bw gain was not affected during the experiment at 350000 ppm. Developmental toxicity At 500000 ppm: - Inc. foetal resorptions and post-implantation losses (stat. sign.) - Inc. minor and major visceral abnormalities (stat. sign.) - Inc. minor skeletal anomalies (stat. sign.). No effects at 350000 ppm.
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Keeling *et al.,* 1986 *Limitations*: Low number of exposed rats per concentration groups; No information on source of test material; Visceral examination not performed; No information if experimenters were blind to treatment; No information on maternal toxicity; Only one concentration level; No information on room temperature and humidity	Sprague-Dawley rats Sacrifice: GD20 N=10 exposed and 23 controls	N_2O (purity not stated) 0; 700000-750000 ppm N_2O Inhalation, whole body exposure 24h on GD8	Maternal toxicity No information. Developmental toxicity - No effects on resorptions, live foetuses and number of implants - Stat. sign. dec. in foetal and placental weight - Stat. sign. inc. in delayed development in N_2O group (decreased mean number of sternebrae and caudal vertebrae) - Stat. sign. inc. in skeletal malformations (e.g., cervical vertebral malformations) - Inc. methyl folate concentration in N_2O exposed group.
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Mazze *et al.,* 1984 *Limitations*: No detailed information on maternal toxicity; No detailed information on the results	Sprague-Dawley rats Sacrifice: GD20 N=25-62/exposed group (pooled results) and 160 controls (pooled results)	N_2O (medical grade; purity not stated) Experiment I: 0; 750000 ppm N_2O Experiment II: 7500; 75000; 750000 ppm	Maternal toxicity No effects up to 250000 ppm At 750000 ppm: impaired food and water consumption, rats were drowsy, impaired motor coordination (no information on statistical significance). Developmental toxicity (750000 ppm)

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
for individual experiments (pooled results)		Experiment III (mated in house): 0; 750000 ppm Experiment IV: 0, 250000 ppm N_2O mixed in oxygen and air (food and water deprivation or food and water *ad libitum*) Inhalation, whole body 24h on GD8	- Inc. in any external abnormalities, runts and major external malformations (stat. sign.) - Inc. in any skeletal abnormalities, major malformations and malformations in rib/vertebra, inc. in variant (extra lumbar rib, cervical rib) (stat. sign.) - Inc. in any major internal malformations (stat. sign.) - Inc. ocular malformation (reported in the text, no tabular information) - Inc. in total, early and late resorptions (stat. sign.) - Dec. number of live foetuses per dams (stat. sign.). At 75000 ppm: stat. sign. inc. in minor skeletal anomalies only (extra lumbar ribs and cervical ribs).
Developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Vieira *et al.,* 1980 *Limitations:* Low number of animals per groups; Size of the chamber not stated; No specification of organs examined (internal or skeletal examination); No details on abnormalities provided (no tabulated data); Few information on maternal toxicity (e.g., no information on weight, clinical signs, behaviour).	Wistar rats N=12/group	N_2O (purity not stated) Inhalation, whole body Control (air), 250, 500 and 1000 ppm 23h/d, GD1-19	<u>Maternal toxicity</u> No effects on food or water consumption. <u>Developmental toxicity</u> - Dec. litter size at 1000 ppm (stat. sign.) - Inc. resorptions at 1000 ppm (stat. sign.) - Dec. crown-rump length at 1000 ppm, no effect on foetal body weight (stat. sign.) - Skeletal abnormalities at 1000 ppm (stat. sign.) (malformation of the vertebrae column and rib).
Developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Vieira *et al.,* 1979 *Limitations:* low number of animals; Only one concentration group; Limited information on environmental conditions; Few internal organs and skeletal examinations; No information on maternal toxicity; No details on skeletal abnormalities (incidences in each group, details of the anomalies)	Wistar rats N=12/group	N_2O (purity not stated) Control (air), 5000 ppm Inhalation, whole body 23h/d, GD1-19	<u>Maternal toxicity</u> No effects on food or water consumption. <u>Developmental toxicity</u> - Stat. sign. dec. in litter size; 4/12 dams had full resorptions (vs 0 in controls) - Stat. sign inc. in skeletal malformations (rib). Foetuses with malformations were smaller than their litter mates or controls - Marked stat. sign. reduction in mean crown-rump length of exposed foetuses - Stat. sign. dec. in mean foetuses weight.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.
** Plug day=day 0 of gestation.
Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant; bw = body weight

Contrary to the DS, RAC used only Klimisch 2 studies in weighing the evidence, noting that studies with Klimisch 3 and 4 are presented in Tables 13-16 in the CLH report. Additionally, the following is considered by RAC:

All animal studies included inhalation exposure using whole body application. The available animal studies were not performed in compliance with OECD TG and GLP and have limitations such as application of a single (high) concentration level, small animal group size and limited number of parameters investigated. Nevertheless, RAC considers that in case observations are noted upon exposure during a critical window in studies having limited parameters or upon a single exposure concentration, such observations still can point towards serious substance-specific adverse effects relevant for classification.

RAC notes that prenatal developmental toxicity studies with repeated intermittent inhalation exposure (i.e., 6-8 hours per day during specific parts or the whole gestation period) are highly relevant for various human exposure regimes. In addition, prenatal developmental toxicity studies with 24h exposure to high concentrations of N_2O during a relevant exposure window and prenatal developmental studies with repeated continuous (24h/d) exposure to low(er) concentrations N_2O are included as additional evidence.

- Experimental data in rats: prenatal developmental toxicity studies, intermittent exposure

The DS presented several rat prenatal developmental toxicity studies with a repeated intermittent inhalation exposure, i.e., 6-8 hours per day during specific parts or the whole gestation period. Only the studies marked with a Klimisch score 2 by the DS are discussed below.

Mazze *et al.* (1986) exposed female Sprague-Dawley rats to 750000 ppm N_2O by inhalation 6h/d, during GD7-9, GD10-12 or GD13-15. A statistically significant increase in resorptions and foetal wastage (dead and resorbed) was noted in dams exposed during GD13-15 (1.32 per litter versus 0.46 in controls for resorptions, and 1.37 per litter versus 0.49 in controls for foetal wastage). This was associated with a decrease in live foetuses/implantation (87.8% versus 96.7% in controls). Foetal weight was significantly reduced in the group exposed during GD13-15, which was accompanied by lower weight gain of the maternal animals. Though not statistically significant, an increase in external malformations in the N_2O group versus control was present in offspring of dams exposed during GD7-9 and skeletal malformations in offspring of dams exposed during GD13-15. Historical control data are lacking, and the type of malformations was not specified. Visceral malformations were not increased in this study. It is noted that the exposure concentration is quite close to the threshold for hypoxia. Regarding maternal toxicity, animals were conscious during the experiment in all groups. Reduced body weight gain was noted in the dams exposed to N_2O, resulting in more than 20% reduction in body weight gain in the GD13-15 group and 5% and 8% for the GD11-13 and GD8-10 groups, respectively. The differences in weight gain in dams exposed during the various stages of development in this study were described by the study author to be due to the difference in their gestational age when they were received from the breeder and first weighed. RAC considers this explanation to be plausible. Further, information on corrected body weight is lacking. All in all, several effects on development were noted but in view of the differences in effects on weight gain in the various groups it is difficult to judge whether these were, at least in part, due to maternal effects. Due to these limitations, RAC gives limited weight to this study in the overall weight of evidence assessment.

Pope *et al.* (1978) exposed Sprague-Dawley rats by inhalation to 10000, 100000 or 500000 ppm N_2O for 8h/d, during the whole gestation. Adverse effects on development included delayed foetal development, specified as statistically significant decrease in foetal weight, accompanied by a decrease in crown-rump length and significant delayed ossification (at ≥100000 ppm). No effects on resorption or number of dead foetuses were observed upon N_2O exposure. Placental weight

was reduced (≥10000 ppm). No effect on maternal toxicity was observed (i.e., no effects on either food consumption or body weight). Only gross skeletal abnormalities were examined, and no visceral examination was performed in the study. This study also included a group, in which animals that got excited were transferred to a so-called stress group. In this group several effects were noted, such as decrease in the number of live litters and increase in foetal loss. Due to these limitations, RAC gives limited weight to this study in the overall weight of evidence assessment.

Vieira *et al.* (1983b) exposed Sprague-Dawley rats by inhalation to 250, 500, 1000 or 5000 ppm for 6h/d, 5d/week during the 3 weeks of gestation. A statistically significant decrease in litter size was present at 5000 ppm, though it is noted this was not observed in the presence of resorptions. There was no evidence of malformations. Crown-rump length and body weight was not affected. Information on maternal toxicity was not available.

***Table**: Developmental findings reported by Vieira et al. (1983b)*

Concentration level (ppm)	No. of foetuses	Litter size (mean±SD)	Range per litter
0	120	11±1.4	9-13
250	119	11±1.3	9-13
500	117	11±1.3	8-13
1000	117	10±1.2	8-13
5000	98	7.0±2.3*	6-10

*p<0.001

- Experimental data in rats: prenatal developmental toxicity studies, continuous exposure

The DS presented multiple prenatal developmental toxicity studies with a single 24h inhalation exposure during a specific day of the gestation period as well as studies with inhalation exposure of 23-24h/d during multiple days of the gestation period or during the whole period of gestation.

Vieira *et al.* (1979; 1980) applied continuous inhalation exposure during the gestation period (23h/d, GD1-19; 250, 500, 1000 ppm in the 1980 study, 5000 ppm in the 1979 study) in Wistar rats. Regarding maternal toxicity, no effect on food or water consumption were noted up to 5000 ppm. Adverse effects on development were noted in both studies with statistical significance (see two next tables below). An increase in resorptions and decreased litter size were noted at ≥1000 ppm N_2O. Skeletal malformations were noted at ≥1000 ppm, specified as rib malformations and abnormal vertebrae columns. In addition, crown-rump length was also decreased at 1000 ppm onward. RAC notes the lower effective exposure concentrations in this study.

***Table:** Litter size, crown-rump measurements and foetal resorption upon exposure to 250, 500, 1000 ppm N_2O (Vieira et al., 1980)*

Concentration level (ppm)	Number of litters	Number of foetuses	Litter size (mean±SD)	Crown-rump measurements (mm, mean±SD)	Resorptions
0	12	120	11±1.4	44±1.4	None
250	12	120	11±1.3	43±1.4	None
500	12	118	11±1.4	43±1.3	None
1000	12	66	6.3±4**	35±1.6*	4**

**p<0.01, *p<0.05

Table: *Litter size, crown-rump measurements and foetal resorption upon exposure to 5000 ppm N_2O (Vieira et al., 1979)*

Maternal rat number												
N_2O group												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Live foetuses	7	9			13			11	11	10	5	11
Foetal weight (g), mean±SD	1.4±0.2	1.3±0.2			1.8±0.0			1.3±0.3	1.5±0.2	1.7±0.1	1.8±0.2	2.1±0.1
Resorption sites	4	1	11	12	2	10	12		1			
Crown-rump length (mm), mean±SD	29±0.2	28±0.2			30±0.2			29±0.2	28±0.2	28±0.2	28±0.2	29±0.2
Live foetuses with abnormalities		1			2			1	1	2		2
No. of live foetuses without abnormality	7	8			11			10	10	8	5	9*
Controls												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Live foetuses	12	12	10	10	10	13	12	12	9	13	12	10
Foetal weight (g) mean±SD	2.0±0.1	3.5±0.2	3.1±0.0	3.3±0.6	3.1±0.2	3.2±0.3	2.6±0.3	2.0±0.1	2.0±0.1	2.0±0.2	2.3±0.2	2.3±0.2
Crown-rump length (mm), mean±SD	44±0.2	44±0.2	44±0.2	43±0.2	42±0.2	42±0.2	44±0.2	44±0.2	42±0.2	43±0.2	44±0.2	44±0.2

*data retrieved from original publication

In a series of 7 studies from the same research group, Sprague-Dawley rats were exposed for 24h via whole body inhalation to N_2O at concentration levels between 7500 and 750000 ppm on a specific day of the gestation (Mazze *et al.*, 1984; 1987; 1988, Fujinaga *et al.,* 1987; 1989; 1990; 1991):

- Fujinaga *et al.* (1987; 1990; 1991) applied a single inhalation exposure on GD8 (24h) using a single concentration level at either 500000 ppm, 600000 or 700000-750000 ppm N_2O.
- Mazze *et al.* (1984; 1987; 1988) applied a single inhalation exposure on GD8 (24h) using several concentration levels to investigate concentration-response, i.e., from 7500 to 750000 ppm N_2O.

- Fujinaga *et al.* (1989) applied a single inhalation exposure (24h) to 600000 ppm on either GD6, 7, 8, 9, 10, 11 or 12.

Animals were sacrificed at GD20, i.e., one day before expected delivery, except for the study of Fujinaga *et al.* (1990) where rats were randomly sacrificed at GD11-16, 18 or 20.

Adverse effects on development were consistently observed in these studies. Such effects included increased early and late resorptions, subsequent decrease in the number of live foetuses per litter, skeletal abnormalities and malformations, visceral abnormalities and malformations.

Regarding the resorptions, a concentration-dependent increase in early and late resorptions was observed in those studies investigating the effect of N_2O following 24h exposure on GD8 (Fujinaga *et al.,* 1987; 1989; 1991, Mazze *et al.,* 1984; 1987; 1988). The effect reached statistical significance at ≥500000 ppm, and a NOAEC of 350000 ppm was identified by the researchers. In the study of Fujinaga *et al.* (1989) at which inhalation exposure to 600000 ppm N_2O was applied on various days of gestation (GD6, 7, 8, 9, 10, 11 or 12), two critical periods of exposure were identified for the occurrence of resorptions. These was one at GD8 and one at GD11. Further, Fujinaga *et al.* (1990) found that upon inhalation exposure to 700000-750000 ppm N_2O, the increase in resorptions was first observed in the animals sacrificed on GD14, and not in animals sacrificed at GD11, 12 or 13. Further on, the rate of resorption remained constant as shown in animals sacrificed on GD15, 16, 18 and 20.

Regarding the skeletal and visceral abnormalities and malformations, a statistically significant marked increase was noted following inhalation exposure on GD8 (24h) at ≥500000 ppm N_2O (Fujinaga *et al.,* 1987; 1989; 1991; Mazze *et al.,* 1984; 1987; 1988). Various types of malformations were observed. In some of the studies, these were specified and included:

- Cardiac anomalies (Fujinaga *et al.,* 1991) or more specifically 'right sided aortic arch' (Fujinaga *et al*., 1989; Mazze *et al.,* 1988; Fujinaga *et al.,* 1987)
- Situs inversus (Fujinaga *et al.,* 1991)
- Ocular malformations (Mazze *et al.,* 1984)
- Hydrocephalus (Fujinaga *et al.,* 1991: 1989)
- Ribs and vertebrae (Fujinaga *et al*., 1989; Mazze *et al*., 1984)
- Limb deformities (Mazze *et al.,* 1984).

Further, body laterality as investigated by Fujinaga *et al.* (1990) was significantly altered compared to controls at all stages of development. This specifically included side of tail flexion, side of the body from which the umbilical artery emerged, side of the body that faced the placenta, and side to which the aortic arch curved.

The main visceral anomalies observed were left-sided umbilical artery (Fujinaga *et al.,* 1991; 1989). In addition, skeletal anomalies such as cervical ribs and 14^{th} rudimentary rib were also increased (Fujinaga *et al*., 1991; 1989), Mazze *et al.,* 1988).

Information on maternal toxicity was, with the exception of Fujinaga *et al.* (1990), available for 6 out of these 7 studies. Mortality was noted in one of these studies (2-4 dams per exposure group; with unknown cause according to the authors) upon 24h inhalation exposure to 600000 ppm N_2O at GD6, 7, 8, 9, 11 or 12 (Fujinaga *et al*., 1989) and in a second study (1/25 exposed dams) upon 24h inhalation exposure to 600000 ppm N_2O at GD8 (Fujinaga *et al.,* 1991). In the other studies no increased mortality or significant morbidity was noted upon 24h inhalation exposure at GD8 up to 750000 ppm (Fujinaga *et al.*, 1987, Mazze *et al.,* 1984; 1987; 1988).

A decreased body weight compared to control was in general noted in most studies upon 24- h inhalation exposure at ≥500000 ppm N_2O. This may be caused by embryo-foetal lethality, though information on corrected body weight was not available in any of the studies. More specifically, no significant effect on body weight was noticed in dams exposed 24h at GD8 to 600000 ppm N_2O in the study of Fujinaga *et al*. (1991). Fujinaga *et al*. (1989) demonstrated, in addition to

the deaths observed, a reduction in mean body weight upon exposure to 600000 ppm N_2O. Furthermore, Mazze *et al.* (1988) found a reduced body weight upon exposure to 500000 ppm or 750000 ppm N_2O (approximately 8% as compared to control). In Fujinaga *et al.* (1987), a decrease in body weight of dams was observed specifically at GD12 and 20 upon inhalation exposure to 500000 ppm. No effect on body weight was noticed at GD9, 14, and 16. See the table below for details on maternal body weight, indicating the decrease in body weight is slight (<6%) in this study of Fujinaga *et al.* (1987).

Table: *Selected maternal and foetal observations (mean ± SD) (Fujinaga et al., 1987)*

N_2O	**Control**	**500000 ppm**
No. of rats examined	37	26
Weight (mean, g)		
- GD6 (on arrival)	211±15	210±12
- GD8 (before exposure)	233±15	231±13
- GD9 (after exposure)	212±15	208±11
- GD12	254±17	241±12*
- GD14	271±18	259±14
- GD16	290±21	279±24
- GD20 (at caesarean section)	348±32	328±24*
Weight loss during the exposure	21±4	24±5
Early resorptions/rat (%)	4.9 ±10.3	18±19.9*
Late resorptions/rat (%)	0	6.8±11.7*
Total foetal wastage/rat (%)	4.9±10.3	25.9±28*
Total live foetuses/rat	11.7±3.4	9.5±3.5

* $p<0.05$ versus control

Finally, Mazze *et al.* (1987) found a difference in body weight of 9% at the end of the study between controls and high dose, which was at least in part due to the increase in post implantations loss and resorptions. A decreased body weight gain was noted in the 500000 ppm group and not in the 350000 ppm group. Like in other studies, information on corrected maternal body weight was not provided, making it uncertain to exclude the decreased litter size as cause of the body weight effects.

Mild sedation was observed during exposure in some of these studies, i.e., upon 24h inhalation exposure at GD8 to 350000; 500000; 600000 or 750000 ppm N_2O in the studies of Fujinaga *et al.* (1987; 1989; 1991) and Mazze *et al*. (1984; 1987).

In summary, for the rat studies with a single 24h exposure, it is considered that maternal toxicity did not occur <350000 ppm. Up to <600000 ppm for 24h, maternal toxicity was limited to reduced body weight and mild sedation. At 600000 ppm for 24h, lethality was observed. It is further noted that information on corrected maternal body weight was not provided, which makes it uncertain to exclude the decreased litter size as cause of the body weight effects in rats.

Overall, RAC agrees with the DS that the available information on maternal toxicity cannot unequivocally explain the adverse effects on development as shown in this series of 7 studies with 24h exposure, at least for the increased incidence of resorptions and malformations at the effective concentration of 500000 ppm. Specifically, the study of Fujinaga *et al.* (1987) is considered the most important. Upon exposure to 500000 ppm N_2O on GD8, a statistically significant increase in early resorptions (18% vs. 4.9% in control) and late resorptions (6.8% vs. 0% in control), total foetal wastage (25.9% vs. 4.9% in control), major visceral malformation (14.9% vs. 0% in control) and skeletal developmental variants (33% vs. 15.2% in control) were observed. The predominant visceral lesion was a right-sided aortic arch, which was present in foetuses from 5 of the 26 litters exposed to 500000 ppm N_2O. Maternal toxicity in this study was limited to mild sedation during exposure and slight decrease in bw (see analysis above). RAC considers that the developmental effects as noted in Fujinaga *et al.* (1987) cannot be explained

by the maternal toxicity. The other studies of this series of 7 are given less weight, as these applied concentration levels at which lethality was observed or information on maternal toxicity was lacking.

In addition to the series of 7 studies as described above, Keeling *et al.* (1986) exposed Sprague-Dawley rats by inhalation for 24h on GD8 to 700000-750000 ppm N_2O in oxygen. Information on maternal toxicity was not provided. No increase in resorptions was noted. Foetal weight and placental weight were decreased in a statistically significant manner compared to control. In addition, a statistically significant increase in skeletal malformations (mainly cervical vertebrae) and delayed development was observed in the N_2O exposed group.

- Experimental data in rats: pre-/postnatal development and postnatal (neuro)developmental toxicity studies (intermittent exposure)

Studies presented in this section include those with a repeated intermittent inhalation exposure, i.e., 6-8 hours per day during specific parts or the whole gestation period and investigating the pre- and/or postnatal (neuro)development.

Holson *et al.* (1995) exposed female Sprague Dawley rats to 0, 1000, 5000 or 10000 ppm N_2O for 6h/d during GD1-21 (3h on GD21). No effect on weight (gain) of the dams or weight of the offspring was noted. No effect on litter size and no treatment-related effects on behaviour in offspring were observed.

Mullenix *et al.* (1986) investigated potential neurodevelopmental toxicity (residential maze activity and time-lapse photography) and exposed Sprague Dawley rats for 8h/d on GD14 or GD13-14 to 750000 ppm N_2O. Exposure on GD13-14 produced significant hyperactivity in the females at 1-month and 5-month timepoints and in males at 1-month timepoint. Exposure on GD14 only produced a tendency to hypoactivity in females and hyperactivity in males.

- Experimental data in other species (mainly intermittent exposure)

In addition to studies in rats, studies with intermittent exposure in hamsters and mice were presented by the DS. Only the two studies in mice with a Klimisch 2 score are discussed below

No developmental effects (resorptions, litter size, malformations) were noted in Swiss/ICR mice in the study of Mazze *et al.* (1982) up to 500000 ppm N_2O (4h/d, GD6-15).

Rice *et al.* (1990) studied behavioural effects in offspring of Swiss mice following exposure by inhalation to 0, 50000, 150000 or 350000 ppm N_2O for 4h/d during GD6-15. Information on maternal toxicity was not available. Exposure to N_2O did not affect reproduction indices and survival or physical milestones of development. On postnatal day (PND)126 or 127 no effect on brain weights were observed. Ability to stay on a rotarod was also not affected by prenatal N_2O exposure. However, prenatal exposure to N_2O resulted in hypo-reactivity of the startle reflex on PND95 for all N_2O-exposed groups.

Discussion on adverse effects on development

RAC considers that a large database is available for the endpoint adverse effects on development, although several of the studies are poorly reported.

The available human data do not provide clear evidence on its own for classification, given the inconsistent findings, and the limitations regarding characterisation of the N_2O exposure and co-exposure and other confounding factors. Therefore, classification in Category 1A is not appropriate.

Animal data provide clear evidence for adverse effects on development upon inhalation exposure of N_2O, which is most evident in rats. Adverse effects are observed in multiple studies upon different exposure regimens.

Indications for adverse effects on development are obtained upon intermittent exposure, i.e., 6-8h/d, to N_2O (Mazze *et al.,* 1986, Pope et al., 1978; Vieira *et al*., 1983b; Mullenix *et al*., 1986; Rice *et al.,* 1990), an exposure regimen which RAC considers most relevant for humans. Such effects include delayed growth, i.e., decreased foetal weight and delayed ossification, (Pope *et al.,* 1978), reduced litter size (though in absence of resorptions; Vieira *et al.,* 1983b), increased resorptions, decrease in live foetuses per implantation and reduced foetal weight (Mazze *et al.,* 1986) and some evidence for effects on reactivity in pups (Rice *et al.,* 1990; Mullenix *et al*., 1986). For many of these studies it was noted that the information available did not allow an in-depth analysis whether the effects observed were in part due to maternal toxicity. Overall, the evidence obtained upon intermittent exposure is considered insufficient for classification in Category 1B on its own.

However, support for adverse effects on development is obtained from studies with a single 24h exposure to high concentrations on a specific day of the gestation (Mazze *et al.,* 1984; 1987; 1988; Fujinaga *et al.,* 1987; 1989; 1990; 1991; Keeling *et al.*, 1986), and moreover, from studies applying continuous 24h exposure to low (and more relevant) concentrations during the whole gestation period (Vieira *et al.,* 1979; 1980). A decreased crown-rump length, an increase in resorptions and a decreased litter size was noted in two studies in rats at ≥1000 ppm N_2O upon 23h/d, GD1-19. Also, skeletal malformations were noted at ≥1000 ppm in both studies, specified as rib malformations and abnormal vertebrae columns (Vieira *et al*., 1979; 1980). RAC notes the lower effective exposure concentrations in these two studies. Regarding the single 24h exposure on a specific day of the gestation, RAC considers the study of Fujinaga *et al.* (1987) to provide clear evidence for adverse effects on development. Exposure of rats to 500000 ppm N_2O for 24h at GD8 resulted in resorptions and malformations. More specifically, a statistically significant increase in early and late resorptions, total foetal wastage, major visceral malformation (predominantly right-sided aortic arch) and skeletal developmental variants was observed. It is noted that in this study maternal toxicity was limited to mild sedation during exposure and slight decrease in body weight, which is not sufficiently severe to discount the development effects observed (e.g., death or severe inanition, Annex I 3.7.2.4.3). Overall, RAC considers that the developmental effects as noted in Fujinaga *et al.* (1987) cannot be explained by the maternal toxicity.

Regarding maternal toxicity, RAC also notes that in the experiment of Mazze *et al.* (1984) the effect of food deprivation on the N_2O-induced adverse effects was investigated. Exposure to 250000 ppm N_2O at GD9 and concurrent withholding of food and water did not result in an increased incidence of abnormalities.

Although in mice some evidence on potential effect on reactivity in pups was obtained, no effect on litter size, foetal growth and teratogenicity were noted in this species. However, the available mouse dataset is less extensive than the dataset for rat. Nevertheless, this divergence between species does not reduce the concern.

It is noted that the adverse effects on development in rats seem to be related to a specific exposure window. The results of Fujinaga *et al.* (1989) show that increased resorptions and major malformations were only noted in dams exposed at GD8, 9 or 11 to 600000 ppm N_2O for 24h and not in dams exposed to the same concentration level on GD6, 7, 10, or 12. It is however noted that in this specific study maternal mortality was noted, i.e., 2-4 dams per exposure group, except in control and GD10 exposed group.

Regarding hypoxia, N_2O was, especially at the high concentration levels in the developmental toxicity studies, mixed with oxygen in order to obtain an adequate test atmosphere (for example the Mazze *et al.* and Fujinaga *et al.* studies). Oxygen concentrations were monitored continuously during exposure in these studies. RAC notes that typical hypoxic effects such as respiratory distress or death from asphyxia were not reported in these developmental toxicity studies with

information on maternal toxicity. Moreover, RAC notes that clear evidence for adverse effects on development is also observed upon much lower effective concentration of N_2O Vieira *et al.* (1979; 1980; 1983b).

It may be questioned whether the observed teratogenic effects are related to the substance itself or are secondary to the anaesthetised state. Regarding this issue, the DS presented several studies investigating the developmental effects of other anaesthetics. Pope *et al*. (1978) noted that high subanaesthetic concentrations of N_2O (up to 500000 ppm), halothane and methoxyflurane (8h/d, during whole gestation) can cause foetal growth retardation unaccompanied by foetal loss or abnormalities and unrelated to a specific agent. They considered that these developmental effects may well act through a general effect of the anaesthetic on the mother and foetus, rather than a toxic effect of the substance itself on the foetus. In contrast, Lane *et al.* (1980) exposed rats to 700000-750000 ppm N_2O or xenon (which has anaesthetic properties similar to those of N_2O) for 24 hours on GD9. Foetal resorption, delayed maturation and anomalies to the skeletal systems were only observed with N_2O and not xenon, suggesting that the observed effects are related to the substance itself rather than its anaesthetic mechanism. In line with this, Mazze *et al.* (1986) did not demonstrate teratogenic findings in rats exposed to intermittent exposure (6h/d, during GD7-9, GD10-12 or GD13-15) to anaesthetics such as isoflurane, enflurane or halothane. In contrast, an increase in foetal loss was observed following exposure to N_2O (750000 ppm), although some questions were posed as to the maternal effects. Overall, the adverse effects on development observed upon inhalation exposure to N_2O seem thus not to be the result of the anaesthetised state but rather an adverse effect of the substance itself, though some uncertainties are noted. Nevertheless, RAC notes that clear evidence for adverse effects on development is also observed upon exposure to much lower effective concentrations of N_2O.

In relation to N_2O-induced cobalamin (Vit. B12) deficiency as a potential mode of action, Mazze *et al.* (1984) exposed rats on GD10 to 250000 ppm N_2O and measured deoxyuridine (DU) suppression values in bone marrow and embryonic cells as marker of cobalamin deficiency. A 2.5-fold increase of DU-suppression was observed upon exposure to N_2O, though unaccompanied by teratogenic changes. Mazze *et al.* (1988) investigated the effect of co-exposure to folinic acid on the teratogenic effect of N_2O and its effect on methionine synthase activity. The developmental effect as induced by 500000-750000 ppm N_2O for 24h at GD8 (i.e., increased incidence of foetal wastage, major visceral malformations, minor skeletal anomalies, skeletal developmental variants) was still present with co-exposure to folinic acid, except for a partial reduction in minor skeletal anomalies, suggesting that cobalamin deficiency may not, alone, explain the teratogenicity induced by N_2O. Further, there was no correlation found with methionine synthase activity. In addition, Keeling *et al.* (1986) noticed that although a reduction in the N_2O-induced developmental effects were noted in presence of folinic acid, a statistically significant increase in major malformations was still observed compared to control.

Overall, no conclusion on the possible mode of action(s) can be drawn from the available information.

In addition, RAC notes that, in the absence of mechanistic information that raises doubt about the relevance of the effect for humans, the strong presumption that the substance has the capacity to interfere with reproduction in humans prevails based on clear evidence from animal studies (Annex I, Table 3.7.1(a)).

Conclusion on adverse effects on development

In summary, adverse effects on development were noted in studies where rats were exposed by inhalation to N_2O. These effects on development were observed upon different exposure regimens. Upon repeated continuous exposure of female rats to low effective concentrations, i.e., 23h/d,

GD1-19, 1000 and 5000 ppm, decreased crown-rump length, increase in resorptions, decreased litter size and also occurrence of skeletal malformations (specified as rib malformations and abnormal vertebrae columns) were noted in two rat studies (Vieira *et al*., 1979; 1980). Intermittent exposure resulted in a statistically significant decrease in litter size at 5000 ppm (Vieira *et a*l., 1983b). Upon single exposure of female rats to 500000 ppm N_2O for 24h at GD8, early and late resorptions, increased total foetal wastage, major visceral malformation (predominantly right-sided aortic arch) and skeletal developmental variants were observed (Fujinaga *et al.,* 1987).

Overall, these data provide clear evidence for an adverse effect on development. RAC considers the observed adverse effects on development serious and relevant for humans and not to be a secondary consequence of other unspecific toxic effect.

Therefore, RAC agrees with the DS and concludes that **classification in Category 1B for adverse effects on development (Repr. 1B; H360D May damage the unborn child) is warranted**.

Given the level of effective concentrations and taking into account that in most studies a single concentration level was applied (which hampers assessment of concentration-response relationship, derivation of ED_{10} values based on single study, and establishing the potency), the available data on development do not support the calculation of an SCL.

Adverse effects on or via lactation

Given the lack of data, RAC considers that **classification for adverse effects on or via lactation is not warranted**.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of hazards to the ozone layer

Summary of the Dossier Submitter's proposal

The DS proposed classification of N_2O as Ozone 1 (H420, Harms public health and the environment by destroying ozone in the upper atmosphere). The DS cited a study in which Ozone Depleting Potential (ODP) for dinitrogen oxide was calculated. Ravishankara *et al*. (2009) used a two-dimensional model to calculate the ODP value of N_2O. The ODP of N_2O under current atmospheric conditions was calculated as 0.017, which is comparable with the ODP of some hydrochlorofluorocarbons (HCFCs) that are considered as hazardous to the ozone layer. Ravishankara *et al*. (2009) took into consideration several key factors that influence the ODP of N_2O: nitrogen oxides contribute most to the ozone depletion in the stratosphere where the ozone concentration is the largest unlike the chlorine-catalysed ozone destruction that take place in the lowest and upper stratospheres. The DS noted the following when concluding on the classification:

- Based on the knowledge that N_2O depletes the stratospheric ozone.
- Based on the World Meteorological Organization scientific assessment of ozone depletion (WMO, 2018).
- Based on the current calculated ozone depletion potential-weighed emission (Ravishankara *et al*., 2009), Joint Research Centre (JRC, 2015) indicated that N_2O is the largest of all ozone depleting substances.

Comments received during consultation

Three comments from one each from industry, an MSCA and an individual were received during the consultation. All the commenters expressed concern regarding the interpretation of the data forming the basis for the proposed classification.

The Individual noted that only 10% of N_2O impacting the ozone layer is deliberately made by man and the amount of ozone harm via N_2O deliberately created by man has only a small impact on the ozone layer when compared to the amount of ozone harm caused by deliberately created halogenated molecules. In their response, the DS noted that a risk or impact assessment is not a part of the CLP Regulation.

The Industry sector pointed out that the literature data used for classification was not new and was already considered by the registrants at the time of registration. The experts concluded the data to be conclusive but not sufficient for classification. The DS noted that there is no explanation why the data from Ravishankara *et al*. (2009) were not considered sufficient for classification.

The MSCA expressed concern regarding the method used for calculation of the N_2O ODP value in Ravishankara *et al*. (2009). The MSCA referred to the cited WMO report (2018) and Montreal Protocol (2018) in which no specific recommendations for the ODP value calculation have been made. Consequently, there is no specific method to be recommended for calculation of an ODP value for N_2O in particular. There is uncertainty about the long-term impact of N_2O on ozone due to changes in stratospheric chemistry and dynamics caused by increasing greenhouse gas concentration. The DS noted that there is also no method recommendation for the ODP calculation in the CLP Regulation or CLP Guidance.

Assessment and comparison with the classification criteria

RAC agrees with the DS that impact assessments and risk related elements raised during the consultation and subsequent discussions are not relevant for hazard assessment under the CLP Regulation. The CLP Regulation (Annex I.5) states that a substance should be classified as hazardous to the ozone layer "*if the available evidence concerning its properties and its predicted or observed environmental fate and behaviour indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer"* (Annex I, Section 5.1.2.1). Furthermore, it is stated in the CLP Guidance (Part 5.1) that any substances having an ODP greater or equal to the lowest ODP (*i.e.,* 0.005) of the substances currently listed in Annex I to Regulation (EC) No 1005/2009 should be classified as Ozone 1 (H420). RAC notes that N_2O is not currently listed in Annex I to Regulation (EC) 1005/2009.

Although the suitability of the data was questioned during the consultation, no reason has been provided as to why the data is not suitable for use under the CLP Regulation. RAC agrees with the DS in noting that there are no particular criteria for evaluation of open literature studies providing ODP values. RAC has assessed the ODP value from Ravishankara *et al*. (2009) and agrees with the DS that this is reliable and the ODP value reported of 0.017 is suitable for comparison with the CLP criteria. RAC further notes that the ODP value provided by Ravishankara *et al*. (2009) is still used in the latest WMO report (2022), where N_2O continues to be considered as an ozone depleting substance, as well as in the previous WMO report (2018) and JRC report (2015), both cited in the CLH report.

The impact of N_2O increase on the ozone layer has also been investigated in other studies, such as Revell *et al*. (2015), which was made available to RAC. In contrast to Ravishankara *et al.* (2009) (which calculated the OPD value of N_2O using the well-established Garcia-Solomon two-dimensional model, considering past and future changes in ozone and includes comprehensive

chemistry, detailed radiative transfer, and dynamics), Revell *et al.* (2015) employed a three-dimensional chemistry-climate model and performed different simulations to explore the range of possible N_2O ODP values under different atmospheric conditions. They calculated ODP values in the range of 0.015 to 0.030 from the year 2000 to 2100. Although a different technique was used to calculate the ODP in Revell *et al*. (2015), a value comparable (0.015 for the year 2000) to that from Ravishankara *et al*. (2009) was derived. Consequently, although Ravishankara *et al*. (2009) and Revell *et al*. (2015) applied different approaches the obtained values are comparable, being 0.017 and 0.015, respectively. Both available studies further indicate that ozone would decrease as stratospheric N_2O increases.

RAC agrees with the DS that the provided ODP value is relevant and reliable and that as the ODP value of 0.017 for N_2O along with the supporting ODP value of 0.015 are greater than the lowest ODP value in Annex I to Regulation (EC) 1005/2009 (0.005 for chlorofluoroethane) (following the CLP Guidance Section 5.1), **classification as Ozone 1 (H420 Harms public health and the environment by destroying ozone in the upper atmosphere) is warranted**.

Additional references

Revell, L. E., Tummon F., Salawitch R. J., Stenke A., and Peter T. (2015), The changing ozone depletion potential of N2O in a future climate, Geophys. Res. Lett., 42, 10,047–10,055, doi:10.1002/2015GL065702.

World Meteorological Organization (WMO). Scientific Assessment of Ozone Depletion: 2022, GAW Report No. 278, 509 pp.; WMO: Geneva, 2022

ANNEXES:

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

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