CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

Dinitrogen oxide

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

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

Index Number: **-**

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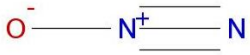
CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substances

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

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Nitrous oxide
Other names (usual name, trade name, abbreviation)	Dinitrogen oxide, laughing gas, hyponitrous oxide, dinitrogen monoxide, nitrogen oxide
EC number	233-032-0
EC name	Dinitrogen oxide
CAS number	10024-97-2
Molecular formula	N_20
Structural formula	
SMILES notation	[N-]=[N+]=O
Molecular weight or molecular weight range	44.013

1.2 Composition of the substance

Table 2: Constituents

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self- classification and labelling (CLP)
Dinitrogen oxide	Mono-constituent	-	Ox. Gas 1, H270 Press. Gas, H281 STOT SE 3, H336 (brain) (inhalation) Acute Tox. 2, H330

Table 3: Impurities if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
No impurities relevant for classification				

No additives if relevant for the classification of the substance.

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 4:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	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	Not available										
Dossier submitters proposal	TBD	Dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT RE 1 STOT SE 3 Ozone 1	H360Df H372 (nervous system) H336 H420	GHS07 GHS08 Dgr	H360fD H372 (nervous system) H336 H420			
Resulting Annex VI entry if agreed by RAC and COM	007-RST-VW-Y	Dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT RE 1 STOT SE 3 Ozone 1	H360Df H372 (nervous system) H336 H420	GHS07 GHS08 Dgr	H360fD H372 (nervous system) H336 H420			

Table 5: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	hazard class not assessed in this dossier	No
Oxidising gases	hazard class not assessed in this dossier	No
Gases under pressure	hazard class not assessed in this dossier	No
Flammable liquids	hazard class not assessed in this dossier	No
Flammable solids	hazard class not assessed in this dossier	No
Self-reactive substances	hazard class not assessed in this dossier	No
Pyrophoric liquids	hazard class not assessed in this dossier	No
Pyrophoric solids	hazard class not assessed in this dossier	No
Self-heating substances	hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	hazard class not assessed in this dossier	No
Oxidising liquids	hazard class not assessed in this dossier	No
Oxidising solids	hazard class not assessed in this dossier	No
Organic peroxides	hazard class not assessed in this dossier	No
Corrosive to metals	hazard class not assessed in this dossier	No
Acute toxicity via oral route	hazard class not assessed in this dossier	No
Acute toxicity via dermal route	hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	hazard class not assessed in this dossier	No
Skin corrosion/irritation	hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	hazard class not assessed in this dossier	No
Respiratory sensitisation	hazard class not assessed in this dossier	No
Skin sensitisation	hazard class not assessed in this dossier	No
Germ cell mutagenicity	hazard class not assessed in this dossier	No
Carcinogenicity	hazard class not assessed in this dossier	No
Reproductive toxicity	harmonised classification proposed: Repr. 1B – H360Df	Yes
Specific target organ toxicity-single exposure	STOT SE 3 – H336 (narcosis)	Yes
Specific target organ toxicity-repeated exposure	STOT RE 1 – H372 (nervous system)	Yes
Aspiration hazard	hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	hazard class not assessed in this dossier	No
Hazardous to the ozone layer	Ozone 1 – H420	yes

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Dinitrogen oxide has not been classified according to the Classification and Labelling of the Dangerous Substance Directive (Dir. 67/548/EEC) and have no entry in Annex VI Tables 3.1 and 3.2 of the Regulation (EC) No. 1272/2008 (CLP Regulation).

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Action is required at Community level based on the need to classify for CMR endpoints.

Furthermore, self-classification STOT SE 3 for brain by inhalation is not sufficient and justify the need for action at Community level since, based on animal and human data, there are evidence that the substance shall be classified for STOT RE 1 for the nervous system in addition to classification STOT SE 3 for narcosis.

In addition, it has been known since 1970 that dinitrogen oxide depletes the stratospheric ozone. According to the world meteorological organization scientific assessment of ozone depletion (WMO, 2018), natural and anthropogenic emissions of dinitrogen oxide make a larger contribution to stratospheric ozone depletion than emissions of any of the individual ozone depleting long-lived halogenated source gases. Based on the current calculated ozone depletion potential-weighed emission (Ravishankara et al., 2009), JRC (2015) indicated that dinitrogen oxide is the largest of all ozone depleting substances. Therefore, action is required at community level and justify the need to classify dinitrogen oxide as hazardous to the ozone layer.

5 IDENTIFIED USES

According to ECHA website, dinitrogen oxide (N_2O) is manufactured and/or imported in the European Economic Area at ≥1000 to < 10 000 tons per year.

N_2O is used for more than 150 years in surgery as an adjuvant in inhalational general anaesthesia. The substance is also used for pain relief during delivery or for short analgesia during minor medical procedure (e.g. dentistry, emergency, veterinary medicine). The substance is commonly used in combination with other anaesthetics.

N_2O is also an industrial chemical used in food industry as a food additive (E942).

Furthermore, N_2O is a propellant in canister used in many preparation and uses (e.g. aerate whipping cream, inflate balloons).

It is also an additive to rocket fuels to increase available oxygen for combustion. In addition, N_2O is used in laboratory as an oxidizing agent in atomic flame absorption spectrometry.

Recreational misuses of the gas, also called “laughing gas”, has been identified as strongly increasing in recent years (ANSES, 2020) due to its euphoric, relaxing and hallucinogenic properties, with various effects for health, including severe ones.

6 DATA SOURCES

Literature search was conducted in the Pubmed database.

Titles, key words and abstracts were screened with the following key words (#1 AND #2 AND #3 NOT #4):

Substance identity #1:

(dinitrogen oxide) OR (nitrous oxide) OR (nitrogen protoxide) OR (laughing gas) OR (10024-97-2) OR (Dinitrogen monoxide) OR (dinitrogenoxide) OR (hyponitrous acid anhydride) OR (N2O)

Toxicokinetics and human health #2 :

(kinetic) OR (metabolism) OR (metabolite) OR (absorption) OR (distribution) OR (excretion) OR (elimination) or (half-life) OR (clearance) OR (model) OR (Health effects) OR (health) OR (developmental toxicity) OR (toxic) OR neurotoxicity OR reproductive toxicity OR toxicity

Population #3

Workers OR adult OR volunteers OR occupation OR occupational OR human OR laboratory animal OR animal experimentation OR models animal OR animal population groups OR vertebrates OR mammals OR primates OR mice OR mus OR mouse OR murine OR rats OR rat OR muridae OR hamster OR hamsters OR rodentia OR rodent OR rodents OR pigs OR pig OR piglets OR piglet OR guinea pigs OR guinea pig OR rabbits OR rabbit OR monkey OR monkeys OR canine OR porcine OR dog OR dogs

Exclusion #4

soil OR denitrification OR greenhouse gas OR emissions

The table below summarised the inclusion/exclusion criteria used for human and animal studies.

Table 6: Literature search inclusion and exclusion criteria

Publication type	IN	Primary research studies
	OUT	Secondary studies (e.g. editorials, conference)
Language	IN	English
	OUT	Other languages
Study design	IN	Human experimental volunteer studies, Cohort studies, Cross-sectional studies, Case-control studies, case studies Experimental animal studies
	OUT	*In vitro* studies *in silico* studies
Population	IN	Adult healthy male and female volunteers, men and women occupationally exposed to N_2O, recreational drug exposure All mammalian animals
	OUT	Patients under anaesthesia
Exposure	IN	Inhalation
	OUT	Mixtures Anaesthesia Air pollution Subdermal route of exposure in animals Pharmacological research (e.g. alcohol withdrawal)
Time	IN	From 1952 – December 2020 for reproductive toxicity From 2001-2021 for STOT
Outcome	IN	Human health endpoints included in the dossier (reproductive toxicity, neurotoxicity)
	OUT	Risk management measures Other Human Health endpoint not assessed in this dossier

All data sources retained in this report are listed in section 15.

Secondary literature were also considered to check for potential additional research studies or mechanistic data not captured during the literature search: IPCS-INCHEM, 1992; INRS, 2010 and 2018; MAK, 1993 and 2015; ACGIH, 2001; ANSES, 2020; HCN, 2000; EIGA, 2008.

Furthermore, REACH registration dossiers (last modified: 25 October 2016) for N_2O available from ECHA's disseminated database (ECHA, 2021) have been taken into account.

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties for dinitrogen oxide

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Colourless gas with sweetish odour	(ECHA, 2021)	No study has been presented. Nevertheless, in NIOSH Handbook (2018) the physical description of dinitrogen oxide is Colourless gas with a slightly sweet odour.
Melting/freezing point	-90.81 °C at 1013.25 hPa	(ECHA, 2021)	in NIOSH Handbook (2018) the freezing point was specify to be at -127 F° equivalent to -52.78°C (at atmospheric pressure).
Boiling point	-88.3 °C at 1013.25 hPa	(ECHA, 2021)	In NIOSH Handbook (2018) the boiling point was determined to be -132 F° equivalent to -55.56°C (at atmospheric pressure).
Relative density	1.2 (-89°C) and 1.99 mg/cm³(0°C)	(ECHA, 2021)	In NIOSH Handbook the relative gas density value was 1.53.
Vapour pressure	42900 mmHg at 25°C	(ECHA, 2021)	In NIOSH Handbook (2018) the vapour pressure was 51.3 atm. equivalent to 38756 mmHg at ambient temperature.
Surface tension	1.75 dynes/cm at 20°C	(ECHA, 2021)	No study has been presented to confirm this surface tension value.
Water solubility	1.5 g/L at 15°C pH not indicated	(ECHA, 2021)	No study has been presented to confirm this water solubility value. In NIOSH Handbook (2018) water solubility was 0.1% at 21.5°C without the precision of the pH.
Partition coefficient n-octanol/water	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Flash point	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Flammability	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property. In NIOSH Handbook (2018)

Property	Value	Reference	Comment (e.g. measured or estimated)
			it is reported that the dinitrogen oxide is Non-flammable Gas, but supports combustion at elevated temperatures.
Explosive properties	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Self-ignition temperature	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Oxidising properties	Oxidizing	(ECHA, 2021)	No data has been provided to confirm the oxidizing classification of Dinitrogen oxide: Ox. Gas 1, H270 Press. Gas, H281.
Granulometry	Not relevant	(ECHA, 2021)	Not relevant as Dinitrogen oxide is a gas substance.
Stability in organic solvents and identity of relevant degradation products	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Dissociation constant	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Viscosity	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.

8 EVALUATION OF PHYSICAL HAZARDS

Not performed for this substance.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Conclusions from studies are summarised in section 9.1			

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

Absorption

In humans, N_2O is mainly absorbed through inhalation. The rate of N_2O uptake during the first 1 or 2 min is about 1.0 L/min (at an inspired concentration of 80%) (INRS, 2018). Due to the high infusibility and low solubility of N_2O, the alveolar concentration is close to the inhaled concentration in less than 5 min (ANSM, 2014[1]). The blood/gas partition is 0.47.

There is no data in animal.

Distribution

In humans and animals, N_2O is rapidly distributed throughout the body (only in dissolved form), particularly in vessel-rich regions, including the brain, heart, kidney, splanchnic circulation, and endocrine glands (ANSM, 2014; INRS, 2018).

Stenqvist et al., 1994, in the table below, report the partition coefficients. The total body uptake of N_2O is relatively smaller than for more soluble anaesthetics like isoflurane and desflurane (13 times more soluble in fat than N_2O).

Table 9: Partition coefficient of N_2O (Stenqvist et al., 1994)

Blood/gas	0.5
Brain/blood	1.1
Muscle/blood	1.2
Fat/blood	2.3

Dinitrogen oxide is able to cross the placental barrier (INRS, 2018).

Metabolism

In humans and animals N_2O is poorly metabolised (0.004 %) by bacterial reductases in the intestines, due to its relatively non-reactivity and low solubility in blood.

As described in MAK report, 1993, dinitrogen oxide is reduced to nitrogen in the reaction with the central Co+ ion of vitamin B12. After a single passage through the liver, however, the concentration of dinitrogen oxide in the blood decreases by only 0.03 %. Formation of radicals has been demonstrated *in vitro* in human intestinal contents incubated with dinitrogen oxide. It has been deduced from *in vitro* investigations that about 0.004 % of the total dinitrogen oxide absorbed is metabolised in humans and animals to nitrogen by bacterial reductases in the intestine. During this process OH radicals are probably formed.

[1] http://agence-prd.ansm.sante.fr/php/ecodex/rcp/R0234390.htm, consulted in May 2021 (only available in French)

Excretion

N_2O is almost completely eliminated unchanged by the lungs (in few minutes); only small amounts pass into the urine, and there is some minimal diffusion through the skin (INRS, 2012). Dinitrogen oxide is eliminated in the urine through a diffusion process determined by the equilibration of partial pressures in urine and plasma (Henderson et al., 2002).

O'Reilly et al. (1983) exposed 20 healthy volunteers, young and elderly males to identify any aged-related differences, under a protocol to mimic a dental operatory. The authors measured dinitrogen oxide in expired gas (at the end of 30 min inhalation and periodically for 70 min after withdrawal). Two elimination phases were identified with half-lives of about 1.8 minutes for the first one and of 20 minutes for the second.

Sixteen hours after the end of exposure, Dinitrogen oxide is completely eliminated from the blood (INRS, 2018).

Conclusion on toxicokinetics

N_2O is very volatile and rapidly absorbed through inhalation. It is rapidly distributed in richly vascularised tissues, and easily penetrates into the brain. It is quickly excreted unchanged by the lungs. It is able to cross the placental barrier (INRS, 2018).

10 EVALUATION OF HEALTH HAZARDS

Nitrous oxide, N_2O and dinitrogen oxide are synonyms.

10.1 Acute toxicity - oral route

Evaluation not performed for this substance.

10.2 Acute toxicity - dermal route

Evaluation not performed for this substance.

10.3 Acute toxicity - inhalation route

Evaluation not performed for this substance.

10.4 Skin corrosion/irritation

Evaluation not performed for this substance.

10.5 Serious eye damage/eye irritation

Evaluation not performed for this substance.

10.6 Respiratory sensitisation

Evaluation not performed for this substance.

10.7 Skin sensitisation

Evaluation not performed for this substance.

10.8 Germ cell mutagenicity

Evaluation not performed for this substance.

10.9 Carcinogenicity

Evaluation not performed for this substance.

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Five published studies in rats and four published studies in mice investigated the potential effects on sexual function and fertility of dinitrogen oxide. The table below summarised the relevant findings. See annex I for detailed summary of the method and results.

Table 10: Summary table of rats studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline paternal study and dominant lethal test GLP: not stated Crl:COBS(SD)BR rats Paternal study: N=12/exposed groups (3 replicates of 4 males or females per dose) Dominant lethal test: 24 males per groups mated with 30 non-exposed females *Limitations:* - few parameters investigated in the study - no information on survival or clinical signs in the sires or dams in the study	Dinitrogen oxide (checked for purity) Whole body inhalation exposure Males: 6h/day, 5d/w for 9 weeks. Mated with non-exposed females (paternal and dominant lethal assay) 0, 1000, 5000, 10,000 ppm N_2O in air	<u>General toxicity</u> No effect on weight of males, no effect on body weight gain in females up to 10,000 ppm <u>Reproductive toxicity</u> - Paternal study: No statistically significant findings in litter size (trend to lower number of pups per litter in dinitrogen oxide groups) - Dominant lethal assay: no statistically significant effect on conception rate, total number of implants, live foetuses. However, a trend to increase in the number of resorption noted at the top dose with slight increase in lethality.	Holson et al., 1995 (substudy ; fertility) Klimisch score 2 WOE
Non-guideline fertility study in female rats GLP: not stated Female Sprague-Dawley rats Group 1: 8 animals/group: hypothalamic LHRH-producing cell counts.	Dinitrogen oxide (no information on purity) 0, 300,000 ppm N_2O in air Inhalation (whole body exposure) 8h/d for 4 days or one ovulatory cycle	<u>General toxicity</u> No information <u>Group 1: Brain study</u> ↑ LHRH (Luteinizing hormone-releasing hormone) cell count in hypothalamus in the 4 animals exposed on proestrus compared to the 4 controls. No effects on LHRH cell count in animals exposed during metestrus compared to control <u>Group 2: Ovulatory cycle study</u>	Kugel et al., 1990 Klimisch score: 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Group 2: 12 animals/group : 6 animals/group treated in proestrus and 6 animals/group treated in random stage of ovulatory cycle Group 3: 12 animals/group: mated with proven male breeder for 4 days. 6 animals/group treated in proestrus and 6 animals/group treated in random stage of ovulatory cycle. Number of pregnancy, litter size and weight were analysed. *Limitations:* - No information on general toxicity in dams. - Only one concentration tested - Low number of animals used in each group - Statistical analysis not performed		**Disrupted cycles** following the first day of exposure and 11 out of the 12 exposed rats went into constant proestrus. This dysfunction resolved itself after approximately 3 weeks. Control animals cycled normally throughout the experiment. <u>Group 3: Fertility study</u> Mating occurred in all animals. All 12 controls gave births. Only 6 out of 12 treated animals (3 in groups treated in proestrus and 3 in random phase of the cycle) gave birth. No effects on litter size and weight of pups.	
Non-guideline fertility study in female rats GLP: no Female rats n=12/group Limitations: - Only short communication of the results in the publication. - no statistical analysis	Dinitrogen oxide 0, 500 ppm N_2O in air Inhalation, 8h/day, 35 days	**Transitory oestral cycle disturbance** in all exposed female rats. Return to normal after 3 weeks. Only 6 out of 12 exposed animals gave birth vs all animals in control. No effects on litter size and weight of pups in both control and exposed groups.	Kugel et al., 1989 Klimisch score: 4 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline fertility study in male rats GLP: no Wistar male rats n= 12/group Mating with non-exposed females: after exposure or after 6-month recovery *Limitations*: - no information on environmental condition (light cycle, temperature) - single dose level - short duration period (< 10 weeks) - few parameters analysed - no information on general toxicity in animals	Dinitrogen oxide (purity not stated) Inhalation, 6h/d, 5d/w, 30 days 0, 5000 ppm N_2O in air	General toxicity: no information Fertility: **Statistically significant decrease in litter size and developmental delay in offspring** (body weight, tail length and body length). No significant effect after a 6-month recovery period.	Vieira et al., 1983a Klimisch score: 2 WOE
Non-guideline testis toxicity study in male rats GLP: no Male LEW/f Mai rats N=4-6 rats/group *Limitations* - Low number of animals per groups - Only one dose level - Duration of exposure is too short (< 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.	Dinitrogen oxide (no information on purity) 0, mixture of 200,000 ppm N_2O: 20% O2:60% N_2 Inhalation, Group 1: 1 to 35 days intermittent (8h/d) exposure, Group 2: 32-day continuous exposure (24h/d), sacrifice after 3, 6 or 10-day post-exposure.	General toxicity: no information Male reproductive organs: Statistically significant **decrease in testis weight in group 1 and 2 compared to controls**. Effect was reversible after 6-day recovery (continuous exposure) Microscopic examination: **injury (damage, destruction) observed in the spermatogenic cells in the seminiferous**. Some reversibility of the effect noted. Effect more severe in group 2 than in group 1. No effects in controls. No effect in Leydig cells and supporting cells within the tubules. No effect on serum testosterone levels	Kripke et al., 1976 Klimisch score: 2 WOE

Table 11: Summary table of mice studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline 14-week repeated dose toxicity study GLP: no Swiss Webster Mice N=15/sex/group Limitations: - Few organs and parameters examined - Low duration of exposure (4h per day)	Dinitrogen oxide (purity not stated) Inhalation, 4h/d, 5d/w (whole body) 14-week exposure, 0, 5000, 50,000, 500,000 ppm N_2O in air	General toxicity All animals survived. Significant decreased in body weight gain by 77% and 63% in males and females, respectively. Organ toxicity No histopathological findings in testes and in ovaries (no other reproductive organ examined).	Rice *et al.*, 1985 Klimisch score 4 WOE
Non-guideline reproductive organ toxicity study GLP: no Swiss Webster mice N=15/sex/group *Limitations:* - Only 4h exposure per day - No information on the source of test material - Low number of animals per groups 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	Dinitrogen oxide (medical grade) Inhalation, 4h/d, 5d/w, 14-week exposure Control, 5000, 50,000, 500,000 ppm N_2O in air Methyl methanesulfonate positive control for male germ cell toxicity Methychloranthrene: positive control for oocyte count	General toxicity No excitement or general anaesthesia seen in animals. Mice behave normally. Reproductive toxicity No effects on testes weight, percentage of abnormal sperm, sperm count or histologic appearance of the testes. No effects on the mean number of oocytes (33.3 ± 14.4 versus 29.8 ± 8.0 in controls) A statistically significant positive response was obtained for each positive control.	Mazze *et al.*, 1983 Klimisch score 2 WOE
Non-guideline fertility study in male mice Swiss/ICR mice GLP: no N=18-21 male mice per groups mated with non-exposed females *Limitations:* - few reproductive parameters investigated - no information on general toxicity	Dinitrogen oxide (purity not stated) Inhalation (whole body), 9 weeks, 4h/day, 5d/week Control (air treatment), control (colony), 5000, 50,000, 500,000 ppm N_2O in air	General toxicity No information Reproductive toxicity No effect on litter size, abilities of males to impregnate females, no effect on foetal wastage (resorption, dead), foetal size	Mazze *et al.*, 1982 Klimisch score 2 WOE
Non-guideline study on	Dinitrogen oxide	No animal died from exposure	Land *et al.*,

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
early spermatogenesis in male mice GLP: no (C57Bl xC3H)F1 mice 5 males/group *Limitations* - Low number of animals - No information on general toxicity - 80% dinitrogen oxide is above threshold leading to hypoxia - Only 5-day exposure does not cover full spermatogenesis	(purity not specified) Inhalation, 4h/day, 5 consecutive day Control (air), 80000, 800,000 ppm N_2O in air	General toxicity not described No effects on abnormal spermatozoa	1981 Klimisch score 3 Disregarded

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Few studies are available on the effect of dinitrogen oxide on sexual function and fertility. None of the studies were performed in compliance with OECD TG for reproductive toxicity. All the studies had limitations (e.g. single dose level, low number of animals, few parameters investigated) and were performed either on males or females.

- **Female rats**

Kugel et al. (1990) exposed female rats to 0 or 300,000 ppm dinitrogen oxide for 4 days (one ovulatory cycle) by inhalation during 8 hours per day and performed 3 different experiments on 3 different groups. Reduction in fertility was noted in the females mated with non-exposed male breeders as only 6/12 females produced litters versus 12/12 in controls. No effect on pup weight or litter size was noted in exposed animals compared to controls. In the second experiment evaluating oestral cycle, 11 out of 12 exposed rats went into constant proestrus whereas all 12 controls had normal cycles. The effect was reversible by 3 weeks. In the experiment measuring various hormones, an increase in total LHRH cell count was seen in the 4 exposed females on the morning of protestrus (~4-fold) compared to the 4 controls. This effect was not observed in the four animals exposed in the morning of metestrus compared to the 4 controls. The increase in LHRH (a decapeptide manufactured by highly specialised neuroendocrine cells, key regulator of the hypothalamic–hypophyseal–gonadal axis, essential for reproductive competence) was interpreted by the authors as a decrease in release of LHRH and a subsequent increase intracellular content, rather than an increase in the actual number of LHRH producing cells.

Kugel et al. (1989) reported a reversible interruption of ovulation and a 50% decrease in fertility (6/12 exposed rats vs 12/12 control rats gave birth) following exposure to dinitrogen oxide at 500 ppm (mixed in compressed air) delivered 8 hours per day for 35 days. As only a short summary of the method and result is available in the publication, it is not possible to assess the reliability of the study.

- Male rats

In Holson et al. (1995), male rats were exposed 5 day per week for 9 weeks, 6 hours per day, at similar dose levels (3 replicates of 4 males per groups) as in the maternal study (0, 1000, 5000, 10000 ppm). Exposed males were mated with non-exposed females (paternal study). No effect on body weight or body weight gain was noted in the males. In addition, no statistically significant effect on litter size was noted compared to control. Nevertheless, the authors reported a **trend to a decrease in the number of pups per litter** in the dinitrogen oxide groups. The male used in this study were also mated with 30 non-exposed females at the end of the exposure period for a dominant lethal assay. Although no statistically significant findings (ANOVA) were noted in the number of implants per litters, number of live foetuses per litters and number of resorptions per litter, the authors noted a **tendency to an increase in the number of resorptions in the top dose group** (table 2 of Annex 1).

In Vieira et al. (1983a), two groups of 12 male Wistar rats were used in the study. One group was exposed to 5000 ppm dinitrogen oxide/air mixture (v/v) and one group was exposed to air only (control). The rats were exposed 6h/day, 5d/w for 30 days. At the end of the exposure period, each male rat was mated with 3 nulliparous female rats. Following mating, the male rats were allowed for recovery period of 6 months. Thereafter, each male rat was mated with 3 female nulliparous rats. At birth, the number of each litter was recorded and all litter mates were examined macroscopically for gross defects. The young rats were weighed and measured at weekly interval for 8 weeks.
A statistically significant decrease in litter size was seen after dinitrogen oxide exposure compared to control. These findings were not observed 6-month after recovery. According to the authors, the litter size showed that in the control group one litter numbered nine offsprings while the remaining 35 mothers had litters ranging from 11-15. This pattern was similar to that in the group following the recovery period, which showed one litter with eight offsprings and the remaining litters ranging from 10-14. In contrast, in the group mated immediately after exposure to dinitrogen oxide one litter comprised 14 offsprings but the remaining 35 litters ranged between two and six offsprings. With regards to body weight, there was a significant difference from week-3 onward in the offsprings belonging to the group exposed to 5000 ppm dinitrogen oxide compared to control. A significant decrease in tail length and body length was also noted in this group. The effects were not observed in the offsprings belonging to the group of exposed males that were allowed 6-month recovery. It may be noted that the effect on litter size was the sole parameter reported and no other parameters (e.g. number of *corporea lutea*, number of implants, early and late resorptions) were reported in the published study to support the interpretation of the toxicological significance of the effect.

Table 12: Summary of litter size in Vieira et al. (1983a)

Group	**Control**	**N_2O, 5000 ppm initial mating**	**N_2O, 5000 ppm 6-month recovery**
No. of litters	36	36	36
No. of born rats	382	252	380
Litter size	Mean: 12 Range: 9-15	Mean: 7* Range: 2-14	Mean: 11 Range: 8-14

*p<0.001

Kripke et al. (1976) exposed male rats (n=4-6 per group) 8h/d (intermittent) for 1 to 35 days of exposure or 24h/d (continuous) for 32 days with 3, 6 or 10-day recovery to a mixture of 200,000 ppm dinitrogen oxide, 20% O_2 and 60% N_2. **Absolute testis weight was decreased in both groups (continuous or intermittent exposure) compared to control. Damage and destruction of spermatogenic cells were observed in the exposed groups. Incidence and severity** was more pronounced in the continuous exposed group compared to the intermittent exposure group. The effects were more frequent and more pronounced with exposure duration and by day 14, the findings were observed in all rats of both exposure groups. No effects were noted in the control group. No effect in Leydig cells and supporting cells within the tubules was reported, as well as for testosterone levels in any group. The study is considered of limited reliability. Indeed, only one dose was used, no information was provided on the source of test material, the duration of exposure was not long enough to cover the whole spermatogenic cycle. However, even with this short duration of exposure, effects on some reproductive parameters were observed. No detailed results (incidence, grade of the lesions) were provided in the publication.

- Mice

There are three studies available in mice, performed in the same laboratory. The fourth study from Land et al., 1981 was disregarded as the study was not found reliable (few animals per group, one dose-level above hypoxia). The three relevant studies did not provide evidence of potential effect of dinitrogen oxide in fertility or sexual function in mice up to 500,000 ppm:

- No effects were observed on testis or ovaries in mice exposed to dinitrogen oxide up to 500,000 ppm during 4h per days, 5 days per week for 14 weeks (Rice et al., 1995).

- No effects compared to controls were noted following a specific investigation of male testes and spermatogenesis and female mice oocyte count in Mazze et al. (1983). The same experimental condition and dose levels as in Rice et al. (1995) were used (14-week exposure, 4h/d, 5d/w) in this study.

- Mazze et al., 1982 did not report effect in the ability of males to impregnate females or in litter size, foetal wastage (dead and resorbed) or foetal size after 4h exposure per day of male mice for 9 weeks (5 days per weeks) up to 500,000 ppm dinitrogen oxide.

No fertility study in female mice exposed to dinitrogen oxide is available.

- Human data

Two human retrospective cohort studies investigated the potential effect of dinitrogen oxide on fertility in midwifes (Alhborg et al., 1996) or in dental assistants (Rowland et al., 1992).

In Alhborg et al. (1996), 3358 women midwives, born between 1940 and 1989 and member of the Swedish Association of Midwives, were included in a retrospective cohort study. Exposure was based on the average number of deliveries per month at which the midwife assisted where N_2O was used and on the type of work and work schedule (full-time or part-time). **In a multivariate analysis, including all the non-occupational variables, it was found that age, pregnancy order, and previous pill use or fertility problems were significantly associated with fecundability.** Midwives who worked rotating shifts had reduced fertility compared to midwives who worked day-time. Midwifes that had assisted at > 30 deliveries with N_2O per month had longer time to pregnancy than those reporting less or no N_2O exposure. Fecundability ratio was calculated to be 0.63 (95% CI: 0.43-0.94). Adjustment was done for several variables (smoking, age, contraceptive pill, history of pelvic inflammatory disease, number of previous sexual partners, frequency of intercourse, race). **Although expected, there was no information on potential co-exposure in the study.**

In Rowland et al. (1992), questionnaires were sent to 7000 female dental assistants, registered in California in 1987 and working full-time (> 30h/week). Only 69% responded to the questionnaire in which the authors noted a considerable amount of missing data. Exposure was determined considering the number of hours of exposure per weeks and the presence or absence of a scavenging system. Several adjustments factors were considered in the study (oral contraceptives, number of cigarettes, age, history of pelvic inflammatory disease, number of sexual partners, frequency of intercourse, race). No relation was found between scavenged N_2O exposure and fecundability. Reduced fertility was only noted in women that reported an exposure more than 5h per week to unscavenged N_2O. The OR was 0.41 (95% CI: 0.23-0.74). Potential co-exposure (e.g. mercury exposure) was not considered in the study.

Overall, although potential fertility effects were seen in two studies, characterisation of N_2O exposure levels and co-exposition impaired a firm conclusion on the observed effects.

- Mechanistic information

N_2O irreversibly inactivates methionine synthase function by oxidation of the Co^+ ion of vitamin B12 in all species. Methionine synthase is a vitamin B12-dependent enzyme involved in folate metabolism. This enzyme converts L-homocysteine and 5-methyltetrahydrofolate into L-methionine and tetrahydrofolate, respectively, *via* a methylation process. Methionine is important for DNA and RNA synthesis, for histone methylation, synthesis of neurotransmitters and myelin, among other products. As a consequence, inactivation of methionine synthase results in a depletion of methionine and tetrahydrofolate, which are required for DNA synthesis and myelin production. There are no data available investigating the inhibition of methionine synthase function on effect on fertility in animals.

According to Kugel et al. (1990), the lack of LHRH release can be explained on the basis of an increase in opioids and substance P in areas of the brain where LHRH is synthesized. The authors noted that dinitrogen

oxide may produce its analgesic and sedative effects through an increase in endogenous opioid which in turn can have an inhibitory effect on the release of LHRH cells in the hypothalamus. Further data would be needed to support this hypothesis.

10.10.3 Comparison with the CLP criteria

For potential classification on sexual function and fertility, criteria from CLP guidance (ECHA, 2017) were applied.

- Adverse effects on sexual function and fertility are described as "*Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems*." *(ECHA, 2017).*
- *Known human reproductive toxicant. "The classification of a substance in this Category 1A is largely based on evidence from humans."*

A few studies are available in human. The positive results (decreased fecundability ratio) obtained in these studies are not sufficient by themselves to serve as basis for a classification. Indeed, potential co-exposure and the absence of quantitative characterisation of N_2O exposure in these studies lead to uncertainties on the results. Therefore, category 1A is not warranted for dinitrogen oxide.

- *Presumed human reproductive toxicant. "The classification of a substance in this Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate*" (ECHA, 2017).
- *Suspected human reproductive toxicant "Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects".*

Two experimental studies from the same authors have investigated effects of dinitrogen oxide in female rat on reproductive function. The studies used different exposure condition: 300,000 ppm dinitrogen oxide, 8h/day for 4 day or 500 ppm, 8h per day for 35 days. Reproductive cycle abnormalities and marked decreased fertility (by 50% vs control) was observed in female rats in both studies (Kugel et al., 1989, 1990). In these studies, there were several limitations: no information on general toxicity in dams, the number of animals per group was low, only one-dose level, few parameters investigated. Regarding parental toxicity, the CLP guidance (ECHA, 2017) stated 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*". In the developmental toxicity studies (see next section), no excessive toxicity was found up to 300,000 ppm in rats. Marked toxicity, as described in the guidance, can therefore be reasonably excluded and the effect on oestrous cycle and fertility is not expected to be a consequence of other unspecific toxic effect. In addition, there is no indication that the effect would not be relevant to human. Although occurring at high dose levels in one study (300,000 ppm), similar findings were noted by the authors at lower dose levels (500 ppm in Kugel et al., 1989).

In mice, no histopathological findings in ovary and no effects were noted in primary oocytes count following repeated exposure up to 500,000 ppm dinitrogen oxide (Rice et al., 1985, Maze et al., 1983). Fertility and

oestrous cycle was not investigated in female mice. Therefore, it is not possible to disregard the effect observed in rat based on the negative results observed in mice.

Studies investigating effects of 200,000 ppm dinitrogen oxide on male rat reproductive organs showed significant effects on sperm and testes (Kripke et al., 1976). The main limitation in this study is the use of a single dose level.

Effects observed in litter size in dams from male rats exposure to 5000 ppm dinitrogen oxide (Vieira et al., 1983a) is of concern. Although no statistical significant effects were noted on litter size in Holson et al., 1995, the trend observed on a decreasing number of pups per litter noted at 10,000 ppm may support an effect of concern although the study raised some uncertainties on NOAEC and LOAEC for this effect.

No effects on male fertility and sexual function (testes, spermatogenesis) were observed in mice in any of the three available studies (Mazze et al., 1983, Mazze et al., 1982, Rice et al., 1985). Nevertheless, only a few parameters were investigated in these studies.

In conclusion, the decreased fertility and oestrous cycle changes observed consistently in females rats in two studies leads to clear concern on female fertility. In addition, the effects observed on testis and spermatogenesis in Kripke et al., 1976 and the decrease in litter size in Vieira et al., 1983a support potential male fertility effects. The DS has no explanation with regards to the absence of effect in mice compared to rats, leading to some uncertainties. Category 2 is therefore considered more appropriate than category 1B due to the limited number of parameters investigated in the studies and the absence of effects in mice. Dinitrogen oxide warrant to be **classify as Repr. 2, H361f.**

10.10.4 Adverse effects on development

Table 13: Summary table of animal studies on adverse effects on development in rats in prenatal developmental toxicity studies, **continuous exposure studies (23-24h per day)** (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline prenatal developmental toxicity study GLP: no Sprague-Dawley rats N=25 in exposed group and 30 in control group Sacrifice: GD 20 *Limitations* - Only one dose 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.	Dinitrogen oxide (medical grade) Inhalation, whole body 24h exposure at GD8 0, 600,000 ppm N_2O mixed with O_2 and air	<u>Maternal toxicity</u> No statistically significant difference in mean bw on GD 20 (334g vs 368 g in controls). Mortality in one out of 25 exposed dam. <u>Developmental effects:</u> - ↑stat. sign. Foetal resorptions/litter (48% vs 5% in controls) - ↓ stat. sign. Live foetuses/litter (mean 6.5±4.1 vs 12±2.1 in controls) - ↓ stat. sign mean number of foetuses/litters - ↑ stat. sign major visceral malformations - ↑ stat. sign minor visceral anomalies - ↑ stat. sign minor skeletal anomalies and variants	Fujinaga et al., 1991 Klimisch 2 WOE
Non-guideline developmental toxicity study GLP: no Sprague-Dawley rats N = 35/group Sacrifice: 4-6 rats on GD11, 12, 13, 14, 15, 16, 18 and GD20 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	Dinitrogen oxide (medical grade) Inhalation, whole-body 24h exposure at GD8 0, 700,000-750,000 ppm N_2O mixed in air and oxygen	<u>Maternal toxicity</u> No information <u>Developmental toxicity</u> Statistically significant increase in embryo-foetal mortality rate on GD 14 and onward compared to control Statistically significantly increase in altered laterality at all stage compared to controls	Fujinaga et al., 1990 Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
not provided. - No information on foetuses weight - Single dose level, single exposure - No information if experimenters were blind to treatment			
Non-guideline developmental toxicity study in rat GLP: no Sprague-Dawley rats 20 rats/group, 30 controls Sacrifice: GD20 Limitations - Low number of litters examined - Lack of details on maternal toxicity (food consumption, clinical signs, weight) - Only one dose level - No information if experimenters were blind to treatment	Dinitrogen oxide (medical-grade) Inhalation, whole-body exposure Exposure: 24h at GD 6, 7, 8, 9, 10, 11 or 12 0, 600,000 ppm N_2O mixed in air and oxygen	<u>Maternal toxicity</u> Mortality in 2 to 4 dams per groups except in control and GD 10 exposed group. Mid sedation. Decrease (stat. sig.) mean maternal body weight at caesarean section in all treated groups (Table 8 in confidential Annex I, no further information) <u>Developmental toxicity</u> - No effects on the number of implantations, live foetuses, mean foetal weight, sex ratio. - Increased % of resorptions per litter on GD8 and 11 (Stat. sign.); - Increased skeletal malformations following exposure on GD 9 (stat. sign.) - Increased skeletal variations following exposure on GD 8 (stat. sign.). - Increased visceral malformations and minor visceral anomalies when dams exposed on GD8 or GD9 (stat. sign.)	Fujinaga et al., 1989 Klimisch 2 WOE
Non-guideline developmental toxicity study in rat GLP: no 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 N2O +	Dinitrogen oxide (medical grade) Experiment 1 and 2: Inhalation, whole body, 24h at GD 8 Control, 500,000 ppm (experiment 1), 750,000 ppm N_2O (experiment 2), folinic acid, 750,000 ppm N_2O + folinic acid Methionine synthase activity: 24h at GD 8 Control, 500,000 ppm N_2O, 500,000 ppm N_2O + halothane, 500,000 ppm N_2O + folinic acid Sacrifice at 24, 48 and 72h post-treatment	<u>Maternal toxicity</u> Mid sedation in both experiments at 500,000 or 750,000 ppm Statistically significant ↓ body weight due to lower number of live foetuses <u>Developmental toxicity</u> - No effect on weight of pups - Increased early and late resorptions (stat. sign.) - Increased visceral malformations (stat. sign.) - Increased minor skeletal anomalies and variants (stat. sign.) Teratogenic effect still observed with co-administration of folinic acid. No correlation with methionine synthase activity	Mazze et al., 1988 Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
halothane - 20 exposed to folinic acid + N_2O - only 5 of each of these groups were killed for the assay. Limitations - Few details on maternal toxicity (survival, food consumption) - Only one dose reported (results at 50% and 75% dinitrogen oxide 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)			
Non-guideline developmental toxicity study in rats GLP: no Sprague-Dawley rats N= 40 in controls and 30 in treated groups Sacrifice: GD 20 *Limitations* - single dose tested - single day of exposure	Dinitrogen oxide (medical grade) Inhalation, whole body 24h on GD-8 0, 500,000 ppm N_2O mixed in oxygen and air	<u>Maternal toxicity</u> No mortality Mild sedation Decreased body weight gain on GD 12 and 20 compared to controls (stat. sign.). No significant effect on GD 6, 8, 9, 14, 16. <u>Developmental toxicity</u> Statistically significant 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)	Fujinaga et al., 1987 Klimisch 2 WOE
Non-guideline developmental toxicity study in rats GLP: no Sprague-Dawley rats N= 34-40 in controls and 24-30 in treated groups Sacrifice: GD 20	Dinitrogen oxide (medical grade) Inhalation, whole body 24h on GD-8 0, 350,000, 500,000 ppm N_2O mixed in O_2 and air	<u>Maternal toxicity</u> - Mild sedation of dams. - Significant decreased body weight gain on GD 6-21 compared to controls (135g in controls compared to 106 g 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 body weight gain was not affected during	Mazze et al., 1987 Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Limitations - Two dose levels - single day of exposure		the experiment at 350,000 ppm. <u>Developmental toxicity</u> At 500,000 ppm: - Increased foetal resorptions and post-implantation losses (stat. sign.) - Increased minor and major visceral abnormalities (stat. sign.) - Increased minor skeletal anomalies (stat. sign.). No effects at 350,000 ppm.	
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=2-10 per groups Limitations - Single dose tested - No information on age or weight of animals at reception - No explanation on the differences on the number of animals between groups - unclear number of animals per dose groups - Very low number of exposed dams per groups (e.g. only 3 dams exposed at GD 11-15) - No information on survival - Visceral examination not performed -limited skeletal examination	Dinitrogen oxide (purity not specified) Inhalation, whole body, 24h on GD10-14 or 15-19 0, 750,000 ppm dinitrogen oxide mixed with oxygen	<u>Maternal toxicity</u> - Statistically significant decrease in body weight following exposure compared to controls (376 g in control vs 323 g in GD 10-14 dinitrogen oxide group and 339g in control vs 273g in dinitrogen oxide GD15-19 group) <u>Developmental toxicity</u> - Statistically significant decreased in foetal weight - No effects on litter size or resorptions	Tassinari et al., 1986 (Sub-study results: continuous exposure) Klimisch 3 Disregarded
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats Sacrifice: GD 20 N= 10 exposed and 23 controls *Limitations:* - Low number of exposed rats per dose groups - No information on source of test material	Dinitrogen oxide (purity not specified) Inhalation, whole body exposure 24h on GD8 0, 700,000-750,000 ppm dinitrogen oxide	<u>Maternal toxicity</u> No information. <u>Developmental toxicity</u> - No effects on resorptions, live foetuses and number of implants. - Statistically significant decrease in foetal and placental weight. - ↑ Statistically significant increase in delayed development in dinitrogen oxide group (decreased mean number of sternebrae and caudal vertebrae). - Stat. sign. increase in skeletal malformations (e.g. cervical vertebral malformations) - Increased methyl folate concentration in	Keeling et al., 1986 Klimisch 2, WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
- Visceral examination not performed - No information if experimenters were blind to treatment - No information on maternal toxicity - Only one dose level - No information on room temperature and humidity		dinitrogen oxide exposed group.	
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats Sacrifice: GD 20 N=25-62/exposed group (pooled results) and 160 controls (pooled results) *Limitations:* - no detailed on maternal toxicity - no detailed on the results for individual experiments (pooled results)	Dinitrogen oxide (medical grade) Inhalation, whole body 24h on GD-8 Experiment I: 0, 750,000 ppm dinitrogen oxide Experiment II: 7500, 75000, 750000 ppm Experiment III (mated in house): 0, 750,000 ppm Experiment IV: 0, 250,000, dinitrogen oxide mixed in oxygen and air (food and water deprivation or food and water *ad libidum*)	<u>Maternal toxicity</u> No effects up to 250,000 ppm At 750,000 ppm: impaired food and water consumption, rats were drowsy, impaired motor coordination (no information on statistical significance) <u>Developmental toxicity</u> (750,000 ppm) - Increased in any external abnormalities, runts and major external malformations (stat. sign.) - Increased in any skeletal abnormalities, major malformations and malformations in rib/vertebra, increased in variant (extra lumbar rib, cervical rib) (stat. sign.) - Increased in any major internal malformations (stat. sign.) - increased ocular malformation (reported in the text, no tabular information) - Increased in total, early and late resorptions (stat. sign.) - Decreased number of live foetuses per dams (stat. sign.) At 75,000 ppm: stat. sign. Increase in minor skeletal anomalies only (extra lumbar ribs and cervical ribs)	Mazze et al., 1984 Klimisch 2 WOE
Non-guideline prenatal developmental toxicity studies Rats N=10 per group Limits: no information on statistical significance, secondary literature	Dinitrogen oxide (no information on purity) Inhalation, Whole-body exposure 24h/day GD1-21 0, 50,000, 150,000, 200,000 ppm dinitrogen oxide	<u>Maternal toxicity:</u> no information <u>Developmental toxicity</u> - Increased resorption rate, - decreased litter size, foetal body length and body weight at ≥150,000 ppm - Increased wavy ribs, fused, additional or absent ribs, separation of vertebral ossification centres at 200,000 ppm.	Rao et al., 1981, Tong et al., 1982 Klimisch 4 (as reported in MAK, 2015) WOE
Non-guideline developmental toxicity study in rats GLP: no Sprague-Dawley rats	Dinitrogen oxide (no information on purity) Inhalation, whole body	Maternal toxicity No information Developmental toxicity - Increased foetal losses (4-fold compared to	Lane et al., 1980 Klimisch 4

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Sacrifice: GD20 N=8/group Limitations: secondary literature (abstract), few information available)	24h on GD8 700,000 to 750,000 ppm dinitrogen oxide	control) - Increased anomalies (encephalocele, hydrocephalus, anophthalmia, microphthalmia, gastroschisis and gonadal agenesis)	WOE
Non-guideline developmental toxicity study in rats GLP: no Wistar rats N=12/group *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)	Dinitrogen oxide (purity not specified) Inhalation, whole body, 23h/d, GD1-19 Control (air), 250, 500 and 1000 ppm	Maternal toxicity No effects on food or water consumption. Developmental toxicity - Decreased litter size at 1000 ppm (stat. sign.) - Increased resorptions at 1000 ppm (stat. sign.) - Decreased 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)	Vieira et al., 1980 Klimisch 2 WOE
Non-guideline developmental toxicity study in rats GLP: no Wistar rats N=12/group Limitations - Low number of animals - Only one dose group - Few information on environmental conditions - Few internal organs and skeletal examinations - No information on maternal toxicity - No details on skeletal abnormalities (incidences in each groups, details of the anomalies)	Dinitrogen oxide (purity not specified) Inhalation, whole body, 23h/d, GD1-19 Control (air), 5000 ppm	Maternal toxicity No reported Developmental toxicity - Statistically significant decrease in litter size 4/12 dams had full resorptions (vs 0 in controls) - Statistically significant increase in skeletal malformations (ribs). Foetuses with malformations were smaller that their litter mates or controls. - Marked statistically significant reduction in mean crown-rump length of exposed foetuses. - Statistically significant decrease in mean foetuses weight.	Vieira et al., 1979 Klimisch 2, WOE
Non-guideline developmental toxicity study in rats GLP: no Rats N=5-10 per groups Limitations	Dinitrogen oxide (purity not specified) Inhalation, whole-body, 24h/day on GD8-13 or 12-19 0, 1000, 15,000 ppm N_2O	Maternal toxicity: No information Developmental toxicity: Increased foetal death compared to controls.	Corbett et al., 1973 (Sub-study results on continuous exposure only)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
- strain, age or weight of rats at the beginning of the study not specified. - Actual dose levels lower than target dose due to leakage in the homemade chambers - low number of animals per groups - lack of methodological details - no information on maternal toxicity and health status of dams - foetal death was the only parameter investigated in the study			Klimisch score 3 Disregarded

Table 14: Summary table of animal studies on adverse effects on development in rats in prenatal developmental toxicity studies, <u>intermittent exposure studies</u> (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N= 19-50/group Limitations: - Single dose level	Dinitrogen oxide (medical grade) Inhalation, 6h/d 0; 750,000 ppm N_2O in oxygen and air GD13-15, GD10-12 or GD7-9	Maternal toxicity: ↓ in body weight gain (statistically significant in dams exposed on GD8-10, by 20%) Developmental toxicity: - ↓ Foetal weight (GD 13-15) - ↑ Resorptions and foetal wastage (dead and resorbed) in dams exposed during GD13-15 window - ↑ Major malformations and external abnormalities in dams exposed on GD 7-9	Mazze et al., 1986 Klimisch 2 WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=2-10 per groups Limitations - Single dose tested - No information on age or weight of animals at reception - No explanation on the differences on the number of animals between groups - Very low number of exposed dams per groups (e.g. only 3 dams exposed at GD 10-14) - No information on survival - Visceral examination not performed -limited skeletal examination - unclear number of animal per dose group	Dinitrogen oxide (no information on purity) Inhalation, 8h/d on GD8-12, GD10-14, GD13-14 or GD14 0; 750,000 ppm N_2O	Maternal toxicity: no effects Developmental toxicity: no effects on the number of foetuses, resorptions, foetal weight.	Tassinari et al., 1986 (Sub-study results: prenatal intermittent exposure) Klimisch 3 Disregarded
Non-guideline prenatal developmental toxicity study in rats GLP: no Female Wistar rats N=12/group 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	Dinitrogen oxide (purity not specified) Inhalation, 6h/d, 5d/w, whole gestation period (3 weeks) 0; 250; 500; 1,000; 5,000 ppm dinitrogen oxide in air	Maternal toxicity: no information Developmental toxicity: - Dose-related ↓ in litter size (statistically significant at 5000 ppm) - no effect on foetal weight or crown-rump length of foetuses. - no malformations reported.	Vieira et al. 1983b Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
pressure is unknown - few details on study method and results			
Non-guideline prenatal developmental toxicity study Sprague-Dawley rats N= 10 per groups Limitations: few information available based on secondary literature	Dinitrogen oxide (no information on purity) Inhalation, whole body 8h per day, 5d/w, GD1-21 0, 200,000 ppm dinitrogen oxide	Maternal toxicity: no information Developmental toxicity: no skeletal findings unlike after continuous exposure	Rao et al., 1981; Tong et al., 1982 Klimisch 4 (as reported in MAK, 2015) WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Rats N=30/group Limitations: few information available based on secondary literature	Dinitrogen oxide (no information on purity) Inhalation, 6-7h/d, whole gestation 0; 1,000 ppm dinitrogen oxide in air	No evidence of maternal toxicity or developmental toxicity	Hardin et al., 1981 Klimisch 4 WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=8-10 per groups *Limitations* - Low number of animals per treatment group - Animals were exposed simultaneously in the chambers - no information on how animals were sacrified - 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	Dinitrogen oxide (purity not specified) Inhalation, 8h/d, whole gestation (GD0-20) 0; 10,000; 100,000; 500,000 ppm dinitrogen oxide in air, additional stress group as control	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 ≥ 100,000 ppm - ↓ placental weight, stat. sign. at ≥ 10,000 ppm - increased foetal loss at the low and mid dose but not statistically significant and inside spontaneous range of the laboratory. No increase in foetal loss at 500,000 ppm.	Pope et al., 1978 Klimisch 2 WOE
Non-guideline developmental toxicity	Dinitrogen oxide (purity not specified)	Maternal toxicity: No information	Corbett et al.,

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
study in rats GLP: no Rats N=5-10 per groups Limitations - strain, age or weight of rats at the beginning of the study not specified. - Actual dose levels lower than target dose due to leakage in the homemade chambers - low number of animals per groups - lack of methodological details - no information on maternal toxicity and health status of dams - foetal death was the only parameter investigated in the study	Inhalation, whole-body, 8h/day on GD8-13, 14-19 or 10-19 0, 1000, 15,000 ppm N_2O mixed in oxygen and balance with nitrogen	Developmental toxicity: Significant increase in foetal death rate in rats exposed to 8 hours to dinitrogen oxide from 6am to 2 pm (group 5 and 7) at 1000 ppm. No increase was observed in group 9 exposed from 2pm to 10pm to a longer period (GD10-19).	1973 Klimisch 3 Disregarded

Table 15: Summary table of animal studies on adverse effects on development in rats in pre-postnatal developmental and postnatal (neuro)developmental toxicity studies (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline behavioral teratogenicity and dominant lethal study Sprague-Dawley rats 12 rats/group Limitations - Low number of animals per groups - limited examinations	Dinitrogen oxide (checked for purity) Inhalation, whole body 0, 1000, 5000, 10,000 ppm 6h/d, 5d/w for 9 weeks in exposed male (paternal study) and GD1-20 in exposed females (maternal study)	<u>Maternal or paternal toxicity</u> No effect on weight reported <u>Maternal and paternal behavioral studies</u>: No differences in litter size and weight of offsprings. No treatment-related effects on behavior in offsprings	Holson et al., 1995 (substudy : postnatal development) Klimisch 2 WOE
Non-guideline prenatal neurodevelopmental toxicity study GLP: no Sprague-Dawley rats N=15 or 5 per groups Limitations - No information on age or weight of animals - only one dose level - Low number of animals for neurobehavioral tests (GD13-14 exposure) - No information if experimenters were blind to treatment	Dinitrogen oxide (purity not specified) Inhalation, whole body 8h on GD14 or 8h on GD13-14 0, 750,000 ppm dinitrogen oxide in oxygen	<u>Maternal toxicity</u> No effects on weight of dams during exposure No information on survival. No effects in offspring dams <u>Neurodevelopmental toxicity</u> *Residential maze activity* - Exposure on GD 14 only: no significant changes in males and females at 1-month age. At 5-month age: significant hypoactivity in females and significant diurnal hyperactivity in males. - Exposure on GD13-14: significant exploratory and diurnal hyperactivity in the females at 1 and 5-months timepoint and in males at 1-month age. No effects in males at 5-month of age. *Time-lapse photography* - Exposure on GD 14 only: no effects in males at 1 or 5-months exposure. In females, slight tendency to hypoactivity at 5-month. - Exposure on GD13-14: In males, no effect at 1-month. At 5-month, changes in body position, ↓ frequency of standing and increased in the average duration of rearing. Change on face washing behavior compared to control. In females, evidence of hyperactivity found at 1-month and 5-months (increase rearing and walking frequencies, shorter duration of standing and sitting. Worst at 5-month)	Mullenix et al., 1986 Klimisch 2 WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=2-10 per groups *Limitations* - single dose levels - No information on age or weight of animals at reception - No explanation on the	Dinitrogen oxide (purity not specified Inhalation, whole body 8h, GD13-14 Sacrifice: PND16, 18 or 21 0, 750,000 ppm in oxygen	<u>Maternal toxicity</u> No information <u>Early postnatal developmental toxicity</u> - No effect on weight of pups. - No significant effects on auditory startle and on eye opening. - Decreased suspension reflex in female only.	Tassinari et al., 1986 (Substudy results: postnatal development) Klimisch 3 Disregarded

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
differences on the number of animals between groups. Unclear number of animals per groups - No information on survival - Visceral examination not performed -limited skeletal examination - Unclear number of dams per group			
Non-guideline pre/postnatal developmental toxicity study in rats GLP: no Wistar rats N=8/group *Limitations* - only one dose tested - Low number of animals/groups - No information on maternal toxicity - poor reporting of study methods and results - few parameters investigated - No information if dinitrogen oxide concentration was checked during exposure	Dinitrogen oxide (purity not specified) Inhalation, 6h/d, 5d/w 3 control groups, N2O 1% groups: Group 1: whole gestation, Group 2: weeks 1 and 2 of gestation Group 3: 1st week of gestation	<u>Maternal toxicity:</u> No information <u>Developmental toxicity</u> Stat. sig. decreased in litter size in the dinitrogen oxide exposure groups Stat. sig. decrease in body and tail length, body weight in dinitrogen oxide exposed groups (pooled results)	Vieira et al., 1978 Klimisch score 3 Disregarded (missing information on study methods)

Table 16: Summary table of adverse effects on development induced by dinitrogen oxide in other species than rats in prenatal developmental toxicity studies (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline Pre-/postnatal developmental toxicity study GLP: no information SW mice 10 litters examined per groups Limitations: - low duration of exposure (4h/day), - low number of animals in the neurobehavioral studies - low level of details	Dinitrogen oxide (no information on purity) Inhalation, GD6-15, control, 50,000, 150,000 and 300,000 ppm in air.	Maternal toxicity: no information Developmental toxicity: - No effect on reproductive indices, survival, physical milestones of development - No change in post-natal brain weight (PND126 or 127) - No effect on rotating rod - Significant hyporeactivity of the startle reflex in response to acoustic or tactile stimuli (PND 95) in all dinitrogen oxide group compared to control	Rice et al., 1990 Klimisch score 2 WOE
Non-guideline pre/post natal developmental toxicity study DUB/ICR mice N=5-6 /group Analysis: physical landmark and behavioral measures Limitations: few information available based on secondary literature	Dinitrogen oxide (no information on purity) Inhalation, GD14, 6h/d in dams, 750,000 ppm Litters PN2 exposed 4h/d	Maternal toxicity: After exposure on GD 14 females resumed activity within a few minutes. Developmental toxicity - No effect on pup body weight; except for an increased pup weight on PN 2 - Ear unfolding retarded after pre- and postnatal exposure air and surface righting were retarded during test period after pre- and postnatal exposure - Locomotion was affected after pre- and postnatal exposure - Total activity was affected after postnatal exposure	Koëter et al., 1986 Klimisch 4 (As reported in HCNL, 2000) WOE
Prenatal developmental toxicity study Guideline: similar to OECD 414 GLP: no Swiss/ICR Mice N=24 to 32 females/group *Limitations* -Exposure only 4h/day	Dinitrogen oxide (purity not specified) Inhalation, GD 6-15, 4h/day Control (air treatment), control (colony) 5000, 50,000, 500,000 ppm Positive control: retinoic acid (gavage), n=11 dams	Maternal toxicity Dams appeared not adversely affected by treatments. No Excitement or general anaesthesia observed. No effects among groups on maternal weight gain. Developmental toxicity No effects on litter size, foetal wastage (dead and resorbed), foetal size. No treatment related abnormalities (external, skeletal, visceral).	Mazze et al., 1982 Klimisch score 2 WOE
Non-guideline prenatal developmental toxicity study GLP: no Golden Sirian Hamster N=5/group Limitations:	Dinitrogen oxide (no information on purity) Inhalation, whole-body, 24h GD 7, 8, 9, 10 or 11 0, 700,000, 800,000, 900,000, 950,000 ppm	Maternal toxicity: no information Developmental toxicity: - Increased number of malformations, not statistically significant, no dose-relation - No effect observed at 700,000 ppm. - Increased number of resorption on GD7, 10 and 11 at ≥900,000 ppm, which is hypoxic.	Shah et al., 1979 Klimisch score 4 Disregarded

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
- few information as secondary literature, based on abstract only, - 800,000 ppm is above the hypoxic concentration of dinitrogen oxide, - only 5 animals per groups			

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

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

- ***Mazze et al., 1984, 1987, 1988 and Fujinaga et al., 1987, 1989, 1990, 1991***

In a series of seven studies from the same laboratory, Sprague-Dawley rats were exposed 24 hours via whole body inhalation to dinitrogen oxide between 7500 to 750,000 ppm on specific days of gestation (Mazze et al., 1984, 1987, 1988, Fujinaga et al., 1987, 1989, 1990, 1991). Administration was done as follow (considering plug day = day 0 of gestation):

- Single administration on day 8 of gestation using single dose level at either 500,000, 600,000 or 700,000-750,000 ppm dinitrogen oxide (Fujinaga et al., 1987, 1990 and 1991),
- Single administration on day 8 of gestation using several dose levels in Mazze et al. (1984, 1987, 1988) to investigate dose-response: from 7,500 to 750,000 ppm dinitrogen oxide.
- Exposure 24h to 600,000 ppm 24h either at GD 6, 7, 8, 9, 10, 11 or12 (Fujinaga et al., 1989).

A similar protocol was used in the seven studies. On the chosen day or days of pregnancy, dinitrogen oxide was administered to the rats. They were placed in the chambers in their cages without food or water. Medical grade dinitrogen oxide and oxygen were delivered to the chambers and were mixed with room air to achieve the desired dinitrogen oxide and oxygen concentrations.
On day 20 of pregnancy, 1 day before delivery was expected, rats were sacrificed by carbon dioxide inhalation and Caesarean sections were performed except in Fujinaga et al., 1990 where rats were randomly killed each days 11-16, 18 and 20 of gestation. The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and each foetus was examined for external abnormalities. Half of the foetuses were examined for skeletal examination and half of the foetuses for visceral examination. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with vital functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies.

In order to exclude some potential bias, Mazze et al. (1984) included additional groups to investigate if starvation, shipping dams while pregnant and restraining the dam during exposure could be a potential cause of effect.
In addition, in order to investigate the potential mode of action, other chemical substances were used in some of these studies either alone or co-administered with dinitrogen oxide: phenoxybenzamine, isoflurane,

fentanyl, halothane, enflurane, folinic acid. Summaries of the author's hypothesis are included in the mode of action section below.

- *Developmental toxicity*

Developmental toxicity was consistently observed in the studies, including increased early and late resorptions, with a resulting decrease in the number of live foetuses per litter, skeletal abnormalities and malformation, visceral abnormalities and malformations.

In the six studies investigating the effect of dinitrogen oxide following 24-hour exposure on GD-8, a dose-related increase in early and late resorptions was observed. The effect was statistically significant at ≥500,000 ppm. The authors of the studies identified 350,000 ppm as a NOAEC for this effect.

Table 17: Resorptions observed in controls following 24-hour air exposure on gestation day 8 and sacrifice at GD 20 (Mazze et al., 1984, 1987, 1988, Fujinaga et al., 1987, 1989, 1991)

	Control[1]	**Control[2]**	**Control[3]**	**Control[4]**	**Control[5]**	**Control[6]**
Dams	160	85	237	67	23	28
Foetuses	1705	954	433	694	261	337
% total resorptions per dams	5.5±12.1	0.59±1.2	4.9±10	7.4±19	3.4	5.0±5.6
% early resorptions per dams	5.2±11.7	na	4.9±10	7.3±19	na	na
% late resorption in dams	0.3±2.3	na	0	0.1±0.8	na	na

*p<0.05 respective to the respective control of the study; na: information not available ;[1] Mazze et al., 1984; [2] Mazze et al., 1987; [3]Fujinaga et al., 1987; [4] Mazze et al., 1988;[5] Fujinaga et al., 1989; [6]Fujinaga et al., 1991

Table 18: Resorptions observed following 24-hour dinitrogen oxide exposure on gestation day 8 and sacrifice at GD 20 (Mazze et al., 1984, 1987, 1988, Fujinaga et al., 1987, 1989, 1991)

Dinitrogen oxide (ppm)	**7500[1]**	**75,000[1]**	**250,000[1]**	**350,000[2]**	**500,000[2]**	**500,000[3]**	**500,000[5]**	**600,000[6]**	**500,000-750,000[4]**	**750,00**
Dams	27	25	49	41	41	26	36	22	46	75
Foetuses	285	293	572	479	306	241	301	150	301	437
% total resorptions per dams	9.2±16	9.3±10	4.4±10	1.5±2.2	10±8.5*	25±31*	37*	48±32*	35±231	39±3
% early resorptions per dams	9.2±16	8.9±10	3.8±10	na	na	18±20*	na	na	35±31*	37±3
% late resorption in dams	0	0.3±1.5	0.6±2.3	na	na	6.8±12*	na	na	8.8±14*	2.5±7.

*p<0.05 respective to the respective control of the study; na: information not available; [1] Mazze et al., 1984; [2] Mazze et al., 1987; [3]Fujinaga et al., 1987; [4] Mazze et al., 1988;[5] Fujinaga et al., 1989; [6]Fujinaga et al., 1991

Fujinaga et al., (1989) identified two critical period of exposure for resorptions, one on day 8 of gestation and one after exposure on gestational day 11. Fujinaga et al., (1990) found that in the dams exposed on gestational day 8 during 24 hours at 700,000-750,000 ppm dinitrogen oxide, the increase in resorptions was first observed in the group sacrified on gestational day 14 (not in animals scarified at GD 11, 12 or 13) and then the rate remained constant (in animals sacrified on each days 15, 16, 18 and 20 of gestation).

As detailed in the table below, a statistically significant marked increase in skeletal minor anomalies and malformations, visceral minor anomalies and malformations were noted following continuous exposure on GD8 at ≥ 500,000 ppm dinitrogen oxide.

Table 19: Summary of the statistically significant effects observed in the studies following dinitrogen oxide exposure on GD8 during 24h

Dinitrogen oxide (ppm)	**7500[1]**	**75,000[1]**	**250,000[1]**	**350,000[2]**	**500,000[2]**	**500,000[3]**	**500,000[5]**	**600,000[6]**	**500,000-750,000[4]**	**750,000[1]**
No. rats examined	27	25	49	41	39	26	13	22	40	62
No. Foetuses examined	285	293	572	479	306	123	100	150	301	437
Visceral major malformations	-	-	-	-	+	+	+	+	+	+
Visceral minor anomalies	-	-	-	-	+	-	+	+	-	-
Skeletal major malformations	-	-	-	-	-	+	-[$]	-	+	+
Skeletal minor anomalies	-	-	-	-	+	+	+	+	+	+
External abnormalities	-	-	-	-	-	-	-	-	-	+

*p<0.05 respective to the respective control of the study; na: information not available; [1] Mazze et al., 1984; [2] Mazze et al., 1987; [3]Fujinaga et al., 1987; [4] Mazze et al., 1988;[5] Fujinaga et al., 1989; [6]Fujinaga et al., 1991; $: increased following exposure to dinitrogen oxide on GD9

Regarding the malformations, some of the studies reported details on the type of malformations observed (See annex I for details on incidences):

- Cardiac anomalies (Fujinaga et al. 1991), specified as “right sided aortic arch” in (Fujinaga et al., 1991, Mazze et al., 1988, Fujinaga et al., 1987),
- Situs inversus (Fujinaga et al., 1991),
- Ocular malformations (Mazze et la., 1984),
- Hydrocephalus (Fujinaga et al., 1991, 1989),
- Ribs and vertebrae (Fujinaga et al., 1989),
- Limb deformities (Mazze et al., 1984).

Alteration of body laterality was specifically investigated in Fujinaga et al. (1990) (Table 7 of Annex I), and was significantly altered compared to controls (side of tail flexion, side of body from which the umbilical artery emerged, side of body that face placenta, side to which the aortic arch curved).

The main minor visceral anomalies cited was left sided umbilical artery (Fujinaga et al, 1991, 1989)
In addition, skeletal anomalies such as cervical ribs and 14th rudimentary rib were also increased (Fujinaga et al., 1991, 1989, Mazze, 1988).

- *Maternal toxicity*

Maternal toxicity was not described in all of the studies but was detailed in some of them. Except in one study (Fujinaga et al., 1989), no increased death or significant morbidity was noted in rats exposed continuously to dinitrogen oxide on GD 8 or other specific day of gestation up to 750,000 ppm.

In Fujinaga et al. (1991), no statistically significant decrease in body weight was noted in dams exposed 24h on GD8 to 600,000 ppm dinitrogen oxide.

Table 20: Summary of maternal weight noted in Fujinaga et al. (1991)

	Control	600,000 ppm dinitrogen oxide
No. rat studied/ examined on day 20	30/30	25/24
Mean bw of rats before exposure (g)	249	248
Mean bw after exposure (g)	226	217
Mean bw on GD 20	368	334

In Fujinaga et al. (1989), in addition to the deaths observed, the mean body weight was decreased in the exposed group (600,000 ppm) compared to controls. The decrease was observed even in the absence of increase in mean % of resorptions per litters. To be noted that the cause of deaths was unknown to the authors.

Table 21: Summary of a selection of maternal variables and % of resorption (Fujinaga et al.; 1989)

Gestation day	Control	6	7	8	9	10	11	12
No rats that died during exposure	0	2	2	2	2	0	4	3
Mean body weight of pregnant rats (g)	352	309*	318*	321*	330**	322*	296*	313*
Mean % resorption per litter	3.4	18.9	5.5	36.8*	11.6	16.5	37.3*	20.1

In Mazze et al. (1988), a decrease in body weight was noted in rats exposed to 500,000 ppm or 750,000 ppm dinitrogen oxide. It is stated by the authors that almost all the difference could be explained by the fewer number of live foetuses that the dams carried. Corrected maternal body weight was however not provided.

Table 22: Body weight changes and body weight in dams (Mazze et al., 1988).

Dinitrogen oxide (ppm)	Control	500,000 or 750,000 ppm
Weight (g), GD4	205	206
Weight (g), GD20	362	332*

In Mazze et al. (1987), dams exposed to dinitrogen oxide appeared only mildly sedated at 350,000 ppm and 500,000 ppm. Decreased body weight gain was significant compared to control in the 500,000 ppm dinitrogen oxide group, but not at 350,000 ppm. No information on corrected maternal body weight was provided in the published study. Thus, it is not possible to exclude that the body weight effects may have been related, at least partly, to the decreased litter size observed at the top dose (7.7 vs 11.2 in controls).

Table 23: Body weight changes and body weight in dams (Mazze et al., 1987).

Dinitrogen oxide (ppm)	Control	350,000	500,000
Weight (g), GD5	213	212	212
Weight (g), GD20	351	357	321*
Weight gain GD6-21	135	139	106*
Weight loss during the 24h exposure	23	24	29*

*p<0.05

In Fujinaga et al. (1987), at 500,000 ppm, a statistically significant decrease in body weight of dams compared to control was noted on GD12 of pregnancy (241g±12 vs 254g ±17) and GD 20 (328g±24 vs 348g±32 in controls). No significant effect were noted on weight on GD9 GD14 and GD16 after exposure compared to control.

Overall, decrease body weight compared to control was noted in all studies at ≥ 500,000 ppm. Part of this effect may have been due to embryo-foetal lethality. Nevertheless, in none of the studies corrected body weight was available.

- ***Keeling et al. (1986)***

Keeling et al. (1986), exposed rats for 24 hours on GD8 (considering vaginal plug = day 0 of pregnancy) to 700,000-750,000 ppm dinitrogen oxide mixed in oxygen. No increase in resorptions was noted in the study. Foetal weight and placental weight was decreased in a statistically significant manner compared to control. In addition, a statistical significant increase in skeletal malformations (mainly cervical vertebrae) and delayed development was observed in the dinitrogen oxide exposed group.

- ***Rao et al. (1981)***

In Rao et al. (1981), dams were continuously exposed during GD1 to 21 to dinitrogen oxide. The following findings were noted at 150,000 ppm: decreased litter size, foetal body length and foetal body weight. In addition, at 200,000 ppm, an increase in the number of skeletal findings was noted (wavy ribs, fused, additional or absent rib, separation of vertebral ossification centres). No information on maternal toxicity was available.

- ***Vieira et al. (1979 and 1980)***

Vieria et al. (1979 and 1980) exposed Wistar female rats to continuous inhalation exposure 23h/d from GD1 to 19. Doses levels were 250, 500 and 1000 ppm in 1980 and 5000 ppm in the 1979 study. No effect on food or water consumption was noted up to 1000 ppm. At 5000 ppm, maternal toxicity was not specified. Increased resorptions and decreased litter size was noted at ≥ 1000 ppm (table 26 and 27 of Annex I). Skeletal malformations were noted at ≥ 1000 ppm (ribs, vertebrae). In addition, crown-rump length was also decreased at 1000 ppm onward.

Table 24: Litter size, crown-rump measurements and foetal resorption in Vieira et al. (1980).

	Number of litters	**Number of foetuses**	**Litter size (mean+/-SD)**	**Crow-rump measurements (mm, mean+/-SD)**	**Resorptions**
Control	12	120	11±1.4	44 ±1.4	None
Dinitrogen oxide, 1000 ppm	12	66	6.3±4**	35±1.6*	4**
Dinitrogen oxide, 500 ppm	12	118	11±1.4	43±1.3	None
Dinitrogen oxide, 250 ppm	12	120	11±1.3	43±1.4	None

**p<0.01, *p<0.05

Table 25: Foetal information (Vieira et al., 1979)

Maternal rat number												
Dinitrogen oxide group												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

Liver 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 (g) mean ± SD	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	11
Controls												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Liver 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

- ***Lane et al. (1980)***

Exposure of pregnant rats to dinitrogen oxide on GD8 of gestation (day of plug=day0 of gestation) to dinitrogen oxide at 700,000/750,000 ppm causes fetal resorption, skeletal anomalies, and macroscopic lesions including encephalocele, anophthalmia, microphthalmia, and gastroschisis.

Experimental data in rats: prenatal developmental toxicity studies, 4 to 8h per day intermittent exposure

- ***Mazze et al. (1986)***

Using a similar test design as the prenatal developmental Mazze toxicity studies described above, Mazze et al. (1986) exposed Sprague-Dawley rats for intermittent 750,000 ppm dinitrogen oxide exposure, during 6h per day, during critical period of gestation: GD 13-15, 10-12 and 7-9. A statistically significant increase in resorptions was noted in rats exposed during GD 13-15 (1.32 per litter compared to 0.46 in controls). This was associated with a decrease in live foetuses per implantation. No statistically significant increase in malformation was noted in the study. Nevertheless, an increase in external abnormalities and malformations was noted in dams exposed on GD 7-9 and skeletal malformation in dams exposed on GD 13-15 was observed in dinitrogen oxide group compared to control. No historical control is available. No increase in visceral malformations was noted in this study. The type of malformation was not further detailed in the publication.

Regards to maternal toxicity, decreased in body weight gain were noted in dams exposed to dinitrogen oxide compared to controls on GD 13-15 only. There is no information on corrected body weight. This would better reflect potential maternal toxicity as the decreased may have been due to the increased foetal wastage (see table below) in the exposed group. Animals were conscious throughout the experiments in all groups.

Table 26: Maternal body weight gain and developmental toxicity in Mazze et al. (1986)

	Period	**Control**	**Dinitrogen oxide (750,000 ppm)**
Dam weight gain (g)	I	84.9	68.5*
	II	95.1	90.9
	III	113	104.2

Foetal weight (g)	I	4.58	4.13*
	II	4.52	4.46
	III	4.49	4.29
Total foetal wastage[1]	I	0.49	1.37*
	II	0.63	0.59
	III	0.62	0.32
Resorptions (no./dam)	I	0.46	1.32*
	II	0.63	0.59
	III	0.56	0.24
Major external malformations	I	0.0±0.0	0.0±0.0
	II	0.0±0.0	0.0±0.0
	III	0.0±0.0	3.2±16
Major skeletal malformations	I	0.0±0.0	1.1±4.6
	II	0.0±0.0	0.0±0.0
	III	0.0±0.0	0.0±0.0

*p<0.05, I: GD13-15, Period II: 10-12, Period III: exposure days 7-9 (plug day = day 0 of pregnancy); [1] number/dam; dead + resorbed

- ***Pope et al. (1978)***

Sprague-Dawley rats were exposed 8 hour per days during the whole gestation period to 10,000, 100,000 or 500,000 ppm dinitrogen oxide in air. Foetal delayed development was reported at ≥ 100,000 ppm. Delayed development consisted of a statistically significant decrease in foetal weight, a decrease in crown rump lengths and was also associated with a significant delay in ossification. The authors did not observe a dose-related effects on resorption and dead foetuses after dinitrogen oxide exposure.

Table 27: Litter sizes, percentage of foetal loss induced by dinitrogen oxide

	No. of pregnant rats	**Live/litter (SE)**	**% Foetal loss (SE)**
Control	8	14.3 (0.7)	0
Dinitrogen oxide, 10,000 ppm	7	12.7 (1.4)	8.2 (3.3)
Control	8	11.3 (1.3)	1.1 (0.8)
Dinitrogen oxide, 100,000 ppm	7	14.3 (0.6)	8.3 (3.7)
Control	10	13.7 (0.6)	8.1 (2.5)
Dinitrogen oxide, 500,000 ppm	10	12 (0.9)	10.4 (2.2)
Stress group	4	1.5(1.5)	91 (8.8)

Table 28: Foetal and placental weight (Pope et al., 1978)

	Foetal weight (g)	**Placental weight (g)**
Control	5.45 (0.04)	0.59 (0.01)
Dinitrogen oxide, 10,000 ppm	5.31 (0.07)	0.51 (0.01)**
Control	5.0 (0.05)	0.45 (0.01)
Dinitrogen oxide, 100,000 ppm	4.22 (0.05)**	0.42 (0.01)*
Control	5.51 (0.04)	0.47 (0.01)
Dinitrogen oxide, 500,000 ppm	4.35 (0.07)**	0.43 (0.01)*
Stress group	3.25 (0.19)	0.31 (0.02)

*p<0.05, **p<0.01

No maternal toxicity was reported as any effects on maternal body weight and food consumption was noted in the study. Only gross skeletal abnormalities were examined and no visceral examination was performed in the study.

- ***Vieira et al. (1983b)***

Decreased litter size was noted in the Vieira et al. (1983b). In this study rats were exposed 6h/d, 5d/w during the 3 weeks of the gestation period at 250, 500, 1000 or 5,000 ppm. A LOAEC based on decreased litter size was identified at 5000 ppm by the authors. The NOAEC was 1000 ppm in the study. No foetal delayed development was noted. The toxicological significance of the decrease litter size in the absence of concomitant findings (e.g. resorptions) is questionable. There was no information on maternal toxicity in this study.

Table 29: Developmental findings reported by Vieira et al. (1983b).

Dose levels (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
1,000	117	10 ± 1.2	8-13
5,000	98	7.0 ± 2.3 ***	6-10

***p<0.001

- ***Rao et al. (1981)***

In contrast with the effects observed after continuous exposure, teratogenic effects, decreased litter size, foetal body length and foetal body weight were not observed after intermittent exposure to 200,000 ppm dinitrogen oxide during the whole gestation period (8h/d, 5d/w).

- ***Hardin et al. (1981)***

No evidence of developmental toxicity was noted in the study of Hardin et al., 1981 up to 1000 ppm dinitrogen oxide, 6-7h/day during the whole gestation period.

Experimental data in rats: pre-/postnatal developmental toxicity studies

In Holson et al., 1995, males were exposed for 9 weeks, 6h/d, 5d/w to dinitrogen oxide at 0, 1000, 5000 or 10000 ppm. At the end of the exposure period, male were mated with non-exposed females. In addition, females were exposed in similar condition during the whole gestation period. No effect on litter size and weight or behaviour of pups was noted in the study. No effect were reported on offsprings from female exposed to dinitrogen oxide during the whole gestation period for 6h/d, 5d/w or from males exposed for 9 weeks. Although some positive results were obtained in some test, such as an increase in developmental activity in females at PND20 at 10,000 ppm, the authors considered that, overall, the full battery of test was negative.

Mullenix et al., 1986 exposed rats on GD 14 or GD 13-14 for 8h/d to 750,000 ppm dinitrogen oxide mixed in oxygen. Exposure on GD13-14 produced hyperactivity in both males and females. In contrast, exposure on GD14 only produced a tendency to hypoactivity in females and hyperactivity in males.

Experimental data in other species

Behavioural effects in offspring of Swiss mice were studied following exposure by inhalation to 0, 5, 15 or 35% N_2O for 4 h/day on days 6 through 15 of gestation (Rice et al., 1990). Exposures did not affect reproduction indices and survival or physical milestones of development. Body weights showed significant exposure effects that could be isolated to specific exposure groups; however, N_2O-exposed mice tended to weight more than control animals. On postnatal days 126 or 127 no effect on brain weights were observed. Ability to stay on a rotarod was not affected by prenatal N_2O exposure. Prenatal exposure to N_2O resulted in hypo-reactivity of the startle reflex on PN95 for all N_2O-exposed groups. Maternal toxicity was not described in this study.

Koëter et al. (1986) studied the effect of 750,000 ppm dinitrogen oxide by inhalation in mice. They reported delayed development, retarded surface air and air righting and total activity were affected by exposure.

No developmental effects (resorptions, litter size, malformations) were noted in mice in the published study of Mazze et al.(1982) up to 500,000 ppm dinitrogen oxide. In this study the mice were exposed 4h/d on GD6-15.

Human data:

A number of studies have been published which examined the developmental toxicity (including teratogenicity) of dinitrogen oxide in humans.

Abortion

Six studies were identified a spontaneous abortion risk specifically related to N_2O exposure. In all the studies, exposure concentrations were poorly defined and no measurement was available. Therefore, these studies are only described briefly for information.

No effect was observed in the two most recent cross-sectional studies (Eftimova et al., 2017 and Uzun et al., 2014) but a high risk of bias was identified in the studies. Notably, no control was used in Uzun et al., 2014 and in Eftimova et al., missing information on exposure and potential cofounding decreased the reliability of the study. No adjustment for potential risk factors was done in these studies.

No increased risk was observed in midwives in a retrospective cohort study of Axelsson et al., 1996. The study population includes all female members of The Swedish Association of Midwives in 1989 and born after 1940. On this population, 84.3% answered the questionnaire and 7599 pregnancies, which began before 1989, were reported by 2786 women. To avoid memory bias, pregnancies that started before 1980 were excluded. Only women that worked more than half time during the first trimester and for which information was completed were included. Final analysis was made among the 1717 pregnancies during which the women worked as a midwife. Exposure was based on three categories (no use, use of N_2O in up to 500,000 ppm of assisted delivery and use of N_2O in more than 50% of assisted deliveries). The nurses were not sure whether scavenging equipment was present. Several adjustment factors were included: age, pregnancy number, previous spontaneous abortion, smoking, infection, analgesic drugs, other anaesthetic gas, work time.

In contrast, in California, Rowland et al. (1995) performed a retrospective cohort study in female dental assistants exposed at least 3 hours per week to unscavenged N_2O. In this study, 7000 dental assistants were selected and 1805 of the respondents had been pregnant at least once and 1465 provided information about the pregnancy. Exposure was assessed based on the date of the woman's last menstrual period and work history information (number of hours per week of exposure, scavenging system). Potential confounding factors were categorised for smoking, age, previous spontaneous abortions, coffee consumption, heavy lifting, infection with high fever, night work, shifts, and shortage of staff, daily contact with other anaesthetic gases, ultrasound equipment, antineoplastic drugs and stress. No information on potential confounders such alcohol use or paternal occupation were taken into account. As the analysis was limited to women working full time. According to the authors potential bias for unhealthy worker effect cannot be excluded in the study. Indeed, the authors commented that in the United States, population of working women tend to be less reproductively healthy than women of similar social class background who work part time or who do not work outside the home. In addition, the authors pointed out that an earlier detection of pregnancy by an exposed group can increase the number of recognised spontaneous abortions and created appearance of an occupational hazard. The authors adjusted for potential co-exposure to mercury. An increased risk of abortion was observed with an odd ratio of 2.6 (CI 95%: 1.3-5). No clear exposure-response effect was observed in this study.

In a retrospective study published by Heidam et al., 1984, a cohort of 772 dental assistants, having being working in dental clinics or in dental school services, were included in the study (1431 employees used as control group). The data were collected via a questionnaire and covered the women's entire reproductive life before May 1980. Dinitrogen oxide effect on abortion was noted in the study. Nevertheless, exposure was not characterised and there were insufficient information on potential other risk factor (e.g. smoking). Potential co-exposure to inorganic mercury was taken into account and odd ratio for specific substance was calculated. A negative information bias was identified by the authors.

In an older study from Cohen et al. (1980) a statistically significant increase in spontaneous abortion (around 2-fold increase) was also observed in dental assistant exposed to dinitrogen oxide. In the study, 15,000 dentists using N_2O and 15,000 dentists not using N_2O were included. The dentists were from 25 hospitals using advance scavenging systems. A statistically significant increase in spontaneous abortion was noted in dinitrogen oxide users compared to controls (16±1.4 vs 5.5 ±0.95, $p<0.01$). Exposure duration-response was observed in this study as the increase was higher when dental assistants were exposed more than 8h per week compared to 1 to 8 hours per week. However, exposure evaluation was based on a questionnaire and not characterised by measurements. Spontaneous abortions were defined as a loss before the 20^{th} week of gestation. The rate was defined as the number of cases per 100 reported pregnancies during the past 10 years. Several adjustment factors were taken into account in the study: maternal age, smoking history, history of previous spontaneous abortion or congenital abnormalities.

A higher incidence of spontaneous abortion was also noted in several other old studies among workers directly exposed to waste anaesthetic gases (ACGIH, 2001; MAK, 1993). Nevertheless, in these studies, N_2O was not specifically investigated and thus, these studies are not further described.

Developmental abnormalities

In the retrospective cohort study from Teschke et al. (2011), 56,213 female nurses registered for at least one year between 1974 and 2000 were included in Canada. The analyses considered singleton birth born live to 943 nurses in the cohort exposed to N_2O and 13,745 singleton births from mothers not exposed to dinitrogen oxide used as control. In this study, halothane, enflurane, isoflurane and dinitrogen oxide were frequently used. Measurements in hospital reported values generally below LOD by the hospitals except dinitrogen oxide exposure that was sometimes reported to be above 25 ppm. Exposure probability was estimated based on employment information retrieved during a telephone survey. To categorise congenital anomalies, they used the Registry classification protocol as follows: nervous system; eye; ear, face, and neck; heart; other circulatory system; respiratory system; cleft lip or palate; alimentary system; other digestive system; urinary system; musculoskeletal system; integumentary system; chromosomal anomalies; multiple anomalies; and other or unspecified anomalies. Only year of birth and mother's age were considered as adjustments factors. Congenital anomalies were reported on 50/517 (9.7%) children of mothers who were potentially exposed to dinitrogen oxide in the calendar year of their first trimester of pregnancy. An increased risk of congenital anomalies was noted in this study for dinitrogen oxide (OR: 1.82 (95%CI: 1.1-2.99)). The anomaly the most frequently associated with N_2O exposure was integument. An increased risk was also noted for halothane (OR=2.61 [1.31-5.18]), isoflurane (OR= 2.82 [1.3-5.82]) and sevoflurane (OR= 4.71, 95%CI: 2.14-10.3). Associations were increased with likelihood of exposure.

A statistically significant increase in congenital anomalies was also noted in US dental assistants exposed to N_2O (Cohen et al., 1980). In this study, as described above, 15,000 users of dinitrogen oxide and 15,000 non-users were included in the study in 25 hospitals equipped with advance scavenging system. The authors took into account potential mercury exposure and same level of exposure to mercury was noted in exposed and non-exposed groups. The rate of congenital abnormalities was based on the number of living babies born with one or more non-skin abnormalities per 100 births. No specific measurements were performed in the study. Light exposure was defined as 1 to 8h exposure per week, considering cumulative exposure 1-2999 hours in the past ten years. Heavy exposure was defined as exposure above 8h per day and cumulative exposure above 3000h in the past decade. A 1.5-fold increase in the rate of musculoskeletal defects was observed in the exposed groups. Only a limited number of adjustment factors were taken into account: maternal age, smoking history, history of previous spontaneous abortion or congenital abnormalities.

In a study by Bodin et al., 1999, new-borns from midwives exposed to dinitrogen oxide had a reduced birth weight when compared to the control group (OR=-77g; 95% CI= -129, -24) and an increase in odds of being small for gestational age (OR: 1.8; 95% CI =1.1, 2.8). In this study exposure was characterised based on the use of dinitrogen oxide (<50% or > 50% of all deliveries). Information on the use of a scavenging system was often missing. Adjustment factors that were considered were the gestational age, the parity (number of time the women has given birth: 1, 2, 3, 4 or ≥ 5), the employment and work schedule. Potential co-exposures to other substances were not addressed in the publication.

Mode of action hypothesis

Dinitrogen oxide probably acts by directly inhibiting vitamin B12 formation and methionine synthase. Methionine synthase inhibition leads to the impairment of the generation of methyl groups for DNA methylation. According to Tserga et al., 2017, disruption on genomic imprinting leads to biallelic expression which may affect disease susceptibility. For example, epigenetic control of some genes may be influenced by maternal plasma folate.

In the review published by Imbard et al., 2013, the authors summarised that neural tube defects (NTDs) have complex and multifactorial etiologies in which both genetic, life style and environmental factors appear to be involved. In addition to genetic factors, environmental influences such as parental occupation, maternal obesity, and maternal nutritional status have been related to NTDs. Particularly, the authors pointed out that it has been suggested more than 40 years ago that maternal folate status is associated with neural tube disease risk. A substantial number of reviews have been published on neural tube diseases and folic acid. Over the years, more and more studies suggested that not only folate but whole methylation metabolism could be involved in the etiology of NTDs. Neural tube disease such as hydrocephaly has been reported by some authors. Guéant et al., 2013 reviewed that early vitamin B12 and folate deprivation during gestation and lactation in rats was associated with long-lasting disabilities of behaviour and memory capacities, with persisting hallmarks related to increased apoptosis, impaired neurogenesis and altered plasticity.

Nevertheless, as demonstrated by Mazze et al., 1988, this mechanism may not be the sole factor of dinitrogen oxide teratogenicity as folinic acid supplementation did not fully prevent the teratogenic potential of the substance. The authors also hypothesised that dinitrogen oxide developmental toxicity may be related to the increased adrenergic tones induced by dinitrogen oxide. They showed that the combination with alpha adrenergic blocking agent reduce the incidence of some of the malformations, presumably by restoring the uterine blood flow to normal. The authors further noted that the reason that toxic reproductive effects in human are not conclusive may be related to the multifactorial etiology of the reproductive effects. Fujinaga et al., 1991, also suggested that uterine blood flow disturbance may account for some of the reproductive toxicity of the substance.
Although biochemical changes occur in rats and in humans after short dinitrogen oxide exposures, a prolonged
period of decreased uterine blood flow may be necessary to produce foetotoxicity (i.e., 24 hours in a 21-day rat gestation Mazze et al., 1988).

The exact mechanism by which dinitrogen oxide act as a teratogen is still not fully understood.

10.10.6 Comparison with the CLP criteria

In human, some studies indicated that dinitrogen oxide may induce congenital abnormalities or reduction of birth weight following high exposure (no measurements available) to dinitrogen oxide or in the absence of appropriate scavenging systems. Nevertheless, the interpretation of the human data is difficult due to potential co-exposure, the absence of reliable characterisation of exposure, and the absence of adjustment for potential other risk factors. It may be noted that according to animal data, co-exposure with other anaesthetic agents (e.g. halothane or isoflurane) may be protective, whereas other anaesthetic could potentiate dinitrogen oxide developmental toxicity. Therefore, the results of the human data may be very difficult to interpret in case of co-exposure and potential bias. Regarding abortion, inconsistent findings were noted in human. Overall, dinitrogen oxide does not fulfill the criteria for category 1A.

In animals, 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. In addition, delayed development and decreased foetal weight was also noted in several studies.

Dinitrogen oxide caused embryotoxicity and teratogenicity in rats exposed during GD8 to 10 of gestation after single 24h administration. A NOAEC for both effect was identified at 350,000 ppm dinitrogen oxide (Mazze et al., 1987). Following continuous exposure during the whole gestation period, a NOAEC for malformations was identified at 500 ppm in Vieira et al., 1980 (LOAEC = 1000 ppm). Maternal toxicity was not described in all the studies. Reported maternal toxicity at up to 750,000 ppm include mostly decreased body weight gain and mild sedation, not indicative of excessive toxicity. There is no data on corrected weight of dams to better characterised true maternal toxicity.

Following intermittent exposure to dinitrogen oxide (4 to 8h/day exposure during critical windows or whole gestation period), embryo-foetotoxicity (decrease foetal weight, resorption or dose related decrease in litter size) was observed. No statistically significant increase in malformations were noted except in one study were an increase in major malformations and external abnormalities was observed (without statistical significance) at 750,000 ppm (Mazze et al., 1986).

Neurobehavioral studies in pups exposed during gestation (Rice et al., 1990, Mullenix et la., 1986, Koëter et al., 1986) provide some evidence on potential effect on reactivity in pups.

As teratogenicity occurred at high concentration, it is questionable if the effects are related to the substance itself or are secondary to the anaesthetised state. In Pope et al., 1978, the authors noted that foetal delayed development was not related to a specific anaesthetic agent in the study as the effect was also observed with halothane or methoxyflurane. Therefore, the authors suggested that the effect may have been due to a general effect of the anaesthetic on the mother and foetuses rather than a specific toxic effect on the foetus. Contradictory to these results, in Lane et al., 1980, rats were exposed to xenon and 750,000 ppm dinitrogen oxide for 24 hours on GD 9. Foetal resorption, delayed maturation and anomalies to the skeletal systems were only observed with dinitrogen oxide and not xenon, suggesting that the observed effects are related to the substance itself rather than its anaesthetic mechanism. In Mazze et al., 1986, the authors also did not demonstrated teratogenic findings in rats exposed to intermittent exposure to anaesthetics such as isoflurane, enflurane or halothane. By contrast an increase in foetal loss was observed following exposure to dinitrogen oxide. Overall, the teratogenic effects observed with dinitrogen oxide is considered as an intrinsic property of the substance.

Although a statistically significant increase in malformations was only noted following continuous exposure to dinitrogen oxide, a trend toward an increase in malformations was noted at least in one study following intermittent exposure (Mazze et al., 1986). Other relevant findings were nevertheless observed in these studies: resorption and dead in utero or dose related decrease in litter size (Vieira et al., 1983b). The lack of sensitivity in the intermittent studies (e.g. low number of dams) may explain the absence of effect or statistical significance.

Regarding maternal toxicity, few information are available in studies. When there are, effects are generally limited to reduced body weight or body weight gain, sedation, sometimes impaired food and water consumption. Some deaths were observed in only one study. Moreover, concerning the decrease in body weight, it can be due to the decrease size of the litter. The CLP guidance (2017) indicates 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*" And "*Developmental effects which occur even in the presence of maternal toxicity are considered to be evidence of developmental toxicity, unless it can be unequivocally demonstrated on a case-by-case basis that the developmental effects are secondary to maternal toxicity*". Therefore, the few data available cannot discount the effect observed on offsprings. In addition, toxicity expected at high dose levels (e.g. neurotoxicity, immunotoxicity), not investigated or reported in the developmental toxicity studies, may not explain the observed malformation as shown by Fujinaga et al., 1979. Although the same dose level was used, malformations and resorptions were only observed following exposure during the critical window of exposure (GD 8, 9 or 11).

Overall, based on the clear embryo-lethality observed following dinitrogen oxide exposure, not secondary to unspecific maternal toxicity, dinitrogen oxide warrants to be classify as Repr. 1B, H360D for developmental toxicity.

10.10.7 Adverse effects on or via lactation

Endpoint not assessed (lack of data).

10.10.8 Conclusion on classification and labelling for reproductive toxicity

Classification for reproductive toxicity addresses adverse effects on sexual function and fertility, developmental effects. Based on the clear embryofoetotoxicity of dinitrogen oxide a classification in category 1B is considered appropriate. Based on the observed effect on fertility in studies having limitations, a classification in category 2 for fertility is considered appropriate.

Dinitrogen oxide warrant to be classified as **Repr. 1B, H360fD.**

10.11 Specific target organ toxicity-single exposure

Table 30: Summary table of animal studies on STOT SE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
RATS			
Non-guideline neurotoxicity study in Fischer-344xBrown Norway F1 hybrids (F344xBN F1/NIA) female rats N=5-10/group Limitations: - no information on GLP status - no information on the origin of the substance - purity of test material not provided - No. of animals at beginning and at the end of the study similar not specified - housing and lighting conditions not specified - no data on general toxicity	Inhalation, 3h exposure, Dinitrogen oxide mixed in oxygen Control, 50,000 to 2,000,000 ppm	EC_{50}: 1,180,000 ppm Neuron vacuolation (posterior cingulate/retrosplenial cortex)	Jevtovic-Todorovic et al., 2005 Klimisch 2 WOE
Non-guideline neurotoxicity study in rats Sprague-Dawley rats N= 6-17 per group Limitations: - No information on GLP status - Purity of test material not provided - Little information on housing conditions - just one dose tested (above hypoxia)	Single exposure: 0, 4, 6, 8, 12 or 16h 1,500,000 ppm N_2O	>3h, reversible vacuolation of neurons >8h exposure: Cell death	Jevtovic-Todorovic et al., 2003 Klimisch 2 WOE
Non-guideline neurotoxicity study in male and female rats Sprague-Dawley (N= 7-9/group) Limitations: - No information on GLP status - Purity of test material not provided - No data on general toxicity - No real materiel and method - No information about housing, lighting and feeding conditions - No information on the origin of the substance - No analytical control of concentrations during inhalation	Inhalation, 3h exposure 0 to 1,800,000 ppm dinitrogen oxide in air	EC_{50}=1,040,000 ppm (males) EC_{50}=1,170,000 ppm (females) Dose-related increase in vacuolated neurons (retrosplenic cortex)	Jevtovic-Todorovic et al., 2000 and 2001 Klimisch 2 WOE

Non-guideline neurotoxicity study in male Long-Evans rats (n=10/group) Limitations: - No information on GLP status - Purity of test material not provided - No data on general toxicity - limitation indicated by the authors: it is possible that the precision of delay measurements (0,1s) was not sufficient for discrimination in reaction time variations of the order of a few milliseconds. For this technical reason they can't confirm that their results agree or disagree with previous studies in humans	Inhalation, single continuous exposure 300,000, 400,000, 500,000, 600,000, 700,000 ppm N_2O	≥ 300,000 ppm Dose-related decrease (stat. sig.) in locomotor activity, alteration of visual detection task	Courtière et al., 1997 Klimisch 2 WOE
Non-guideline neurotoxicity study in male rats (strain not specified) (n=8-10/group) Limitations: - No information on GLP status - Purity of test material not provided - No analytical control of concentrations during inhalation - information on the source/origin of the dinitrogen oxide - Just one dose tested	Single continuous exposure (24h) 700,000 ppm mixed in O_2	Transient decreased in visual evoked potential amplitude. Decreased in nocturnal locomotion. Tolerance observed during the following light-dark cycle.	Dzoljic et al., 1994 Klimisch 2 WOE
MICE			
Non-guideline neurotoxicity study in male NIH Swiss mice (n=12-15/group) Limitations: - No information on GLP status - Purity of test material not provided - Age of rats not specified - No data on general toxicity - No information on exposure duration	0, 250,000; 500,000; 750,000 ppm N_2O mixed in O_2 Unknown exposure duration	At 500,000 ppm: Increased (stat. sign.) in the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner	Li et al., 2001 Klimisch 3 WOE
Non-guideline neurotoxicity study in male Swiss-Webster mice mice (n=15-20/group) Limitations: - No information on GLP status - Age of rats not specified - No information about housing, lighting and feeding conditions - Purity of test material not provided - No data on general toxicity - Only one dose tested	35-60 min single exposure 500,000 ppm N_2O	Increased behavioural anxiolytic effects	Caton et al., 1994 Klimisch 2 WOE

Non-guideline neurotoxicity study in male mice (strain not specified) (n=4/group) Limitations: - No information on GLP status - Purity of test material not provided - No analytical control of concentrations during inhalation - information on the source/origin of the N_2O unspecified - information on the source/origin of mice unspecified - type of statistical test performed not specified - No information about housing, lighting and feeding conditions - No data on general toxicity - Only one dose tested - few number of animals per group	1h single exposure 500,000 ppm N_2O	Increased locomotor activity	Dorris et al., 1993 Klimisch 3 Disregarded

EC_{50}: Median effective concentration

Table 31: Summary table of human data on STOT SE – Human volunteer studies

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); 100,000; 200,000; 300,000; 400,000 ppm N_2O	Significant impairment on auditory reaction time and eye-hand coordination. No acute tolerance to N_2O LOAEC: 300,000 NOAEC: 200,000	Yajnik et al., 1996
Human volunteer study	8 ♂ + 4 ♀ Chamber (nasal mask) 5 sessions (~ 60 min): air; 50,000; 100,000; 200,000; 400,000 ppm N_2O	Significant differences: impairment of reaction time and attention LOAEC: 100,000 NOAEC: 50,000	Fagan et al., 1994
Human volunteer study	15 ♂ Chamber (nasal mask) 4 sessions (duration not specified): air (training session & control session); 200,000; 400,000 ppm N_2O	Impairment on psychomotor tests: - Symbol digit - Finger tapping - Test response latency LOAEC= 200,000 ppm	Mahoney et al., 1988
Human volunteer study	6 (sex not specified) Chamber (nasal mask) 4 sessions (10 + 20 min): air; 100,000; 200,000; 400,000 ppm N_2O in O_2	Impairment on psychomotor tests: - Continuous performance test - Finger tapping LOAEC = 100,000 ppm	Estrin et al., 1988
Human volunteer study	24 ♂ Chamber (nasal mask) 4h-exposure: placebo, 50 ppm N_2O	Psychomotor performance: No effect Mood: No statistical difference NOAEC = 50 ppm	Venables et al., 1983
Human volunteer study	20 ♂/group Chamber (nasal mask) 4h-exposure, twice: - 25 ppm N_2O + 0.5 ppm of halothane, - 50 ppm of N_2O, - 50 ppm + 1 ppm halothane, - 500 ppm N_2O,	Impairment on psychomotor tests : - Memory - Visual acuity - Audio-visual capacity NOAEC = 50 ppm N_2O	Bruce and Bach, 1976

	- 500 ppm N_2O + 10 ppm halothane		
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), 200,000 ppm N_2O + 30% O_2 + room air	Significant activation in the anterior cingulate cortex and deactivation in the posterior cingulate hippocampus LOAEC = 200,000 ppm	Gyulai et al., 1996
Human volunteer study	N= 15 (sex not specified) Facial mask, 15-min exposure: 100,000; 300,000; 500,000 ppm N_2O mixed in O_2	Significant decrease in cerebral function analysing monitor LOAEC = 300,000 ppm	William et al., 1984

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – single exposure

Human data

- *Effects on cognitive function*

In 1976, Bruce and Bach investigated effects of N_2O on behavioural performance at low doses levels exposing 100 male volunteers. Twenty (20) subjects per group were tested twice at one week interval. Ten naïve subjects received air first and ten received anaesthetic first. They were exposed during 4 hours, via a mask, to:
- 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.

The authors evaluated the subject performance in several tests, including visual acuity, time reaction, vigilance, manual dexterity, memory, etc., at different time periods after the beginning of exposure (starting 2h after the beginning of exposure). The results were reported for each exposure in a table by the authors according to the tests conducted.

Table 32: Summary of results observed in Bruce and Bach (1976) with N_2O alone

	Test results (%)	
N_2O (ppm)	50	500
Tachistoscope	-	-7*
Raven matrices	-	-9*
O'Connor Dexterity	-	-
3-min audio-visual	-5*	-17**
60-min vigilance	-	-14*
7-min audio-visual	-5*	-17**
Digit span	-	-12***

- : no changes reported, *p<0.05; **p<0.01; ***p<0.001;

Audio-visual capacity was slightly impaired after exposure to N_2O at 50 ppm (5% change). At 500 ppm N_2O, visual acuity, audio-visual capacity, immediate memory and vigilance response were altered. Only manual dexterity seemed to be resistant to N_2O exposure according to the authors. They observed that the changes were very small. They noted that repeated prolonged exposure would be needed to take into account tolerance to the effects and to conclude on adverse performance of workers occupationally exposed to N_2O. Regarding the gas analysis, the authors noted considerable variations in the anaesthetic concentrations of

end-of-exposure expired air samples. In two previous studies from the same team (Bruce et al., 1974; Bruce and Bach, 1975), digit span response was affected following 4h exposure to 500 ppm N_2O.

In a letter published several years later, in 1991, Bruce indicated that most of the dental student subjects used in the Bruce and Bach (1976) study were Mormons who might have been abnormally sensitive to depressant drugs. Bruce considered the results on performance, derived from the study, wrong due to an inadvertent sampling bias as the subject chosen in the study may not be representative of the general population.

In an experimental study, twenty-four (24) male volunteers were exposed twice to 0 ("placebo") and 50 ppm of N_2O in an exposure chamber during 4 hours to investigate effects on psychomotor performance (Venables et al., 1983). The subjects were tested at the same time, *i.e.* morning and afternoon (four volunteers in the chamber during each session of exposure). Performance testing took place during the final 40 min in the chamber. The authors conducted the following psychomotor tests: audio-visual task, simple reaction time, four choice reaction time, stressalyser[2]. They performed also a mood test, using a visual analogic scale. The authors compared the scores at the beginning and at the end of exposure. Venables et al. (1983) didn't find difference in the mean performances for the four psychomotor tests. Concerning the visual analogic scale scores, the authors showed an impairment in mood on all four dimensions assessed (sleepiness, physical tiredness, mental tiredness and general good health) at 50 ppm of N_2O exposure, but the difference was not statistically significant.

Table 33: Mean changes in the visual analogue scores for 0 and 50 ppm N_2O (Venables et al., 1983)

N_2O (ppm)	0	50
Sleepiness	27 ± 59	50 ± 55
Physical tiredness	17 ± 51	34 ± 47
Mental Tiredness	22 ± 54	33 ± 41
General good health	10 ± 55	17 ± 28

Mahoney et al. (1988) evaluated the validity of neurobehavioral tests using N_2O as a model. The authors exposed fifteen volunteers to N_2O (duration of exposure was not stated) *via* a scavenging mask and asked them to breath only through their noses. The subjects were tested with a neurobehavioral evaluation system (NES) test battery on 4 separated sessions (training session, at 0, 200,000 and 400,000 ppm N_2O), NES combines 10 tests (continuous performance test, hand-eye coordination test, serial digit learning, symbol-digit substitution test, pattern recognition and memory, finger tapping, switching attention, mood scale). The switching attention task was performed under three separate conditions (Switching time, switching direction or in a more "complex condition" using both switching time or direction).

The authors observed a significant impairment on performance at 200,000 ppm for 2 tests of psychomotor speed (symbol digit and finger tapping), and effect on performance test response latency (p=0.055). The switching attention task was also impaired from 200,000 ppm onward (when tests were applied in a "complex condition").

In 1988, Estrin et al., developed neurophysiological techniques for measuring cognitive performances in a standardised, objective, and reproducible manner to quantify the transient cognitive dysfunction induced by the administration of N_2O. Six volunteers (27-35 years) were exposed *via* a nasal mask to 0; 100,000; 200,000 and 400,000 ppm of N_2O for 10 minutes initially and remained at that dose for 20 minutes during which the subtests of the computerised psychometric test battery were given and the P-300 determination was made. Respective simultaneous administration of O_2 was 0, 100, 90, 80 and 40%. The authors measured the effects using the P300 Evoked Potential[3] and administered psychometric tests (symbol digit, continuous performance test, finger tapping). The results showed a trend in all variables (excepted symbol digit test) at 100,000 ppm and significant correlations between standardised measures of psychomotor testing and P-300 event-related potential latency and/or amplitude.

[2] Task in a form of a stress analyser (Buck et al. 1981)

[3] P300 is a neurophysiological technique used decision-making research

Table 34: Summary of results observed in Estrin et al. (1988), n=6

N_2O (ppm)	Control	100,000	200,000	400,000
CPT mean latency (msec)	360	385.0¤	381.8**	401.8*
FTT (N°/10sec)	56.3	55	53.3**	48.8*
SDT (sec/pair)	1.88	1.89	1.88	2.23*
P-300 latency (msec)	301.2	312.8	330.5	377.3*
P-300 amplitude (µU)	1.47	1.32	1.30	0.96*

¤ Dunnet's t test, p<0,05
*P<0.01, repeated-measures ANOVA
***P<0.06, repeated-measures ANOVA
CPT: Continuous Performance Test; FTT: Finger Tapping Test; SDT: Symbol Digit Test;

Fagan et al. (1994) investigated the effects of N_2O on psychological performance and mood in twelve volunteers successively exposed during 1 hour to 0, 50,000; 100,000; 200,000 and 400,000 ppm N_2O. Order of treatment was randomised and each session was performed on a separate day. The authors performed a battery of tests to evaluate effect on performance including memory, attention, and reaction time. To evaluate effects on mood the authors used a subjective test, and a visual analogue scale. Almost all tests showed an effect at 400,000 ppm exposure, and no change was observed at the lowest dose level of 50,000 ppm N_2O. Significant differences were reported at 100,000 ppm in some functions (reaction time and attention).

Yajnik et al. (1996) studied the phenomenon of acute tolerance, which is defined as a change of "sensitivity to a drug within the duration of one continuous drug exposure" (definition from Kalant et al., 1971). For this, the authors exposed eleven volunteers through a facial mask to N_2O (0, 100,000, 200,000, 300,000, 400,000 ppm, during 120 minutes) for five time periods separated by at least one week. The subjects were selected according to medical history (e.g. no significant psychiatric disorders or history of neurologic, cardiac, pulmonary, hepatic or renal disease). The effects were measured using a self-reported questionnaire, cognitive and psychomotor tests and physiological analyses (which consisted in electrocardiogram, peripheral oxygen saturation, blood pressure). The tests were conducted during the exposure session, 15, 40, 60, 80, 85 and 105 minutes after initiation of N_2O exposure and 5, 30, 60 min after cessation of exposure.

Concerning the physiological measures, no effect was observed with N_2O exposure. The results of subjective measures showed a significant and dose-related increase in ratings of feel drugs effects with no evidence of a lessening of drug effect during the exposure session. Significant impairments of auditory reaction time, eye–hand coordination, and number of symbols correctly completed on the Digit Symbol Substitution Test (DSST), starting at ≥ 300,000 ppm were observed. The authors indicated that these impairments seemed to be concentration-dependent and there was no evidence of acute tolerance to N_2O exposure on these effects. They concluded a lack of acute tolerance to the psychomotor impairment effects of N_2O, and they reported that the recovery of psychomotor and cognitive functions was rapid (by 5 minutes after exposure for most of the measurements).

- *Effects on nervous conduction*

Gyulai et al. (1996) analysed the effects of N_2O exposure in eight subjects. They measured regional cerebral flow (rCBF) changes and regional cerebral metabolic rate (rCMR) which both reflect changes in neuronal activity, in 4 subjects for each test. Both were separately assayed under control and N_2O condition (200,000 ppm N_2O, 20% O_2 and balance room air) by the mean of a Positron Emission Tomography (PET) used during exposure sessions which begun at least 15 minutes before PET to map the brain areas. The volunteers were exposed *via* a facial mask, and some physiological parameters were measured (blood pressure, electrocardiogram, arterial oxygen saturation and end tidal carbon). The authors found a significant activation in the anterior cingulate cortex, which is associated to the psychomotor and cognitive processes. Moreover, deactivation was observed in the posterior cingulate hippocampus, parahippocampal gyrus and visual association cortices in both hemispheres, these regions are known to mediate learning and memory.

Fifteen volunteers were exposed to room air, 100% of O_2 (during 10 minutes) and 100,000, 300,000 and 500,000 ppm of N_2O (mix with O_2) during 15 minutes, via a fitting facial mask (Williams et al., 1984). Their cerebral activity was then tested by the Cerebral Function Analysing Monitor (CFAM) which is a microprocessor device based on cerebral function monitoring through an electroencephalographic signal

derived from a single pair of surface electrodes. During the session, blood pressure and respiratory rate, and CO_2 concentration in exhaled air were measured. Data on only nine of the fifteen subjects were analysed (because of a lack of cooperation or unpleasant feelings). The authors observed a significant reduction in CFAM amplitude at 3000000 and 500000 ppm of N_2O (but no change was observed in the frequency distribution of the weighted EEG signal). Moreover, the subjects reported subjective effects whilst breathing N_2O, as hyperacusis (9/9), emotional states (fear and panic), and euphoria.

Animal data

Rats

In a series of restrictions reported by Jevtovic-Todorovic et al. (2000, 2001, 2003 and 2005), following acute 3-hour exposure to 500,000 to 2,000,000 ppm N_2O (exposure were conducted under hyperbaric conditions), vacuolation of cerebrocortical neurons was observed. EC_{50} for vacuolated neurons per section cut though cortex (posterior cingulate/retrosplenial cortex) was 1,040,000 ppm and 1,170,000 ppm N_2O for males and females, respectively. When N_2O was terminated at 3 h and the rats were killed 1 hour later, the vacuole reaction was markedly diminished and when the rats were killed 3 hours later the vacuole reaction had completely disappeared. Prolonged exposure to 1,500,000 ppm N_2O (for 8 hours or more) caused neuronal cell death, detectable 32h later. The authors concluded that short-term exposure of adult rats to N_2O causes injury to neurons that is rapidly reversible, and prolonged N_2O exposure causes neuronal cell death.

Courtière et al. (1997) evaluated vigilance performance task in rats. The rats were required to respond to slight luminous increment of the house-light. A statistically significant dose-related decrease of correct response was observed in rats exposed to 300,000 to 700,000 ppm N_2O. Concomitant decrease response time was observed at ≥ 600,000 ppm and omission was strongly increased at 700,000 ppm N_2O. Moreover, in this study, a statistically significant dose-related decrease in locomotor activity was noted at ≥ 400,000 ppm N_2O.

Decreased locomotion followed by a development of a tolerance and unaltered motor activity during withdrawal was noted following continuous 24h exposure to 700,000 ppm N_2O in rats (Dzoljic et al., 1994). Similarly, an initial decrease of visual evoked potential amplitudes was followed by tolerance to N_2O.

Mice

Behavioural anxiolytic effect of N_2O was investigated in a light/dark exploration test in male mice (Li et al., 2001). In this study, mice were exposed once to 0; 250,000; 500,000 or 700,000 ppm N_2O by inhalation, mixed in O_2. Duration of N_2O exposure is unclear in the study and thus the study was rated unreliable. N_2O increased the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner. The increase was statistically significant at ≥ 500,000 ppm.

Caton et al. (1994) investigated mice exploratory and locomotor activity following 35-60 minutes exposure to 500,000 ppm N_2O. Mice exposed to 500,000 ppm N_2O exhibited significant increases in both the percent of entries into open arms and the percent time spent in open arms of the elevated plus-maze. N_2O produced an overall net increase in the mean number of overall (open plus enclosed) arm entries, indicating an increase in locomotor activity.

10.11.2 Comparison with the CLP criteria

According to the CLP criteria, substances are classified in category 1 for specific target organ toxicity (single exposure) on the basis of:
"a. *reliable and good quality evidence from human cases or epidemiological studies; or*
b. *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."*

The criteria for classification in category 2 are based on:
"Substances are classified in Category 2 for specific target organ toxicity (single exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided […] in order to help in classification.
In exceptional cases, human evidence can also be used to place a substance in Category 2."

The criteria for classifying substances as Category 3 for narcotic effects are:
"(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 effects are not transient in nature, then they shall be considered to support classification for Category 1 or 2 specific target organ toxicity single exposure."*

According to the ECHA guidance document on classification, the assignment of STOT-SE Category 1 or 2 is independent to the assignment of Category 3. Therefore, a substance may be classified in both category 1/2 and category 3 if the respective criteria are met, for instance, in the case of a neurotoxic substance that also causes transient narcotic effects.

In several studies in rats (Jevtovic-Todorovic et al., 2000, 2001, 2003 and 2007), significant and severe changes in the nervous system were observed. Indeed, brain histopathological findings and/or behavioural changes were reported after single exposure to ≥ 300,000 ppm dinitrogen oxide. Behavioral changes were also noted in the three described mice studies at ≥ 250,000 ppm. Nevertheless, although the effects were significant and severe enough to fulfil the criteria for STOT SE 1 or 2, no experimental studies were found at dose levels and exposure duration that would fulfil the criteria for classification STOT SE (C < 20,000 ppmV/4h).

In human volunteer experimental studies, Bruce and Bach, 1976 reported a slight impairment of audio-visual capacity at 50 ppm and lower performance in visual acuity, audio-visual capacity, immediate memory and vigilance response following 4-hours exposure to N_2O at 500 ppm. On the other hand, Venables et al., 1983 and other authors failed to reproduce the results obtained at 50 ppm. Nevertheless, according to the authors, the volunteers used may have been particularly sensitive to the cognitive effects of dinitrogen oxide and may not have been representative of the general population. At 500 ppm, effects on memory (digit span test) were reported by Bruce et al., 1975. Other acute studies were performed at higher dose levels and also reported effects on the nervous system. A LOAEC of 50-500 ppm can be identified for these effects.

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 control exposure levels, classification of the substance in category 3 (narcosis) for STOT SE is considered relevant. 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 dinitrogen oxide were rapidly reversible. In addition, ataxia were also noted in animals and were reversible, supporting a classification (Singh et al., 2015, detailed in STOT RE section) STOT SE 3 rather than STOT SE 1 or 2.

In addition, with regards to an additional classification for STOT SE 1 or 2, the observed effects in human volunteer studies may not be sufficiently convincing but point toward potential significant toxicity of the substance following repeated exposure (See section on STOT RE).

10.11.3 Conclusion on classification and labelling for STOT SE

As detailed, the effects observed in human on the nervous system, supported by the animal data, are not sufficiently convincing to propose a classification of dinitrogen oxide for STOT SE. However, based on narcotic findings in human volunteer studies, a classification as STOT SE 3, H336 is warranted.

10.12 Specific target organ toxicity-repeated exposure

Table 35: Summary table of animal studies on STOT RE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
RATS			
Non-guideline neurotoxicity study in rats Wistar rats Male N=5-10/group Limitations: - no information on GLP status - no information on the origin of the substance - purity of test material not provided - only one dose - no data on general toxicity - unclear dose levels	N_2O mixed in O_2 Inhalation, 60-day exposure, 2h/d 0, 500,000 ppm	**<u>At 500,000 ppm:</u>** - No effect on survival - Statistically significant decrease in bw gain at the end of the experiment (200.6 mg vs 245.6 mg in controls) - Necropsy: Brain, spinal cord Astrocytes activation in brain and spinal cord (GFAP phenotype analysis) - Spontaneous locomotor activity: Significant reduction in total distance travelled, time moving, number of rearing significant increase in resting time. No changes in stereotypic counts - Significant reduction in grip strength compared to controls - Homocysteine levels, glutamate and malondialdehyde (MDA) levels were significantly increased, however GSH and total antioxidant capacity (TAC) level decreased in dinitrogen oxide exposed group compared to the controls.	Misra et al., 2020 Klimisch 2
Non-guideline neurotoxicity study in rats Wistar Rats, Male N= 6/group Limitations: - no information on GLP status - no information on the origin of the substance - purity of test material not provided - only one dose level - no data on general toxicity aside weight loss	Dinitrogen oxide Inhalation, 1.5h/d, 60-day exposure 500,000 ppm N_2O mixed in O_2	**<u>At 500,000 ppm:</u>** General toxicity: There was progressive weight loss in exposed group compared to controls (303.8±14.11 vs 244.6±8.75 g; p = 0.0073). Clinical signs: After exposure the rats appeared sluggish, lethargic and developed limb weakness, which was more marked in the hind limbs. These symptoms recovered in 1–1.5 h. Necropsy: Cerebrum neuronal degeneration, focal demyelination in the outer layer of the cerebral cortex, degenerative changes in pyramidal layer; Spongy vacuolation, myelin damage, throughout the white matter in spinal cord. No effect observed in the control group Behavioral studies: the rats appeared sluggish, lethargic and developed predominantly hind limb weakness for 1–1.5 h. In the exposed group, the total distance travelled (2001.66± 118.27 cm; p = 0.037), time moving (80.16± 5.7 s; p = 0.028), number of rearing (10.33 ±1.45; p = 0.014) and grip strength (1042.40 ±51.3 N; p = 0.041) were significantly decreased whereas, resting time significantly increased (219.83 ±5.7 s; p = 0.030) compared to controls. No changes in stereotypic counts Biochemical markers: Serum vitamin B12 level was below 150 ng/dl in 5 of 6 rats in both exposed and control groups. Homocysteine level was significantly increased in the exposed group compared to control (20.56±1.30:10.40±1.42 mm/ml; p = 0.0007)	Singh et al., 2015 Klimisch 2

Non-guideline neurotoxicity study in rats Rats Sex: not specified N=18/group	Dinitrogen oxide 4h/d, 5d/w, 6-month exposure 0, 700,000 ppm N_2O	**<u>At 700,000 ppm</u>** - No morphometric and teased fibre abnormalities in peripheral nerves - No changes in electromyography, nerve conduction velocity or axonal flow	Dyck et al., 1980 Klimisch score 2
Non-guideline neurotoxicity study in rats Sex: both sexes No. of animals not specified	Dinitrogen oxide Inhalation, 8h/d 7 or 14-d exposure 400,000 ppm N_2O	**<u>At 400,000 ppm</u>** Cortical cell count changes in brain (frontal parietal and occipital portions)	Hayden et al., 1974 Klimisch score 4
MICE			

Non-guideline neurotoxicity study Swiss Mice Group: 8 controls and 7 per exposed group Sex: Male adults Analysis: - Locomotor activity: measured one-day after exposure (horizontal activity was measured) at 10 min. for 2h (10, 30, 60, 90, 120 min time-points). - Anxiety assessment was investigated during 30 min. Side changes, dark-like area time spent measured - Stereotypy: total number of stereotypies at 10 intervals for 2 hours. - Coronal sections of the occipital lobe of control and animals exposed to 2000 ppm dinitrogen oxide were examined under light microscopy. The large cells (neural cells) and small cells (neuroglial cells were counted in a 0.03 mm2 area.) - Motor coordination: rolling-roller performance step (rotating rod) Limitations: - males only Low number of animals per groups	Dinitrogen oxide 0, 1000, 2000 ppm N_2O Inhalation, 8h/d 8-day exposure	No changes in body weight of mice between groups. **At 1000 ppm:** - Dinitrogen oxide-exposed mice showed reduced locomotor activity compared to control animals; however, with the exception of the longest time period (120 minutes), this decrease was not statistically significant - Dose-related reduction in stereotypic behaviour - No effect on motor coordination or anxiety level - no significant difference in the number of neural cells, neuroglial cells, or total cells counted in the control tissue, as compared to the neural tissue from mice exposed to dinitrogen oxide.	Fung et al., 1993 Klimisch score 2
Non-guideline repeated dose toxicity study in mice Non GLP Swiss webster mice Male and female N=15/sex/group Limitation: limited number of parameters investigated	Dinitrogen oxide (purity not specified) 14-week whole body exposure, Inhalation, 4h/d, 5d/w 0, 5000, 50,000, 500,000 ppm N_2O Vehicle: air	- All animals survived to necropsy - Statistically significant treatment-related decrease in body weight at the top dose (decreased in body weight gain in males and females by 77 and 63% vs control, respectively). - No effect on brain weight, no histopathologic findings in brain.	Rice et al., 1985 Klimisch score 2

GFAP: glial fibrillary acidic protein

Table 36: Summary table of human data on STOT RE – recreational use

Type of study/report	Test substance, Route of exposure, relevant information about the study	Observations	Reference
Case report	Case: 29 year-old man Exposure: dinitrogen oxide, 60 dinitrogen oxide canisters (whippets) per day for the first 5 days followed by use every 2-3 days thereafter. No regular medication.	Symptoms: ascending lower limb numbness, pins and needles, difficulty walking 3 day after stopping exposure. Examination: unsteady gait, reduced sensation to light touch and pinprick Clinical chemistry: vit. B12 deficiency, elevated homocysteine levels, MRI: normal Follow up: immediate improvement of symptoms after treatment.	Thayabaran et al., 2021
Retrospective case series report	Retrospective evaluation of neurological disorders attributed to dinitrogen oxide recreational use Cases from January 2018 to June 2019 Participants: 43 patients, 15-30 year-old, Exposure: mean usage rate of 10.8 times per month (1-28) for 10.7 months (0.7-48)	Symptoms: limb numbness and weakness (98%), unsteady gait (70%), anxiety (14%) followed by insomnia, hallucination, lethargy Clinical chemistry: vit. B12 deficiency (63%), hyperhomocysteinemia (93%), anaemia (19%), Electromyography: sensory and motor nerve impairments (100%), mixed axonal and demyelinating neuropathy (93%) more prominent in lower limbs. MRI: changes in the dorsal column of the cervical spinal cord (77%), cervical and thoracic spinal cord changes in one patient. One patient with diffuse brain atrophy. Diagnoses: peripheral neuropathy (10/43), myeloneuropathy (30/43), combined myeloneuropathy and toxic encephalopathy (3/43) <u>Follow up</u> after treatment and cessation of exposure (4-32 month): - full recovery: 5 (11.6%) - marked improvement: 36 (83.7 %) - No improvement in 2 patients (4.7%)	Zheng et al., 2020
Case report	Participants: 1 patients, 21 year-old, Exposure: dinitrogen oxide capsule used for whipped cream recharging, a few times daily for a month for anxiety relief. Last use three days before hospitalisation.	Neurologic symptoms: lower extremity weakness, double vision, falls, dizziness, anxiety Neurological exam.: positive Romberg test, 4/5 strength in lower extremities Clinical chemistry: Vit. B12 deficiency MRI: normal (brain and spinal cord) <u>Follow up</u> after treatment and cessation of exposure: partial resolution	Lundin et al., 2019
Retrospective case series report	Retrospective evaluation of subacute degeneration of the spinal cord attributed to dinitrogen oxide exposure	Neurologic symptoms: numbness (8/9), hyperesthesia (3/9), muscle weakness (all), ataxia (8/9)	Lan et al., 2019

	Cases from march 2012 to January 2018 Participants: 9 patients, 14-19 year-old Exposure: History of dinitrogen oxide abuse 6 months before the onset of symptoms and degeneration predominantly restricted to posterior and lateral column. 7/8 has used dinitrogen oxide more than 6 months Data collection: clinical history, Clinical symptoms, onset and duration, laboratory data, nerve conductive velocities, somatosensory evoked potential and spinal magnetic resonance images.	Neurological exam. Absence of deep tendon reflex (all), proprioception defect (8/9), decreased muscle power (all). Clinical chemistry: Vit. B12 deficiency (4/8), elevated serum homocysteine level (8/9), anaemia (2/9). Neurophysiology: demyelinated features, spinal cord involvement (4/6) and axonal degeneration (1/6) MRI: degeneration of cervical area (6/6), thoracic regions (1/6), simultaneous cervicothoracic involvement (other) Follow up after treatment: - Muscle power recovery within 2-month, - Persistent sensory deficit (5/9), sensory ataxia (1/9)	
Case report	Participant: 45 year-old man Exposure: 100 canisters of whipped cream chargers per week for approx. 4 weeks	Symptoms: progressive worsening numbness and tingling of extremities, ataxic gait, instability. Examination.: 4/5 strength, decrease sensation to light touch in extremities and vibration sense, dysmetria in finger to nose testing, positive Romberg test Clinical chemistry: Vit. B12 deficiency, hyperhomocysteinemia MRI: Changes in the dorsal column of the cervical and thoracic spinal cord Follow up after treatment: improvement at day 14	Shah et al., 2019
Case report	Participant: 21 year-old woman Exposure: habitual inhalation of dinitrogen oxide over the past year, with increased consumption over the preceding weeks of up to 300 canisters/week	Symptoms: confusion, gait ataxia, impaired insight, orientation, short-term memory, attention Neurological exams: impair limb proprioception with sensory ataxia, positive Romberg test. Decrease limb strength, globally depressed reflexes	Jonhson et al., 2018
Case series report	Retrospective evaluation of subacute degeneration of the spinal cord attributed to dinitrogen oxide exposure Cases from November 2016 to May 2017 Participants: 10 (3 women and 7 men), median age of 22 (range 17-26) Exposure: dinitrogen oxide 2-3 times per weeks, 75 to 2000 canisters per week	Neurological symptoms and exams: altered sensation in the limbs (10/10), gait ataxia (8/10), falls (3/10), Romberg's signs (6/10), Pseudoathetosis (5/10), Lhermitte's phenomenon (1/10), Uhtoff's phenomenon (1/10), segmental myoclonus (1/10) Clinical chemistry: Vit. B12 deficiency (4/10), elevated MMA (7/8 patients), MRI: Changes in the dorsal column of the cervical spinal cord consistent with subacute degeneration Follow up after average 14 month (5-27 months): - Full recovery (3/6) - residual paraesthesia, gait ataxia and sensory	Keddie et al., 2018

		loss (3/6) - Persisting MRI changes (2/4)	
Case report	Case: 22 year-old man Exposure: high-volume recreational use of dinitrogen oxide	Severe peripheral polyneuropathy, vit. B12 deficiency, partial recovery at most recent follow-up	Middleton et al. 2018
Case report	Case: 24 year-old women Exposure: recreational "whippets" exposure to dinitrogen oxide	Examination: unsteady gait, positive Rhomberg sign Clinical chemistry: macrocytic erythropoiesis, vit. B12 deficiency, elevated homocysteine and MMA levels. MRI: degeneration of the posterior spinal column.	Egan et al., 2018
Case report	Case: 27 year-old man Exposure: pain management in the emergency department (> 50 occasion over a 5 year period)	Symptoms: paresthesia, numbness, distal sensory loss Nerve conduction studies: mild large fibre, length-dependent axonal sensorimotor polyneuropathy Clinical chemistry: Normal Hb and MCV MRI: normal Diagnoses: functional leg spasm and sensorimotor neuropathy secondary to functional B12 deficiency related to controlled dinitrogen oxide administration. Follow-up: improvement of sensory symptoms, normal vit. B12, reflexes remained absent and the neurophysiological findings absent.	Kaski et al., 2017
Case report	Case: 23-year old women Exposure: recreational use of dinitrogen oxide (no further information)	Clinical exams: symmetrical weakness of the iliopsoas muscle an quadriceps, paralysis of dorsal flexors of the feet, areflexia of the legs and feet, loss of vibration sense Clinical chemistry: haemolytic anaemia, leukopenia Electromyography revealed axonal polyneuropathy with demyelination MRI: normal cerebrum and spine Diagnostic: leukopenia and severe neurological signs as a result of a severe vitamin B12	Glijn et al., 2017

		deficiency due to recreational use of N_2O Follow up: after 6- month treatment: - slight improvement of paraparesis but the patient is only capable of walking within her own with a walking frame	
Case report	Case: 29 year-old woman Exposure: Inhalation of dinitrogen oxide in A&E as an opioid-sparing agent Other: Poor nutritional status	Symptoms: leg numbness, altered sensation of extremities, 2-month history of increasing falls and urinary frequency Neurological exams.: peripheral neuropathy including 4/5 strength of lower limbs, impaired joint position and pain perception MRI: posterior spinal cord lesions (cervical and thoracic) Diagnose: subacute combined degeneration of the spinal cord (dinitrogen oxide myelopathy) Follow up: improvement but no full recovery after 2-year	Sleeman et al., 2016
Case report	Case: 20 year-old women Exposure: 2-year exposure to dinitrogen oxide and ketamine for recreational purposes, no information on dosage and frequency Patient taken vit. B12 complex before admission	At presentation: one-month unsteady gait, involuntary movement in the four limbs and mild tingling sensation in a stocking glove distribution, difficulty to walk. Neurological exam.: mild stuttering in speech and dystonia in the facial muscle and tongue, full muscle strength, dystonia-like posture in four limbs and athetoid movements in fingers and toes, worsened by eye closure, impaired vibration and proprioception distal to wrists and ankles. Nerve conduction study: sensory motor polyneuropathy. Visual evoked potentials: impaired visual conduction pathway. Impaired somatosensory evoked potential (central and peripheral sensory conduction). Clinical chemistry: anaemia, normal serum vit. B12, iron deficiency. MRI: cervical spinal cord lesions. No effect in brain spinal cord Diagnose: dinitrogen oxide induced spinal cord degeneration Follow up: 3 weeks later - Gradual improvement of gait and involuntary movement - walk slowly independently	Chen et al., 2016
Retrospective case report series	Retrospective evaluation of patients with dinitrogen oxide induced myeloneuropathy Cases from 2005-2015 Participants: 33 (19 men and 14 women), median age of 22 (range 17-26). 56 healthy controls' nerve	Nerve conduction velocity studies: abnormal results in at least one motor or sensory nerve (97% of patients), conduction slowing in peroneal (36%) and tibial (30%) nerves were the most frequently encountered features. Electromyography: active denervation changes	Li et al., 2016

	conduction studies collected for comparison analysis Exposure: 20.9 ±5.5 month (range: 1-120), last dinitrogen oxide exposure to symptom onset: 10.2±1.5 days (range: 1-28). 14 patients used multiple illicit substances	(11/18 patients)	
Case report	Case: 36 year-old man Exposure: Habitual dinitrogen oxide inhalation from « whippits » (300 whippits per day). Potential co-exposure: positive screening results for amphetamine	Symptoms: ascending limb paraesthesiae, progressive balance difficulties. Neurological examination: pseudoathetosis in upper limbs, flexor plantars, reduced vibration sensation to the hips bilaterally and inhability to stand due to sensory ataxia. Diagnostic: myeloneuropathy Follow-up: at 4 and 8 weeks - short distance mobility using zimmer frame 4 weeks after treatment - independent mobility and significant improvement of spinal cord MRI ty 12 weeks after vit. B12 treatment	Massey et al., 2016
Case reports	Cases 1: 25 year -old worker Exposure: mask connected to a plastic bag connected with a whipped cream pump loaded with dinitrogen oxide. History of dinitrogen oxide abuse. Case 2: 35 year-old man Exposure: gas mask connected to a gas cylinder marked dinitrogen oxide. No further information.	Case 1: found dead in his home. At autopsy, no pathological findings, no indication of alcohol, licit or illicit drugs. Case 2: dead at home. No further information	Bäckström et al., 2015
Case study	Case: 27 year-old women Exposure: dinitrogen oxide abuse over 3 years (average of 100-200 “whippit” cartridges daily on 3 or 4 days per week) Medication: vit. B12	Symptoms: abdominal pain and inability to urinate, lower extremities weakness, pins and needles sensation in lower extremities for approximately 1 year Clinical chemistry: vit. B12 deficiency MRI: indicative of spinal cord degeneration Lost to follow up	Pugliese et al., 2015
Case report	Case: 20 year-old women Exposure: Dinitrogen oxide whippets, no further information	Symptoms: bilateral paraesthesia of the lower extremities, limb weakness, difficulty walking, numbness in the hands, bowel incontinence Clinical examination: bilateral hip flexor weakness, thoracic sensory-level deficit, reduced proprioception distally in the bilateral lower extremities MRI: consistent with demyelination of the dorsal regions of the spinal column and demyelination around the central canal. Negative brain MRI. Nerve conduction study: in line with demyelinating polyneuropathy Follow up: 1-month follow-up	Duque et al., 2015

		- the patient showed remarkable improvement of her neurologic symptoms with intact neurological sensitivity and no symptoms of muscular weakness	
Case study	Case: 35 year-old man with dinitrogen oxide-related myeloneuropathy Exposure: daily consumption of over 100 canisters of dinitrogen oxide (so-called "whippets"). Coexposure with 6 mg Xanax and 3-6 beers daily	Symptoms: complaint of disorientation, worsening weakness, flexion contracture of the fingers, urinary urgency, low of balance Clinical chemistry: Normal vit. B12, other parameters not assessed (MMA, homocysteine). Examination: positive Babinski signs, abnormally brisk reflexes of four limbs, diminish sensation in a stocking and glove distribution, ataxia MRI: Cervical spinal cord lesions in dorsal column Follow up: recovery has been slow at the date of publication (no further information)	Rhainbolt et al., 2015
Case study	Case: 19 year-old man Exposure: occasional abused oxycodone, alcohol, huffed dinitrogen oxide canisters several times per week (20 pound canisters of dinitrogen oxide at one setting).	Symptoms: progressive numbness and weakness in all extremities. Progression to an inability to deambulate and numbness in the distal extremities. Examination: proximal weakness of 3/5 in the deltoids bilaterally and distally of 2/5. In the lower extremities, weak iliopsoas muscle group (4/5) and distal groups were 3/5., hyperreflexia, absent sensation to touch and pin prick in the distal extremities as well as vibration and proprioception. Clinical chemistry: no effect on Vit. B12 and folate levels MRI: cervical spinal cord lesions. Follow-up: atypical complete resolution 36 hours after methylprednisolone treatment	Ghobrial et al., 2012
Case report	Case: 19 year-old man Exposure: recreational use of dinitrogen oxide over a period of 2 months up until the time paraesthesia become worse. Approximately 500-600 cartridges from dinitrogen oxide filled balloons during 5-6-hour sessions, 4-5 times per 3-year smoking history.	Symptoms: 1-month history of limb numbness and gait imbalance. Expansion of numbness to upper limb and entire body. Examination: absent of brachioradialis and knee jerck reflexes, negative plantar response, reduced muscle strength (4/5), positive Romberg sign, ataxia, decreased pinprick and temperature sensation in distal extremities. Clinical chemistry: megaloblastic red blood cells, vit. B12 deficiency other parameters were normal. Motor nerve conduction: prolonged distal latency in bilateral median nerves, prolonged F latency, reduced sensory nerve action potential. Electromyography: no spontaneous activity in some muscles. Decreased number of motor unit potentials in these muscles. MRI: cervical spinal cord lesions (posterior and anterior column), no findings in lateral column Final diagnosis: polyneuropathy and myelopathy.	Hsu et al., 2012

		Follow-up: - One week after start of treatment: decreased numbness, mild sensory ataxic gait remained - Full recovery after cessation of exposure without any neurologic sequela	
Case report	Case: 28 year-old man Exposure: 2-year history of recreational abuse. Dinitrogen oxide bulb use. At least 80 bulb per day (1 bulb = 8g dinitrogen oxide under pressure)	Symptoms: bilateral numbness and weakness in both lower limbs, difficulty walking and maintaining balance Examination: absent Lhermitte's sign, subjective sensory deficit to soft touch, decrease proprioception in both feet and vibration sense to the level of both knees. Romberg's negative. Clinical chemistry: increased homocysteine levels, normal vit. B12 levels No other test to exclude other cause than dinitrogen oxide abuse of vit. B12 deficiency. Follow up: - no improvement after 3-month (denies ongoing use of dinitrogen oxide)	Richardson et al., 2010
Case report	Case: 41 year-old man Exposure: dinitrogen oxide abuser: 4-5 cans per day, about 2000 ml/can for more than 10 years.	Symptoms: motor clumsiness and distal paresthesia in the four limbs. Clinical chemistry: abnormal megaloblastic red blood cells and vit. B12 deficiency Motor conduction: sensory-motor axonal polyneuropathy Electromyography: Abnormal visual, brainstem and somatosensory evoked potential MRI: Spinal cord degeneration in the posterior and lateral columns (cervical)	Lin et al., 2007
Case report	Case: 23 year-old female Exposure: Dinitrogen oxide "nanging" initial consumption of 1 box of 10 whipped cream whippet bulbs per day to 13 boxes per days for 6 weeks. History of intravenous drug abuse	Symptoms: profound tetraparesis. Examination: absent pelvic reflexes. Clinical chemistry: raised creatinine and urea (acute renal failure), normocytic anaemia, depressed vit. B12 level Nerve conduction studies: peripheral axonal sensorimotor neuropathy. Encephalogram: severe diffuse encephalopathy MRI: cervico-thoracic spinal cord lesions Diagnostic: toxic myeloneuropathy due to dinitrogen oxide	Shulman et al., 2007
Case report	Case: 33 year-old man Exposure: 4-week exposure to dinitrogen oxide "whippits", daily inhalation.	Symptoms: fixed delusions No motor or sensory deficit Clinical chemistry, increased MMA and homocysteine levels, normal vit. B12 level Follow-up: 2-weeks after start of treatment: fixed delusion resolved. Lost to further follow-up	Sethi et al., 2006
Case report	Case: 26 year-old woman	Symptoms: weakness and numbness of lower	Wu et al.,

	Exposure: weekly dinitrogen oxide inhalation for 2-3 month for recreational purpose	limbs Examination: Strength was 3/5 for lower limbs and 4/5 for upper limbs. Absent of tendon reflex and plantar response, decreased vibration sensation in the feet and legs, respiratory difficulty Nervous conduction and electromyography: sensory-motor demyelinating polyneuropathy. Clinical chemistry: increased MCV, vit. B12 deficiency MRI: abnormal demyelinating lesion affecting the posterior column (cervical spine) Follow-up: symptoms resolved completely after 2 month treatment	2006
Case report	Case: 23 year-old patient Exposure: dinitrogen oxide abuse	Symptoms: diffuse paresthesia and sensory loss Mild reduction of vit. B12, high levels of MMA and homocysteine Resolution after cessation of exposure	Waclawik et al., 2003
Case report	Case: 55 year-old man	Multiple neurological abnormalities, low serum vit. B12. Improvement after cessation of exposure and vit. B12 treatment.	Iwata et al., 2001

MRI: magnetic resonance imaging, MMA: Methylmalonic acid; MCV: mean corpuscular volume

Table 37: Summary table of human data on STOT RE – Occupational exposure

Type of study/report	Test substance, Route of exposure, relevant information about the study	Observations	Reference
Observational study: Experimental at workplace	**Participants:** 30 exposed to dinitrogen oxide (13 ♂, 17 ♀) (1 week with gaseous anaesth. vs 1 week with non-gaseous anaesth. with a 2-week interval) and 20 controls **Exposure:** [N2Oa] (ppm) breathing zone during 3 hours - SW: 50.9 (20.8) - EW : 54.2 (22.1) [N2Ou] (µg/L) - SW : 21.54 (9.2) - EW: 25.67 (10.88)	**LOAEC [N_2Oa] = 54.2 ppm** **[N_2Ou] = 25.67 µg/L** SRT significantly increased after gaseous anaesthesia (*vs* non-gaseous anaesthesia and *vs* controls) at EW. Effects reversible (not seen at the beginning of the week). Other results : - No effect on cortisol but effects on prolactin (associated with SRT; r=0.3, p=0.001) - Correlation between N_2Ou and N_2Oa (r=0.89, p=0.0001)	Lucchini et al., 1996

Observational study: Cross-sectional multi-centre study	**Participants:** 112 exposed and 135 controls **Exposure:** [N_2Oa] (ppm) *Stationary sampling* - SW: 23.2 (3-183) - EW : 20.6 (4-154) [N_2Ou] (µg/L) - SW : 7.1 (1.5-43) - EW: 7.8 (1.0-73) Isoflurane: 1.8 µg/L (0.5 ppm)	**NOAEC [N_2Oa] = 25 ppm** **[N_2Ou] = 13 µg/L** No statistically significant difference between groups in colour word vigilance test, EUROQUEST questionnaire and block design test.	Lucchini et al., 1997
Observational longitudinal study	2 working weeks in a 1-year study **Participants:** 38 exposed (17 ♂, 21 ♀) and 23 control (2 ♂, 21 ♀) **Exposure:** [N_2Ou] (µg/L) ES Monday & Friday - unexposed - <13 - ≥ 13 - <27 - ≥ 27 Workshift = 7h12/day Isoflurane < 3.32 µg/L	**NOAEC [N_2Ou]: <27 µg/L** **LOAEC [N_2Ou]: 27 µg/L** Corresponding to 50 ppm N_2O (Imbriani et al., 1995) Lower performance in colour word vigilance test	Scapellato et al., 2008

SRT: Simple reaction time; SW: start of week, EW: end of week, GM: geometric mean, SD: Standard deviation

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Animal data

Misra et al., 2020, reported in Wistar rats (n=6 male/group) exposed 2h/d for 60 days at 500,000 ppm and O_2 in ratio 1:1 an alteration of spontaneous locomotor activity (reduction in total distance, time moving, number of rearings and time resting) and grip strength. Time rearing and the number of stereotypic counts were not statistically significantly changed. The same conditions of exposure were used in the Singh et al., 2015 study, except that exposure was 2 hours per day instead of 1.5 hour per day. Decreased body weight gain was also seen in the exposed animals. Blood homocysteine levels was significantly increased whereas no significant changes was noted for vitamin B12 or folic acid levels. Although glutathione and total antioxidant capacity were decreased in the plasma of exposed rats, serum malondialdehyde (MDA) and cerebral cortex glutamate levels were higher in the control group. In this study, glutamate level, glial fibrillary acidic protein (GFAP) expression were increased in brain and spinal cord, and related to the behavioural changes. According to the authors, this may suggest that clinical dysfunction may be related to astrocytes proliferation, related to oxidative stress and glutamate neurotoxicity.

In the same laboratory, similar results were obtained by Singh et al. (2015). They investigated behavioural and histopathological changes in male Wistar rats. Rats (6 males per groups) were exposed by inhalation 90 min/day during one month to a mixture of 500,000 ppm N_2O mixed in O_2 in 1:1 ratio. The rats appeared sluggish, lethargic and developed predominantly hind limb weakness after exposure. These effects recovered 1–1.5 h after cessation of exposure. In the exposed group, the total distance travelled, time moving, number of rearings and grip strength were significantly decreased, whereas resting time significantly increased compared to controls. Stereotypic counts were not statistically significantly increased. At this dose, weight loss was noted in the exposed animals. Blood homocysteine level was significantly increase in the exposure group compared to the control group. Nevertheless, serum folic acid and vitamin B12 levels were not significantly different. Glutathione and TAC level significantly decreased following N_2O exposure and histopathological changes were observed in the meninges, brain and the spinal cord. Such changes were not observed in the control group. No abnormalities were noted in the cerebellum. The meninges showed localised capillary congestion with vascular dilatation. Neuronal degeneration (shrinkage and vacuolation)

was observed in the brain. Thickening of vascular endothelial wall with infiltration of mononuclear and polynuclear cells were noted. This finding was more marked in the subarachnoid space. Focal demyelination (depletion of myelin, vacuolation) was noted in the outer layer of the cerebral cortex. Degenerative changes such as neurophagia and satellitosis were observed in the external pyramidal layer. In the spinal cord, multiple demyelinated areas were noted, consisting of vacuolation and depletion of myelin throughout the white matter. The authors suggested that the neurobehavioral changes were due to cobalamin deficiency rather than a direct effect of N_2O. Blood oxidative stress parameters (glutathione levels, total antioxidant capacity) were significantly decreased in exposed group and correlated with the behavioural changes observed.

Following rat exposure to 700,000 ppm N_2O, 4h/day, 5d/w for six months, Dyck et al., 1980, did not found any disturbance in conduction velocity, axonal flow or morphological abnormalities in peripheral nerves in rats. In this study, sign and neurological symptoms, nerve conduction, electromyographic abnormalities in caudal nerve and morphometric and teased fibre abnormalities (from sural nerve and tibial nerve branch) were recorded. The authors concluded that it is unlikely that N_2O is a peripheral nerve neurotoxin in rats.

Hayden et al. (1974) also found damage in cortical cell count following 400,000 ppm N_2O, 7 to 14-day exposure, 8h/day, in rats (abstract only).

In mice, Fung et al., 1993 used lower levels of N_2O. Mice were exposed 8h per day for 8 consecutive days at 1000 or 2000 ppm. At the end of the exposure period, mice were tested for motor coordination, locomotor activity, stereotypic behaviour and anxiety level. No effect was found on motor coordination or anxiety level. Mice exposed to N_2O showed reduced locomotor activity compared to control animals; however, with the exception of the longest time period (120 minutes), this decrease was not statistically significant and not dose-related. Mice exposed to N_2O showed a dose-dependent reduction in stereotypic behaviour. Necropsy was performed in the 2,000 ppm group. Although the number of neural cell counts was less in the N_2O exposed group compared to control mice, this was not statistically significant (206±8 cells in control vs 186±7.2 in 0.2% N_2O group). No significant differences were seen in the number of neuroglial cells or total cells counted in the control tissues, as compared to the neural tissues from mice exposed to N_2O. The authors suggested that short-term exposure to N_2O might alter central dopaminergic neuronal activities in striatal and mesolimbic regions.

No change in brain weight or histology was noted in repeated-dose toxicity studies (Rice et al., 1985) up to 500,000 ppm N_2O when male and female mice were exposed 14-week, 4h/d, 5d/w.

In summary in rats, clinical signs (ataxia), structural changes in brain and spinal cord (vacuolation of neurons, demyelination), neurochemical (hyperhomocystinaemia, decrease in methionine synthase activity, decrease glutamate levels), and behavioural changes (locomotor activity, grip-strength) have been reported following repeated high dose exposure to N_2O. In some studies (Singh et al., 2015), concomitant structural changes, behavioural changes and neurochemical changes were observed in the animals providing support of the neurotoxic potential of N_2O. These effects were investigated and reported only at very high dose levels ≥ 400,000 ppm not relevant for classification STOT RE. No data are available at lower dose levels that would be relevant for classification.

Only two mouse studies investigated the potential effect of N_2O (Rice et al., 1985 and Fung et al., 1993). Rice et al. (1985) did not report histopathological or weight changes in the brain at concentrations up to 500,000 ppm N_2O in a 14-week inhalation study. In a 8-day sub-acute study, Fung et al. reported doubtful effects on the locomotor activity of animals and dose-related changes in the stereotypic behaviour of mice at ≥ 1000 ppm. No such change on stereotypic behaviour was noted in rats at higher exposure levels (up to 500,000 ppm) in Singh et al. (2015) and Misra et al. (2020). Nevertheless, differences in methods (duration of exposure) and test animals (different strain), make the comparison difficult. In addition, the observed effects in Fung et al., 1993 were all observed at dose levels relevant for classification STOT RE (≤2500 ppmV for STOT RE 2 classification for a 8-day study).

Severe ataxia, peripheral nerve degeneration and spinal cord degeneration and demyelination was noted by following 18, 30, 35 and 56-day continuous exposure to 150,000 ppm dinitrogen oxide in monkeys (n=1/group) (Dinn et al., 1980). Central grey matter and anterior horn cells were normal in the spinal cord. Brain and motor nerves were normal in these monkeys. No haematological changes were noted in this study. The study is considered of low reliability as only 1 animal per group was used and as no concurrent control

were used in the study. Nevertheless, the changes were very consistent with the observed effects in other animal species and human.

Human data

Information regarding specific target organ toxicity following repeated exposure in humans is mainly provided by extensive literature concerning adverse health effect in occupational setting and dinitrogen oxide recreational use. For the purpose of classification, evidence from epidemiological studies and case reports are considered in a weight-of-evidence approach.

Human data - Dinitrogen oxide abuse/recreational use

- Poison control centre data

Dinitrogen oxide, commonly known as "laughing gas", is used among other things in whipped cream siphon cartridges. Recreational inhalation has been on the rise since 2018. Following a request by the French Health Products Safety Agency (ANSM), the French poison control centres (PCCs) analysed 66 cases recorded between 1 January 2017 and 31 December 2019. They concerned young people, in a mostly festive context, consuming quantities ranging from just a few cartridges to several hundred a day, over several months. The most frequently reported effects were neurological and neuromuscular disorders (paraesthesia, tremor in the extremities and muscle pain). Four people reported symptoms suggestive of peripheral neuropathy following chronic inhalation of dinitrogen oxide.

A total of 63 medical records (66 individuals) concerned exposure to dinitrogen oxide in a context of recreational use/ addiction. Of these, 39 were men and 27 were women. This equated to a M/F sex ratio of 1.4, indicating a male preponderance. Users were young, with a median age of 21 years old, ranging from 14 to 49 years, and 54.5% of cases concerned people between 20 and 25 years of age. The vast majority of exposures occurred in 2019, the last year of the study, with 46 cases compared to 10 cases for the years 2017 and 2018 respectively, underlining the increase in consumption. The regions of Hauts-de-France (northern France) and Île-de-France (including Paris) were the most concerned, each accounting for a quarter of cases. When this information was reported, i.e. in only 57.6% of cases, the type of dinitrogen oxide consumed was almost exclusively dinitrogen oxide contained in cartridges for food use, available over the counter and inhaled via balloons. The duration and history of consumption varied widely. These ranged from occasional consumption at a party to consumption several times a day for months. Similarly, the individuals reported taking quantities ranging from just a few cartridges to several hundred a day, with great variety in the total quantities. Lastly, in 47% of cases, the dinitrogen oxide was inhaled in the home of the exposed person or of their family or friends. In 13.6% of cases, consumption took place in a nightclub/bar, 10.6% at a party where the location was not specified, and 6.1% during a student integration weekend. Fifty-nine people reported adverse symptoms following inhalation of dinitrogen oxide. Neurological and neuromuscular problems were the most common signs. 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 this gas. Half of the 42 cases suffered from at least one symptom such as headaches / dizziness / balance disorders.

Of the 59 cases with adverse symptoms, 40 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 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 dinitrogen oxide – around ten cartridges a day for one, and around forty a day for the other – at home and without taking any other psychoactive substances. Both had neurological symptoms. Since the beginning of 2020, more new cases have been reported to the French poison control centres (PCCs). Some presented serious neurological signs, confirmed by medical imaging. These involved regular consumers occasionally increasing their intake, a situation that had neurological consequences.

Because the quantities consumed were not systematically reported to PCCs, it was not always possible to link the reported symptoms to the claimed quantities. One way to quantify this exposure would be to measure dinitrogen oxide levels in urine, but these tests are rarely performed and their interpretation is

subject to great uncertainty. These tests cannot therefore provide routine assurance of actual exposure to dinitrogen oxide (ANSES, 2020, report published in French).

- Published case reports

According to the systematic review of case reports in Medline (Oussalah et al., 2019), 100 cases with individual data were published from 1966 to 2018. Median age was 27 years, the male: female distribution ratio was 60:40. The case included in the review was repeatedly exposed to dinitrogen oxide with a minimum consumption of one cartridge per month. 76% had regular exposure. Patients were exposed to dinitrogen oxide in the setting of recreational use (57%) or surgery (25%). Median number of cartridge per day: 25, median duration of exposure: 0.7 year.

The three main diagnoses were subacute combined degeneration (28%), myelopathy (26%) and generalised demyelinating polyneuropathy (23%). In patients that underwent MRI, changes in the spinal cords (T2 signal hyperintensity) was 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 (MMA) and homocysteine levels in 90 and 94% of the patients, respectively.

Univariate analysis of the data by Oussalah et al., 2019 found that among all the investigated variable, age (> 40-year), vit. B12 concentration (≤ 74 pmol/L) and MCV (> 100fL) were associated with a short dinitrogen oxide exposure, mostly associated with surgery and a more severe clinical picture.

Data on the amount of dinitrogen oxide exposure was available in 28 patients. The amount of dinitrogen oxide was not significantly correlated with any biological variable. 75% of the regular users were recreational users.

The neurological disorders reported in other case reports were similar to the ones include in this systematic review study. Almost all the cases reported in the table above were included in the systematic review except some published paper not including data on biological parameters or where preventive treatment with vit. B12 before dinitrogen oxide exposure was used.

Several follow-up studies revealed persistent symptoms. The duration of follow-up was in most of the cases only of few month. Nevertheless, in Sleeman et al., 2016, full recovery was not observed after 2-year follow-up. Among 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 focus on neurological sequel and psychiatric disorders following dinitrogen oxide abuse. In the 59 cases studied by the authors for whom follow-up information was available, neurological symptoms improved in 46 cases and persisted in 3 cases. Symptoms fully resolved in 10 cases. In one case the patient's symptoms almost entirely resolved, but he later committed suicide after relapsing. It has to be noted that as the substance is use in the context of abuse, it can be difficult to quantify truly and accurately dinitrogen oxide exposure and they may be uncertainties on the stop of exposure.

Human data- Occupational exposure

- Cases report:

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 room (where air conditioning was deficient during three years). CTE was characterised as an impairment of cognitive functions on at least 3 specific behavioural domains among 6 (attention, memory, executive skills, dexterity, visuospatial organization and psychomotor slowness). The authors reported high levels of anaesthetics gases with mean concentration of N_2O (311 ppm, peaks 1,600 ppm) and halogenated gases (16 ppm, peaks 1,600 ppm).

A direct relationship between N_2O exposure and CTE is uncertain in these 3 cases, because of anaesthetists are also exposed to many other neurotoxic agents including halogenated anaesthetic gases. Also in these individual cases, other non-occupational risk factors could have played a role in CTE development.

- Studies carried out at workplace (operating theatres):

Lucchini et al. (1996) conducted a study in an Italian hospital. In this study, authors examined 30 operating room workers. The group of volunteers represented 80% of the entire personnel of the department and was composed of surgeons, anaesthetists, operating room nurses and technicians. A control group consisted of 20 subjects randomly selected among medical and paramedical personal in other departments in the same hospital. N_2O atmospheric concentration was measured using personal sampling during a 3 hours period of time. Urinary N_2O was also measured in urine at the end of the shift. Simple reaction time (SRT) test was selected as psychomotor test and was performed at two different times: first, during a week with constant use of non-gaseous anaesthesia and secondly, during a week with constant use of gaseous anaesthesia, with a two-week interval between these weeks. In addition, biological measurement were performed: serum cortisol as a biological stress indicator and serum prolactin to investigate interference with the dopaminergic system. The authors used a –so-called "double-blind testing condition": as a matter of fact, only the 4 (exposed) anaesthetists knew during which week gaseous anaesthesia was used. Potential confounding factors such as age of alcohol consumption were checked. No information on potential co-exposures was provided in this study. On the last day of the gaseous anaesthesia week, mean N_2O air concentration was 54.2 (SD= 22.8) ppm and mean urine concentration was 25.6 (SD= 22.1) µg/L. A good correlation between N_2O in air and in urine (r=0.89; p=0.0001) was found. The study shows a prolonged reaction time and increased serum prolactin levels in exposed workers only when they worked with gaseous anaesthesia. No effect of N_2O exposure was observed for serum cortisol levels. The authors concluded that their results indicate neurobehavioural effects of N_2O exposure below 100 ppm. However these results should be considered with caution, as co-exposures were not taken into account and the number of workers included in the study was small.

In order to better define a safe exposure level, Lucchini et al. (1997), conducted a multi-centre study in Italy evaluating neuropsychological symptoms, subjective stress and response speed functions in subjects occupationally exposed to low levels of anaesthetic gases. A group of 112 operating theatre workers from 10 Italian hospitals was exposed to anaesthetic gases (N_2O and isoflurane), and 135 non-exposed workers were used as control group. The workers were examined before and after the shift on the first and the last day of the working week. The testing comprised a complex reaction time test (the Stroop Colour Word) and a subjective mood scale. The week preceding the first testing a training session was organized in order to limit the learning effect of neurobehavioral testing. During this session, a questionnaire for neuropsychological symptoms (EURO-QUEST) was administered together with the block design subtest from the WAIS battery measuring visuospatial and motor skills, Mood scale measuring stress and arousal state, Colour word vigilance test, which is a complex reaction time test. The aims of these supplementary tests were to examine basic intellectual abilities of the participants. Biological and atmospheric indicators of exposure were measured at the beginning and the end of the working week for N_2O and isoflurane. The results of these measurements indicated moderate exposures: for atmospheric N_2O geometric mean and 95^{th} percentile were 23.2 ppm and 127 ppm on the 1^{st} day and 20.6 ppm and 114 ppm on the last one; the corresponding values for isoflurane were 0.4 ppm and 3.8 ppm, and 0.3 ppm and 2.7 ppm. For end-of-shift urine N_2O concentrations geometric means and 95^{th} percentiles were 7.1 µg/L and 12.4 µg/L on the 1^{st} day, 7.8 µg/L and 21.5 µg/L on the last one. No statistical difference was observed between exposed and control subjects for neurobehavioral effects, stress and arousal levels. The authors concluded that the biological exposure limits of 13 µg/L for urine N_2O concentration (corresponding to 25 ppm for TWA air concentration) is adequately protective for the integrity of workers neurobehavioral functions, as measured with the tests used.

In an Italian hospital, operating-theatre workers exposed to anaesthetic gases (N=38) and 23 unexposed nurses participated in a longitudinal study (Scapellato et al., 2008) during one year to investigate effects on neurobehavioral functions. Neurobehavioral functions were assessed using a battery of tests: Euroquest self-administered questionnaire, exploring symptoms, Block design subtest (WAIS) measuring visuospatial and motor skills, Mood scale measuring stress and arousal state, Colour word vigilance test, which is a complex reaction time test. The study was designed to consider potential pre-existing abilities and potential changes over time (repeated cross-sectional study). Three measures were taken for each subject at each of four time points: before and after work shift on Monday and Friday of a working week, twice a year. To attenuate learning effects, the subjects were allowed to practice the tests before the experimental session. The colour word vigilance (CWV) test was the endpoint used to appraise short-term effects induced by N_2O and isoflurane. Exposure was assessed *via* biological concentration in urine (urinary N_2O and isoflurane) at the end of work shift (Monday and Friday), twice a year (not stated if tests are performed on the same week). Contamination of urine was avoided and urine was analysed using gas chromatography with electron capture detection. The authors gathered information on the subjects to identify potential confounding factors. The subjects were classified into 4 groups (A: unexposed, B: <13; C: ≥ 13 to <27 and D: ≥ 27 µg/L). The urinary concentrations of 13 and 27 µg/L correspond respectively to air concentrations of 25 and 50 ppm. For the analysis of repeated measures of CWV, a model of two-stage regression was used, which was built as follows. In the first-stage, reaction times (or CWV test results) were plotted against time in each subject, obtaining through the simple regression analysis a slope (or coefficient of regression), which expressed the individual change of CWV test results over a working week. At the second-stage, the slope was the dependent variable in a multiple regression analysis in order to select factors which affected longitudinal changes in reaction times among the following variables: general characteristics of subjects (age, gender, years of schooling, alcohol and coffee consumption, smoking habit, length of work); subjective symptoms (EQscores); basic cognitive abilities (BD test results); Monday morning CWV test result (the baseline value, conveying the pre-existing ability of the subjects); and occupational exposure. This approach consists therefore of a regression model for the average response over time and the effects of covariates on this average response. Stress and arousal were taken concurrently with CWV and, since the contingent mood state could affect CWV, they were analysed simultaneously using a multiple analysis of variance for repeated measures. Although the overall means were below the reference values of 27 µg/l for N_2O and 3.32 µg/l for isoflurane, urinary concentrations of N_2O exceeded the biological exposure limit in 12 out of 38 exposed subjects (32%), and that of isoflurane in 4 out of 38 (11%). No significant difference was found for all variables except sex (effect of sex distribution on reaction time was of borderline significance). There was no significant correlation between urinary levels of N_2O and end-shift CWV values, separately on Monday and Friday. With respect to the unexposed group, CWV test results over a working week were significantly ($p < 0.020$) higher in the Group D, but not in Group B nor C. There was a rough dose–effect relationship between increasing N_2O level of exposure and impairment of neurobehavioral performance. Since they were not significantly different, Groups B and C were considered equal to Group A and the two groups were collapsed into a single unit. Therefore, subjects were then categorized in two classes, according to the level of N_2O urinary concentrations being below or above 27 µg/L. The weekly profiles of reaction times for the two groups were not parallel. In subjects with urinary concentrations of N_2O below 27 µg/l, there was a linear decrease in reaction times from Monday morning to Friday evening, indicating a learning effect. In subjects with N_2O urinary concentrations above 27 µg/l, the means of the CWV were essentially steady across a work week, indicating that performances may have been impaired. The highest difference is located between Monday end-shift and Friday before shift. For arousal, the tests of within-subjects contrasts were significant at ‘‘trial 1 vs. 2’’ ($F = 9.845$; $p < 0.003$) and at ‘‘trial 3 vs. 4’’ ($F = 5.719$; $p < 0.020$). In subjects with N_2O urinary concentrations above 27 µg/L, arousal was low on Monday morning, increased at end of the workshift, and remained high until Friday evening. In subjects with urinary N_2O below 27 µg/l, arousal was high before workshift, and low after workshift, on both Monday and Friday. It seems, therefore, that

significant changes in reaction times and arousal occur from Monday to Friday, thus suggesting a cumulative effects of anaesthetic gases over a week of exposure. No contrast was significant for the stress. According to the authors, for N_2O urine concentration at the end of shift, 27 µg/L is a threshold under which vigilance alteration is not expected in exposed workers; it corresponds to 50 ppm for TWA air concentration. However, their results should be considered with caution, due to the small number of workers concerned in their study and also because occupational co-exposures were not taken into account.

In summary, information on low-dose human exposure is available. The effects observed in three studies of good quality consistently showed that neurobehavioral changes could occur following repeated low exposure levels to dinitrogen oxide. However, there are some uncertainties concerning the strength of the effects due to co-exposure with other anaesthetic gases at low concentration. Although cumulative effects were noted thorough a working weeks, the effects were reversible following the week-end.

Mechanism of action

The neurological effects are the results 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. Nevertheless, the time course for N_2O inactivation of methionine synthase has been identified to be species dependent. In rats exposed to N_2O, the half-time of hepatic methionine synthase inactivation is 5 minutes (Royston et al., 1983). After cessation of exposure, recovery takes 3 to 44 days because the vitamin B12 cofactor is irreversibly oxidized and covalently bound to the enzyme. New enzyme must be synthesized before the restauration of the activity. In humans, the half-life of inactivation is about 45 min (in biopsied liver cells). 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.

10.12.2 Comparison with the CLP criteria

In rats, severe and adverse effects in the meninges, brain and the spinal cord were noted in several studies. Nevertheless, only high concentrations of dinitrogen oxide were tested, not relevant for classification STOT RE 2.

In mice, no histopathological findings in brain were noted up to 500,000 ppm in Rice et al. (1985). Nevertheless, the behavioral changes observed in Fung et al. after 8-day exposure to 1000 ppm could support a classification of dinitrogen oxide as STOT RE 2.

In human, there are numerous case of reported demyelinated polyneuropathy, subacute combined degeneration of the spinal cord. Although some of the cases were due to recreational use, in some cases, severe effects were seen under controlled dinitrogen oxide conditions (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. Epidemiological studies from occupational exposure although support that neurobehavioral effects may occur at low dose levels.

According to the CLP criteria, substances are classified in category 1 if they met the following criteria: *Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies*.
Specific target organ toxicity (repeated exposure) means specific, target organ toxicity arising from a repeated exposure to a substance. All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed are included.

Overall, based on good quality evidence from human cases and epidemiological studies, dinitrogen oxide induce after repeated exposure effects on the central nervous system. A classification of dinitrogen oxide as STOT RE for the nervous system is thus warranted.

Nevertheless, according to the ECHA guidance on classification criteria, "*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 recreation abuse cases support that repeated-dose exposure to dinitrogen oxide can lead to exacerbation of toxicity as shown by the severe effects such as myelopathy with potential irreversible consequences. The available mode of action also support that STOT RE may be more relevant that STOT SE as it will be the accumulation of homocysteine levels and/or deficiency of methyl constituent that will be responsible of the observed effects.

10.12.3 Conclusion on classification and labelling for STOT RE

Based on significant adverse health effects in nervous system of humans exposed to dinitrogen oxide, classification of dinitrogen oxide 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 is considered appropriate.

An SCL is not proposed as dinitrogen oxide did not induce target organ toxicity at a dose level clearly below the guidance values according to CLP regulation.

10.13 Aspiration hazard

Evaluation not performed for this substance.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Evaluation not performed for this substance

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

Dinitrogen oxide is a greenhouse gas and one of the most important gaseous species currently leading to stratospheric ozone depletion (ozone-depleting gas). Dinitrogen oxide is the dominant source of reactive nitrogen to the stratosphere.
Indeed, N_2O is transported to the stratosphere and broken down in the middle stratosphere and above via photolysis (Portmann et al., 2012):
$N_2O + h\nu \rightarrow N_2 + O^{1D}$
$N_2O + O^{1D} \rightarrow N_2 + O_2$
$N_2O + O^{1D} \rightarrow 2\ NO$
The NO produced in reaction to the above reaction is the primary source of reactive nitrogen (i.e. NO_x: ozone depleting nitrogen oxide) in the lower and middle stratosphere (transport of mesospheric NO_x can be significant in the upper stratosphere). The formation of reactive nitrogen can lead to the catalytic destruction of ozone:
$NO + O_3 \rightarrow NO_2 + O_2$
$NO_2 + O \rightarrow NO + O_2$
$O_3 + O \rightarrow 2\ O_2$
NO_2 formed can also photolyse to restore NO, or react with chlorinated species. Thus, as briefly summarised in JRC, 2015 dinitrogen oxide leads to stratospheric ozone depleting nitrogen oxides (NO_x) and free radical reservoirs (e.g. HNO3) in the stratosphere (Crutzen, 1970).

N_2O has a very long atmospheric lifetime. SPARC (2013) summarized the SPARC lifetimes assessment which estimated the lifetime of dinitrogen oxide to be 123 (104-152) years (2-sigma "most likely" range). Based on observations from the Microwave Limb Sounder (MLS) and a radiative transfer model, Prather et al. (2015) recommend a lifetime of 116 ± 9 years, which is lower than the maximum likelihood SPARC estimate but within their uncertainties.

According to the classification criteria, a substance shall be classified as Hazardous to the Ozone Layer (Category 1) 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.

The ozone-depleting potential (ODP) is used as a metric to compare the impact of various gas emissions on ozone and as a classification criteria in the CLP regulation. As indicated in the ECHA CLP guidance document, any substances having an ODP greater or equal to the lowest ODP of the substances currently listed in Annex I to Regulation (EC) No 1005/2009 (i.e., ODP 0.005 for Chlorofluoroethane) should be classified as hazardous to the ozone layer (category 1).

The ODP provides the global mean ozone loss for a gas per unit mass emission relative to chlorofluorocarbure CFC-11. The ODP is defined as the time-integrated global ozone depletion induced by a perturbation of an equal mass emission of gas X relative to a reference gas (always taken to be CFC-11, labelled F11 below):

$$\mathrm{ODP_X} = \frac{\int_0^\infty [\Delta \mathrm{O_3}]_\mathrm{X}^P \mathrm{d}t}{\int_0^\infty [\Delta \mathrm{O_3}]_\mathrm{F11}^P \mathrm{d}t},$$

where [□O3] is the global mean total ozone change induced by the perturbation (the P superscript refers to the pulse emission and the subscript is the perturbation compound) (Portmann et al., 2012).
According to Portmann et al., 2012, the ODP is rarely calculated using the formula above. Instead, it can be re-written in steady-state form:

$$\mathrm{ODP_X} = \frac{m_\mathrm{F11}}{m_\mathrm{X}} \frac{\Delta\mu_\mathrm{F11}}{\Delta\mu_\mathrm{X}} \frac{\tau_\mathrm{X}}{\tau_\mathrm{F11}} \frac{[\Delta \mathrm{O_3}]_\mathrm{X}}{[\Delta \mathrm{O_3}]_\mathrm{F11}},$$

where m is the mass, Δm the mixing ratio perturbation, t the lifetime and [ΔO3] is the steady-state annual and global mean total ozone change induced by the perturbation. This steady-state formulation is valid for source gases characterized by a first-order decay process. It also assumes that the ozone change is linear over the range of changes of gas X and CFC-11, which is valid for the sizes of perturbations considered here.

The ODP of N_2O has been calculated using a well-established methodology published in Ravishankara et al., 2009. The authors used the Garcia-Solomon two dimensional model. The model is described in Garcia et al. (1992), Solomon et al. (1998) and Portmann et al. (1999). This model has been used to study past and future changes in ozone and includes comprehensive chemistry, detailed radiative transfer, and dynamics including planetary and gravity wave breaking schemes. The model, under the conditions of year 2000, indicates an ODP value of dinitrogen oxide of **0.017** (Ravishankara et al., 2009). Although the value may be conservative, it is well above 0.005.

As noted in WMO (2018), ODPs depend on the background atmosphere. Revell et al. (2015) have confirmed that this is especially the case for N_2O, where ODP values are likely to be larger (by as much as a factor of two depending on levels of chlorine and methane in the stratosphere) for 2100 than in the present day.

This ODP value of N_2O is comparable to the ODP of many hydrochlorofluorocarbons that are currently being phased out under the Montreal Protocol, such as HCFC-123 and HCFC-124.

Therefore, as N_2O has an ODP of 0.017, it warrants classification as hazardous to the ozone layer in category 1, H420.

13 ADDITIONAL LABELLING

14 ANNEXES

Annex I: Non-confidential annex documenting the key studies for assessment.

15 REFERENCES

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