15th July 2022

CONSULTATION ON HARMONISED CLASSIFICATION OF DINITROGEN OXIDE RESPONSE BY MEMBERS OF EUROPEAN CYLINDER MAKERS ASSOCIATION

About ECMA

The European Cylinder Makers Association, ECMA, represents the majority of gas cylinder manufacturers across Europe. The Association can trace its origins back to the early 1990s. ECMA's objective is for safe and efficient European gas cylinders and to support this ECMA members actively participate in the development of international standards and ECMA is present at various international regulatory forums such as the United Nations Dangerous Goods Transport Committees.

Executive Summary

ECHA have issued a consultation on the harmonised classification and labelling for dinitrogen oxide, (nitrous oxide).

Several members of the European Cylinder Makers Association are actively involved in the manufacture and supply of small cylinders that contain dinitrogen oxide. They have commissioned response to the proposed changes for a harmonized classification to dinitrogen oxide. This response is Annex 1 of this document.

The report does not support the proposed changes to the harmonized classification of dinitrogen oxide and on behalf of its affected members ECMA requests that there are no changes made to the existing classification of dinitrogen oxide.

Comments to ECMA Member's Report

Reproductive toxicity

All of the animal studies have substantial limitations (non-GLP, not test guideline compliant as recommended by the European authority, single dose level, overtly high concentrations dosed, small group size) that make it difficult to interpret the data. It is further prudent to note that the overtly high concentrations used would have displaced oxygen levels, with no understanding of hypoxia compared to toxicity induced by exposure alone to dinitrogen oxide. The overtly high concentrations used in the animal studies exceeded the maximum recommended concentration as detailed in OECD TG for inhalation studies (20,000 ppm).

It is furthermore important to acknowledge that with only limited reporting of maternal toxicity, an assumed secondary non-specific consequence of other toxic effects cannot be excluded.

The human data provided by ANSES was only discussed briefly due to the lacking reliability of the results. These were retrospective studies which investigated the effect of dinitrogen oxide on midwives and dental assistants, respectively. In the first study the exposure was estimated based on the average deliveries per month and in the second study the exposure was derived from the total scavenged dinitrogen oxide during dental therapies. The main bias in both studies was that a co-exposure to other factors influencing reproduction could not be excluded, e.g., smoking,

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contraceptives, exposure to other anaesthetics, lifestyle, underlying disease etc. Thus, a clear causal relation cannot be established between dinitrogen oxide and fertility.

It should also be noted that there has been no discussion or consideration of other potential modes of action (hypoxia, cobalamin deficiency).

In conclusion, a classification of dinitrogen oxide neither in Category 1A or 1B (missing evidence in humans) nor in Category 2 with regard to toxicity to reproduction is not justified.

Specific target organ toxicity

Single exposure: from the available human volunteer studies provided in the CLH Annex, the limited reporting of the data does not provide a reliability assessment. With the limited reporting of methodology, limited test article characterisation of the retrospective application of the ToxRTool to assess reliability of the data (as detailed in Schneider et al (2009) Toxicology Letter, 189(20) pp 138-144) cannot be applied.

Specific target organ toxicity – repeated exposure

When considering STOT RE classification, solely based on animal data defined cut off values are provided in the CLP guidance. When administration via inhalation is the route of exposure the guidance provides the following:

- Category 1: (rat [6 h], gas): C≤50 ppm;
- Category 2: (rat [6 h], gas) C>50, but <250 ppm)

With the same limitations as described above there is also relevance for the animal studies used to conclude on STOT RE, with reliability of the data used to drive STOT RE deemed to be questionable. Adversity effects were observed at concentrations that exceeded the maximum recommended concentration for repeat dose inhalation studies (20,000 ppm), without consideration of hypoxic effects or other potential mode(s) of action.

ANSES also considered human data as a weight of evidence approach for nervous system effects. It however is pertinent to note that these data were taken from case studies reporting the misuse of dinitrogen oxide. The cohort of people that misused dinitrogen oxide, other confounding factors are not neither discussed or considered i.e.the misuse of other psychotic or mood disorder drugs or substances of abuse which cause CNS/PNS effects. Reliability assessments have not been undertaken (as previously detailed) on these studies.

In conclusion, the concentrations used in all the animal studies considered for STOT RE exceed the CLP pre-defined cut-off values and therefore animal data should not be used to classify for either Category 1 or 2. It is further added that for the reasons discussed above the human data should not be used in a weight of evidence approach for STOT RE classification. Therefore, a STOT RE classification based on the animal and human data is not supportable.

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Annex 1 to ECMA Submission
CONSULTATION ON HARMONISED CLASSIFICATION OF DINITROGEN OXIDE
RESPONSE BY MEMBERS OF EUROPEAN CYLINDER MAKERS ASSOCIATION

Reproductive toxicity:

Maternal Toxicity

All studies listed by ANSES that are supposed to provide evidence for reproductive/developmental toxicity are not sufficient to derive a Harmonized Classification and Labelling for toxicity to reproduction. Predominantly, general toxicity, i.e., maternal toxicity was not detailed in the available publications. In order to determine an actual specific reproductive effect, maternal toxicity has to be excluded.

Regulation (EU) No. 1272/2008 (CLP) states:

Data from animal studies ideally shall provide clear evidence of specific reproductive toxicity in the absence of other systemic toxic effects.....If appropriate information is available, it is important to try to determine whether developmental toxicity is due to a specific maternally mediated mechanism or to a non-specific secondary mechanism, like maternal stress and the disruption of homeostasis……. In some situations it can be assumed that reproductive toxicity is due to a secondary consequence of maternal toxicity and discount the effects, if the substance is so toxic that dams fail to thrive and there is severe inanition, they are incapable of nursing pups; or they are prostrate or dying.

Data quality

Furthermore, ANSES proposes a classification on a Weight of Evidence (WoE) approach. According to CLP regulation this approach is influenced by several factors like:

the quality of the studies, consistency of results, nature and severity of effects, the presence of maternal toxicity in experimental animal studies, level of statistical significance for inter-group differences, number of endpoints affected, relevance of route of administration to humans and freedom from bias.

In contrast to the conclusion of the proposal for the harmonized classification and labelling of chemicals and as can be seen from the listed studies above, the majority of these studies cited by ANSES does not provide consistent results and due to the different degree of detailedness of the reported results the reason for inconsistency in the results cannot be evaluated (CHL report for dinitrogen oxide; Proposal for Harmonised Classification and Labelling; Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2.; version No. 2; p. 18 (April 2022).

Moreover, the two studies that were mentioned in the proposal as reliable database for toxic effect to reproduction indeed showed effects on female fertility but did not provide any statistical examination or showed deficiencies in the description of methods (Kugel et al., 1989, 1990).

Other limitations of these studies include:

- only one concentration tested;(Kugel et al., 1990; 1989, Kripke et al. 1976, Vieira et al., 1983)
- no general toxicity was reported; (Kugel et al., 1990, Kripke et al. 1976, Vieira et al., 1983, Mazze et al., 1982, Land et al., 1981)
- and the methods, e.g. the inhalation chamber, was not sufficiently described, small animal number, only a few parameters examined (Kugel et al., 1990, 1989, Kripke et al., 1976, Vieira et al., 1983, Rice et al., 1985, Mazze et al., 1983, 1982; Land et al., 1981)

Thus, a clear causal correlation which leads to a concern on female fertility cannot be derived.

In two studies, only a trend towards a decrease in litter size was reported, the statistical examination was also missing here (Kugel et al., 1989, 1990).

The CLP Regulation states:

If, in some reproductive toxicity studies in experimental animals the only effects recorded are considered to be of low or minimal toxicological significance, classification may not necessarily be the outcome. These effects include small changes in semen parameters or in the incidence of spontaneous defects in the foetus, small changes in the proportions of common foetal variants such as are observed in skeletal examinations, or in foetal weights, or small differences in postnatal developmental assessments.

Concentrations

The concentrations used in the mentioned studies ranged from 0.1% to 80% N_2O with varying exposure times and durations. Many concentrations used were far above the exposure concentration applied even in operating theaters (dentists, anesthetists etc.). N_2O is no longer used for anesthesia in general but a concentration of 50% N_2O in air was identified to be sufficient for short-term anesthesia i.e., several minutes.

One important aspect of general parental toxicity is not described in sufficient detail in all the publications included into the weight of evidence approach by ANSES. High concentrations (>50%) of N_2O lead to hypoxia. since there was no description of maternal toxicity, hypoxia cannot be ruled out as primary source of a reproductive effect. However, even when no ouvert toxic signs were observed in the dams it should be assumed that exposure to N_2O leads at least to a partial tissue hypoxia and that the reproductive tissues and the developing fetus are particularly vulnerable towards hypoxia.

Mode of action

Possible effects that can result from hypoxia during the reproductive cycle are damages to testicular seminiferous tubule leading to the disorder of seminiferous epithelium and shedding of spermatogenic cells, estrus cycle irregularity, morphological alterations of the uterus and ovary. (Li et al., 2021, Liu et al., 2020). It was also reported that the number of pups per litter were reduced by hypoxia and this effect was markedly increased if both parental animals were held under hypoxic conditions (Cikutovic et al.2009). Based on this information the alterations detected in rats and mice exposed to N_2O cannot be exclusively assigned specifically to N_2O.

Maternal toxicity – toxicity to reproduction

Only Rice et al. (1985) and Mazze et al. (1983) gave a short, very limited insight into maternal toxicity, i.e. stated that the animals survived. However, this description is not sufficient to exclude relevant maternal toxicity as mortality is the final toxic sign. Clinical signs like sedation or anesthesia, maternal behaviour is expected to be reported in reliable studies. Further parameters like body weight, body weight gain, organ weights (total and relative to body weight), food consumption and/or water intake are necessary to determine the magnitude of maternal toxicity and the severity of the detected effects. For example, if there is a reduction in food consumption, decreased pup weights can occur which are not based on developmental toxicity. Besides, also the concentration of N_2O is an important factor. None of the mentioned studies provided a dose-range finding. The concentrations used in the animal studies however are based on observations made in clinical trials examining the role of N_2O in medical usage and the suspected correlated decrease of pregnancy in nurses etc. (see also human data).

Concentrations - toxicity to reproduction

Low concentrations as used by Vieira et al (1983), Holson et al. (1990), Kugel et al. (1989), Rice et al. (1985) and Mazze et al. (1983 and 1982) showed only a marginal if any and reversible effect on reproduction: i.e. decreased litter size (reversible), oestal cycle disturbance (reversible), the latter one

cannot be assigned to the detected hormonal changes (increase in LHRH). At concentrations above 50% (Kripke et al. (1976), Land et al. (1981)) for varying exposure durations (60%: 8h/d-35 days or 24h/d; 80%: 4h/d-5 days) more severe effects were determined like injuries in spermatogenic cells. In these cases, the reversibility was not reported. Kugel et al. (1990) investigated the effect of 30% N_2O on female rats and found disrupted cycles following the first day of exposure and 11 out of the 12 exposed rats went into constant proestrus. But again, this effect was reversible within three weeks after the end of exposure. It cannot be ruled out that hypoxia of varying degree occurred and is responsible for the secondary reproductive effects.

Moreover, only the minority of the presented studies were conducted with at least three different concentrations as recommended by the current guidelines for reproductive and developmental toxicity. In most studies only one high concentration was used and as explained above, hypoxia was not excluded. From these studies no concentration-response relation can be derived and it remains unclear whether there is a concentration without an effect.

Methodologic limitations - toxicity to reproduction

Further limitations diminish the reliability of the presented results. The number of animals used was in general below the numbers required by current guidelines and thereby making reliable statistical analysis at least difficult. Webman et al. (1990) used 5-22 animals per group with the control group exhibiting the lowest number of animals. Also, Kugel et al. (1990) employed varying number of animals per group with 6 animals in the control group. Kripke et al. (1976), Mazze et al. (1983) and Land et al. (1981) conducted studies with a very low number of animals, thus a statistic significant effect, if detected, is also considered untenable.

Furthermore, all discussed studies showed limitations in the description of methods and/or in the reporting of results, hence, possible confounding factors are not disclosed. Especially for inhalation studies the function and structure of the inhalation chamber should be described in detail, otherwise an irregular exposure to the reported concentrations cannot be excluded.

With regard to the studies used to determine the toxicity to reproduction only one author reported effects on body weight gain in dams (Rice et al. 1985). In no other study information about the maternal body weight or the body weight gain were provided. The determination of body weight is essential for the detection of any changes in relative organ weight, thus, changes in organ weight cannot be fully taken into account. Furthermore, a reduction in body weight gain or a reduced food consumption can indicate signs of toxicity in the parental generation. Even in case of sedation which is likely when animals are exposed to concentrations above 30% N_2O for several hours, may result in a reduced body weight gain and subsequently also in disturbances of reproductive performance.

Human data - toxicity to reproduction

The French MSCA also provides two of several available clinical studies in humans which are only briefly discussed. Both studies are retrospective studies which investigated the effect of N_2O on midwives and dental assistants, respectively. In the first study the exposure was estimated based on the average deliveries per month and in the second study the exposure was derived from the total scavenged N_2O during dental therapies. The main bias in both studies was that a co-exposure to other factors influencing reproduction could not be excluded, e.g., smoking, contraceptives, exposure to other anesthetics etc.. Thus, a clear causal relation could not be established.

In conclusion, a classification of N_2O neither in Category 1A or 1B (missing evidence in humans) nor in Category 2 with regard to toxicity to reproduction is not justified. The latter classification is considered appropriate only if:

Such effects… 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. (Regulation (EC) No 1272/2008, Annex I, section 3.7.2.1.1, Table 3.7.1 (a)).

Based on the missing information on maternal toxicity and in this special case, possible hypoxia in the dams due to exposure to N_2O, the classification into Category 2 is also not appropriate.

Studies central to classification:

Kugel *et al.,* (1989) [1]**:**

Guideline: Non-guideline (fertility study), non GLP.
Test methodology: Female rats (strain unknown, 12 animals/group) exposed *via* inhalation (whole body) at 0, 500 ppm, for 8 h/day for 35 days.
ANSES conclusions: A transitory oestral cycle disturbance in all exposed females was observed, only 6 of 12 exposed females gave birth
ANSES Klimisch score: ANSES concluded the reliability of the study could not be assessed, with a Klimisch 4 (not assignable) score.
Concerns raised: Limited reporting of data; consequently inadequate characterisation of test article; no assessment of maternal toxicity reported; details of inhalation chamber and flow rate not provided. A single dose level was used, consequently no concentration-response relationship can be established.
According to OECD TG 414 [2] 'group[s] should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy. Groups with fewer than 16 animals with implantation sites may be inappropriate.' In this study 12 animals/group were used.

Kugel *et al.,* (1990) [3]**:**

Guideline: Non-guideline, non GLP.
Test methodology: Female Sprague Dawley rats (6-12 animals/group) exposed *via* inhalation (whole body) at 0 or 300,000 ppm for 4 days or 1 ovulatory cycle (8 h/days). Animals were sampled for LHRH (Group 1, 8 females/group); ovulatory cycle assessment (Group 2, 6 females/group treated in proestrus; 6 females/group treated in random stage of ovulatory cycle); Fertility assessment: same number animals as Group 2, with females mated with proven males.
ANSES conclusions: *Group 1*: Increases in LHRH cell counts were observed in 4/8 animals*; Group 2:* disrupted cycles following the 1st day of exposure in 11/12 animals went into constant proestrus. Dysfunction resolved after 3 weeks. Control animals cycled normally throughout the experiment. *Group 3*: all 12 control animals gave birth. Only 6/12 animals only gave birth (3 in groups treated in proestrus and 3 in random phase of the cycle) gave birth. No effect on litter size or pup weight observed.
ANSES Klimisch score: Klimisch 2 (reliable with restrictions).
Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; no assessment of maternal toxicity reported. A single dose level was used, consequently no concentration- response relationship can be established.
According to OECD TG 414 [2] 'group[s] should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy. Groups with fewer than 16 animals with implantation sites may be inappropriate.' In this study 6-12 animals/group were used.
The maximum concentration of 300,000 ppm, exceeds the limit concentration (20,000 ppm for gases) in the sub-chronic inhalation test guideline (OECD TG 413 [8]). The data should be viewed with caution when applying CLP where adversity was observed at a concentration which exceeds the recommended limit concentration.

Kripke *et al*., (1976) [4]:

Guideline: Non-guideline (testis toxicity study), non GLP.
Test methodology: Male LEW/f Mai rats (4-6 animals/group) exposed *via* inhalation (whole body) at 0 and mixture of 200,000 ppm containing 20% N_2O: 20% O_2; 60% O_2 for 1-35 days (Group 1, for 8 h/day) or 32 days (Group 2 for 24 h/day). Therefore, the amount of dinitrogen oxide administered was equivalent to 40,000 ppm.

ANSES conclusions: Statistically significant decrease in testis weights were observed, with associated spermatogenic cell damage of the seminiferous tubules.
ANSES Klimisch score: ANSES concluded this to be of limited reliability, however they have assigned a Klimisch 2 (reliable with restrictions) score
Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; no

assessment of general toxicity reported; inadequate justification of an extension of the typical 6 h/day exposure, as detailed in OECD TG 412 [5] to 8 h/day up to a continuous exposure (24 h/day).
A single dose level was used, consequently no concentration-response relationship can be established. According to OECD TG 412 [5] *'Each group consists of a minimum of 5 males…rodents that are exposed to the test chemical for 6 hours per day on a 5 day per week...*'' In this study 4-6 animals/group we used.
The maximum concentration of 40,000 ppm, exceeds the limit concentration (20,000 ppm for gases) in the sub-acute inhalation test guideline (OECD TG 412 [5]). The data should be viewed with caution when applying CLP where adversity was observed at a concentration which exceeds the recommended limit concentration.

Vieira *et al.,* (1983) [22]:

Guideline: Non-guideline (fertility study), non GLP.
Test methodology: Male Wistar rats (12 animals/group) exposed *via* inhalation (whole body) at 0, 5,000 ppm for 6 h/day, 5 day/wk for 30 days. At the end of the exposure period males were mated with unexposed, nulliparous females; this was repeated 6 months post a recovery period.
ANSES conclusions:. Statistically significant decrease in litter size and developmental delay in offspring were observed. No significant effects were observed after a 6 month recovery.
ANSES Klimisch score: Klimisch 2 (reliable with restrictions) score
Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; no assessment of general toxicity reported (therefore effects reported cannot be contextualized); details of inhalation chamber and flow rate not provided. Exposure to males was limited to 30 days, and therefore not covering a complete spermatogenic cycle (10 wks). The effects reported are not interpreted in the context of background laboratory historical control data.
The effects reported in the study contradict the conclusions reached in the Holson *et al* study [34]: (KL.2 and whilst detailed and discussed in the CLH dossier but not considered by ANSES in the context of driving classification), where both males and females were exposed up 10,000 ppm with no statistically significant. change in litter size observed.
These data from Vieira *et al.,* 1983 should be viewed with caution as the effects observed are not replicated at concentrations 2-fold greater.

Additional studies not considered for classification:

Holson *et al.,* (1995) [34]:

Guideline: Non-guideline (parental and dominant lethal study), non GLP.
Test methodology: Parental and dominant lethal test. *Parental*: Male Sprague Dawley rats (4 animals/group) exposed *via* inhalation (whole body) at 0, 1,000, 5,000, 10,000 ppm for 6 h/day, 5 days/wk for 9 weeks mated with non-exposed females (4 animals/group). *Dominant lethal*: Male rats (24 animals/group) exposed *via* the same route. concentrations and duration as previously listed were mated with non-exposed females (30 animals/group).
ANSES conclusions: No effect on weight of males, no effect on body weight gain in females up to 10,000 ppm. *Parental*: no statistically significant findings in litter size (trend to lower numbers of pups/litter in the treated groups). *Dominant lethal*: no statistically significant effect on conception rate, total number of implants, live fetuses. However, a trend to increase in the number of resorptions noted at the top dose with slight increase in lethality
ANSES Klimisch score: Klimisch 2 (reliable with restrictions) score
Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; limited assessment of general toxicity reported. Although it was reported that a trend of lower numbers of pups/litter were observed, this was only limited to the dominant lethal form of the study (live fetus/litter at 0, 1,000, 5,000, 10,000 ppm: 14.4, 13.8, 13.3, 13.0, respectively), and was not replicated in the paternal/maternal component for the study (live fetus/litter at 0, 1000, 5000, 10,000 ppm: for exposed females: 14.0, 12.3, 13.8, 13.9; for exposed males: 14.4, 12.8, 13.4, 13.3, respectively). In the absence of historical control data it is

unclear if these could be attributed to biological variation.

Rice *et al.,* (1985) [6]:

Guideline: Non-guideline (repeat dose 14 week study), non GLP.

Test methodology: Swiss Webster mice (15 animals/sex/group) exposed *via* inhalation (whole body) at 0, 5,000 ppm for 4 h/day, 5 day/wk for 14 weeks.
ANSES conclusions: Significant decreases in body weight gain in males (77%) and females (63%). No histopathological findings observed in testes or ovaries.
ANSES Klimisch score: Klimisch 4 (reliable with restrictions) score

Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; limited assessment of general toxicity reported. Histopathological assessment was limited to testes and ovaries, with no assessment of target organ exposure (lungs). Inadequate justification of the low duration of exposure (4 h/day) rather than the required 6 h/day exposure, as detailed in OECD TG 413 [8].

Mazze *et al.,* (1983) [7]**:**

Guideline: Non-guideline (reproductive study), non GLP.

Test methodology: Swiss Webster mice (15 animals/sex/group) exposed *via* inhalation (whole body) at 0, 5,000, 50,000, 500,000 ppm for 4 h/day, 5 day/wk for 14 weeks. MMS and 3MC included as positive controls for male germ cell and oocyte count assessments, respectively.

ANSES conclusions: no excitement or general anaesthesia observed, with mice behaving normally. No effect on testes weights, % abnormal sperm, sperm count or histological appearance of the testes. No effects on the mean number of oocytes.

ANSES Klimisch score: Klimisch 2 (reliable with restrictions) score

Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; limited assessment of general toxicity reported. Histopathological assessment was limited, with no assessment of target organ exposure (lungs). Inadequate justification of the low duration of exposure (4 h/day) rather than the required 6 h/day exposure, as detailed in OECD TG 413 [8]

Mazze *et al.,* (1982) [9]]**:**

Guideline: Non-guideline (fertility study), non GLP.

Test methodology: *Experiment 1*: Untreated male Swiss ICR mice mated with untreated females (1 male:2 females). Females exposed *via* inhalation (whole body) at 0 (untreated colony control [UCC], inhalation chamber control [ICC]), 5,000, 50,000, 500,000 ppm for 4 h/day over GD 6-15. Group sizes were 32 [UCC], 34 [ICC] and 24-27 for dinitrogen oxide treatment groups. Females killed on GD 18.

Experiment 2: Swiss ICR mice (18-21 males/group) exposed *via* inhalation (whole body) at 0, 5,000, 50,000, 500,000 ppm for 4 h/day, 5 day/wk for 9 weeks and mated with non-exposed females (each treated male mated with two untreated females). Females killed on GD 18

ANSES conclusions: no information on general toxicity provided. No effect on litter size, abilities of males to impregnate females, no effect on fetal wastage (resorption, dead), fetal size.

ANSES Klimisch score: Klimisch 2 (reliable with restrictions) score

Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; limited assessment of general toxicity reported. Reproductive parameters assessed were limited.

Land *et al*., (1981) [10)]**:**

Guideline: Non-guideline (spermatogenesis study), non GLP.

Test methodology: Male C57B1 xC3H mice (5 animals/group) exposed *via* inhalation (whole body) at 0, 80,000, 800,000 ppm for 4 h/day for 5 day/wks.

ANSES conclusions: no animal died from exposure, with no effects on abnormal spermatozoa.

ANSES Klimisch score: Klimisch 3 (disregarded) score

Concerns raised: Limited reporting of data; consequently, inadequate characterisation of test article; no assessment of general toxicity reported; inadequate justification of an extension of the typical 6 h/day exposure, as detailed in OECD TG 412 [5] to 8 h/day up to a continuous exposure (24 h/day). Exposure to males was limited to 5 days, and therefore not covering a complete spermatogenic cycle (10 wks).

Human data

Two human retrospective cohort studies investigated the potential effect of dinitrogen oxide on fertility in midwives [11] and dental assistants [12]. In midwives, exposure was based on the average number of deliveries performed/month at which the labour was assisted with dinitrogen oxide. For dental assistants' exposure was determined considering the number of hours of exposure per weeks and the presence or absence of a scavenging system. ANSES concluded that potential fertility effects were seen in both studies, however both studies are bias with other co-exposures which can influence fertility not excluded, *e.g.* lifestyle (smoking, diet), job role (exposure to other anaesthetics), age, medical history (medication, pre-existing medical conditions), environmental conditions (level of air pollution where they live/work).

Developmental effects:

The results presented by ANSES for developmental effects were obtained to a large extent from the same studies that also investigated the toxicity to reproduction, i.e. showing the same limitations as described above. However, in the proposal for classification and labelling of chemicals from ANSES the detected effects were distinguished by the type of exposure, either continuous or intermittent exposure and the type of effect, i.e. fetal or embryo toxicity and pre- and postnatal development.

From the available publications it becomes obvious, that the occurrence of effects on fetuses depends on the time-point of exposure. Effects were mainly observed when the animals were exposed at GD 8 (beginning of organogenesis). Several of the mentioned studies used an exposure duration of 24h/d at different time-points in gestation (Fujinaga et al., 1987, 1989,1990, 1991; Mazze et al., 1984, 1987, 1988). After continuous exposure an increase in early and late resorptions was detected at > 50% N_2O. The NOEC was determined at 35% N_2O. As expected, exposure to N_2O at gestation day (GD) 8, i.e., at the beginning of organogenesis, appeared most critical. Resorptions and skeletal and visceral malformation or anomalies were detected. This study has also considerable limitations.

Maternal toxicity – developmental toxicity

The proposal of the French authority states: *Maternal toxicity was not described in all of the studies but was detailed in some of them.*

We cannot agree with this explanation. In Mazze et al., 1984, 1987 and 1988 only a mild sedation of the dams was reported and in one of these studies the body weight was mentioned. However, no data was presented, and no other signs of toxicity were recorded.

In Fujinaga et al., 1987, 1989 and 1991 also a mild sedation and a decrease in maternal body weight was reported but no data was provided. In Fujinaga et al. 1990 no information about maternal toxicity was provided.

The description as mildly sedated also seems quite understated as the animals were treated with concentrations of 50% or more of N_2O, the typical concentration for anesthesia in operating theatres. Additionally, no information about the food consumption or water intake was provided which also diminishes the reliability of the reported results. It can be reasonably expected that animals exposed to 50%-75% or even starting already at lower concentrations are somehow sedated and subsequently also faced with hypoxia either by respiratory depression or oxygen displacement. This might also be an explanation for the reduced body weight in dams which will lead to a decrease in pup weight at birth or even trigger resorptions. On the other hand, weight loss can also be explained by a reduced food intake which would occur also in dams that show no decrease in litter size.

Concentrations and effects - developmental toxicity

Vieira et al., 1980, showed in their results that exposure to low concentrations (0.025 – 0.1%) of N_2O does not result in developmental toxicity in rats. The majority of the developmental toxicity studies

also only used one high concentration (50, 60, 70 or 75%), thus, a dose dependence could not be confirmed.

Holson et al., 1990 showed in their study that there were no signs of toxicity neither to reproduction nor in development. Sprague Dawley rats were exposed with up to 1% N_2O for 9 weeks for 6h/day. The studies of Rao et al., 1981 and Tong et al., 1982, although disregarded based on the limited information provided, also used several concentrations of 20, 15 and 5% N_2O for a continuous exposure (24h GD 1-21). Effects on development were only reported for the high concentration groups (20 and 15%).

Several of the mentioned studies used an exposure duration of 24h/d at different time-points in

gestation (Fujinaga et al., 1987, 1989,1990, 1991; Mazze etal., 1984, 1988; Tassinari et al., 1986; Keeling et al., 1986; Lane et al., 1980; Vieira et al., 1979, 1980; Corbett et al., 1973). Independent of the concentration used in these settings, different adverse effects like a decrease in litter size, skeletal malformations, smaller pups or increased incidence of resorptions (Vieira et al., 1979, 1980) were reported, even for low concentration of N_2O (Vieira et al., 1979; 0.5%). In the study by Keeling et al. (1986) rats were exposed for 24h on GD 8 with 70 – 75% N_2O. Although several effects on fetuses were detected, like a decrease in fetal weight or a delayed development (decreased number of sternebrae and caudal vertebrae), these effects cannot be exclusively correlated to exposure of N_2O but could also result from hypoxic conditions on GD 8. However, in this study there was again no reporting about maternal effects, thus, the reliability of the study is strongly diminished, and it should be disregarded for the evaluation of developmental toxicity.

The notion that hypoxia may be the basis of the effects observed can be substantiated by the results of the studies that were conducted with an intermittent exposure to N_2O.

Especially when exposed to lower N_2O concentrations no malformations, no decrease in fetal numbers or increase of resorptions (Tassinari et al., 1986; Rao et al. 1981; Tong et al., 1982, Hardin et al., 1981) occurred.

From the seven studies that were conducted with intermittent exposure only three studies mentioned that there is either a decrease in maternal body weight (Mazze et al., 1986) or no maternal toxicity observed (Hardin et al., 1981; Pope et al., 1978). Mazze et al. (1986) found a decrease in fetal weight, an increase in resorptions and fetal wastage and skeletal malformations, however, the exposure concentration although intermittently applied was high (75%) and the oxygen supply was reduced in the critical gestation days 7-9.

Vieira et al., 1983 reported that the litter size after exposure to 0.5% N_2O over the whole gestation period was reduced, no other effects in the pups were observed, no information about maternal signs of toxicity were reported. The exposure schedule was 6h per day at 5 days per week, thus, the expected partial oxygen reduction was returned to normal supply and thus, the fetuses were normally oxygenated at least for 2 days a week and developed normally.

Pope et al., 1978 treated rats with up to 50% N_2O for 8h per day over the whole gestation period, thus, oxygen was reduced over the whole experimental time which can be correlated to increased fetal losses and delayed development. Strikingly, the effects were only observed in the mid concentration group (i.e., 10% N2O) and not at 50% N_2O exposure. There was no dose- response relation which further questions the relevance of the observed effects.

Corbett et al., 1973 reported an increased fetal loss in animals exposed to 0.1% N_2O during early and late gestation days for 5 days, but no effect was observed if the animals were exposed for a longer time, i.e., GD 10-19. Overall, these studies could not substantiate a toxic effect on development resulting from exposure to N_2O, rather it is assumed that even a partial decrease in oxygen supply adversely influenced the developing embryo.

Further four studies were used to elucidate the presumed adverse effects of N_2O on pre- postnatal and postnatal neurodevelopment. Only two studies with the highest concentration applied (75% N_2O) intermittently on GD 13-14 for 8h and with sacrifice on PND 16, 18, or 21, respectively showed an effect on pups' behaviour or neurodevelopmental effects. As stated above, in these studies no information on maternal effects were reported, and the concentration was sufficiently high to provide a profound hypoxia in the dams independently from the intermittent exposure. Hence, a clear correlation to N_2O exposure is also not provided by these studies.

<u>Hypoxia - developmental toxicity</u>

It is well known that hypoxia also results in teratogenic effects like shortening of the snout and extremities, polydactyly, syndactyly, and eye defects. Degeneration was also seen in the basal ganglia, cerebral cortex, and anterior horns of the spinal cord of some fetuses (Webster and Abela, 2007). Webster et al. (2006) reviewed the drug induced hypoxia on the development of cleft lips in rodents. They pointed out a distinct effect on embryonic heart inhibition and subsequently a decreased supply of oxygen. A prolonged embryonic hypoxia was correlated to this malformation.

In another review (Adam et al., 2021) hypoxia was referred to as a teratogen due to the malformations, dysplasias and congenital disruptions that are related to this state of low oxygen. Additionally, the foetal wastage resulting from hypoxia during gestation was also investigated. Wang et al. (2004) examined the effect of oxygen depletion on litter size as well as on molecular mechanisms underlying fetal growth restriction. The animals were exposed to hypoxic conditions over 11 days from GD 9 onwards. Cesarean sections were performed at GD 20 and organs were dissected and weighted Exposure resulted also in a decreased food consumption but the main result was a decreased litter size, an increase in resorptions, a decreased fetal weight and a decreased placental weight. These results are in line with the effects detected in the studies cited by the French authority.

In the study of Van Geijn et al. (1980) pregnant Sprague Dawley rats were exposed to hypoxia at gestation day 10 to day 22. On day 22 the pups were resected, and body, brain, liver, and placenta weights of each fetus were measured. Food and water intake were markedly decreased in the hypoxic animals and the litter size and fetal body weight was reduced.

Also, the organ weights were decreased as compared to the normoxic control. Although also this study has its limitations (small animal number, no malformations examined etc.) it can be used to substantiate the similarity between the results from the studies conducted with N_2O and those using simply hypoxic conditions.

Although hypoxia was applied late in gestation Williams et al. (2005) showed that rats when exposed to hypoxia at GD 15 lost significantly more pups during late pregnancy than the control animals. This result is also in line with the presented results gathered from the studies conducted with N_2O exposure.

The presented results from the hypoxia studies confirm the thesis that there is no clear causal correlation of the developmental effects detected in the N_2O studies. Based on the similarity of the results it cannot be excluded that the effects result from hypoxia not from N_2O exposure.

A secondary effect on development rather resulting from hypoxia cannot be ruled out.

Further information was provided from studies conducted with mice. However, it appears that mice are less vulnerable against the exposure to N_2O or against the simultaneously occurring oxygen reduction during gestation. Only in one study (Shah et al., 1979) in which extremely high concentrations were used (i.e., up to 90% N_2O, which is obviously a hypoxic condition) malformations and a reduced litter size was observed.

<u>Human data - developmental toxicity</u>

Classification according to Regulation (EC) No 1272/2008 (CLP) warrants at least:

<u>Suspected human reproductive toxicant</u>

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.

The human data provided by ANSES was only discussed briefly due to the lacking reliability of the results: *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.*

Although in some studies confounding factors were included, i.e., smoking, infection, analgesic drugs other anesthetic gases etc. the missing information on the exposure concentrations and the lack of measurement data disqualify the provided information.

Also, in the study of Axelsson et al., 1996 bias like unhealthy worker effect, i.e. poor health condition probably caused by lifestyle (smoking habits, nutrition, alcohol consumption etc.) but also by work itself (stress, working in night shifts etc.), could not be excluded thus a risk for abortion due to N_2O exposure could not be established. Also, in other studies other factors that influence development were not evaluated, e.g., smoking behaviour. Studies conducted on the basis of a questionnaire are also not appropriate because clear measurements of exposure concentrations are missing and the possible use of other anesthetic gases which also influence reproduction and development were not considered. In the study of Bodin et al., 1999, a reduced birth weight was reported for newborns of midwives, however, the exposure to other substances was not considered, thus, overall none of the human data is considered to be sufficient for classification purposes. There is no clear causal correlation to the exposure with N_2O.

Prenatal developmental toxicity studies: continuous exposure:

In a collection of four studies conducted by Fujinaga *et al.,* ([13], [14], [15], [16]) and three studies conducted by Mazze *et al*., ([17], [18], [19]), Sprague Dawley rats were exposed to a single administration (unless otherwise indicated) of dinitrogen oxide administered via inhalation (whole body) at overtly high concentrations (*i.e.* greater than the recommended 20,000 ppm) for a 24 h/day exposure period at different timepoints during gestion, typically centered around the beginning of organogenesis (GD 8). Collectively for all these studies a mild sedation in dams was reported at concentrations which exceed that used in operating theatres to achieve anaesthesia (≥500,000 ppm). Other parameters used to assess maternal toxicity (food/water consumption, clinical signs, body weight, hypoxic effects) were either not measured, or limited assessments were undertaken, raising

concerns over the reliability of the data in the context of evaluating development toxicity in the absence of maternal toxicity assessments. Where developmental effects were determined (early/later resorptions, skeletal and visceral malformations or anomalies), these were observed at exposure concentrations exceeding 500,000 ppm, a concentration 25-fold greater than the maximum recommended concentration for inhalation studies. All these studies, singly and collectively have considerable limitations.

The two studies conducted by Vieira *et al* ([20], [21]) were undertaken at lower concentrations for 23 h/day from GD 1-19, with Wistar rats exposed *via* inhalation (whole body) at 0, 5,000 ppm [20] or 0, 250, 500, 1000 ppm [21]. Groups numbers of both studies were limited to 12 animals/ group, with chamber sizes and environmental conditions not reported. No effects on food or water consumption were observed up 1,000 ppm [21], with no assessment of maternal toxicity undertaken in the first study [20]. Whilst developmental toxicity was observed in both studies at 1,000 (where tested) and 5,000 ppm and included decreased litter size, increased resorptions, skeletal malformations, decreased crown-rump length; these data contradict the Vieira *et al*., (1983) [22] (exposure for 6 h/day, 5 days/wk) and the Holson *et al*., [34] (exposure for 6 h/day for 9 wks) studies when dinitrogen

oxide was tested up to 5,000 or 10,000 ppm, respectively with no developmental effects reported.

The data generated by Rao *et al.,* [23]; Tong *et al.,* [24] and Lane *et al.,* [25] have not been considered as the data reported were limited, with concentrations tested inexcess of the maximum recommended concentration for repeat dose inhalation studies.

The study conducted by Corbett *et al.,* [26] provides contradictory data with increased incidence of fetal loss in dams exposed for 5 days at 1,000 ppm during both early and late stages of gestation. However, no effects were observed in animals exposed for 9 days at 1,000 ppm. The study details reported were limited, fetal death was the only parameter assessed, with no assessment of maternal toxicity reported. Group numbers ranged from 6-12 and a lack of methodology details raise concerns over data reliability.

Prenatal developmental toxicity studies: intermittent exposure:

Three studies ([27], [28] [29]) are considered to be reliable with restrictions (Klimisch 2) with five studies disregarded ([26], [30]) or unreliable ([23], [24],[31]).

With similar exposure levels in the both the Mazze *et al.,* [27] and the Pope *et al.,* [29] studies, maternal effects were either reductions in body weight gain in dams exposed over GD 8-10 [27], or no effects reported following exposure over the complete gestation period [29]. Mazze *et al.,* [27] reported reductions in fetal weight, increased resorptions and fetal wastage, with major malformation and external abnormalities at a concentration of 750,000 ppm. These findings are in contrast to the findings of Pope *et al.,* [29] which showed delayed development and fetal loss at the two lowest dose groups (10,000 and 100,000 ppm), but not in the high dose group (500,000 ppm). With three dose groups included, evidence of a dose-response was absence.

In the Vieira *et al.,* study [28], Wistar rats (12 animals/group) were exposed *via* inhalation (whole body) at 0, 250, 500, 1,000, 5,000 ppm for 6 h/day, 5/days/wk over the entire gestation period. No information regarding maternal toxicity was reported. Dose related reductions in litter size were reported at 5,000 ppm, however there was no effect on fetal weight and crown-rump length and no malformations were observed. It is prudent to acknowledge in this study that dams were only exposed 5 days/wk, therefore dams were devoid of oxygen depletion on 2 days of each week during gestation with fetuses developing normally.

In the disregarded [30] and unreliable studies ([23], [24],[31]) animals were dosed at concentrations ranging from 1,000 to 750,000 ppm with dosing occur at specific timepoints of gestation or over the entire gestation period and confirmed no change in fetal numbers, resorptions, fetal weight and no malformations observed.

In the Corbett *et al.,* [26] study fetal death was the only parameter assessed. Significant increases in fetal death were observed at 1,000 ppm animals exposed during both early and late phases of gestation following a 5 day exposure. This effect was not replicated in animals exposed for a longer time period (GD 10-19).

Prenatal developmental toxicity studies: pre-, postnatal developmental and postnatal neurodevelopment:

Four studies were reported which examined the effects of dinitrogen oxide on prenatal neurodevelopmental [32], behavioural developmental and dominant lethal [34], postnatal developmental toxicity [30] and pre/postnatal developmental toxicity [33].

In the Mullenix *et al.,* [32] study, reporting was limited, with no body weight effects in dams dosed at 750,000 ppm, 8 h on GD 14 or 8 h on GD 13-14 were observed. Neurodevelopmental effects were

observed in female offspring of dams exposed on GD 13-14, with assessments made at 1 and 5 months of age.

In the Holson *et al.,* [34] study, maternal effects were not reported in dams dosed up to 10,000 ppm, 6 h/day over the entire gestation period. Litter size and weight of offspring, along with offspring behaviour were unaffected.

The Tassinari *et al.,* [30] study assessed early postnatal developmental effects in offspring following exposure of dams to 750,000 ppm dinitrogen oxide over GD 13 – 14. No significant effects on auditory startle or eye opening was observed; however female offspring exhibited decreased suspension reflex. The Vieira *et al.,* [33] study exposed dams 6 h/day, 5 days/wk for either the whole of gestation, weeks 1 and 2 gestation or the 1st week of gestation. From the limited information reported, data were pooled with decreased litter sizes, body and tail length and body weight. Due to the limitations in these studies, ANSES disregarded these.

STOT RE 1

With defined cut off values for STOT RE following inhalation, the CLP guidance dose/ concentration values are provided below:

- Category 1: (rat [6 h], gas): C≤50 ppm;
- Category 2: (rat [6 h], gas) C>50, but <250 ppm),

The concentrations used in all the animal data considered for STOT RE exceed the cut-off values and therefore animal data should not be used to classify for either Category 1 or 2.

The human data considered for as a weight of evidence approach for nervous system effects are taken from a collection of case study reports following misuse of dinitrogen oxide do not consider co- exposure to other factors (*e.g*. other drugs which induce a state of stupor) which may suppress the nervous system.

STOT SE 3

With limited reporting of the human volunteer studies (inadequate test article characterisation, methodology, acceptance / evaluation criteria) that are fulcrum to STOT SE classification, a reliability assessment cannot be made as detailed in Schneider *et al* [35].

Conclusions in regard to reproductive, developmental and STOT classification

From the available animal data many of the studies were undertaken with a single dinitrogen oxide dose group, thereby unable to establish a concentration-response relationship. Animal group numbers were generally below the recommended test guideline group numbers, thereby making statistical analysis (if undertaken) difficult to interpret. Where adversity was observed this occurred at concentrations that exceeded the maximum recommended concentration for repeat dose inhalation studies (20,000 ppm), without consideration of hypoxic effects or maternal toxicity. Collectively concerns are raised over the reliability of the data used to derive the classification.

The human data considered as a weight of evidence approach for fertility effects are of limited scientific use and do not consider co-exposure to other factors which may impair fertility.

From the animal and human data presented uncertainties exist, with the data deemed insufficient to serve as a basis for classification for sexual function, fertility or developmental toxicity (Repr. 1B, H360Df), STOT RE 1 or STOT SE 3.

Conclusions on literature summary performed by ANSES

The literature search, which under pins the CLH dossier undertaken and prepared by ANSES was limited to a single database (Pubmed). The disadvantage to this approach is that the data obtained are restricted. EFSA recommends that literature searches are undertaken using 3 different platforms (equating to *ca.* 4 different databases).

This restricted literature search raises concerns over a single database used to derive the reproductive toxicity classification, at concentrations which exceed the recommended limit for animal inhalation (gas) studies.

Whilst there is insufficient time to undertake a new literature search, these comments should be raised during the commenting period for reasons above.

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