Proposal for Harmonised Classification and Labelling of Dinitrogen Oxide: position paper discussing reproductive and developmental CLH proposals

FINAL REPORT

Date: 7 July 2022

Report No: 0649151-TOX 1

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1 Introduction

Dinitrogen oxide/nitrous oxide (N_2O) is commonly used in medical-, technical- and food- applications. Although the CLH process does not take into consideration the application field, it is worth mentioning that the submitted studies focus solely on inhalation disregarding all the other application fields. KAYSER Berndorf GmbH has been processing N_2O solely as a food additive which is acknowledged as such in Europe and internationally according to JECFA and Codex Alimentarius (INS 942) as well as in EU Regulations (178/2002, 1333/2008, 1129/2011, 2016/324) and the USA-FDA (Sec. 184.1545). Within this application scope, it is globally approved, evidentially safe and, most importantly, irreplaceable.

This position paper has been prepared by Environmental Resources Management Limited (ERM), on behalf of KAYSER Berndorf GmbH. ERM is a leading global provider of environmental, health, safety, risk, social and sustainability consulting services (www.erm.com). Our team of regulatory experts across Europe and the globe are suitably experienced in the regulation, processes and features of the CLP Regulation and harmonized classification process. ERM's regulatory team has extensive experience in the strategy, development and toxicology aspects for classification

2 Background to the CLH process within the European Union

It is prudent to acknowledge that classification and labelling within the EU process under Regulation (EC) No 1272/2008 is solely driven by hazard assessment, with risk assessment (*i.e.* route of exposure where toxic effects are observed *vs.* how the consumer is exposed to) playing no part in the process. This is contradictory to the US regulatory process.

The JCEFA review [1] of dinitrogen oxide (see below, with reference made to TAP review [2]), in terms of use as a food additive this use is deemed safe, with no specific intake limit (*quantum satis*). it is prudent to acknowledge that this review is based on risk assessment (*i.e.* a quantifiable hazard).

In terms of reviewing the animal toxicity data in the context of inhalation exposure, the maximum recommended dose for gases is 20,000 ppm for both an acute (OECD TG 403 [3]) and sub-chronic (OECD TG 413 [4]) exposures. These values are prudent as they provide a context for adversity seen at high dose levels *i.e.* less weight should be placed on classification where adversity is observed above these concentrations, when based on animal data alone. It is further prudent to acknowledge that many of the studies do not report maternal toxicity effects, were conducted at a single dose level, thereby making dose response interpretation non-existent; were conducted at overtly high dose levels where hypoxic effects cannot be excluded, not conducted to OECD test guidelines, with limited reporting of the data.

This position paper critically reviews the study data that are fulcrum to the proposed classification for dinitrogen oxide in the CLH report, prepared by ANSES (on behalf of the French MSCA) [5], with a focus on hazard classes Repro. 1B H360fD and STOT RE 1.

3 Reproductive toxicity: sexual function and fertility

Repr 2. H361f proposed classification is driven by four animal studies ([6], [7], [8],[9]) which are deemed to show decreased fertility and oestrous cycle changes. An additional five studies have also

been included ([10], [11], [12],[13], [14), but are not consider critical for classification. All studies are briefly discussed below:

3.1 Studies central to classification:

Kugel *et al.,* (1989) [6]**:**

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 [15] '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) [7]**:**

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 [15] '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 [4]). 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) [8]:

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 [16] 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 [16] *'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 [16). 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) [9]**:**

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 [10]: (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.

3.2 Additional studies not considered for classification:

Holson *et al.,* (1995) [10]:

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) [11]**:**

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 [4].

Mazze *et al.,* (1983) [12]**:**

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 [4].

Mazze *et al.,* (1982) [13]**:**

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) [14]**:**

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 [16] 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).

3.3 Human data

Two human retrospective cohort studies investigated the potential effect of dinitrogen oxide on fertility in midwives [17] and dental assistants [18]. 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 anesthetics), age, medical history (medication, pre-existing medical conditions), environmental conditions (level of air pollution where they live/work).

4 Developmental toxicity

A number of the studies used to assess developmental effects (Repro. 1B H360D) were also used for the assessment of reproductive effects, consequently the deficiencies detailed previously are also relevant here. ANSES distinguish between intermittent (4-8 h) and continuous (23 or 24 h) exposure and the type of effect (prenatal or postnatal development).

4.1 Prenatal developmental toxicity studies: continuous exposure:

In a collection of four studies conducted by Fujinaga *et al.,* ([19], [20], [21], [22]) and three studies conducted by Mazze *et al*., ([23], [24], [25]), 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* ([26], [27]) were undertaken at lower concentrations for 23 h/day from GD 1-19, with Wistar rats exposed *via* inhalation (whole body) at0, 5,000 ppm [26] or 0, 250, 500, 1000 ppm [27]. 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 [27], with no assessment of maternal toxicity undertaken in the first study [26]. 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) [9] (exposure for 6 h/day, 5 days/wk) and the Holson *et al*., [10] (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.,* [28]; Tong *et al.,* [29] and Lane *et al.,* [30] have not been considered as the data reported were limited, with concentrations tested in excess of the maximum recommended concentration for repeat dose inhalation studies.

The study conducted by Corbett *et al.,* [31] 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.

4.2 Prenatal developmental toxicity studies: intermittent exposure:

Three studies ([32], [33] [34]) are considered to be reliable with restrictions (Klimisch 2) with five studies disregarded ([31], [35]) or unreliable ([28], [29],[36]).

With similar exposure levels in the both the Mazze *et al.,* [32] and the Pope *et al.,* [34] studies, maternal effects were either reductions in body weight gain in dams exposed over GD 8-10 [32], or no effects reported following exposure over the complete gestation period [34]. Mazze *et al.,* [32] 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.,* [34] 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 [33], 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 [35] and unreliable studies ([28], [29],[36]) 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.,* [31] 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).

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

Four studies were reported which examined the effects of dinitrogen oxide on prenatal neurodevelopmental [37], behavioural developmental and dominant lethal [10], postnatal developmental toxicity [35] and pre/postnatal developmental toxicity [38].

In the Mullenix *et al.,* [37] 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.,* [10] 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.,* [35] 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.,* [38] 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.

5 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* [39].

6 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.

7 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) or STOT RE 1 or STOT SE 3.

8 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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