Comments on Proposal for Harmonised Classification and Labelling, Nitroethane

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The conclusions in the Report titled Comments on Proposal for Harmonised Classification and Labelling, Nitroethane are Integral Consulting's professional opinion, as of the time of the Report, and concerning the scope described in the Report. The opinions in the document are based on conditions and information existing at the time the scope of work was conducted and do not take into account any subsequent changes.

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Principal

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Table 1. DS Proposed and Recommended CLH Classifications for NE

ACRONYMS AND ABBREVIATIONS

1-NP	1-nitropropane
CLH	Harmonized Classification and Labelling
CLP	Classification, Labelling and Packaging
DS	dossier submitter
ECHA	European Chemicals Agency
IRIS	Integrated Risk Information System
IUR	inhalation unit risk
MTD	maximum tolerable dose
NE	nitroethane
NM	nitromethane
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
ppm	parts per million
RAAF	Read-Across Assessment Framework
WoE	weight of evidence

1 INTRODUCTION

A recently published proposal for Harmonized Classification and Labelling (CLH) of Nitroethane (NE; EC number: 201-188-9; CAS number: 79-24-3) notably recommended classification of NE as Carc. 1B, H350 and Repr. 1B, H360Df. The dossier submitter (DS) used weight of evidence (WoE) and read-across between three nitroalkanes to justify the proposed classifications: NE itself; 1-nitropropane (1-NP; EC number: 203-544-9; CAS number: 108-03-2); and nitromethane (NM; EC number: 200-876-6; CAS number: 75-52-5).

The opinions summarized herein pertain to Chapters 10.9 (Carcinogenicity) and 10.10 (Reproductive Toxicity) of the CLH Report. Specifically, our comments are as follows:

Chapter 10.9 Carcinogenicity

- The read-across from NM used by the DS to classify NE as Carc. 1B does not follow Read-Across Assessment Framework (RAAF) principles and is scientifically invalid.
- The NM studies used for read-across do not provide conclusive evidence of the need for a carcinogenicity classification.
- Available data for 1-NP and NE are sufficient to conclude that neither of them should be classified as a carcinogen.
- Genotoxicity findings do not suggest a genotoxic potential for NE.

Chapter 10.10 Reproductive Toxicity

- Read-across from NM used to classify NE does not follow RAAF principles and is scientifically invalid.
- The sperm effects observed in NM and NE studies are secondary to systemic toxicity in studies without investigation of reproductive function and hence do not require a classification for effects on reproduction.
- The Organisation for Economic Co-operation and Development (OECD) 422 study on the analogue 1-NP allows to conclude that NE should not be classified as a developmental toxicant.
- The OECD 422 study on the analogue 1-NP allows to conclude that NE should not be classified as a reproductive toxicant.

Given the points outlined above and described herein, we propose the classifications outlined in Table 1.

Table 1. DS Proposed and Recommended CLH Classifications for NE

Toxicity Endpoint	DS Proposed Classification	Classification Proposed Herein
Carcinogenicity	Carc. 1B (H350)	No Classification
Reproductive Toxicity	Repr. 1B (H360Df)	No Classification

2 CLH REPORT CHAPTER 10.9 – CARCINOGENICITY

The DS has proposed a CLH classification of Carc. 1B (H350) for NE (CLH Report for NE, 2023: pp. 3, 75). As per Classification, Labelling and Packaging (CLP) criteria, for a classification of Category 1B, evidence is needed from "*(a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols*" (CLH Report for NE, 2023: p. 73). The DS used read-across from NM data to arrive at this proposed classification. As discussed in greater detail below, this read-across is invalid when considering the differential metabolisms of NE and NM. The available carcinogenicity study for NE and read-across from carcinogenicity data for 1-NP are sufficient to support no carcinogenicity classification for NE.

2.1 THE READ-ACROSS FROM NM USED BY DS TO CLASSIFY NE AS CARC. 1B DOES NOT FOLLOW RAAF PRINCIPLES AND IS SCIENTIFICALLY INVALID

The DS has proposed a CLH classification of Carc. 1B (H350) for NE (CLH Report for NE, 2023: pp. 3, 75). In Section 10.9 of the CLH Report, the DS considered available carcinogenicity data for NE and structurally similar compounds NM and 1-NP when applying the Carc. 1B classification. The DS considered one carcinogenicity study for NE (cited in the CLH report as "Anonymous 35, 1986") in which no treatment-related increase in tumor incidence was observed, but stated:

> "The classification proposal for carcinogenicity of nitroethane and nitropropane is fully based on read-across from nitromethane because the available studies on nitroethane and 1-nitropropane are uninformative due to too low dosing and too low animal number. Thus, the key studies for the assessment of carcinogenicity are the 2-year studies in mice and rats on nitromethane (NTP, 1997)." (CLH Report for NE, 2023: p. 74)

The DS's assessment of NE carcinogenicity based on read-across from NM carcinogenicity data is inappropriate and invalid for several reasons.

2.1.1 The DS Did Not Follow RAAF Guidelines in Its Read-Across Approach

According to the European Chemicals Agency (ECHA 2017) RAAF, a category approach to read-across can be used when *"physico-chemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern"* (ECHA 2017: p. 25). According to the RAAF document, substances can be considered as a category if they are structurally similar, with one measure of structural similarity listed as the likelihood of common breakdown products (ECHA 2017: p. 6).

The use of a category approach to read-across from NM to NE is inappropriate based on qualitative differences in the aldehyde metabolites formed and quantitative differences in relative tissue reactivity of those metabolites, between NE and NM. As described in Garnick et al. (2021), cytochrome P450-mediated oxidative denitrification of the primary nitroalkanes, including NE, results in the formation of nitrite (CAS number: 14797-65-0) and an aldehyde with corresponding chain length (p. 19). For 1-NP, NE, and NM, these aldehyde metabolites are propionaldehyde (CAS number: 123-38-6), acetaldehyde (CAS number: 75-07-0), and formaldehyde (CAS number: 50-00-0), respectively. The formation of formaldehyde as a metabolite by P450-mediated oxidative denitrification is a metabolic pathway specific to NM, not NE or other primary nitroalkanes, and is a significant operative mechanism by which NM imparts carcinogenic and genotoxic potential (Garnick et al. 2021, p. 19). See further discussion of the variability of known toxicity of these metabolites in Section 2.1.2.

According to the RAAF, the likelihood of common breakdown products is a measure of structural similarity (ECHA 2017: p. 6). The DS provided no consideration of the differential breakdown products of NE or NM when utilizing NM carcinogenicity data to classify NE. The RAAF also states:

- "The category hypothesis should apply in an unambiguous manner to all the category members. Only category members that are covered by the category hypothesis can be involved in the read-across" (ECHA 2017: p. 26).
- "A common mechanism linking the presence of the compounds driving the effects with the prediction needs to be identified, and should also link the structures of the compounds under consideration with the possibility to predict qualitatively similar effects for the target substance" (ECHA 2017: p. 24).
- "Other compounds than those linked to the prediction in the read-across hypothesis may be formed through other (bio)transformation pathways or may be intermediates/metabolites of the identified pathway… Exposure to these compounds has to be considered in the justification" (ECHA 2017: p. 24).

The DS did not provide any hypothesis or rationale in performing its read-across as to how NM, NE, and 1-NP would exhibit the same properties in terms of carcinogenicity. In fact, the DS incorrectly relied upon the current categorization of formaldehyde as supporting evidence of NE's potential carcinogenicity. The DS states:

> "IARC classified nitromethane for carcinogenicity in category 2B 'possibly carcinogenic to humans'. Furthermore, the DS notes as supporting evidence that the metabolism of nitromethane leads to the formation of formaldehyde which has a harmonised classification as Carc. 1B, H350… ." (CLH Report for NE, 2023: p. 75)

The DS failed to consider that formaldehyde is not a metabolite or breakdown product of NE, or consider the relevance of this discrepancy in justifying its read-across approach. Therefore, the

DS's read-across from NM to classify NE as Carc. 1B (H350) is invalid and disregards RAAF principles published by ECHA.

2.1.2 NM-Specific Metabolite Formaldehyde Makes Read-Across to NE Scientifically Invalid

Cytochrome P450-mediated oxidative denitrification of the primary nitroalkanes, including NE, results in the formation of nitrite and an aldehyde with corresponding chain length. For NE and NM, these aldehyde metabolites are acetaldehyde (CAS number: 75-07-0) and formaldehyde (CAS number: 50-00-0), respectively. Formaldehyde generation through the oxidative denitrification of NM is a metabolic process that is specific to NM and would not be relevant to the genotoxic or carcinogenic potential of other nitroalkanes such as NE. Formaldehyde has a toxicological profile distinct from that of acetaldehyde, the NE oxidative denitrification metabolite.

The RAAF states that one can substantiate read-across on the basis of two hypotheses: 1) that the substances (bio)transform to common compound(s), or 2) that different compounds have the same effect (ECHA 2017: p. 11). In its classification of NE using read-across to NM data, the DS provided insufficient substantiation for either hypothesis. As a matter of fact, neither hypothesis applies. First, the biotransformation products of NE and NM are acetaldehyde and formaldehyde, respectively, dismissing the use of the read-across hypothesis based on transformation to common compounds. Second, when considering the potential read-across of the biotransformation products acetaldehyde and formaldehyde themselves, the available scientific literature indicates that there are distinct differences in their effects that do not substantiate that the two compounds would "have the same type of effect" on "a mechanistic level" (ECHA 2017: p. 11). The RAAF also states that "for a category approach, there is a need to take further account of whether or not quantitative variations in the properties are observed among the category members" (ECHA 2017: p. 11).

With regard to the metabolites of NM and NE—formaldehyde and acetaldehyde, respectively—there are mechanistic differences in toxicity as well as potency differences. Notably, differences in their potency demonstrate that the carcinogenic effects observed for these compounds are not comparable. Both formaldehyde and acetaldehyde have been shown to cause respiratory tract tumors in rodents, but formaldehyde demonstrated higher potency (Morris 1997; Roe and Wood 1992; Woutersen et al. 1986). In a review of the carcinogenicity of acetaldehyde, Morris (1997) indicated that rats exposed to 1,000 parts per million (ppm) acetaldehyde via inhalation demonstrated the same level of DNA-protein cross-links in respiratory mucosa as exposure to 5.6 ppm formaldehyde, suggesting that acetaldehyde (NE metabolite) is 180 times less potent than formaldehyde (NM metabolite) from this perspective. A comparison of the inhalation unit risks (IURs) calculated by the U.S. Environmental Protection Agency Integrated Risk Information System (IRIS) for formaldehyde and for acetaldehyde, both based on observations of respiratory tract tumors in rats, shows an approximately 17-fold difference in cancer potency between the two aldehydes (IUR for

formaldehyde = 1.3E-5 per µg/m^3; IUR for acetaldehyde = 2.2E-6 per µg/m^3)(U.S. EPA 1991a,b). *In vitro,* this potency difference was confirmed and was considerably higher than *in vivo*. Kuykendall and Bogdanffy (1992) found that a DNA damage level of 1 crosslink per 2.7 kbp of DNA was reached at 1.5 µM formaldehyde and 115 mM acetaldehyde, and that the concentration of formaldehyde needed to give 1 crosslink per molecule was 10^5 times less than that of acetaldehyde. These data all confirm that the DNA damage potential of the NM metabolite (formaldehyde) is at least 180-fold higher than that of the NE metabolite (acetaldehyde). This constitutes a highly significant quantitative difference in properties, making the read-across from NM to NE scientifically invalid.

NM is the only nitroalkane that would generate formaldehyde as a metabolite, and formaldehyde is a potent carcinogen and mutagen too. Using NM as a read-across source to any nitroalkane that does not metabolize into formaldehyde, such as NE, leads to overconservative conclusions, leading to bias for overclassification. No scientifically sound conclusion about carcinogenicity classification can be derived when using such a scientifically irrelevant approach.

2.2 THE NM STUDIES USED FOR READ-ACROSS DO NOT PROVIDE CONCLUSIVE EVIDENCE OF THE NEED FOR A CARCINOGENICITY CLASSIFICATION FOR NM

The available NM carcinogenicity studies are not considered relevant for carcinogenicity classification of NE due to invalid read-across as shown under Section 2.1. However, even if these NM studies were to be considered in an overconservative approach disregarding both RAAF and toxicological principles, then it should be emphasized that available NM data to not provide conclusive evidence to classify NM as a carcinogen.

2.2.1 The NM Rat Carcinogenicity Study Used for Read-Across to NE Does Not Demonstrate a Carcinogenic Potential

The National Toxicology Program (NTP) study (NTP 1997) of NM carcinogenicity, which the DS relies on for read-across information to NE carcinogenicity, has significant limitations. The NTP (1997) study found that mammary tumors increased in a dose-dependent manner among female F344 rats. However, the incidence of these mammary tumors stayed within the historical control range (Garnick et al. 2021: p. 5-6), meaning that the observed effect was reflecting biological variability rather than NM exposure.

In addition, the high background rate of tumors within the F344 strain led NTP to phase out the use of this strain in 2-year chronic toxicity and carcinogenicity studies, beginning in 2006 (Garnick et al. 2021: p. 5). Thus, it has been scientifically known for two decades that interpretation of tumor incidence data from this rat strain should be done with great care.

2.2.2 The NM Mouse Carcinogenicity Study Used for Read-Across to NE Shows Effects Secondary to Local Toxicity and Formation of NM-Specific Metabolite Formaldehyde

The findings in the evaluation of NM carcinogenicity in B6C3F1 mice performed by NTP (1997) are consistent with formaldehyde-related toxicity and further demonstrate that use of NM carcinogenicity data is not appropriate for read-across to NE. We agree with the DS that harderian tumors have limited relevance for human health (CLH Report for NE, 2023: pp. 68, 74; Garnick et al. 2021: p. 6). As the DS notes, the incidence of the observed liver tumors was within the historical control range for this strain, so these liver tumors were not treatment-related and do not provide evidence of a carcinogenic potential (CLH Report for NE, 2023: p. 74).

Increased lung tumors were observed in the NTP (1997) mouse study at the high dose of 750 ppm (carcinomas in males, combined lung adenoma and carcinoma in females; CLH Report for NE, 2023: p. 63). However, the 750-ppm high dose was associated with respiratory tract non-neoplastic effects, suggesting that pulmonary tumors were secondary to cytotoxicity. Formaldehyde is a respiratory tract tumorigen at high concentrations by this mechanism and has a CLP harmonized classification as Carcinogen 1B in the European Union (U.S. EPA 1991a; ECHA 2024a).

To the extent tumors were seen outside of the respiratory tract, they were likely secondary to general toxicity. The observation of respiratory tract tumors in the mouse NM cancer study is consistent with the formation of the formaldehyde metabolite and further stresses the irrelevance of using these data for read-across to NE, because NE does not metabolize into formaldehyde.

2.3 AVAILABLE DATA FOR 1-NP AND NE ARE SUFFICIENT TO CONCLUDE THAT NEITHER OF THEM SHOULD BE CLASSIFIED AS A CARCINOGEN

In a WoE assessment, data for the chemical of interest should be considered first, followed by the most related members of the category (ECHA 2011; WHO 2021). Our prior argument discussed in Section 2.1 indicates that the use of NM data for read-across was inappropriate because of the differential metabolism of NE and NM and the distinct toxicological properties of the NM metabolite formaldehyde. A proper WoE assessment would consider the available data for NE and, at best, consider read-across to the more relevant NE data set.

The DS inappropriately dismissed a relevant inhalation study for NE, Griffin et al. (1988) (cited as “Anonymous 35, 1986” in the CLH Report) because “only two doses were tested and no systemic effects were reported at the highest dose (200 ppm nitroethane)” (CLH Report for NE, 2023: p. 74). Griffin et al. (1988) was a full 2-year study of guideline-compliant duration to assess carcinogenicity and utilized sufficiently high doses. The DS’s assertion that the highest

concentration tested was too low is incorrect. The 200-ppm concentration caused significant body weight decreases in the animals and, thus, reached the maximum tolerable dose (MTD) as suggested by OECD 451 (OECD 2018). Furthermore, the study evaluated numbers approaching OECD 451 guidelines at 40/sex/group (OECD 2018; Griffin et al. 1988). Therefore, Griffin et al. (1988) provides sufficient information to determine that NE is not classifiable as a carcinogen using a WoE approach.

As a WoE, in addition to NE data showing no need for NE cancer classification, a supportive argument could be read-across from 1-NP. Indeed, this read-across seems more appropriate than read-across from NM, given the similarities in structure (close molecular weight) and metabolism end products between 1-NP and NE (as described in Garnick et al. 2021). The DS inappropriately dismissed a relevant chronic inhalation study for 1-NP, Griffin et al. (1982), that found no significant dose-related increase in tumors in male and female rats exposed to 1-NP at concentrations of 0 or 100 ppm for up to 21.5 months of exposure (10 rats/sex/group). Griffin et al. (1982) is an informative negative carcinogenicity study. The duration of the study was 21.5 months, which is near the standard length for animal cancer studies of 24 months. Further, doses provided to rats in the study were up to 100 ppm, which is much greater than human-relevant doses and equal to the inhalation dose level Carney et al. (2004) determined to be appropriate for the maximum inhalation dose based on a range-finding study. An oral 1-NP carcinogenicity study by Hadidian et al. (1968) was also negative. Therefore, as with NE, 1-NP shows no indication of a carcinogenic potential.

Taken together, when the negative carcinogenicity data for NE is supplemented with the negative carcinogenicity data for 1-NP, a proper WoE conclusion is that there is no evidence of NE carcinogenicity (ECHA 2011; WHO 2021).

2.3.1 Genotoxicity Findings Do Not Suggest a Genotoxic Potential for NE

Our conclusion that NE is not classifiable as a carcinogen is further supported by evidence of the non-genotoxicity of NE. The CLH Report on NE states that the *"data are inconclusive for germ cell mutagenicity*" (CLH Report for NE, 2023: p. 50). This is inconsistent with a WoE evaluation showing that NE is negative for mutagenicity. The evidence has been integrated in Garnick et al. (2021), in which the authors concluded that NE is not mutagenic according to numerous well-documented bacterial mutagenicity assays (p. 15). Specifically, NE was negative in an *in vivo* micronucleus assay in CD-1 mice (Hite and Skeggs 1979). In a bacterial mutation assay conducted by Löfroth et al. (1986), results were considered to be of low reliability in the WoE evaluation due to potential contamination of the low concentration NE with 2-nitropropane (Garnick et al. 2021: p. 15). Further, mechanistic data provide no support for direct DNA interaction under physiological conditions (Garnick et al. 2021).

3 CLH REPORT CHAPTER 10.10 - REPRODUCTIVE TOXICITY

Concerning **development**, the CLH report recommends a classification of Repr. 1B (H360D) for NE based on data on NM (CLH Report for NE, 2023: pp. 102, 104), stating:

> "The classification proposal is based on the read-across with nitromethane as there is no prenatal developmental toxicity study performed on 1-nitropropane and nitroethane. In the available prenatal developmental toxicity study performed with nitromethane (Anonymous 36, 2017), clear evidence of effects on developmental parameters were observed considered not secondary to maternal toxicity which is in line with a classification in category 1B." (CLH Report for 1-NE, 2023: p. 101)

Concerning **fertility**, the DS concluded that sperm effects seen in NE subchronic studies warrants a classification of Repr. 2 (H361f) for NE (CLH Report of NE, 2023: p. 90), stating:

> "The classification proposal for sexual function and fertility of nitromethane, nitropropane and nitroethane is based on the overall WoE from all category members. There is no EOGRTS or 2-generation study on any of the category member, and thus only limited aspects of potential effects on sexual function and fertility have been investigated in the available data set. However, spermatotoxic effects were reported on nitromethane (90-day NTP studies in rats and mice) and nitroethane (90-day NTP study in rats) and these findings are supported by nitrate/nitrite-mediated spermatotoxic and fertility related effects involving NO redox cycle. As indicated above, nitrite is the common metabolite for nitromethane, nitroethane, and 1-nitropropane. In addition, the OECD TG 422 on 1-nitropropane showed that 2 females at the mid- and high dose groups failed to become pregnant. Overall, these data are considered to support Repr. 2; H361f for nitromethane, nitroethane and 1- nitropropane and these classifications are proposed in individual dossiers." (CLH Report of NE, 2023: p. 11)

The DS did not follow appropriate standards for the assessment of reproductive and developmental toxicity in determining these classifications. The DS inappropriately used read-across to NM data in its developmental toxicity assessment. Read-across to NM data is invalid because of qualitative and quantitative differences in the production of and toxicity profiles of the relevant biotransformation products. Read-across to a guideline-compliant OECD 422 study of 1-NP is more appropriate when using a WoE approach to classification of NE. There is greater similarity in nitrite formation kinetics between NE and 1-NP (Sakurai et al. 1980; Lai et al. 1982). This OECD 422 study (Carney et al. 2004, referenced as "Anonymous 37, 2003" in the CLH Report) did not show that 1-NP is a reproductive or developmental toxicant.

Based on a WoE approach and disregarding read-across to NM, no classification is warranted for NE. However, because the DS used a questionable read-across approach that was aimed at

addressing limitations in the NE data set, additional considerations related to the available evidence are described further below.

3.1 READ-ACROSS FROM NM USED TO CLASSIFY NE DOES NOT FOLLOW RAAF PRINCIPLES AND IS SCIENTIFICALLY INVALID

The DS included systemic toxicity studies on NM as part of the rationale for NE classification for reproductive effects (Category 2) (CLH Report for NE: pp. 88-90). The sperm effects observed were attributed to the shared metabolite nitrite (CLH Report of NE, 2023: p. 11).

This read-across approach is not appropriate for several reasons. First, although NM, NE, and 1-NP could all have a common nitrite metabolite, it is not known that said metabolite is the underlying cause of the observed sperm effects for NM and NE. Furthermore, even if that was an operative mode of action, there are quantitative differences in nitrite formation and elimination between NM, NE, and 1-NP. Lai et al. (1982) acknowledged the potential discrepancies in nitrite formation among the nitroalkanes, stating, "Nitrite is the major metabolite found in the blood, urine, and various organs after administration of NE, 1-NP, or 2-NP but not NM" (p. 11). Likewise, Sakurai et al. (1980) noted that NM had a unique binding affinity, compared to other nitroalkanes, in rat liver microsomes.

The read-across approach utilized by the DS in the classification has significant uncertainty and does not fully follow RAAF principles. The RAAF requires demonstrative evidence that two compounds, or a class of compounds, share properties, stating:

- "The category hypothesis should apply in an unambiguous manner to all the category members. Only category members that are covered by the category hypothesis can be involved in the read-across" (ECHA 2017: p. 26).
- "A common mechanism linking the presence of the compounds driving the effects with the prediction needs to be identified, and should also link the structures of the compounds under consideration with the possibility to predict qualitatively similar effects for the target substance" (ECHA 2017: p. 24).
- "Other compounds than those linked to the prediction in the read-across hypothesis may be formed through other (bio)transformation pathways or may be intermediates/metabolites of the identified pathway… Exposure to these compounds has to be considered in the justification" (ECHA 2017: p. 24).
- "[F]or a category approach, there is a need to take further account of whether or not quantitative variations in the properties are observed among the category members" (ECHA 2017: p. 11).

The DS failed to demonstrate the causal relationship between shared metabolite formation and toxic effect in its read-across from NM to NE. Furthermore, the DS did not take into account kinetic or quantitative differences in rate of metabolite, specifically nitrite, formation.

Therefore, the DS's read-across of NM data when classifying NE as CLH classification Repr. 2 is invalid and disregards RAAF principles published by ECHA.

3.2 THE SPERM EFFECTS OBSERVED IN NM AND NE STUDIES ARE SECONDARY TO SYSTEMIC TOXICITY AND HENCE DO NOT REQUIRE A CLASSIFICATION FOR EFFECTS ON REPRODUCTION

The available NM studies are not considered relevant for reproductive toxicity classification of NE due to invalid read-across. However, even if considering the NM and NE studies with equal weight in an overconservative approach disregarding both RAAF and toxicological principles, then it should be emphasized that available data do not provide conclusive evidence to classify these substances for reproductive toxicity.

The DS focused on sperm effects as the primary basis for the reproductive toxicity classification. One hypothesis is that the sperm effects were secondary to hypoxia, an effect of nitrite (Reyes et al. 2012, as described in Garnick et al. 2021: p. 20). In addition, the observed sperm effects for NM and NE come from repeat-dose toxicity studies without any evaluation of reproductive function and, hence, cannot be used for classification.

Overall, while there is a potential for coincident occurrence of systemic toxicity and effects on sperm for nitroalkanes (a plausible mode of action secondary to hypoxia) in subchronic studies, these data do not meet criteria to classify either NM or NE for fertility effects, in absence of evaluation of reproductive function.

3.3 THE OECD 422 STUDY ON THE ANALOGUE FOR 1-NP ALLOWS TO CONCLUDE THAT NE SHOULD NOT BE CLASSIFIED AS A DEVELOPMENTAL TOXICANT

The DS proposes a classification of Repr. Cat. 1B, H360D for developmental toxicity (CLH Report for NE, 2023: p. 102). The classification proposal is based on a read-across from NM, as the DS states that "*there is no prenatal developmental toxicity study performed on 1-nitropropane and nitroethane*" (CLH Report for NE, 2023: p. 102). However, the assessment for developmental toxicity presented by the DS does not appropriately weigh the evidence. We recommend no classification for NE based on the absence of observed effects for 1-NP in a guideline compliant OECD 422 study.

This OECD 422 study, Carney et al. (2004) and referenced as "Anonymous 37, 2003" in the CLH Report, is available for structurally similar compound 1-NP. This study reported a developmental no-observed-adverse-effect concentration of 100 ppm, the highest concentration tested. Read-across from 1-NP to NE is appropriate because there are data to substantiate greater similarity in the formation kinetics of nitrite, the metabolite suspected of

mediating developmental toxicity, between NE and 1-NP (Sakurai et al. 1980; Lai et al. 1982). Further, while NE and 1-NP are metabolized to form different aldehyde metabolites, acetaldehyde and propionaldehyde, neither are classified for reproductive or developmental toxicity under the harmonized CLP (ECHA 2024b,c). Thus, the different aldehyde metabolites between 1-NP and NE does not disqualify read-across from 1-NP to NE for this classification endpoint.

The OECD 422, while not considered as robust as OECD 414, 415, 416, or 443 studies, is designed to screen for potential reproductive effects of a test chemical for the purpose of hazard assessment. OECD states that the 422:

> "...comprises a reproduction/developmental toxicity screening test and, therefore, can also be used to provide initial information on possible effects on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition, either at an early stage of assessing the toxicological properties of test chemicals, or on test chemicals of concern.." (OECD 2016, p. 22)

With regard to the DS's primary criticisms of the Carney et al. (2004) 1-NP study, both the Carney et al. (2004) study and the prenatal development study of NM relied upon by the DS cover an overlapping exposure period. In fact, the 1-NP study covers premating and post-parturition periods not covered in the NM study. Thus, in terms of periods of susceptibility, the 1-NP study is superior to NM data. Further, while Carney et al. (2004) did not assess fetal variations, there are many effects that were fully examined in both the NM and 1-NP studies. In the NM study, fetal morphological changes occurred at the same concentration as many other effects that were examined in the 1-NP study. In other words, it is not likely that the design of the OECD 422 study for 1-NP would have missed a potential developmental effect related to fetotoxicity that was uniquely identified in the NM study. If anything, this gives further support for the use of the 1-NP study as the primary evidence when determining the classification of NE.

The DS also raised concerns regarding the highest dose tested in the Carney et al. (2004) study of 1-NP. While this study did not observe *"severe"* or frank observable clinical effects at 100 ppm, the study was sufficient. This reflects two key points. First, a range finding study at 100, 250, 500 ppm found that at the 250-ppm concentration, females had clear onset of clinical signs, and that this dose would have exceeded the MTD (Carney et al. 2004: p. 15). Thus, 100 ppm is likely appropriately close to the onset dose for severe effects. Second, as described by Lewis et al. (2024), maximum doses for a reproductive study need to consider other biological response mechanisms that induce developmental toxicity secondary to toxicity in the dams. One specific mechanism noted by Lewis et al. (2024) is anemia and hypoxia, both of which are known effects of 1-NP at high doses (Garnick et al. 2021). Although, the 100-ppm concentration in the Carney et al. study did not show evidence of methemoglobinemia or hematological changes, the goal of the OECD 422 study is to test as close to but before the onset of such effects. Therefore, the highest concentration tested is appropriate in the

consideration of avoiding hypoxia. Further, the 100-ppm concentration did induce target organ effects, including decreased bodyweight in males and nasal histopathological changes in females suggestive of doses that would induce animal stress.

Because of the similarities in structure and metabolism kinetics, conclusions regarding classification of NE could be drawn based on data for 1-NP. Overall, Carney et al. (2004) evidenced an absence of developmental toxicity for 1-NP, and, using read-across, this conclusion applies to NE as well.

3.4 THE OECD 422 STUDY ON THE ANALOGUE 1-NP ALLOWS TO CONCLUDE THAT NE SHOULD NOT BE CLASSIFIED AS A REPRODUCTIVE TOXICANT

The DS recommended Repr. 2 (H361f) classification for adverse effects on sexual function and fertility (CLH Report for NE, 2023: p. 90). As explained under Sections 3.1 and 3.2, this conclusion is scientifically invalid and biased towards overclassification because it takes into account inadequate read-across from NM, and because it assumes that effects on sperm seen in subchronic studies on NM and NE without any investigation of reproductive function warrant a classification for reproductive effects. This does not follow CLP classification criteria.

We recommend no classification for NE based on the absence of observed effects for 1-NP in a guideline compliant OECD 422 study.

This OECD 422 study, Carney et al. (2004) and referenced as “Anonymous 37,2003” in the CLH Report, is available for the structurally similar compound 1-NP. This study reported a reproductive no-observed-adverse-effect concentration of 50 ppm due to decreased litter size secondary to maternal toxicity. Read-across from 1-NP to NE is appropriate because there are data to substantiate greater similarity in the formation kinetics of nitrite, the metabolite suspected of mediating reproductive toxicity, between NE and 1-NP (Sakurai et al. 1980; Lai et al. 1982). Further, while NE and 1-NP are metabolized to form different aldehyde metabolites, acetaldehyde and propionaldehyde, neither are classified for reproductive or developmental toxicity under the harmonized CLP (ECHA 2024b,c). Thus, the different aldehyde metabolites between 1-NP and NE does not disqualify read-across from 1-NP to NE for this classification endpoint.

The OECD 422 study, while not considered as robust as OECD 414, 415, 416, or 443 studies, is designed to screen for potential reproductive effects of a test chemical for the purpose of hazard assessment. OECD states that the 422:

> “comprises a reproduction/developmental toxicity screening test and, therefore, can also be used to provide initial information on possible effects on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition, either at an early

> stage of assessing the toxicological properties of test chemicals, or on test chemicals of concern." (OECD 2016, p. 22)

Therefore, negative findings observed in Carney et al (2004) should support hazard assessment findings.

Carney et al. (2004) found no treatment-related effect on time to mating, gestation length, post-implantation loss, pup survival, or pup-sex ratio in an OECD 422 study of 1-NP in Sprague-Dawley rats exposed at concentrations of 0, 25, 50, or 100 ppm. Two females in the mid- and high-dose levels failed to be pregnant, but the CLH Report concluded it "cannot be stated if the reduced fertility index can be attributed to male, female or unspecific causes. Plus, the reduction is still comprised within the historical control data range" (CLH Report for NE, 2023: p. 86).

As it relates to sperm effects, the OECD 422 study did assess the effects of chemical exposure in the premating period in males; therefore, any effects on sperm quality or quantity would have resulted in fertility changes, and no such changes occurred in the study (Carney et al. 2004: p. 12).

Overall, the Carney et al. (2004) study is the only appropriate and guideline-compliant reproductive toxicity study available for classification of NE, and it demonstrated that 1-NP does not produce treatment-related reproductive effects. The potential decrease in fertility in the 1-NP study was not statistically significant or outside of historical control data (Carney et al. 2004). The interpretation of the changes in estrous cycle were unclear given the variability in this measure, and there is no evidence for endocrine related activity as a predicate for such an effect. Thus, based on a guideline-compliant OECD 422 study of 1-NP, NE should not be classified as a reproductive toxicant.

4 CONCLUSION

In conclusion, we encourage the DS to reconsider the proposed classifications for NE regarding carcinogenicity and reproductive toxicity. Specifically, we believe that the read-across approach from NM used by the DS is not in line with scientific and RAAF principles and that a scientifically sound WoE approach warrants "no classification" for carcinogenicity and reproductive toxicity of NE.

Overall, the read-across approach between the three primary nitroalkanes utilized by the DS did not respect RAAF principles and should not be considered valid in the classification of NE as Carc. 1B and Repr. 1B. There are qualitative and quantitative differences between the three primary nitroalkanes; hence, direct use of NM data is not appropriate to conclude for NE. For carcinogenicity, data for NE itself should take precedence over data for analogs NM and 1-NP, because they derive from studies of adequate design and quality to assess carcinogenicity. The DS did not seem to take into account a detailed WoE analysis presented in a critical review published in 2021 in the peer-reviewed *Journal of Applied Toxicology* (Garnick et al. 2021).

For developmental or reproductive hazards, read-across from NM data is invalid due to qualitative and quantitative differences in the production of and toxicity profiles of the relevant biotransformation products. Read-across from a guideline-compliant study of 1-NP would be more appropriate when using a WoE approach to classification of NE. The OECD 422 study of 1-NP is sufficient to conclude that NE should not be classified as a developmental or reproductive toxicant.

Considering mode of action is critical for each endpoint. In the case of NE, data should be weighed as NE > 1-NP >> NM. There is scientific evidence that the differential metabolisms of the three nitroalkanes contribute to distinct toxicological profiles for each compound. Additionally, there may be quantitative differences in rate and degree of metabolism of the compounds that have not been assessed (yielding different systemic dose patterns of nitrite, a key metabolite in the mode of action for potential reproductive effects). In addition, as noted by the DS, the aldehyde metabolite arising from each compound differs, indicating a qualitative and quantitative difference in potential effect potency for carcinogenicity.

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