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REVIEW ARTICLE

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Hazard characterization of carcinogenicity, mutagenicity, and reproductive toxicity for short chain primary nitroalkanes

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Abstract

Nitroalkanes are organic aliphatic hydrocarbon compounds with a nitro moiety that are commonly used as solvents or intermediates to synthesize a variety of organic compounds due to their inherent reactivity. In June 2020, a harmonized classification and labeling (CLH) proposal was submitted to the European Chemicals Agency (ECHA) for the following harmonized carcinogenicity, mutagenicity, and reproductive toxicity ("CMR") classifications for nitromethane (NM), nitroethane (NE), and 1-nitropropane (1-NP): NM Carc. 1B and Repr. 1B; NE Repr. 1B; and 1-NP Repr. 2. In this assessment, a weight of evidence (WoE) evaluation of studies on animal carcinogenicity and reproductive and developmental toxicity, genotoxicity, and mode of action for these three nitroalkanes was performed to critically assess the relevance of the proposed CMR classifications. Overall, the WoE indicates that NM, NE, and 1-NP are not carcinogenic, genotoxic, nor selective reproductive or developmental toxicants. Based on our analysis, classifying NM, NE, and 1-NP as Category 2 reproductive toxicants is most appropriate. Furthermore, not classifying NE and 1-NP with respect to their carcinogenicity is appropriate based on the available studies for this endpoint coupled with negative results in genotoxicity studies, metabolism data, and in silico predictions. We determined that the classification for NM of Carc. 1B is not appropriate, based on the fact that rat mammary and harderian tumors are likely not relevant to humans and lung and liver tumors reported in mice were equivocal in their dose–response and statistical significance.

KEYWORDS
carcinogenicity, CMR, mutagenicity, nitroalkanes, primary nitroalkanes, reproductive toxicity

1 | INTRODUCTION

Nitroalkanes are organic chemicals with a nitro moiety bound to an aliphatic hydrocarbon chain. The primary short chain nitroalkanes include nitromethane (NM; CAS number 72-52-5), nitroethane (NE; CAS number 79-24-3), and 1-nitropropane (1-NP; CAS number 108-03-2) (Table 1). These compounds possess a combination of physical and chemical properties that make them useful for many diversified applications. As such, nitroalkanes are commonly used as a solvent in a variety of industrial applications including extractions, fractionating petroleum distillate oils, dewaxing diesel fuels, and chemical intermediates for organic synthesis in the development and manufacture of products, such as pharmaceuticals, pesticides, fibers, coatings, and explosives (Markofsky, 2011). Nitroalkanes are manufactured in large volumes and/or used for various applications in commerce in both the European Union and North American markets (European Chemicals Agency [ECHA], 2021a, 2021b, 2021c).

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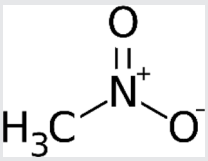
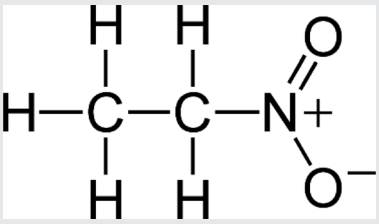
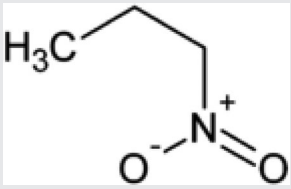
TABLE 1 Physicochemical properties of primary nitroalkanes

Chemical name	Nitromethane	Nitroethane	1-Nitropropane
Chemical abstracts no.	79-24-3	75-52-5	108-03-2
Chemical structure			
Chemical formula	CH_3NO_2	$C_2H_5NO_2$	$C_3H_7NO_2$
Molecular weight (g/mol)	61.04	75.067	89.094

The hazard characterization of these nitroalkanes has been evaluated to a variable extent over the years (ECHA, 2021a, 2021b, 2021c). Due to potential occupational and consumer exposures to nitroalkanes, there is public health value in critically evaluating the hazard characterizations for these compounds. In November 2018, a revised classification proposal was submitted to ECHA for harmonized classifications for NM, NE, and 1-NP. The initial proposal was to classify NM, NE, and 1-NP as germ cell mutagens, reproductive toxicants, and carcinogens. In the initial classification, a read-across approach was utilized to determine the carcinogenic and reproductive classifications for both NE and 1-NP based on the lack of adequate data on these endpoints. However, in June of 2020, an updated harmonized classification and labeling (CLH) proposal was submitted, and the proposed germ cell mutagenicity classification was removed for all three chemicals. Additionally, cancer classifications for NE and 1-NP were no longer considered, and the proposed reproductive toxicity classification for 1-NP was lowered to Category 2.[1] To date, the proposed reproductive toxicity classifications for NE and NM remain Category 1B.[2] This comparative evaluation provides an opportunity to support increased clarity and harmonization among the current CLH hazard conclusions for these nitroalkane compounds.

The objective of this evaluation was to conduct a comprehensive state of the science review of the hazard data for NM, NE, and 1- NP in light of the revised harmonized CLH proposed for carcinogenicity, mutagenicity, and reproductive/developmental toxicity, as well as the conclusions published in the ECHA dossiers for these chemicals. The assessment incorporates a mode of action (MoA) and data quality informed process for integrating and evaluating evidence for each endpoint. Since each of these chemicals has limited toxicological data, using read-across from short chain nitroalkanes (from 2-NP to the primary nitroalkanes as well as across each of the primary nitroalkanes) based on MoA considerations was also considered in the weight of evidence (WoE) integration.

2 | METHODS

2.1 | Literature identification

The assessment included data identified from key studies reported on carcinogenic, mutagenic, and/or reproductive toxicity responses in the ECHA toxicological profiles for three nitroalkane compounds: NM, NE, and 1-NP. A supplementary literature search was conducted through August 2020 in the PubMed database to identify additional human and animal studies following exposure to each of the chemical compounds.

2.2 | Structure activity relationships

Structure activity relationship (SAR) predictions were developed for endpoints relevant to carcinogenicity, genotoxicity, and reproductive toxicity using profilers in the OECD ToolBox (version 4.4.1), Derek Nexus v6.0.1 (Lhasa Limited), and the USEPA CompTox Chemicals Dashboard.

2.3 | WoE evaluation

A WoE evaluation was performed for the toxicity studies of each chemical. The studies were qualitatively ranked according to their relative significance and relevance to humans. Specifically, a simple weighting paradigm using principles of the OHAT approach for systematic review was used for animal studies in consideration of factors such as assay quality, quantity and type(s), the consistency of results across studies, and biological plausibility and relevance (NTP, 2019a, 2019b). Study quality was also quantitatively assessed via Klimisch scores (K-scores) (Klimisch et al., 1997) determined utilizing the ToxRTool.

TABLE 2 In vivo chronic and subchronic carcinogenicity studies for NM

Reference	Chemical purity	Route	Animal/model	Dose	Duration	Effect level	Result and effects	Confidence score (OHAT/Klimisch)
Griffin et al., 1996	96.26% (2.79% NE; 0.62% 2-NP)	Inhalation	Long–Evans rats	100 or 200 ppm	7 h/day, 5 days/week, 2 years	NOAEC: 200 ppm	**Negative.** No significant, dose-related effects reported	**High/1**
Lewis et al., 1979	96.5% (1.5% NE; 1.4% 2-NP; 0.5% propionitrile)	Inhalation	Sprague–Dawley rats and New Zealand rabbits (males)	98 or 745 ppm	7 h/day, 5 days/week, 6 months	NOAEC: 745 ppm	**Negative.** No significant, dose-related effects reported	**Moderate/1** (Insufficient study duration to assess carcinogenicity endpoint)
NTP, 1997	~98%	Inhalation	F344/N rats	94, 188, or 375 ppm	6 h/day, 5 days/week, 103 weeks	NOAEC: 94 ppm; LOAEC: 188 ppm (female only)	**Positive.** Significantly increased incidence of mammary tumors in exposed female rats compared with controls Fibroadenomas were increased in 188- and 375-ppm exposure groups while carcinomas were increased in the 375-ppm group	**High/1**
NTP, 1997	~98%	Inhalation	B6C3F1 mice	188, 375, or 750 ppm	6 h/day, 5 days/week, 103 weeks	Hepatocellular tumors - LOAEC: 188 ppm (female only) Lung tumors - NOAEC: 188 ppm; LOAEC: 375 ppm (females). NOAEC: 375 ppm; LOAEC: 750 ppm (males) Harderian gland tumors - NOAEC: 188 ppm; LOAEC: 375 (males and females)	**Positive.** Hepatocellular adenoma and adenoma/carcinoma combined were significantly increased in female mice in the 188- and 750-ppm groups Female mice and male mice had significantly increased alveolar/bronchial carcinomas in the 375- and 750-ppm dose groups, respectively. Combined alveolar/bronchiolar carcinomas and adenomas were increased for females in the 750-ppm group For both male and female mice, harderian gland adenomas as well as combined adenomas/carcinomas were	**High/2** (Based on lack of complete dose–response for liver and lung tumors in female mice and the absence of liver tumors in male mice. A lack dose–response was also reported for lung tumors in female mice)

(Continues)

TABLE 2 (Continued)

Reference	Chemical purity	Route	Animal/model	Dose	Duration	Effect level	Result and effects	Confidence score (OHAT/Klimisch)
							significantly increased in the 375- and 740-ppm dose groups	
OECD ToolBox (version 4.4.1) (QSAR)	-	N/A	N/A	N/A	N/A	N/A	Positive prediction of carcinogenicity from "potentially oncogenic and mutagenic" nitroaliphatic class	-

2.4 | Read-across evaluation

Data gap filling using read across from 2-nitropropane (2-NP) to the primary nitroalkanes as well as among the three primary nitroalkanes was completed to address mode of action hypotheses for mutagenicity and cancer. The general principles described in current guidance from ECHA and OECD and as summarized by Patlewicz et al. (2018) was used.

3 | RESULTS AND DISCUSSION

3.1 | Carcinogenicity

The in vivo carcinogenicity studies for NM, NE, and 1-NP are summarized in Table 2. No human studies evaluating the carcinogenicity of these nitroalkanes were identified.

3.1.1 | NM carcinogenicity studies

To date, a total of four studies have evaluated the carcinogenic hazard potential of NM in rodents via inhalation (Table 2). Findings from these studies have reported conflicting results with respect to the carcinogenicity of NM. For example, the 1997 two-year bioassay on NM by the National Toxicology Program (NTP) reported a statistically significant increase in mammary gland tumors (fibroadenomas; and fibroadenomas or carcinomas combined) in exposed female F344/N rats compared with controls (Table 3). Of note, no increased tumor incidence was observed in male rats in any tissue evaluated. Based on these findings, the NTP concluded that there was "clear evidence of carcinogenicity" for NM in female rats due to the observed increased incidence of mammary tumors (NTP, 1997). Of note, the background rate in untreated female F344 of mammary gland fibroadenomas rats was reported to be up to 54%, and carcinomas was reported to be up to 10% (similar to that reported in the NTP study); data for fibroadenomas or carcinomas combined was not available (Haseman et al., 1998).

A separate 2-year chronic NM bioassay (with overlapping exposure concentrations) performed in Long–Evans rats (Griffin et al., 1996) did not show statistically significant increases in benign or malignant tumors in any tissue evaluated in either male or female rats. Further, Lewis et al. (1979) reported a lack of carcinogenicity in male Sprague–Dawley rats and New Zealand rabbits exposed via inhalation to 98- or 745-ppm NM for 6 months. Although it is possible that the exposure duration may have been insufficient to observe potential tumorigenic effects in these animal models if the tumorigenic potency was low, there is evidence to suggest such results are predictive if specific conditions are met (e.g., nongenotoxic compound and no preneoplastic lesions). Specifically, Woutersen et al. (2016) evaluated whether preneoplastic lesions in subchronic toxicity studies could predict cancer outcomes in chronic studies for 163 nongenotoxic chemicals. Of the 148 compounds that were negative for

TABLE 3 Key carcinogenicity findings for NM from the NTP (1997) study

Endpoint	Animal	Tumor type (overall rate)	Dose (ppm)			
			0	94	188	375
Mammary gland	F344 rat (female)	Fibroadenoma	**19/50 (38%)****	21/50 (42%)	**33/50 (66%)****	**36/50 (72%)****
		Carcinoma	**2/50 (4%)****	7/50 (14%)	1/50 (2%)	**11/50 (22%)***
		Fibroadenoma or carcinoma	**21/50 (42%)****	25/50 (50%)	**34/50 (68%)****	**41/50 (82%)****
			0	**188**	**375**	**750**
Harderian gland	B6C3F1 mouse (female)	Adenoma	**5/50 (10%)****	7/50 (14%)	**16/50 (32%)****	**19/50 (38%)****
		Carcinoma	1/50 (2%)	2/50 (4%)	4/50 (8%)	3/50 (6%)
		Adenoma or carcinoma	**6/50 (12%)****	9/50 (18%)	**20/50 (40%)****	**21/50 (42%)****
	B6C3F1 mouse (male)	Adenoma	**9/50 (18%)****	10/50 (20%)	**19/50 (38%)***	**32/50 (64%)****
		Carcinoma	**1/50 (2%)***	1/50 (2%)	6/50 (12%)	5/50 (10%)
		Adenoma or carcinoma	**10/50 (20%)****	11/50 (22%)	**25/50 (50%)****	**37/50 (74%)****
			0	**188**	**375**	**750**
Liver	B6C3F1 mouse (female)	Adenoma	**14/50 (28%)****	**25/49 (51%)***	17/49 (35%)	**35/50 (70%)****
		Carcinoma	10/50 (20%)	14/49 (29%)	8/49 (16%)	12/50 (24%)
		Adenoma or carcinoma	**19/50 (38%)****	**34/49 (69%)****	22/49 (45%)	**40/50 (80%)****
			0	**188**	**375**	**750**
Lung (alveoli and bronchioles)	B6C3F1 mouse (female)	Adenoma	**3/50 (6%)***	3/50 (6%)	2/49 (4%)	9/50 (18%)
		Carcinoma	0/50 (0%)	3/50 (6%)	**5/49 (10%)***	3/50 (6%)
		Adenoma or carcinoma	**3/50 (6%)****	6/50 (12%)	6/49 (12%)	**12/50 (24%)***
	B6C3F1 mouse (male)	Adenoma	11/50 (22%)	10/50 (20%)	9/50 (18%)	12/50 (24%)
		Carcinoma	**2/50 (4%)****	3/50 (6%)	3/50 (6%)	**11/50 (22%)****
		Adenoma or carcinoma	13/50 (26%)	13/50 (26%)	12/50 (24%)	20/50 (40%)

Note: In the control column, the *p* values are associated with the Cochran–Armitage trend test, which directly compares the proportion of tumor bearing animals in dosed and control groups. In the exposed group columns, the *p* values are corresponding to the pairwise comparisons between the controls and that exposed group. Bold results are statistically significant.
*$p \leq 0.05$.
**$p \leq 0.01$.

preneoplastic lesions in subchronic studies, 75% were also negative in the carcinogenicity studies, and this predictivity was improved to 97% when relevance of animal tumors was taken into account (Woutersen et al., 2016). Of note, no histological evidence of neoplasticity was observed in the tissues of animals in either the Griffin et al. (1996) or Lewis et al. (1979) studies.

Nonetheless, the observed discrepant mammary tumorigenesis findings for NM in female rats in the NTP study relative to the other studies warrant additional consideration. The two rat species received similar exposures (188 ppm in NTP and 200 ppm in Griffin et al.) over comparable dosing regimens in the two studies. Alternative hypotheses for the apparent inconsistent findings could include the following: (1) that the increased tumor incidence in the NTP study was driven by differences in impurities (i.e., presence of the known rodent tumorigen 2-NP) in the NM used in each study or (2) the observed findings reflect differential strain sensitivities to mammary tumor formation.

It is unlikely that 2-NP contamination would explain the conflicting results in these studies. The NM used by Griffin et al. reportedly contained 2.79% NE and 0.62% 2-NP. The NTP used two discrete batches of NM: (1) NM with a reported purity of 99.3% was used for the beginning of the two-year study and (2) NM with a reported purity of 99% was used for the study's remainder. The first batch was identified by the supplier as containing 0.27% nitroethane while the second batch reportedly contained 0.25% nitroethane and 0.03% 2-nitropropane. Based on the impurities of constituents reported by the suppliers, 2-NP levels in the test article are lower in the NTP study than in the NM procured by Griffin et al. (1996), inconsistent with the comparative tumor data between the studies since 2-NP is an identified rodent tumorigen (Gadagbui et al., 2020).

A second hypothesis for differences in rat mammary tumor findings between the NTP and Griffin studies is that F344 rats used in the NTP (1997) carcinogenicity study may have increased sensitivity to spontaneous mammary tumor formation relative to the Long–Evans rats. The F344 strain has been identified as having a high susceptibility to various tumors, which ultimately prompted the NTP in 2006 to phase out use of the strain in 2-year chronic toxicity and carcinogenicity studies (King-Herbert & Thayer, 2006; Maronpot et al., 2016). Of note, the third highest incidence of spontaneous tumors found in

F344 rats in the NTP 2-year bioassays were mammary tumors; typically, the observed spontaneous mammary tumors were benign in nature (e.g., fibroadenomas and fibromas) as opposed to malignant tumors such as adenocarcinomas, which are the most frequent tumors induced by chemical carcinogens (Russo & Russo, 1996). Given the facts that (1) spontaneous mammary fibroadenomas have been very common in female F344 rats in NTP bioassays, (2) the F344 strain was discontinued in NTP 2-year carcinogenicity bioassays, and (3) known chemical carcinogens are more likely to result in malignant mammary tumors (e.g., adenocarcinomas), it is likely that the observed benign mammary tumors in the 1997 NTP NM bioassay are not treatment related. The lack of mammary (or any) tumors in other rodent strains (e.g., Long–Evans) exposed to comparable levels of NM in 2-year chronic studies support that the observed mammary tumors in the female F344 rats in the NTP study were likely spontaneous in nature. These results are consistent with absence of expected endrocrine activity based on in silico predictions as described below.

In B6C3F1 mice, the NTP reported that chronic NM exposure resulted in statistically significant increases in the incidences of hepatic tumors in female mice, as well as lung and harderian gland tumors in both male and female mice (Table 3). Specifically, it was reported that female mice had increased incidences of hepatocellular adenomas as well as combined adenomas and carcinomas in only the lowest (188 ppm) and highest (750 ppm) dose groups (Table 3). The increased liver adenoma incidences reported in female B6C3F1 mice exceeded the historical range of 0%–18% reported in untreated controls across 59 NTI/NTP carcinogenicity studies (Maronpot et al., 1987), but the absence of a clear increase in incidence with exposure concentration hinders interpretation. The study also reported an increase in lung tumors in exposed groups of males and females versus controls, but there was not a clear concentration-response pattern for the lung tumor incidence. Of note, the background rate of lung tumors in untreated mice was reported to be approximately 20% in male mice and 8% in female mice, similar to the rate reported in the NTP study (Haseman et al., 1998). Both sexes showed significantly increased formation of harderian gland adenomas. Notably, no statistically significant increases in harderian gland carcinomas were reported for either sex. Nonetheless, the NTP concluded that there was "clear evidence of carcinogenicity" in both male and female mice, based on the increased incidence of harderian gland tumors in males and of harderian gland and liver tumors in females (NTP, 1997). Although statistically significant increases in harderian gland tumors were reported in both male and female mice in the middle and high dose groups, these findings may not always be relevant to human hazard (Albert et al., 1986). There has been no evidence of either transiently present or vestigial harderian glands in humans (Albert et al., 1986). It is noteworthy that previous researchers have reported that harderian tumors do not have relevance to humans for non-DNA reactive substances (Cohen, 2004; Mortelmans et al., 1986; NTP, 1997). This reflects that tumor site concordance is an important consideration when a tissue-specific mode of action is operative but not for a mode of action that could theoretically be operative regardless of tissue physiology (i.e., genotoxicity). As detailed later in this evaluation, NM has been shown to be negative in various Ames assays at doses as high as 10,000 μg/plate (Cohen, 2004; Mortelmans et al., 1986; NTP, 1997), and as such, the increased harderian gland adenomas in mice resulting from chronic NM exposure is not relevant to humans.

3.1.2 | NE carcinogenicity studies

One chronic carcinogenicity study of NE was identified in the published literature (Griffin et al., 1996) (Table 4). No significant increases in tumor incidences were reported for any organ following NE exposure in the animals.

3.1.3 | 1-NP carcinogenicity studies

One study that evaluated the carcinogenicity of 1-NP was identified in the published literature (Hadidian et al., 1968) (Table 4). Fischer F344 rats were administered 1-NP daily, by gavage, at dose levels of 0.3, 3, or 10 mg/day for 52 weeks. Neoplastic lesions were reported in numerous organs in the dose groups as well as in controls. Overall, there was a lack of dose–response of neoplastic lesion incidence across the exposure groups. Furthermore, there may be limited relevance of an oral dosing study when assessing chronic inhalation cancer risk. Specifically, the EPA has previously noted that substances may be carcinogenic by one route of exposure and considered noncarcinogenic by other routes (U.S. Environmental Protection Agency, 1986) depending on differences in tissue dose and the mode of action.

3.1.4 | Structure activity relationship results for carcinogenicity

All three nitroalkanes were evaluated for carcinogenic hazard potential using the OECD ToolBox. The carcinogenic hazard prediction from the ToolBox identified NM as positive based on a "potentially oncogenic and mutagenic nitroaliphatic class" using the Benigni decision rules. Regarding the predictive carcinogenicity of NE and 1-NP via ToolBox, no alerts were found for genotoxic or nongenotoxic carcinogenicity using the ISS database.

Derek modeling resulted in no genotoxicity alerts for NM, NE, or 1-NP. Regarding carcinogenicity, Derek modeling predicted that NM was a "probable" carcinogen, based on the fact that NM is classified as a 2B carcinogen by International Agency for Research on Cancer (IARC). NE and 1-NP were predicted to be "plausible" carcinogens based on a structural match with other aliphatic nitro compounds, including NM and 2-NP.

As detailed in this analysis, available empirical evidence does not support these carcinogenicity predictions for NM.

While the Quantitative structure–activity relationship (QSAR) predictions provide one line of evidence, the potential for a genotoxic

TABLE 4 In vivo chronic carcinogenicity studies for NE and 1-NP

Chemical	Reference	Chemical purity	Route	Animal/model	Dose	Duration	Effect level	Result and effects	Confidence score (OHAT/Klimisch)
NE	Griffin et al., 1988	97.92% (0.01% NM; 2.07% 2-NP)	Inhalation	Long–Evans rat	100 or 200 ppm	7 h/day, 5 days/week, 2 years	NOAEC: 200 ppm	**Negative.** No significant, dose-related effects reported	**High/1**
1-NP	Hadidian et al., 1968	-	Gavage	Fischer F344 rats	0.3, 3, or 10 mg/day	5 days per week for 52 weeks	NOAEL: 10 mg/day	**Negative.** No increase in tumor incidence; one esophageal tumor reported in male rat in middle dose group	**High/1**

MoA was also evaluated based on available empirical data below. The empirical data are given greater weight, when studies are considered reliable.

3.1.5 | Carcinogenicity synthesis of overall results

To date, there is limited to no evidence available to support that the three nitroalkane compounds evaluated are definitively carcinogenic to humans. Of the three primary nitroalkanes, only NM was shown to induce tumors (mammary, liver, lung, and harderian gland) in vivo following chronic inhalation exposure. However, it is important to note that the statistically significant increased incidence of mammary fibroadenomas in female F344 rats reported in the NTP (1997) study may be attributed to the strain's increased tumor susceptibility, as similar NM exposures did not induce any tumors in the other evaluated strains or species (Griffin et al., 1996; NTP, 1997). Reported mouse liver and lung tumors following NM exposure occurred in tissues with high background, lacked a clear concentration-response, and have no compelling MoA hypothesis. Moreover, the mouse harderian gland tumors have questionable relevance since tumor site concordance is an important consideration for chemicals that lack genotoxicity.

Given the lack of consistent carcinogenic findings in available NE and 1-NP studies, and the limited to no evidence for the carcinogenic hazard for NM, available animal data would support that at this time, these primary nitroalkanes would not be expected to be carcinogenic to humans.

Based on available animal studies, a carcinogenic classification for NM of 1B is not appropriate. This conclusion is based on the fact that rat mammary and harderian tumors are likely not relevant. Furthermore, lung and liver tumors in mice are equivocal in their dose–response and statistical significance. Additionally, based on a lack of likely genotoxic mode of action (as explained below), NM is not expected to act as a multiorgan carcinogen.

3.2 | Genotoxicity

3.2.1 | Synthesis of empirical studies and QSAR for genotoxicity

The genotoxicity and mutagenicity of NM, NE, and 1-NP have been extensively evaluated (Tables 5–7). These compounds were not classified for mutagenicity in their respective ECHA dossiers.

The genotoxicity of NM was evaluated by numerous studies that assessed the chemical's mutagenicity as well as its ability to impair chromosome function and induce cell transformation (Table 5). Of the eight Ames assays, six of the studies indicate that the compound is not mutagenic. One of the studies reported positive results for one of the four strains of Salmonella tested (Ong et al., 1980); however, it is noteworthy that neither the dosing regimen in the study nor the statistical significance of the findings were reported. All but one of the other genotoxicity studies of NM were also negative.

TABLE 5 Genotoxicity studies for NM

Reference	Endpoint	Study type	Chemical purity	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
Chiu et al., 1978	AMES	In vitro	-	*S. typhimurium* strains (TA98, TA100)	0.1, 1, or 10 μmol/plate (without metabolic activation)	72-h incubation	HID: 610 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of positive control)
Dayal et al., 1989	AMES	In vitro	-	*S. typhimurium* strains (TA100, TA98, TA102)	Exact dose(s) not presented. Estimates from figure 3: 25, 50, 100, or 200 μmol/plate (without metabolic activation)	-	HID: 12,200 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of documentation of controls)
Dellarco & Prival, 1989	AMES	In vitro	>99% purity	*S. typhimurium* strains (TA98, TA100)	0.1, 1, or 10 μmol/plate (without metabolic activation)	48-h incubation	HID: 10 μmol/plate (610 μg/plate)	**Negative.** No significant, dose-related increase in mutation frequency	**1** (reliable)
Gocke et al., 1981	AMES	In vitro	-	*S. typhimurium* strains (TA100, TA1535, TA1537, TA1538, TA98)	Maximum dose 'usually' up to 3600 μg/plate (with and without metabolic activation)	-	HID: 3600 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of documentation of dosing regimen; no specification of controls)
Löfroth et al., 1986	AMES	In vitro	~99.7%	*S. typhimurium* strains (TA100, TA1535, TA98)	Maximum of 20,000 or 50,000 μg/plate (with and without metabolic activation)	-	HID: 20,000 or 50,000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of positive control and documentation of dosing regimen; statistical significance not reported)
Mortelmans et al., 1986	AMES	In vitro	-	*S. typhimurium* strains (TA1535, TA1537, TA97, TA98, and TA100)	0.100, 0.333, 1.000, 3.333, or 10.000 mg/plate (with and without metabolic activation	NR	HID: 10,000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	Not assignable; original data unavailable

TABLE 5 (Continued)

Reference	Endpoint	Study type	Chemical purity	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
NTP, 1997	AMES	In vitro	-	*S. typhimurium* strains (TA98, TA100, TA1535)	100, 333.31000, 3333.3, 10,000 μg/plate (with and without metabolic activation)	48-h incubation	HID: 10,000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**2** (reliable with restriction) (Statistical significance of findings not reported; lack of of information on test system characterization)
Ong et al., 1980	AMES	In vitro	-	*S. typhimurium* strains (TA98, TA100, TA1535, TA1537)	50 μl/plate (with and without metabolic activation). Other doses not characterized	72-h incubation	HID: 40 μl/plate, LED: 50 μl/plate	**Positive** (for TA1535 only)	**3** (not reliable) (Based on lack of documentation of dosing regimen; statistical significance not reported)
Kerckaert et al., 1996	Cell transformation	In vitro	-	Syrian hamster embryo cells	2000, 2500, 3000, 3500, 4000, or 4500 μg/ml (without exogenous metabolic system)	24-h treatment	HID: 3000 μg/ml; LED: 3500 μg/ml	**Positive.** Statistically significant positive responses in two highest dose groups	**1** (reliable)
NTP, 1997	Chromosomal aberration	In vitro	-	Chinese hamster ovary cells	1077, 2316, or 4980 μg/ml (with and without metabolic activation)	2-h chemical treatment followed by 11.5-h incubation	HID: 4980 μg/ml	**Negative.** No significant, dose-related increase in mutation frequency	**2** (reliable with restriction) (Based on lack of test substance identification, including purity)
Gibson et al., 1997	Micronucleus	In vitro	-	Syrian hamster embryo (SHE) cells	5.0, 5.5 or 6.0; 3500, 4000, or 5000 μg/ml	24-h treatment	HID: 5000 μ g/ml	**Negative**. No significant, dose-related increase in mutation frequency	**1** (reliable)
Gocke et al., 1981	Micronucleus	In vivo (I.P. injection)	-	Micronucleated bone marrow erythrocytes from NMRI mice	Cumulative doses of 410, 1830, or 3660 mg/kg/day (without metabolic activation)	Two doses administered in 24 h; cells harvested 6 h after second dose	HID: 1830 mg/kg/day	**Negative**. No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of positive control)

(Continues)

TABLE 5 (Continued)

Reference	Endpoint	Study type	Chemical purity	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
NTP, 1997	Micronucleus	In vivo (inhalation) /in vitro	-	Micronucleated erythrocytes in peripheral blood from B6C3F1 mice	94, 188, 375, 750, or 1500 ppm (without metabolic activation)	13-week in vivo exposures	HID: 1500 ppm	**Negative**. No significant, dose-related increase in mutation frequency	**2** (reliable with restriction) (Based on lack of test substance identification, including purity)
Gocke et al., 1981	Sex-linked mutations	In vivo (oral)/ in vitro	-	Basc test in *D. melanogaster*	Cumulative doses of 410, 1830, or 3660 mg/kg (without metabolic activation)	One dose	HID: 3660 mg/kg	**Negative**. No significant, dose-related increase in mutation frequency	**1** (reliable)
NTP, 1997	Sister chromatid exchange	In vitro	-	Chinese hamster ovary cells	497, 1655, or 4965 μg/ml (with and without metabolic activation)	2-h treatment followed by initial incubation of 26 h	HID: 4965 μg/ml	**Negative**. No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of documentation of positive control)

TABLE 6 Genotoxicity studies for NE

Reference	Endpoint	Study type	Chemical purity	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
Dayal et al., 1989	AMES	In vitro	-	*S. typhimurium* strains (TA100, TA98, TA102)	Exact dose(s) not presented. Estimates from figure 3: 25, 50, 100, or 200 μmol/plate (without metabolic activation)	-	HID: 200 μmol/plate (15,014 μg/plate)	**Negative.** No significant, dose-related increase in mutation frequency	**3** (not reliable) (Based on lack of documentation of controls)
Hite & Skeggs, 1979	AMES	In vitro	-	*S. typhimurium* strains (TA92, TA98, TA100, TA1537)	0.0037, 0.011, or 0.03 ml/plate (with and without activation)	72-h incubation	HID: 0.03 ml/plate (31,500 μg/plate)	**Negative.** One elevated mutation frequency (twice the control) in TA92 at 0.011 ml/plate was not reproducible.	**2** (reliable with restriction) (Statistical significance of findings not reported; lack of information on test substance characterization, including purity)
Löfroth et al., 1986	AMES	In vitro	~91.4% (8.6% 2-NP) and >99.5%	*S. typhimurium* strains (TA100, TA1535, TA98)	Maximum of 20,000 or 50,000 μg/plate (with and without metabolic activation)	-	LED: 20,000 or 50,000 μg/plate	**Positive.** Low mutagenic response at 20,000 or 50,000 μg/plate	**3** (not reliable) (Based on lack of positive control and documentation of dosing regimen; statistical significance not reported)
Mortelmans et al., 1986	AMES	In vitro	-	*S. typhimurium* strains (TA1535, TA1537, TA97, TA98, and TA100)	0.100, 0.333, 1.000, 3.333, or 10.000 mg/plate (with and without metabolic activation	Pending	HID: 10,000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	Not assignable; original data unavailable
Hite & Skeggs, 1979	Micronucleus	In vivo (gavage) /in vitro	-	CD-1 mouse erythrocytes	0.25, 0.50 or 1.00 ml/kg/day	Cells harvested 6 h after exposure	HID: 1.00 ml/kg/day	**Negative.** No significant, dose-related increase in micronucleated cells	**1** (reliable)

TABLE 7 Genotoxicity studies for 1-NP

Reference	Endpoint	Chemical purity	Study type	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
Conaway, Hussain, et al., 1991	AMES	-	In vitro	*S. typhimurium* (TA98, TA100, TA102)	10, 20, 40, or 80 μmol/plate	48-h incubation	HID: 80 μmol/plate (7127.2 μg/plate)	**Negative.** No significant, dose-related increase in mutation frequency	**1** (reliable)
Thompson, 1995 (Dow Chemical Company)	AMES	~99%	In vitro	*S. typhimurium* (TA1535, TA1537, TA98, TA10); *E. coli* (WP2uvrA-)	312.5, 625, 1250, 2500, or 5000 μg/plate (with and without S9 activation)	48-h incubation	HID: 5000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**1** (reliable)
Thompson, 1996 (Dow Chemical Company)	AMES	~99%	In vitro	*S. typhimurium* (TA1535, TA1537, TA98, TA100); *E. coli* (WP2uvrA-)	150, 500, 1500, or 5000 μg/plate (with and without S9 activation) in TA100 and E. coli	48-h incubation	HID: 5000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**1** (reliable)
Haworth et al., 1983	AMES	97%	In vitro	*S. typhimurium* (TA1535, TA1537, TA98, TA100)	100, 333, 1000, 3333, 9770 μg/plate	48-h incubation	HID: 10,000 μg/plate	**Negative.** No significant, dose-related increase in mutation frequency	**2** (reliable with restrictions) Authors concluded that 1-NP was negative. However, a dose-dependent revertant frequency was reported in TA1535 with metabolic activation (9, 7, 8, 9, 11, and 16 revertants/plate at 0, 100, 333, 1000, 3333, and 9770 μg/plate). In their methods, authors listed having at least one dose-dependent increase as a "questionable" finding, but did not report assign this classification.

TABLE 7 (Continued)

Reference	Endpoint	Chemical purity	Study type	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
Hite & Skeggs, 1979	AMES	-	In vitro	*S. typhimurium* (TA1537, TA92, TA98, TA100)	0.011, 0.030, or 0.100 ml/plate (with and without activation)	72-h incubation	HID: 0.1 ml/plate (99,800 μg/plat)	**Negative.** No significant, dose-related increase in mutation frequency	**2** (reliable with restriction) (Statistical significance of findings not reported; lack of information on test substance characterization, including purity)
Roscher et al., 1990	Mutagenicity	97.4% (2.3% 2-NP)	In vitro	Chinese hamster V79 lung cells; H4IIECG3/G^{-} rat liver cells	1, 3, or 10 mM Hepatoma cell lines received 24-h pretreatment of DEX to induce monooxygenase activity	3-h (lung cells) or 24-h (hepatoma cells) durations	LED: 1 mM in lung cells	**Positive.** Doses of 1, 3, and 10 mM 1-NP induced mutation frequencies (30 M, 54 M, 50 M) in lung cells compared with controls (12 M). No increased mutation frequency in hepatoma cells with or without metabolic activation	**3** (not reliable) (Based on lack of positive and negative controls; statistical significance not reported for elevated revertant frequencies)
Speck et al., 1982	AMES	-	In vitro	*S. typhimurium* strains (TA98, TA100)	100, 330, 1000, 3300, 10,000, or 20,000 μg/plate (with and without metabolic activation)	48-h incubation		**Positive.** Marginally elevated revertant frequencies reported in TA100 and TA98 with metabolic activation at unspecified dose(s)	**3** (not reliable) (Based on lack of positive control; statistical significance not reported for elevated revertant frequencies)

(Continues)

TABLE 7 (Continued)

Reference	Endpoint	Chemical purity	Study type	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
Durward, 1995 (Dow Chemical Company)	Chromosome aberration	98.5%	In vitro	Chinese hamster lung cells	156.25, 312.5, 625, 1250, or 5000 μg/plate (with S9 activation) 312.5, 625, 1250, 2500, and 5000 μg/plate (without S9 activation); concentrations differed with 6-h vs. 24- and 48-h exposure durations)	6-, 24-, and 48-h exposures without metabolic activation 6-h exposure with metabolic activation	HID: 2500 μg/ml	**Negative.** No toxicologically significant, dose-related increases in cells with aberrations	**1** (reliable)
Kliesch & Adler, 1987	Micronucleus	>98%	In vivo (I.P injection) /in vitro	101/E1 × C3H/E1 mouse bone marrow cells	100, 200, or 300 mg/kg	Cells harvested after 18, 24, and 30 h for low-dose; 24 and 72 h for middle-dose; and 24 for high-dose groups	HID: 300 mg/kg	**Negative.** No significant, dose-related increase in micronucleated cells	**1** (reliable)
Roscher et al., 1990	Micronucleus	97.4% (2.3% 2-NP)	In vitro	Chinese hamster V79 lung cells; H4IIECG3/G^{-}, 2sFou, and C2rev7 rat liver cells	1, 3, 6, or 10 mM Hepatoma cell lines received 24-h pretreatment of DEX to induce monooxygenase activity	3-h (lung cells) or 24-h (hepatoma cells) durations	HID: 1 mM; LED: 3 mM in lung cells	**Positive.** Increased micronuclei induction at 3 mM concentration and above in lung cells No proliferation of micronuclei in hepatoma cells at concentration up to 10 mM with or without metabolic activation	**3** (not reliable) (Based on lack of positive and negative controls; statistical significance not reported for elevated revertant frequencies)
George et al., 1989	Micronucleus and DNA synthesis	-	In vivo (gavage)/ in vitro	Bone marrow cells from male Sprague–Dawley rat	100, 200, 300, or 400 mg/kg	One dose	Preliminary study - HID: 200 mg/kg; LED: 300 mg/kg Repeated study - HID:	**Positive.** Statistically significant increase in micronucleated cells (1.58/1000 PE) in 300 mg/kg dose group (with	**1** (reliable)

TABLE 7 (Continued)

Reference	Endpoint	Chemical purity	Study type	Animal/model	Dose	Duration	Effect level	Result and effects	Klimisch score
							400 mg/kg	indication of cytotoxicity) Repeat study at 300 and 400 mg/kg doses did not increase micronuclei frequency. No induction of unscheduled DNA synthesis (liver).	

NM did significantly increase cell transformation in Syrian hamster embryo (SHE) cells in one study; morphological transformation frequencies were significantly increased in the two highest dose groups, 4000 (p = 0.0291) and 5000 μg/ml (p = 0.0027) (Kerckaert et al., 1996). However, results from cell transformation studies must be interpreted with caution, due to the subjectivity of identifying morphologically transformed cells; cell transformation assays are not usually relied upon to make regulatory decisions (Creton et al., 2012). Therefore, cell transformation assays are typically used in conjunction with other genotoxicity assays as well as animal bioassays to confirm genotoxic potential (Creton et al., 2012).

Given the facts that the vast majority of genotoxicity studies were negative, and NM was predicted to be negative for various genotoxicity endpoints using profilers in the OECD ToolBox; the WoE supports that NM does not have mutagenic potential.

NE was negative for mutagenicity in three studies with *Salmonella typhimurium* (Dayal et al., 1989; Hite & Skeggs, 1979; Mortelmans et al., 1986) (Table 6). Another study with limited documentation showed negative results for high purity NE but was positive for NE containing 2-NP (Löfroth et al., 1986). In an in vivo micronucleus assay in CD-1 mice, NE did not cause a significant increase in the number of micronucleated polychromatic erythrocytes (Hite & Skeggs, 1979). Furthermore, no in vivo micronucleus alerts by ISS or protein binding alerts for chromosomal aberration by ISS were reported for NE via QSAR. Although some of the QSAR predictions suggested that NE may have the potential to induce DNA damage through a "radical mechanism via indirect ROS formation as well as SN2 nucleophilic substitution after nitrate formation" (OECD ToolBox, version 4.4.1), the available empirical data do not support that the compound is genotoxic.

The genotoxicity studies for 1-NP are summarized in Table 7; 1-NP did not induce mutagenicity in the well-documented bacterial mutagenicity assays (Conaway, Hussain, et al., 1991; Haworth et al., 1983; Thompson, 1995, 1996). A "marginal" positive response was reported in one assay, but none of the elevated mutation frequencies exceeded two times the rate of the negative control; however, this study did not utilize a positive control and therefore has limited reliability (Speck et al., 1982). The mutagenicity of 1-NP was also assessed in Chinese hamster lung cells (V79) with induced monooxygenase activity and rat hepatoma cell lines (H4IIEC3/G^{-}) (Roscher et al., 1990); 1-NP was not mutagenic in hepatoma cells but increased the mutation frequencies in V79 lung cells; 2-NP, which was run as an internal standard, showed a similar mutagenic efficiency as a mid-concentration of 1-NP in this study. Of note, the 1-NP used in this study was contaminated with 2.3% 2-NP. The negative result in liver cells is consistent with the results of Fiala et al. (1995) in which DNA repair was not induced in exposed rat hepatocytes (Fiala et al., 1995). Based on negative or marginal findings in two in vivo micronucleus assays (George et al., 1989; Kliesch & Adler, 1987) and in an in vitro chromosomal aberration study (Thompson, 1995), the WoE suggests that 1-NP does not induce chromosomal effects. Further, no alerts were reported for 1-NP under the following OECD ToolBox genotoxicity endpoints: DNA alerts for Ames, CA, and NMT by OASIS;

in vivo micronucleus alerts by ISS; or protein binding alerts for chromosomal aberration by ISS (OECD 2020). Subsequently, the current WoE for 1-NP is that the chemical is not genotoxic based on robust data from bacterial mutagenicity assays and lack of consistent chromosomal effects.

3.2.2 | Theoretical carcinogenic mode of action evaluation

When evaluating the potential for carcinogenic hazard, WoE conclusions are often drawn from a combination of long-term rodent bioassays and genotoxicity data. However, assessments on available mechanistic data are also used by various agencies and organizations to inform on the potential biological plausibility of a carcinogenic mode of action (Clewell et al., 2019; U.S. Environmental Protection Agency, 2005; Meek et al., 2014). Various agencies have relied on read-across information from 2-NP for their respective MoA evaluations for nitroalkanes (e.g., NM) (IARC, 1999, 2000; Lai et al., 1982; NTP, 1997). Notably, 2-NP is considered carcinogenic by a genotoxic mechanism of action (IARC, 1999), and recent safety assessments for 2-NP have applied methods consistent with that assumption (Gadagbui et al., 2020). Currently, three primary mechanisms are proposed by which 2-NP can form electrophiles, which could result in DNA damage and ultimately genotoxicity and carcinogenicity (Figure 1). Although there is substantial evidence supporting a lack of mutagenicity and genotoxicity of the primary nitroalkanes, the observation of tumors in the NTP study for NM, as well as the limited marginal or positive evidence of genotoxicity in some assays for NM, NE, and 1-NP, led to an assessment of potential relationships between 2-NP and the mechanisms that might be operative for primary nitroalkanes.

3.2.3 | Tautomerization

It has been proposed that 2-NP can exert its mutagenic and potentially carcinogenic activity via direct nonenzymatic reaction with DNA, which is based on the observation that metabolic activation is not required for mutagenicity or alkylation (Lai et al., 1982; Speck et al., 1982). The DNA reactivity of 2-NP has been attributed to the electrophilicity of its nitroalkene tautomers (propane-2-nitronic acid and propane-2-nitronate), as the nitronate anion form is more physicochemically reactive than 2-NP (IARC, 1999; Lai et al., 1982). Similarly, the nitronate forms of other secondary nitroalkanes (e.g., 2-nitrobutane, 3-nitropentane, and nitrocyclopentane) have been shown to be more mutagenic than the parent compounds (Conaway, Hussain, et al., 1991; IARC, 1999). Further, available evidence indicates that propane-2-nitronate is more mutagenic than 2-NP in *S. typhimurium*, and thus, it has been proposed that propane-2-nitronate may act as an intermediate in 2-NP-induced genotoxicity and carcinogenicity (Dayal et al., 1989; Fiala et al., 1987; IARC, 1999; Kohl et al., 1994; Löfroth et al., 1986). In support of this hypothesis, reduced bacterial mutagenicity and induction of unscheduled DNA synthesis have been demostrated in rat hepatocytes under conditions that limit the formation of propane-2-nitronate (IARC, 1999; Kohl et al., 1994).

This mechanism of electrophile generation by tautomerization is also relevant to NM, NE, and 1-NP. Like the secondary nitroalkanes

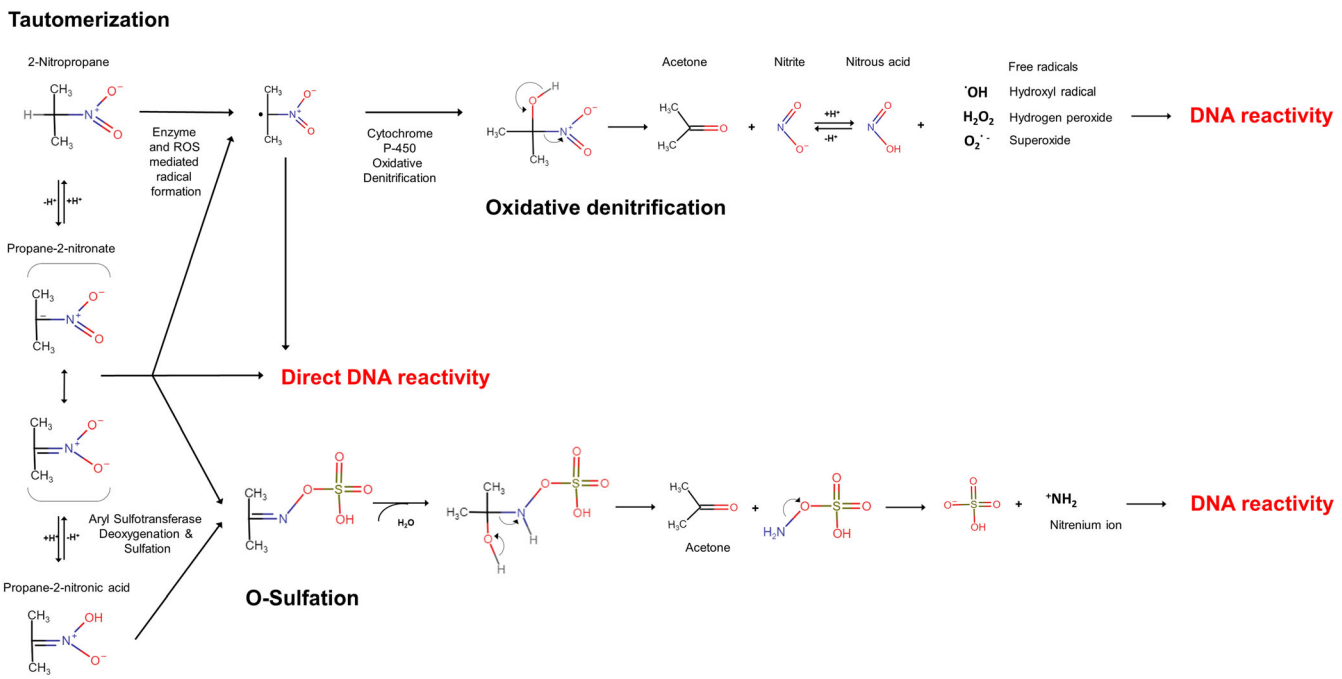


FIGURE 1 Mechanisms of electrophile generation

TABLE 8 Developmental and reproductive toxicity studies for NM

Reference	Species	Route	Dose	Duration	Effect level	Effects	Confidence score (OHAT/Klimisch)
NTP, 1997	Fischer 344/N rats (female and male)	Inhalation	375, 750, or 1500 ppm	6 h/day, 5 days/week, 13 weeks	LOAEC: 750 ppm	Statistically significant decrease in sperm motility was seen in male rats in the 750- and 1500-ppm dose groups. Male rats in 1500-ppm group had statistically significant decreased caudal, epididymal, and testicular weights	**High/1**
NTP, 1997	B6C3F1 mice (female and male)	Inhalation	375, 750, or 1500 ppm	6 h/day, 5 days/week, 13 weeks	LOAEC: 375 ppm	Statistically significant decrease in sperm motility was seen in male mice in all dose groups. Statistically significant increase in estrous cycle length was reported for all dose levels in female mice.	**High/1**
Theuns-van Vliet, 2017	Wistar rats (female)	Inhalation	300, 600, or 1200 ppm	6 h/day from GD 6 to GD 20	NOAEC (maternal toxicity and developmental toxicity): 600 ppm	Statistically significant maternal toxicity seen in 1200-ppm dose group (lower mean body weight, lower food consumption) Statistically significant developmental toxicity reported in 1200-ppm dose group (higher mean number of fetal resorptions and losses; decreased fetal body weights; delay in ossification; skeletal and visceral variations observed in fetuses); suggested as secondary to maternal toxicity	**High/1**

(Continues)

TABLE 8 (Continued)

Reference	Species	Route	Dose	Duration	Effect level	Effects	Confidence score (OHAT/Klimisch)
Whitman et al., 1977	Charles River albino rats (female)	Intraperitoneal injection	0.5 ml of 1.5 M nitromethane in 0.9% sodium chloride (approximately 184 mg/kg)	Injection every third day, 1 week prior to breeding and throughout gestation	NA	No differences between exposure and control groups reported for litter size, death rate of pups, mothering behaviors, or birth weight.	**Low/3** (missing purity information, missing individual outcome data; lack of directness and applicability)

(e.g., 2-NP), in aqueous solution, the primary nitroalkanes also exist in a state of equilibrium between the protonated and neutral nitroalkane parent compounds as well as the nitronic acid and nitronate forms (NTP, 1997). However, at cellular pH, the relative concentration of the nitronate anion and nitronic acid forms are much higher for the secondary nitroalkanes than the primary nitroalkanes (IARC, 1999). Specifically, roughly half of 2-NP is present as the nitronate under physiological conditions; however, the equilibrium for primary nitroalkanes, such as 1-NP, overwhelmingly favors the nitro form, suggesting primary nitroalkanes are less reactive than 2-NP (Linhart et al., 1991).

The observed lack of DNA reactivity and mutagenicity of the nitronates of the primary nitroalkanes has been attributed to their relatively short half-lives (e.g., 0.26 h for 1-NP) and rapid reprotonation to the parent forms under physiological conditions; comparatively, the nitronates of the secondary nitroalkanes have relatively long half-lives (e.g., 25.8 h for 2-NP) and slow rate of reprotonation (20-fold-less than primary nitroalkanes at pH 7.7) (Conaway, Hussain, et al., 1991; Conaway, Nie, et al., 1991; Nielsen, 1989).

The relative tendency of 2-NP versus 1-NP to form electrophilic tautomers has been demonstrated functionally. Further, while alteration of sedimentation of purified single-stranded DNA on a sucrose gradient was observed after in vitro reaction with 2-NP, the sedimentation profile for 1-NP was identical to that of control (Speck et al., 1982). These data suggest that alkylation of purine moieties of DNA is not a relevant mechanism for 1-NP.

Overall, these observations are consistent with the data that secondary nitroalkanes (e.g., 2-NP) produce DNA damage and genotoxicity, whereas the primary nitroalkanes (e.g., 1-NP) do not (IARC, 1999).

In conclusion, while direct DNA reactivity of primary nitroalkanes via reactive nitronate tautomers appears qualitatively possible, in contrast to 2-NP, this MoA is not expected to be quantitatively relevant for NM, NE, and 1-NP based on the equilibrium heavily favoring the parent compound under physiological conditions.

3.2.4 | Oxidative denitrification

Denitrification is another potential molecular mechanism involved in the genotoxicity and carcinogenesis of nitroalkanes, including 2-NP (Lai et al., 1982). Specifically, it has been demonstrated that many nitroalkanes are metabolized by cytochrome P450 in rodent NADPH-dependent hepatic microsomes (Dayal et al., 1991; Marker & Kulkarni, 1986; NTP, 1997; Sakurai et al., 1980; Ullrich et al., 1978). Oxidative denitrification is thought to be proceeded by enzyme and reactive oxygen species (ROS)-mediated radical formation (Fiala et al. 1987; Kuo & Fridovich, 1986; Sakurai et al., 1980). Notably, the affinity of nitroalkanes toward the microsomal mixed function oxidase system decreases with decreasing chain length, resulting in higher relative rates of oxidative denitrification for longer alkanes than shorter alkanes (Dayal et al., 1991; Lai et al., 1982; NTP, 1997; Sakurai et al., 1980; Ullrich et al., 1978). These findings are consistent with

the fact that 2-NP is a more potent mutagen and carcinogen than the primary nitroalkanes (Lai et al., 1982; NTP, 1997). In fact, Lai et al. (1982) cited the low rate of denitrification of nitromethane as a possible factor underlying its lack of mutagenicity and carcinogenicity. Thus, while oxidative denitrification of primary nitroalkanes as a mechanism of electrophile generation is qualitatively possible, it appears to be less quantitatively relevant than for secondary nitroalkanes due to their relative affinities in the mixed function oxidase system.

Additionally, the rate of metabolic nitrite generation from propane-2-nitronate was found to be five to 10 times greater than that of 2-NP in mouse microsomes (IARC, 1999), indicative of preference for the tautomer over the parent compound for oxidative denitrification. The low propensity of primary nitroalkanes to form the nitronate tautomer further reduces the relevance of this mechanism of electrophile generation.

Oxidative denitrification of 2-NP results in formation of nitrite and acetone, whereas the oxidative denitrification of primary nitroalkanes results in the formation of nitrite and the corresponding aldehyde (IARC, 1999; Lai et al., 1982; NTP, 1997). Based on the results of mutagenicity studies in various strains of *Salmonella*, nitrite is not the major cause of mutagenicity of nitroalkanes, suggesting that the other metabolites may be driving ROS generation in the secondary nitroalkanes (Löfroth et al., 1986; NTP, 1997).

Notably, NM's substrate binding spectrum was found to be different from that of other nitroalkanes in rat liver microsomes, with formation of a putative cytochrome P450-NO complex catalyzing production of formaldehyde in an NADPH-independent reaction (NTP, 1997). Oxidative denitrification of nitromethane results in the formation of formaldehyde and possibly nitric oxide (Sakurai et al., 1980). Because this is a specific NM metabolic pathway, a contribution of formaldehyde synthesis would not be relevant to NE or 1-NP genotoxicity or carcinogenicity. Although this metabolic pathway could generate formaldehyde, it is not expected to be significant operative mechanism because the oxidative process is not favored for short chain primary nitroalkanes and the empirical data suggest negative genotoxicity of NM (whereas formaldehyde is genotoxic).

3.2.5 | *O*-sulfation

Sulfation reactions of 2-NP and its nitronate may cause DNA damage (i.e., 8-aminodeoxyguanosine) via *O*-sulfation of the *N*-hydroxy form, resulting in formation of an amino radical and acetone (IARC, 1999; Sodum et al., 1994). It has been proposed that the nitronate anion can undergo aryl sulfotransferase-mediated conversion to hydroxylamine-*O*-sulfonate, resulting in formation of a highly reactive nitrenium ion $NH2^{+}$ (Sodum et al., 1993, 1994). The activation of 2-NP to an aminating species by rat liver aryl sulfotransferase has been demonstrated in vitro and in vivo (Sodum et al., 1994). Specifically, the reactive species generated from the activation of 2-NP and propane-2-nitronate by aryl sulfotransferase aminates guanosine at the C8 position, forms 8-aminodeoxyguanosine as well as 8-oxoguanosine. Formation of these compounds from 2-NP nitronate has been found to be three to five times greater than that of 2-NP (pKa of 7.63), consistent with their relative mutagenicity (Sodum et al., 1994) and suggestive that *O*-sulfation of 2-NP proceeds via tautomerization to 2-NP nitronate.

This mechanism of electrophile generation is not relevant to the primary nitroalkanes. Specifically, when tested under the same in vitro conditions as 2-NP and its nitronate, 1-NP and its nitronate did not result in the formation of 8-aminoguanosine or an increased level of 8-oxoguanosine. These data indicate that 1-NP (pKa of 8.98) and 1-NP nitronate are not substrates of rat liver aryl sulfotransferase, which is consistent with the differences in mutagenicity and genotoxicity between 2-NP and 1-NP as well as their nitronates (Sodum et al., 1994). The lack of sulfotransferase-mediated activation for 1-NP can likely be attributed to the equilibrium greatly favoring 1-NP over its nitronate under physiological conditions. Although no information was identified for NM and NE, this mechanism of electrophile generation is not expected to be relevant based on their relatively low propensity to form and relative instability of their nitronate tautomers.

3.2.6 | Other mechanisms

In addition to the three mechanisms of electrophile generation that have been outlined by other organizations (IARC, 1999; Lai et al., 1982; NTP, 1997), other potential mechanisms of electrophile generation are possible that have not been explored in the context of cancer risk assessments, including formation of acetone oxime following the addition of water to o-sulfone, formation of the nitrosonium ion from nitrous acid, and formation of hydroxylamine from the nitro reductase mediated reduction of nitrone.

Taken together the reactivity of 2-NP and its ability to participate in each of these mechanisms for generation of electrophilic metabolites is fully consistent with its demonstrated rodent tumorigenicity. These mechanisms have been shown to be nonoperative or significantly less likely for the primary nitroalkanes, NM, NE, and 1-NP, which is consistent with their overall lack of genotoxicity. Of the metabolic processes investigated, only the oxidative denitrification and possible formation of formaldehyde presents a plausible connection carcinogenicity, which are relevant only to NM. The absence of genotoxicity for NM suggests this is not a significantly operative pathway.

3.3 | Reproductive and developmental toxicity

3.3.1 | NM reproductive and developmental toxicity studies

Three studies were available for review with data relevant to the reproductive toxicity of NM (Table 8).

A 13-week inhalation study was conducted in rats and mice to evaluate toxic effects of repeated exposure to NM (NTP, 1997) (Table 9). In rats, a decrease in sperm motility was seen in the 750- and 1500-ppm dose groups; in mice, a decreased sperm motility was seen in all dose groups. No effects were reported on spermatid head measurements or spermatid counts for any dose group of rats or mice. In rats, decreased caudal, epididymal, and testicular weights were reported in the 1500-ppm group, whereas no significant effects on testicular weights were reported for any mouse dose group. Additionally, an increase in estrous cycle length was reported for all dose levels in female mice; however, no changes in estrous cycle length were reported in treated rats (Table 9).

The estrous cycle length for all dose groups of female mice was significantly higher than in controls; however, the length of the cycles was still within the standard range for mice (Caligioni, 2009). As reported in many studies, the estrous cycle length in rats and mice averages 4 to 5 days and occasionally 6 days, with factors such as light, age, temperature, noise, nutrition, stress, and social relationships influencing cycle length (Cora et al., 2015). Furthermore, mice are more likely than rats to have a 4-day estrous cycle followed by a 5-day cycle (Nelson et al., 1982). After analyzing over fifty 13-week NTP studies, Morrissey et al. (1988) suggested that "[m]ore data from breeding studies in which female estrous cycle length is measured are needed to assess fully the association of cycle length with reproductive outcome; stages of the estrous cycle are so variable that they may not be useful in assessing potential toxicity."

With regard to the sperm motility effect reported in the NTP (1997) study, the ECHA registrant determined that this effect was not toxicologically relevant because "there was significant systemic toxicity particularly at the 1500 ppm concentration, which was clearly causing secondary effects in a number of organs." Additionally, the NTP researchers indicated that the decreased testicular weights observed in the 1500-ppm group of rats were not biologically significant. Of note, the overall body weight of the male rats also decreased and the relative weight of the testes compared with the body did not change. Furthermore, the ECHA registrant noted that "there were no male reproductive organ histopathologic effects in the [two]-year studies." Specifically, it was reported that "[t]here were no treatment-related increases in the incidences of neoplasms or non-neoplastic lesions at any site in the male rats" in a 2-year NTP study which included doses of 94, 188, and 375 ppm (p. 66). Sperm motility and testicular weights were not examined in this 2-year study. Based on the lack of histopathologic changes seen in the 375-ppm group and below, the sperm effects are likely secondary to systemic toxicity at higher doses. As reported in Reyes et al. (2012), hypoxia can lead to adverse effects on spermatogenesis. It was noted in the NTP's 13-week study that "concentration-dependent, microcytic, responsive anemia" was seen in male and female rats in the 375-ppm and higher dose groups, along with the presence of schistocytes, Heinz bodies, and spherocytes, and an increase in hemoglobin and methemoglobin concentration. These effects are all indicative of hypoxia, which has been shown to reduce sperm motility and sperm count (Reyes et al., 2012).

A developmental toxicity study (OECD 414) was performed by Theuns-van Vliet (2017) in which female Wistar rats were exposed to 300-, 600-, or 1200-ppm NM for 6 h per day from gestation day 6 to 20. Statistically significant maternal toxicity effects, including lower mean body weight, lower body weight gain at the end of gestation, and lower food consumption, were reported in the 1200-ppm dose

TABLE 9 Key reproductive findings for NM from the NTP (1997) study

Endpoint	Animal/model	Dose (ppm) 0	375	750	1500
Sperm motility	Male rat	94.57 ± 1.30	92.16 ± 1.90	**87.11 ± 1.88****	**75.45 ± 2.78****
	Male mice	93.50 ± 0.46	**85.09 ± 1.21****	**86.47 ± 1.17****	**82.41 ± 1.30****
Sperm count	Male rat	64.33 ± 3.89	62.75 ± 3.63	62.68 ± 3.02	68.95 ± 3.14
	Male mice	82.80 ± 1.89	75.85 ± 1.80	77.43 ± 3.11	82.48 ± 3.14
Necropsy body weight	Male rat	338 ± 7	341 ± 4	331 ± 4	**299 ± 11****
L. cauda weight		0.207± 0.004	0.210 ± 0.004	0.204 ± 0.006	**0.177 ± 0.009****
L. epididymis weight		0.467 ± 0.009	0.458 ± 0.006	0.444 ± 0.009	**0.412 ± 0.0013**
L. testis weight		1.39 ± 0.03	1.36 ± 0.01	1.34 ± 0.02	**1.20 ± 0.02****
Necropsy body weight	Male mice	36.1 ± 0.5	36.3 ± 0.6	35.2 ± 0.4	34.7 ± 0.5
L. cauda weight		0.020 ± 0.001	0.019 ± 0.001	0.020 ± 0.001	0.018 ± 0.001
L. epididymis weight		0.049 ± 0.002	0.047 ± 0.002	0.050 ± 0.002	0.050 ± 0.003
L. testis weight		0.118 ± 0.003	0.121 ± 0.002	0.117 ± 0.003	0.121 ± 0.001
Estrous cycle length (days)	Female mice	4.00 ± 0.00[a]	**4.33 ± 0.14*[b]**	**4.50 ± 0.21***	**4.71 ± 0.26**[c]**
	Female rat	4.89 ± 0.07[b]	4.75 ± 0.16[a]	5.00 ± 0.14[b]	5.00 ± 0.15

[a]Estrous cycle was longer than 12 days or unclear in two of 10 animals.
[b]Estrous cycle was longer than 12 days or unclear in one of 10 animals.
[c]Estrous cycle was longer than 12 days or unclear in three of 10 animals.
*$p \leq 0.05$.
**$p \leq 0.01$.

TABLE 10 Developmental and reproductive toxicity studies for NE

Reference	Species	Route	Dose	Duration	Effect level	Effects	Confidence score (OHAT/Klimisch)
Beliles et al., 1978	Swiss mice (female)	Inhalation	14.3-ppm nitroethane and 8.9-ppm diethlyhydroxylamine (DEHA)	8.25 ± 2.25 h/day, gestation day 6 to 17. Continuous exposure to diethylamine hydrogen sulfite 24 h/day	NOAEC: 8.25 ppm	No reproductive or developmental effects reported in dams or offspring	**Low/2** (lack of applicability-two chemicals combined, no dose–response, risk of bias due to no randomization)
Heicklen et al., 1979	Swiss mice (female)	Inhalation	8.6- to 14.3-ppm nitroethane and 6.6- to 9.0-ppm diethlyhydroxylamine (DEHA)	Exposure to nitroethane and DEHA for 8.25 ± 2.25 h/day, for 6–8 weeks. Continuous exposure to diethylamine hydrogen sulfite 24 h/day Two litters from each mated pair of mice were produced in generations 2 and 3, the offspring in generation 2B used to produce generations 3A and 3B.	NOAEC: 8.25 ppm	No reproductive or developmental effects reported in dams or offspring	**Low/2** (lack of applicability-two chemicals combined, no dose–response, risk of bias due to no randomization)
Gushow et al., 1982 (Dow Chemical Company) **High/1**	Fischer 344 rats (male and female)	Inhalation	100-, 350-, or 1000-ppm nitroethane (0.3, 1.0, or 3.0 mg/L) (15/sex)	6 h/day, 5 days/week for 92 days (13 weeks) (interim kill at 30 days)	NOAEC: 1000 ppm	No reproductive effects reported	
Gushow et al., 1982 (Dow Chemical Company)	B6C3F1 mice (male and female)	Inhalation	100-, 350-, or 1000-ppm nitroethane (0.3, 1.0 or 3.0 mg/L) (15/sex)	6 h/day, 5 days/week for 93 days (13 weeks) (interim kill at 29 days)	NOAEC: 350 ppm	Mice exposed to 1000 ppm showed multinucleated spermatids in the testes	**High/1**

group. Statistically significant findings of developmental toxicity were observed in the 1200-ppm dose group, including a higher mean number of fetal resorptions and losses, decreased fetal body weights, delay in ossification, and skeletal and visceral variations observed in fetuses. The authors concluded that delayed ossification and skeletal/visceral variations were "indicative [of] fetal toxicity or considered secondary to the fetal toxicity observed in this group" and noted that the skeletal variations, including wavy ribs as well as bent radius and ulna bones, are "commonly observed … in this strain of rats." They also noted that the decreased fetal body weights in the high-dose group were "related [to] and indicative [of] maternal toxicity." As reported in Black and Marks (1992), "fetal effects that are commonly associated with maternal toxicity" include reduced fetal body weight, delayed ossification, and bent ribs. Furthermore, there were clear indications of maternal toxicity. Furthermore, a recent review has noted that "maternal feed restriction resulted in a reduction of fetal body weight that was sometimes accompanied by delayed ossification in both species." (Nitzsche, 2017). OECD guidelines state that maternal toxicity can be assessed by reductions in food or water intake, decreased maternal body weight gain, and occurrence of maternal deaths or clinical signs of overt toxicity (Paumgartten, 2010). In this case, lower mean body weight and decreased food consumption were observed in the 1200-ppm group. There was also a reported increase in fetal resorptions and losses in the 1200-ppm group, which is indicative of maternal toxicity severe enough to alter the homeostasis of the pregnant animal (Black & Marks, 1992).

It was stated in the ECHA dossier that developmental effects were observed in the 1200-ppm group but were a "secondary non-specific consequence of maternal toxicity effects." The ECHA registrant proposed to classify NM as a Repro Tox Cat. 2 toxicant according to CLP criteria based on postimplantation loss/late resorptions, fetus weight, delay in ossification, and skeletal/visceral observations seen in the high concentration group.

A 1977 study was conducted in which female Charles River albino rats were injected intraperitoneally with 0.5 ml of 1.5 M (approximately 184 mg/kg^3) NM every third day, 1 week prior to breeding and throughout gestation. They reported no reproductive or developmental differences between the control or exposure groups (Whitman et al., 1977). However, confidence scoring for this study is low due to the lack of individual outcome data.

Taken together, the evidence supports the conclusion that the reproductive and developmental effects reported in NM exposure studies are coincident with and may result from maternal and paternal systemic toxicity. Because the effects are not driven by a MoA specific to reproductive or developmental molecular targets, read-across to other primary nitroalkanes is not adequately supported by the existing data.

3.3.2 | NE reproductive and developmental toxicity studies

Based on limited data in laboratory animals, NE is not a reproductive or developmental toxicant (Table 10). Two multigenerational reproductive toxicity tests were identified in which pregnant female mice were exposed via inhalation to a mixture of NE, diethlyhydroxylamine (DEHA), and diethylamine hydrogen sulfite; no reproductive or developmental effects were noted (Beliles et al., 1978; Heicklen et al., 1979). However, it is noteworthy that when assessing the hazard potential of NE, these studies have limitations due to the simultaneous exposure of mice to multiple chemicals as well as a lack of dose–response data (only one concentration was tested).

A separate 13-week inhalation study was identified in which male and female F344 rats and B6C3F1 mice were exposed to 100-, 350-, or 1000-ppm NE (Gushow et al., 1982). No adverse effects of the reproductive organs were reported for any dose group of rats. Rats in the 1000-ppm group also had elevated methemoglobin levels and increased Heinz bodies in the blood, which as noted above, are signs of hypoxia that can affect spermatogenesis. In the Dow 1982 NE study, mice in the 1000-ppm group showed multinucleated spermatids in the testes. However, it is unclear whether these results were statistically significant as only five animals were tested in the control and 1000-ppm groups; none were examined in the 100- or 350-ppm group. Additionally, the multinucleated spermatid effects were reported to be "very slight" or "slight" in the highest dose group; there were no differences in the testes weights between groups; and testicular lesions were noted in one animal in the control group. Furthermore, the reproductive implications of abnormal sperm morphology are unclear (U.S. Environmental Protection Agency, 1996).

3.3.3 | 1-NP reproductive and developmental toxicity studies

Based on limited animal data, it was shown that 1-NP is not a reproductive or developmental toxicant (Table 11). No long-term study was available for review. Two subchronic studies were available: in one, groups of female and male CD rats were administered 1-NP daily, by gavage, at dose levels of 10, 50, or 90 mg/kg/day (Zablotny et al., 2004). However, due to excessive toxicity, the study was terminated on the eighth test day with no further collection of data. No relevant reproductive effects were seen.

In a range-finding study, five male and five female CD rats were exposed to 100-, 250-, or 500-ppm 1-NP. Early in the test period, three rats in the 500-ppm group were found dead and significant signs of toxicity were seen in the remaining males and females that received this treatment, including decreased or absent activity/reactivity, dilated pupils, decreased quantity of feces, uncoordinated gait, head tilt, splayed hindlimbs, and periocular or perioral soiling. In the 250-ppm female exposure group, uncoordinated gait, decreased quantity of feces, perioral/periocular soiling, decreased feed consumption, and decreased body weights were reported. No treatment-related observations were reported in the 250-ppm male group or the 100-ppm male or female groups (Carney et al., 2004).

In a follow-up to the range-finding study, groups of male and female CD rats were exposed via inhalation to 25-, 50-, or 100-ppm

TABLE 11 Developmental and reproductive toxicity studies for 1-NP

Reference	Species	Route	Dose	Duration	Effect level	Effects	Confidence score (OHAT/Klimisch)
Zablotny et al., 2004 (Dow Chemical Company)	CD rats (male and female)	Gavage	10, 50, or 90 mg/kg/day	8 days (study terminated due to excessive toxicity)	LOAEL: 50 mg/kg/day (in male rats)	Decreased reactivity and perioral soiling reported in males in 50 mg/kg/day group. Adverse clinical signs, including CNS and eye effects, reported in males in 90 mg/kg/day group on day 7. No adverse effects reported in females	**Low/2** (no individual outcome data, lack of applicability - gavage)
Carney et al. 2004 (Dow Chemical Company)	CD rats (male and female)	Inhalation	0, 25, 50, 100 ppm	6 h/day, 7 days/week. Females: 14 days prior to mating, during mating until gestation day 19. Males: 14 days prior to mating and for 28 days	NOAEC (fetotoxicity): 50 ppm	No developmental or reproductive effects observed. Reduced litter size in the 100-ppm group (not statistically significant); suggested to be secondary to maternal toxicity or stress in dams.	**High/1**

1-NP through mating and gestation (Carney et al., 2004). The uteri of all females were examined, and the number of implantation sites were recorded. The testes, epididymides, and ovaries, along with other organs, were weighed, and organ to body weight ratios were calculated. A histopathological examination of the testes included a qualitative staging of spermatogenesis. Reproductive indices, such as mating index and post implantation loss, were also calculated. No treatment-related effects were reported for any reproductive metrics, including time to mating, gestation length, pup survival and growth, pup sex ratio, neurologic function, clinical chemistry, or hematology. There was a slight decrease in litter size in the 100-ppm exposure group, but the difference was not statistically significant, and the authors suggested it was due to maternal toxicity and/or stress in the high-dose females. Males exposed to 100-ppm 1-NP had statistically significant higher testes weights, but the authors noted that this finding was reflective of the treatment-related lower body weights at that dose level. No differences were reported for spermatic elements. In general, decreases in body weight were seen in male rats exposed to 100 ppm, and histopathologic effects were noted in the nasal tissues of males exposed to 100 ppm and females exposed to either 50 or 100 ppm.

3.3.4 | Structure activity relationship results for reproductive and developmental toxicity

Derek modeling resulted in no alerts for developmental toxicity, teratogenicity, or testicular toxicity for NM, NE, and 1-NP (Derek). Furthermore, OECD Toolbox returned no structural or mechanistic alerts for reproductive or developmental toxicity, and EPA CompTox results were negative for estrogen and androgen receptor activity, as well as thyroid and steroidogenesis activities in the EDSP21 assay set for all three nitroalkanes (Toolbox; Comptox).

3.3.5 | Reproductive and developmental toxicity synthesis of overall results

Overall, there is insufficient evidence that NM, NE, or 1-NP causes female reproductive effects or in utero or postnatal developmental effects (Table 12). The following criteria can be used to evaluate the potential for a chemical to produce reproductive toxicity: the data exhibit a dose–response relationship, the results are statistically significant and biologically plausible, and the results are consistent across studies (U.S. Environmental Protection Agency, 1996; Meek et al., 2003). Based on the extant data, some evidence for male reproductive effects were observed at high concentrations for NM and NE, but there was inconsistency among related endpoints, the effects were limited in degree, and at least one plausible hypothesis of effects secondary to systemic toxicity was identified. Effects related to female reproduction were limited to NM; however, the changed estrous cycle length in mice were within levels of historical controls and were not supported by relevant MoA data or predictions. Of the available data, it appears that developmental effects were observed

TABLE 12 Summary of evidence for reproductive and developmental toxicity

Chemical	Male reproductive	Female reproductive	In utero developmental	Postnatal developmental
Nitromethane	Some evidence of concern/ high confidence in data (decreased sperm motility; decreased testicular weights—NTP, 1997)	No evidence of concern/high confidence in data (increased estrous cycle but within normal range—NTP, 1997)	Some evidence of concern/ high confidence in data (decreased fetal body weights, delay in ossification, and skeletal and visceral variations; likely due to maternal toxicity—NTP, 1997)	No data
Nitroethane	Some evidence of concern/ high confidence in data (multinucleated spermatids—Dow 1982)	No evidence of concern/high confidence in data	No data	No evidence of concern/low confidence in data (Beliles et al., 1978; Heicklen et al., 1979)
1-Nitropropane	No evidence of concern/ medium confidence in data	No evidence of concern/ medium confidence in data	No evidence of concern/ medium confidence in data	No evidence of concern/ medium confidence in data

only in the presence of maternal toxicity. Developmental effects in pups were not consistently observed across studies for NM, with Theuns-van Vliet (2017) reporting delayed ossification and decreased body weights but Whitman et al. (1977) reporting no effects. Although the effects reported by Theuns-van Vliet (2017) did follow a dose–response relationship, the effects were consistent with maternal toxicity. The only studies available to evaluate the developmental effects of NE have limited relevance due to the simultaneous exposure of mice to multiple chemicals as well as a lack of dose–response data (only one concentration was tested). No developmental effects were noted in a study with 1-NP. Thus, there is no robust evidence for any of the three primary nitroalkanes for reproductive or development toxicity, although the potential that these effects resulted from paternal or maternal systemic toxicity is consistent with the data.

Based on data quality assessment, MoA considerations, and evidence integration, classifying NM, NE, and 1-NP as Category 2 reproductive toxicants is most appropriate. According to CLP guidelines, "Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1." Furthermore, the guidelines state that "when a substance is so toxic that maternal death or severe inanition results, or the dams are prostrate and incapable of nursing the pups, it is reasonable to assume that developmental toxicity is produced solely as a secondary consequence of maternal toxicity and discount the developmental effects. Classification is not necessarily the outcome in the case of minor developmental changes, when there is only a small reduction in foetal/pup body weight or retardation of ossification when seen in association with maternal toxicity" (Annex I: 3.7.2.4.3).

4 | CONCLUSION

In 2018, ECHA proposed a harmonized CMR classification for three specific nitroalkanes: NM, NE, and 1-NP. Specifically, the organization proposed to classify all three chemicals as germ cell mutagens, reproductive toxicants, and carcinogens. In June 2020, a revised proposal was submitted, recommending the following harmonized classifications: NM Carc. 1B and Repr. 1B; NE Repr. 1B; and 1-NP Repr. 2. In this analysis, a comprehensive state of the science review of the hazard data for NM, NE, and 1- NP was conducted in light of the harmonized CLH proposed for CMR endpoints. Based on the results of the current evaluation, the WoE indicates that NM, NE, and 1-NP are not carcinogenic, genotoxic, or reproductive or developmental toxicants. As such, the following conclusions regarding the appropriate classifications are warranted based on currently available data:

- Carcinogenicity:
  - 1B is not appropriate for NM with respect to carcinogenicity. Of the three primary nitroalkanes, only NM was shown to induce tumors in vivo following chronic exposure, which was hypothesized to be due to impurities in the NM used in each study, or differential strain sensitivities to mammary tumor formation. Furthermore, rat mammary and mouse harderian tumors are likely not relevant to humans, and the reported lung and liver tumors in mice were equivocal in their dose response and statistical significance.
  - Classification for NE or 1-NP with respect to carcinogenicity is not warranted at this time, due to the absence of positive studies identifying a carcinogenic hazard coupled with the reported negative findings in available genotoxicity assays.
- Mutagenicity:
  - Classification for NM, NE, or 1-NP with respect to mutagenicity is not warranted at this time, based on negative empirical data and lack of expected significant electrophile generation under physiological conditions.
- Reproductive and developmental toxicity:
  - Category 2 is an appropriate classification for NM, NE, and 1-NP with respect to reproductive and developmental toxicity. As detailed in the analysis, the observed responses are likely occurring due to systemic paternal toxicity in the animals.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ENDNOTES

[1] Category 2: Suspected human reproductive toxicant, based on some evidence from human or animal studies. Evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects, the adverse effect on reproduction is considered not to be a secondary nonspecific consequence of other toxic effects.

[2] Category 1B: Presumed human reproductive toxicant, largely based on data from animal studies. 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 1A (known human reproductive toxicant).

[3] Calculated based on molarity of solution and an assumed average female rat weight of 0.25 kg (weight not specified in study)

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