anses

Valeurs limites d'exposition
en milieu professionnel

Le protoxyde d'azote

Évaluation des effets sur la santé et des méthodes de mesure

Avis de l'Anses
Rapport d'expertise collective

Février 2024

Connaître, évaluer, protéger

Avis de l'Anses
Saisine n°2020-SA-0042

Le directeur général

Maisons-Alfort, le 8 février 2024

AVIS
de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

relatif à l'expertise en vue de la fixation de valeurs limites d'exposition à des agents chimiques en milieu professionnel

Evaluation des effets sur la santé et des méthodes de mesure des niveaux d'exposition sur le lieu de travail

pour le protoxyde d'azote
(CAS n° 10024-97-2)

L'Anses met en œuvre une expertise scientifique indépendante et pluraliste.
L'Anses contribue principalement à assurer la sécurité sanitaire dans les domaines de l'environnement, du travail et de l'alimentation et à évaluer les risques sanitaires qu'ils peuvent comporter.
Elle contribue également à assurer d'une part la protection de la santé et du bien-être des animaux et de la santé des végétaux et d'autre part à l'évaluation des propriétés nutritionnelles des aliments.
Elle fournit aux autorités compétentes toutes les informations sur ces risques ainsi que l'expertise et l'appui scientifique technique nécessaires à l'élaboration des dispositions législatives et réglementaires et à la mise en œuvre des mesures de gestion du risque (article L.1313-1 du code de la santé publique).
Ses avis sont publiés sur son site internet.

L'Anses a été saisie le 16 mars 2020 par la Direction générale du travail (DGT) pour la réalisation de l'expertise suivante : Evaluation des effets sur la santé et des méthodes de mesure des niveaux d'exposition sur le lieu de travail pour le protoxyde d'azote en vue de la fixation de valeurs limites d'exposition à des agents chimiques en milieu professionnel.

1. CONTEXTE ET OBJET DE LA SAISINE

Dans le cadre du protocole d'accord entre l'Anses et le ministère du travail pour la mise en œuvre du programme de travail d'expertise scientifique en matière de valeurs limites atmosphériques et biologiques pour les expositions professionnelles, établi en juillet 2018, la DGT a saisi l'Anses afin de mener les travaux d'expertise nécessaires à la fixation de valeurs limites d'exposition professionnelle (VLEP) fondées sur des considérations sanitaires pour le protoxyde d'azote (N_2O).

La France ne dispose à ce jour d'aucune valeur limite d'exposition professionnelle pour cette substance.

2. ORGANISATION DE L'EXPERTISE

L'expertise a été réalisée dans le respect de la norme NF X 50-110 « Qualité en expertise – Prescriptions générales de compétence pour une expertise (Mai 2003) ».

L'expertise relève du domaine de compétences du comité d'experts spécialisé (CES) « Valeurs sanitaires de référence » (CES VSR).

Pour conduire ces travaux d'expertise, différents collectifs ont été mobilisés :

- le CES VSR a réalisé l'évaluation des effets sanitaires du protoxyde d'azote et a recommandé des VLEP,
- le groupe de travail « Métrologie » a réalisé l'évaluation des méthodes de mesures atmosphériques dans les lieux de travail au regard des VLEP recommandées.

Les travaux d'expertise ont été soumis régulièrement au CES tant sur les aspects méthodologiques que scientifiques.

Le présent avis se fonde pour les aspects scientifiques sur un rapport, rédigé en anglais[1], intitulé « *Expert appraisal on recommending occupational exposure limits for chemical agents - Assessment of health effects and methods for the measurement of exposure levels in workplace atmospheres for nitrous oxide* » *(november 2023).* »

Le rapport en anglais, ainsi que la synthèse et les conclusions de l'expertise collective en français, ont été adoptés par le CES VSR le 1er juillet 2022 pour mise en consultation. Ces documents ont fait l'objet d'une consultation publique du 22 juin au 15 septembre 2023. Les personnes ou organismes ayant contribué à la consultation publique sont listés en annexe 6 du rapport. Les commentaires reçus ont été examinés et discutés par le CES VSR qui a adopté le rapport en anglais ainsi que la synthèse et les conclusions de l'expertise collective en français le 9 novembre 2023.

L'Anses analyse les liens d'intérêts déclarés par les experts avant leur nomination et tout au long des travaux, afin d'éviter les risques de conflits d'intérêts au regard des points traités dans le cadre de l'expertise.

Les déclarations d'intérêts des experts sont publiées sur le site internet : https://dpi.sante.gouv.fr/.

3. ANALYSE ET CONCLUSIONS DU CES

Le profil toxicologique réalisé pour le protoxyde d'azote a été rédigé sur la base de rapports de synthèse réalisés par des organismes reconnus (IPCS-INCHEM, 1992 ; INRS, 2010 et 2018 ; DFG, 1993 et 2015 ; ACGIH, 2018 ; Anses, 2020a ; HCN, 2000 ; EIGA, 2008) et d'une recherche bibliographique effectuée jusqu'en décembre 2020 dans la base de données

[1] Une synthèse de l'argumentaire et des conclusions est disponible en français en introduction du rapport qui est rédigé en anglais pour faciliter un portage de l'expertise au niveau européen.

PubMed®. Les détails de la recherche bibliographique (requête, principaux mots-clés, critères d'inclusion et d'exclusion) sont décrits dans l'annexe 1 du rapport.

■ Informations générales

Le protoxyde d'azote (N_2O ou oxyde nitreux) est utilisé depuis plus de 150 ans en chirurgie comme adjuvant de l'anesthésie générale par inhalation. La substance est également utilisée pour soulager la douleur lors de l'accouchement ou pour une courte analgésie lors d'interventions médicales mineures (par exemple en dentisterie, urgence, médecine vétérinaire). Elle est couramment utilisée en association à d'autres gaz anesthésiants. Le N_2O est utilisé dans l'industrie alimentaire comme additif alimentaire (E942). C'est un gaz propulseur utilisé dans de nombreux produits (par exemple, pour aérer de la crème fouettée, gonfler des ballons). C'est également un additif des carburants pour fusées afin d'augmenter l'oxygène disponible pour la combustion. Le N_2O est également utilisé en laboratoire comme agent oxydant en spectrométrie d'absorption atomique en mode flamme.

L'abus en usage récréatif du gaz, également appelé « gaz hilarant », s'est accru ces dernières années en raison de ses propriétés euphorisantes, relaxantes et hallucinogènes. Un rapport d'étude de toxicovigilance, élaboré avec l'appui des centres anti-poison, met en évidence des effets indésirables pouvant être sévères tels que des troubles du rythme cardiaque, un risque d'asphyxie, des troubles psychiques et des atteintes neurologiques (Anses, 2020a).

Le N_2O est naturellement émis par les sols et les océans. Les émissions de N_2O d'origine humaine sont principalement dues aux activités agricoles et, dans une moindre mesure, à d'autres sources telles que le secteur de la santé. C'est un gaz qui s'accumule dans l'atmosphère (durée de vie atmosphérique d'environ 114 ans) et qui contribue à l'effet de serre et à la destruction de la couche d'ozone.

■ Éléments de proposition pour fixer des VLEP

- **Valeur limite d'exposition professionnelle sur 8 heures (VLEP-8h)**

  - *Choix de l'effet critique*

L'analyse de l'ensemble des études de toxicité répétée réalisées par inhalation identifie les systèmes nerveux, hématologique, immunitaire et reproducteur, comme les plus sensibles.

Des effets sur la fertilité et le développement ont été rapportés chez le rat mais sans établir de relation dose-réponse. Bien qu'observés chez l'Homme et l'animal, les effets hématologiques et immunitaires n'ont pas été retenus comme effets critiques, du fait d'absence de relation dose-réponse, de l'existence de co-expositions non prises en compte ou de résultats équivoques.

L'effet critique du protoxyde d'azote, c'est-à-dire l'effet apparaissant aux concentrations les plus faibles, a été identifié comme étant l'altération des performances cognitives.

L'altération des performances cognitives est donc retenue comme effet critique pour la construction de la VLEP-8h.

  - *Choix des études clés*

En ce qui concerne les données humaines, considérées comme les plus adéquates pour l'élaboration de valeurs de référence (Anses, 2023 à venir), la majorité des études de toxicité répétée, en milieu professionnel, sont des études transversales. A noter qu'aucune étude n'a caractérisé les effets à long terme de l'exposition au N_2O.

Compte tenu des données disponibles, les études de Scapellato et *al.* de 2008 (étude réalisée sur 1 an avec 2 périodes d'observation) et celles de Lucchini et *al.* de 1995, 1996 et 1997, décrivant les effets observés en termes de performances neurocomportementales (altération de la vigilance) sont considérées comme les plus pertinentes pour l'établissement de la VLEP-8h. Elles ont donc été retenues comme études clés car :

- l'exposition des travailleurs au N_2O dans ces études est considérée comme représentative des expositions professionnelles ;
- ces études complémentaires ont montré des effets similaires en utilisant des méthodologies différentes ;
- de nombreuses variables et biais potentiels ont été pris en compte et ces études ont permis de caractériser la relation dose-réponse par rapport à l'effet critique retenu ;
- néanmoins, certaines limites ont été identifiées :
    - l'existence d'une co-exposition à de faibles concentrations avec d'autres gaz anesthésiants ;
    - une possible sous-estimation des niveaux d'exposition liée à la méthode analytique utilisée.

- *Choix du point de départ (PoD)*

Concernant les niveaux de dose, une altération significative des performances cognitives a été notée à la concentration urinaire de 27 µg/L, correspondant à une concentration dans l'air de 50 ppm[2] en présence de 1,3 ppm d'isoflurane dans l'étude de Scapellato *et al.*. Ces altérations ont été observées à 54,2 ppm et 62,6 ppm en fin de semaine respectivement dans les études de Lucchini *et al.* de 1995 et 1996, en présence de 1,5 ppm d'isoflurane.

Les études de Lucchini *et al.* de 1997 et de Scapellato *et al.* n'ont pas identifié d'effet sur les performances cognitives respectivement pour des expositions à 23,2 ppm de N_2O ou à moins de 27 µg/L de N_2O dans l'urine.

Sur la base des effets précédemment décrits, une LOAEC[3] de 50 ppm (90 mg/m^3) est identifiée. La NOAEC[4] de 23,2 ppm, arrondie à 25 ppm (45 mg/m^3), est retenue comme PoD.

- *Choix des facteurs d'incertitude*

Un facteur d'incertitude global de 1 a été retenu car :

- la VLEP-8h est élaborée à partir de données humaines, de plusieurs études incluant une étude multicentrique, dont les sujets sont des hommes et des femmes ;
- une NOAEC a été identifiée ;
- les travailleurs exposés professionnellement à la substance ont été suivis pendant un an ;
- bien qu'il existe des incertitudes dans la relation dose-réponse pour la toxicité pour la reproduction, les effets retenus comme effets critiques (effets sur les performances neurocomportementales) étaient les plus sensibles[5].

[2] Selon les auteurs (sur la base de la corrélation entre les concentrations urinaires et atmosphériques de l'étude d'Imbriani *et al.* de 1995)

[3] Lowest Observed Adverse Effect Concentration (= concentration minimale entraînant un effet néfaste observé)

[4] No Observed Adverse Effect Concentration (= concentration maximale n'entraînant pas d'effet néfaste observé)

[5] Compte tenu d'un point de départ de 5 000 ppm identifié chez l'animal pour le retard de développement fœtal, la méthodologie de construction des valeurs de référence appliquée à l'Anses (Anses, 2023) conduirait à une VLEP-8h de 33 ppm, valeur supérieure à celle recommandée pour les effets sur les performances neurocomportementales.

Une VLEP-8h de 45 mg/m³ (25 ppm) est donc recommandée.

- **Valeur limite d'exposition professionnelle sur 15 minutes (VLEP-15min)**

Conformément à la méthodologie de dérivation de valeurs de référence (Anses, 2023 à paraitre) et en l'absence de données disponibles permettant de caractériser les relations dose-effet à court terme du protoxyde d'azote, il est recommandé **de ne pas dépasser sur une période de 15 minutes la valeur de 5 fois la valeur de la VLEP-8h**, soit 225 mg/m³ (125 ppm), afin de limiter l'amplitude et le nombre des pics d'exposition.

Une **VLCT-15 min pragmatique** de **225 mg/m³** (soit 125 ppm**)** est donc recommandée.

- **mention « peau »**

En l'absence de donnée, la mention « peau » n'est pas recommandée.

- **mention « bruit »**

En l'absence de donnée sur d'éventuelles interactions lors de co-expositions au bruit et au protoxyde d'azote, la mention « bruit » n'est pas recommandée.

Conclusion et recommandations

Type de VLEP		**VLEP-8h**	**VLCT-15 min pragmatique**
VR	Organisme	Anses	Anses
	Année	2023	2023
	Valeur	**45 mg/m³ équivalent à 25 ppm**	**225 mg/m³ équivalent à 125 ppm**
Population cible		Travailleurs	Travailleurs
Effet critique		Altération subaiguë réversible de la performance des fonctions cognitives	**Par défaut, en l'absence de donnée disponible, recommandation de ne pas dépasser, sur une période de 15 minutes, 5 fois la valeur de la VLEP-8h**
Etude clé	Référence	1. Scapellato et al., 2008 2. Lucchini et al., 1995, 3. Lucchini et al., 1996 4. Lucchini et al., 1997	
	Population de l'étude	Travailleurs	
	Exposition (durée, voie)	1. 1 an 2. 3h 3. 3h 4. Non indiquée	
Point de départ (PoD)		NOAEC = 23,2 ppm	
Ajustement temporel		/	
Ajustement allométrique		/	
Facteurs d'incertitude (FI)		1 (FI_H : 1 ; FI_S : 1 ; $FI_{L/B}$: 1 ; FI_D : 1)	
Mention « peau »		Non recommandée	
Mention « bruit »		Non recommandée	

La VLEP-8h recommandée devrait également protéger des effets délétères hématologiques, sur le système immunitaire et le développement. Néanmoins, il n'est pas possible de déterminer si cette valeur protège des effets sur la fertilité en l'absence de données humaine et animale fiables.

- **Éléments de proposition pour fixer une méthode de mesure**

Les experts ont évalué les méthodes de référence applicables pour la mesure des niveaux d'exposition du protoxyde d'azote sur le lieu de travail. La qualité de ces méthodes et leur applicabilité à la mesure des expositions aux fins de comparaison à une VLEP sont évaluées et classées au regard des exigences de performances indiquées notamment dans la norme NF EN 482 : « Atmosphère des lieux de travail – Exigences générales concernant les performances des modes opératoires de mesurage des agents chimiques » et des critères de décision détaillés dans le rapport méthodologique (Anses, 2020b).

Le classement de ces méthodes est réalisé de la manière suivante :

- catégorie 1A : méthodes validées (l'ensemble des critères de performance sont satisfaits) ;
- catégorie 1B : méthodes partiellement validées (les critères essentiels de performance sont satisfaits) ;
- catégorie 2 : méthodes indicatives (des critères essentiels de validation ne sont pas suffisamment explicités, ou bien la méthode nécessite des ajustements devant faire l'objet d'une validation) ;
- catégorie 3 : méthodes non recommandées car inadaptées (des critères essentiels de validation ne sont pas remplis)
- catégorie 3* : méthodes non recommandées car non évaluables (des critères essentiels de validation ne sont pas documentés).

NB : Pour la mesure d'aérosols ou de substances en phase mixte, un premier classement est établi au regard des critères de performance portant sur la méthode de prélèvement. Un second classement est établi au regard des critères de performance portant sur la méthode d'analyse. Le classement final de la méthode de mesure correspond au classement le plus défavorable des deux classements.

Une étude comparative et détaillée des méthodes classées en catégorie 1A, 1B et 2 est réalisée au regard des différentes données de validation et de la faisabilité technique, de manière à recommander la ou les méthodes les plus appropriées pour la mesure des concentrations aux fins de comparaison aux VLEP.

Conclusions et recommandations

Six méthodes de mesure du N_2O dans l'air des lieux de travail ont été identifiées et évaluées au regard de la VLEP-8h et de la VLCT-15min pragmatique :

- méthode 1 : Prélèvement passif sur support adsorbant suivi d'une désorption thermique et d'une analyse par spectroscopie infrarouge ;
- méthode 2 : Prélèvement actif sur support adsorbant suivi d'une désorption thermique et d'une analyse par chromatographie gazeuse (GC) couplée à un détecteur à capture d'électrons (ECD) ou détecteur de conductivité thermique (TCD)
- méthode 3 : Échantillonnage passif sur un support adsorbant suivi d'une désorption thermique et d'une analyse par GC-ECD ou TCD ;

- méthode 4 : Analyseur infrarouge à transformée de Fourier en continu ;
- méthode 5 : Instrument de lecture directe - détection photo-acoustique (ILD-PA) ;
- méthode 6 : Échantillonnage actif à l'aide d'un sac Tedlar, analyse par GC-ECD.

Les méthodes 4, 5 et 6 sont classées en catégorie 3 et ne sont pas recommandées pour le contrôle technique réglementaire de la VLEP-8h, le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme. En effet, ces méthodes sont adaptées pour des mesures d'ambiance, mais ne permettent pas d'effectuer des mesures individuelles. A noter que la méthode 4 (analyseur infrarouge en continu) et la méthode 5 (instrument à lecture directe - détection photoacoustique (ILD-PA)) permettent de suivre l'évolution des concentrations et d'identifier les périodes de forte exposition. De plus, elles peuvent être utilisées pour obtenir rapidement des informations sur l'air ambiant en différents endroits.

Pour le contrôle technique réglementaire de la VLEP-8h :

- la méthode 1 est classée en catégorie 1B en raison de nombreux critères essentiels de validation qui répondent aux exigences de performances indiquées notamment dans la norme NF EN 482, notamment au travers des données mentionnées dans le protocole OSHA ID 166 obtenues pour des durées de 4 à 7 heures ;
- la méthode 2 est classée en catégorie 2 dans les conditions du protocole DFG Method 3 en raison des hypothèses formulées sur le volume d'air prélevé pour l'évaluation des données de validation, mais en catégorie 3 dans les conditions du protocole INRS MétroPol M-416 en raison d'une durée de prélèvement qui ne peut excéder 30 minutes sur le support adsorbant préconisé ;
- la méthode 3 est classée en catégorie 1A dans les conditions préconisées par le protocole INRS MétroPol M-415 avec deux prélèvements successifs de 4 heures. Le protocole DFG Method 3 mettant en œuvre un support adsorbant différent n'a pas été évalué compte tenu de l'absence de données de validation relatives à ce support.

Ainsi, les méthodes 1 (dans les conditions du protocole ; OSHA ID 166) et 3 (dans les conditions du protocole INRS MétroPol M-415 avec deux prélèvements successifs de 4 heures) sont recommandées.

Pour le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme :

- la méthode 1 est classée en catégorie 3 en raison d'un débit d'échantillonnage variable avec la durée d'échantillonnage et d'un manque de données sur la stabilité de ce débit sur 15 min ;
- la méthode 2 est classée en catégorie 1B dans les conditions du protocole INRS MétroPol M 416 ;
- la méthode 3 est classée en catégorie 3 dans les conditions du protocole INRS MétroPol M-415 en raison d'une durée de prélèvement qui ne peut être inférieure à 1 heure. Les données du protocole DFG Method 3 n'ont pas été prises en compte dans l'évaluation en raison d'un manque d'information sur les durées d'échantillonnage associées aux données de validation mentionnées.

Ainsi **la méthode 2, dans les conditions du protocole INRS MétroPol M 416, est recommandée**.

Le tableau ci-dessous présente de façon synthétique les méthodes recommandées pour la mesure du protoxyde d'azote dans l'air des lieux de travail aux fins de comparaison avec les VLEP recommandées.

Figure 1 : Méthodes recommandées pour la mesure du protoxyde d'azote dans l'air des lieux de travail au regard de la VLEP-8h et de la VLCT-15 min pragmatique

Méthodes		Protocoles	VLEP-8h	VLCT-15min pragmatique	
			Contrôle technique réglementaire	Contrôle technique réglementaire	Suivi des expositions court terme
1	Prélèvement passif sur support adsorbant Désorption thermique Analyse par spectroscopie infrarouge (IR)	OSHA ID-166 (1994)	1B	3 (non recommandée)	
2	Prélèvement actif sur support adsorbant Désorption thermique Analyse par chromatographie gazeuse (GC) couplée à un détecteur à capture d'électrons (ECD) ou détecteur de conductivité thermique (TCD)	INRS MétroPol M-416 (2022)	3 (non recommandée)	1B	
3	Prélèvement passif sur support adsorbant Désorption thermique Analyse par GC-ECD ou TCD	INRS MétroPol M-415 (2022)	1A	3 (non recommandée)	

4. CONCLUSIONS ET RECOMMANDATIONS DE L'AGENCE

Conformément aux conclusions de son Comité d'Experts Spécialisés (CES) « Valeurs Sanitaires de Référence », l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail recommande la fixation d'une VLEP-8h de 45 $mg.m^{-3}$ et d'une VLCT-15min pragmatique de 225 $mg.m^{-3}$. En l'état des connaissances, la mention « peau » et la mention « bruit » ne sont pas recommandées.

Au regard de l'évaluation des méthodes de mesure du protoxyde d'azote dans l'air des lieux de travail, l'Anses recommande :

- pour le contrôle technique réglementaire de la VLEP-8H, les méthodes 1 (dans les conditions du protocole OSHA ID 166) et 3 (dans les conditions du protocole INRS MétroPol M-415 avec deux prélèvements successifs de 4 heures), respectivement classées en catégorie 1B et 1A, et consistant à effectuer un prélèvement passif sur support adsorbant, une désorption thermique puis une analyse par spectroscopie

infrarouge IR ou une analyse par chromatographie en phase gazeuse couplée avec un détecteur à capture d'électrons ou de conductivité thermique ;

- pour le contrôle technique réglementaire de la VLCT-15min pragmatique, la méthode 2 (dans les conditions du protocole INRS MétroPol M 416), classée en catégorie 1B et consistant à effectuer un prélèvement actif sur support adsorbant, une désorption thermique suivie d'une analyse par chromatographie gazeuse couplée à un détecteur à capture d'électrons ou de conductivité thermique.

L'Anses indique que la VLEP-8h recommandée a été établie pour prévenir l'altération des performances cognitives et qu'elle devrait également protéger des effets néfastes sur les systèmes hématologique et immunitaire ainsi que sur le développement. Elle souligne qu'il n'est toutefois pas possible de déterminer si cette valeur protège des effets sur la fertilité en l'absence de données humaine et animale fiables.

Par ailleurs, l'Anses rappelle que si le protoxyde d'azote ne dispose pas encore à ce jour d'une classification harmonisée selon le règlement CLP, elle a soumis à l'Agence européenne des produits chimiques (ECHA) une proposition de classification harmonisée notamment comme substance toxique pour la reproduction de catégorie 1B (H360Df[6]) qui a été confirmée par le comité d'évaluation des risques de l'ECHA dans son avis du 16 mars 2023[7]. En conséquence, l'Anses recommande que l'exposition au protoxyde d'azote, lorsqu'elle ne peut être évitée, soit réduite au niveau le plus faible possible.

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[6] H360Df : Peut nuire au fœtus. Susceptible de nuire à la fertilité.

[7] https://echa.europa.eu/documents/10162/f205cf23-1a5c-6cad-a379-0812b512c3f5, consulté le 12/12/2023

MOTS-CLÉS

VLEP, valeurs limites, niveaux d'exposition, milieu professionnel, agents chimiques, effets sur la santé, métrologie, méthodes de mesure, lieux de travail, valeur référence, protoxyde d'azote, oxyde nitreux, oxyde de diazote

KEY WORDS

OEL, limit values, exposure levels, occupational, chemical agents, health effects, metrology, measurement methods, workplaces, reference value, nitrous oxide, dinitrogen oxide

CITATION SUGGÉRÉE

Anses. (2023). Avis de l'agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail relatif à l'expertise en vue de la fixation de valeurs limites d'exposition à des agents chimiques en milieu professionnel - Évaluation des effets sur la santé et des méthodes de mesure des niveaux d'exposition sur le lieu de travail pour le protoxyde d'azote. (saisine 2020-SA-0042). Maisons-Alfort : Anses, 10 p.

Expert appraisal on recommending occupational exposure limits for chemical agents

Assessment of health effects and methods for the measurement of exposure levels in workplace atmospheres for nitrous oxide (CAS n°10024-97-2)

Expertise en vue de la fixation de valeurs limites d'exposition à des agents chimiques en milieu professionnel

Évaluation des effets sur la santé et des méthodes de mesure des niveaux d'exposition sur le lieu de travail pour le protoxyde d'azote (CAS n°10024-97-2)

Mission permanente VLEP / OEL Permanent Mission
Saisine n°2020-SA-0042 / Request n°2020-SA-0042

Rapport d'expertise collective
Collective expert appraisal

Comité d'experts spécialisé « Valeurs sanitaires de référence »

Expert Committee on « health reference values »

Groupe de travail « Métrologie » / Working group « metrology »

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Mots clés

VLEP, valeurs limites, niveaux d'exposition, milieu professionnel, agents chimiques, effets sur la santé, métrologie, méthodes de mesure, lieux de travail, valeur de référence, protoxyde d'azote, oxyde nitreux

Key words

OEL, limit values, exposure levels, occupational, chemical agents, health effects, metrology, measurement methods, workplaces, reference value, nitrous oxide, dinitrogen oxide

Presentation of participants

PREAMBULE : The expert members of the Expert Committees and Working Groups or designated rapporteurs are all appointed in a personal capacity, intuitu personae, and do not represent their parent organisations.

EXPERT COMMITTEE (CES)

The work carried out as part of this report was adopted by:

- The “health reference values” Committee (2021-2024)

Chair

Mr Fabrice MICHIELS – Occupational physician-toxicologist, Intercompany association for occupational health, Corrèze and Dordogne (SPST 19-24) – Expertise: occupational medecine, occupational and environmental toxicology.

Vice-Chair

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Members

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- The working group “metrology” (2020-2023)

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Vice-Chairman

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Members

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Mrs. Caroline RIO – Head of Interregional Chemistry Laboratory (LIC) - Skills: ambient and indoor air quality, physical chemistry, organic aerosol, metrology.

Mrs Dominique SAURAT – Military pharmacist (Ministère des armées, service de santé des armées, currently LERES) – skills : air sampling and analysis.

Mrs. Sophie SOBANSKA – Research Officer (Centre national de la recherche scientifique (CNRS)) - Skills: Air quality, biochemistry, particles, metals.

Mr. THIAULT Guénaël – Section Head (CPPL) - Skills: indoor and workplace air quality, metrology, chemistry.

ANSES PARTICIPATION

Scientific Coordination

Mrs Nathalie PRINTEMPS. Resignation in September 2022.

Mrs Farida LAMKARKACH. Resignation in April 2023.

Scientific Contribution

Mrs Nathalie PRINTEMPS. Resignation in September 2022.

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Ce document synthétise les travaux du comité d'experts spécialisés « Valeurs sanitaires de référence » (CES VSR) et du groupe de travail « Métrologie ».

Présentation de la question posée

Dans le cadre du protocole d'accord entre l'Anses et le ministère du travail pour la mise en œuvre du programme de travail d'expertise scientifique en matière de valeurs limites atmosphériques et biologiques pour les expositions professionnelles (VLEP), établi en juillet 2018, la direction générale du travail (DGT) a saisi l'Anses afin de mener les travaux d'expertise nécessaires à la fixation de valeurs limites d'exposition professionnelle (VLEP) fondées sur des considérations sanitaires pour le protoxyde d'azote (N_2O).

La France ne dispose à ce jour d'aucune valeur limite d'exposition professionnelle pour cette substance.

Contexte scientifique

Les VLEP telles que recommandées par le CES sont des niveaux de concentration en polluants dans l'atmosphère des lieux de travail à ne pas dépasser sur une période de référence déterminée et en deçà desquels le risque d'altération de la santé est négligeable. Même si des modifications physiologiques réversibles sont parfois tolérées, aucune atteinte organique ou fonctionnelle de caractère irréversible ou prolongée n'est admise à ce niveau d'exposition pour la grande majorité des travailleurs. Ces niveaux de concentration sont déterminés en considérant que la population exposée (les travailleurs) est une population qui ne comprend ni enfants ni personnes âgées.

Ces niveaux de concentrations sont déterminés par les experts du CES à partir des informations disponibles dans des études épidémiologiques, cliniques, de toxicologie animale, etc. L'identification de ces concentrations sécuritaires pour la santé humaine nécessite généralement d'appliquer des facteurs d'ajustement aux valeurs identifiées directement par les études. Ces facteurs permettent de prendre en compte un certain nombre d'éléments d'incertitude inhérents à la démarche d'extrapolation conduite dans le cadre d'une évaluation des effets sanitaires des substances chimiques sur l'Homme.

Trois types de valeurs sont recommandées par le CES :

- valeur limite d'exposition 8 heures (VLEP-8h) : il s'agit de la limite de la moyenne pondérée en fonction du temps de la concentration atmosphérique d'un agent chimique

dans la zone de respiration d'un travailleur au cours d'un poste de 8 heures. Dans l'état actuel des connaissances scientifiques (en toxicologie, médecine, épidémiologie, etc.), la VLEP-8h est censée protéger d'effets sur la santé à moyen et long termes, les travailleurs exposés régulièrement et pendant la durée d'une vie de travail à l'agent chimique considéré ;

- valeur limite d'exposition à court terme (VLCT-15min) : il s'agit de la limite de la moyenne pondérée en fonction du temps de la concentration atmosphérique d'un agent chimique dans la zone de respiration d'un travailleur sur une période de référence de 15 minutes pendant le pic d'exposition quelle que soit sa durée. Elle vise à protéger les travailleurs des effets néfastes sur la santé (effets toxiques immédiats ou à court terme, tels que des phénomènes d'irritation), dus à des pics d'exposition ;

- valeur plafond : il s'agit de la limite de la concentration atmosphérique d'un agent chimique dans la zone de respiration d'un travailleur, qui ne doit être dépassée à aucun moment de la période de travail. Cette valeur est appliquée aux substances reconnues comme irritant fort ou corrosif ou pouvant causer un effet grave potentiellement irréversible, à très court terme.

Ces trois types de valeurs sont exprimés :

- soit en $mg.m^{-3}$, c'est-à-dire en milligrammes d'agent chimique par mètre cube d'air et en ppm (parties par million), c'est-à-dire en centimètres cube d'agent chimique par mètre cube d'air, pour les gaz et les vapeurs ;

- soit en $mg.m^{-3}$ uniquement, pour les aérosols liquides et solides ; soit en $f.cm^{-3}$, c'est-à-dire en fibres par cm^3 pour les matériaux fibreux.

La valeur de la VLEP-8h peut être dépassée sur de courtes périodes pendant la journée de travail à condition toutefois :

- que la moyenne pondérée des valeurs sur l'ensemble de la journée de travail ne soit pas dépassée ;
- de ne pas dépasser la valeur de la VLCT si elle existe.

En plus des VLEP, le CES évalue la nécessité d'attribuer ou non une mention « peau », lorsqu'une pénétration cutanée significative a été identifiée (Anses,à paraître). Cette mention indique la nécessité de prendre en compte la voie d'exposition cutanée dans l'évaluation de l'exposition et, le cas échéant, de mettre en œuvre des mesures de prévention appropriées (telles que le port de gants de protection). En effet, la pénétration cutanée des substances n'est pas prise en compte pour la détermination des niveaux de valeurs limites atmosphériques et peut donc potentiellement entraîner des effets sanitaires indépendamment du respect de ces dernières.

Le CES évalue également la nécessité d'attribuer ou non une mention « bruit » signalant un risque d'atteinte auditive en cas de co-exposition au bruit et à la substance en dessous des limites d'exposition recommandées afin que les préventeurs mettent en place des mesures appropriées (collectives, individuelles et médicales) (Anses, à paraître).

Le CES évalue également les méthodes de référence applicables pour la mesure des niveaux d'exposition sur le lieu de travail. La qualité de ces méthodes et leur applicabilité à la mesure des expositions aux fins de comparaison à une VLEP ont été évaluées notamment sur leur conformité aux exigences de performance de la NF EN 482 et de leur niveau de validation.

Organisation de l'expertise

L'Anses a confié au comité d'experts spécialisés « Valeurs sanitaires de référence » (CES VSR), l'instruction de cette saisine.

Pour conduire ces travaux d'expertise, différents collectifs ont été mobilisés :

- le CES VSR a réalisé l'évaluation des effets sanitaires du protoxyde d'azote et a recommandé des VLEP,
- le groupe de travail « Métrologie » a réalisé l'évaluation des méthodes de mesures atmosphériques dans les lieux de travail au regard des VLEP recommandées.

Les travaux d'expertise ont été soumis régulièrement au CES VSR tant sur les aspects méthodologiques que scientifiques. Le rapport produit tient compte des observations et éléments complémentaires transmis par les membres du CES.

Le rapport en anglais, ainsi que la synthèse et les conclusions de l'expertise collective en français, ont été adoptés par le CES VSR le 1er juillet 2022 pour mise en consultation. Ces documents ont fait l'objet d'une consultation publique du 22 juin au 15 septembre 2023. Les personnes ou organismes ayant contribué à la consultation publique sont listés en annexe 6. Les commentaires reçus ont été examinés et discutés par le CES VSR qui a adopté le présent document le 9 novembre 2023.

Ces travaux d'expertise sont ainsi issus d'un collectif d'experts aux compétences complémentaires. Ils ont été réalisés dans le respect de la norme NF X 50-110 « qualité en expertise ».

Prévention des risques de conflits d'intérêts

L'Anses analyse les liens d'intérêts déclarés par les experts avant leur nomination et tout au long des travaux, afin d'éviter les risques de conflits d'intérêts au regard des points traités dans le cadre de l'expertise.

Les déclarations d'intérêts des experts sont publiées sur le site internet : https://dpi.sante.gouv.fr/.

Description de la méthode

- Pour l'évaluation des effets sur la santé

Un profil toxicologique a été élaboré par l'Anses et soumis au CES VSR qui l'a commenté et complété.

Le profil toxicologique est issu d'éléments bibliographiques prenant en compte la littérature scientifique parue sur cette substance jusqu'en décembre 2020. La recherche bibliographique a été effectuée dans la base de données PubMed®. La requête bibliographique, les principaux mots-clés et les critères d'inclusion et d'exclusion de la recherche bibliographique sont décrits en Annexe 1.

La littérature secondaire de l'IPCS-INCHEM (1992), de l'INRS (2010 et 2018), de la DFG (1993 et 2015), de l'ACGIH (2018), de l'Anses (2020a), de l'HCN (2000) et de l'EIGA (2008)[1] ont également été prises en compte.

[1] IPCS-INCHEM : International Programme on Chemical Safety- International Chemistry; INRS : Institut national de la recherche et de sécurité pour la prévention des accidents et maladies professionnelles ;

Pour toutes les études pertinentes retenues dans la synthèse, la validité interne des études a été vérifiée et notée (Annexe 2).

- Pour l'évaluation des méthodes de mesure des niveaux d'exposition sur le lieu de travail

Un rapport de synthèse a été élaboré par le GT « Métrologie » et soumis au CES VSR qui l'a commenté.

Le rapport de synthèse présente les différents protocoles de mesure du protoxyde d'azote dans l'air des lieux de travail recensés et regroupés en fonction des méthodes mises en œuvre. Ces dernières ont ensuite été évaluées et classées au regard des exigences de performances indiquées notamment dans la norme NF EN 482 : « Atmosphère des lieux de travail – Exigences générales concernant les performances des modes opératoires de mesurage des agents chimiques » et des critères de décision détaillés dans le rapport méthodologique (Anses, 2020b).

La liste des principales sources consultées est précisée dans le rapport méthodologie (Anses, 2020b).

Le classement de ces méthodes est réalisé de la manière suivante :

- catégorie 1A : méthodes validées (l'ensemble des critères de performance sont satisfaits) ;
- catégorie 1B : méthodes partiellement validées (les critères essentiels de performance sont satisfaits) ;
- catégorie 2 : méthodes indicatives (des critères essentiels de validation ne sont pas suffisamment explicités, ou bien la méthode nécessite des ajustements devant faire l'objet d'une validation) ;
- catégorie 3 : méthodes non recommandées car inadaptées (des critères essentiels de validation ne sont pas remplis)
- catégorie 3* : méthodes non recommandées car non évaluables (des critères essentiels de validation ne sont pas documentés).

NB : Pour la mesure d'aérosols ou de substances en phase mixte, un premier classement est établi au regard des critères de performance portant sur la méthode de prélèvement. Un second classement est établi au regard des critères de performance portant sur la méthode d'analyse. Le classement final de la méthode de mesure correspond au classement le plus défavorable des deux classements.

Une étude comparative et détaillée des méthodes classées en catégorie 1A, 1B et 2 est réalisée au regard des différentes données de validation et de la faisabilité technique, de manière à recommander la ou les méthodes les plus appropriées pour la mesure des concentrations aux fins de comparaison aux VLEP.

DFG : Deutsche Forschungsgemeinschaft (Fondation de la recherche Allemande) ; ACGIH : American conference of governmental industrial hygienists ; HCN : Health Council of the Netherlands ; EIGA : European industrial gases association

Résultat de l'expertise collective concernant les effets sur la santé

- **Information générale**

Le protoxyde d'azote (N_2O) est un gaz incolore ayant une odeur perçue comme agréable.

Le N_2O est utilisé depuis plus de 150 ans en chirurgie comme adjuvant de l'anesthésie générale par inhalation. La substance est également utilisée pour soulager la douleur lors de l'accouchement ou pour une courte analgésie lors d'interventions médicales mineures (par exemple en dentisterie, urgence, médecine vétérinaire). La substance est couramment utilisée en association à d'autres gaz anesthésiants.

Par ailleurs, le N_2O est une substance chimique enregistrée au titre du règlement REACh et est fabriqué et/ou importé dans l'espace économique européen à raison de 1 000 à 10 000 tonnes par an. Cette substance est également enregistrée en tant qu'intermédiaire.

Le N_2O est utilisé dans l'industrie alimentaire comme additif alimentaire (E942). C'est un gaz propulseur utilisé dans de nombreux produits (par exemple, pour aérer de la crème fouettée, gonfler des ballons).

C'est également un additif des carburants pour fusées afin d'augmenter l'oxygène disponible pour la combustion. De plus, le N_2O est utilisé en laboratoire comme agent oxydant en spectrométrie d'absorption atomique en mode flamme.

L'abus en usage récréatif du gaz, également appelé « gaz hilarant », s'est accru ces dernières années en raison de ses propriétés euphorisantes, relaxantes et hallucinogènes. Le rapport d'étude de toxicoviligance, élaboré avec l'appui des centres anti-poison, met en évidence des effets indésirables pouvant être sévères tels que des troubles du rythme cardiaque, un risque d'asphyxie, des troubles psychiques et des atteintes neurologiques (Anses, 2020a).

Le N_2O est naturellement émis par les sols et les océans. Les émissions de N_2O d'origine humaine sont principalement dues aux activités agricoles et dans une moindre mesure à d'autres sources telles que le secteur de la santé. C'est un gaz qui s'accumule dans l'atmosphère (durée de vie atmosphérique de 114 ans) et qui contribue à l'effet de serre et à la destruction de la couche d'ozone.

- **Revue des recommandations en matière de valeurs limites d'exposition professionnelle**

L'Institut national américain pour la sécurité et la santé au travail (NIOSH) a recommandé en 1977 une limite d'exposition (REL-TWA) de 25 ppm lors de l'administration du N_2O en tant qu'anesthésique. L'objectif de cette limite est de prévenir l'altération des performances mentales, des capacités audiovisuelles et de la dextérité manuelle lors d'expositions au N_2O. Le NIOSH a noté qu'afin de fixer une limite pour prévenir les effets néfastes sur la reproduction (développement et fertilité), davantage de données seraient nécessaires.

En 1993, la DFG (« Deutsche Forschungsgemeinschaft » ou fondation allemande pour la recherche) a recommandé une valeur MAK (« Maximale Arbeitsplatz-Konzentration » ou concentration maximale des lieux de travail) provisoire de 100 ppm (180 mg/m^3) basée sur

l'induction du système enzymatique microsomal observée chez des personnes exposées à 200 ppm de N_2O (360 mg/m^3). Dans l'addendum de 2007 (publié en 2015) sur la toxicité pour la reproduction, une NOAEC de 10 000 ppm a été retenue pour la toxicité sur le développement sur la base des études de Holson *et al.* de 1995 et Pope *et al.* de 1978. Le comité allemand a conclu qu'aucune toxicité sur le développement n'était attendue à la valeur MAK de 100 ppm (180 mg/m^3).

Aux États-Unis, l'ACGIH (« American Conference of Governmental Industrial Hygienists ») recommande une valeur limite sur 8 heures (ou TLV-TWA) de 50 ppm (90 mg/m^3) (ACGIH, 2018 ; dernière mise à jour en 1996). L'ACGIH a considéré que les systèmes reproducteur, hématologique et nerveux humains étaient les organes cibles les plus sensibles au protoxyde d'azote. L'ACGIH a conclu que la valeur limite de 50 ppm devrait protéger contre la toxicité potentielle embryofœtale, le risque accru d'avortement spontané, la dépression médullaire et les fonctions psychomotrices et cognitives. L'ACGIH n'a pas recommandé de limite d'exposition à court terme sur 15 minutes ou de notations spécifiques.

- **Données de toxicocinétique**

Le N_2O est très volatil et rapidement absorbé par inhalation. Il est rapidement distribué dans les tissus richement vascularisés et pénètre facilement dans le cerveau. Il est rapidement excrété sous sa forme inchangée par les poumons. Le N_2O est également capable de franchir la barrière placentaire (INRS, 2018).

- **Données de toxicité**
  - ***Toxicité aiguë***
    - Système nerveux

Données chez l'Homme

Dans des études expérimentales sur des volontaires sains, Bruce et Bach ont signalé une légère altération de la capacité audiovisuelle à 50 ppm et une diminution des performances de l'acuité visuelle, de la mémoire immédiate et de la vigilance après une exposition de 4 heures au N_2O à 500 ppm (Bruce et Bach, 1976). En revanche, Venables *et al.* (Venables *et al.*, 1983) et d'autres auteurs n'ont pas réussi à reproduire les résultats obtenus à 50 ppm par Bruce et Bach (Bruce et Bach, 1976). Néanmoins, dans une correspondance, Bruce émet l'hypothèse que les volontaires utilisés dans son étude de 1976 ont pu être particulièrement sensibles aux effets cognitifs du N_2O et ne sont donc peut- être pas représentatifs de la population générale (Bruce, 1991). D'autres études, réalisées à des niveaux de doses plus élevés ont également rapporté des effets sur les fonctions cognitives en termes de capacité audiovisuelle, de mémoire et de vigilance, de temps de réaction. En ce qui concerne la réversibilité de ces effets, bien qu'elle n'ait pas été spécifiquement évaluée dans les études, Fagan *et al.* ont signalé que les étourdissements, les paresthésies et l'euphorie observés chez les volontaires exposés au N_2O étaient rapidement réversibles (Fagan *et al.*, 1994).

Des effets sur la conduction nerveuse ont également été rapportés chez des volontaires sains exposés au N_2O pendant 15 minutes à 200 000 ppm (Gyulai *et al.*, 1996, William *et al.*, 1984).

Données animales

Dans une série d'études réalisées chez le rat (Jevtovic-Todorovic *et al.*, 2000, 2001, 2003 et 2007), des modifications significatives et sévères du système nerveux ont été observées après une exposition unique. En effet, des modifications histopathologiques du cerveau et/ou des changements de comportement ont été rapportés après une seule exposition supérieure ou égale 300 000 ppm de protoxyde d'azote. Des changements de comportement ont également été notés chez la souris à des concentrations supérieures ou égales à 250 000 ppm (Li *et al.*, 2001, Caton *et al.*, 1994). Aucune étude expérimentale chez l'animal n'a été trouvée à des niveaux de dose et des durées d'exposition correspondant aux expositions pouvant survenir en milieu professionnel.

- ***Irritation et sensibilisation***

Pas de donnée disponible.

- ***Toxicité subchronique et chronique***
  - Système nerveux

Données chez l'Homme

Lucchini *et al.* ont conduit une série d'études (transversales et expérimentales) mesurant le N_2O dans l'air (prélèvements individuels ou ambiant pour Lucchini *et al.*, 1997) et dans les urines (fin de poste) de personnels médicaux (Lucchini *et al.*, 1995, 1996 et 1997).

En 1995, Lucchini *et al.*, ont rapporté une différence statistiquement significative entre le groupe contrôle (42 infirmiers/infirmières) et le groupe exposé (62 infirmiers/infirmières) (moyenne géométrique dans l'air = 62,6 [7-553] ppm et moyenne géométrique dans les urines = 26,8 [4-297] µg/L) concernant la diminution du temps de réaction (test de vigilance ou *simple reaction time* (SRT)) en fin de poste, fin de semaine. Ils ont conclu qu'une exposition inférieure à 100 ppm n'avait pas eu d'effet chronique sur la fonction psychomotrice, mais que des effets réversibles sur la vigilance et la vitesse de réaction pouvaient être attendus (temporaires car observés le dernier jour et non au début de la semaine) (Lucchini *et al.*, 1995).

En 1996, ces mêmes auteurs ont également mesuré le taux de cortisol sérique en tant qu'indicateur biologique du stress et de la prolactine sérique afin d'investiguer l'interférence du N_2O avec le système dopaminergique sur 30 infirmiers/infirmières, exposés en double aveugle pendant une semaine à des gaz anesthésiants et une autre semaine à des anesthésiants non gazeux (à deux semaines d'intervalle) et un groupe témoin de 20 sujets. Ils ont observé un temps de réaction (SRT) prolongé pendant la semaine d'exposition au N_2O ainsi qu'une augmentation de la concentration en prolactine sérique (pas d'effet sur la concentration de cortisol). Les auteurs concluent que des effets peuvent survenir à des concentrations moyennes de 54,2 ±22,8 ppm et de 25,6 ±22,1 µg/L respectivement pour le N_2O dans l'air et les urines, respectivement. Cependant, ils soulignent que ces résultats doivent être considérés avec précaution en raison du faible nombre d'individus exposés et des co-expositions possibles (Lucchini *et al.,*1996*)*.

En 1997, Lucchini *et al.*, ont conduit une étude multicentrique sur 112 sujets exposés (130 contrôles) avec des critères d'inclusion plus stricts (consommation d'alcool, café, médicaments…). Ils ont mesuré le N_2O dans les urines et dans l'air ambiant (prélèvement stationnaire) et évalué les effets sur la fonction cognitive à l'aide de tests plus complexes. Les auteurs n'ont observé aucun effet à des concentrations urinaires de N_2O de 13 µg/L (25 ppm) (Lucchini *et al.*, 1997).

Plus récemment, Scapellato *et al.,* ont procédé à l'évaluation des effets de l'exposition au N_2O sur la fonction neurocomportementale, par le biais d'une batterie de tests (début et fin de poste, début et fin de semaine). Pour cela, ils ont suivi une équipe de 38 infirmier(e)s exposé(e)s et 23 non exposé(e)s, sur laquelle ils ont mené une série de tests deux fois sur une année. Ils ont rapporté une relation dose-réponse entre l'augmentation de l'exposition au N_2O et les performances neurocomportementales. Les auteurs ont initialement classé les sujets en 4 groupes d'exposition (A) non exposés ; B) <13 µg/L; C) ≥13 à <27 µg/L et D) ≥27 µg/L) puis les groupes B et C ont été regroupés en un seul groupe (<27 µg/L). Les auteurs ont observé des changements significatifs dans le temps de réaction et dans l'humeur des sujets au cours de la semaine chez les sujets ayant une concentration urinaire ≥27 µg/L (correspondant à 50 ppm[2]) (Scapellato *et al.,* 2008). Comme pour l'étude de Lucchini *et al.* de 1997, les co-expositions ainsi que le faible effectif de l'étude doivent conduire à considérer les résultats avec prudence.

Des études conduites en milieu professionnel sont disponibles mais présentent des limites (notamment en raison de données imprécises sur les concentrations de N_2O dans l'air, l'absence de mesures biologiques et de biais de sélection). Parmi ces études (décrites dans le tableau 9 du rapport), l'étude de Stollery *et al.* rapporte des effets (non significatifs) sur la fonction cognitive à des concentrations de 58 ppm (prélèvement individuel) par rapport au groupe contrôle. Concernant ce groupe contrôle, les auteurs précisent qu'il n'est pas considéré comme non exposé (15 ppm de N_2O) (Stollery *et al.* ,1998).

Données animales

Chez le rat, des effets graves et indésirables au niveau des méninges, du cerveau et de la moelle épinière ont été notés dans plusieurs études après une exposition répétée. Les durées d'exposition étaient comprises entre 7 jours à 6 mois, et entre 1,5 à 8 heures par jour. Néanmoins, seules des concentrations élevées de protoxyde d'azote ont été testées (≥ 400 000 ppm), peu pertinente au regard de l'exposition en milieu professionnel.

Chez la souris, aucune constatation histopathologique dans le cerveau n'a été notée jusqu'à 500 000 ppm dans une étude de toxicité répétée de 14 semaines de Rice *et al.*, (Rice *et al.*, 1985). Néanmoins, des changements de comportement ont été observés dans l'étude de Fung *et al.* 1993 après 8 jours d'exposition, 8 heures par jour à 1 000 ppm de protoxyde d'azote chez la souris (Fung *et al.,*1993).

- <u>Système hématologique et fonction immune</u>

Données chez l'Homme

Des modifications de l'hémogramme (notamment au niveau du nombre total de lymphocytes et de globules rouges, de l'hémoglobine, de l'hématocrite, du volume globulaire moyen et de la concentration corpusculaire moyenne en hémoglobine) ont été observées dans sept études observationnelles transversales chez des travailleurs en salle d'opération ou chez des dentistes exposés de façon répétée au N_2O. Cependant, à l'exception de l'étude de Bargellini (Bargellini *et al.,* 2001), la caractérisation de l'exposition était inadéquate et les facteurs de confusion étaient insuffisamment analysés. Bargellini *et al.,* ont constaté chez des

[2] Selon les corrélations entre les concentrations atmosphérique et urinaire de N_2O rapportées par Imbriani *et al.* (1995) : $[N_2O]_{urine} = 0,57x[N_2O]_{atmo} + 1,584$ ($r = 0,89$; $p < 0,001$).

anesthésistes une diminution des cellules T (CD3+), en corrélation avec une diminution des cellules T auxiliaires (CD4). La diminution des cellules T auxiliaires était dose-dépendante (dépendante de l'intensité et/ou de la durée). Cependant, la conception de cette étude ne permet pas d'établir une relation quantitative exposition-réponse et les travailleurs étaient également co-exposés à d'autres gaz anesthésiants et à des rayons-X.

Données animales

Le N_2O est immunotoxique chez les rats et les souris après une exposition répétée. Des lésions de la moelle osseuse, telles qu'une diminution de la cellularité des séries érythroïde, myéloïde et lymphoïde, ont été observées après une exposition continue de 24 heures pendant plusieurs jours à des doses élevées (≥ 200 000 ppm) de N_2O.

Chez la souris, après une exposition de 6 heures par jour, 5 jours par semaine pendant 13 semaines au N_2O (ce qui est plus représentatif de l'exposition en milieu professionnel), une diminution du nombre de globules blancs a été observée, sans relation dose-réponse, à partir de 50 ppm (Healy *et al.*, 1990). Cependant, dans l'étude de Rice *et al.* de 1985, aucun effet sur les paramètres hématologiques n'a été observé chez la souris après une exposition pendant 14 semaines, 4 heures par jour, 5 jours par semaine jusqu'à 500 000 ppm de N_2O. De même, aucun effet n'a été observé chez la souris après une exposition de 10 000 ppm au N_2O pendant 4 heures par jour pendant 104 semaines dans l'étude de Baden *et al. de* 1986. La différence de résultat pourrait être liée en partie à une différence de susceptibilité des souches de souris. L'immunotoxicité, incluant les effets du N_2O sur la réponse humorale, a été spécifiquement testée par Healy *et al.* chez la souris (Healy *et al.,* 1990). Dans cette étude, une diminution de la fonction immunitaire a été observée à 5 000 ppm. Une diminiution de la désoxyuridine (réalisée pour quantifier la conversion de la désoxyuridine en désoxythymidine dans les cellules de la moelle osseuse), a été notée après exposition au N_2O à des concentrations supérieures à 500 ppm.

Chez le rat mâle, après une exposition au protoxyde d'azote à 1 000 ppm, 5 jours par semaine, 6 heures par jour, pendant 6 semaines à 6 mois, aucune leucocytopénie n'a été observée (Cleaton-Jones *et al.*, 1977). À cette dose, seuls des effets transitoires sur la concentration d'hémoglobine et le volume cellulaire ont été notés et une augmentation du nombre de réticulocytes après une exposition de 5 mois. Les résultats de l'analyse de la moelle osseuse étaient similaires à ceux des témoins, à l'exception d'une augmentation des mastocytes dans la moelle osseuse des exposés.

En conclusion, des effets sur les paramètres hématologiques ont été observés chez l'Homme et l'animal. Cependant, les données disponibles ne permettent pas de caractériser avec certitude la relation dose-réponse pour ces effets.

- <u>Système hépatique</u>

Données chez l'Homme

Une induction d'enzymes hépatiques (mesurée indirectement par l'excrétion urinaire d'acide D-glucarique) a été rapportée chez des patients recevant des mélanges d'halothane et de N_2O. Sur la base de l'induction d'enzymes microsomales hépatiques à 200 ppm, une VLEP provisoire de 100 ppm avait été adoptée par la DFG en 1993. Néanmoins, les auteurs ont indiqué que l'effet pouvait être dû à l'exposition à l'halothane.

Plus récemment, Scapellato *et al.* ont cherché à savoir si l'excrétion d'acide D-glucarique pouvait être un biomarqueur d'effet pour la surveillance des travailleurs exposés aux gaz

anesthésiants. Les auteurs ont mesuré l'excrétion d'acide D-glucarique chez 229 travailleurs avant et après une séance opératoire et 229 travailleurs ont été utilisés comme témoins. Le N_2O et l'isoflurane ont été mesurés après au moins 4 heures d'exposition. Les travailleurs ont été classés par catégorie d'exposition en fonction du niveau urinaire de N_2O. Les auteurs ont constaté que l'induction enzymatique microsomale (augmentation de l'excrétion urinaire d'acide D-glucarique) était augmentée uniquement dans le groupe le plus exposé (N_2O > 50 ppm, isoflurane > 1 ppm) et seulement chez certains des travailleurs de ce groupe. Les auteurs ont conclu que l'induction enzymatique des microsomes hépatiques ne peut pas être utilisée comme biomarqueur d'effet chez les travailleurs (Scapellato *et al.*, 2001).

- ***Génotoxicité***

Chez l'Homme, l'exposition professionnelle au N_2O a été associée à des dommages à l'ADN avec une dose sans effet autour de 96 ppm (Wrońska-Noffer *et al.,* 2009). Une étude suggère que ces dommages à l'ADN pourraient être de nature oxydative. Des échanges de chromatides sœurs ont été observés chez les hommes mais pas chez les femmes dans une étude portant sur des travailleurs exposés à de faibles concentrations (11,9 ppm) de N_2O. Néanmoins, ce type de dommages à l'ADN s'est avéré réversible, suggérant l'activation d'un mécanisme de réparation après l'arrêt de l'exposition. Des effets clastogènes (augmentation des micronoyaux, aberrations chromosomiques) ont été observés à des doses plus élevées, autour de 180 ppm mais en présence d'autres gaz anesthésiants tels que l'isoflurane et le sevoflurane.

Bien que de faibles effets génotoxiques aient été observés chez l'Homme, les modes d'action potentiels étaient tous indirects, notamment le stress oxydatif, la carence en folates et la toxicité de l'homocystéine (O'Donovan et Hammond, 2015). En effet, une carence en vitamine B12 et des taux plasmatiques élevés d'homocystéine sont associés à une formation accrue de micronoyaux (Mogsenzadegan *et al.,* 2020). Ainsi, un seuil est attendu. À l'exception de quelques résultats équivoques sur les échanges de chromatides sœurs, aucun effet génotoxique n'a été observé chez l'Homme à des doses inférieures à 96 ppm.

Des données expérimentales fiables et adéquates sur le protoxyde d'azote *in vitro* et *in vivo* chez l'animal sont disponibles.

In vitro, le N_2O n'induit pas de mutations géniques chez les bactéries (*S. Typhimurium* TA98 et TA100) après 8 heures d'exposition au N_2O sous pression (Baden *et al.*, 1981). Aucun essai fiable de cytogénicité ou de mutation génique sur cellules de mammifères *in vitro* avec le N_2O seul n'a été identifié.

In vivo, des résultats négatifs ont été obtenus dans un essai de mutations létales dominantes chez le rat (Holson *et al.,* 1995) et dans un essai de mutation létale récessive liée au sexe chez *Drosophila melanogaster* (Kundomal *et al.*, 1985). Aucun autre test *in vivo* sur cellules somatiques, utilisant le N_2O seul, n'a été identifié.

En conclusion, les données sont insuffisantes pour pouvoir conclure sur le potentiel génotoxique du N_2O.

- ***Cancérogénicité***

Données chez l'Homme

Certaines études épidémiologiques ont mis en évidence une tendance à l'augmentation de l'incidence des tumeurs chez les travailleurs exposés professionnellement à des gaz anesthésiants (Cohen *et al.,* 1980 ; Corbett *et al.*, 1973a). Cependant, dans ces études, les expositions étaient mal ou pas du tout caractérisées. Par ailleurs, aucune donnée épidémiologique portant spécifiquement sur l'exposition au N_2O n'a été identifiée.

Données animales

Dans une étude expérimentale, l'incidence des tumeurs n'a pas été significativement augmentée par rapport au groupe témoin, chez des souris après une exposition au N_2O à des concentrations de 100 000 ppm ou 400 000 ppm, 4 heures par jour, 5 jours par semaine pendant 78 semaines, suivies d'une période de récupération de 4 semaines (Baden *et al.*, 1986).

Dans l'ensemble, bien que les données soient limitées, il n'y a aucune preuve, à partir des données actuellement disponibles, que le N_2O puisse être cancérogène.

- ***Toxicité sur la reproduction et le développement***
  - Effets sur la fertilité

Données humaines

Deux études épidémiologiques menées auprès de travailleurs sont disponibles chez l'Homme. Les résultats positifs, obtenus dans ces études, notamment concernant la diminution du taux de fécondité, ne sont pas suffisants pour conclure à un effet chez l'Homme (Alhborg *et al.*, 1996 ; Rowland *et al.*, 1992). En effet, la co-exposition potentielle et l'absence de caractérisation quantitative des expositions au N_2O entraînent des incertitudes dans l'interprétation des résultats.

Données animales

- Données chez le rat

Deux études expérimentales menées par le même laboratoire ont examiné les effets du N_2O sur la fonction de reproduction chez les rattes. Les études ont utilisé différentes conditions d'exposition : d'une part 300 000 ppm de N_2O, 8 heures par jour pendant 4 jours d'autre part 500 ppm, 8 heures par jour pendant 35 jours. Douze animaux ont été testés dans chaque groupe. Des anomalies du cycle œstral et une diminution de 50 % de la fertilité par rapport aux témoins ont été observées chez les rats femelles dans les deux études (Kugel *et al.,* 1989 et 1990). Ces études comportaient plusieurs limites : aucune information sur la toxicité maternelle, le petit nombre d'animaux par groupe, un seul niveau de dose et peu de paramètres étudiés. De plus, seul un résumé de l'étude de Kugel *et al.* de 1989 a été publié, entraînant une incertitude sur les résultats.

Une étude portant sur les effets sur les organes reproducteurs de rats mâles, exposés à 200 000 ppm de protoxyde d'azote, a montré des effets significatifs sur le sperme et les testicules (Kripke *et al.*, 1976). L'utilisation d'une dose unique dans l'étude ne permet pas d'établir une relation dose-réponse.

Chez des rats mâles exposés à 5 000 ppm de N_2O pendant 30 jours, 6 heures par jour, 5 jours par semaine, Vieira *et al.* ont noté une diminution de la taille des portées chez les mères non exposées qui ont été accouplées avec les rats mâles exposés au N_2O comparativement aux rattes accouplées à des rats mâles non exposés. Les effets observés sur la taille des portées sont préoccupants. Cependant, l'étude présente des limites car peu de paramètres ont été analysés (aucune information sur les résorptions, nombre de corps jaunes, etc.). De plus, la dose-réponse n'a pas été étudiée (niveau de dose unique) (Vieira *et al.*,1983a). Bien qu'aucun effet statistiquement significatif sur la taille des portées n'ait été noté dans l'étude de Holson *et al.* de 1995, la tendance à la diminution du nombre de petits par portée chez les femelles accouplées avec des rats mâles exposés à 10 000 ppm de N_2O, pendant 9 semaines, 6 heures par jour, 5 jours par semaine, soutient un effet potentiel de la substance chez le rat mâle. Cependant, il existe des incertitudes sur le niveau de dose avec effet.

- Données chez la souris

Chez la souris, après une exposition répétée jusqu'à 500 000 ppm de N_2O pendant 13 semaines, 4 heures par jour, 5 jours par semaine, aucun effet histopathologique n'a été observé au niveau des ovaires, des testicules, des vésicules séminales, sur la spermatogénèse ou sur le nombre d'ovocytes primaires (Rice *et al.*, 1985 ; Mazze *et al.*, 1983). Dans l'étude de Mazze *et al.*, aucun effet sur la taille des portées ou le poids des petits n'a été observé après exposition des mâles pendant 9 semaines, 4 heures par jour, 5 jours par semaine (Mazze *et al.*,1982). La fertilité et le cycle œstral n'ont pas été étudiés chez les souris femelles et peu de paramètres ont été analysés chez les mâles. Par conséquent, l'absence d'effet observé chez la souris ne remet pas en cause les effets observés chez les rats.

En conclusion, la diminution de la fertilité et les modifications du cycle œstral observées chez les rattes dans deux études soulèvent des préoccupations claires quant à la fertilité des femelles exposées au N_2O. De plus, les effets observés chez les rats mâles sur les testicules et la spermatogenèse dans l'étude de Kripke *et al.* (Kripke *et al.*,1976) et la diminution de la taille des portées dans l'étude de Vieira *et al.* (Vieira *et al.*, 1983a) soutiennent des effets potentiels sur la fertilité des mâles. Cependant, il existe des incertitudes quant au niveau de dose avec et sans effet chez les rats ainsi que sur la relation dose-réponse en raison des limites des études disponibles, notamment l'utilisation d'une dose unique ou le petit nombre de paramètres analysés. Bien que les effets observés chez les rats ne soient pas remis en question, l'absence d'effet chez la souris n'est pas expliquée.

- <u>Effets sur le développement</u>

Données chez l'Homme

Trois études observationnelles en milieu professionnel ont indiqué que le N_2O peut induire des anomalies congénitales ou un poids réduit à la naissance après une forte exposition au N_2O (Teschke *et al*, 2011 ; Cohen *et al.,* 1980 ; Bodin *et al.*, 1999). Néanmoins, l'interprétation des données est difficile en raison des co-expositions potentielles, du manque de caractérisation fiable de l'exposition et de l'absence d'ajustement sur d'autres facteurs de risques potentiels.

Des résultats contradictoires ont été rapportés sur le risque d'avortement spontané dans six études observationnelles en milieu professionnel chez l'Homme. Cependant, dans ces études, l'exposition au N_2O était faiblement caractérisée (Eftimova *et al.*, 2017 ; Uzun *et al.*, 2014 ; Axelsson *et al.*, 1996 ; Rowland *et al.*, 1995 ; Heidam *et al.*, 1984 ; Cohen *et al.*, 1980).

Données animales

Plusieurs équipes ont étudié les effets de l'exposition au N_2O chez les rattes gestantes. Dans une série d'études, le N_2O a induit une embryotoxicité et une tératogénicité chez les rats exposés pendant les jours 8, 9 ou 10 de la gestation après une seule administration de 24 heures. Une concentration sans effet a été identifiée à 350 000 ppm de N_2O. Suite à une exposition continue de 23 ou 24 heures par jour pendant toute la période de gestation, une augmentation des malformations a été observée à partir de 1 000 ppm et la concentration sans effet était de 500 ppm. Cependant, comme une exposition continue de 24 heures ne correspond pas à un scénario d'exposition professionnelle, ces études n'ont pas été utilisées pour établir la valeur limite d'exposition professionnelle pour le N_2O.

Cinq études ont étudié les effets du N_2O chez le rat après une exposition de 4 à 8 heures par jour pendant des jours spécifiques de la gestation ou pendant toute la période de gestation :

- Mazze *et al.* ont observé une augmentation significative des résorptions, associée à une diminution du nombre de fœtus vivants par implantation, après exposition au N_2O à 750 000 ppm, 6 heures par jour, pendant les jours 13 à 15 de la gestation. Une augmentation non significative des malformations a également été notée dans l'étude (Mazze *et al.*,1986) ;
- un retard du développement fœtal (poids, taille, ossification) a été observé à 100 000 ppm de N_2O chez les rattes exposées 8 heures par jour pendant toute la durée de gestation. Les auteurs n'ont pas observé d'augmentation des résorptions ou des fœtus morts suite à l'exposition *in utero* au N_2O (Pope *et al.,*1978) ;
- Vieira *et al.* ont observé une diminution de la taille des portées après une exposition 6 heures par jour, 5 jours par semaine à 5 000 ppm de N_2O pendant toute la durée de gestation. La pertinence toxicologique de l'effet pose question en l'absence d'autres effets concomitants (ex : résorptions). Aucun effet n'a été observé aux doses de 500 ou 1 000 ppm dans cette étude (Vieira *et al.*, 1983b) ;
- le N_2O n'a pas induit de toxicité développementale après exposition jusqu'à 1 000 ppm, 6 à 7 heures par jour pendant toute la période de gestation (Hardin *et al.*,1981) ;
- le N_2O n'a pas induit de toxicité sur le développement après une exposition des rattes à 200 000 ppm de N_2O, 8 heures par jour, 5 jours par semaine pendant toute la période de gestation (Rao *et al.*, 1981).

L'étude neurocomportementale, chez les petits exposés *in utero* aux jours 13 ou 14-15 de la gestation à 750 000 ppm de N_2O, 8 heures par jour, indique un effet potentiel sur la réactivité des petits (Mullenix *et al.*, 1986). A doses plus faibles, aucun effet sur le comportement des petits exposés *in utero*, sur la taille des portées ou le poids des petits, n'a été rapporté par Holson *et al.* jusqu'à 10 000 ppm (6 heures par jour, 5 jours par semaine) (Holson *et al.*,1995).

Dans l'ensemble, les effets sévères sur le développement ont été observés à des doses élevées ou après une exposition continue de 23-24 heures d'exposition continue au N_2O. Après une exposition pendant 4 à 8 heures par jour pendant la gestation, les effets les plus sensibles observés étaient la diminution de la taille de la portée à 5 000 ppm de N_2O (Vieira *et al.*,1983b), en l'absence d'autres effets sur le développement, et des retards de développement à 100 000 ppm dans l'étude de Pope *et al.* de 1978.

- **Construction des VLEP**
  - ***VLEP-8h***
    - *Choix de l'effet critique*

L'analyse de l'ensemble des études de toxicité répétée réalisées par inhalation identifie les systèmes nerveux, hématologique, immunitaire et reproducteur comme les plus sensibles.

Des effets sur la fertilité et le développement ont également été rapportés chez le rat mais sans établir de relation dose-réponse. Bien qu'observés chez l'Homme et l'animal, les effets hématologiques et immunitaires n'ont pas été retenus comme effet critique du fait d'absence de relation dose-réponse, de l'existence de co-expositions non prises en compte ou de résultats équivoques.

L'effet critique, c'est-à-dire l'effet apparaissant aux concentrations les plus faibles, du protoxyde d'azote a été identifié comme étant l'altération des performances cognitives.

L'altération des performances cognitives est donc retenue comme effet critique pour la construction de la VLEP-8h.

- *Choix de l'étude clé*

Les données humaines sont considérées plus adéquates que les données chez l'animal.

La majorité des études de toxicité répétée, en milieu professionnel, sont des études transversales. A noter qu'aucune étude n'a caractérisé les effets à long terme de l'exposition au N_2O. Compte tenu des données disponibles, les études de Scapellato *et al.* de 2008 (étude réalisée sur 1 an avec 2 périodes d'observation) et celles de Lucchini *et al.* de 1995, 1996 et 1997 décrivant les effets observés en termes de performances neurocomportementales (altération de la vigilance) sont considérées comme les plus pertinentes pour l'établissement de la VLEP-8h. Elles ont donc été retenues comme études clés car :

- ces études complémentaires ont montré des effets similaires en utilisant des méthodologies différentes ;
- de nombreuses variables et biais potentiels ont été pris en compte et ces études ont permis d'étudier la relation dose-réponse par rapport à l'effet observé.
- Néanmoins, certaines limites ont été identifiées :
  - l'existence d'une co-exposition à de faibles concentrations à d'autres gaz anesthésiants ;
  - une possible sous-estimation des niveaux d'exposition liée à la méthode analytique utilisée.

Dans l'étude longitudinale d'un an de Scapellato *et al.* de 2008, l'exposition au N_2O a été mesurée dans les urines en fin de poste le lundi et le vendredi sans mesurage des niveaux de concentrations atmosphériques. Tous les sujets ont travaillé 7 heures et 12 minutes par jour pendant toute de la semaine d'observation. Dans l'étude de Lucchini *et al.* de 1996, les travailleurs ont été exposés soit une semaine à des anesthésiants non gazeux, soit à une utilisation constante d'anesthésiant gazeux (protoxyde d'azote). La concentration atmosphérique de N_2O a été mesurée par prélèvement individuel sur une durée de 3 heures au début de la semaine et le dernier jour de la semaine de travail dans les études de Lucchini *et al.* de 1995 et de 1996. Dans l'étude de Lucchini *et al.* de 1997, les auteurs ont procédé à des prélèvements d'air ambiant (stationnaire).

Dans ces quatre études, les auteurs ont effectué des prélèvements biologiques et mesuré la concentration de N_2O dans les urines en début et fin de poste (début et fin de semaine).

Globalement, l'exposition des travailleurs au N_2O dans ces études est considérée comme représentative des expositions professionnelles et pertinente pour la dérivation de la VLEP-8h.

Compte tenu de ces données et des autres études disponibles, les études de Scapellato *et al.* de 2008, de Lucchini *et al.* de 1995, 1996 et 1997 sont les études les plus pertinentes pour l'établissement de la VLEP-8h et sont donc retenues comme études clés.

- *Choix du point de départ (PoD)*

Concernant les niveaux de dose, une altération significative des performances cognitives a été notée à la concentration urinaire de 27 µg/L, correspondant à une concentration dans l'air de 50 ppm[3] en présence de 1,3 ppm d'isoflurane (dans Scapellato *et al.* de 2008). Ces altérations ont été observées à 54,2 ppm et 62,6 ppm en fin de semaine respectivement dans les études de Lucchini *et al.* de 1995 et 1996, en présence de 1,5 ppm d'isoflurane.

Les études de Lucchini *et al.* de 1997 et de Scapellato *et al.* de 2008 n'ont pas identifié d'effet sur les performances cognitives respectivement pour des expositions à 23,2 ppm de N_2O ou à moins de 27 µg/L de N_2O dans l'urine.

Sur la base des effets précédemment décrits, une LOAEC de 50 ppm (90 mg/m³) est identifiée. La NOAEC de 23,2 ppm, arrondie à 25 ppm (45 mg/m³), est retenue comme PoD.

- *Choix des facteurs d'incertitude*

Le calcul de la VLEP-8h à partir de la NOAEC a été effectué à l'aide des facteurs d'incertitude présentés dans le tableau suivant.

[3] Selon les auteurs (sur la base de la corrélation entre les concentrations urinaires et atmosphériques de l'étude d'Imbriani *et al.* de 1995).

Facteurs d'incertitude	Justification	Valeur
Variabilité inter-espèces (FI_A)	La VLEP se base sur des données humaines. Pas de FI_A à appliquer.	1
Variabilité inter-individuelle (FI_H)	La VLEP se base sur plusieurs études incluant une étude multicentrique, dont les sujets sont des hommes et des femmes. De plus, l'effet critique choisi est un paramètre très sensible. Pas de FI_H à appliquer.	1
Transposition subchronique – chronique (FI_S)	Dans l'une des études clé, les travailleurs exposés professionnellement à la substance ont été suivis pendant un an. Pas de FI_H à appliquer.	1
Utilisation d'une BMDL, LOAEC ou NOAEC (FI_L)	Le point de départ retenu est une NOAEC. Pas de facteur additionnel à appliquer. Sévérité des effets : les effets étaient réversibles. Pas de FI_L à appliquer.	1
Incertitudes dues aux lacunes de la base de données (FI_D)	Bien qu'il existe des incertitudes dans la relation dose-réponse pour la toxicité pour la reproduction, compte tenu d'un point de départ de 5 000 ppm identifié chez l'animal pour le retard de développement fœtal, une VLEP-8h de 33 ppm serait dérivée en considérant un FA total de 150 (10 pour FI_A, 5 pour FI_H et 3 pour l'extrapolation LOAEC à NOAEC). Par conséquent, l'effet sur le système nerveux est considéré comme l'effet le plus sensible. Cet effet a été largement étudié dans la littérature. Pas de FI_D à appliquer.	1

Le facteur d'incertitude global pour la dérivation de la VLEP-8h est donc de 1.

- *Proposition de VLEP-8h*

Une **VLEP-8h de 45 mg/m³** (25 ppm) est donc recommandée.

La VLEP-8h recommandée devrait également protéger des effets délétères hématologiques, sur le système immunitaire et le développement. Néanmoins, il n'est pas possible de déterminer si cette valeur protège des effets sur la fertilité en l'absence de données humaine et animale fiables.

○ *VLCT-15min*

Faute de données disponibles quant aux effets toxiques à court terme du protoxyde d'azote, le CES VSR recommande, conformément à sa méthodologie (Anses, à paraître), de ne pas dépasser sur une période de 15 minutes la valeur de 5 fois la valeur de la VLEP-8h, soit 225 mg/m³ (125 ppm), afin de limiter l'amplitude et le nombre des pics d'exposition.

Le CES VSR recommande une **VLCT-15 min pragmatique de 225 mg/m³**.

○ *Mention « peau »*

En l'absence de donnée, la mention « peau » n'est pas recommandée.

○ *Mention « bruit »*

En l’absence de donnée sur d’éventuelles interactions lors de co-expositions au bruit et au protoxyde d’azote, la mention « bruit » n’est pas recommandée.

Résultat de l'expertise collective concernant les méthodes de mesure atmosphérique dans les lieux de travail

- **Évaluation des méthodes de mesure du protoxyde d'azote dans l'air des lieux de travail**

Six méthodes de mesure du protoxyde d'azote dans l'air des lieux de travail ont été recensées et évaluées (Tableau 1)

Tableau 1 : Recensement et classement des méthodes de mesure du protoxyde d'azote dans l'air des lieux de travail au regard de la VLEP-8h et de la VLCT-15min pragmatique recommandées

	Méthodes	Protocoles	VLEP-8h	VLCT-15min pragmatique	
			Contrôle technique réglementaire	Contrôle technique réglementaire	Suivi des expositions court terme
1	Prélèvement passif sur support adsorbant Désorption thermique Analyse par spectroscopie infrarouge (IR)	DFG Method 2 (2006) OSHA ID-166 (1994)	1B	3	3
2	Prélèvement actif sur support adsorbant Désorption thermique Analyse par chromatographie gazeuse (GC) couplée à un détecteur à capture d'électrons (ECD) ou détecteur de conductivité thermique (TCD)	DFG Method 3 (2006)	2	2	2
		INRS MétroPol M-416 (2022)	3	1B	1B
3	Prélèvement passif sur support adsorbant Désorption thermique Analyse par GC-ECD ou TCD	DFG Method 3 (2006)	3*	3*	3*
		INRS MétroPol M-415 (2022)	1A	3	3
4	Analyseur infrarouge à transformée de Fourier en continu	DFG Method 1 (1989) NIOSH 3800 (2016) NIOSH 6600 (1994)	3	3	3
5	Instrument à lecture directe – détecteur photo-acoustique (ILD-PA)	IRSST 320-1 (non daté)	3	3	3
6	Prélèvement actif à l'aide d'un sac Tedlar – analyse par GC-ECD	INSHT MTA/MA-020/A91 (1991)	3	3	3

Les deux figures suivantes présentent les plages pour lesquelles les différentes méthodes ont été validées et leur limite de quantification au regard de la VLEP- 8h et

de la VLCT-15min pragmatique recommandées par le CES. Les méthodes classées en catégorie 3, sur la base de critères d'exclusion, ne sont pas représentées.

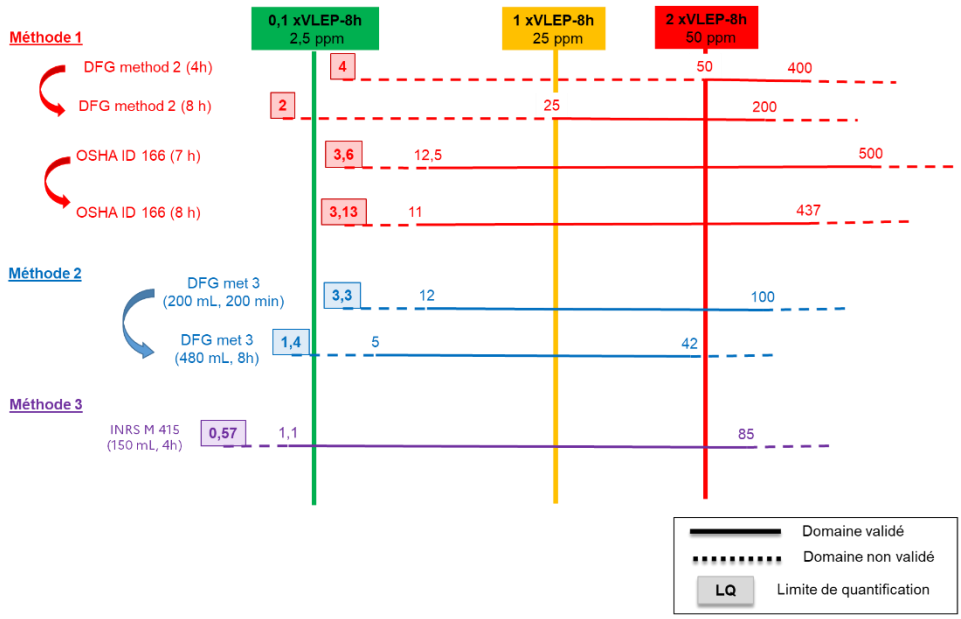

Figure 1 : Domaine de validité et limites de quantification des méthodes comparés au domaine 0,1 à 2 fois la VLEP-8h proposée par le CES

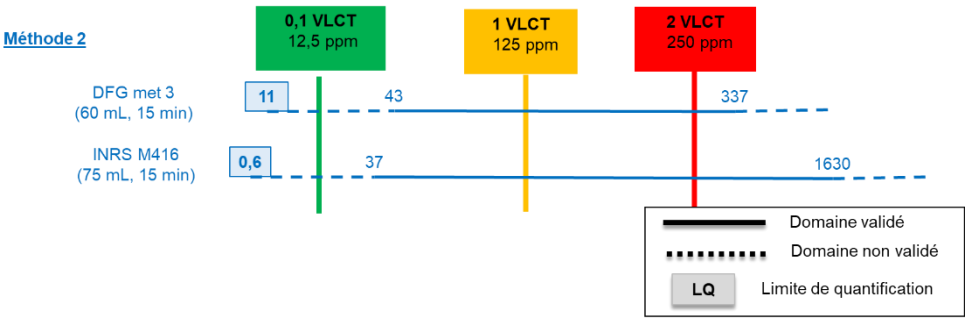

Figure 2 : Domaine de validité et limites de quantification des méthodes comparés au domaine 0,1 à 2 fois la VLCT-15min pragmatique proposée par le CES

- Méthode 1

La méthode consiste à effectuer un prélèvement passif sur tube adsorbant constitué de tamis moléculaire, puis une désorption thermique et une analyse par spectroscopie infrarouge. La méthode est décrite dans les protocoles DFG Method 2 et OSHA-ID166.

De nombreux critères de validation essentiels sont disponibles et répondent aux exigences, notamment grâce aux données mentionnées dans le protocole OSHA ID 166. La méthode permet de couvrir la gamme de concentration de 2,5 à 50 ppm (correspondant à 0,1 à 2 fois la VLEP-8h) avec un prélèvement de 8 heures, bien que les données de validation aient été établies pour des durée de 4 ou 7 heures. Cette méthode est donc classée en catégorie 1B pour le contrôle technique réglementaire de la VLEP-8h.

Toutefois, le débit de diffusion varie avec la durée d'échantillonnage et aucune donnée sur la stabilité de ce débit n'est disponible pour des durées inférieures à 2 heures. Ainsi la méthode

est classée en catégorie 3 pour le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme.

- Méthode 2

La méthode consiste à effectuer un prélèvement actif au travers d'un support adsorbant, puis une désorption thermique et une analyse par chromatographie gazeuse (GC) couplée à un détecteur par capture d'électron (ECD) ou un détecteur de conductivité thermique (TCD). La méthode est décrite dans les protocoles DFG Method 3 et INRS MétroPol M-416, qui préconisent des supports différents : le protocole DFG Method 3 recommande un support constitué de tamis moléculaire 5Å alors que le protocole INRS MétroPol M-416 recommande un tube contenant 750 mg de zéolithe BaZSM5.

De nombreux critères essentiels de validation sont disponibles et répondent aux exigences. Néanmoins, des hypothèses ont été émises concernant le volume d'air prélevé lors des tests de validation dans le protocole DFG Method 3 pour l'analyse des données et la durée de prélèvement qui ne doit pas excéder 30 min dans les conditions du protocole INRS MétroPol M-416.

La méthode 2 est donc classée, dans les conditions du protocole DFG Method 3, en catégorie 2 pour le contrôle technique réglementaire de la VLEP-8h, le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme. Dans les conditions du protocole INRS MétroPol M-416, cette méthode est classée en catégorie 1B pour le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme, mais en catégorie 3 pour le contrôle technique réglementaire de la VLEP- 8h.

- Méthode 3

La méthode consiste à effectuer un prélèvement passif sur un support adsorbant, suivi d'une désorption thermique puis d'une analyse par GC-ECD ou TCD. La méthode est décrite dans les protocoles DFG Method 3 et INRS MétroPol M-415, qui préconisent des supports différents : respectivement un tamis moléculaire 5Å et 750 mg de zéolithe BaZSM5.

Pour le protocole DFG Method 3, une durée d'échantillonnage de 4 à 8 heures est recommandée. Les données de validation rapportées par ce protocole ne précisent pas toujours la durée d'échantillonnage associée ou mentionnent une durée de « 4 à 8h ». Or, la nature de l'adsorbant étant identique à la méthode 1, le taux de diffusion varie avec la durée de prélèvement. C'est pourquoi, les données de ce protocole n'ont pas été prises en compte dans l'évaluation de la méthode qui repose donc uniquement sur les données du protocole INRS MétroPol M-415.

L'ensemble des critères essentiels de validation sont disponibles dans le protocole INRS MétroPol M-415 et répondent aux exigences. Dans les conditions de ce protocole, la méthode 3 couvre la gamme de concentration de 2,5 à 50 ppm (correspondant à 0,1 à 2 fois la VLEP-8h) avec deux prélèvements successifs de 4 heures, mais cette méthode ne permet pas d'effectuer un prélèvement d'une durée inférieure à une heure. Cette méthode est donc classée en catégorie 1A pour le contrôle technique réglementaire de la VLEP-8h, mais en catégorie 3 pour le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme.

- Méthode 4

La méthode, décrite par les protocoles DFG Method 1 (1989), NIOSH 3800 (2016) et NIOSH 6600 (1994), met en œuvre un instrument portable à lecture directe dont le débit dépend du système, entre 0,1 et 20 $L.min^{-1}$. La technique d'analyse est la spectroscopie infrarouge à transformée de Fourier.

Cette méthode est adaptée pour des mesures ambiantes mais pas des mesures individuelles. Les spectres des échantillons doivent être acquis à chaque point d'échantillonnage pendant une période de temps qui n'est pas inférieure au temps de réponse du système (dépendant de l'équipement). Cette méthode permet d'obtenir l'évolution des concentrations et d'identifier les périodes de forte exposition. Elle peut également être utilisée pour obtenir rapidement des informations sur l'air ambiant en différents endroits.

La méthode 4 est donc classée en catégorie 3 pour le contrôle technique réglementaire de la VLEP-8h et de la VLCT-15min pragmatique ainsi que pour le suivi des expositions court terme.

- Méthode 5

La méthode est décrite dans le protocole IRSST 320-1 et met en œuvre un instrument à lecture directe avec détection photo-acoustique. La valeur minimale rapportée est de 0,05 ppm. Cette valeur semble couvrir l'extrémité inférieure des plages de concentration de la VLEP-8h et de la VLCT-15min pragmatique. Cette méthode ne permet pas de réaliser des mesures individuelles et aucune donnée de validation n'est disponible.

En l'état, la méthode 5 est donc classée en catégorie 3 pour le contrôle technique réglementaire de la VLEP- 8h et de la VLCT-15min pragmatique ainsi que pour le suivi des expositions court terme.

- Méthode 6

La méthode est décrite dans le protocole INSST MTA/MA-020/A91. Elle consiste à effectuer un prélèvement actif à l'aide d'un sac Tedlar d'une capacité de 5 litres, puis une analyse par GC-ECD.

De nombreux critères essentiels de validation de la méthode ne sont pas renseignés dans le protocole INSST MTA/MA-020/A91. De plus, cette méthode est plus adaptée aux mesures d'ambiance qu'aux mesures individuelles. La méthode 6 est donc classée en catégorie 3 pour le contrôle technique réglementaire de la VLEP- 8h et de la VLCT-15min pragmatique ainsi que pour le suivi des expositions court terme

- **Conclusions et recommandations**

Six méthodes de mesure du N_2O dans l'air des lieux de travail ont été identifiées et évaluées :

- méthode 1 : Prélèvement passif sur support adsorbant suivi d'une désorption thermique et d'une analyse par spectroscopie infrarouge ;
- méthode 2 : Prélèvement actif sur support adsorbant suivi d'une désorption thermique et d'une analyse par GC-ECD ou TCD ;
- méthode 3 : Échantillonnage passif sur un support adsorbant suivi d'une désorption thermique et d'une analyse par GC-ECD ou TCD ;

- méthode 4 : Analyseur infrarouge à transformée de Fourier en continu ;
- méthode 5 : Instrument de lecture directe - détection photo-acoustique (ILD-PA) ;
- méthode 6 : Échantillonnage actif à l'aide d'un sac Tedlar, analyse par GC-ECD.

Les méthodes 4, 5 et 6 sont classées en catégorie 3 et ne sont pas recommandées pour le contrôle technique réglementaire de la VLEP-8h, le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme du fait que ces méthodes sont adaptées pour des mesures d'ambiance, mais ne permettent pas d'effectuer des mesures individuelles. A noter que la méthode 4 (analyseur infrarouge en continu) et la méthode 5 (instrument à lecture directe - détection photoacoustique (ILD-PA)) permettent de suivre l'évolution des concentrations et d'identifier les périodes de forte exposition. De plus, elles peuvent être utilisées pour obtenir rapidement des informations sur l'air ambiant en différents endroits.

Pour le contrôle technique réglementaire de la VLEP-8h :

- la méthode 1 est classée en catégorie 1B en raison de nombreux critères essentiels de validation qui répondent aux exigences, notamment au travers des données mentionnées dans le protocole OSHA ID 166 obtenues pour des durées de 4 à 7 heures ;
- la méthode 2 est classée en catégorie 2 dans les conditions du protocole DFG Method 3 en raison des hypothèses formulées sur le volume d'air prélevé pour l'évaluation des données de validation, mais en catégorie 3 dans les conditions du protocole INRS MétroPol M-416 en raison d'une durée de prélèvement qui ne peut excéder 30 minutes sur le support adsorbant préconisé ;
- la méthode 3 est classée en catégorie 1A dans les conditions préconisées par le protocole INRS MétroPol M-415 avec deux prélèvements successifs de 4 heures. Le protocole DFG Method 3 mettant en œuvre un support adsorbant différent n'a pas été évalué compte tenu de l'absence de données de validation relatives à ce support.

Ainsi, les méthodes 1 (dans les conditions du protocole OSHA ID 166) et 3 (dans les conditions du protocole INRS MétroPol M-415 avec deux prélèvements successifs de 4 heures) sont recommandées.

Pour le contrôle technique réglementaire de la VLCT-15min pragmatique et le suivi des expositions court terme :

- la méthode 1 est classée en catégorie 3 en raison d'un débit d'échantillonnage variable avec la durée d'échantillonnage et d'un manque de données sur la stabilité de ce débit sur 15 min ;
- la méthode 2 est classée en catégorie 1B dans les conditions du protocole INRS MétroPol M 416, mais en catégorie 2 dans les conditions du protocole DFG Method 3 en raison des hypothèses formulées sur le volume d'air prélevé pour l'analyse des données de validation ;
- la méthode 3 est classée en catégorie 3 dans les conditions du protocole INRS MétroPol M-415 en raison d'une durée de prélèvement qui ne peut être inférieure à 1 heure. Les données du protocole DFG Method 3 n'ont pas été prises en compte dans l'évaluation en raison d'un manque d'information sur les durées d'échantillonnage associées aux données de validation mentionnées.

Ainsi, **la méthode 2, dans les conditions du protocole INRS MétroPol M 416, est recommandée**.

Le tableau suivant présente les méthodes de mesure du protoxyde d'azote recommandées.

Tableau 2 : Méthodes recommandées pour la mesure du protoxyde d'azote dans l'air des lieux de travail au regard de la VLEP-8h et de la VLCT-15 min pragmatique

Méthodes		Protocoles	VLEP-8h	VLCT-15min pragmatique	
			Contrôle technique réglementaire	Contrôle technique réglementaire	Suivi des expositions court terme
1	Prélèvement passif sur support adsorbant Désorption thermique Analyse par spectroscopie infrarouge (IR)	OSHA ID-166 (1994)	1B	3 (non recommandée)	
2	Prélèvement actif sur support adsorbant Désorption thermique Analyse par chromatographie gazeuse (GC) couplée à un détecteur à capture d'électrons (ECD) ou détecteur de conductivité thermique (TCD)	INRS MétroPol M-416 (2022)	3 (non recommandée)	1B	
3	Prélèvement passif sur support adsorbant Désorption thermique Analyse par GC-ECD ou TCD	INRS MétroPol M-415 (2022)	1A	3 (non recommandée)	

Collective expert appraisal report

Acronyms and abbreviations

ACGIH:	American Conference of Governmental Industrial Hygienists
AChE:	Acetylcholinesterase
Anses :	*Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail* [French agency for food, environmental and occupational health & safety]
ANSM:	*Agence nationale de sécurité du médicament et des produits de santé* [French agency for the safety of medicines and health products]
BW:	Body weight
CA:	Chromosomal aberration
CAS:	Chemical abstract service
CFAM:	Cerebral function analyzing monitor
CI:	Confidence interval
CNS:	Central nervous system
CPT:	Continuous performance test
CTE:	Chronic toxic encephalopathy
DFG:	Deutsche Forschungsgemeinschaft [German research foundation]
DNA:	Deoxyribonucleic acid
DRI-PAD:	Direct reading instrument - photoacoustic detection
dU:	Deoxyuridine
ECD:	Electron capture dection
DSST:	Digit symbol substitution test
DUST:	Deoxyuridine suppression test
EC50:	Median effective dose
ECHA:	European chemicals agency
EIGA:	European Industrial Gases Association
EINECS:	European Inventory of Existing Chemical Substances
EW:	End of week
FPG:	Formamidopyrimidine glycosylase
FTIR:	Fourier-transform infrared spectroscopy
FTT:	Finger tapping test
GC:	Gas chromatography
GD:	Gestation day
GFAP:	Glial fibrillary acidic protein
GM:	Geometric mean
Hb:	Haemoglobin
HCN:	Health council of the Netherlands
Ht:	Haematocrit

HRV:	Health reference value
INRS:	*Institut national de recherche et de sécurité pour la prévention des accidents du travail et des maladies professionnelles [French institute for research and security*]
INCHEM:	Internationally peer reviewed chemical safety information
INSHT:	Instituto Nacional de Seguridad e Hygiene en el Trabajo (now called INSST: Instituto Nacional de Seguridad y Salud en el Trabajo)
IPCS:	International programme on chemical safety
IR:	Infrared
IRSST:	Institut de recherche Robert-Sauvé en santé et en sécurité du travail
IUPAC:	International Union of Pure and Applied Chemistry
LOAEC:	Lowest observed adverse effect concentration
LOD:	Level of detection
LOQ:	Limit of quantification
MAC:	Minimal alveolar concentration
MAK:	Maximale Arbeitsplatzkonzentration [maximum workplace concentration]
MCH:	Mean corpuscular haemoglobin
MCHC:	Mean cell heamoglobin concentration
MCV:	Mean corpuscular volume
MLC:	Mix lymphocyte culture
MLR:	Mixed lymphocyte reaction
MN:	Micronucleus
MoA:	Mode of action
NA:	Not applicable
NB:	Nota bene
NES:	Neurobehavioural evaluation system
NMDA:	N-methyl-D-aspartate
NIOSH:	U.S. National institute for occupational safety and health
NK:	Natural killer
NOAEC:	No observed adverse effect concentration
NS:	Not specified
NTP-OHAT:	National toxicology program – office of health assessment and translation
OEL:	Occupational exposure limit
OELV:	Occupational exposure limit value
OSHA	U.S. Occupational Safety and Health Administration
PET:	Positron emission tomography
PFC	Plaque forming cell
PHA:	Phytohaemagglutinin
PND	Postnatal day

ppm:	Parts per million
RBC:	Red blood cell
rCBF:	Regional cerebral flow
rCMR:	Regional cerebral metabolic rate
RAC:	Risk assessment committee
REACh:	Regulation (EC) No 1907/206 of 18/12/06 concerning the registration, evaluation, and authorisation of chemicals (REACh)
REL:	Recommended exposure limit
RH:	Relative humidity
SCOEL:	Scientific committee on occupational exposure limits
STOT SE:	Specific target organ toxicity - Single exposure
STOT RE:	Specific target organ toxicity - Repeated exposure
OR:	Odd ratio
RoB:	Risk of bias
SCE:	Sister chromatid exchange
SD:	Standard deviation
SDT:	Symbol digit test
STEL:	Short term exposure limit
SRBC:	Sheep red blood cell
SRT:	Simple reaction time
SW:	Start of week
TCD:	Thermal conductivity detection
THF:	Tetrahydrofolic acid
TLV:	Threshold limit value
TWA:	Time weighted average
UF:	Uncertainty factor
US:	United States
WBC:	White blood cell

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Preambule

The French system for establishing Occupational Exposure Limits (OELVs) has three clearly distinct phases:

- Independent scientific expertise (the phase entrusted to ANSES);
- Proposal by the Ministry of Labour of a draft regulation for the establishment of limit values, which may be binding or indicative;
- Stakeholder consultation during the presentation of the draft regulation to the French Steering Committee on Working Conditions. The aim of this phase is to discuss the effectiveness of the limit values and if necessary to determine a possible implementation timetable, depending on any technical and economic feasibility.

The organisation of the scientific expertise phase required for the establishment of Occupational Exposure Limits Values (OELVs) was entrusted to the agency in the framework of the French 2005-2009 Occupational Health Plan.

The OELs, as proposed by the “Health reference values” Committee (HRV Committee), are concentration levels of pollutants in workplace atmospheres that should not be exceeded over a determined reference period and below which the risk of impaired health is considered as negligible. Although reversible physiological changes are sometimes tolerated, no organic or functional damage of an irreversible or prolonged nature is accepted at this level of exposure for the large majority of workers. These concentration levels are determined by considering that the exposed population (the workers) is one that excludes both children and the elderly.

These concentration levels are determined by the HRV Committee experts based on information available from epidemiological, clinical and animal toxicology studies. Identifying concentrations that are safe for human health are the results of correction factors applied to the values identified directly by the studies. These corrections factors take into account a number of uncertainties inherent to the extrapolation process conducted as part of an assessment of the health effects of chemicals on humans.

The Committee recommends the use of three types of values:

- 8-hour occupational exposure limit (8h-OEL): this corresponds to the limit of the time-weighted average (TWA) of the concentration of a chemical in the worker's breathing zone over the course of an 8-hour work shift. In the current state of scientific knowledge (toxicology, medicine and epidemiology), the 8h-OEL is designed to protect workers exposed regularly and for the duration of their working life from the medium- and long-term health effects of the chemical in question;
- Short-term exposure limit (15min-STEL): this corresponds to the limit of the time-weighted average (TWA) of the concentration of a chemical in the worker's breathing zone over a 15-minute reference period during the peak of exposure, irrespective of its duration. It aims to protect workers from adverse health effects (immediate or short-term toxic effects such as irritation phenomena) due to peaks of exposure;
- Ceiling value: this is the limit of the concentration of a chemical in the worker's breathing zone that should not be exceeded at any time during the working period. This value is recommended for substances known to be highly irritating or corrosive or likely to cause serious potentially irreversible effects after a very short period of exposure.

These three types of values are expressed:

- in $mg.m^{-3}$, i.e. in milligrams of chemical per cubic meter of air, or in ppm (parts per million), i.e. in cubic centimetres of chemical per cubic meter of air, for gases and vapours;

- or in $mg.m^{-3}$ only for liquid (fog) and solid (fumes) aerosols;
- or in $f.cm^{-3}$, i.e. in fibres per cubic centimetre for fibrous materials.

The 8h-OELV may be exceeded for short periods during the working day provided that:

- the weighted average of levels calculated over the entire working day is not exceeded;
- The short term exposure limit (15min-STEL) value, when one exists, is not exceeded.

In addition to the OELs, the HRV Committee assesses the need to assign a “skin” notation, when significant penetration through the skin is possible. This notation indicates the need to consider the dermal route of exposure in the exposure assessment and, where necessary, to implement appropriate preventive measures (such as wearing protective gloves). Skin penetration of substances is not taken into account when determining the atmospheric limit levels, even it can potentially cause health effects even when the atmospheric levels are respected.

The HRV Committee assesses the need to assign a “noise” notation indicating a risk of hearing impairment in the event of co-exposure to noise and the substance below the recommended OELs, to enable OSH experts to implement appropriate measures (collective, individual and/or medical) (Anses to be released).

The HRV Committee also assesses the applicable reference methods for the measurement of exposure levels in the workplace. The quality of these methods and their applicability to the measurement of exposure levels for comparison with an OEL are assessed, particularly with regards to their compliance with the performance requirements in the NF-EN 482 Standard and the decision-making criteria listed in the methodology report (ANSES, 2020b)[4]. Once they have been assessed, these methods can be classified into one of the following categories:

- category 1A: validated methods (all of the performance criteria are met);
- category 1B: partially validated methods (the essential performance criteria are met);
- category 2: indicative methods (essential criteria for validation are not clear enough or else the method requires adjustments that need to be validated);
- category 3 : methods not recommended because they are unsuitable (essential validation criteria are not fulfilled)
- category 3*: methods not recommended because they cannot be evaluated (essential validation criteria are not documented).

NB : For the measurement of aerosols and substances in mixed phases, an initial classification is established with regard to the performance criteria for sampling methods. A second classification is then established with regard to the performance criteria for analytical methods. The final classification of the method corresponds to the least favourable of these two classifications.

A detailed comparative study of the methods in categories 1A, 1B and 2 was conducted with respect to their various validation data and technical feasibility, in order to recommend the most suitable method(s) for measuring concentrations for comparison with OELs.

Organisation of the expert appraisal

[4] NF EN 482 : "Workplace atmospheres - General requirements for the performance of procedures for the measurement of chemical agents"

ANSES entrusted examination of this request to the expert committee on health reference value (HRV Committee). The « metrology » working group was mandated for the assessment of the measurement methods in workplace air.

The methodological and scientific aspects of the work were regularly submitted to the Expert Committee.

The report produced takes account of observations and additional information provided by the Committee members.

The collective expert appraisal work in English and the conclusions and recommendations in French were adopted for public consultation by the HRV Committee on 1st July 2022. These documents were submitted for public consultation from 22 June to 15 September 2023. The people or organizations who contributed to the public consultation are listed in appendix 6.The comments received were reviewed by the HRV Committee who finally adopted this document on 9 November 2023.

This expert appraisal was therefore conducted by a group of experts with complementary skills. It was carried out in accordance with the French Standard NF X 50-110 “Quality in Expertise Activities”.

Preventing risks of conflicts of interest

ANSES analyses interests declared by the experts before they are appointed and throughout their work in order to prevent potential conflicts of interest in relation to the points addressed in expert appraisals.

The experts’ declarations of interests are published on the website https://dpi.sante.gouv.fr/.

Description of the method

For the assessment of the health effects, a toxicological profile was prepared by Anses's officers and submitted to the HRV Committee, which commented on it and added to it.

The toxicological profile is mainly based on literature search that is described in Annex I.

For all the relevant studies retained in the synthesis, internal validity of the studies were checked and scored as described in Annex 2.

For the assessment of the measurement methods, a summary report was prepared by the WG on Metrology and submitted to the HRV Committee, which added its own comments.

The summary report presents the various protocols for measuring nitrous oxide in workplace atmospheres, which were identified and grouped according to the methods used. These methods were then assessed and classified based on the performance requirements set out particularly in the French Standard NF EN 482: "Workplace atmospheres - General requirements for the performance of procedures for the measurement of chemical agents" and the decision-making criteria listed in the methodology report (ANSES, 2020b).

A list of the main sources consulted is detailed in the methodology report (ANSES, 2020b).

These methods were classified as follows:

- category 1A: validated methods (all of the performance criteria are met);
- category 1B: partially validated methods (the essential performance criteria are met);

- category 2: indicative methods (essential criteria for validation are not clear enough or else the method requires adjustments that need to be validated);
- category 3 : methods not recommended because they are unsuitable (essential validation criteria are not fulfilled)
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NB : For the measurement of aerosols and substances in mixed phases, an initial classification is established with regard to the performance criteria for sampling methods. A second classification is then established with regard to the performance criteria for analytical methods. The final classification of the method corresponds to the least favourable of these two classifications.

A detailed comparative study of the methods in categories 1A, 1B and 2 was conducted with respect to their various validation data and technical feasibility, in order to recommend the most suitable method(s) for measuring concentrations for comparison with OELs.

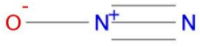
Part A – Assessment of health effects

1 General information

Nitrous oxide and N_2O are used interchangeably in the report.

1.1 Substance identification

Table 1: Substance identity and structural formula of the substance

IUPAC name	**Nitrous oxide**
CAS Number	10024-97-2
EINECS Number	233-032-0
Molecular formula	N_2O
Structural formula	
Synonyms	Dinitrogen monoxide Laughing gas Nitrogen hypoxide Nitrogen protoxide Hyponitrous acid anhydride

1.2 Physico-chemical properties

In normal condition, nitrous oxide (N_2O) is a colourless, non-flammable gas, with a pleasant smell. A summary of its physico-chemical properties is provided in the table below.

Table 2: Summary of physico-chemical properties of N_2O (ACGIH, 2018 and INRS, 2018)

Physical appearance at 20°C, 1013.25hPa	Gas, colourless, sweetish odour
Molecular weight ($g.mol^{-1}$) :	44.02
Melting point (°C) :	-90.81 °C
Boiling point (°C):	-88.46 °C
Vapour pressure	5070 to 5850 kPa at 20°C
Relative density	1.5 (Air =1)
Solubility	Soluble in water, alcohol, ether, oils, sulfuric acid
Log Pow	0.4 at 25°C
Oxidizing properties	Oxidizing
Conversion factors at 25°C and 1023 hPa :	1 $mL.m^{-3}$ (ppm) = 1.80 $mg.m^{-3}$ 1 $mg.m^{-3}$ = 0.556 $mL.m^{-3}$ (ppm)

1.3 European classification

N_2O has no European harmonised classification under Regulation (EC) no. 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation).

Nevertheless, France has submitted a proposal to ECHA for harmonised classification on 25 April 2022 as Repr. 1B, H360Df “May damage the unborn child, Suspected of damaging fertility”, STOT SE 3 (H336) “may cause drowsiness or dizziness”, STOT RE 1 (H372) “Cause damage to organs (nervous system) through prolonged or repeated exposure” and Ozone 1 (H420) “Harms public health and the environment by destroying ozone in the upper atmosphere”. The final deadline for the adoption of the opinion by the risk assessment committee is October 25th, 2023.

1.4 Major uses and sources

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

N_2O is also an industrial chemical used in food industry as a food additive (E942). N_2O is a propellant in canister used in many preparations (e.g. aerate whipping cream, inflate balloons).

In addition, N_2O is used in laboratory as an oxidizing agent in atomic flame absorption spectrometry.

It is also an additive to rocket fuels to increase available oxygen for combustion.

Recreational abuse of the gas, also called “laughing gas”, has been increased in recent years due to its euphoric, relaxing and hallucinogenic properties. A toxicoviligance report, developed with the support of poison control centers, highlights adverse effects that can be severe, such as heart rhythm disorders, risk of asphyxiation, mental disorders and neurological damage (ANSES, 2020a).

N_2O is naturally emitted from soils and oceans. Emission of N_2O from human sources is mainly due to agricultural activities and to a lesser extent to other sources such as the health sector. It is a long-lived (atmospheric lifetime of 114 years) greenhouse gas that accumulates in the atmosphere and contribute to global warming.

2 Overview of existing recommended occupational limit values

In Europe, there is no recommendation for N_2O from the Scientific Committee on Occupational Exposure Limits (SCOEL) or the Risk Assessment Committee (RAC) of the European Chemicals Agency (ECHA) available to date.

In 1993, the German DFG ("Deutsche Forschungsgemeinschaft") set a provisional MAK[5] (« Maximale Arbeitsplatz-Konzentration » or maximum workplace concentration) value at 100 ppm (180 mg/m^3) based on effect on the microsomal enzyme system observed in persons exposed to 200 ppm N_2O. In the addendum of 2007 (published in 2015) on reproductive toxicity, a NOAEC of 10 000 ppm was retained for developmental toxicity based on Holson *et al.*, 1995 and Pope *et al.*, 1978. It was concluded by the German committee that no developmental toxicity was expected at the MAK value of 100 ppm (180 mg/m^3).

In the United States (US), the American Conference of Governmental Industrial Hygienists (ACGIH) recommended an 8-hour threshold limit value – time weighed average (TLV-TWA) of 50 ppm (90 mg.m^{-3}) (ACGIH, 2018; last update in 1996). ACGIH considered that the human reproductive, hematologic and nervous systems were the most susceptible target organs. ACGIH concluded that this value should be protective for potential embryofoetal toxicity, increased risk of spontaneous abortion, bone marrow depression and psychomotor and cognitive functions. ACGIH did not recommend a short-term exposure limit (STEL) or specific notations.

In 1977, the U.S. National Institute for Occupational Safety and Health (NIOSH) set a REL of 25 ppm (TWA over the time exposed) to prevent decreases in mental performance, audio-visual ability, and manual dexterity during exposures to N_2O. The NIOSH REL for nitrous oxide is only intended for exposure to waste anesthetic gases (Niosh Pocket guide[6]).

[5] The MAK value was published in 1998

[6] NIOSH pocket guide entry for nitrous oxide, https://www.cdc.gov/niosh/npg/npgd0465.html consulted in October 2023

3 Toxicokinetics

3.1 Absorption

In humans, N_2O is mainly absorbed through inhalation. The rate of N_2O uptake during the first 1 or 2 minutes is about 1.0 L/min at an inspired concentration of 80% (INRS, 2018[7]). Due to the high infusibility and low solubility of N_2O, the alveolar concentration reaches the inhaled concentration in less than 5 min (National Agency for Medicines and Health Products' Safety (ANSM), 2014[8]). The blood/gas partition is 0.47.

There is a lack of data in experimental animals.

3.2 Distribution

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

Partition coefficients, as reported by Stenqvist *et al.*, are presented in table below (Table 3) (Stenqvist *et al.*, 1994). The total body uptake of N_2O is relatively smaller than for more soluble anaesthetics like isoflurane and desflurane (13 times more soluble in fat than N_2O).

Table 3 : Partition coefficient of N_2O (Stenqvist *et al.*, 1994)

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

N_2O is able to cross the placental barrier (INRS, 2018).

3.3 Metabolism

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

As described in MAK report (DFG, 1993), N_2O is reduced to nitrogen in the reaction with the central Co^+ ion of vitamin B12 (Banks *et al.*, 1968; Hong *et al.*, 1980). After a single passage through the liver, however, the concentration of N_2O in the blood decreases by only 0.03 %. Formation of radicals has been demonstrated *in vitro* in human intestinal contents incubated with N_2O (Bösterling *et al.*, 1980).

[7] French National Research and Safety Institute for prevention of Occupational Accidents and diseases
[8] http://agence-prd.ansm.sante.fr/php/ecodex/rcp/R0234390.htm, consulted in May 2021 (only available in French)

3.4 Excretion

N_2O is almost completely eliminated unchanged by the lungs (in few minutes); only small amounts pass into the urine, and there is some minimal diffusion through the skin (INRS, 2018). N_2O is eliminated in the urine through a diffusion process determined by the equilibration of partial pressures in urine and plasma (Henderson *et al.*, 2002).

O'Reilly *et al.* exposed 20 healthy volunteers to sedative concentration of N_2O/O_2, young and elderly males to identify any aged-related differences, under a protocol to mimic a dental operatory (O'Reilly *et al.*, 1983). The authors measured N_2O in expired gas (at the end of 30 min inhalation and periodically for 70 min after withdrawal). Two elimination phases were identified with half-lives of about 1.8 minutes for the first one and of 20 minutes for the second.

Sixteen hours after the end of exposure, N_2O is completely eliminated from the blood (INRS, 2018).

3.5 Conclusion on toxicokinetics

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

4 Mechanism of action

Inactivation of methionine synthase

N_2O irreversibly inactivates methionine synthase function by oxidation of the Co^+ ion of vitamin B12. Methionine synthase is a vitamin B12-dependent enzyme involved in folate metabolism. This enzyme converts L-homocysteine and 5-methyltetrahydrofolate into L-methionine and tetrahydrofolate, respectively, *via* a methylation process. Methionine is important for DNA and RNA synthesis, for histone methylation, synthesis of neurotransmitters and myelin, among other products. As a consequence, inactivation of methionine synthase results in a depletion of methionine and tetrahydrofolate, which are required for DNA synthesis and myelin production. In turn, this depletion results in a clinical picture that resembles the characteristic findings of pernicious anaemia (bone marrow depression and polyneuropathy) (Garakani *et al.*, 2016). In parallel, rise in homocysteine levels are observed and promote varying neurological and cardiovascular symptoms (Weimann *et al.*, 2003).

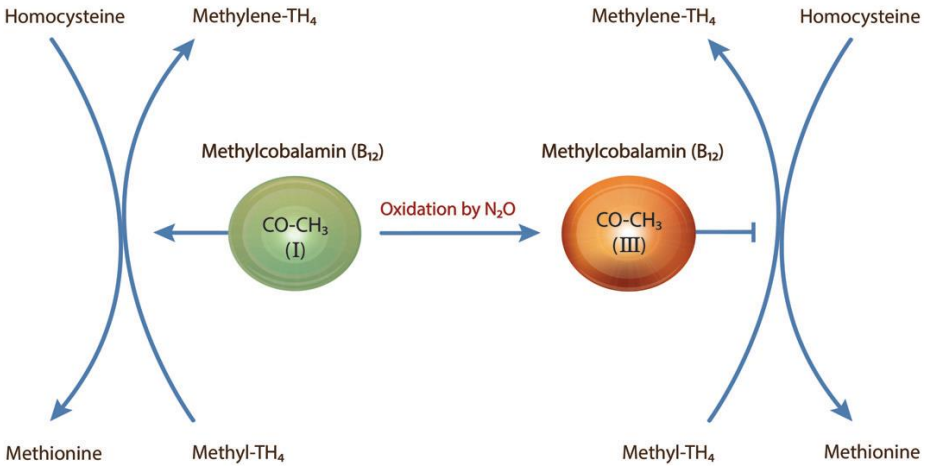


Figure 3: Methionine and folate metabolism (Figure published in Mohsenzadegan *et al.*, 2020); TH4: methyl tetrahydrofolate.

As summarised by the European Industrial Gases Association (EIGA, 2008), markers such as red cell folate, serum folate, vitamin B12 and homocysteine levels have all been used to monitor the effects of N_2O exposure as well as direct measurements of methionine synthase activity. Studies have shown strong associations between depletion of methionine synthase activity and both serum homocysteine level increase and DNA synthesis decrease (as indicated by a deoxyuridine [dU] suppression test) in bone marrow. These are generally the preferred markers of vitamin B12 deficiency as in a large number of studies, serum vitamin B12 was not a sensitive parameter.

Irreversible inactivation of methionine synthase by N_2O is identical between species. Nevertheless, the time course for N_2O inactivation of methionine synthase has been identified to be species dependent (Stewart *et al.*, 2019). In rats exposed to N_2O, the half-time of hepatic methionine synthase inactivation is 5 minutes (Royston *et al.*, 1983). After cessation of exposure, recovery takes 3 to 44 days because the vitamin B12 cofactor is irreversibly oxidized and covalently bound to the enzyme. New enzyme must be synthesized before the restauration

of the activity. In humans, the half-life of inactivation is about 45 min (in biopsied liver cells). Stewart *et al.* in 2019 highlighted that it is still unknown whether deficiency of methyl substituents, necessary for synthesis of myelin, DNA other essential reactions, or accumulation of homocysteine to toxic levels, accounts for the pathophysiology alone or in concert.

In rats, the NOAEC for dU-suppression resulting from methionine synthase inactivation was found to be 500-1 000 ppm after continuous exposure (EIGA, 2008). In mice, a LOAEC of 500 ppm for dU-suppression was identified in Healy *et al.* (1990) after a 13-week intermittent exposure.

In 2007, Krajewski *et al.* provided evidence for the first time that N_2O exposure leads to alteration of vitamin B12 plasma levels in humans (Krajewski *et al.*, 2007). In a cross-sectional study, they investigated the effect of exposure to N_2O on vitamin B12 metabolism, in female nurses (with 5-26 years of exposure). The exposed group was constituted of 95 nurses among an operating theatre staff and the control group was constituted of 90 unexposed nurses. Blood and air samples were collected on the same day. For air sampling, static monitoring was used for N_2O and individual dosimeters were used for other volatile anaesthetics. Folic acid, total homocysteine and vitamin B12 were also measured in blood (end of day). Vitamin B12 level was categorised according to the concentration as: low (150–250 pmol/L), border low (250–300 pmol/L), medium (250–350 pmol/L), or high (350 pmol/L). The authors reported lower serum concentration of vitamin B12 in highly exposed workers *vs* low or non-exposed workers. No differences were observed between groups for folic acid concentrations. The authors reported a significant negative correlation between N_2O and vitamin B12 levels (r=-0.22; P=0.038) and a significant positive correlation between N_2O and total homocysteine (r=0.51, P<0.001). The authors noted that there was no clear-cut correlation between duration of employment and the degree of abnormalities, albeit a tendency towards higher homocysteine levels among nurses with longest occupational exposure.

Table 4: Summary of cobalamin metabolic status in subject exposed to N_2O (Krajewski *et al.*, 2007)

	Control (n=90)	Low exposure (n=46)	High exposure (n=49)
N_2O concentration (mg/m^3)	-	185 (110)	753 (293)
Vitamin B12 (pmol/L)	437 (13.2)	402 (21.3)	342 (17.7)**
Folic acid (nmol/L)	8.1 (0.3)	8.4 (0.6)	9.2 (0.4)
Homocysteine (µmol/L)	8.9 (0.5)	9.6 (0.7)	12.9 (0.7)*

*p<0.05; **p<0.01

In 2016, Staubli *et al.* did not find any differences for the parameters of the vitamin B12 status between workers exposed to N_2O and a control group (medical workers not exposed to N_2O). These authors analysed vitamin B12 status by measuring homocysteine plasma levels. There was no air monitoring of N_2O, the authors only mentioned the use of N_2O in 2% to 3% on 37 000 paediatric patients per year.

Analgesic and anaesthetic effects Mode of Action

Effects on neurotransmitter receptors activity have been reported in the literature in *in vitro* and *in vivo* experiments. Non-competitive inhibition on the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors has been reported to be an important mediator of the effects of N_2O (Jevtovic-todorovic *et al.*, 1998; Ranft *et al.*, 2007), involving the inhibition of glutamatergic transmission (Georgiev *et al.*, 2010). This is the main theory for its anaesthetic action. Evidence also implicates $GABA_A$ receptors as mediators of N_2O's effects (Ohashi *et al.*, 2003; Hashimoto *et al.*, 2001; Hapfelmeier *et al.*, 2000). Opioid receptors and noradrenergic neurons have been implicated as being involved in the analgesic and antinociceptive properties of N_2O (Fugakawa *et al.*, 2014; Koyama *et al.*, 2010, Emmanouil *et al.*, 2008; Quock *et al.*, 1990) which may be provoked by antagonism of the NMDA receptor (Sanders *et al.*, 2009). N_2O exposure results in release of serotonin in the rat spinal cord (Richardson *et al.*, 2015; Emmanouil *et al.*, 2008), and other data suggest that the anxiolytic and antinociceptive effects of N_2O may involve a serotonergic mechanism. After exposure of rats to 60% N_2O, a progressive elevation of dialysate dopamine levels, reaching a statistical significance after 40 min of N_2O exposure was noted (Sakamoto *et al.*, 2006). A maximum of 144% of the basal level was reached in the third perfusate sample (60 – 80 min time period of N_2O exposure). After the exposure to N_2O was discontinued, the dopamine level gradually decreased to the basal level. The pharmacologic properties of N_2O are complex and different receptor systems may be involved depending on the response that is being examined (Richardson *et al.*, 2015).

5 Toxicity data

5.1 Acute toxicity

5.1.1 Nervous system

5.1.1.1 Human data

N_2O is an asphyxiant and induce hypoxia. In humans, following acute exposure, the most frequently reported effects were headache, nausea, vomiting, altered vision, ataxia, and hallucination.

Effects on nervous system were also documented through studies conducted in healthy volunteers or with subjects exposed at workplace (most often on operating theatre personnel). These studies used a battery of tests described in annex 3 and aimed to assess effects on psychomotor and cognitive system and on mood.

Experimental studies in volunteers

- *Effects on cognitive function*

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

- 25 ppm N_2O + 0.5 ppm of halothane,
- 50 ppm of N_2O,
- 50 ppm + 1 ppm halothane,
- 500 ppm N_2O,
- 500 ppm N_2O + 10 ppm halothane.

The authors evaluated the subject performance in several tests, including visual acuity, time reaction, vigilance, manual dexterity, memory, etc. at different time periods after the beginning of exposure (starting 2 hours after the beginning of exposure). The results were reported for each exposure in a table by the authors according to the tests conducted (Table 5).

Table 5: Summary of '*performance test*' results (in %) observed in Bruce and Bach (1976) in 20 male subjects exposed for 4 hours to N_2O alone

	Performance assessed	N_2O (ppm)	
		50	500
Tachistoscope	Visual perception and acuity	-	-7%* (p< 0.025)
Raven matrices	logical reasoning	-	-9%* (p< 0.05)
O'Connor Dexterity	Fine motor coordination	-	-
3-min audio-visual	Reaction to audio-visual stimuli	-5%* (p< 0.05)	-17%* (p< 0.005)
60-min vigilance	Vigilance	-	-14* (p< 0.05)
7-min audio-visual	Reaction to audio-visual stimuli	-5%* (p< 0.05)	-17* (p< 0.005)
Digit span	Immediate memory	-	-12* (p< 0.001)

%: mean per cent decrease in performance in anaesthetic condition; -: no changes reported, *: significance on anaesthetic effect on performance

Audio-visual capacity was slightly impaired after exposure to N_2O at 50 ppm (5% change). At 500 ppm N_2O, visual acuity, audio-visual capacity, immediate memory and vigilance response were altered. Only manual dexterity seemed to be resistant to N_2O exposure according to the authors. They observed that the changes were very small. They noted that repeated prolonged exposure would be needed to take into account tolerance to the effects and to conclude on adverse performance of workers occupationally exposed to N_2O. Regarding the gas analysis, the authors noted considerable variations in the anaesthetic concentrations of end-of-exposure expired air samples. In two previous studies from the same team (Bruce *et al.*, 1974; Bruce and Bach, 1975), digit span response was affected following 4 hours exposure to 500 ppm N_2O.

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

In an experimental study, twenty-four male volunteers were exposed twice to 0 ("placebo") and 50 ppm of N_2O in an exposure chamber during 4 hours to investigate effects on psychomotor performance (Venables *et al.*, 1983). The subjects were tested at the same time, *i.e.* morning and afternoon (four volunteers in the chamber during each session of exposure). Performance testing took place during the final 40 min in the chamber. The authors conducted the following psychomotor tests: audio-visual task, simple reaction time, four choice reaction time, stressalyser[9]. They performed also a mood test, using a visual analogic scale. The authors compared the scores at the beginning and at the end of exposure. Venables *et al.* didn't find differences in the mean performances for the four psychomotor tests. Concerning the visual analogic scale scores, the authors showed an impairment in mood on all four dimensions assessed (sleepiness, physical tiredness, mental tiredness and general good health) at

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

50 ppm of N_2O exposure, but the difference was not statistically significant (Venables *et al.*, 1983).

Table 6: Mean changes in the visual analogue scores for 0 and 50 ppm N_2O (Venables *et al.*, 1983)

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

Mahoney *et al.* evaluated the validity of neurobehavioural tests using N_2O as a model. The authors exposed fifteen volunteers to N_2O (duration of exposure was not stated) *via* a scavenging mask and asked them to breath only through their noses. The subjects were tested with a neurobehavioural evaluation system (NES) test battery on 4 separated sessions (training session, at 0, 200 000 and 400 000 ppm N_2O), NES combines 10 tests (continuous performance test, hand-eye coordination test, serial digit learning, symbol-digit substitution test, pattern recognition and memory, finger tapping, switching attention, mood scale). The switching attention task was performed under three separate conditions (switching time, switching direction or in a more "complex condition" using both switching time or direction) (Mahoney *et al.*, 1988). The authors observed a significant impairment on performance at 200 000 ppm for 2 tests of psychomotor speed (symbol digit and finger tapping), and effect on performance test response latency (p=0.055). The switching attention task was also impaired from 200 000 ppm onward (when tests were applied in a "complex condition") (Table 7).

Table 7: Effects of experimental N_2O exposure on NES performance in normal subjects (n = 16)

NES test (measure)	Experimental condition (% N_2O)			
	Training	0%	20%	40%
Finger tapping (mean no.)	112.60 (14.79)	117.47*** (12.53)	112.00* (14.52)	109.60* (13,20)
CPT latency (msec)	364.67 33.82)	354.76* (39.90)	360.44 (40.91)	384.24* (44.94)
Pattern Memory (No correct)	13.00 (1.46)	12.93 (1.33)	13.07 (1.39)	11.53* (1.46)
Handeye coordination (RMS)	3.24 (0.61)	2.89** (0.56)	2.85 (0.47)	3.26* (0.67)
Symbol Digit (s/symbol)	1.97 (0.29)	1.83** (0.31)	1.95* (0.34)	2.22* (0.93)
Switching Attention (msec)				
(latency side)	295.02 (47.52)	289.43 (51.36)	311.26 (62.35)	318.18* (62.59)
(latency dir)	460.93 (49.09)	465.47 (70.37)	469.33 (74.81)	476.93* (125.03)
(latency swide)	496.07 (108.26)	442.67** (102.89)	514.20* (148.28)	573.84* (209.74)
(latency swdir)	617.07 (100.49)	563.53** (121.15)	625.87* (182.68)	663.47* (195.64)
Mood Scales				
(Confusion)	1.87 (0.64)	1.68 (0.55)	2.17* (0.51)	2.72* (0.66)
(Tension)	2.05 (0.75)	1.85 (0.51)	1.89 (0.61)	1.88 (0.55)
(Depression)	1.44 (0.38)	1.33 (0.21)	1.40 (0.29)	1.33 (0.21)
(Anger)	1.08 (0.18)	1.05 (0.12)	1.13 (0.26)	1.03 (0.10)
(Fatigue)	2.36	2.31	2.43	2.43

	(0.75)	(0.87)	(0.68)	(0.63)
Serial Digit Learning	3.13 (3.58)	2.47 (2.92)	2.73 (1.71)	4.80 (3.86)

Data are means (SD); * significant drug effect compared to 0% N_2O (control); ** Significant "practice" effect. Control session compared to training session.

In 1988, Estrin *et al.* developed neurophysiological techniques for measuring cognitive performances in a standardised, objective, and reproducible manner to quantify the transient cognitive dysfunction induced by the administration of N_2O. Six volunteers (27-35 years) were exposed *via* a nasal mask to 0, 100 000, 200 000 and 400 000 ppm of N_2O for 10 minutes and remained to the dose for 20 minutes (tests were conducted during this second period). Respective simultaneous administration of O_2 was 100, 90, 80 and 60%. The authors measured the effects using the P300 Evoked Potential[10] and administered psychometric tests (symbol digit, continuous performance test, finger tapping). The results showed a trend in all variables (excepted symbol digit test) at 100 000 ppm and significant correlations between standardised measures of psychomotor testing and P-300 event-related potential latency and/or amplitude.

Table 8: Summary of results observed in Estrin et al. (1988), n=6

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

¤ Dunnet's t test, p<0.05; *p<0.01, repeated-measures ANOVA; ***p<0.06, repeated-measures ANOVA
CPT: Continuous Performance Test; FTT: Finger Tapping Test; SDT: Symbol Digit Test;

Moreover, they found that the decrease of P-300 amplitude and the increase of P-300 latency were correlated with increased N_2O concentration (respectively with r= -0.57 and r= 0.73).

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

Yajnik *et al.* studied the phenomenon of acute tolerance which is defined as a change of "sensitivity to a drug within the duration of one continuous drug exposure" (definition from Kalant *et al.*, 1971). For this, the authors exposed eleven volunteers through a facial mask to N_2O (0, 100 000, 200 000, 300 000, 400 000 ppm, during 120 minutes) for five time periods separated by at least one week. The subjects were selected according to medical history (e.g.

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

no significant psychiatric disorders or history of neurologic, cardiac, pulmonary, hepatic or renal disease). The effects were measured using a self-reported questionnaire, cognitive and psychomotor tests and physiological analyses (which consisted in electrocardiogram, peripheral oxygen saturation, blood pressure). The tests were conducted during the exposure session, 15, 40, 60, 80, 85 and 105 minutes after initiation of N_2O exposure and 5, 30, 60 min after cessation of exposure (Yajnik *et al.*, 1996).

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

Table 9: Summary of human experimental studies on cognitive function effects following N_2O exposure

Reference Design	Results	NOAEC/LOAEC (ppm)	Reliability (RoB)[11]
Yajnik *et al.*, 1996 5 ♂ + 6 ♀ Chamber (nasal mask) 5 sessions (~190 min) : air (control session); 100 000; 200 000; 300 000; 400 000 ppm N_2O	Significant impairment on auditory reaction time and eye-hand coordination. No acute tolerance to N_2O	**LOAEC: 300 000** **NOAEC: 200 000**	2
Fagan *et al.*, 1994 8 ♂ + 4 ♀ Chamber (nasal mask) 5 sessions (~ 60 min): air; 50 000; 100 000; 200 000; 400 000 ppm N_2O	Significant differences: impairment of reaction time and attention	**LOAEC: 100 000** **NOAEC: 50 000**	2
Mahoney *et al.*, 1988 15 ♂ Chamber (nasal mask) 4 sessions (duration not specified): air (training session & control session); 200 000; 400 000 ppm N_2O	Impairment on psychomotor tests: - symbol digit - finger tapping - test response latency	**LOAEC: 200 000**	3
Estrin *et al.*, 1988 6 (sex not specified) Chamber (nasal mask) 4 sessions (10 + 20 min): air; 100 000; 200 000; 400 000 ppm N_2O in O_2	Impairment on psychomotor tests: - continuous performance test - finger tapping	**LOAEC: 100 000**	3
Venables *et al.*, 1983 24 ♂ Chamber (nasal mask) 4h-exposure: placebo, 50 ppm N_2O	Psychomotor performance: No effect Mood: No statistical difference	**NOAEC: 50**	2
Bruce and Bach, 1976 20 ♂/group Chamber (nasal mask) 4h-exposure, twice: - 25 ppm N_2O + 0.5 ppm halothane, - 50 ppm of N2O, - 50 ppm + 1 ppm halothane, - 500 ppm N_2O, - 500 ppm N_2O + 10 ppm halothane	Impairment on psychomotor tests: - memory - visual acuity - audio-visual capacity	**LOAEC: 50** **NOAEC: 25 (+0.5 halothane)**	3
Bruce and Bach, 1975 30 ♂ Chamber (ambient air) 4h-exposure: air, 500 ppm N_2O	Impairment on psychomotor test: - digit-span test	**LOAEC: 500**	2

[11] Risk of Bias assessment, according to OHAT approach (See Annex 2)

- *Effects on nervous conduction*

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

Fifteen volunteers were exposed to room air, 100% of O_2 (during 10 minutes) and 100 000, 300 000 and 500 000 ppm of N_2O (mix with O_2) during 15 minutes, via a fitting facial mask (Williams *et al.*, 1984). Their cerebral activity was then tested by the Cerebral Function Analysing Monitor (CFAM) which is a microprocessor device based on cerebral function monitoring through an electroencephalographic signal derived from a single pair of surface electrodes. During the session, blood pressure and respiratory rate and CO_2 concentration in exhaled air were measured. Data on only nine of the fifteen subjects were analysed (because of a lack of cooperation or unpleasant feelings). The authors observed a significant reduction in CFAM amplitude at 30 and 50% (but no change was observed in the frequency distribution of the weighted EEG signal). Moreover, the subjects reported subjective effects whilst breathing N_2O, as hyperacusis (9/9), emotional states (fear and panic) and euphoria.

5.1.1.2 Animal data

- **Rats**

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

Courtière *et al.* evaluated vigilance performance task in rats. The rats were required to respond to slight luminous increment of the house-light. A statistically significant dose-related decrease of correct response was observed in rats exposed to 300 000 to 700 000 ppm N_2O. Concomitantly, a decrease in correct anticipatory responses was observed up to 600 000 ppm and an increase of omissions was strongly increased at 700 000 ppm N_2O. Moreover, in this

study, a statistically significant dose-related decrease in locomotor activity was noted at ≥ 400 000 ppm N_2O (Courtière *et al.*, 1997).

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

- **Mice**

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

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

Stimulation of locomotion was also reported in male mice exposed 1 hour to 500 000 ppm N_2O (Dorris *et al.*, 1993). This study presents several limitations[12] such as a few number of animals (n = 4/group).

[12] Limitations : no information on GLP status, purity of test material not provided, no analytical control of concentrations during inhalation, information on the source/origin of the N_2O unspecified, information on the source/origin of mice unspecified, type of statistical test performed not specified, no information about housing, lighting and feeding conditions, no data on general toxicity, only one dose tested, few number of animals per group

Table 10: Summary of animal experimental studies on neurotoxicity following acute N_2O exposure

Methods	Results	Reliability (Klimisch) [13]	Reference
RATS			
Female rats (n=5-10/group) 500 000 to 2 000 000 ppm N_2O Inhalation, 3h exposure	EC_{50}: 1 180 000 ppm Neuron vacuolation (posterior cingulate/ /retrosplenial cortex)	2	Jevtovic-Todorovic *et al.*, 2005
Female rats (n=6-17/group) Single 1-16h exposure 1 500 000 ppm N_2O	>3h, reversible vacuolation of neurons >8h exposure: Cell death	2	Jevtovic-Todorovic *et al.*, 2003
Male and female rats (7-9/group) 500 000 to 2 000 000 ppm N_2O Inhalation, 3h exposure	EC_{50}=1 040 000 ppm (males) EC_{50}=1 170 000 ppm (females) Dose-related increase in vacuolated neurons (retrosplenic cortex)	2	Jevtovic-Todorovic *et al.*, 2000 and 2001
Male rats (n=10/group) 300 000 to 700 000 ppm N_2O Inhalation, single exposure	≥ 300 000 ppm Dose-related decrease in locomotor activity, alteration of visual detection task	2	Courtière *et al.*, 1997
Male rats (n=8-10/group) Single continuous exposure 700 000 ppm mixed in O_2	Transient decreased in visual evoked potential amplitude. Decreased in nocturnal locomotion. Tolerance observed during the following light-dark cycle.	2	Dzoljic *et al.*, 1994
MICE			
Male mice (n=12-15/group) 0, 250 000; 500 000; 750 000 ppm N_2O mixed in O_2 Unknown exposure duration	At 500 000 ppm: Increased in the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner	3	Li *et al.*, 2001
Male mice (n=15-20/group) 35-60 min single exposure 500 000 ppm N_2O	Reduced level of anxiety	2	Caton *et al.*, 1994
Male mice (n=4/group) 1h single exposure 500 000 ppm N_2O	Increased locomotor activity	3	Dorris *et al.*, 1993

EC_{50}: Median effective concentration

[13] See Annex 2

5.1.1.3 Summary and discussion

In humans, following single acute experimental exposure in volunteers, Bruce and Bach in 1976 published psychomotor alteration below exposure concentration of 1 000 ppm. Indeed, in two separate studies, they found memory alteration in digit span test after 4 hours exposure to 500 ppm N_2O. In a 1976 experiment, minor effects on audio-visual capacity were observed at dose levels as low as 50 ppm (but not at 25 ppm). Nevertheless, according to the authors, the study population in this study may have been particularly sensitive to the effects of N_2O. At 50 ppm, no author was able to reproduce these results.

In several studies in rats (Jevtovic-Todorovic *et al.*, 2000, 2001, 2003 and 2005; Courtière *et al*., 1997; Dzoljic *et al*., 1994), brain histopathological findings, locomotor activity and/or behavioural changes were reported after single exposure ≥ 300 000 ppm N_2O. Behavioral changes were also noted in the three described mice studies at ≥ 500 000 ppm (Li *et al*., 2001; Caton *et al*., 1994; Dorris *et al*., 1993). No study was found at lower dose exposure levels.

5.1.2 Haematopoietic system and immune function

There are inconsistent results on the potential effects on N_2O on immune function after acute exposure. In a controlled trial, Chen *et al.* reported that in patients undergoing colorectal surgery (n=91), DNA damage in leucocytes and risk of wound infection were increased in the group treated with 700 000 ppm N_2O for 2 hours (Chen *et al.*, 2013). In one hand, these results suggest potential effects of N_2O on the immune system. On the other hand, in a randomised trial from Fleischmann *et al.*, where 418 patients underwent colorectal surgery, no difference in infection rate, ASEPSIS wound healing score, wound collagen deposition was found between an anaesthetized group with N_2O and a group exposed to nitrogen, remifencil and isoflurane (Fleischmann *et al.*, 2005).

No acute toxicity study in experimental animals investigating specific effects of N_2O on haematological system and immune function was found.

5.2 Irritation and sensitisation

No data available on irritation and sensitisation.

5.3 Repeated-dose toxicity

5.3.1 Nervous system

5.3.1.1 Human data

- *Cases report*

Dreyfus *et al.* reported the cases of two anaesthetists who developed a chronic toxic encephalopathy (CTE) after many years of exposure to anaesthetic gases in operating room

(where air conditioning was deficient during three years). CTE was characterised as an impairment of cognitive functions on at least 3 specific behavioural domains among 6 (attention, memory, executive skills, dexterity, visuospatial organization and psychomotor slowness). The authors reported high levels of anaesthetics gases with mean concentration of N_2O (311 ppm, peaks 1 600 ppm) and halogenated gases (16 ppm, peaks 1 600 ppm) (Dreyfus *et al.*, 2008).

A direct relationship between N_2O exposure and CTE is uncertain in these 2 cases. Indeed, anaesthetists are also exposed to many other neurotoxic agents including halogenated anaesthetic gases. Also in these individual cases, other non-occupational risk factors could have played a role in CTE development.

- *Studies carried out at workplace (operating theatres)*

At workplace, eight relevant published studies report effects on the nervous system after repeated exposure to N_2O. In these cross-sectional studies, N_2O was measured in air and/or urine. A high risk of bias was noted in four of these studies. The reference threshold for N_2O air concentration was 50 ppm in most cases (which corresponds to OEL recommended or set in some countries[14]) or 27 µg/L in urine (corresponding to biological limit value set in Italy and to 50 ppm in indoor air at the working place, according to Imbriani *et al.* in 1995[15]). The authors analysed psychomotor and cognitive effects and effects on mood. These effects were reported and assessed though a battery of tests and self-questionnaires (similar test use in experimental studies described for acute effects). Although N_2O exposure was well assessed (personal sampling and/or biomonitoring), the studies were conducted in study populations in the healthcare field, exposed to many other agents including other anaesthetic gases.

  - *Highest quality studies*

In 1995, Lucchini *et al.* studied acute, sub-chronic and chronic effects on central nervous system following exposure to N_2O in 62 exposed nurses (vs 46 non-exposed nurses, used as controls). The authors measured atmospheric N_2O in the breathing zone and in urine[16]. They reported geometric means (GM) of N_2O levels in air and in urine of 62.6 ppm (7-553 ppm) and of 26.8 (4-297) µg/L respectively, with the highest levels found on the last day of the working week. Ethrane® (enflurane) was also measured in air with a mean concentration of 1.3 ppm. It was estimated that the correlation between air and urine N_2O concentration was good (first day: r^2=0.4; p= 0.0001, N=29 and last day: r^2= 0.8; p=0.0001; N=32). Acute effects were explored through vigilance testing (simple reaction time) at four different times (before and after shift, the first and last day of the working week). To reduce the potential learning effects, half of the volunteers performed the test before the shift and then after the shift and half was tested first after a shift and then before a shift. To investigate sub-chronic and chronic effects, the authors also performed a battery of psychomotor tests (simple reaction time or SRT, profile of mood state for evaluating mood state, visual digit span for memory, Benton visual retention for visual memory, digit serial for visual learning ability, digit symbol for coding speed, aming pursuit for motor speed and steadiness). Effects were observed only on the SRT. The decrease in mean SRT test performance was statistically significant in exposed subjects compared to

[14] Gestis website: https://limitvalue.ifa.dguv.de/ consulted in January 2021.

[15] $[N_2O]_{urine} = 0.57x[N_2O]_{atmo} + 1.584$ ($r = 0.89$; $p < 0.001$).

[16] In Italy, the Ministry of healthset a biological value for nitrous oxide in urine of 27 µg/L corresponding to 50 ppm of N_2O in air (Scapellato *et al.*, 2008).

control group after the shift on the last day of the working week. The alteration was also observed on subjects with N_2O < 55 µg/L (corresponding to the previous regulatory Italian limit value of 100 ppm). A different trend of SRT test performance was observed as exposed subject's performance decreased progressively and remained stable in the control subjects.

Table 11: Simple reaction time in exposed and control group before and after the shift on the first and last day of the working week

	Exposed group		Control group	
	n	Mean ± SD	n	Mean ± SD
First day				
Before shift	61	273 ± 32	44	274 ± 29
After shift	61	274 ± 38	45	268 ± 31
Last day				
Before shift	61	269 ± 36	39	262 ± 35
After shift	59	284 ± 45	39	266 ± 34*

*p<0.05

The authors concluded that exposure under the current limit value (100 ppm) did not cause chronic effect on psychomotor function but reversible acute effects on vigilance and response speed can be expected (temporary because observed on the last day not at the beginning of the week).

To assess the effects of stress and work organisation on these results, in 1996, the same authors conducted a similar study in the same hospital (N_2O measured in air and urine). In this study, Lucchini *et al.* examined 30 operating room workers. The group of volunteers represented 80% of the entire personnel of the department and was composed of surgeons, anaesthetists, operating room nurses and technicians. A control group consisted of 20 subjects randomly selected among medical and paramedical personal in other departments in the same hospital. N_2O atmospheric concentration was measured using personal sampling during a 3 hour period of time. Urinary N_2O was also measured in urine at the end of the shift. Simple reaction time (SRT) test was selected as psychomotor test and was performed at two different times: first, during a week with constant use of non-gaseous anaesthesia and secondly, during a week with constant use of gaseous anaesthesia, with a two-week interval between these weeks. In addition, biological measurements were performed: serum cortisol as a biological stress indicator and serum prolactin to investigate interference with the dopaminergic system. The authors used a –so-called "double-blind testing condition": as a matter of fact, only the 4 (exposed) anaesthetists knew during which week gaseous anaesthesia was used. Potential confounding factors such as age of alcohol consumption were checked. No information on potential co-exposures was provided in this study (Lucchini *et al.*, 1996).

On the last day of the gaseous anaesthesia week, mean N_2O air concentration was 54.2 ppm (SD=22.8 ppm) and mean urine concentration was 25.6 µg/L (SD=22.1 µg/L). A good correlation between N_2O in air and in urine (r=0.89; p=0.0001) was found. The study showed a prolonged reaction time and increased serum prolactin levels in exposed workers only when they worked with gaseous anaesthesia. No effect of N_2O exposure was observed for serum cortisol levels.

The authors concluded that their results indicate neurobehavioural effects of N_2O exposure below 100 ppm. However, these results should be considered with caution, as co-exposures were not taken into account and the number of workers included in the study was small.

In order to better define a safe exposure level, Lucchini *et al.* conducted a multi-centre study in Italy evaluating neuropsychological symptoms, subjective stress and response speed functions in subjects occupationally exposed to low levels of anaesthetic gases. A group of 112 operating theatre workers from 10 Italian hospitals was exposed to anaesthetic gases (N_2O and isoflurane) and 135 non-exposed workers were used as control group. People with daily alcohol intake exceeding 80 g were excluded as well as those with daily coffee consumption exceeding 5 cups, and/or with CNS medications, and/or neurological or psychiatric disorders and/or occupational or non-occupational exposure to neurotoxic metals or solvents and/or aged of more than 59 years. The workers were examined before and after the shift on the first and the last day of the working week. The testing comprised a complex reaction time test (the Stroop Colour Word) and a subjective mood scale. The week preceeding the first testing a training session was organized in order to limit the learning effect of neurobehavioural testing. During this session, a questionnaire for neuropsychological symptoms (EURO-QUEST) was administered together with the block design subtest from the WAIS battery. The aims of these supplementary tests were to examine basic intellectual abilities of the participants. Biological and atmospheric indicators of exposure were measured at the beginning and the end of the working week for N_2O and isoflurane. The results of these measurements indicated moderate exposures: for atmospheric N_2O geometric mean and 95th percentile were 23.2 ppm and 127 ppm on the 1st day and 20.6 ppm and 114 ppm on the last one; the corresponding values for isoflurane were 0.4 ppm and 3.8 ppm, and 0.3 ppm and 2.7 ppm. For end-of-shift urine N_2O concentrations geometric means and 95th percentiles were 7.1 µg/L and 12.4 µg/L on the 1st day, 7.8 µg/L and 21.5 µg/L on the last one (Lucchini *et al.*, 1997).

No statistical difference was observed between exposed and control subjects for neurobehavioural effects, stress and arousal levels.

The authors concluded that the biological exposure limits of 13 µg/L for urine N_2O concentration (corresponding to 25 ppm for TWA air concentration) is adequately protective for the integrity of workers neurobehavioural functions, as measured with the tests used.

In an Italian hospital, operating-theatre workers exposed to anaesthetic gases (n=38) and 23 unexposed nurses participated in a longitudinal study (Scapellato *et al.*, 2008) during one year to investigate effects on neurobehavioural functions. Neurobehavioural functions were assessed using a battery of tests:

- Euroquest self-administered questionnaire, exploring symptoms,
- Block design subtest (WAIS) measuring visuospatial and motor skills,
- Mood scale measuring stress and arousal state,
- Colour word vigilance (CWV) test, which is a complex reaction time test.

The study was designed to consider potential pre-existing abilities and potential changes over time (repeated cross-sectional study). Three measures were taken for each subject at each of four time points: before and after work shift on Monday and Friday of a working week, twice a year. To attenuate learning effects, the subjects were allowed to practice the tests before the experimental session. The CWV test was the endpoint used to appraise short-term effects induced by N_2O and isoflurane. Exposure was assessed *via* biological concentration in urine (urinary N_2O and isoflurane) at the end of work shift (Monday and Friday), twice a year (not stated if tests are performed on the same week). Contamination of urine was avoided and urine was analysed using gas chromatography (GC) with electron capture detection. The authors gathered information on the subjects to identify potential confounding factors. The subjects

were classified into 4 groups (A: unexposed, B: <13; C: ≥ 13 to <27 and D: ≥ 27 µg/L). The urinary concentrations of 13 and 27 µg/L correspond respectively to air concentrations of 25 and 50 ppm[17] (Imbriani *et al.*, 1995). The correlation between urinary N_2O excretion and end shift CWV was investigated by using the analysis of simple linear regression. For the analysis of repeated measures of CWV, a model of two-stage regression was used, which was built as follows. In the first-stage, reaction times (or CWV test results) were plotted against time in each subject, obtaining through the simple regression analysis a slope (or coefficient of regression), which expressed the individual change of CWV test results over a working week. At the second-stage, the slope was the dependent variable in a multiple regression analysis in order to select factors which affected longitudinal changes in reaction times among the following variables: general characteristics of subjects (age, gender, years of schooling, alcohol and coffee consumption, smoking habit, length of work); subjective symptoms (EQscores); basic cognitive abilities (BD test results); Monday morning CWV test result (the baseline value, conveying the pre-existing ability of the subjects) and occupational exposure. This approach consisted therefore of a regression model for the average response over time and the effects of covariates on this average response. Stress and arousal were taken concurrently with CWV and, since the contingent mood state could affect CWV, they were analysed simultaneously using a multiple analysis of variance for repeated measures.

Although the overall means were below the reference values of 27 µg/L for N_2O and 3.32 µg/L for isoflurane, urinary concentrations of N_2O exceeded the biological exposure limit in 12 out of 38 exposed subjects (32%), and that of isoflurane in 4 out of 38 (11%). No significant difference was found for all variables except sex (effect of sex distribution on reaction time was of borderline significance). There was no significant correlation between urinary levels of N_2O and end-shift CWV values, separately on Monday and Friday). With respect to the unexposed group, CWV test results over a working week were significantly ($p < 0.020$) higher in the Group D, but not in Group B nor C (Scapellato *et al.*, 2008).

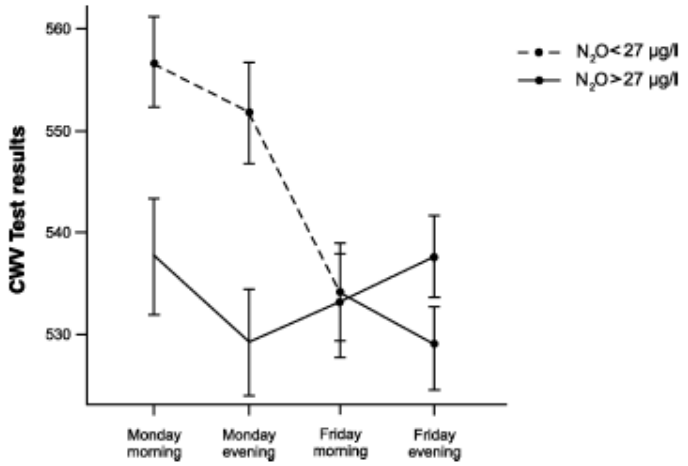


Figure 4: Means of colour word vigilance test (Scapellato *et al*., 2008)

[17] The concentrations of anesthetics such as N_2O in the ambient atmosphere were determined in 190 operating theaters of 41 hospitals in Italy. Anesthetics was detected in the urine of 1521 exposed workers (anesthetists, surgeons, and nurses). Significant correlations were found between the anesthetics concentration in urine produced during the shift collected after a 4-h exposure (µg/L) and anesthetics environmental concentration (ppm). According to Imbriani *et al*., the urinary anesthetic concentration can be used as an appropriate biological exposure index. The urinary concentration values proposed for N_2O are 25 µg/L for an environmental value of 50 ppm, based on the 95% lower confidence limit. Therefore it "should be considered as a protection for the individual, especially if each biological value is corrected according to analytical variability of the measurements".

In subjects with urinary concentrations of N_2O below 27 µg/L, there was a linear decrease in reaction times from Monday morning to Friday evening, indicating a learning effect. In subjects with N_2O urinary concentrations above 27 µg/L, the means of the CWV were essentially steady across a work week, indicating that performances may have been impaired. The highest difference is located between Monday end-shift and Friday before shift. For arousal, the tests of within-subjects contrasts were significant at ‘‘trial 1 vs. 2’’ ($F = 9.845$; $p < 0.003$) and at ‘‘trial 3 vs. 4’’ ($F = 5.719$; $p < 0.020$). In subjects with N_2O urinary concentrations above 27 µg/L, arousal was low on Monday morning, increased at end of the workshift, and remained high until Friday evening. In subjects with urinary N_2O below 27 µg/L, arousal was high before workshift, and low after workshift, on both Monday and Friday. It seems, therefore, that significant changes in reaction times and arousal occur from Monday to Friday, thus suggesting a cumulative effect of anaesthetic gases over a week of exposure. No contrast was significant for the stress.

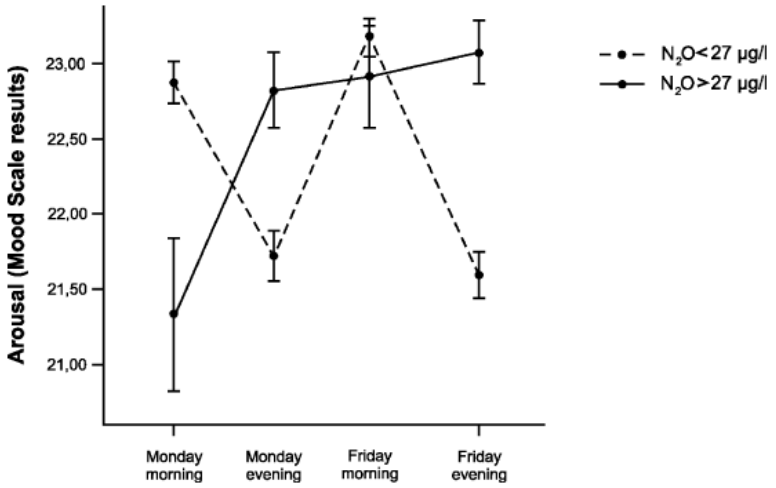


Figure 5: Means (and standard error of the mean) of arousal across a working week in subjects with urinary N_2O above or below 27 µg/L (Scapellato *et al*., 2008)

According to the authors, for N_2O urine concentration at the end of shift, 27 µg/L is a threshold under which vigilance alteration is not expected in exposed workers; it corresponds to 50 ppm for TWA air concentration. However, their results should be considered with caution, due to the small number of workers concerned in their study and also because occupational co-exposures were not taken into account.

Table 12: Summary of *human occupational exposure* studies (operating rooms) on effects of N_2O exposure on cognitive function, highest quality studies

Reference **Design**	**Exposure** GM (SD ; Range)	**Results**	**Reliability (RoB)**
Lucchini *et al.*, 1995 Cross-sectional study 62 exposed (40 ♂, 22 ♀) 46 controls (28 ♂, 18 ♀)	[N_2Oa] (ppm) *breathing zone during 3 hours* - SW: 45 (67.2 ; 12-333) - EW: 62.6 (129.6; 7-553) [N_2Ou] (µg/L) - SW: 20.7 (30.2 ; 4-133) - EW: 26.8 (71.7; 4-297) Enflurane: 1 ppm	**LOAEC [N_2Oa] = 62.6 ppm** **[N_2Ou] = 26.8 µg/L** SRT: significantly increased in exposed group compared to controls at EW, ES Impairment on SRT test also reported in the group of workers exposed to less than 55 µg/L	Tier 2
Lucchini *et al.*, 1996 Experimental at workplace 30 exposed (13 ♂, 17 ♀) (1 week with gaseous anaesth. vs 1 week with non-gaseous anaesth. with a 2-week interval) 20 controls	[N_2Oa] (ppm) *breathing zone during 3 hours* - SW: 50.9 (20.8) - EW : 54.2 (22.1) [N_2Ou] (µg/L) - SW : 21.54 (9.2) - EW: 25.67 (10.88)	**LOAEC [N_2Oa] = 54.2 ppm** **[N_2Ou] = 25.67 µg/L** SRT significantly increased after gaseous anaesthesia (vs non gaseous anaesthesia and vs controls) at EW. Effects reversible (not seen at the beginning of the week). Other results : - No effect on cortisol but effects on prolactin (associated with SRT; r=0.3, p=0.001) - Correlation between N_2Ou and N_2Oa (r=0.89, p=0.0001)	Tier 2
Lucchini *et al.*, 1997 Cross-sectional multi-centre study 112 exposed 135 controls	[N_2Oa] (ppm) *Stationary sampling* - SW: 23.2 (3-183) - EW : 20.6 (4-154) [N_2Ou] (µg/L) - SW : 7.1 (1.5-43) - EW: 7.8 (1.0-73) Isoflurane: 1.8 µg/L (0.5 ppm)	**NOAEC [N_2Oa] = 25 ppm** **[N_2Ou] = 13 µg/L** No statistically significant difference between groups in colour word vigilance test, EUROQUEST questionnaire and block design test.	Tier 2
Scapellato *et al.*, 2008 Longitudinal study 38 exposed (17 ♂, 21 ♀) 23 control (2 ♂, 21 ♀)	[N_2Ou] (µg/L) ES Monday & Friday - unexposed - <13 - ≥ 13 - <27 - ≥ 27 Workshift = 7h12/day Isoflurane < 3.32 µg/L	**NOAEC [N_2Ou]: <27 µg/L** **LOAEC [N_2Ou]: 27 µg/L** Corresponding to 50 ppm N_2O (Imbriani *et al.*, 1995) Lower neurobehavioral performance in colour word vigilance test	Tier 2

SW: start of week, EW: end of week, GM: geometric mean, SD: Standard deviation, u: urinary, a: air, SRT: Simple reaction time

- *Lowest quality studies (risk of bias assessment (RoB) according to OHAT approach, see Annex 2)*

Stollery *et al.* investigated the effects on mood and cognitive functions of N_2O exposure, in 22 anaesthetics workers in operating theatres. After a training session organised in order to limit

the learning effect of neurobehavioural testing, each subject was tested during two successive one-day experimental sessions: one with and one without exposure to anaesthetic gases. One group (11/22) was exposed (in an operating theatre) during the first session and the second group (11/22) was exposed during the second session. The subjects were examined via psychological tasks: syntactic reasoning, SRT, category-search and free-recall, visual-spatial memory, mood self-reported effects (mood, stress, arousal). Exposure to N_2O was measured by personal sampling with a threshold weighted average (TWA) of 58 ppm in the operating theatre (exposed session), and 15 ppm in the intensive care unit (unexposed session). The corresponding levels for halothane co-exposure were 1.4 ppm and 0.03 ppm. The test results showed no difference in mean stress (p=0.20); arousal reached a peak on the middle of the exposed day (which was not dependent on anaesthetic exposure). Concerning the cognitive tests, only performance on the serial reaction time task varied according to exposure sessions in both groups (Stollery *et al.*, 1988).

The significance of these results is limited, as the reference condition (intensive care) cannot be considered as a non-exposure condition. Moreover, the subjects were also exposed to halothane (to 1.4 ppm in operating theatres and to 0.03 ppm in intensive care).

In a mail survey, Brodsky *et al.* reported an increased incidence of neurologic complaints in dental professional who were heavily exposed to N_2O. The survey was initiated by the American Dental Association. A random sampling of 15,000 inhalation anaesthetic users and 15,000 nonusers were used. The investigation was done via questionnaire, with detailed questions concerning the type and hours per week of inhalation anaesthetic agents used, the usage by year over the decade 1968-1978. The results were adjusted for significant covariates (age, smoking history and mercury exposure). Neurologic disease incidences were rated based on the number of cases over the decade per 100 respondents. The level of anaesthetics exposure was calculated by cumulative exposure (hours during the period hours) < 2999 hours for the light exposure group and > 3000 hours for the heavy exposure group. The cut-off point corresponds to 6 hours per week over a decade. The response rate in female chair-side assistants and male dentists was above 70%. In dentists (men and women) heavily exposed to N_2O, the rate of complaints was more than 4 times greater than unexposed dentists. The highest difference was noted for reported symptoms of numbness, tingling and weakness without specific diagnosis (Brodsky *et al.*, 1981).

N_2O in air was measured at various sites in and around the operating rooms of Minnesota Hospitals (Snyder *et al.*, 1978). Twenty exposed operating room personnel (student nurse anaesthetists, registered nurse anaesthetists, anaesthesiologists) were matched with 20 non-exposed nurses or physicians of the same hospitals (12 nurses and 8 physicians). The group were matched for age, education, years of working, complexity of tasks, and as far as possible level of stress associated with the job. Subjects taking drugs, having history of central nervous system illness, ill or on night shift the preceding week were excluded from the study. A battery of 18 tests was used to assess mnemonic and intellectual functions (logical memory, direct or delayed recall, porteus maze test, four-choice reaction time). Some tests were specific to temporal and prefrontal dysfunction (e.g. Thomas imagery battery). All subjects were administered the battery on a Monday morning and again on a Friday afternoon. Half of each group started on Monday and the other half on Friday. According to the authors, this allows to estimate cumulative effects over a short period of time and reversibility over a weekend. Each volunteer was tested in a 30-min individual session followed by a 30-min group session.

Regards to exposure, background gas levels were measured in the operating suites and non-operating room of the hospitals. Means of air N_2O levels were measured between 109 and 176 ppm and halothane levels between 1 and 3.9 ppm. The authors pointed out that any toxic effects could not be directly related to the presence of N_2O or halothane as other pollutants may have been present (other pollutants were not specified by the authors). No evidence for acute reversible intoxication of operating room personal was found (the exposed group did not do better on Monday morning than on Friday afternoon). There was no significant difference between the experimental and the control group on Friday and only two marginally significant differences on Monday. However, a strong trend toward better performance was noted in the control subject compared to the exposed personnel on both Monday and Friday afternoon, suggesting a deterioration in the experimental group. Nevertheless, only one (test of recent Memory) out of 18 tests showed significative correlation with years of exposure. The authors concluded that there was subtle and inconclusive evidence for chronic cumulative toxicity in the exposed group.

Korttila *et al.* studied 19 operating room nurses working in three operating rooms in Helsinki central Hospital. Nitrous oxide was used in all the rooms whereas halothane was used in 2 of the 3 rooms. The nurses had worked for an average of 9 years in operating rooms. Eleven ward nurses from the same clinics served as control. N_2O and halothane were sampled in ambient air (in front of both anaesthetic and instrument nurses) throughout a working day in consecutive samples of 0.5 to 1-hour periods. Blood and end-tidal air samples were taken from subjects approximately 15 and 60 min after leaving the operating room. Air concentration vary greatly between the rooms with mean range of 721, 397 and 265 ppm in room 1, 2 and 3, respectively. Each subject was tested on perceptual and psychomotor tests and on driving simulator between 3.45 and 4.30 pm after a usual working day. The following tests were used: perceptual speed, Santa Ana dexterity test, tapping speed, reaction skills, simulated driving.Tests started approximately 15 min after work. The driving simulator was used first, and the perceptual and psychomotor test were carried out after (around 45 min later). No major difference was noted in psychomotor tests and simulated driving. The authors suggested that tolerance to N_2O may occur in these nurses. Nevertheless, there were a lot of potential bias in the study (e.g. operating room nurses were significantly older than the ward control nurses) (Korttila *et al.*, 1978).

Table 13: Summary of human occupational exposure studies on effects of N_2O exposure on cognitive function (studies with high risk of bias)

Method	Exposure	Results	Reliability (RoB)[18]	Reference
Experimental study at workplace N= 22 exposed *2 experimental sessions :* *11 exposed 1st day and 11 exposed on the 2nd day*	[N_2Oa] (ppm) *Personal sampling* *Exposed : TWA = 58 ppm* *Not exposed (not really unexposed) = 15 ppm*	Decision gaps, movement gaps, movement times were affected but not significantly No impairment for subjects considered not exposed (but TWA =15 ppm) NOAEC: 58 ppm	Tier 3	Stollery *et al.,* 1988
Retrospective cohort study N= 30 000 dentists (15 000 users and 15 000 non users) Mail survey	Questionnaire: non exposed, <2999 hr/10yr, > 3000 hr/10yr	Neurologic disease complaints	Tier 3	Brodsky *et al.*, 1981
Cross-sectional N= 20 exposed N= 20 controls	Ambient air : 0 – 176 ppm	Mnemonic and intellectual functions. No acute findings, equivocal chronic effects	Tier 3	Snyder *et al.,* 1978
Cross-sectional N= 19 exposed N= 11 controls	Ambient air: 245 - 1200 ppm Up to 15 ppm halothane	Psychomotor and driving experience. No findings	Tier 3	Korttila *et al.*, 1978

5.3.1.2 Animal data

Eleven animal studies (4 in rats, 3 in mice, 2 in monkeys, 2 in pigs) were retained in the database on the investigation of structural, neurochemical, neurophysiological and/or behavioural effects of N_2O. Almost all these studies were considered reliable though some limitations were noted (e.g. low number of animals, insufficient reporting of the results). Studies performed using repeated continuous exposure (e.g. pigs and monkeys) are considered of low relevance for workers occupationally exposed to N_2O and are not further detailed in this report (see annex 4 for references).

Rats

- *Sub-acute exposure*

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

[18] Risk of Bias assessment, according to OHAT approach (See Annex 2)

- *Sub-chronic exposure*

Singh *et al.* investigated behavioural and histopathological changes in male Wistar rats. Wistar rats (6 males per group) were exposed by inhalation 90 min/day during one month to a mixture of 500 000 ppm N_2O mixed in O_2 in 1:1 ratio. The rats appeared sluggish, lethargic and developed predominantly hind limb weakness after exposure. These effects recovered 1–1.5 hour after cessation of exposure. In the exposed group, the total distance travelled, time moving, number of rearings and grip strength were significantly decreased, whereas resting time significantly increased compared to controls. Stereotypic counts were not statistically significantly increased. At this dose, weight loss was noted in the exposed animals. Blood homocysteine level was significantly increase in the exposure group compared to the control group. Nevertheless, serum folic acid and vitamin B12 levels were not significantly different. Glutathione and total antioxidant capacity level significantly decreased following N_2O exposure and histopathological changes were observed in the meninges, brain and the spinal cord. Such changes were not observed in the control group. No abnormalities were noted in the cerebellum. The meninges showed localised capillary congestion with vascular dilatation. Neuronal degeneration (shrinkage and vacuolation) was observed in the brain. Thickening of vascular endothelial wall with infiltration of mononuclear and polynuclear cells were noted. This finding was more marked in the subarachnoid space. Focal demyelination (depletion of myelin, vacuolation) was noted in the outer layer of the cerebral cortex. Degenerative changes such as neurophagia and satellitosis were observed in the external pyramidal layer. In the spinal cord, multiple demyelinated areas were noted, consisting of vacuolation and depletion of myelin throughout the white matter. The authors suggested that the neurobehavioural changes were due to cobalamin deficiency rather than a direct effect of N_2O. Blood oxidative stress parameters (glutathione levels, total antioxidant capacity) were significantly decreased in exposed group and correlated with the behavioural changes observed (Singh *et al.*, 2015).

In the same laboratory, Misra *et al.*confirmed their previous results on the alteration of spontaneous locomotor activity (reduction in total distance, time moving, number of rearings and time resting) and grip strength in Wistar rats (n=6 male/group) at 500 000 ppm and O_2 in ratio 1:1. Time rearing and the number of stereotypic counts were not statistically, significantly changed. The same conditions of exposure of the study of Singh *et al.* from 2015 were used, except that exposure was 2 hours per day instead of 1.5 hour per day. Decreased body weight was also seen in the exposed animals. Similar findings were also obtained for homocysteine, vitamin B12 and folic acid levels. Although glutathione and total antioxidant capacity were decreased in the plasma of exposed rats, serum malonodialdehyde (MDA) and cerebral cortex glutamate levels were higher in the control group. In this study, glutamate level, glial fibrillary acidic protein (GFAP) expression, were increased in brain and spinal cord, and related to the behavioural changes. According to the authors, this may suggest that clinical dysfunction may be related to astrocytic proliferation, related to oxidative stress and glutamate neurotoxicity (Misra *et al.*, 2020).

- *Chronic exposure*

Following rat exposure to 700 000 ppm N_2O, 4 hours/day, 5 days/week for six months, Dyck *et al.* did not found any disturbance in conduction velocity, axonal flow or morphological abnormalities in peripheral nerves in rats. In this study, sign and neurological symptoms, nerve conduction, electromyographic abnormalities in caudal nerve and morphometric and teased

fibre abnormalities (from sural nerve and tibial nerve branch) were recorded. The authors concluded that it is unlikely that N_2O is a peripheral nerve neurotoxin in rats (Dyck *et al.*, 1980).

Table 14 : Summary of *in vivo* effects in rat studies indicative of neurotoxicity

Methods	Results	Reliability (Klimisch [19]	Reference
Sub-acute exposure			
Rats Sex: both sexes N= no information Inhalation, 8h/d 7 or 14-d exposure 400 000 ppm N_2O	At 400 000 ppm: Cortical cell count changes in brain (frontal parietal and occipital portions)	4	Hayden *et al.*, 1974
Sub-chronic exposure			
Wistar rats Male Control; 500 000 ppm N_2O and O_2 N=5-10/group 60-day exposure, 2h/d	At 500 000 ppm: - necropsy: Brain, spinal cord astrocytes activation in brain (GFAP phenotype analysis) - Spontaneous locomotor activity: significant reduction in total distance travelled, time moving, number of rearing significant increase in resting time. No changes in stereotypic counts - significant reduction in grip strength	2	Misra *et al.*, 2020
Wistar Rats, Male Control; 500,000 ppm N_2O and O_2 N=6/group 1.5h/d, 60-day exposure	At 500 000 ppm: - Weight loss and clinical signs - Necropsy: cerebrum neuronal degeneration, focal demyelination in the outer layer of the cerebral cortex, degenerative changes in pyramidal layer; Spongy vacuolation, myelin damage, throughout the white matter in spinal cord - Spontaneous locomotor activity: significant reduction in total distance travelled, time moving, number of rearing significant increase in resting time. No changes in stereotypic counts - significant reduction in grip strength	2	Singh *et al.*, 2015
Rats Sex: n.a. N=18/group 4h/d, 5d/w, 6-month exposure 0, 700 000 ppm N_2O	- No morphometric and teased fibre abnormalities in peripheral nerves - No changes in electromyography, nerve conduction velocity or axonal flow	2	Dyck *et al.*, 1980

GFAP: glial fibrillary acidic protein; n.a.: not available;

[19] According to Klimisch cotation (See annex 2)

Mice

- *Sub-acute exposure*

Fung *et al.* used lower levels of N_2O. Mice were exposed 8 hours per day for 8 consecutive days at 1 000 or 2 000 ppm. At the end of the exposure period, mice were tested for motor coordination, locomotor activity, stereotypic behaviour and anxiety level. No effect was found on motor coordination or anxiety level. Mice exposed to N_2O showed reduced locomotor activity compared to control animals; however, with the exception of the longest time period (120 minutes), this decrease was not statistically significant and not dose-related. Mice exposed to N_2O showed a dose-dependent reduction in stereotypic behaviour. Necropsy was performed in the 2 000 ppm group. Although the number of neural cell counts was less in the N_2O exposed group compared to control mice, this was not statistically significant (206±8 cells in control vs 186±7.2 in 0.2% N_2O group). No significant differences were seen in the number of neuroglial cells or total cells counted in the control tissues, as compared to the neural tissues from mice exposed to N_2O. The authors suggested that short-term exposure to N_2O might alter central dopaminergic neuronal activities in striatal and mesolimbic regions (Fung *et al.*, 1993).

- *Sub-chronic exposure*

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

Table 15: Summary of in vivo effects in mice studies indicative of neurotoxicity

Methods	Results	Reliability (Klimisch)[20]	Reference
Sub-acute toxicity			
Mice Group: 8/group Sex: Males 0, 1 000, 2 000 ppm N_2O Inhalation, 8h/d 8-day exposure	At 1 000 ppm: - Reduced locomotor activity (not statistically significant, not dose-related), - dose-related change in stereotypic behaviour - No effect on motor coordination or anxiety level - no significant difference in the number of neural cells, neuroglial cells, or total cells counted in the control tissue, as compared to the neural tissue from mice exposed to nitrous oxide.	2	Fung *et al.*, 1993
Sub-chronic toxicity			
Mice (male and female) N=15/group 14-week exposure, Inhalation, 4h/d, 5d/w 0, 5 000, 50 000, 500 000 ppm N_2O	No mortality, decreased body weight gain No effect on brain weight, no histopathologic findings in brain.	4	Rice *et al.*, 1985

[20] According to Klimisch cotation (See annex 2)

5.3.1.3 Summary and discussion

In summary in rats, clinical signs (ataxia), structural changes in brain and spinal cord (vacuolation of neurons, demyelination), neurochemical (hyperhomocystinaemia, decrease in methionine synthase activity, decrease glutamate levels), and behavioural changes (locomotor activity, grip-strength) have been reported following repeated high dose exposure to N_2O. In some studies, (Singh *et al.*, 2015), concomitant structural changes, behavioural changes and neurochemical changes were observed in the animals providing support of the neurotoxic potential of N_2O. These effects were investigated and reported only at very high dose levels ≥ 400 000 ppm.

Only two mouse studies investigated the potential effect of N_2O (Rice *et al.*, 1985 and Fung *et al.*, 1993). Rice *et al.* did not report histopathological or weight changes in the brain at concentrations up to 500 000 ppm N_2O. Fung *et al.* reported doubtful effects on the locomotor activity of animals and dose-related changes in the stereotypic behaviour of mice at ≥ 1 000 ppm (0.1%). No such change on stereotypic behaviour was noted in rats at higher exposure levels (up to 500 000 ppm) by Singh *et al.* (Singh *et al.*, 2015) and Misra *et al.* (Misra *et al.*, 2020). Nevertheless, differences in methods (duration of exposure) and test animals (different strain), make the comparison difficult.

In humans occupationally exposed to N_2O, sub-acute effects on reaction time were noted in the most recent studies investigating psychomotor function (Scapellato *et al.*, 2008; Lucchini *et al.*, 1995 and 1996). The effects were seen at the end of the working week. The data do not support a chronic cumulative toxicity of N_2O as in these studies, performance of the exposed groups was not impaired on Monday morning. Regarding the dose levels, these acute findings were noted above 27 µg/L corresponding to an air concentration of 50 ppm in presence of isoflurane (Scapellato *et al.*, 2008). In studies published by Lucchini *et al.*, effects were reported at geometric mean of 62.6 ppm N_2O in presence of 1.3 ppm enflurane. Both authors, reported that no effect was seen below 50 ppm of N_2O. Co-exposure with halogenated anaesthetics may worsen the effects seen with N_2O. Nevertheless, no quantitative data is available between potential co-exposed workers or workers exposed to N_2O only.

Analytical method used in Lucchini *et al.* studies can lead to uncertainties in the measurement. Regards to atmospheric monitoring, the authors referred to Imbriani *et al.*'s study from 1988. There is a lack of information on the passive sampler used, badge type and debit flow. These uncertainties may underestimate N_2O level reported by the authors.

5.3.2 Haematopoietic system and immune function

5.3.2.1 Human data

Already in 1956, Lassen *et al.* reported severe bone marrow depression in severely ill patients after prolonged N_2O anaesthesia. Depending on time frequency and duration, reversible bone marrow depression with aplastic anaemia, leucopoenia and thrombopoenia have been reported in patients after prolonged or repeated anaesthesia. In most patients, the deoxyuridine suppression test (DUST) has yielded positive results, even after short-term

exposure. Administration of vitamin B12 or folate was found to accelerate regression or may prevent the symptoms (MAK, 1993).

- Chronic occupational exposure

In occupational settings, nine cross-sectional studies were retained from the literature. In most of these studies, a high risk of bias was noted for potential co-exposure and confounding factors (e.g. no adjustments for relevant variables). In addition, in most of these studies, lack of contextual information on measurements, such as sampling time period and method used, provide uncertainties on exposure characterisation. Dose-response was examined and adjustments were performed only in Bargellini *et al.*'s study from 2001.

No change in haematological parameters was noted by De Zotti *et al.* (De Zotti *et al.*, 1983) or Salo *et al.* (Salo *et al*, 1984) in operating room workers. Nevertheless, two cases of macrocytosis and one case of segmented neutrophils were reported among eight exposed male anaesthetists (Salo *et al.*, 1984). Due to the very low number of individuals and as these findings were not observed in the other part of the study (exposed nurses), no conclusion was drawn by the authors. Normal levels of vitamin B12 and folate levels were measured in exposed individuals.

Sweeney *et al.* reported positive results in deoxyuridine suppression test (as *a measure of synthesis of DNA dependent on vitamin B12, folic acid)* in 3 out of 20 exposed dentists, two had also a number of hypersegmented neutrophils. These three dentists were exposed to 1 900, 2 500 and 1 800 ppm (TWA exposure). No correlation with exposure and duration was seen. No change in vitamin B12 or folate concentration was noted in this study (Sweeney *et al.*, 1985).

Significant reduction in total leucocyte count was reported by Perić *et al.* (Perić *et al.*, 1991 and 1994, which are based on the same data) in operating room workers (anaesthetists), with a stronger effect in youngest staff. A significant decrease in CD21 lymphocyte (B-subtype) was seen in the oldest workers compared to controls and youngest staff, no recovery was observed during holidays. In addition, significant differences in erythrocyte count, haemoglobin concentration and haematocrit in anaesthetic staff were also seen before and after vacations. No change in natural killer function was observed in this study. Operating theatres were improperly ventilated and no scavenging systems were present. 8h-TWA N_2O exposure was between 85 and 1 600 ppm in the operating theatres with concomitant halothane levels between 10 and 350 mg/m^3 TWA. Another limitation of this study was the small size of the groups.

Bargellini *et al.* investigated the effect of N_2O in 51 anaesthetists (27 males and 24 females) compared to 21 controls from the same hospital in Italy (Bargellini *et al.*, 2001). Lymphocyte count, lymphocyte sub-populations and the natural killer activity were investigated. Exposure was evaluated as follow:

- Short term exposure: activity in operating room during the last 15 days (yes/no);
- Long-term exposure: number of days in operating rooms in the last semester: low (< 40 days), medium (40-80 days) and high (> 80 days);

- Individual exposure scores: one point for each month in operating rooms with means N_2O concentration < 50 ppm; 2 points when N_2O concentration was between 50 and 100 ppm and 3 points in rooms with N_2O concentration > 100 ppm. Three categories were assigned according to the global score: low (<2 points), medium (3-6.5 points) and high (> 6.5 points). Exposure levels during the last semester were provided by the hospital;
- Exposure to x-ray (yes/no).

Percentages of T cells (CD3) cells were significantly decreased in the exposed group compared to control. Natural killer (NK) cells were increased without effect on NK activity. The results are summarized in table below.

Table 16: Immunological findings in anaesthetists compared to controls (Bargellini *et al.,* 2001)

	Controls	Anaesthetists
Lymphocytes (%)	34 ± 7.9	36.9 ± 7.2
CD3 (T) (%)	73.8 ± 8.9	68.1 ± 88 *
CD19 (B) (%)	9.45 ± 3.0	9.60 ± 3.06
NK (%)	12.2 ± 7.8	16.3 ± 9.4**

p< 0.05, p< 0.01

After correction for confounders, short-term exposure was found to alter the percentage of total T lymphocytes (CD3) and T helper (CD4) lymphocytes compared to controls and T helper only compared to non-recently exposed anaesthetists. Percentage of T helper cells decreased significantly with the individual exposure scores. After adjustment for covariates, mean percentages of T helper cells were 45% (S.E. = 1.84 %) in controls, 44 % (S.E. = 2.64 %) in low scored anaesthetists, 40 % (S.E. = 1.68 %) in medium-scored ones, and 38.5 % (S.E. = 1.98 %) in those having the highest scores (> 6.5 points, p<0.05). Furthermore, a trend (p=0.08) in T helper lymphocyte percentages was found according to long-term exposure, mean corrected values were 44.1% (1.86 %) in control, 42.66% (2.97 %) in the low exposure group, 39.32 % (2.02 %) in the medium exposure group and 38.56 % (2.03 %) in the high exposure group (> 80 days of exposure). Among covariates, X-ray exposure was clearly associated with the NK lymphocytes activity. In this study, volunteers were also exposed to isoflurane, but this co-exposure was not taken into account in the analysis and isoflurane exposure characterisation was not provided.

In the Krajewsky *et al.*'s study from 2007, detailed in the mode of action section above, no haematological findings were noted.

Increased blood IL-8 was reported, by Chaoul *et al.*, in operating room medical workers exposed to anaesthetic gases including N_2O (≥ 100 ppm) for 3 years (15 exposed and 15 controls) (Chaoul *et al.*, 2015).

Slight statistically significant decreases in haemoglobin, haematocrit, mean cell haemoglobin concentration and red blood cell count were reported in a cross-sectional study conducted by

Amiri *et al.* in operating room medical workers exposed to a mixture of anaesthetic gases including N_2O (52 exposed and 52 controls) (Amiri *et al.*, 2018).

The table below presents the results of studies investigating effects of N_2O exposure on haematopoiesis in humans.

Table 17: Summary of studies investigating effects of N_2O exposure on human haematopoiesis in occupational settings

Reference	Study type	Study participants	Exposed group/control	Exposure	Results	Co-exposure	Reliability[21]
Amiri *et al.*, 2018	Cross-sectional	Operating room workers	N=52 exposed, N= 52 non-exposed	> 1 year exposure Mean N_2O concentration: 850 ppm (10-2 895 ppm)	Slight Stat. sign. ↓ Hb, Ht, MCH, MCHC, red blood cell count	Isoflurane, sevoflurane (and other agents usually present in operating room : e.g. ionizing radiations)	2
Chaoul *et al.*, 2015	Cross-sectional	Operating room workers	N=15 exposed, 15 controls	≥100 ppm	↑ blood cytokine Il-8	Isoflurane, sevoflurane, > 7 ppm (and other agents usually present in operating room : e.g. ionizing radiations)	3
Krajewski *et al.*, 2007	Cross-sectional	Operating room workers (nurses)	N=95 exposed, 90 unexposed	260 ppm [20-835], 15.8 year exposure	No effect on RBC, Hb, Ht, MCH, MCHC, Vitamin B12	Sevoflurane, isoflurane, halothane (and other agents usually present in operating room e.g. ionizing radiations)	2
Bargellini *et al.*, 2001	Cross-sectional	Anaesthetists	N=51 exposed, 21 non-exposed	3 categories : <50 ppm, 50-100 ppm, > 100 ppm Short-term to long-term exposure	Decreased percentage of total T lymphocytes and CD4 lymphocytes Increased NK cells related to X-ray exposure. No effect on total lymphocyte count	X-ray, isoflurane	2
Peric *et al.*, 1991; 1994	Cross-sectional	Anaesthetists	N= 21 exposed, 35 control	TWA: 85 - 1 500 ppm	Decreased leucocyte count, B sub-type lymphocytes more affected than T lymphocytes,	Halothane (TWA: 10-350 ppm) (and other agents usually present in operating room: e.g. ionizing radiations)	3
Sweeney *et al.*, 1985	Cross-sectional	Dentists	N=20	TWA: 159 - 4 600 ppm	Positive results in DUST in 3 exposed dentists, 2	Not investigated	2

[21] See annex 2

					had ↑ number of hypersegmented neutrophils, no clear correlation with exposure and duration		
De Zotti *et al.*, 1983	Cross-sectional	Operating room workers (anaesthetists, surgeons, nurses)	N=61 exposed, 156 controls 6-12 year of exposure	500 - 1 272 ppm (mean)	No change in Hb, Ht, RBC count, WBC count and differentiation, platelet count.	Enflurane (17.3-22.6 ppm) (and other agents usually present in operating room: e.g. ionizing radiations)	2

DUST: deoxyuridine suppression test; RBC: red blood cell, Hb: haemoglobin, Ht: haematocrit, MCH: mean cell haemoglobin, MCV: mean cell volume, MCHC: mean cell haemoglobin concentration, Stat. Sign. : Statistically significant; WBC: White blood cells

5.3.2.2 Animal data

As observed in humans, after repeated-exposure to high concentration of N_2O, haematological findings were reported in experimental animals. As repeated continuous exposure is of low relevance for human occupational exposure, only intermittent exposure studies are summarized below (see annex 4 for references on continuous exposure).

- **Rats**

Following exposure to 1 000 ppm N_2O in air, 5d/w, 6h/d, for up to 6 months, no leukocytopenia was observed in male Wistar rats (Cleaton-Jones *et al.*, 1977). At this concentration, only transient changes in haemoglobin concentration and pack cell volume were noted and an increase in the number of reticulocytes was present, following 5-month of exposure. Bone marrow analysis was similar to controls except that an increase in mast cells in bone marrow was noted. This finding was considered of unknown biological relevance by the authors.

Kripke *et al.* did not observed bone marrow alteration and leucopoenia after exposure to 200 000 or 400 000 ppm N_2O (and 20% O_2)(Kripke *et al.*, 1977).

- **Mice**

A decrease of total white blood cell count was noted, following 13-week exposure (6h/d, 5d/w), in CD-1 mice. This decrease was not dose-related but statistically significant $p< 0.01$ at all doses tested (50, 500, 5 000 ppm). One of the limitations of the study was that only 6 animals per dose group were used (Healy *et al.*, 1990).

Healy *et al.* also performed immunologic assessments. Humoral response as measured by a plaque forming cell (PFC) assay and by Elisa was not affected after 2-week exposure up to 5 000 ppm. PFC/10^6 splenic cells count was statistically significantly decreased at the top dose (5 000 ppm) following 13-week exposure. Anti-sheep red blood cell-immunoglobulin M levels were also statistically significantly decreased after a 13-week exposure to 5 000 ppm. Splenic Lymphocyte Proliferative Responses to Selected Mitogens (LPS, Con A, PWM, PHA) were also tested and a Mix lymphocyte culture assay (MLC) was performed in the same study. Concerning the uptake of [^{3}H]-thymidine by splenic cells, the results were biphasic with a decreased response after 2 weeks of exposure ($p< 0.05$ for LPS, $p<0.01$ for ConA, PHA and PWm), and an increased response after 13 weeks exposure at the top dose (not statistically significant). This response was associated with an increased mitogen-induced proliferation in cultures without mitogens. Exposure to N_2O has no effect on mixed lymphocyte reaction (MLR). Decreased thymidylate synthetase (dTMPS) pathway in bone marrow, as measured by a deoxyuridine suppression test was noted following 13-week exposure at the top dose of 5 000 ppm (dose-dependent suppression of dU uptake). After 2-week exposure no trend in the cellularity of the bone marrow was noted. Nevertheless, DUST uptake was supressed about 10 % in all dose groups. In conclusion, significant effects on immune function were observed at 5 000 ppm in this study (Healy *et al.*, 1990).

In contrast, in a 13-week study (Rice *et al.*, 1985) and in a 2-year study (Baden *et al.*, 1986), neither leucopoenia nor bone marrow damage were reported in mice exposed up to 500 000

ppm and 400 000 ppm, respectively. In these studies, mice were treated for 4h/d and 5 days per week.

Table 18: Summary of studies investigating effects on haematopoiesis in rodents

Methods	Results	Reliability (Klimisch)[22]	Reference
Wistar rats N=12-18/groups 6 weeks to 6-month study Inhalation, 6h/d, 5d/w 0, 1 000 ppm N_2O	At 1 000 ppm: - isolated changes in Hb concentration, - mast cells in bone marrow, - ↓ reticulocyte count at 5 months	2	Cleaton-Jones *et al.*, 1977
Rats N=18-25/group 21-35 days Intermittent exposure 0; 200 000 ppm in O_2	No findings	2	Kripke *et al.*, 1977
CD-1 mice N=6/group 2 weeks, 6h/d, 5d/w or 13 weeks, 6h/d, 5d/w 0, 50, 500 or 5 000 ppm	At 5 000 ppm (2-week exposure) : - ↓ WBC (non-statistically significant) At 5 000 ppm (13-week exposure): - immunotoxicity (PFC activity, antibody levels SRBC), - ↓ DUST At ≥ 50 ppm (13-week exposure): ↓**WBC, no dose-response, only 6 animals per dose	2	Healy *et al.*, 1990
Swiss Webster mice 104-week N=75-91/sex/group Inhalation, 4h/d, 5d/w 0, 100 000 or 400 000 ppm	No bone marrow depression or haematological findings	2	Baden *et al.*, 1986
Swiss Webster mice 14 weeks, N=15/sex/group Inhalation 4h/d, 5d/w 0, 5 000, 50 000 and 500 000 ppm	No haematological findings	2	Rice *et al.*, 1985

Hb: haemoglobin; WBC: white blood cells, PFC: plaque forming cell, SRBC: sheep red blood cell; DUST: deoxyuridine suppression Test

5.3.2.3 Summary and discussion

In human, changes in haematological parameters (i.e. decreased total lymphocyte count, Hb, Ht, MCH, MCHC, and/or red blood cell count) were observed in workers of operating room or dentists. Nevertheless, except in the Bargellini ***et al.'s*** study from 2001, in the cross-sectional studies, exposure characterisation was insufficient and confounding factors were insufficiently analysed. Bargellini *et al.* found that T cells (CD3+) were decreased, in correlation with a decrease in T helper cells (CD4). The decrease in T helper cells was dose-related (intensity and/or duration dependent). However, this study does not allow to establish a quantitative exposure-response relationship.

[22] See Annex 2

N_2O was shown to be an immunotoxicant in rats and mice. *In vivo*, leucotoxicity has been observed in rats and mice exposed to N_2O. Although not consistently seen in the studies, white blood cell count changes were already observed at 50 ppm in one study in mice (Healy *et al.*, 1990). Bone marrow damages, such as decreased cellularity of the erythroid, myeloid and lymphoid series have been observed following continuous high dose exposure to N_2O for few days. No such changes were noted following intermittent exposure. Immunotoxicity, notably humoral response, was tested by Healy *et al. in* 1990. In this study, immune function was observed at 5 000 ppm (PFC-assay, SRBC assay) in mice (but not at 500 ppm). A dose-related deoxyuridine suppression (DUST test), performed to quantify the conversion of deoxyuridine into deoxythymidine in bone marrow cells, was noted after N_2O exposure and statistically significant at ≥ 500 ppm.

5.3.3 Other target organs

Liver enzyme induction (indirectly measured by urinary excretion of D-glucaric acid) has been reported in patients receiving mixtures of halothane and N_2O. Based on liver microsomal enzyme induction at 200 ppm, a provisional OEL of 100 ppm was adopted by DFG in 1993. Nevertheless, it was stated that the effect may have been due to halothane exposure.

More recently, Scapellato *et al.* investigated whether D-glucuric acid (DGA) excretion could be a biomarker of effect in monitoring workers exposed to anaesthetic gases (Scapellato *et al.*, 2001). The authors measured excretion of D-glucaric acid in 229 workers before and after an operating session and 229 workers were used as controls. N_2O and isoflurane were measured after at least 4 hours exposure. The workers were categorized in exposure category based on urinary levels. They found that microsomal enzyme induction (increased D-glucaric acid urine excretion) was only increased in the highest exposure group (N_2O > 50 ppm, isoflurane > 1 ppm) and only in some, but not all, workers of this group. The authors concluded that liver microsome enzyme induction cannot be used as a biomarker of effect in workers.

5.4 Genotoxicity

5.4.1 Human data

Sixteen human studies were retrieved from the literature search based on full-text articles. Most of human data were cross-sectional studies assessing workers occupationally exposed to N_2O in hospital operating rooms (anaesthetists, nurses, surgeons, technical assistants).

Three studies were excluded as the main exposure may have been other anaesthetic gases and workers may not have been exposed to N_2O (Costa-Paes *et al.*, 2014; Karelova *et al.*, 1992 and Lamberti *et al.*, 1989).

Direct DNA damages were measured in several comet assays in lymphocytes of exposed workers. Positive results were observed at ≥ 180 ppm. A dose-related increase in DNA damage was noted in Wrońska-Nofer *et al.* with a NOAEC at 96 ppm (Wrońska-Nofer *et al.*, 2009).

Negative results were also observed at 140 ppm (Aun *et al.*, 2018). The mode of action of these DNA damages was investigated using a modified comet assay employing formamidopyrimidine glycosylase (FPG) to assess oxidative damage. Positive correlation was observed between N_2O levels in the ambient air and oxidative DNA damages. Oxidative stress might thus be linked with DNA damage induced by N_2O (Wrońska-Nofer *et al.*, 2012).

Regarding the damage at chromosomal levels, Kargar Sgouroki *et al.* reported an increase in micronuclei and chromosomal aberration at 850 ppm (Kargar Sgouroki *et al.*, 2019). No increase was observed at 180 ppm in another study (Braz *et al.*, 2020).

Increase in sister chromatid exchange (SCE) in males but not in females was noted at ≥ 11.9 ppm (Hoerauf *et al.*, 1999). A reversible increase in SCE damages was noted at 119 ppm in a prospective cohort (Ergoglu *et al.*, 2006).

The main identified bias was the co-exposure to other inhalational anaesthetics in most of the studies (mainly isoflurane and sevoflurane). Operating room workers may also be exposed to other genotoxic agents (ionizing radiations, anticancer drugs, etc.). Adjustment for these other relevant exposures was not performed in the published studies. In addition, according to the available studies, smoking habit, age and gender play a role in the observed genetic damage and should be taken into consideration when interpreting the results. In a few studies, N_2O air concentrations were measured and some indicated a dose-response relationship.

Table 19: Summary of genotoxicity data available for N_2O exposure in humans

(Reference) Method	Results	Reliability (RoB)[23]	Co-exposure
Direct DNA damage			
(Braz *et al.*, 2020) Cross-sectional study N= 31 controls and 32 exposed physicians (males and females) Comet assay in lymphocytes Oxidative stress and inflammatory markers	LOAEC = 180 ppm (air samples in the breathing zone, 8h TWA) Statistically significant increase in DNA damage and Il-17	2	Isoflurane (5.3 ppm), sevoflurane (9.7 ppm)
(Aun *et al.*, 2018) Cross-sectional study N =26 exposed male and female subjects (also controls) Apoptosis and comet assay in lymphocytes	No effect on apoptosis and in the comet assay NOAEC = 140 ppm (air samples in the breathing zone, 8h TWA)	2	Halofurane, desflurane, sevoflurane, isoflurane (9.9 ppm)
(Wrońska-Noffer *et al.*, 2012) Cross-sectional study N= 36 exposed and 36 control nurses Oxidative stress, comet and modified comet assay (FPG) in lymphocytes	LOAEC = 100-826 ppm (air static monitoring) Significant increase in DNA damage, more significant with FPG	2	Isoflurane (0.05-1.9 ppm), sevoflurane (0.06-1.6 ppm)
(Wrońska-Noffer *et al.*, 2009) Cross-sectional study N= 83 exposed operating room personnel and 83 controls (males and females) Comet assay in lymphocytes	LOAEC = 388 ppm in high dose group (air personal and static monitoring) Dose-related increase in DNA damages. Statistically significant in the high dose group.	2	Isoflurane (5.2 ppm), sevoflurane (4.7 ppm)
Damage at chromosomal levels (MN, CA, SCE)			
(Eroglu *et al.*, 2006) Prospective cohort study N= 25 exposed and 25 control male physicians Sister chromatid exchanges assay	LOAEC= 119 ppm (air measurements in the breathing zone) Statistically significant increase in SCE, reversible effect after 2 months, no correlation with the duration of working	2	Sevoflurane (9 ppm)
(Braz *et al.*, 2020) Cross-sectional study	NOAEC = 180 ppm (air samples in the breathing zone, 8h TWA) Increased micronucleus in buccal cells in exposed group not statistically significant	2	Isoflurane (5.3 ppm), sevoflurane (9.7 ppm)

[23] Risk of Bias assessment, according to OHAT approach (See Annex 2)

N= 31 controls and 32 exposed physicians (males and females) Micronucleus study in buccal cells			
(Kargar Shouroki *et al.*, 2019) Cross-sectional study N= 60 exposed and 60 control operating room personnels (males and females) Micronucleus and chromosome aberration assays	LOAEC = 850 ppm (air sampling in the breathing zone, as 8h TWA) Increased chromosomal aberration and micronucleus, statistically significant in technicians and nurses but not in surgeons	2	Isoflurane (2.4 ppm), sevoflurane (0.18 ppm)
(Lewinska *et al.*, 2005) Cross-sectional study N=46 exposed and 28 control female nurses Micronucleus assay+FISH in lymphocytes	LOAEC = 20 – 1 270 ppm (stationary air monitoring) Increased in micronuclei frequency, correlation with duration of exposure.	2	Isoflurane, sevoflurane (< 2.3 ppm)
(Hoerauf *et al.*, 1999) Cross-sectional study 27 exposed and 27 control male and female physicians SCE assay	Equivocal at 11.8 ppm (Air monitoring in the breathing zone, as 8h TWA) Increased SCE in male only	2	Isoflurane (0.5 ppm)
(Rosenberg *et al.*, 1977) Cross-sectional study N= 20 exposed and 20 control male and female nurses Chromosome aberration assay in lymphocytes	NOAEC at 470-2 000 ppm	2	Halothane (0-0.22 ppm)
(Chang *et al.*, 1996) Cross-sectional study N= 18 exposed and 18 control female nurses Micronuclei assay	Exposure poorly characterised Statistically significant increase in micronuclei frequency, correlated with exposure duration in years	3	No information
(Sardas *et al.*, 1992) Cross-sectional study N= 67 exposed and 50 control operating room personnels SCE in peripheral blood lymphocytes	No monitoring of exposure Significant increase in SCE	3	Halothane, isoflurane
(Husum *et al.*, 1986) Cross-sectional study N= 142 exposed male and female dentists SCE in peripheral blood lymphocytes	No monitoring (equipped with scavenging system) No increase in SCE	3	No information

CA: Chromosomal aberration, FPG: Formamidopyrimidine glycosylase LOAEC: Lowest observed adverse effect concentration, MN: Micronucleus, NOAEC: No observed adverse effect concentration, SCE: Sister chromatid exchange, TWA: Time weighted average

5.4.2 *In vitro* and *in vivo* data

In vitro, negative responses were obtained in bacteria after 8 hours exposure to N_2O under pressure (Baden *et al.*, 1981) in *S. thyphymurium* TA 98 and TA 100. One study suggested that the substance may enhance the clastogenic effect of halothane (Sturrock and Nunn, 1976 as cited in MAK, 1993). In addition, N_2O did not induce sister chromatid exchange in chinese hamster ovary cells exposed 1 hour to 1 minimal alveolar concentration (MAC) of N_2O (White *et al.*, 1979).

Few studies were available from the literature search *in vivo* with N_2O tested as sole substance.

In a dominant lethal assay performed in rats (Holson *et al.*, 1995), no statistically significant effect on conception rate, total number of implants and live foetuses was noted in the study up to 10 000 ppm (1% N_2O). Nevertheless, a trend on an increase in the number of resorptions with N_2O exposure was noted in the study. Negative results were also obtained in a sex-linked recessive lethal test in drosophila melanogaster (Kundomal *et al.*, 1985).

5.4.3 Summary and discussion

Reliable *in vitro* and *in vivo* experimental data are lacking. It is notable that the package for genotoxicity studies either under REACh regulation or for the registration of a novel pharmaceutical has not been performed.

Based on human data at the workplace, N_2O exposure was associated with DNA damages with a NOAEC observed at around 96 ppm (Wrońska-Noffer *et al.*, 2009, Braz *et al.*, 2020). One study also suggests that the DNA damages were likely of oxidative type. SCE were observed in males but not in females in one study of workers exposed to low concentration (11.9 ppm) (Hoerauf *et al.*, 1999). No study allows identifying a potential NOAEC or dose-response for SCE. Nevertheless, this type of damage was shown to be reversible suggesting that this type of lesion may be repaired after exposure cessation. Clastogenicity (increased MN, CA) was observed at higher concentrations than DNA damages and SCE lesions. No statistically significant increase in micronuclei was noted in workers exposed at 180 ppm.

N_2O was often combined with other anaesthetics and important confounding and modifying variables were mostly not taken into account. Moreover, the postulated modes of action (MoA) are all indirect, including oxidative stress, folate deficiency and homocysteine toxicity.

Although weak genotoxic effects have been observed in human, postulated mode of action were all indirect, including oxidative stress, folate deficiency and homocysteine toxicity (O'Donovan and Hammond, 2015). Indeed, vitamin B12 deficiency and high plasma homocysteine levels are associated with an increase in micronucleus formation (Mogsenzadegan *et al.*, 2020).

Relevant *in vitro* or *in vivo* data are lacking on N_2O as only a negative Ames assay has been found in the literature.

A threshold is thus expected. Except some equivocal findings on SCE, no genotoxic effect was observed in humans at dose levels below 96 ppm.

5.5 Carcinogenicity

5.5.1 Human

Some epidemiological studies pointed out a trend toward increased incidence of tumours[24] in workers occupationally exposed to anaesthetics (Corbett *et al.*, 1973a). Nevertheless, in this study, no information on the exposure was available. Cohen *et al.* reported a 2.4-fold increase in cervix cancer among female chairside assistants, heavily exposed to anaesthetics compared to non-exposed female assistants. No analysis according to N_2O and other agent exposures was performed in this study. No other epidemiological data focusing specifically on N_2O exposure was found (Cohen *et al.*, 1980).

5.5.2 Animal experimental data

The incidence of tumours in male and female Swiss Webster mice treated with N_2O at concentrations of 10% (100 000 ppm) or 40% (400 000 ppm), 4h/d, 5d/wk for 78 weeks, followed by a 4-week recovery period, was not statistically significantly increased compare to controls (Baden *et al.*, 1986).The authors noted that the background incidence of lung adenoma in mice was high and that the statistical power for the analysis of this tumour type incidence was low.

Table 20: Percentage of mice with tumours observed by gross examination (Baden et al., 1986)

	% of mice with tumours observed by gross examination					
N_2O (ppm)	**Control**		**100 000**		**400 000**	
Tumours	Males	Females	Males	Females	Males	Females
Lung adenomas	37.4	28.4	37.3	31.2	21.1	41.3
Liver adenomas	12.1	4.5	10.6	2.6	7.6	2.7
others	9.9	21.6	13.3	14.3	10.5	14.6

Other studies examining the carcinogenic effect of N_2O also produced negative results, however the exposure period was too short in the study of Eger *et al.* from 1978 and/or co-administration with halothane was used in the study of Coate *et al.* from 1979. These studies were thus considered of low weight for the current analysis

Overall, there is no evidence, in the current literature, that N_2O could be carcinogenic in humans or animals.

5.5.3 Summary

Regards to carcinogenicity, two epidemiological studies pointed toward an increased risk of tumours. Nevertheless, in these studies, exposure to other anaesthetics was not taken into account and no exposure characterisation was performed. Furthermore, no animal study

[24] Tumor type: breast, cervix, thyroid, endometrial, pancreas, hepatocellular, skin, malignant thymoma, leiomyosarcoma, malignant melanoma, leukaemia

demonstrated a carcinogenic potential of the substance. **Overall, there is no evidence of a carcinogenic potential** of N_2O.

5.6 Reproductive toxicity

5.6.1 Fertility and sexual function

5.6.1.1 Human data

Two human retrospective cohort studies specifically investigated N_2O effects on fertility either in midwifes (Alhborg *et al.*, 1996) or in dental assistants (Rowland *et al.*, 1992).

3 358 female midwives, born between 1940 and 1989 and member of the Swedish Association of Midwives, were included in a retrospective cohort study (Alhborg *et al.*, 1996). Exposure was based on the average number of deliveries per month at which the midwife assisted where N_2O was used and on the type of work and work schedule (full-time or part-time). In a multivariate analysis, including all the non-occupational variables, it was found that age, pregnancy order, and previous pill use or fertility problems were still significantly associated with fecundability. Midwives who worked rotating shifts had reduced fertility compared to midwives who worked day-time. Midwifes that had assisted at > 30 deliveries with N_2O per month had longer time to pregnancy than those reporting less or no N_2O exposure. Fecundability ratio was calculated to be 0.63 (95% CI: 0.43-0.94). Adjustment was done for several variables (smoking, age, contraceptive pill, history of pelvic inflammatory disease, number of previous sexual partners, frequency of intercourse, race). There was no information on potential co-exposure in the study.

In another study, questionnaires were sent to 7 000 female dental assistants, registered in California in 1987 and working full-time (> 3h/week) (Rowland *et al.*, 1992). Only 69% responded to the questionnaire in which the authors noted a considerable amount of missing data. Exposure was determined considering the number of hours of exposure per weeks and the presence or absence of a scavenging system. Several adjustments factors were considered in the study (oral contraceptives, number of cigarettes, age, history of pelvic inflammatory disease, number of sexual partners, frequency of intercourse, race). No relation was found between scavenged N_2O exposure and fecundability. Reduced fertility was only noted in women reported more than 5 hours per week to unscavenged N_2O. The odd ratio (OR) was 0.41 (95% CI: 0.23-0.74). Potential co-exposure (e.g. mercury exposure) was not considered in the study.

Overall, although potential fertility effects were seen in two studies, characterisation of N_2O exposure levels and co-exposition are needed to confirm the observed effects and exposure levels.

Table 21: Summary of fertility studies in women occupationally exposed to N_2O

Method	Results	Reliability[25]	Reference
Retrospective cohort study Midwives N=380 exposed, 346 non-exposed	Fecundability ratio: 0.63 (95% CI: 0.43-0.94) at reported high and frequent exposure	2	Alhborg *et al.*, 1996
Retrospective cohort study in US Female dental assistants N=181 exposed (121 scavenged and 60 unscavenged) and 203 unexposed	OR fecundability ratio= 0.41 (95% CI: 0.23-0.74) in exposure group ≥ 5 hours/week to unscavenged N_2O	3	Rowland *et al.*, 1992

OR: odd ratio; CI: confidence interval

5.6.1.2 Animal data

In rats, three studies were identified investigating fertility and sexual function. All these studies had limitations (one dose level, low number of animals). None of the studies was in compliance with OECD TG for reproductive toxicity.

- Studies investigating male reproductive function and fertility
    - *Rats*

In a study by Kripke *et al.*, male rats (n=6-8 per group) were exposed 8h/d or 24h/d during 28 or 38 days with 6-day recovery to 200 000 ppm N_2O. Absolute testis weight was decreased in both groups. Damage and destruction of spermatogenic cells were observed. No effect in Leydig cells and supporting cells within the tubules was reported, as well as for testosterone levels. The study is considered of limited reliability due to the very low number of animals. In addition, only one dose was used, no information was provided on the source of test material, the duration of exposure was not long enough to cover the whole spermatogenic cycle and no information was provided if the experiment were performed under blinded conditions (Kripke *et al.*,1976).

In one study of Vieira *et al.*, two groups of 12 male Wistar rats were used. One group was exposed to 5 000 ppm N_2O/air mixture (v/v) and one group was exposed to air only (control) (Vieira *et al.*, 1983a). The rats were exposed for 6h/d, 5d/w for 30 days. The concentration of N_2O was checked. At the end of the exposure period, each male rat was mated with 3 nulliparous female rats. Following mating, the male rats were allowed for recovery period of 6 months. Thereafter, each male rat was mated with 3 female nulliparous rats. At birth, the number of each litter was recorded and all litter mates were examined macroscopically for gross defects. Thereafter the young rats were weighed and measured at weekly interval for 8 weeks. The study is considered reliable with limitations due to the low number of animals per groups, as only one dose was tested, no information was provided on general toxicity, due to the short period of exposure, the lack of reporting of environmental conditions, as no information on source of test material.

A statistically significant decrease in litter size and delayed development was seen after exposure compared to control. These findings were not observed 6 months after recovery.

[25] See annex 2

According to the authors, the litter size showed that in the control group one litter numbered nine offsprings while the remaining 35 mothers had litters ranging from 11-15. This pattern was similar to that in the group following the recovery period which showed one litter with eight offsprings and the remaining litters ranging from 10-14. In contrast, in the group mated immediately after exposure to N_2O one litter comprised 14 offsprings but the remaining 35 litters ranged between two and six offsprings. Regards to body weight of offsprings, there was a significant difference from week-3 onward in weight of the offsprings belonging to the group exposed to 5 000 ppm N_2O. A significant decrease in tail length and body length was also noted in this group. The effects were not observed in the offsprings belonging to the group of males that were allowed 6 months recovery.

Results obtained in the study are summarized in the table below.

Table 22: Summary of litter size results in Vieira *et al.*, 1983a

Group	Control	N_2O, 5 000 ppm initial mating	N_2O, 5 000 ppm 6-month recovery
Number of born rats	382	252	380
Litter size	Mean: 12 Range: 9-15	Mean: 7* Range: 2-14	Mean: 11 Range: 8-14

*p<0.001

Male rats were exposed 5 day per week for 9 weeks, 6 hours per day (3 replicates of 4 males per groups) 0, 1 000, 5 000, 10 000 ppm (Holson *et al.*, 1995). Exposed males were mated with non-exposed females (paternal study). No effect on body weight or body weight gain was noted in the males. In addition, no statistically significant effect on litter size was noted compared to control. Nevertheless, the authors reported a trend to a decrease in the number of pups per litter in the nitrous oxide groups. The male used in this study were also mated with 30 non-exposed females at the end of the exposure period for a dominant lethal assay. Although no statistically significant findings (ANOVA) were noted in the number of implants per litters, number of live foetuses per litters and number of resorptions per litter, the authors noted a tendency to an increase in the number of resorptions in the top dose group.

- *Mice*

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

- No effects were observed on testis or ovaries in mice exposed to nitrous oxide up to 500 000 ppm during 4 hours per days, 5 days per week for 14 weeks (Rice *et al.*, 1995);
- In a study by Mazze *et al.*, no effects were noted on male testes and spermatogenesis and on female mice oocyte count with the same experimental conditions and dose levels as in Rice *et al.*, 1995 (14-week exposure, 4h/d, 5d/w) (Mazze *et al.*,1983);
- Mazze *et al.* did not report effects on the ability of males to impregnate females or on litter size, foetal wastage (dead and resorbed) or foetal size after 4h exposure per day

of male mice for 9 weeks (5 days per weeks) up to 500 000 ppm nitrous oxide (Mazze *et al.* (1982).

Table 23: Summary of fertility effects induced by N_2O in males

Method	Results	Reliability (Klimisch)	Reference
RATS			
LEW/f Male rats Inhalation, 8h/d (intermittent group) or 24h/day (continuous group). N=4-6 rats/groups 200 000 ppm (20%) N_2O, 20% O_2 and 60% N_2 Group 1: 28 days, group 2: 32 days, group 3: 38 days + 6 days recovery Sacrifice: 1 to 42 days	General toxicity: no information Male reproductive organs: Statistically significant decrease in testis absolute weight in group 1 and 2. Microscopic examination: injury (damage, destruction) observed in the spermatogenic cells in the seminiferous tubules all animals. No effect in Leydig cells and supporting cells within the tubules. No effect on serum testosterone levels	2	Kripke *et al.*, 1976
Wistar male rats N= 12/group Inhalation, 6h/d, 5d/w, 30 days Mating with non-exposed females: after exposure or after 6-month recovery 0, 5 000 ppm N_2O (0.5%) in air	General toxicity: no information Male findings: Statistically significant decrease in litter size and developmental delay in offspring of the male rats exposed to N_2O at 5 000 ppm. No significant effect after recovery period.	2	Vieira *et al.*, 1983a
Sprague-Dawley rats N=12/groups (3 replicates of 4 males per dose) Whole body inhalation exposure Males: 6h/day, 5d/w for 9 weeks. Mated with non-exposed females 0, 1 000, 5 000, 10 000 ppm N_2O in air	General toxicity: no effect on weight of males Paternal study: No statistically significant findings in litter size (trend to lower number of pups per litter in nitrous oxide groups)	2	Holson *et al.*, 1995
MICE			
Mice N=18-20 male/group Inhalation, 4h/d, 5d/w, 9-week exposure 0, 5 000, 50 000 or 500 000 ppm	No effect on litter size or growth	2	Mazze *et al.*, 1982
Swiss Webster mice N=15/sex/group Inhalation, 4h/d, 5d/w, 14-week exposure Control, 0.5%, 5% or 50% nitrous oxide (5 000, 50 000, 500 000 ppm).	No effect on testes weight and histopathology. No effect on oocyte population.	2	Mazze *et al.*, 1983
Swiss Webster Mice N=15/sex/group Inhalation, 4h/d, 5d/w, 90-day study Doses: 0, 0.5% (5 000 ppm), 5% (50 000 ppm), 50% (500 000ppm)	No effect on testis, seminal vesicle or ovary at necropsy	4	Rice *et al.*, 1985

- Studies investigating female reproductive function and fertility

According to the short summary provided in Kugel *et al.*, 24 virgin female rats were used to investigate potential effects of N_2O on fertility (Kugel *et al.*, 1989). Daily vaginal smears were taken and rats with 2 consecutive normal cycles were used. Twelve rats were places in an environmental chamber with a mix of 500 ppm N_2O/compressed air delivered 8 hours/day for 35 days (controls received compressed air). All exposed rats had disrupted ovulatory cycles consisting of constant proestrus (day of ovulatory surge) and lasting 3 weeks. Ovulatory cycle gradually returned to normal. Control rats had normal cycles. All rats were mated with proven male breeders. 6 to 12 N_2O exposed and 12 on 12 control rats gave birth. No difference was noted in birth weight or in litter size. According to the short abstract available, the authors suggested that the results may be explained by disruption of luteinising hormone releasing hormone LHRH cells in the hypothalamus; LHRH increases LH release and in turn causes ovulation. These results should be used with care due to the lack of reporting on the method used in the study.

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

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

Table 24: Summary of fertility effects induced by N_2O in females

Methods	Results	Reliability (Klimisch)	References
Sprague-Dawley female rats Inhalation, whole body exposure 0 or 300 000 ppm N_2O, 8h/day for 4 days Fertility study N=12 animals/group mated with proven male breader. 6 animals/group treated in proestrus and 6 animals/group treated in random stage of ovulatory cycle	Maternal toxicity No information Fertility study Mating occurred in all animal. All 12 controls gave births. Only 6 out of 12 treated animals (3 in groups treated in proestrus and 2 in random phase of the cycle) gave birth. No effect on litter size and weight of pup Ovulatory cycle study (n= 12/group) Disrupted cycles in 11/12 (constant proestrus). Reversible after approximately 3 weeks. No effect in controls Brain study (n=8/group) ↑ LHRH cells in brains of N_2O exposed females	2	Kugel *et al.*, 1990
Non-guideline fertility study in rats N=24 female rats Inhalation, 35 days, 8h/d, 5d/w 500 ppm N_2O (0.05 %) Klimisch score 4 (insufficient reporting, abstract only available)	Transitory oestral cycle disturbance in all treated female rats. All 12 controls gave births. Only 6 out of 12 treated animals. No effect on litter size and weight of pups.	4	Kugel *et al.*, 1989

5.6.1.3 Summary and discussion

The available animal studies did not sufficiently investigate the sexual and reproduction function following N_2O exposure. Fertility effects have been observed at exposure levels of 300 000 ppm and were reported at dose levels as low as 500 ppm in female rats (Kugel *et al.*, 1989). Nevertheless, this study was insufficiently reported and additional data would be needed to confirm the LOAEC for these effects. In male rat, exposure to N_2O at 5 000 ppm produced effect on the litter sizes and delayed the development in pups. Testicular damages were observed in male rats after exposure to 200 000 ppm for 28-38 days. No effect on testis and/or male fertility was observed in mice after exposure to 800 000 ppm.

Few studies investigated N_2O fertility effects in human. Co-exposure and absence of characterisation of N_2O exposure lead to uncertainties in the fertility findings.

5.6.2 Developmental toxicity

5.6.2.1 Human data

Abortion

Six studies were identified on spontaneous abortion risk specifically related to N_2O exposure. In all the studies, exposure concentrations were poorly defined and no measurement was available.

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

No increased risk was observed in midwives in a retrospective cohort study of Axelsson *et al.* (Axelsson *et al.,* 1996). The study population includes all female members of The Swedish Association of Midwives in 1989 and born after 1940. On this population, 84.3% answered the questionnaire and 7 599 pregnancies, which began before 1989, were reported by 2 786 women. To avoid memory bias, pregnancies that started before 1980 were excluded. Only women that worked more than half time during the first trimester and for which information was completed were included. Final analysis was made among the 1 717 pregnancies during which the women worked as a midwife. Exposure was based on three categories (no use, use of N_2O in up to 50% of assisted delivery and use of N_2O in more than 50% of assisted deliveries). The nurses were not sure whether scavenging equipment was present. Several adjustment factors were included: age, pregnancy number, previous spontaneous abortion, smoking, infection, analgesic drugs, other anaesthetic gas, work time.

In contrast, in California, Rowland *et al.* performed a retrospective cohort study in female dental assistants exposed at least 3 hours per week to unscavenged N_2O (Rowland *et al.,* 1995). In this study, 7 000 dental assistants were selected, 1 805 of the respondents had been pregnant at least once and 1 465 provided information about the pregnancy. Exposure was assessed based on the date of the woman's last menstrual period and work history information (number of hours per week of exposure, scavenging system). Potential confounding factors were categorised for smoking, age, previous spontaneous abortions, coffee consumption, heavy lifting, infection with high fever, night work, shifts, and shortage of staff, daily contact with other anaesthetic gases, ultrasound equipment, antineoplastic drugs and stress. No information on potential confounders such as alcohol use or paternal occupation were taken into account. An increase in the risk of abortion was observed with an odd ratio of 2.6 (CI 95%: 1.3-5). No clear exposure-response effect was observed in this study. However, it was noted by the authors that in the United Sates, full-time working women tend to be less reproductively healthy than women of similar social class background who work part time or who do not work outside the home (women who have child often work part time). As the analysis was limited to women working full time, an "unhealthy worker effect" may be mistaken for an occupational exposure effect. In addition, the authors pointed out that an earlier detection of pregnancy by an exposed group can increase the number of recognised spontaneous abortions and created appearance of an occupational hazard. The authors adjusted for potential co-exposure to mercury.

In a retrospective study published by Heidam *et al.*, a cohort of 772 dental assistants, working in dental clinics or in dental school services, were included in the study (1 431 employees used as control group) (Heidam *et al.*, 1984). The data were collected via a questionnaire and covered the women's entire reproductive lives before May 1980. No excess of abortions was noted in the study. Nevertheless, exposure was not characterised and there was insufficient information on potential other risk factors (e.g. smoking). Potential co-exposure to inorganic mercury was taken into account and odds ratio for specific substance were calculated. A negative information bias was identified by the authors.

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

A higher incidence of spontaneous abortion was also noted in several other old studies among workers directly exposed to waste anaesthetic gases (ACGIH, 2018; MAK, 1993). Nevertheless, in these studies, N_2O was not specifically investigated and thus, these studies are not further described.

Table 25: Summary of N_2O effects on abortions in women occupationally exposed to N_2O

Methods	Results	Reliability [26]	References
Cross-sectional study n= 23 exposed (operating room workers) n= 20 unexposed from internal medicine department Co-exposure: unknown	No association	3	Eftimova *et al.*, 2017
Cross-sectional study n= 60 exposed (operating room nurses), no control Co-exposure: isoflurane, sevoflurane	No association	3	Uzun *et al.*, 2014
Retrospective cohort study N= 1 119 exposed, 598 unexposed (midwives) Co-exposure: unknown	No association	2	Axelsson *et al.*, 1996
Retrospective cohort study Dental assistants N=424 exposed, 559 unexposed Co-exposure (both group): mercury	OR (abortion) =2.6 (95% CI: 1.3-5), exposure ≥ 3 hours/week to unscavenged N_2O. No effect if scavenged system is used	2	Rowland *et al.*, 1995
Retrospective cohort study Dental assistants N=807 exposed, 3 184 unexposed Co-exposure (both group): mercury	1-8h/week exposure: 1.75 fold increase in spontaneous abortion. > 8h/week: 2.25-fold increase	2	Cohen *et al.*, 1980
Retrospective cohort study Dental assistants N=22 exposed, 97 unexposed Co-exposure (both group): mercury, organic solvent	No association	2	Heidam *et al.*, 1984

Developmental abnormalities

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

[26] See annex 2

(95%CI: 1.1-2.99)). The anomaly the most frequently associated with N_2O exposure was integument. An increased risk was also noted for halothane (OR=2.61 [1.31-5.18]), isoflurane (OR= 2.82 [1.3-5.82]) and sevoflurane (OR= 4.71, 95%CI: 2.14-10.3). Associations were increased with likelihood of exposure.

A statistically significant increase in congenital anomalies was also noted in US dental assistants exposed to N_2O (Cohen *et al.*, 1980). In this study, as described above, 15 000 users of N_2O and 15 000 non-users were included in the study in 25 hospitals equipped with advance scavenging system. The authors took into account potential mercury exposure and same level of exposure to mercury was noted in exposed and non-exposed groups. The rate of congenital abnormalities was based on the number of living babies born with one or more non-skin abnormalities per 100 births. No specific measurements were performed in the study. Light exposure was defined as 1 to 8 hours exposure per week, considering cumulative exposure 1-2 999 hours in the past ten years. Heavy exposure was defined as exposure above 8 hours per day and cumulative exposure above 3000 hours in the past decade. A 1.5-fold increase in the rate of musculoskeletal defects was observed in the exposed groups. Only a limited number of adjustment factors were taken into account: maternal age, smoking history, history of previous spontaneous abortion or congenital abnormalities.

In a study by Bodin *et al.*, new-borns from midwives exposed to N_2O had a reduced birth weight when compared to the control group (OR=-77g; 95% CI= -129, -24) and an increase in odds of being small for gestational age (OR: 1.8; 95% CI =1.1, 2.8) (Bodin *et al.*, 1999). In this study, exposure was characterised based on the use of N_2O (<50% or > 50% of all deliveries). Information on the use of a scavenging system was often missing. Adjustment factors that were considered were the gestational age, the parity, the employment and work schedule. Potential co-exposures to other substances were not addressed in the publication.

Table 26: Summary of N_2O effects on abortions in women occupationally exposed to N_2O

Methods	Results	Reliability[27]	Reference
Retrospective cohort study Nurses N= 934 exposed, 13 735 unexposed Co-exposure: halofuran, enflurane, isoflurane (<LOD)	OR (congenital anomaly) = 1.82 (95%CI: 1.1-2.99)	2	Teschke *et al.*, 2011
Retrospective cohort study Dental assistants N=807	1.5 fold increase in congenital abnormalities (statistically significant)	2	Cohen *et al.*, 1980
Retrospective cohort study Midwives N=761 exposed and 855 non-exposed	Reduction in birth weight (OR=-77g, 95% CI: -129, -24) and increase infants being small for gestational age (OR=1.8, CI=1.1,2.8)	2	Bodin *et al.*, 1999

LOD: limit of detection; OR: Odd Ratio, CI: Confidence Interval

[27] See annex 2

5.6.2.2 Animal data

5.6.2.2.1 Rats

- *Prenatal developmental toxicity*

In rats, developmental prenatal toxicity studies were found in the literature and analysed.

In most of the studies, continuous (23-24 hours per day) exposure to N_2O during specific period of gestation was used. Based on these studies, a LOAEC of 1 000 ppm and a NOAEC of 500 ppm could be identified based on risks of resorptions, decreased litter size, delayed development and malformations (Vieira *et al.*, 1980). A decrease in implantations per rat was also observed by Corbett *et al.* at the same dose level (Corbett *et al.*, 1973b). As this type of exposure is not considered representative of occupational exposure, these studies are just presented in the Annex 4 for references.

Six studies were conducted using intermittent exposure. The results of these studies are summarised in the table below.

Table 27: Summary of prenatal developmental toxicity studies in rats following N_2O intermittent exposure

Methods	Results	Reliability (Klimisch)	Reference
EXPOSURE DURING WHOLE GESTATION PERIOD			
Wistar female rats N=12/group Inhalation, 6h/d, 5d/w, whole gestation period (3 weeks) 0; 250; 500; 1 000; 5 000 ppm N_2O in air	Maternal toxicity: no information Developmental toxicity: - Dose-related ↓ in litter size (statistically significant at 5 000 ppm) - No effect on foetal weight or crown-rump length of foetuses. - No malformations reported.	2	Vieira *et al.*, 1983b
Sprague-Dawley female rats N= 10 per groups Inhalation, whole body 8h per day, 5d/w, GD1-21 0, 200 000 ppm nitrous oxide	Maternal toxicity: no information Developmental toxicity: no skeletal findings unlike after continuous exposure	4	Rao *et al.*, 1981; Tong *et al.*, 1982 as reported in MAK, 2015
Female rats N=30/group Inhalation, 6-7h/d, whole gestation 0; 1 000 ppm N_2O in air	Maternal toxicity: no evidence Developmental toxicity: no evidence	4	Hardin *et al.*, 1981
Sprague-Dawley female rats N=8-10/group Inhalation, 8h/d, whole gestation (GD 0-20) 0; 10 000; 100 000; 500 000 ppm N_2O in air, additional stress group as control	Maternal toxicity: no effect (body weight, food consumption) Developmental toxicity: - delayed development (foetal weight, crown-rump length, ossification), stat. sign. at ≥ 100 000 ppm - ↓ placental weight, stat. sign. at ≥ 10 000 ppm - increased foetal loss at the low and mid dose but not statistically significant and inside spontaneous range of the laboratory. No increase in foetal loss at 500 000 ppm.	2	Pope *et al.*, 1978
EXPOSURE DURING SPECIFIC WINDOW OF THE GESTATION PERIOD			
Sprague-Dawley female rats N=3-7/group Inhalation, 8h/d 0; 750 000 ppm N_2O in air GD9-11, GD9-13, GD14-15	Maternal toxicity: no effect on body weight Developmental toxicity: ↑ resorptions	3	Tassinari *et al.*, 1986
Sprague-Dawley female rats N=19-50 per groups Inhalation, 6h/d 0; 750 000 ppm N_2O mixed in air and oxygen GD14-16, GD11-13 or GD8-10	Maternal toxicity: ↓ in body weight gain (statistically significant) Developmental toxicity: - ↓ foetal weight - ↑ resorptions and foetal wastage (dead and resorbed) in dams exposed during GD14-16 window - ↑ major malformations and external abnormalities in dams exposed on GD 8-10	2	Mazze *et al.*, 1986
Female rats N=6-12/group Inhalation, 8h/d, whole gestation 0; 100; 1 000 ppm N_2O in air GD10-13, GD14-19 or GD10-19	Maternal toxicity: no information Developmental toxicity: no effect	3	Corbett *et al.*, 1973

GD : Gestation day

Mazze *et al.* exposed Sprague-Dawley rats to 750 000 ppm nitrous oxide, during 6 hours per day, during critical periods of gestation GD 14-16, 11-13 and 8-10 (plug day = day 1 of pregnancy) (Mazze *et al.*,1986). A statistically significant increase in resorptions was noted in rats exposed during GD 14-16 (1.32 per litter compared to 0.46 in controls). This was associated with a decrease in live fetuses/implantations. No statistically significant increase in malformations was noted. Nevertheless, an increase in external abnormalities and malformations was noted in dams exposed on GD 8-10 and of skeletal malformations in dams exposed on GD 14-16 was observed in nitrous oxide group compared to control. No historical control is available. No increase in visceral malformations was noted in this study. The type of malformation was not further detailed in the publication.

Regards to maternal toxicity, decreased in body weight gain were noted in dams exposed to nitrous oxide compared to controls on GD 14-16 only. There is no information on corrected body weight. This would better reflect potential maternal toxicity as the decreased may have been due to the increased foetal wastage in the exposed group. Animals were conscious throughout the experiments in all groups.

In another study, Sprague-Dawley rats were exposed 8 hours per day during the whole gestation period to 10 000, 100 000 or 500 000 ppm nitrous oxide in air (Pope *et al.*, 1978). Foetal delayed development was reported at ≥ 100 000 ppm. Delayed development consisted of a statistically significant decrease in foetal weight, a decrease in crown rump lengths; it was associated with a significant delay in ossification. The authors did not observe dose-related effects on resorption and foetus death after nitrous oxide exposure. No maternal toxicity was reported. Only gross skeletal abnormalities were examined and no visceral examination was performed in the study.

Decreased litter size was noted in a study where rats were exposed 6 hours/day, 5 days/week during the 3 weeks of the gestation period at 250, 500, 1 000 or 5 000 ppm (Vieira *et al.*, 1983b). A LOAEC based on decreased litter size was identified at 5 000 ppm by the authors. The NOAEC was 1 000 ppm. No foetal delayed development was noted. There was no information on maternal toxicity in this study.

Table 28: Developmental findings reported by Vieira et al., 1983b.

Dose levels (ppm)	No. of foetuses	Litter size (Mean ± SD)	Range per litter
0	120	11 ± 1.4	9-13
250	119	11 ±1.3	9-13
500	117	11 ±1.3	8-13
1 000	117	10 ± 1.2	8-13
5 000	98	7.0 ± 2.3 ***	6-10

***p<0.001

In another study, neither teratogenic effects, nor decreased litter size, or decreased foetal body length and weight were observed after 8 hours/day, 5 days/week exposure to 200 000 ppm nitrous oxide during the whole gestation period (Rao *et al.*, 1981).

Overall, three main effects were induced by N_2O exposure in these studies following 6 to 8 hours/day exposure to nitrous oxide during gestation: delayed development, decreased litter size and resorptions. No malformation was reported following 6 to 8 hours exposure to N_2O in contrast to continuous (23-24 hours exposure). An increase in resorptions were noted following high exposure levels at 750 000 ppm N_2O in 2 studies (Mazze *et al.*, 1986; Tassinari *et al.*, 1986). Decreased litter size was noted in the study conducted during the whole gestation period at ≥ 5 000 ppm (Vieira *et al.*, 1983b). A NOAEC was identified at 1 000 ppm by the authors. Hardin *et al.* did not found effect on litter size at 1 000 ppm (single dose tested) (Hardin *et al.*, 1981). However, Pope *et al.* did not reported effect on the number of live foetuses per litter, up to 500 000 ppm (Pope *et al.*, 1978). In the same way, decrease in litter size was not observed following exposition during a specific period of gestation at 750 000 ppm (Mazze *et al.*, 1986 and Tassinari *et al.*, 1986). The adversity and the biological relevance of the decreased litter size in the absence of concomitant findings and reproducibility in another laboratory is questionable.

- *Pre-/Post-natal developmental toxicity studies*

Male rats were exposed for 9 weeks, 6 hours/day, 5 days/week to nitrous oxide at 0, 1 000, 5 000 or 10 000 ppm (Holson *et al.*, 1995). At the end of the exposure period, males were mated with non-exposed females. In addition, females were exposed in similar condition during the whole gestation period. No effects on litter size, and weight or behaviour of pups were noted. No effects were reported on offsprings from female exposed to nitrous oxide during the whole gestation period for 6 hours/day, 5 days/week or from males exposed for 9 weeks. Offspring from treated adults was subjected to an extensive behavioural test battery. Although some positive results were obtained in some tests, such as an increase in developmental activity in females at PND20 at 10 000 ppm, the authors considered that, overall, the full battery of test was negative.

Mullenix *et al.* exposed rats on GD 13 or GD 14-15 for 8 hours/day to 750 000 ppm nitrous oxide mixed in oxygen. Exposure on GD14-15 produced hyperactivity in both males and females. In contrast, exposure on GD14 only produced a tendency to hypoactivity in females and hyperactivity in males (Mullenix *et al.*, 1986).

In contrast, twelve Wistar female rats per groups were exposed to N_2O in air at 10 000 ppm during the whole gestation, during the first two weeks of gestation or during the first week of gestation only. At birth, all litter mates were examined for gross defects and the number of each litter was recorded. At weekly interval, the young rats were weighed and tail and body length were measured. A decrease in litter size was noted at 10 000 ppm in all exposed groups. Decreased postnatal growth was also noted in all exposed groups when compared to control. Nevertheless, the results of this study were rated in Klimisch score 3 due to insufficient reporting on study method and results (Vieira *et al.*, 1978).

Table 29: Developmental findings reported by Vieira et al., 1978.

Period of exposure	No. of offspring	Litter size (Mean ± SD)
None	248	10.4 ± 2.3
3 weeks	66	8.3 ± 3.2***
1st and 2nd weeks	850	6.3 ± 2.6***
1st week	846	5.7 ± 1.6***

***p<0.001

5.6.2.2.2 Mice, rabbits, guinea-pigs and hamsters

- Mice

In mice, four studies were retrieved through the literature search.

In a prenatal developmental toxicity study, mice were exposed 4 hours/day during GD 6-15 to 0, 5 000, 50 000 or 500 000 ppm. No maternal toxicity was noted in the dams. No developmental effect was noted up to the highest dose tested (Mazze *et al.*, 1982).

Female SW mice (10 litters per groups) were exposed to 0, 5 000 ppm, 150 000 ppm or 500 000 ppm N_2O during GD6-15, for 4 hours per day. Post-natal developmental effects were investigated. No effect on survival or in motor coordination was noted in pups. Hyporeactivity as shown by a statistically significant decrease in startle response (auditory, tactile) in all dosed group was noted at postnatal day (PND) 95. Nevertheless, no dose-response was observed (Rice *et al.*, 1990).

In Rodier *et al.* (Rodier *et al.*, 1985) and Koëter *et al.* (Koëter *et al.*, 1986), mice were exposed to 750 000 ppm N_2O, 6 hours on GD14 or 4 hours at PND 2. Disturbed activity was noted in pups at PND 8, 13 and 20-23 but not at PND45-48 or at 6-month. Only the abstract was available for these studies.

- Rabbits

One developmental toxicity study was available in rabbits (Hardin, 1981). Due to the lack of reporting, the study was rated in Klimisch 4. In this study, no developmental or maternal effects were observed at 1 000ppm N_2O in air following 6-7 hours exposure during the whole gestation period. Higher dose level was not investigated in rabbits.

- Guinea-pigs and hamsters

In a study following a single 24 hours exposure to 700 000 to 950 000 ppm N_2O during gestation, increased incidences of resorptions and malformations were seen. In contrast, no effect was observed in hamsters continuously exposed to 500 000 ppm for 4-5 days during gestation and the authors concluded that it was unsure if the effects observed in guinea pigs were due to N_2O, hypoxia or a combination of both (Shah *et al.*, 1979).

5.6.2.3 Summary and discussion

In experimental animals, using exposure period of 6 or 8 hours/day during gestation, the lowest NOAEC was 1 000 ppm based on decreased litter size and 5 000 ppm based on delayed foetal development (Vieira *et al.*, 1983b). The effects on litter size were not observed in other laboratories, questioning the toxicological relevance. Therefore, based on delayed development, a NOAEC of 5,000 ppm could be retained for developmental toxicity.

Neurobehavioral studies in pups exposed during gestation provide some evidence on potential effect on reactivity in pups at 750 000ppm nitrous oxide (Mullenix *et al.*, 1986).

In humans, some studies indicated that N_2O may induce congenital abnormalities or reduction of birth weight following high exposure (no measurements available) or in the absence of appropriate scavenging systems. Nevertheless, the interpretation of the human data is difficult due to potential co-exposure, the absence of reliable characterisation of exposure, and the absence of adjustment for potential other risk factors. It may be noted that according to animal data, co-exposure with other anaesthetic agents (e.g. halothane or isoflurane) may be protective, whereas other anaesthetic could potentiate N_2O developmental toxicity. Therefore, the results of the human data may be very difficult to interpret in case of co-exposure. Regarding abortion, inconsistent findings were noted in humans.

6 Construction of the OELs

6.1 Construction of an 8 hour occupational exposure limit (8 hour-OEL)

6.1.1 Choice of the critical effect

Regards to carcinogenicity, two epidemiological studies pointed toward an increased risk of tumours (Corbett *et al.*, 1973a; Cohen *et al.*, 1980). Nevertheless, in these studies, exposure to other anaesthetics was not taken into account and no exposure characterisation was performed. Furthermore, no animal study demonstrated a carcinogenic potential of the substance. Overall, there is no evidence of a carcinogenic potential of N_2O.

The review of all repeated toxicity studies by inhalation identified nervous system, blood and immune systems and reproductive system as the most sensitive.

Regards to reproductive toxicity, according to human cohort studies, there is an indication that nitrous oxide exposure during pregnancy is associated with a decreased fecundity ratio, with an increased incidence of congenital abnormalities and a reduced body weight at birth. Nevertheless, interpretation of human data is difficult due to potential co-exposures (mercury, halogenated anaesthetics, ionizing radiations, heat, prolonged standing…) and other confounding factors (few adjustments in the studies). In addition, in none of the studies exposure was sufficiently characterised.

N_2O was teratogenic in rats after continuous exposure of 23 or 24 hours per day during critical periods of gestation. Malformations, reduced litter size, resorptions and delayed development were also observed. Using intermittent exposure of 4 to 8 hours per day during gestation, more relevant for occupational workplace exposure, delayed development was observed in rats, at 10 000 ppm and decrease litter size at ≥ 5 000 ppm (Vieira *et al.*, 1983b). Pope *et al.* (Pope *et al.*,1978) and Holson *et al.* (Holson *et al.*, 1995) studies did not report these effects at 10 000 ppm, leading to uncertainties on dose-response and on the toxicological relevance of the decreased litter size in absence of other critical findings (e.g. resorptions). Overall, based on the most sensitive effect observed in the Vieira *et al.*'s study a LOAEC of 5 000 ppm can be considered for delayed development.

Fertility effects have been insufficiently investigated in animals. In experimental animals, the decreased fertility and changes in the oestrus cycle observed in female rats in two studies raise clear concerns about the fertility of women exposed to N_2O. In addition, the effects observed in male rats on testes and spermatogenesis (Kripke *et al.*, 1976) and the reduction in litter size (Vieira *et al.*, 1983a) support potential effects on male fertility. However, there are uncertainties on dose-response due to the limitations of the available studies, notably the use of a single dose level or the low number. Although not questioning the effects observed in rats, the lack of effect in mice is not explained.

Although reported in rats, effects on fertility and development were not retained as critical effect due to the absence of a dose-response relationship.

Blood and immune systems toxicity

Recent studies of effects on blood and immune systems in human after repeated-exposure have been identified. There are some evidence that N_2O exposure affects haematological parameters, notably lymphocyte sub-populations (Bargellini *et al.*, 2001). Nevertheless, in these studies, no quantitative exposure-response association or correlation with duration of exposure could be identified and co-exposure to other substances was almost always present (e.g. X-ray, halothane, etc.). In animals, immune function was tested in a 90-day study and altered humoral response was observed at 5 000 ppm (Healy *et al.*, 1990). Inconsistent results were obtained on white blood cell parameter in the studies after repeated-dose administration that may be due to strain differences in sensitivity.

Although observed in humans and animals, effects on hematological and immune systems y were not retained as critical effect due to the absence of a dose-response relationship, the existence of co-exposures not taken into account or equivocal results.

Neurotoxicity

- **Human data**

Nervous conduction

Acute exposure. Two human volunteer studies reported impairments of nervous conduction at exposure ≥ 200,000 ppm (Gyulai *et al.*, 1996; Williams *et al.*, 1984). No study was found at dose levels below this value.

Cognitive performance

Acute exposure.

In human volunteer experimental studies, data are equivocal. In one hand, a slight impairment of audio-visual capacity at 50 ppm and lower performance in visual acuity, audio-visual capacity, immediate memory and vigilance response were reported after 4-hour exposure to 500 ppm N_2O (Bruce and Bach, 1976). In the other hand, Venables *et al.* failed to reproduce the results obtained at 50 ppm (Venables *et al.*, 1983). At 500 ppm, effects on memory (digit-span test) were also previously reported by Bruce and Bach (Bruce and Bach, 1975). Other acute studies were performed at higher dose levels (Yajnik *et al*., 1996; Fagan *et al*., 1994; Mahoney *et al*., 1988; Estrin *et al*., 1988). A LOAEC of 50-500 ppm can be identified for these effects. Nevertheless, according to the authors, the volunteers used for the Bruce and Bach study may have been particularly sensitive to the cognitive effects of N_2O and may not have been representative of the general population (Bruce and Bach, 1976).

Sub-acute exposure

In humans, occupationally exposed to N_2O, sub-acute effects on reaction time were noted in the most reliable studies investigating psychomotor function (Scapellato *et al.*, 2008; Lucchini *et al.*, 1995, 1996 and 1997). The effects on neurobehavioural performance were seen at the end of the working week. The study results support that these effects were reversible, as no impairments were detected on the next Monday morning. Nevertheless, potential chronic neurotoxic effects of nitrous oxide were not investigated. Regarding the dose levels, these acute findings were associated with a mean 27 µg/L urine N_2O level, corresponding to an air concentration of 50 ppm in the presence of 1.3 ppm isoflurane (Scapellato *et al.*, 2008). The effects were noted at 54.2 ppm and 62.6 ppm in the end of the working week in the presence of 1.5 ppm isoflurane (Luccini *et al.*, 1995 and 1996). Lucchini *et al.* (Lucchini *et al.*, 1997) and

Scapellato *et al.* (Scapellato *et al.*, 2008) did not find effect on neurobehavioural performance at 23 ppm of N_2O or below 27 µg/L in urine, respectively. A NOAEC of 25 ppm and a LOAEC of 50 ppm can be retained from these studies based on neurobehavioural performance (rounded values).

- **Animal data**

Acute exposure.

In several studies in rats (Jevtovic-Todorovic *et al.*, 2000, 2001, 2003 and 2005; Courtière *et al*., 1997; Dzoljic *et al*., 1994), brain histopathological findings, locomotor activity and/or behavioural changes were reported after single exposure ≥ 300 000 ppm N_2O. Behavioral changes were also noted in the three described mice studies at ≥ 500 000 ppm (Li *et al*., 2001; Caton *et al*., 1994; Dorris *et al*., 1993). No study was found at lower dose exposure levels.

Sub-acute exposure

Only one repeated-dose toxicity study was identified in animals at dose levels below 100 000 ppm (Fung *et al.*, 1993). At 1 000 ppm a slight effect on stereotypy was noted in rats. Nevertheless, as effect on stereotypy was not observed at higher dose levels and as only 6 animals per group were used in the study, confirmation of an effect at this dose level is needed.

Sub-chronic/chronic exposure. In animals, species differences have been noted. In rats, after one-month exposure, brain and spinal cord degeneration were noted at ≥ 500 000 ppm N_2O (Singh *et al.*, 2015; Misra *et al.*, 2020). In mice, no weight or histopathological changes were noted up to 500 000 ppm in a 14-week repeated-dose exposure study (Rice *et al.*, 1985).

Overall, the most sensitive effect in humans was the sub-acute impairment of cognitive performance in workers established in several published studies.

The effects on vigilance may be of importance for tasks where attention is needed (such as in operating room theatre). In addition, no data are available regarding possible long-term effects on the central nervous system functions. Therefore, impairment of neurobehavioural function is considered as the critical effect for N_2O.

It is expected that the 8h-OEL based on this critical effect would also prevent potential effects on immune function, blood system and developmental toxicity. **Nevertheless, it is not possible to know whether the OEL could prevent potential fertility effects of the substance as both robust human and animal data are lacking.**

6.1.2 Choice of the key studies

According to the Anses OEL derivation methodology (Anses, to be released), human data are considered more adequate than animal data.

Most occupational studies of repeated toxicity are cross-sectional studies. No study has characterized the long-term effects of N_2O exposure. Among all these studies showing effects on nervous system on workers (effects on vigilance), 4 studies are identified as the most relevant and robust studies for OEL setting:

- Scapellato *et al.*, 2008,
- Lucchini *et al.*, 1995, 1996 and 1997.

These four studies are chosen as key studies because:

- workers' exposure to N_2O is considered to be representative of occupational exposures in these studies;
- they consistently indicate potential neurobehavioural function impairments in humans. Although different designs were used, similar results were observed;
- many potential biases were taken into account and these studies made it possible to identify the dose-response relationship for the critical effect;
- nevertheless, some limits were identified:
    - co-exposure at low concentrations with other anaesthetic gases,
    - although exposure was well characterised, the analytical method used in these studies may lead to an under-estimation of exposure levels..

These studies are complementary and have the following advantages and limits.

Table 30: Advantages and limits of key studies

	Advantages	Limits
Scapellato *et al.*, 2008	- longitudinal study over one-year - categorisation in several dose-levels - numerous variables and potential confounders investigated	No air measurement (biomonitoring only) Presence of isoflurane
Lucchini *et al.*, 1997	- multi-centre study - male and female workers	Cross-sectional study Static air monitoring
Lucchini *et al.*, 1996	- air concentration in the breathing zone of workers - numerous variables and potential confounders investigated - double-blind condition - experimental condition	Cross-sectional study No information on potential co-exposure Measurements during 3 hours
Lucchini *et al.*, 1995	- air measurements in the breathing zone of workers	Cross-sectional study Stress and work organisation not taken into account Air measurements during 3 hours Presence of Enflurane

In the 1-year longitudinal study by Scapellato *et al.* (Scapellato *et al.*, 2008), exposure to N_2O was measured in urine at the end of work shift on Monday and Friday. All workers worked for 7 hours and 12 minutes per day throughout the week of observation. In Lucchini and collaborators (Lucchini *et al.*, 1995), nurses were exposed to N_2O measured in the breathing zone during a 3 hour period. In Lucchini and collaborators (Lucchini *et al.*, 1996), workers were exposed every two weeks to N_2O (and performed nongaseous anaesthesia the other week) or had a constant use of N_2O anaesthesia. N_2O concentration was measured during a 3 hour period at the beginning of the week and at the last day of the week. In Lucchini and collaborators (Lucchini *et al.*, 1997), urinary N_2O levels were measured on the first and last day of the working week.

In these four studies, the authors took biological samples and measured N_2O urinary concentrations at the start and end of shifts (beginning and end of week).

Overall, worker exposure in these studies is considered relevant for the derivation of the 8h-OEL.

Therefore, the studies by Scapellato *et al*. in 2008 and by Lucchini *et al*. in 1995, 1996 and 1997 are the most relevant studies for the establishment of the 8h-OEL and are retained as key studies.

6.1.3 Identification of point of departure (PoD)

Cognitive effects were observed at 54.2 ppm and 62.6 ppm at weekends respectively in the 1995 and 1996 Lucchini et al. studies, in the presence of 1.5 ppm isoflurane. In the study of Scapellato et al., significant impairment of cognitive performance was noted at a urinary concentration of 27 µg/L, corresponding to an air concentration of 50 ppm in the presence of 1.3 ppm isoflurane.

The studies by Lucchini et al. in 1997 and Scapellato et al. identified no effect on cognitive performance for exposures to 23.2 ppm N_2O or less than 27 µg/L N_2O in urine respectively.

On the basis of the effects described above, the LOAEC retained is therefore 50 ppm (90 mg/m3) and the NOAEC 23.2 ppm, rounded to 25 ppm (45 mg/m3).

Based on the effects described above, a LOAEC of 50 ppm (90 $mg.m^{-3}$) is identified. **The NOAEC of 23.2 ppm, rounded to 25 ppm (45 $mg.m^{-3}$), can be retained as PoD.**

6.1.4 Application of uncertainty factors

The 8h-OEL was calculated using uncertainty factors (UF) described in the following table.

Table 31: Uncertainty factors

Uncertainty factors	Justification	Value
Inter-species (UF_A)	The OEL is based on human data. No factor.	1
Intra-individual (UF_H)	The key studies are based on human data (in males and females), including multicenter study and there is a large number of studies examining effects on the central nervous system (on animals and humans). The critical effect is a very sensitive endpoint. In an acute volunteer study, including highly sensitive subjects, effects were not observed at the NOAEC of 25 ppm (Bruce and Bach, 1976). Acknowledging the differences in exposure (acute), these results may support the absence of additional factor for the OEL. Overall, no factor is considered needed for differences in susceptibility.	1
Subchronic to chronic transposition (UF_S)	In one of the key studies, workers occupationally exposed to the substance were followed during one year. No correction for exposure duration is considered necessary.	1
Use of LOAEC/severity of effects ($UF_{L/B}$)	NOAEC/LOAEC: The retained point of departure is a NOAEC, no additional factor is needed.	1

	Severity of effects: behavioural function impairments were of low severity and may be reversible. No additional factor is considered necessary for the severity of the effect	
Quality of the database (UF_D)	The substance is a data-rich substance and numerous studies were published on the potential effects of N_2O on the nervous system. Although there are uncertainties in the dose-response for reproductive toxicity, considering a conservative PoD of 5,000 ppm for delayed development, a 8h-OEL of 33 ppm would be derived considering UF 150 (10 for UF_A, 5 for UF_H and 3 for LOAEC to NOAEC). Therefore, the effect on nervous system is considered the most sensitive effect, which was extensively studied.	1

The overall uncertainty factor for deriving the 8h-OEL is 1. Therefore, the HRV committee recommends an 8h-OEL of 45 mg/m^3 (i.e. 25 ppm).

6.2 Construction of a short-term exposure level (15min-STEL)

Although slight impairment of audio-visual capacity was reported at 50 ppm in a human volunteer study (Bruce and Bach, 1976) after 4 hours exposure to N_2O, these findings were not reproduced by other authors at this dose level. The authors acknowledge that the volunteers in their study were particularly sensitive and that the results may not be extrapolated to other populations.

Therefore, regarding the lack of relevant data on short-term effects of N_2O for the construction of a 15min-STEL, and to limit the magnitude and the number of peaks of exposure, the committee recommends, according to its methodology (Anses, to be released), not to exceed 5 times the 8h-OEL, i.e. 125 ppm, over a period of 15 minutes.

The HRV committee recommends a pragmatic 15min-STEL of 225 mg/m^3 (125 ppm).

6.3 “Skin” notation

In the absence of data on dermal absorption, **no "skin” notation** has been assigned for this substance.

6.4 “Noise” notation

As there are no data on possible interactions during co-exposure to noise and N_2O, **the "noise" notation is not recommended**.

7 Conclusions of the collective expert appraisal

Table 32: Summary table of threshold OEL

Type of OEL		**8h-OEL**	**Pragmatic 15 min-STEL**
RV	Organism	Anses	Anses
	Year	2023	2023
	Value	**45 mg/m³ equivalent to 25 ppm**	**225 mg/m³ equivalent to 125 ppm**
Target population		Workers	Workers
Critical Effect		Reversible sub-acute impairment of cognitive function performance	**By default of available data, recommendation not to exceed over a 15 minutes period 5 times the value of the 8h-OEL**
Key study	Reference	1. Scapellato et al., 2008 2. Lucchini et al., 1995, 3. Lucchini et al., 1996 4. Lucchini et al., 1997	
	Population of the study or species	Workers	
	Exposure (time, route)	1. 1-year 2. 3h 3. 3h 4. Not mentioned	
Point of departure (PoD)		NOAEC = 23.2 ppm	
Time Adjustement		/	
Dosimetric Adjustement		/	
Uncertainty Factors (UF)		1 (UF_H: 1 ; UF_S: 1 ; $UF_{L/B}$: 1 ; UF_D: 1)	
« skin » notation		None	
« noise » notation		None	

The recommended 8h-OEL should also protect against hematological deleterious effects, effects on immune system and on development. However, it is not possible to know whether the 8h-OEL would prevent fertility effects as both reliable human and animal data are lacking.

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Part B – Report on the assessment of methods for measurement of exposure levels in workplace atmospheres

1 Mapping measurement methods

The following table presents the methods and protocols for measuring N_2O concentration in workplace air. Details in terms of sampling media, sample processing, analysis and validation data are given in Annex 5.

Table 33: Summary table of methods for measuring N_2O in workplace air

Method		Protocols			
N°	Description	Reference	Support	Desorption	Analyse
1	Passive sampling on adsorbent media followed by thermal desorption and analysis by infrared spectroscopy	DFG Method 2 (2006)	Molecular sieve tube	Thermal	IR
		OSHA ID-166 (1994)			
2	Active sampling on adsorbent support followed by thermal desorption and analysis by gas chromatography and electron capture detection (GC-ECD) or thermal conductivity detection (TCD)	DFG Method 3 (2006)	Molecular sieve tube	Thermal	GC - ECD
		INRS MetroPol M-416 (2022)	Zeolithe BaZSM5 tube	Thermal	GC-TCD (microcatharometre)
3	Passive sampling on adsorbent media followed by thermal desorption and analysis by GC-ECD or TCD	DFG Method 3 (2006)	Molecular sieve tube	Thermal	GC - ECD
		INRS MetroPol M-415 (2022)	Zeolithe BaZSM5 tube	Thermal	GC-TCD (microcatharometre)
4	Continuous Fourier-transform infrared spectroscopy (FTIR) analyser	DFG Method 1 (1989) NIOSH 3800 (2016)	Continuous FTIR analyser		
		NIOSH 6600 (1994)	Long pathlength portable infrared spectrophotometer (field readout)		
5	Direct reading instrument - photoacoustic detection (DRI-PAD)	IRSST 320-1 (undated)	Direct reading instrument - photoacoustic detection (DRI-PAD)		
6	Active sampling in tedlar bag, analysis by GC-ECD	INSHT MTA/MA-020/A91 (1991)	tedlar bag	NA	GC-ECD

2 Detailed assessment of the methods

Requirements: Considering the 8h-OEL and the pragmatic 15min-STEL recommended by the Committee, methods should be validated in the following concentration range:

- 0.1 to 2 *8h-OEL: 4.5 – 90 mg.m^{-3} (or 2.5 – 50 ppm) (for the technical regulatory control)
- 0.1 to 2 *15min-STEL: 22.5 – 450 mg.m^{-3} (or 12.5 – 250 ppm) (for the technical regulatory control)
- 0.5 to 2 *15min-STEL: 112.5 – 450 mg.m^{-3} (or 62.5 – 250 ppm) (for the monitoring of short exposure)

The following table presents the rating of identified methods relevant to measure worker's N_2O exposure (Table 34). The evaluation is described in the following paragraphs.

Table 34: Rating of monitoring methods for workplace N_2O assessment

Method		Protocols	8h-OEL	Pragmatic 15min-STEL	
N°	Description	Reference	Technical regulatory control	Technical regulatory control	Short term exposure monitoring
1	Passive sampling on adsorbent media - thermal desorption – analysis by IR spectroscopy	DFG Method 2 (2006)	1B	3	3
		OSHA ID-166 (1994)			
2	Active sampling on adsorbent support media - thermal desorption - analysis by GC-ECD or TCD	DFG Method 3 (2006)	2	2	2
		INRS MetroPol M-416 (2022)	3	1B	1B
3	Passive sampling on adsorbent media - thermal desorption - analysis by GC-ECD or TCD	DFG Method 3 (2006)	3*	3*	3*
		INRS MetroPol M-415 (2022)	1A	3	3
4	Continuous FTIR analyser	DFG Method 1 (1989) NIOSH 3800 (2016)	3	3	3
		NIOSH 6600 (1994)			
5	Direct reading instrument - photoacoustic detection (DRI-PAD)	IRSST 320-1 (undated)	3	3	3
6	Active sampling in tedlar bag, analysis by GC-ECD	INSHT MTA/MA-020/A91 (1991)	3	3	3

The following figures present the ranges for which the various methods were tested and their limit of quantification for the 8h-OEL and the 15min STEL recommended by the Committee (Figure 6 and Figure 7). The methods classified as category 3, based on exclusion criteria, are not represented.

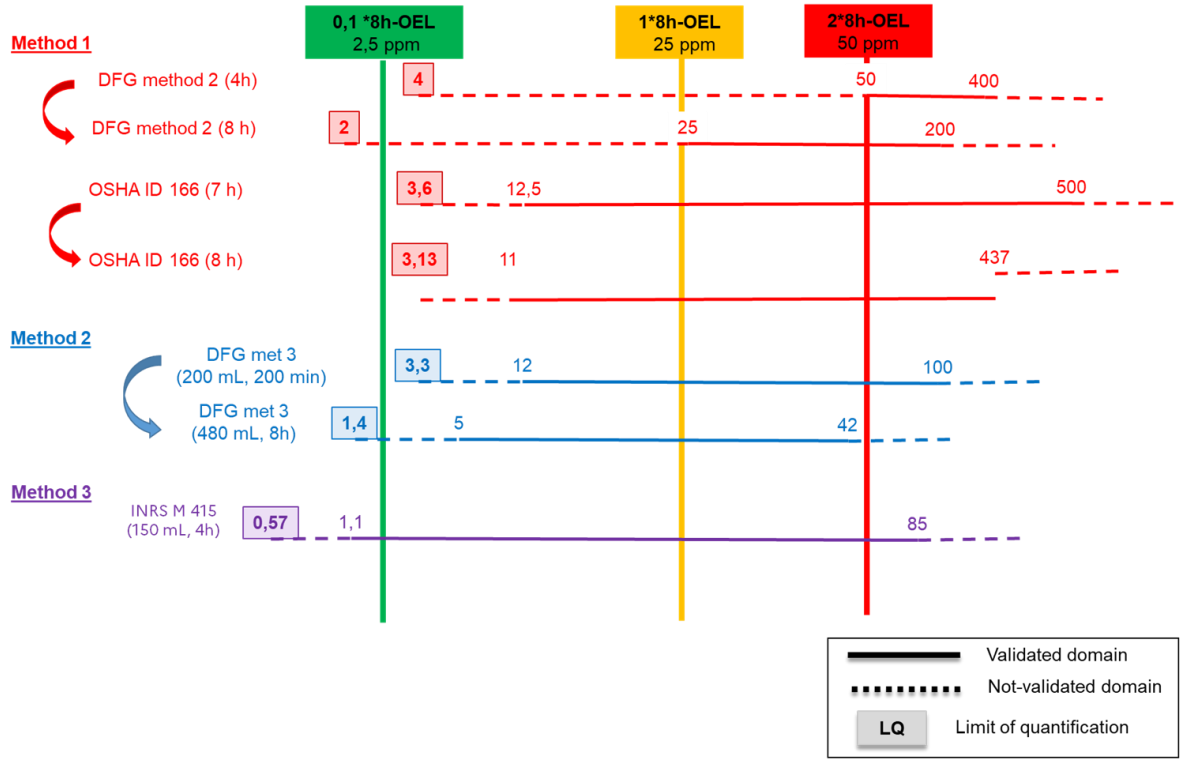

Figure 6: Range of validity and limit of quantification of the measurement methods compared to 0.1 to 2 times the OEL-8h proposed by the Expert committee

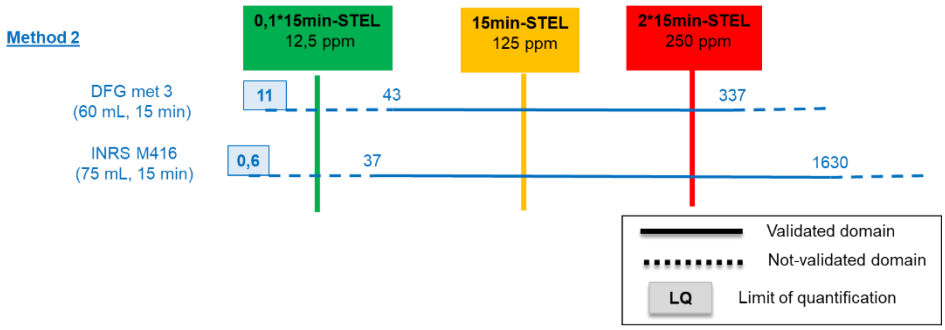

Figure 7: Range of validity and limit of quantification of the measurement methods compared to 0.1 to 2 times the pragmatic 15min-STEL proposed by the Expert committee

2.1 Method 1: Passive sampling on adsorbent media followed by thermal desorption and infrared spectroscopy

Description: Method 1 is described in the DFG Method 2 and OSHA-ID166 protocols.

In the DFG Method 2 protocol, the passive sampling media are Draëger Safety diffusive tubes, No. 8101471, containing 5Å molecular sieve. The uptake rate is not specified in the protocol. The sampling time recommended is between 1 and 8 hours.

For the OSHA-ID166 protocol, the sampling media are NITROX passive monitor containing molecular sieve. The protocol specifies that other nitrous oxide passive monitors with molecular sieve can be used, and are analyzed in the same manner as the tubes. However, the validation data mentioned in the protocol only concern the NITROX passive monitor. The uptake rate is not indicated in the protocol but can be deduced from validation data. The uptake rate varies with the duration of sampling: 0.64 $mL.min^{-1}$ for 120 min, 0.60 $mL.min^{-1}$ for 300 min, 0.58 $mL.min^{-1}$ for 420 min. The protocol mentions that sampling can be performed for a period of up to 8 hours.

For both protocols, the tubes are thermally desorbed (temperature of 220°C for 5 minutes for the DFG Method 2 protocol; conditions are not indicated for the OSHA-ID166 protocol). The analysis was carried out using a Fourier transform infrared spectrometer (e.g. FTIR spectrometer type Nicolet 20 DX-B for the DFG Method 2 protocol with an absorption measured between 4.42 and 4.63 µm). Linearity was checked. For the OSHA-ID166 protocol, analyses are conducted by the tube manufacturer.

Desorption efficiency:

The desorption coefficients are 99% for the DFG Method 2 protocol (obtained from doped tubes). For the OSHA-ID166 protocol, the desorption coefficients are higher than 93%.

Storage stability:

Samples are stable 30 days at room temperature (20 to 25°C)(DFG Method 2 and OSHA 166). Storage stability test were performed in the OSHA ID166 protocol: Four sets of six tubes were exposed to 25 ppm. Each set was analysed at 2, 7, 15 and 30 days. The mean sample recovery after 30 days of storage was within ±10% of results at Day 2.

Environmental conditions impact:

For the OSHA-ID166 protocol, tests were conducted to verify the impact of high humidity values (90%). Measurements were made at concentrations of 500 ppm. No impact of relative humidity was found. The protocol states that the diffusion tubes can be used at 90% RH for 8 hours. The DFG Method 2 protocol does not mention an impact study of environmental conditions.

Selectivity / Interference:

In the DFG Method 2 protocol, the tubes are fitted with a filter layer which retains any interfering components such as CO_2 and H_2O. The 5 Å molecular sieve avoids adsorption of compounds such as halothane, isoflurane, desflurane, sevoflurane, enflurane, 2-propanol, formaldehyde and glutaraldehyde. The selected FTIR absorption range also avoids interferents.

The OSHA-ID166 protocol mentions that no known interferences were reported by the manufacturer.

Reverse Diffusion:

No data is available for the DFG Method 2 protocol.

In the OSHA-ID166 protocol, a reverse diffusion study was performed: one set of 6 tubes were exposed for 2 hours to 25 ppm and one set of 6 tubes were exposed for 2 hours to 25 ppm and then 4 hours to 0 ppm. The difference between the means of the two sets in terms of ppm-hour is less than 8%. Reverse diffusion would not be a significant problem for at least an 8-hour sampling duration.

Uptake rate stability:

No data is available for the DFG Method 2 protocol or for the OSHA ID 166 protocol. But, the uptake rate can be deduced from precision and accuracy data in OSHA ID-166. The uptake rate varies with the duration of sampling: 0.64 $mL.min^{-1}$ for 120 min, 0.60 $mL.min^{-1}$ for 300 min, 0.58 $mL.min^{-1}$ for 420 min.

No data is available for 15 min sampling.

Uncertainty:

The DFG Method 2 protocol mentions a standard deviation between 2.3 and 4.4% and a mean variation between 5.9 and 11.3% for a concentration range between 50 and 400 ppm for exposure times of 1 and 4 hours.

For the OSHA-ID166 protocol, the overall error determined using data derived from 5 sample sets (12.5, 25, 50, 110 ppm, and one 500 ppm set) is ± 21.5 %.

Validation range:

For the DFG Method 2 protocol, the validation range is 5 to 300 ppm without indication of the corresponding sampling time. Precision data are obtain upon the range 50 to 400 ppm for duration between1 and 4 hours.

For the OSHA-ID166 protocol, the validation range is 12.5 to 500 ppm for 7 hours of sampling. The sampling times given in both protocols do not allow a comparison to the pragmatic 15min-STEL.

Quantification limit:

The quantification limit given by the DFG Method 2 protocol is 15 ppm*h, i.e. 15 ppm for 1 hour and 2 ppm for 8 hours.

A limit of detection of 2 µg is given (manufacturer's data) in the OSHA ID-166 protocol, but the protocol mentions that the recommended minimum dose is 25 ppm*h, which corresponds to 3.6 ppm for a 7 hours sample, or 3.125 ppm for a 8 hours sample.

Breakthrough or sampler capacity

Breakthrough is not studied, but the method is validated beyond 2*8h-OEL with an 8 hours sample.

The concentration range of 2.5 to 50 ppm (corresponding to 0.1 to 2 times the 8h-OEL) is covered for an 8 hours sample.

Accessible measurement range:

Taking into account the limit of quantification and the upper bound of the validation range, the accessible measurement range with an 8-hour sampling duration is 2 – 200 ppm in the conditions of DFG Method 2 protocol or 3.125 – 437 ppm in the conditions of OSHA ID-166 protocol. Then the accessible measurement range covers 0.1 to 2 times the 8h-OEL.

Many essential validation criteria are available and meet the requirements, in particular through the data mentioned in the OSHA ID 166 protocol. Method 1 covers the concentration range from 2.5 to 50 ppm (corresponding to 0.1 to 2 times the 8h-OEL). This method is classified as category 1B for the technical regulatory control of the 8h-OEL.

The uptake rate varies with the sampling time and the sampling time cannot be less than 1 hour. It is therefore classified as category 3 for the technical regulatory control of the pragmatic 15min-STEL and the short term exposure assessment.

2.2 Method 2: Active sampling on adsorbent media followed by thermal desorption and analysis by GC-ECD or TCD

Method 2 is described in the DFG Method 3 and in INRS MetroPol M-416 protocols.

In the DFG Method 3 protocol, the active sampling medium is a stainless-steel tube (6.3 mm x 89 mm, 5 mm inner diameter) filled with 5 Å molecular sieve. Prior to use, the tubes are heated to 250°C in the thermal desorber for 30 minutes and then closed with swagelocks or aluminium caps.

The recommended sampling rate is 1 to 4 $mL.min^{-1}$ for sampling times of 50 to 200 minutes. The maximum volume recommended is 200 mL, i.e. sampling at 1 $mL.min^{-1}$ for 200 min (or sampling at 4 $mL.min^{-1}$ for 50 min).

For the INRS MetroPol M-416 protocol, the sampling medium is a steel tube containing 750 mg of BaZSM5 zeolite closed with swagelock-type metal caps. The tubes are conditioned for 1 hour at 350°C under nitrogen flow.

The recommended sampling rate is 5 $mL.min^{-1}$. The protocol mentions that the described method concerns short sampling times of less than 30 minutes. The recommended sampling time is 15 minutes or a volume of 75 mL.

For both protocols, the tubes are thermally desorbed at 250°C. Analysis is performed by gas chromatography coupled to an electron capture detector for the DFG Method 3 protocol and by gas chromatography coupled to a microcatharometer for the INRS MetroPol M-416 protocol. Analytical parameters are documented in both protocols. Linearity was checked.

Desorption efficiencies:

Desorption coefficients are 99% for the DFG Method 3 protocol (obtained from spiked tubes). For the INRS MetroPol M-416 sheet, the desorption coefficients are above 95%.

Sample storage:

Data is available for sample preservation. The DFG Method 3 protocol states that no loss was observed after 1 week of storage at 20-25°C. The INRS MetroPol M-416 protocol states that tubes can be stored for up to 5 days at room temperature and 21 days at 4°C. Recoveries of over 97% were obtained.

Environmental conditions impact:

The DFG Method 3 protocol was tested for humidity conditions between 5 and 80%. For the INRS MetroPol M-416 protocol, tests were performed at 50% humidity. This molecular sieve is not very sensitive to moisture. Therefore, a study of its behaviour at high HR values is not necessary. No studies of environmental conditions are mentioned.

Selectivity / interference:

In the DFG Method 3 protocol, the use of an electron capture detector eliminates interference with carbon dioxide. The protocol indicates a very high selectivity.

Selectivity is not addressed in the INRS MetroPol M-416 protocol. It should be noted that in the INRS MetroPol M-415 protocol (identical method with passive sampling tube), an interference study with ethanol and isoflurane co-pollution is mentioned.

Estimation of the expanded uncertainty:

The DFG Method 3 protocol mentions a standard deviation between 0.8 to 2.0% and a mean variation between 7.5 to 15% for a concentration range of 12.8 to 109 ppm without specifying the exposure time. The INRS MetroPol M-416 protocol indicates an expanded uncertainty of 20.7% according to EN482 with an expansion factor of 2.

Limit of quantification and validation range:

For the DFG Method 3 protocol, the detection limit is 1 ppm for a 200 mL sample, i.e. an assessed limit of quantification of 3.3 ppm (i.e. 1.188 µg on tube). The upper bound of the validation range is not stated but the precision of the method were determined with a loaded atmosphere between 12.8 and 101 ppm and recoveries has been determined up to 500 ppm. Neither the sampling time neither the sampled volume for the precision study and for the recoveries study are mentioned. But because the detection limit is given for a 200 mL sample, the working group assumes that these data were obtained with the same volume of 200 mL. The validation range covers 0.5 to 4*8h-OEL with a sampled volume of 200 mL

In the INRS MetroPol M-416 protocol, the validation range is from 5 to 220 µg on the support, i.e., 37 to 1630 ppm for 15-minute samples (75 mL). The limit of quantification is 0.6 ppm. The INRS MetroPol M-416 protocol therefore covers the area for comparison with the pragmatic 15min-STEL.

Accessible measurement range:

For comparison to the 8h-OEL:

Taking into account the limit of quantification and the upper bound of the validation range, the accessible measurement range with an 8-hour sampling duration would cover 1.375 to 42 ppm, i.e. 0.06 to 1.68 * 8h-OEL in the conditions of the DFG Method 3 protocol. As the recoveries test were performed up to 500 ppm, with a presumed volume of 200 mL, the method would cover 2*8h-OEL with an 8h-sampling.

The INRS MetroPol M-416 protocol cannot be used for an 8h-OEL comparison because the sampling time must not exceed 30 minutes.

For a comparison to the pragmatic 15min-STEL:

Considering a 15 minutes sampling time at 4 $mL.min^{-1}$, the LOQ mentioned in the DFG Method 3 protocol and the upper bound of the validation range, the accessible measurement range would be 11 to 337 ppm, i.e. 0.09 to 2.7*pragmatic 15min-STEL.

Method 2 is described by 2 protocols (DFG Method 3 and INRS MetroPol M-416) using different media. Many essential validation criteria are available and meet the requirements. Nevertheless, assumptions have been made concerning the air volume sampled during the validation tests in the DFG Method 3 protocol, and the sampling time must not exceed 30 minutes in the condition of the INRS MetroPol M-416.

Then, method 2 is classified, in the conditions of the DFG Method 3 protocol, as category 2 for the technical regulatory control of the 8h-OEL, the technical regulatory control of the pragmatic 15min-STEL and the short term exposure assessment. In the conditions of the INRS MetroPol M-416 protocol, this method is classified as category 1B for the technical regulatory control of the pragmatic 15min-STEL and the short term exposure assessment, but as category 3 for the technical regulatory control of the 8h-OEL.

2.3 Method 3: Passive sampling on adsorbent media followed by thermal desorption and analysis by GC-ECD or TCD

Method 3 is described in the DFG Method 3 protocol and the INRS MetroPol M-415 protocol.

For the DFG Method 3 protocol, a sampling time of 4 to 8 hours is recommended. The reported validation data do not always specify the sampling duration or mention a duration of 4 to 8 hours. However, as the diffusion medium is the same as for method 1, the uptake rate varies with the sampling time (see § 4.2.1). Thus, the data from this protocol were not taken into account in the evaluation of the method. Only the data from the INRS MetroPol M-415 protocol is taken into account and reported below.

The sampling medium is a steel tube containing 750 mg of BaZSM5 zeolite closed with swagelock-type metal caps. The tubes are conditioned for 1 hour at 350°C under nitrogen flow. A diffusion cap is placed at one end during sampling.

The protocol mentions that sampling must not be less than 1 hour and must not exceed 4 hours. The uptake rate varies according to the sampling time and the average moisture content. If the sampling time is between 1 and 2 hours, the uptake rate is 0.63 $mL.min^{-1}$. For times longer than 2 hours, the uptake rate is 0.63 $mL.min^{-1}$ for moisture content below 50% and 0.5 $mL.min^{-1}$ for moisture content above 50%. Measurement of the moisture content during sampling is necessary.

The tubes are thermally desorbed at 250°C. The analysis is performed by gas chromatography coupled with a microcatharometer. Analytical parameters are documented. Linearity was verified.

Desorption efficiency:

Desorption coefficients are above 95%.

Storage stability:

Tubes can be stored for up to 5 days at room temperature and 21 days at 4°C. Recovery rates of over 97% were obtained.

Environmental conditions impact, selectivity, interference and reverse diffusion:

An experimental design according to EN 838 was carried out to determinate the uptake rate. 16 tests have been realized with temperature between 18 and 25°C and humidity between 30 and 60%, a reverse diffusion study, nitrous oxide concentrations between 5 and 50 ppm, sampling times between 1 and 4 hours and ethanol and isoflurane co-pollution. Impact of duration and moisture content have been shown. For sampling times longer than 2 hours, the uptake rate is 0.63 $mL.min^{-1}$ for moisture content below 50% and 0.5 $mL.min^{-1}$ for moisture content above 50%. No effects of temperature, concentrations levels and ethanol and isoflurane co-pollution have been shown. Reverse diffusion seems insignificant.

Uncertainty:

The expanded uncertainty of 18.5% according to EN482 with a coverage factor of 2.

Validation range and quantification limit:

The MetroPol M-415 protocol indicates sampling times of less than 4 hours, but more than 1 hour. Then the method can't be used for the technical regulatory control of the pragmatic 15min-STEL or the short term exposure assessment. And two successive 4-hour samples should therefore be taken for a comparison with the 8h-OEL. It indicates a quantification limit of 0.156 µg on substrat, i.e. 0.57 ppm for a 4h-sample.

The validation range is from 0.3 to 23 µg on the substrate, i.e., for 4-hour samples, measurement ranges of 1.1 to 85 ppm for a humidity level below 50% or 1.4 to 108 ppm for a humidity level above 50%. These ranges cover the concentration range of 2.5 to 50 ppm corresponding to 0.1 to 2 times the 8h-OEL.

Too much information is missing from the DFG Method 3 protocol to evaluate the method on the basis of these data. Under the conditions of this protocol, the method is therefore classified as category 3*.

However, all essential validation criteria are available in the INRS MetroPol M-415 protocol and meet the requirements. In the condition of the INRS MetroPol M-415 protocol, Method 3 covers the concentration range from 2.5 to 50 ppm (corresponding to 0.1 to 2 times the 8h-OEL) with two successive 4 hours samples, but the sampling time cannot be less than 1 hour. Then this method is classified as category 1A for the technical regulatory control of the 8h-OEL, but as category 3 for the technical regulatory control of the pragmatic 15min-STEL and the short term exposure assessment.

2.4 Method 4: Continuous FTIR analyser

Method 4 is described by the DFG Method 1 (1989), NIOSH 3800 (2016) and NIOSH 6600 (1994) protocols. This method concerns ambient air measurements or analysis of gas collecting tube or gas collecting bags.

The method uses a portable direct-reading instrument with a flow rate dependent of the system, between 0.1 to 20 $L.min^{-1}$. The analytical technique is extractive Fourier Transform Infrared (FTIR) spectrometry. The range depends of absorption path length. For example, in NIOSH 3800 protocol, measurements range is 0.36 to 904 ppm for a 10 m absorption path length. The sample temperature needs to be within 10 to 30°C.

For direct measurements of ambient air, the air is pumped at 1cell volume/min through the spectrometer sample cell (NIOSH 6600 protocol). Thus, this method is not a really continuous measurement. Several punctual measurements are realised, depending of volume cell, sampling flow and system response time.

This method is suitable for ambient measurements but not for individual measurement. It is indicated that the sampling location may be changed as desired. Sample spectra must be acquired at each sampling location for a time period no less than the system response time (dependent of the equipment).

This method allows to obtain the concentrations evolution and can identify period of high exposition. Moreover, it can be used to obtain quickly information for ambient air in different locations.

As it stands, method 4 is therefore classified as category 3 for the technical regulatory control of the 8h-OEL and of the pragmatic 15min-STEL, and for the short term exposure assessment.

2.5 Method 5: Direct reading instrument - photoacoustic detection (DRI-PAD)

Method 5 is described in the IRSST 320-1 protocol and implements a direct reading instrument - photoacoustic detection. The minimum value reported is 0.05 ppm. This value seems to cover the lower end of the concentration ranges of the 8h-OEL and the pragmatic 15min-STEL.

This method is not suitable for individual measurements and no validation data are available.

As it stands, method 5 is therefore classified as category 3 for the technical regulatory control of the 8h-OEL and of the pragmatic 15min-STEL, and for the short term exposure assessment.

2.6 Active sampling in Tedlar bag, analysis by GC-ECD

Method 6 is described in the INSST MTA/MA-020/A91 protocol of 1991. It consists of active sampling using a 5 litres Tedlar bag, followed by GC-ECD analysis.

The validation range indicated is 10 to 1200 ppm, however the sampling time is not indicated.

The inert material bag is filled using a pump with a constant flow at ± 5%. The sampling flow is not mentioned. It must be adapted to the sampling time in order to respect the capacity of the bag. For sampling times of 8 hours (technical regulatory control of the 8h-OEL) the maximum flow rate would be around 1 $mL.min^{-1}$.

Storage stability:

The protocol indicates that inert bag can be stored for up to 3 weeks with variation coefficient between 0.1 to 3.1 %. The study is not detailed. The protocol specifies that bags have to be closed carefully. Then sample stability seems to be sufficient.

Uncertainty:

The protocol indicates a standard deviation calculated with results of interlaboratory, less than 6%.

Many essential criteria for the validation of the method are not filled in the INSST MTA/MA-020/A91. Moreover, this method is more suitable for ambient measurements than individual measurements. As it stands, method 6 is therefore classified as category 3 for the technical regulatory control of the 8h-OEL and of the pragmatic 15min-STEL, and for the short term exposure assessment.

3 Conclusions and recommendations

Six methods for measuring N_2O in workplace air have been identified and evaluated:

- Method 1 : Passive sampling on adsorbent media followed by thermal desorption and analysis by infrared spectroscopy
- Method 2 : Active sampling on adsorbent support followed by thermal desorption and analysis by GC-ECD or TCD
- Method 3 : Passive sampling on adsorbent media followed by thermal desorption and analysis by GC-ECD or TCD
- Method 4 : Continuous FTIR analyser
- Method 5 : Direct reading instrument - photoacoustic detection (DRI-PAD)
- Method 6 : Active sampling in Tedlar bag, analysis by GC-ECD

Methods 4, 5 and 6 are classified as category 3 and are not recommended for the technical regulatory control of the 8h-OEL, the technical regulatory control of the pragmatic 15min-STEL and the monitoring of short-term exposures because these methods are suitable for ambient measurements, but not for individual measurements. It should be noted that method 4 (continuous infrared analyser) and method 5 (direct reading instrument - photoacoustic detection (DRI-PAD)) enable to monitor the concentration trends and to identify periods of high exposure. In addition, they can be used to quickly obtain information on ambient air in different locations.

For the technical regulatory control of the 8h-OEL:

- Method 1 is classified as category 1B due to many of the key validation criteria meeting the requirements, particularly through the data mentioned in the OSHA ID 166 protocol and obtained for durations of 4 hours to 7 hours,
- Method 2 is classified as category 2 under the conditions of the DFG Method 3 protocol because of the assumptions made on the sampled air volume for the validation data assessment, but as category 3 under the conditions of the INRS MetroPol M-416 protocol because of a sampling time that cannot exceed 30 minutes on the recommended sorbent medium,
- Method 3 is classified as category 1A under the conditions recommended by the INRS MetroPol M-416 protocol with two successive 4-hours sampling. The DFG Method 3 protocol using a different adsorbent medium was not evaluated due to the lack of validation data for this medium,
- Thus methods 1 (under the conditions of the protocol; OSHA ID 166, with one 8-hours sampling) and 3 (under the conditions of the INRS MetroPol M-415 protocol with two successive 4-hours sampling) are recommended.

For the technical regulatory control of the pragmatic 15min-STEL and the monitoring of short-term exposure:

- Method 1 is classified as category 3 due to a variable uptake rate with the sampling duration and a lack of data on the stability of this rate over 15 minutes,

- Method 2 is classified as category 1B under the conditions of the INRS MetroPol M 416 protocol, but as category 2 under the conditions of the DFG Method 3 protocol due to the assumptions made on the sampled air volume for the validation data assessment,
- Method 3 is classified as category 3 under the conditions of the INRS MetroPol M-415 protocol due to a sampling time that cannot be less than 1 hour. The data from the DFG Method 3 protocol were not taken into account in the assessment due to a lack of information on the sampling times associated with the validation data mentioned,
- Thus method 2, under the conditions of the INRS MetroPol M 416 protocol, is recommended.

The following table shows the recommended nitrous oxide measurement methods in workplace air.

Table 3: recommended nitrous oxide measurement methods in workplace air

Method		Protocols	8h-OEL	Pragmatic 15min-STEL	
N°	Description	Reference	Technical regulatory control	Technical regulatory control	Short term exposure monitoring
1	Passive sampling on adsorbent media followed by thermal desorption and infrared analysis	OSHA ID-166 (1994)	1B	3 (not recommended)	
2	Active sampling on adsorbent support followed by thermal desorption and analysis by GC-ECD or TCD	INRS MetroPol M-416 (2022)	3 (not recommended)	1B	1B
3	Passive sampling on adsorbent media followed by thermal desorption and analysis by GC-ECD or TCD	INRS MetroPol M-415 (2022)	1A	3 (not recommended)	

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ANNEXES

Annex 1 : Bibliographic Search

Study question: Identification of the toxicological studies performed with nitrous oxide

The goal of this review was to derived short-term and chronic toxicological reference values by inhalation with N_2O.

Description of the review method

Litterature search was conducted in the Pubmed database.

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

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

Substance identity #1:

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

Toxicokinetics and human health #2 :

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

Population #3

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

Exclusion #4

soil OR denitrification OR greenhouse gas OR emissions

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

Litterature search inclusion and exclusion criteria:

Publication type	IN	Primary research studies
	OUT	Secondary studies (e.g. editorials, conference)
Language	IN	English, French
	OUT	Other languages
Study design	IN	Human experimental volunteer studies Cohort studies Cross-sectional studies Case-control studies Experimental animal studies not examining mode of action
	OUT	In vitro studies (except genotoxicity studies) In silico studies Experimental mechanistic animal studies
Population	IN	Adult healthy male and female volunteers, men and women occupationally exposed to N2O All mammalian animals
	OUT	Patients under anaesthesia Children
Exposure	IN	All route of exposure relevant to occupational exposure Young mammalian animals under development
	OUT	Mixtures* Anesthesia in human Air pollution Recreational exposure/abuse Subdermal route of exposure in animals Pharmacological research (e.g. alcohol withdrawal)
Time	IN	From 1952 – December 2020
Outcome	IN	Human health
	OUT	Risk management measures

**for human data, the study was considered out if N2O was not the main exposure or if N2O exposure was not specified (e.g. anaestetic without further information)*

6619 studies were screened. Relevant studies are included in the synthesis.

Annex 2 : Assessment of the reliability of the toxicological data

Selected relevant studies included in the synthesis were checked for reliability using methodology described below.

Human data

Assessment was performed using OHAT Approach for Systematic Review and Evidence Integration (NTP-OHAT, 2015). The same questions and domains (e.g. selection, detection, attrition, etc.) were used to appraise the studies and the risk of bias. For each question, the response options were definitely low Risk of Bias (RoB) (++)", "Probably low RoB (+)", "Probably high RoB (-)", "Definitely high RoB (--)". Based on key questions, the studies were rated in Tier 1 (low concern for bias), Tier 2 or Tier 3 (high concern for bias), as described on pages 37 and 38 of the NTP-OHAT document (NTP-OHAT, 2015).

Animal data

All the animal studies (except mechanistic studies) were rated using ToxRtool (Toxicological data Reliability assessment Tool)[28] to assess their reliability. Based on the questions of the tool, the studies were categorized according to Klimisch score 1, 2 or 3. In case of secondary litterature or if abstract only was available, the studies were rated in Klimisch score 4.

[28] https://joint-research-centre.ec.europa.eu/scientific-tools-and-databases/toxrtool-toxicological-data-reliability-assessment-tool_en, consulted in October 2023

Annex 3 : Behavioral and neurological test battery

Endpoint	Test	Basis	Measure	Reference
Cognitive function	Simple reaction time	Subjects press button in response to a red light which appeared in a stimulus window.	Reaction time	Venables *et al.*, 1983 Lucchini *et al.*, 1995 Lucchini *et al.*, 1996
Cognitive function	Complex Reaction time Color Word vigilance	The test measures the speed at which subject recognizes the correct association between colors and words	Reaction time	Scapellato *et al.*, 2008 Lucchini *et al.*, 1997
Cognitive function	Four Choice reaction time	4 lights in a square configuration, 4 response buttons being arranged in the same way under the lights. Subject has to extinguish the light pressing the response button which corresponds to it geometrically. ==> Task of speeded repetitive decision making and performance	Correct reaction time	Venables *et al.*, 1983
Cognitive function	Continuous performance test: attention	Subjects press button in response to a large letter "S" appearing randomly, as part of 5 minutes series of other letters (one per sec)	Reaction time and response error	Mahoney *et al.*, 1988 Estrin *et al.*, 1988
Cognitive function	Symbol-digit substitution test	9 pairs of symbols and digits are presented at the top of the screen. Subject respond by matching digits to nine symbols reordered at the bottom of the screen. 4 sets are presented.	Reaction time and response error	Mahoney *et al.*, 1988 Estrin *et al.*, 1988
Cognitive function	Finger tapping test : hand speed	Subjects press a button with the index finger of the left hand as many times as possible in a 10 second interval, repeat this task with the right index finger and then press 2 buttons alternately with the index of the preferred hand.	Reaction time	Mahoney *et al.*, 1988 Estrin *et al.*, 1988
Cognitive function	Raven Matrices	Recognize a pattern of changes and find logically which pattern would be expected to appear next. 20 patterns	Ability to reason logically	Bruce and Bach, 1976

		selected, 10 for each of the 2 sessions. Each pattern of one set was matched with one on the second set for which the scores accompanying the set indicated a pattern of equal difficulty.		
Cognitive function	O'Connor dexterity test	Subjects place 3 pegs in each of 100 holes as rapidly as possible using only his preferred hand. Time = 3 min	Number of holes filled correctly during the time	Bruce and Bach, 1976
Cognitive function	Vigilance test	T = 60 minutes S watch a continuous display of a normal ecg recorded from an ecg simulator (oscilloscope). 12 times during this hour at intervals from 3 to 8 minutes a brief (1-2s) change to a pattern of atrial fibrillation was interposed among the otherwise normal complexes.	Mean reaction time	Bruce and Bach, 1976
Cognitive function	Audio-visual task	Simultaneously auditory and a visual stimulus with four combinations: flat line, fast clicking, flat line, slow clicking, sine wave, slow clicking, sine wave, fast clicking (changing after 4.5s and randomly presented). S task was to indicate as quickly as possible each time there was a change of stimulus combination by pressing the appropriate button on a response panel (7.5 minutes during which 100 changes of stimulus combination took place, 25 each of four combination	Time taken from the change of stimulus combination to the subject's pressing of the correct response button was measured for all 100 changes, and the overall mean reaction time was computed	Venables *et al.*, 1983 (based on Bruce and Bach, 1976)

Cognitive function	Tachistoscope	Subjects see a 9-square grid containing 4, 5 or 6 filled black circles. After each presentation, Subject enter the position of the circles on a blank grid.	Visual perception	Bruce and Bach, 1976
Cognitive function	Pattern memory test	Short term visual memory - S are briefly presented a block-like pattern and then required to identify which of three subsequently presented patterns s identical to the original pattern. The similarity of the three test patterns to each other is increased over the fifteen trials, so that the choice between them becomes progressively more difficult.	Number of correct responses and the response time (for each trial)	Mahoney *et al.*, 1988
Cognitive function	Digit span test	Subjects are given a standard series of number (Wechsler Adults Intelligence Scale) which had to be repeated correctly to the experimenter. Series increased in length.	Mean number of digits recalled correctly	Bruce and Bach, 1976
Cognitive function	Mood scales	Subjects describe their mood during 40 minutes test session using a five-point scale to indicate the degree to which each of 25 adjectives presented described their emotional state.		Greenberg_1986/Mahoney_1988
Cognitive function	Mood scales	12 adjectives to qualify the mood state	Mood	Scapellato *et al.* 2008
Cognitive function	EUROQUEST (self administered questionnaire)	9 items with x questions each (exploring neurological and psychosomatic symptoms, memory, contration, sleeping disorders ==> chronic effects; irritation of mucous tissues ==> acute effects; oversensitivity to nose and light, anxiety and mood changes ==> personality disorders)	Score for each question	Scapellato *et al.*, 2008; Lucchini *et al.*, 1997
Nervous conduction	Positron emission Tomography (PET)	Measure of regional cerebral blood flow (rCBF)/ regional cerebral metabolic rate (rCMR)	Nervous conduction	Guylai *et al.*, 1996

Annex 4 : References included in the analysis but not considered relevant for occupational exposure to N_2O

Acute toxicity (nervous system)

The following studies were not retained due to experimental coexposure with other anaesthetics than N_2O:

Cook, M.D., M. Smith, J.A. Strakweather, P.M. Winter, M.D. Edmond, M.D. Eger. Behavioral Effects of Trace and Subanaesthetic Halothane and nitrous Oxide in Man. Anaesthesiology 49; 419-424 (1978).

Smith, G., and A. W. Shirley. "Failure to demonstrate effect of trace concentrations of nitrous oxide and halothane on psychomotor performance". *Br. J. Anaesth*. (1977), 49, 65.

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Korttila K,. Operating room nurses' psychomotor and driving skills after occupational exposure to halothane and nitrous oxide. Acta Anaesthesiol Scand. 1978;22(1):33-9. https://doi: 10.1111/j.1399-6576.1978.tb01277.x.

Neurotoxicity (repeated-exposure)

The following studies were analysed but were not considered relevant due to continuous exposure to N_2O:

Weir, D. G., S. Keating, A. Molloy, J. McPartlin, S. Kennedy, J. Blanchflower, D. G. Kennedy, D. Rice, et J. M. Scott. « Methylation Deficiency Causes Vitamin B12-Associated Neuropathy in the Pig ». *Journal of Neurochemistry* 51, nº 6 (1988): 1949-52. https://doi.org/10.1111/j.1471-4159.1988.tb01184.x.

Weir, D. G., A. M. Molloy, J. N. Keating, P. B. Young, S. Kennedy, D. G. Kennedy, et J. M. Scott. « Correlation of the Ratio of S-Adenosyl-L-Methionine to S-Adenosyl-L-Homocysteine in the Brain and Cerebrospinal Fluid of the Pig: Implications for the Determination of This Methylation Ratio in Human Brain ». *Clinical Science (London, England: 1979)* 82, nº 1 (1992): 93-97. https://doi.org/10.1042/cs0820093.

Dinn, J. J., D. G. Weir, S. McCann, B. Reed, P. Wilson, and J. M. Scott. 'Methyl Group Deficiency in Nerve Tissue: A Hypothesis to Explain the Lesion of Subacute Combined Degeneration'. *Irish Journal of Medical Science* 149, no. 1 (December 1980): 1–4. https://doi.org/10.1007/BF02939099.

Scott, J. « Pathogenesis of subacute combined degeneration: a result of methyl group deficiency ». *The Lancet* 318, nº 8242 (1981): 334-37. https://doi.org/10.1016/S0140-6736(81)90649-8.

Immunotoxicity

The following studies were analysed but were not considered relevant due to continuous exposure to N_2O:

Green, C. D., and D. W. Eastwood. 'Effects of Nitrous Oxide Inhalation on Hemopoiesis in Rats'. *Anesthesiology* 24 (June 1963): 341–45. https://doi.org/10.1097/00000542-196305000-00015.

Parbrook, G. D. « Exposure of Experimental Animals to Nitrous-Oxide-Containing Atmospheres ». *British Journal of Anaesthesia* 39, nº 2 (1967): 114-18. https://doi.org/10.1093/bja/39.2.114.

Green, C. D. « Strain Sensitivity of Rats to Nitrous Oxide ». *Anesthesia and Analgesia* 47, nº 5 (1968): 509-14.

Reproductive toxicity

The following studies were not considered relevant due to continuous exposure to N_2O:

Fink, B. R., T. H. Shepard, et R. J. Blandau. « Teratogenic Activity of Nitrous Oxide ». *Nature* 214, nº 5084 (1967): 146-48. https://doi.org/10.1038/214146a0.

Fujinaga, M., J. M. Baden, et R. I. Mazze. « Susceptible Period of Nitrous Oxide Teratogenicity in Sprague-Dawley Rats ». *Teratology* 40, nº 5 (1989): 439-44. https://doi.org/10.1002/tera.1420400505.

Fujinaga, M., J. M. Baden, T. H. Shepard, et R. I. Mazze. « Nitrous Oxide Alters Body Laterality in Rats ». *Teratology* 41, nº 2 (1990): 131-35. https://doi.org/10.1002/tera.1420410202.

Fujinaga, M., J. M. Baden, A. Suto, J. K. Myatt, et R. I. Mazze. « Preventive Effects of Phenoxybenzamine on Nitrous Oxide-Induced Reproductive Toxicity in Sprague-Dawley Rats ». *Teratology* 43, nº 2 (1991): 151-57. https://doi.org/10.1002/tera.1420430207.

Fujinaga M, Baden JM, Yhap EO, Mazze RI. Reproductive and teratogenic effects of nitrous oxide, isoflurane, and their combination in Sprague-Dawley rats. Anesthesiology. 1987 Dec;67(6):960-4. doi: 10.1097/00000542-198712000-00014. PMID: 3688539.

Lane, G. A., M. L. Nahrwold, A. R. Tait, M. Taylor-Busch, P. J. Cohen, et A. R. Beaudoin. « Anaesthetics as Teratogens: Nitrous Oxide Is Fetotoxic, Xenon Is Not ». *Science (New York, N.Y.)* 210, nº 4472 (1980): 899-901. https://doi.org/10.1126/science.7434002.

Mazze, R. I., M. Fujinaga, et J. M. Baden. « Halothane Prevents Nitrous Oxide Teratogenicity in Sprague-Dawley Rats; Folinic Acid Does Not ». *Teratology* 38, nº 2 (1988): 121-27. https://doi.org/10.1002/tera.1420380204.

Mazze RI, Fujinaga M, Baden JM. Reproductive and teratogenic effects of nitrous oxide, fentanyl and their combination in Sprague-Dawley rats. Br J Anaesth. 1987 Oct;59(10):1291-7. doi: 10.1093/bja/59.10.1291. PMID: 3676057.

Mazze RI, Wilson AI, Rice SA, Baden JM. Reproduction and fetal development in rats exposed to nitrous oxide. Teratology. 1984 Oct;30(2):259-65. doi: 10.1002/tera.1420300213. PMID: 6495226.

Keeling, P. A., D. A. Rocke, J. F. Nunn, S. J. Monk, M. J. Lumb, et M. J. Halsey. « Folinic Acid Protection against Nitrous Oxide Teratogenicity in the Rat ». *British Journal of Anaesthesia* 58, nº 5 (1986): 528-34. https://doi.org/10.1093/bja/58.5.528.

Vieira, E. « Effect of the Chronic Administration of Nitrous Oxide 0.5% to Gravid Rats ». *British Journal of Anaesthesia* 51, nº 4 (1979): 283-87. https://doi.org/10.1093/bja/51.4.283

Annex 5 : Technical support - Details of analytical methods for workplace assessment

Table 35: Descriptive parameters of the method 1

		DFG Method 2 (2006)	OSHA ID-166
Gas /vapour - Aerosol - Combined		**Gas /vapour**	
Sampling	**Active / passive**	Passive	
	Sampler	Draëger Safety diffusion tubes, n° 8101471 5Å molecular sieve. Tubes with CO_2 and H_2O anti-interference filters	NITROX diffusion tube. Molecular sieve
	Uptake rate	NS	NS
	Duration	1 to 8 hours	Up to 8 hours
	Volume	NS	NS
	Sampling treatment	Thermal desorption at 220 °C for 5 minutes	thermal desorption. Temperature and time not specified
Analysis	**Analytical technique**	Fourier transform infrared spectrometer (ex FTIR spectrometer type Nicolet 20 DX-B)	FTIR
	Analytical parameters	measured absorption measured between 4.42 and 4.63 µm (wave number 2260 to 2160 cm^{-1})	NS

Table 36 : Validation data of the method 1

	DFG Method 2 (2006)	OSHA ID-166
Working range	concentration range: 5 to 300 ppm	10 to 500 ppm
Sampling and recovery efficiencies	analysis of spiked tubes. 99% recovery in the range 50 to 1600 mLxm-3xh	> 93%
Uptake rate experimental validation data	NS	NS
Uptake rate stability data	NS	NSR
Reverse diffusion	NS	6 tubes exposed for 2 hours to 25 ppm and 6 tubes exposed for 2 hours to 25 ppm then 4 hours to 0 ppm. Difference between the 2 batches less than 8%. Reverse diffusion without impact for the 8-hour passive samples.

		DFG Method 2 (2006)	OSHA ID-166
Capacity limit		NS	NS
Detector response linearity		Linear calibration curve between 25 and 1600 mLxm3xh	NS
Storage stability		It is recommended that the tubes be analysed no later than 4 weeks after collection. Storage at room temperature. Closed tubes with caps.	Storage at room temperature (20 to 25°C) in the laboratory for 30 days. 4 sets of 6 tubes were exposed to 25 ppm. 1 tube from each set was analysed at 2, 7, 15 and 30 days. A difference of ±10% is observed between the analysis at 30 days compared to the analysis at 2 days."
Environmental condition		NS	Tests conducted to verify impact of high humidity values (90%). Measurements at 500 ppm. No impact found. The protocol indicates that the diffusion tubes can be used at 90% RH for 8 hours
Selectivity / Interfering		The tubes are fitted with filters to avoid interference from CO_2 and H_2O. The 5 A molecular sieve avoids adsorption of compounds such as halothane, isoflurane, desflurane, sevoflurane, enflurane, 2-propanol, formaldehyde and glutardialdehyde. The selected FTIR absorption range also avoids interferents.	NS
Speciation		yes	yes
Conditions for the 8h-OEL or 15min-STEL determination	**Estimated expanded uncertainty**	Deviation standard 2.3 - 4.4% mean variation : 5.9 - 11.3 % for concentrations between 50 and 400 ppm	Overall error : +- 21.5%
	Detection limit	5 ppm for 1 hour 0.7 ppm for 8 hours	NS
	Quantification limit	15 ppm for 1 hour 2 ppm for 8 hours	2 µg (manufacturer data)
Additional details		/	

Table 37: Descriptive parameters of the method 2

		DFG Méthod 3 (2006)	INRS Métropol M-416 (2022)
Gas /vapour - Aerosol - Combined		Gas /vapour	
Sampling	**Active / passive**	Active	
	Sampler	Stainless steel tube (6.3 mm x 89 mm, 5 mm internal diameter) filled with 5 A molecular sieve. Before use, the tubes are heated to 250°C in the thermal desorber for 30 minutes. Tubes closed with swagelock or aluminium caps.	Steel tube containing 750 mg of BaZSM5 zeolite closed with swagelock type metal caps. Tubes conditioned for 1 hour at 350°C under nitrogen flow.
	Flow rate	1 to 4 mL/min	5 mL/min
	Duration	50 to 200 minutes	Sampling should not exceed 15 min
	Volume	200 mL (i.e. max duration 200 minutes at 1L/min)	75 mL
Analysis	**Sampling treatment**	Thermal desorption at 250°C for 5 minutes.	Thermal desorption at 250°C for 10 minutes.
	Analytical technique	GC-ECD	GPC with microcatharometer
	Analytical parameters	0.32 mm diameter quartz column, 27.5 m length. External calibration (range from 10 to 300% of the limit value i.e. 10 to 300 ppm).	Porous polymeric divinylbenzene column, diameter 0.53 mm, length 30 m. External calibration.

Table 38 : Validation data of the method 2

	DFG Méthod 3 (2006)	INRS Métropol M-416 (2022)
Working range	1 ppm to 109 ppm (or 500 ppm if we consider breakthrough tests	5 to 220 µg on the support, i.e. 40 to 1760 ppm if 75 mL sample is taken
Sampling and recovery efficiencies	Charged tubes 0.1 to 5 times the limit value (i.e. 10 to 500 ppm). Recovery rate at 0.99	Tests carried out with 5 to 210 µg collected on the support. Recovery rate higher than 95%.
Uptake rate experimental validation data	NA	NA
Uptake rate stability data	NA	NA
Reverse diffusion	NA	NA
Capacity limit	Rrecovery rate greater than 99%, so no breakthrough for 500 ppm	Recovery rate tested at 20°C and 50% RH.

		DFG Méthod 3 (2006)	INRS Métropol M-416 (2022)
Detector response linearity		Linear relationship between peak area and concentration. The protocol indicates that a non-linear calibration curve should be investigated for high concentration values (not precise).	linearity checked between 0 and 220 µg on the support
Storage stability		No loss observed after 1 week of storage at 20-25°C	Storage for a maximum of 5 days at room temperature and 21 days at 4°C. Recovery rates higher than 97%.
Environmental condition		Method tested for humidity conditions between 5 and 80%.	NS
Selectivity / Interfering		Selectivity depends on the column and the type of detector used. The use of an ECD eliminates interference with CO_2. very high selectivity.	NS
Speciation		Yes	Yes
Conditions for the 8h-OEL or 15min-STEL determination	**Estimated expanded uncertainty**	Standard deviation: 0.8 to 3%. mean variation: 7.5 to 15%. for concentration range 12.8 to 109 ppm	20.7% according to EN482 with an expansion factor of 2.
	Detection limit	1 ppm per 200 mL sampled (i.e. 0.36µg) 3.3 ppm per 60 mL sampled (15 minutes to 4 mL/min)	NS
	Quantification limit	3 ppm per 200 mL (deduced from LOD)	0.6 ppm
Additional details		/	

Table 39: Descriptive parameters of the method 3

		DFG Méthod 3 (2006)	INRS Métropol M-415 (2022)
Gas /vapour - Aerosol - Combined		Gas /vapour	
Sampling	**Active / passive**	passive	
	Sampler	Stainless steel tube (6.3 mm x 89 mm, 5 mm internal diameter) filled with molecular sieve. Before use, the tubes are heated to 250°C in the thermal desorber for 30 minutes.	Steel tube containing 750 mg of BaZSM5 zeolite closed with swagelock type metal caps. Tubes conditioned for 1 hour at 350°C under nitrogen flow. A

		DFG Méthod 3 (2006)	INRS Métropol M-415 (2022)
		Tubes closed with swagelock or aluminium caps. A diffusion cap is positioned at one end during sampling	diffusion plug is placed at one end during sampling.
	Uptake rate	"0.645 mL/min for 60 minutes (38.7 mL) 0.577 mL/min for 120 minutes (69.2 mL) 0.540 mL/min for 180 minutes (97.2 mL) 0.516 mL/min for 240 minutes (123.8 mL) 0.498 mL/min for 300 minutes (149.4 mL) 0.483 mL/min for 360 minutes (173.9 mL) 0.472 mL/min for 420 minutes (198.2 mL) 0.462 mL/min for 480 minutes (221.8 mL)	0.63 mL/min for 60 to 120 minutes 0.63 mL/min for sampling time > 120 minutes and RH < 50 % 0.5 mL/min for sampling time > 120 minutes and RH > 50%.
	Duration	4 to 8 hours	the sampling time must not be less than 1 hour and must not exceed 4 hours
	Volume	for 60 minutes: 38.7 mL for 120 minutes: 69.2 mL for 180 minutes: 97.2 mL for 240 minutes: 123.8 mL for 300 minutes: 149.4 mL for 360 minutes: 173.9 mL for 420 m minutes in: 198.2 mL for 480 m minutes in: 221.8 mL	for 60 minutes: 37.8 mL for 120 minutes: 75.6 mL for 180 minutes: 113.4 mL (RH < 50%) for 240 m minutes in: 151.2 mL (RH < 50%) for 180 minutes: 90 mL (RH > 50%) for 240 minutes: 120 mL (RH > 50%)
Analysis	**Sampling treatment**	Thermal desorption at 250°C for 5 minutes.	Thermal desorption at 250°C for 10 minutes.
	Analytical technique	GC-ECD	GPC with microcatharometer
	Analytical parameters	0.32 mm diameter quartz column, 27.5 m long. External calibration (range from 10 to 300% of the limit value i.e. 10 to 300 ppm).	Porous polymeric divinylbenzene column, diameter 0.53 mm, length 30 m. External calibration.

Table 40 : Validation data of the method 3

		DFG Méthod 3 (2006)	INRS Métropol M-415 (2022)
Working range		-	0.3 to 23 µg on the carrier, i.e. 2.2 to 170 ppm for 2-hours sampling (75 mL) or 1.1 to 85 ppm for 4-hours sampling (150 mL)
Sampling and recovery efficiencies		Tubes spiked with 0.1 to 5 times the limit value (i.e. 10 to 500 ppm). Recovery rate : 99%	Tests carried out with 5 to 210 µg collected on the support. Recovery rate higher than 95% (M-416 data)
Uptake rate experimental validation data		Diffusion rate determined for durations of 1 to 8 hours. At 15, 43 and 80% RH.	Experimental design according to EN 838 with 16 tests temperature between 18 and 25°C and humidity between 30 and 60% 50/50 reverse diffusion concentration between 5 and 50 ppm sampling time between 1 and 4 hours
Uptake rate stability data		Diffusion rate is dependent on sampling time	Uptake rate depends on sampling time and RH
Reverse diffusion		NS	studied in the experimental plan.
Capacity limit		NS	NS
Detector response linearity		Linear relationship between peak area and concentrations. The protocol indicates that a non-linear calibration curve should be investigated for high concentration values (not specified).	Linearity verified between 0 and 23 µg on support
Storage stability		no loss observed after 1 week of storage at 20-25°C	Storage 5 days maximum at room temperature then 21 days at 4°C. Recovery rates higher than 97%.
Environmental condition		Diffusion coefficient determined at 5, 43 and 80% RH	Method applies for a temperature between 18 and 25°C and a humidity between 30 and 60%.
Selectivity / Interfering		Selectivity depends on the column and detector type used. The use of an ECD eliminates interference with CO_2. very high selectivity.	NS
Speciation		yes	yes
Conditions for the 8h-OEL or 15min-STEL	**Estimated expanded uncertainty**	Standard deviation: 0.5 to 4.7%. mean variation: 10 to 13%.	Expanded uncertainty of 18.5% according to EN482 with a coverage factor of 2.

		DFG Méthod 3 (2006)	INRS Métropol M-415 (2022)
determination		for concentration range 12.8 to 109 ppm	
	Detection limit	2 ppm for 100 mL (i.e. 3 hours, i.e. 0.36 µg on a carrier) we therefore have 0.9 ppm for 8 hours	-
	Quantification limit	NS	0.156 µg on substrat, i.e. 0.57 ppm for a 4h-sample.
Additional details		/	

Annex 6: Public consultation

This report and the conclusions were the subject of a public consultation from 22/06/2023 to 15/09/2023

The following individuals or organizations provided comments during the consultation phase:

- NIOSH (U.S. National Institute for Occupational Safety and Health)

Annex 7: Following up of the modification of the report

Date	Version	Description de la modification
01 July 2022	V01	Validation by the committee for public consultation
9 November 2023	V02	Validation by the committee Modifications : - additional details on the NIOSH REL - additional tables describing the results of neurological tests (tables 5 and 7) - additional footnote for the description of the study Imbriani et al, 1995 (page 63) - additional summary on carcinogenicity - clarification of the rationale for not recommending a noise notation - reorganization of the part relating to the construction of the OELs - deletion of the reference to the 2003 NIOSH 3800 protocol - addition of DFG method 3 category 3* classification (Table 34, and page 134)) - correction of LQ and validation domain for method 1 (figure 1 and figure 6) - addition of a clarification on sampling media (page debit media (page 128) - correction of the overall error for OSHA ID 166 protocol (page 129) - correction of the LQ mentioned in the "Accessible measurement range" paragraph (page 130) - for passive sampling method, replacement of the term "flow rate" by "uptake rate". - Corrections of the entries in the columns for INRS Metropol M-416 in table 38 (annex 5)

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