Committee for Risk Assessment

RAC

Annex 1
Background document
to the Opinion proposing harmonised classification and labelling at EU level of

dinitrogen oxide

EC Number: 233-032-0
CAS Number: 10024-97-2

CLH-O-0000007281-79-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

16 March 2023

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

Dinitrogen oxide

EC Number: **233-032-0**

CAS Number: **10024-97-2**

Index Number: **-**

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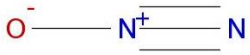
Version number: 02 **Date: April 2022**

CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substances

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Nitrous oxide
Other names (usual name, trade name, abbreviation)	Dinitrogen oxide, laughing gas, hyponitrous oxide, dinitrogen monoxide, nitrogen oxide
EC number	233-032-0
EC name	Dinitrogen oxide
CAS number	10024-97-2
Molecular formula	N_20
Structural formula	
SMILES notation	[N-]=[N+]=O
Molecular weight or molecular weight range	44.013

1.2 Composition of the substance

Table 2: Constituents

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self- classification and labelling (CLP)
Dinitrogen oxide	Mono-constituent	-	Ox. Gas 1, H270 Press. Gas, H281 STOT SE 3, H336 (brain) (inhalation) Acute Tox. 2, H330

Table 3: Impurities if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
No impurities relevant for classification				

No additives if relevant for the classification of the substance.

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 4:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	Not available										
Dossier submitters proposal	TBD	Dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT RE 1 STOT SE 3 Ozone 1	H360Df H372 (nervous system) H336 H420	GHS07 GHS08 Dgr	H360fD H372 (nervous system) H336 H420			
Resulting Annex VI entry if agreed by RAC and COM	007-RST-VW-Y	Dinitrogen oxide	233-032-0	10024-97-2	Repr. 1B STOT RE 1 STOT SE 3 Ozone 1	H360Df H372 (nervous system) H336 H420	GHS07 GHS08 Dgr	H360fD H372 (nervous system) H336 H420			

Table 5: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	hazard class not assessed in this dossier	No
Oxidising gases	hazard class not assessed in this dossier	No
Gases under pressure	hazard class not assessed in this dossier	No
Flammable liquids	hazard class not assessed in this dossier	No
Flammable solids	hazard class not assessed in this dossier	No
Self-reactive substances	hazard class not assessed in this dossier	No
Pyrophoric liquids	hazard class not assessed in this dossier	No
Pyrophoric solids	hazard class not assessed in this dossier	No
Self-heating substances	hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	hazard class not assessed in this dossier	No
Oxidising liquids	hazard class not assessed in this dossier	No
Oxidising solids	hazard class not assessed in this dossier	No
Organic peroxides	hazard class not assessed in this dossier	No
Corrosive to metals	hazard class not assessed in this dossier	No
Acute toxicity via oral route	hazard class not assessed in this dossier	No
Acute toxicity via dermal route	hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	hazard class not assessed in this dossier	No
Skin corrosion/irritation	hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	hazard class not assessed in this dossier	No
Respiratory sensitisation	hazard class not assessed in this dossier	No
Skin sensitisation	hazard class not assessed in this dossier	No
Germ cell mutagenicity	hazard class not assessed in this dossier	No
Carcinogenicity	hazard class not assessed in this dossier	No
Reproductive toxicity	harmonised classification proposed: Repr. 1B – H360Df	Yes
Specific target organ toxicity-single exposure	STOT SE 3 – H336 (narcosis)	Yes
Specific target organ toxicity-repeated exposure	STOT RE 1 – H372 (nervous system)	Yes
Aspiration hazard	hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	hazard class not assessed in this dossier	No
Hazardous to the ozone layer	Ozone 1 – H420	yes

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Dinitrogen oxide has not been classified according to the Classification and Labelling of the Dangerous Substance Directive (Dir. 67/548/EEC) and have no entry in Annex VI Tables 3.1 and 3.2 of the Regulation (EC) No. 1272/2008 (CLP Regulation).

RAC general comment

The scope of the CLH dossier and current RAC opinion is focussed on the hazard classes STOT SE, STOT RE, reproductive toxicity and hazardous to the ozone layer.

Use and application

Dinitrogen oxide (N_2O) has many uses and applications:

- N_2O is used in surgery as an adjuvant in inhalational anaesthesia, for pain relief during delivery, or for short analgesia during minor medical procedures (e.g., dentistry). The dossier submitter (DS) provided an indication for the concentration used for anaesthesia (50% N_2O and 50% O_2).
- N_2O is used as a food additive (E942).
- N_2O is an in-canister propellant used in many preparations and uses (e.g., aerate whipping cream, inflate balloons).

"Recreational" misuse of the gas has been identified as strongly increasing in recent years.

RAC notes that it has the task to evaluate the information presented to it for the hazard classes included in the CLH dossier and which were open for consultation; this may include human data from clinical case studies. However, this evaluation is independent of the uses of the substance.

Toxicokinetics and proposed mechanism of action

N_2O is rapidly absorbed through inhalation. The alveolar concentration is similar to the inhaled concentration within 5 minutes and the blood/gas partition coefficient is 0.47 (ANSM, 2014). N_2O is rapidly distributed throughout the body in dissolved form, especially to richly vascularised tissues. It easily penetrates into the brain and is able to cross the placental barrier (INRS, 2018). N_2O is poorly metabolised (0.004%). However, N_2O is reduced to nitrogen in the reaction with the cobalt (Co^{2+}) of vitamin B12 (MAK, 1993). N_2O is mostly eliminated unchanged through the lungs within a few minutes, and only small amounts are eliminated via the urine.

As indicated by the DS, N_2O probably acts by directly inhibiting vitamin B12 formation and inactivating methionine synthase through oxidation of the Co^{2+}. Methionine synthase inhibition leads to the impairment of the generation of methyl groups for DNA methylation. This results in the decrease in the synthesis of deoxythymidine, thymidine and DNA among other products. Stewart *et al.* (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. The specific impacts of the proposed mechanism of action are further discussed under the relevant hazard classes in question. However, it is noted that the CLP Regulation does not require a mode of action to be conclusively demonstrated for classification in these hazard classes.

Test concentrations in animal studies

For the human health endpoints, the DS presented animal data derived from inhalation studies using whole body exposure, in addition to human data. Regarding the level of applied test concentration in these animal studies, RAC notes the following:

- OECD test guidelines, such as TG 412/413 (section 13) state that '*The maximum concentration tested should consider: 1) the maximum attainable concentration, 2) the need to maintain an adequate oxygen supply, and/or 3) animal welfare considerations. In the absence of data-based limits, the acute limits of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals [GHS] may be used (i.e., up to a maximum concentration of 5 mg/L for aerosols, 20 mg/L for vapours, and 20000 ppm for gases). Justification should be provided if it is necessary to exceed these limits when testing gases or highly volatile test chemicals (e.g. refrigerants).*'

- OECD TG 414 (section 17) states that '*Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test chemical often may indicate the maximum attainable level of exposure (for example, dermal application should not cause severe localised toxicity).*'

- Specific information on reproductive toxicity testing is also stated in the CLP Regulation (EC) 1272/2008 Annex I (part 3: Health Hazards, chapter 3.7: Reproductive toxicity, section 3.7.2.5.7) as '*There is general agreement about the concept of a limit dose, above which the production of an adverse effect is considered to be outside the criteria which lead to classification, but not regarding the inclusion within the criteria of a specific dose as a limit dose. However, some guidelines for test methods, specify a limit dose, others qualify the limit dose with a statement that higher doses may be necessary if anticipated human exposure is sufficiently high that an adequate margin of exposure is not achieved. Also, due to species differences in toxicokinetics, establishing a specific limit dose may not be adequate for situations where humans are more sensitive than the animal model.*'

RAC has taken note of the various exposure concentrations applied in the available animal studies. These can reach concentrations far above the indicated GHS level of 20000 ppm. In line with the OECD TGs/GD (guidance document) and CLP Regulation (Annex I, 3.7.2.5.7), the adverse effects observed at high test concentrations (i.e., above 20000 ppm) are considered relevant for classification by RAC because the anticipated human exposure is high, as indicated by the use as an anaesthetic and foreseeable recreational use. When following the 20000 ppm guidance value, an adequate margin of exposure is not likely to be reached. However, RAC further notes that OECD GD 39 (section 61) states that '*An oxygen concentration of at least*

19%, a carbon dioxide concentration not exceeding 1%, and an evenly distributed exposure atmosphere should be ensured[1]*.'*, leaving a maximum of 80% (800000 ppm) for the test item.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Action is required at Community level based on the need to classify for CMR endpoints.

Furthermore, self-classification STOT SE 3 for brain by inhalation is not sufficient and justify the need for action at Community level since, based on animal and human data, there are evidence that the substance shall be classified for STOT RE 1 for the nervous system in addition to classification STOT SE 3 for narcosis.

In addition, it has been known since 1970 that dinitrogen oxide depletes the stratospheric ozone. According to the world meteorological organization scientific assessment of ozone depletion (WMO, 2018), natural and anthropogenic emissions of dinitrogen oxide make a larger contribution to stratospheric ozone depletion than emissions of any of the individual ozone depleting long-lived halogenated source gases. Based on the current calculated ozone depletion potential-weighed emission (Ravishankara et al., 2009), JRC (2015) indicated that dinitrogen oxide is the largest of all ozone depleting substances. Therefore, action is required at community level and justify the need to classify dinitrogen oxide as hazardous to the ozone layer.

5 IDENTIFIED USES

According to ECHA website, dinitrogen oxide (N_2O) is manufactured and/or imported in the European Economic Area at ≥1000 to < 10 000 tons per year.

N_2O is used for more than 150 years in surgery as an adjuvant in inhalational general anaesthesia. The substance is also used for pain relief during delivery or for short analgesia during minor medical procedure (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).

Furthermore, N_2O is a propellant in canister used in many preparation and uses (e.g. aerate whipping cream, inflate balloons).

It is also an additive to rocket fuels to increase available oxygen for combustion. In addition, N_2O is used in laboratory as an oxidizing agent in atomic flame absorption spectrometry.

Recreational misuses of the gas, also called "laughing gas", has been identified as strongly increasing in recent years (ANSES, 2020) due to its euphoric, relaxing and hallucinogenic properties, with various effects for health, including severe ones.

6 DATA SOURCES

Literature search was conducted in the Pubmed database.

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 :

[1] OECD GD 39 (section 60) contains a similar statement for nose only exposure: '*Each exposure port should have similar exposure conditions with an oxygen concentration of at least 19% and a carbon dioxide concentration not exceeding 1%*.'

(kinetic) OR (metabolism) OR (metabolite) OR (absorption) OR (distribution) OR (excretion) OR (elimination) or (half-life) OR (clearance) OR (model) OR (Health effects) OR (health) OR (developmental toxicity) OR (toxic) OR neurotoxicity OR reproductive toxicity 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 summarised the inclusion/exclusion criteria used for human and animal studies.

Table 6: Literature search inclusion and exclusion criteria

Publication type	IN	Primary research studies
	OUT	Secondary studies (e.g. editorials, conference)
Language	IN	English
	OUT	Other languages
Study design	IN	Human experimental volunteer studies, Cohort studies, Cross-sectional studies, Case-control studies, case studies Experimental animal studies
	OUT	*In vitro* studies *in silico* studies
Population	IN	Adult healthy male and female volunteers, men and women occupationally exposed to N_2O, recreational drug exposure All mammalian animals
	OUT	Patients under anaesthesia
Exposure	IN	Inhalation
	OUT	Mixtures Anaesthesia Air pollution Subdermal route of exposure in animals Pharmacological research (e.g. alcohol withdrawal)
Time	IN	From 1952 – December 2020 for reproductive toxicity From 2001-2021 for STOT
Outcome	IN	Human health endpoints included in the dossier (reproductive toxicity, neurotoxicity)
	OUT	Risk management measures Other Human Health endpoint not assessed in this dossier

All data sources retained in this report are listed in section 15.

Secondary literature were also considered to check for potential additional research studies or mechanistic data not captured during the literature search: IPCS-INCHEM, 1992; INRS, 2010 and 2018; MAK, 1993 and 2015; ACGIH, 2001; ANSES, 2020; HCN, 2000; EIGA, 2008.

Furthermore, REACH registration dossiers (last modified: 25 October 2016) for N_2O available from ECHA's disseminated database (ECHA, 2021) have been taken into account.

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties for dinitrogen oxide

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Colourless gas with sweetish odour	(ECHA, 2021)	No study has been presented. Nevertheless, in NIOSH Handbook (2018) the physical description of dinitrogen oxide is Colourless gas with a slightly sweet odour.
Melting/freezing point	-90.81 °C at 1013.25 hPa	(ECHA, 2021)	in NIOSH Handbook (2018) the freezing point was specify to be at -127 F° equivalent to -52.78°C (at atmospheric pressure).
Boiling point	-88.3 °C at 1013.25 hPa	(ECHA, 2021)	In NIOSH Handbook (2018) the boiling point was determined to be -132 F° equivalent to -55.56°C (at atmospheric pressure).
Relative density	1.2 (-89°C) and 1.99 mg/cm³(0°C)	(ECHA, 2021)	In NIOSH Handbook the relative gas density value was 1.53.
Vapour pressure	42900 mmHg at 25°C	(ECHA, 2021)	In NIOSH Handbook (2018) the vapour pressure was 51.3 atm. equivalent to 38756 mmHg at ambient temperature.
Surface tension	1.75 dynes/cm at 20°C	(ECHA, 2021)	No study has been presented to confirm this surface tension value.
Water solubility	1.5 g/L at 15°C pH not indicated	(ECHA, 2021)	No study has been presented to confirm this water solubility value. In NIOSH Handbook (2018) water solubility was 0.1% at 21.5°C without the precision of the pH.
Partition coefficient n-octanol/water	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Flash point	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Flammability	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property. In NIOSH Handbook (2018)

Property	Value	Reference	Comment (e.g. measured or estimated)
			it is reported that the dinitrogen oxide is Non-flammable Gas, but supports combustion at elevated temperatures.
Explosive properties	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Self-ignition temperature	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Oxidising properties	Oxidizing	(ECHA, 2021)	No data has been provided to confirm the oxidizing classification of Dinitrogen oxide: Ox. Gas 1, H270 Press. Gas, H281.
Granulometry	Not relevant	(ECHA, 2021)	Not relevant as Dinitrogen oxide is a gas substance.
Stability in organic solvents and identity of relevant degradation products	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Dissociation constant	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.
Viscosity	No data	(ECHA, 2021)	No data was provided to assess this physicochemical property.

8 EVALUATION OF PHYSICAL HAZARDS

Not performed for this substance.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Conclusions from studies are summarised in section 9.1			

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Absorption

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

There is no data in animal.

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).

Stenqvist et al., 1994, in the table below, report the partition coefficients. 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 9: 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

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

Metabolism

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

As described in MAK report, 1993, dinitrogen oxide is reduced to nitrogen in the reaction with the central Co+ ion of vitamin B12. After a single passage through the liver, however, the concentration of dinitrogen oxide in the blood decreases by only 0.03 %. Formation of radicals has been demonstrated *in vitro* in human intestinal contents incubated with dinitrogen oxide. It has been deduced from *in vitro* investigations that about 0.004 % of the total dinitrogen oxide absorbed is metabolised in humans and animals to nitrogen by bacterial reductases in the intestine. During this process OH radicals are probably formed.

[2] http://agence-prd.ansm.sante.fr/php/ecodex/rcp/R0234390.htm, consulted in May 2021 (only available in French)

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, 2012). Dinitrogen oxide 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. (1983) exposed 20 healthy volunteers, young and elderly males to identify any aged-related differences, under a protocol to mimic a dental operatory. The authors measured dinitrogen oxide 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, Dinitrogen oxide is completely eliminated from the blood (INRS, 2018).

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).

10 EVALUATION OF HEALTH HAZARDS

Nitrous oxide, N_2O and dinitrogen oxide are synonyms.

10.1 Acute toxicity - oral route

Evaluation not performed for this substance.

10.2 Acute toxicity - dermal route

Evaluation not performed for this substance.

10.3 Acute toxicity - inhalation route

Evaluation not performed for this substance.

10.4 Skin corrosion/irritation

Evaluation not performed for this substance.

10.5 Serious eye damage/eye irritation

Evaluation not performed for this substance.

10.6 Respiratory sensitisation

Evaluation not performed for this substance.

10.7 Skin sensitisation

Evaluation not performed for this substance.

10.8 Germ cell mutagenicity

Evaluation not performed for this substance.

10.9 Carcinogenicity

Evaluation not performed for this substance.

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Five published studies in rats and four published studies in mice investigated the potential effects on sexual function and fertility of dinitrogen oxide. The table below summarised the relevant findings. See annex I for detailed summary of the method and results.

Table 10: Summary table of rats studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline paternal study and dominant lethal test GLP: not stated Crl:COBS(SD)BR rats Paternal study: N=12/exposed groups (3 replicates of 4 males or females per dose) Dominant lethal test: 24 males per groups mated with 30 non-exposed females *Limitations:* - few parameters investigated in the study - no information on survival or clinical signs in the sires or dams in the study	Dinitrogen oxide (checked for purity) Whole body inhalation exposure Males: 6h/day, 5d/w for 9 weeks. Mated with non-exposed females (paternal and dominant lethal assay) 0, 1000, 5000, 10,000 ppm N_2O in air	<u>General toxicity</u> No effect on weight of males, no effect on body weight gain in females up to 10,000 ppm <u>Reproductive toxicity</u> - Paternal study: No statistically significant findings in litter size (trend to lower number of pups per litter in dinitrogen oxide groups) - Dominant lethal assay: no statistically significant effect on conception rate, total number of implants, live foetuses. However, a trend to increase in the number of resorption noted at the top dose with slight increase in lethality.	Holson et al., 1995 (substudy ; fertility) Klimisch score 2 WOE
Non-guideline fertility study in female rats GLP: not stated Female Sprague-Dawley rats Group 1: 8 animals/group: hypothalamic LHRH-producing cell counts.	Dinitrogen oxide (no information on purity) 0, 300,000 ppm N_2O in air Inhalation (whole body exposure) 8h/d for 4 days or one ovulatory cycle	<u>General toxicity</u> No information <u>Group 1: Brain study</u> ↑ LHRH (Luteinizing hormone-releasing hormone) cell count in hypothalamus in the 4 animals exposed on proestrus compared to the 4 controls. No effects on LHRH cell count in animals exposed during metestrus compared to control <u>Group 2: Ovulatory cycle study</u>	Kugel et al., 1990 Klimisch score: 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Group 2: 12 animals/group : 6 animals/group treated in proestrus and 6 animals/group treated in random stage of ovulatory cycle Group 3: 12 animals/group: mated with proven male breeder for 4 days. 6 animals/group treated in proestrus and 6 animals/group treated in random stage of ovulatory cycle. Number of pregnancy, litter size and weight were analysed. *Limitations:* - No information on general toxicity in dams. - Only one concentration tested - Low number of animals used in each group - Statistical analysis not performed		**Disrupted cycles** following the first day of exposure and 11 out of the 12 exposed rats went into constant proestrus. This dysfunction resolved itself after approximately 3 weeks. Control animals cycled normally throughout the experiment. <u>Group 3: Fertility study</u> Mating occurred in all animals. All 12 controls gave births. Only 6 out of 12 treated animals (3 in groups treated in proestrus and 3 in random phase of the cycle) gave birth. No effects on litter size and weight of pups.	
Non-guideline fertility study in female rats GLP: no Female rats n=12/group Limitations: - Only short communication of the results in the publication. - no statistical analysis	Dinitrogen oxide 0, 500 ppm N_2O in air Inhalation, 8h/day, 35 days	**Transitory oestral cycle disturbance** in all exposed female rats. Return to normal after 3 weeks. Only 6 out of 12 exposed animals gave birth vs all animals in control. No effects on litter size and weight of pups in both control and exposed groups.	Kugel et al., 1989 Klimisch score: 4 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline fertility study in male rats GLP: no Wistar male rats n= 12/group Mating with non-exposed females: after exposure or after 6-month recovery *Limitations*: - no information on environmental condition (light cycle, temperature) - single dose level - short duration period (< 10 weeks) - few parameters analysed - no information on general toxicity in animals	Dinitrogen oxide (purity not stated) Inhalation, 6h/d, 5d/w, 30 days 0, 5000 ppm N_2O in air	General toxicity: no information Fertility: **Statistically significant decrease in litter size and developmental delay in offspring** (body weight, tail length and body length). No significant effect after a 6-month recovery period.	Vieira et al., 1983a Klimisch score: 2 WOE
Non-guideline testis toxicity study in male rats GLP: no Male LEW/f Mai rats N=4-6 rats/group *Limitations* - Low number of animals per groups - Only one dose level - Duration of exposure is too short (< 10 week necessary to cover the whole spermatogenic cycle) - no detailed results (incidence, grade of the lesions) - although several organs were sampled, only findings in testes were published.	Dinitrogen oxide (no information on purity) 0, mixture of 200,000 ppm N_2O: 20% O2:60% N_2 Inhalation, Group 1: 1 to 35 days intermittent (8h/d) exposure, Group 2: 32-day continuous exposure (24h/d), sacrifice after 3, 6 or 10-day post-exposure.	General toxicity: no information Male reproductive organs: Statistically significant **decrease in testis weight in group 1 and 2 compared to controls**. Effect was reversible after 6-day recovery (continuous exposure) Microscopic examination: **injury (damage, destruction) observed in the spermatogenic cells in the seminiferous**. Some reversibility of the effect noted. Effect more severe in group 2 than in group 1. No effects in controls. No effect in Leydig cells and supporting cells within the tubules. No effect on serum testosterone levels	Kripke et al., 1976 Klimisch score: 2 WOE

Table 11: Summary table of mice studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline 14-week repeated dose toxicity study GLP: no Swiss Webster Mice N=15/sex/group Limitations: - Few organs and parameters examined - Low duration of exposure (4h per day)	Dinitrogen oxide (purity not stated) Inhalation, 4h/d, 5d/w (whole body) 14-week exposure, 0, 5000, 50,000, 500,000 ppm N_2O in air	General toxicity All animals survived. Significant decreased in body weight gain by 77% and 63% in males and females, respectively. Organ toxicity No histopathological findings in testes and in ovaries (no other reproductive organ examined).	Rice *et al.*, 1985 Klimisch score 4 WOE
Non-guideline reproductive organ toxicity study GLP: no Swiss Webster mice N=15/sex/group *Limitations:* - Only 4h exposure per day - No information on the source of test material - Low number of animals per groups for oocyte examination (n=6) - Only few organs and parameters examined. - Low level of information on general toxicity - no information if experimenters were blind to treatment	Dinitrogen oxide (medical grade) Inhalation, 4h/d, 5d/w, 14-week exposure Control, 5000, 50,000, 500,000 ppm N_2O in air Methyl methanesulfonate positive control for male germ cell toxicity Methychloranthrene: positive control for oocyte count	General toxicity No excitement or general anaesthesia seen in animals. Mice behave normally. Reproductive toxicity No effects on testes weight, percentage of abnormal sperm, sperm count or histologic appearance of the testes. No effects on the mean number of oocytes (33.3 ± 14.4 versus 29.8 ± 8.0 in controls) A statistically significant positive response was obtained for each positive control.	Mazze *et al.*, 1983 Klimisch score 2 WOE
Non-guideline fertility study in male mice Swiss/ICR mice GLP: no N=18-21 male mice per groups mated with non-exposed females *Limitations:* - few reproductive parameters investigated - no information on general toxicity	Dinitrogen oxide (purity not stated) Inhalation (whole body), 9 weeks, 4h/day, 5d/week Control (air treatment), control (colony), 5000, 50,000, 500,000 ppm N_2O in air	General toxicity No information Reproductive toxicity No effect on litter size, abilities of males to impregnate females, no effect on foetal wastage (resorption, dead), foetal size	Mazze *et al.*, 1982 Klimisch score 2 WOE
Non-guideline study on	Dinitrogen oxide	No animal died from exposure	Land *et al.*,

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
early spermatogenesis in male mice GLP: no (C57Bl xC3H)F1 mice 5 males/group *Limitations* - Low number of animals - No information on general toxicity - 80% dinitrogen oxide is above threshold leading to hypoxia - Only 5-day exposure does not cover full spermatogenesis	(purity not specified) Inhalation, 4h/day, 5 consecutive day Control (air), 80000, 800,000 ppm N_2O in air	General toxicity not described No effects on abnormal spermatozoa	1981 Klimisch score 3 Disregarded

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Few studies are available on the effect of dinitrogen oxide on sexual function and fertility. None of the studies were performed in compliance with OECD TG for reproductive toxicity. All the studies had limitations (e.g. single dose level, low number of animals, few parameters investigated) and were performed either on males or females.

- **Female rats**

Kugel et al. (1990) exposed female rats to 0 or 300,000 ppm dinitrogen oxide for 4 days (one ovulatory cycle) by inhalation during 8 hours per day and performed 3 different experiments on 3 different groups. Reduction in fertility was noted in the 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 rats went into constant proestrus whereas all 12 controls had normal cycles. The effect was reversible by 3 weeks. In the experiment measuring various hormones, an increase in total LHRH cell count was seen in the 4 exposed females on the morning of protestrus (~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 specialised neuroendocrine cells, key regulator of the hypothalamic–hypophyseal–gonadal axis, essential for reproductive competence) was interpreted by the authors as a decrease in release of LHRH and a subsequent increase intracellular content, rather than an increase in the actual number of LHRH producing cells.

Kugel et al. (1989) reported a reversible interruption of ovulation and a 50% decrease in fertility (6/12 exposed rats vs 12/12 control rats gave birth) following exposure to dinitrogen oxide at 500 ppm (mixed in compressed air) delivered 8 hours per day for 35 days. As only a short summary of the method and result is available in the publication, it is not possible to assess the reliability of the study.

- Male rats

In Holson et al. (1995), male rats were exposed 5 day per week for 9 weeks, 6 hours per day, at similar dose levels (3 replicates of 4 males per groups) as in the maternal study (0, 1000, 5000, 10000 ppm). 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 dinitrogen 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** (table 2 of Annex 1).

In Vieira et al. (1983a), two groups of 12 male Wistar rats were used in the study. One group was exposed to 5000 ppm dinitrogen oxide/air mixture (v/v) and one group was exposed to air only (control). The rats were exposed 6h/day, 5d/w for 30 days. 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. The young rats were weighed and measured at weekly interval for 8 weeks.
A statistically significant decrease in litter size was seen after dinitrogen oxide exposure compared to control. These findings were not observed 6-month after recovery. 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 dinitrogen oxide one litter comprised 14 offsprings but the remaining 35 litters ranged between two and six offsprings. With regards to body weight, there was a significant difference from week-3 onward in the offsprings belonging to the group exposed to 5000 ppm dinitrogen oxide compared to control. 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 exposed males that were allowed 6-month recovery. It may be noted that the effect on litter size was the sole parameter reported and no other parameters (e.g. number of *corporea lutea*, number of implants, early and late resorptions) were reported in the published study to support the interpretation of the toxicological significance of the effect.

Table 12: Summary of litter size in Vieira et al. (1983a)

Group	**Control**	**N_2O, 5000 ppm initial mating**	**N_2O, 5000 ppm 6-month recovery**
No. of litters	36	36	36
No. of born rats	382	252	380
Litter size	Mean: 12 Range: 9-15	Mean: 7* Range: 2-14	Mean: 11 Range: 8-14

*$p<0.001$

Kripke et al. (1976) exposed male rats (n=4-6 per group) 8h/d (intermittent) for 1 to 35 days of exposure or 24h/d (continuous) for 32 days with 3, 6 or 10-day recovery to a mixture of 200,000 ppm dinitrogen oxide, 20% O_2 and 60% N_2. **Absolute testis weight was decreased in both groups (continuous or intermittent exposure) compared to control. Damage and destruction of spermatogenic cells were observed in the exposed groups. Incidence and severity** was more pronounced in the continuous exposed group compared to the intermittent exposure group. The effects were more frequent and more pronounced with exposure duration and by day 14, the findings were observed in all rats of both exposure groups. No effects were noted in the control group. No effect in Leydig cells and supporting cells within the tubules was reported, as well as for testosterone levels in any group. The study is considered of limited reliability. Indeed, 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. However, even with this short duration of exposure, effects on some reproductive parameters were observed. No detailed results (incidence, grade of the lesions) were provided in the publication.

- Mice

There are three studies available in mice, performed in the same laboratory. The fourth study from Land et al., 1981 was disregarded as the study 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 dinitrogen oxide in fertility or sexual function in mice up to 500,000 ppm:

- No effects were observed on testis or ovaries in mice exposed to dinitrogen oxide up to 500,000 ppm during 4h per days, 5 days per week for 14 weeks (Rice et al., 1995).

- No effects compared to controls were noted following a specific investigation of male testes and spermatogenesis and female mice oocyte count in Mazze et al. (1983). The same experimental condition and dose levels as in Rice et al. (1995) were used (14-week exposure, 4h/d, 5d/w) in this study.

- Mazze et al., 1982 did not report effect in the ability of males to impregnate females or in 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 dinitrogen oxide.

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

- Human data

Two human retrospective cohort studies investigated the potential effect of dinitrogen oxide on fertility in midwifes (Alhborg et al., 1996) or in dental assistants (Rowland et al., 1992).

In Alhborg et al. (1996), 3358 women midwives, born between 1940 and 1989 and member of the Swedish Association of Midwives, were included in a retrospective cohort study. 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 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). **Although expected, there was no information on potential co-exposure in the study.**

In Rowland et al. (1992), questionnaires were sent to 7000 female dental assistants, registered in California in 1987 and working full-time (> 30h/week). 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 that reported an exposure more than 5h per week to unscavenged N_2O. The 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 impaired a firm conclusion on the observed effects.

- Mechanistic information

N_2O irreversibly inactivates methionine synthase function by oxidation of the Co^+ ion of vitamin B12 in all species. 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. There are no data available investigating the inhibition of methionine synthase function on effect on fertility in animals.

According to Kugel et al. (1990), the lack of LHRH release can be explained on the basis of an increase in opioids and substance P in areas of the brain where LHRH is synthesized. The authors noted that dinitrogen

oxide may produce its analgesic and sedative effects through an increase in endogenous opioid which in turn can have an inhibitory effect on the release of LHRH cells in the hypothalamus. Further data would be needed to support this hypothesis.

10.10.3 Comparison with the CLP criteria

For potential classification on sexual function and fertility, criteria from CLP guidance (ECHA, 2017) were applied.

- Adverse effects on sexual function and fertility are described as "*Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems*." *(ECHA, 2017).*
- *Known human reproductive toxicant. "The classification of a substance in this Category 1A is largely based on evidence from humans."*

A few studies are available in human. The positive results (decreased fecundability ratio) obtained in these studies are not sufficient by themselves to serve as basis for a classification. Indeed, potential co-exposure and the absence of quantitative characterisation of N_2O exposure in these studies lead to uncertainties on the results. Therefore, category 1A is not warranted for dinitrogen oxide.

- *Presumed human reproductive toxicant. "The classification of a substance in this Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate*" (ECHA, 2017).
- *Suspected human reproductive toxicant "Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects".*

Two experimental studies from the same authors have investigated effects of dinitrogen oxide in female rat on reproductive function. The studies used different exposure condition: 300,000 ppm dinitrogen oxide, 8h/day for 4 day or 500 ppm, 8h per day for 35 days. Reproductive cycle abnormalities and marked decreased fertility (by 50% vs control) was observed in female rats in both studies (Kugel et al., 1989, 1990). In these studies, there were several limitations: no information on general toxicity in dams, the number of animals per group was low, only one-dose level, few parameters investigated. Regarding parental toxicity, the CLP guidance (ECHA, 2017) stated that "*Adverse effects on fertility and reproductive performance seen only at dose levels causing marked systemic toxicity (e.g. lethality, dramatic reduction in absolute body weight, coma) are not relevant for classification purposes*". In the developmental toxicity studies (see next section), no excessive toxicity was found up to 300,000 ppm in rats. Marked toxicity, as described in the guidance, can therefore be reasonably excluded and the effect on oestrous cycle and fertility is not expected to be a consequence of other unspecific toxic effect. In addition, there is no indication that the effect would not be relevant to human. Although occurring at high dose levels in one study (300,000 ppm), similar findings were noted by the authors at lower dose levels (500 ppm in Kugel et al., 1989).

In mice, no histopathological findings in ovary and no effects were noted in primary oocytes count following repeated exposure up to 500,000 ppm dinitrogen oxide (Rice et al., 1985, Maze et al., 1983). Fertility and

oestrous cycle was not investigated in female mice. Therefore, it is not possible to disregard the effect observed in rat based on the negative results observed in mice.

Studies investigating effects of 200,000 ppm dinitrogen oxide on male rat reproductive organs showed significant effects on sperm and testes (Kripke et al., 1976). The main limitation in this study is the use of a single dose level.

Effects observed in litter size in dams from male rats exposure to 5000 ppm dinitrogen oxide (Vieira et al., 1983a) is of concern. Although no statistical significant effects were noted on litter size in Holson et al., 1995, the trend observed on a decreasing number of pups per litter noted at 10,000 ppm may support an effect of concern although the study raised some uncertainties on NOAEC and LOAEC for this effect.

No effects on male fertility and sexual function (testes, spermatogenesis) were observed in mice in any of the three available studies (Mazze et al., 1983, Mazze et al., 1982, Rice et al., 1985). Nevertheless, only a few parameters were investigated in these studies.

In conclusion, the decreased fertility and oestrous cycle changes observed consistently in females rats in two studies leads to clear concern on female fertility. In addition, the effects observed on testis and spermatogenesis in Kripke et al., 1976 and the decrease in litter size in Vieira et al., 1983a support potential male fertility effects. The DS has no explanation with regards to the absence of effect in mice compared to rats, leading to some uncertainties. Category 2 is therefore considered more appropriate than category 1B due to the limited number of parameters investigated in the studies and the absence of effects in mice. Dinitrogen oxide warrant to be **classify as Repr. 2, H361f.**

10.10.4 Adverse effects on development

Table 13: Summary table of animal studies on adverse effects on development in rats in prenatal developmental toxicity studies, **continuous exposure studies (23-24h per day)** (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline prenatal developmental toxicity study GLP: no Sprague-Dawley rats N=25 in exposed group and 30 in control group Sacrifice: GD 20 *Limitations* - Only one dose level, single day of exposure - No information if experimenters were blind to treatment and control groups - Few details on maternal toxicity (only bw before and after exposure and on GD20 was provided), no information on clinical signs. - It is not specified if the death observed in the treated group was treatment related.	Dinitrogen oxide (medical grade) Inhalation, whole body 24h exposure at GD8 0, 600,000 ppm N_2O mixed with O_2 and air	Maternal toxicity No statistically significant difference in mean bw on GD 20 (334g vs 368 g in controls). Mortality in one out of 25 exposed dam. Developmental effects: - ↑stat. sign. Foetal resorptions/litter (48% vs 5% in controls) - ↓ stat. sign. Live foetuses/litter (mean 6.5±4.1 vs 12±2.1 in controls) - ↓ stat. sign mean number of foetuses/litters - ↑ stat. sign major visceral malformations - ↑ stat. sign minor visceral anomalies - ↑ stat. sign minor skeletal anomalies and variants	Fujinaga et al., 1991 Klimisch 2 WOE
Non-guideline developmental toxicity study GLP: no Sprague-Dawley rats N = 35/group Sacrifice: 4-6 rats on GD11, 12, 13, 14, 15, 16, 18 and GD20 Only survival and *situs inversus* in foetuses examined Limitations - No information on the age and body weight of the animals at the start of the study - Body weight of dams analysed but results were not reported. - General toxicity of dam	Dinitrogen oxide (medical grade) Inhalation, whole-body 24h exposure at GD8 0, 700,000-750,000 ppm N_2O mixed in air and oxygen	Maternal toxicity No information Developmental toxicity Statistically significant increase in embryo-foetal mortality rate on GD 14 and onward compared to control Statistically significantly increase in altered laterality at all stage compared to controls	Fujinaga et al., 1990 Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
not provided. - No information on foetuses weight - Single dose level, single exposure - No information if experimenters were blind to treatment			
Non-guideline developmental toxicity study in rat GLP: no Sprague-Dawley rats 20 rats/group, 30 controls Sacrifice: GD20 Limitations - Low number of litters examined - Lack of details on maternal toxicity (food consumption, clinical signs, weight) - Only one dose level - No information if experimenters were blind to treatment	Dinitrogen oxide (medical-grade) Inhalation, whole-body exposure Exposure: 24h at GD 6, 7, 8, 9, 10, 11 or 12 0, 600,000 ppm N_2O mixed in air and oxygen	<u>Maternal toxicity</u> Mortality in 2 to 4 dams per groups except in control and GD 10 exposed group. Mid sedation. Decrease (stat. sig.) mean maternal body weight at caesarean section in all treated groups (Table 8 in confidential Annex I, no further information) <u>Developmental toxicity</u> - No effects on the number of implantations, live foetuses, mean foetal weight, sex ratio. - Increased % of resorptions per litter on GD8 and 11 (Stat. sign.); - Increased skeletal malformations following exposure on GD 9 (stat. sign.) - Increased skeletal variations following exposure on GD 8 (stat. sign.). - Increased visceral malformations and minor visceral anomalies when dams exposed on GD8 or GD9 (stat. sign.)	Fujinaga et al., 1989 Klimisch 2 WOE
Non-guideline developmental toxicity study in rat GLP: no Sprague-Dawley Rats Experiment 1: 90 rats: - 20 animals exposed to N_2O or folinic acid or folinic acid + N_2O - 30 controls (air) Experiment 2: 116 rats - 37 controls (air) - 26 animals exposed to N_2O - 27 animals exposed to folinic acid - 26 animals exposed to folinic acid + N_2O Methionine synthase activity: 65 non-pregnant rats - 5 controls (air) - 20 exposed to N_2O- 20 exposed to N2O +	Dinitrogen oxide (medical grade) Experiment 1 and 2: Inhalation, whole body, 24h at GD 8 Control, 500,000 ppm (experiment 1), 750,000 ppm N_2O (experiment 2), folinic acid, 750,000 ppm N_2O + folinic acid Methionine synthase activity: 24h at GD 8 Control, 500,000 ppm N_2O, 500,000 ppm N_2O + halothane, 500,000 ppm N_2O + folinic acid Sacrifice at 24, 48 and 72h post-treatment	<u>Maternal toxicity</u> Mid sedation in both experiments at 500,000 or 750,000 ppm Statistically significant ↓ body weight due to lower number of live foetuses <u>Developmental toxicity</u> - No effect on weight of pups - Increased early and late resorptions (stat. sign.) - Increased visceral malformations (stat. sign.) - Increased minor skeletal anomalies and variants (stat. sign.) Teratogenic effect still observed with co-administration of folinic acid. No correlation with methionine synthase activity	Mazze et al., 1988 Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
halothane - 20 exposed to folinic acid + N_2O - only 5 of each of these groups were killed for the assay. Limitations - Few details on maternal toxicity (survival, food consumption) - Only one dose reported (results at 50% and 75% dinitrogen oxide were pooled) - No information if experimenters were blind to treatment - no justification of differences in the number of animals per groups. - unclear why only 5 out of 20 exposed animals for methionine synthase activity were selected/ - Controls may not have been exposed to air in inhalation chamber as the exposed animals (unclear in the paper)			
Non-guideline developmental toxicity study in rats GLP: no Sprague-Dawley rats N= 40 in controls and 30 in treated groups Sacrifice: GD 20 *Limitations* - single dose tested - single day of exposure	Dinitrogen oxide (medical grade) Inhalation, whole body 24h on GD-8 0, 500,000 ppm N_2O mixed in oxygen and air	<u>Maternal toxicity</u> No mortality Mild sedation Decreased body weight gain on GD 12 and 20 compared to controls (stat. sign.). No significant effect on GD 6, 8, 9, 14, 16. <u>Developmental toxicity</u> Statistically significant effects: increased early and late resorption, increased foetal wastage (dead and resorbed) and increase major visceral malformations (right side aortic arch, in 5/26 litters)	Fujinaga et al., 1987 Klimisch 2 WOE
Non-guideline developmental toxicity study in rats GLP: no Sprague-Dawley rats N= 34-40 in controls and 24-30 in treated groups Sacrifice: GD 20	Dinitrogen oxide (medical grade) Inhalation, whole body 24h on GD-8 0, 350,000, 500,000 ppm N_2O mixed in O_2 and air	<u>Maternal toxicity</u> - Mild sedation of dams. - Significant decreased body weight gain on GD 6-21 compared to controls (135g in controls compared to 106 g in exposed group) and to mean body weight at caesarean section compared to controls (321g vs 351g in controls). Mean weight at caesarean section and body weight gain was not affected during	Mazze et al., 1987 Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Limitations - Two dose levels - single day of exposure		the experiment at 350,000 ppm. <u>Developmental toxicity</u> At 500,000 ppm: - Increased foetal resorptions and post-implantation losses (stat. sign.) - Increased minor and major visceral abnormalities (stat. sign.) - Increased minor skeletal anomalies (stat. sign.). No effects at 350,000 ppm.	
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=2-10 per groups Limitations - Single dose tested - No information on age or weight of animals at reception - No explanation on the differences on the number of animals between groups - unclear number of animals per dose groups - Very low number of exposed dams per groups (e.g. only 3 dams exposed at GD 11-15) - No information on survival - Visceral examination not performed -limited skeletal examination	Dinitrogen oxide (purity not specified) Inhalation, whole body, 24h on GD10-14 or 15-19 0, 750,000 ppm dinitrogen oxide mixed with oxygen	<u>Maternal toxicity</u> - Statistically significant decrease in body weight following exposure compared to controls (376 g in control vs 323 g in GD 10-14 dinitrogen oxide group and 339g in control vs 273g in dinitrogen oxide GD15-19 group) <u>Developmental toxicity</u> - Statistically significant decreased in foetal weight - No effects on litter size or resorptions	Tassinari et al., 1986 (Sub-study results: continuous exposure) Klimisch 3 Disregarded
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats Sacrifice: GD 20 N= 10 exposed and 23 controls *Limitations:* - Low number of exposed rats per dose groups - No information on source of test material	Dinitrogen oxide (purity not specified) Inhalation, whole body exposure 24h on GD8 0, 700,000-750,000 ppm dinitrogen oxide	<u>Maternal toxicity</u> No information. <u>Developmental toxicity</u> - No effects on resorptions, live foetuses and number of implants. - Statistically significant decrease in foetal and placental weight. - ↑ Statistically significant increase in delayed development in dinitrogen oxide group (decreased mean number of sternebrae and caudal vertebrae). - Stat. sign. increase in skeletal malformations (e.g. cervical vertebral malformations) - Increased methyl folate concentration in	Keeling et al., 1986 Klimisch 2, WOE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
- Visceral examination not performed - No information if experimenters were blind to treatment - No information on maternal toxicity - Only one dose level - No information on room temperature and humidity		dinitrogen oxide exposed group.	
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats Sacrifice: GD 20 N=25-62/exposed group (pooled results) and 160 controls (pooled results) *Limitations:* - no detailed on maternal toxicity - no detailed on the results for individual experiments (pooled results)	Dinitrogen oxide (medical grade) Inhalation, whole body 24h on GD-8 Experiment I: 0, 750,000 ppm dinitrogen oxide Experiment II: 7500, 75000, 750000 ppm Experiment III (mated in house): 0, 750,000 ppm Experiment IV: 0, 250,000, dinitrogen oxide mixed in oxygen and air (food and water deprivation or food and water *ad libidum*)	<u>Maternal toxicity</u> No effects up to 250,000 ppm At 750,000 ppm: impaired food and water consumption, rats were drowsy, impaired motor coordination (no information on statistical significance) <u>Developmental toxicity</u> (750,000 ppm) - Increased in any external abnormalities, runts and major external malformations (stat. sign.) - Increased in any skeletal abnormalities, major malformations and malformations in rib/vertebra, increased in variant (extra lumbar rib, cervical rib) (stat. sign.) - Increased in any major internal malformations (stat. sign.) - increased ocular malformation (reported in the text, no tabular information) - Increased in total, early and late resorptions (stat. sign.) - Decreased number of live foetuses per dams (stat. sign.) At 75,000 ppm: stat. sign. Increase in minor skeletal anomalies only (extra lumbar ribs and cervical ribs)	Mazze et al., 1984 Klimisch 2 WOE
Non-guideline prenatal developmental toxicity studies Rats N=10 per group Limits: no information on statistical significance, secondary literature	Dinitrogen oxide (no information on purity) Inhalation, Whole-body exposure 24h/day GD1-21 0, 50,000, 150,000, 200,000 ppm dinitrogen oxide	<u>Maternal toxicity:</u> no information <u>Developmental toxicity</u> - Increased resorption rate, - decreased litter size, foetal body length and body weight at ≥150,000 ppm - Increased wavy ribs, fused, additional or absent ribs, separation of vertebral ossification centres at 200,000 ppm.	Rao et al., 1981, Tong et al., 1982 Klimisch 4 (as reported in MAK, 2015) WOE
Non-guideline developmental toxicity study in rats GLP: no Sprague-Dawley rats	Dinitrogen oxide (no information on purity) Inhalation, whole body	Maternal toxicity No information Developmental toxicity - Increased foetal losses (4-fold compared to	Lane et al., 1980 Klimisch 4

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Sacrifice: GD20 N=8/group Limitations: secondary literature (abstract), few information available)	24h on GD8 700,000 to 750,000 ppm dinitrogen oxide	control) - Increased anomalies (encephalocele, hydrocephalus, anophthalmia, microphthalmia, gastroschisis and gonadal agenesis)	WOE
Non-guideline developmental toxicity study in rats GLP: no Wistar rats N=12/group *Limitations:* - Low number of animals per groups - Size of the chamber not stated - No specification of organs examined (internal or skeletal examination - No details on abnormalities provided (no tabulated data) - Few information on maternal toxicity (e.g. no information on weight, clinical signs, behaviour)	Dinitrogen oxide (purity not specified) Inhalation, whole body, 23h/d, GD1-19 Control (air), 250, 500 and 1000 ppm	Maternal toxicity No effects on food or water consumption. Developmental toxicity - Decreased litter size at 1000 ppm (stat. sign.) - Increased resorptions at 1000 ppm (stat. sign.) - Decreased crown-rump length at 1000 ppm, no effect on foetal body weight (stat. sign.) - Skeletal abnormalities at 1000 ppm (stat. sign.) (malformation of the vertebrae column)	Vieira et al., 1980 Klimisch 2 WOE
Non-guideline developmental toxicity study in rats GLP: no Wistar rats N=12/group Limitations - Low number of animals - Only one dose group - Few information on environmental conditions - Few internal organs and skeletal examinations - No information on maternal toxicity - No details on skeletal abnormalities (incidences in each groups, details of the anomalies)	Dinitrogen oxide (purity not specified) Inhalation, whole body, 23h/d, GD1-19 Control (air), 5000 ppm	Maternal toxicity No reported Developmental toxicity - Statistically significant decrease in litter size 4/12 dams had full resorptions (vs 0 in controls) - Statistically significant increase in skeletal malformations (ribs). Foetuses with malformations were smaller that their litter mates or controls. - Marked statistically significant reduction in mean crown-rump length of exposed foetuses. - Statistically significant decrease in mean foetuses weight.	Vieira et al., 1979 Klimisch 2, WOE
Non-guideline developmental toxicity study in rats GLP: no Rats N=5-10 per groups Limitations	Dinitrogen oxide (purity not specified) Inhalation, whole-body, 24h/day on GD8-13 or 12-19 0, 1000, 15,000 ppm N_2O	Maternal toxicity: No information Developmental toxicity: Increased foetal death compared to controls.	Corbett et al., 1973 (Sub-study results on continuous exposure only)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
- strain, age or weight of rats at the beginning of the study not specified. - Actual dose levels lower than target dose due to leakage in the homemade chambers - low number of animals per groups - lack of methodological details - no information on maternal toxicity and health status of dams - foetal death was the only parameter investigated in the study			Klimisch score 3 Disregarded

Table 14: Summary table of animal studies on adverse effects on development in rats in prenatal developmental toxicity studies, <u>intermittent exposure studies</u> (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N= 19-50/group Limitations: - Single dose level	Dinitrogen oxide (medical grade) Inhalation, 6h/d 0; 750,000 ppm N_2O in oxygen and air GD13-15, GD10-12 or GD7-9	Maternal toxicity: ↓ in body weight gain (statistically significant in dams exposed on GD8-10, by 20%) Developmental toxicity: - ↓ Foetal weight (GD 13-15) - ↑ Resorptions and foetal wastage (dead and resorbed) in dams exposed during GD13-15 window - ↑ Major malformations and external abnormalities in dams exposed on GD 7-9	Mazze et al., 1986 Klimisch 2 WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=2-10 per groups Limitations - Single dose tested - No information on age or weight of animals at reception - No explanation on the differences on the number of animals between groups - Very low number of exposed dams per groups (e.g. only 3 dams exposed at GD 10-14) - No information on survival - Visceral examination not performed -limited skeletal examination - unclear number of animal per dose group	Dinitrogen oxide (no information on purity) Inhalation, 8h/d on GD8-12, GD10-14, GD13-14 or GD14 0; 750,000 ppm N_2O	Maternal toxicity: no effects Developmental toxicity: no effects on the number of foetuses, resorptions, foetal weight.	Tassinari et al., 1986 (Sub-study results: prenatal intermittent exposure) Klimisch 3 Disregarded
Non-guideline prenatal developmental toxicity study in rats GLP: no Female Wistar rats N=12/group Limitations - Low number of animals per groups - Size of the inhalation chamber not specified - No information on maternal toxicity - Potential effects of high latitude (1700m) and	Dinitrogen oxide (purity not specified) Inhalation, 6h/d, 5d/w, whole gestation period (3 weeks) 0; 250; 500; 1,000; 5,000 ppm dinitrogen oxide in air	Maternal toxicity: no information Developmental toxicity: - Dose-related ↓ in litter size (statistically significant at 5000 ppm) - no effect on foetal weight or crown-rump length of foetuses. - no malformations reported.	Vieira et al. 1983b Klimisch 2 WOE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
pressure is unknown - few details on study method and results			
Non-guideline prenatal developmental toxicity study Sprague-Dawley rats N= 10 per groups Limitations: few information available based on secondary literature	Dinitrogen oxide (no information on purity) Inhalation, whole body 8h per day, 5d/w, GD1-21 0, 200,000 ppm dinitrogen oxide	Maternal toxicity: no information Developmental toxicity: no skeletal findings unlike after continuous exposure	Rao et al., 1981; Tong et al., 1982 Klimisch 4 (as reported in MAK, 2015) WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Rats N=30/group Limitations: few information available based on secondary literature	Dinitrogen oxide (no information on purity) Inhalation, 6-7h/d, whole gestation 0; 1,000 ppm dinitrogen oxide in air	No evidence of maternal toxicity or developmental toxicity	Hardin et al., 1981 Klimisch 4 WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=8-10 per groups *Limitations* - Low number of animals per treatment group - Animals were exposed simultaneously in the chambers - no information on how animals were sacrified - results for maternal liver and kidney weight not provided, representative maternal tissues fixed for microscopic examination not specified - Detailed results of skeletal examination not provided. - no analysis of visceral abnormalities - unknown if the 5-6 foetuses selected for skeletal examination was randomly done	Dinitrogen oxide (purity not specified) Inhalation, 8h/d, whole gestation (GD0-20) 0; 10,000; 100,000; 500,000 ppm dinitrogen oxide in air, additional stress group as control	Maternal toxicity: no effect on body weight or on food consumption Developmental toxicity: - delayed development (foetal weight, crown-rump length, delayed 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.	Pope et al., 1978 Klimisch 2 WOE
Non-guideline developmental toxicity	Dinitrogen oxide (purity not specified)	Maternal toxicity: No information	Corbett et al.,

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
study in rats GLP: no Rats N=5-10 per groups Limitations - strain, age or weight of rats at the beginning of the study not specified. - Actual dose levels lower than target dose due to leakage in the homemade chambers - low number of animals per groups - lack of methodological details - no information on maternal toxicity and health status of dams - foetal death was the only parameter investigated in the study	Inhalation, whole-body, 8h/day on GD8-13, 14-19 or 10-19 0, 1000, 15,000 ppm N_2O mixed in oxygen and balance with nitrogen	Developmental toxicity: Significant increase in foetal death rate in rats exposed to 8 hours to dinitrogen oxide from 6am to 2 pm (group 5 and 7) at 1000 ppm. No increase was observed in group 9 exposed from 2pm to 10pm to a longer period (GD10-19).	1973 Klimisch 3 Disregarded

Table 15: Summary table of animal studies on adverse effects on development in rats in pre-postnatal developmental and postnatal (neuro)developmental toxicity studies (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Non-guideline behavioral teratogenicity and dominant lethal study Sprague-Dawley rats 12 rats/group Limitations - Low number of animals per groups - limited examinations	Dinitrogen oxide (checked for purity) Inhalation, whole body 0, 1000, 5000, 10,000 ppm 6h/d, 5d/w for 9 weeks in exposed male (paternal study) and GD1-20 in exposed females (maternal study)	<u>Maternal or paternal toxicity</u> No effect on weight reported <u>Maternal and paternal behavioral studies</u>: No differences in litter size and weight of offsprings. No treatment-related effects on behavior in offsprings	Holson et al., 1995 (substudy : postnatal development) Klimisch 2 WOE
Non-guideline prenatal neurodevelopmental toxicity study GLP: no Sprague-Dawley rats N=15 or 5 per groups Limitations - No information on age or weight of animals - only one dose level - Low number of animals for neurobehavioral tests (GD13-14 exposure) - No information if experimenters were blind to treatment	Dinitrogen oxide (purity not specified) Inhalation, whole body 8h on GD14 or 8h on GD13-14 0, 750,000 ppm dinitrogen oxide in oxygen	<u>Maternal toxicity</u> No effects on weight of dams during exposure No information on survival. No effects in offspring dams <u>Neurodevelopmental toxicity</u> *Residential maze activity* - Exposure on GD 14 only: no significant changes in males and females at 1-month age. At 5-month age: significant hypoactivity in females and significant diurnal hyperactivity in males. - Exposure on GD13-14: significant exploratory and diurnal hyperactivity in the females at 1 and 5-months timepoint and in males at 1-month age. No effects in males at 5-month of age. *Time-lapse photography* - Exposure on GD 14 only: no effects in males at 1 or 5-months exposure. In females, slight tendency to hypoactivity at 5-month. - Exposure on GD13-14: In males, no effect at 1-month. At 5-month, changes in body position, ↓ frequency of standing and increased in the average duration of rearing. Change on face washing behavior compared to control. In females, evidence of hyperactivity found at 1-month and 5-months (increase rearing and walking frequencies, shorter duration of standing and sitting. Worst at 5-month)	Mullenix et al., 1986 Klimisch 2 WOE
Non-guideline prenatal developmental toxicity study in rats GLP: no Sprague-Dawley rats N=2-10 per groups *Limitations* - single dose levels - No information on age or weight of animals at reception - No explanation on the	Dinitrogen oxide (purity not specified Inhalation, whole body 8h, GD13-14 Sacrifice: PND16, 18 or 21 0, 750,000 ppm in oxygen	<u>Maternal toxicity</u> No information <u>Early postnatal developmental toxicity</u> - No effect on weight of pups. - No significant effects on auditory startle and on eye opening. - Decreased suspension reflex in female only.	Tassinari et al., 1986 (Substudy results: postnatal development) Klimisch 3 Disregarded

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
differences on the number of animals between groups. Unclear number of animals per groups - No information on survival - Visceral examination not performed -limited skeletal examination - Unclear number of dams per group			
Non-guideline pre/postnatal developmental toxicity study in rats GLP: no Wistar rats N=8/group *Limitations* - only one dose tested - Low number of animals/groups - No information on maternal toxicity - poor reporting of study methods and results - few parameters investigated - No information if dinitrogen oxide concentration was checked during exposure	Dinitrogen oxide (purity not specified) Inhalation, 6h/d, 5d/w 3 control groups, N2O 1% groups: Group 1: whole gestation, Group 2: weeks 1 and 2 of gestation Group 3: 1st week of gestation	<u>Maternal toxicity:</u> No information <u>Developmental toxicity</u> Stat. sig. decreased in litter size in the dinitrogen oxide exposure groups Stat. sig. decrease in body and tail length, body weight in dinitrogen oxide exposed groups (pooled results)	Vieira et al., 1978 Klimisch score 3 Disregarded (missing information on study methods)

Table 16: Summary table of adverse effects on development induced by dinitrogen oxide in other species than rats in prenatal developmental toxicity studies (plug day = day 0 of pregnancy)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Non-guideline Pre-/postnatal developmental toxicity study GLP: no information SW mice 10 litters examined per groups Limitations: - low duration of exposure (4h/day), - low number of animals in the neurobehavioral studies - low level of details	Dinitrogen oxide (no information on purity) Inhalation, GD6-15, control, 50,000, 150,000 and 300,000 ppm in air.	Maternal toxicity: no information Developmental toxicity: - No effect on reproductive indices, survival, physical milestones of development - No change in post-natal brain weight (PND126 or 127) - No effect on rotating rod - Significant hyporeactivity of the startle reflex in response to acoustic or tactile stimuli (PND 95) in all dinitrogen oxide group compared to control	Rice et al., 1990 Klimisch score 2 WOE
Non-guideline pre/post natal developmental toxicity study DUB/ICR mice N=5-6 /group Analysis: physical landmark and behavioral measures Limitations: few information available based on secondary literature	Dinitrogen oxide (no information on purity) Inhalation, GD14, 6h/d in dams, 750,000 ppm Litters PN2 exposed 4h/d	Maternal toxicity: After exposure on GD 14 females resumed activity within a few minutes. Developmental toxicity - No effect on pup body weight; except for an increased pup weight on PN 2 - Ear unfolding retarded after pre- and postnatal exposure air and surface righting were retarded during test period after pre- and postnatal exposure - Locomotion was affected after pre- and postnatal exposure - Total activity was affected after postnatal exposure	Koëter et al., 1986 Klimisch 4 (As reported in HCNL, 2000) WOE
Prenatal developmental toxicity study Guideline: similar to OECD 414 GLP: no Swiss/ICR Mice N=24 to 32 females/group *Limitations* -Exposure only 4h/day	Dinitrogen oxide (purity not specified) Inhalation, GD 6-15, 4h/day Control (air treatment), control (colony) 5000, 50,000, 500,000 ppm Positive control: retinoic acid (gavage), n=11 dams	Maternal toxicity Dams appeared not adversely affected by treatments. No Excitement or general anaesthesia observed. No effects among groups on maternal weight gain. Developmental toxicity No effects on litter size, foetal wastage (dead and resorbed), foetal size. No treatment related abnormalities (external, skeletal, visceral).	Mazze et al., 1982 Klimisch score 2 WOE
Non-guideline prenatal developmental toxicity study GLP: no Golden Sirian Hamster N=5/group Limitations:	Dinitrogen oxide (no information on purity) Inhalation, whole-body, 24h GD 7, 8, 9, 10 or 11 0, 700,000, 800,000, 900,000, 950,000 ppm	Maternal toxicity: no information Developmental toxicity: - Increased number of malformations, not statistically significant, no dose-relation - No effect observed at 700,000 ppm. - Increased number of resorption on GD7, 10 and 11 at ≥900,000 ppm, which is hypoxic.	Shah et al., 1979 Klimisch score 4 Disregarded

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
- few information as secondary literature, based on abstract only, - 800,000 ppm is above the hypoxic concentration of dinitrogen oxide, - only 5 animals per groups			

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

Experimental data in rats: prenatal developmental toxicity studies, continuous exposure

- ***Mazze et al., 1984, 1987, 1988 and Fujinaga et al., 1987, 1989, 1990, 1991***

In a series of seven studies from the same laboratory, Sprague-Dawley rats were exposed 24 hours via whole body inhalation to dinitrogen oxide between 7500 to 750,000 ppm on specific days of gestation (Mazze et al., 1984, 1987, 1988, Fujinaga et al., 1987, 1989, 1990, 1991). Administration was done as follow (considering plug day = day 0 of gestation):

- Single administration on day 8 of gestation using single dose level at either 500,000, 600,000 or 700,000-750,000 ppm dinitrogen oxide (Fujinaga et al., 1987, 1990 and 1991),
- Single administration on day 8 of gestation using several dose levels in Mazze et al. (1984, 1987, 1988) to investigate dose-response: from 7,500 to 750,000 ppm dinitrogen oxide.
- Exposure 24h to 600,000 ppm 24h either at GD 6, 7, 8, 9, 10, 11 or12 (Fujinaga et al., 1989).

A similar protocol was used in the seven studies. On the chosen day or days of pregnancy, dinitrogen oxide was administered to the rats. They were placed in the chambers in their cages without food or water. Medical grade dinitrogen oxide and oxygen were delivered to the chambers and were mixed with room air to achieve the desired dinitrogen oxide and oxygen concentrations.
On day 20 of pregnancy, 1 day before delivery was expected, rats were sacrificed by carbon dioxide inhalation and Caesarean sections were performed except in Fujinaga et al., 1990 where rats were randomly killed each days 11-16, 18 and 20 of gestation. The uterus was examined and the number and position of live and dead foetuses, resorptions and implantations were recorded. The weight and sex of each live foetus were determined and each foetus was examined for external abnormalities. Half of the foetuses were examined for skeletal examination and half of the foetuses for visceral examination. Foetal morphological abnormalities that altered general body conformation, disrupted or interfered with vital functions, or generally were incompatible with life were categorized as major malformations. Abnormalities in anatomical structure that were considered to have no significant biological effects on the rats' health or on their body conformity and represented only slight deviations from normal were categorized as developmental variants. Abnormalities which were not within the strict definition of major malformations, but which clearly were not developmental variants, were categorized as minor anomalies.

In order to exclude some potential bias, Mazze et al. (1984) included additional groups to investigate if starvation, shipping dams while pregnant and restraining the dam during exposure could be a potential cause of effect.
In addition, in order to investigate the potential mode of action, other chemical substances were used in some of these studies either alone or co-administered with dinitrogen oxide: phenoxybenzamine, isoflurane,

fentanyl, halothane, enflurane, folinic acid. Summaries of the author's hypothesis are included in the mode of action section below.

- *Developmental toxicity*

Developmental toxicity was consistently observed in the studies, including increased early and late resorptions, with a resulting decrease in the number of live foetuses per litter, skeletal abnormalities and malformation, visceral abnormalities and malformations.

In the six studies investigating the effect of dinitrogen oxide following 24-hour exposure on GD-8, a dose-related increase in early and late resorptions was observed. The effect was statistically significant at ≥500,000 ppm. The authors of the studies identified 350,000 ppm as a NOAEC for this effect.

Table 17: Resorptions observed in controls following 24-hour air exposure on gestation day 8 and sacrifice at GD 20 (Mazze et al., 1984, 1987, 1988, Fujinaga et al., 1987, 1989, 1991)

	Control[1]	**Control[2]**	**Control[3]**	**Control[4]**	**Control[5]**	**Control[6]**
Dams	160	85	237	67	23	28
Foetuses	1705	954	433	694	261	337
% total resorptions per dams	5.5±12.1	0.59±1.2	4.9±10	7.4±19	3.4	5.0±5.6
% early resorptions per dams	5.2±11.7	na	4.9±10	7.3±19	na	na
% late resorption in dams	0.3±2.3	na	0	0.1±0.8	na	na

*p<0.05 respective to the respective control of the study; na: information not available ;[1] Mazze et al., 1984; [2] Mazze et al., 1987; [3]Fujinaga et al., 1987; [4] Mazze et al., 1988;[5] Fujinaga et al., 1989; [6]Fujinaga et al., 1991

Table 18: Resorptions observed following 24-hour dinitrogen oxide exposure on gestation day 8 and sacrifice at GD 20 (Mazze et al., 1984, 1987, 1988, Fujinaga et al., 1987, 1989, 1991)

Dinitrogen oxide (ppm)	**7500[1]**	**75,000[1]**	**250,000[1]**	**350,000[2]**	**500,000[2]**	**500,000[3]**	**500,000[5]**	**600,000[6]**	**500,000-750,000[4]**	**750,00**
Dams	27	25	49	41	41	26	36	22	46	75
Foetuses	285	293	572	479	306	241	301	150	301	437
% total resorptions per dams	9.2±16	9.3±10	4.4±10	1.5±2.2	10±8.5*	25±31*	37*	48±32*	35±231	39±3
% early resorptions per dams	9.2±16	8.9±10	3.8±10	na	na	18±20*	na	na	35±31*	37±3
% late resorption in dams	0	0.3±1.5	0.6±2.3	na	na	6.8±12*	na	na	8.8±14*	2.5±7

*p<0.05 respective to the respective control of the study; na: information not available; [1] Mazze et al., 1984; [2] Mazze et al., 1987; [3]Fujinaga et al., 1987; [4] Mazze et al., 1988;[5] Fujinaga et al., 1989; [6]Fujinaga et al., 1991

Fujinaga et al., (1989) identified two critical period of exposure for resorptions, one on day 8 of gestation and one after exposure on gestational day 11. Fujinaga et al., (1990) found that in the dams exposed on gestational day 8 during 24 hours at 700,000-750,000 ppm dinitrogen oxide, the increase in resorptions was first observed in the group sacrified on gestational day 14 (not in animals scarified at GD 11, 12 or 13) and then the rate remained constant (in animals sacrified on each days 15, 16, 18 and 20 of gestation).

As detailed in the table below, a statistically significant marked increase in skeletal minor anomalies and malformations, visceral minor anomalies and malformations were noted following continuous exposure on GD8 at ≥ 500,000 ppm dinitrogen oxide.

Table 19: Summary of the statistically significant effects observed in the studies following dinitrogen oxide exposure on GD8 during 24h

Dinitrogen oxide (ppm)	**7500[1]**	**75,000[1]**	**250,000[1]**	**350,000[2]**	**500,000[2]**	**500,000 [3]**	**500,000[5]**	**600,000[6]**	**500,000-750,000[4]**	**750,000[1]**
No. rats examined	27	25	49	41	39	26	13	22	40	62
No. Foetuses examined	285	293	572	479	306	123	100	150	301	437
Visceral major malformations	-	-	-	-	+	+	+	+	+	+
Visceral minor anomalies	-	-	-	-	+	-	+	+	-	-
Skeletal major malformations	-	-	-	-	-	+	-[$]	-	+	+
Skeletal minor anomalies	-	-	-	-	+	+	+	+	+	+
External abnormalities	-	-	-	-	-	-	-	-	-	+

*p<0.05 respective to the respective control of the study; na: information not available; [1] Mazze et al., 1984; [2] Mazze et al., 1987; [3]Fujinaga et al., 1987; [4] Mazze et al., 1988;[5] Fujinaga et al., 1989; [6]Fujinaga et al., 1991; $: increased following exposure to dinitrogen oxide on GD9

Regarding the malformations, some of the studies reported details on the type of malformations observed (See annex I for details on incidences):

- Cardiac anomalies (Fujinaga et al. 1991), specified as “right sided aortic arch” in (Fujinaga et al., 1991, Mazze et al., 1988, Fujinaga et al., 1987),
- Situs inversus (Fujinaga et al., 1991),
- Ocular malformations (Mazze et la., 1984),
- Hydrocephalus (Fujinaga et al., 1991, 1989),
- Ribs and vertebrae (Fujinaga et al., 1989),
- Limb deformities (Mazze et al., 1984).

Alteration of body laterality was specifically investigated in Fujinaga et al. (1990) (Table 7 of Annex I), and was significantly altered compared to controls (side of tail flexion, side of body from which the umbilical artery emerged, side of body that face placenta, side to which the aortic arch curved).

The main minor visceral anomalies cited was left sided umbilical artery (Fujinaga et al, 1991, 1989)
In addition, skeletal anomalies such as cervical ribs and 14th rudimentary rib were also increased (Fujinaga et al., 1991, 1989, Mazze, 1988).

- *Maternal toxicity*

Maternal toxicity was not described in all of the studies but was detailed in some of them. Except in one study (Fujinaga et al., 1989), no increased death or significant morbidity was noted in rats exposed continuously to dinitrogen oxide on GD 8 or other specific day of gestation up to 750,000 ppm.

In Fujinaga et al. (1991), no statistically significant decrease in body weight was noted in dams exposed 24h on GD8 to 600,000 ppm dinitrogen oxide.

Table 20: Summary of maternal weight noted in Fujinaga et al. (1991)

	Control	600,000 ppm dinitrogen oxide
No. rat studied/ examined on day 20	30/30	25/24
Mean bw of rats before exposure (g)	249	248
Mean bw after exposure (g)	226	217
Mean bw on GD 20	368	334

In Fujinaga et al. (1989), in addition to the deaths observed, the mean body weight was decreased in the exposed group (600,000 ppm) compared to controls. The decrease was observed even in the absence of increase in mean % of resorptions per litters. To be noted that the cause of deaths was unknown to the authors.

Table 21: Summary of a selection of maternal variables and % of resorption (Fujinaga et al.; 1989)

Gestation day	Control	6	7	8	9	10	11	12
No rats that died during exposure	0	2	2	2	2	0	4	3
Mean body weight of pregnant rats (g)	352	309*	318*	321*	330**	322*	296*	313*
Mean % resorption per litter	3.4	18.9	5.5	36.8*	11.6	16.5	37.3*	20.1

In Mazze et al. (1988), a decrease in body weight was noted in rats exposed to 500,000 ppm or 750,000 ppm dinitrogen oxide. It is stated by the authors that almost all the difference could be explained by the fewer number of live foetuses that the dams carried. Corrected maternal body weight was however not provided.

Table 22: Body weight changes and body weight in dams (Mazze et al., 1988).

Dinitrogen oxide (ppm)	Control	500,000 or 750,000 ppm
Weight (g), GD4	205	206
Weight (g), GD20	362	332*

In Mazze et al. (1987), dams exposed to dinitrogen oxide appeared only mildly sedated at 350,000 ppm and 500,000 ppm. Decreased body weight gain was significant compared to control in the 500,000 ppm dinitrogen oxide group, but not at 350,000 ppm. No information on corrected maternal body weight was provided in the published study. Thus, it is not possible to exclude that the body weight effects may have been related, at least partly, to the decreased litter size observed at the top dose (7.7 vs 11.2 in controls).

Table 23: Body weight changes and body weight in dams (Mazze et al., 1987).

Dinitrogen oxide (ppm)	Control	350,000	500,000
Weight (g), GD5	213	212	212
Weight (g), GD20	351	357	321*
Weight gain GD6-21	135	139	106*
Weight loss during the 24h exposure	23	24	29*

*p<0.05

In Fujinaga et al. (1987), at 500,000 ppm, a statistically significant decrease in body weight of dams compared to control was noted on GD12 of pregnancy (241g±12 vs 254g ±17) and GD 20 (328g±24 vs 348g±32 in controls). No significant effect were noted on weight on GD9 GD14 and GD16 after exposure compared to control.

Overall, decrease body weight compared to control was noted in all studies at ≥ 500,000 ppm. Part of this effect may have been due to embryo-foetal lethality. Nevertheless, in none of the studies corrected body weight was available.

- ***Keeling et al. (1986)***

Keeling et al. (1986), exposed rats for 24 hours on GD8 (considering vaginal plug = day 0 of pregnancy) to 700,000-750,000 ppm dinitrogen oxide mixed in oxygen. No increase in resorptions was noted in the study. Foetal weight and placental weight was decreased in a statistically significant manner compared to control. In addition, a statistical significant increase in skeletal malformations (mainly cervical vertebrae) and delayed development was observed in the dinitrogen oxide exposed group.

- ***Rao et al. (1981)***

In Rao et al. (1981), dams were continuously exposed during GD1 to 21 to dinitrogen oxide. The following findings were noted at 150,000 ppm: decreased litter size, foetal body length and foetal body weight. In addition, at 200,000 ppm, an increase in the number of skeletal findings was noted (wavy ribs, fused, additional or absent rib, separation of vertebral ossification centres). No information on maternal toxicity was available.

- ***Vieira et al. (1979 and 1980)***

Vieria et al. (1979 and 1980) exposed Wistar female rats to continuous inhalation exposure 23h/d from GD1 to 19. Doses levels were 250, 500 and 1000 ppm in 1980 and 5000 ppm in the 1979 study. No effect on food or water consumption was noted up to 1000 ppm. At 5000 ppm, maternal toxicity was not specified. Increased resorptions and decreased litter size was noted at ≥ 1000 ppm (table 26 and 27 of Annex I). Skeletal malformations were noted at ≥ 1000 ppm (ribs, vertebrae). In addition, crown-rump length was also decreased at 1000 ppm onward.

Table 24: Litter size, crown-rump measurements and foetal resorption in Vieira et al. (1980).

	Number of litters	**Number of foetuses**	**Litter size (mean+/-SD)**	**Crow-rump measurements (mm, mean+/-SD)**	**Resorptions**
Control	12	120	11±1.4	44 ±1.4	None
Dinitrogen oxide, 1000 ppm	12	66	6.3±4**	35±1.6*	4**
Dinitrogen oxide, 500 ppm	12	118	11±1.4	43±1.3	None
Dinitrogen oxide, 250 ppm	12	120	11±1.3	43±1.4	None

**p<0.01, *p<0.05

Table 25: Foetal information (Vieira et al., 1979)

Maternal rat number												
Dinitrogen oxide group												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

Liver foetuses	7	9			13			11	11	10	5	11
Foetal weight (g), mean ± SD	1.4 ±0.2	1.3 ±0.2			1.8 ±0.0			1.3 ±0.3	1.5 ±0.2	1.7 ±0.1	1.8 ±0.2	2.1 ±0.1
Resorption sites (g) mean ± SD	4	1	11	12	2	10	12		1			
Crown-rump length (mm), mean ± SD	29± 0.2	28± 0.2			30± 0.2			29± 0.2	28± 0.2	28± 0.2	28± 0.2	29± 0.2
Live foetuses with abnormalities		1			2			1	1	2		2
No. of live foetuses without abnormality	7	8			11			10	10	8	5	11
Controls												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Liver foetuses	12	12	10	10	10	13	12	12	9	13	12	10
Foetal weight (g) mean ± SD	2.0± 0.1	3.5± 0.2	3.1± 0.0	3.3± 0.6	3.1± 0.2	3.2± 0.3	2.6± 0.3	2.0± 0.1	2.0± 0.1	2.0± 0.2	2.3± 0.2	2.3± 0.2
Crown-rump length (mm), mean ± SD	44± 0.2	44± 0.2	44± 0.2	43± 0.2	42± 0.2	42± 0.2	44± 0.2	44± 0.2	42± 0.2	43± 0.2	44± 0.2	44± 0.2

- ***Lane et al. (1980)***

Exposure of pregnant rats to dinitrogen oxide on GD8 of gestation (day of plug=day0 of gestation) to dinitrogen oxide at 700,000/750,000 ppm causes fetal resorption, skeletal anomalies, and macroscopic lesions including encephalocele, anophthalmia, microphthalmia, and gastroschisis.

Experimental data in rats: prenatal developmental toxicity studies, 4 to 8h per day intermittent exposure

- ***Mazze et al. (1986)***

Using a similar test design as the prenatal developmental Mazze toxicity studies described above, Mazze et al. (1986) exposed Sprague-Dawley rats for intermittent 750,000 ppm dinitrogen oxide exposure, during 6h per day, during critical period of gestation: GD 13-15, 10-12 and 7-9. A statistically significant increase in resorptions was noted in rats exposed during GD 13-15 (1.32 per litter compared to 0.46 in controls). This was associated with a decrease in live foetuses per implantation. No statistically significant increase in malformation was noted in the study. Nevertheless, an increase in external abnormalities and malformations was noted in dams exposed on GD 7-9 and skeletal malformation in dams exposed on GD 13-15 was observed in dinitrogen 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 dinitrogen oxide compared to controls on GD 13-15 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 (see table below) in the exposed group. Animals were conscious throughout the experiments in all groups.

Table 26: Maternal body weight gain and developmental toxicity in Mazze et al. (1986)

	Period	**Control**	**Dinitrogen oxide (750,000 ppm)**
Dam weight gain (g)	I	84.9	68.5*
	II	95.1	90.9
	III	113	104.2

Foetal weight (g)	I	4.58	4.13*
	II	4.52	4.46
	III	4.49	4.29
Total foetal wastage[1]	I	0.49	1.37*
	II	0.63	0.59
	III	0.62	0.32
Resorptions (no./dam)	I	0.46	1.32*
	II	0.63	0.59
	III	0.56	0.24
Major external malformations	I	0.0±0.0	0.0±0.0
	II	0.0±0.0	0.0±0.0
	III	0.0±0.0	3.2±16
Major skeletal malformations	I	0.0±0.0	1.1±4.6
	II	0.0±0.0	0.0±0.0
	III	0.0±0.0	0.0±0.0

*p<0.05, I: GD13-15, Period II: 10-12, Period III: exposure days 7-9 (plug day = day 0 of pregnancy); [1] number/dam; dead + resorbed

- ***Pope et al. (1978)***

Sprague-Dawley rats were exposed 8 hour per days during the whole gestation period to 10,000, 100,000 or 500,000 ppm dinitrogen oxide in air. 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 and was also associated with a significant delay in ossification. The authors did not observe a dose-related effects on resorption and dead foetuses after dinitrogen oxide exposure.

Table 27: Litter sizes, percentage of foetal loss induced by dinitrogen oxide

	No. of pregnant rats	**Live/litter (SE)**	**% Foetal loss (SE)**
Control	8	14.3 (0.7)	0
Dinitrogen oxide, 10,000 ppm	7	12.7 (1.4)	8.2 (3.3)
Control	8	11.3 (1.3)	1.1 (0.8)
Dinitrogen oxide, 100,000 ppm	7	14.3 (0.6)	8.3 (3.7)
Control	10	13.7 (0.6)	8.1 (2.5)
Dinitrogen oxide, 500,000 ppm	10	12 (0.9)	10.4 (2.2)
Stress group	4	1.5(1.5)	91 (8.8)

Table 28: Foetal and placental weight (Pope et al., 1978)

	Foetal weight (g)	**Placental weight (g)**
Control	5.45 (0.04)	0.59 (0.01)
Dinitrogen oxide, 10,000 ppm	5.31 (0.07)	0.51 (0.01)**
Control	5.0 (0.05)	0.45 (0.01)
Dinitrogen oxide, 100,000 ppm	4.22 (0.05)**	0.42 (0.01)*
Control	5.51 (0.04)	0.47 (0.01)
Dinitrogen oxide, 500,000 ppm	4.35 (0.07)**	0.43 (0.01)*
Stress group	3.25 (0.19)	0.31 (0.02)

*p<0.05, **p<0.01

No maternal toxicity was reported as any effects on maternal body weight and food consumption was noted in the study. Only gross skeletal abnormalities were examined and no visceral examination was performed in the study.

- ***Vieira et al. (1983b)***

Decreased litter size was noted in the Vieira et al. (1983b). In this study rats were exposed 6h/d, 5d/w during the 3 weeks of the gestation period at 250, 500, 1000 or 5,000 ppm. A LOAEC based on decreased litter size was identified at 5000 ppm by the authors. The NOAEC was 1000 ppm in the study. No foetal delayed development was noted. The toxicological significance of the decrease litter size in the absence of concomitant findings (e.g. resorptions) is questionable. There was no information on maternal toxicity in this study.

Table 29: 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

- ***Rao et al. (1981)***

In contrast with the effects observed after continuous exposure, teratogenic effects, decreased litter size, foetal body length and foetal body weight were not observed after intermittent exposure to 200,000 ppm dinitrogen oxide during the whole gestation period (8h/d, 5d/w).

- ***Hardin et al. (1981)***

No evidence of developmental toxicity was noted in the study of Hardin et al., 1981 up to 1000 ppm dinitrogen oxide, 6-7h/day during the whole gestation period.

Experimental data in rats: pre-/postnatal developmental toxicity studies

In Holson et al., 1995, males were exposed for 9 weeks, 6h/d, 5d/w to dinitrogen oxide at 0, 1000, 5000 or 10000 ppm. At the end of the exposure period, male were mated with non-exposed females. In addition, females were exposed in similar condition during the whole gestation period. No effect on litter size and weight or behaviour of pups was noted in the study. No effect were reported on offsprings from female exposed to dinitrogen oxide during the whole gestation period for 6h/d, 5d/w or from males exposed for 9 weeks. Although some positive results were obtained in some test, 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., 1986 exposed rats on GD 14 or GD 13-14 for 8h/d to 750,000 ppm dinitrogen oxide mixed in oxygen. Exposure on GD13-14 produced hyperactivity in both males and females. In contrast, exposure on GD14 only produced a tendency to hypoactivity in females and hyperactivity in males.

Experimental data in other species

Behavioural effects in offspring of Swiss mice were studied following exposure by inhalation to 0, 5, 15 or 35% N_2O for 4 h/day on days 6 through 15 of gestation (Rice et al., 1990). Exposures did not affect reproduction indices and survival or physical milestones of development. Body weights showed significant exposure effects that could be isolated to specific exposure groups; however, N_2O-exposed mice tended to weight more than control animals. On postnatal days 126 or 127 no effect on brain weights were observed. Ability to stay on a rotarod was not affected by prenatal N_2O exposure. Prenatal exposure to N_2O resulted in hypo-reactivity of the startle reflex on PN95 for all N_2O-exposed groups. Maternal toxicity was not described in this study.

Koëter et al. (1986) studied the effect of 750,000 ppm dinitrogen oxide by inhalation in mice. They reported delayed development, retarded surface air and air righting and total activity were affected by exposure.

No developmental effects (resorptions, litter size, malformations) were noted in mice in the published study of Mazze et al.(1982) up to 500,000 ppm dinitrogen oxide. In this study the mice were exposed 4h/d on GD6-15.

Human data:

A number of studies have been published which examined the developmental toxicity (including teratogenicity) of dinitrogen oxide in humans.

Abortion

Six studies were identified a spontaneous abortion risk specifically related to N_2O exposure. In all the studies, exposure concentrations were poorly defined and no measurement was available. Therefore, these studies are only described briefly for information.

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 Uzun et al., 2014 and in Eftimova et al., missing information on exposure and potential cofounding decreased the reliability of the study. No adjustment for potential risk factors was done in these studies.

No increased risk was observed in midwives in a retrospective cohort study of 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 7599 pregnancies, which began before 1989, were reported by 2786 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 1717 pregnancies during which the women worked as a midwife. Exposure was based on three categories (no use, use of N_2O in up to 500,000 ppm 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. (1995) performed a retrospective cohort study in female dental assistants exposed at least 3 hours per week to unscavenged N_2O. In this study, 7000 dental assistants were selected and 1805 of the respondents had been pregnant at least once and 1465 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 alcohol use or paternal occupation were taken into account. As the analysis was limited to women working full time. According to the authors potential bias for unhealthy worker effect cannot be excluded in the study. Indeed, the authors commented that in the United States, population of 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. 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. An increased 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.

In a retrospective study published by Heidam et al., 1984, a cohort of 772 dental assistants, having being working in dental clinics or in dental school services, were included in the study (1431 employees used as control group). The data were collected via a questionnaire and covered the women's entire reproductive life before May 1980. Dinitrogen oxide effect on abortion was noted in the study. Nevertheless, exposure was not characterised and there were insufficient information on potential other risk factor (e.g. smoking). Potential co-exposure to inorganic mercury was taken into account and odd ratio for specific substance was calculated. A negative information bias was identified by the authors.

In an older study from Cohen et al. (1980) a statistically significant increase in spontaneous abortion (around 2-fold increase) was also observed in dental assistant exposed to dinitrogen oxide. In the 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 dinitrogen oxide 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 8h 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 20th 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, 2001; MAK, 1993). Nevertheless, in these studies, N_2O was not specifically investigated and thus, these studies are not further described.

Developmental abnormalities

In the retrospective cohort study from Teschke et al. (2011), 56,213 female nurses registered for at least one year between 1974 and 2000 were included in Canada. 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 dinitrogen oxide used as control. In this study, halothane, enflurane, isoflurane and dinitrogen oxide were frequently used. Measurements in hospital reported values generally below LOD by the hospitals except dinitrogen oxide exposure that was sometimes reported to be above 25 ppm. Exposure probability was 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 dinitrogen oxide in the calendar year of their first trimester of pregnancy. An increased risk of congenital anomalies was noted in this study for dinitrogen oxide (OR: 1.82 (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 dinitrogen oxide 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 8h exposure per week, considering cumulative exposure 1-2999 hours in the past ten years. Heavy exposure was defined as exposure above 8h per day and cumulative exposure above 3000h 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., 1999, new-borns from midwives exposed to dinitrogen oxide 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). In this study exposure was characterised based on the use of dinitrogen oxide (<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 (number of time the women has given birth: 1, 2, 3, 4 or ≥ 5), the employment and work schedule. Potential co-exposures to other substances were not addressed in the publication.

Mode of action hypothesis

Dinitrogen oxide probably acts by directly inhibiting vitamin B12 formation and methionine synthase. Methionine synthase inhibition leads to the impairment of the generation of methyl groups for DNA methylation. According to Tserga et al., 2017, disruption on genomic imprinting leads to biallelic expression which may affect disease susceptibility. For example, epigenetic control of some genes may be influenced by maternal plasma folate.

In the review published by Imbard et al., 2013, the authors summarised that neural tube defects (NTDs) have complex and multifactorial etiologies in which both genetic, life style and environmental factors appear to be involved. In addition to genetic factors, environmental influences such as parental occupation, maternal obesity, and maternal nutritional status have been related to NTDs. Particularly, the authors pointed out that it has been suggested more than 40 years ago that maternal folate status is associated with neural tube disease risk. A substantial number of reviews have been published on neural tube diseases and folic acid. Over the years, more and more studies suggested that not only folate but whole methylation metabolism could be involved in the etiology of NTDs. Neural tube disease such as hydrocephaly has been reported by some authors. Guéant et al., 2013 reviewed that early vitamin B12 and folate deprivation during gestation and lactation in rats was associated with long-lasting disabilities of behaviour and memory capacities, with persisting hallmarks related to increased apoptosis, impaired neurogenesis and altered plasticity.

Nevertheless, as demonstrated by Mazze et al., 1988, this mechanism may not be the sole factor of dinitrogen oxide teratogenicity as folinic acid supplementation did not fully prevent the teratogenic potential of the substance. The authors also hypothesised that dinitrogen oxide developmental toxicity may be related to the increased adrenergic tones induced by dinitrogen oxide. They showed that the combination with alpha adrenergic blocking agent reduce the incidence of some of the malformations, presumably by restoring the uterine blood flow to normal. The authors further noted that the reason that toxic reproductive effects in human are not conclusive may be related to the multifactorial etiology of the reproductive effects. Fujinaga et al., 1991, also suggested that uterine blood flow disturbance may account for some of the reproductive toxicity of the substance.
Although biochemical changes occur in rats and in humans after short dinitrogen oxide exposures, a prolonged
period of decreased uterine blood flow may be necessary to produce foetotoxicity (i.e., 24 hours in a 21-day rat gestation Mazze et al., 1988).

The exact mechanism by which dinitrogen oxide act as a teratogen is still not fully understood.

10.10.6 Comparison with the CLP criteria

In human, some studies indicated that dinitrogen oxide may induce congenital abnormalities or reduction of birth weight following high exposure (no measurements available) to dinitrogen oxide 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 dinitrogen oxide developmental toxicity. Therefore, the results of the human data may be very difficult to interpret in case of co-exposure and potential bias. Regarding abortion, inconsistent findings were noted in human. Overall, dinitrogen oxide does not fulfill the criteria for category 1A.

In animals, developmental findings were observed in numerous available developmental toxicity studies in rats, mice or hamsters. The main findings were the increase in resorptions and in malformations. In addition, delayed development and decreased foetal weight was also noted in several studies.

Dinitrogen oxide caused embryotoxicity and teratogenicity in rats exposed during GD8 to 10 of gestation after single 24h administration. A NOAEC for both effect was identified at 350,000 ppm dinitrogen oxide (Mazze et al., 1987). Following continuous exposure during the whole gestation period, a NOAEC for malformations was identified at 500 ppm in Vieira et al., 1980 (LOAEC = 1000 ppm). Maternal toxicity was not described in all the studies. Reported maternal toxicity at up to 750,000 ppm include mostly decreased body weight gain and mild sedation, not indicative of excessive toxicity. There is no data on corrected weight of dams to better characterised true maternal toxicity.

Following intermittent exposure to dinitrogen oxide (4 to 8h/day exposure during critical windows or whole gestation period), embryo-foetotoxicity (decrease foetal weight, resorption or dose related decrease in litter size) was observed. No statistically significant increase in malformations were noted except in one study were an increase in major malformations and external abnormalities was observed (without statistical significance) at 750,000 ppm (Mazze et al., 1986).

Neurobehavioral studies in pups exposed during gestation (Rice et al., 1990, Mullenix et la., 1986, Koëter et al., 1986) provide some evidence on potential effect on reactivity in pups.

As teratogenicity occurred at high concentration, it is questionable if the effects are related to the substance itself or are secondary to the anaesthetised state. In Pope et al., 1978, the authors noted that foetal delayed development was not related to a specific anaesthetic agent in the study as the effect was also observed with halothane or methoxyflurane. Therefore, the authors suggested that the effect may have been due to a general effect of the anaesthetic on the mother and foetuses rather than a specific toxic effect on the foetus. Contradictory to these results, in Lane et al., 1980, rats were exposed to xenon and 750,000 ppm dinitrogen oxide for 24 hours on GD 9. Foetal resorption, delayed maturation and anomalies to the skeletal systems were only observed with dinitrogen oxide and not xenon, suggesting that the observed effects are related to the substance itself rather than its anaesthetic mechanism. In Mazze et al., 1986, the authors also did not demonstrated teratogenic findings in rats exposed to intermittent exposure to anaesthetics such as isoflurane, enflurane or halothane. By contrast an increase in foetal loss was observed following exposure to dinitrogen oxide. Overall, the teratogenic effects observed with dinitrogen oxide is considered as an intrinsic property of the substance.

Although a statistically significant increase in malformations was only noted following continuous exposure to dinitrogen oxide, a trend toward an increase in malformations was noted at least in one study following intermittent exposure (Mazze et al., 1986). Other relevant findings were nevertheless observed in these studies: resorption and dead in utero or dose related decrease in litter size (Vieira et al., 1983b). The lack of sensitivity in the intermittent studies (e.g. low number of dams) may explain the absence of effect or statistical significance.

Regarding maternal toxicity, few information are available in studies. When there are, effects are generally limited to reduced body weight or body weight gain, sedation, sometimes impaired food and water consumption. Some deaths were observed in only one study. Moreover, concerning the decrease in body weight, it can be due to the decrease size of the litter. The CLP guidance (2017) indicates that "*Adverse effects on fertility and reproductive performance seen only at dose levels causing marked systemic toxicity (e.g. lethality, dramatic reduction in absolute body weight, coma) are not relevant for classification purposes*" And "*Developmental effects which occur even in the presence of maternal toxicity are considered to be evidence of developmental toxicity, unless it can be unequivocally demonstrated on a case-by-case basis that the developmental effects are secondary to maternal toxicity*". Therefore, the few data available cannot discount the effect observed on offsprings. In addition, toxicity expected at high dose levels (e.g. neurotoxicity, immunotoxicity), not investigated or reported in the developmental toxicity studies, may not explain the observed malformation as shown by Fujinaga et al., 1979. Although the same dose level was used, malformations and resorptions were only observed following exposure during the critical window of exposure (GD 8, 9 or 11).

Overall, based on the clear embryo-lethality observed following dinitrogen oxide exposure, not secondary to unspecific maternal toxicity, dinitrogen oxide warrants to be classify as Repr. 1B, H360D for developmental toxicity.

10.10.7 Adverse effects on or via lactation

Endpoint not assessed (lack of data).

10.10.8 Conclusion on classification and labelling for reproductive toxicity

Classification for reproductive toxicity addresses adverse effects on sexual function and fertility, developmental effects. Based on the clear embryofoetotoxicity of dinitrogen oxide a classification in category 1B is considered appropriate. Based on the observed effect on fertility in studies having limitations, a classification in category 2 for fertility is considered appropriate.

Dinitrogen oxide warrant to be classified as **Repr. 1B, H360fD.**

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The DS proposed classification as Repr. 1B (H360Df) for N_2O.

Regarding **adverse effects on sexual function and fertility**, the DS considered that:

- None of the animal studies were performed in compliance with OECD TG. All animal studies had limitations, e.g., single concentration level, low number of animals, few parameters investigated, and were either performed on male or female animals.
- Decreased fertility and oestrous cycle changes observed consistently in female rats in two studies (Kugel *et al.*, 1990: 1989) leads to a clear concern for female fertility.
- The effects observed on testis and spermatogenesis in a rat study (Kripke *et al*., 1976) and the decrease in litter size upon exposure of male animals in another rat study (Vieira *et al.*, 1983a) support concern for potential male fertility effects.
- Adverse effects were only observed in rats and not in mice. The DS had no explanation for the absence of effects in mice compared to rats, leading to some uncertainties. However, the DS noted that fertility and oestrus cycle were not investigated in female mice. Therefore, the DS considered it not possible to disregard the effect observed in rats based on the negative results observed in mice.
- Although fertility effects were seen in the two human studies (Ahlborg *et al*., 1996; Rowland *et al.*, 1992), limited characterisation of N_2O exposure levels and co-exposure impaired a firm conclusion on the observed effects in humans.

Overall, the DS considered Category 2 for adverse effects on sexual function and fertility as more appropriate than Category 1B due to the limited number of parameters investigated in the animal studies and the absence of effects in mice.

Whether an SCL or generic concentration limit (GCL) should be applied to the data, was not specifically discussed by the DS.

Regarding the mode of action, the DS hypothesised that the effect of N_2O on fertility may be related to disturbance of folate metabolism via inactivation of methionine synthase induced by N_2O. However, the DS considered that data investigating this are not available.

Regarding **adverse effects on development**, the DS considered the following:

- Developmental findings were observed in numerous available developmental toxicity studies in rats, mice or hamsters. The main findings were the increase in resorptions and in malformations, and further, delayed development and decreased foetal weight.
- N_2O caused embryotoxicity and teratogenicity in rats during gestation day (GD)8 to GD10 after single 24h exposure with a NOAEC for both effects of 350000 ppm (Mazze *et al.*, 1987). Following continuous exposure during the whole gestation period, a NOAEC for malformations of 500 ppm was identified (Vieira *et al.*, 1980). Following intermittent exposure to N_2O (4-8h/d exposure during critical window or whole gestation period), embryofoetal toxicity (decrease foetal weight, resorption or dose related decrease in litter size) was observed. No significant increase in malformations upon intermittent exposure was noted, though in one study an increase in major malformations and external malformations were observed (without statistical significance) at 750000 ppm (Mazze *et al.*, 1986).
- Maternal toxicity was not described in all the studies. Reported maternal toxicity at up to 750000 ppm included mostly decreased body weight gain and mild sedation, not indicative of excessive toxicity.
- The teratogenic effects in animals were considered not to be secondary to maternal toxicity including the mild sedation.
- Inconsistent findings were noted regarding abortion in humans. Although some human studies indicated that N_2O may induce congenital abnormalities or reduced birth weight, interpretation of the human data is difficult due to co-exposure and bias.

Overall, the DS proposed classification in Category 1B given the clear embryo-lethality observed in animals following N_2O exposure, which was considered not to be secondary to unspecific maternal toxicity.

Whether an SCL or GCL should be applied to the data, was not specifically discussed by the DS.

Regarding the mode of action, the DS hypothesised that the effect of N_2O on teratogenicity may be related to inhibition of vitamin B12 formation and methionine synthase and involvement of folate. However, the DS considered that the exact mechanism by which N_2O acts as a teratogen is still not fully understood.

Adverse effects on or via lactation was not assessed by the DS due to lack of data.

Comments received during consultation

A total of ten comments were received, with one comment from an individual, six comments from industry representatives and three comments from MSCAs.

Two MSCAs supported the proposed overall classification as Repr. 1B (H360Df). A third MSCA

supported the proposed Category 2 for adverse effects on sexual function and fertility. With regards to adverse effects on development this MSCA indicated to have a slight preference for Category 2 rather than Category 1B, given the omission of data on maternal toxicity in some of the available studies. This issue was also mentioned by one of the other two MSCA (though supporting Category 1B).

The individual considered the wording in section 4 of the CLH report ('Justification that action is needed at community level') to be misleading as they considered it untrue that there is evidence of reproductive harm in humans.

All industry representatives disagreed with the proposed classification, both for adverse effects on sexual function and fertility and for adverse effects on development. Their comments focussed on:

- Quality of the animal studies, i.e., studies being non-guideline/non-GLP, low number of animals and lack of statistical analysis, single concentration group (so no assessment of concentration-response relationship), lack of details in study methodology, few parameters included.
- (Ir)relevance of high concentration levels.
    - Concentration levels being above a limit concentration.
    - Considering the role of hypoxia.
- Lack of information on general toxicity or maternal toxicity in the animal studies.
- Adverse effects being the consequence of the anaesthetised state.
- Quality of the human data, i.e., limited quantitative characterisation of N_2O exposure, lack of information on co-exposures and confounding factors.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

Table: *Summary of the available data on* ***female rats*** *for adverse effects on sexual function and fertility*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
FEMALE RAT			
Study examining effects on brain (1), ovulatory cycle (2) and fertility (3) Non-guideline; GLP not stated; Klimisch 2 Kugel et al., 1990 Limitations: No information on general toxicity in	Sprague-Dawley rat Experiment 1: 8 females/exposure group, divided as 4/group in proestrus and 4/group in metestrus Experiment 2: 12 females/exposure group** Experiment 3: 12	N_2O (purity not stated) 0; 300000 ppm N_2O in (compressed) air* Inhalation, whole body 8h/d for 4 days or one ovulatory cycle	<u>General toxicity</u> No information provided. <u>Reproductive toxicity</u> Experiment 1 (brain): increase in LHRH (luteinising hormone-releasing hormone) cell counts in hypothalamus in animals exposed on proestrus. No effects in animals exposed in metestrus. Experiment 2 (ovulatory cycle): disrupted cycles following the first day of

dams; Only one concentration tested; Low number of animals used in each group; No statistical analysis performed	females/exposure group, divided as 6 animals/group in proestrus and 6 animals/group in random stage of ovulatory cycle. Mated with non-exposed males.		exposure and 11 out of 12 exposed rats went into constant proestrus. Effects resolved within 3 weeks. Normal cycle in controls. Experiment 3 (fertility): mating occurred in all animals. Only 6 out of 12 treated animals (3 in group treated in proestrus and 3 in random phase of the cycle) gave birth vs. all animals in control. No effects on litter size and weight***

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

** based on the original publication

*** in the original publications this is literally presented as "No significant difference was noted in litter size and weight (between exposed and control animals)." RAC assumes that this refers to those animals that gave birth.

Table: *Summary of the available data on* ***male rats*** *for adverse effects on sexual function and fertility.*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
MALE RAT			
Dominant lethal test & paternal study (including behavioural examination) Non-guideline; GLP not stated; Klimisch 2 Holson et al., 1995 (sub-study: fertility) Limitations: Few parameters investigated in the study; No information on survival or clinical signs in the sires or dams in the study	Sprague-Dawley rat 12 (paternal study) and 24 (dominant lethal test) males per exposure group Mated with non-exposed females (dominant lethal test and paternal study)	N_2O (purity checked though not stated) 0, 1000; 5000; 10000 ppm N_2O in air* Inhalation, whole body 6h/d, 5d/week for 9 weeks	<u>General toxicity</u> No effect on bw <u>Reproductive toxicity</u> Dominant lethal test: no effect on conception rate, total number of implants/litter or live foetuses/litter. Concentration-related trend (not stat. sign.) to an inc. in the number of resorptions at the highest concentration. Paternal study: no effect on litter size, no behavioural effect on offspring. Slight tendency (not stat. sign.) towards fewer pups per litter in the N_2O-exposed groups.
Fertility study Non-guideline; non-GLP; Klimisch 2 Vieira et al., 1983a Main limitations: Single concentration; Exposure period too short to cover	Wistar rat 12 males/exposure group Mated with non-exposed females, directly upon exposure and after a 6-month recovery period.	N_2O (purity not stated) 0; 5000 ppm N_2O in air* Inhalation, whole body 6h/d, 5d/week for 30 days	<u>General toxicity</u> No information provided. <u>Reproductive toxicity</u> Dec. in litter size and developmental delay in offspring (i.e., bw, tail length and body length) upon mating directly at the end of exposure period. No effect on offspring upon mating after a 6-month recovery period.

whole spermatogenic cycle; Few parameters examined; No information on general toxicity			
Study focusing on testis toxicity Non-guideline; non-GLP; Klimisch 2 Kripke et al., 1976 Limitations: Low number of animals per group; Only one concentration level; Too short exposure period (<10 week necessary to cover the whole spermatogenic cycle); No detailed results (incidence, grade of the lesions); Although several organs were sampled, only findings in testes were published.	LEW/f Mai rat 4-6 males/exposure group	N_2O (purity not stated) 0; 200000 ppm N_2O in air* Inhalation, whole body Experiment 1: 8h/d for 1 to 35 days Experiment 2: 24h/d for 32 days; sacrifice after 3, 6 or 10 days recovery	General toxicity No information provided. Reproductive toxicity Dec. absolute testis weight (experiment 1 and 2). Effect reversed after 6d recovery (experiment 2). Dec. in the number of spermatogenic cells with some disorganisation of the normal architecture (experiment 1). Damage and destruction of spermatogenic cells with increasing severity and frequency with exposure duration (experiment 2). No effect in Leydig cells and supporting cells within the tubules, no effect on serum testosterone levels.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant; bw = body weight

Table: *Summary of the available data on* ***mice*** *for adverse effects on sexual function and fertility*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
MOUSE			
Subchronic inhalation toxicity study Non-guideline, non-GLP; Klimisch 2 Rice et al., 1985 Limitations: Few organs and parameters examined; Short exposure duration (4h/d)	Swiss Webster mouse 15/sex/exposure group	N_2O (purity not stated) 0; 5000; 50000; 500000 ppm N_2O in air* Inhalation, whole body 4h/d, 5d/week for 14 weeks	General toxicity All animals survived. Dec. bw gain (M: 77%, F: 63%). Reproductive toxicity No histopathological changes in testes and ovaries.

Study focusing on germ cell toxicity Non-guideline, non-GLP; Klimisch 2 Mazze et al., 1983 Limitations: Short exposure duration (4h/d); No information on the source of test material; Low number of animals per group for oocyte examination (n=6); Only few organs and parameters examined; Low level of information on general toxicity; No information if experimenters were blind to treatment	Swiss Webster mouse 15/sex/exposure group	N_2O (medical grade; purity not stated) 0; 5000; 50000; 500000 ppm N_2O in air* Inhalation, whole body 4h/d, 5d/week for 14 weeks Positive controls used for male germ cell toxicity (methyl methanesulfonate) and oocyte count (methylchloranthrene)	General toxicity Normal behaviour. No excitement or general anaesthesia. Reproductive toxicity No effect on testis weight, percentage abnormal sperm, sperm count or histopathological appearance of testis. Number of oocytes unaffected. Positive response obtained for positive controls.
Fertility study Non-guideline, non-GLP; Klimisch 2 Mazze et al., 1982 Limitations: Few reproductive parameters investigated; No information on general toxicity	Swiss/ICR mouse 18-21 males/exposure group Mated with non-exposed females	N_2O (medical grade; purity not stated) 0; 5000; 50000; 500000 ppm N_2O in air* Inhalation, whole body 4h/d, 5d/week for 9 weeks	General toxicity No information Reproductive toxicity No effect on abilities of males to impregnate females. No effect on litter size, foetal wastage (resorption, death), foetal growth.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

bw = body weight

***Table**: Summary of the available human data for adverse effects on sexual function and fertility*

Study, reference	**Characteristics**	**Results**
Retrospective cohort study Ahlborg et al., 1996 Limitations: No information on co-exposure; Quantitative characterisation of N_2O exposure limited	Questionnaire sent to 3985 women midwifes born in 1940 and thereafter; 84% responded (n=3358). N_2O exposure based on average number of deliveries per month at which the midwife assisted where N_2O was used, and the type of work and work schedule. Detailed information on number of menstrual cycles required to achieve pregnancy and working conditions during that period were obtained concerning the most recent planned pregnancy. Probability of becoming pregnant (per cycle) calculated.	No effect of N_2O exposure on fecundability noted, except for the small group of midwifes that assisted >30 deliveries with N_2O per month showing a reduced fecundability ratio (0.64; 95% CI: 0.44-0.95)

	Fecundability ratio (as relation to the unexposed) determined.	
Retrospective cohort study Rowland et al., 1992 Limitations: No information on co-exposure; Quantitative characterisation of N_2O exposure limited	Screening questionnaires sent to 7000 women dental assistants aged 18 to 39; questionnaire completed by 4856. 459 women met criteria set by study authors and were followed up by detailed telephone interviews, resulting in a total of 418 women. N_2O exposure based on number of hours of exposure per weeks in room where N_2O was being used and the presence or absence of scavenging systems. Fertility measured by the number of menstrual cycles without contraception that the women required to become pregnant.	No relation between scavenged N_2O exposure and fertility. Reduced fertility only noticed in women with >5 h per week exposure to unscavenged N_2O.

The available human data (Ahlborg *et al.*, 1996; Rowland *et al.*, 1992) do not provide clear evidence for classification on its own, given the limitations regarding characterisation of the N_2O exposure and co-exposure. Therefore, classification in Category 1A is not appropriate.

All animal studies included inhalation exposure using whole body application. Studies with Klimisch score 2 are used in the RAC assessment and included in the table above. RAC notes that studies with Klimisch score 3 or 4 were included in the CLH report (table 11). The available animal studies were not performed in compliance with OECD TG and GLP and have limitations such as application of a single (high) concentration level, small animal group size and limited number of parameters investigated. Furthermore, either male or female animals were exposed. Nevertheless, RAC considers that in case observations are noted in studies with limited parameters or applying a single exposure concentration, they can in a weight of evidence approach, still point towards serious substance-specific adverse effects relevant for classification.

Evidence for an adverse effect on sexual function and fertility is primarily obtained from rat studies. Regarding female fertility, the results of the rat study by Kugel *et al.* (1990) point towards decreased fertility. Following inhalation exposure to 300000 ppm N_2O 8h/d for 4 days or one ovulatory cycle, only half of the exposed females (6/12) gave birth after mating with non-exposed males compared to 12/12 in controls. In addition, disrupted oestrus cycles immediately following day 1 of exposure were observed with 11/12 exposed female rats being into constant proestrus. Though the adverse effects on oestrus cyclicity resolved, it required 3 weeks for normalisation.

Regarding male fertility, results of the rat studies of Kripke *et al.* (1976) and Vieira *et al.* (1983a) provide supportive evidence. Inhalation exposure of male rats to 200000 ppm N_2O in air, being intermittent for 8h/d for 1-35 days or continuous for a total of 32 days, resulted in reduced absolute testis weight (data on relative testis weight not presented). Also, damage and destruction of the spermatogenic cells was shown, with both incidence and severity being more pronounced in continuously exposed rats compared to intermittently exposed rats (Kripke *et al.*, 1976). It is noted that the exposure duration is too short to cover the full period of spermatogenesis; nevertheless effects on some reproductive parameters were observed.

In the rat study of Vieira *et al.* (1983a), a statistically significant reduction in litter size was seen after inhalation exposure of male animals to 5000 ppm N_2O 6h/d, 5 d/week for 30 days

and subsequent mating with non-exposed females. This effect on litter size was not observed after a 6-month recovery of the exposed males followed by mating with non-exposed females. Nevertheless, the reduced litter size itself is considered an irreversible effect. In the control group one litter comprised of nine offspring while the remaining 35 litters included 11-15 offspring (mean number of offspring per litter: 12). A similar pattern was observed in the recovery group with one litter with eight offspring and the remaining 35 litters ranging from 10-14 offspring (mean number of offspring per litter: 11). In the group mated directly upon exposure to N_2O one litter comprised of 14 offspring but the remaining 35 litters ranged between two and six offspring (mean number of offspring per litter: 7 ($p<0.001$)). In addition, a significant decrease in body weight, tail length and body length were noticed in offspring of this latter group compared to control. Such effects were not observed in the recovery group.

In the rat study of Holson *et al.* (1995), no effect on conception rate, total number of implants/litter or live foetuses/litter (dominant lethal test), no effect on litter size and no behavioural effect on offspring (paternal test) was observed upon inhalation exposure of male animals up to 10000 ppm N_2O 6h/d, 5/week for 9 weeks and subsequent mating with non-exposed females. A concentration-related trend to an increase in the number of resorptions at the highest concentration (dominant lethal test) and a slight tendency towards fewer pups per litter in the N_2O-exposed groups (paternal test) were noted, though these trends were not statistically significant.

RAC notes an apparent inconsistency between the rat studies that investigated male fertility. Kripke *et al.* (1976) applied a high exposure concentration and observed clear adverse effects on spermatogenesis, though they did not include mating in the study design. While in the study of Vieira *et al.* (1983a) a significantly reduced litter size was observed upon mating of exposed males with non-exposed females, this was not the case for the study of Holson *et al.* (1995) despite that in this latter study a 2× higher exposure concentration and 1.5× longer exposure duration was applied when compared to the study of Veira *et al.* (1983a). It is noted that different rat strains were used in both studies, i.e., Wistar in Vieira *et al.* (1983a) and Sprague-Dawley in Holson *et al.* (1995), which might explain the observed difference.

The CLP guidance states that '*Adverse effects on fertility and reproductive performance seen only at dose levels causing marked systemic toxicity (e.g. lethality, dramatic reduction in absolute body weight, coma) are not relevant for classification purposes.*' Information on general toxicity was not provided for the rat studies that demonstrated adverse effects on sexual function and fertility. Concentration levels pointing towards adverse effects on sexual function and fertility in rats were up to 300000 ppm. However, adverse effects on fertility were also observed after repeated exposure of rats to lower concentrations (e.g., 5000 ppm in Vieira *et al.*, 1983a). In the developmental toxicity studies (see below), maternal toxicity did not occur <350000 ppm for 24h in rats (Mazze *et al.*, 1984; 1987). Maternal toxicity was limited, when exposed up to 600000 N_2O ppm for 24h, to reduced body weight and mild sedation. However, at 600000 ppm lethality was observed. In the repeated dose studies (see section STOT RE), serious general toxicity was not reported between 400000 and 700000 ppm in rats (Hayden *et al.*, 1974; Singh *et al.*, 2015; Mistra *et al.*, 2020; Dyck *et al.*, 1980). Lower concentration levels were not tested. Overall, RAC agrees with the DS that the presence of marked systemic toxicity can reasonably be excluded.

The studies performed in mice do not provide evidence of an adverse effect on sexual function or fertility. Upon repeated inhalation exposure up to 500000 ppm, 4h/d, 5d/week for 9-14 weeks, no adverse effect on testes, spermatogenesis and fertility were noted in male mice nor was an adverse effect on ovaries and oocytes noticed in female mice (Rice *et al.*, 1985; Mazze

et al., 1982; Mazze *et al.*, 1983). As already noted by the DS, fertility and oestrus cyclicity were not investigated in female mice.

In summary, adverse effects on sexual function and fertility were noted in studies where rats were exposed by inhalation to N_2O. However, the available studies are not OECD TG- or GLP-compliant and included only limited parameters. Upon exposure of female animals, serious effects included oestrus cycle disturbances and a marked decreased (50%) fertility upon exposure to 300000 ppm N_2O, 8h/d for 4 days (Kugel *et al.*, 1990). Upon exposure of male animals, adverse effects on spermatogenesis were noted at 200000 ppm N_2O, being intermittent for 8h/d for 1-35 days or continuous for a total of 32 days (Kripke *et al*., 1976). In addition, marked reduced litter size was observed upon 5000 ppm N_2O 6h/d, 5d/week for 30 days and subsequent mating to non-exposed females (Vieira et al., 1983a). RAC further noted an inconsistency as in the third study investigating male rat fertility and applying higher exposure concentration and longer exposure duration no such significant effects were noted (Holson *et al*., 1995). This could be due to strain differences or other effects and introduces an uncertainty.

RAC considers the evidence for adverse effect on fertility and sexual function to be stronger for female than for male rats. Further, RAC considers that the negative findings in the mouse studies do not negate the positive findings in rat. The diverse findings in these two species might point towards species differences in sensitivity but is considered not to reduce the concern. The CLP guidance does indicate that in the absence of mechanistic information showing otherwise, humans are generally assumed to be the most sensitive species. RAC notes that there is no information available to disregard the positive findings in rat. Further, the absence of adverse effects in mice is not considered to reduce the concern and the human data are considered supportive.

Overall, these data provide evidence for an adverse effect on fertility and sexual function. RAC considers the observed adverse effects on fertility and sexual function relevant for humans and not to be a consequence of other unspecific toxic effects. However, considering that the available data are limited, and the apparent inconsistent findings in rats introduces uncertainties, RAC agrees with the DS and concludes that **classification for adverse effects on sexual function and fertility in Category 2 (Repr. 2; H361f Suspected of damaging fertility) is warranted**.

Given the effective concentration levels, and also taking into account that in most studies a single concentration level was applied (which hampers assessment of concentration-response relationship, derivation of ED_{10} values and establishing the potency), the available data on sexual function and fertility do not support the calculation of an SCL.

Adverse effects on development

Human data

The DS presented a number of studies investigating the developmental toxicity of N_2O in humans.

Several studies investigated the potential risk for spontaneous abortion related to N_2O exposure. However, the interpretation of the results of these studies were hampered by poorly defined exposure concentrations and lack of measurements, and the results being inconsistent. No effect was observed in the two most recent cross-sectional studies, though a high risk of bias was identified, with either no control groups or missing information on exposure and potential confounding factors hampering the reliability of the studies (Eftimova *et al.*, 2017;

Uzun *et al.*, 2014). In the retrospective cohort study of Axelsson *et al.* (1996), no increased risk for abortion was observed in midwives. In contrast, in the retrospective cohort study of Rowland *et al.* (1995) an increased risk of abortion was observed among female dental assistants exposed at least 3 hours per week to unscavenged N_2O, though no clear concentration-response was noticed in this study. Furthermore, Heidam *et al.* (1984) identified an effect of N_2O on abortion among dental assistants, though the characterisation of the exposure was limited. Finally, Cohen *et al.* (1980) observed a significant increase in spontaneous abortions in dental assistants exposed to N_2O versus controls. However, exposure evaluation was based on questionnaires rather than measurements.

Regarding developmental abnormalities, Teschke *et al.* (2011) identified an increased risk of congenital abnormalities in offspring among female nurses exposed to N_2O. Exposure probability was estimated based on employment information retrieved during a telephone survey. Cohen *et al.* (1980) found an increase in congenital anomalies in offspring to dental assistants exposed to N_2O. A 1.5-fold increase in the rate of musculoskeletal defects was observed in the exposed group. However, adjustment for potential other risk factors was limited. A reduced birth weight and an increase in odds of being small for gestational age was noticed in offspring of midwives exposed to N_2O (Bodin *et al.*, 1999). Exposure was not quantified, and co-exposure was not considered in this study.

Overall, the available human data do not provide clear evidence on its own for classification, given the inconsistent findings, the limitations regarding characterisation of the N_2O exposure, co-exposure and other confounding factors.

Animal data

The DS presented multiple animal studies for the endpoint adverse effect on development:

- Rat prenatal developmental toxicity studies with a repeated intermittent inhalation exposure, i.e., 6-8 hours per day during specific parts of/or the whole gestation period (see table below).
- Rat prenatal developmental toxicity studies with a single 24h inhalation exposure during a specific day of the gestation period as well as studies with inhalation exposure of 23-24h/d during multiple days of the gestation period or during the whole period of gestation (see table below).
- Rat studies investigating the pre- and/or postnatal (neuro)development upon intermittent exposure (see Background document for details).
- Prenatal developmental toxicity studies in other species (see Background document for details).

Table**: Detailed summary of the available animal data on N_2O for the endpoint adverse effects on development: **rat prenatal developmental toxicity studies with intermittent exposure

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
Prenatal developmental toxicity study in rats Non-guideline; Non-GLP; Klimisch 2 (DS)/3 (RAC)	Sprague-Dawley rats N=19-50/group	N_2O (medical grade; purity not stated) 0; 750000 ppm N_2O	Maternal toxicity: dec. in body weight gain (stat. sign. in dams exposed on GD13-15). Developmental toxicity:

Mazze et al., 1986 Limitations: Single concentration level		in oxygen and air Inhalation, whole body 6h/d on GD13-15, GD10-12 or GD7-9	- Dec. foetal weight (GD13-15) - Inc. resorptions and foetal wastage (dead and resorbed) in dams exposed during GD13-15 window - Inc. major malformations and external abnormalities in dams exposed on GD7-9 and skeletal malformations in dams exposed during GD13-15 (not stat. sign.).
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Vieira et al., 1983b Limitations: Low number of animals per groups; Size of the inhalation chamber not specified; No information on maternal toxicity; Potential effects of high latitude (1700m) and pressure is unknown; Few details on study method and results	Female Wistar rats N=12/group	N_2O (purity not stated) 0; 250; 500; 1000; 5000 ppm N_2O in air Inhalation, whole body 6h/d, 5d/week, whole gestation period (3 weeks)	Maternal toxicity: No information Developmental toxicity: Dose-related dec. in litter size (stat. sign. at 5000 ppm). No effect on foetal weight or crown-rump length of foetuses. No malformations reported.
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 (DS)/3 (RAC) Pope et al., 1978 Limitations: Low number of animals per treatment group; Animals were exposed simultaneously in the chambers; No information on how animals were sacrificed; Results for maternal liver and kidney weight not provided, representative maternal tissues fixed for microscopic examination not specified; Detailed results of skeletal examination not provided; No analysis of visceral abnormalities; Unknown if the 5-6 foetuses selected for skeletal examination was randomly done	Sprague-Dawley rats N=8-10 per groups	N_2O (purity not stated) 0; 10000; 100000; 500000 ppm N_2O in air, additional stress group as control Inhalation, whole body 8h/d, whole gestation (GD0-20)	Maternal toxicity: no effect on body weight or on food consumption Developmental toxicity: - Delayed development (foetal weight, crown-rump length, delayed ossification), stat. sign. at ≥100000 ppm - Dec. placental weight, stat. sign. at ≥10000 ppm - Inc. foetal loss at the low and mid dose but not stat. sign. and inside spontaneous range of the laboratory. No increase in foetal loss at 500000 ppm.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.

** Plug day=day 0 of gestation.

Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant; bw = body weight

Table: *Summary of the available animal data on N_2O for the endpoint adverse effects on development:* ***rat prenatal developmental toxicity studies with continuous exposure (23-24h/d)***

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels*, Duration of exposure****	**Results**
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Fujinaga et al., 1991 Limitations: Only one concentration level; Single day of exposure; No information if experimenters were blind to treatment and control groups; Few details on maternal toxicity (only bw before and after exposure and on GD20 was provided); No information on clinical signs; It is not specified if the death observed in the treated group was treatment related.	Sprague-Dawley rats N=25 in exposed group and 30 in control group	N_2O (medical grade; purity not stated) 0; 600000 ppm N_2O mixed with O_2 and air Inhalation, whole body 24h exposure at GD8 Sacrifice: GD20	<u>Maternal toxicity</u> Exposed dams appeared mildly sedated and rested quietly during exposure period. No stat. sign. difference in mean bw on GD20 (334 g vs 368 g in controls). Mortality in one out of 25 exposed dams. <u>Developmental effects</u> - Stat. sign. inc. foetal resorptions/litter (48% vs 5% in controls) - Stat. sign. dec. live foetuses/litter (mean 6.5±4.1 vs 12±2.1 in controls) - Stat. sign. dec. mean number of foetuses/litters - Stat. sign. inc. major visceral malformations - Stat. sign. inc. minor visceral anomalies - Stat. sign. inc. minor skeletal anomalies and variants.
Prenatal developmental toxicity study Non-guideline, Non-GLP; Klimisch 2 Fujinaga et al., 1990 Only survival and situs inversus in foetuses examined Limitations: No information on the age and body weight of the animals at the start of the study; Body weight of dams analysed but results were not reported; General toxicity of dam not provided; No information on foetuses weight; Single dose level, single exposure; No information if experimenters were blind to treatment	Sprague-Dawley rats N=35/group	N_2O (medical grade; purity not stated) 0; 700000-750000 ppm N_2O mixed in air and oxygen Inhalation, whole-body 24h exposure at GD8 Sacrifice: 4-6 rats on GD11, 12, 13, 14, 15, 16, 18 or 20 Only survival and situs inversus in foetuses examined	<u>Maternal toxicity</u> No information <u>Developmental toxicity</u> Stat. sign. inc. in embryo-foetal mortality rate on GD14 and onward compared to control. Stat. sign. inc. in altered laterality at all stages of development compared to controls.
Prenatal developmental toxicity study in rat Non-guideline; Non-GLP; Klimisch 2 Fujinaga et al., 1989 Limitations: Low number of litters examined; Lack of details on maternal toxicity (food consumption, clinical signs, weight); Only one concentration level; No	Sprague-Dawley rats 20 females/N_2O exposure group, 30 controls	N_2O (medical grade; purity not stated) 0; 600000 ppm N_2O mixed in air and oxygen Inhalation, whole-body Exposure: 24h at GD6, 7, 8, 9, 10, 11 or 12	<u>Maternal toxicity</u> Mortality in 2 to 4 dams per group except in control and GD10 exposed group. Mild sedation during exposure. Dec. (stat. sig.) mean maternal bw at caesarean section in all treated groups (Table 8 in confidential Annex I, no further information). <u>Developmental toxicity</u> - No effects on the number of implantations, live foetuses, mean

information if experimenters were blind to treatment		Sacrifice: GD20	foetal weight, sex ratio - Inc. % of resorptions per litter on GD8 and 11 (stat. sign.) - Inc. skeletal malformations following exposure on GD9 (stat. sign.) - Inc. skeletal variations following exposure on GD8 (stat. sign.) - Inc. visceral malformations and minor visceral anomalies when dams exposed on GD8 or GD9 (stat. sign.).
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Mazze et al., 1988 Limitations: Few details on maternal toxicity (survival, food consumption); Only one concentration reported (results at 50% and 75% N_2O were pooled); No information if experimenters were blind to treatment; No justification of differences in the number of animals per groups; Unclear why only 5 out of 20 exposed animals for methionine synthase activity were selected; Controls may not have been exposed to air in inhalation chamber as the exposed animals (unclear in the paper)	Sprague-Dawley rats Experiment 1: 90 rats: - 20 animals exposed to N_2O or folinic acid or folinic acid + N_2O - 30 controls (air) Experiment 2: 116 rats - 37 controls (air) - 26 animals exposed to N_2O - 27 animals exposed to folinic acid - 26 animals exposed to folinic acid + N_2O Methionine synthase activity: 65 non-pregnant rats - 5 controls (air) - 20 exposed to N_2O- 20 exposed to N_2O + halothane - 20 exposed to folinic acid + N_2O - only 5 of each of these groups were killed for the assay.	N_2O (medical grade; purity not stated) Experiment 1 and 2: Control; 500000 ppm (experiment 1); 750000 ppm N_2O (experiment 2); folinic acid; 750000 ppm N_2O + folinic acid Methionine synthase activity: Control; 500000 ppm N_2O; 500000 ppm N_2O + halothane; 500000 ppm N_2O + folinic acid Inhalation, whole body, 24h at GD8 Sacrifice at 24, 48 and 72h post-treatment	<u>Maternal toxicity</u> Mild sedation in both experiments at 500000 and 750000 ppm Stat. sign. dec. bw due to lower number of live foetuses <u>Developmental toxicity</u> - No effect on weight of pups - Inc. early and late resorptions (stat. sign.) - Inc. visceral malformations (stat. sign.) - Inc. minor skeletal anomalies and variants (stat. sign.) Teratogenic effect still observed with co-administration of folinic acid. No correlation with methionine synthase activity.
Developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Fujinaga et al., 1987 Limitations: Single concentration tested; Single day of exposure	Sprague-Dawley rats N=40 females in controls and 30 in treated groups Sacrifice: GD20	N_2O (medical grade; purity not stated) 0; 500000 ppm N_2O mixed in oxygen and air Inhalation, whole body 24h on GD8	<u>Maternal toxicity</u> No mortality Mild sedation during exposure Dec. body weight gain on GD12 and 20 compared to controls (stat. sign.). No significant effect on GD6, 8, 9, 14, 16. <u>Developmental toxicity</u> Stat. sign. effects: increased early and late resorption, increased foetal

			wastage (dead and resorbed) and increase major visceral malformations (right side aortic arch, in 5/26 litters).
Developmental toxicity study in rats Non-guideline; Non-GLP; Klimisch 2 Mazze et al., 1987 Limitations: Two concentration levels; Single day of exposure	Sprague-Dawley rats N=34-40 in controls and 24-30 in treated groups	N_2O (medical grade) 0; 350000; 500000 ppm N_2O mixed in O_2 and air Inhalation, whole body 24h on GD8 Sacrifice: GD20	Maternal toxicity - Mild sedation of dams (350000 and 500000 ppm) - Sign. dec. bw gain on GD6-21 compared to controls (135g in controls compared to 106g in exposed group) and to mean body weight at caesarean section compared to controls (321g vs 351g in controls). Mean weight at caesarean section and bw gain was not affected during the experiment at 350000 ppm. Developmental toxicity At 500000 ppm: - Inc. foetal resorptions and post-implantation losses (stat. sign.) - Inc. minor and major visceral abnormalities (stat. sign.) - Inc. minor skeletal anomalies (stat. sign.). No effects at 350000 ppm.
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Keeling et al., 1986 Limitations: Low number of exposed rats per concentration groups; No information on source of test material; Visceral examination not performed; No information if experimenters were blind to treatment; No information on maternal toxicity; Only one concentration level; No information on room temperature and humidity	Sprague-Dawley rats Sacrifice: GD20 N=10 exposed and 23 controls	N_2O (purity not stated) 0; 700000-750000 ppm N_2O Inhalation, whole body exposure 24h on GD8	Maternal toxicity No information. Developmental toxicity - No effects on resorptions, live foetuses and number of implants - Stat. sign. dec. in foetal and placental weight - Stat. sign. inc. in delayed development in N_2O group (decreased mean number of sternebrae and caudal vertebrae) - Stat. sign. inc. in skeletal malformations (e.g., cervical vertebral malformations) - Inc. methyl folate concentration in N_2O exposed group.
Prenatal developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Mazze et al., 1984 Limitations: No detailed information on maternal toxicity; No detailed information on the results for individual experiments (pooled results)	Sprague-Dawley rats Sacrifice: GD20 N=25-62/exposed group (pooled results) and 160 controls (pooled results)	N_2O (medical grade; purity not stated) Experiment I: 0; 750000 ppm N_2O Experiment II: 7500; 75000; 750000 ppm Experiment III (mated in house): 0; 750000 ppm Experiment IV: 0, 250000 ppm N_2O mixed in oxygen	Maternal toxicity No effects up to 250000 ppm At 750000 ppm: impaired food and water consumption, rats were drowsy, impaired motor coordination (no information on statistical significance). Developmental toxicity (750000 ppm) - Inc. in any external abnormalities, runts and major external malformations (stat. sign.) - Inc. in any skeletal abnormalities, major malformations and malformations in rib/vertebra, inc. in variant (extra lumbar rib, cervical rib)

		and air (food and water deprivation or food and water ad libitum) Inhalation, whole body 24h on GD8	(stat. sign.) - Inc. in any major internal malformations (stat. sign.) - Inc. ocular malformation (reported in the text, no tabular information) - Inc. in total, early and late resorptions (stat. sign.) - Dec. number of live foetuses per dams (stat. sign.). At 75000 ppm: stat. sign. inc. in minor skeletal anomalies only (extra lumbar ribs and cervical ribs).
Developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Vieira et al., 1980 Limitations: Low number of animals per groups; Size of the chamber not stated; No specification of organs examined (internal or skeletal examination); No details on abnormalities provided (no tabulated data); Few information on maternal toxicity (e.g., no information on weight, clinical signs, behaviour).	Wistar rats N=12/group	N_2O (purity not stated) Inhalation, whole body Control (air), 250, 500 and 1000 ppm 23h/d, GD1-19	<u>Maternal toxicity</u> No effects on food or water consumption. <u>Developmental toxicity</u> - Dec. litter size at 1000 ppm (stat. sign.) - Inc. resorptions at 1000 ppm (stat. sign.) - Dec. crown-rump length at 1000 ppm, no effect on foetal body weight (stat. sign.) - Skeletal abnormalities at 1000 ppm (stat. sign.) (malformation of the vertebrae column and rib).
Developmental toxicity study Non-guideline; Non-GLP; Klimisch 2 Vieira et al., 1979 Limitations: low number of animals; Only one concentration group; Limited information on environmental conditions; Few internal organs and skeletal examinations; No information on maternal toxicity; No details on skeletal abnormalities (incidences in each group, details of the anomalies)	Wistar rats N=12/group	N_2O (purity not stated) Control (air), 5000 ppm Inhalation, whole body 23h/d, GD1-19	<u>Maternal toxicity</u> No effects on food or water consumption. <u>Developmental toxicity</u> - Stat. sign. dec. in litter size; 4/12 dams had full resorptions (vs 0 in controls) - Stat. sign inc. in skeletal malformations (rib). Foetuses with malformations were smaller than their litter mates or controls - Marked stat. sign. reduction in mean crown-rump length of exposed foetuses - Stat. sign. dec. in mean foetuses weight.

* test concentrations were analytically verified. Moreover, for gases, the efficiency for dynamic test atmosphere generation is expected to be near 100%.
** Plug day=day 0 of gestation.
Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant; bw = body weight

Contrary to the DS, RAC used only Klimisch 2 studies in weighing the evidence, noting that studies with Klimisch 3 and 4 are presented in Tables 13-16 in the CLH report. Additionally, the following is considered by RAC:

All animal studies included inhalation exposure using whole body application. The available animal studies were not performed in compliance with OECD TG and GLP and have limitations such as application of a single (high) concentration level, small animal group size and limited

number of parameters investigated. Nevertheless, RAC considers that in case observations are noted upon exposure during a critical window in studies having limited parameters or upon a single exposure concentration, such observations still can point towards serious substance-specific adverse effects relevant for classification.

RAC notes that prenatal developmental toxicity studies with repeated intermittent inhalation exposure (i.e., 6-8 hours per day during specific parts or the whole gestation period) are highly relevant for various human exposure regimes. In addition, prenatal developmental toxicity studies with 24h exposure to high concentrations of N_2O during a relevant exposure window and prenatal developmental studies with repeated continuous (24h/d) exposure to low(er) concentrations N_2O are included as additional evidence.

- Experimental data in rats: prenatal developmental toxicity studies, intermittent exposure

The DS presented several rat prenatal developmental toxicity studies with a repeated intermittent inhalation exposure, i.e., 6-8 hours per day during specific parts or the whole gestation period. Only the studies marked with a Klimisch score 2 by the DS are discussed below.

Mazze *et al.* (1986) exposed female Sprague-Dawley rats to 750000 ppm N_2O by inhalation 6h/d, during GD7-9, GD10-12 or GD13-15. A statistically significant increase in resorptions and foetal wastage (dead and resorbed) was noted in dams exposed during GD13-15 (1.32 per litter versus 0.46 in controls for resorptions, and 1.37 per litter versus 0.49 in controls for foetal wastage). This was associated with a decrease in live foetuses/implantation (87.8% versus 96.7% in controls). Foetal weight was significantly reduced in the group exposed during GD13-15, which was accompanied by lower weight gain of the maternal animals. Though not statistically significant, an increase in external malformations in the N_2O group versus control was present in offspring of dams exposed during GD7-9 and skeletal malformations in offspring of dams exposed during GD13-15. Historical control data are lacking, and the type of malformations was not specified. Visceral malformations were not increased in this study. It is noted that the exposure concentration is quite close to the threshold for hypoxia. Regarding maternal toxicity, animals were conscious during the experiment in all groups. Reduced body weight gain was noted in the dams exposed to N_2O, resulting in more than 20% reduction in body weight gain in the GD13-15 group and 5% and 8% for the GD11-13 and GD8-10 groups, respectively. The differences in weight gain in dams exposed during the various stages of development in this study were described by the study author to be due to the difference in their gestational age when they were received from the breeder and first weighed. RAC considers this explanation to be plausible. Further, information on corrected body weight is lacking. All in all, several effects on development were noted but in view of the differences in effects on weight gain in the various groups it is difficult to judge whether these were, at least in part, due to maternal effects. Due to these limitations, RAC gives limited weight to this study in the overall weight of evidence assessment.

Pope *et al.* (1978) exposed Sprague-Dawley rats by inhalation to 10000, 100000 or 500000 ppm N_2O for 8h/d, during the whole gestation. Adverse effects on development included delayed foetal development, specified as statistically significant decrease in foetal weight, accompanied by a decrease in crown-rump length and significant delayed ossification (at ≥100000 ppm). No effects on resorption or number of dead foetuses were observed upon N_2O exposure. Placental weight was reduced (≥10000 ppm). No effect on maternal toxicity was observed (i.e., no effects on either food consumption or body weight). Only gross skeletal abnormalities were examined, and no visceral examination was performed in the study. This

study also included a group, in which animals that got excited were transferred to a so-called stress group. In this group several effects were noted, such as decrease in the number of live litters and increase in foetal loss. Due to these limitations, RAC gives limited weight to this study in the overall weight of evidence assessment.

Vieira *et al.* (1983b) exposed Sprague-Dawley rats by inhalation to 250, 500, 1000 or 5000 ppm for 6h/d, 5d/week during the 3 weeks of gestation. A statistically significant decrease in litter size was present at 5000 ppm, though it is noted this was not observed in the presence of resorptions. There was no evidence of malformations. Crown-rump length and body weight was not affected. Information on maternal toxicity was not available.

***Table**: Developmental findings reported by Vieira et al. (1983b)*

Concentration level (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
1000	117	10±1.2	8-13
5000	98	7.0±2.3*	6-10

*p<0.001

- Experimental data in rats: prenatal developmental toxicity studies, continuous exposure

The DS presented multiple prenatal developmental toxicity studies with a single 24h inhalation exposure during a specific day of the gestation period as well as studies with inhalation exposure of 23-24h/d during multiple days of the gestation period or during the whole period of gestation.

Vieira *et al.* (1979; 1980) applied continuous inhalation exposure during the gestation period (23h/d, GD1-19; 250, 500, 1000 ppm in the 1980 study, 5000 ppm in the 1979 study) in Wistar rats. Regarding maternal toxicity, no effect on food or water consumption were noted up to 5000 ppm. Adverse effects on development were noted in both studies with statistical significance (see two next tables below). An increase in resorptions and decreased litter size were noted at ≥1000 ppm N_2O. Skeletal malformations were noted at ≥1000 ppm, specified as rib malformations and abnormal vertebrae columns. In addition, crown-rump length was also decreased at 1000 ppm onward. RAC notes the lower effective exposure concentrations in this study.

***Table:** Litter size, crown-rump measurements and foetal resorption upon exposure to 250, 500, 1000 ppm N_2O (Vieira et al., 1980)*

Concentration level (ppm)	Number of litters	Number of foetuses	Litter size (mean±SD)	Crown-rump measurements (mm, mean±SD)	Resorptions
0	12	120	11±1.4	44±1.4	None
250	12	120	11±1.3	43±1.4	None
500	12	118	11±1.4	43±1.3	None
1000	12	66	6.3±4**	35±1.6*	4**

**p<0.01, *p<0.05

Table: *Litter size, crown-rump measurements and foetal resorption upon exposure to 5000 ppm N_2O (Vieira et al., 1979)*

Maternal rat number												
N_2O group												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Live foetuses	7	9			13			11	11	10	5	11
Foetal weight (g), mean±SD	1.4±0.2	1.3±0.2			1.8±0.0			1.3±0.3	1.5±0.2	1.7±0.1	1.8±0.2	2.1±0.1
Resorption sites	4	1	11	12	2	10	12		1			
Crown-rump length (mm), mean±SD	29±0.2	28±0.2			30±0.2			29±0.2	28±0.2	28±0.2	28±0.2	29±0.2
Live foetuses with abnormalities		1			2			1	1	2		2
No. of live foetuses without abnormality	7	8			11			10	10	8	5	9*
Controls												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Live foetuses	12	12	10	10	10	13	12	12	9	13	12	10
Foetal weight (g) mean±SD	2.0±0.1	3.5±0.2	3.1±0.0	3.3±0.6	3.1±0.2	3.2±0.3	2.6±0.3	2.0±0.1	2.0±0.1	2.0±0.2	2.3±0.2	2.3±0.2
Crown-rump length (mm), mean±SD	44±0.2	44±0.2	44±0.2	43±0.2	42±0.2	42±0.2	44±0.2	44±0.2	42±0.2	43±0.2	44±0.2	44±0.2

*data retrieved from original publication

In a series of 7 studies from the same research group, Sprague-Dawley rats were exposed for 24h via whole body inhalation to N_2O at concentration levels between 7500 and 750000 ppm on a specific day of the gestation (Mazze *et al.*, 1984; 1987; 1988, Fujinaga *et al.,* 1987; 1989; 1990; 1991):

- Fujinaga *et al.* (1987; 1990; 1991) applied a single inhalation exposure on GD8 (24h) using a single concentration level at either 500000 ppm, 600000 or 700000-750000 ppm

N_2O.

- Mazze *et al.* (1984; 1987; 1988) applied a single inhalation exposure on GD8 (24h) using several concentration levels to investigate concentration-response, i.e., from 7500 to 750000 ppm N_2O.
- Fujinaga *et al.* (1989) applied a single inhalation exposure (24h) to 600000 ppm on either GD6, 7, 8, 9, 10, 11 or 12.

Animals were sacrificed at GD20, i.e., one day before expected delivery, except for the study of Fujinaga *et al.* (1990) where rats were randomly sacrificed at GD11-16, 18 or 20.

Adverse effects on development were consistently observed in these studies. Such effects included increased early and late resorptions, subsequent decrease in the number of live foetuses per litter, skeletal abnormalities and malformations, visceral abnormalities and malformations.

Regarding the resorptions, a concentration-dependent increase in early and late resorptions was observed in those studies investigating the effect of N_2O following 24h exposure on GD8 (Fujinaga *et al.,* 1987; 1989; 1991, Mazze *et al.,* 1984; 1987; 1988). The effect reached statistical significance at ≥500000 ppm, and a NOAEC of 350000 ppm was identified by the researchers. In the study of Fujinaga *et al.* (1989) at which inhalation exposure to 600000 ppm N_2O was applied on various days of gestation (GD6, 7, 8, 9, 10, 11 or 12), two critical periods of exposure were identified for the occurrence of resorptions. These was one at GD8 and one at GD11. Further, Fujinaga *et al.* (1990) found that upon inhalation exposure to 700000-750000 ppm N_2O, the increase in resorptions was first observed in the animals sacrificed on GD14, and not in animals sacrificed at GD11, 12 or 13. Further on, the rate of resorption remained constant as shown in animals sacrificed on GD15, 16, 18 and 20.

Regarding the skeletal and visceral abnormalities and malformations, a statistically significant marked increase was noted following inhalation exposure on GD8 (24h) at ≥500000 ppm N_2O (Fujinaga *et al.,* 1987; 1989; 1991; Mazze *et al.,* 1984; 1987; 1988). Various types of malformations were observed. In some of the studies, these were specified and included:

- Cardiac anomalies (Fujinaga *et al.,* 1991) or more specifically 'right sided aortic arch' (Fujinaga *et al*., 1989; Mazze *et al.,* 1988; Fujinaga *et al.,* 1987)
- Situs inversus (Fujinaga *et al.,* 1991)
- Ocular malformations (Mazze *et al.,* 1984)
- Hydrocephalus (Fujinaga *et al.,* 1991: 1989)
- Ribs and vertebrae (Fujinaga *et al*., 1989; Mazze *et al*., 1984)
- Limb deformities (Mazze *et al.,* 1984).

Further, body laterality as investigated by Fujinaga *et al.* (1990) was significantly altered compared to controls at all stages of development. This specifically included side of tail flexion, side of the body from which the umbilical artery emerged, side of the body that faced the placenta, and side to which the aortic arch curved.

The main visceral anomalies observed were left-sided umbilical artery (Fujinaga *et al.,* 1991; 1989). In addition, skeletal anomalies such as cervical ribs and 14th rudimentary rib were also increased (Fujinaga *et al*., 1991; 1989), Mazze *et al.,* 1988).

Information on maternal toxicity was, with the exception of Fujinaga *et al.* (1990), available for 6 out of these 7 studies. Mortality was noted in one of these studies (2-4 dams per exposure group; with unknown cause according to the authors) upon 24h inhalation exposure to 600000 ppm N_2O at GD6, 7, 8, 9, 11 or 12 (Fujinaga *et al*., 1989) and in a second study (1/25 exposed dams) upon 24h inhalation exposure to 600000 ppm N_2O at GD8 (Fujinaga *et al.,* 1991). In the other studies no increased mortality or significant morbidity was noted upon 24h inhalation exposure at GD8 up to 750000 ppm (Fujinaga *et al.*, 1987, Mazze *et al.,* 1984; 1987; 1988).

A decreased body weight compared to control was in general noted in most studies upon 24- h inhalation exposure at ≥500000 ppm N_2O. This may be caused by embryo-foetal lethality, though information on corrected body weight was not available in any of the studies. More specifically, no significant effect on body weight was noticed in dams exposed 24h at GD8 to 600000 ppm N_2O in the study of Fujinaga *et al*. (1991). Fujinaga *et al*. (1989) demonstrated, in addition to the deaths observed, a reduction in mean body weight upon exposure to 600000 ppm N_2O. Furthermore, Mazze *et al.* (1988) found a reduced body weight upon exposure to 500000 ppm or 750000 ppm N_2O (approximately 8% as compared to control). In Fujinaga *et al.* (1987), a decrease in body weight of dams was observed specifically at GD12 and 20 upon inhalation exposure to 500000 ppm. No effect on body weight was noticed at GD9, 14, and 16. See the table below for details on maternal body weight, indicating the decrease in body weight is slight (<6%) in this study of Fujinaga *et al.* (1987).

Table: *Selected maternal and foetal observations (mean ± SD) (Fujinaga et al., 1987)*

N_2O	**Control**	**500000 ppm**
No. of rats examined	37	26
Weight (mean, g)		
- GD6 (on arrival)	211±15	210±12
- GD8 (before exposure)	233±15	231±13
- GD9 (after exposure)	212±15	208±11
- GD12	254±17	241±12*
- GD14	271±18	259±14
- GD16	290±21	279±24
- GD20 (at caesarean section)	348±32	328±24*
Weight loss during the exposure	21±4	24±5
Early resorptions/rat (%)	4.9 ±10.3	18±19.9*
Late resorptions/rat (%)	0	6.8±11.7*
Total foetal wastage/rat (%)	4.9±10.3	25.9±28*
Total live foetuses/rat	11.7±3.4	9.5±3.5

* p<0.05 versus control

Finally, Mazze *et al.* (1987) found a difference in body weight of 9% at the end of the study between controls and high dose, which was at least in part due to the increase in post implantations loss and resorptions. A decreased body weight gain was noted in the 500000 ppm group and not in the 350000 ppm group. Like in other studies, information on corrected maternal body weight was not provided, making it uncertain to exclude the decreased litter size as cause of the body weight effects.

Mild sedation was observed during exposure in some of these studies, i.e., upon 24h inhalation exposure at GD8 to 350000; 500000; 600000 or 750000 ppm N_2O in the studies of Fujinaga *et al.* (1987; 1989; 1991) and Mazze *et al*. (1984; 1987).

In summary, for the rat studies with a single 24h exposure, it is considered that maternal toxicity did not occur <350000 ppm. Up to <600000 ppm for 24h, maternal toxicity was

limited to reduced body weight and mild sedation. At 600000 ppm for 24h, lethality was observed. It is further noted that information on corrected maternal body weight was not provided, which makes it uncertain to exclude the decreased litter size as cause of the body weight effects in rats.

Overall, RAC agrees with the DS that the available information on maternal toxicity cannot unequivocally explain the adverse effects on development as shown in this series of 7 studies with 24h exposure, at least for the increased incidence of resorptions and malformations at the effective concentration of 500000 ppm. Specifically, the study of Fujinaga *et al.* (1987) is considered the most important. Upon exposure to 500000 ppm N_2O on GD8, a statistically significant increase in early resorptions (18% vs. 4.9% in control) and late resorptions (6.8% vs. 0% in control), total foetal wastage (25.9% vs. 4.9% in control), major visceral malformation (14.9% vs. 0% in control) and skeletal developmental variants (33% vs. 15.2% in control) were observed. The predominant visceral lesion was a right-sided aortic arch, which was present in foetuses from 5 of the 26 litters exposed to 500000 ppm N_2O. Maternal toxicity in this study was limited to mild sedation during exposure and slight decrease in bw (see analysis above). RAC considers that the developmental effects as noted in Fujinaga *et al.* (1987) cannot be explained by the maternal toxicity. The other studies of this series of 7 are given less weight, as these applied concentration levels at which lethality was observed or information on maternal toxicity was lacking.

In addition to the series of 7 studies as described above, Keeling *et al.* (1986) exposed Sprague-Dawley rats by inhalation for 24h on GD8 to 700000-750000 ppm N_2O in oxygen. Information on maternal toxicity was not provided. No increase in resorptions was noted. Foetal weight and placental weight were decreased in a statistically significant manner compared to control. In addition, a statistically significant increase in skeletal malformations (mainly cervical vertebrae) and delayed development was observed in the N_2O exposed group.

- <u>Experimental data in rats: pre-/postnatal development and postnatal (neuro)developmental toxicity studies (intermittent exposure)</u>

Studies presented in this section include those with a repeated intermittent inhalation exposure, i.e., 6-8 hours per day during specific parts or the whole gestation period and investigating the pre- and/or postnatal (neuro)development.

Holson *et al.* (1995) exposed female Sprague Dawley rats to 0, 1000, 5000 or 10000 ppm N_2O for 6h/d during GD1-21 (3h on GD21). No effect on weight (gain) of the dams or weight of the offspring was noted. No effect on litter size and no treatment-related effects on behaviour in offspring were observed.

Mullenix *et al.* (1986) investigated potential neurodevelopmental toxicity (residential maze activity and time-lapse photography) and exposed Sprague Dawley rats for 8h/d on GD14 or GD13-14 to 750000 ppm N_2O. Exposure on GD13-14 produced significant hyperactivity in the females at 1-month and 5-month timepoints and in males at 1-month timepoint. Exposure on GD14 only produced a tendency to hypoactivity in females and hyperactivity in males.

- <u>Experimental data in other species (mainly intermittent exposure)</u>

In addition to studies in rats, studies with intermittent exposure in hamsters and mice were presented by the DS. Only the two studies in mice with a Klimisch 2 score are discussed below

No developmental effects (resorptions, litter size, malformations) were noted in Swiss/ICR mice in the study of Mazze *et al.* (1982) up to 500000 ppm N_2O (4h/d, GD6-15).

Rice *et al.* (1990) studied behavioural effects in offspring of Swiss mice following exposure by inhalation to 0, 50000, 150000 or 350000 ppm N_2O for 4h/d during GD6-15. Information on maternal toxicity was not available. Exposure to N_2O did not affect reproduction indices and survival or physical milestones of development. On postnatal day (PND)126 or 127 no effect on brain weights were observed. Ability to stay on a rotarod was also not affected by prenatal N_2O exposure. However, prenatal exposure to N_2O resulted in hypo-reactivity of the startle reflex on PND95 for all N_2O-exposed groups.

Discussion on adverse effects on development

RAC considers that a large database is available for the endpoint adverse effects on development, although several of the studies are poorly reported.

The available human data do not provide clear evidence on its own for classification, given the inconsistent findings, and the limitations regarding characterisation of the N_2O exposure and co-exposure and other confounding factors. Therefore, classification in Category 1A is not appropriate.

Animal data provide clear evidence for adverse effects on development upon inhalation exposure of N_2O, which is most evident in rats. Adverse effects are observed in multiple studies upon different exposure regimens.

Indications for adverse effects on development are obtained upon intermittent exposure, i.e., 6-8h/d, to N_2O (Mazze *et al.,* 1986, Pope et al., 1978; Vieira *et al*., 1983b; Mullenix *et al*., 1986; Rice *et al.,* 1990), an exposure regimen which RAC considers most relevant for humans. Such effects include delayed growth, i.e., decreased foetal weight and delayed ossification, (Pope *et al.,* 1978), reduced litter size (though in absence of resorptions; Vieira *et al.,* 1983b), increased resorptions, decrease in live foetuses per implantation and reduced foetal weight (Mazze *et al.,* 1986) and some evidence for effects on reactivity in pups (Rice *et al.,* 1990; Mullenix *et al*., 1986). For many of these studies it was noted that the information available did not allow an in-depth analysis whether the effects observed were in part due to maternal toxicity. Overall, the evidence obtained upon intermittent exposure is considered insufficient for classification in Category 1B on its own.

However, support for adverse effects on development is obtained from studies with a single 24h exposure to high concentrations on a specific day of the gestation (Mazze *et al.,* 1984; 1987; 1988; Fujinaga *et al.,* 1987; 1989; 1990; 1991; Keeling *et al.*, 1986), and moreover, from studies applying continuous 24h exposure to low (and more relevant) concentrations during the whole gestation period (Vieira *et al.,* 1979; 1980). A decreased crown-rump length, an increase in resorptions and a decreased litter size was noted in two studies in rats at ≥1000 ppm N_2O upon 23h/d, GD1-19. Also, skeletal malformations were noted at ≥1000 ppm in both studies, specified as rib malformations and abnormal vertebrae columns (Vieira *et al*., 1979; 1980). RAC notes the lower effective exposure concentrations in these two studies. Regarding the single 24h exposure on a specific day of the gestation, RAC considers the study of Fujinaga *et al.* (1987) to provide clear evidence for adverse effects on development. Exposure of rats to 500000 ppm N_2O for 24h at GD8 resulted in resorptions and malformations. More specifically, a statistically significant increase in early and late resorptions, total foetal wastage, major visceral malformation (predominantly right-sided aortic arch) and skeletal developmental variants was observed. It is noted that in this study maternal toxicity was limited to mild sedation during exposure and slight decrease in body weight, which is not sufficiently severe to discount the development effects observed (e.g., death or severe inanition, Annex I 3.7.2.4.3). Overall, RAC considers that the developmental effects as noted in Fujinaga *et al.* (1987)

cannot be explained by the maternal toxicity.

Regarding maternal toxicity, RAC also notes that in the experiment of Mazze *et al.* (1984) the effect of food deprivation on the N_2O-induced adverse effects was investigated. Exposure to 250000 ppm N_2O at GD9 and concurrent withholding of food and water did not result in an increased incidence of abnormalities.

Although in mice some evidence on potential effect on reactivity in pups was obtained, no effect on litter size, foetal growth and teratogenicity were noted in this species. However, the available mouse dataset is less extensive than the dataset for rat. Nevertheless, this divergence between species does not reduce the concern.

It is noted that the adverse effects on development in rats seem to be related to a specific exposure window. The results of Fujinaga *et al.* (1989) show that increased resorptions and major malformations were only noted in dams exposed at GD8, 9 or 11 to 600000 ppm N_2O for 24h and not in dams exposed to the same concentration level on GD6, 7, 10, or 12. It is however noted that in this specific study maternal mortality was noted, i.e., 2-4 dams per exposure group, except in control and GD10 exposed group.

Regarding hypoxia, N_2O was, especially at the high concentration levels in the developmental toxicity studies, mixed with oxygen in order to obtain an adequate test atmosphere (for example the Mazze *et al.* and Fujinaga *et al.* studies). Oxygen concentrations were monitored continuously during exposure in these studies. RAC notes that typical hypoxic effects such as respiratory distress or death from asphyxia were not reported in these developmental toxicity studies with information on maternal toxicity. Moreover, RAC notes that clear evidence for adverse effects on development is also observed upon much lower effective concentration of N_2O Vieira *et al.* (1979; 1980; 1983b).

It may be questioned whether the observed teratogenic effects are related to the substance itself or are secondary to the anaesthetised state. Regarding this issue, the DS presented several studies investigating the developmental effects of other anaesthetics. Pope *et al*. (1978) noted that high subanaesthetic concentrations of N_2O (up to 500000 ppm), halothane and methoxyflurane (8h/d, during whole gestation) can cause foetal growth retardation unaccompanied by foetal loss or abnormalities and unrelated to a specific agent. They considered that these developmental effects may well act through a general effect of the anaesthetic on the mother and foetus, rather than a toxic effect of the substance itself on the foetus. In contrast, Lane *et al.* (1980) exposed rats to 700000-750000 ppm N_2O or xenon (which has anaesthetic properties similar to those of N_2O) for 24 hours on GD9. Foetal resorption, delayed maturation and anomalies to the skeletal systems were only observed with N_2O and not xenon, suggesting that the observed effects are related to the substance itself rather than its anaesthetic mechanism. In line with this, Mazze *et al.* (1986) did not demonstrate teratogenic findings in rats exposed to intermittent exposure (6h/d, during GD7-9, GD10-12 or GD13-15) to anaesthetics such as isoflurane, enflurane or halothane. In contrast, an increase in foetal loss was observed following exposure to N_2O (750000 ppm), although some questions were posed as to the maternal effects. Overall, the adverse effects on development observed upon inhalation exposure to N_2O seem thus not to be the result of the anaesthetised state but rather an adverse effect of the substance itself, though some uncertainties are noted. Nevertheless, RAC notes that clear evidence for adverse effects on development is also observed upon exposure to much lower effective concentrations of N_2O.

In relation to N_2O-induced cobalamin (Vit. B12) deficiency as a potential mode of action, Mazze *et al.* (1984) exposed rats on GD10 to 250000 ppm N_2O and measured deoxyuridine (DU) suppression values in bone marrow and embryonic cells as marker of cobalamin deficiency. A

2.5-fold increase of DU-suppression was observed upon exposure to N_2O, though unaccompanied by teratogenic changes. Mazze *et al.* (1988) investigated the effect of co-exposure to folinic acid on the teratogenic effect of N_2O and its effect on methionine synthase activity. The developmental effect as induced by 500000-750000 ppm N_2O for 24h at GD8 (i.e., increased incidence of foetal wastage, major visceral malformations, minor skeletal anomalies, skeletal developmental variants) was still present with co-exposure to folinic acid, except for a partial reduction in minor skeletal anomalies, suggesting that cobalamin deficiency may not, alone, explain the teratogenicity induced by N_2O. Further, there was no correlation found with methionine synthase activity. In addition, Keeling *et al.* (1986) noticed that although a reduction in the N_2O-induced developmental effects were noted in presence of folinic acid, a statistically significant increase in major malformations was still observed compared to control.

Overall, no conclusion on the possible mode of action(s) can be drawn from the available information.

In addition, RAC notes that, in the absence of mechanistic information that raises doubt about the relevance of the effect for humans, the strong presumption that the substance has the capacity to interfere with reproduction in humans prevails based on clear evidence from animal studies (Annex I, Table 3.7.1(a)).

Conclusion on adverse effects on development

In summary, adverse effects on development were noted in studies where rats were exposed by inhalation to N_2O. These effects on development were observed upon different exposure regimens. Upon repeated continuous exposure of female rats to low effective concentrations, i.e., 23h/d, GD1-19, 1000 and 5000 ppm, decreased crown-rump length, increase in resorptions, decreased litter size and also occurrence of skeletal malformations (specified as rib malformations and abnormal vertebrae columns) were noted in two rat studies (Vieira *et al*., 1979; 1980). Intermittent exposure resulted in a statistically significant decrease in litter size at 5000 ppm (Vieira *et a*l., 1983b). Upon single exposure of female rats to 500000 ppm N_2O for 24h at GD8, early and late resorptions, increased total foetal wastage, major visceral malformation (predominantly right-sided aortic arch) and skeletal developmental variants were observed (Fujinaga *et al.,* 1987).

Overall, these data provide clear evidence for an adverse effect on development. RAC considers the observed adverse effects on development serious and relevant for humans and not to be a secondary consequence of other unspecific toxic effect.

Therefore, RAC agrees with the DS and concludes that **classification in Category 1B for adverse effects on development (Repr. 1B; H360D May damage the unborn child) is warranted**.

Given the level of effective concentrations and taking into account that in most studies a single concentration level was applied (which hampers assessment of concentration-response relationship, derivation of ED_{10} values based on single study, and establishing the potency), the available data on development do not support the calculation of an SCL.

Adverse effects on or via lactation

Given the lack of data, RAC considers that **classification for adverse effects on or via lactation is not warranted**.

10.11 Specific target organ toxicity-single exposure

Table 30: Summary table of animal studies on STOT SE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
RATS			
Non-guideline neurotoxicity study in Fischer-344xBrown Norway F1 hybrids (F344xBN F1/NIA) female rats N=5-10/group Limitations: - no information on GLP status - no information on the origin of the substance - purity of test material not provided - No. of animals at beginning and at the end of the study similar not specified - housing and lighting conditions not specified - no data on general toxicity	Inhalation, 3h exposure, Dinitrogen oxide mixed in oxygen Control, 50,000 to 2,000,000 ppm	EC_{50}: 1,180,000 ppm Neuron vacuolation (posterior cingulate/retrosplenial cortex)	Jevtovic-Todorovic et al., 2005 Klimisch 2 WOE
Non-guideline neurotoxicity study in rats Sprague-Dawley rats N= 6-17 per group Limitations: - No information on GLP status - Purity of test material not provided - Little information on housing conditions - just one dose tested (above hypoxia)	Single exposure: 0, 4, 6, 8, 12 or 16h 1,500,000 ppm N_2O	>3h, reversible vacuolation of neurons >8h exposure: Cell death	Jevtovic-Todorovic et al., 2003 Klimisch 2 WOE
Non-guideline neurotoxicity study in male and female rats Sprague-Dawley (N= 7-9/group) Limitations: - No information on GLP status - Purity of test material not provided - No data on general toxicity - No real materiel and method - No information about housing, lighting and feeding conditions - No information on the origin of the substance - No analytical control of concentrations during inhalation	Inhalation, 3h exposure 0 to 1,800,000 ppm dinitrogen oxide in air	EC_{50}=1,040,000 ppm (males) EC_{50}=1,170,000 ppm (females) Dose-related increase in vacuolated neurons (retrosplenic cortex)	Jevtovic-Todorovic et al., 2000 and 2001 Klimisch 2 WOE

Non-guideline neurotoxicity study in male Long-Evans rats (n=10/group) Limitations: - No information on GLP status - Purity of test material not provided - No data on general toxicity - limitation indicated by the authors: it is possible that the precision of delay measurements (0,1s) was not sufficient for discrimination in reaction time variations of the order of a few milliseconds. For this technical reason they can't confirm that their results agree or disagree with previous studies in humans	Inhalation, single continuous exposure 300,000, 400,000, 500,000, 600,000, 700,000 ppm N_2O	≥ 300,000 ppm Dose-related decrease (stat. sig.) in locomotor activity, alteration of visual detection task	Courtière et al., 1997 Klimisch 2 WOE
Non-guideline neurotoxicity study in male rats (strain not specified) (n=8-10/group) 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 dinitrogen oxide - Just one dose tested	Single continuous exposure (24h) 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.	Dzoljic et al., 1994 Klimisch 2 WOE
MICE			
Non-guideline neurotoxicity study in male NIH Swiss mice (n=12-15/group) Limitations: - No information on GLP status - Purity of test material not provided - Age of rats not specified - No data on general toxicity - No information on exposure duration	0, 250,000; 500,000; 750,000 ppm N_2O mixed in O_2 Unknown exposure duration	At 500,000 ppm: Increased (stat. sign.) in the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner	Li et al., 2001 Klimisch 3 WOE
Non-guideline neurotoxicity study in male Swiss-Webster mice mice (n=15-20/group) Limitations: - No information on GLP status - Age of rats not specified - No information about housing, lighting and feeding conditions - Purity of test material not provided - No data on general toxicity - Only one dose tested	35-60 min single exposure 500,000 ppm N_2O	Increased behavioural anxiolytic effects	Caton et al., 1994 Klimisch 2 WOE

Non-guideline neurotoxicity study in male mice (strain not specified) (n=4/group) 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	1h single exposure 500,000 ppm N_2O	Increased locomotor activity	Dorris et al., 1993 Klimisch 3 Disregarded

EC_{50}: Median effective concentration

Table 31: Summary table of human data on STOT SE – Human volunteer studies

Type of study/report	Test substance, Route of exposure, relevant information about the study	Observations	Reference
Human volunteer study	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	Yajnik et al., 1996
Human volunteer study	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	Fagan et al., 1994
Human volunteer study	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 ppm	Mahoney et al., 1988
Human volunteer study	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 ppm	Estrin et al., 1988
Human volunteer study	24 ♂ Chamber (nasal mask) 4h-exposure: placebo, 50 ppm N_2O	Psychomotor performance: No effect Mood: No statistical difference NOAEC = 50 ppm	Venables et al., 1983
Human volunteer study	20 ♂/group Chamber (nasal mask) 4h-exposure, twice: - 25 ppm N_2O + 0.5 ppm of halothane, - 50 ppm of N_2O, - 50 ppm + 1 ppm halothane, - 500 ppm N_2O,	Impairment on psychomotor tests : - Memory - Visual acuity - Audio-visual capacity NOAEC = 50 ppm N_2O	Bruce and Bach, 1976

	- 500 ppm N_2O + 10 ppm halothane		
Human volunteer study	30 ♂ Chamber 4h-exposure: air, 500 ppm N_2O	Impairment on psychomotor test : - digit-span test LOAEC = 500 ppm	Bruce and Bach, 1975
Human volunteer study	5 ♀ + 3 ♂ Facial mask 2 sessions (15-min exposure) air (control session), 200,000 ppm N_2O + 30% O_2 + room air	Significant activation in the anterior cingulate cortex and deactivation in the posterior cingulate hippocampus LOAEC = 200,000 ppm	Gyulai et al., 1996
Human volunteer study	N= 15 (sex not specified) Facial mask, 15-min exposure: 100,000; 300,000; 500,000 ppm N_2O mixed in O_2	Significant decrease in cerebral function analysing monitor LOAEC = 300,000 ppm	William et al., 1984

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – single exposure

Human data

- *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 (20) 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 2h after the beginning of exposure). The results were reported for each exposure in a table by the authors according to the tests conducted.

Table 32: Summary of results observed in Bruce and Bach (1976) with N_2O alone

	Test results (%)	
N_2O (ppm)	50	500
Tachistoscope	-	-7*
Raven matrices	-	-9*
O'Connor Dexterity	-	-
3-min audio-visual	-5*	-17**
60-min vigilance	-	-14*
7-min audio-visual	-5*	-17**
Digit span	-	-12***

- : no changes reported, *p<0.05; **p<0.01; ***p<0.001;

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 4h 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 (1976) study 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 (24) 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[3]. 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. (1983) didn't find difference 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 50 ppm of N_2O exposure, but the difference was not statistically significant.

Table 33: 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. (1988) evaluated the validity of neurobehavioral 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 neurobehavioral 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).

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").

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 initially and remained at that dose for 20 minutes during which the subtests of the computerised psychometric test battery were given and the P-300 determination was made. Respective simultaneous administration of O_2 was 0, 100, 90, 80 and 40%. The authors measured the effects using the P300 Evoked Potential[4] 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.

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

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

Table 34: 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;

Fagan et al. (1994) 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).

Yajnik et al. (1996) 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. 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.

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 were observed. 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).

- *Effects on nervous conduction*

Gyulai et al. (1996) analysed the effects of N_2O exposure in eight subjects. They measured regional cerebral flow (rCBF) changes and 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.

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 3000000 and 500000 ppm of N_2O (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.

Animal data

Rats

In a series of restrictions reported by 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 h 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 32h 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. (1997) 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. Concomitant decrease response time was observed at ≥ 600,000 ppm and omission 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.

Decreased locomotion followed by a development of a tolerance and unaltered motor activity during withdrawal was noted following continuous 24h 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 and thus the study was rated unreliable. 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. (1994) 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.

10.11.2 Comparison with the CLP criteria

According to the CLP criteria, substances are classified in category 1 for specific target organ toxicity (single exposure) on the basis of:
"a. *reliable and good quality evidence from human cases or epidemiological studies; or*
b. *observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations."*

The criteria for classification in category 2 are based on:
"Substances are classified in Category 2 for specific target organ toxicity (single exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided […] in order to help in classification.
In exceptional cases, human evidence can also be used to place a substance in Category 2."

The criteria for classifying substances as Category 3 for narcotic effects are:
"(a) central nervous system depression including narcotic effects in humans such as drowsiness, narcosis, reduced alertness, loss of reflexes, lack of coordination, and vertigo are included. These effects can also be manifested as severe headache or nausea, and can lead to reduced judgment, dizziness, irritability, fatigue, impaired memory function, deficits in perception and coordination, reaction time, or sleepiness.
(b) *narcotic effects observed in animal studies may include lethargy, lack of coordination, loss of righting reflex, and ataxia. If these effects are not transient in nature, then they shall be considered to support classification for Category 1 or 2 specific target organ toxicity single exposure."*

According to the ECHA guidance document on classification, the assignment of STOT-SE Category 1 or 2 is independent to the assignment of Category 3. Therefore, a substance may be classified in both category 1/2 and category 3 if the respective criteria are met, for instance, in the case of a neurotoxic substance that also causes transient narcotic effects.

In several studies in rats (Jevtovic-Todorovic et al., 2000, 2001, 2003 and 2007), significant and severe changes in the nervous system were observed. Indeed, brain histopathological findings and/or behavioural changes were reported after single exposure to ≥ 300,000 ppm dinitrogen oxide. Behavioral changes were also noted in the three described mice studies at ≥ 250,000 ppm. Nevertheless, although the effects were significant and severe enough to fulfil the criteria for STOT SE 1 or 2, no experimental studies were found at dose levels and exposure duration that would fulfil the criteria for classification STOT SE (C < 20,000 ppmV/4h).

In human volunteer experimental studies, Bruce and Bach, 1976 reported a slight impairment of audio-visual capacity at 50 ppm and lower performance in visual acuity, audio-visual capacity, immediate memory and vigilance response following 4-hours exposure to N_2O at 500 ppm. On the other hand, Venables et al., 1983 and other authors failed to reproduce the results obtained at 50 ppm. Nevertheless, according to the authors, the volunteers used may have been particularly sensitive to the cognitive effects of dinitrogen oxide and may not have been representative of the general population. At 500 ppm, effects on memory (digit span test) were reported by Bruce et al., 1975. Other acute studies were performed at higher dose levels and also reported effects on the nervous system. A LOAEC of 50-500 ppm can be identified for these effects.

Based on the effects observed on cognitive function (e.g. lower performance in audio-visual capacity, memory and vigilance, reaction time), in human volunteer studies using control exposure levels, classification of the substance in category 3 (narcosis) for STOT SE is considered relevant. The effects are expected to be reversible. Although not assessed, it was reported by Fagan et al., 1994 that dizziness, paraesthesia and euphoria observed in volunteers exposed to dinitrogen oxide were rapidly reversible. In addition, ataxia were also noted in animals and were reversible, supporting a classification (Singh et al., 2015, detailed in STOT RE section) STOT SE 3 rather than STOT SE 1 or 2.

In addition, with regards to an additional classification for STOT SE 1 or 2, the observed effects in human volunteer studies may not be sufficiently convincing but point toward potential significant toxicity of the substance following repeated exposure (See section on STOT RE).

10.11.3 Conclusion on classification and labelling for STOT SE

As detailed, the effects observed in human on the nervous system, supported by the animal data, are not sufficiently convincing to propose a classification of dinitrogen oxide for STOT SE. However, based on narcotic findings in human volunteer studies, a classification as STOT SE 3, H336 is warranted.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The DS proposed to classify N_2O as STOT SE 3 (H336).

The DS noted the following when concluding on the classification:

- In several studies in rats (Jevtovic-Todorovic *et al.,* 2000; 2001; 2003; 2005), significant and severe changes in the nervous system were observed >300000 ppm.
- In mice significant behavioural changes were observed >250000 ppm
- In a human volunteer study, Bruce and Bach (1976) reported a slight impairment of audio-visual capacity at 50 ppm, and lower performance in visual acuity, audio-visual capacity, immediate memory and vigilance response following 4h exposure to N_2O at 500 ppm. However, the volunteers may not have been representative for the general population, as indicated by a letter published in 1991 by the same authors. Several other acute studies which were performed at higher concentrations reported effects on the nervous system. A LOAEC of 50-500 ppm can be identified for these effects.

Although effects in some studies were considered by the DS to be sufficiently severe to fulfil the criteria for STOT SE 1 or 2, no experimental studies were found at concentration levels and exposure duration that would fulfil the criteria for classification for STOT SE (Cat 2. C ≤20000 ppmV/4h).

Based on the effects observed on cognitive function (e.g., lower performance in audio-visual capacity, memory and vigilance, reaction time) in human volunteer studies using controlled exposure levels, classification of the substance in category 3 (narcotic effects) for STOT SE was considered relevant by the DS. The effects are expected to be reversible. Although not assessed, it was reported by Fagan *et al.* (1994) that dizziness, paraesthesia and euphoria observed in volunteers exposed to N_2O were rapidly reversible. In addition, reversible ataxia was also noted in animals (Singh *et al.,* 2015, detailed in STOT RE section), supporting a classification as STOT SE 3 for narcotic effects.

Comments received during consultation

Two Member State Competent Authorities (MSCAs) commented and agreed with the proposed classification.

Six industry representatives disagreed with the proposal based on several arguments which questioned the quality and validity of the data, including:

- lack of substance characterisation
- inadequate methodology and acceptance/evaluation criteria
- limited reporting of methodology

- data search only in a single database (PubMed); however, RAC notes that secondary literature and the REACH database were consulted as well

The industry representatives all considered that the reliability of the human volunteer studies cannot be assessed when considering their limitations.

Assessment and comparison with the classification criteria

The DS discussed eight non-guideline neurotoxicity animal studies with exposure via inhalation, five in rats and three in mice. All of these had limitations which included lack of information on purity, substance origin, GLP status, housing conditions and sometimes animal age, exposure duration, analytical control and information on general toxicity. RAC notes that no information was provided on the exposure method (whole body or nose only).

Three studies by the same author (Jevtovic-Todorovic *et al*., 2000; 2001; 2003; 2005) exposed rats to concentrations above 800000 ppm N_2O under hyperbaric conditions (higher pressure allows higher concentrations). The artificial nature of these studies and unclear oxygen levels makes it difficult to assess the relevance of the toxic effect of N_2O for humans. The carbon dioxide levels may also have exceeded the recommended 1% in these studies (1-3%). The effects reported in the studies included (reversible) neuron vacuolation (>500000 ppm) and neuronal cell death at very high concentrations (>1000000 ppm). Overall, the results of these studies by Jevtovic-Todorovic *et al.* are only used as supportive evidence for classification, as they add information to the general picture of neuronal effects caused by N_2O.

A summary of the animal studies relevant for classification is presented in the table below.

***Table**. Summary of the relevant animal studies for STOT SE*

Method, Guideline, GLP status, Reliability, Reference	**Species, Strain, Sex, No/ group**	**Test substance, Concentration levels, Duration of exposure**	**Results**
Rats			
Non-guideline neurotoxicity study Klimisch score: 2 Courtière et al., 1997	Male Long-Evans rats N=10/group	Inhalation, single continuous exposure 300000; 400000; 500000; 600000; 700000 ppm N_2O	No information on general toxicity ≥300000 ppm: Dose-related dec. performance (stat. sign.) in visual vigilance performance task ≥400000 ppm: Dose-related dec. (stat. sign.) in locomotor activity
Non-guideline neurotoxicity study Klimisch score: 2 Dzoljic et al., 1994	Male rats (strain not specified) N=8-10/group	Single continuous exposure (24h) 700000 ppm mixed in O_2	Transient dec. in visual evoked potential amplitude. Dec. in nocturnal locomotion. Tolerance observed during the following light-dark cycle.

Mice			
Non-guideline neurotoxicity study Klimisch score: 3 Li et al., 2001	Male NIH Swiss mice N=12-15/group	0; 250000; 500000; 750000 ppm N_2O mixed in O_2 Unknown exposure duration	No data on general toxicity. At 500000 ppm: Inc. (stat. sign.) in the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner.
Non-guideline neurotoxicity study Klimisch score: 2 Caton et al., 1994	Male Swiss-Webster mice N=15-20/group	35-60 min single exposure 500000 ppm N_2O	No data on general toxicity. Inc. behavioural anxiolytic effects.
Non-guideline neurotoxicity study Klimisch score: 3 Dorris et al., 1993	Male mice, strain not specified	1h single exposure 500000 ppm N_2O	No data on general toxicity. Inc. locomotor activity.

Inc. = increase or increased; dec. = decrease or decreased; stat. sign. = statistically significant

In rats, neurological effects observed included:

- ≥300000 ppm (exposure duration not reported): Dose-related decrease in locomotor activity, alteration of visual detection task (Courtière *et al.,* 1997).
- 700000 ppm: Transient decrease in visual evoked potential amplitude. Decrease in nocturnal locomotion. Effect on tolerance observed during the following light-dark cycle (Dzoljic *et al.,* 1994).
- Supportive: >500000 ppm: reversible neuron vacuolation (studies by Jevtovic-Todorovic *et al.,* 2000; 2001; 2003; 2005) conducted under hyperbaric conditions.

In mice, neurological effects observed included:

- 500000 ppm: Increased behavioural anxiolytic effects (Caton *et al.,* 1994), increased locomotor activity (Dorris *et al.,* 1993, but disregarded by the DS because of poor reporting/many limitations).
- >500000 ppm (no information on exposure duration): Increased time spent in the light compartment and in the number of intercompartmental transitions. (Li *et al.*, 2001).

In addition to the animal studies, nine studies with human volunteers were presented by the DS. These studies had limitations including a limited number of volunteers per study and non-standardised study designs. However, RAC notes that such limitations are usually present with human volunteer studies and the number of participants in some of the studies and the total number of human volunteers cannot be considered limited.

Table: *Summary of the human volunteer studies for STOT SE*

Type of study/report	**Test substance, Route of exposure, relevant information about the study**	**Observations**	**Reference**

Human volunteer study	5 ♂ + 6 ♀ Chamber (nasal mask) 5 sessions (~190 min): air (control session); 100000; 200000; 300000; 400000 ppm N_2O	Significant impairment on auditory reaction time and eye-hand coordination. No acute tolerance to N_2O LOAEC=300000 ppm Rapid recovery (5 min)	Yajnik *et al.,* 1996
Human volunteer study	8 ♂ + 4 ♀ Chamber (nasal mask) 5 sessions (~ 60 min): air; 50000; 100000; 200000; 400000 ppm N_2O	Significant differences: impairment of reaction time and attention LOAEC=100000 ppm Rapid recovery (few minutes)	Fagan *et al.,* 1994
Human volunteer study	15 ♂ Chamber (nasal mask) 4 sessions (duration not specified): air (training session & control session); 200000; 400000 ppm N_2O	Impairment on psychomotor tests: - Symbol digit - Finger tapping - Test response latency LOAEC=200000 ppm	Mahoney *et al.,* 1988
Human volunteer study	6 (sex not specified) Chamber (nasal mask) 4 sessions (10 + 20 min): air; 100000; 200000; 400000 ppm N_2O in O_2	Impairment on psychomotor tests: - Continuous performance test - Finger tapping LOAEC=100000 ppm	Estrin *et al.,* 1988
Human volunteer study	24 ♂ Chamber (nasal mask) 4h exposure: placebo, 50 ppm N_2O	Psychomotor performance: No effect Mood: effects observed but no statistical difference NOAEC=50 ppm	Venables *et al.,* 1983
Human volunteer study	20 ♂/group Chamber (nasal mask) 4h exposure, twice: - 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	Impairment on psychomotor tests: - Memory - Visual acuity - Audio-visual capacity NOAEC=50 ppm N_2O	Bruce and Bach, 1976
Human volunteer study	30 ♂ Chamber 4h exposure: air, 500 ppm N_2O	Impairment on psychomotor test: - digit-span test LOAEC=500 ppm	Bruce and Bach, 1975
Human volunteer study	5 ♀ + 3 ♂ Facial mask 2 sessions (15-min exposure) air (control session), 200000 ppm N_2O + 30% O_2 + room air	Significant activation in the anterior cingulate cortex and deactivation in the posterior cingulate hippocampus LOAEC=200000 ppm	Gyulai *et al.,* 1996
Human volunteer study	N=15 (sex not specified) Facial mask, 15-min exposure: 100000; 300000; 500000 ppm N_2O mixed in O_2	Significant decrease in cerebral function analysing monitor LOAEC=300000 ppm	William *et al.,* 1984

In summary, the neurological effects observed in nine studies with human volunteers included:

- 500 ppm: small reversible changes in audio visual acuity/capacity, digit span, memory and vigilance response (Bruce and Bach, 1974; 1975; 1976). Studies may have been affected with sampling bias of sensitive subjects as noted in a letter by the authors a few years after publication.
- ≥100000 ppm for 1h: impairment of reaction time and attention (Fagan *et al.,* 1994).

- ≥100000 ppm, several sessions, exposure duration not specified: impairment on psychomotor tests including test response latency, finger tapping (Mahoney *et al.,* 1988, Estrin *et al.,* 1988).
- 200000 ppm: significant activation in the anterior cingulate cortex and deactivation in the posterior cingulate hippocampus (Gyulai *et al.,* 1996).
- >300000 ppm for 190 min: significant impairment on auditory reaction time and eye-hand coordination. (Yajnik *et al.,* 1996). Rapid reversibility (<5min after exposure).
- ≥300000 ppm: significant decrease in cerebral function analysing monitor (William *et al.,* 1984).

RAC notes that N_2O is used for inhalational anaesthesia in human medicine, implying it has known sedative properties on the nervous system. In addition, similar effects on the nervous system have also been observed after repeated exposure (see section on STOT RE).

Applicability of STOT SE 1 or 2

The CLP criteria allow classification for STOT SE 1 or 2 based on animal studies if adverse non-lethal effects are observed at concentrations below 20000 ppm for 4h (CLP Regulation, Annex I, 3.8.1 and Table 3.8.2). In the available studies, animals were not exposed at concentrations below this level. Therefore, a classification for STOT SE 1 or 2 is not warranted based on the information from animal studies.

There are no guidance values (GVs) for classification for STOT SE 1 or 2 based on human studies.

According to the CLP Regulation, substances may be classified for STOT SE 1 based on human data *"if substances that have produced significant toxicity in humans after single exposure."* Further the CLP Regulation Annex I 3.8.2.1.7.1. states that *"Classification is supported by evidence associating single exposure to the substance with a consistent and identifiable toxic effect."*

The neurological effects observed in human volunteers (and supported by findings in animals), i.e., mostly reductions in psychomotor activity, reaction times, attention and coordination, are signs of nervous system depression, which is considered as a consistent finding between studies. RAC considers these findings to be transient and reversible in nature, rather than "significant" and "adverse". The reversibility was fast in the human volunteer studies where this information was reported, which is supported by the fast toxicokinetic of N_2O after single exposure.

Overall, the effects after single exposure in animal studies occur only at concentrations above the guidance values and the neuronal effects in humans are considered transient and reversible in nature. The toxic effects cannot be considered "significant" or sufficiently adverse to warrant a classification as STOT SE 1 or 2. In conclusion, RAC agrees with the DS that a classification as STOT SE 1 or 2 is not warranted.

Applicability of STOT SE 3

The CLP criteria (Annex I, Section 3.8.2.2.2) indicate that for classification as STOT SE 3 is based on: `

(a)central nervous system depression including narcotic effects in humans such as drowsiness, narcosis, reduced alertness, loss of reflexes, lack of coordination, and

vertigo are included. These effects can also be manifested as severe headache or nausea, and can lead to reduced judgment, dizziness, irritability, fatigue, impaired memory function, deficits in perception and coordination, reaction time, or sleepiness;

(b) *narcotic effects observed in animal studies may include lethargy, lack of coordination, loss of righting reflex and ataxia. If these are not transient in nature, they shall be considered to support classification for Category 1 or 2 specific target organ toxicity single exposure*.'

All the studies with human volunteers presented by the DS have limitations. Most notably, the study quality and reliability are difficult to assess for various reasons including non-standardised study designs and limited number of volunteers in the individual studies. This hampers the interpretation of the individual results. However, since many of these studies consistently indicate (narcotic) neurological effects, and that overall, the total number of human volunteers in these studies is rather high, it is unlikely an effect of chance or study design. In further support, the substance is used in human medicine as an anaesthetic and the observed effects are of similar nature, although less severe, as the reported effects from recreational use (presented with STOT RE). Therefore, RAC considers that the narcotic effects observed in the human volunteer studies are relevant for classification.

Narcotic effects following a single inhalation exposure were noted in human volunteers supported with similar findings in studies with rats and mice. These narcotic effects included impaired locomotor/psychomotor activity, reaction time, attention and coordination. They are considered the be transient in nature starting at concentrations of 100000 ppm in the presented human volunteer studies.

In conclusion, RAC agrees with the DS that **classification as STOT SE 3; H336 (May cause drowsiness or dizziness) is warranted**.

10.12 Specific target organ toxicity-repeated exposure

Table 35: Summary table of animal studies on STOT RE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
RATS			

Non-guideline neurotoxicity study in rats Wistar rats Male N=5-10/group Limitations: - no information on GLP status - no information on the origin of the substance - purity of test material not provided - only one dose - no data on general toxicity - unclear dose levels	N_2O mixed in O_2 Inhalation, 60-day exposure, 2h/d 0, 500,000 ppm	**<u>At 500,000 ppm:</u>** - No effect on survival - Statistically significant decrease in bw gain at the end of the experiment (200.6 mg vs 245.6 mg in controls) - Necropsy: Brain, spinal cord Astrocytes activation in brain and spinal cord (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 compared to controls - Homocysteine levels, glutamate and malondialdehyde (MDA) levels were significantly increased, however GSH and total antioxidant capacity (TAC) level decreased in dinitrogen oxide exposed group compared to the controls.	Misra et al., 2020 Klimisch 2
Non-guideline neurotoxicity study in rats Wistar Rats, Male N= 6/group Limitations: - no information on GLP status - no information on the origin of the substance - purity of test material not provided - only one dose level - no data on general toxicity aside weight loss	Dinitrogen oxide Inhalation, 1.5h/d, 60-day exposure 500,000 ppm N_2O mixed in O_2	**<u>At 500,000 ppm:</u>** General toxicity: There was progressive weight loss in exposed group compared to controls (303.8±14.11 vs 244.6±8.75 g; p = 0.0073). Clinical signs: After exposure the rats appeared sluggish, lethargic and developed limb weakness, which was more marked in the hind limbs. These symptoms recovered in 1–1.5 h. 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. No effect observed in the control group Behavioral studies: the rats appeared sluggish, lethargic and developed predominantly hind limb weakness for 1–1.5 h. In the exposed group, the total distance travelled (2001.66± 118.27 cm; p = 0.037), time moving (80.16± 5.7 s; p = 0.028), number of rearing (10.33 ±1.45; p = 0.014) and grip strength (1042.40 ±51.3 N; p = 0.041) were significantly decreased whereas, resting time significantly increased (219.83 ±5.7 s; p = 0.030) compared to controls. No changes in stereotypic counts Biochemical markers: Serum vitamin B12 level was below 150 ng/dl in 5 of 6 rats in both exposed and control groups. Homocysteine level was significantly increased in the exposed group compared to control (20.56±1.30:10.40±1.42 mm/ml; p = 0.0007)	Singh et al., 2015 Klimisch 2
Non-guideline neurotoxicity study in rats Rats Sex: not specified N=18/group	Dinitrogen oxide 4h/d, 5d/w, 6-month exposure 0, 700,000 ppm N_2O	**<u>At 700,000 ppm</u>** - No morphometric and teased fibre abnormalities in peripheral nerves - No changes in electromyography, nerve conduction velocity or axonal flow	Dyck et al., 1980 Klimisch score 2

Non-guideline neurotoxicity study in rats Sex: both sexes No. of animals not specified	Dinitrogen oxide Inhalation, 8h/d 7 or 14-d exposure 400,000 ppm N_2O	**<u>At 400,000 ppm</u>** Cortical cell count changes in brain (frontal parietal and occipital portions)	Hayden et al., 1974 Klimisch score 4
MICE			
Non-guideline neurotoxicity study Swiss Mice Group: 8 controls and 7 per exposed group Sex: Male adults Analysis: - Locomotor activity: measured one-day after exposure (horizontal activity was measured) at 10 min. for 2h (10, 30, 60, 90, 120 min time-points). - Anxiety assessment was investigated during 30 min. Side changes, dark-like area time spent measured - Stereotypy: total number of stereotypies at 10 intervals for 2 hours. - Coronal sections of the occipital lobe of control and animals exposed to 2000 ppm dinitrogen oxide were examined under light microscopy. The large cells (neural cells) and small cells (neuroglial cells were counted in a 0.03 mm2 area.) - Motor coordination: rolling-roller performance step (rotating rod) Limitations: - males only Low number of animals per groups	Dinitrogen oxide 0, 1000, 2000 ppm N_2O Inhalation, 8h/d 8-day exposure	No changes in body weight of mice between groups. **At 1000 ppm:** - Dinitrogen oxide-exposed mice 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 - Dose-related reduction 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 dinitrogen oxide.	Fung et al., 1993 Klimisch score 2

Non-guideline repeated dose toxicity study in mice Non GLP Swiss webster mice Male and female N=15/sex/group Limitation: limited number of parameters investigated	Dinitrogen oxide (purity not specified) 14-week whole body exposure, Inhalation, 4h/d, 5d/w 0, 5000, 50,000, 500,000 ppm N_2O Vehicle: air	- All animals survived to necropsy - Statistically significant treatment-related decrease in body weight at the top dose (decreased in body weight gain in males and females by 77 and 63% vs control, respectively). - No effect on brain weight, no histopathologic findings in brain.	Rice et al., 1985 Klimisch score 2

GFAP: glial fibrillary acidic protein

Table 36: Summary table of human data on STOT RE – recreational use

Type of study/report	Test substance, Route of exposure, relevant information about the study	Observations	Reference
Case report	Case: 29 year-old man Exposure: dinitrogen oxide, 60 dinitrogen oxide canisters (whippets) per day for the first 5 days followed by use every 2-3 days thereafter. No regular medication.	Symptoms: ascending lower limb numbness, pins and needles, difficulty walking 3 day after stopping exposure. Examination: unsteady gait, reduced sensation to light touch and pinprick Clinical chemistry: vit. B12 deficiency, elevated homocysteine levels, MRI: normal Follow up: immediate improvement of symptoms after treatment.	Thayabaran et al., 2021
Retrospective case series report	Retrospective evaluation of neurological disorders attributed to dinitrogen oxide recreational use Cases from January 2018 to June 2019 Participants: 43 patients, 15-30 year-old, Exposure: mean usage rate of 10.8 times per month (1-28) for 10.7 months (0.7-48)	Symptoms: limb numbness and weakness (98%), unsteady gait (70%), anxiety (14%) followed by insomnia, hallucination, lethargy Clinical chemistry: vit. B12 deficiency (63%), hyperhomocysteinemia (93%), anaemia (19%), Electromyography: sensory and motor nerve impairments (100%), mixed axonal and demyelinating neuropathy (93%) more prominent in lower limbs. MRI: changes in the dorsal column of the cervical spinal cord (77%), cervical and thoracic spinal cord changes in one patient. One patient with diffuse brain atrophy. Diagnoses: peripheral neuropathy (10/43), myeloneuropathy (30/43), combined myeloneuropathy and toxic encephalopathy (3/43) <u>Follow up</u> after treatment and cessation of exposure (4-32 month): - full recovery: 5 (11.6%)	Zheng et al., 2020

		- marked improvement: 36 (83.7 %) - No improvement in 2 patients (4.7%)	
Case report	Participants: 1 patients, 21 year-old, Exposure: dinitrogen oxide capsule used for whipped cream recharging, a few times daily for a month for anxiety relief. Last use three days before hospitalisation.	Neurologic symptoms: lower extremity weakness, double vision, falls, dizziness, anxiety Neurological exam.: positive Romberg test, 4/5 strength in lower extremities Clinical chemistry: Vit. B12 deficiency MRI: normal (brain and spinal cord) Follow up after treatment and cessation of exposure: partial resolution	Lundin et al., 2019
Retrospective case series report	Retrospective evaluation of subacute degeneration of the spinal cord attributed to dinitrogen oxide exposure Cases from march 2012 to January 2018 Participants: 9 patients, 14-19 year-old Exposure: History of dinitrogen oxide abuse 6 months before the onset of symptoms and degeneration predominantly restricted to posterior and lateral column. 7/8 has used dinitrogen oxide more than 6 months Data collection: clinical history, Clinical symptoms, onset and duration, laboratory data, nerve conductive velocities, somatosensory evoked potential and spinal magnetic resonance images.	Neurologic symptoms: numbness (8/9), hyperesthesia (3/9), muscle weakness (all), ataxia (8/9) Neurological exam. Absence of deep tendon reflex (all), proprioception defect (8/9), decreased muscle power (all). Clinical chemistry: Vit. B12 deficiency (4/8), elevated serum homocysteine level (8/9), anaemia (2/9). Neurophysiology: demyelinated features, spinal cord involvement (4/6) and axonal degeneration (1/6) MRI: degeneration of cervical area (6/6), thoracic regions (1/6), simultaneous cervicothoracic involvement (other) Follow up after treatment: - Muscle power recovery within 2-month, - Persistent sensory deficit (5/9), sensory ataxia (1/9)	Lan et al., 2019
Case report	Participant: 45 year-old man Exposure: 100 canisters of whipped cream chargers per week for approx. 4 weeks	Symptoms: progressive worsening numbness and tingling of extremities, ataxic gait, instability. Examination.: 4/5 strength, decrease sensation to light touch in extremities and vibration sense, dysmetria in finger to nose testing, positive Romberg test Clinical chemistry: Vit. B12 deficiency, hyperhomocysteinemia MRI: Changes in the dorsal column of the cervical and thoracic spinal cord Follow up after treatment: improvement at day 14	Shah et al., 2019
Case report	Participant: 21 year-old woman Exposure: habitual inhalation of dinitrogen oxide over the past year, with increased consumption over the preceding weeks of up to 300 canisters/week	Symptoms: confusion, gait ataxia, impaired insight, orientation, short-term memory, attention Neurological exams: impair limb proprioception with sensory ataxia, positive Romberg test. Decrease limb strength, globally depressed reflexes	Jonhson et al., 2018

Case series report	Retrospective evaluation of subacute degeneration of the spinal cord attributed to dinitrogen oxide exposure Cases from November 2016 to May 2017 Participants: 10 (3 women and 7 men), median age of 22 (range 17-26) Exposure: dinitrogen oxide 2-3 times per weeks, 75 to 2000 canisters per week	Neurological symptoms and exams: altered sensation in the limbs (10/10), gait ataxia (8/10), falls (3/10), Romberg's signs (6/10), Pseudoathetosis (5/10), Lhermitte's phenomenon (1/10), Uhtoff's phenomenon (1/10), segmental myoclonus (1/10) Clinical chemistry: Vit. B12 deficiency (4/10), elevated MMA (7/8 patients), MRI: Changes in the dorsal column of the cervical spinal cord consistent with subacute degeneration Follow up after average 14 month (5-27 months): - Full recovery (3/6) - residual paraesthesia, gait ataxia and sensory loss (3/6) - Persisting MRI changes (2/4)	Keddie et al., 2018
Case report	Case: 22 year-old man Exposure: high-volume recreational use of dinitrogen oxide	Severe peripheral polyneuropathy, vit. B12 deficiency, partial recovery at most recent follow-up	Middleton et al. 2018
Case report	Case: 24 year-old women Exposure: recreational "whippets" exposure to dinitrogen oxide	Examination: unsteady gait, positive Rhomberg sign Clinical chemistry: macrocytic erythropoiesis, vit. B12 deficiency, elevated homocysteine and MMA levels. MRI: degeneration of the posterior spinal column.	Egan et al., 2018
Case report	Case: 27 year-old man Exposure: pain management in the emergency department (> 50 occasion over a 5 year period)	Symptoms: paresthesia, numbness, distal sensory loss Nerve conduction studies: mild large fibre, length-dependent axonal sensorimotor polyneuropathy Clinical chemistry: Normal Hb and MCV MRI: normal Diagnoses: functional leg spasm and sensorimotor neuropathy secondary to functional B12 deficiency related to controlled dinitrogen oxide administration. Follow-up: improvement of sensory symptoms, normal vit. B12, reflexes remained absent and the neurophysiological findings absent.	Kaski et al., 2017
Case report	Case: 23-year old women Exposure: recreational use of dinitrogen oxide (no further information)	Clinical exams: symmetrical weakness of the iliopsoas muscle an quadriceps, paralysis of dorsal flexors of the feet, areflexia of the legs and feet, loss of vibration sense Clinical chemistry: haemolytic anaemia, leukopenia Electromyography revealed axonal polyneuropathy with demyelination	Glijn et al., 2017

		MRI: normal cerebrum and spine Diagnostic: leukopenia and severe neurological signs as a result of a severe vitamin B12 deficiency due to recreational use of N_2O Follow up: after 6- month treatment: - slight improvement of paraparesis but the patient is only capable of walking within her own with a walking frame	
Case report	Case: 29 year-old woman Exposure: Inhalation of dinitrogen oxide in A&E as an opioid-sparing agent Other: Poor nutritional status	Symptoms: leg numbness, altered sensation of extremities, 2-month history of increasing falls and urinary frequency Neurological exams.: peripheral neuropathy including 4/5 strength of lower limbs, impaired joint position and pain perception MRI: posterior spinal cord lesions (cervical and thoracic) Diagnose: subacute combined degeneration of the spinal cord (dinitrogen oxide myelopathy) Follow up: improvement but no full recovery after 2-year	Sleeman et al., 2016
Case report	Case: 20 year-old women Exposure: 2-year exposure to dinitrogen oxide and ketamine for recreational purposes, no information on dosage and frequency Patient taken vit. B12 complex before admission	At presentation: one-month unsteady gait, involuntary movement in the four limbs and mild tingling sensation in a stocking glove distribution, difficulty to walk. Neurological exam.: mild stuttering in speech and dystonia in the facial muscle and tongue, full muscle strength, dystonia-like posture in four limbs and athetoid movements in fingers and toes, worsened by eye closure, impaired vibration and proprioception distal to wrists and ankles. Nerve conduction study: sensory motor polyneuropathy. Visual evoked potentials: impaired visual conduction pathway. Impaired somatosensory evoked potential (central and peripheral sensory conduction). Clinical chemistry: anaemia, normal serum vit. B12, iron deficiency. MRI: cervical spinal cord lesions. No effect in brain spinal cord Diagnose: dinitrogen oxide induced spinal cord degeneration Follow up: 3 weeks later - Gradual improvement of gait and involuntary movement - walk slowly independently	Chen et al., 2016
Retrospective case report series	Retrospective evaluation of patients with dinitrogen oxide induced myeloneuropathy	Nerve conduction velocity studies: abnormal results in at least one motor or sensory nerve (97% of patients), conduction slowing in peroneal (36%) and tibial (30%) nerves were the most	Li et al., 2016

	Cases from 2005-2015 Participants: 33 (19 men and 14 women), median age of 22 (range 17-26). 56 healthy controls' nerve conduction studies collected for comparison analysis Exposure: 20.9 ±5.5 month (range: 1-120), last dinitrogen oxide exposure to symptom onset: 10.2±1.5 days (range: 1-28). 14 patients used multiple illicit substances	frequently encountered features. Electromyography: active denervation changes (11/18 patients)	
Case report	Case: 36 year-old man Exposure: Habitual dinitrogen oxide inhalation from « whippits » (300 whippits per day). Potential co-exposure: positive screening results for amphetamine	Symptoms: ascending limb paraesthesiae, progressive balance difficulties. Neurological examination: pseudoathetosis in upper limbs, flexor plantars, reduced vibration sensation to the hips bilaterally and inhability to stand due to sensory ataxia. Diagnostic: myeloneuropathy Follow-up: at 4 and 8 weeks - short distance mobility using zimmer frame 4 weeks after treatment - independent mobility and significant improvement of spinal cord MRI ty 12 weeks after vit. B12 treatment	Massey et al., 2016
Case reports	Cases 1: 25 year -old worker Exposure: mask connected to a plastic bag connected with a whipped cream pump loaded with dinitrogen oxide. History of dinitrogen oxide abuse. Case 2: 35 year-old man Exposure: gas mask connected to a gas cylinder marked dinitrogen oxide. No further information.	Case 1: found dead in his home. At autopsy, no pathological findings, no indication of alcohol, licit or illicit drugs. Case 2: dead at home. No further information	Bäckström et al., 2015
Case study	Case: 27 year-old women Exposure: dinitrogen oxide abuse over 3 years (average of 100-200 "whippit" cartridges daily on 3 or 4 days per week) Medication: vit. B12	Symptoms: abdominal pain and inability to urinate, lower extremities weakness, pins and needles sensation in lower extremities for approximately 1 year Clinical chemistry: vit. B12 deficiency MRI: indicative of spinal cord degeneration Lost to follow up	Pugliese et al., 2015
Case report	Case: 20 year-old women Exposure: Dinitrogen oxide whippets, no further information	Symptoms: bilateral paraesthesia of the lower extremities, limb weakness, difficulty walking, numbness in the hands, bowel incontinence Clinical examination: bilateral hip flexor weakness, thoracic sensory-level deficit, reduced proprioception distally in the bilateral lower extremities MRI: consistent with demyelination of the dorsal regions of the spinal column and demyelination	Duque et al., 2015

		around the central canal. Negative brain MRI. Nerve conduction study: in line with demyelinating polyneuropathy Follow up: 1-month follow-up - the patient showed remarkable improvement of her neurologic symptoms with intact neurological sensitivity and no symptoms of muscular weakness	
Case study	Case: 35 year-old man with dinitrogen oxide-related myeloneuropathy Exposure: daily consumption of over 100 canisters of dinitrogen oxide (so-called "whippets"). Coexposure with 6 mg Xanax and 3-6 beers daily	Symptoms: complaint of disorientation, worsening weakness, flexion contracture of the fingers, urinary urgency, low of balance Clinical chemistry: Normal vit. B12, other parameters not assessed (MMA, homocysteine). Examination: positive Babinski signs, abnormally brisk reflexes of four limbs, diminish sensation in a stocking and glove distribution, ataxia MRI: Cervical spinal cord lesions in dorsal column Follow up: recovery has been slow at the date of publication (no further information)	Rhainbolt et al., 2015
Case study	Case: 19 year-old man Exposure: occasional abused oxycodone, alcohol, huffed dinitrogen oxide canisters several times per week (20 pound canisters of dinitrogen oxide at one setting).	Symptoms: progressive numbness and weakness in all extremities. Progression to an inability to deambulate and numbness in the distal extremities. Examination: proximal weakness of 3/5 in the deltoids bilaterally and distally of 2/5. In the lower extremities, weak iliopsoas muscle group (4/5) and distal groups were 3/5., hyperreflexia, absent sensation to touch and pin prick in the distal extremities as well as vibration and proprioception. Clinical chemistry: no effect on Vit. B12 and folate levels MRI: cervical spinal cord lesions. Follow-up: atypical complete resolution 36 hours after methylprednisolone treatment	Ghobrial et al., 2012
Case report	Case: 19 year-old man Exposure: recreational use of dinitrogen oxide over a period of 2 months up until the time paraesthesia become worse. Approximately 500-600 cartridges from dinitrogen oxide filled balloons during 5-6-hour sessions, 4-5 times per 3-year smoking history.	Symptoms: 1-month history of limb numbness and gait imbalance. Expansion of numbness to upper limb and entire body. Examination: absent of brachioradialis and knee jerck reflexes, negative plantar response, reduced muscle strength (4/5), positive Romberg sign, ataxia, decreased pinprick and temperature sensation in distal extremities. Clinical chemistry: megaloblastic red blood cells, vit. B12 deficiency other parameters were normal. Motor nerve conduction: prolonged distal latency in bilateral median nerves, prolonged F latency, reduced sensory nerve action potential. Electromyography: no spontaneous activity in some muscles. Decreased number of motor unit	Hsu et al., 2012

		potentials in these muscles. MRI: cervical spinal cord lesions (posterior and anterior column), no findings in lateral column Final diagnosis: polyneuropathy and myelopathy. Follow-up: - One week after start of treatment: decreased numbness, mild sensory ataxic gait remained - Full recovery after cessation of exposure without any neurologic sequela	
Case report	Case: 28 year-old man Exposure: 2-year history of recreational abuse. Dinitrogen oxide bulb use. At least 80 bulb per day (1 bulb = 8g dinitrogen oxide under pressure)	Symptoms: bilateral numbness and weakness in both lower limbs, difficulty walking and maintaining balance Examination: absent Lhermitte's sign, subjective sensory deficit to soft touch, decrease proprioception in both feet and vibration sense to the level of both knees. Romberg's negative. Clinical chemistry: increased homocysteine levels, normal vit. B12 levels No other test to exclude other cause than dinitrogen oxide abuse of vit. B12 deficiency. Follow up: - no improvement after 3-month (denies ongoing use of dinitrogen oxide)	Richardson et al., 2010
Case report	Case: 41 year-old man Exposure: dinitrogen oxide abuser: 4-5 cans per day, about 2000 ml/can for more than 10 years.	Symptoms: motor clumsiness and distal paresthesia in the four limbs. Clinical chemistry: abnormal megaloblastic red blood cells and vit. B12 deficiency Motor conduction: sensory-motor axonal polyneuropathy Electromyography: Abnormal visual, brainstem and somatosensory evoked potential MRI: Spinal cord degeneration in the posterior and lateral columns (cervical)	Lin et al., 2007
Case report	Case: 23 year-old female Exposure: Dinitrogen oxide "nanging" initial consumption of 1 box of 10 whipped cream whippet bulbs per day to 13 boxes per days for 6 weeks. History of intravenous drug abuse	Symptoms: profound tetraparesis. Examination: absent pelvic reflexes. Clinical chemistry: raised creatinine and urea (acute renal failure), normocytic anaemia, depressed vit. B12 level Nerve conduction studies: peripheral axonal sensorimotor neuropathy. Encephalogram: severe diffuse encephalopathy MRI: cervico-thoracic spinal cord lesions Diagnostic: toxic myeloneuropathy due to dinitrogen oxide	Shulman et al., 2007
Case report	Case: 33 year-old man Exposure: 4-week exposure to dinitrogen oxide "whippits", daily	Symptoms: fixed delusions No motor or sensory deficit Clinical chemistry, increased MMA and	Sethi et al., 2006

	inhalation.	homocysteine levels, normal vit. B12 level Follow-up: 2-weeks after start of treatment: fixed delusion resolved. Lost to further follow-up	
Case report	Case: 26 year-old woman Exposure: weekly dinitrogen oxide inhalation for 2-3 month for recreational purpose	Symptoms: weakness and numbness of lower limbs Examination: Strength was 3/5 for lower limbs and 4/5 for upper limbs. Absent of tendon reflex and plantar response, decreased vibration sensation in the feet and legs, respiratory difficulty Nervous conduction and electromyography: sensory-motor demyelinating polyneuropathy. Clinical chemistry: increased MCV, vit. B12 deficiency MRI: abnormal demyelinating lesion affecting the posterior column (cervical spine) Follow-up: symptoms resolved completely after 2 month treatment	Wu et al., 2006
Case report	Case: 23 year-old patient Exposure: dinitrogen oxide abuse	Symptoms: diffuse paresthesia and sensory loss Mild reduction of vit. B12, high levels of MMA and homocysteine Resolution after cessation of exposure	Waclawik et al., 2003
Case report	Case: 55 year-old man	Multiple neurological abnormalities, low serum vit. B12. Improvement after cessation of exposure and vit. B12 treatment.	Iwata et al., 2001

MRI: magnetic resonance imaging, MMA: Methylmalonic acid; MCV: mean corpuscular volume

Table 37: Summary table of human data on STOT RE – Occupational exposure

Type of study/report	Test substance, Route of exposure, relevant information about the study	Observations	Reference
Observational study: Experimental at workplace	**Participants:** 30 exposed to dinitrogen oxide (13 ♂, 17 ♀) (1 week with gaseous anaesth. vs 1 week with non-gaseous anaesth. with a 2-week interval) and 20 controls **Exposure:** [N2Oa] (ppm) breathing zone during 3 hours - SW: 50.9 (20.8) - EW : 54.2 (22.1) [N2Ou] (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)	Lucchini et al., 1996

Observational study: Cross-sectional multi-centre study	**Participants:** 112 exposed and 135 controls **Exposure:** [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.	Lucchini et al., 1997
Observational longitudinal study	2 working weeks in a 1-year study **Participants:** 38 exposed (17 ♂, 21 ♀) and 23 control (2 ♂, 21 ♀) **Exposure:** [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 performance in colour word vigilance test	Scapellato et al., 2008

SRT: Simple reaction time; SW: start of week, EW: end of week, GM: geometric mean, SD: Standard deviation

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Animal data

Misra et al., 2020, reported in Wistar rats (n=6 male/group) exposed 2h/d for 60 days at 500,000 ppm and O_2 in ratio 1:1 an alteration of spontaneous locomotor activity (reduction in total distance, time moving, number of rearings and time resting) and grip strength. Time rearing and the number of stereotypic counts were not statistically significantly changed. The same conditions of exposure were used in the Singh et al., 2015 study, except that exposure was 2 hours per day instead of 1.5 hour per day. Decreased body weight gain was also seen in the exposed animals. Blood homocysteine levels was significantly increased whereas no significant changes was noted for vitamin B12 or folic acid levels. Although glutathione and total antioxidant capacity were decreased in the plasma of exposed rats, serum malondialdehyde (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 astrocytes proliferation, related to oxidative stress and glutamate neurotoxicity.

In the same laboratory, similar results were obtained by Singh et al. (2015). They investigated behavioural and histopathological changes in male Wistar rats. Rats (6 males per groups) 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 h 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 TAC 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 neurobehavioral 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.

Following rat exposure to 700,000 ppm N_2O, 4h/day, 5d/w for six months, Dyck et al., 1980, 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.

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

In mice, Fung et al., 1993 used lower levels of N_2O. Mice were exposed 8h per day for 8 consecutive days at 1000 or 2000 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.

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.

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 not relevant for classification STOT RE. No data are available at lower dose levels that would be relevant for classification.

Only two mouse studies investigated the potential effect of N_2O (Rice et al., 1985 and Fung et al., 1993). Rice et al. (1985) did not report histopathological or weight changes in the brain at concentrations up to 500,000 ppm N_2O in a 14-week inhalation study. In a 8-day sub-acute study, Fung et al. reported doubtful effects on the locomotor activity of animals and dose-related changes in the stereotypic behaviour of mice at ≥ 1000 ppm. No such change on stereotypic behaviour was noted in rats at higher exposure levels (up to 500,000 ppm) in Singh et al. (2015) and Misra et al. (2020). Nevertheless, differences in methods (duration of exposure) and test animals (different strain), make the comparison difficult. In addition, the observed effects in Fung et al., 1993 were all observed at dose levels relevant for classification STOT RE (≤2500 ppmV for STOT RE 2 classification for a 8-day study).

Severe ataxia, peripheral nerve degeneration and spinal cord degeneration and demyelination was noted by following 18, 30, 35 and 56-day continuous exposure to 150,000 ppm dinitrogen oxide in monkeys (n=1/group) (Dinn et al., 1980). Central grey matter and anterior horn cells were normal in the spinal cord. Brain and motor nerves were normal in these monkeys. No haematological changes were noted in this study. The study is considered of low reliability as only 1 animal per group was used and as no concurrent control

were used in the study. Nevertheless, the changes were very consistent with the observed effects in other animal species and human.

Human data

Information regarding specific target organ toxicity following repeated exposure in humans is mainly provided by extensive literature concerning adverse health effect in occupational setting and dinitrogen oxide recreational use. For the purpose of classification, evidence from epidemiological studies and case reports are considered in a weight-of-evidence approach.

Human data - Dinitrogen oxide abuse/recreational use

- Poison control centre data

Dinitrogen oxide, commonly known as "laughing gas", is used among other things in whipped cream siphon cartridges. Recreational inhalation has been on the rise since 2018. Following a request by the French Health Products Safety Agency (ANSM), the French poison control centres (PCCs) analysed 66 cases recorded between 1 January 2017 and 31 December 2019. They concerned young people, in a mostly festive context, consuming quantities ranging from just a few cartridges to several hundred a day, over several months. The most frequently reported effects were neurological and neuromuscular disorders (paraesthesia, tremor in the extremities and muscle pain). Four people reported symptoms suggestive of peripheral neuropathy following chronic inhalation of dinitrogen oxide.

A total of 63 medical records (66 individuals) concerned exposure to dinitrogen oxide in a context of recreational use/ addiction. Of these, 39 were men and 27 were women. This equated to a M/F sex ratio of 1.4, indicating a male preponderance. Users were young, with a median age of 21 years old, ranging from 14 to 49 years, and 54.5% of cases concerned people between 20 and 25 years of age. The vast majority of exposures occurred in 2019, the last year of the study, with 46 cases compared to 10 cases for the years 2017 and 2018 respectively, underlining the increase in consumption. The regions of Hauts-de-France (northern France) and Île-de-France (including Paris) were the most concerned, each accounting for a quarter of cases. When this information was reported, i.e. in only 57.6% of cases, the type of dinitrogen oxide consumed was almost exclusively dinitrogen oxide contained in cartridges for food use, available over the counter and inhaled via balloons. The duration and history of consumption varied widely. These ranged from occasional consumption at a party to consumption several times a day for months. Similarly, the individuals reported taking quantities ranging from just a few cartridges to several hundred a day, with great variety in the total quantities. Lastly, in 47% of cases, the dinitrogen oxide was inhaled in the home of the exposed person or of their family or friends. In 13.6% of cases, consumption took place in a nightclub/bar, 10.6% at a party where the location was not specified, and 6.1% during a student integration weekend. Fifty-nine people reported adverse symptoms following inhalation of dinitrogen oxide. Neurological and neuromuscular problems were the most common signs. At least one neurological and neuromuscular symptom was reported in 42 cases (71.2%). Among them, 73.8% had at least one motor or sensory symptom such as paraesthesia, tremor in the extremities, or muscle pain. Four people reported symptoms suggestive of peripheral neuropathy following chronic use of this gas. Half of the 42 cases suffered from at least one symptom such as headaches / dizziness / balance disorders.

Of the 59 cases with adverse symptoms, 40 were mild, 14 were moderate and 5 were severe. For three of them, the symptoms were due to consumption with concurrent use of one or more psychoactive substances (alcohol with or without drugs) at a party. For one person suffering from cardiorespiratory arrest, heart disease was later discovered in hospital. The two others experienced convulsive episodes, with one person falling into a coma and suffering myoclonus. The two other severe cases involved chronic consumption of dinitrogen oxide – around ten cartridges a day for one, and around forty a day for the other – at home and without taking any other psychoactive substances. Both had neurological symptoms. Since the beginning of 2020, more new cases have been reported to the French poison control centres (PCCs). Some presented serious neurological signs, confirmed by medical imaging. These involved regular consumers occasionally increasing their intake, a situation that had neurological consequences.

Because the quantities consumed were not systematically reported to PCCs, it was not always possible to link the reported symptoms to the claimed quantities. One way to quantify this exposure would be to measure dinitrogen oxide levels in urine, but these tests are rarely performed and their interpretation is

subject to great uncertainty. These tests cannot therefore provide routine assurance of actual exposure to dinitrogen oxide (ANSES, 2020, report published in French).

- Published case reports

According to the systematic review of case reports in Medline (Oussalah et al., 2019), 100 cases with individual data were published from 1966 to 2018. Median age was 27 years, the male: female distribution ratio was 60:40. The case included in the review was repeatedly exposed to dinitrogen oxide with a minimum consumption of one cartridge per month. 76% had regular exposure. Patients were exposed to dinitrogen oxide in the setting of recreational use (57%) or surgery (25%). Median number of cartridge per day: 25, median duration of exposure: 0.7 year.

The three main diagnoses were subacute combined degeneration (28%), myelopathy (26%) and generalised demyelinating polyneuropathy (23%). In patients that underwent MRI, changes in the spinal cords (T2 signal hyperintensity) was noted in 68% of the patients.

Neurological symptoms were reported in 96% of the patients, including paraesthesia in extremities (80%), walking impairment or unsteady gait (58%), weaknesses (43%) fallings or equilibrium disorders (24%), Lhermitte's signs (15%) and ataxia (12%).

Clinical chemistry analysis revealed that at least 72% of the patients had haematological abnormalities (e.g. low haemoglobin level). Vitamin B12 deficiency was noted in 71% of the patients and elevated methylmalonic acid (MMA) and homocysteine levels in 90 and 94% of the patients, respectively.

Univariate analysis of the data by Oussalah et al., 2019 found that among all the investigated variable, age (> 40-year), vit. B12 concentration (≤ 74 pmol/L) and MCV (> 100fL) were associated with a short dinitrogen oxide exposure, mostly associated with surgery and a more severe clinical picture.

Data on the amount of dinitrogen oxide exposure was available in 28 patients. The amount of dinitrogen oxide was not significantly correlated with any biological variable. 75% of the regular users were recreational users.

The neurological disorders reported in other case reports were similar to the ones include in this systematic review study. Almost all the cases reported in the table above were included in the systematic review except some published paper not including data on biological parameters or where preventive treatment with vit. B12 before dinitrogen oxide exposure was used.

Several follow-up studies revealed persistent symptoms. The duration of follow-up was in most of the cases only of few month. Nevertheless, in Sleeman et al., 2016, full recovery was not observed after 2-year follow-up. Among the reported rates for persistent numbness and accidental injury were 4.3% and 1.2%, respectively (Oussalah et al., 2019). Garakani et al., 2016 also focus on neurological sequel and psychiatric disorders following dinitrogen oxide abuse. In the 59 cases studied by the authors for whom follow-up information was available, neurological symptoms improved in 46 cases and persisted in 3 cases. Symptoms fully resolved in 10 cases. In one case the patient's symptoms almost entirely resolved, but he later committed suicide after relapsing. It has to be noted that as the substance is use in the context of abuse, it can be difficult to quantify truly and accurately dinitrogen oxide exposure and they may be uncertainties on the stop of exposure.

Human data- Occupational exposure

- Cases report:

Dreyfus et al. (2008) 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).

A direct relationship between N_2O exposure and CTE is uncertain in these 3 cases, because of 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):

Lucchini et al. (1996) conducted a study in an Italian hospital. In this study, authors 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 hours 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 measurement 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. On the last day of the gaseous anaesthesia week, mean N_2O air concentration was 54.2 (SD= 22.8) ppm and mean urine concentration was 25.6 (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 shows 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. (1997), 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. 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 preceding the first testing a training session was organized in order to limit the learning effect of neurobehavioral testing. During this session, a questionnaire for neuropsychological symptoms (EURO-QUEST) was administered together with the block design subtest from the WAIS battery measuring visuospatial and motor skills, Mood scale measuring stress and arousal state, Colour word vigilance test, which is a complex reaction time test. 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 95^{th} percentile were 23.2 ppm and 127 ppm on the 1^{st} 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 95^{th} percentiles were 7.1 µg/L and 12.4 µg/L on the 1^{st} day, 7.8 µg/L and 21.5 µg/L on the last one. No statistical difference was observed between exposed and control subjects for neurobehavioral 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 neurobehavioral 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 neurobehavioral functions. Neurobehavioral 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 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 colour word vigilance (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 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. 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 consists 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. There was a rough dose–effect relationship between increasing N_2O level of exposure and impairment of neurobehavioral performance. Since they were not significantly different, Groups B and C were considered equal to Group A and the two groups were collapsed into a single unit. Therefore, subjects were then categorized in two classes, according to the level of N_2O urinary concentrations being below or above 27 µg/L. The weekly profiles of reaction times for the two groups were not parallel. 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 effects of anaesthetic gases over a week of exposure. No contrast was significant for the stress. 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.

In summary, information on low-dose human exposure is available. The effects observed in three studies of good quality consistently showed that neurobehavioral changes could occur following repeated low exposure levels to dinitrogen oxide. However, there are some uncertainties concerning the strength of the effects due to co-exposure with other anaesthetic gases at low concentration. Although cumulative effects were noted thorough a working weeks, the effects were reversible following the week-end.

Mechanism of action

The neurological effects are the results of the **irreversible inactivation of methionine synthase function** by oxidation of the Co^+ ion of vitamin B12. This results in the decrease in the synthesis of deoxythymidine and thymidine and DNA synthesis and myelin among other products. 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. 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., 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.

10.12.2 Comparison with the CLP criteria

In rats, severe and adverse effects in the meninges, brain and the spinal cord were noted in several studies. Nevertheless, only high concentrations of dinitrogen oxide were tested, not relevant for classification STOT RE 2.

In mice, no histopathological findings in brain were noted up to 500,000 ppm in Rice et al. (1985). Nevertheless, the behavioral changes observed in Fung et al. after 8-day exposure to 1000 ppm could support a classification of dinitrogen oxide as STOT RE 2.

In human, there are numerous case of reported demyelinated polyneuropathy, subacute combined degeneration of the spinal cord. Although some of the cases were due to recreational use, in some cases, severe effects were seen under controlled dinitrogen oxide conditions (e.g. pain management). The full reversibility of the effect is questionable and longer follow-up would be needed to conclude on this point with more certainty. Epidemiological studies from occupational exposure although support that neurobehavioral effects may occur at low dose levels.

According to the CLP criteria, substances are classified in category 1 if they met the following criteria: *Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies*.
Specific target organ toxicity (repeated exposure) means specific, target organ toxicity arising from a repeated exposure to a substance. All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed are included.

Overall, based on good quality evidence from human cases and epidemiological studies, dinitrogen oxide induce after repeated exposure effects on the central nervous system. A classification of dinitrogen oxide as STOT RE for the nervous system is thus warranted.

Nevertheless, according to the ECHA guidance on classification criteria, "*where the same target organ toxicity of similar severity is observed after single and repeated exposure to a similar dose, it may be concluded that the toxicity is essentially an acute (i.e. single exposure) effect with no accumulation or exacerbation of the toxicity with repeated exposure. In such a case classification with STOT-SE only would be appropriate*". Human recreation abuse cases support that repeated-dose exposure to dinitrogen oxide can lead to exacerbation of toxicity as shown by the severe effects such as myelopathy with potential irreversible consequences. The available mode of action also support that STOT RE may be more relevant that STOT SE as it will be the accumulation of homocysteine levels and/or deficiency of methyl constituent that will be responsible of the observed effects.

10.12.3 Conclusion on classification and labelling for STOT RE

Based on significant adverse health effects in nervous system of humans exposed to dinitrogen oxide, classification of dinitrogen oxide for the nervous system as primary target organ system is considered relevant. As the most severe effects on the nervous system are noted following repeated exposure in human, classification as STOT RE 1 is considered appropriate.

An SCL is not proposed as dinitrogen oxide did not induce target organ toxicity at a dose level clearly below the guidance values according to CLP regulation.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS proposed to classify N_2O as STOT RE 1; H372 (nervous system) based on neurological effects after repeated exposure in humans.

The DS considered the following arguments when concluding on the classification:

- In rats, severe adverse effects in the meninges, brain and the spinal cord were noted in several studies. Since only high concentrations of N_2O were tested, these effects are not relevant for classification as STOT RE 2.
- In mice, no histopathological findings in brain were noted up to 500000 ppm in Rice *et al.* (1985). Nevertheless, the behavioural changes observed in Fung *et al.* (1993) after 8-d exposure to 1000 ppm could support a classification of N_2O as STOT RE 2.
- In humans, there are numerous cases of reported subacute combined degeneration of the spinal cord. Although some of the cases were due to recreational use, severe effects were also seen under controlled conditions of N_2O exposure (e.g., pain management). The full reversibility of the effect is questionable and longer follow-up would be needed to conclude on this point with more certainty.
- Results from occupational exposure studies support that these neurobehavioral effects may also occur at low concentration levels.
- According to the ECHA guidance on the application of the CLP criteria (CLP guidance, 2017), "*where the same target organ toxicity of similar severity is observed after single and repeated exposure to a similar dose, it may be concluded that the toxicity is*

essentially an acute (i.e. single exposure) effect with no accumulation or exacerbation of the toxicity with repeated exposure. In such a case classification with STOT SE only would be appropriate". Human recreational abuse cases support that repeated exposure to N_2O can lead to exacerbation of toxicity as shown by the severe effects such as myelopathy with potential irreversible consequences. The mode of action (irreversible inactivation of vitamin B12 through oxidation of the cobalt ion) proposed by the DS also supports that STOT RE may be more relevant than STOT SE as the accumulation of homocysteine levels and/or deficiency of methyl constituent will be responsible for the observed effects.

Based on significant adverse health effects in the nervous system of humans exposed to N_2O, classification of N_2O for the nervous system as primary target organ system is considered relevant. As the most severe effects on the nervous system are noted following repeated exposure in human, classification as STOT RE 1 was considered appropriate by the DS.

A specific concentration limit (SCL) was not proposed by the DS as N_2O did not induce target organ toxicity at a concentration level clearly below the guidance values according to the CLP Regulation.

Comments received during consultation

Two MSCAs agreed with the proposed classification.

Comments from six industry representatives argued against the proposed classification based on effect concentrations found in animals above the guidance values for STOT RE. Regarding the studies, the industry representatives considered there were several issues:

- All of the available animal data had limitations. These studies were not conducted according to GLP or any relevant test guideline.
- Many studies included a single concentration and as a result, no concentration-response relationship can be established.
- The number of animals per group were generally lower than recommended in test guidelines, thereby making a statistical analysis (if undertaken) difficult to interpret.
- Where adversity was observed this occurred at concentrations that exceeded the maximum recommended concentration for repeated dose inhalation studies, without consideration of hypoxic effects.
- Similar to the human volunteer studies under STOT SE, limitations hamper a reliability assessment for the human data. These limitations include no standardised studies, unclear exposure, potential co-exposure and other possible confounders.
- The effects observed in the case studies may have been confounded with factors such as co-exposure to other drugs. These confounders have not been discussed or considered.
- No reliability assessments were or can be undertaken for the human data.

Assessment and comparison with the classification criteria

Animal data

The DS discussed seven non-guideline inhalation neurotoxicity studies in animals, four in rats,

two in mice and one with monkeys. All of these had limitations including lack of information on GLP status, unclear substance purity and origin and lack of information on general toxicity. The rats were exposed (whole body) for >400000 ppm and exposure duration varied from 1.5h/d to 8h/d from 7 days to six months. As the exposure levels were clearly above the GV for STOT RE, only the main findings are presented. These studies provide supportive information on possible effects after repeated exposure to N_2O.

At 500000 ppm (whole body, for 1.5 - 2h/d for 60 days), clinical signs (ataxia), structural changes in brain and spinal cord (vacuolation of neurons, demyelination) were observed as well as neurochemical effects (hyperhomocysteinemia, decrease in methionine synthase activity, decreased glutamate and vitamin B12 levels) (Singh *et al.,* 2015; Mistra *et al.,* 2020). Both Singh *et al.* (2015) and Mistra *et al.* (2020) reported behavioural changes, including reductions in locomotor activity and grip strength, following repeated high concentration exposure to N_2O. Dyck *et al.* (1980) found no changes after exposing rats to 700000 ppm for 4h/d, 5d/week for 6 months, but the investigation was limited to recording nerve conduction, electromyographic abnormalities in caudal nerve and morphometric and teased fibre abnormalities. Hayden *et al.* (1974) found cortical cell count changes in the brain at 400000 ppm after exposure 8h/d for 7-14 days. However, this information could only be obtained from an abstract and is therefore of limited value.

Mice were exposed to lower concentrations (i.e., below GV for STOT RE) by Fung *et al.* (1993), at 1000-2000 ppm (whole body) N_2O for 8h/d for 8 days. Reduced locomotor activity and neural cell counts were noted at the end of the period, but the changes were not statistically significant. Rice *et al.* (1985) exposed mice to 500000 ppm for 4h/d, 5 d/week for 14 weeks but did not find any statistically significant changes apart from lower body weight gain.

In monkeys, continuously exposed to 150000 ppm for 56 days, severe ataxia, peripheral nerve degeneration, spinal cord degeneration and demyelination was observed (Dinn *et al.,* 1980). However, only a single monkey/group was used in this study which means it has a low reliability.

Human data

The DS summarised numerous case studies and retrospective case series which reported on recreational use of N_2O. In addition, a poison centre analysis and three occupational exposure studies in the form of observational studies were included.

Poison control centre data

The French poison control centres analysed 66 cases, including 39 men and 27 women, recorded between 1 January 2017 and 31 December 2019. The median age was 21 years, ranging from 14 to 49 years, and 54.5% of the cases concerned people between 20 and 25 years of age. The type of N_2O consumed was almost exclusively N_2O contained in cartridges for food use, available over the counter and inhaled via balloons. The duration and history of consumption varied widely, ranging from occasional consumption to several times a day for months. The individuals reported taking quantities ranging from just a few cartridges to several hundred a day, and with great variety in the total quantities. Fifty-nine people reported adverse symptoms following inhalation of N_2O. Neurological and neuromuscular problems were the most common signs. Forty were mild, 14 were moderate and 5 were severe. For three of them, the symptoms were due to consumption with concurrent use of one or more psychoactive substances (alcohol with or without drugs) at a party. For one person suffering from cardiorespiratory arrest, heart disease was later discovered in the hospital. The two others experienced convulsive episodes, with one person falling into a coma and suffering

myoclonus. The two other severe cases involved chronic consumption of N_2O – around ten cartridges a day for one person, and around forty a day for the other – at home and without taking any other psychoactive substances. Both had neurological symptoms. At least one neurological and neuromuscular symptom was reported in 42 cases (71.2%). Among them, 73.8% had at least one motor or sensory symptom such as paraesthesia, tremor in the extremities, or muscle pain. Four people reported symptoms suggestive of peripheral neuropathy following chronic use of N_2O. Half of the 42 cases suffered from at least one symptom such as headaches, dizziness or balance disorders.

Case report series

Oussalah *et al*. (2019) reviewed 100 cases published between 1966 and 2018 where individuals were exposed to at least one cartridge/month between 1966 and 2018. 76% had regular exposure. The three main diagnoses were subacute combined degeneration of the spinal cord (28%), myelopathy (26%) and generalised demyelinating polyneuropathy (23%). In patients that underwent Magnetic Resonance Imaging (MRI), changes in the spinal cord (T2 signal hyperintensity) were noted in 68% of the patients.

Neurological symptoms were reported in 96% of the patients, including paraesthesia in extremities (80%), walking impairment or unsteady gait (58%), weaknesses (43%), fallings or equilibrium disorders (24%), Lhermitte's signs (15%) and ataxia (12%).

Clinical chemistry analysis revealed that at least 72% of the patients had haematological abnormalities (e.g., low haemoglobin level). Vitamin B12 deficiency was noted in 71% of the patients and elevated methylmalonic acid and homocysteine levels in 90 and 94% of the patients, respectively.

The neurological disorders reported in other case reports were similar to the ones included in this systematic review study. Almost all the case reports presented by the DS were included in the systematic review by Oussalah *et al.* (2019) except some published papers not including data on biological parameters or where preventive treatment with vitamin B12 before N_2O exposure was used.

Several follow-up studies revealed persistent symptoms. The duration of follow-up was in most of the cases only a few months. Nevertheless, in Sleeman *et al.* (2016), full recovery was not observed after 2-year follow-up. However, this was based on a single case report of a 29-year-old woman. The reported rates for persistent numbness and accidental injury were 4.3% and 1.2%, respectively (Oussalah *et al.,* 2019). Garakani *et al.* (2016) also focussed on neurological sequelae and psychiatric disorders following N_2O abuse. In the 59 cases for which follow-up information was available, neurological symptoms improved in 46 cases and persisted in 3 cases. Symptoms fully resolved in 10 cases. It is noted that there may be uncertainties surrounding the full stop of exposure in these follow-up studies. Overall, it seems most symptoms resolved after a while, but there are exceptions where some of the symptoms persisted.

Occupational exposure

Dreyfus *et al.* (2008) reported the cases of two anaesthetists who developed a chronic toxic encephalopathy (CTE) after many years of exposure to anaesthetic gases in operating rooms (where ventilation was deficient for three years). The authors reported high levels of anaesthetics gases such as N_2O (mean 311 ppm, peaks 1600 ppm) and halogenated gases (mean 16 ppm, peaks 1600 ppm). A direct relationship between N_2O exposure and CTE is uncertain in these cases, because of anaesthetists are also exposed to many other neurotoxic

agents including halogenated anaesthetic gases.

Lucchini *et al.* (1996) conducted a study in an Italian hospital, examining 30 operating room workers. The workers were exposed to N_2O for 1 week with gaseous anaesthetics and given a personal sampling device for N_2O for 3 hours. On the last day of the gaseous anaesthesia week, mean N_2O air concentration was 54.2 (SD=22.8) ppm and mean urine N_2O concentration was 25.6 (SD=22.1) µg/L. The study showed a prolonged reaction time and increased serum prolactin levels in exposed workers only when they worked with gaseous anaesthesia. Notably, no information on potential co-exposure was provided and therefore the effects measured may not be solely caused by N_2O.

Lucchini *et al.* (1997) also conducted a multi-centre study in Italy with a group of 112 workers from 10 Italian hospitals who were exposed to anaesthetic gases (N_2O and isoflurane). For atmospheric N_2O, the geometric mean and 95th percentile were 23.2 ppm and 127 ppm, respectively, on the 1st day before the shift and 20.6 ppm and 114 ppm, respectively, on the last day of the shift; the corresponding values for isoflurane were 0.4 ppm and 3.8 ppm, and 0.3 ppm and 2.7 ppm. No statistical difference was observed between exposed and control subjects for neurobehavioral effects, stress and arousal levels.

Scapellato *et al.* (2008) investigated effects of N_2O exposure on behavioural functions in workers of an Italian hospital for one year. In subjects with urinary concentrations of N_2O below 27 µg/L, there was a linear decrease in reaction times in the colour word vigilance test results from Monday morning to Friday evening, indicating an effect on learning. In subjects with N_2O urinary concentrations above 27 µg/L, the mean reaction time results were essentially steady across a work week, indicating that learning performances may have been impaired. A similar effect was observed for 'arousal' in the mood scale measures suggesting a cumulative effect of anaesthetic gases over a week of exposure.

In summary, neurobehavioral changes could occur following repeated low exposure levels to N_2O. However, there was possible co-exposure with other anaesthetic gases. In addition, the cumulative effects during a working week were reversible following the weekend.

RAC notes that human data is often hampered by unclear exposure levels and potential confounders. In some but not all of the available studies, confounding factors were identified. Although the value of individual data is limited, the high number of case reports can be considered sufficient evidence to support effects after repeated exposure in a weight of evidence assessment.

<u>Proposed mechanism of action</u>

The neurological effects are proposed to be the result of the irreversible inactivation of methionine synthase function by oxidation of the Co^+ ion of vitamin B12. This results in the decrease in the synthesis of deoxythymidine and thymidine and DNA synthesis and myelin among other products. Irreversible inactivation of methionine synthase by N_2O is identical between species.

RAC recognises that studies in both animals and humans indicate that exposure to N_2O is associated with deficiency in vitamin B12. Vitamin B12 deficiency relates amongst others to neuro(developmental) effects. Therefore, it is plausible that the resulting neurological effects seen after N_2O exposure are at least partly a consequence of the vitamin B12 deficiency. It is also noted there is no clear evidence that the effects could be attributed to hypoxia in the presented studies. RAC notes that it not necessary to demonstrate a mode of action for classification.

Applicability of classification as STOT RE 1 or 2

According to the CLP Regulation, *'Substances are to be classified for target organ toxicity after repeated exposure in category 1 on the basis of:*

- *reliable and good quality evidence from human cases or epidemiological studies; or*
- *observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.'*

Guidance values are not applicable for effects observed after repeated exposure in humans. According to the CLP Regulation, Annex I, section 3.9.2.7.3, the following effects shall be taken into consideration for classification (only relevant criteria cited):

'*(b) significant functional changes in the central or peripheral nervous systems or other organ systems, including signs of central nervous system depression and effects on special senses (e.g. sight, hearing and sense of smell);'*

-> After N_2O exposure, neurological effects are consistently observed in humans including subacute combined degeneration of the spinal cord, myelopathy and generalised demyelinating polyneuropathy. A significant number also suffered from changes in the spinal cord observed with MRI techniques.

Neurological symptoms included paraesthesia in extremities, locomotor activity impairment such as walking impairment or unsteady gait, weaknesses, fallings or equilibrium disorders, Lhermitte's signs and ataxia. In some cases, effects were irreversible or only partly reversible.

-> In support of these findings, changes (mostly suppression of) in locomotor activity were noted such as lower grip strength. Nerve and spinal cord degeneration were also observed in animals (rats and a monkey).

'*(c) any consistent and significant adverse change in clinical biochemistry, haematology, or urinalysis parameters;'*

-> The most notable and consistent effects in both humans and animals are vitamin B12 deficiency and increased homocysteine levels. Vitamin B12 deficiency can be related to neurological findings.

Such effects observed in humans can normally be considered sufficiently clear and adverse for classification. However, RAC has also considered some additional factors in their assessment.

In relation to whether effects arising from the recreational use of a substance should be considered for classification, RAC notes the provisions in CLP Article 9(5): '*When evaluating the available information for the purposes of classification, the manufacturers, importers and downstream users shall consider the forms or physical states in which the substance or mixture is placed on the market and in which it can reasonably be expected to be used'.*

Although there were some comments received during consultation of the CLH report indicating that non-intentional use should not be taken into account, Article 9(5) indicates that the *form* for its intended use must be considered in the evaluation for classification. Clearly the form of N_2O as placed on the market is the same as in the provided studies and recreational cases. Therefore, the studies with this form of the substance, whether it is from recreational use or not, can be used to support classification.

Considering the limitations of the case reports, such as unknow exposure levels, evidence from a single case report would be considered insufficient to support classification. This is also

mentioned in the CLP guidance: *'A single case report from deliberate exposure (i.e., abuse) is unlikely to provide sufficiently robust evidence to support classification without other evidence'*. In this case, the number of case reports is large. The French poison centres summarised over 66 cases and the reports series review by Oussalah *et al.* (2019) covered about 100 cases. RAC considers such a large number of case reports indicating similar, neurological effects to provide sufficiently strong evidence for classification of the intrinsic properties of N_2O.

Apart from the recreational use, there is no information on the exposure duration and the frequency. RAC notes the effects after recreational use/repeated exposure are more severe compared to a single high exposure (STOT SE). After a short/single medical or recreational inhalation exposure, there is a neurological effect that quickly (seconds to minutes) disappears after the end of the exposure (see the toxicokinetic section). These neurological effects might be more related to mechanisms like reversible receptor binding. However, the neurological effects after prolonged inhalation exposure do not reverse quickly after the end of exposure. It is a slower process in the range of days – months rather than minutes. This is likely more related to the decrease in vitamin B12 and its slow recovery and aligns with the proposed mechanism of action.

Overall, the neuronal effects observed in humans after repeated exposure are clear and sometimes irreversible. There is no indication that the severity of these effects could be obtained after a single high exposure in humans. Therefore, RAC considers it most appropriate to evaluate the findings relevant in the presented context, i.e., effects after prolonged and repeated exposure.

The available animal data indicate adverse biological and behavioural neuronal effects after repeated N_2O exposure at high concentrations, most notably in rats. These effects occurred only after exposure levels above the guidance values for classification as STOT RE in the CLP Regulation, Annex I, Table 3.9.2 and Table 3.9.3. The non-statistically significant reduced locomotor activity and neural cell counts observed in mice after exposure to low levels of N_2O (below the GV of 2500 ppm) is not considered sufficiently significant and severe for classification for STOT RE. Overall, the animal studies provide further support for the effects on the neuronal system observed in human case reports and occupational exposure data.

In summary, RAC considers that there is sufficient evidence from the large number of human case studies, reporting severe, sometimes irreversible neurological effects including subacute combined degeneration of the spinal cord and suppression of locomotor activity. These effects are supported with similar findings in animal studies. Therefore, RAC agrees with the DS and concludes that **classification as STOT RE 1; H372 (Causes damage to the nervous system through prolonged or repeated exposure) is warranted**.

Since effects were either above the guidance values or the exposure was not clear, no SCL can be derived.

10.13 Aspiration hazard

Evaluation not performed for this substance.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Evaluation not performed for this substance

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

Dinitrogen oxide is a greenhouse gas and one of the most important gaseous species currently leading to stratospheric ozone depletion (ozone-depleting gas). Dinitrogen oxide is the dominant source of reactive nitrogen to the stratosphere.
Indeed, N_2O is transported to the stratosphere and broken down in the middle stratosphere and above via photolysis (Portmann et al., 2012):
$N_2O + h\nu \rightarrow N_2 + O^{1D}$
$N_2O + O^{1D} \rightarrow N_2 + O_2$
$N_2O + O^{1D} \rightarrow 2\ NO$
The NO produced in reaction to the above reaction is the primary source of reactive nitrogen (i.e. NO_x: ozone depleting nitrogen oxide) in the lower and middle stratosphere (transport of mesospheric NO_x can be significant in the upper stratosphere). The formation of reactive nitrogen can lead to the catalytic destruction of ozone:
$NO + O_3 \rightarrow NO_2 + O_2$
$NO_2 + O \rightarrow NO + O_2$
$O_3 + O \rightarrow 2\ O_2$
NO_2 formed can also photolyse to restore NO, or react with chlorinated species. Thus, as briefly summarised in JRC, 2015 dinitrogen oxide leads to stratospheric ozone depleting nitrogen oxides (NO_x) and free radical reservoirs (e.g. HNO3) in the stratosphere (Crutzen, 1970).

N_2O has a very long atmospheric lifetime. SPARC (2013) summarized the SPARC lifetimes assessment which estimated the lifetime of dinitrogen oxide to be 123 (104-152) years (2-sigma "most likely" range). Based on observations from the Microwave Limb Sounder (MLS) and a radiative transfer model, Prather et al. (2015) recommend a lifetime of 116 ± 9 years, which is lower than the maximum likelihood SPARC estimate but within their uncertainties.

According to the classification criteria, a substance shall be classified as Hazardous to the Ozone Layer (Category 1) if the available evidence concerning its properties and its predicted or observed environmental fate and behaviour indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer.

The ozone-depleting potential (ODP) is used as a metric to compare the impact of various gas emissions on ozone and as a classification criteria in the CLP regulation. As indicated in the ECHA CLP guidance document, any substances having an ODP greater or equal to the lowest ODP of the substances currently listed in Annex I to Regulation (EC) No 1005/2009 (i.e., ODP 0.005 for Chlorofluoroethane) should be classified as hazardous to the ozone layer (category 1).

The ODP provides the global mean ozone loss for a gas per unit mass emission relative to chlorofluorocarbure CFC-11. The ODP is defined as the time-integrated global ozone depletion induced by a perturbation of an equal mass emission of gas X relative to a reference gas (always taken to be CFC-11, labelled F11 below):

$$\mathrm{ODP_X} = \frac{\int_0^\infty [\Delta \mathrm{O_3}]_{\mathrm{X}}^{P} \mathrm{d}t}{\int_0^\infty [\Delta \mathrm{O_3}]_{\mathrm{F11}}^{P} \mathrm{d}t},$$

where [□O3] is the global mean total ozone change induced by the perturbation (the P superscript refers to the pulse emission and the subscript is the perturbation compound) (Portmann et al., 2012).
According to Portmann et al., 2012, the ODP is rarely calculated using the formula above. Instead, it can be re-written in steady-state form:

$$\mathrm{ODP_X} = \frac{m_{\mathrm{F11}}}{m_{\mathrm{X}}} \frac{\Delta\mu_{\mathrm{F11}}}{\Delta\mu_{\mathrm{X}}} \frac{\tau_{\mathrm{X}}}{\tau_{\mathrm{F11}}} \frac{[\Delta \mathrm{O_3}]_{\mathrm{X}}}{[\Delta \mathrm{O_3}]_{\mathrm{F11}}},$$

where m is the mass, Δm the mixing ratio perturbation, t the lifetime and [ΔO3] is the steady-state annual and global mean total ozone change induced by the perturbation. This steady-state formulation is valid for source gases characterized by a first-order decay process. It also assumes that the ozone change is linear over the range of changes of gas X and CFC-11, which is valid for the sizes of perturbations considered here.

The ODP of N_2O has been calculated using a well-established methodology published in Ravishankara et al., 2009. The authors used the Garcia-Solomon two dimensional model. The model is described in Garcia et al. (1992), Solomon et al. (1998) and Portmann et al. (1999). This model has been used to study past and future changes in ozone and includes comprehensive chemistry, detailed radiative transfer, and dynamics including planetary and gravity wave breaking schemes. The model, under the conditions of year 2000, indicates an ODP value of dinitrogen oxide of **0.017** (Ravishankara et al., 2009). Although the value may be conservative, it is well above 0.005.

As noted in WMO (2018), ODPs depend on the background atmosphere. Revell et al. (2015) have confirmed that this is especially the case for N_2O, where ODP values are likely to be larger (by as much as a factor of two depending on levels of chlorine and methane in the stratosphere) for 2100 than in the present day.

This ODP value of N_2O is comparable to the ODP of many hydrochlorofluorocarbons that are currently being phased out under the Montreal Protocol, such as HCFC-123 and HCFC-124.

Therefore, as N_2O has an ODP of 0.017, it warrants classification as hazardous to the ozone layer in category 1, H420.

RAC evaluation of hazards to the ozone layer

Summary of the Dossier Submitter's proposal

The DS proposed classification of N_2O as Ozone 1 (H420, Harms public health and the environment by destroying ozone in the upper atmosphere). The DS cited a study in which Ozone Depleting Potential (ODP) for dinitrogen oxide was calculated. Ravishankara *et al*. (2009) used a two-dimensional model to calculate the ODP value of N_2O. The ODP of N_2O under current atmospheric conditions was calculated as 0.017, which is comparable with the ODP of some hydrochlorofluorocarbons (HCFCs) that are considered as hazardous to the ozone layer. Ravishankara *et al*. (2009) took into consideration several key factors that influence the ODP of N_2O: nitrogen oxides contribute most to the ozone depletion in the stratosphere where the ozone concentration is the largest unlike the chlorine-catalysed ozone destruction that take place in the lowest and upper stratospheres. The DS noted the following when concluding on the classification:

- Based on the knowledge that N_2O depletes the stratospheric ozone.
- Based on the World Meteorological Organization scientific assessment of ozone depletion (WMO, 2018).
- Based on the current calculated ozone depletion potential-weighed emission (Ravishankara *et al*., 2009), Joint Research Centre (JRC, 2015) indicated that N_2O is the largest of all ozone depleting substances.

Comments received during consultation

Three comments from one each from industry, an MSCA and an individual were received during the consultation. All the commenters expressed concern regarding the interpretation of the data forming the basis for the proposed classification.

The Individual noted that only 10% of N_2O impacting the ozone layer is deliberately made by man and the amount of ozone harm via N_2O deliberately created by man has only a small impact on the ozone layer when compared to the amount of ozone harm caused by deliberately created halogenated molecules. In their response, the DS noted that a risk or impact assessment is not a part of the CLP Regulation.

The Industry sector pointed out that the literature data used for classification was not new and was already considered by the registrants at the time of registration. The experts concluded the data to be conclusive but not sufficient for classification. The DS noted that there is no explanation why the data from Ravishankara *et al*. (2009) were not considered sufficient for classification.

The MSCA expressed concern regarding the method used for calculation of the N_2O ODP value in Ravishankara *et al*. (2009). The MSCA referred to the cited WMO report (2018) and Montreal Protocol (2018) in which no specific recommendations for the ODP value calculation have been made. Consequently, there is no specific method to be recommended for calculation of an ODP value for N_2O in particular. There is uncertainty about the long-term impact of N_2O on ozone due to changes in stratospheric chemistry and dynamics caused by increasing greenhouse gas concentration. The DS noted that there is also no method recommendation for the ODP calculation in the CLP Regulation or CLP Guidance.

Assessment and comparison with the classification criteria

RAC agrees with the DS that impact assessments and risk related elements raised during the consultation and subsequent discussions are not relevant for hazard assessment under the CLP Regulation. The CLP Regulation (Annex I.5) states that a substance should be classified as hazardous to the ozone layer "*if the available evidence concerning its properties and its predicted or observed environmental fate and behaviour indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer"* (Annex I, Section 5.1.2.1). Furthermore, it is stated in the CLP Guidance (Part 5.1) that any substances having an ODP greater or equal to the lowest ODP (*i.e.,* 0.005) of the substances currently listed in Annex I to Regulation (EC) No 1005/2009 should be classified as Ozone 1 (H420). RAC notes that N_2O is not currently listed in Annex I to Regulation (EC) 1005/2009.

Although the suitability of the data was questioned during the consultation, no reason has been provided as to why the data is not suitable for use under the CLP Regulation. RAC agrees with the DS in noting that there are no particular criteria for evaluation of open literature studies providing ODP values. RAC has assessed the ODP value from Ravishankara *et al*. (2009) and agrees with the DS that this is reliable and the ODP value reported of 0.017 is suitable for comparison with the CLP criteria. RAC further notes that the ODP value provided by Ravishankara *et al*. (2009) is still used in the latest WMO report (2022), where N_2O continues to be considered as an ozone depleting substance, as well as in the previous WMO report (2018) and JRC report (2015), both cited in the CLH report.

The impact of N_2O increase on the ozone layer has also been investigated in other studies, such as Revell *et al*. (2015), which was made available to RAC. In contrast to Ravishankara *et*

al. (2009) (which calculated the OPD value of N_2O using the well-established Garcia-Solomon two-dimensional model, considering past and future changes in ozone and includes comprehensive chemistry, detailed radiative transfer, and dynamics), Revell *et al.* (2015) employed a three-dimensional chemistry-climate model and performed different simulations to explore the range of possible N_2O ODP values under different atmospheric conditions. They calculated ODP values in the range of 0.015 to 0.030 from the year 2000 to 2100. Although a different technique was used to calculate the ODP in Revell *et al*. (2015), a value comparable (0.015 for the year 2000) to that from Ravishankara *et al*. (2009) was derived. Consequently, although Ravishankara *et al*. (2009) and Revell *et al*. (2015) applied different approaches the obtained values are comparable, being 0.017 and 0.015, respectively. Both available studies further indicate that ozone would decrease as stratospheric N_2O increases.

RAC agrees with the DS that the provided ODP value is relevant and reliable and that as the ODP value of 0.017 for N_2O along with the supporting ODP value of 0.015 are greater than the lowest ODP value in Annex I to Regulation (EC) 1005/2009 (0.005 for chlorofluoroethane) (following the CLP Guidance Section 5.1), **classification as Ozone 1 (H420 Harms public health and the environment by destroying ozone in the upper atmosphere) is warranted**.

13 ADDITIONAL LABELLING

14 ANNEXES

Annex I: Non-confidential annex documenting the key studies for assessment.

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Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification: pethoxamid (ISO); 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenylprop-1-enyl)acetamide

EC Number:

CAS Number: **106700-29-2**

Index Number: **616-145-00-3**

Contact details for dossier submitter:

Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology

Radetzkystraße 2; 1030 Vienna

Austria

Version number: 3.0 **Date: 08.03.2022**

ANNEX TO ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON PETHOXAMID (ISO); 2-CHLORO-*N*-(2-ETHOXYETHYL)-*N*-(2-METHYL-1-PHENYLPROP-1-ENYL)ACETAMIDE

CONTENTS

1 PHYSICAL HAZARDS

Studies on the physical chemical properties of pethoxamid have been previously reviewed and are included in the EU draft Renewal Asssessment Report Volume 3 -B.2 (AS) (August 2016).

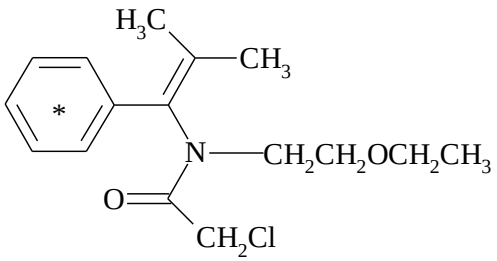
2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1 Anonymous, 2000

Reference: Pethoxamid: Metabolism in the rat
Author(s), year: Anonymous, 2000
Report/Doc. number: 60 PXA (TOX2001-275) / TON '007/974328
Guideline(s): EPA FIFRA 85-1 ≈ 94/79/EEC ≈ JMAFF 59 Nohsan No. 4200; considered equivalent to OECD 417 (1984)
GLP: Yes
Deviations: Weight variation of animals exceeds 20%
Acceptability: Yes

Material and Methods:

Test material:



*[phenyl-U-14C]pethoxamid:

Specific activity: 1.11 GBq/mmol, Batch no.: CFQ9694, Radiochemical purity: >98 %.

Test animals: Sprague- Dawley rats (CRL:CD® BR), Charles River, Margate, UK.

In a pilot experiment no detectable radioactivity was found in the expired air traps, so these were not included in the main study. Furthermore, based on this pilot study, dose levels for the main study and sacrifice times for the tissue distribution experiments were selected.

Excretion studies:
Three groups of 5 male and 5 female rats were given a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw or 300 mg/kg bw, or fourteen consecutive daily oral doses of ^{14}C-pethoxamid at 8 mg/kg bw. Cooled urine was collected at 6, 12, and 24 hours and at 24-hour intervals up to 96 hours after dosing. Faeces were collected with 24-hour intervals up to 96 hours after final dosing. In the multiple dosing study, additional urine and faeces were collected at 24-hour intervals throughout dosing. Blood was sampled immediately prior to sacrifice. Upon sacrifice the residual carcass and organs were taken for analysis.

Biliary excretion was measured in another group of 5 male and 5 female rats after receiving a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw. Bile was collected and deep frozen at 1, 2, 4, 6, 8, 12, 24 and 48 hours after

dosing. Urine was collected at 12, 24 and 48 hours after dosing and faeces were collected at 24 hour intervals until sacrifice at 48 hours after dosing. Residual carcass was taken for analysis.

Blood/plasma kinetics:
Groups of 5 male and 5 female rats received a single oral dose of 8 mg/kg bw or 300 mg/kg bw of ^{14}C-pethoxamid. At 4, 12, 48, 96, 120 and 168 hours after dosing blood samples were taken.

Tissue distribution studies:
Groups of 12 male and 12 female rats received a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw or 300 mg/kg bw. Groups of animals (3/sex) were sacrificed at 12, 48, 120 and 168 hours after dosing. Upon sacrifice the residual carcass and the organs were taken for analysis.

Whole-body autoradiography:
Four male and four female rats received a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw. Pairs of animals (1/sex) were sacrificed at 12, 48, 120 and 168 hours after dosing and used for autoradiography.

Analysis:
Radioactivity was measured using LSC. Tissue concentrations were determined by either combustion or solubilisation of replicate subsamples. Whole-blood concentrations were determined by direct combustion and radioassay. Urine, cage washes, plasma and bile concentrations were determined by direct radioassay of aliquots. Faeces were solvent extracted and aliquots of the extract and combustion of the residues were undertaken to determine residue levels. Subsequent tissue extracts, faeces extracts, bile and urine were analysed either by HPLC and TLC to assess the nature and proportion of any metabolites.

Results:

Excretion balance:
After a single oral dose of 8 mg/kg bw of ^{14}C-pethoxamid, the urinary excretion accounted for means of 35.0% and 31.4% in male and female rats, respectively, during 0-96 hours and 39.4% and 36.8%, respectively, after the high dose level of 300 mg/kg bw (Table 2.1.1-1). The majority of the remaining radioactivity was excreted via the faeces. The excretion pattern for both single dose levels, 8 and 300 mg/kg bw, was very similar. The majority of the urinary excretion took place in the first 24 hours, while the excretion in the faeces was mainly within 48 hours. After 96 hours the total recovery (urine, cagewash, faeces, carcass and tissue) was 95.9 (female) and 96.9% (male). After administration of 8 mg/kg bw/d for 14 days the excretion pattern was similar to that seen after a single dose.

Concentrations of radioactivity were measured in tissues at 96 hours (Table 2.1.1-6). The highest concentrations were found in the livers, lungs and spleens for all dosing regimes. Concentrations in the tissues from rats receiving 14 doses (8 mg/kg bw/d) were 3-16 fold higher than at the same time after a single dose (8 mg/kg bw).

Biliary excretion:
Rats with cannulated bile ducts excreted 73.2% of the radioactivity in females, and 75.6% in males through the bile (Table 2.1.1-2), with the majority occurring between 0 and 4 hours after dosing (37.3% for males and 48.1% for females). Recovery after 48 hours was >90%.

Pharmacokinetic parameters:
After a single low or high dose, the concentration of radioactivity in whole-blood and plasma was higher in males than in females, with maximum radioactivity 12 hours after dosing. At both dose levels the decline in radioactivity was monoexponential. The terminal half-lives of both whole-blood and plasma did not vary significantly between males and females or between the doses administered. However, the $t_{1/2}$ from whole-blood was much longer than from plasma (Table 2.1.1-3).

Tissue distribution:
Upon a single dose, the tissue distribution was similar between males and females. However, the values were slightly higher in males than in females. Highest concentrations in all tissues were 12 hours after dosing. The highest radioactivity occurred in whole-blood, plasma, liver and kidneys after single low or high dose administration. After high dose administration, the lung also contained high radioactivity (Table 2.1.1-5). Mean tissue concentrations of radioactivity occurring 96 hours after a single oral dose (8 and 300 mg/kg bw), or 96 hours after the last dosing at 8 mg/kg bw for 14 days are shown in Table 2.1.1-6. The tissue concentrations of radioactivity in rats after 14 daily doses were 3-16 fold higher than after a single dose.

Whole-body autoradiography:
Tissue concentrations determined by luminography were generally in good agreement with those obtained by liquid scintillation counting. Differences occurred, may be attributed to the use of single animals, for luminography.

Metabolic pathway:
In urine up to eleven metabolites (all < 10% of the applied dose) were identified up to 48 hours after dosing. The single low and high dose and the 14 consecutive daily low doses showed a similar profile. In bile up to 24 hours, 10 metabolites were detected after administration of a single low level dose, of which polar metabolites accounted for 39.8% and 25.5% of the applied dose in males and females, respectively. In faeces extracts, up to 13 components were detected, generally accounting for <5% of the applied dose up to 48 hours (Table 2.1.1-7).

Pethoxamid is metabolised by glutathione-S-transferase to give a number of methylthio- metabolites, which are further oxidised to sulphonyl derivatives. This pathway is well known for herbicides which contain a chloroacetamide group. Metabolism also occurred by cleavage of the N-(2-ethoxyethyl) group and by oxidation of the methyl groups attached to the ethylenic bond. The sulphonic acid metabolite of pethoxamid (MET-42) was identified in faeces (eluted in F2-F4 region (Table 2.1.1-7) and accounted for maxima of 5.3% and 4.9% of the dose in females at 8 mg/kg bw and 300 mg/kg bw dose level, respectively. No metabolites derived from oxidation of the benzene ring were detected at all, in contrast to other well known chloroacetamide herbicides. A proposed metabolic pathway is shown in Figure B.6.1-1.

Table 2.1.1-1: Mean excretion and retention after single low and high dose and repeated (14-daily) low dose administration of pethoxamid

Dose level	8 mg/kg bw		300 mg/kg bw		8 mg/kg bw/d	
Sample	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Urine	35.0	31.4	39.4	36.8	34.4	31.1
Cagewash	0.3	0.3	0.4	0.4	1.2	1.9
Faeces	57.6	62.7	53.2	58.0	60.5	62.4
Carcass	1.8	1.2	1.6	1.1	0.7	0.6
Total recovery	94.5	95.6	94.6	96.3	96.9	95.9

Results expressed as % administered dose, 96 hours after dosing

Table 2.1.1-2: Mean excretion and retention in bile-duct cannulated rats after single low dose administration of pethoxamid

Dose level	8 mg/kg bw	
Sample	**Male**	**Female**
Urine	6.6	10.6
Cagewash	0.1	0.1
Faeces	8.5	6.1
Bile	75.6	73.2
Carcass	1.5	1.7
Total recovery	92.4	91.5

Results expressed as % administered dose, up to 48 hours after dosing

Table 2.1.1-3: Pharmacokinetic parameters of pethoxamid in plasma and whole-blood following a single low or high oral administration of ^{14}C-pethoxamid

	Plasma				Whole-blood			
Dose level	**8 mg/kg bw**		**300 mg/kg bw**		**8 mg/kg bw**		**300 mg/kg bw**	
Parameter	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
$t_{1/2}$ (hours)	43.7	46.7	41.0	45.2	145.7	122.5	149.2	148.2
AUC_{168}	55.9	49.3	2297.8	1840.9	286.8	175.3	14753.8	8779.0

Half life values calculated between 12 and 168 hours, except for male (whole-blood) 8 mg/kg bw, calculated between 48 and 168 hours.

Table 2.1.1-4: Mean tissue concentrations of radioactivity following a single oral dose of ^{14}C-pethoxamid at a nominal level of 8 mg/kg bw

Sacrifice time	**12 hours**		**48 hours**		**120 hours**		**168 hours**	
Animal numbers	**73M - 75M**	**76F - 78F**	**79M - 81M**	**82F - 84F**	**85M - 87M**	**88F - 90F**	**91M - 93M**	**94F - 96F**
Adrenal glands	0.385	0.305	0.130	0.125	0.064	0.069	0.099	0.053
Bone	0.059	0.069	nd	0.029	0.014	nd	0.015	nd
Brain	0.126	0.105	0.055	0.040	0.063	0.032	0.050	0.028
Carcass	0.890	1.316	0.209	0.285	0.105	0.095	0.066	0.058
GIT	62.577	42.909	3.555	3.786	0.306	0.396	0.172	0.143
Heart	0.438	0.329	0.232	0.168	0.168	0.082	0.126	0.070
Kidney	1.360	1.175	0.379	0.388	0.223	0.158	0.176	0.112
Liver	2.882	2.056	0.766	0.678	0.398	0.348	0.305	0.184
Lung	0.563	0.478	0.324	0.317	0.255	0.162	0.195	0.113
Muscle (skeletal)	0.233	0.187	0.082	0.066	0.060	0.029	0.043	0.023
Ovary	ns	0.367	ns	0.141	ns	0.052	ns	0.039
Plasma	0.795	0.773	0.329	0.381	nd	nd	0.067	0.051
Spleen	0.540	0.399	0.288	0.278	0.251	0.162	0.201	0.114
Testis	0.207	ns	0.067	ns	0.032	ns	0.021	ns
Thyroid	0.584	0.305	0.243	0.161	0.137	0.082	0.077	nd
Whole-blood	2.276	1.591	1.810	1.417	1.664	0.984	1.384	0.805
Fat (abdominal)	0.222	0.224	0.056	0.054	0.037	0.018	0.026	0.011

Results are expressed as µg pethoxamid equivalents/g; ns: no sample; nd: not detected

Table 2.1.1-5: Mean tissue concentrations of radioactivity following a single oral dose of ^{14}C-pethoxamid at a nominal level of 300 mg/kg bw

Sacrifice time	**12 hours**		**48 hours**		**120 hours**		**168 hours**	
Animal numbers	**133M - 135M**	**136F - 138F**	**139M - 141M**	**142F - 144F**	**145M - 147M**	**148F - 150F**	**151M - 153M**	**154F - 156F**
Adrenal glands	16.269	24.408	4.785	4.625	3.948	3.392	3.558	3.132
Bone	4.173	3.556	1.170	0.756	0.632	0.555	0.596	nd
Brain	6.266	9.306	2.588	1.572	1.819	1.713	1.750	1.300
Carcass	17.434	35.006	7.020	7.025	3.929	5.046	2.842	3.421
GIT	1632.5	1926.4	65.075	65.380	5.340	7.376	2.680	3.087
Heart	16.375	23.025	8.377	4.893	5.950	4.406	5.318	3.724
Kidney	53.574	52.933	12.869	11.338	8.672	7.616	5.825	5.795
Liver	74.446	87.842	15.679	12.670	9.736	8.025	6.516	5.854
Lung	22.841	30.391	12.429	10.292	8.960	7.997	7.131	7.233
Muscle (skeletal)	9.099	12.478	3.215	1.881	2.342	1.547	1.865	1.315
Ovary	ns	19.657	ns	4.340	ns	3.009	ns	1.645
Plasma	25.836	33.614	12.127	10.304	4.199	4.749	2.032	2.606
Spleen	19.876	22.322	11.697	8.646	8.643	7.350	10.113	6.877
Testis	8.111	ns	2.097	ns	1.085	ns	0.717	ns
Thyroid	15.258	19.416	7.486	6.178	5.679	4.159	4.841	3.907
Whole-blood	74.576	76.418	70.582	51.676	56.611	46.254	49.628	43.666
Fat (abdominal)	12.445	24.053	1.809	1.985	1.155	1.272	0.671	0.869

Results are expressed as µg pethoxamid equivalents/g; ns: no sample; nd: not detected

Table 2.1.1-6: Mean tissue concentrations at 96 hours after single low or high dose and repeated low dose administration of TKC-94

Dose level	8 mg/kg bw		300 mg/kg bw		8 mg/kg bw/d	
Tissue	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Adrenal gland	0.093	0.074	8.156	4.209	1.126	0.797
Bone	0.023	nd	1.071	0.929	0.142	0.103
Brain	0.051	0.026	3.170	2.053	0.599	0.302
GIT	0.465	0.503	6.564	5.183	0.830	0.743
Heart	0.153	0.087	10.044	4.640	1.352	1.024
Kidney	0.229	0.183	12.139	7.706	1.976	1.295
Liver	0.425	0.312	11.447	7.311	2.629	1.681
Lung	0.251	0.173	15.482	9.868	2.609	1.975
Muscle (skeletal)	0.069	0.033	4.039	1.672	0.458	0.230
Ovary	ns	0.069	ns	2.977	ns	0.500
Plasma	0.168	0.178	8.998	5.680	0.520	0.520
Spleen	0.316	0.222	15.653	7.570	2.825	1.878
Testis	0.037	ns	2.064	ns	0.187	ns
Thyroid	0.112	0.071	15.401	13.692	1.430	1.126
Whole-blood	1.468	0.945	96.029	53.432	15.951	10.400
Fat (abdominal)	0.042	0.027	1.965	1.957	0.197	0.140

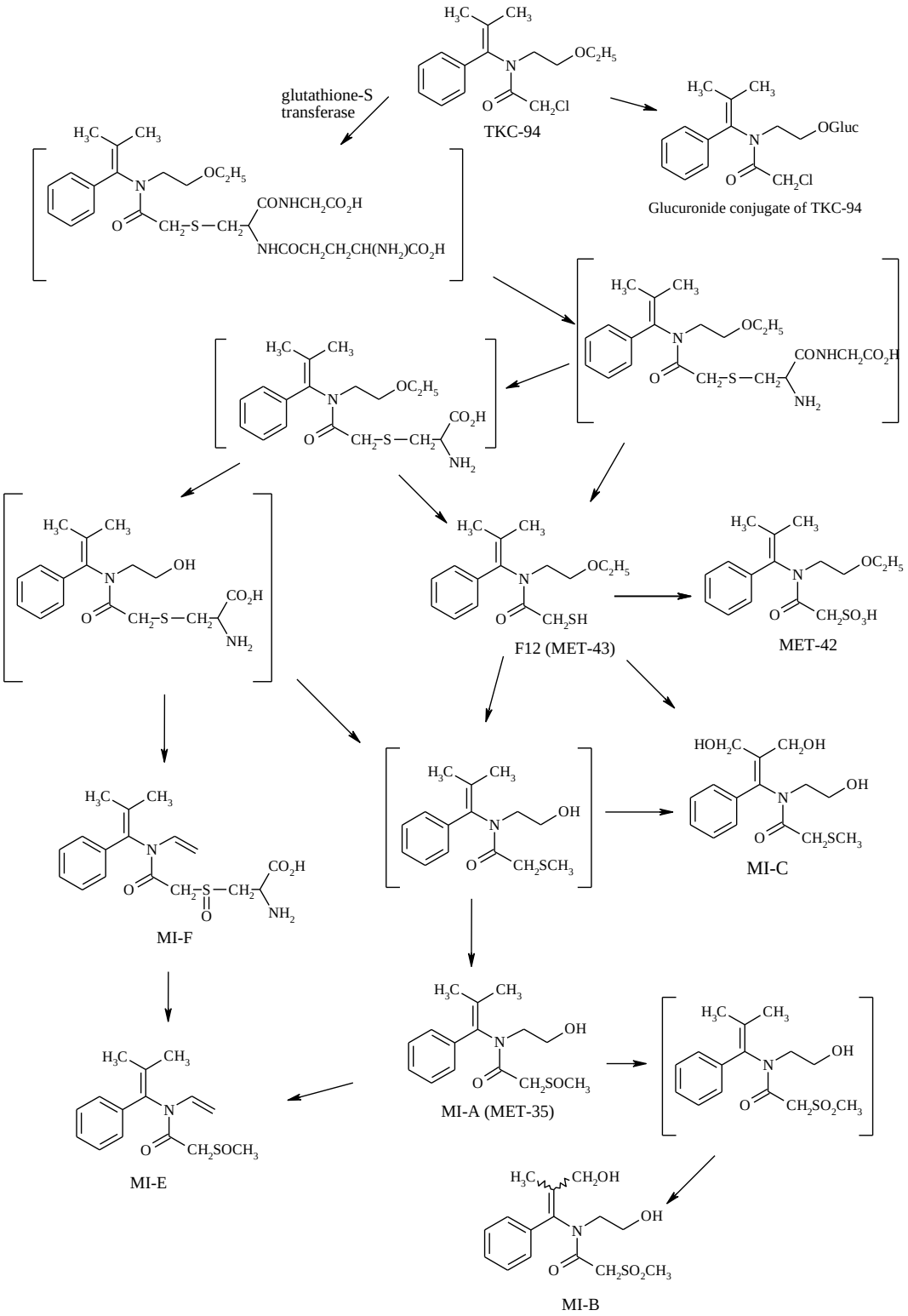
Results expressed as µg test compound equivalents/g; ns: no sample; nd: not detected

Table 2.1.1-7: Radioactive components expressed as % of the administered dose in urine, faeces and bile after oral administration of ^{14}C-pethoxamid to rats[1]

Component	8 mg/kg bw		300 mg/kg bw		8 mg/ kg bw/d	
	Male	Female	Male	Female	Male	Female
Urine						
U1	0.7	1.0	1.4	0.7	0.1	0.5
U2	1.4	2.1	nd	1.5	0.4	0.4
U3	1.9	1.0	4.1	2.2	0.5	0.2
U4	nd	1.7	nd	2.0	0.3	0.3
MI-F	2.7	5.0	5.2	4.5	0.5	0.6
MI-E	1.4	2.8	1.2	4.5	0.2	1.0
MI-D	1.9	3.7	3.3	5.7	0.6	0.5
U8	0.8	nd	2.3	1.4	nd	nd
MI-C	9.2	1.7	6.7	2.2	0.1	0.2
MI-B	5.4	2.6	5.8	2.9	1.3	0.2
MI-A	2.6	2.3	3.7	4.2	0.7	0.5
Pethoxamid	2.0	1.5	0.8	1.1	0.5	0.2
Others[2]	3.9	3.9	3.8	2.7	0.1	0.4
Faeces[3]						
F1	0.7	2.2	3.7	5.3	2.7	2.6
F2	3.6	2.7	1.3	2.3	nd	2.3
F3	2.5	4.0	1.2	1.6	nd	0.3
F4	1.2	3.0	1.3	2.5	0.3	nd
F5	0.5	4.9	nd	nd	1.4	0.3
F6	nd	2.4	nd	nd	0.5	0.2
F7	0.8	2.1	0.3	6.4	0.5	<0.1
F8	5.5	1.4	0.3	1.3	0.3	<0.1
F9	3.5	0.7	5.9	nd	0.1	0.1
F10	2.2	0.2	2.0	0.9	0.2	0.4
F11	0.9	1.5	3.2	nd	0.2	nd
F12	1.0	2.0	3.7	1.9	0.6	0.6
F13	0.9	1.4	2.4	nd	nd	nd
Pethoxamid	1.5	0.4	6.8	11.0	nd	nd
Others[1]	6.9	5.7	7.8	10.2	1.0	1.2
Unextracted	22.3	25.3	10.8	11.4	nd	nd
Bile						
B1	6.0	8.7				
B2	8.6	4.2				
B3	19.2	4.7				
B4	6.0	7.9				
MI-F	6.1	11.8				
MI-E	0.8	5.8				
MI-D	16.6	4.6				
MI-C	1.9	2.0				
MI-B	1.2	1.1				
MI-A	0.5	0.9				
Pethoxamid	0.9	5.2				
Others [2]	7.0	15.4				

[1] Sampling: Single administration: Urine and faeces 0-48 hours; bile 0-24 hours; 14 days administration: Urine 0-15 days, faeces 0-14 days; [2] Radioactivity not associated with specific components; [3] MET-42 (in F2-F4 region eluting): 8 mg/kg bw: 0.5% (males), 5.3% (females), 300 mg/kg bw: 1.5% (males), 4.9% (females); nd: not detected.

Figure 2.1.1-1: Proposed metabolic pathway of pethoxamid in the rat



3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Anonymous, 1994a

Reference: Acute oral toxicity to the rat of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 63 PXA (TOX2001-280)/ TKS '15/932187/AC
Guideline(s): OECD 401 (1987)
GLP: Yes
Deviations: No
Acceptability: Yes

Materials and methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: Groups of 5 male and 5 female CD Sprague-Dawley rats, seven to ten weeks old, 204 to 248 g from Harlan Olac Ltd.,Bicester, Oxon, England.

Test method: The test material was administered as supplied to the rats by oral gavage at dose levels of 800, 1260 and 2000 mg/kg bw (the maximal application volume was 1.8 mL/kg bw). The observation period was 14 days post-exposure.

Results:

Table 3.1.1-1: Mortality

Sex	**Dose (mg/kg bw)**			**LD50 (95% C.I.) (mg/kg bw)**
	800	1260	2000	
Male	2/5	3/5	5/5	983 (623 to 1360)
Female	0/5	2/5	4/5	1472 (1039 to 2235)
Male and Female	2/10	5/10	9/10	1196 (878 to 1579)

Table 3.1.1-2: Incidence of clinical observations

Finding	**Dose level (mg/kg bw)**					
	800		1260		2000	
	male	female	male	female	male	female
Piloerection	5	5	5	5	5	5
Abnormal body carriage (hunched posture)	5	5	5	5	5	5
Abnormal gait (waddling)	5	5	5	5	5	5

Lethargy	5	5	5	5	5	5
Decreased respiratory rate	5	0	3	0	5	5
Partially closed eyelids	5	0	1	0	2	5
Pallor of the extremities	5	0	5	5	5	5
Soft or liquid faeces	5	5	5	3	5	4
Increased salivation	5	5	2	5	4	5
Unsteadiness	0	5	0	0	0	1
Body tremors	0	0	0	0	2	1
Dilation of pupils	0	0	0	0	5	4
Cold to the touch	0	0	0	0	1	0
Staining of the ano-genital region	0	0	2c	2b	1b	2c
Staining on cage litter tray	0	0	5a	0	0	1d
a: Yellow staining; b: Yellow/orange staining; c: Yellow/red staining; d: Red/brown staining						

Body weight: The rats that died showed bodyweight loss, and revealed changes to subcutaneous tissue, liver, spleen, kidneys, stomach, intestines and testes at macroscopic examination.
All surviving rats were recovered at day 6. Slightly low bodyweight gains were recorded on day 8 for two males dosed at 800 and 1260 mg/kg bw, and a slight bodyweight loss for one female at 2000 mg/kg bw.

Necropsy: At macroscopic examination no abnormalities were recorded for the surviving animals.

Conclusion: The acute oral median lethal dose (LD_{50}) and its 95% confidence limits in male rats was estimated to be 983 (623 to 1360) mg/kg bw. Therefore, according to the criteria in Council Directive 67/548/EEC, the test material is classified as harmful (Xn) and labelled with R22, "harmful if swallowed".

According to Annex VI of Regulation (EC) 1272/2008, ATP01 (Regulation (EC) 790/2009), pethoxamid is classified as **Acute Tox. Cat. 4 and labelled with H302, "Harmful if swallowed".**

3.1.2 Human data

No relevant studies.

3.1.3 Other data

No relevant studies.

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Anonymous (1994b)

Reference: Acute dermal toxicity to the rat of NSK-68
Author(s), year: Anonymous, 1994

Report/Doc. number: 64 PXA (TOX2001-277) / TKS '16/932186/AC
Guideline(s): OECD 402 (1981)
GLP: Yes
Deviations: No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: 5 male, 5 female CD Sprague-Dawley rats.

Test method: The test material was administered as supplied to the rats at 2000 mg/kg bw to the shaved skin of each animal (application volume 1.8 mL/kg). A gauze pad was then placed over the treated area and held in place with a non-irritative dressing for 24 hours. The observation period was 14 days post-exposure.

Findings:
Clinical findings: No mortalities were observed. No clinical signs were noted during the study. No irritation or other dermal changes were observed in any animal throughout the observation period.

Body weight: Slightly low bodyweight gains were recorded for all five males and two females on Day 8. In addition, at this time, two females showed either no change or a slight body weight loss. By Day 15, three males had achieved the anticipated body weight gains. Slightly low body weight gains were than evident in two males and three females. One female achieved the anticipated body weight gain throughout the study.

Necropsy: No abnormalities were noted at necroscopy.

Conclusion: The acute dermal median lethal dose (LD_{50}) in rats was found to be greater than 2000 mg/kg bw. Therefore according to the criteria in Council Directive 67/548/EEC classification is not required.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding acute dermal toxicity is not required.

3.2.2 Human data

No relevant studies.

3.2.3 Other data

No relevant studies.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Anonymous (1994)

Reference: Acute inhalation toxicity to the rat of NSK-68
Author(s), year: Anonymous, 1994

Report/Doc. number: 65 PXA (TOX2001-278) / TKS '14/932316
Guideline(s): OECD 403 (1981)
GLP: Yes
Deviations from OECD 403 (2009): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: Albino rats, (Sprague Dawley); one control group and one test group each of 5 male and 5 female rats, six and eight weeks old respectively, 200 g of weight, from Charles River UK Ltd, Manston Road, Margate,Kent, UK.

Test method: The rats were exposed to the test material or to clean dried air by continuous whole body exposure for 4 hours by inhalation. The test atmosphere contained a liquid droplet aerosol generated from the melted test substance. After the observation period of 14 days post exposure, the animals were macroscopically examined. The lungs were also examined microscopically.

Table 3.3.1-1: Exposure parameters

Parameter		Value
Chamber concentration (mg/L)	Chemical analysis	4.16
	Gravimetric analysis	4.38
Particle size (µm)	MMAD ± GSD	3.3 ± 2.14
	% respirable (<7 µm)	84.1
Chamber air temperature (°C)	Control group	24
	Test group	23
Relative humidity (%)	Control group	45
	Test group	96
MMAD: Mass median aerodynamic diameter, GSD: Standard geometric deviation		

Results:
Clinical observations: No mortalities were observed. Signs seen during exposure to the test substance included partial closing of the eyes, wetness around the eyes, snout and mouth and matted fur. During the observation period, signs seen in rats exposed to pethoxamid included residues of the test material on the fur, brown discharge from the eyes, wet fur and brown staining around the snout, jaws and on the underbody, and matted fur. Exaggerated respiratory movements were observed in 1 male rat. Recovery from the effects was evident from Day 6 in male rats and Day 8 in female rats. All rats were recovered on Day 11.

Body weight: Reduced body weight or decreased rate of body weight gain was observed in treated rats for one day. Subsequently, weight gain was similar to that of control rats.

Pathological examination: At necropsy, the lungs of one female rat exposed to pethoxamid had dark subpleural foci in all lobes. These foci are commonly found in rats. There were no macroscopic abnormalities in any other test or control rat. There were also no microscopic abnormalities observed that were considered treatment-related.

Conclusion: The acute inhalation median lethal concentration (LC_{50}, 4 hours) in the rat was greater than 4.16 mg/L of air. This was the highest attainable analytical concentration under the test conditions, the exposure level achieved was far in excess of any exposure levels that could be generated accidentally. Only a transient effect on bodyweight with no other effects was observed, and therefore according to the criteria in Council Directive 67/548/EEC classification is not required.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding acute inhalation toxicity is not required.

3.3.2 Human data

No relevant studies.

3.3.3 Other data

No relevant studies.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Anonymous (1994a)

Reference: Skin irritation to the rabbit of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 66 PXA (TOX2001-279) / TKS '17/932160/SE
Guideline(s): OECD 404 (1992)
GLP: Yes
Deviations from OECD (2002): No
Acceptability: Yes

Material and Methods:
Test Material: Pethoxamid; Batch TB 930727; Purity: 95%.
Test Animals: Six New Zealand white male rabbits, 10 to12 weeks of age, 2.3 to 2.7 kg of weight, from Froxfield (U.K.) Ltd. Petersfield, Hampshire, England.

Test method: The rabbits received each 0.5 mL of the test substance which was administered as supplied to the shaved skin of each animal. A 2.5 x 2.5 cm gauze pad was then placed over the area and covered with an elastic adhesive dressing (semi-occlusive) for four hours.

Results:
Clinical findings: Very slight erythema was seen in all animals on Day 1. This persisted in one animal only, accompanied by very slight oedema on Days 2 and 3. All reactions had completely resolved by Day 5.

There were no signs of toxicity or ill health in any rabbit during the observation period.

Table 3.4.1-1: Individual and mean skin irritation scores

	Erythema						Oedema					
	Days					Mean score	Days					Mean score
Animal no.	0.5h	24h	48h	72h	96h	24-72h	0.5h	24h	48h	72h	96h	24-72h
2575	1	0	0	0	0	0	0	0	0	0	0	0
2576	1	0	0	0	0	0	0	0	0	0	0	0
2577	1	0	0	0	0	0	0	0	0	0	0	0
2578	1	0	0	0	0	0	0	0	0	0	0	0
2579	1	1	1	1	0	1	0	1	1	0	0	0.67
2580	1	0	0	0	0	0	0	0	0	0	0	0

Conclusion: On the basis of the skin reactions observed (mean skin irritation scores 24-72 hours after removal of the test article are 0.0-1) and the criteria specified in Council Directive 67/548/EEC, the test compound does not have to be classified as a skin irritant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid as skin irritant is not required.

3.4.2 Human data

No relevant studies.

3.4.3 Other data

No relevant studies.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Anonymous (1994b)

Reference: Eye irritation to the rabbit of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 67 PXA (TOX2001-280) / TKS '18/932199/SE
Guideline(s): OECD 405 (1987)
GLP: Yes
Deviations from OECD (2012): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: Six New Zealand white rabbits, 13 to 15 weeks of age, 3.1 to 3.6 kg of weight, from Froxfield (U.K.) Ltd., Petersfield, Hampshire, England.

Test method: The rabbits each received 0.1 mL of the test substance, by a singular ocular instillation without irrigation to the lower everted lid of one eye of each animal.

Results:
Clinical findings: The eye findings are tabulated below. The well-defined conjunctival irritation occurring in all animals and the dulling of the cornea (5/6 rabbits) were confined to the one hour assessment. Remaining slight reactions had resolved one or two days after instillation.

There were no signs of toxicity or ill health in any rabbit during the observation period.

Table 3.5.1-1: Individual and mean eye irritation scores

Animal no.	**Region of eye**	**1h**	**24h**	**48h**	**72h**	**4 days**	**7days**	**Mean score 24-72h**
4203	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	1	0	0	0	0	0.33
	discharge	2	0	0	0	0	0	0
2624	Cornea density	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	0	0	0	0	0	0
	discharge	2	0	0	0	0	0	0
2625	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	1	0	0	0	0	0.33
	discharge	3	0	0	0	0	0	0
2626	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	0	0	0	0	0	0
	chemosis	2	0	0	0	0	0	0
	discharge	2	0	0	0	0	0	0
2501	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	1	0	0	0	0	0.33
	discharge	2	0	0	0	0	0	0
2506	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	0	0	0	0	0	0
	discharge	2	0	0	0	0	0	0

Conclusion: Instillation of the test compound into the rabbit eye elicited a transient well-defined conjunctival irritation. On the basis of the eye reactions observed (mean eye irritation scores 0.0-0.33) and the criteria specified in Council Directive 67/548/EEC and Regulation 1272/2008, the test compound does not have to be classified as an eye irritant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid as eye irritant is not required.

3.5.2 Human data

No relevant studies.

3.5.3 Other data

No relevant studies.

3.6 Respiratory sensitisation

3.6.1 Animal data

No relevant studies.

3.6.2 Human data

No relevant studies.

3.6.3 Other data

No relevant studies.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Anonymous (1998)

Reference: TKC-94 : Skin Sensitisation in the Guinea-Pig, (incorporating a positive control using hexyl cinnamic aldehyde (HCA))
Author(s), year: Anonymous, 1998
Report/Doc. number: 68 PXA (TOX2001-281) / TON '017/983095/SS
Guideline(s): OECD 406 (1992)
GLP: Yes
Deviations: No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-960306; Purity: 95%.

Test animals: Sixty male albino guinea pigs of Dunkin/ Hartley strain, four to seven weeks old, 444 to 538 g of weight, from D. Hall Newchurch, Staffordshire, England.

Test method: The guinea pigs were allocated to four groups:
1. Control for group 2; 20 animals
2. Pethoxamid group; 20 animals
3. Control for group 4; 10 animals
4. Hexyl cinnamic aldehyde group (HCA; positive control); 10 animals

Based on the results of a preliminary study, the following dose levels were selected:

Study phase	Pethoxamid	HCA, positive control
Induction intradermal injection	0.5% v/v in Alembicol D	10% v/v in Alembicol D
Induction topical application	As supplied	As supplied
Topical challenge	25 and 12.5% v/v in Alembicol D	As supplied and 50% v/v in Alembicol D

The induction and challenge applications were held in place with semi-occlusive dressing for 48 and 24 hours respectively. After removal of the patches, the challenge sites were evaluated after 24 and 48 hours.

Results:
Clinical findings: No signs or effects on body weight were recorded. Dermal reactions were seen following the induction applications. After the challenge, dermal reactions were seen in nineteen of the twenty test animals.

Table 3.7.1-1: Number of animals exhibiting skin reactions after challenge with pethoxamid

Group	Treatment	Incidence of erythema				Incidence of oedema			
		24 hours		48 hours		24 hours		48 hours	
		anterior	posterior	anterior	posterior	anterior	posterior	anterior	posterior
Control	Freund's treated controls	3/20	2/20	3/20	2/20	0/20	0/20	0/20	0/20
Test	Pethoxamid	20/20	19/20	20/20	19/20	19/20	14/20	19/20	16/20
Control	Freund's treated controls	0/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
Positive control	HCA	10/10	10/10	10/10	10/10	9/10	4/10	9/10	2/10

Conclusion: Due to the positive responses seen in 19 out of 20 test animals, pethoxamid does have to be classified as sensitizing and labelled with R43 according to the criteria specified in Council Directive 67/548/EEC.

According to Annex VI of Regulation (EC) 1272/2008, ATP01 (Regulation (EC) 790/2009), pethoxamid is classified as **Skin Sens. Cat.1 and labelled with H317, "May cause an allergic skin reaction".**

3.7.2 Human data

No relevant studies.

3.7.3 Other data

No relevant studies.

3.8 Germ cell mutagenicity

3.8.1 In vitro data

3.8.1.1 Anonymous (1994)

Reference: Pethoxamid: Bacterial mutation assay
Author(s), year: Anonymous, 1994
Report/Doc. number: 75 PXA (TOX2001-290) / TKS '22/941449
Guideline(s): OECD 471 (1983), OECD 472 (1983)
GLP: Yes
Deviations from OECD 471 (1997): - no inclusion of S. typhimurium TA102 or E.coli WP2 uvrA
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %; Solvent: DMSO.

Test method: Four strains of *Salmonella typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) were dosed with a single application of 0.1 mL/plate of the test substance, with and without S9 mix (rat Aroclor). From a preliminary test, it was concluded that the test compound was not toxic up to 5000 mg/plate and therefore this dose level was chosen as top dose level in the main test. Dose levels used in the main test were as follows: 0, 50, 150, 500, 1500, 5000 mg/plate in triplicate. Positive controls were included. Revertant colonies per plate following a 72 hour incubation period were counted. The main test was repeated.

Results: None of the bacterial strains, tested with the test substance, showed any significant increases in the number of revertant colonies at any dose level, in the presence or absence of the S9 mix. Toxicity was observed following treatment with 5000 mg/plate in the first main test only in the presence of S9 mix, but in the second main test both in the presence and absence of S9 mix. All positive control compounds induced marked increases in the number of revertants.

Conclusion: The test material was found to be non-mutagenic under the conditions of this *in vitro* bacterial system.

3.8.1.2 Anonymous (2012)

Reference: Reverse Mutation Assay using Bacteria (Salmonella typhimurium and Escherichia coli) with Pethoxamid Technical

Author(s), year: Anonymous, 2012

Report/Doc. number: 647 PXA / BSL 115522

Guideline(s): OECD 471 (1997)

GLP: Yes

Deviations from guideline: - none

Acceptability: Yes

Material and Methods:
Test material: Pethoxamid technical

Lot/batch number: P1351-BKA-65

Purity: 93.5% (w/w) (dose calculation was not adjusted for purity)

Stability of test item: 17 October 2012 (stored at ambient temperature). NB: stable during the conduct of the study.

Storage conditions: At room temperature.

Test method: The test item pethoxamid was investigated for its potential to induce bacterial gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and tester strain E. coli WP2 uvrA.

In two independent experiments several concentrations of the test item were used. Each assay was conducted with and without metabolic activation. The concentrations, including the controls, were tested in triplicate.

The following concentrations were used in experiments I and II: 3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate.

Revertant colonies were automatically counted using a ProtoCol counter. For tester strains where the spontaneous mutant frequency is low (TA1535 and TA1537) revertant colonies were counted manually. Statistical analysis was not deemed necessary.

Results: No precipitation of the test item was observed in any tester strain used in experiment I and II (with and without metabolic activation). In experiment I toxic effects of the test item were observed at concentrations of 1000 µg/plate and higher (without metabolic activation) and at concentrations of 2500 µg/plate and higher (with metabolic activation), depending on the particular tester strain. In experiment II toxic effects of the test item were noted at concentrations of 316 µg/plate and higher (without metabolic activation) and at concentrations of 1000 µg/plate (with metabolic activation), depending on the particular tester strain.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with pethoxamid at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II. The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments.

Conclusion: In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, pethoxamid did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used. Therefore, pethoxamid is considered to be non-mutagenic in this bacterial reverse mutation assay.

3.8.1.3 Anonymous (1994)

Reference: Pethoxamid: Metaphase chromosome analysis of human lymphocytes cultured in vitro
Author(s), year: Anonymous, 1994
Report/Doc. number: 76 PXA (TOX2001-291) / TKS 12/931249
Guideline(s): OECD 473 (1983)
GLP: Yes

Deviations from OECD 473 (1997): none
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %; Solvent: DMSO.

Test method: Cultured human lymphocytes were exposed to the test substance, in the presence and absence of S9 mix (rat Aroclor). Without S9 mix, cells were exposed continuously for 18 hours. In the presence of S9 mix, exposure was limited to three hours, cells were harvested 15 hours after exposure. The following concentrations were selected for the duplicate tests:

- Experiment 1 without S9-mix: 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 µg/mL
- Experiment 1 with S9-mix: 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 µg/mL
- Experiment 2 without S9-mix: 3.75, 7.5, 15, 20, 25, 37.5, 50, 75, 100, 150 µg/mL
- Experiment 2 with S9-mix: 3.75, 7.5, 15, 30, 45, 60, 80, 100, 200 µg/mL

Solvent controls and positive controls were included. The mitotic index was recorded. The dose level causing approximately 50% decrease, was used as highest dose for metaphase analysis. Additionally, an intermediate and low dose level was selected.

Findings: In the first test no cells survived, both in the presence and absence of S9 mix, at concentrations of 62.5 µg/mL and above. In the second test, death of all cells was seen at a concentration of 75 µg/mL and above in the absence of S9 mix, and at 200 µg/mL in the presence of S9 mix.

In the first test, in the absence of S9 mix, a statistically significant increase in chromosomal aberrations occurred at the highest dose level, indicative of a clastogenic activity. In the presence of S9 mix, a statistically significant increase in chromosomal aberrations (gaps only) occurred at the intermediate dose level.

In the second test in the absence of S9 mix, statistically significant increases in chromosomal aberrations occurred at the intermediate and high dose levels. In the presence of S9 mix, a statistically significant increase in chromosomal aberrations occurred at all dose levels analysed.

The positive control compounds produced clear increases in chromosome aberrations.

Table 3.8.1-1: Summary of cytogenetic tests

Expt	Treatment (dose in µg/mL)	S9-mix	Mean MI (%)	Cells with aberrations including gaps (%)			Cells with aberrations excluding gaps (%)		
				range	mean	statistical significance	range	mean	statistical significance
1	DMSO	-	100	4-8	5.5	-	3-8	5.25	-
	Pethoxamid (2)	-	116	4-4	4.0	ns	4-4	4.0	ns
	Pethoxamid (7.8)	-	100	4-4	4.0	ns	4-4	4.0	ns
	Pethoxamid (15.6)	-	65	13-10	11.5	**	12-10	11.0	**
	EMS (500)	-	nd	17-24	20.5	***	23-17	20.0	***
	DMSO	+	100	1-5	2.5	-	1-5	2.5	-
	Pethoxamid (3.9)	+	102	4-2	3.0	ns	2-4	3.0	ns
	Pethoxamid (15.6)	+	98	6-6	6.0	*	5-6	5.5	ns

	Pethoxamid (31.3)	+	61	2-9	5.5	ns	2-8	5.0	ns
	CP (10)	+	nd	37-27	32.0	***	37-26	31.5	***
2	DMSO	-	100	0-3	1.0	-	1-0	0.25	-
	Pethoxamid (3.75)	-	100	1-2	1.5	ns	2-1	1.5	ns
	Pethoxamid (20)	-	49	4-9	6.5	***	4-7	5.5	***
	Pethoxamid (37.5)	-	41	16-16	16.0	***	15-16	15.5	***
	EMS (500)	-	nd	11-13	12.0	***	13-7	10.0	***
	DMSO	+	100	4-1	2.5	-	1-4	2.5	-
	Pethoxamid (7.5)	+	111	6-6	6.0	*	6-6	6.0	*
	Pethoxamid (45)	+	95	11-18	14.5	***	11-17	14.0	***
	Pethoxamid (80)	+	55	42-42	42.0	***	42-42	41.5	***
	CP (10)	+	-	43-39	41.0	***	43-39	40.5	***

MI: Mean mitotic index compared to negative control values; EMS: Ethylmethanesulphonate; CP: Cyclophosphamide

ns not significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; nd: not determined;

Conclusion: Pethoxamid demonstrated **clastogenic activity** both in the absence and presence of S9 mix in this *in vitro* mammalian cytogenicity assay.

3.8.1.4 Anonymous (2015)

Reference: In vitro Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/TK+/-) in Mouse Lymphoma L5178Y Cells with Pethoxamid technical
Author(s), year: Anonymous, 2015
Report/Doc. number: 1449 PXA / Eurofins BioPharma Product Testing study No: 150778
Guideline(s): OECD 476 (1997)
GLP: Yes
Deviations from guideline: No
Acceptability: Yes

Materials and methods:
Test material: Pethoxamid technical

Lot/batch number: P1351-BKA-65

Purity: 94.5% (w/w) (dose calculation was not adjusted for purity)

Stability of test item: 27 February 2017 (stored at ambient temperature). NB: stable during the conduct of the study

Storage conditions: At room temperature

Test method: The test item Pethoxamid technical was assessed for its potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The selection of the concentrations used in the main experiments was based on data from the pre-experiments up to a maximum concentration of 10 mM. Experiment I without and with metabolic

activation and experiment II with metabolic activation were performed as a 4 h short-term exposure assay. Experiment II without metabolic activation was performed as a 24 h long-term exposure assay.

The test item was investigated at the following concentrations:
Experiment I without metabolic activation: 25, 50, 100, 150, 200, 250, 300 and 350 µM
Experiment I with metabolic activation: 50, 100, 150, 200, 250, 300, 350, 400 and 450 µM
Experiment II without metabolic activation:0.5, 1, 5, 10, 25, 50, 75 and 100 µM
Experiment II with metabolic activation:60, 120, 170, 230, 280, 330, 360 and 390 µM

According to OECD Guidelines at least 8 concentrations of the test item were set up in the experiments without and with metabolic activation.

The positive controls used for experiments without metabolic activation were EMS (ethylmethanesulfonate) and MMS (methylmethanesulfonate), and for experiments with metabolic activation B[α]P (benzo[α]pyrene).

The test item is considered mutagenic if the following criteria are met:
-The induced mutant frequency (IMF) meets or exceeds the Global Evaluation factor (GEF) of 126 mutants per 10^6 cells. The GEF is defined as the mean of the negative/vehicle mutant frequency plus one standard deviation; data are gathered from ten laboratories. For the microwell method the GEF was defined to be 126.
-A dose-dependent increase in mutant frequency is detected.

Besides, combined with a positive effect in the mutant frequency, an increased occurrence of small colonies (≥40% of total colonies) is an indication for potential clastogenic effects and/or chromosomal aberrations.

A test item is considered to be negative if the induced mutant frequency is below the GEF and the trend of the test is negative.

Results:
Toxicity: No precipitation of the test item was noted in the main experiments.

Growth inhibition was observed in experiment I and II without and with metabolic activation.

In experiment I without metabolic activation the relative total growth (RTG) was 16.7% for the highest concentration (350 µM) evaluated. The highest concentration evaluated with metabolic activation was 450 µM with a RTG of 8.7%.

In experiment II without metabolic activation the relative total growth (RTG) was 11.6% for the highest concentration (100 µM) evaluated. The highest concentration evaluated with metabolic activation was 390 µM with a RTG of 10.0%.

Mutagenicity: In experiments I and II no biologically relevant increase of mutants was found after treatment with the test item (without and with metabolic activation). The Global Evaluation Factor (GEF; defined as the mean of the negative/vehicle mutant frequency plus one standard deviation; data gathered from ten laboratories) was not exceeded by the induced mutant frequency (IMF) at any concentration.

EMS, MMS and B[α]P were used as positive controls and showed distinct and biologically relevant effects in mutation frequency.

Clastogenicity: Colony sizing was performed for the highest concentrations of the test item and for the negative and positive controls. An extension of the GEF by the induced mutant frequency in combination with an increased occurrence of small colonies (defined by slow growth and/or morphological alteration of the cell clone) is an indication for potential clastogenic effects and/or chromosomal aberrations.

In experiment I (without and with metabolic activation) and experiment II (without metabolic activation), colony sizing showed no clastogenic effects. In experiment II with metabolic activation a higher number of small colonies induced by the test item was noted. However, since no mutagenicity was found at that dose, all dose groups were considered as not clastogenic.

The positive controls MMS (8 and 10 µg/mL) and B[α]P (3.5 µg/mL) induced a significant increase in mutant frequency and a biologically significant increase of small colonies (≥40%), thus proving the ability of the test system to indicate potential clastogenic effects.

Table 3.8.1-2: Summary table: Experiment I and II, without metabolic activation

Experiment	Group	Concn (µM)	RCE[a] (%)	RTG[b] (%)	MF[c] (mutants/10^6 cells)	IMF[d] (mutants/10^6 cells)	GEF[e] exceeded	Statistically significant increase [f]	Precipitate
I	C1	0	100.0	100.0	62.2	/	/	/	-
	C2	0	100.0	100.0	52.1	/	/	/	-
	3	25	103.3	91.9	60.3	3.1	-	-	-
	4	50	122.7	110.6	51.9	-5.3	-	-	-
	5	100	100.0	50.6	57.6	0.5	-	-	-
	6	150	87.1	48.0	61.3	4.2	-	-	-
	7	200	105.0	50.6	100.9	43.8	-	+	-
	8	250	116.2	45.1	78.8	21.6	-	-	-
	9	300	92.5	19.5	104.1	47.0	-	+	-
	10	350	108.5	16.7	161.3	104.1	-	+	-
	EMS	300 µg/mL	77.4	58.5	670.0	612.8	+	+	-
	MMS	10 µg/mL	74.2	54.1	514.8	457.6	+	+	-
II	C1	0	100.0	100.0	53.3	/	/	/	-
	C2	0	100.0	100.0	52.7	/	/	/	-
	3	0.5	109.4	109.4	53.1	0.1	-	-	-
	4	1	93.2	81.1	60.8	7.8	-	-	-
	5	5	121.5	87.1	38.1	-14.9	-	-	-
	6	10	96.1	86.3	63.5	10.5	-	-	-
	7	25	121.5	93.2	34.6	-18.4	-	-	-
	8	50	107.6	49.2	33.5	-19.5	-	-	-
	9	75	100.8	29.1	59.3	6.3	-	-	-
	10	100	94.7	11.6	62.9	9.9	-	-	-

	EMS	200 µg/mL	57.9	30.5	1901.3	1848.3	+	+	-
	MMS	8 µg/mL	49.4	33.8	1018.6	965.6	+	+	-

C: Negative Controls

a: Relative Cloning Efficiency, RCE = [(CE $_{\text{dose group}}$ / CE $_{\text{of corresponding controls}}$) x 100]. Cloning Efficiency, CE = ((-LN (((96 - (mean P1,P2)) / 96)) / 1.6) x 100)

b: Relative Total Growth, RTG = (RSG x RCE)/100. Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]

c: Mutant Frequency, MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non-selective medium)]}x800

d: Induced Mutant Frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls

e: Global Evaluation Factor, GEF (126); +: GEF exceeded, -: GEF not exceeded

f: statistical significant increase in mutant frequency compared to negative controls (Mann Whitney test , $p<0.05$).

+: significant; -not significant

EMS: Ethylmethanesulfonate [200 and 300 µg/mL]

MMS: Methylmethanesulfonate [8 and 10 µg/mL]

Table 3.8.1-3: Summary table: Experiment I and II, with metabolic activation

Experiment	**Group**	**Concn (µM)**	**RCE[a] (%)**	**RTG[b] (%)**	**MF[c] (mutants /10^6 cells)**	**IMF[d] (mutants /10^6 cells)**	**GEF[e] exceeded**	**Statistically significant increase[f]**	**Precipitate**
I	C1	0	100.0	100.0	71.0	/	/	/	-
	C2	0	100.0	100.0	77.5	/	/	/	-
	4	50	102.3	96.6	57.9	-16.3	-	-	-
	5	100	146.9	119.4	26.5	-47.8	-	-	-
	6	150	112.1	70.4	42.0	-32.2	-	-	-
	7	200	112.1	71.3	55.8	-18.4	-	-	-
	8	250	117.5	68.7	97.0	22.8	-	-	-
	9	300	84.7	35.0	114.2	39.9	-	+	-
	10	350	112.1	41.5	73.1	-1.1	-	-	-
	11	400	105.4	24.9	108.0	33.8	-	-	-
	12	450	72.4	8.7	188.5	114.3	-	+	-
	B[α]P	3.5 µg/mL	76.6	37.2	546.9	472.7	+	+	-
II	C1	0	100.0	100.0	89.1	/	/	/	-
	C2	0	100.0	100.0	56.4	/	/	/	-
	2	60	90.2	84.5	69.4	-3.3	-	-	-
	3	120	119.0	94.3	85.5	12.7	-	-	-

	4	170	83.7	58.4	94.2	21.4	-	-	-
	5	230	91.6	48.6	98.8	26.0	-	-	-
	6	280	99.0	43.5	128.8	56.0	-	+	-
	7	330	88.9	22.0	152.9	80.2	-	+	-
	8	360	76.7	15.3	158.8	86.0	-	+	-
	9	390	85.0	10.0	153.5	80.7	-	+	-
	B[α]P	3.5 µg/mL	88.9	47.3	666.9	594.1	+	+	-

C: Negative Controls

a: Relative Cloning Efficiency, RCE = [(CE $_{\text{dose group}}$ / CE $_{\text{of corresponding controls}}$) x 100]. Cloning Efficiency, CE = ((-LN (((96 - (mean P1,P2)) / 96)) / 1.6) x 100)

b: Relative Total Growth, RTG = (RSG x RCE)/100. Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]

c: Mutant Frequency, MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non-selective medium)]}x800

d: Induced Mutant Frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls

e: Global Evaluation Factor, GEF (126); +: GEF exceeded, -: GEF not exceeded

f: statistical significant increase in mutant frequency compared to negative controls (Mann Whitney test , $p<0.05$).

+: significant; -not significant

B[*α*]P: Benzo[*a*]pyrene [3.5 µg/mL]

Conclusion: In conclusion, in the described mutagenicity test under the experimental conditions reported, the test item pethoxamid is considered to be non-mutagenic in the *in vitro* mammalian cell gene mutation assay (thymidine kinase locus) in mouse lymphoma L5178Y cells.

3.8.1.5 Anonymous (1992)

Reference: Mutagenicity study of pethoxamid in mammalian cells (V79) in vitro (HGPRT-test)
Author(s), year: Anonymous, 1992
Report/Doc. number: 77 PXA (TOX2001-292) / LPT 7328/92
Guideline(s): OECD 476 (1984)
GLP: Yes
Deviations from OECD 476 (1997): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: 0592; Purity: 98.6 %; Solvent: DMSO.

Test method: Cultured mammalian cells (V79). The cells were exposed to the test substance, both in the presence and absence of S9 mix (rat Aroclor) in two independent experiments. Without S9 mix, cells were exposed for 24 hours, while in the presence of S9 mix, the exposure was limited to two hours. From a preliminary test, it was concluded that the test compound was completely cytotoxic from 30 mg/ml onwards without S9 mix, and from 1000 mg/mL onwards with S9 mix. The following concentrations were used: 0, 1, 3, 10, 20, 30 µg/mL (triplicate cultures without S9 mix) and 0, 10, 30, 100, 200, 300 µg/mL (triplicate cultures with S9 mix). Positive controls were included.

Results: Cytotoxicity was observed starting at concentrations between 20 and 30 mg/mL without (between 100 and 200 mg/mL) and with metabolic activation. The V79 cells tested with the test substance showed mutation frequencies within the normal range of the solvent controls at any dose level,

in the presence or absence of S9 mix. No significance was observed according to the criteria for assay evaluation. The positive control compounds produced clear increases in mutation frequency.

Table 3.8.1-5: Results of HGPRT test

Pethoxamid concentration (µg/mL)	S9-mix	Plating efficiencies				Mutation frequency[1] x10^{-6}	
		Experiment 1		Experiment 2		Experiment 1	Experiment 2
		PE_1	PE_2	PE_1	PE_2		
0	-	0.87	0.70	0.96	0.89	7.4	4.7
1	-	1.18	0.98	0.79	1.08	6.9	4.3
3	-	0.72	0.53	0.74	0.97	3.4	4.7
10	-	0.70	0.69	0.62	0.80	4.3	4.8
20	-	1.01	0.73	0.73	0.94	3.8	4.0
30	-	0.34	0.64	0.17	0.69	3.1	12.2
EMS (600)	-	<0.10	0.82	0.05	0.42	250.7	1002.4
EMS (700)	-	<0.10	0.71	0.11	0.34	206.8	1281.6
0	+	1.08	0.83	0.73	0.65	2.4	5.5
10	+	0.80	0.54	0.72	0.82	8.1	4.9
30	+	0.88	0.88	0.83	0.97	7.5	8.9
100	+	0.92	0.90	0.88	0.61	5.6	6.6
200	+	0.69	0.78	0.77	0.81	7.2	8.4
300	+	0.41	0.90	0.33	0.85	5.3	11.5
DMBA (20)	+	0.61	0.66	0.72	0.73	341.2	301.4
DMBA (30)	+	0.54	0.74	0.54	0.68	201.1	226.8

PE1: Plating efficiency at the end of the exposure time; PE2: Plating efficiency at the time of selection; [1] Mutation frequency defined as total number of thioguanine-resistant cells; EMS: Ethyl methanesulphonate (positive control); DMBA: 9,10-dimethyl-1,2-benzanthracene (positive control), HGPRT: Hypoxanthine-guanidinephosphoribosyl-transferase

Conclusion: Pethoxamid did not show any evidence of exerting mutagenic effects in the HGPRT-test with V 79 cells (forward mutation system).

3.8.2 Animal data

3.8.2.1 Anonymous (1994)

Reference: Pethoxamid: Mouse micronucleus test
Author(s), year: Anonymous, 1994
Report/Doc.number: 78 PXA (TOX2001-293) / TKS '25/941500
Guideline(s): OECD 474 (1983)
GLP: Yes
Deviations from OECD 476 (1997): - The presence of micronuclei in 1000 polychromatic erythrocytes instead of at least 2000 was evaluated
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %.

Test animals: Four groups of 15 female and 15 male CD-1 mice of Swiss origin, specific pathogen free, 22-26 grams of weight, approximately 35 days old, from Charles River (UK) Limited, Margate, Kent, England.

Test method: Based on the results from a preliminary test, single doses of 0, 320, 640 and 1280 mg/kg bw were used. The mice were dosed by intragastric gavage and groups of 5 female and 5 male mice were killed 24, 48 and 72 hours after dosing. The mice of the positive control group were sacrificed after 24 hours. The incidence of micronucleated cells and the proportion of polychromatic erythrocytes was assessed for each animal.

Results:

Mortality: Eight female and 3 male mice died after treatment with the high dose. At post-mortem examination, none of these animals showed signs of misdosing. As at the highest dose 5 extra female and 5 extra male mice were treated, 10 animals were replaced as follows: 5 females, 2 males instead of 8 females and 3 males.

Clinical signs: Within the first hour after dosing in all groups, included hunched posture and piloerection. Recovery for the low and intermediate dose groups was within 3 hours. The high dose group also showed lethargy and ptosis. Recovery was complete in all mice by the end of Day 1.

Erythrocyte count: No significant increase in the number of micronucleated immature erythrocytes was observed at 24, 48 or 72 hours. No effect was observed on the proportion of immature erythrocytes, indicating no bone marrow toxicity. The positive control group did show an increased frequency of micronucleated cells.

Table 3.8.2-1: Results of the micronucleus test

Treatment	Dose level (mg/kg bw)	Sampling time	Frequency of micronucleated polychromatic cells[1]		P:M ratio	
			mean incidence	range	mean ratio	range
Methylcellulose	1%	24 h	0.9	0-2	0.817	0.464-1.343
Pethoxamid	320		0.8	0-2	0.894	0.591-1.270
	640		0.6	0-2	0.974	0.645-1.574
	1280		1.2	0-5	0.940	0.495-1.242
Mitomycin C	12		55.1*	30-95	0.839	0.474-1.087
Methylcellulose	1%	48 h	0.7	0-3	0.932	0.616-1.440
Pethoxamid	320		0.5	0-2	0.843	0.618-1.098
	640		0.6	0-2	0.820	0.488-1.197
	1280		0.8	0-	0.768	0.328-1.352
Methylcellulose	1%	72 h	0.6	0-2	0.993	0.644-1.415

1.415Pethoxami0.451-1.783d	320		1.3	0-4	0.946	0.451-1.783
	640		1.1	0-3	1.163	0.554-2.146
	1280		0.4	0-2	0.937	0.533-1.477

[1] Number of micronucleated cells observed per 1000 immature erythocytes examined; P:M: Proportion of immature (polychromatic) erythrocytes to mature (normochromatic) erythrocytes; Methylcellulose (vehicle); Mitomycin C (positive control);

Statistical significance: * $p<0.001$

Conclusion: Pethoxamid did not show any evidence of chromosomal or other damage leading to micronucleus formation in this *in vivo* test.

3.8.2.2 Anonymous (1994)

Reference: Pethoxamid: In vivo rat liver DNA repair test;
Author(s), year: Anonymous, 1994
Report/Doc. number: 79 PXA (TOX2001-294) / TKS '25/941500
Guideline(s): OECD 482 (1986), OECD 486 (1997, Draft)
GLP: Yes
Deviations from OECD 486 (1997): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %.

Test animals: Albino Hsd/Ola Sprague-Dawley male rats, specific pathogen free, 140-149 grams of weight and five weeks old, from Harlan Olac (UK), LTD., Bicester, Oxon, England.

Test method: From the results of a preliminary toxicity study, in which minor toxicity at the maximum dose level of 2000 mg/kg bw was observed, single doses of 600, 1200 (provisional group, not evaluated for unscheduled DNA synthesis) and 2000 mg/kg bw were used. Groups of 4 rats were administered the test compound orally by intragastric gavage. Hepatocytes were isolated at 2 and 14 hours after the administration. Positive controls were included. Gross nuclear grain counts (silver grains overlying the nucleus) and net nuclear grain counts (cytoplasmatic grain count subtracted from gross nuclear grain count) were assessed. On completion of the grain count analysis, the stained autoradiographs from the 14 hours expression were recorded for S-phase cells (for each animal 1000 hepatocytes from several randomly fields of view).

Results:
Mortality and clinical signs: No mortalities occurred. For the 2 hours expression time group within the first hour after dosing, slight piloerection was observed at 600 and 2000 mg/kg bw. Recovery was complete by the second hour. No clinical signs were recorded for any of the rats in the 14 hours expression time group.
Grain count: The test compound did not cause any significant increase in either the gross or net nuclear grain count at both dose levels at the 2 hours expression time At the 14 hours expression time, statistically significant increases in gross nuclear grain counts were obtained at dose levels of 600 and 2000 mg/kg bw but not in net nuclear grain counts, i.e. not indicative for unscheduled DNA synthesis. Positive control group animals showed a significant increase in the gross and net nuclear grain count.

During analysis, it was noted that some slices had an unusually high number of S phase cells (easily recognizable by their heavily grained appearance). Therefore for all groups (including the provisional group) the S-phase cells after 14 hours of expression were determined. Only the group which received 600 mg/kg bw showed statistical significance. The increases of S-phase cells are indicative of an increased cell proliferation.

Table 3.8.2-2: Results of the liver DNA repair test

Treatment	Dose level (mg/kg bw)	Expression time	Nuclear grain count		S-phase cells (cells/1000 hepatocytes)
			Mean gross	Mean net	
Methylcellulose	1%	2 h	15.5	-3.7	nd
Pethoxamid	600		15.6	-2.6	nd
	2000		16.2	-4.1	nd
Dimethylnitrosamine	4		53.8**	39.00**	nd
Methylcellulose	1%	14 h	8.7	-2.7	9.3

Pethoxamid	600		12.4**	-2.0	98.8**
	1200		nd	nd	47.0
	2000		11.3*	-2.3	47.3
2-Acetylaminofluorene	50		26.3**	15.9**	20.0[1]

Methylcellulose (vehicle); Dimethylnitrosamine (positive control); 2-Acetylaminofluorene (positive control); nd: not determined;

[1] not statistically evaluated because of insufficient variance to allow analysis;

Statistical significance: * $p<0.01$, ** $p<0.001$

Conclusion: Pethoxamid did not elicit unscheduled DNA synthesis in the rat liver in this *in vivo* test system.

3.8.3 Human data

No relevant studies.

3.8.4 Other data

No relevant studies.

3.9 Carcinogenicity

3.9.1 Animal data

3.9.1.1 Anonymous (2000a)

Reference: TKC-94 Pethoxamid: Potential tumorigenic and toxic effects in prolonged dietary administration to rats
Author(s), year: Anonymous., 2000a
Report/Doc. number: 80 PXA (TOX2001-295) / TON 6/974064
Guideline(s): OECD 453 (1981)
GLP: Yes
Deviations from OECD 453 (2009): Haematology: mean corpuscular hemoglobin (MCH) not tested; No organ weight of uterus; No preservation of the coagulating gland, peripheral nerve
Acceptability: Yes

Reference: Amendment 1 toTKC-94 Pethoxamid: Potential Tumorigenic and Toxic Effects in Prolonged Dietary Administration to Rats
Author(s), year: Anonymous., 2000-amdt-1
Report/Doc. number: 80 PXA amdt-1 (TOX2001-295) / TON 6/974064
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

In addition to the usual investigations, electron microscopical investigations of liver tissue were performed on satellite groups (0 and 1600ppm). Results were integrated in the main study report (L. Anonymous (2000a, 80 PXA (TOX2001-295) / TON '6/974064))

Reference: Supplement 1 to TKC-94/Pethoxamid - TON 6/974064: Organ weight data report
Author(s), year: Anonymous, 2003

Report/Doc. number: 80 PXA suppl-1 (TOX2001-295)
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Raw data of individual organ weights of animals used in Anonymous (2000a, 80 PXA (TOX2001-295) / TON '6/974064).

Reference: Supplement 2 to Historical histopathology data and historical organ weight data
Author(s), year: Anonymous, 2016
Report/Doc. number: 80 PXA suppl-2
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Historical Control Data is presented for studies starting between 1996 and 1999 which is considered relevant for Anonymous (2000a;80 PXA). Data on neoplastic findings in thyroid and pancreas, as well as organ weight of thyroids are presented.

Reference: Position paper - Pethoxamid: EU-Wirkstoffprüfung zur Aufnahme von Wirkstoffen in Anhang I der Richlinie 91/414/EWG - Neuer Wirkstoff: Pehoxamid, Kenn-nr. WNL 005072-00/00 : Erstellung eines Monographie-Addendums (Sektion Toxicologie und Metabolismus)
Author(s), year: Anonymous, 2003
Report/Doc. number: Cheminova A/S Report No.: 203 PXA Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Calculations of thyroid weights relative to body weights were presented. Furthermore, statistical analyis of pancreas islet cell tumour rates were shown (pair wise comparison; trend test)

Deviations from study protocol: On occasions, the temperature and humidity exceeded the pre-set limits, which were generally transient. Diet usage records indicate that some rats from the 25 ppm group may have been fed small amounts of the diet containing 400 ppm for a maximum of 3 days. These deviations were not considered to affect the integrity and/or validity of the study.

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306; Purity: 95.0 %.

Test animals: Four groups of 80 male and 80 female Crl:CD BR (IGS) rats, 43 days old, 176-260 g (males) and 128-189 g (females) of weight, from Charles River Laboratories, Manston Road, Margate, Kent, UK. The main groups (50 rats/sex/group) received the test material by dietary administration at concentrations of 0, 25, 400 and 1600 ppm for 104 weeks. The doses were based on the 13 weeks study (Report no. TKS 24/961565). From the satellite groups (30/sex/group), up to 10 rats/sex/group were pathologically examined after the completion of 26, 52 and 78 weeks of treatment.

In order of increasing doses the treated rats ingested the equivalent of 1.0, 17.0 and 70 mg/kg bw/day for males and 1.4, 23.3 and 99 mg/kg bw/day for females.

All animals were subjected to body weight, clinical sign, food and water consumption, ophthalmoscopy, haematology, biochemistry, urinalysis, organ weight, macro- and microscopic pathology assessments.

Furthermore, electron microscopy was performed with liver tissue from satellite groups sacrificed after 26 and 52 weeks of treatment.

The homogeneity and stability of the formulation was regularly checked by chemical analysis.

Analytical chemistry: The mean concentrations of pethoxamid in the test diet formulations were between +9.5% (one exception +15.6%) and -13 % of nominal concentrations which were within the acceptable limits (+10%/-15%).

Mortality, clinical and ophthalmologic findings: Neither treatment related deaths nor treatment related clinical and ophthalmologic signs occurred.

Body weight, body weight gain and food consumption: The body weights at the terminal kill were not significantly different from control values. Lower body weight gains were measured from 400 ppm, significant at 1600 ppm. For females, a treatment-related effect at 400 ppm cannot be excluded in view of the consistency between the main and satellite groups and the magnitude of differences from control values.

At 400 ppm, the males showed a significant lower food intake only during the first 4 weeks of the study. At 1600 ppm, the lower food intake was slight but consistent throughout the study. At this dose, the females consumed less food only during Week 1 to 4.

Table 3.9.1-1: Body weight, body weight gain and food consumption data

Parameter	Dose (ppm)							
	Males				Females			
	0	25	400	1600	0	25	400	600
Body weight terminal kill (g)	771	785	748	722	466	526	509	440
Body weight gain (g/rat) Week 0-4 (main)	176	178	170	149**	75	77	74	64**
Body weight gain (g/rat) Week 0-88 (main)	631	635	612	547**	376	377	336	290**
Body weight gain (g/rat) Week 0-104 (main)	560	579	540	506	308	375	349	283
Body weight gain (g/rat) Week 0-4 (satellite)	182	178	170	150**	72	78	71	66*
Food consumption (g/rat) Week 1-4 (main)	851	837	814**	779**	591	577	568	556**
Food consumption (g/rat) Week 1-104 (main)	21387	21034	20860	20121**	16483	16640	16140	15950
Food consumption (g/rat) Week 1-4 (satellite)	847	825	824	792**	577	581	563	550**
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$								

Haematology: At 1600 ppm males showed consistently lower mean reticulocyte values. However, in the absence of a similar finding for females or any corroborative blood film or microscopic findings, this difference in males was not considered to be of toxicological importance.

Clinical chemistry and urinalysis: At 1600 ppm in all weeks of investigations, both sexes showed statistically significantly higher mean cholesterol values, except for males in Week 78. Males showed

statistically significantly higher g-GT activities values in all weeks of investigation and females showed slightly, but consistently higher mean globulin and concomitant total protein values, statistical significance being attained for the globulin values except for Weeks 78 and 104.

At urinalysis, no treatment-related findings were observed.

Table 3.9.1-2: Clinical chemistry

Parameter	**Week**	**Dose (ppm)**							
		Males				**Females**			
		0	25	400	1600	0	25	400	1600
Cholesterol (mg/dL)	13	87	75	84	122**	81	93	85	108**
	26	98	82	90	119*	90	105	102	127**
	52	108	95	100	130*	107	114	109	156**
	78	121	111	125	141	122	114	129	178*
	104	111	128	139	158*	128	123	153	187*
γ-glutamic transferase (mU/mL)	13	<1	<1	<1	<4**	1	1	1	1
	26	<2	<1	<2	<3**	2	1	1	2
	52	<2	<1	<2*	4**	2	2	2	2
	78	<1	<1	<1	<4**	1	1	1	2
	104	<1	<2	<2	<8**	1	1	1	1
Globulin (g/dL)	13	3.8	3.8	3.7	4.0	3.5	3.7	3.5	3.8*
	26	3.8	3.8	3.7	3.8	3.5	3.6	3.5	3.8*
	52	4.1	3.9	4.1	4.1	3.8	3.9	3.9	4.3**
	78	4.3	4.1	4.0	4.1	3.9	3.8	3.9	4.1
	104	3.9	3.9	4.0	3.9	4.0	3.9	4.1	4.3
Total protein (g/dL)	13	6.8	6.8	6.7	7.0	6.9	7.1	6.0	7.2
	26	6.7	6.6	6.6	6.6	7.0	7.3	7.0	7.3
	52	7.1	7.1	7.2	7.2	7.7	7.8	7.9	8.5**
	78	7.3	7.1	6.9	7.2	7.8	7.6	7.7	7.9
	104	6.4	6.4	6.6	6.6	7.3	7.0	7.3	7.6
Statistical significance *p<0.05; **p<0.01									

Pathological examinations:
Necroscopy: Only the male rats from the main group revealed changes at the high dose

Table 3.9.1-3: Necropsy findings (decedent and terminal sacrifice animals)

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	25	400	1600	0	25	400	1600

Liver, enlarged	2/50	4/50	1/50	6/50	2/50	1/50	4/50	2/50
Thyroid, enlarged	4/50	8/50	6/50	12/50	4/50	2/50	3/50	2/50

Organ weights: The higher thyroid weights in treated animals at some occasions - thus not consistent with time - were not supported by any microscopic or biochemical changes. In contrast, the generally increased liver weights at the high dose were accompanied by the typical histopathological findings.

Table 3.9.1-4: Organ weights

Organ		**Week**	**Dose (ppm)**							
			Males				**Females**			
			0	25	400	1600	0	25	400	1600
Liver	adjusted (g)	27	24.5	23.5	24.3	26.9* +10%	11.8	11.8	12.2	13.8** +17%
		53	25.1	25.0	24.4	28.1* +12%	14.6	13.6	12.5	14.9 +2%
		79	25.0	23.6	26.3	29.1 +16%	16.7	16.2	14.9	18.7 +12%
		105	25.6	25.1	25.8	28.5 +11%	17.5	17.7	18.0	20.3** +16%
Thyroid	absolute (mg)	53	29.6	38.3 +30%	35.3 +19%	38.7 +30%	25.7	27.2	29.5	24.3
	adjusted (mg)		26.9	37.1** +38%	37.5** +39%	40.3** +50%	25.6	26.2	28.8	26.0
	relative (%)		3.7	5.0 +35%	5.1 +38%	5.5 +49%	6.8	6.9	7.5	7.0
	absolute (mg)	79	35.9	35.8	50.0 +39%	44.5 +24%	38.1	30.0	31.0	34.4
	adjusted (mg)		34.4	36.3	51.4* +49%	45.1** +31%	n.a	n.a	n.a	n.a
	relative (%)		4.4	4.7	6.7 +52%	5.8 +32%	7.5	6.1	7.1	8.4
	absolute (mg)	105	61.6	45.1	49.9	51.7	41.8	43.2	36.9	36.6
	adjusted (mg)		n.a	n.a	n.a	n.a	42.2	41.4	35.8	37.9
	relative (%)		8.0	5.7	6.7	7.2	9.0	8.2	7.2	8.3

n.a: not applicable

Statistical significance: *≤ 0.05, **≤ 0.01

Table 3.9.1-5: Historical Control Data - Range of thyroid weights from performing laboratory

Organ weight	Week	Male	Female
Absolute weight (mg)	52	31.3-35.0	27.2-32.0
Realative weight (%)	52	4.3-5.1	7.1-7.6
Absolute weight (mg)	104	40.0-61.3	35.0-44.8
Realative weight (%)	104	5.5-8.9	7.2-8.9
Week 52 data derived from 2 studies performed in 1997 and 1999 Week 104 data derived from 10 studies performed between 1996 and 1999			

Histopathology, interim kills: At the high dose, both sexes showed a statistically significant increased incidence of centrilobular hepatocyte hypertrophy in Week 26, males also in Weeks 52 and 78. Males also showed an increased incidence of concentric intracytoplasmic inclusions (mainly periportal zones) in Weeks 26, 52 and 78. The light microscopic findings were supported by electron microscopy, done at Weeks 26 and 52, in which an increase of smooth endoplasmic reticulum was found (centrilobular: minimal for both sexes; periportal: minimal for females and moderate for males) and in which the inclusions in males were recognized as multilamellar.

Histopathology, terminal kill - neoplastic findings: At 1600 ppm, a slightly higher incidence of follicular cell adenomas of the thyroid was seen in male rats. The incidence falls outside the background control range. The test for trend was statistically significant. The pairwise comparison between control and high dose groups (adenoma and carcinoma combined) was not statistically significant. The incidence of follicular carcinoma was two in the control and zero in all treated groups. The adenomas were all within a similar morphological and size range and occurred generally singly in the affected animals.

Slightly higher incidences of pancreas islet cell tumours were seen in main group male rats from the 25ppm and 1600ppm groups compared to controls. However, no statistically significant differences were noted in the incidences and distribution, and the incidences were not dose dependent. Though the findings were slightly above the range of historical control data, they were considered to be incidential and not related to treatment.

Histopathology, terminal kill - non-neoplastic findings: The main groups - as the satellite groups - showed hepatocyte hypertrophy. Associated findings were focal clear cell hepatocytes, focal cystic degeneration, and hepatocytes with concentric intracytoplasmic inclusions (mainly in periportal zones).

In the thyroid, a small and not significant increase in the incidence of follicular cell hyperplasia and follicular cell cystic hyperplasia was seen in male rats at the high dose.

No treatment related effects were recognized to be contributory to death.

Table 3.9.1-6: Histopathology findings

Finding	Dose level (ppm)							
	Males				Females			
	0	25	400	1600	0	25	400	1600
Neoplastic findings								

Thyroid								
- Follicular cell adenoma, main[1]	2/50 4%	2/50 4%	2/49 4.1%	9/50[2] 18%	1/50	0/50	2/50	2/50
- Follicular cell carcinoma, main[1]	2/50	0/50	0/49	0/50	0/50	0/50	0/50	0/50
Pancreas								
- Islet cell adenoma, main[1]	4/49 8.2%	8/50 16%	6/50 12%	8/50 16%	3/50	3/40	2/33	2/50
- Islet cell carcinoma, main[1]	0/49 0%	2/50 4%	1/50 1%	2/50 4%	0/50	0/40	0/33	0/50
Non-neoplastic findings								
Thyroid								
- Follicular cell hyperplasia	0/50	0/50	1/49	4/50	0/50	0/50	0/50	0/50
- Follicular cell cystic hyperplasia	4/50	1/50	4/49	7/50	1/50	0/50	0/50	1/50
Liver								
- Centrilobular hepatoctic hypertrophy, main	0/50	0/50	0/50	11/50**	0/50	0/50	0/50	8/50**
- Centrilobular hepatoctic hypertrophy, satellite, week 26	0/10	0/10	0/10	7/10	0/10	0/10	0/10	5/10
- Centrilobular hepatoctic hypertrophy, satellite, week 52	0/10	0/10	0/10	6/10	0/10	0/10	0/10	0/10
- Centrilobular hepatoctic hypertrophy, satellite, week 78	0/10	0/9	0/4	3/8	0/7	0/8	0/8	0/10
- Concentric intracytoplasmic inclusions, main[1]	0/50	0/50	0/50	10/50**	0/50	0/50	0/50	0/50
Concentric intracytoplasmic inclusions, week 26	0/10	0/10	0/10	4/10	0/10	0/10	0/10	0/10
- Concentric intracytoplasmic inclusions, main[1], week 52	0/10	0/10	0/10	5/10	0/10	0/10	0/10	0/10
- Concentric intracytoplasmic inclusions, main[1], week 78	0/10	0/9	0/4	2/8	0/7	0/8	0/8	0/10
- Cystic degeneration, main[1]	12/50	5/50	14/50	24/50*	1/50	2/50	0/50	0/50

- Clear cell hepatocytes, main[1]	6/50	6/50	6/50	15/50*	3/50	5/50	4/50	2/50
[1] Decedents and terminal sacrificed [2] Trend test statistically significant; Statistical significance: *≤ 0.05, **≤ 0.01								

Table 3.9.1-7: Historical control data from performing laboratory

Finding	Males		Females	
	Totals (affected/examined)	Range	Totals (affected/examined)	Range
Thyroid				
- Follicular cell adenoma	30/954; 3.14%	0.0-12%	16/951; 1.68%	0.0-8.3%
- Follicular cell carcinoma	7/954; 0.73%	0.0-5.1%	4/951; 0.42%	0.0-1.7%
Pancreas				
- Islet cell adenoma	85/954; 8.91%	1.7-13.8%	not available – not necessary	
- Islet cell carcinoma	30/954; 3.14%	0.0-11.7%	not available – not necessary	
Historical control data derived from 16 studies performed between 1996 and 1999				

Conclusion: The NOAEL is considered to be 25 ppm (1.0 mg/kg bw/d), based on the decreased body weight gain in females at 400 ppm (approximately 11% compared with the control group; weeks 0 - 88) which has been evaluated as an adverse effect. The increase of thyroid weights in males (weeks 53 and 79) is considered to be caused by a rodent-specific mechanism which is not relevant to humans. This effect is therefore not considered for setting a NOAEL for this study.

The only evidence of tumourigenicity was a higher incidence of thyroid follicular cell adenoma in males at the high dose level of 1600 ppm. Mechanistic studies have postulated a phenobarbitone-like mode of action for pethoxamid leading to increased incidences of thyroid follicular cell tumours, particularly in the male rat. Due to differences in thyroid physiology between rodents and humans, thyroid tumours in rodents consequent to the phenobarbitone-like mode of action are not considered relevant to humans.

According to the criteria specified in Regulation (EC) 1272/2008, **classification of pethoxamid regarding carcinogenicity is not required.**

3.9.1.2 Anonymous (2000b)

Reference: Pethoxamid: Carcinogenicity study by administration to CD-1 mice for at least 80 weeks
Author(s), year: Anonymous, 2000b
Report/Doc. number: 82 PXA (TOX2001-296) / TON 014/973848
Guideline(s): OECD 453 (1981)
GLP: Yes
Deviations from OECD 453 (2009):
- slight exceedance of weight variation in females
- no organ weights of spleen, uterus
- no preservation of the coagulating gland, peripheral nerves
- haematology: only blood smears were prepared
- no clinical biochemistry measurements conducted

Acceptability: Yes

Reference: Supplement 1, Historical histopathology data CD-1 Mice studies of 78 to 106 weeks duration
Author(s), year: Anonymous, 2016
Report/Doc. number: 82 PXA suppl-1
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Historical Histopathology Data is presented for studies starting between 1996 and 1999 which is considered relevant for Anonymous (2000b; 82 PXA). Data on neoplastic findings in the liver of male mice are presented.

Reference: TKC-94 : Carcinogenicity Study by Administration to CD-1 Mice for at least 80 Weeks. Photomicrographic Report
Author(s), year: Anonymous, 2001
Report/Doc. number: 83 PXA (TON 082/000175)
Guideline(s): -
GLP: Yes
Deviations: -
Acceptability: Yes

The objective of this study was to illustrate microscopic findings in the duodenum and jejunum of selected animals from the carcinogenicity study by administration to CD-1 mice for at least 80 weeks. Low and high magnification photomicrographs of the duodenum and jejunum of selected animals, killed at termination, are presented.

Reference: Position paper. Carcinogenicity in mice statement
Author(s), year: Anonymous, 2001
Report/Doc. number: 1484 PXA
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

A statement is given regarding the gastro-intestinal effects in the long-term toxicity study in mice (Anonymous (2000b); 82 PXA). It is stated that the metabolism study revealed that 33 to 66% of Pethoxamid is excreted by faeces, therefore a huge amount of metabolites is passing the gastro-intestinal tract. As the same findings are observed in the satellite group (terminated at week 52) and the main group (terminated at week 95/92) the severity of effects has not been increased between termination of the satellite and the main group. Moreover, no cancer could be observed in that region. This occurrence did not shorten the life of animals. It is further stated that Pethoxamid is assumed to be a Phenobarbital type inducer and this reaction might be an adaptive response to the hepatic enzyme induction. Furthermore, this is claimed a rodent-specific effect and not of concern for humans.

Reference: TKC-94 : Additional study on cell proliferation in the liver to Carcinogenicity Study by dietary Administration to CD-1 Mice for at least 80 Weeks
Author(s), year: Anonymous, 2001
Report/Doc. number: 1241 PXA / IET 00-0138
Guideline(s): -
GLP: Yes

Deviations: -
Acceptability: Yes

In order to evaluate the effect of pethoxamid on hepatic cell proliferation, paraffin sections of the liver obtained from the original paraffin blocks used in the carcinogenicity study of the test substance by dietary administration to CD-1 mice for at least 80 weeks (TON 014) were subjected to immunohistochemistry for proliferating cell nuclear antigen (PCNA). PCNA labeling index (LI) was determined for each animal by counting the number of PCNA-positive (S-phase) cells per approximately 1000 hepatocytes. Cell proliferation was evaluated on the basis of PCNA LI for each dose group at 0, 30, 400, and 5000 ppm after 52 and 95 weeks of treatment. There were no significant changes in PCNA LI considered to be treatment-related in any dose group after 52 or 95 weeks of treatment. Based on the results described above, it has been suggested that TKC-94 appears to have no influence on cell proliferation in the liver when administered in feed to male mice at 30, 400 and 5000 ppm for 52 or 95 weeks.

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306 and TB-960306-C; Purity: 95.0% and 94.8%.

Test animals: Groups of 60 male and 60 female CD-1 BR mice, 6 weeks old and 25-37 g (males) and 19-30 g (females) of weight, from Charles River Laboratories, Margate, Kent, England.

Test method: The mice received the test material by dietary administration at concentrations of 0, 30, 400 or 5000 ppm. Main group animals (50/sex/group) were treated until one of the groups in each sex reached 50% survival rate, namely for up to 92 weeks (females) or 95 weeks (males) for assessment of tumorigenic potential. Satellite animals (10/sex/group) were treated for up to 52 weeks and were used for assessment of chronic toxicity. In order of increasing doses the treated mice ingested the equivalent of 4.0, 56.8 and 982 mg/kg bw/d for males and 5.0, 68 and 1068 mg/kg bw/d for females.

All animals were subjected to body weight, clinical signs, food consumption, haematology (blood smears), organ weights, macro- and microscopic pathology assessments.

The treatment levels were based on the results from the 4-week (Report no. TON 3/960337) and the 13 week study (Report no. TON 5/971279).

The homogeneity and stability of the formulation was checked by chemical analysis.

Results:

Analytical chemistry: Analysis indicated that the samples taken from the formulations were within 10% deviation of nominal values.

Mortality and clinical signs: There was no effect of treatment on survival. No clinical signs were observed, indicative of a reaction to treatment.

Body weight, body weight gain and food consumption: The body weights were significantly lower in the animals of the high dose groups at the end of the study. As well, throughout the treatment period, the body weight gains were significantly decreased. This was accompanied for males by a statistically significant higher food consumption.

Table 3.9.1-8: Body weight development and food consumption

Parameter	Dose (ppm)

	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Body weight (g)[1]	50	53	51	42**	41	42	44	34**
Body weight gain (g/mouse) – week 0-92/95 (main)[1]	19.1	22.2	19.2	11.5**	17.2	18.0	19.2	11.3**
Body weight gain (g/mouse) – week 0-52 (satellite)	23.0	18.1	24.6	9.1**	21.3	13.9	15.4	9.4**
Food consumption (g/mouse)	4579	4512	4687	5527**	4365	4025	4348	4598
[1] Males at Week 95, females at Week 93 Statistical significance: *p<0.05; **p<0.01								

Pathological examinations:
Necroscopy: A higher incidence of liver masses, granulation of the kidneys and kidney cysts were noted in males at 5000 ppm at the end of the study. No treatment-related findings were noted at the interim kill.

Table 3.9.1-9: Macroscopic pathology, terminal kill

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver								
- masses	11/27	13/28	12/25	25/34	1/25	2/30	0/31	0/32
Kidneys								
- granulation	0/27	1/28	2/25	17/34	0/25	0/30	1/31	0/32
- cysts	4/27	2/28	6/25	9/34	0/25	0/25	0/25	0/25

Organ weights: From 400 ppm, the adjusted liver and kidney weights were increased and additionally at 5000 ppm, the thyroid weights were increased in both sexes and the adrenal weights in males.

Table 3.9.1-10: Organ weights

Parameter	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver (g), terminal, adjusted[2]	2.70	2.88 +7%	3.09 +14%	4.78** +77%	2.01	2.02	2.22* +10%	2.73** +36%
Liver, interim, adjusted[2]	2.64	2.61	2.60	3.37** +28%	1.97	2.04	2.21 +12%	2.58* +31%
Kidney (g), term., adjusted[2]	0.915	0.890	0.951	0.992	0.511	0.522 +2%	0.569** +11%	0.629** +23%

Kidney, interim, adjusted[2]	0.930	1.029	0.893	0.982	0.496	0.558 +13%	0.552 +11%	0.592* +19%
Thyroid (mg), terminal, absolute	7.0	6.7	7.2	8.5* +21%	7.8	8.0	7.9	9.7 +24%
Thyroid, terminal, adjusted[2]	na	na	na	na	7.8	7.8	7.6	10.1** +29%
Adrenals (mg), terminal, adjusted[2]	7.0	6.7	7.0	8.8* +26%	na	na	na	na
[2] Adjusted for body weight; na not applicable Statistical significance: *p<0.05; **p<0.01								

Histopathology, interim kill: Hepatocyte hypertrophy was observed in females at 400 ppm. At the high dose, it was seen in both sexes. Some males showed at 30 ppm intestinal swelling and rarefaction of villous epithelial cells in the duodenum and jejunum. At the higher doses, the finding occurred in both sexes and was in males accompanied by hypertrophy. From 400 ppm, a not dose related and not significant increase in follicular cell hypertrophy in the thyroid was observed.

Table 3.9.1-11: Microscopic pathology, interim kill

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver								
hepatocyte hypertrophy, generalised	0/8	0/8	0/10	9/9***	0/10	0/8	0/10	0/10
hepatocyte hypertrophy, periportal	0/8	0/8	0/10	0/9	0/10	0/8	2/10	9/10***
Duodenum								
swelling/rarefaction of villous epithelium	0/8	5/8*	8/10**	9/9***	0/10	0/8	6/10*	10/10***
villous hypertrophy	0/8	0/8	2/10	7/9**	0/10	0/8	0/10	7/10**
Jejunum								
swelling/rarefaction of villous epithelium	0/8	4/8	6/10*	8/9***	0/10	0/8	5/10*	10/10***
villous hypertrophy	0/8	0/8	3/10	6/9**	0/10	0/8	0/10	7/10**
Thyroids								
follicular cell hypertrophy	2/8	0/8	5/10	5/9	2/10	1/8	2/10	7/10
Statistical significance: *p<0.05; **p<0.01; *** p<0.001								

Histopathology, terminal kill - neoplastic findings: At 5000 ppm, treatment-related hepatocellular adenomas appeared in male mice. There was also a slightly higher but not statistically significant number of malignant hepatocellular tumors.

Table 3.9.1-12: Microscopic pathology – neoplastic findings, terminal kill

Finding	Dose (ppm)							
	Males				Females			
	0	30	400	5000	0	30	400	5000
Liver number examined	50	50	50	50	50	50	50	50
- hepatocellular adenoma	19 (38%)	15 (30%)	18 (36%)	34** (68%)	3	1	3	0
- hepatocellular carcinoma	3 (6%)	3 (6%)	4 (8%)	6 (12%)	1	0	0	0

Histopathology, terminal kill - non-neoplastic findings: At 5000 ppm, hepatocyte hypertrophy (generalized in males and periportal in females) was seen in both sexes. It is likely that the hypertrophy was due to the induction of drug metabolizing hepatic enzymes. At both kills, the severity of this finding was mainly moderate (no evidence of progression). The incidence of vascular pooling in centrilobular areas, commonly occurring in ageing mice, was only increased in males.

In the kidney, a higher incidence of some findings, which commonly occur in older mice, was found. Males additionally showed an increased incidence of cortical tubular cell hypertrophy (slight), cortical cysts, and cortical fibrosis with tubular collapse and basophilia.

The incidence of swelling and rarefaction of villous epithelial cells of the duodenum and jejunum was increased at all doses. At higher doses this finding was accompanied by villous hypertrophy. The severity of these findings in animals killed at the end of the treatment period was not increased compared to that in mice killed after 52 weeks of treatment.

Table 3.9.1-13: Microscopic pathology, non-neoplastic findings - terminal kill

Finding	Dose (ppm)							
	Males				Females			
	0	30	400	5000	0	30	400	5000
Liver number examined	50	50	50	50	50	50	50	50
- hepatocyte hypertrophy – generalised	0	0	0	39***	0	0	0	0
- - hepatocyte hypertrophy - periportal	0	0	0	1	0	0	0	42***
- vascular pooling in centrilobular areas	0	2	0	10	4	4	6	0
Duodenum number examined	48	47	47	49	45	50	50	49
- swelling/rarefaction of villous epithelium	0	6*	29***	42***	0	3	12***	18***
- villous hypertrophy	0	0	9**	27***	0	0	0	5
Jejunum number examined	48	47	48	49	46	50	50	49
- swelling/rarefaction of villous epithelium	0	4	25***	35***	0	2	7*	14***

- villous hypertrophy	0	0	8**	16***	0	0	0	2
Kidney number examined	50	50	50	50	50	50	50	50
- cortical tubular cell hypertrophy (slight)	0	0	0	8**	0	0	0	0
- cortical fibrosis with tubular collapse and basophilia	13	13	15	37***	8	4	6	4
- cortical cysts	20	15	15	31*	7	4	7	5
- cortical tubules - basophilic	33	26	35	43*	12	13	14	41***
- medullary tubules dilated with eosinophilic casts	27	23	30	42**	14	14	13	32***
- cortical mineralisation	32	34	40	46**	3	0	1	26***
- medullary mineralisation	6	8	13	44***	0	0	0	31***
- papillary mineralisation	11	4	10	36***	3	6	1	30***
Statistical significance: *p<0.05; **p<0.01; *** p<0.001								

Table 3.9.1-14: Historical control data for hepatocellular adenomas and carcinomas in males

Finding	Totals (affected/examined)	Range
Hepatocellular adenoma	199/867; 22.95%	8.3 – 42%
Hepatocellular carcinoma	70/867; 8.07%	3.6 – 22%
Historical control data derived from 16 studies performed between 1996 and 1999		

Conclusion: The only evidence of tumourigenicity was an increased number of males showing benign hepatocellular liver tumours at the high dose of 5000ppm. The no effect level for tumourigenicity was set at 400ppm (56.8 mg/kg bw/d). Mechanistic studies have postulated a phenobarbitone-like mode of action for pethoxamid involving liver enzyme induction leading to increased liver tumour formation, particularly in males. This type of tumour formation is considered rodent-specific and not relevant to humans.

Non-neoplastic microscopic changes occurred in the duodenum and jejunum. The swelling and rarefaction of villous epithelial cells was of higher incidence in males than in females. In the duodenum, it was significantly increased in males at 30 ppm in the interim and the terminal kills. Since effects on the GIT were also noted in other toxicity studies, the LOAEL was considered to be < 30ppm (< 4 mg/kg bw/d). No NOAEL could be derived from this study.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding carcinogenicity is not required.

3.9.2 Human data

No relevant studies.

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests)

No relevant studies.

3.9.4 Other data (e.g. studies on mechanism of action)

3.9.4.1 Anonymous (2001a)

Reference: TKC-94: Additional study on cell proliferation in the liver to Carcinogenicity Study by dietary Administration to CD-1 Mice for at least 80 Weeks.
Author(s), year: Anonymous, 2001a
Report/Doc. number: 1241 PXA / IET 00-0138
Guideline(s): Not applicable
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

In order to evaluate the effect of pethoxamid on hepatic cell proliferation, paraffin sections of the liver obtained from the original paraffin blocks used in the carcinogenicity study of the test substance by dietary administration to CD-1 mice for at least 80 weeks (TON 014) were subjected to immunohistochemistry for proliferating cell nuclear antigen (PCNA). PCNA labeling index (LI) was determined for each animal by counting the number of PCNA-positive (S-phase) cells per approximately 1000 hepatocytes. Cell proliferation was evaluated on the basis of PCNA LI for each dose group at 0, 30, 400, and 5000 ppm after 52 and 95 weeks of treatment. There were no significant changes in PCNA LI considered to be treatment-related in any dose group after 52 or 95 weeks of treatment. Based on the results described above, it has been suggested that TKC-94 appears to have no influence on cell proliferation in the liver when administered in feed to male mice at 30, 400 and 5000 ppm for 52 or 95 weeks.

3.9.4.2 Anonymous (2000)

Reference: Investigation of the potential effects of dietary administration of pethoxamid on thyroid function in male rats using the perchlorate discharge test
Author(s), year: Anonymous, 2000
Report/Doc. number: 94 PXA (TOX2001-304) / TON '072/002273
Guideline(s): Not applicable, non-guideline study
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

Material and Methods:

Principle of the method: The test uses the ability of perchlorate (a competitive inhibitor of thyroidal iodine transport) to discharge free ^{125}iodide from the thyroid. The presence of free ^{125}iodide within the thyroid is a consequence of the inhibition of thyroidperoxidases, which are responsible for the incorporation of ^{125}iodide into organic compounds, by substances as propylthiouracil (positive control substance). As a further positive control substance can serve phenobarbitone which acts indirectly on the thyroid by inducing hepatic microsomal enzymes including the thyroxine metabolizing UDP glucuronyl transferase (see also 3.12.1.2 and 3.12.1.4).

Test material: Pethoxamid; Batch: TB960306I; Purity: 94.8% (formulation checked by HPLC).

Test animals: Five groups of 16 male Sprague-Dawley CD albino rats each, 28 (+/-2 days) old, 14 g of weight, from Charles River UK Ltd, Margate, Kent. Only male animals were used because follicular cell adenomas were observed in this sex only in the carcinogenicity study (Report no. TON 6/974064, point 3.9.1.1).

The rats were treated for 28 consecutive days with pethoxamid by dietary administration of 0, 1600 or 5000 ppm. The dose levels and the route of administration were those previously used in toxicity studies.

The positive control groups were administered the commonly used doses of 75 mg/kg bw/d phenobarbitone or 200 mg/kg bw/d propylthiouracil by oral gavage.

Before treatment (Day 1) and on Days 12 and 24 a blood sample was taken for the measurement of the plasma concentrations of the hormones tri-iodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH). After 28 days of treatment, sodium^{125}Iodide was administered intraperitoneally to each animal and 6 hours later, potassium perchlorate or saline was administered separately to 6 animals from each group by intraperitoneal injection. Exactly two and a half minutes after the injections of potassium perchlorate or saline, each animal was anaesthetized and a blood sample was collected for the measurement of radioactivity. Immediately after sampling, all animals were sacrificed. The thyroid gland was removed by dissection and the amount of radioactivity measured.

Results:

Formulation analysis and substance intake: The mean concentrations of pethoxamid in the test diet were within 1.5% of target concentrations. The animals consumed 155 and 462 mg/kg bw/d at the administered doses of 1600 and 5000 ppm.

Mortality and clinical signs: There were no treatment-related deaths in any group.

Two rats were sacrificed on Days 17 and 20 of treatment because of corneal damage caused by blood sampling from the orbital sinus.

Pethoxamid: There were no clinical signs indicative of a reaction to treatment.

Propylthiouracil: Post-dosing salivation was recorded for the majority of the animals. Furthermore, piloerection and hair loss were noted in some animals.

Phenobarbitone: Signs included underactive behaviour, abnormal gait, hunched posture, shallow respiration and prostration occurring about 1 hour after dosing. All animals appeared to have fully recovered before the next dosing.

Body weight and food consumption:

Pethoxamid: There was a statistically significant reduction in body weight gain in comparison with controls during the first 4 days of treatment (Table 3.9.4-1). From Day 4 to 29, body weight gain continued to be lower than for the control group but to a lesser degree. This was accompanied by reduced food consumption, especially in the first week of treatment (palatability effect, also observed in other studies).

Propylthiouracil: A large reduction in body weight gain and food consumption and, after Day 11, weight loss was observed.

Phenobarbitone: Body weight gain was slightly decreased and food consumption increased.

Hormone concentrations (T3, T4, TSH):

Pethoxamid: No statistically significant differences were found for the T3 and for the T4 levels (Table 3.9.4-1). The mean TSH concentrations were slightly increased at both dose levels, but statistical significance was only reached on Day 12 in TSH level at concentration 1600 ppm.

Propylthiouracil: A marked decrease in the T3 and T4 levels was noted which was attributable to the direct effect on the thyroid. A large statistically significant increase in mean plasma concentration of TSH, apparent after treatment for 12 days, due to the resulting negative feedback was also observed.

Phenobarbitone: A statistically significant increase in the T3 levels was noted on Day 24. An increase in TSH levels over the time course is likely to be age related. From the start of treatment until Day 24, a slight increase in TSH levels was noted for the phenobarbitone treated group (6.7 ng/dl), but also for the control group (3.3 ng/dl). However, the increase was higher, although not statistically significant, for the phenobarbitone treated animals. The significant decrease in the T4 level at Day 12 was most likely to be due to the relatively high T4 level in control animals at this day.

Thyroid weights:

Pethoxamid: A slight, but not statistically significant, increase in thyroid weight was observed, similarly to phenobarbitone treated animals.

Propylthiouracil: A large, statistically significant increase in thyroid weight was seen.

Phenobarbitone: A slight, but not statistically significant, increase in thyroid weight was observed.

Radioactivity measurements:

Thyroid radioactivity:

Pethoxamid: The total radioactivity content in the thyroid (% of dose, Table 3.9.4-1) was statistically significantly increased at the high dose (saline and perchlorate). No significant change in the thyroid radioactivity concentration (total % dose/g) was seen.

Propylthiouracil: A statistically significant decrease in thyroid radioactivity concentration was observed, especially for the perchlorate treated rats.

Phenobarbitone: A statistically significant increase in the total radioactivity content but not in the radioactivity concentration was noted (saline and perchlorate).

Whole-blood radioactivity:

Pethoxamid: A statistically significant decrease in the radioactivity concentration (% of dose per g blood, Table 3.9.4-1) was observed for the high dose, and in the total radioactivity content (% of dose) for both dose levels (saline and perchlorate; not tabulated).

Propylthiouracil: The concentration of radioactivity was statistically significantly higher (saline and perchlorate). The total radioactivity content in blood was lower (saline and perchlorate; not tabulated) because of the decreased body weight and thus blood volume.

Phenobarbitone: A statistically significant decrease was noted in the total radioactivity content and in the radioactivity concentration (saline and perchlorate).

Perchlorate administration:

Pethoxamid: No significant differences occurred between the saline and perchlorate treated animals for the determinations of radioactivity in blood as well as thyroid.

Propylthiouracil: The mean concentration and amount of radioactivity in the thyroid in saline compared with perchlorate treated animals show that about 60% of thyroid radioactivity was displaced by perchlorate. Thus, a large amount of free ^{125}iodide must have been present in the thyroid.

Phenobarbitone: The concentration and total content in blood were significantly higher in perchlorate treated groups, but this was not the case for both values for the thyroid.

Net result of the investigations:

After administration of propylthiouracil, the large displacement of thyroid radioactivity by perchlorate was a consequence of the inhibition of the peroxidases. The much lower thyroid/blood concentration ratio reflects a lower ^{125}iodide uptake and metabolization in the thyroid.

After administration of phenobarbitone, no significant discharge of thyroid radioactivity occurred by perchlorate (no inhibition of peroxidases and thus, absence of significant amounts of free ^{125}iodide). The decrease in blood radioactivity (concentration and total amount) is likely to be the result of an enhancement of the uptake and organification of ^{125}iodide by the thyroid. The enhancement could be the result of the slightly increased TSH release from the pituitary followed by some thyroid hypertrophy (indicated by the slightly increased thyroid weight).

Pethoxamid (as phenobarbitone) did not cause a significant discharge of thyroid radioactivity by perchlorate; thus, the activity of thyroid peroxidases was obviously not reduced.

The substance did not affect the T3 levels, which indicates that it does not inhibit the 5'- mono de-iodonase enzymes (conversion T4 to T3). Since there was no decline in TSH levels and no consistent elevation in T3 and T4 levels, it is unlikely that pethoxamid has an agonistic action at the TSH receptor.

Table 3.9.4-1: Administration of thyroid-active compounds to male rats - effects on parameters of general health and thyroid function (see next page)

	Control	**Pethoxamid 1600 ppm**	**Pethoxamid 5000 ppm**	**Phenobarbitone 75 mg/kg bw/d**	**Propylthiouracil 200 mg/kg bw/d**
General health parameters					
Body weight gain (g)					
Day 1-4	23.6	8.6**	-27.0**	24.8	14.4*
Day 4-29	143.7	125.9	102.0**	125.4	19.3**
Food consumption					
Week 1 (g/rat)	182	159**	86**	221**	198*
Week 2-4 (g/rat)	555	541	478**	600*	350**
Hormone levels					
T3 (ng/dl)					
Prior to treatment	131.5	134.2	124.0	112.9**	102.4**
Day 12	87.1	91.6	90.3	81.9	25.1**
Day 24	77.8	85.7[1]	87.6	94.3**	37.9[1]**
T4 (µg/dl)					
Prior to treatment	5.2	5.5	5.5	5.1	4.6*
Day 12	7.6	8.2	6.6	5.0**	0.0[2]**
Day 24	5.3	6.0[1]	4.9	5.3	0.3[1]**
TSH (ng/ml)					
Prior to treatment	5.9	6.1	6.2	5.2	5.2
Day 12	7.5	10.8*	9.0	9.5	23.7**
Day 24	9.2	12.6[1]	13.0	11.9	34.5[1]**
Thyroid weight (g)					
Saline treated	0.0146	0.0165	0.0172	0.0187	0.0452**
Perchlorate treated	0.0172	0.0186	0.0179	0.0183	0.0547**
Radioactivity measurements					
Whole-blood (% of dose/g)					
Saline treated	0.281	0.264	0.228**	0.200**	0.331**
Perchlorate treated	0.297	0.294	0.236**	0.257**	0.414**
Thyroid (total % of dose)					
Saline treated	6.24	6.82	7.58*	7.96*	3.26**
Perchlorate treated	6.25	8.38	8.12*	8.02*	1.27**
Thyroid (% of dose/g)					
Saline treated	427.4	408.0	442.7	425.7	71.9**
Perchlorate treated	364.9	461.4	454.7	440.0	23.0**

Thyroid : blood radioactivity ratio[3]					
Saline treated	1520	1549	1940**	2145**	222**
Perchlorate treated	1264	1576	1926**	1713**	56**

[1] Mean for 15 rats; [2] Values below the limit of accurate quantification (0.2 ug/dl) were taken as zero for calculation of mean and standard deviations; [3] expressed as dpm/g; Statistical significance: *p<0.05; **p<0.01

Conclusion: Comparing the data for pethoxamid with that for propylthiouracil, it suggests that pethoxamid did not directly affect the thyroid function.

The data obtained for pethoxamid at the high dose level is similar to that for phenobarbitone (TSH levels, thyroid and whole-blood radioactivity); **thus, the mechanism of action of pethoxamid is apparently similar to that of phenobarbitone.**

3.9.4.3 Anonymous (2001b)

Reference: TKC-94: 2-Week Hepatic Drug-Metabolizing Enzyme Induction and Cell Proliferation Study in Mice.

Author(s), year: Anonymous, 2001b
Report/Doc. number: 98 PXA / IET-00-0118
Guideline(s): Not applicable, non-guideline study
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

Executive summary:
In order to evaluate effects of TKC-94 on hepatic drug-metabolizing enzyme induction and cell proliferation, the test substance was administered in feed to male ICR (Crj:CD-1) mice at dose levels of 0, 30, 400 or 5000 ppm for 2 weeks. In addition, cell to cell communication in the liver was evaluated by counting the number of gap junction protein connexion 32 (Cx 32) spots per hepatocyte.

Clinically there were neither treatment-related abnormalities nor deaths in any dose group during the study. There were no significant difference in body weight change between the treated and control groups during the study. Food consumption by the 5000 ppm group was decreased (about 37% less than the control value) at the 1st week of treatment, but recovered at the 2nd week. The average food consumption by this group during the study was slightly lower (about 14% less) than that by the control group.

The average test substance intake for each group during the study was 3.92, 49.1 and 541 mg/kg bw/day for the 30, 400 and 5000 ppm dose levels, respectively.

At necropsy, enlarged and/or dark-coloured livers were frequently observed in the 5000 ppm group. Organ weight measurements revealed significant increases in both absolute and relative (ratio to body weight) liver weights in the 5000 ppm group when compared to the control group.

Analysis of hepatic microsomal samples disclosed significant increases in microsomal protein content, cytochrome P-450 content, and pentoxyresorufin O-dealkylase activity in the 5000 ppm group. In addition, P-450 isoenzyme contents of CYP1A, CYP2B, CYP3A2, and CYP4A1 were significantly increased in this group and the increase in CYP2B content was most evident. A significant increase in

pentoxyresorufin O-dealkylase activity was also noted in the 400 ppm group. In addition, a significant decrease in CYP1A was noted, but no changes in other parameters.

Measurement of cell proliferation in the liver revealed a significant increase in PCNA labelling index in the 5000 ppm group after 3 and 7 days of treatment, but not after 14 days of treatment.

Regarding cell to cell communication in the liver, a dose-dependent decrease in the number of Cx32 spots per hepatocyte was observed in the treated groups. In the 5000 and 400 ppm groups, significant decreases (20-30%) in this parameter were observed at all sampling times. In the 30 ppm group, a mild (12-17%) but significant decrease was noted after 3 and 7 days of treatment, but not after 14 days of treatment.

Based on the results observed, the overall profile of effects are suggestive that TKC-94 may be a phenobarbital-type enzyme inducer which can increase cell proliferation in the liver during the initial stages of exposure when administered at 5000 ppm in the diet. In addition, the test substance may inhibit gap junctional intercellular communication.

MATERIALS AND METHODS

Materials:

Test material:	Pethoxamid
Lot/batch number:	TB-960306 I
Purity:	95.0% (w/w) (dose calculation was not adjusted to purity)
Stability of test item:	30 September 2000 (stored at approximately 4°C in the dark) NB: stable during the conduct of the study
Storage conditions:	Refrigeration (approximately 4°C) in the dark
CAS#:	1006700-29-2

Study Design:
The objective of this study was to investigate effects of TKC-94 on hepatic drug-metabolizing enzyme induction and cell proliferation in mice following dietary administration for 2 weeks. This study was conducted as part of mechanistic studies to clarify the mechanism(s) for the development of hepatocellular tumours that increased in high-dose male mice in the carcinogenicity study.

Specific-pathogen-free (SPF) male ICR (crj:CD-1) mice were purchased from Hino Breeding Centre (Hino-cho, Gamoh-gun, Shiga) of Charles River Japan, Inc. The CD-1 mouse was chosen because this strain of mice was used in the previously conducted toxicity studies of TKC-94 in mice.

The test substance was administered orally by incorporating it into the basal diet for 2 weeks. 18 male mice/group were used. Dose levels were 0, 30, 400 or 5000 ppm in diet. After 3, 7 and 14 days of treatment, 6 animals from each group were killed and subjected to necropsy, measurement of liver weights, and measurements of cell proliferation and cell to cell communication. In addition, measurement of hepatic drug-metabolising enzymes was performed on the animals killed after 14 days of treatment.

Preparation of the treated diet was undertaken on 2 occasions at approximately a weekly interval, to fit with established stability data.

Mice were subject to daily clinical signs, weekly body weight measurement and weekly food consumption measurement. Post-life measurements entailed; liver weight recording, cell proliferation measurement, gap junction protein CX32 and hepatic drug-metabolizing enzyme measurement.

The following microsomal enzymes were measured; protein content, cytochrome P-450 content, Pentoxyresorufin O-dealkylase activity, CYP1A, CYP2B, CYP3A2, and CYP4A1.

Cell proliferation was determined by immunohistochemistry measurement of proliferating cell nuclear antigen (PCNA). Additional duodenum samples were taken and similarly processed to serve as positive controls for PCNA staining. Approximately 1000 hepatocytes for each animal were examined using an image analyser.

Frozen sections were obtained from the liver tissue embedded in a Tissue Mount for each animal at each scheduled sacrifice and subjected to immunohistochemistry for hepatic gap junction protein connexion 32(CX32) using rat monoclonal CX32 antibodies. The number of CX32 spots per hepatocyte was calculated.
Appropriate statistical analysis was undertaken on relevant data.

Results:
Achieved intake: The achieved intake is given below:

Table 3.9.4-2: Achieved intake

Dose in diet (ppm)	Average achieved intake (mg/kg bw/day)
30	3.92
400	49.1
5000	541

Clinical signs, body weight and food consumption: There were no treatment-related clinical signs observed. There were no noteworthy effects on body weight between the groups. Food consumption was lower during the first week of treatment (approximately 37%) in the high dose mice when compared with the concurrent control values but recovered at the 2nd week. The average weight gain per week was 5.1, 5.5, 5.0 and 4.4 g/mouse/day for 0, 30, 400 and 5000 ppn, respectively.

Necropsy: At necropsy, in the 5000 ppm group, enlarged and/or dark-colored livers were frequently observed after 7 and 14 days of treatment.

Organ weights: Absolute and relative liver weights in the 5000 ppm group after 3, 7 and 14 days of treatment were significantly increased when compared with concurrent controls. No similar findings were observed at <5000 ppm.

Table 3.9.4-3: Liver weights

Dose (ppm)	Absolute liver weight			Relative liver weight		
	3 days	7 days	14 days	3 days	7 days	14 days
30	104	97	102	105	95	98
400	106	93	108	105	98	107
5000	123**	131**	137**	129**	133**	136**
Values represent percentage to the control values. **: Significantly different from the control at 1% level of probability (Dunnett´s test)						

Microsomal protein content and enzyme activity: Microsomal protein content, cytochrome P-450 content, and pentoxyresorufin O-dealkylase activity which were measured for each dose group after 14 days of treatment were significantly higher in the 5000 ppm group when compared with concurrent

controls. In the 400 ppm group, pentoxyresorufin O-dealkylase activity was significantly increased, but there were no significant changes in other parameters. In the 30 ppm group, there were no significant changes in any parameter.

Table 3.9.4-4: Microsomal protein content, cytochrome P-450 content, and pentoxyresorufin O-dealkylase activity

Dose (ppm)	Microsomal protein content	Cytochrome P-450 content	Pentoxyresorufin O-dealkylase activity
30	105	93	367
400	107	100	867*
5000	121**	195**	5667**
Values represent percentage to the control values. *, **: Significantly different from the control at 5% and 1% level of probability, respectively (Dunnett´s test)			

Significant increases in cytochrome P-450 isoenzyme contents of CYP-1A (4.3 times higher), CYP2B (8.5 times higher), CYP3A2 (2.1 times higher), and CYP4A1 (1.6 times higher) were observed in the 5000 ppm group when compared to the control group. In the 400 ppm group, a significant decrease in CYP-1A content was noted, but there were no significant changes in other parameters. In the 30 ppm group, there were no significant changes in any isoenzyme content.

Table 3.9.4-5: Cytochrome P-450 isozyme contents

Dose (ppm)	Cytochrome P-450 isozyme contents			
	CYP1A	CYP2B	CYP3A2	CYP4A1
30	81	95	95	100
400	81**	105	98	120
5000	429**	845**	214**	164**
Values represent percentage to the control values. **: Significantly different from the control at 1% level of probability (Student´s t-test or Aspin-Welch test)				

Cell proliferation: In the 5000 ppm group, significant increases in PCNA LI (labelling index) were observed after 3 and 7 days of treatment, but the value after 14 days of treatment was comparable to the controls. In other dose groups, there were no significant differences in PCNA LI between the treated and control groups.

Table 3.9.4-6: PCNA labelling index

Dose (ppm)	PCNA LI on the liver		
	3 days	7 days	14 days
30	108	122	231
400	135	74	131
5000	377*	781*	163
Values represent percentage to the control values. *: Significantly different from the control at 5% level of probability (Dunnett´s test)			

In the 5000 ppm group, significant decreased (about 30% lower) number of CX32 spots were observed after 3, 7 and 14 days of treatment when compared to the controls. In the 400 ppm group, significant

decreased (about 20% lower) number of CX32 spots were also observed after 3, 7 and 14 days of treatment when compared to the controls. In the 30 ppm group, mild (12-17%) but significant decreased number of CX32 spots were noted after 3 and 7 days of treatment when compared to the controls, but not after 14 days of treatment.

Table 3.9.4-7: Number Cx32 spots per hepatocyte

Dose (ppm)	No. of Cx32 spots per hepatocyte		
	3 days	7 days	14 days
30	88*	83*	87
400	81**	82**	81*
5000	71**	69**	71**
Values represent percentage to the control values. *, **: Significantly different from the control at 5% and 1% level of probability, respectively (Dunnett´s test)			

Conclusion: Based on the results observed, the overall profile of effects are suggestive that TKC-94 may be a phenobarbital-type enzyme inducer which can increase cell proliferation in the liver during the initial stages of exposure when administered at 5000 ppm in the diet. In addition, the test substance may inhibit gap junctional intercellular communication (CJIC) in the liver. In consideration of these effects the no-observed-adverse-effect level (NOAEL) for this study was determined to be 30 ppm (3.92 mg/kg-bw/day) because there were no significant changes in any parameters in the 30 ppm group after 14 days of treatment, although a mild but statistically significant decrease in GJIC was noted after 3 and 7 days of treatment.

3.9.4.4 Anonymous (2016a)

Reference: Pethoxamid Technical: Evaluation Of Liver And Thyroid Effects And Their Potential Reversibility After Dietary Exposure In Mice And Rats
Author(s), year: Anonymous, 2016
Report/Doc. number: 1538 PXA
Guideline(s): Mechanistic study: Guidelines considered in the study design rather than adhered with. OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 407): Health Effects, Repeated Dose 28-Day Oral Toxicity Study in Rodents (2008). U.S. EPA Health Effects Test Guidelines, OPPTS 870.3050, "Repeated Dose 28-Day Oral Toxicity Study in Rodents", (1998)
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

EXECUTIVE SUMMARY:

The objective of this study was to generate liver samples from mice, and liver, thyroid, and serum samples (for thyroid hormone assessment) from rats, which were administered a diet containing pethoxamid technical. In order for changes in the liver and thyroid (including hormone levels) to be appropriately characterized, mice were dosed for a period of 7 days and rats for a period of 14 days. Liver and thyroid samples (including serum thyroid hormone levels) were used to evaluate biochemical effects and correlating macroscopic and microscopic toxicological effects on these organs. BrdU (5-bromo-2'-deoxyuridine) was administered to enable assessment of DNA replication (indicative of cell division) while potential reversibility was evaluated after a 42-day recovery period. The Recovery group animals in both species were provided with basal diet during the 42-day recovery period.

Mice in Groups 1 through 5 and the first eight rats in Groups 6 through 10 were fitted with an osmotic pump, containing 15 mg/mL BrdU in sterile saline, implanted subcutaneously between the shoulder blades. The pumps, calibrated to deliver 1 µL/hr for seven days, were surgically implanted in all mice from Groups 1-3 on Day 0 and in Groups 4-5 on Day 42. Rats in Groups 6-8 (0, 400, and 1600 ppm) received 10 µL/hr BrdU in sterile saline from Day 7 to Day 14 (scheduled sacrifice). Rats in Groups 9-10 (0 and 1600 ppm, respectively) received the same dosing regimen from Day 49 (pump implantation) to Day 56. Incorporation of BrdU into hepatocytes was then measured using a mouse monoclonal anti-BrdU antibody and light microscopy.

At terminal sacrifice (Day 7 for Groups 1-3, Day 14 for Groups 6-8, Day 49 for Groups 4-5, and Day 56 for Groups 9-10); all animals were euthanized by CO_2 asphyxiation and subjected to a gross necropsy. The liver was harvested from all mice in Groups 1-5. The mouse liver samples were collected, weighed and stored for enzyme induction analysis, general histopathology, and IHC evaluation. The liver and thyroid glands were harvested from the first eight rats in Groups 6-10. A section of the rat livers was sent for enzyme induction analysis at another laboratory under a separate study number. The thyroids were collected, fixed overnight, weighed and stored for general histopathology and IHC evaluation. Serum was collected from all rats (Groups 6-10) for thyroid hormone analysis.

There were no mortalities during this study. There were no clinical signs considered attributable to the administration of pethoxamid technical.

There were no changes in body weight, mean daily body weight gain or food consumption considered attributable to pethoxamid technical administration seen in mice.

An initial slight decrease in bodyweight gain and food consumption (Days 0-7), that did not impact on the overall body weight values during the dosing period, was observed in rats consuming diet containing 1600 ppm pethoxamid technical (Groups 8 and 100). As the lower initial food consumption did not have any noteworthy impact on body weights, this observation was considered to be likely related to treated diet palatability and of limited toxicological relevance.

Macroscopic findings in mice were limited to ‘Pale liver’ observed in one animal treated at 5000 ppm for 14 days. This finding correlated microscopically with hepatocyte hypertrophy, grade 2. There were no test substance-related macroscopic observations in rats.

Increased absolute and relative liver weights were observed in mice treated at 5000 ppm for 14 days. Liver weights (absolute and relative to body weight) were slightly higher than concurrent controls in mice after a 42-day recovery period; however, there were no microscopic correlates, the difference was minor and only the liver-to-body weight ratio achieved statistical significance. This observation is therefore considered of limited toxicological significance.

In rats, increased absolute and relative thyroid weights were observed in animals treated at 400 and 1600 ppm for 14 days. Thyroid weight values after a 42-day recovery period were comparable to concurrent controls. No test substance-related liver weight changes were observed in rats.

Thyroid hormone levels (TSH, T4, and Free T3) for rats treated with pethoxamid technical were generally comparable to the respective control group.

Evaluation on Day 7 of liver from mice treated with pethoxamid technical at 5000 ppm demonstrated hepatocellular hypertrophy and an increase of the number of BrdU positive cells when compared with mice treated with either basal diet or pethoxamid technical at 400 ppm. These increases were completely reversible in recovery animals evaluated on Day 49.

Rat thyroid glands evaluated on Day 14, treated with pethoxamid technical at 1600 ppm, demonstrated an increase in the BrdU labeling index when compared to the rats treated with basal diet or pethoxamid technical at 400 ppm. This increase appeared to be completely reversible in recovery animals evaluated on Day 56. Thyroid follicular epithelium hypertrophy (grade 1) was observed in 2 out of 8 animals treated with 1600 ppm pethoxamid technical on Day 14, while this change was not observed in any of the recovery animals regardless of treatment consistent with full reversibility.

Under the conditions of this study and based on the toxicological and histopathological endpoints evaluated, in addition to increased absolute and relative liver weights, pethoxamid technical administered to mice in their diet at concentrations of 5000 ppm caused reversible changes in centrilobular hepatocytes including hypertrophy as well as increased hepatocyte proliferation. Pethoxamid Technical administered to rats in their diet at concentrations of 1600 ppm caused increased absolute and relative thyroid weights and reversible increases in follicular thyroid cell proliferation. There were no noteworthy effects of pethoxamid on circulating thyroid hormone levels in rats. Only selected minor effects across the end-points assessed were observed at the low, non-oncogenic dose level of 400 ppm, in both rats and mice.

MATERIALS AND METHODS:

Materials:

Test material:	Pethoxamid technical
Lot/batch number:	P1351-JaK-T2-23-6
Purity:	92.6% (w/w) (dose calculation was not adjusted to purity)
Stability of test item:	28 November 2015 (stored at ambient temperature) *NB: stable during the conduct of the study*
Storage conditions:	At room temperature, protected from light

Forty healthy male mice and seventy-five healthy male rats were selected for the test and distributed into 10 groups in two cohorts of animals (Main Toxicity and Recovery). The Main Toxicity cohort consisted of six groups (three per species) and the Recovery cohort consisted of four groups (two per species). The mice were provided with treated diet at dietary levels of 0 ppm (Groups 1 and 4, basal diet control, main and recovery, respectively), 400 ppm (Group 2), and 5000 ppm (Groups 3 and 5, main and recovery, respectively) of pethoxamid technical. The rats were provided with treated diet at dietary levels of 0 ppm (Groups 6 and 9, basal diet control, main and recovery, respectively), 400 ppm (Group 7), and 1600 ppm (Groups 8 and 10, main and recovery, respectively) of pethoxamid technical. All treated groups were fed the treated diet for 7 (mice) or 14 days (rats). The control groups were fed the basal diet for the entirety of the study. The Recovery group animals in both species were provided with basal diet during the 42-day recovery period.

Forty (40) mice were randomly assigned to one of the following groups:

Group	No. mice/Group	Treatment - Dietary Concentration (ppm)	% in Diet	Target Dosage (mg/kg/day)[a]
1	8	Basal Diet (Control) – 0	0	0
2	8	Pethoxamid Technical – 400	0.04	107
3	8	Pethoxamid Technical – 5000	0.5	1333
4	8	Basal Diet (Control) – 0 (Recovery)	0	0
5	8	Pethoxamid Technical – 5000 (7 days dosing, 42 days Recovery)	0.5	1333

[a] Based on 30 gram male CRL: CD 1 (ICR) mice eating 8 g/day. Doses calculated without correcting for purity.

Seventy-five (75) rats were randomly assigned to one of the following test groups:

Group	No. Rats/Group	Treatment - Dietary Concentration (ppm)	% in Diet	Target Dosage (mg/kg/day)[a]
6	15	Basal Diet (Control) – 0	0	0

7	15	Pethoxamid Technical – 400	0.04	33.3
8	15	Pethoxamid Technical – 1600	0.16	133
9	15	Basal Diet (Control) – 0 (Recovery)	0	0
10	15	Pethoxamid Technical – 1600 (14 days dosing, 42 days Recovery	0.16	133

[a] Based on 300 gram male Crl: CD® SD rats eating 25 g/day. Doses calculated without correcting for purity.

The test substance and control diets were presented to their respective groups on Day 0 of the study. Additional diet was provided as needed throughout the study to ensure *ad libitum* feeding. All animals were observed daily for viability, signs of gross toxicity, and behavioral changes. Body weights were recorded two times during the acclimation period, prior to test initiation (Day 0), and weekly thereafter for all surviving animals and just prior to scheduled sacrifice. Individual food consumption was also recorded to coincide with body weight measurements. Dietary intake of pethoxamid (mg/kg/day) was calculated for all test groups.

Mice in Groups 1 through 5 and the first eight rats in Groups 6 through 10 were fitted with an osmotic pump, containing 15 mg/mL BrdU in sterile saline, implanted subcutaneously between the shoulder blades. The pumps, calibrated to deliver 1 µL/hr for seven days, were surgically implanted in all mice from Groups 1-3 on Day 0 and in Groups 4-5 on Day 42. Rats in Groups 6-8 (0, 400, and 1600 ppm) received 10 µL/hr BrdU in sterile saline from Day 7 to Day 14 (scheduled sacrifice). Rats in Groups 9-10 (0 and 1600 ppm, respectively) received the same dosing regimen from Day 49 (pump implantation) to Day 56. Incorporation of BrdU into hepatocytes was then measured using a mouse monoclonal anti-BrdU antibody and light microscopy.

At terminal sacrifice (Day 7 for Groups 1-3, Day 14 for Groups 6-8, Day 49 for Groups 4-5, and Day 56 for Groups 9-10); all animals were euthanized by CO_2 asphyxiation and subjected to a gross necropsy. The liver was harvested from all mice in Groups 1-5. The mouse liver samples were collected, weighed and stored for enzyme induction analysis, general histopathology, and IHC evaluation. The liver and thyroid glands were harvested from the first eight rats in Groups 6-10. A section of the rat livers was sent for enzyme induction analysis at another laboratory under a separate study number. The thyroids were collected, fixed overnight, weighed and stored for general histopathology and IHC evaluation. Serum was collected from all rats (Groups 6-10) for thyroid hormone analysis.

RESULTS AND DISCUSSION:

Mortality and clinical signs: There were no mortalities during this study. There were no clinical signs considered attributable to the administration of pethoxamid technical.

Body weights and food consumption: There were no changes in body weight, mean daily body weight gain or food consumption considered attributable to pethoxamid technical administration seen in mice.

An initial slight decrease in bodyweight gain and food consumption (Days 0-7), that did not impact on the overall body weight values during the dosing period, was observed in rats consuming diet containing 1600 ppm pethoxamid technical (Groups 8 and 10). As the lower initial food consumption did not have any noteworthy impact on body weights, this observation was considered to be likely related to treated diet palatability and of limited toxicological relevance.

Macroscopic observations: Macroscopic findings in mice were limited to 'Pale liver' observed in one animal treated at 5000 ppm for 14 days. This finding correlated microscopically with hepatocyte hypertrophy, grade 2. There were no test substance-related macroscopic observations in rats.

Organ weight evaluation: Increased absolute and relative liver weights were observed in mice treated at 5000 ppm for 14 days. Liver weights (absolute and relative to body weight) were slightly higher than concurrent controls in mice after a 42-day recovery period; however, there were no microscopic

correlates, the difference was minor and only the liver-to-body weight ratio achieved statistical significance. This observation is therefore considered of limited toxicological significance.

Table 3.9.4-8: Summary of terminal body and liver weights (mice)

Parameter		Group				
		1	2	3	4	5
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	5000 ppm	0 ppm	5000 ppm
BW (g)	Mean	31.9	31.3	30.6	36.6	39.0
	SD	1.1	2.3	1.6	1.8	3.0
	N	8	8	8	8	8
Liver Weight (g)	Mean	2.09	1.90	2.59*	1.94	2.28
	SD	0.66	0.27	0.27	0.22	0.18
	N	8	8	8	8	8
Liver-to-BW Ratio (%)	Mean	0.07	0.06	0.08*	0.05	0.06#
	SD	0.02	0.01	0.01	0.00	0.00
	N	8	8	8	8	8

*Statistically significant from Group 1 Control, $p<0.05$, by Dunn's Multiple Comparisons Test

#Statistically significant from recovery Group 4 Control, $p<0.05$, by the Mann-Whitney Test.

In rats, increased absolute and relative thyroid weights were observed in animals treated at 400 and 1600 ppm for 14 days. Thyroid weight values after a 42-day recovery period were comparable to concurrent controls. No test substance-related liver weight changes were observed in rats.

Hormone level (measured in rats only): Thyroid hormone levels (TSH, T4, and Free T3) for rats treated with pethoxamid technical were generally comparable to the respective control group.

Table 3.9.4-9: Summary of terminal body and thyroid/liver weights (rats)

Parameter		Group				
		6	7	8	9	10
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	1600 ppm	0 ppm	1600 ppm
BW (g)	Mean	327.1	321.7	319.7	451.6	454.1
	SD	16.6	13.3	15.9	43.1	57.0
	N	15	15	15	15	15
Liver Weight (g)	Mean	15.11	14.53	16.87	16.65	16.38
	SD	1.75	1.51	1.67	2.82	2.00
	N	8	8	8	8	8
Liver-to-BW Ratio (%)	Mean	0.05	0.04	0.05	0.04	0.04
	SD	0.00	0.00	0.00	0.01	0.01
	N	8	8	8	8	8
Thyroid Weight (g)	Mean	0.014	0.018**	0.020**	0.030	0.025
	SD	0.003	0.002	0.002	0.003	0.002
	N	8	8	8	8	8
Thyroid-to-BW Ratio (%)	Mean	$4.30x10^{-5}$	$5.56x10^{-5}$**	$6.23x10^{-5}$**	$6.67x10^{-5}$	$5.50x10^{-5}$
	SD	$9.54x10^{-6}$	$4.86x10^{-6}$	$5.19x10^{-6}$	$6.37x10^{-6}$	$4.38x10^{-6}$
	N	8	8	8	8	8

**Statistically significant from Group 1 Control, $p<0.01$, by Dunnett's Multiple Comparisons Test

Table 3.9.4-10: Summary of thyroid hormone assessment (rats)

Parameter		Group				
		6	7	8	9	10
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	1600 ppm	0 ppm	1600 ppm
TSH (Ng/mL)	Mean	5.788	5.194	6.507	5.345	4.927
	SD	3.482	2.896	3.132	4.132	3.373
	N	15	15	15	15	15
Total T4 (µg/mL)	Mean	4.662	5.115	5.504*	5.795	5.293
	SD	0.768	0.569	1.143	1.877	1.304
	N	15	15	15	15	15
Free T3 (pg/mL)	Mean	3.547	3.585	3.754	4.043	3.441
	SD	0.89	0.408	0.763	1.235	0.914
	N	15	15	15	15	15

*Statistically significant from Group 1 Control, $p < 0.05$, by Dunnett Multiple Comparisons Test

Histopathology: Evaluation on Day 7 of liver from mice treated with pethoxamid technical at 5000 ppm demonstrated hepatocellular hypertrophy and an increase of the number of BrdU positive cells when compared with mice treated with either basal diet or pethoxamid technical at 400 ppm. These increases were completely reversible in recovery animals evaluated on Day 49.

Table 3.9.4-11: BrDU assessment (mice)

Parameter	Animal Sequence per Group	Group				
		1	2	3	4	5
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	5000 ppm	0 ppm	5000 ppm
Individual Mean BrdU Values	1	0.3	0.3	14.4	0.0	0.1
	2	0.2	0.1	13.8	0.0	0.1
	3	0.1	1.5	14.0	0.2	0.0
	4	0.3	0.1	14.0	0.5	0.2
	5	0.2	0.9	8.8	0.2	0.0
	6	0.1	0.1	10.8	0.0	0.1
	7	0.3	0.3	13.0	0.0	0.0
	8	0.4	1.3	9.1	0.1	0.0
Mean		0.2	0.6	12.2 ***	0.1	0.1
SD		0.1	0.6	2.3	0.1	0.1
N		8	8	8	8	8

*** Statistically significant from Group 1 Control, $p<0.001$, by Dunn's Multiple Comparison

Rat thyroid glands evaluated on Day 14, treated with pethoxamid technical at 1600 ppm, demonstrated an increase in the BrdU labeling index when compared to the rats treated with basal diet or pethoxamid technical at 400 ppm. This increase appeared to be completely reversible in recovery animals evaluated on Day 56. Thyroid follicular epithelium hypertrophy (grade 1) was observed in 2 out of 8 animals treated with 1600 ppm pethoxamid technical on Day 14, while this change was not observed in any of the recovery animals regardless of treatment consistent with full reversibility.

Table 3.9.4-12: BrDU assessment (rats)

Parameter	Animal Sequence per Group	Group				
		6	7	8	9	10
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	1600 ppm	0 ppm	1600 ppm
Individual Mean brdU Values	1	1.8	4.1	8.8	1.6	2.7
	2	5.6	1.8	21.0	0.3	1.7
	3	2.6	3.8	5.5	0.7	0.6
	4	5.9	2.7	5.4	0.4	0.9
	5	1.8	3.4	1.7	0.9	1.8
	6	2.1	2.9	2.3	0.6	0.3
	7	2.9	1.0	5.3	2.3	0.8
	8	1.2	4.0	7.8	0.7	1.3
Mean		3.0	3.0	7.2	0.9	1.3
SD		1.8	1.1	6.1	0.7	0.8
N		8	8	8	8	8

No statistical significance between dose groups and controls.

CONCLUSION: Under the conditions of this study and based on the toxicological and histopathological endpoints evaluated, in addition to increased absolute and relative liver weights, pethoxamid technical administered to mice in their diet at concentrations of 5000 ppm caused reversible changes in centrilobular hepatocytes including hypertrophy as well as increased hepatocyte proliferation.

Pethoxamid Technical administered to rats in their diet at concentrations of 1600 ppm caused increased absolute and relative thyroid weights and reversible increases in follicular thyroid cell proliferation. There were no noteworthy effects of pethoxamid on circulating thyroid hormone levels in rats. Only selected minor effects across the endpoints assessed were observed at the low, non-oncogenic dose level of 400 ppm, in both rats and mice.

3.9.4.5 Anonymous (2016b)

Reference: Ex Vivo Evaluation of Pethoxamid as an Inducer of Liver Microsomal Cytochrome P450 and UDP Glucuronosyltransferase (UGT) Expression in Male Rats and Mice
Author(s), year: Anonymous, 2016
Report/Doc. number: 1539 PXA
Guideline(s): Non-specific; mechanistic study
GLP: Yes
Deviations from OECD 416 (2001): Not applicable
Acceptability: Yes

EXECUTIVE SUMMARY:

The objective of this study was to evaluate the effect of Pethoxamid on liver microsomal uridine diphosphate glucuronosyltransferase (UGT) activity and mRNA levels toward the thyroid hormone thyroxine (T4) in male rats and on liver microsomal cytochrome P450 (CYP) enzyme activity and mRNA levels in male mice. The effects of Pethoxamid on the specific content of liver microsomal cytochrome b_5 and cytochrome P450 were also evaluated in both male rats and male mice.

The most pronounced effects of Pethoxamid were the statistically significant and dose-dependent increases in UGT1A6 mRNA levels in male rats and the statistically significant and dose-dependent increases in Cyp3a11/13 activity and Cyp2b10, Cyp3a11 and Cyp4a10 mRNA levels in male mice. Of the CYP end-points assessed the induction of Cyp2b10 mRNA at 5000 ppm was the highest (115-fold concurrent control). Results following the observance of a 42-day recovery period illustrated no notable persisting effects (similar to concurrent control) on the end-points assessed.

MATERIALS AND METHODS:

The objective of this study was to evaluate the effect of Pethoxamid on liver microsomal uridine diphosphate glucuronosyltransferase (UGT) activity and mRNA levels toward the thyroid hormone thyroxine (T4) in male rats and on liver microsomal cytochrome P450 (CYP) enzyme activity and mRNA levels in male mice. The effects of Pethoxamid on the specific content of liver microsomal cytochrome b_5 and cytochrome P450 were also evaluated in both male rats and male mice.

In a separate *in-vivo* study male rats were treated with a control or one of two different dosages of Pethoxamid (equivalent to 400 and 1600 ppm in diet) for 14 days. At the end of the treatment period, or following a 42-day recovery period, the animals were euthanized and livers were removed, frozen and stored at −70°C. The frozen liver tissues were shipped to the Testing Facility, where microsomes were isolated and assayed for enzyme activity of thyroxine glucuronide (UGT1A1/6). A portion of liver tissue from the same rats was harvested and stored in RNA*later* RNA stabilization reagent at 4 C overnight prior to storage at −70°C and shipped to the Testing Facility, where it was lysed with TRIzol to isolate RNA, which was analyzed by qRT PCR to assess the effect of Pethoxamid on UGT1A1 and UGT1A6 mRNA levels.

In a separate *in-vivo* study male mice were treated with a control or one of two different dosages of Pethoxamid (equivalent to 400 and 5000ppm) for 7 days. At the end of the treatment period, or following a 42-day recovery period, the animals were euthanized and livers were removed, frozen and stored at −70°C. The frozen liver tissues were shipped to the Testing Facility, where microsomes were isolated and assayed for enzyme activities known to be relatively specific markers of CYP enzymes, namely 7-ethoxyresorufin O-dealkylation (Cyp1a1/2), testosterone hydroxylation (Cyp2b10 and Cyp3a11/13) and lauric acid 12-hydroxylation (Cyp4a10/12). A portion of liver tissue from the same mice were harvested and stored in RNA*later* RNA stabilization reagent at 4°C overnight prior to storage at −70°C and shipped to the Testing Facility, where it was lysed with TRIzol to isolate RNA, which was analyzed by qRT PCR to assess the effect of Pethoxamid on Cyp1a2, Cyp2b10, Cyp3a11 and Cyp4a10 mRNA levels.

RESULTS AND DISCUSSION:

Treatment of male rats with prototypical enzyme inducers caused anticipated increases in UGT activities. Treatment with β-naphthoflavone caused an increase of 7.14-fold in UGT1A1/6 activity when compared with concurrent control values.

Treatment of male mice with prototypical enzyme inducers caused anticipated increases in CYP activities. Treatment with β-naphthoflavone caused increases of 9.82- and 11.9-fold in Cyp1a1/2 activity when compared with concurrent control values. Treatment with phenobarbital caused increases of 2.94-, 3.44- and 3.28-fold in Cyp2b10 activity, while treatment with dexamethasone caused increases of 11.5-, 11.3- and 12.4-fold in Cyp3a11/13 activity when compared with concurrent control values. Finally, treatment with clofibric acid caused increases of 4.43- and 6.02-fold and perfluorodecanoic acid (PFDA) caused increases of 11.7- and 11.4-fold in Cyp4a10/12 activity when compared with concurrent control values.

Treatment of male rats with Pethoxamid for 14 days caused statistically significant increases in cytochrome b_5 content (1.22-fold, compared with concurrent control, at 1600 ppm), cytochrome P450 content (1.17- and 1.63-fold, compared with concurrent control, at 400 and 1600 ppm), thyroxine glucuronidase (UGT1A1/6) activity (1.62-fold, compared with concurrent control, at 1600 ppm), UGT1A1 mRNA levels (1.23-fold, compared with concurrent control, at 1600 ppm) and UGT1A6

mRNA levels (1.82- and 3.78-fold, compared with concurrent control, at 400 and 1600 ppm, respectively). Following a 42 day recovery period, measured values were generally comparable between rats previously treated at 1600 ppm Pethoxamid and concurrent controls. Cytochrome b_5 content was similar to the concurrent control even though statistical significance was achieved in previously treated rats (1.09-fold). The statistical significance may be attributed to the low variability observed in values within the treatment group rather than reflecting a test item related increase.

Treatment of male mice with Pethoxamid for 7 days caused statistically significant increases in cytochrome b_5 content (1.39-fold, compared with concurrent control, at 5000 ppm), cytochrome P450 content (1.22- and 1.50-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), 7-ethoxyreorufin-O-dealkylation (Cyp1a1/2) activity (1.69- and 1.54-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), testosterone 16β-hydroxylase (Cyp2b10) activity (1.46- and 1.70-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), testosterone 6β-hydroxylase (Cyp3a11/13) activity (4.39-fold, compared with concurrent control, at 400 ppm), Cyp1a2 mRNA levels (1.97-fold, compared with concurrent control, at 5000 ppm), Cyp2b10 mRNA levels (12.4- and 115-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), Cyp3a11 mRNA levels (6.90-fold, compared with concurrent control, at 5000 ppm) and Cyp4a10 mRNA levels (9.00-fold, compared with concurrent control, at 5000 ppm), and had little or no effect on lauric acid 12-hydroxylase (Cyp4a10/12) activity. Following a 42 day recovery period, measured values were generally comparable between mice previously treated at 5000 ppm Pethoxamid and concurrent controls. Testosterone 16β-hydroxylase (Cyp2b10) activity was 0.813-fold (achieving statistical significance) that of the concurrent controls. The statistical significance may be attributed to the low level of variability in values within the treatment group rather than reflecting a test item related decrease.

Table 3.9.4-13: Summary of Results in rats and mice

End-point	Pethoxamid concentration	Fold vs. concurrent controls	Considered notable Pethoxamid related effect[#]	Effects considered reversible following 42-day observation period
Rats				
UGT1A1/6 activity	400 ppm	1.14	No	NA
	1600 ppm	1.62 §	No	NA
UGT1A1 mRNA levels	400 ppm	1.05	No	NA
	1600 ppm	1.23 §	No	NA
UGT1A6 mRNA levels	400 ppm	1.82 §	No	NA
	1600 ppm	3.78 §	Yes	Yes
Mice				
Cyp1a1/2 activity	400 ppm	1.69 §	No	NA
	5000 ppm	1.54 §	No	NA
Cyp2b10 activity	400 ppm	1.46 §	No	NA
	5000 ppm	1.70 §	No	NA
Cyp3a11/13 activity	400 ppm	4.39 §	Yes	Yes
	5000 ppm	1.24	No[*]	NA
Cyp4a10/12 activity	400 ppm	0.924	No	NA
	5000 ppm	1.22	No	NA

Cyp1a2 mRNA levels	400 ppm	1.06	No	NA
	5000 ppm	1.97 §	No	NA
Cyp2b10 mRNA levels	400 ppm	12.4 §	Yes	Yes
	5000 ppm	115 §	Yes	Yes
Cyp3a11 mRNA levels	400 ppm	1.21	No	NA
	5000 ppm	6.90 §	Yes	Yes
Cyp4a10 mRNA levels	400 ppm	2.21	Yes	Yes
	5000 ppm	9.00 §	Yes	Yes

[#]'Yes' designation reflects > 2-fold. The 2-fold criteria was used as a numerical framework for discerning notable test item related changes from expected inter-individual variability. An increase of ≥ 2-fold was applied to reflect the EMA 2013 guidance that states that the observation of a ≥ 100% increase in enzyme level can routinely be considered as a positive indication of enzyme induction. It is acknowledged that the EMA guidance primarily relates to *in-vitro* test systems.§ Significantly different from the vehicle control (0 ppm Pethoxamid) as a result of One-way Analysis of Variance ($p < 0.05$).
[*]The reason for the apparent inverse dose-response relationship remains uncertain within the context of this study.
NA: Not applicable

CONCLUSION: The most pronounced effects of Pethoxamid were the statistically significant and dose-dependent increases in UGT1A6 mRNA levels in male rats and the statistically significant and dose-dependent increases in Cyp3a11/13 activity and Cyp2b10, Cyp3a11 and Cyp4a10 mRNA levels in male mice. Of the CYP end-points assessed the induction of Cyp2b10 mRNA at 5000 ppm was the highest (115-fold concurrent control). Results following the observance of a 42-day recovery period illustrated no notable persisting effects (similar to concurrent control) on the end-points assessed.

3.9.4.6 Anonymous (2019a)

Reference: TKC-94 : Pethoxamid technical: I*n-vitro* inhibition of non-juvenile male SD rat thyroperoxidase (TPO)-catalysed guaiacol oxidation
Author(s), year: Anonymous, 2019
Report/Doc. number: 2018TOX-PXA4481 / CLS4_0023_0001
Guideline(s): None
GLP: Yes
Acceptability: Yes

JUSTIFICATION FOR TEST SYSTEM SELECTION: The guaiacol assay of TPO activity was used for this study (***Chang and Doerge, 2000, Paul et al., 2013 and Paul et al., 2014***). The known TPO inhibitor, PTU, was used as a positive control substance. The potency of pethoxamid, and the positive control, as inhibitors of TPO were quantified in terms of half maximal concentrations (IC_{50}) values, where applicable. Rat thyroid microsomes are a well-established source used for the evaluation of TPO activity.

EXECUTIVE SUMMARY
The objective of this study was to assess the potential for pethoxamid to inhibit thyroperoxidase (TPO) activity in pooled thyroid microsomes prepared from male Sprague Dawley rats. The guaiacol assay of TPO activity was used for this study. The known TPO inhibitor, 6-propyl-2-thiouracil (PTU), was used as a positive control substance.

The positive control, PTU, was assayed at the following concentrations: 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50, 100 and 200 µM. PTU was a potent inhibitor of TPO activity, exhibiting a half maximal inhibitory concentration (IC_{50}) value of 3.4 µM, 95% CI: 2.5 to 5.2 µM. This estimate is similar to a previously published estimate of 1.3 µM, 95% CI: 0.5 to 3.2 µM (***Paul et al., 2013***), validating the performance of the assay.

Pethoxamid was assayed at the following concentrations: 0, 0.01, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 µM. Pethoxamid did not inhibit TPO activity at any of the tested concentrations.

Conclusion: Pethoxamid is not an inhibitor of TPO activity.

MATERIALS AND METHODS

Materials:

Test Material:	Pethoxamid
Description:	Brown liquid
Lot/Batch number:	21082018
Purity:	97.7 %

Vehicle / positive control: DMSO / 6-propyl-2-thiouracil (PTU).

Study Design and Methods:
Experimental dates: Start: 06 December 2018, End: 12 December 2018.

Test item and positive control stock preparations: A solubility test was carried out to confirm that a 100 mM stock solution could be prepared for assaying the test item. The test item was made up fresh on the day of the assay by performing serial dilutions from a freshly prepared 100 mM stock solution. Positive control concentrations were made up fresh on the day of the assay by performing serial dilutions from a 100 mM stock solution. In each case there were a total of 10 concentrations and a blank.

Characterization of Test System: The test system, rat thyroid microsomes, was prepared from a pool of non-juvenile male Sprague Dawley rat thyroid glands (rats between 10 and 16 weeks of age) Thyroids were stored at approximately -80°C.

Protein Determination: The protein concentration of the pooled thyroid microsomes was determined in aqueous solutions using a modification of the method of ***Lowry et al., (1951)*** and bovine serum albumin standards. Samples were analysed using a Hitachi UV-Vis spectrophotometer.

TPO Activity: This method exploits the ability of TPO to catalyse the oxidation of guaiacol (a naturally occurring organic compound described as a colourless to light yellow and pink liquid) to a coloured product. The rate of production of the coloured product was determined spectrophotometrically (OD_{450}) and TPO activity was then expressed in units of ΔOD_{450}/min/mg protein. Thyroid microsomal samples were analysed in 6 replicates per concentration of test item or positive control, using a Hidex Sense Microplate reader. Test item or positive control (2 µL) was administered directly into each well of a 96-well plate containing 198 µL of microsomal assay preparation.

Acceptability of the assay: Of the six replicates performed, at least 3 are required for analysis (all 6 used if possible). The CV of replicate activity measurements will not exceed 15%. The IC_{50} value for PTU must be approximately 2 µM (similar to previously published value in ***Paul et al., 2013***).

Statistical Analysis: TPO activity (expressed as a percentage of control activity) was determined as a function of Test Item or positive control concentration, and IC50 parameters were estimated by fitting a four-parameter logistic model to the resulting data set. Model fitting was performed using GraphPad Prism® (Version 7.04, GraphPad Software Inc, San Diego, California, USA).

Calculation of TPO Enzyme Activity: TPO enzyme activity was calculated and expressed as ΔOD_{450}/min/mg protein. For each well, the plot of OD_{450} versus time was examined to establish the period of time the OD_{450} changed in a linear manner. The start of this period was defined as T_1 (in seconds) and the end of this period was defined as T_2 (in seconds); the corresponding OD_{450}s were

defined as OD_1 and OD_2, respectively. TPO enzyme activity was then calculated according to the following equation:

$$\text{TPO activity} \quad \frac{\frac{\Delta OD_{450}}{\text{min}}}{\text{mg protein}} = \frac{(60 \times (OD_2 - OD_1)) \times \frac{1000}{C}}{T_2 - T_1}$$

Where C = amount of microsomal protein (in µg) in the incubation.

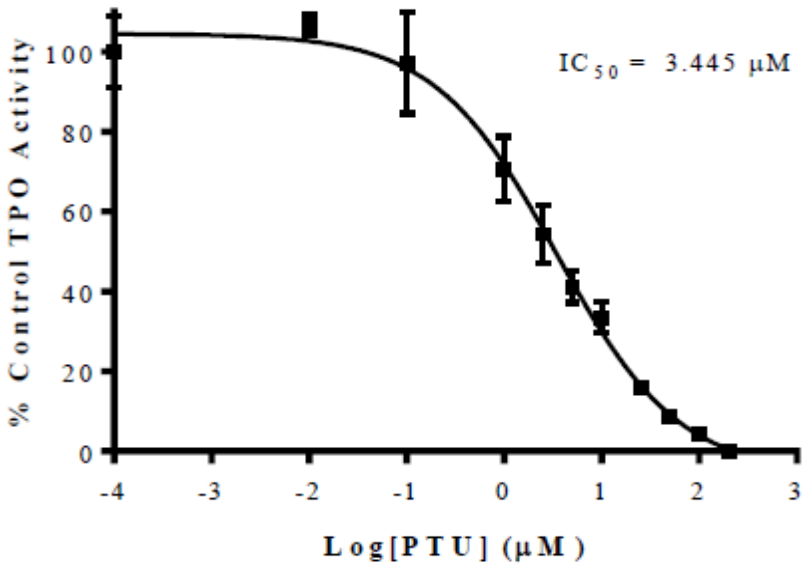
Data were then further processed and expressed as a percentage of control; these data were then used to estimate IC_{50} values using GraphPad Prism®.

RESULTS AND DISCUSSION

The positive control, PTU, was assayed at the following final concentrations: 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50, 100 and 200 µM. PTU was a potent inhibitor of TPO activity, exhibiting an IC_{50} value of 3.4 µM, 95% CI: 2.5 to 5.2 µM. This estimate is similar to a previously published estimate of 1.3 µM, 95% CI: 0.5 to 3.2 µM (***Paul et al., 2013***) validating the performance of the assay.

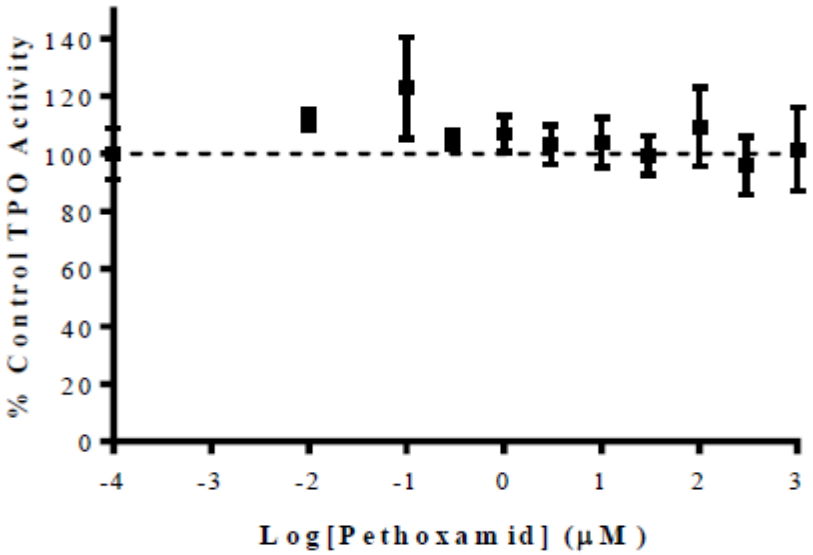
Pethoxamid was assayed at the following final concentrations: 0, 0.01, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 µM. Pethoxamid did not inhibit TPO activity at any of the tested concentrations.

Figure 3.9.4-1: Inhibition of rat TPO activity by PTU



TPO activity (expressed as a percentage of control activity) plotted as a function of PTU concentration. Data are presented as mean ± SD of up to 6 replicates per concentration. A four parameter logistic model was fitted to the data (best-fit curve shown). Error bars are small and in general are obscured by the data symbols.

Figure 3.9.4-2: Inhibition of rat TPO activity by pethoxamid



TPO activity (expressed as a percentage of control activity) plotted as a function of pethoxamid concentration. Data are presented as mean ± SD of up to 6 replicates per concentration. The dotted line is to highlight the scatter of the data points around the 100% value.

CONCLUSION: Pethoxamid is not an inhibitor of TPO activity.

References:

Chang, H.C. and Doerge, D.R. (2000). Dietary genistein inactivates rat thyroid peroxidase in vivo without apparent hypothyroid effect. *Toxicol. Appl. Pharmacol.* **168**: 244-252.

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Paul KB, Hedge JM, Rotroff DM, Hornung MW, Crofton KM and Simmons SO (2014). Development of a thyroperoxidase inhibition assay for high-throughput screening. *Chem. Res. Toxicol.* **27:** 387-399.

3.9.4.7 Anonymous (2020)

Reference: Pethoxamid: A 90-Day Oral (Dietary) Thyroid Mechanistic Study in Rats **Author(s), year**: Anonymous, 2020 **Report/Doc. number**: Laboratory Project ID: 00206020 / Report No.: 2018TOX-PXA4560 **Guideline(s)**: None **GLP**: Yes **Acceptability**: Yes

Justification for test system selection: The Crl:CD(SD) rat was chosen as it is an accepted rodent species for preclinical toxicity testing by regulatory authorities. In addition, this strain was used in an earlier chronic/carcinogenicity study and, therefore, enables a comparison of outcome. The number of

animals was the minimum required to properly characterize the effects as, at this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals do not currently exist. Oral administration was selected as this is the most likely route of human exposure.

EXECUTIVE SUMMARY

This study was designed to evaluate potential mechanisms underlying thyroid gland changes seen when pethoxamid was administered via the diet to male Sprague Dawley rats for at least 27 or at least 90 days.

Three groups of 20 male rats were fed diet containing 400, 1600 or 5000 ppm pethoxamid. An additional group of 20 male rats received basal diet and served as controls and another group of 20 male rats received 1000 ppm phenobarbital and served as positive controls. The rats were fed their diets for 29 or 92/93 consecutive days. Five rats/group were killed after 29 days. Clinical signs, body weights, body weight gains, food consumption, thyroid hormone assessment, gross necropsy findings, liver and thyroid weights, UDP-glucuronyl transferase (UGT) enzyme induction and histopathology of liver, thyroid and pituitary were evaluated during the study.

The overall mean daily intake of pethoxamid was 24, 96 and 308 mg/kg bw/day for the 400, 1600 and 5000 ppm groups, respectively.

There were no test-substance related mortalities, clinical observations, effects on food consumption or macroscopic findings.

At 5000 ppm pethoxamid, mean body weight gains were generally lower throughout the study which resulted in lower mean body weights and lower cumulative body weight gains.

There was a higher mean TSH value at 1600 and 5000 ppm on days 15, 29, 57 and 89 and a time-dependent decrease in total T4 relative to pretreatment values during the first 29 days of the study. There was no effect on total T3 or rT3.

There were higher mean thyroid/parathyroid weights at 5000 ppm on days 30 and 93/94 and higher mean liver weights at 5000 ppm on day 30 and at 1600 and 5000 ppm on day 93/94.

Test substance-related microscopic findings included follicular cell hypertrophy at 1600 and 5000 ppm on day 93/94 and hepatocellular hypertrophy at 5000 ppm on days 30 and 93/94.

T_4-glucuronidation activity was increased at 1600 and 5000 ppm on days 30 and 93/94. T_3-glucuronidation activity was increased at 1600 and 5000 ppm on day 30 and at 5000 ppm on day 92/93.

Thyroid follicular cell hypertrophy/hyperplasia and liver centrilobular hypertrophy, associated increased mean thyroid/parathyroid and liver weights were seen in the positive controls, along with increased TSH values on days 15, 29, 57 and 89, increased total T3 and rT3 values on day 89, lower mean total T4 values on days 15 and 29 and increased T_4- and T_3-glucuronidation activity on days 30 and 93/94. These changes are consistent with well recognised phenobarbital-related changes.

Conclusion: Based on the results of this study, dietary administration of pethoxamid to Crl:CD(SD) rats at dosage levels of 1600 and 5000 ppm for a minimum of 90 days resulted in liver enzyme induction leading to an increase in T4 glucuronidation and clearance of T4 which elicited a feedback response on the thyroid via an increase in TSH. It can be concluded from the hormone data that the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid treated rats.

MATERIALS AND METHODS

Materials:

Test Material:	Pethoxamid (IN-45263-006)
Description:	Technical material, dark brown solid
Lot/Batch number:	21082018
Purity:	99.4%
CAS#:	Not reported
Stability of test compound:	Expiry date 13 February 2022 (stored at 18-24ºC)
Positive control:	Phenobarbital sodium salt
Description:	White powder
Lot/Batch number:	SLBX0037
Purity:	99.9%
Stability of test compound:	Retest date March 2020 (stored at 18-24ºC)

Vehicle: Basal Diet, test substance administered via the diet.

Test Animals:	
Species	Rat
Strain	Crl:CD(SD)
Age/weight at dosing	7-8 weeks / 210-272 g
Source	Charles River Laboratories Inc., Raleigh, NC, USA.
Housing	2-3/cage in solid-bottom cages with appropriate bedding
Acclimatisation period	13 days
Diet	Treated or basal PMI Nutrition International, LLC Certified Rodent LabDiet®5002 (meal) *ad libitum*.
Water	Municipal tap water from the public supply *ad libitum*.
Environmental conditions	Temperature: 20-26°C
	Humidity: 30-70%
	Air changes: at least 10/hour
	Photoperiod: 12 hours light and 12 hours dark

Study Design and Methods:

In-life dates: Start: 04 March 2019, End: 05 June 2019.

Dose level selection rationale: The dose levels corresponded to levels used in an earlier subchronic and chronic/carcinogenicity studies with pethoxamid. It was anticipated that the highest concentration would show test substance related effects but not produce mortalities sufficient to prevent meaningful evaluation.

Test item and control preparations: Test and positive control dietary preparations were prepared as follows. An appropriate quantity of test substance together with an appropriate amount of acetone and basal diet was mixed in a Hobart mixer to form a pre-mix. The remainder of the basal diet to achieve the desired concentration on a w/w basis was mixed with the pre-mix in a V blender. The diet was blended to achieve a total batch of homogeneous diet at the appropriate concentration/group; the acetone was evaporated overnight prior to feeding the diet to the rats. Test diets, containing pethoxamid, were prepared weekly and stored at 18-24°C. Positive control diets were prepared daily for the initial 5 days

and every 3 days thereafter and stored at 18-24°C. An appropriate quantity of basal diet was weighed out approximately weekly for the controls and stored at 18-24°C.
Samples of the diets were collected and analysed by HPLC, using a validated analytical procedure, to determine achieved concentrations, homogeneity and stability of the test substance in diet at the following times:

Day	Achieved concentration	Homogeneity	Stability
-1	all groups	all groups except control	n/a
1	n/a	n/a	positive controls
5	n/a	positive controls	n/a
28	all groups except positive control	n/a	n/a
29	positive control	n/a	n/a
84	all groups except positive control	n/a	n/a
89	positive control	n/a	n/a
n/a = not applicable			

For concentration analyses, a single (50 g) sample of each dietary preparation was collected; duplicate samples were analysed. Concentration results were considered acceptable if mean sample concentration results were 100±15% of theoretical.

For homogeneity analyses, a single (50 g) sample of each dietary preparation was collected from each stratum; duplicate samples from each stratum were analysed. Homogeneity results were considered acceptable if the relative standard deviation of the mean concentration value at each sampling location was ≤ 10% and if mean sample concentration results were 100±15% of theoretical.

Stability of pethoxamid in diet was not assessed as dietary preparations have been shown previously to be stable for at least 10 days at room temperature (18 to 24°C) and frozen (-10 to -50°C). Stability of positive control dietary preparations were determined after 1, 3 and 7 days at 8-24°C. Samples (50 g) from each stratum were combined; duplicate samples were analysed. Stability results were considered acceptable if analysis results were ≥ 90% of pre-storage concentrations.

Analytical results: The dietary preparations contained 95.1-114% of the target concentrations and were homogeneous. Test substance dietary preparations have been shown previously to be stable and homogeneous over the range of concentrations (300 and 6000 ppm) used on this study for at least 8 days at 18-24°C.

The analysed dietary formulations contained 88.8-111% of the target concentration of the positive control (1000 ppm phenobarbital) and were homogeneous. Positive control formulations, however, were not stable at 18-24°C for 7 days but were for 3 days and, therefore, were prepared every 3 days.

No test substance was detected in control diet.

Animal assignment and treatment: The rats were assigned to groups by a stratified randomisation scheme designed to achieve similar group mean body weights. Basal diet (controls) or dietary preparations containing 400, 1600 or 5000 ppm pethoxamid or 1000 ppm phenobarbital (positive controls) was administered to groups of 20 male rats for 29 or 92/93 consecutive days. Five rats/group were killed after 29 days.

Table 3.9.4-14: Study design

Group	Treatment	Dietary concentration (ppm)	Number of males
1 (control)	basal diet	0	20
2 (low)	pethoxamid	400	20
3 (mid)		1600	20
4 (high)		5000	20
5 (positive control)	phenobarbital	1000	20

Mortality and clinical observations: Animals were checked for general health/mortality and moribundity twice daily, once in the morning and once in the afternoon. Cage-side observations were recorded daily throughout the study starting on day 1. The animals were removed from their cages, and a detailed clinical observation was performed 1 week (±2 days) prior to randomisation, on the day of randomisation, on day 1 (prior to dosing), weekly (±2 days) during the study period and on the day of scheduled termination.

Body weight: Animals were weighed 1 week (±2 days) prior to randomisation, on the day of randomisation, on day 1 (prior to dosing), weekly (±2 days) during the study period, on the day prior to scheduled termination and on the day of scheduled termination.

Food consumption and achieved dose: Food consumption was measured once following randomisation (all groups) and weekly (±2 days) during the study period (groups 1-4) or daily (group 5). The mean amounts of basal diet and dietary preparations containing the test or positive control substance consumed (mg/kg bw/day) per group were calculated.

Thyroid hormone assessment: Blood samples were collected from all animals, via the jugular vein, without anticoagulant on days -3, 15, 29/30, 57 and 89. The blood was allowed to clot for 30 minutes and then all samples were centrifuged (3000 rpm; 2056xg) for 10 minutes at 4°C within 2 hours of collection and divided into aliquots. Samples were stored at -55 to -85°C prior to analysis.

Analyses to determine total T3 and T4 concentrations were conducted using a validated UPHLC/MS/MS assay. Analyses to determine TSH (rTSH [Rat Thyroid Stimulating Hormone] RIA Kit and rT3 (rT3 RIA Kit) concentrations were conducted using qualified radioimmunoassays.

Termination and pathology: A necropsy was conducted on the one rat that died on study and specified tissues were saved. Animals surviving until scheduled termination (days 30 or 93/94) were fasted overnight, weighed and killed by carbon dioxide inhalation followed by exsanguination.

Macroscopic findings: Animals were subjected to a complete necropsy examination, which included evaluation of all external surfaces and orifices; the cranial cavity and external surfaces of the brain; and thoracic, abdominal and pelvic cavities with their associated organs and tissues.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

liver pituitary	thyroid with parathyroids (post-fixation)

Paired organs were weighed together. Organ to body weight ratios, using terminal body weights, were calculated.

Tissue submission: Representative samples of the following tissues were collected from all animals and preserved in 10% neutral buffered formalin:

pituitray	abnormal tissue
liver	thyroid with parathyroids [1]

[1] parathyroids were examined if in the plane of section and in all cases where a gross lesion of the organ was present

Microscopic examination: All tissues listed above were processed, sectioned and stained with hematoxylin and eosin and examined histopthologically.

Determination of UDP-glucuronosyltransferase (UGT) enzyme induction: Liver was collected at the interim and terminal necropsies for UGT analysis from all animals/group. Two (2-3 g) samples of liver were excised from all animals/group and saved separately in RNAse-free tubes and immediately snap-frozen in liquid nitrogen for analysis of UGT. Two additional (20-30 mg) samples were excised from the left lobe and saved separately in RNAse-free tubes and immediately snap-frozen in liquid nitrogen for possible future analysis.

UGT induction *ex vivo*: The enzymatic activity in liver collected from all animals at the interim necropsy and 5 animals/group at terminal necropsy was evaluated.

Enzymatic activity	Substrate	Metabolite
T3-glucuronidation	T3	T3-glucuronide
T4-glucuronidation	T4	T4-glucuronide

Liver samples were thawed at room temperature and homogenised in ice-cold 0.1 M Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose. The homogenate was centrifuged for 30±1 minute at 12000 g (4°C). The resulting supernatant was centrifuged at 105000 g for 75±1 minutes (4°C). Subsequently the microsomal pellet was resuspended in 0.01 M Tris buffer pH 7.4 containing 3 mM EDTA. Microsomes were stored in aliquots of 0.05 mL and 0.25 mL (for protein and the UGT activity determinations, respectively) at ≤75°C.

Protein concentrations were measured in liver microsomes from each animal, in duplicate, by the method of Lowry (***Lowry et al, 1951***) using bovine serum albumin (BSA) as a standard.

Stock solutions of T3 and T4 substrates were prepared. Incubation mixtures were prepared on ice, in duplicate, by mixing 0.1 M Tris-HCl buffer pH 7.4 with rat liver microsomes and UGT reaction mixtures. After shaking, the mixtures were pre-incubated for 15±1 minutes on ice. Subsequently, the mixtures were pre-incubated for 5±1 minutes at 37±1°C in a water bath. The reaction was started by the addition of the appropriate (T3 or T4) spiking solution. After incubation at 37±1°C for a defined time, the samples were transferred to ice and a protein precipitation solution was added to precipitate proteins. Subsequently, a sample pre-treatment procedure was used to remove precipitated proteins and the samples were analysed by UPLC-PDA-MS.

Statistics: All statistical tests were conducted at the 5% significance level. All pairwise comparisons used two-sided tests and were reported at the 1% and 5% levels. Numerical data collected were analysed as indicated at each interval. Inferential statistics were performed when possible, but excluded semi-quantitative data, and any group with <3 observations. A parametric (WTDMS^{TM}) method was used for body weight, body weight gain, food consumption, TSH and rT3 data and total T3 vand T4 data. A parametric (Provantis or Nevis) method was used for organ weights and organ weights relative to body weight. Pairwise comparisons of groups 2, 3, 4 and 5 versus group 1 (controls) were made. Datasets were compared using an overall one-way ANOVA *F*-test. If the overall *F*-test was found to be significant, then the above pairwise comparisons were conducted using Dunnett's test.

RESULTS AND DISCUSSION

Mortality and clinical observations: One 1600 ppm rat was found dead on day 30 shortly after blood collection. The cause of death was acute haemorrhage and was not test substance related. There were no other mortalities.

There were no test substance related clinical observations.

Body weights: Treatment-related lower body weights were noted at 5000 ppm throughout the study and were statistically significantly different from control values during weeks 2-5, 7-10 and 12-13. Mean body weight was 7.5% lower than controls at the end of the study.

Treatment-related lower body weight gains were noted at 5000 ppm throughout the study and were statistically significantly different from control values during weeks 1-2, 4-5 and 6-7. Statistically significantly lower mean cumulative body weight gains, compared to controls, were noted at 5000 ppm throughout the study (14% lower), most notably during weeks 1-5 and 5-9 (20.7% and 19% lower, respectively).

There were no treatment-related effects on body weight at 400 or 1600 ppm pethoxamid or in the positive control group.

Table 3.9.4-15: Intergroup comparison of bodyweights and body weight change - selected timepoints

Week	Dietary Concentration pethoxamid (ppm)				Positive control (1000 ppm phenobarbital)
	0 (control)	400	1600	5000	
Body weights [g]					
1	240	240	240	240	238
2	303	304	301	279**	301
4	389	388	381	358**	385
8	492	499	490	454*	500
12	556	563	550	509*	561
14	569	581	568	527	584
Body weight changes [g]					
1-2	63	63	61	39**	63
4-5	43	46	42	35**	41
6-7	29	29	28	21**	30
Cumulative body weight changes [g]					
1-5	193	194	183	153**	188
1-9	275	281	268	229**	280
1-14	332	342	327	285*	341
5-9	88	88	83	71*	90
9-14	57	61	59	56	61

* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)

** Statistically significant difference from control group mean, p<0.01 (Dunnett's test)

Food consumption and achieved dose: There were no test substance related effects on food consumption.

The mean dose received (based on nominal dietary levels of pethoxamid) is shown below.

Table 3.9.4-16: Overall mean dose received (mg/kg/day)

Group	Treatment	Dietary concentration (ppm)*	Mean test substance consumption (mg/kg bw/day)
1 (control)	basal diet	0	0
2 (low)	pethoxamid	400	24
3 (mid)		1600	96
4 (high)		5000	308
5 (positive control)	phenobarbital	1000	62

*No correction factor was used for test substance purity.

Thyroid hormone assessment: Test substance related higher mean TSH values were noted at 1600 and 5000 ppm on days 15, 29, 57 and 89 and increased in a time-dependent manner on day 15 and 29. The changes were statistically significant compared to the controls, with the exception at 1600 ppm on day 89.

There were no statistically significant and/or biologically relevant differences in total T3 levels between test substance and control groups.

There was a trend towards slightly higher total T4 levels at 5000 ppm compared to controls on days 15, 57 and 89. However, the pre-treatment values for total T4 at 5000 ppm were higher relative to controls (P<0.01). T4 levels at 5000 ppm decreased in a time-dependent manner on days 15 and 29 relative to pre-test values and returned to near pre-test values on day 57. The time course related decrease in total T4 levels at 5000 ppm correlated with the increase in TSH over the same time interval. As there was no impact on T3 levels at 5000 ppm, the changes in T4 were considered not to be adverse.

In the positive control group, higher mean TSH values were noted on days 15, 29, 57 and 89 and increased in a time-dependent manner on days 15 and 29. Statistically significant higher mean total T3 and rT3 values were noted on day 89. Total T4 levels decreased in a time-dependent manner on days 15 and 29, compared to pre-treatment values, and corresponded to the increase in TSH levels during the same time interval. These changes were statistically significantly different from the control group values on day 29.

There were no other test substance-related effects on thyroid hormones. Other values that achieved statistical significance were considered not test or positive control-related as they were prior to test substance initiation, there was a lack of dose-response and most values were within the range of concurrent control values.

Table 3.9.4-17: Mean thyroid hormone parameters (% difference from controls)

Day	Dietary Concentration pethoxamid (ppm)				Positive control (1000 ppm phenobarbital)
	0 (control)	400	1600	5000	
TSH (ng/mL)					
-3	4.9	8.1* (65.3)	6.6 (34.7)	8.9** (81.6)	8.5* (73.5)
15	7.3	8.3 (13.7)	14.3* (95.9)	17.8** (143.8)	18.6** (154.8)
29	11.7	14.9 (27.4)	22.1* (88.9)	24.6** (110.3)	26.5** (126.5)
57	7.9	9.9 (25.3)	16.6* (110.1)	20.8** (163.3)	24.4** (208.9)
89	12.0	12.3 (2.5)	19.3 (60.8)	24.3** (102.5)	27.2** (126.7)
Total T3 (pg/mL)					
-3	874	897 (2.6)	869 (-0.6)	866 (-0.9)	803 (-8.1)
15	686	648 (-5.5)	559** (-18.5)	678 (-1.2)	601 (-12.4)
29	709	678 (-4.4)	663 (-6.5)	720 (1.6)	642 (-9.4)
57	769	655 (-14.8)	795 (3.4)	810 (5.3)	756 (-1.7)
89	582	610 (4.8)	578 (-0.7)	677 (16.3)	729* (25.3)
Total T4 (pg/mL)					
-3	62995	60665 (-3.6)	71940 (14.3)	82600** (31.2)	78225** (24.3)
15	51195	50690 (-1.0)	52010 (1.6)	61485** (20.1)	45390 (-11.3)
29	58840	52350 (-11.0)	52250 (-11.2)	52720 (-10.4)	35590** (-39.5)
57	65993	67820 (2.8)	72043 (9.2)	79620** (20.6)	55513 (-15.3)
89	36493	38840 (6.4)	35171 (-3.6)	50287** (37.8)	36900 (1.1)
rT3 (ng/mL)					
-3	0.179	0.148* (-17.3)	0.142** (-20.7)	0.181 (1.1)	0.171 (-4.5)
15	0.141	0.094** (-33.3)	0.092** (-34.8)	0.144 (2.1)	0.108* (-23.4)
29	0.091	0.141** (54.9)	0.133** (46.2)	0.106 (16.5)	0.101 (11.0)
57	0.105	0.091 (-13.3)	0.078* (-25.7)	0.133* (26.7)	0.110 (4.8)
89	0.103	0.098 (-4.9)	0.076 (-26.2)	0.134 (30.1)	0.199** (93.2)

* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)

** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test)

Figures in bold = values considered to be test substance-related

Macroscopic findings: No test or positive control substance-related macroscopic findings were seen on day 30 or day 93/94. Macroscopic findings observed were incidental, of the type commonly seen in this strain and age of rat and/or were of similar incidence in control and treated animals.

Organ weights:

Day 30: Increased liver and thyroid/parathyroid weights (absolute and/or relative to body weight) were seen at 5000 ppm pethoxamid and were considered to be test substance-related. The increased liver weight correlated with microscopic findings of hepatocellular hypertrophy.

Increased liver and thyroid/parathyroid weights (absolute and/or relative to body weight) were also seen in the positive controls and correlated with microscopic findings of hepatocellular hypertrophy and thyroid follicular cell hypertrophy.
No other test or positive control substance-related changes were seen. There were other isolated organ weight values that were statistically significantly different from controls but there were no patterns, trends or correlating data to suggest these values were toxicologically relevant and they were, therefore, considered incidental.

Day 93/94: The increased liver weights (absolute and relative to body weight) in the 1600 and 5000 ppm test substance groups and the increased thyroid/parathyroid weight (absolute and relative to body weight) in the 5000 ppm test substance group were considered to be test substance-related. The increased liver and thyroid/parathyroid weights at 5000 ppm correlated with microscopic findings of hepatocellular hypertrophy and thyroid follicular cell hypertrophy, respectively.

Increased liver and thyroid/parathyroid weights (absolute and/or relative to body weight) were also seen in the positive controls and correlated with microscopic findings of hepatocellular hypertrophy and thyroid follicular cell hypertrophy/hyperplasia.

No other test or positive control substance-related changes were seen. There were other isolated organ weight values that were statistically significantly different from controls but there were no patterns, trends or correlating data to suggest these values were toxicologically relevant and they were, therefore, considered incidental.

Table 3.9.4-18: Summary of mean organ weight data (% difference from controls)

	Dietary Concentration pethoxamid (ppm)				Positive control (1000 ppm phenobarbital)
	0 (control)	400	1600	5000	
Day 30					
No. animals/group	5	5	5	5	5
Terminal body weight (g)	432.4	416.6 (-3.7)	397.0 (-8.2)	350.6*** (-18.9)	384.4* (-11.1)
Liver – absolute weight (g)	17.6174	16.5890 (-5.8)	17.7314 (0.6)	18.6318 (5.8)	20.6154* (17.0)
Liver - % of body weight	4.07300	3.98076 (-2.3)	4.46751 (9.7)	5.31128** (30.4)	5.36565** (31.7)
Thyroid/parathyroid – absolute weight (g)	0.0156	0.0172 (10.3)	0.0170 (9.0)	0.0204* (30.8)	0.0238*** (52.6)
Thyroid/parathyroid - % body weight	0.00364	0.00411 (13.0)	0.00426 (17.1)	0.00576** (58.3)	0.00615** (69.2)
Day 93/94					
No. animals/group	15	15	14	15	15
Terminal body weight (g)	550.9	558.6 (1.4)	547.4 (-0.6)	504.5* (-8.4)	552.3 (0.2)
Liver – absolute weight (g)	17.0991	18.4860 (8.1)	19.7418* (15.5)	23.0585***(34.9)	25.9685***(51.9)
Liver - % of body weight	3.10376	3.30747 (6.6)	3.61093** (16.3)	4.57668** (47.5)	4.71391** (51.9)
Thyroid/parathyroid – absolute weight (g)	0.0183	0.0205 (12.4)	0.0183 (0.1)	0.0231** (26.6)	0.0248*** (35.8)

Thyroid/parathyroid - % body weight	0.00332	0.00365 (10.1)	0.00334 (0.5)	0.00460** (38.6)	0.00451** (35.8)

* Statistically significant difference from control group mean, p<0.05 (Dunnett's test)

** Statistically significant difference from control group mean, p<0.01 (Dunnett's test)

*** Statistically significant difference from control group mean, p<0.001 (Dunnett's test)

Histopathology:

Day 30: Liver centrilobular hypertrophy was seen at 5000 ppm pethoxamid and was considered to be test substance-related.

Thyroid follicular cell hypertrophy, liver centrilobular hypertrophy and pituitary gland hypertrophy of the pars distalis (one animal only) seen in the positive control group were considered to be treatment-related and were consistent with well-recognised phenobarbital effects.

Other microscopic findings seen were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and were, therefore, considered unrelated to treatment.

Day 93/94: Thyroid follicular cell hypertrophy was seen at 1600 and 5000 ppm and liver centrilobular hypertrophy was seen at 5000 ppm and were considered to be treatment-related. Pituitary gland hypertrophy of the pars distalis was seen in one animal at 5000 ppm was potentially test substance-related.

Thyroid follicular cell hypertrophy and hyperplasia, liver centrilobular hypertrophy and pituitary gland hypertrophy of the pars distalis (one animal only) seen in the positive control group were considered to be treatment-related and were consistent with well-recognised phenobarbital effects.

Other microscopic findings seen were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and were, therefore, considered unrelated to treatment.

Table 3.9.4-19: Summary of incidence of selected microscopic findings

		Dietary Concentration pethoxamid (ppm)				**Positive control (1000 ppm phenobarbital)**
		0 (control)	**400**	**1600**	**5000**	
Day 30						
No. tissues examined/group		5	5	5	5	5
Pituitary – hypertrophy, pars distalis	minimal	0	0	0	0	1
	total	0	0	0	0	1
Thyroid – hypertrophy, follicular cell	minimal	0	0	0	0	2
	total	0	0	0	0	2
Liver – hypertrophy, centrilobular	mild	0	0	0	3	0
	moderate	0	0	0	0	3
	marked	0	0	0	0	2
	total	0	0	0	3	5
Day 93/94						

No. tissues examined/group		15	15	14	15	15
Pituitary – hypertrophy, pars distalis	minimal	0	0	0	0	1
	mild	0	0	0	1	0
	total	0	0	0	1	1
Thyroid – hypertrophy, follicular cell	minimal	0	0	3	1	0
	moderate	0	0	0	4	0
	severe	0	0	0	10	15
	total	0	0	3	15	15
Thyroid – hyperplasia, follicular cell	minimal	0	0	0	0	3
	mild	0	0	0	0	2
	total	0	0	0	0	5
Liver – hypertrophy, centrilobular	minimal	0	0	0	4	0
	mild	0	0	0	1	0
	moderate	0	0	0	0	12
	marked	0	0	0	0	3
	total	0	0	0	5	15

UDP-glucuronosyltransferase (UGT) induction: Dose-dependent increases in T_4-glucuronidation activity were observed in the pethoxamid groups and were statistically significantly different from controls at 1600 and 5000 ppm at days 30 and 93/94 (4.9 and 3.2-fold increases at 5000 ppm compared to control values, respectively).

Increases in T_3-glucuronidation activity were also noted in the pethoxamid groups, although the dose-response was not as prominent as it was for T_4-glucuronidation activity and was statistically significant at 1600 ppm at day 30 and at 5000 ppm at day 93/94 (1.5 to 1.9-fold increase at day 30 and 1.0 to 1.93-fold at day 93/94).

A statistically significant increase in liver microsomal protein content was observed at 5000 ppm at day 30 (1.5-fold increase compared to controls) and was consistent with induction of liver metabolising enzymes.

T_4-glucuronidation activity (4.1 and 2.4-fold) and T_3-glucuronidation activity (3.7 and 1.9-fold) were statistically significantly increased in the positive controls at day 30 and day 93/94, respectively. Liver microsomal protein content was elevated at day 30 only (1.6-fold). These changes were consistent with the well-recognised increases in liver metabolising enzymes and UDP-glucuronyl transferase activity in rodents induced by phenobarbital.

Table 3.9.4-20: Protein content and enzyme activities (fold induction compared to control)

Group	**Treatment**	**Protein content**	**T_3-glucuronidation activity**	**T_4-glucuronidation activity**
Day 30				
2 (low)	400 ppm pethoxamid	1.2	1.5	2.0

3 (mid)	1600 ppm pethoxamid	1.1	1.9	2.7
4 (high)	5000 ppm pethoxamid	1.5	1.7	4.9
5 (positive control)	1000 ppm phenobarbital	1.6	3.7	4.1
Day 93/94				
2 (low)	400 ppm pethoxamid	0.9	1.4	1.5
3 (mid)	1600 ppm pethoxamid	0.9	1.0	1.9
4 (high)	5000 ppm pethoxamid	1.0	1.9	3.2
5 (positive control)	1000 ppm phenobarbital	1.0	1.9	2.4

CONCLUSION: Based on the results of this study, dietary administration of pethoxamid to Crl:CD(SD) rats at dosage levels of 1600 and 5000 ppm for a minimum of 90 days resulted in liver enzyme induction leading to an increase in T4 glucuronidation and clearance of T4 which elicited a feedback response on the thyroid via an increase in TSH. It can be concluded from the hormone data that the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid treated rats.

Reference:
Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265-275.

3.9.4.8 Anonymous (2019b)

Reference: Effect of pethoxamid on biliary excretion of [^{125}I]thyroxine and metabolites in rats
Author(s), year: Anonymous, 2019
Report/Doc. number: 2018MET-PXA4538 / 037182-1
Guideline(s): None
GLP: Yes
Acceptability: Yes

Justification for Test System Selection: The objective of this study was to determine the effect of pethoxamid on Thyroxine (T4) clearance by determining the levels of [^{125}I]Thyroxine ([^{125}I]T4) and its metabolites in serum and bile in bile-duct cannulated rats treated with pethoxamid for 7 days followed by subsequent treatment with [^{125}I]T4 at a single dose level.

EXECUTIVE SUMMARY
Bile-duct cannulated rats were pre-treated with 1% (w/v) methylcellulose with 0.5% Tween 80 in water (negative control), phenobarbital (PB, positive control), or pethoxamid once daily for 7 consecutive days. On Day 8, approximately 15 minutes prior to the [^{125}I]T4 dose, the rats were dosed with 2 mg/kg of potassium iodide. A single IV dose of [^{125}I]T4 in sterile saline was administered by intravenous injection to each male bile duct and jugular vein cannulated rats. The study design for the induction phase is summarised below.

Table 3.9.4-21: Induction phase

Group	Inducer	Inducer dose level (mg/kg)	Inducer dose concentration (mg/mL)	Inducer dose volume (mL/kg)	Number of males
1	1% (w/v) methylcellulose (MC) with 0.5% (w/v) Tween 80 in water (Negative Control)	0	0	10	6
2	Phenobarbital (PB) (Positive Control)	100	20	5	6
3	Pethoxamid (PXA)	300	30	10	6

Blood was collected from the jugular cannula for serum at 6 time points following IV dose administration. Bile was collected at 2 time points (0-2 and 2-4 hours) following IV dose administration.

The mean serum Tmax values for total radioactivity were 0.42 ± 0.14 hours, 0.40 ± 0.14 hours, and 0.25 ± 0.00 hours for Groups 1, 2, and 3, respectively. The total radioactivity Cmax and AUC0-4 values for Group 2 (PB treatment) and Group 3 (pethoxamid treatment) were decreased relative to Group 1 (control group), suggesting greater metabolism and subsequent elimination. The mean Cmax values were 0.715 ± 0.085 ng-equiv/mL, 0.427 ± 0.039 ng-equiv/mL, and 0.541 ± 0.084 ng-equiv/mL for Groups 1, 2, and 3, respectively. The mean AUC0-4 values were 1.742 ± 0.210 hr*ng-equiv/ml, 1.083 ± 0.168 hr*ng-equiv/mL, and 1.285 ± 0.186 hr*ng-equiv/mL for Groups 1, 2, and 3, respectively.

Mean liver weights in the PB and pethoxamid groups increased relative to the control group, consistent with the induction of metabolizing enzymes in these two groups

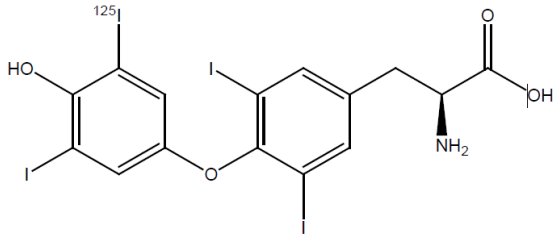
T4 was the major radioactive component in the pooled serum extracts. A peak corresponding in HPLC retention time to T4 Glucuronide was observed in the serum extracts from control rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.00% at 0.25 hours to 1.77% at 4 hours. This same peak was also observed in the serum extracts of pethoxamid -treated rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.44% at 0.25 hours to 6.76% at 4 hours. No T4 Glucuronide was found in the serum extracts from PB-treated rats, possibly due to rapid elimination in the bile.

The mean percentages of total radioactivity (expressed as the percent of administered dose or % AD) excreted in bile were 7.96 ± 0.38%, 16.13 ± 5.46%, and 11.99 ± 2.80% for Groups 1, 2, and 3, respectively. The % AD excreted in bile for Group 2 (PB-treated) and Group 3 (pethoxamid -treated) were approximately 2.0-fold higher and 1.5-fold higher, respectively, than the % AD in bile for Group 1 (control).

Bile samples from Groups 1, 2, and 3 were analyzed by HPLC/RAD to determine the distribution of [^{125}I]T4 and its metabolites. A peak corresponding in retention time to T4 Glucuronide was the major radioactive peak in the bile samples. There were greater quantities of T4 Glucuronide and T3 sulfate conjugates in bile from the PB- and pethoxamid -treated groups than in the control group. T4 Glucuronide accounted for 4.66 ± 0.30%, 10.61 ± 4.42%, and 7.44 ± 2.24% AD for Groups 1, 2, and 3, respectively. T3 Sulfate accounted for 0.62 ± 0.10%, 1.18 ± 0.15%, and 1.01 ± 0.24% AD for Groups 1, 2, and 3, respectively. Peaks corresponding in retention time to T4, T4 4'-O-Sulfate, and iodide anion were also observed. The increase in the amount of T4 Glucuronide and T3 Sulfate following PB and pethoxamid treatment was presumably due to the induction of the enzyme(s) responsible for formation of T4 Glucuronide and T3 Sulfate.

Conclusion: PB and pethoxamid treatment resulted in greater overall clearance of thyroxine due to induced T4 glucuronidation and in part induced T3 sulfation in rat liver. This induction correlated with the increase in liver weight in PB- and pethoxamid -treated rats.

MATERIALS AND METHODS
Materials:

Test Material:	[^{125}I] thyroxine ([^{125}I]T4)
Lot/Batch number:	AU52490
Specific activity:	969 Ci/mmol
Radiochemical purity:	≥95%
Structure:	

Storage:	2-10ºC
Reference standard:	Unlabeled L-thyroxine
Lot/Batch number:	BCBV2496
Purity:	≥98%
Stability:	Retest date April 2020
Reference standard:	T4 Glucuronide
Lot/Batch number:	6-EKP-111-3
Purity:	99.1%
Stability:	Retest date 31 July 2022
Reference standard:	Thyroxine 4'- *O* Sulfate
Lot/Batch number:	M17Q08157
Purity:	98.5%
Stability:	Retest date August 2019
Reference standard:	Thyroxine T_3 Sulfate
Lot/Batch number:	M17Q06160
Purity:	≥95%
Stability:	Retest date June 2019
Inducer:	Pethoxamid technical
Lot/Batch number:	21082018
Purity:	99.4%
Stability:	Expiry date 13 February 2022
Inducer:	Phenobarbital sodium salt
Lot/Batch number:	SLBX0037
Purity:	99.9%
Stability:	Retest date March 2020
Negative control:	1% methylcellulose with 0.5% Tween 80 in water solution
Vehicle::	[^{125}I]T4 was dissolved in saline

Test Animals:	
Species	Rat (bile-duct and jugular vein-cannulated for the collection of serial bile and blood samples).
Sex:	Male.
Strain	Sprague-Dawley
Age/weight at dosing	10.4 weeks/297.0-355.7 g
Source	Charles River Breeding Laboratories, Inc.
Housing	Individually housed in Nalgene metabolism cages
Acclimatisation period	1 day
Diet	Certified rodent diet *ad libitum*
Water	Tap water *ad libitum*
Environmental conditions	Temperature: 68-79°F Humidity: 30-70% Air changes: At least 10/hour

Study Design and Methods:
Experimental dates: Start: 04 April 2019, End: 07 May 2019.

Induction phase: A 1% (w/v) methylcellulose with 0.5% Tween 80 in water solution (Group 1, negative control) was administered by oral gavage (10 mL/kg) daily for 7 days to the animals in Group 1. Phenobarbital sodium salt was dissolved in 0.9% saline to yield a final concentration of 20 mg/mL (Group 2, Positive Control). This solution was administered via oral gavage (5 mL/kg, 100 mg/kg) daily for 7 days to the animals in Group 2. Pethoxamid technical was suspended in 1% (w/v) methylcellulose with 0.5% Tween 80 in water to yield a concentration of 30 mg/mL This suspension was administered via oral gavage (10 mL/kg, 300 mg/kg) daily for 7 days to the animals in Group 3.

[^{125}I]T4 Dose Level and Vehicle: [^{125}I]T4 was dissolved in saline. [^{125}I]T4 in vehicle was administered by bolus intravenous injection to each experimental animal at a dose level of 30 µCi/animal.

[^{125}I]T4 Dose Analysis: The radioconcentration (dpm/g) and homogeneity (CV) of the dose formulations were determined prior to dosing. Three aliquots of the dosing formulation were analysed for the determination of radiolabel concentration and homogeneity by gamma counting before and after dosing. Radiochemical purity of each dose formulation was determined before and after dosing.

[^{125}I]T4 Dosing Procedure: KI in sterile saline (2 mg/mL) was administered via bolus intravenous injection into the tail vein of each rat at 2 mg/kg (1 mL/kg) approximately 15 minutes prior to [^{125}I]T4 dosing to prevent uptake of [^{125}I]T4 by the thyroid. Then, [^{125}I]T4 was administered via bolus intravenous injection into the tail vein of each rat at a dose level of 30 µCi/animal.

Daily Observations: Animals were observed for viability at least 4 hours apart (once in the morning and once in the afternoon), throughout the study.

Body Weights: All animals were weighed prior to initial dose administration.

Termination: At the end of the exposure period at 4 hours post-dose, rats were killed by an overdose of CO_2 followed by exsanguination. The liver was removed from each animal weighed and stored at approximately -70°C for potential future analysis.

Sample Collection and Analyses: Blood samples (target of 250 µL per sample) were collected from the jugular cannula from each animal at 0.25, 0.5, 1, 1.5, 2, and 4 hours. The blood samples were placed in tubes without anticoagulant. Following each sample collection, the rats were infused with an equal volume of saline to maintain blood volume. Immediately following blood collection, the samples were held on wet ice for 15 minutes for coagulation before centrifugation. Samples were centrifuged within

60 minutes of collection under refrigeration (5°C for 10 minutes at 2000g). Aliquots of serum samples were analysed for total radioactivity by gamma counting. Remaining serum samples were stored at approximately -70°C for metabolite profiling.

Bile samples were collected at 2 hours (0-2 hour collection) and 4 hours (2-4 hour collection) from the bile duct cannula. Aliquots of bile samples were analysed for total radioactivity by gamma counting. Remaining bile samples were stored at approximately -70°C for metabolite profiling.

Radioactivity in all samples was quantitated by gamma counting using a Packard Cobra II Gamma Counter. Formulation samples, bile samples, and serum extract samples were diluted with 0.5 mL of HPLC water prior to analysis. HPLC fractions were analyzed without dilution. Dose formulations, bile samples, and serum extracts were analyzed by HPLC/RAD. Unlabeled reference standards of T4 and potential metabolites were analyzed with each HPLC run. The UV retention times of the standards were determined.

Metabolite Profiling: Bile samples were diluted with HPLC mobile phase A (20 mM ammonium acetate in water, pH 4.0) and analysed by HPLC/RAD. Individual animal bile samples containing sufficient amounts of radioactivity were analysed. Serum samples from the animals were pooled by group and time point by combining equal volumes of serum from each animal. Serum samples were extracted with an equal volume of acetonitrile. The samples were centrifuged at 5°C at 2000g for 10 minutes. Aliquots of the serum extracts were analyzed by gamma counting to determine extractablity and by HPLC/RAD to detect and quantitate [^{125}I]T4 and metabolites.

Calculations: The radiolabel concentration in serum (as determined by gamma counting) was calculated as nanogram equivalents/mL by dividing the cpm/mL by the specific activity of the [^{125}I]T4 test substance. Data from the analyses of the serum collected from the animals was used to calculate the following parameters for both total radioactivity concentrations in serum: 1) the time (Tmax) to reach peak concentrations of radiolabel in the serum, 2) the concentration (Cmax) of radiolabel in the serum at Tmax, 3) the total area under the serum total radioactivity concentration versus time curve (AUC), and 4) the elimination half-life of total radioactivity. WinNonlin version 6.2 (Pharsight Corp.), operating as a validated software system was used to determine the pharmacokinetic parameters using noncompartmental analysis. The excretion of radioactivity in bile was calculated as the percentage of administered dose by dividing the quantity of radioactivity in each bile sample by the administered dose.

RESULTS AND DISCUSSION

Dose analyses: Radiochemical purity and homogeneity of the [^{125}I]T4 formulations are shown below:

Table 3.9.4-22: Radiochemical purity (%)

Group	Pre-dose	Post-dose
1, 2	90.78	90.05
3	85.62	93.11

Table 3.9.4-23: Homogeneity (cpm/µL dose formulation)

Group	Pre-dose	Post-dose	Coefficient of variation (%)
1, 2	196,473	9,966	5.07
3	204,426	10,019	4.90

Administered doses: The amounts of radioactivity dosed were 26.550 µCi for Groups 1 and 2 and 27.625 µCi for Group 3.

Serum pharmacokinetics: The mean T_{max} values for total radioactivity were 0.42 ± 0.14 hours, 0.40 ± 0.14 hours, and 0.25 ± 0.00 hours for Groups 1, 2, and 3, respectively. Terminal elimination half-lives were not reported for 2 of 3 animals in Group 1 and for all animals in Groups 2 and 3 due to extensive extrapolation (>30%) for the elimination phase.

The total radioactivity C_{max} and AUC_{0-4} values for Group 2 (PB treatment) and Group 3 (pethoxamid treatment) were decreased relative to Group 1 (control group), suggesting greater metabolism and elimination. The mean C_{max} values were 0.715 ± 0.085 ng-equiv/ml, 0.427 ± 0.039 ng-equiv/mL, and 0.541 ± 0.084 ng-equiv/mL for Groups 1, 2, and 3, respectively. The mean AUC_{0-4} values were 1.742 ± 0.210 hr*ng-equiv/ml, 1.083 ± 0.168 hr*ng-equiv/mL, and 1.285 ± 0.186 hr*ng-equiv/mL for Groups 1, 2, and 3, respectively. The results for Groups 1 and 2 are similar to those previously reported in the literature (Kato *et al.*, 2010).

Table 3.9.4-24: Serum total radioactivity concentrations (ng-eq/g) following a single IV dose of [^{125}I]T4

Time (h)	Total radioactivity concentration (ng-eq/g)					
	Group 1		Group 2		Group 3	
	1% MC/0.5% Tween 80 in Water (Negative Control)		PB (Positive Control) 100 mg/kg bw/day		Group 3, Pethoxamid 300 mg/kg bw/day	
	mean	SD	mean	SD	mean	SD
0.25	0.668	0.125	0.389	0.080	0.541	0.084
0.5	0.662	0.100	0.345	0.145	0.480	0.057
1.0	0.553	0.084	0.328	0.065	0.366	0.058
1.5	0.418	0.052	0.293	0.025	0.313	0.048
2.0	0.411	0.047	0.208	0.070	0.251	0.034
4.0	0.236	0.045	0.190	0.015	0.240	0.046

n=3 for Groups 1 and 3 and n=5 for Group 2.

T4 was the major radioactive component in the pooled serum extracts. A peak corresponding to iodide anion (I^-), presumably formed by radiolytic decomposition of [^{125}I]T4, was observed in all serum extract samples. A peak corresponding in HPLC retention time to T4 Glucuronide was observed in the serum extracts from control rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.13% at 0.25 hours to 1.77% at 4 hours. This same peak was also observed in the serum extracts of pethoxamid-treated rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.44% at 0.25 hours to 6.76% at 4 hours. No T4 Glucuronide was found in the serum extracts from PB-treated rats.

Table 3.9.4-25: Quantitation of T4 and metabolites in pooled serum extracts

Group	Treatment		% HPLC					
			0.25 h	0.5 h	1 h	1.5 h	2 h	4 h
1	1% MC/0.5% Tween 80 in water (negative control)	Iodide	18.12	28.29	31.03	29.16	29.79	31.82
		T4 glucuronide	0.13	0.08	0.47	0.33	0.63	1.77
		T4	81.11	71.10	67.94	69.87	68.78	64.72
2	PB (Positive Control) 100 mg/kg bw/day	Iodide	31.03	32.68	30.11	32.31	31.28	37.74
		T4 glucuronide	0.00	0.02	0.00	0.00	0.00	0.00
		T4	68.70	66.93	68.80	67.13	68.10	61.59
3	Pethoxamid 300 mg/kg bw/day	Iodide	24.85	25.84	29.40	30.64	27.42	26.72
		T4 glucuronide	0.44	0.77	1.98	2.70	3.23	6.76
		T4	74.37	72.01	67.68	65.67	68.48	65.18

Liver weight: Mean liver weights in the PB and pethoxamid groups increased relative to the control group.

Table 3.9.4-26: Mean liver weights (g)

Mean liver weight (g)					
Group 1		**Group 2**		**Group 3**	
1% MC/0.5% Tween 80 in Water (Negative Control)		**PB (Positive Control) 100 mg/kg bw/day**		**Group 3, Pethoxamid 300 mg/kg bw/day**	
mean	**SD**	**mean**	**SD**	**mean**	**SD**
13.9302	0.9773	17.0702	0.6986	19.7548	1.7509

n=3 for Groups 1 and 3 and n=5 for Group 2.

Excretion of Administered Radioactivity in Bile: The mean percentages of administered dose (% AD) excreted in bile were 7.96 ± 0.38%, 16.13 ± 5.46%, and 11.99 ± 2.80% for Groups 1, 2, and 3, respectively. The % AD excreted in bile for Group 2 (PB-treated) and Group 3 (pethoxamid -treated) were approximately 2.0-fold higher and 1.5-fold higher, respectively, than the % AD in bile for Group 1 (control).

Table 3.9.4-27: Percentage Recovery of the Administered Radiolabeled Dose ([^{125}I]T4) in Bile

Time (h)	**Percentage of administered radiolabeled dose**					
	Group 1		**Group 2**		**Group 3**	
	1% MC/0.5% Tween 80 in Water (Negative Control)		**PB (Positive Control) 100 mg/kg bw/day**		**Group 3, Pethoxamid 300 mg/kg bw/day**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
2	3.89	1.43	7.77	3.50	6.96	1.61
4	4.06	1.10	8.35	2.12	5.02	1.32
Total	7.96	0.38	16.13	5.46	11.99	2.80

n=3 for Groups 1 and 3 and n=5 for Group 2.

Bile samples from Groups 1, 2, and 3 were analyzed by HPLC/RAD to determine the distribution of [^{125}I]T4 and metabolites in the bile samples. A peak corresponding in retention time to T4 Glucuronide was the major radioactive peak in the bile samples. There was significantly more T4 Glucuronide in bile from the PB- and pethoxamid-treated groups than for the control group. T4 Glucuronide accounted for 4.66 ± 0.30%, 10.61 ± 4.42%, and 7.44 ± 2.24% AD for Groups 1, 2, and 3, respectively. T3 Sulfate accounted for 0.62 ± 0.10%, 1.18 ± 0.15%, and 1.01 ± 0.24% AD for Groups 1, 2, and 3, respectively. The increase in the amount of T4 Glucuronide and T3 Sulfate following PB and pethoxamid treatment was presumably due to the induction of the enzyme(s) responsible for formation of T4 Glucuronide and T3 Sulfate.

Table 3.9.4-28: Quantitation of T4 and metabolites in bile samples

Group	**Treatment**	**Time (h)**		**% AD**	
				mean	**SD**
1	**1% MC/0.5% Tween 80 in Water (Negative Control)**	Total % AD	Bile	7.96	0.38
			Iodide	0.46	0.12
			T4 glucuronide	4.66	0.30
			T3 sulfate	0.62	0.10
			T4 4'-*O*-Sulfate	0.61	0.10
			T4	0.54	0.16
2	**PB (Positive control) 100 mg/kg bw/day**	Total % AD	Bile	16.13	5.46
			Iodide	1.30	0.38
			T4 glucuronide	10.61	4.42
			T3 sulfate	1.18	0.15
			T4 4'-*O*-Sulfate	0.88	0.25
			T4	0.70	0.24
3	**Pethoxamid 300 mg/kg bw/day**	Total % AD	Bile	11.99	2.80
			Iodide	0.79	0.16

			T4 glucuronide	7.44	2.24
			T3 sulfate	1.01	0.24
			T4 4'-*O*-Sulfate	0.59	0.15
			T4	0.95	0.16

n=3 for Groups 1 and 3 and n=5 for Group 2.

CONCLUSION: PB and pethoxamid treatment resulted in greater clearance of thyroxine due to induced T4 glucuronidation in rat liver. The induction of T3 sulfation by PB and pethoxamid was also observed. This induction correlated with the increase in liver weight in PB- and pethoxamid -treated rats.

Reference:
Y. Kato, *et al.*, *Toxicology and Applied Pharmacology*, **249 (2010)**, 238-246

3.9.4.9 Anonymous (2019c)

Reference: Pethoxamid technical: mRNA and DNA-synthesis induction in cultured mouse and human hepatocytes
Author(s), year: Anonymous, 2019
Report/Doc. number: 2018TOX-PXA4482 / CLS4_0023_0006
Guideline(s): None
GLP: Yes
Acceptability: Yes

Justification for Test System Selection: The hypothesis under investigation was that the mode of action (MoA) for pethoxamid-induced mouse liver tumor formation was similar to that of phenobarbital (PB). PB is an activator of the constitutive androstane receptor (CAR) and to a weaker extent pregnane X receptor (PXR) (***Elcombe et al, 2014***). The test system used was male CD-1 mouse primary hepatocytes and primary cryopreserved hepatocytes from three male human donors.

EXECUTIVE SUMMARY
This study was designed to test the hypothesis that pethoxamid induces mouse liver tumors by a phenobarbital (PB)-like mode of action (MoA) (***Elcombe et al., 2014***). CYP2B and CYP3A expression is induced by the constitutive androstane receptor (CAR) and to a weaker extent pregnane X receptor (PXR). In rodents, activation of CAR by PB leads to hepatocellular tumors that are not evident in hamsters, guinea pigs or primates including humans (***Elcombe et al., 2014***). While CAR/PXR induced gene expression is conserved across species, differences in replicative DNA synthesis (RDS) [S-phase of the cell cycle], a key event associated with liver tumor formation, occurs in rodent but not in human hepatocytes.

Isolated primary male CD-1 mouse hepatocytes or male primary human hepatocytes (3 donors) were exposed in culture to pethoxamid, PB or epidermal growth factor (EGF) for approximately 96 hours after which cell cytotoxicity was evaluated by quantification of ATP levels, or the cells were harvested and processed for mRNA analysis of Cyp2b10 and Cyp3a11 (mouse) and CYP2B6 and CYP3A4 (human), or cells were processed for assessment of replicative DNA synthesis.

Following a preliminary cytotoxicity assessment where cytotoxicity was observed at dosing concentrations ≥ 30 µM (1.3% of control ATP content), mouse hepatocytes were exposed to pethoxamid at dosing concentrations of 1, 3, 10 and 20 µM. Human hepatocytes were exposed to pethoxamid (1, 3, 10 and 20 µM, donor 385) and (0.3, 1, 3 and 10 µM, donors 8210 and 8219). Pethoxamid caused overt cytotoxicity (as defined by a decrease in intracellular ATP content of >20% of control levels) in human donor 385 (20 µM, 70.7% of control ATP levels), donor 8210 (10 µM, 62.2% of control ATP concentration) and donor 8219 (10 µM, 75.4% of control ATP levels).

In mouse hepatocytes, PB induced Cyp2b10 and Cyp3a11 to approximately 2.1- and 4.3 fold control, but high variance resulted in a lack of statistical significance. Pethoxamid induced a somewhat lower increase in Cyp2b10 or Cyp3a11 relative to PB of ~ 1.4-1.5-fold (at 3 µM) in mouse hepatocytes, but this response also lacked statistical significance due to variance in the data.

In all 3 human donors, PB (1000 µM) treatment resulted in statistically significant induction of CYP2B6 mRNA (donor 385, 20.2-fold; donor 8210, 5.6-fold; donor 8219, 5.0-fold. Likewise, CYP3A4 mRNA induction by PB (1000 µM) was statistically significant in all 3 donors, (donor 385, 8.8-fold; donor 8210, 9.8-fold; donor 8219, 13.8-fold).

Pethoxamid induced dose related increases in CYP2B6 in human hepatocytes, reaching 4.3-fold at 20 µM (donor 385), and 2.0- and 3.2-fold at 10 µM (donors 8210 and 8219, respectively). CYP3A4 was similarly increased reaching 1.86-fold at 20 µM (donor 385) and 2.4- and 2.53-fold at 10 µM (donors 8210 and 8219, respectively).
Overt cytotoxicity was observed for all human donor hepatocytes at either 10 (donors 8210 and 8219) or 20 µM (donor 385) pethoxamid. The only exception to this was treatment with pethoxamid at 3 µM in donor 8219, where a statistically significant induction in CYP3A4 (2.50-fold) was observed in the absence of cytotoxicity.

In cultures of primary male CD-1 mouse hepatocytes, the positive control EGF (25 ng/mL) induced RDS (6.2-fold). As expected in mouse hepatocytes, PB (1000 µM) also induced RDS (1.9-fold). Pethoxamid induced RDS at all concentrations tested (1 µM, 1.7-fold; 3 µM, 2.8-fold; 10 µM, 2.0-fold; 20µM, 2.2-fold).

In human hepatocytes, the positive control EGF (25 ng/mL) induced RDS in all 3 human hepatocyte donors (donor 385, 5.1-fold; donor 8210, 8.7-fold; donor 8219, 13.5-fold).
Neither pethoxamid nor PB caused an increase in RDS in hepatocytes from any human donor tested.

Conclusion: Pethoxamid is a weak inducer of CAR and/or PXR in vitro. Pethoxamid induced RDS in mouse hepatocytes but not in human hepatocytes. The response observed with pethoxamid is qualitatively similar to that of PB. The results in this study provide data supporting the lack of human relevance for mouse liver tumor formation following treatment with pethoxamid.

MATERIALS AND METHODS
Materials:

Test Material:	Pethoxamid
Description:	Brown solid when frozen
Lot/Batch number:	21082018
Purity:	97.7%
Stability of test compound:	Expiry date 12 October 2020 (stored at approximately -20ºC)

Control items: Phenobarbital, sodium salt (PB); Epidermal Growth Factor (EGF)

Vehicle: Dimethyl sulfoxide (DMSO) for test item the test item was formulated in DMSO and administered such that the final DMSO concentration in all cultures was 0.1% (v/v) (i.e. 1 µL DMSO/mL medium).

Study Design and Methods:
Experimental dates: Start: 10 December 2018. End: 08 August 2019.

Test item and control preparations: A solubility test confirmed that pethoxamid was soluble in dimethyl sulfoxide (DMSO) at 1M. When the subsequent dilutions were made in culture medium, pethoxamid precipitated at final concentrations at or exceeding 100µm. During the main experiments,

where final Test Item concentrations did not exceed 20 mM, no undissolved Test Item was observed by microscopy any tested concentrations. The test item was formulated in DMSO and administered such that the final DMSO concentration in all cultures was 0.1% (v/v) (i.e. 1 µL DMSO/mL medium).

PB was prepared in sterile water at a concentration of 1M, filter sterilized through a 0.2 µm filter and stored at approximately 4ºC.

EGF was reconstituted according to the manufacturer's instructions to 25 ng/mL and stored at approximately -70ºC. The aliquot of EGF used for treating hepatocyte cultures was stored at approximately -20ºC.

A vehicle control with DMSO (0.1% v/v) only was included in all cultures.

Characterization of Test System; The test system used was male CD-1 mouse primary hepatocytes (*Mus musculus*) and primary cryopreserved hepatocytes from three male human donors (*Homo sapiens*). Hepatocytes were isolated from male CD-1 mice using a two-step collagenase perfusion method according to ***Mitchell et al, (1984)***. In brief, mice were terminally anesthetised using Euthatal®, the livers were perfused with Krebs Ringer phosphate buffer, followed by Krebs Ringer hydrogen carbonate buffer. Human hepatocytes were sourced from Corning Life Sciences, Fogostraat 12, 1060 LJ Amsterdam, Netherlands (donor 385) or Life Technologies, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR (donors 8210 and 8219). Viability of all cell preparations was greater than 70%.

Hepatocyte culture conditions: Primary mouse hepatocytes were cultured in CL-15 media. Freshly isolated hepatocytes were initially cultured in CL-15 media for approximately 4 hours to allow adherence to the tissue culture plates in a humidified incubator at 37ºC under atmospheric air. Cells were seeded in 96-well plates at a density of 2×10^4 cells per well (cytotoxicity) or 6-well plates at a density of 0.8×10^6 cells per well for mRNA analysis and RDS.

Human hepatocytes were cultured in HCL-15 media (CL15-media + ascorbic acid (0.3 mM). Human hepatocytes were cultured on collagen coated tissue culture plates. Human hepatocytes were thawed in Cryopreserved Hepatocytes Recovery Medium (CHRM, Life Technologies), then cultured in Cryopreserved Hepatocytes Plating Medium (CHPM, Life Technologies) for approximately 6 hours to allow adherence in a humidified incubator at 37ºC under 95% air /5% CO_2. Cells were seeded in 96-well plates at a density of 4×10^4 cells per well (cytotoxicity) or 6-well plates at a density of 1.6×10^6 cells per well for mRNA analysis and RDS.

Cytotoxicity: In the preliminary study, a preliminary cytotoxicity assessment was carried out to evaluate the potential cytotoxicity of pethoxamid and to select appropriate concentrations of pethoxamid for use in the main study for analysis of mRNA expression and replicative DNA-synthesis. Pethoxamid was evaluated at 9 concentrations (1, 3, 10, 30, 100, 300, 600, 900 and 1000 µM). A vehicle control (DMSO 0.1% (v/v)) was included. The medium including test item or vehicle control was replenished daily for 3 days following the initial dosing and cells were cultured as detailed above. Cells were cultured for a total of approximately 96 hours prior to evaluation of cytotoxicity.

In the main study, pethoxamid cytotoxicity was evaluated at 4 or 5 concentrations outlined below, while PB cytotoxicity was evaluated at 1000 µM. The vehicle control was DMSO (0.1% v/v). Mouse hepatocytes were cultured with pethoxamid (1, 3, 10 and 20 µM), PB (1000 µM) or vehicle control (DMSO 0.1% (v/v)). Human hepatocytes were cultured with pethoxamid (donor 385: 0.3, 1, 3, 10 and 20 µM; donors 8210 and 8219: 0.3, 1, 3, and 10 µM), PB (1000 µM) and vehicle control (DMSO 0.1% (v/v)). The medium including pethoxamid, PB or vehicle control was replaced daily for 3 days following the initial dosing and cells were cultured as detailed above. Cells were cultured for a total of approximately 91-96 hours prior to evaluation of cytotoxicity.

Hepatocytes were plated in 6 replicates for each concentration in 96-well plates for cytotoxicity (ATP) measurements.

Hepatocyte culture mRNA analysis: Mouse or human hepatocytes were seeded in 6 well plates (n=3 per treatment). Mouse hepatocytes were cultured with pethoxamid (1, 3, 10 and 20 µM), PB (1000 µM) or vehicle control (DMSO 0.1% (v/v)). Human hepatocytes were cultured with pethoxamid (donor 385: 0.3, 1, 3, 10 and 20 µM; donors 8210 and 8219: 0.3, 1, 3, and 10 µM), PB (1000 µM) and vehicle control (DMSO 0.1% (v/v)). To keep a consistent number of dosing concentrations for evaluation across all human donors, the 0.3 µM dosing samples for donor 385 were not processed to measures of mRNA abundance.Following approximately 93-96 hours in culture, cells were processed for mRNA analysis.

Hepatocyte culture RDS (S-phase): Mouse or human hepatocytes were seeded in 6 well plates and incubated for 96 hours (n=5 per treatment). 5-bromo-2'-deoxyuridine (BrdU) was added to the culture medium for the last 3 days of culture. BrdU is a thymidine analogue and is incorporated into newly synthesized DNA. BrdU incorporation can be determined immunocytochemically and quantified.

Mouse hepatocytes were cultured with pethoxamid (1, 3, 10 and 20 µM), PB (1000 µM), EGF (25 ng/mL) or vehicle control (DMSO 0.1% (v/v)). Human hepatocytes were cultured with pethoxamid (donor 385: 0.3, 1, 3, 10 and 20 µM; donors 8210 and 8219: 0.3, 1, 3, and 10 µM), PB (1000 µM), EGF (25 ng/mL) or vehicle control (DMSO 0.1% (v/v)).

Cytotoxicity as evaluated by intracellular ATP concentrations: Cell toxicity was assessed following approximately 91 to 96 hours of culture as indicated by ATP depletion. Cellular ATP was determined by luminometry and analyzed using a Hidex Sense Microplate reader with Hidex Sense Plate Reader Software version 0.5.55.0. The bioluminescent determination of ATP release from viable cells was carried out using an assay kit supplied by Promega (CellTiter-Glo luminescent cell viability assay) according to manufacturer's instructions. Results were expressed as a percentage of the amount of ATP released as compared to the control cells.

Taqman® Analysis of mRNA expression: RNA was extracted from cultured hepatocytes using an RNeasy mini kit according to manufacturer's instructions. cDNA was synthesized from all available RNA samples using Qiagen QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions and quality checked using ThermoFisher NanoDrop 1000. Taqman® analysis was performed on all available samples using primer/probe sets specific for murine Cyp2b10 and Cyp3a11, or human CYP2B6 and CYP3A4 (Assay-on-demand kits, Applied Biosystems). Beta-actin was used as the internal standard (Assay-on-demand kits, Applied Biosystems). Data was analyzed by generation of threshold cycle (C_T) and delta delta C_T values for both the internal standard and cytochrome P450 genes.

Replicative DNA synthesis (S-phase): The number of cells undergoing RDS in any given cell population was determined by the incorporation of BrdU and was analysed immunocytochemically. Immunostaining was performed after fixation, using a mouse monoclonal anti-bromodeoxyuridine Clone Bu20a primary antibody (Agilent, M0744, Santa Clara, California, USA) and a polyclonal rabbit anti-mouse immunoglobulins/house radish peroxidase secondary antibody (Agilent, P0260, Santa Clara, California, USA). The number of hepatocytes in S-Phase was assessed by manual counting of four fields of view from five independent wells and recorded as the labelling index [(number of labelled hepatocyte / total number of hepatocytes) x 100]. Cells deemed morphologically abnormal at any concentration were not counted. Typically, 300 – 400 total cells were counted per field of view.

Statistical analysis: Statistical comparisons between hepatocytes treated with test item and the vehicle control group were undertaken using a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. Statistical comparisons between hepatocytes treated with each control item (PB or EGF) and the vehicle control group were undertaken using a student's t-test. Analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

Mouse hepatocytes:

Cytotoxicity: The preliminary investigation of pethoxamid cytotoxicity in male CD-1 mouse hepatocytes demonstrated that pethoxamid was tolerated up to a concentration of 10 µM, as cytotoxicity was observed at dosing concentrations ≥ 30 µM (1.3% of control ATP content). Therefore, concentrations of 1, 3, 10 and 20 µM pethoxamid was tested in the subsequent experiments and no significant decreases in cell viability were observed.

Table 3.9.4-29: Effect of pethoxamid on ATP levels in primary male CD-1 mouse hepatocytes (preliminary study)

Test Item & Concentration	ATP Content (Luminescence Units)	
	Mean ± SD	Fold Control Mean ± SD
Vehicle control (0.1% [v/v] DMSO)	348000 ± 59000 (100 ± 17)	-
Pethoxamid 1 µM	405000 ± 11000 (116.4 ± 3.1)***	1.16 ± 0.20
Pethoxamid 3 µM	407400 ± 9500 (117.1 ± 2.7)***	1.17 ± 0.20
Pethoxamid 10 µM	410000 ± 20000 (117.9 ± 5.9)***	1.18 ± 0.21
Pethoxamid 30 µM	4510 ± 600 (1.30 ± 0.17)***	0.0130 ± 0.0028
Pethoxamid 100 µM	515 ± 34 (0.1481 ± 0.0097)***	0.00148 ± 0.00027
Pethoxamid 300 µM	371 ± 74 (0.107 ± 0.021)***	0.00107 ± 0.00028
Pethoxamid 600 µM	298 ± 60 (0.086 ± 0.017)***	0.00086 ± 0.00023
Pethoxamid 900 µM	245 ± 20 (0.0704 ± 0.0057)***	0.00070 ± 0.00013
Pethoxamid 1000 µM	216 ± 13 (0.0621 ± 0.0036)***	0.00062 ± 0.00011
ATP values are Mean ± SD, n = 6 (expressed as percentage in parentheses). The error estimates in the fold control values were calculated by error propagation from the SD associated with the numerator and denominator values used to calculate the mean fold control value. In all cases, the number of significant figures reflects the error in the measurement. A one way-ANOVA was performed on the results followed by a Dunnett's multiple comparison test; *** statistically different from control $P < 0.001$.		

Induction of Cyp2b10 and Cyp3a11 in primary male mouse CD-1 hepatocytes: PB (1000 µM) induced Cyp2b10 (2.1-fold) and Cyp3a11 (4.3-fold) mRNA expression, but statistical significance was not reached due to variability in the data.

Pethoxamid induces a somewhat lower increase in Cyp2b10 and Cyp3a11 mRNA expression relative to PB in primary male mouse CD-1 hepatocytes. The highest induction of Cyp2b10 and Cyp3a11 was observed at 3 µM (1.41-1.508-fold, respectively). However, due to variability in the data, these responses lacked statistical significance.

Table 3.9.4-30: Taqman® analysis of Cyp2b10 and Cyp3a11 mRNA in primary mouse hepatocytes from male CD-1 mice

Test Item & Concentration	Cyp2b10	Cyp3a11

Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.37	1.00 ± 0.12
PB 1000 µM	2.1 ± 1.5	4.3 ± 2.5
Pethoxamid 1 µM	0.99 ± 0.66	1.20 ± 0.49
Pethoxamid 3 µM	1.41 ± 0.54	1.508 ± 0.079
Pethoxamid 10 µM	1.22 ± 0.59	0.92 ± 0.17
Pethoxamid 20 µM	0.64 ± 0.16	0.76 ± 0.10
Data expressed as fold change over the mean vehicle control. Values are Mean ± SD; n = 3 per group. The number of significant figures is dictated by the error in the measurement. A one-way ANOVA was performed on the results followed by a Dunnett's multiple comparison test. Δ A Student's t test was performed on the results for PB treated hepatocytes. No statistical differences were observed compared to DMSO control.		

Replicative DNA synthesis (S-phase) mouse hepatocytes: In cultures of primary male CD-1 hepatocytes, the positive control EGF (25 ng/mL) induced RDS (6.2-fold, $P < 0.001$). As expected in mouse hepatocytes PB (1000 µM) also induced RDS (1.90-fold, $P < 0.01$). Pethoxamid induced RDS at all concentrations tested (1 µM, 1.71-fold, $P < 0.01$; 3 µM, 2.78-fold, $P < 0.001$; 10 µM, 2.03-fold, $P < 0.001$; 20 µM, 2.15-fold, $P < 0.001$)

Table 3.9.4-31:Effect of pethoxamid, PB or EGF on replicative DNA synthesis (S-Phase) in primary mouse hepatocytes from male CD-1 mice

Test Item & Concentration	**S-Phase Labelling Index**	
	Mean ± SD	**Mean % control ± SD**
Vehicle control (0.1% [v/v] DMSO)	0.230 ± 0.060 (100 ± 26)	–
PB 1000 µM	0.44 ± 0.11 (190 ± 50)**	1.90 ± 0.70
Pethoxamid 1 µM	0.394 ± 0.056 (171 ± 24)**	1.71 ± 0.50
Pethoxamid 3 µM	0.64 ± 0.10 (278 ± 45)***	2.78 ± 0.85
Pethoxamid 10 µM	0.468 ± 0.056 (203 ± 24)***	2.03 ± 0.58
Pethoxamid 20 µM	0.496 ± 0.057 (215 ± 25)***	2.15 ± 0.61
EGF 25 ng/mL $^{\Delta}$	1.43 ± 0.14 (620 ± 62)***	6.2 ± 1.7
S-Phase values are Mean ± SD, n = 5 (expressed as percentage in parentheses). The error estimates in the fold control values were calculated by error propagation from the SD associated with the numerator and denominator values used to calculate the mean fold control value. In all cases, the number of significant figures reflects the error in the measurement. A one-way ANOVA was performed on the results, followed by a Dunnett's multiple comparison test; ** statistically different from control $P < 0.01$; *** $P < 0.001$. Δ A Student's t test (two-tailed) was performed on the results for PB or EGF treated hepatocytes; ** statistically different from control $P < 0.01$; *** $P < 0.001$		

Human hepatocytes:

Cytotoxicity: The preliminary evaluation of pethoxamid toxicity in primary cultures of male human hepatocytes demonstrated cytotoxicity above 10 µM for donor 385 and above 3 µM for donors 8210 and 8219. Subsequently, pethoxamid concentrations chosen for evaluation in donor 385 were 0.3, 1, 3, 10 and 20 µM, and 0.3, 1, 3 and 10 µM for donors 8210 and 8219.

In the main experiment, overt cytotoxicity was observed in response to pethoxamid (20 µM) in donor 385 (70.7% of control ATP levels ($P < 0.001$)). In donors 8210 and 8219, pethoxamid (10 µM) also caused overt cytotoxicity (62.2% of control ATP ($P < 0.001$) and 75.4% of control ATP levels ($P <$

0.01), respectively). No cytotoxicity was detected at any other concentration tested in all three donors (Viability > 90%).

Table 3.9.4-32: Effects of pethoxamid or PB on ATP levels in primary human hepatocytes from three male donors (main study)

Test Item & Concentration	ATP Content (Luminescence Units)		
	Donor 385	**Donor 8210**	**Donor 8219**
Vehicle control (0.1% [v/v] DMSO)	587000 ± 25000 (100.0 ± 4.2)	306000 ± 24000 (100.0 ± 7.8)	274000 ± 33000 (100 ± 12)
PB 1000 µM Δ	541000 ± 28000 (92.3 ± 4.7)*	296000 ± 35000 (97 ± 11)	299000 ± 35000 (109 ± 13)
Pethoxamid 0.3 µM	604000 ± 46000 (103.0 ± 7.9)	295000 ± 26000 (96.5 ± 8.6)	296000 ± 34000 (108 ± 12)
Pethoxamid 1 µM	600000 ± 34000 (102.3 ± 5.8)	322000 ± 40000 (105 ± 13)	306000 ± 37000 (111 ± 14)
Pethoxamid 3 µM	601000 ± 39000 (102.5 ± 6.7)	303000 ± 31000 (99.0 ± 10.0)	300000 ± 32000 (109 ± 12)
Pethoxamid 10 µM	549000 ± 27000 (93.6 ± 4.7)	190000 ± 15000 (62.2 ± 5.1)***	207000 ± 15000 (75.4 ± 5.3)**
Pethoxamid 20 µM	415000 ± 33000 (70.7 ± 5.7)***	Not tested	Not tested
ATP values are Mean ± SD, n = 6 (expressed as percentage in parentheses). In all cases, the number of significant figures reflects the error in the measurement. A one way-ANOVA was performed on the results followed by a Dunnett's multiple comparison test; ** statistically different from control P < 0.01; *** P < 0.001. Δ A Student's t test (two-tailed) was performed on the results for PB treated hepatocytes; * statistically different from control P < 0.05			

Induction of CYP2B6 and CYP3A4 in human hepatocytes: PB (1000 µM) induced CYP2B6 mRNA expression in all 3 human donors (donor 385: 20.2-fold, P < 0.05; donor 8210: 5.6-fold, P < 0.05; donor 8219: 4.96-fold, P < 0.01). Pethoxamid (20 µM) induced CYP2B6 mRNA expression (4.3-fold, P < 0.01) in donor 385. In donor 8219, pethoxamid (10 µM) induced CYP2B6 mRNA expression (3.2-fold, P < 0.05). CYP2B6 mRNA expression was increased (2.0-fold) in donor 8210, although this increase was not statistically significant.

PB (1000 µM) induced CYP3A4 mRNA expression in all 3 donors (donor 385: 8.8-fold, P < 0.05; donor 8210: 9.8-fold, P < 0.01; donor 8219: 13.8-fold, P < 0.001). Pethoxamid (3 and 10µM) induced a statistically significant increase in CYP3A4 mRNA expression in donor 8219 (2.50-fold (P < 0.01) and 2.53-fold (P < 0.01), respectively). While increases in CYP3A4 were observed in donor 385 at 3, 10 and 20 µM (1.89-, 1.88-, 1.86-fold, respectively) and in donor 8210 at 1, 3, and10 µM (1.50-, 2.37-, 2.4-fold, respectively), these changes did not reach statistical significance.

Table 3.9.4-33: Taqman® analysis of CYP2B6 and CYP3A4 mRNA in human hepatocytes from three male donors

Test Item & Concentration	Donor 385		Donor 8210		Donor 8219	
	CYP2B6	**CYP3A4**	**CYP2B6**	**CYP3A4**	**CYP2B6**	**CYP3A4**
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.42	1.00 ± 0.45	1.00 ± 0.39	1.00 ± 0.38	1.00 ± 0.27	1.00 ± 0.24
PB 1000 µM Δ	20.2 ± 9.0*	8.8 ± 4.1*	5.6 ± 2.6*	9.8 ± 1.8**	4.96 ± 1.00**	13.8 ± 1.6***

Pethoxamid 0.3 µM	Not tested	Not tested	1.14 ± 0.46	1.18 ± 0.49	1.58 ± 0.17	1.56 ± 0.19
Pethoxamid 1 µM	1.59 ± 0.68	1.17 ± 0.57	1.32 ± 0.66	1.50 ± 0.62	1.87 ± 0.57	1.64 ± 0.42
Pethoxamid 3 µM	2.44 ± 0.58	1.89 ± 0.71	1.88 ± 0.75	2.37 ± 0.83	2.27 ± 0.27	2.50 ± 0.17**
Pethoxamid 10 µM	2.9 ± 1.2	1.88 ± 0.96	2.0 ± 1.1	2.4 ± 1.1	3.2 ± 1.5*	2.53 ± 0.79**
Pethoxamid 20 µM	4.3 ± 1.7**	1.86 ± 0.87	Not tested	Not tested	Not tested	Not tested
Data expressed as fold change over mean vehicle control. Values are Mean ± SD; n = 3 per group. The number of significant figures is dictated by the error in the measurement. A one-way ANOVA was performed on the results followed by a Dunnett's multiple comparison test; * statistically different from control $P < 0.05$; ** $P < 0.01$. Δ A Student's t test was performed on the results for PB treated hepatocytes; * statistically different from control $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.						

Replicative DNA synthesis (S-phase) human hepatocytes: The positive control EGF (25 ng/mL) induced RDS in all 3 human hepatocyte donors; (donor 385, 5.09-fold, $P < 0.001$), (donor 8210, 8.7-fold, $P < 0.001$) and (donor 8219, 13.5-fold, $P < 0.001$). PB had no effect on RDS in primary hepatocytes from any human donor.

Pethoxamid had no effect on RDS in primary hepatocytes from any human donor at all concentrations tested.

CONCLUSION: Pethoxamid is a weak inducer of CAR and/or PXR in vitro. Pethoxamid induced RDS in mouse hepatocytes but not in human hepatocytes. The response observed with pethoxamid is qualitatively similar to that of PB. The results in this study provide data supporting the lack of human relevance for mouse liver tumor formation following treatment with pethoxamid.

References:

Elcombe, C. R., Peffer, R. C., Wolf, D. C., Bailey, J., Bars, R., Bell, D., Cattley, R. C., Ferguson, S. S., Geter, D., Goetz, A., Goodman, J. I., Hester, S., Jacobs, A., Omiecinski, C. J., Schoeny, R., Xie, W. & Lake, B. G. 2014. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol*, 44, 64-82.

Mitchell, A. M., Bridges, J. W. & Elcombe, C. R. 1984. Factors influencing peroxisome proliferation in cultured rat hepatocytes. *Arch Toxicol*, 55, 239-46.

3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 Anonymous (1998a)

Reference: TKC-94 : Preliminary Study of Effects on Reproductive Performance in CD Rats by Dietary Administration
Author(s), year: Anonymous, 1998
Report/Doc. number: 84 PXA (TOX2001-297) / TON013/980047
Guideline(s): OECD 416 (1983)
GLP: Yes
Deviations from OECD 416 (2001): Dose range finding study, with low number of animals and limited parameters assessed
Acceptability: Yes; limited information

Material and Methods:
Test material: Pethoxamid, Batch: TB-960306; Purity 95.0%.

Test animals: Four groups of 6 male and 6 female of the CD strain Sprague Dawley rats, 215-291 g (males), 137-217 g (females), 7 weeks of age, from Charles River UK Limited, Margate Kent, England to form the F0 generation.

Test method: The animals received the test material by dietary administration at concentrations of 0, 25, 100, 400 or 1600 ppm (F0: 15 days prior and through pairing, gestation and lactation; selected F1: until about 6 weeks of age).

Findings and conclusion:

Mortality, clinical signs, body weight and food consumption: No treatment related clinical signs were observed and there were no deaths. At 1600 ppm, F0 females showed the lowest body weight gain before pairing. It was not affected during gestation and gains were superior to the control group during lactation. Food consumption was unaffected as well reproduction parameters and litter size and survival.

Absolute body weights of offspring at birth were lower for the groups receiving 100, 400 and 1600 ppm than for the control group. This was considered to be to the slightly larger litter size at birth in these groups. Body weight gains from Day 4 of age were lower at 400 and 1600 ppm. At the selection of the F1 generation, this resulted in lower mean body weights for the 1600 ppm group than for the control group. Body weights to six weeks of age were similar for the 1600 ppm and control groups.

Pathological examination:
Necropsy: Necroscopy revealed no treatment related findings in the F0 and F1 generations.

Organ weights: In the F1 generation, liver weights (absolute and body weight relative) were increased at 1600 ppm.

Conclusion: It was concluded, that treatment at 1600 ppm (F0: 127-172 mg/kg bw/d; F1: 240-296 mg/kg bw/d) resulted in a decreased body weight gain of the offspring and 25 ppm was estimated to be a no effect level.

3.10.1.2 Anonymous (2000)

Reference: TKC-94 : Study of Reproductive Performance in CD Rats treated continuously through two successive generations by Dietary Administration;
Author(s), year: Anonymous, 2000
Report/Doc. number: 85 PXA (TOX2001-298) / TON015/992242
Guideline(s): OECD 416 (1983)
GLP: Yes
Deviations from OECD 416 (2001): - no pituitary and thyroid weight of parental animals recorded (- no functional investigations in offspring; only *recommended* endpoint in OECD 416)
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid, Batch: TB-960306; Purity 95.0%.

Test animals: Four groups of 28 male and 28 female of the CD strain Sprague Dawley rats, 215-291 g (males), 137-217 g (females), 7 weeks of age, from Charles River UK Limited, Margate Kent, England to form the F0 generation.

Test method: Based on a preliminary study [Report no. TON 013/980047], the animals received the test material by dietary administration at concentrations of 0, 25, 200 or 1600 ppm throughout two

generations. Both the F0 and F1 generation received the treated diet for a minimum of 10 weeks from selection throughout pairing, gestation and lactation.

All adult animals were subjected to a detailed necropsy and the reproductive organs and selected potential target organs were weighed and retained. Histopathological examinations were performed on tissues from control and high dose animals. Surplus F1 offspring and F2 offspring were killed on Day 25 of age and selected organs from one pup per sex per litter were weighed and retained in fixative.

Investigated endpoints (parental):
Clinical Signs
Mortality
Body weight: Males: Beginning of treatment and weekly thereafter. Females: Beginning of treatment and weekly thereafter; then Days 0, 7, 14, 20, 21 after mating and Days 1, 4, 7, 14 and 21 of lactation
Food consumption
Pre-coital interval (females only): time between initial pairing and detection of mating
Parturition and duration of gestation
Maternal behaviour
Macroscopic pathology
Oestrous cycles (F0, F1)
Mating performance and fertility: percentage mating, conception rate, fertility index, gestation index
Sperm numbers (F0 and F1 males): sperm motility, sperm count, sperm morphology, homogenisation-resistant spermatids
Organ weights: adrenals, brain, epididymides (L+R), kidneys, liver, ovaries with oviduct (L+R), prostate, seminal vesicles and coagulation glands, spleen, testes (L+R), thymus, uterus with cervix
Histopathology: animals from control and high dose group: abnormalities, adrenal glands, brain, epididymis (R), kidneys, liver, mammary glands caudal, ovaries with oviduct (L+R), pituitary, prostate, seminal vesicles and coagulation gland, spleen, testis (R), thymus, uterus, cervix, vagina

Investigated endpoints (offspring):
Mortality and Litter size: group mean litter size, survival indices
Clinical signs
Sex ratio
Sexual maturation of F1: vaginal opening, preputial separation
Organ weights: brain, liver, kidneys (L+R), spleen, thymus
Histopathology: abnormalities, adrenal glands, brain, epididymis (L+R), kidneys, liver, ovaries with oviduct (L+R), prostate, seminal vesicles and coagulation gland, spleen, testis (L+R), thymus, uterus, cervix, vagina

Results:
F0 generation

Mortality and clinical signs: There were no treatment-related clinical signs and mortalities.

Body weight gain and food consumption: There were no effects on body weight or body weight gain before pairing. At 1600 ppm, females showed a reduced body weight gain during gestation with recovery during the lactation phase. The food consumption was not adversely affected.

Pathological examinations:
Necropsy: Necropsy revealed no treatment related effects.

Organ weights: At 1600 ppm, increases in both absolute and body weight related liver weights and decreases in absolute and body weight related spleen weights in females were observed.

Histopathology and sperm evaluation: No treatment related effects were observed.

Reproduction and litter parameters (estrus cycles, pre-coital interval, mating, fertility, gestation length, parturition, litter size, sex ratio, survival of offspring): These parameters were not influenced by the administration of the test substance.

Table 3.10.1-1: Overview of toxicity in F0 rats

Parameter	Dose (ppm)							
	Male				Female			
	0	25	200	1600	0	25	200	1600
Achieved intake (mg/kg bw/day)								
- before pairing		2.7-1.3	22-11	170-85		2.9-1.8	24-14	181-117
- during gestation						2.1-1.7	17-14	132-112
- during lactation						2.5-4.3	24-38	180-295
Body weight (g)	not applicable							
- Day 0 gestation					310	303	305	299
- Day 7 gestation					345	337	337	331
- Day 14 gestation					381	368	368	366
- Day 20 gestation					459	441	445	433
Body weight gain (g)	not applicable							
- Weeks 0-7 gestation					35	34	33	31
- Weeks 0-14 gestation					71	65	63	66
- Weeks 0-20 gestation					149	138	141	133**
Body weight (g)	not applicable							
- Day 1 lactation					328	323	322	319
- Day 4 lactation					355	336	337	338
- Day 7 lactation					364	351	349	353
- Day 14 lactation					380	367	369	364
- Day 21 lactation					365	362	354	366
Absolute organ weights								
- Liver (% control)	100	98	98	113**	100	97	99	112**
- Spleen (% control)	100	97	95	93	100	95	94	95*
Relative organ weights								
- Liver (% control)	100	100	101	118**	100	99	102	112**
- Spleen (% control)	100	100	98	97	100	96	97	90**

* statistically different from control $P < 0.05$; ** $P < 0.01$

F1 generation

Mortality and clinical signs:
There were no treatment related clinical signs and mortalities. Sexual maturation was not influenced.

Body weight gain and food consumption: At 1600 ppm, body weight was significantly decreased at day 21 in male and female pups. At 1600 ppm, during the last week before weaning (Days 14 to 21), both sexes showed a slightly but statistically significantly reduced weight gain. Then, the pups had already access to the treated diet and chemical intake is expected to be at its highest in this period. From weaning until Week 10, the body weight gains were further lower at 1600 ppm. However, the control females gained relatively more weight than the treated females in this period and showed an unusual high body weight at Week 10 (background control data from 5 studies: 297 to 319 g, mean: 305 g, SD: 8.5 g). Thus, the body weights of all treated female groups appeared low and the lower body weight gain for females at the middle dose was considered to be due to this unusual high weight gain in control females. The divergence of the control group mean body weight from background control data continued during gestation (Day 20: 443 g, SD: 7 g (5 studies); concurrent control group 472 g, SD 42 g) and lactation phases (Day 21: 355 g, SD: 3 g (4 studies); concurrent control group 379 g, SD 33 g). Therefore, the body weights of females appeared to be low but they generally lay within the background control data.

The food consumption was not adversely affected.

Pathological examinations:
Necropsy: Necropsy of F1 pups that died before weaning revealed absence of milk in the stomach as the only consistent finding. Necropsy of F1 pups at 25 days of age revealed no evident changes that could be related to treatment. Necropsy of the F1 adults revealed no treatment related effects.

Organ weights: In female offspring, at 200 ppm, and in both sexes at 1600 ppm, liver weights were significantly increased. At the high dose, spleen weights were lower in both sexes. In the adults, at 1600 ppm, relative liver weights were increased. The absolute thymus weights were significantly decreased (compared to the control group at 200 ppm: 85% for females, 1600 ppm: 86 and 78% for males and females).

Histopathology and sperm evaluation: No treatment related effects were observed.

Reproduction and litter parameters (sexual maturation: vaginal opening and preputial separation, estrus cycles, pre-coital interval, mating, fertility, gestation length, parturition, litter size, sex ratio, survival of offspring): These parameters were not influenced by the administration of the test substance.

Table 3.10.1-2: Overview of toxicity in F1 rats

Parameter	**Dose (ppm)**							
	Male				**Female**			
	0	**25**	**200**	**1600**	**0**	**25**	**200**	**1600**
Achieved intake (mg/kg bw/day)								
- before pairing		4.3-1.5	34-12	291-97		4.7-1.9	36-16	303-123
- during gestation						2.0-1.8	17-14	138-121
- during lactation						2.4-4.5	18-37	139-312
Body weight offspring F1 (g)								
- Day 1	6.0	6.4	6.2	6.3	5.7	5.9	5.9	6.0

- Day 4 (before cull)	8.3	8.9	8.8	8.9	8.0	8.4	8.4	8.4
- Day 7	13.9	14.5	14.6	14.2	13.1	13.8	14.0	13.4
- Day 14	30.9	31.1	32.0	30.2	29.9	30.0	31.0	29.0
- Day 21	51.9	52.7	53.1	48.6*	50.1	50.3	51.3	46.7*
Body weight gain offspring F1 (g)								
- Days 1-4	2.2	2.6	2.5	2.4	2.1	2.5	2.5	2.3
- Days 1-7	7.7	8.1	8.4	7.7	7.3	7.9	8.1	7.4
- Days 1-14	24.7	24.8	25.7	23.7	24.0	24.1	25.0	23.0
- Days 1-21	45.8	46.3	46.8	42.2*	44.2	44.3	45.4	40.8*
Body weight parent F1 (g)								
- Week 0	92	88	94	86	84	80	86	81
- Week 1	146	143	151	138	128	122	130	123
- Week 2	211	204	211	198*	165	157	164	158
- Week 3	276	268	275	257**	196	186	190	185*
- Week 4	338	331	336	312**	224	212*	215	211*
- Week 5	390	381	382	356**	245	238	236	230*
- Week 6	429	421	419	392**	267	253	250*	247**
- Week 7	462	454	448	424**	283	270	268*	263**
- Week 8	491	476	471	447**	297	282	276*	
- Week 9	514	498	490	464**	315	295*	294*	285**
- Week 10	531	518	506	479**	328	305*	306*	295**
- Week 11	540	527	518	489**		-	-	-
- Week 12	556	546	534	507**	-	-	-	-
- Week 13	567	559	547	519**	-	-	-	-
- Week 14	585	577	561	530**	-	-	-	-
- Week 15	604	590	577	548**	-	-	-	-
- Week 16	617	604	592	557**	-	-	-	-
- Week 17	629	619	600	568**	-	-	-	-
- Week 18	637	627	608	573**	-	-	-	-
Body weight gain parent F1 (g)								
- Weeks 0-3	184	180	181	171*	112	107	104*	104*
- Weeks 0-10	440	430	413	393**	244	225	220*	214**
- Weeks 0-18	545	540	514	487**	-	-	-	-
Body weight F1 (g)	not applicable							
- Day 0 gestation					331	306**	309**	299**
- Day 7 gestation					362	337**	336**	326**
- Day 14 gestation					398	368**	368**	361**
- Day 20 gestation					472	441**	430**	432**

Body weight gain F1 (g)	not applicable							
- Weeks 0-7 gestation					31	31	28	27
- Weeks 0-14 gestation					67	63	59	62
- Weeks 0-20 gestation					141	136	122**	133**
Body weight F1 (g)	not applicable							
- Day 1 lactation					343	311*	314*	305**
- Day 4 lactation					362	337*	331**	318**
- Day 7 lactation					372	343**	339**	328**
- Day 14 lactation					388	361**	361**	349**
- Day 21 lactation					379	355*	362*	356*
Adults F1 Absolute organ weights								
- Liver (% control)	100	100	98	103	100	91	93	99
- Thymus (% control)	100	110	100	86*	100	87	85*	78**
Adults F1 Relative organ weights								
- Liver (% control)	100	101	104	115**	100	99	99	108*
- Thymus (% control)	100	112	105	97	100	93	89	85*
Offspring F1 absolute organ weights								
- Liver (% control)	100	103	107	110*	100	105	109*	112**
- Spleen weight (% control)	100	97	97	82**	100	103	106	87*
Offspring F1 relative organ weights								
- Liver (% control)	100	101	105	119**	100	104	106*	117**
- Spleen weight (% control)	100	96	97	89**	100	102	103	91*
Adults F0 Reproductive organs								
Absolute organ weights								
Epididymides weight (g)	1.456	1.379	1.448	1.402	-	-	-	-
Prostate (g)	0.789	0.806	0.791	0.803	-	-	-	-
Seminal vesicles (g)	2.71	2.67	2.70	2.60	-	-	-	-
Testes (g)	3.87	3.59	3.89	3.83	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.147	0.132	0.142	0.132
Uterus+cervix (g)	-	-	-	-	0.57	0.48*	0.51	0.49
Relative organ weights								
Epididymides weight (g)	0.2243	0.2181	0.2308	0.2261	-	-	-	-
Prostate (g)	0.1226	0.1281	0.1251	0.1296	-	-	-	-
Seminal vesicles (g)	0.416	0.422	0.433	0.420	-	-	-	-
Testes (g)	0.597	0.567	0.621	0.619	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.0419	0.0381	0.0420	0.0374 *

Uterus+cervix (g)	-	-	-	-	0.165	0.139*	0.149	0.140
Adults F1 Reproductive organs								
Absolute organ weights								
Epididymides weight (g)	1.405	1.328	1.401	1.366	-	-	-	-
Prostate (g)	0.733	0.690	0.713	0.659	-	-	-	-
Seminal vesicles (g)	2.39	2.39	2.54	2.33	-	-	-	-
Testes (g)	3.83	3.61*	3.84	3.77	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.144	0.125	0.135	0.117**
Uterus+cervix (g)	-	-	-	-	0.49	0.48	0.46	0.39**
Relative organ weights								
Epididymides weight (g)	0.2222	0.2157	0.2330	0.2432*	-	-	-	-
Prostate (g)	0.1155	0.1117	0.1185	0.1170	-	-	-	-
Seminal vesicles (g)	0.377	0.388	0.423*	0.414	-	-	-	-
Testes (g)	0.606	0.586	0.639	0.672**	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.0395	0.0367	0.0389	0.0348
Uterus+cervix (g)	-	-	-	-	0.135	0.139	0.133	0.115
Statistical significance: *p<0.05; **p<0.01								

F2 generation

Body weight gain and food consumption: At 1600 ppm, body weight was significantly decreased at day 21 in male and female pups. Furthermore, during the last week before weaning (Days 14 to 21), both sexes showed a slightly but statistically significantly reduced weight gain. Then, the pups had already access to the treated diet and chemical intake is expected to be at its highest in this period.

Pathological examinations:
Necropsy: Necropsy of F2 pups that died before weaning revealed absence of milk in the stomach as the only consistent finding. Necropsy of F2 pups at 25 days of age revealed no evident change that could be related to treatment.

Organ weights: At 200 ppm, the male pups and at 1600 ppm both sexes showed an increased relative liver weight.

Table 3.10.1-3: Overview of toxicity in F2 rats

Parameter	**Dose (ppm)**							
	Male				**Female**			
	0	**25**	**200**	**1600**	**0**	**25**	**200**	**1600**
Body weight offspring F2 (g)								
- Day 1	6.1	6.1	6.1	5.9	5.8	5.7	5.7	5.5
- Day 4 (before cull)	8.6	8.7	8.8	8.2	8.2	8.3	8.1	7.8

- Day 7	14.2	14.0	14.2	13.2	13.6	13.4	13.2	12.7
- Day 14	31.3	30.6	31.0	29.2	30.0	29.5	29.4	27.9
- Day 21	53.2	51.3	51.6	47.0**	50.9	49.1	49.1	45.0**
Body weight gain offspring F2 (g)								
- Days 1-4	2.5	2.6	2.5	2.3	2.4	2.4	2.4	2.4
- Days 1-7	8.0	7.9	7.9	7.3	7.7	7.6	7.4	7.2
- Days 1-14	25.1	24.4	24.8	23.3	24.1	23.6	23.6	22.4
- Days 1-21	47.0	45.2	45.3	41.2**	45.1	43.2	43.3	39.5**
Absolute liver weight (% control)	100	99	103	108	100	95	96	103
Relative liver weight (% control)	100	101	106*	119**	100	99	103	116**
Statistical significance: *p<0.05; **p<0.01								

Conclusion: Up to 1600 ppm (85 mg/kg bw/d), pethoxamid had no adverse effects upon reproductive performance of rats through two successive generations. Because of the slightly reduced body weight gain of the pups (F1, F2) at the end of the lactation phase, the NOAEL (NOEL) for offspring toxicity is considered to be 200 ppm (equivalent to 11 mg/kg bw/d for the F0- and F1-generation). This dose is also considered to be a NOAEL for parental toxicity, based on decreased body weight gain during gestation and increased liver weight and decreased weight of thymus and spleen.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (adverse effects on sexual function and fertility) is not required.

3.10.1.3 Anonymous (1996)

Reference: TKC-94 : Preliminary Study of Embryo-foetal Toxicity in the CD Rat by Oral Gavage Administration
Author(s), year: Anonymous, 1996
Report/Doc. number: 86 PXA (TOX2001-299) / TNP001/0116
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001): Limited number (six) of animals per group; Shorter administration period (day 6 to 15); Limited parameters retrieved (no skeletal or soft tissue examination of foetuses done)
Acceptability: Yes (limited information); range finding study

Material and Methods:
Test material: Pethoxamid; Batch: TB-951005; Purity: 95.0%.

Test animals: Four groups each of 6 pregnant female Crl:CD rats, weighing from 213 to 263 g (approx. 10 to 11 weeks of age).

Test method: The rats received the test compound at dosages of 0, 8, 80, 400 or 800 mg/kg bw/d by oral gavage from Day 6 to 15 of gestation.

Summarized findings: At 800 mg/kg bw/d, adverse substance related maternal toxicity was observed, as mortality (Day 9 and 10: 2 dams died and 1 killing *in extremis*, respectively) and reduced body weight

gain. The dams which survived showed a general condition similar to the control group. All animals of the high dose group were pregnant. Apart from salivation which was observed at 80, 400 and 800 mg/kg bw/d, no treatment related effects were observed for animals receiving 8, 80 and 400 mg/kg bw/d.

No adverse effects on the litter responses and foetal development were observed.

Conclusion: A dosage between 400 and 800 mg/kg bw/d was concluded suitable as the highest dose in the main study.

3.10.1.4 Anonymous (1997a)

Reference: TKC-94 : Study of Embryo-foetal Toxicity in the CD Rat by Oral Gavage Administration
Author(s), year: Anonymous, 1997a
Report/Doc. number: 87 PXA (TOX2001-300)/ TNP002/0696
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001): - Shorter administration period (day 6 to 15 of gestation); - 40% (10/25) of the dams of the high dose group died.
Acceptability: Yes, number of animals (15) in the high dose is slightly lower than the lowest number of animals (16) to be achieved according to OECD 414

Material and Methods:

Test material: Pethoxamid; Batch: TB-951005; Purity: 95.0%.

Test animals: Four groups each of 25 pregnant female Crl:CD rats, weighing from 245 to 298 g (approx. 11 to 12 weeks of age).

Test method: From a preliminary study [Report no. 96/TNP001/0116], it was concluded that the highest dose considered suitable should be between 400 and 800 mg/kg bw/d. Thus, the rats received the test compound at dosages of 0, 8, 80 or 600 mg/kg bw/d by oral gavage from Day 6 to 15 of gestation.

Results:

Maternal responses
Mortality and clinical signs: At 600 mg/kg bw/d, 10 early decedents were recorded. The animals were killed after showing a significant adverse reaction following dose administration. Signs prior killing included piloerection, unresponsiveness to stimuli and hunched posture. In all animals of the high dose group, salivation was observed after all dose administrations. Apart from that, the surviving animals showed no treatment related effects. Post-dosing salivation was also seen at some occasions following the administration of 80 mg/kg bw/d.

Bodyweight gain and food consumption: At 600 mg/kg bw/d, the body weight gain was essentially static during the first three days of treatment and was lower at Day 9 and 10 when compared to the control group. The body weight gain increased after cessation of treatment. This was not accompanied by changes in food consumption. Some decedents showed overnight weight loss.
Pathological examination: The majority of the early decedents exhibited disturbances to the gastrointestinal tract as devoid of or with reduced content and pale appearance to the liver. All dams were confirmed as pregnant.

The surviving animals did not show treatment-related effects.

Litter responses: No treatment-related findings were recorded for the number of implantations and viable young, extent of pre-and post-implantation losses, foetal and placental weights and foetal anogenital distance.

No treatment-related effects were recorded at macroscopic, visceral and skeletal examinations.

Table 3.10.1-4: Results in the rat teratogenicity study

	Dose level (mg/kg bw/day)			
	0	**8**	**80**	**600**
Number of Dams	25	25	25	25
Number surviving to Day 20 with live litters	25	24	25	15
Killed *in extremis*	0	0	0	9 (8,9,10,11,14,17)[1]
Found dead	0	0	0	1 (11)
Not pregnant	0	1	0	0
Body weight gain Days 6-9 gestation (g)	11	13	14	4**
Body weight gain Days 6-15 gestation (g)	48	51	53	48
Body weight gain Days 6-20 gestation (g)	120	131	129	125
[1] (day of death, days 9, 10 and 11 2 animals killed *in extremis*) Statistical significance: **p<0.01				

Conclusion: Significant maternal toxicity was seen at 600 mg/kg bw/d. The dose of 8 mg/kg bw/d is considered to be the maternal NOAEL, based on the salivation which occurred from 80 mg/kg bw/d onwards. The NOAEL (NOEL) for developmental toxicity was at 600 mg/kg bw/d.

Although more than 10% of the dams died at the high dose, the study is considered to be acceptable because a high number of dams (15) were available for the investigations at the end of the study, and there was no evidence of developmental toxicity. In the preliminary study, at 400 mg/kg bw/d, no substance related effects (apart from salivation) were observed on the dams and up to the highest dose (800 mg/kg bw/d), no developmental toxicity was seen.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.1.5 Anonymous (2014a)

Reference: A developmental toxicity study of pethoxamid by oral (gavage) administration in rats
Author(s), year: Anonymous, 2014
Report/Doc. number: 1138 PXA / 20039155
Guideline(s): OECD 414 (2001)
GLP: Yes
Deviations from OECD 414 (2001): -
Acceptability: Yes

Material and Methods:

Test material: Pethoxamid technical
Lot/batch number: P1351-JaK-T2-23-6

Purity: 95.80% (w/w) (dose calculation was not adjusted to purity)
Stability of test item: 06 January 2014 (stored at ambient temperature). *NB: stable during the conduct of the study*
Storage conditions: At room temperature, protected from light

Test method: From the results of a previously conducted study performed at Huntingdon Life Sciences Ltd., UK, [Report No. 96/TNP002/0695 02.12.96], it was concluded that the highest dose considered suitable should be between 400 and 600 mg/kg bw/day due to mortality observed at 600 mg/kg bw/day.

Pregnant female Crl:CD(SD) rats (25 or 30/group) were orally administered pethoxamid or the vehicle control (1% (w/v) methylcellulose (400 cP) and 0.5% (v/v) Tween® 80 in reverse osmosis membrane-processed deionized water) once daily by oral gavage on gestation days 6 through 20 (GD 6- 20) at dose levels of 0, 10, 75 or 500 mg/kg bw/day. The dosing volume was 5 mL/kg bw. There were 25 rats per group, except at the high dose which contained 30 rats.

Due to mortality and/or adverse clinical signs of toxicity in 7 rats at the 500 mg/kg bw/day dose level within the first 3 to 10 days of dosing, the high-dose level was reduced to 350 mg/kg bw/day. Mortality occurred in 3 additional rats at the 350 mg/kg bw/day dose level, which resulted in a subsequent reduction of the dose level to 250 mg/kg bw/day. There were 2 additional deaths in the 250 mg/kg bw/day dose group that occurred on GD 21 after the last dose on GD 20.

The following parameters and end points were evaluated in this study: viability, clinical signs, body weights, food consumption, necropsy observations, uterine contents (including uterine weights) and foetal external, visceral, and skeletal alterations.

Results:

Mortality: At 500/350/250 mg/kg bw/day, there was an increase or a statistically significant increase in the total number of female rats that were either found dead or euthanized due to adverse clinical signs during the study. There was also one female rat in the vehicle control group that was euthanized due to an injury to the left hind paw. Common clinical signs in these rats included effects on gait (e.g., ataxia; low carriage; decreased motor activity; lost righting reflex), respiratory effects (e.g. hyperpnea, bradypnea, dyspnea, open-mouthed breathing, gasping), and additional adverse clinical signs (e.g. discolored faeces [light brown], paleness in the ears, eyes and extremities, hunched posture, ungroomed coat, coldness to the touch). All other female rats survived until scheduled euthanasia.

Clinical signs: There was a statistically significant increase in the number of rats in the 500/350/250 mg/kg bw/day dose group (21 to 26) observed with hunched posture; light brown faeces; mild dehydration (based on skin turgor); and the total number of rats observed with dehydration (mild, moderate and/or severe). In the 500/350/250 mg/kg bw/day dose group, there was also a statistically significant increase in the number of rats (4 to 9 per group) observed with moderate dehydration; slightly pale and/or pale ears; ungroomed coat; ptosis; thin body condition; urine staining; slight excess salivation; decreased motor activity; pale extremities (both forelimbs, both forepaws, both hind limbs and/or both hind paws); coldness to the touch; scant faeces; and ataxia. There was also an increase (1 to 3 per group) in the number of rats in the 500/350/250 mg/kg bw/day dose group observed with impaired righting reflex; bradypnea; hyperpnea; paleness in the body and/or whole body; red perivaginal area fur and/or brown-yellow perioral fur; soft or liquid faeces; urine-stained abdominal fur; low carriage; brown and dried perioral substance; open mouth breathing; splayed hind limbs; prostate; vocalization in the home cage; pale eyes; abdominal distention; red substance in the cage; active vaginal bleeding; dyspnea; gasping; tachypnea; and piloerection.

Body weight: Body weights were reduced or statistically significantly reduced in the 500/350/250 mg/kg bw/day dose group at all intervals during the dose period (GDs 6 through 21). In the 500/350/250

mg/kg bw/day dose group, there was a statistically significant reduction in body weight gain observed for the entire dose period (calculated as GDs 6 to 21) as well as the overall study period (calculated as GDs 0 to 21). Reductions or statistically significant reductions in body weight gains and/or statistically significant body weight losses were observed at all tabulated intervals (with the exception of GDs 12 to 15) in the 500/350/250 mg/kg bw/day dose group. Uterine weights in the 500/350/250 mg/kg bw/day dose group were slightly reduced (8%) in comparison with the vehicle control group value.

Food consumption: Correlating with concurrent reductions in body weight gains and body weight losses, mean absolute and relative food consumption values at 500/350/250 mg/kg bw/day were reduced. In this dose group, mean absolute and relative food consumption values were reduced or significantly reduced after the initiation of dose administration.

Maternal necropsy: There was a statistically significant reduction in the number of rats in the 500/350/250 mg/kg bw/day dose group that appeared normal at the time of necropsy. There was also a statistically significant increase in the number of rats in the 500/350/250 mg/kg bw/day dose group observed with all lobes of the liver mottled, tan, red, dark red and/or brown. In this dose group, there were also one or two rats observed with numerous pitted areas on the liver; all lobes of the lungs spongy, pale and/or dark red; intestines distended with gas; and/or small spleen.

Caesarean-sectioning and litter observations: Pregnancy occurred in 25 (100%), 23 (92.0%), 25 (100%) and 29 (96.7%) rats in Groups 1 through 4, respectively. Due to the previously described mortality, Caesarean sectioning observations on GD 21 were based on 24, 23, 25 and 17 pregnant rats in the 0 (Vehicle Control), 10, 75 and 500/350/250 mg/kg bw/day dose groups, respectively. Although more than 10% mortality of the dams occurred in the 500/350/250 mg/kg bw/day dose group, the study was considered to be acceptable because a sufficiently high number of dams (n=17) were available for the investigations at the end of the study.

A statistically significant reduction in foetal body weights (male, female and combined) occurred in the 500/350/250 mg/kg bw/day dose group. No additional test substance-related Caesarean-sectioning or litter parameters were affected by doses of the test substance as high as 500/350/250 mg/kg bw/day.

In the 10 mg/kg bw/day dose group, there was a statistically significant increase in the litter averages for resorptions, early resorptions, postimplantation loss, dams with any resorptions and percent resorbed conceptuses per litter in comparison with the vehicle control group values. These values were not considered to be test substance-related because they were not dose-dependent. There was also a slight increase in the percent postimplantation loss observed in the 500/350/250 mg/kg bw/day dose group at the time of Caesareansectioning; however, this value (7.4%) was primarily the result of one dam that had all resorbed conceptuses. When this dam was removed from the summary, the percent postimplantation loss changed from 7.4% to 1.6%, which was comparable with the vehicle control group value (2.8%). The litter averages for corpora lutea, implantations, preimplantation loss, litter sizes, live foetuses and percent live male foetuses were comparable among the four dose groups.

Foetal alterations: Foetal alterations were defined as: 1) malformations (irreversible changes that occur at low incidences in this species and strain); or 2) variations (common findings in this species and strain and reversible delays or accelerations in development). Litter averages were calculated for specific foetal ossification sites as part of the evaluation of the degree of foetal ossification.

No foetal gross external, soft tissue or skeletal alterations (malformations or variations) were considered to be test substance-related.

Table 3.10.1-5: Findings in the rat teratogenicity study

Parameter	Dose level (mg/kg/day)

	0	**10**	**75**	**500/350/250**
Dam data				
Number of animals	25	25	25	30
Surviving to Day 21 with live litters	24	23	25	17
Killed *in extremis*	1	0	0	4
Found dead	0	0	0	8**
Not pregnant	0	2	0	1
Mean body weight gain (g)				
Days 6-9	11.0	12.1	13.8	-6.6**
Days 6-21	146.4	146.4	152.3	104.8**
Days 0-21	175.9	175.1	182.9	133.6**
Mean corrected[1] body weight gain (g)				
Days 6-21	41.3	44.0	45.0	12.5**
Days 0-21	70.8	72.7	75.6	41.3**
Mean food consumption (g/day)				
Days 6-21	23.5	23.1	23.7	19.5**
Days 0-21	22.6	22.3	22.8	19.8**
Litter responses				
% Postimplantation loss	2.8	6.5*	2.9	7.4
Foetal evaluation				
Foetal body weight (g)	5.65	5.56	5.58	5.08**
Foetal body weight – male (g)	5.81	5.70	5.71	5.18**
Foetal body weight – female (g)	5.48	5.42	5.44	4.96**
Foetal abnormalities	None	None	None	None

*p<0.05; **p<0.01

[1] Corrected body weight gain = gestation day 21 body weight minus the gravid uterine weight

Conclusion: The maternal NOAEL for pethoxamid is 75 mg/kg bw/day. Mortality, clinical signs, reductions in body weight, body weight gain and/or body weight losses and reductions in absolute and/or relative feed consumption values occurred in the 500/350/250 mg/kg bw/day dose group.

The developmental NOAEL is also 75 mg/kg bw/day. Reductions in gravid uterine weights and foetal body weights occurred in the 500/350/250 mg/kg bw/day dose group.

Pethoxamid is not a selective developmental toxicant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.1.6 Anonymous (1997b)

Reference: TKC-94 : Study of Tolerance in the Rabbit by Oral Gavage Administration
Author(s), year: Anonymous, 1997
Report/Doc. number: 89 PXA (TOX2001-301) / TON008/970270
Guideline(s): Not applicable
GLP: Yes
Acceptability: Yes (tolerance study)

Material and Methods:

Test material: Pethoxamid; Batch: TB-960306; Purity: 95.0%.

Test animals: Non-pregnant female New Zealand White rabbits (2 for the staircase phase weighing 3.92 and 3.98 kg and 2 for the constant dosage study weighing 4.49 and 4.64 kg) from an accredited closed colony, Froxfield Farms UK Limited, Hampshire, England.

Test method:

Staircase phase: Pethoxamid was administered by oral gavage to 2 rabbits, beginning with 50 mg/kg bw/d for 2 days. In the absence of any adverse response, the doses were doubled every two days (until Day 10, second day of dosing at 800 mg/kg bw/d).

Constant dosage study: Two females received 300 mg/kg bw/d for 7 consecutive days from Day 6 of gestation. Thereafter animals were killed and examined for reactions to the treatment.

Results:
Staircase phase: At 800 mg/kg bw/d, adverse toxicity occurred.

Constant dosage study: Body weight loss was recorded in both animals at 300 mg/kg bw/d. No other abnormalities were detected. Only one female was pregnant.

Conclusion: It was concluded that the highest dose in a preliminary teratogenicity study on rabbits should be at or less than 300 mg/kg bw/d.

3.10.1.7 Anonymous (1998b)

Reference: TKC-94 : Preliminary Embryo-foetal Toxicity Study in the Rabbit by Oral Gavage Administration
Author(s), year: Anonymous, 1998
Report/Doc. number: 90 PXA (TOX2001-302) / TON009/972410
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001):
- Limited number (4) of animals per group
- Shorter administration period (day 6 to 19)
- Limited parameters retrieved (no skeletal or soft tissue examination of foetuses assessed, no weight of gravid uteri recorded)

Acceptability: Yes (limited information); range finding study

Material and Methods:

Test material: Pethoxamid; Batch: TB-960306; Purity: 95.1%.

Test animals: Four groups of 4 pregnant female New Zealand White rabbits, 3.3-4.3 kg of weight and 18-26 weeks old, from an accredited closed colony, Froxfield Farms UK Limited, Hampshire, England.

Test method: The rabbits received the test compound at doses of 0, 30, 100 or 300 mg/kg bw/d, administered by oral gavage from Day 6 to 19 of gestation.

Results: The general condition was unaffected by the treatment. There were no death or treatment related pregnancy failures. The dams of the high dose group exhibited body weight loss and reduced food intake. Macroscopic examination on Day 29 of gestation revealed no effects related to treatment.

Conclusion: It was concluded that the highest dose in the main study should be between 100 and 300 mg/kg bw/d.

3.10.1.8 Anonymous (1998c)

Reference: TKC-94 : Study of Embryo-foetal Toxicity in the Rabbit by Oral Gavage Administration
Author(s), year: Anonymous, 1998
Report/Doc. number: 88 PXA (TOX2001-303) / TON012/982289
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001): Shorter administration period (day 6 to 19 of gestation)
Acceptability: Yes

Material and Methods:

Test material: Pethoxamid; Batch: TB-960306; Purity: 95.1%.

Test animals: Four groups of 20 pregnant female New Zealand White rabbits, 3,1-4,9 kg of weight and 18-26 weeks old, from an accredited closed colony, Charles River UK Limited, Margate, Kent, England.

Test method: Based on the effects observed in preliminary studies [Report no. TON008/970270 and TON009/972410], the rabbits received the test compound at doses of 0, 12.5, 50 or 200 mg/kg bw/d, administered by oral gavage from Day 6 to 19 of gestation. To ensure there were sufficient numbers of litters available for foetal pathology examination, a second set of four groups each of 5 pregnant rabbits were given pethoxamid at the same dosages and underwent the same procedures as conducted for Groups 1 to 4. These animals were not needed for the investigations.

Results:

Maternal reponses
General condition: One female receiving 50 mg/kg bw/d was killed for humane reasons on Day 23 of gestation following significant body weight loss. At necropsy, findings revealed a deflated lung and clear fluid within the thoracic cavity; this death was not considered to be related to treatment. One female in each of the groups receiving 12.5 or 200 mg/kg bw/d aborted; these abortions were not considered to be treatment-related as two females in the control group also aborted at about the same stage of pregnancy.

Body weight gain and food consumption: During the second half of treatment, at 50 mg/kg bw/d a stasis and at 200 mg/kg bw/d a stasis followed by a reduction in body weight gain were observed. After termination of the treatment, the body weight gain rapidly increased in both groups.

During the second half of the treatment period, the food consumption was slightly lower at 50 mg/kg bw/d and markedly lower at 200 mg/kg bw/d (approximately 76% of the control value). Once treatment had ended, diet intake recovered and was greater than for the control group at the end of the gestation period.

Pathological examination: No treatment-related findings were observed.

Litter responses: No treatment-related findings were recorded for the litter responses as assessed by the number of implantations and viable young, extent of pre- and post-implantation losses, final live litter size, foetal and placental weights.

No treatment-related findings were recorded at macroscopic, visceral and skeletal examinations.

Table 3.10.1-6: Data for dams in the rabbit teratogenicity study

Doses (mg/kg bw/d)	**0**	**12.5**	**50**	**200**
Number of dams	20	20	20	20
Number surviving to Day 29 with live litters	15	17	16	15
Number with only resorptions *in utero*	0	0	1	1
Not pregnant	3	2	2	3
Number aborting	2	1	0	1
Body weight gain (kg) Days 14-20 of gestation	0.08	0.08	0.04	-0.01
Food intake (g/rabbit/day) Days 13-19 of gestation	147	144	132	111

Conclusion: At 200 mg/kg bw/d and 50 mg/kg bw/d (the latter not that pronounced, hence not considered adverse), a reduced body weight gain and a lower food intake was observed during the treatment period. Therefore, the dose of 50 mg/kg bw/d is considered to be the maternal NOAEL.

No dose was associated with adverse effects on *in utero* survival or embryo-foetal development; thus the NOAEL (NOEL) for developmental toxicity is at 200 mg/kg bw/d.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.1.9 Anonymous (2014b)

Reference: A developmental toxicity study of pethoxamid by oral (gavage) administration in rabbits
Author(s), year: Anonymous, 2014
Report/Doc. number: 1139 PXA / Study No. 20039156
Guideline(s): OECD Guideline 414 (2001)
GLP: Yes
Deviations from OECD 414 (2001): -
Acceptability: Yes

Material and methods:

Test material: Pethoxamid technical
Lot/batch number: P1351-JaK-T2-23-6
Purity: 95.80% (w/w) (dose calculation was not adjusted to purity)

Stability of test item: 06 January 2014 (stored at ambient temperature). *NB: stable during the conduct of the study*
Storage conditions: At room temperature, protected from light

Test method: In a previously conducted study performed at Huntingdon Life Sciences Ltd., UK, [Report No. TON012/982289 October 5, 1998], a dose level of 200 mg/kg bw/day resulted in decreased body weights and reduced food consumption. No effects were observed on the foetuses. Therefore, four groups of pregnant female New Zealand white female rabbits received the test compound by gavage at daily dose levels of 0, 12.5, 50 or 200 mg/kg bw/day from Day 6 to 28 of gestation mating. There were 25 rabbits/group.

The vehicle control substance was 1% (w/v) methylcellulose (400 cP) and 0.5% (v/v) Tween® 80 in Reverse osmosis membrane-processed deionized water. The dose volume was 5 mL/kg.

The following parameters and end points were evaluated in this study: viability, maternal clinical signs, maternal body weights, maternal body weight changes, maternal food consumption, maternal gross observations, gravid uterine weights, ovarian and uterine examinations, foetal sex, foetal body weights and foetal external, visceral and skeletal morphology.

Results:

Mortality: At 200 mg/kg bw/day, there was a statistically significant increase in the number of rabbits that aborted (four rabbits). Rabbits observed to abort were subsequently euthanized during the study. There was also an additional rabbit in the 200 mg/kg bw/day dose group that delivered on GD 29 and was subsequently euthanized. Common clinical signs in the rabbits observed to abort included: scant faeces, ungroomed coat, thin body condition, mild dehydration and red substance in the cage pan. All other females survived until scheduled euthanasia.

Clinical signs: There was a statistically significant increase in the number of does in the 200 mg/kg bw/day dose group observed with scant faeces during the dose period. In the 200 mg/kg bw/day dose group, there was an increase or statistically significant increase in the number of does (3 to 6 per category) observed with thin body condition; ungroomed coat; red substance in the cage pan; red substance on the fur of the lower midline or perivaginal area; and the total amount of sparse hair coat observed on study (on the back and the underside). There was also an increase in the number of does (2) observed with no faeces in the cage pan and mild dehydration (based on skin turgor).

Body weights: Maternal body weights were statistically significantly reduced in the 200 mg/kg bw/day dose group beginning on GD 15 and continuing for the remainder of the dose period. In the 200 mg/kg bw/day dose group, there was also a statistically significant reduction in body weight gain observed for the entire dose period as well as the overall study period. Reductions or statistically significant reductions in body weight gains, reductions in body weight losses or statistically significant body weight losses were observed at all tabulated intervals in the 200 mg/kg bw/day dose group. Although not statistically significant, uterine weights in the 200 mg/kg bw/day dose group were slightly reduced (88% of the vehicle control group value). Corrected body weights (body weight on GD 29 minus the gravid uterine weight) were statistically significantly reduced in the 200 mg/kg bw/day dose group. Corrected mean body weight gains (body weights on GDs 6 to 29 and 0 to 29 minus the gravid uterine weight) were also statistically significantly reduced in the 200 mg/kg bw/day dose group.

Food consumption: Correlating with concurrent reductions in body weight gains and body weight losses, mean absolute and relative food consumption values at 200 mg/kg bw/day were also reduced. In the 200 mg/kg bw/day dose group, mean absolute food consumption values were reduced or significantly reduced at all intervals after the initiation of dose administration. The relative food

consumption values were also decreased or statistically significantly decreased at all intervals during the dose period.

Maternal necropsy: There were no necropsy findings that were considered to be treatement related.

Caesarean-sectioning and litter observations: Pregnancy occurred in 22 (88.0%), 25 (100%), 23 (92.0%) and 21 (84.0%) does in the 0, 12.5, 50 and 200 mg/kg bw/day dose groups, respectively. Reflecting the four does that aborted and were subsequently euthanized and the one doe that delivered early at 200 mg/kg bw/day described previously, Caesarean-sectioning observations were based on 22, 25, 23 and 16 pregnant rabbits in Groups 1 through 4, respectively. Average foetal body weights (total, male and female) were statistically significantly reduced in the 200 mg/kg bw/day dose group.

No other Caesarean-sectioning or litter parameters were affected by doses of pethoxamid as high as 200 mg/kg bw/day. The litter averages for corpora lutea, implantations, preimplantation loss, litter sizes, live foetuses, early resorptions, late resorptions, postimplantation loss and percent male foetuses were comparable among the four dose groups. All placentae appeared normal.

Foetal alterations: Foetal alterations were defined as: 1) malformations (irreversible changes that occur at low incidences in this species and strain); or 2) variations (common findings in this species and strain and reversible delays or accelerations in development). Litter averages were calculated for specific foetal ossification sites as part of the evaluation of the degree of foetal ossification.

The 200 mg/kg bw/day dose group had statistically significant increases in the incidence of supernumerary thoracic ribs with associated statistically significant increases and decreases in the numbers of thoracic and lumbar vertebrae, respectively, a common variation observed at maternally toxic doses. Similar findings were observed in the 50 mg/kg bw/day dose group; the incidences were within historical control values but still considered to be the starting point of effects, being more severe at the high dose.

No additional foetal gross external, soft tissue or skeletal alterations (malformations or variations) were considered to be test substance related.

No additional gross external, soft tissue or skeletal alterations (malformations or variations) were considered to be test substance-related.

Table 3.10.1-7: Findings in the rabbit teratogenicity study

Parameter	Dose Level (mg/kg bw/day)			
	0	**12.5**	**50**	**200**
Dam Data				
Number of animals	25	25	25	25
Aborted and euthanized	0	0	0	4**
Delivered and euthanized	0	0	0	1
Number found dead	0	0	0	0
Number pregnant	22	25	23	25[1]
Clinical signs	-	-	-	scant feces, ungroomed coat, thin body condition, mild dehydration, sparse

				hair coat and red substance in the cage pan and on the fur
Body weight gain				
Mean (kg) Days 9-12	0.05	0.05	0.04	0.00**
Mean (kg) Days 6-29	0.35	0.33	0.38	0.09**
Mean k(g) Days 0-29	0.48	0.46	0.48	0.20**
Corrected body weight gain				
Mean (g) Days 6-21	-0.20	-0.19	-0.16	-0.40**
Mean (g) Days 0-21	-0.07	-0.07	-0.05	-0.28**
Food consumption				
Mean (g/day) Days 6-29	142.9	137.9	148.7	101.9**
Mean (g/kg/day) Days 6-29	37.5	36.5	39.4	27.9**
Litter responses	No treatment-related effects			
Foetal evaluation				
Foetal body weights (g) Total	44.05	42.42	44.11	35.81**
Males	44.98	43.29	45.15	36.43**
Females	42.81	41.80	43.49	35.09**
Foetal ossification sites/foetus/litter				
Vertebrae:				
Thoracic (±SD)				
MIN	12.43 ±0.30	12.55 ±0.27	12.63*[2] ±0.31	12.70** ±0.27
MAX	12	12	12	12
Lumbar (±SD)	13	13	13	13
MIN	6.56 ±0.31	6.44 ±0.27	6.35*[2] ±0.31	6.30** ±0.27
MAX	6	6	6	6
	7	7	7	7
Ribs (pairs) (±SD)	12.38 ±0.30	12.45 ±0.25	12.54 ±0.30	12.64** ±0.28
MIN	12	12	12	12
MAX	13	13	13	13

[1]Inclduing the does that aborted or delivered early and were euthanized.

[2]Incidence within historical control values: vertebrae thoracic range 12.40 – 12.66; vertebrae lumbar 6.34 – 6.60; ribs (pairs) 12.34 – 12.58.

*p<0.05; **p<0.01.

CONCLUSION: The maternal NOAEL for pethoxamid is 50 mg/kg bw/day. Significant maternal toxicity was seen at the highest dose level of 200 mg/kg bw/day. At this dose level, 4 rabbits aborted, and clinical signs, reduced body weight gain and reduced food consumption were observed.

The developmental NOEL is 12.5 mg/kg bw/day. In the 200 mg/kg dose group, foetuses showed reductions in foetal body weight and statistically significant increases in the incidence of supernumerary thoracic ribs with associated statistically significant increases and decreases in the numbers of thoracic and lumbar vertebrae, resp., a common variation observed at maternally toxic doses.

The developmental NOAEL is 12.5 mg/kg bw/day. In the 200 mg/kg dose group, fetuses showed reductions in fetal body weight and statistically significant increases in the numbers of ossified thoracic ribs with associated statistically significant increases and decreases ($p \leq 0.01$) in the numbers of ossified thoracic and lumbar vertebrae, resp., a common variation observed at maternally toxic doses. In the 50 mg/kg bw/day dose group, there were statistically significant increases and decreases ($p \leq 0.05$) in the numbers of ossified thoracic and lumbar vertebrae, respectively. Though the effects at 50 mg/kg were still within the historical control data of the Testing Facility, 50 mg/kg were considered to be the starting point of effects, being more severe at the high dose.

Pethoxamid is not a selective developmental toxicant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.2 Human data

No relevant studies.

3.10.3 Other data (e.g. studies on mechanism of action)

No relevant studies.

3.11 Specific target organ toxicity – single exposure

3.11.1 Animal data

3.11.1.1 Anonymous (2014c)

Reference: An acute neurotoxicity study of Pethoxamid by oral gavage in rats.
Author(s), year: Anonymous, 2014
Report/Doc. number: 1137 PXA / Testing Facility Study No. 20039155
Guideline(s): OECD Guideline 424 (1997)
GLP: Yes
Deviations from OECD 486 (1997): No
Acceptability: Yes

EXECUTIVE SUMMARY

The overall objective of this study was to evaluate the potential of pethoxamid to cause neurotoxic effects in Crl:CD(SD) rats after a single oral exposure. The study contains two parts—Part A, range-finding study and Part B, definitive acute neurotoxicity study. The objectives of Part A of the study were as follows: 1) to determine the high dose to be tested in the definitive acute neurotoxicity study (Part B); and 2) to establish the appropriate time point based on clinical signs of toxicity for conducting Functional Observational Battery (FOB) and motor activity evaluations in the definitive study. The objective of Part B of this study was to perform an overall neurotoxicologic evaluation of the rats after acute oral exposure to pethoxamid, including FOB and motor activity assessments, at the time-of-peak effect and a neuropathologic examination centered on the central and peripheral nervous system.

In Part A, male and female rats (5/sex/group) were administered pethoxamid or the vehicle control substance once by oral gavage at dose levels of 0, 600, and 800 mg/kg bw. The vehicle used was 1% methylcellulose and 0.5% Tween® 80 in reverse osmosis deionized water. The dose volume was 5 mL/kg. For Part A of the study, the following parameters and end points were evaluated: viability, clinical signs including detailed clinical observations, body weights, food consumption, and necropsy observations.

No mortality was observed in Part A of the study. In the 800 mg/kg bw dose group, one male rat was observed with decreased motor activity, ptosis, pale right and left ears, mild and/or moderate dehydration, bradypnea and thin body condition. Body weight loss was observed in the 600 and 800 mg/kg bw dose groups in both sexes on Days 1 to 2, which was statistically significant in males at the high dose and in females at the mid and high dose compared with the vehicle control values. There was also a statistically significant reduction in absolute and relative food consumption values observed in male and female rats at 600 and 800 mg/kg bw on the day following dose administration compared with the vehicle control values. In the male rats at 800 mg/kg bw, absolute and relative food consumption values remained reduced on Days 2 to 3.

In the male and female rats, detailed clinical signs at 600 and 800 mg/kg bw that were considered to be related to the test substance included the following: hunched posture, vocalization to the touch, chromorhinorrhea, pale ears, red urine, coldness to the touch, ptosis, mild or moderate dehydration and whole body tremors. These detailed clinical signs were most apparent between the 12- and 16-hour time points; some clinical signs remained apparent at the 24-hour time point. Based on these data, 16 hours was selected as the time-of-peak effect (TOPE) for evaluating FOB and motor activity for the definitive acute neurotoxicity study.

In Part B, male and female rats (10/sex/group) were administered pethoxamid or the vehicle control substance once by oral gavage on Day 1 at dose levels of 0, 100, 300, or 800 mg/kg bw. The vehicle used was 1% methylcellulose and 0.5% Tween® 80 in reverse osmosis deionized water. The dose volume was 5 mL/kg. The following parameters and end points were evaluated: viability, clinical signs, body weights, food consumption, necropsy observations and brain weights. An FOB, followed by motor activity evaluation was performed on all rats prior to dose administration, on the day of dose administration at the estimated TOPE, 7 days after dose administration and 14 days after dose administration. Neurohistopathological examinations were performed on selected tissues (central and peripheral nervous system, skeletal muscles and eyes with retinas and optic nerves) from 5 rats per sex from the vehicle control and the 800 mg/kg bw.

Two female rats in the 800 mg/kg bw dose group were found dead on either Days 2 or 3. The death of these rats was attributed to the test substance. The adverse clinical signs observed in these rats included the following: decreased motor activity; ptosis; mild and moderate dehydration; hunched posture; pale right and left ears and extremities; bradypnea; scant feces; and ungroomed coat.

A decrease or statistically significant decrease in body weight gain occurred in male and female rats at 300 mg/kg bw on Days 1 to 2 compared with vehicle control values. In the male and female rats at 800 mg/kg bw, a statistically significant loss in body weight occurred on Days 1 to 2. There was also a decrease or statistically significant decrease in absolute and relative food consumption values observed in male and female rats at 300 and 800 mg/kg bw on Days 1 to 2.

Adverse clinical signs during the FOB evaluation (i.e., hunched posture, bradypnea and pale right and left ears and extremities) were observed in one female rat that was subsequently found dead. Although these observations occurred in only a single female rat, they were considered to be test substance related. The other found dead female did not survive to the FOB evaluation performed on Day 2. There were no statistically significant or biologically important effects of pethoxamid on the FOB parameters in the

male or female rats at any time point that were considered to be test substance related. No effects on brain weight or gross pathology of the cranial cavity were observed in either sex. No test substance-related microscopic lesions were apparent in the neurohistopathological evaluation of the central or peripheral nervous system, eyes with retinas and optic nerves and skeletal muscle.

On the basis of these data, the no-observable-adverse-effect-level (NOAEL) for systemic toxicity of pethoxamid following a single oral gavage dose is considered to be 100 mg/kg bw for both male and female rats based on body weight and food consumption effects at 300 mg/kg bw and mortality, clinical observations (in the two female rats that were subsequently found dead), body weight and food consumption effects at 800 mg/kg bw. There was no evidence of neurotoxicity in either sex at the highest dose tested; therefore, the NOAEL for neurotoxicity is considered to be 800 mg/kg bw for male and female rats.

MATERIALS AND METHODS

Materials:

Test material:	Pethoxamid technical
Lot/batch number:	P1351-JaK-T2-23-6
Purity:	95.80% (w/w) (dose calculation was not adjusted to purity)
Stability of test item:	06 January 2014 (stored at ambient temperature) *NB: stable during the conduct of the study*
Storage conditions:	At room temperature, protected from light

Study Design:

Groups of 5 male and 5 female Crl:CD(SD)BR rats were administered pethoxamid once by gavage at dose levels of 0, 100, 300 or 800 mg/kg bw. The vehicle used was 1% methylcellulose and 0.5% Tween® 80 in reverse osmosis deionized water. Dose levels and the time to peak effect (TOPE) were determined in a preliminary study. In this study, rats (10/sex/group) were administered pethoxamid once by gavage at dose levels of 0, 600 or 800 mg/kg bw. No mortality occurred; however, body weight loss and clinical signs of toxicity (including but not limited to hunched posture, vocalization, cold to touch and whole body tremors) were observed at both dose levels. The clinical signs occurred predominately between 12 and 16 hours after dose administration. Some signs were still apparent at 24 hours. Based on the results of the preliminary study, a 16-hour TOPE and dose levels of 0, 100, 300 and 800 mg/kg bw were selected for the definitive study.

RESULTS AND DISCUSSION

Mortality: 800 mg/kg bw: Two female rats were found dead, one on Day 2 and one on Day 3.

Clinical signs: 800 mg/kg bw: Clinical signs of toxicity were observed only in the two female rats that were found dead. Signs included decreased motor activity, ptosis, mild and moderate dehydration, hunched posture, pale ears and extremities, bradypnea, scant feces and/or ungroomed coat.

Body weight gain: 800 mg/kg bw: Body weight loss (statistically significant) was observed in male and female rats on Days 1 to 2 after which body weight rebounded. 300 mg/kg bw: Statistically significantly decreased body weight gain was observed in male rats from Day 1 to 2. Body weight gain was reduced in females on Day 1 to 2; however, the effect was not statistically significant.

Food consumption: 800 mg/kg bw: Absolute (g/day) and relative food consumption (g/kg/day) were statistically significantly decreased in males and females from Day 1 to 2. 300 mg/kg bw: Relative food consumption (g/kg/day) was statistically significantly decreased in both sexes from Day 1 to 2.

Functional Observational Battery: 800 mg/kg bw: At the TOPE, hunched posture, bradypnea and pale ears and extremities were observed in one female rat that was subsequently found dead.

Motor Activity: No treatment-related effects observed.

Brain weights: No treatment-related effects observed.

Macroscopic findings: No treatment-related effects found in the two females that were found dead.

Neurohistopathology: No treatment-related effects observed.

Table 3.11.1-1: Summary of acute neurotoxicity study findings-Part B—Definitive Study

Parameter	**Males**			
	Dose Levels (mg/kg bw)			
	0	**100**	**300**	**800**
Mortality	0	0	0	0
Body Weight				
Day 1	315.0	312.2	304.8	305.8
Day 2	321.6	318.6	306.8	302.9
Day 3	330.1	326.0	316.7	309.7
Body Weight Gain				
Days 1 to 2	6.6	6.4	2.0*	-2.9**
Food Consumption				
Absolute Food Consumption (g/day)				
Days 1 to 2	25.5	25.0	21.8	16.2**
Relative Food Consumption (g/kg/day)				
Days 1 to 2	80.2	79.2	72.1*	52.9**
Functional Observation Battery	No treatment-related effects			
Motor Activity	No treatment-related effects			
Neurohistopathology	No treatment-related effects			
Parameter	Females			
	Dose Levels (mg/kg bw)			
	0	100	300	800
Mortality	0	0	0	2
Body Weight				
Day 1	219.0	218.4	222.1	223.3
Day 2	223.1	220.4	222.3	219.9
Day 3	225.8	223.7	227.5	227.6
Body Weight Gain				
Days 1 to 2	4.1	2.0	0.2	-4.2**

Food Consumption **Absolute Food Consumption (g/day)** **Days 1 to 2**	16.0	15.5	12.7	8.8**
Relative Food Consumption (g/kg/day) **Days 1 to 2**	72.5	70.7	56.8*	39.0**
Functional Observation Battery	-	-	-	At TOPE: hunched posture, bradypnea and pale ears and extremities were observed in one female rat that was subsequently found dead
Motor Activity	No treatment-related effects			
Neurohistopathology	No treatment-related effects			

*p<0.05; **p<0.01

CONCLUSION: The no-observable-adverse-effect-level (NOAEL) for systemic toxicity for pethoxamid following a single gavage dose is considered to be 100 mg/kg bw for male and female rats based on decreased body weight gain and relative food consumption at 300 mg/kg bw and mortality, clinical observations (in the two female rats that were subsequently found dead), body weight loss and decreased absolute and relative food consumption at 800 mg/kg bw. No clear evidence of neurotoxicity was observed in either sex at the highest dose tested; therefore, the NOAEL for neurotoxicity is considered to be 800 mg/kg bw for male and female rats.

3.11.2 Human data

No relevant studies.

3.11.3 Other data

No relevant studies.

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

3.12.1.1 Anonymous (1994)

Reference: Toxicity to rats by dietary administration for 4 weeks
Author(s), year: Anonymous, 1994
Report/Doc. number: 69 PXA (TOX2001-282)/ TKS '13/932511
Guideline(s): OECD Guideline 407 (1981)
GLP: Yes

Deviations from OECD 407 (2008): Yes (Haematology: reticulocytes and bile acids not examined; Pathology: no weight of thymus, prostate + seminal vesicles with coagulating glands recorded; Histopathology: no preservation of seminal vesicles with coagulating glands, peripheral nerve (sciatic or tibial); no full histopathology carried out on the preserved organs)

Acceptability: Yes

Deviations from study protocol: Due to a technical error, the water residue was not measured at the seventh day of Week 3 and the water consumption was calculated on a 6 day basis. This deviation is not considered to affect the integrity of the study.

Material and Methods:

Test material: Pethoxamid; Batch TB-930727: Purity: 95.2%.

Test animals: Groups of 5 male and 5 female Crl: CD(SD)BR rats, 28 days old, 187 g to 253 g (males), 151 g to 191 g (females), from Charles River (U.K.), Ltd, Margate, Kent, England.

The rats received the test material by dietary administration at concentrations of 0, 500, 2500, 5000 and 7500 ppm for a duration of 4 weeks. This was equal to 45.3, 227, 482 and 699 mg/kg bw/d for males and 52.9, 266, 535 and 737 mg/kg bw/d for females.

Results:

Clinical findings:

No mortalities occurred in the study. Treatment-related clinical signs or opthalmological findings were not observed.

Body weight, body weight gain, food and water consumption:

Findings on body weight, body weight gain and food consumption were observed from 2500 ppm, partly attaining statistical significance (Table 3.12.1-1). At 7500 ppm body weight losses were noted for both sexes in Week 1. Males showed a notably lower water consumption at this dose.

Table 3.12.1-1: Rat oral 28-day: Body weight, body weight gain, food and water consumption

Sex	**Male**					**Female**				
Dose (ppm)	0	500	2500	5000	7500	0	500	2500	5000	7500
Body weight, term. kill (g)	361	355	326	299*	234**	239	239	221	218*	190**
Body weight gain										
Week 0-1 (g/rat)	55	47	27**	2**	-30**	24	24	15	3**	-13**
Week 1-4 (g/rat)	89	92	83	80	47**	49	49	42	48	39
Week 0-4 (g/rat)	144	139	110**	83**	16**	73	72	56	51**	26**
Food consumption[1]										
Week 1 (g/rat)	193	179	171	166	79	133	142	139	139	93
Week 1-4 (g/rat)	776	741	691	679	557	576	611	581	573	468
Water consumption[1]										
Week 3 (g/rat/week)	212	180	197	199	159	170	169	157	152	174

[1] Only measured in Week 3, statistical analysis not possible due to there only being one cage/sex/group;

Statistical significance: $^{*}p\leq0.05$; $^{**}p\leq0.01$

Haematology:

At 5000 and 7500 ppm, both sexes showed lower mean total white blood cell counts associated with lower lymphocyte counts achieving statistical significance in males (Table 3.12.1-2). In females, the hemoglobin values were significantly lower. The decrease of the concomitant lower mean corpuscular hemoglobin concentrations was only significant at 7500 ppm.

Table 3.12.1-2: Rat oral 28-day: Haematology

Sex	**Male**					**Female**				
Dose (ppm)	0	500	2500	5000	7500	0	500	2500	5000	7500
Platelets ($x10^3/mm^3$)	1016	879	824*	875*	870*	950	810	621	781	820
WBC ($x10^3/mm^3$)	11.6	10.2	10.5	9.6	8.0*	6.9	8.7	7.8	5.9	5.4
Lymphocytes ($x10^3/mm^3$)	10.16	8.35	8.81	7.08**	6.47**	5.97	7.27	6.67	4.63	4.52
Hemoglobin (g/dl)	15.5	16.0	15.6	15.0	14.7	15.8	16.0	15.6	14.8*	14.7**
MCHC (%)	29.2	29.0	28.4	29.0	29.0	30.1	30.0	29.3	29.1	28.7*

WBC: White blood cells; MCHC: Mean corpuscular hemoglobin concentrations;

Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$

Clinical chemistry:

The main finding was a dose related increase in mean cholesterol values from 500 ppm (Table 3.12.1-3). Values for other parameters (ALT, globulin, phosphorus and glucose) varied from the control values at 5000 and 7500 ppm.

Table 3.12.1-3: Rat oral 28-day: Clinical chemistry

Sex	**Male**					**Female**				
Dose (ppm)	0	500	2500	5000	7500	0	500	2500	5000	7500
Cholesterol (mg/dl)	58	90*	128**	152**	190**	77	84	117*	167**	210**
ALT (mU/ml)	26	24	22	27	31	21	24	20	30	35*
Glucose (mg/dl)	118	114	104	120	99*	118	113	107	99*	93**
Globulin (g/dl)	3.5	3.8	3.8	4.2**	3.9**	3.4	3.5	3.6	4.0**	4.1**
Phosphorus (mEq)	4.8	4.5	4.5	4.3**	3.9**	3.7	3.8	3.6	3.4	3.6

ALT: Alanine amino transferase; Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$

Pathological examinations:

Necroscopy: No treatment related changes were found.

Organ weights: Generally, the liver weights were increased, beginning in males from 500 ppm (Table 3.12.1-4). Statistically significant was this finding for the adjusted weights only. In males, the absolute spleen weights were decreased from 500 ppm onwards (11, 17, 33% of the mean control value).

Histopathology: From 2500 ppm, histopathologic findings as centrilobular enlargement of hepatocytes and intracytoplasmic eosinophilic inclusions accompanied the increase of liver weights.

Table 3.12.1-4: Rat oral 28-day: Liver weight and liver histopathology

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**500**	**2500**	**5000**	**7500**	**0**	**500**	**2500**	**5000**	**7500**
Liver weight										
Absolute liver weight (g)	16,4	18,3 +12%	22,1 +35%	22,3 +36%	17,6 +7%	11,4	11,4 +0%	12,0 +5%	15,8 +39%	14,9 +31%
Adjusted liver weight (g)[1]	13.7	15.9 +16%	21.6** +58%	23.1** +69%	22.3** +63%	10.9	11.0 +1%	12.1 +11%	15.8** +45%	15.7** +44%
Liver histopathology										
Centrilobular enlargement of hepatocytes	0/5	0/5	1/5	5/5**	5/5**	0/5	0/5	0/5	3/5	5/5**
Periportal hepatocytes with intracytoplasmic eosinophilic inclusions	0/5	0/5	4/5*	3/5	4/5*	0/5	0/5	0/5	1/5	3/5

[1] Adjusted to body weight; Statistical significance: *p≤0.05; **p≤0.01

Conclusion:

At the lowest dose, effects already occurred – although mostly without statistical significance. Thus, based upon the data obtained from this study, it was recommended that a suitable low level for further investigation should be below 500 ppm, whilst a suitable high level should be 5000 ppm or above, but not approaching 7500 ppm.

3.12.1.2 Anonymous (1996a)

Reference: Palatability/Preliminary dose range finding study in mice by dietary administration for 4 weeks
Author(s), year: Anonymous 1996a
Report/Doc. number: 70 PXA (TOX2001-283) / TON '3/960337
Guideline(s): OECD 407 (1981)
GLP: Yes
Deviations from OECD 407 (2008): Yes (Pathology: No weight of thymus recorded, no preservation of peripheral nerve (sciatic or tibial); no full histopathology carried out on the preserved organs, only for liver and thyroid; no haematology measurements recorded; no clinical chemistry measurements (with the exception of drug metabolizing liver enzymes) recorded).

Acceptability: Yes (limited information); range-finding test

Material and Methods:

Test material: Pethoxamid; Batch: TB-930727; Purity: 95%.
Test animals: Groups of 16 male and 16 female Crl: CD-1 (ICR) BR mice, 6 weeks of age, 26-38 g (males), 22-29 g (females), from Charles River, UK, Ltd, Margate, Kent, England.
The mice received the test material by dietary administration at concentrations of 0, 100, 500, 3000 and 10000 ppm for a duration of 4 weeks. This was equal to 17, 85, 539 and 1786 mg/kg bw/d for males and 22, 114, 679 and 2206 mg/kg bw/d for females.

Results:

Mortality and clinical findings: Treatment related clinical signs were not observed. No mortalities were recorded.

Body weight, Body weight gain and food consumption: From 3000 ppm, the food consumption was significantly lower in Week 1 (Table 3.12.1-5). This was accompanied in males (at 10000 ppm also in females) by a body weight loss. The body weight gain was reduced in males over the entire dosing period.

Pathological examinations:
Necropsy: No treatment related findings were detected.
Organ weights: The body weight adjusted liver weights were significantly increased, beginning in males from 500 ppm (Table 3.12.1-5). An increase in body weight adjusted kidney weights from this dose onwards was also observed (males: 17, 20, 10%; females: 6, 11, 6% above the mean control values).

Histopathology: Hepatocellular hypertrophy was observed.

Hepatic enzyme activities: In mice as in rats, pethoxamid was found to be acting as a phenobarbitone-type inducer of liver enzymes starting already at the 100 ppm dose level (larger induction of 7-pentoxyresorufin O-dependentylase activity (CYP2B) and smaller induction of other P450-related enzyme activities as well as induction of p-nitrophenol UDP-glucuronyltransferase).

Table 3.12.1-5: Mouse oral 28-day study (data summary)

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**100**	**500**	**3000**	**10000**	**0**	**100**	**500**	**3000**	**10000**
Body weight, term. kill	39	39	38	35	31	27	28	29	27	25
Body weight gain										
Week 0-1 (g/mouse)	2.3	2.5	2.3	-0.2**	-2.5**	0.6	0.3	0.5	0.3	-1.2**
Week 1-4 (g/mouse)	4.4	4.3	4.4	3.4*	1.8**	1.1	2.4*	2.8*	2.7*	1.6*
Week 0-4 (g/mouse)	6.7	6.8	6.7	3.2**	-0.8**	1.7	2.8	3.2	3.0	0.4
Food consumption										
Week 1 (g/mouse)	40	42	39	32**	28**	37	38	37	32**	28**
Week 2-4 (g/mouse)	125	134	129	127	118	131	125	132	130	116**
Organ weights										
Absolute liver weight (g)	2,06	2,18 +6%	2,21 +7%	2,20 +7%	2,32 +13%	1,46	1,50 +3%	1,62 +11%	1,69 +16%	1,77 +21%
Adjusted liver weight (g)[1]	1.85	1.98 +7%	2.10** +14%	2.35** +27%	2.69** +45%	1.47	1.47 +0%	1.53 +4%	1.71** +16%	1.87** +27%
Hepatocellular hypertrophy										
-Centrilobular	1/16	0/16	4/16	5/16	7/16*	0/16	0/16	0/16	0/16	0/16
-Generalised	0/16	0/16	2/16	5/16*	9/16**	0/16	0/16	0/16	0/16	0/16
-Periportal	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	14/16**	16/16**

[1] Body weight adjusted; Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$

Conclusion:

The NOAEL is 100 ppm corresponding to 17 mg/kg bw/d based on liver weight increase and liver histopathology findings at the dose level of 500 ppm (85 mg(kg bw/d).

At 100 ppm, phenobarbitone-type liver enzyme induction was already observed, but this was not considered as an adverse effect on its own.

3.12.1.3 Anonymous (1996b)

Reference: Maximum Tolerated Dose and Four Week Constant Dose Study in Dogs
Author(s), year: Anonymous, 1996b
Report/Doc. number: 72 PXA (TOX2001-284) / TON '2/960653
Guideline(s): No OECD guideline for 28 day non-rodents available, based on OECD 407
GLP: Yes
Deviations from OECD 407: Yes (No concurrent controls; Microscopic examinations were limited to liver and thyroid; the number of animals was anyhow too low for any other information but estimation of approximate dose for further testing).

Acceptability: Yes (limited information); range-finding test

Deviations from study protocol: Hepatic drug metabolizing enzymes were tested. The food ration offered to the pair of dogs receiving 400 mg/kg bw/day was modified on occasion in an attempt to stimulate appetite and to improve general condition.

Material and Methods:
Test material: (1): Pethoxamid; Batch:TB 930727; Purity: 96.0%.
(2): Pethoxamid; Batch: TB 951005; Purity 95.1%.
Test animals: Four male and 4 female pure bred beagle dogs, 19-24 weeks old, 6.5-10.9 kg of weight, from Consort Limited, Harewood Park, Harewood End, Herefordshire, HR2 8 TS.
Maximum tolerated dose (MTD) phase: A pair of beagle dogs was given a series of escalating doses of pethoxamid (25, 50, 100, 200, 400, 800, 1000 and 1600 mg/kg bw/d), orally by capsule. The dose level was increased every 3 to 4 days. Based on the results of the MTD phase, three dose levels were chosen to dose further pairs of dogs for 28 days.
Repeat dose phase: Initially, 1 male and 1 female animal received 50, 200 or 800 mg/kg bw/d. However, the pair receiving the highest dosing level showed adverse signs and dosing was suspended after 7 days. Following a period of 7 days off dose, this pair was dosed at 400 mg/kg bw/d for 28 days. Control data were recruited from historical data.

Results:
MTD phase:
In view of the findings (inappetance) seen, it was considered that dose levels of 50, 200 and 800 mg/kg bw/d were suitable for the 28-day study.
Repeat dose phase:
Clinical findings:
No mortalities occurred. The clinical findings comprehended liquid/mucoid faeces, vomiting, salivation, subdued behaviour and tucked up appearance (Table 3.12.1-6). These symptoms were dose related.
Body weight, body weight gain and food consumption:
At 200 mg/kg bw/d, a minor effect on body weight in the female dog was recorded (Table 3.12.1-6). At 400 mg/kg bw/d, both animals recorded overall weight gain but it was necessary to manipulate the diet because of the immediate decrease in appetite. According to the marked reduction in food consumption, a marked loss of body weight was recorded at 800 mg/kg bw/d.
Haematology:
At the high dose (800/400mg/kg bw/d), a slight decrease in red blood cell parameters and increases in reticulocytes and platelets were observed. The blood smear showed slight hypochromasia and/or macrocytosis from Day 20.

Clinical chemistry:
At 800/400 mg/kg bw/d, a dose related decrease in cholesterol levels was noted. In this dose group, the values for most of the measured parameters of hepatic drug metabolism (microsomal protein- and cytochrome P450 concentration, 7-ethyoxyresorufin O-deethylase, 7-pentoxyreso-rufin O-dependentylase, lauric acid 11-hyroxylase, lauric acid 12-hydroxylase, p-nitrophenol UDP-glucuronyltransferase) were below the range of historical data. This decrease is obviously related to the hepatotoxic effect of pethoxamid.
Pathological examinations:
Necropsy: Accompanying the increased absolute and relative liver weights in the female dog, an enlarged liver was found at 800/400 mg/kg bw/d.
Organ weights: The liver weight was above the normal historical upper limit of 4% of the terminal body weight.
Histopathology: A minimal centrilobular hepatocyte hypertrophy was observed; at 200 mg/kg bw/d in both sexes and at 800/400 mg/kg bw/d in the female only.

Table 3.12.1-6: Overview of 28-day toxicity in dogs treated orally with pethoxamid

Sex	**Male**					**Female**				
Dose (mg/kg bw/day)	**0**	**50**	**200**	**800**	**400**	**0**	**50**	**200**	**800**	**400**
Clinical signs										
Vomiting	HD		+	+	+	HD		+	+	+
Liquid/mucoid faeces	HD	+	+	+	+	HD	+	+	+	+
Subdued behaviour	HD			+	+	HD		+	+	+
Tucked up appearance	HD			+	+	HD		+	+	+
Salivation	HD				+	HD				
Body weight gain										
Day 1-29 (g/dog)	HD	0.7	0.6	–	–	HD	0.7	-0.2	–	–
Day 1-8 (g/dog)	HD	–	–	-1.8	–	HD	–	–	-1.6	–
Day 8-14 (g/dog)	HD	–	–	0.7	–	HD	–	–	1.1	–
Day 15-43 (g/dog)	HD	–	–	–	0.2	HD	–	–	–	1.7
Food consumption										
Day 1-28 (g/dog)	HD	400	369	–	–	HD	400	378	–	–
Day 1-7 (g/dog)	HD	–	–	160	–	HD	–	–	151	–
Day 15-42 (g/dog)	HD	–	–	–	[1]	HD	–	–	–	[1]
Haematology[2]										
PCV (%)	HD	40.2	44.1	40.1	31.9	HD	44.0	43.1	39.5	35.9
Hb (g/dl)	HD	12.7	14.1	13.1	9.7	HD	14.0	13.6	12.7	11.0
RBC ($x10^{12}$/l)	HD	5.86	6.32	6.09	4.12	HD	6.11	5.89	5.38	4.72
Reticulocytes (%)	HD	0.5	0.5	<0.1	3.2	HD	1.2	1.1	0.1	3.8
Platelets (10^9/l	HD	290	427	385	592	HD	341	473	431	566
Hypochromasia	HD				+	HD				+
Macrocytosis	HD				+	HD				+
Clinical chemistry										
Cholesterol (mg/dl)	HD	165	144		86	HD	134	101	102	
Organ weights										
Absolute liver weight (g)	HD	383.9	384.7	393.8		HD	334.6	358.3	492.4	
Relative liver weight (%)[3]	HD	3.49	4.01	3.82		HD	3.64	4.03	5.02	
Macroscopic pathology										
Enlarged liver	No treatment-related findings					HD	0/1	0/1	1/1	
Liver histopathology										
-Min. centrilob. hypertrophy	HD	0/1	1/1	0/1		HD	0/1	1/1	1/1	

HD: Historical data used; [1] Range of 100 to 550 g food/dog for male and 150 to 550 g food/dog for female due to loss of appetite and measures to improve palatability; [2] Animals treated with 800/400 mg/kg bw/d were measured at Day 10 for 800 mg/kg bw/d and Day 42 for 400 mg/kg bw/d; [3] Relation to body weight; +: present; -: not calculated for this time interval; PCV: Packed cell volume; Hb: hemoglobin; RBC: Red blood cell count.

Conclusion:
The findings in this study indicate that dosages of 400 mg/kg bw/d and above are not suitable for further investigations on dogs. No induction of drug metabolizing enzymes - as in rats and mice - was found in dogs.

3.12.1.4 Anonymous (1996)

Reference: Toxicity to rats by dietary administration for 13 weeks
Author(s), year: Anonymous, 1996
Report/Doc. number: 61 PXA (TOX2001-285) / TKS '24/951565
Guideline(s): OECD 408 (1981)
GLP: Yes
Deviations from OECD 408 (1998): Yes (Pathology: no weight of thymus recorded; Histology: no full histopathology of accessory sex organs, no histopathology of skin and peripheral nerve)
Acceptability: Yes

Deviations study protocol: In addition, levels of drug metabolizing enzymes were measured.

Material and Methods:
Test material: Pethoxamid, Batch TB-930727; Purity: 95.2%.
Test animals: Groups of 10 male and 10 female Crl:CD BR rats, six weeks old, 179 g to 224 g (males), 135 g to 171 g (females) of weight, from Charles River U.K., Ltd., Margate, Kent.
The rats received the test compound by dietary administration at concentrations of 0, 100, 500, 2500 and 5000 ppm for a duration of 13 weeks. This was equal to 7.5, 36.2, 196 and 388 mg/kg bw/d for males and 8.0, 41.6, 207 and 426 mg/kg bw/d for females.

Results:
Mortality, clinical and ophthalmologic findings:
No deaths occurred. No treatment related clinical and ophthalmoscopic findings were detectable.

Body weight, body weight gain, food- and water consumption:
The absolute body weights were dose related but not statistically significantly lowered. However, the body weight gains were statistically significantly lowered in males from 500 ppm. At 5000 ppm, even a body weight loss occurred. The food- and water consumption were dose related reduced, partly attaining statistical significance.

Table 3.12.1-7: Rat oral 90-day study (Body weight, body weight gain, food- and water consumption)

Sex	Male					Female				
Dose (ppm)	**0**	**100**	**500**	**2500**	**5000**	**0**	**100**	**500**	**2500**	**5000**
Body w., term. kill (g)	543	521	496	457	438	292	301	288	272	255
Body weight gain										
Week 0-1 (g)	60	58	50*	28**	-9**	23	25	23	18	3**
Week 1-13 (g)	278	260	246	228*	240*	118	125	113	102	104
Week 0-13 (g)	338	317	296*	256**	232**	141	150	136	120*	106**
Food consumption										
Week 1 (g/rat)	222	214	191	170*	113**	132	129	133	128	100*
Week 1-13 (g/rat)	2729	2622	2457	2470	2312*	1830	1773	1786	1707	1645
Water consumption										
Week 12 (g/rat)[1]	254	249	219	203*	214*	203	192	181	177	170

[1] Only measured in Week 12; Statistical significance: * p≤0.05, ** p≤0.01

Haematology:
From 2500 ppm, both sexes showed lower mean platelet values, which was statistically significant for males.

Clinical chemistry:
From 2500 ppm, the cholesterol values were statistically significantly increased. At 5000 ppm, the total protein values were significantly higher and the glucose values lower (significant in males only) as the control values. Values for parameters of drug metabolism in the liver altogether increased from 2500 ppm; especially the 7-pentoxyresorufin O-depentylase activity in females. This indicates pethoxamid acting as a phenobarbitone-type inducer of drug metabolizing enzymes in rats.

Table 3.12.1-8: Rat oral 90-day study: Haematology and clinical chemistry

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**100**	**500**	**2500**	**5000**	**0**	**100**	**500**	**2500**	**5000**
Haematology										
Mean platelet value ($x10^3/mm^3$)	794	752	773	695*	706*	772	722	728	681	681
Clinical chemistry										
Cholesterol (mg/dl)	59	65	67	86**	123**	76	77	85	104**	133**
Total mean protein (g/dl)	6.6	6.7	6.6	6.9	7.3**	7.1	7.1	7.1	7.4	7.6*
Mean glucose (mg/dl)	117	116	112	111	102**	123	120	125	115	109
Liver, parameters of drug metabolism										
Cytochrome P450[1] (nmoles/g liver)	1	0.9	1.1	1.7**	2.1**	1	1.0	1.3	1.6**	2.3
7-Ethoxyresorufin O-deethylase[1] (nmol/min/g liver)	1	1.2	2.2**	4.0**	5.6**	1	1.2	1.8*	2.8**	3.2
7-Pentoxyresorufin O-depentylase[1] (nmol/min/g liver)	1	0.9	2.1**	7.8**	12.1**	1	1.0	12.4**	276**	791.6**
Lauric acid 11-hydroxylase (nmol/min/g liver)	1	1.0	1.1	1.6**	1.8**	1	1.1	1.3*	1.7**	1.9*
Lauric acid 12-hydroxylase (nmol/min/g liver)	1	1.2	1.0	1.2	1.3	1	1.2	1.3*	1.5*	1.2*
p-Nitrophenol UDP-glucuronyltransferase[1] (µmoles/hr/g liver)	1	0.9	1.3	3.5**	5.8**	1	1.0	1.5*	3.0**	8.1**

[1] Statistical analysis performed on logarithmically transformed data; Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$.

Pathological examinations:
Necroscopy: No treatment-related findings were observed.
Organ weights: The body weight adjusted liver weights were significantly increased in both sexes from 2500 ppm, and the body weight adjusted thyroid weight in females at 5000 ppm (Table 3.12.1-9).
Histopathology: The single incidences of fat deposition among rats up to 500 ppm were within the background control range. From 2500 ppm, treatment-related changes in the liver comprehended periportal hepatocytes with margination of cytoplasm, fat deposition and concentric intracytoplasmic inclusions. At 5000 ppm, the hepatocytes were generally minimally enlarged in some animals.
In the thyroids, follicular cell hypertrophy was significantly increased in males (from 2500 ppm) and females (5000 ppm).

Table 3.12.1-9: Rat oral 90-day study: Findings on liver and thyroid (organ weights and histological findings)

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**100**	**500**	**2500**	**5000**	**0**	**100**	**500**	**2500**	**5000**
Liver										
Abs. Liver weight (g)	21,3	20,1 -6%	20,6 -3%	22,9 +8%	24,8 +16%	10,6	11,0 +4%	10,6 +0%	12,2 +15%	13,7 +29%
Adj. liver weight (g)[1]	19.1	18.8 -2%	20.3 +6%	24.3** +27%	27.1** +42%	10.3	10.4 +1%	10.4 +1%	12.5** +21%	14.6** +42%
Periportal hepatocyte margination of cytoplasm	0/10	0/10	0/10	9/10**	10/10**	0/10	0/10	0/10	1/10	10/10**
Occasional concentric intracytoplasmic inclus.	0/10	0/10	0/10	3/10	7/10**	0/10	0/10	0/10	0/10	2/10
Minimally generalised hepatocyte enlargement	0/10	0/10	0/10	0/10	4/10*	0/10	0/10	0/10	0/10	4/10*
Fat deposition in periportal hepatocytes	0/10	0/10	2/10	5/10*	6/10**	1/10	3/10	4/10	1/10	1/10
Thyroid										
Abs. thyroid weight (mg)	21.7	22.8 +5%	20.1 -7%	25.4 +17%	24.9 +15%	14.4	17.1 +19%	17.3 +20%	16.7 +16%	17.8 +24%
Adj. thyroid weight (mg)[1]	[2]					14.0	16.3 +16%	17.1 +22%	17.0 +21%	18.8** +34%
Follic. cell hypertrophy	2/10	0/10	0/10	7/10*	9/10**	0/10	0/10	0/10	2/10	4/10*
Sparse colloid	1/10	0/10	0/10	3/10	7/10**	0/10	0/10	0/10	0/10	2/10

[1] Body weight adjusted; [2] Not adjusted as not appropriate; Statistical significance: *p≤0.05; **p≤0.01

Conclusion:
The NOAEL (NOEL) in this study was at 100 ppm corresponding to 7.5 mg/kg bw/d based on decreased body weight gain and food intake. The liver and thyroid findings at higher doses indicate an effect on the liver-thyroid axis known for phenobarbitone-type inducers of drug metabolizing enzymes in the liver.

3.12.1.5 Anonymous (1998)

Reference: Toxicity to mice by dietary administration for 13 weeks

Author(s), year: Anonymous, 1998
Report/Doc. number: 71 PXA (TOX2001-286) / TON '5/971279
Guideline(s): OECD 408 (1981)
GLP: Yes
Deviations from OECD 408 (1981): Yes (Haematology: no haematocrit measured; no organ weights of uterus and thymus recorded; - no histopathological examinations of aorta, gall bladder, skin recorded; not all accessory sex organs preserved; - no organ weights of uterus and thymus recorded; - no histopathological examinations of aorta, gall bladder, skin recorded; not all accessory sex organs preserved).
Acceptability: Yes (limited information)

Supplementary Evaluation of 13-week mice study:

Reference: Toxicity to mice by dietary administration for 13 weeks – Electron Microscopy Report
Author(s), year: Anonymous, 1997
Report/Doc. number: 1288 PXA (TOX2001-286) / TON '5/970411
Guideline(s): Not applicable
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306; Purity: 95.0%.
Test animals: 10 male and 10 female Crl:CD-1 (ICR) BR mice
Based on the results in the 28-day study (Report no. TON 3/960337), the mice received the test material by dietary administration at concentrations of 0, 50, 400, 3000 and 10000 ppm for a duration of 13 weeks in order to select appropriate dosages for the carcinogenicity study. This was equal to 9.1, 70.5, 610 and 2354 mg/kg bw/d for males and 12.0, 93, 724 and 2492 mg/kg bw/d for females.

Results:
Mortality, clinical and ophthalmologic findings:
There were three deaths which were not treatment-related. One mouse from the control group was found dead during Week 13 due to urogenital tract lesions. Two mice (one male and one female from 3000 ppm) were found dead during Week 13 and 14 due to an anaesthetic accident.
No treatment-related clinical effects and no ophthalmologic effects were observed.

Body weight, body weight gain and food consumption:
No treatment-related effects on food consumption were recorded.
The mice gained less weight than the control animals from 3000 ppm and lost weight at 10000 ppm.

Table 3.12.1-10: Mouse oral 90-day study: Body weight and body weight gain

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Body weight, term. kill	39	42	43	37	29	30	32	29	28	26
Body weight gain										
Week 0-1 (g)	2.3	2.8	2.3	0.7**	-2.7**	1.2	1.4	1.2	0.3	-1.1**
Week 1-12 (g)	7.7	11.3	9.4	8.6	2.5**	6.6	7.2	5.2	4.3	2.8**
Week 0-12 (g)	9.9	14.1	11.7	9.2	-0.2**	7.9	8.6	6.4	4.6*	1.6**

Statistical significance: $^{*}p\leq0.05$; $^{**}p\leq0.01$

Haematology:
At the highest dose, both sexes showed lower mean packed cell volume, hemoglobin and red blood cell values, with males also showing higher mean corpuscular volume values and lower mean corpuscular hemoglobin concentration values. Males showed statistically significant lower mean total white blood cell counts, associated mainly with lower lymphocyte counts.

Table 3.12.1-11: Mouse oral 90-day study: Haematology

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
PCV (%)	41.7	43.8	42.7	42.8	38.2*	43.4	44.8	44.4	42.6	40.5**
Hb (g/dl)	13.7	14.4	14.0	14.0	12.2**	14.2	14.7	14.7	14.0	13.1**
RBC (1012/l)	8.80	9.05	9.08	8.60	7.71**	8.78	9.09	9.11	8.83	8.12**
MCV (fl)	47.4	48.4	47.1	49.8**	49.7**	49.5	49.3	48.7	48.3	49.9
MCHC (g/dl)	32.9	32.8	32.8	32.5	31.8**	32.8	32.7	33.1	33.0	32.3
WBC (109/l)	5.54	5.74	6.45	5.67	3.68*	3.16	3.31	3.82	3.80	3.43
Lymphocyte counts (10^9/l)	4.14	4.26	5.48	4.61	2.43*	2.60	2.64	3.32	3.30	2.97

PCV: Packed cell volume; RBC: Red blood cell count; MCV: Mean corpuscular volumes; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell count; Statistical significance: $^{*}p\leq0.05$; $^{**}p\leq0.01$

Clinical chemistry and urinalysis:
The main findings at the two high doses were reduced protein values and increased cholesterol values. The electrolyte values varied slightly from control values in males. Higher alkaline phosphate values were noted for a few individual males at 10000 ppm, but the group mean value was not statistically different from the control data and no histopathological findings could be associated with this effect.
In females, at the two high doses, marked ketonuria was found. There was no histopathological correlate to this finding and it was considered to be of unlikely toxicological importance. Lower urinary protein values were noted for both sexes, achieving statistical significance in females.

Table 3.12.1-12: Mouse oral 90-day study: Clinical chemistry and urinalysis

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Clinical chemistry										
Total protein (g/dl)	5.4	5.4	5.3	5.2	4.8**	5.4	5.3	5.2	5.4	5.1*
Albumin (g/dl)	2.9	2.8	2.9	2.6*	2.6**	3.2	3.1	3.1	3.0**	2.8**
Globulin (g/dl)	2.6	2.6	2.4	2.5	2.3*	2.2	2.2	2.1	2.4*	2.3*
K (mEq/l)	5.1	4.7	5.5	5.2	4.3*	4.5	4.5	4.5	4.6	4.6
Ca (mEq/l)	5.1	5.0	5.0	4.9*	4.7**	5.0	5.0	4.9	5.0	4.9*
P (mEq/l)	3.9	3.6	3.6	4.1	4.5*	4.0	3.9	3.6	4.1	4.1
Cl (mEq/l)	111	110	111	114**	115**	113	113	113	113	114
Cholesterol (mg/dl)	124	150	139	165**	165**	88	99	109	153**	183**
ALP (mU/ml)	90	65	93	61	140	117	105	108	97	88
Urinalysis										
Ketonuria (samples)	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	3/5	5/5
Protein (mg/dl)	668	1102	870	1018	298	199	201	133	101	49**

K: Potassium; Ca: Calcium; P: Phosphorus; Cl: Choride; ALP: Alkaline Phosphatase; Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$

Pathological examinations:
Necroscopy: At 10000 ppm, a reduction in adipose tissue was noted in the majority of male mice compared to control mice (Table 3.12.1-13).
Organ weights: The body weight adjusted liver weights were dose related increased, attaining statistical significance at the two high doses. In females, the body weight adjusted thyroid weights were statistically significantly increased, too. A decrease of the absolute spleen weight occurred in both sexes at 10000 ppm.

Table 3.12.1-13: Mouse oral 90-day study: Absolute and relative[1] organ weights, necroscopy findings

Sex	Male					Female				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Organ weights										
Abs.liver wt.(g)	1,87	1,99 +6%	2,09 +12%	2,25 +20%	2,27 +21%	1,55	1,66 +7%	1,45 -6%	1,84 +19%	2,24 +45%
Adj. liver wt. (g)	1.82	1.88 +3%	1.98 +9%	2.29** +26%	2.51** +38%	1.55	1.55 +0%	1.46 -6%	1.89** +22%	2.29** +48%
Abs. thyroid wt.(mg)	4,5	5,1 +13%	5,0 +11%	4,6 +2%	4,7 +4%	4,0	4,2 +5%	4,0 +0%	4,7 +18%	4,8 +20%
Adj. thyroid wt.(mg)	[2]	[2]	[2]	[2]	[2]	4.0	4.0 +0%	4.0 +0%	4.8* +20%	4.9** +23%
Abs. spleen wt. (g)	0.123	0.120	0.122	0.124	0.082** -33%	0.142	0.147 +4%	0.107 -25%	0.128 -10%	0.104* -27%
Adj. spleen wt. (g) [2]										
Necroscopy findings										
Adipose tissue reduced	0/9	0/10	1/10	2/9	6/10	4/10	1/10	2/10	4/9	4/10

[1] Body weight adjusted; [2] Not adjusted as not appropriate; Statistical significance: *$p \leq 0.05$; **$p \leq 0.01$

Histopathology: Generally, from 3000 ppm, hepatocyte hypertrophy was seen. The light microscopic finding was confirmed by electron microscopy and was concomitant to the higher cholesterol values and relative liver weights. The findings in the spleen at 10000 ppm (increased incidence of hemosiderosis, decreased degree of extramedullary hemopoiesis, reduced cellularity of the marginal zone of the white pulp) were consistent with the slight anaemia. In males, the incidence and degree of involution/atrophy of the thymus were increased. In the small intestine, swelling and cytoplasmic rarefaction of villous epithelial cells were seen at high doses.

Table 3.12.1-14: Mouse oral 90-day study (Histopathology)

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Liver										
Hepatocyte hypertrophy										
- generalised	0/9	0/10	0/10	1/9	2/10	0/10	0/10	0/10	0/10	0/10
- centrilobular midzonal	0/9	0/10	0/10	1/9	8/10**	0/10	0/10	0/10	0/10	0/10
- centrilobular	0/9	0/10	0/10	3/9	0/10	0/10	0/10	0/10	0/10	0/10
- periportal	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	9/9**	10/10**
Spleen										
Reduced cellularity of the white pulp – marginal zone										
- Total	1/9	3/10	3/10	6/9	9/10**	1/10	1/10	1/10	2/9	2/10
- Slight	1/9	3/10	2/10	5/9	1/10	1/10	0/10	1/10	1/9	0/10
- Moderate	0/9	0/10	1/10	1/9	8/10*	0/10	1/10	0/10	1/9	2/10
Extramedullary hemopoiesis										
- Total	9/9	10/10	10/10	9/9	10/10	10/10	10/10	10/10	9/9	10/10
- Minimal	0/9	0/10	0/10	0/9	4/10*	0/10	0/10	0/10	0/9	2/10
- Slight	4/9	6/10	7/10	3/9	4/10	5/10	6/10	8/10	5/9	8/10
- Moderate	5/9	4/10	3/10	6/9	2/10	5/10	3/10	2/10	3/9	0/10
- Marked	0/9	0/10	0/10	0/9	0/10	0/10	1/10	0/10	1/9	0/10
Hemosiderosis										
- Total	3/9	5/10	4/10	4/9	10/10**	7/10	9/10	9/10	8/9	9/10
- Minimal	1/9	3/10	2/10	2/9	2/10	2/10	2/10	2/10	3/9	0/10
- Slight	2/9	2/10	2/10	2/9	8/10*	5/10	6/10	5/10	4/9	5/10
- Moderate	0/9	0/10	0/10	0/9	0/10	0/10	1/10	2/10	1/9	4/10*
Thymus										
Involution/atrophy										
- Total	5/9	4/10	6/9	7/8	9/9	8/10	0/10	0/10	0/10	5/10
- Minimal	4/9	1/10	5/9	4/8	3/9	5/10	0/10	0/10	0/10	4/10
- Slight	0/9	3/10	0/9	2/8	4/9	3/10	0/10	0/10	0/10	1/10
- Moderate	1/9	0/10	1/9	1/8	2/9	0/10	0/10	0/10	0/10	0/10
Duodenum										
Villous epithelial cells swollen with cytoplasmic rarefaction	0/9	0/10	0/10	6/9**	10/10**	0/10	0/10	0/10	3/9	9/10**
Jejunum										
Villous epithelial cells swollen with cytoplasmic rarefaction	0/9	0/10	0/10	0/9	4/10	0/10	0/10	0/10	0/9	4/10*

Statistical significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$

Conclusion:
The NOAEL (NOEL) was at 400 ppm (70.5 mg/kg bw/d) based on decreased body weight gain, increased cholesterol, increased organ weights of liver and thyroid and hepatocyte hypertrophy swelling.

3.12.1.6 Anonymous (1997b)

Reference: Toxicity to Dogs by Repeated Oral Administration for 13 Weeks
Author(s), year: Anonymous, 1997
Report/Doc. number: 73 PXA (TOX2001-288) / TON '4/970936
Guideline(s): OECD 409 (1981)
GLP: Yes
Deviations from OECD 409 (1998): Yes (Haematology: no haematocrit measurements; Clinical chemistry: no ornithine decarboxylase measurements).
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306: Purity: 95.0%.
Test animals: Four male and four female pure bred beagle dogs, 18-15 weeks old, 6.4-9.7 kg of weight, from Huntington Life Sciences supplied by Interfauna UK Limited.
Based on a preliminary study (Report no. TON 2/960653), the dogs were administered pethoxamid by oral capsule at dosages of 0, 8, 50 and 300 mg/kg bw/d for 13 weeks. Due to the decline in the clinical condition of the animals receiving 300 mg/kg bw/d, treatment stopped on Day 4 of Week 2 and following a recovery period of 4 days, dosing was at 200 mg/kg bw/d for the remainder of the study.

Results:
Mortality, clinical and ophthalmologic findings:
There were no treatment-related mortalities.
From 8 mg/kg bw/d, a slightly higher - but dose-related - incidence of post-dosing liquid faeces was noted. This is seen commonly in control dogs, too. Although this finding is considered to be of minor importance, it is associated with treatment. Additional symptoms at the middle and high dose were salivation and vomiting. No treatment-related ophthalmologic effects were evident.

Body weight, body weight gain and food consumption:
At the middle dose, lower weight gain and at the high dose, weight loss (all animals) occurred in Week 1. According to the significant lower food intake at the high dose, the treatment resulted in significant lower mean body weight gains throughout the whole study.

Haematology:
At 300/200 mg/kg bw/d, at both measure times in Week 6 and in Week 12, all animals showed higher platelet and reticulocyte values (significant only in Week 12) and a significantly lower haemoglobin concentration. In Week 12, macrocytosis and hypochromasia were evident in males and in both sexes lower red blood cell packed cell volume counts. The haematological findings were indicating a slight anaemia.

Clinical chemistry and urinalysis:
At the high dose in both Weeks 6 and 12, statistically lower mean albumin and ALT values were evident for both sexes and lower protein levels in Week 6. Significantly lower phosphorus values were noted for males in Weeks 6 and 12 and for females in Week 6 only. This is considered likely to be associated with the clinical conditions of the dogs and therefore not a direct effect of treatment.
At the high dose, abnormal urine colouration and in individual males higher urinary protein levels were noted in Weeks 6 and 12. However, no histopathological findings were found in the kidneys.

Pathological examinations:
Necroscopy: No treatment-related effects were evident.
Organ weights: At the high dose, the following changes in organ weights compared to the control values were observed for both sexes: a lower absolute heart (23%), lung (ca. 20%) and spleen weight (for males 24%, for females 35%), a higher absolute (ca 15%) and body weight adjusted (22%) adrenal weight as well as a higher body weight adjusted liver (19%) and pancreas weight (for males 24%, for females 59%). For males only were observed: a lower absolute brain (7%), prostate (61%) and gonad weight (4%) as well as a higher body weight adjusted kidney weight (39%). The lower organ weights reflect the weight loss at this dose.
Histopathology: The histopathological findings which occurred in other doses than the high dose are summarized in the table below. The vacuolation of the cortical tubules at 8 mg/kg bw/d was only of trace nature. At any dose, this lesion had no inflammatory component and there was no evidence of a progressive degeneration. Because vacuolation is a normal physiological finding in the dog, primarily in the female, the increased incidence of this finding was considered of no toxicological significance. The involution of the thymus which was found in the majority of the animals at the high dose is commonly encountered in animals which show poor condition and/or a dramatic effect on body weight gain. Only at the high dose, both sexes showed glycogen depletion in the liver (8/8) and myeloid atrophy in the bone marrow (7/8). Males showed reduced lymphoid cellularity in the lymph nodes (2/4), immaturity of prostate (3/4) and testes (2/4) with an absence of spermatozoa in the epididymides (2/4) and diffuse vacuolation of the zonae fasciculata and reticularis in adrenals (4/4). These effects were considered to be related to the poor clinical condition of the dogs.

Table 3.2.1-15: Overview of 90-day toxicity in dogs treated orally with pethoxamid

Sex	**Male**				**Female**			
Dose (mg/kg bw/d)	**0**	**8**	**50**	**300/200**	**0**	**8**	**50**	**300/200**
Clinical signs								
Liquid faeces	1	6	19	22	0	6	17	23
Salivation	0	0	2	14	0	0	0	20
Vomiting	0	0	0	1	0	1	0	7
Body weight, term. kill	11.7	11.5	11.2	8.8	11.2	11.1	11.2	9.3
Body weight gain								
Week 0-1 (kg)	0.4	0.4	0.2	-0.3**	0.4	0.4	0.2	-0.8**
Week 0-13 (kg)	3.7	3.4	2.7	0.9**	3.2	3.0	3.1	1.3**
Food consumption								
Week 1 (g/dog/week)	2675	2535	2580	2265**	2685	2585	2618	1440**
Week 3-13 (g/dog/week)	2782	2780	2690	2603	2746	2758	2753	2516**
Haematology (Week 12)								
Platelets (10^9/l)	342	368	322	457**	355	311	394	488*
Reticulocytes (%)	0.2	0.4	0.5	0.9*	0.2	0.5	0.3	1.1*
MCHC (g/dl)	32.5	32.8	32.8	31.4*	32.6	32.6	32.7	32.1
Hb (g/dl)	12.9	12.5	12.2	11.0*	14.1	12.6	13.2	12.3*
PCV (%)	39.6	38.1	37.1	35.2	43.3	38.5	40.3	38.3*
RBC (10^{12}/l)	5.49	5.41	5.17	4.63*	6.08	5.39*	5.57*	5.21**
Urinalysis (Week 12)								
Abnormal urine colouration	0/4	0/4	0/4	2/4	0/4	0/4	1/4	2/4
Higher protein levels	0/4	0/4	0/4	2/4	0/4	0/4	0/4	0/4
Organ weights								
Abs. liver weight	395.6	415.5 +5%	417.1 +5%	355.6 -10%	364.1	368.7 +1%	384.3 +6%	369.9 +2%
Adj. liver weight[1)]	361.6	388.1 +7%	403.9 +12%	430.2 +19%	348.2	357.4 +3%	368.4 +6%	413.0 +19%
Abs. thyroid weight[2)]	0.70	0.84	0.92	0.75	0.73	0.74	0.78	0.66
Abs. heart weight	82.4	83.4	82.7	63.0	85.6	81.6	77.3	66.0
Adj. heart weight[1)]	76.9	78.9	80.5	75.2	83.0	79.7	74.7	73.1
Abs. lung weight	106.7	103.9	100.2	83.6	103.1	100.3	94.3	88.3
Adj. lung weight[1)]	101.6	99.8	98.2	94.8	100.2	98.1	92.5	94.8
Abs. spleen weight	71.1	76.3	74.3	54.0	63.3	70.0	71.3	71.2
Adj. spleen weight[1)]	88.8	95.9	77.8	57.2*	2)			
Abs. adrenal weight	1.22	1.19	1.17	1.42*	1.24	1.20	1.22	1.36

Adj.adrenal weight[1)]	2)				1.21	1.18	1.19	1.44*
Abs. pancreas weight	28.2	27.5	25.4	24.5	23.2	27.5	25.9	26.6
Adj. pancreas weight[1)]	25.1	25.0	24.2	31.2	20.8	25.8	23.6	33.0
Abs. brain weight	82.6	78.6	79.4	76.7**	75.2	75.4	77.0	74.4
Adj. brain weight[1)]	2)				74.3	74.8	76.1	76.7
Abs. prostate weight[2)]	3.04	2.79	5.37	1.18	-	-	-	-
Abs. testes+epid. weight[2)]	20.55	17.81	19.95	13.22**	-	-	-	-
Abs. kidney weight	47.6	51.4	50.8	54.6	47.6	48.8	51.2	50.7
Adj.kidney weight[1)]	44.4	48.9	49.6	61.6*	[2)]			
Histopathology								
Kidney								
Vacuolation cort. tubules	2/4	4/4	4/4	4/4	2/4	4/4	3/4	4/4
Thymus								
– Involution (Total)	0/4	0/4	1/4	3/4	0/4	0/4	2/4	3/4
Spleen								
– Minimal hemosiderosis	1/4	0/4	1/4	2/4	1/4	0/4	1/4	3/4

MCHC: Mean corpuscular hemoglobin concentration; Hb: Hemoglobin; PCV: Packed cell volume; RBC: Red blood cell count; Statistical significance: $^{*}p\leq0.05$; $^{**}p\leq0.01$
[1] Body weight adjusted; [2] Relative organ weight not adjusted as not appropriate

Conclusion:
The alteration of the dosage level from 300 mg/kg bw/d to 200 mg/kg bw/d was not considered to have unduly affected the assessment/interpretation of the data obtained. The effects indicative of anaemia, the lower organ weights and the histopathological findings on adrenal, prostate and gonad at this dose range were characteristic for the poor condition of the dogs.
The NOAEL was set at 8 mg/kg bw/d; based on post dose liquid faeces together with a decreased body weight gain in males at the higher dose of 50 mg/kg bw/d.

3.12.1.7 Anonymous (1999)

Reference: A 12-Month Chronic Toxicity Study of TKC-94 Administered Orally to Beagles
Author(s), year: Anonymous. 1999
Report/Doc. number: 74 PXA (TOX2001-289)/ SBL '98-08
Guideline(s): OECD 452 (1981)
GLP: Yes
Deviations from OECD 4452 (2009): No
Acceptability: Yes

Material and Methods:

Test material: Pethoxamid, Batch number: TB-960306, Purity: 95.0%.

Test animals: Four groups of four male and four female beagle dogs from Shin Nippon Biomedical Laboratories, Ltd., 7.2 to 10.0 kg (male), 5.9 to 8.4 kg (females).

The dogs were administered pethoxamid by oral capsule at dosages of 0, 2, 20 and 150 mg/kg bw/d for 12 months.

Results:

Mortality, clinical and ophthalmologic findings:
At 150 mg/kg bw/d, one male and one female were sacrificed due to moribundity on Day 322 (week 47) and 268 (week 38) respectively, preceded by loss of appetite, bloody stool, salivation, dehydration signs, bradypnea, hypothermia, pale oral mucosa, pale conjunctiva, emaciation, decrease in spontaneous activity, prone and lateral position.
At 20 mg/kg bw/d and for the surviving animals at 150 mg/kg bw/d, increases in the frequency of soft stool and diarrhoea were observed throuout the study period. No treatment-related ophthalmologic effects were observed.

Body weight, body weight gain and food consumption:
At the high dose, the body weights were decreased (Table 3.12.1-16, sacrificed animals excluded). The final body weights of the sacrificed animals were 52 (male) and 64% (female) of their maximum values. Concerning the food consumption, no treatment-related effects were recorded for the surviving animals.

Table 3.12.1-16: 1-year oral dog study: Mortality, clinical signs and terminal body weight

Sex	**Male**				**Female**			
Dose (mg/kg bw/day)	0	2	20	150	0	2	20	150
Mortality	0/4	0/4	0/4	1/4	0/4	0/4	0/4	1/4
Clinical signs[1]								
Soft stool	+	+	++	+++	+	+	++	+++
Diarrhoea	–	–	+	++	–	–	+	++
Vomiting	+	+	+	++	+	+	+	++
Bodyweight[1]								
Week 54 (kg)	14.18	13.08	13.05	13.00	12.83	12.33	12.85	10.83*

[1] Sacrificed animals not included;
Frequency and severity of occurrence:- Normal, + Slight, ++ Moderate, +++ Severe;
Statistical significance: *p<0.05

Haematology:
Many haematological changes were observed for the two sacrificed animals, but these were considered to be related to a nutritional disorder caused by anorexia.
For the surviving animals of this dose group, a slight hypochromasia was noted for 2 males and 1 female at Week 39, and all 3 males and 2 females at Week 53.

Clinical chemistry and urinalysis:
For the sacrificed animals, high values of alkaline phosphatase, AST (male, week 39: 673% of pre-dosing value), ALT (male, week 39: 1439% of pre-dosing value), γ-glutamyl transferase, total bilirubin, total cholesterol, triglyceride and/or blood urea nitrogen were noted. These changes were considered to be related to the disorders of the gastrointestinal tract, kidney and/or liver observed on the histopathology examination. Activity increases in alkaline phosphatase were seen from 20 mg/kg bw/day at different occasions (Table 3.12.1-17).

Urinalysis:
The decreases in urinary excretion of potassium and chloride at the high dose were due to the loss of electrolytes caused by diarrhoea.

Table 3.12.1-17: 1-year oral dog study: Clinical chemistry and urinalysis

Sex	**Male**				**Female**			
Dose (mg/kg bw/day)	**0**	**2**	**20**	**150**	**0**	**2**	**20**	**150**
Clinical chemistry								
ALP (IU/l) Week 26	100.8	110.5	136.0	184.8	93.5	102.0	145.3	218.5*
Week 39	100.8	110.5	151.5	222.3*	100.0	106.5	161.8	218.3*1
Week 53	107.8	114.8	147.0	176.3[1]	104.3	112.0	165.0	261.3*1
Urinalysis								
Potassium (mEq) Week 13	43.0	43.5	41.0	23.5*	41.3	37.8	40.8	32.3
Week 26	37.3	50.3	29.8	13.0	43.3	38.5	45.8	17.8*
Chloride (mEq) Week 13	30.5	31.8	34.3	9.5**	28.0	26.3	34.3	24.5
Week 26	17.0	24.5	20.3	10.0	23.0	24.0	26.8	6.0*
Week 39	19.3	32.0**	38.8**	8.0*	25.3	26.0	20.3	8.7[1]
Week 53	18.0	27.0	23.8	13.0[1]	26.0	18.3	26.0	17.3[1]

[1] Surviving animals;
ALP: Alkaline Phosphatase;
Statistical significance: *p<0.05; **p<0.01

Pathological examinations:
Necroscopy: For the unscheduled sacrificed animals atrophy of the spleen, pancreas, thymus and bilateral testes, enlargement of the bilateral adrenals and accentuated lobular pattern in the liver were observed.
No abnormal changes were observed for the surviving animals.

Organ weights: Compared to the control values, the organ weight increases in females were seen at the mid and high dose for the liver (absolute 44 and 41%, relative 43 and 67% respectively) and for the kidneys (absolute 18 and 34%, relative 18 and 59% respectively; not statistically significant at the mid dose). In males, a significant increase of the liver weights (absolute 28%, relative 38%) was only seen at the high dose.
For the sacrificed animals a high absolute weight of the bilateral adrenals, and low absolute weights of the spleen, testes, bilateral epididymis, heart, lung and unilateral submandibular gland for the male and low absolute weights of the spleen, thymus, heart and bilateral submandibular glands for the female were noted.

Histopathology: The main findings considered substance related concerned the gastrointestinal tract. For the unscheduled sacrificed animals were found: Vacuolation, atrophy, and mononuclear cell infiltration in the muscle layer of the small and large intestines; in the stomach atrophy of the chief cells in the fundus and in the mucosal epithelium in the pylorus, and only in the male swelling of the parietal cells in the fundus; erosion and inflammatory cell infiltration of the mucosa in the oesophagus.
For the kidney, vacuolation and regeneration of the tubular epithelium were noted for both animals, and necrosis and black-blue pigment in the tubular epithelium, debris and hemorrhage in the tubule were noted for the male. The vacuolation in the tubular epithelium was severe. Focal necrosis and foamy change in the hepatocytes, and brown pigment in the hepatocytes and Kupffer cells of the liver were noted for the male. Further changes were considered to be related to the nutritional disorder caused by anorexia.
From the surviving dogs of the high dose group, two males showed very slight to slight findings in the large intestines and one of these dogs additionally findings in stomach and small intestine.

Table 3.12.1-18: 1-year oral dog study: Organ Weights and Histopathology[1,2]

Sex	**Male**				**Female**			
Dose (mg/kg bw/day)	**0**	**2**	**20**	**150**	**0**	**2**	**20**	**150**
Organ weights								
Abs. liver weight (g)	372	338	353	476** +28%	273	290 +6%	392* +44%	386* +41%
Rel. liver weight (g/kg)	26.5	26.0	27.4	36.6** +38%	21.3	23.5 +10%	30.4** +43%	35.7** +67%
Abs. kidney weight (g)	58	54	51	64 +10%	46	47 +2%	55 +18%	62** +34%
Rel. kidney weight (g/kg)	4.2	4.2	3.9	5.0 +19%	3.6	3.8 +6%	4.3 +18%	5.8** +59%
Stomach (pylorus)								
Mucosa, micronecrosis	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Mucosa, epithel. hyperplasia	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Vacuolation and mononuclear cell infiltration in muscle layer	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Small intestine								
Vacuolation and mononuclear cell infiltration in muscle layer	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Large intestine								
Vacuolation and mononuclear cell infiltration in muscle layer	0/4	0/4	0/4	2/3	0/4	0/4	0/4	0/3

[1] Sacrificed animals were not included in this table;
[2] All findings very slight to slight;
Statistical significance: $^{*}p<0.05$; $^{**}p<0.01$.

Conclusion:

In this study, the target organ identified was the gastrointestinal tract. The NOAEL in this study was at 2 mg/kg bw/d based on increased absolute and relative liver weight in females and slightly increased frequency of diarrhoea.

3.12.1.8 Anonymous (2000a)

See section 3.9.1

3.12.1.9 Anonymous (2000b)

See section 3.9.1

3.12.1.10 Anonymous (2014d)

Reference: A 28-day dermal toxicity study of pethoxamid in rats

Author(s), year: Anonymous, (2014d)
Report/Doc. number: 1216 PXA / Charles Rivers, Testing Facility Study Number 20039159
Guideline(s): OECD 410 (1981)
GLP: Yes
Deviations from OECD 410 (1981): No
Acceptability: Yes

EXECUTIVE SUMMARY

Rats (Crl:CD (SD)) were administered pethoxamid or the control substance once daily for a 6-hour exposure period for 28 consecutive days at dose levels of 0, 100, 300 or 1000 mg/kg bw/day. The following parameters and end points were evaluated in this study: viability, clinical signs, dermal scoring, body weights, body weight changes, food consumption, ophthalmic examinations, clinical pathology evaluations, necropsy examinations, organ weights and histopathological evaluations.

Dermal exposure to pethoxamid at dose levels as high as 1000 mg/kg bw/day for 28 consecutive days did not result in any adverse clinical signs, differences in body weights, feed consumption values, any of the variables evaluated during the detailed clinical observations or the ophthalmologic examination performed at the end of the exposure period for both male and female rats. There was a statistically significant increase in the number of male rats at 1000 mg/kg bw/day observed with slight erythema and flaking compared with the control group. Slight erythema and flaking were observed in females at all exposure levels (including controls). No test substance-related or adverse changes were observed in haematology or clinical chemistry. At necropsy, there were no test substance-related adverse gross lesions noted. Both absolute and relative liver weights were increased in the high dose males by 9%, reaching statistical significance only for the relative liver weights. As these findings were not accompanied by clinical chemistry changes or histopathological findings, they were not considered adverse.

All of the microscopic alterations apparent during the histopathological evaluation of the study were considered to represent common background lesions that were not relevant to dermal exposure to pethoxamid. Decreased numbers of anagen-phase follicles with some associated minimal to mild hyperkeratosis were noted within sections of treated skin from the male and female rats in the 1000 mg/kg bw/day dose group, but these findings were considered to most likely be due to treatment-related localized irritation and/or increased grooming behaviour.

In conclusion, repeated dermal exposure to pethoxamid resulted in some localized skin irritation (at the site of exposure) in male rats at 1000 mg/kg bw/day and in female rats at all dose levels (including controls). The no-observed-adverse-effect-level (NOAEL) for systemic toxicity produced by dermal exposure to pethoxamid for 28 days is considered to be 1000 mg/kg bw/day for male and female rats.

MATERIALS AND METHODS

Materials:

Test material:	Pethoxamid technical
Lot/batch number:	P1351-JaK-T2-23-6
Purity:	95.80% (w/w) (dose calculation was not adjusted to take account of purity)
Stability of test item:	06 January 2014 (stored at ambient temperature) *NB: stable during the conduct of the study*
Storage conditions:	At room temperature, protected from light

Study Design:

The test material was applied to the shaved back of male and female Crl:CD(SD) rats (10/sex/group) at dose levels of 0, 100, 300 or 1000 mg/kg bw/day for a 6-hour exposure period for 28 days. The test material

was applied neat and was covered with a semi-occlusive wrap. After 6 hours, the wrap was removed and the site was washed.

RESULTS AND DISCUSSION

Mortality: No mortalities occurred.

Clinical signs: No treatment-related findings were observed.

Skin reactions: 1000 mg/kg bw/day: A statistically significant increase in the number of male rats with grade 1 erythema and flaking was observed.
Body weight gain: No treatment-related findings were observed.

Food consumption: No treatment-related findings were observed.

Haematology: No treatment-related findings were observed.

Clinical Chemistry: No treatment-related findings were observed.

Ophthalmology: No treatment-related findings were observed.

Organ Weights: 1000 mg/kg bw/day: An increase in absolute and relative liver weight was observed in males, being statistically significant only for the relative liver weight. This finding was not accompanied by clinical chemistry findings or liver histopathology; therefore it was not considered adverse.

Macroscopic findings: No treatment-related findings were observed.

Microscopic findings: No treatment-related findings were observed.

Table 3.12.1-19: Summary of findings from 28-day dermal toxicity study in rats

Parameter	**Males**			
	Dose Level (mg/kg bw/day)			
	0	**100**	**300**	**1000**
Mortality	0	0	0	0
Body Weight (g)				
Day 1	302.3	302.7	302.9	306.3
Day 28	407.7	412.1	406.3	410.8
Body Weight Gain (g)				
Days 1 to 28	105.4	109.4	103.4	104.5
Food Consumption				
Absolute Food Consumption (g/day)				
Days 1 to 28	27.2	26.9	27.3	26.6
Relative Food Consumption (g/kg/day)				
Day 1 to 28	74.8	73.8	75.4	73.4
Skin findings				
Residue[1]	0/0[2]	52/9**	51/10**	55/8**

Erythema, grade 1	8/3	0/0	3/3	21/7*
Flaking, grade 1	6/2	0/0	0/0	21/6**
Back: Scab(s)	0/0	0/0	0/0	45/4**
Ophthalmology findings	No treatment-related effects			
Haematology	No treatment-related effects			
Clinical chemistry	No treatment-related effects			
Organ weights (g)				
Liver (absolute)	10.91	11.08 (+ 2%)	11.12 (+ 2%)	11.9 (+ 9%)
Liver (relative)	2.842	2.851 (+ 0%)	2.898 (+ 2%)	3.100* (+ 9%)
Necropsy findings	No treatment-related findings			
Microscopic findings	No treatment-related findings			
Parameter	Females			
	Dose Level (mg/kg bw/day)			
	0	100	300	1000
Mortality	0	0	0	0
Body Weight (g)				
Day 1	226.9	226.9	226.5	228.6
Day 28	257.3	259.8	257.0	265.6
Body Weight Gain (g)				
Days 1 to 28	30.4	32.9	30.5	37.0
Food Consumption				
Absolute Food Consumption (g/day)				
Days 1 to 28	19.0	18.6	18.4	19.2
Relative Food Consumption (g/kg/day)				
Days 1 to 28	78.2	75.6	75.9	77.6
Skin findings				
Residue[1]	0/0[2]	109/9**	179/10**	164/10**
Erythema, grade 1	63/8	16/5	5/3	14/4
Erythema, grade 2	12/2	5/1	0/0	0/0
Flaking, grade 1	56/7	14/2**	4/1**	20/5
Flaking, grade 2	2/1	0/0	0/0	0/0
Back: Scab(s)	47/5	13/3	11/2	57/7
Ophthalmology findings	No treatment-related effects			
Haematology	No treatment-related effects			
Clinical chemistry	No treatment-related effects			
Organ weights	No treatment-related effects			
Necropsy findings	No treatment-related findings			

Microscopic findings	No treatment-related findings

[1] Presumed to be test material residue that couldn't be washed off.
[2] Number of observations/number of animals. *p<0.05; **p<0.01.

CONCLUSION

In conclusion, repeated dermal exposure to pethoxamid resulted in some localized skin irritation (at the site of exposure) in male rats at 1000 mg/kg bw/day and in female rats at all dose levels (including controls). The no-observed-adverse-effect-level (NOAEL) for systemic toxicity produced by dermal exposure to pethoxamid for 28 days is considered to be 1000 mg/kg bw/day for male and female rats.

3.12.2 Human data

No relevant studies.

3.12.3 Other data

No relevant studies.

3.13 Aspiration hazard

3.13.1 Animal data

No relevant studies.

3.13.2 Human data

No relevant studies.

3.13.3 Other data

No relevant studies.

4 ENVIRONMENTAL HAZARDS

4.1 Degradation

4.1.1 Ready biodegradability

4.1.1.1 144 PXA (1999)

Reference: TKC-94 Assessment of ready biodegradability: modified sturm test
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 044/984510, 144 PXA
Guideline(s): OECD Test Guideline 301, 1992; US EPA OPPTS 835.3110, 1998
GLP: Yes
Deviations: No
Acceptability: Yes

Materials:

Lot/batch number:	TB-9960306-C
Purity:	94.8 %

The study was conducted to a current test method (OECD Test Guideline 301, 1992) and no major study deficiencies were identified. The data in 144 PXA (1999) indicated that pethoxamid is not readily biodegradable. Therefore, re-analysis was not considered necessary and the following EU endpoint is proposed: Pethoxamid is not readily biodegradable.

4.1.2 BOD_5/COD

No relevant studies.

4.1.3 Hydrolysis

4.1.3.1 42 PXA (1999)

Reference: TKC-94 Hydrolysis under Laboratory Conditions
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 023/983760, WAS2001-56, 42 PXA
Guideline(s): US EPA, N, 161-1, 1982; SETAC, 1995
GLP: Yes
Deviations: No
Acceptability: Yes

Materials:

Lot/batch number:	TP-960418A
Purity:	99.9 %

Although the study was not conducted to the current test method (OECD Test Guideline 111, 2004), no major study deficiencies were identified. Data in 42 PXA (1999) indicate that pethoxamid is stable to hydrolysis at all pH values investigated. Therefore, re-analysis was not considered necessary and the following EU endpoints are proposed: Pethoxamid is stable under conditions of abiotic hydrolysis at pH 4, 7 and 9 (buffer solutions, 50 °C and sterile conditions).

4.1.4 Water, water-sediment and soil degradation studies

4.1.4.1 1443 PXA (2015)

Reference: Aerobic Mineralization of [^{14}C]Pethoxamid in Surface Water
Author(s), year: Anonymous, 2015
Report/Doc. number: 2517W-1, 1443 PXA
Guideline(s): OECD Test Guideline 309, 2004
GLP: Yes
Deviations: No
Acceptability: Yes

Executive Summary:

An aerobic mineralisation study was conducted with [phenyl-^{14}C]pethoxamid using aerobic surface water (pelagic test). Pethoxamid was applied at two concentrations, 102.3 µg / L (high dose) and 10.3 µg / L (low dose) and the samples were incubated in the dark under aerobic conditions at 20 °C. In addition, reference and sterile control samples were incubated to confirm the microbial activity of the

test water and examine possible abiotic degradation, respectively. The test was performed in flow-through systems allowing humidified air to pass over the sample headspace and through traps to collect volatile organic components (foam bung) and $^{14}CO_2$ (NaOH). Sample aliquots were taken at eight time points throughout the study and analysed by LSC. The distribution of radioactivity between pethoxamid and its degradates was determined in the high dose samples (only) by HPLC. Mass balance averaged 95.2 % AR and 94.9 % AR for the high and low dose samples, respectively. At the end of the study $^{14}CO_2$ averaged 5.7 % AR and 4.9 % AR for the high and low dose samples, respectively, demonstrating that mineralisation of pethoxamid was not dose dependent. Pethoxamid degraded moderately under the conditions of the test and represented an average of 57.3 % AR at the end of the study. The main metabolite observed was MET-30 (a cysteine conjugate), which represented an average of 19.6 % AR at the end of the incubation period. The degradation rate of pethoxamid was determined with the Hockey Stick (HS) model and the half-life was 138 days.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid
Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi/mmol
Radiochemical purity: 96.3 %
2. Test systems: One US natural surface water characterised by AGVISE laboratories

Table 4.1.4-1: Physical and chemical properties of the water used

Water	pH	Hardness (mg $CaCO_3$ / L)	Conductivity (mmhos / cm)	Dissolved Solids (ppm)	Turbidity (NTU)	Ca (ppm)	Mg (ppm)	Na (ppm)
Lake Tuckahoe	7.5	68	0.21	56	1.06	16	6.4	7.5

Study Design:

1. Experimental conditions

The aerobic mineralisation of [^{14}C]pethoxamid was studied in lake water (pelagic test). Pethoxamid was applied at the rate of 102.3 µg / L in three aqueous samples (high dose samples) and 10.3 µg / L in three aqueous samples (low dose samples). Two additional samples were dosed with 11.3 µg / L [^{14}C]benzoic acid as positive controls. One final sample was prepared using lake water sterilised by autoclave, dosed at a rate of 20.5 µg / L as an abiotic control. The water was dispensed into amber glass flasks and traps (polyurethane foam bungs and sodium hydroxide) were attached to the outlet of each treated flask (except the abiotic controls) to trap volatiles. Solutions of the test substance in acetonitrile were prepared, diluted and dispensed into each unit. The organic solvent added was equivalent to 0.05 % (high concentration) or 0.005 % (low concentration) by volume. Following treatment, each flask was incubated at 20 ºC and stirred continuously throughout the course of the study. Humidified air was pulled over the samples and through the traps. The kinetic modelling followed the guidance of FOCUS kinetics employing the software tool for kinetic evaluation KinGUI (v.2).

2. Sampling

Sub-samples from each test system were removed at each sampling interval through a sampling port. Sampling for the high and low dose samples was conducted at zero time and the following intervals; 7, 13, 21, 28, 67 and 102 days. Additional water samples were also removed and analysed for entrained CO_2 by acidification. [^{14}C]benzoic acid dosed vessels were sampled after 13 and 21 days of incubation. Sterile vessels were sampled after 13 days incubation. At 49, 67 and 102 day sampling occasions, the dissolved oxygen (DO) of the water and the pH of the high and low dose samples were recorded. NaOH

traps were analysed at each sampling occasion. Foam bung traps were analysed at the end of the incubation.

3. Description of analytical procedures

Aliquots of aqueous samples (and NaOH traps) were quantified by LSC. Foam bung traps were extracted with acetonitrile prior to sampling for LSC. High dose samples (only) were further analysed by HPLC by direct injection with co-chromatography with reference standard solutions. Peak assignments for parent and degradates were based on co-elution with reference standards. Metabolite confirmation was performed by LC/MS analysis of selected samples.

Results and Discussion:

Results of the [^{14}C]benzoic acid dosed vessels at 13 and 21 days show that the system was viable during the conduct of the study. Dissolved oxygen measurements indicate that the system remained aerobic throughout the study period (6.31 – 7.84 ppm). pH measurements ranged from 7.31 to 8.54 and averaged 7.74. The stability and homogeneity of the test substance under conditions of administration were confirmed by LSC and HPLC analysis. Total mass balances for the high dose and low dose samples averaged 95.2 % AR and 94.9 % AR, respectively. Mass balance for the sterile vessel was 97.5 % AR. Volatile compounds accounted for < 6.3 % AR for all samples tested. Results of acidified samples showed < 2 % AR in the system was attributable to dissolved CO_2. The distribution of radioactivity between pethoxamid and its degradates was determined in the high dose samples only. Pethoxamid represented an average of 57.3 % AR at the end of the test. An unknown metabolite (U-1) was observed in the course of the study (max. 19.6 % AR at the end of the test). LC/MS/MS analysis of a representative aqueous sample identified the metabolite as MET-30 (a cysteine conjugate of pethoxamid), and its identity was confirmed by co-chromatography with an authentic MET-30 reference standard by HPLC. Another minor unknown metabolite (U-2) was observed at a maximum of 4.2 % AR after 102 days. At the end of the study $^{14}CO_2$ averaged 5.7 % AR and 4.9 % AR for the high and low dose samples, respectively, demonstrating that mineralisation of pethoxamid was not dose dependent.

Table 4.1.4-2: Mass balance of [^{14}C]pethoxamid expressed as % AR for low dose samples

% of AR	Incubation Time (days)							
	0	7	13	21	28	49	67	102
Water	96.1	96.7	94.7	93.0	91.1	89.3	90.6	89.1
NaOH	n.a.	0.4	1.0	1.8	2.4	3.6	4.1	4.9
Foam Plug	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.0
Total	96.1	97.0	95.8	94.8	93.5	92.9	94.7	94.0

Table 4.1.4-3: Mass balance of [^{14}C]pethoxamid expressed as % AR for high dose samples

% of AR	Incubation Time (days)							
	0	7	13	21	28	49	67	102
Water	95.2	96.0	95.6	92.0	92.3	92.1	89.1	89.5
NaOH	n.a.	0.3	1.2	1.9	2.4	3.7	4.5	5.7
Foam Plug	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.0
Total	95.2	96.4	96.8	93.9	94.7	95.8	93.6	95.2

Table 4.1.4-4: Product balance of [^{14}C]pethoxamid expressed as % AR for high dose samples (metabolites > 5 % shaded in grey)

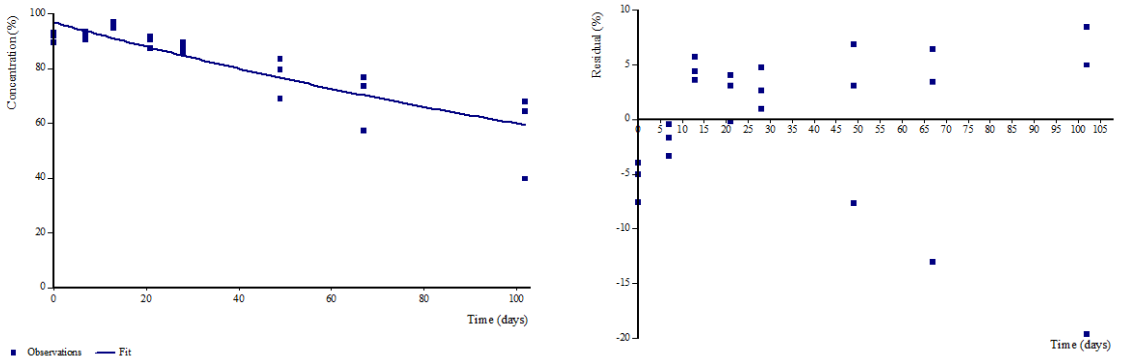
% of AR	Incubation Time (days)							
	0	7	13	21	28	49	67	102
Pethoxamid	91.3	91.8	95.6	89.9	87.4	77.3	69.1	57.3
MET-30	0.0	0.0	0.0	0.9	2.9	8.8	11.9	19.6

U-2	0.0	0.0	0.0	0.0	0.0	2.6	2.5	4.2
Others[a]	3.8	4.2	0.0	1.2	2.0	3.4	5.6	8.4
CO_2	NA	0.3	1.2	1.9	2.4	3.7	4.5	5.7

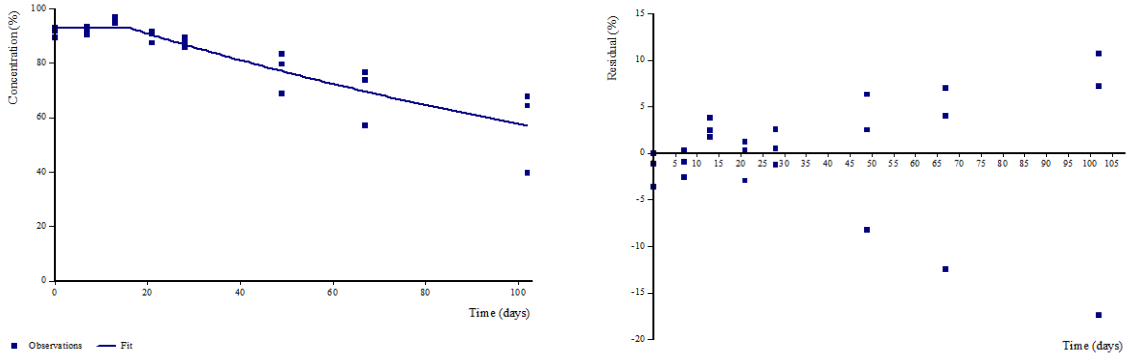
[a] individual peaks represent < 5 % AR

The *DT50* and *DT90* of pethoxamid in aerobic surface water (high dose samples only) were calculated using the Hockey-Stick (HS) kinetic model and based on FOCUS guidance. The HS model represented the best fit for the data.

Figure 4.1.4-1: Kinetic fit of pethoxamid to residues (% AR) measured in aerobic water (high dose samples)



Aerobic water, Lake Tuckahoe – SFO



Aerobic water, Lake Tuckahoe – HS

Table 4.1.4-5: Summary of pethoxamid dissipation kinetics in aerobic water (high dose samples, persistence triggering endpoints in bold)

Test medium	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DT50* / *DT90* (d)
Aerobic water, Lake Tuckahoe	SFO	k (d^{-1})	0.0048	0.0006	2.9	< 0.001	144 / 480
	HS	k_1 (d^{-1})	0.0[a]	-	1.2	-	-
		k_2 (d^{-1})	0.0057	0.0008		< 0.001	-
		t_{split} (d)	16.1	6.5		nd	-
		Overall	-	-		-	**138 / 423**

[a] Fixed to 0

Conclusions:

Pethoxamid degraded moderately in aerobic surface water to one main metabolite, MET-30 (a cysteine conjugate). MET-30 represented a maximum of 19.6 % AR after 102 days. Production of $^{14}CO_2$ in high and low dose samples averaged 5.7 % and 4.9 % AR, respectively, demonstrating that the mineralisation of pethoxamid was not dose dependent.

Comments (RMS AT):

Kinetic fitting was redone by the applicant in accordance with pertinent FOCUS guidance using CAKE 3.1. Only this reassessment is shown in the study evaluation above. The kinetic re-assessment is considered acceptable. The applicant claims to use results from the HS fit (lower χ^2 error) as a persistent endpoint whereas the more simple SFO fit (*DT50* = 144 days) is considered equally appropriate by the RMS AT.

4.1.4.2 1444 PXA (2015)

Reference: Aerobic aquatic metabolism of [^{14}C]pethoxamid
Author(s), year: Anonymous, 2015
Report/Doc. number: 2518W-1, 1444 PXA
Guideline(s): OECD Guideline 308, 2002
GLP: Yes
Deviations: No
Acceptability: Yes
Status: New submission

Executive Summary:

An aerobic aquatic metabolism study was conducted with [phenyl-U-^{14}C]pethoxamid using two water/sediment systems; Golden Lake (GL) and Goose River (GR). Pethoxamid was applied to the water layers at the rate of 0.12 µg/mL (GL) or 0.11 µg/mL (GR) and the samples were incubated in the dark under aerobic conditions at 20 °C for up to 102 days. The test was performed in amber bottles, each connected to a set of traps. Humidified air was gently bubbled through the samples and then through the traps to collect volatile organic components (ethylene glycol) and $^{14}CO_2$ (NaOH). The dissolved oxygen, pH and redox potential of the test systems were measured throughout the study. At each time point the water layers were decanted and the sediments were extracted three times with acetonitrile:water (4:1, v/v). Selected sediment samples were also subject to harsh microwave extraction with the same solvent. Samples were analysed by LSC and the distribution of radioactivity between pethoxamid and its degradates was determined by HPLC and, in selected samples, by 2-D TLC. Mass balances averaged 98.1 % AR (GL) and 99.0 % AR (GR). Pethoxamid degraded quickly throughout the study in both water/sediment systems tested. MET-6 was the major metabolite observed, reaching a maximum of 9.4 % AR at 14 days (GL). MET-2, MET-3, MET-22 and MET-42 were also observed, but represented less than 5 % AR throughout the study. An unknown metabolite was detected in both test systems at a maximum of 8.8 % AR (GL). The unknown metabolite was identified by high-resolution accurate mass LC-MS and named MET-104. The rate of degradation of pethoxamid in the total system was determined using SFO kinetics, which represented the best fit to the data. DT50 and DT90 values were 7.0 days and 23.1 days, respectively, for the GL test system, and 13.0 days and 43.1 days, respectively, for the GR test system.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid

Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide

Lot/Batch #: CFQ41685
Specific Activity: 29 mCi/mmol
Radiochemical purity: 100 %
Reference standards: Pethoxamid, MET-1, MET-2, MET-3, MET-4, MET-5, MET-6, MET-22, MET-31, MET-42, MET-46 (all unlabelled)

2. Test systems: Two US water/sediment systems characterised by AGVISE laboratories.

Table 4.1.4-6: Physical and chemical properties of the water/sediment systems used

Sediment									
System	pH (H_2O)	OC (%)	Sand (%)	Silt (%)	Clay (%)	CEC (mEq / 100 g)	Biomass (mg C / 100 g sediment)	Sediment texture USDA	Bulk density (g / cm^3)
Golden Lake	8.2	0.8	88	8	4	9.5	Start: 329 End: 218	Sand	1.19
Goose River	7.8	3.1	32	38	30	19.3	Start: 389 End: 249	Clay Loam	0.97

Water								
System	pH	Hardness (mg $CaCO_3$ / L)	Conductivity (mmhos / cm)	Dissolved Solids (ppm)	Turbidity (NTU)	Ca (ppm)	Mg (ppm)	Na (ppm)
Golden Lake	8.7	679	1.43	1170	10.5	110	97	97
Goose River	8.1	897	1.74	1496	16.0	192	100	117

CEC = Cation exchange capacity, OC = Organic carbon

Study Design:

1. Experimental conditions

The aerobic aquatic metabolism of [^{14}C]pethoxamid was studied in two water sediment systems. Pethoxamid was applied at the target rate of 0.12 µg/mL, equivalent to 1200 g a.s./ha assuming a water depth of 1 m, and samples were incubated under aerobic conditions at 20 °C for up to 102 days. The sediment and water were dispensed (approximately 3:1 volume ratio) into amber bottles and the samples were allowed to acclimatise under study conditions for 10 days prior to treatment. Ethylene glycol and NaOH solutions were attached to each treated flask to trap volatiles. Solutions of the test substance in acetonitrile were prepared, diluted and dispensed into each unit. Following treatment, humidified air was gently bubbled through the samples and then through the traps to collect volatile organic components and $^{14}CO_2$ throughout the course of the study. The sediment biomass was also monitored over the period of the study by the substrate induced respiration (SIR) method. The kinetic modelling followed the guidance of FOCUS kinetics employing the software tool for kinetic evaluation KinGUI (v. 2)

2. Sampling

Duplicate samples of each test system were removed at each sampling interval. Sampling was conducted

at time 0 and after 3, 7, 14, 31, 60 and 102 days of incubation. At each sampling occasion, the dissolved oxygen (DO) of the water and the pH and redox potential (ORP) of the water and sediment were recorded. Water and sediment layers were separated prior to analysis. Sample processing began immediately following sampling.

3. Description of analytical procedures

Aliquots of water layers were analysed by LSC. Sediment extraction was performed by shaking with 100 mL of acetonitrile:water (4:1, v/v) followed by centrifugation (three times). The extracts were combined before sampling for LSC analysis. Water and sediment extracts were further analysed by HPLC analysis (where appropriate) with co-chromatography with reference standard standards solutions. Peak assignments for parents and degradates were based on co-elution with reference standard, and identities confirmed by 2-D TLC in selected samples. $BaCl_2$ precipitation was used for CO_2 confirmation for selected trapping samples. Selected post-extracted sediment samples were subjected to microwave accelerated extraction and analysed by LSC. Residual sediment samples were homogenised and combusted for analysis by LSC. Post-extracted samples from 102 days were selected for humic acid/fulvic acid partitioning.

Results and Discussion:

Results of the biomass at the start and the end of the study show that both sediments were viable during the conduct of the study. Dissolved oxygen and redox potential measurements indicate that the systems remained aerobic throughout the study period. The stability of the test substances under conditions of administration was confirmed by HPLC analysis. Total mass balances for the Golden Lake (GL) and Goose River (GR) systems averaged 98.1 % AR and 99.0 % AR, respectively. Bound residues in the GL system increased during the study period to a maximum of 63 % AR at 60 days and decreased to an average of 51.7 % AR by 102 days. In the GR system the bound residues increased to a maximum of 64.9 % AR at 101 days. Pethoxamid in the total water/sediment system represented an average of 0.9 % AR (GL) and 1.0 % AR (GR) at the end of the study. The major metabolite observed was MET-6, which reached a maximum of 9.4 % AR (GL) and 8.0 % AR (GR) at 14 days and 31 days, respectively. MET-2 was present at a maximum of 3.0 %AR (GL) and 4.5 % AR (GR) at 60 days and 31 days, respectively. MET-3, MET-22 and MET-42 were also detected throughout the study period, but these metabolites averaged less than 3.3 % AR. The second major metabolite observed was an unknown which did not co-elute with any of the supplied reference standards (Unknown 1). Unknown 1 represented a maximum of 8.8 % AR (GL) and 4.2 % AR at 31 days.

A sample containing pethoxamid, MET-6 and Unknown 1 was prepared by fraction collecting multiple HPLC injections of a sediment extract. The sample was analysed by high-resolution accurate mass LC/MS which allowed structural elucidation of the metabolite. The structure has been named MET-104, and its identity was confirmed by a small scale chemical synthesis.

Table 4.1.4-7: Product balance of [^{14}C]pethoxamid expressed as % AR for Golden Lake system (mean of two replicates, metabolites > 5 % shaded in grey)

Substance	Compartment	Incubation Time (days)						
		0	**3**	**7**	**14**	**31**	**60**	**102**
Pethoxamid	Water	95.1	86.9	39.1	17.2	1.5	0.3	0.2
	Sediment	4.1	5.3	11.5	3.6	2.3	0.6	0.7
	Total system	99.2	92.2	50.6	20.8	3.8	0.9	0.9
MET-6	Water	0.0	0.0	1.8	3.8	0.4	0.5	0.6
	Sediment	0.0	1.6	6.2	5.7	6.2	3.4	3.8
	Total system	0.0	1.6	8.0	9.4	6.6	3.9	4.4

MET-104	Water	0.0	0.0	0.0	0.0	0.0	0.3	0.0
	Sediment	0.0	0.0	0.6	6.2	8.8	0.4	0.8
	Total system	0.0	0.0	0.6	6.2	8.8	0.7	0.8
MET-2	Water	0.0	0.0	0.0	0.0	0.6	0.2	0.2
	Sediment	0.0	0.0	0.0	0.8	0.0	2.8	1.7
	Total system	0.0	0.0	0.0	0.8	0.6	3.0	1.9
MET-3	Water	0.0	0.0	0.0	0.0	0.0	0.3	0.2
	Sediment	0.0	0.0	0.0	0.0	0.0	0.5	0.2
	Total system	0.0	0.0	0.0	0.0	0.0	0.8	0.4
MET-22	Water	0.0	0.0	0.0	0.0	2.3	0.6	0.8
	Sediment	0.0	0.0	0.0	0.0	0.0	1.7	1.5
	Total system	0.0	0.0	0.0	0.0	2.3	2.3	2.3
MET-42	Water	0.0	0.0	0.0	0.0	0.0	1.3	2.0
	Sediment	0.0	0.0	0.0	0.0	0.0	0.6	0.2
	Total system	0.0	0.0	0.0	0.0	0.0	1.9	2.2
Others*	Water	0.0	0.0	0.0	0.0	4.6	3.4	3.4
	Sediment	0.0	0.0	0.0	1.3	0.0	4.0	4.7
	Total system	0.0	0.0	0.0	1.3	4.6	7.4	8.1
CO_2 trapped in water layers	Water	na	na	na	na	1.2	0.5	0.6
Bound Residues	Sediment	0.0	6.3	45.0	51.3	59.7	63.0	57.1
Volatile Organics	Total system	na	0.0	0.0	0.0	0.0	0.0	0.0
CO_2	Total system	na	0.3	1.2	3.5	9.9	12.1	16.3

* Individual peaks represent < 4.5% AR (total system)

Table 4.1.4-8: Product balance of [^{14}C]pethoxamid expressed as % AR for Goose River system (mean of two replicates, metabolites > 5 % shaded in grey)

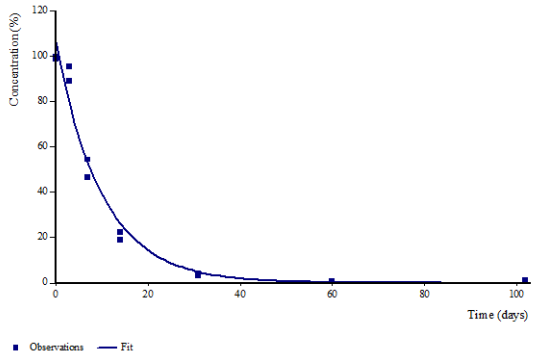
Substance	**Compartment**	**Incubation Time (days)**						
		0	**3**	**7**	**14**	**31**	**60**	**102**
Pethoxamid	Water	96.2	67.6	76.9	29.7	12.1	1.0	0.2
	Sediment	4.9	14.6	8.9	12.4	5.9	1.8	0.8
	Total system	101.1	82.2	85.8	42.1	18.0	2.8	1.0
MET-6	Water	0.0	0.0	0.0	0.0	1.8	0.8	0.3
	Sediment	0.0	1.9	1.3	4.1	6.2	5.3	3.0
	Total system	0.0	1.9	1.3	4.1	8.0	6.1	3.3
MET-104	Water	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sediment	0.0	0.0	0.0	3.5	4.2	0.5	0.7
	Total system	0.0	0.0	0.0	3.5	4.2	0.5	0.7
MET-2	Water	0.0	0.0	0.0	0.0	3.6	0.5	0.3

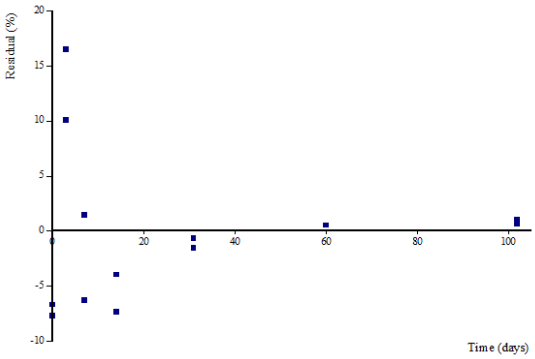
	Sediment	0.0	0.0	0.0	0.0	0.9	2.3	0.9
	Total system	0.0	0.0	0.0	0.0	4.5	2.8	1.2
MET-3	Water	0.0	0.0	0.0	0.0	0.0	0.3	0.3
	Sediment	0.0	0.0	0.0	0.0	0.0	0.2	0.4
	Total system	0.0	0.0	0.0	0.0	0.0	0.5	0.7
MET-22	Water	0.0	0.0	0.0	0.0	1.2	1.1	0.5
	Sediment	0.0	0.0	0.0	0.0	1.1	2.2	1.4
	Total system	0.0	0.0	0.0	0.0	2.3	3.3	1.9
MET-42	Water	0.0	0.0	0.0	0.0	1.1	0.8	1.2
	Sediment	0.0	0.0	0.0	0.0	0.0	1.1	1.0
	Total system	0.0	0.0	0.0	0.0	1.1	1.9	2.2
Others*	Water	0.0	0.0	0.0	0.0	4.1	4.1	2.3
	Sediment	0.0	0.3	0.0	0.0	0.0	4.1	7.1
	Total system	0.0	0.3	0.0	0.0	4.1	8.2	9.4
CO_2 trapped in water layers	Water	NA	NA	NA	NA	3.3	0.8	0.3
Bound Residues	Sediment	0.1	13.5	11.1	50.1	50.4	56.8	64.9
Volatile Organics	Total system	NA	0.0	0.0	0.0	0.0	0.0	0.0
CO_2	Total system	NA	0.5	0.7	2.7	5.0	11.4	13.4

* Individual peaks represent < 3.1% AR (total system)

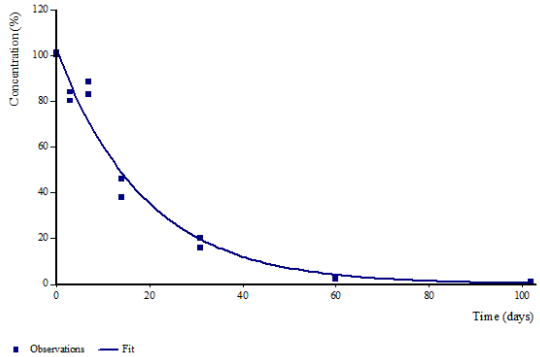
The *DT50* and *DT90* of pethoxamid in the total water/sediment system were calculated using the SFO and FOMC kinetic models and based on FOCUS guidance. The SFO model represented the best fit for the data.

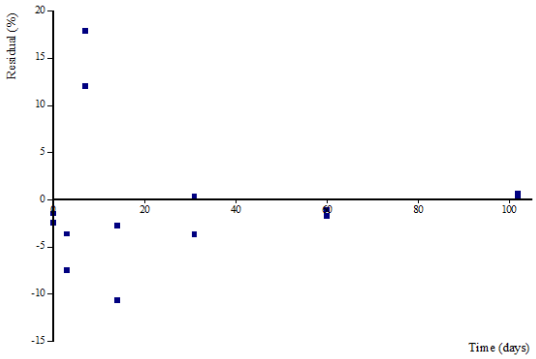
Figure 4.1.4-2: Kinetic fit (all SFO) of pethoxamid to residues (% AR) measured in two water/sediment systems (<u>total system</u>)





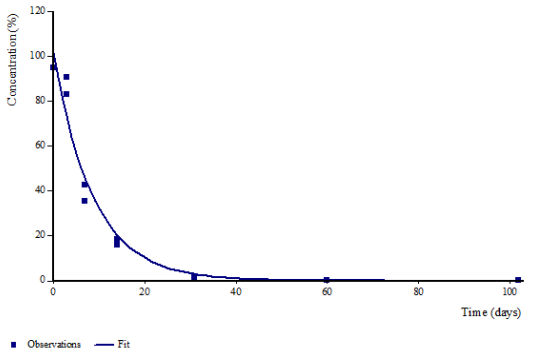
Golden Lake – Total system – SFO

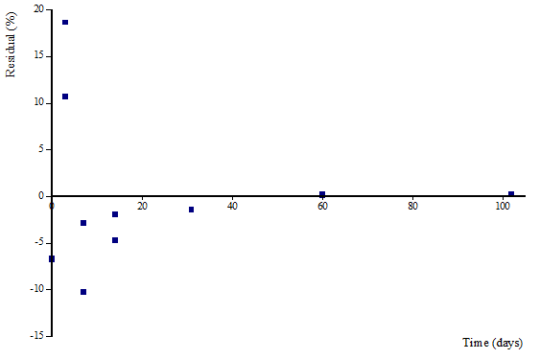




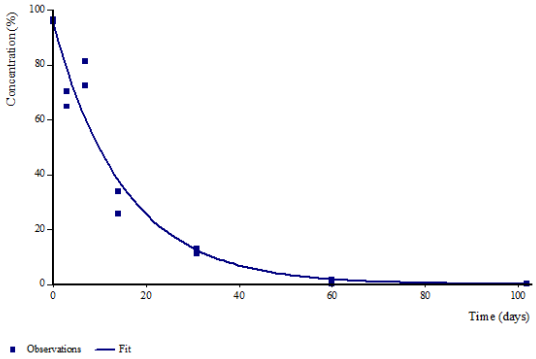
Goose River – Total system – SFO

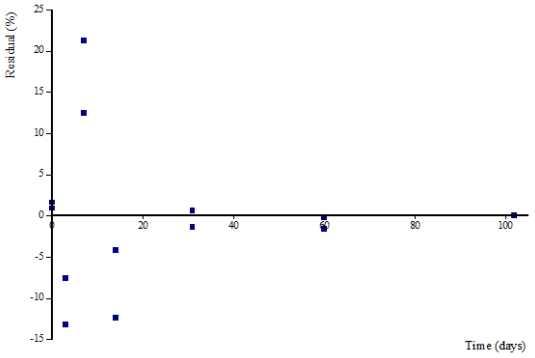
Figure 4.1.4-3: Kinetic fit (all SFO) of pethoxamid to residues (% AR) measured in two water/sediment systems (<u>water phase</u>)





Golden Lake – Water phase – SFO





Goose River – Water phase – SFO

Table 4.1.4-9: Summary of pethoxamid degradation/dissipation kinetics in two water/sediment systems (all SFO kinetics)

Test medium	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DT50 / DT90* (d)
Golden Lake – Total system	SFO	*k* (d^{-1})	0.100	0.010	12.9	< 0.001	7.0 / 23.1
Goose River – Total system	SFO	*k* (d^{-1})	0.053	0.006	11.1	< 0.001	13.0 / 43.1
Golden Lake – Water phase	SFO	*k* (d^{-1})	0.115	0.013	15.6	< 0.001	6.0 / 20.1
Goose River – Water phase	SFO	*k* (d^{-1})	0.065	0.010	16.0	< 0.001	10.6 / 35.2

Conclusions:

Pethoxamid degraded quickly in aerobic water/sediment systems to two main metabolites, MET-6 and MET-104, MET-6 representing a maximum of 9.4 % AR at 14 days and MET-104 representing a maximum of 8.8 % AR at 31 days. Using SFO kinetics, the *DT50* values for pethoxamid in the total water/sediment systems were between 7.0 and 13.0 days. *DT90* values were between 23.1 and 43.1 days.

4.1.4.3 134 PXA (2000)

Reference: (^{14}C)-TKC-94: Soil Metabolism and Degradation
Author(s), year: Anonymous, 2000
Report/Doc. number: CLE 1465/2-D2142, 134 PXA
Guideline(s): SETAC, 1995; JMAFF, 1985
GLP: Yes
Deviations: No major deviations
Acceptability: Yes

The rate of degradation of ^{14}C-pethoxamid (TKC-94) was studied in four UK soils (PT 102, PT 103, PT 070 and SK 961089) under aerobic conditions in darkness at 45 % of the soils maximum water holding capacity at ca. 20 ºC, in PT 102 also at ca. 10 ºC. Separate samples (50 g dry weight) of the test soils (2 mm sieved) were acclimatised for 7 to 12 days under test conditions prior to test substance application. Soils were treated with a single application of ^{14}C-pethoxamid in acetonitrile at a nominal rate of 0.06 mg/50 g dry weight soil. ^{14}C-pethoxamid was radiolabelled uniformly in the phenyl ring. To determine the microbial biomass of the soils at the end of the incubation period, control soil samples were treated with non-radiolabelled test compound and incubated under the same conditions as for the labelled soil samples.

The water content of the test soils was maintained at 45 % of their maximum water holding capacity during the incubation period. Samples were taken at 0, 1, 2, 3, 6, 10, 30, 59, 90 and 120 days after application.

Soil samples were extracted with acetonitrile and aqueous acetonitrile, combined, concentrated and analysed by TLC and HPLC. Unextractable bound residues were air-dried, combusted in oxygen and quantified by LSC. Radiolabelled volatile degradation products were trapped and quantified. The soil microbial biomass was determined prior to application, and in control vessels sampled at the end of the incubation period.

Table 4.1.4-10: Characteristics of the test soil

Parameter	**PT 102**	**PT 103**	**PT 070**	**SK 961089**
Clay (< 2 µm)	9.6	11.5	11.4	28.1
Silt (2 – 63 µm)	44.5	12.4	62.8	42.8
Sand (63 µm – 2 mm)	46.0	76.1	25.9	29.1
Textural class (UK)	Sandy silt loam	Sandy loam	Sandy silt loam	Clay loam
Textural class (BBA)	Silty loam sand	Loamy sand	Sandy loam silt	Clay loam
Textural class (USDA)[a]	Loam	Sandy loam	Silt loam	Clay loam
Soil pH (1:2.5 v/v in water)	7.2	5.3	6.6	7.8
Soil pH (KCl)	6.8	4.6	5.9	7.2
Organic carbon (%)	1.7	1.0	1.9	3.5
Organic matter[b] (%)	2.9	1.7	3.3	6.0
Cation exchange capacity (mEq/100g)	14.9	10.0	17.0	38.2
MWHC (% w/w dry soil)	58.4	45.6	63.5	81.1
WHC at pF 2.5 (% w/w dry soil)	17.4	10.5	20.6	31.0
Microbial biomass (µg C per g soil)				
Pre-study	227.3	263.5	250.1	734.3
Post-study	273.2 (20 °C) 368.9 (10 °C)	123.3	141.2	883.2

[a] Estimated USDA texture (refer to comment section)
[b] Calculated as organic carbon content × 1.724

Results:

The mean recovery of applied radioactivity from the soil samples in each treatment group was in the range 85.5 to 98.5 %. The distribution of the applied radioactivity between the extractable residues, non-extractable residues and evolved volatiles was similar for each soil type. Extractable radioactivity decreased in the 20 ºC soils from 96 to 98 % at time zero to ca. 12 to 26 % after 120 days. Unextractable radioactivity increased during the incubation period representing ca. 21 to 35 % of the applied radioactivity after 120 days. This non-extractable radioactivity was found upon further fractionation to be evenly distributed between the humin, humic acids and fulvic acids fractions at the 10 and 90 day sample points for each soil. Volatile radioactivity recovered in the sodium hydroxide trapping solution increased throughout the course of the study and represented 34 to 46 % of the applied radioactivity after 120 days. This was later confirmed to be from mineralised $^{14}CO_2$.

Analysis of the concentrated soil extracts from the 20 ºC incubation indicated that pethoxamid rapidly declined to between 24 and 40 % after 10 days and thereafter to < 2 % after 120 days.

Degradation products formed at 20 °C were at levels < 10 % of the applied radioactivity. The most significant of these products, unk@51'30 (confirmed by LC-MS/MS to be MET-42) and unk@47'58 were present at maximum levels of 9.7 and 9.0 %, respectively, 30 days after application. Other degradation products, MET-2, unk@1'20 and unk@36'20 were present at maximum levels of 3.2, 2.9 and 4.4 % of the applied radioactivity. Other unidentified degradation products were present at low levels.

Degradation of pethoxamid in soil incubated at 10 ºC resulted in the formation of one major degradation product, unk@51'30 (MET-42), which represented a maximum of 11.2 % of the applied radioactivity. At least 4 other degradation products (MET-2, unk@1'20, unk@36'20, and unk@47'58) were present at maximum levels of 2.1, 2.0, 9.0 and 4.9 % of the applied radioactivity.

Table 4.1.4-11: Extraction and recovery of radioactivity from soil PT 102 (20 °C and 10 °C) after application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates)

DAT	20 °C					10 °C				
	Extracted	Not extracted	Volatiles		Total	Extracted	Not extracted	Volatiles		Total
			Organic	$^{14}CO_2$				Organic	$^{14}CO_2$	
0	97.60	0.10	n.a	n.a	97.70	98.02	0.08	n.a	n.a	98.10
1	93.72	3.42	n.d	1.36	98.50	95.46	1.73	n.d	0.81	97.99
2	89.50	5.75	n.d	2.33	97.58	93.59	2.88	n.d	1.36	97.82
3	84.02	9.25	0.02	3.41	96.69	91.17	3.91	n.d	1.56	96.63
6	72.34	16.67	0.08	6.87	95.95	86.98	7.15	n.d	2.48	96.61
10	62.52	21.81	0.10	10.99	95.41	81.12	11.26	0.02	4.55	96.94
30	43.00	30.60	0.09	20.09	93.77	60.74	23.01	n.d	11.97	95.72
59	36.52	31.68	0.07	25.42	93.69	47.19	27.83	n.d	19.59	94.61
90	29.23	32.43	0.06	31.59	93.30	41.93	29.50	0.02	21.60	93.05
120	20.60	35.14	0.07	34.21	90.02	38.31	32.06	n.d	24.99	95.36

n.a – not applicable, n.d – not detected

Table 4.1.4-12: Extraction and recovery of radioactivity from soil PT 103 and PT 070 after application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates)

DAT	PT 103					PT 070				
	Extracted	Not extracted	Volatiles		Total	Extracted	Not extracted	Volatiles		Total
			Organic	$^{14}CO_2$				Organic	$^{14}CO_2$	
0	96.86	n.d	n.a	n.a	96.86	96.34	n.d	n.a	n.a	96.34
1	92.18	2.67	n.d	0.63	95.47	93.06	2.32	n.d	0.17	95.54
2	87.07	5.65	0.01	2.47	95.19	88.33	4.82	n.d	1.66	94.80
3	83.20	8.93	n.d	3.68	95.80	87.58	7.58	n.d	1.23	96.38
6	72.84	15.22	n.d	7.44	95.50	77.28	13.15	n.d	5.11	95.54
10	59.91	22.45	n.d	11.48	93.83	63.69	22.70	n.d	8.61	94.99
30	41.34	28.30	n.d	22.95	92.58	40.53	31.88	n.d	18.15	90.56
59	34.29	25.70	n.d	30.61	90.59	30.91	31.65	n.d	28.90	91.47
90	29.59	24.84	n.d	35.88	90.31	24.06	32.85	n.d	35.96	92.87
120	26.01	20.65	0.01	38.85	85.51	20.22	31.58	n.d	35.96	87.75

n.a – not applicable, n.d – not detected

Table 4.1.4-13: Extraction and recovery of radioactivity from soil SK 961089 after application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates)

DAT	SK 961089				
	Extracted	Not extracted	Volatiles		Total
			Organic	$^{14}CO_2$	
0	97.71	0.05	n.a	n.a	97.76
1	90.48	4.19	n.d	0.72	95.38
2	87.00	7.34	n.d	1.55	95.88
3	82.69	9.23	n.d	1.93	93.85
6	74.15	15.15	n.d	2.39	91.68
10	55.29	26.71	n.d	4.48	86.47
30	31.25	33.74	0.17	27.08	92.22
59	20.00	34.81	n.d	37.57	92.38
90	13.66	34.67	n.d	42.55	90.88
120	11.80	33.29	n.d	46.20	91.28

n.a – not applicable, n.d – not detected

Table 4.1.4-14: HPLC profile of extractable radioactivity from soil PT 102 maintained at 20 ºC following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	92.39	nd	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	5.21	97.60
1	87.33	0.60	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.92	1.34	3.87	93.72
2	77.79	0.78	n.d	n.d	n.d	0.84	n.d	n.d	n.d	3.76	1.91	6.32	89.50
3	65.92	0.79	n.d	n.d	n.d	2.41	n.d	n.d	n.d	9.52	3.19	5.38	84.02
6	50.39	1.42	n.d	n.d	n.d	0.38	3.68	5.37	n.d	7.66	2.94	3.42	72.34
10	24.23	1.67	n.d	n.d	n.d	1.35	5.56	8.58	1.99	14.70	3.74	4.45	62.52
30	3.83	2.20	n.d	0.40	n.d	2.36	5.45	9.67	4.41	12.12	3.70	2.56	43.00
59	2.39	2.13	n.d	n.d	n.d	1.86	4.83	8.07	2.89	10.85	2.96	3.49	36.52
90	1.72	2.05	n.d	n.d	n.d	2.19	3.30	6.07	2.52	9.75	2.33	1.63	29.23
120	0.46	1.32	n.d	0.66	n.d	2.30	1.47	3.84	3.10	4.95	1.79	2.48	20.60

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-15: HPLC profile of extractable radioactivity from soil PT 102 maintained at 10 ºC following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	92.82	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	5.20	98.02
1	91.72	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	3.74	95.46
2	83.70	0.44	n.d	n.d	n.d	0.60	n.d	n.d	n.d	3.03	1.66	5.82	93.59
3	78.65	0.77	n.d	n.d	n.d	0.74	n.d	n.d	n.d	6.07	2.41	4.94	91.17
6	72.73	0.45	n.d	n.d	n.d	1.08	n.d	n.d	n.d	7.43	3.91	5.30	86.98
10	59.42	0.90	n.d	n.d	n.d	n.d	2.37	5.91	1.10	8.89	2.43	2.52	81.12
30	21.95	1.08	n.d	1.17	n.d	1.34	4.86	11.17	4.64	12.02	4.81	2.51	60.74
59	6.71	2.08	n.d	0.42	n.d	1.64	3.49	8.90	8.14	12.71	3.92	3.10	47.19
90	3.71	2.02	n.d	n.d	n.d	1.52	3.72	9.29	2.34	16.69	6.05	2.64	41.93
120	1.94	1.78	n.d	n.d	n.d	2.03	2.61	7.86	9.02	9.96	3.46	3.11	38.31

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-16: HPLC profile of extractable radioactivity from soil PT 103 following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	95.01	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.85	96.86
1	89.87	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	2.30	92.18
2	77.81	2.07	n.d	1.25	0.79	0.36	n.d	n.d	n.d	2.38	1.18	2.41	87.07
3	69.79	2.02	n.d	n.d	n.d	0.38	n.d	n.d	n.d	7.63	1.96	3.38	83.20
6	48.75	2.40	n.d	n.d	n.d	2.13	n.d	n.d	n.d	15.39	3.95	4.15	72.84
10	30.31	1.49	n.d	n.d	n.d	0.66	7.14	2.49	n.d	15.65	3.43	2.18	59.91
30	4.52	2.18	1.05	0.99	n.d	1.48	9.01	4.04	n.d	15.86	3.37	3.58	41.34
59	2.33	2.35	0.31	1.25	n.d	1.53	6.07	2.73	n.d	15.74	3.64	2.00	34.29
90	1.45	2.00	0.33	n.d	n.d	1.21	5.99	1.98	n.d	14.79	2.14	1.84	29.59
120	1.09	1.91	0.36	n.d	n.d	1.19	5.76	1.93	n.d	12.58	2.30	1.18	26.01

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-17: HPLC profile of extractable radioactivity from soil PT 070 following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
7	95.54	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.80	96.34
1	91.71	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.35	93.06
2	81.10	1.15	n.d	0.81	n.d	0.87	n.d	n.d	n.d	2.21	1.29	2.17	88.33
3	74.37	1.42	n.d	n.d	n.d	0.78	n.d	n.d	n.d	5.34	2.08	5.66	87.58
6	59.31	1.54	n.d	n.d	n.d	2.74	n.d	n.d	n.d	10.55	3.05	3.12	77.28
10	40.18	0.62	n.d	n.d	n.d	0.36	5.85	3.41	0.38	8.84	2.86	4.06	63.69
30	5.80	1.99	n.d	n.d	n.d	1.21	7.93	5.37	3.56	11.93	3.87	2.75	40.53
59	2.92	2.26	n.d	n.d	n.d	1.41	3.94	5.12	2.57	10.94	2.72	1.74	30.91
90	2.07	1.44	n.d	n.d	n.d	1.27	2.65	2.94	3.22	8.68	1.64	1.80	24.06
120	1.29	1.58	n.d	n.d	n.d	1.70	1.62	2.80	3.22	7.10	1.33	0.89	20.22

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-18: HPLC profile of extractable radioactivity from soil SK 961089 following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	96.55	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.16	97.71
1	86.57	1.01	n.d	n.d	n.d	0.27	n.d	n.d	n.d	2.07	1.24	0.54	90.48
2	77.83	0.87	n.d	0.47	n.d	0.59	n.d	n.d	n.d	6.14	2.78	1.10	87.00
3	65.23	1.53	n.d	n.d	n.d	0.38	n.d	n.d	n.d	11.41	4.04	4.12	82.69
6	48.79	1.52	n.d	n.d	n.d	2.24	n.d	n.d	n.d	18.09	6.22	3.51	74.15
10	25.84	1.27	n.d	n.d	n.d	0.39	4.43	6.73	0.86	10.24	2.83	5.53	55.29
30	4.78	3.19	n.d	0.14	n.d	1.62	3.31	5.18	1.90	10.10	2.55	1.02	31.25
59	3.72	3.24	n.d	n.d	n.d	2.14	1.76	3.38	0.39	3.42	0.92	1.95	20.00
90	2.75	2.48	n.d	n.d	n.d	2.73	1.01	1.53	n.d	2.30	0.70	0.86	13.66
120	1.47	2.07	n.d	n.d	n.d	2.87	0.91	1.54	n.d	2.27	0.70	0.67	11.80

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Summary:

In soil under aerobic conditions, pethoxamid is initially metabolised via glutathione conjugation with subsequent loss of glycine and glutamic acid to form an intermediate cysteine conjugate, followed by formation of a thiol via beta lyase cleavage – all of which are transitory. Subsequent oxidation gives MET-101 or a sulfonic acid, MET-42. MET-42 is then degraded to MET-100.

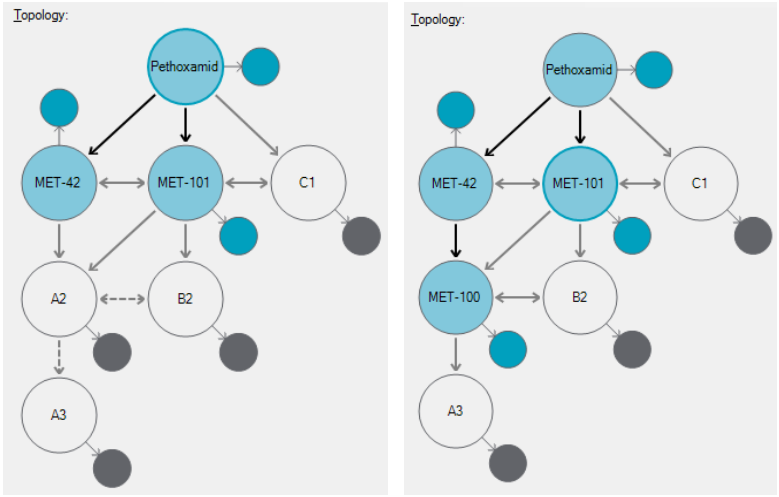
Similar degradation pathways have been documented for compounds containing the chloroacetyl group.

4.1.4.4 1475 PXA (2015)

Reference: Kinetic Analysis of Pethoxamid and its Metabolites in Aerobic Soil Degradation Studies **Author(s), year**: Anonymous, 2015 **Report/Doc. number**: CHA 100625, 1475 PXA **Guideline(s)**: Not applicable **GLP**: No **Deviations**: Not applicable **Acceptability**: Yes

The rate of degradation of pethoxamid and the rates of formation and degradation of its metabolites MET-42, MET-101 and MET-100 in the laboratory aerobic soil degradation study 134 PXA (2000) have been subject to kinetic analysis based on the recommendations of the current EC guidance document in order to derive trigger and modelling endpoints. Calculations were performed using the kinetic evaluation tool CAKE (v. 3.1). The data were evaluated with the SFO model and two degradation schemes (one for studies conducted at 20 °C and one for the study conducted at 10 °C). The results of the kinetic analysis for trigger and modelling endpoints are summarised in the table below. In this case, trigger and modelling endpoints are the same because the SFO model provided an acceptable fit to all data sets.

Figure 4.1.4-4: Degradation schemes applied (left: 20 °C studies; right: 10 °C study)



The study of 134 PXA (2000) was conducted at a soil water content of 45 % maximum water holding capacity (MWHC). Therefore, the $DegT50_{lab}$ values calculated above require normalisation to reference conditions (20 °C and pF 2) to be appropriate as trigger or modelling endpoints. Normalisation to reference conditions was conducted following the EC guidance document (Generic Guidance for Tier 1 FOCUS Groundwater Assessments (version 2.2). Since field capacity (pF 2) was not measured for the soils used, FOCUS default values of water holding capacity for the corresponding soil type were used for the calculation. No correction was necessary with respect to temperature as the incubation studies were conducted at 20 °C.

Table 4.1.4-19: Correction factors for soil moisture and temperature for normalization of degradation rates to reference conditions (pF 2, 20 °C)

Soil name	Soil type (USDA)[a]	MWHC (% w/w)	Study MC, 45 % MWHC (% w/w)	WHC at pF 2 (% w/w)[b]	Moisture correction factor (-)[c]	Study temperature (°C)	Temperature correction factor (-)[d]
PT 102	Loam	58.4	26.3	25	1.0	20	1.0
PT 103	Sandy loam	45.6	20.5	19	1.0	20	1.0
PT 070	Silt loam	63.5	28.6	26	1.0	20	1.0
SK 961090	Clay loam	81.1	36.5	28	1.0	20	1.0

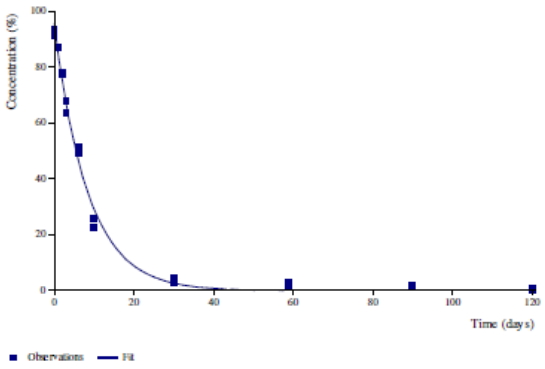
[a] Estimated; also refer to 134 PXA (2000)
[b] Generic Guidance for Tier 1 FOCUS Groundwater Assessments (version 2.2)
[c] Moisture correction factor is 1.0 if WHC at study conditions is above WHC at pF 2, otherwise moisture correction factor is (WHC at study conditions / WHC at pF2)$^{0.7}$
[d] Temperature correction factor based on Q_{10} value of 2.58 is $e^{0.0948 \times (Tref - Tact)}$, where T_{act} is the temperature at study conditions and T_{ref} is the temperature at reference conditions (20 °C)

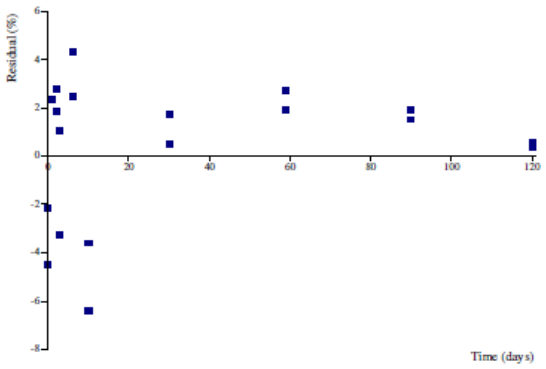
Results:

The SFO model provides a visually and statistically acceptable fit to the data for pethoxamid, MET-42 and MET-101 for all soils. It was not possible to derive reliable kinetic endpoints for MET-100 from the data available.

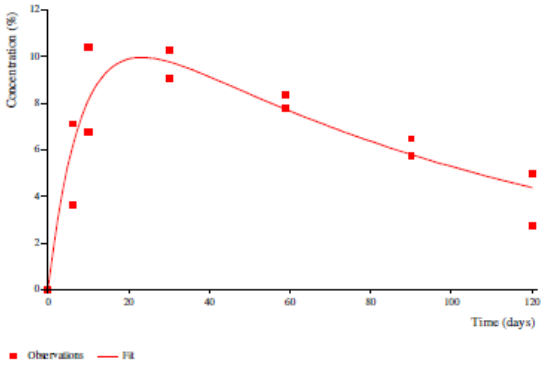
Figure 4.1.4-5: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 102 (20 °C)

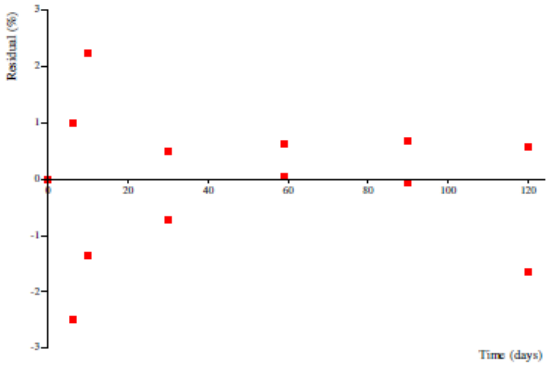
Compartment Parent:



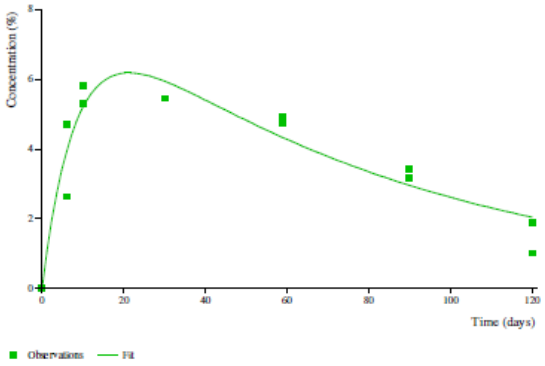


Compartment A1:





Compartment B1:



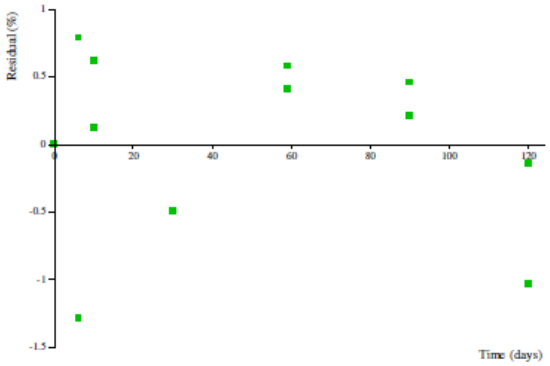
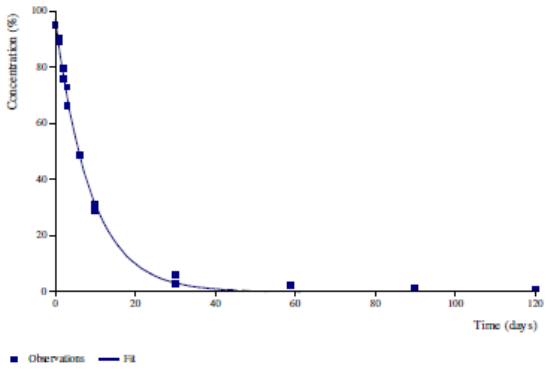
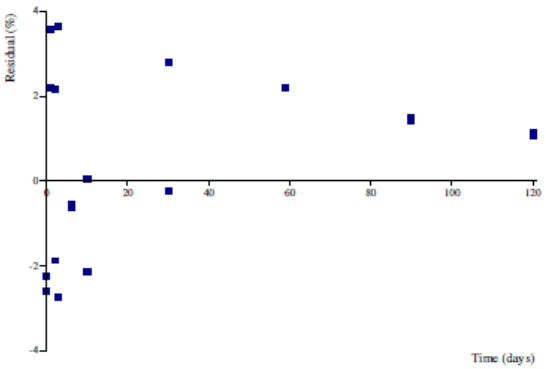


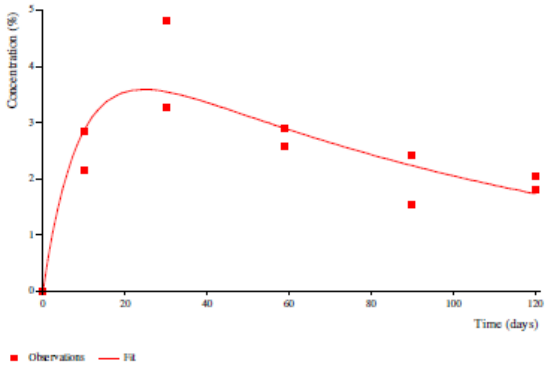
Figure 4.1.4-6: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 103

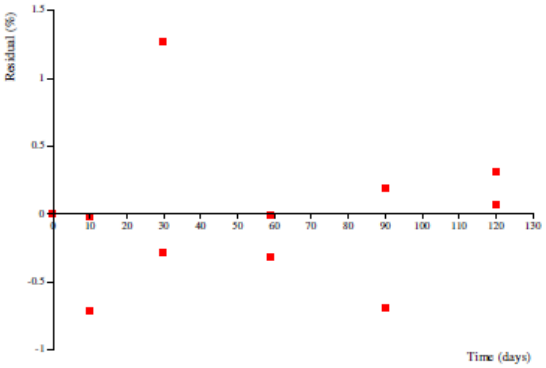
Compartment Parent:



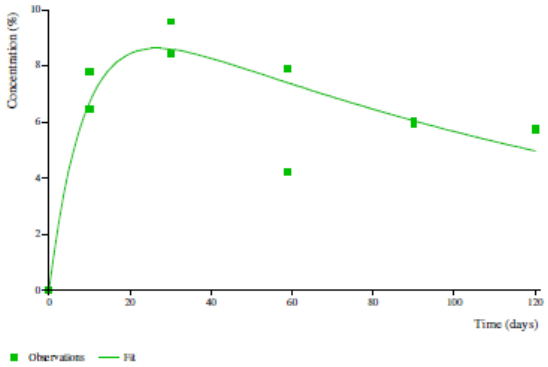


Compartment A1:





Compartment B1:



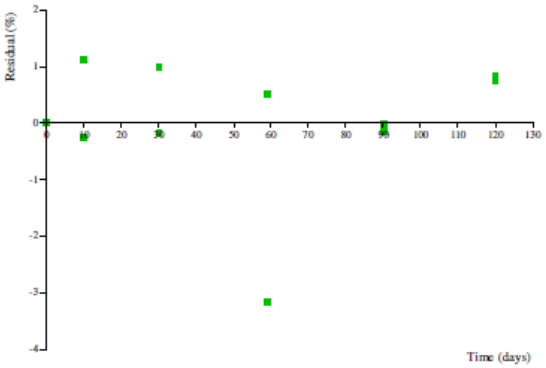


Figure 4.1.4-7: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 070

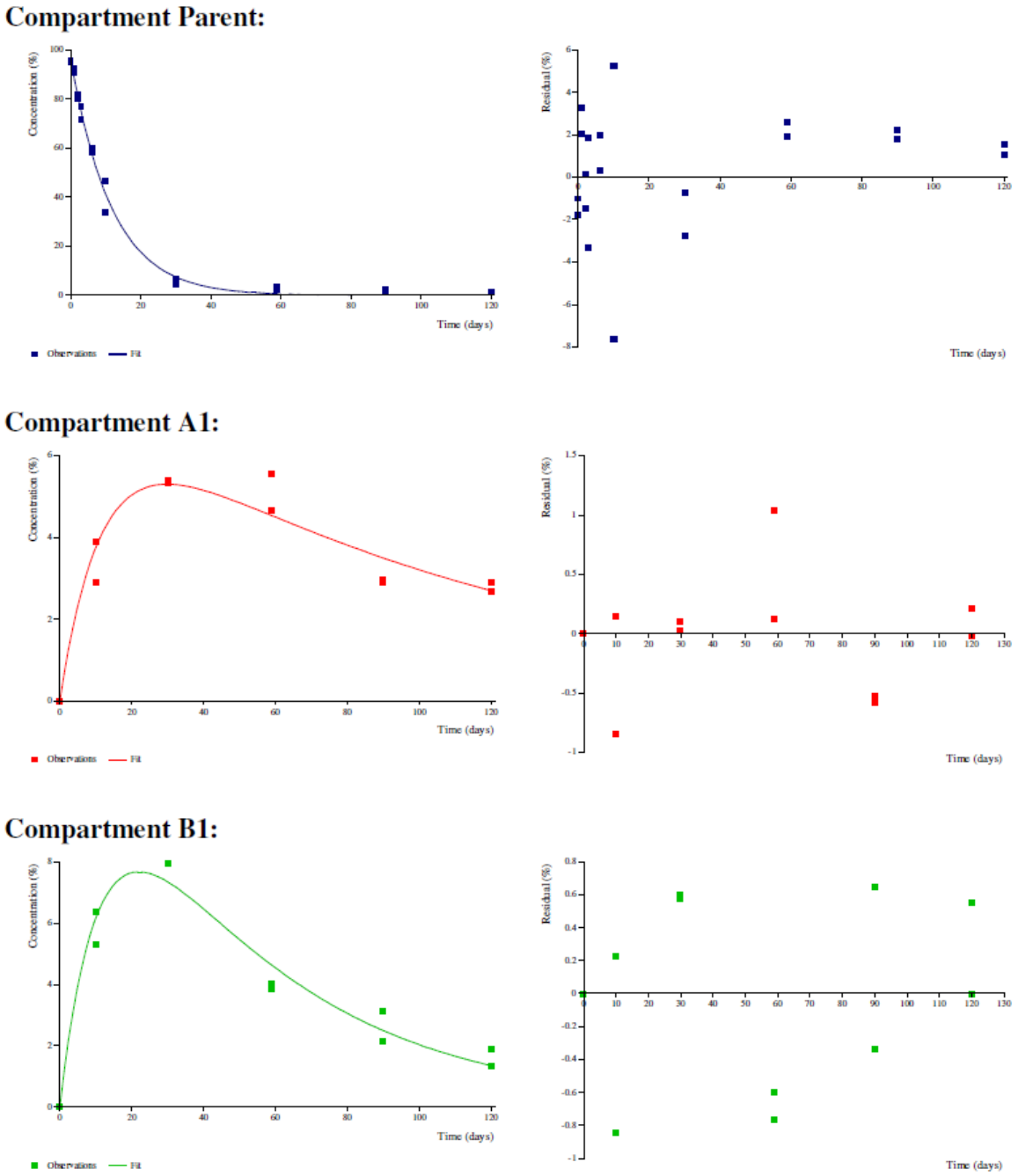
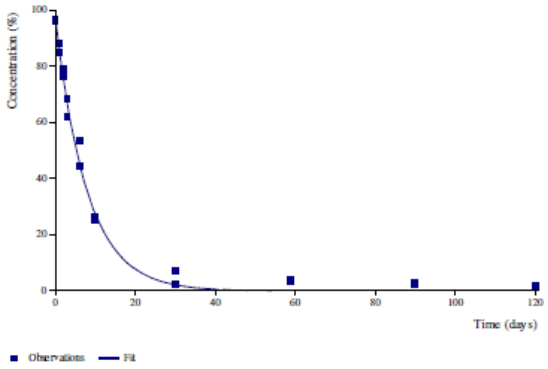
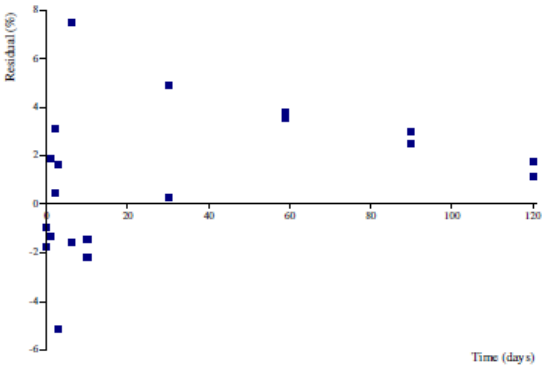


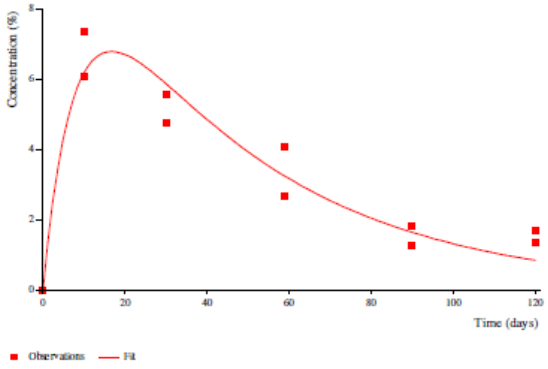
Figure 4.1.4-8: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil SK 961089

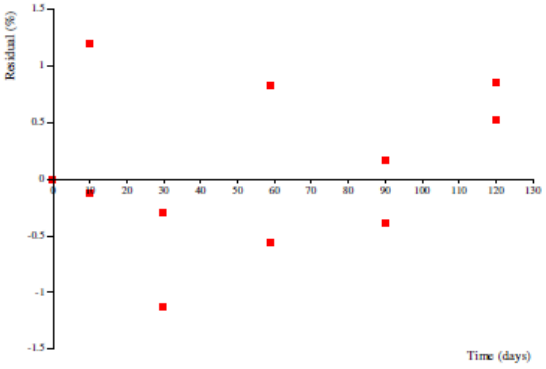
Compartment Parent:



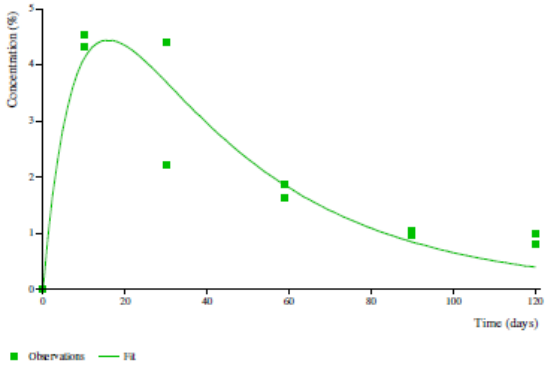


Compartment A1:





Compartment B1:



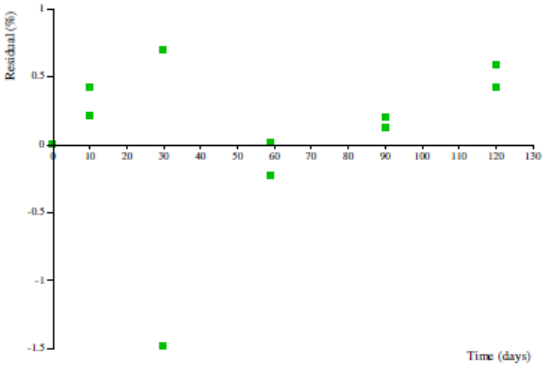
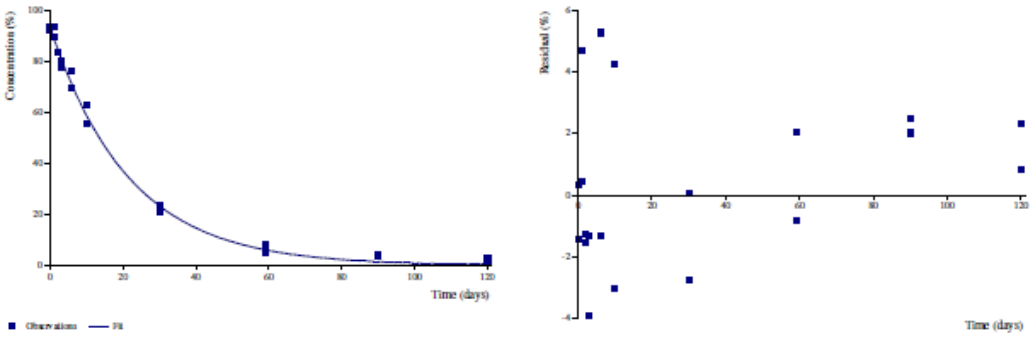
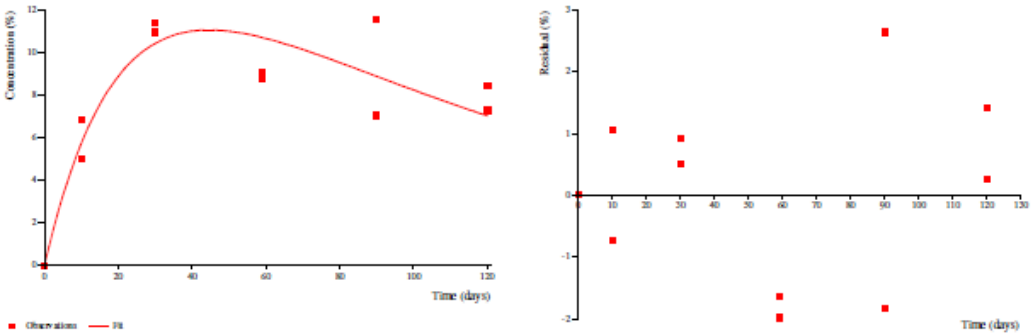


Figure 4.1.4-9: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1), MET-100 (compartment A2) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 102 (10 °C)

Compartment Parent:



Compartment A1:



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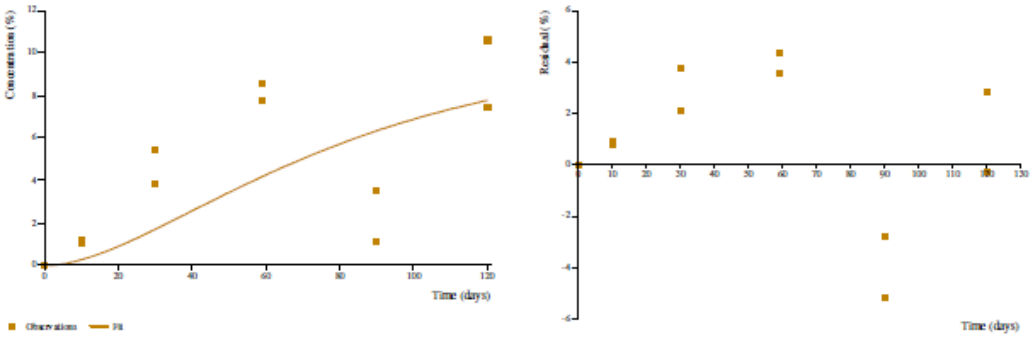


Compartment B1:

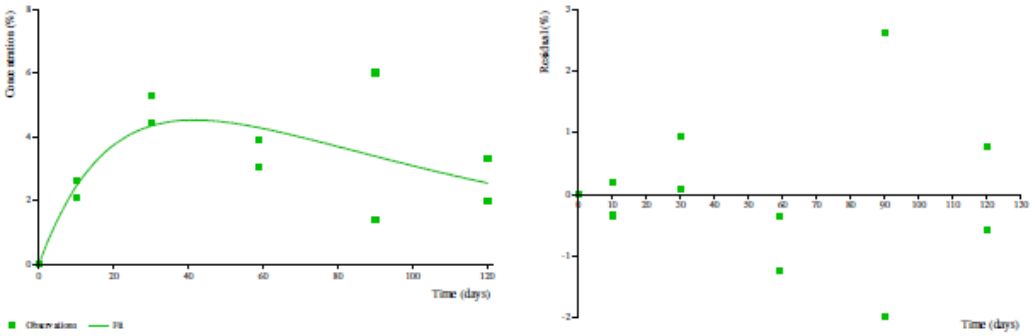


Table 4.1.4-20: Summary of pethoxamid, MET-42 and MET-101 degradation kinetics in aerobic soils at laboratory and reference conditions (20 °C and pF 2) <u>conducted at 20 °C</u> (all SFO kinetics)

Soil name	Compound	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90 (d)*	*DegT50* (d) 20 °C, pF2
PT 102	Pethoxamid	SFO	*k* (d^{-1})	0.119	0.006	5.2	< 0.001	5.9 / 19.4	5.9
	MET-42	SFO	*k* (d^{-1})	0.00935	0.00170	5.3	< 0.001	74.1 / 246	74.1
			ff (-)	0.13	0.01		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0123	0.0017	8.6	< 0.001	56.2 / 187	56.2
			ff (-)	0.08	0.01		nd	-	-
PT 103	Pethoxamid	SFO	*k* (d^{-1})	0.113	0.004	3.1	< 0.001	6.1 / 20.3	6.1
	MET-42	SFO	*k* (d^{-1})	0.00845	0.00206	9.6	< 0.001	82.0 / 272	82.0
			ff (-)	0.05	0.005		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.00654	0.00170	8.1	< 0.001	106 / 352	106
			ff (-)	0.11	0.01		nd	-	-
PT 070	Pethoxamid	SFO	*k* (d^{-1})	0.0852	0.0042	2.9	< 0.001	8.1 / 27.0	8.1
	MET-42	SFO	*k* (d^{-1})	0.00868	0.00135	8.0	< 0.001	79.9 / 265	79.9
			ff (-)	0.07	0.01		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0205	0.0020	8.1	< 0.001	33.8 / 112	33.8
			ff (-)	0.12	0.01		nd	-	-
SK 961089	Pethoxamid	SFO	*k* (d^{-1})	0.127	0.007	4.4	< 0.001	5.5 / 18.2	5.5
	MET-42	SFO	*k* (d^{-1})	0.0220	0.0029	11.1	< 0.001	31.5 / 105	31.5
			ff (-)	0.10	0.01		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0254	0.0044	11.6	< 0.001	27.3 / 90.8	27.3
			ff (-)	0.07	0.01		nd	-	-

Table 4.1.4-21: Summary of pethoxamid, MET-42, MET-101 and MET-100 degradation kinetics in one aerobic soil at laboratory and reference conditions (20 °C and pF 2) <u>conducted at 10 °C</u> (all SFO kinetics)

Soil name	Compound	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90 (d)*	*DegT50* (d) 20 °C, pF2
PT 102	Pethoxamid	SFO	*k* (d^{-1})	0.0462	0.0022	2.7	< 0.001	15.0 / 49.8	-
	MET-42	SFO	*k* (d^{-1})	0.00862	0.00209	9.0	< 0.001	80.4 / 267	-
			ff (-)	0.17	0.02		nd	-	-
	MET-100	SFO	*k* (d^{-1})	0.00292	0.0165	46.2	0.430	238 / 790	-
			ff (-)	1.00	0.83		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0104	0.0043	10.6	0.011	66.6 / 221	-
			ff (-)	0.07	0.02		nd	-	-

4.1.4.5 138 PXA (2000)

Reference: (^{14}C)-TKC-94: Metabolism in Flooded Soil
Author(s), year: Anonymous, 2000
Report/Doc. number: CLE 1465/3-D2142, 138 PXA
Guideline(s): SETAC, 1995; US EPA Subdivision N, Section 162-3,1982; JMAFF, 1985
GLP: Yes
Deviations: None
Acceptability: Yes

Test system:

The anaerobic route and rate of degradation of pethoxamid in a flooded UK sandy silt loam soil maintained at 20 °C, in darkness, under anaerobic conditions was studied over an incubation period of 365 days. Prepared individual flooded soil systems were incubated under study conditions for 32 days prior to test substance application in order to establish anaerobic conditions. ^{14}C-pethoxamid (uniform phenyl labelling) was applied to the surface water overlying the soil samples at a rate of 0.061 mg / 50 g soil, equivalent to a field application rate of 1.22 kg ai/ha. To allow determination of the soil microbial biomass at the end of the incubation period, control samples were treated with non-radiolabelled pethoxamid at the same rate and incubated under the same conditions as for the main study samples.

Duplicate treated samples were taken for analysis at predetermined intervals. Sampled water and soil were separated; the water acidified and partitioned with ethyl acetate, the soil was extracted with acetonitrile. Quantification of metabolites in water and soil was by HPLC. Selected samples were analysed by LC/MS to obtained further information on the chemical structures of significant metabolites. Radiolabelled volatile degradation products were trapped and quantified. Extracted soils were air-dried and combusted prior to radioassay. The unextracted soil residues from 30 and 59 day samples were Soxhlet extracted then fractionated into humin, humic acids, and fulvic acids.

Table 4.1.4-22: Soil characteristics

Parameter	Test soil
Particle size distribution (%) USDA	
Clay (< 2 µm)	10.0
Silt (2 – 50 µm)	43.2
Sand (50 – 2 mm)	46.8
Particle size distribution (%) UK, BBA	
Clay (< 2 µm)	9.6
Silt (2 – 63 µm)	44.5
Sand (63 – 2 mm)	46.0
Textural class (USDA)	Loam
Textural class (UK)	Sandy silt loam
Textural class (BBA)	Silty loam sand
Soil pH (1:2.5 v/v in water)	7.2
Soil pH (1:2.5 KCl)	6.8
Organic carbon (%)	1.7
Organic matter (%)	2.9
Cation exchange capacity (CEC, mEq / 100 g)	14.9
Water holding capacity at pF 0, MWHC (% w/w dry soil)	58.4
Water holding capacity at pF 2.5 (% w/w dry soil)	17.4

Microbial biomass (µg C / g)		
	Pre-study	227.3
	Post-study	324.7

Findings:

The overall recovery of radioactivity in all samples was in the range from 91 to 98 %. Over the incubation period, the quantity of radioactivity in the surface water decreased to 12 % AR. The quantity of extractable radioactivity from the soil increased to 28 % AR after 14 days and thereafter represented 19 % AR after 365 days. The level of unextractable radioactivity represented 61 % AR after 365 days. Volatile radioactivity represented 5 % AR after 365 days.

Pethoxamid was degraded in the test system representing < 1 % AR in the surface water after 30 days and 1 % AR in the soil extracts after 59 days.

Pethoxamid degraded to MET-22, 5 principal unknown components (1 – 8 % AR) and numerous minor components. MET-22 accounted for up to 8.8 % AR and Unk 1 (the largest unknown) accounted for up to 8.1 % AR from the total system.

The unextractable soil residues from the 30 and 59 day sampling intervals were Soxhlet extracted and then fractionated into humin, humic acids and fulvic acids. There was an approximately even distribution of radioactivity across the Soxhlet extract and three residue fractions. The activity in the Soxhlet extract comprised of Unk 3 (32 % of extract, *ca* 3 %AR) and other minor components. The organic phase phase after partition of the fulvic acid fractions comprised of one unknown component. The aqueous phase after partition of the humic acid fraction consisted of at least three poorly resolved components.

LC/MS analysis confirmed the presence of pethoxamid and MET-22 in selected samples. A structure was proposed for the unknown product Unk 1.

Table 4.1.4-23: Extraction and recovery of radioactivity from anaerobic soil after application of ^{14}C-pethoxamid (% of applied radioactivity, mean of two replicates)

DAT	Surface water	Soil extracts	Not Extracted	Volatiles		Total
				Organic	NaOH	Recovery
0	97.09	0.76	0.03	n.a	n.a	97.88
1	90.86	4.85	0.35	n.d	n.d	96.06
3	84.69	10.36	1.00	n.d	n.d	96.05
7	62.28	28.32	5.87	n.d	0.01	96.48
14	46.02	27.67	22.30	n.d	0.05	96.04
30	22.92	21.82	49.91	n.d	0.17	94.82
59	12.91	19.54	60.98	n.d	0.45	93.88
120	12.67	17.44	64.75	n.d	1.60	96.46
181	11.76	18.66	60.82	n.d	2.70	93.94
269	12.23	15.11	60.91	n.d	3.39	91.64
302[a]	13.36	13.86	60.31	n.d	3.80	91.33
330[a]	15.77	15.68	56.84	n.d	4.16	92.45
365	11.62	17.24	60.48	n.d	4.63	93.97

[a] Individual vessels
n.s – no sample, n.d – not detected

Table 4.1.4-24: Proportions of radioactive components in anaerobic soil and overlying surface water (total system) after application of ^{14}C-pethoxamid (% of applied radioactivity, mean of two replicates, metabolite > 5 % shaded in grey)

DAT	Pethoxamid	MET-22	Unk 1 [MET-46]	Unk 2	Unk 3	Unk 4	Unk 5	Other unknowns	Max 'other unknowns'	Unresolved Background	Total
0	93.67	n.d	0.38	n.d	n.d	n.d	n.d	0.36	016	0.34	94.76
1	94.06	n.d	0.47	n.d	n.d	n.d	n.d	0.51	0.29	0.94	95.98
3	92.98	n.d	0.23	0.13	n.d	n.d	n.d	0.08	0.08	0.97	94.41
7	84.85	0.69	1.02	0.59	0.16	0.06	n.d	0.14	0.09	0.24	87.76
14	52.24	3.19	5.51	1.25	1.06	0.46	0.08	3.90	0.98	0.61	68.32
30	10.62	7.17	8.10	1.68	1.00	0.97	n.d	8.25	1.50	0.32	38.11
59	1.48	6.67	4.77	3.44	1.40	1.48	0.50	8.90	1.68	0.37	29.01
120	0.56	7.53	6.02	3.95	1.49	1.24	0.72	5.82	1.07	0.23	27.57
181	0.43	8.77	4.11	3.32	1.15	2.28	1.94	4.41	1.09	0.19	26.61
269	0.31	7.26	5.79	3.96	0.68	1.68	1.22	3.87	1.16	0.08	24.88
302*	0.38	7.84	3.62	2.61	1.45	2.55	1.77	4.38	1.25	0.12	24.71
330*	0.50	8.08	6.79	5.01	0.93	2.46	1.51	4.19	1.31	0.31	29.78
365	0.19	6.78	7.23	3.98	1.35	1.30	1.01	5.21	1.36	0.33	27.40

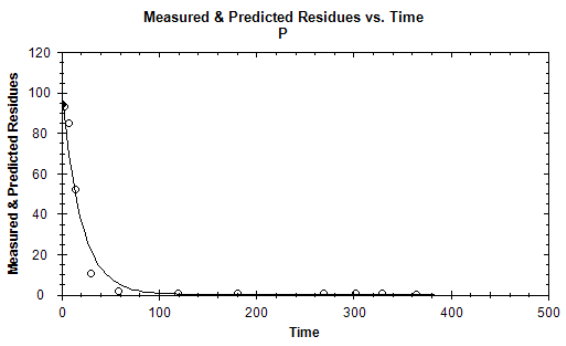
n.d – not detected
Max other unknowns – the largest single component found amongst 'Other unknowns'
Mean values of duplicate samples except where indicated '*'

Summary:

Pethoxamid was metabolised in soil under anaerobic conditions. Radioactive residues were incorporated into the soil matrix and evenly distributed between the humin, humic acid and fulvic acid fractions. The main degradation products were identified as MET-22 and Unk1 (later confirmed as MET-46) and represented a maximum of 8.8 and 8.1 % AR, respectively. Other minor degradates represented < 5 % AR.

Comments (RMS AT):

- Soil *DegT50* of pethoxamid under anaerobic conditions was recalculated by the RMS AT following pertinent FOCUS guidance using KinGUI 2:



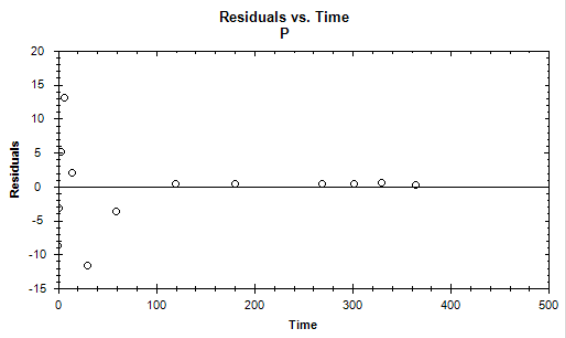


Figure 4.1.4-10: Kinetic fit (SFO) of pethoxamid to residues (% AR) measured in anaerobic soil – RMS AT assessment.

Table 4.1.4-25: Summary of pethoxamid degradation kinetics in anaerobic soil – RMS AT assessment

Soil	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90* (d)
UK sandy silt loam	SFO	*k* (d^{-1})	0.0507	0.0059	14.9	< 0.001	13.7 / 45.4

DegT50 of pethoxamid in soil under anaerobic conditions is considered to be 13.7 days.

4.1.4.6 135 PXA (2000)

Reference: Terrestrial Field Dissipation Study with TKC-94 EC60 Applied to Bare Soil in Spain and France in 1998 and 1999
Author(s), year: Anonymous, 2000
Report/Doc. number: TON 021/002214, 135 PXA
Guideline(s): SETAC, 1995; OEPP/EPPO Bulletin 23, 27-49, 1993; BBA Guidelines, Part IV, 4-1, 1986
GLP: Yes
Deviations: No major deviations
Acceptability: Yes

Test system:

Three terrestrial field dissipation trials were performed to establish the decline of residues of pethoxamid and its metabolite MET-42 resulting from a single application to bare soil at the maximum labelled rate of TKC-94 EC60 (ASU 95620 H, batch no. 9802, content: 599 g/l). A fourth trial (TON/021-02) was terminated 1998, because residues of pethoxamid were found in all the horizons within the soil cores at the 0 DAT event, which was thought to be due to the sandy soil type. When the soil cores were taken, a small gap was evident between

the soil core and the inside of the acetate tube. This allowed the sandy soil to run freely up and down the core, thus contaminating the whole sample.

Trials TON/021-01 (sandy clay loam, Spain), TON/021-03 (silty sandy loam, France) and TON/021-04 (sandy clay, Spain) were sprayed in May 1998, June 1998 and April 1999 respectively. The soil types of each trial were representative of crop production areas for the intended use of TKC-94 EC60. Details of the soil characteristics and microbial biomass of each trial site are given below.

Table 4.1.4-26: Characteristics of the soils from TKC-94 dissipation sites

Parameter	TON/021-01 (Spain)	TON/021-03 (France)	TON/021-04 (Spain)
pH (KCl)	7.5	6.9	7.3
Organic carbon (%)	0.8	0.8	0.8
Water holding capacity (at 0.001 bar suction (pF0) % w/w of dry soil)	57.8	59.5	49.7
Cation exchange capacity (mEq/100g)	19.1	14.8	16.3
Clay (%)	37	24	31
Silt (%)	23	46	16
Sand (%)	40	30	53
Classification (BBA)	Sandy clay loam	Silty sandy loam	Sandy clay
Microbial biomass (mg C / 100 g soil) (-1 DAT) (1)	10.88	3.06	204.13
Microbial biomass (mg C / 100 g soil) (120 DAT) (1)	7.00	5.02	5.40

(1)Mean value for the treated plots

The trial design at each site consisted of a non-treated plot (Plot 1) and two replicated treated plots (Plots 2 and 3). TKC-94 EC60 (emulsifiable concentrate at a nominal concentration of 600 g/l, actual concentration 599 g/l) was applied once at a nominal rate of 2 l/ha (1200 g ai/ha) by research backpack boom sprayer. The nominal application volume was 250 l/ha. The treatment rate was verified by the analysis of spray target cards placed on the test site prior to treatment. Adequate herbicides were applied to each plot for the duration of the study to maintain bare soil conditions.

Soil cores were taken at predetermined intervals and maintained frozen until analysed. Each sample consisted of twenty soil cores, collected using hydraulic soil coring equipment with 5 cm (diameter) × 50 cm (length) acetate tubes. Prior to analysis, the soil cores were cut into 0-10 and 10-20 cm horizons. For trial TON/021-01, as pethoxamid residues were found in the 10-20 cm horizons for sample points 0, 3, 7, 16, 26, 60 and 90 days after treatment, the 20-30 cm horizons for these time points were also analysed. Soil samples for microbial biomass determination were collected prior to treatment and nominally at 120 days after treatment.

Each soil sample (20 cores) was bulked according to horizon and sieved to remove any debris. Soil was extracted by shaking with aqueous acetone and an aliquot of the resulting extract reduced to dryness by rotary film evaporation. Quantification was performed by liquid chromatography using mass spectroscopy. Control soil samples were fortified with either pethoxamid or MET-42 to validate the analytical method. The LOD and LOQ were set at 2 ng/g and 10 ng/g respectively in soil for both compounds.

Findings:

Analysis of the deposition cards from the treated plots during application indicated a favourable comparison with the target application rate of 3.8 mg/5 deposition cards. Values ranged from 2.8 mg/5 deposition cards to 3.8 mg/5 deposition cards with a mean of 3.3 mg/5 deposition cards. This corresponded to 74-100 % (mean 86 %) of the actual application rate as determined by timed spray passes.

Procedural recoveries for pethoxamid and MET-42 in soil all fell within the acceptable range of 70 – 110 %. No residues of either pethoxamid or MET-42 were detected in any soil horizons taken from the untreated plot of any of the three trials.

Trial TON/021-01: A mean pethoxamid residue of 308 ng/g was detected in the soil horizon (0 – 30 cm) from the sample taken at the time of treatment. Thereafter the level of pethoxamid residues declined to less than the limit of detection (2 ng/g) in soil sampled 360 days after treatment (DAT). The presence of pethoxamid residues in the lower horizons up to 90 DAT was considered to be due to contamination occurring during the coring procedure. Mean residues of MET-42 reached a maximum of 30 ng/g in the 0 – 10 cm horizon of soils sampled 185 DAT and thereafter declined to less than the limit of detection in soils 360 DAT. No residues of MET-42 were at quantifiable levels in the 10–20 cm horizon of soils from this trial.

Trial TON/021-03: A mean pethoxamid residue of 746 ng/g was detected in the upper horizon (0 – 10 cm) of soils sampled at the time of treatment and thereafter declined to less than the limit of quantification in soils sampled 179 DAT. No residues of pethoxamid were detected in the lower horizon (10 – 20 cm) of soils in this trial. Mean residues of MET-42 represented a maximum of 37 ng/g in the 0 – 10 cm horizon of soils sampled 96 DAT declining to less than the limit of quantification in the corresponding horizons of soils sampled 122 DAT. No residues of MET-42 were detected in the 10 – 20 cm horizons of soils from this trial.

Trial TON/021-04: A mean pethoxamid residue of 831 ng/g in the upper horizon of soils sampled at the time of treatment declined to less than the limit of quantification in soils sampled 183 DAT. The presence of pethoxamid residues at detectable levels in the lower soil horizons was considered to be due to contamination during sampling. Mean residues of MET-42 represented a maximum of 33 ng/g in the 0 – 10 cm horizon of soils sampled 59 DAT, declining to 19 ng/g in the corresponding horizon of soils sampled 183 DAT. No residues of MET-42 were detected in the 10 – 20 cm horizon of soils from this trial.

Table 4.1.4-27: Summary TON/021-01 (Spain) findings

Plot number	Soil sample	Amount of TKC-94 (ng/g) (0 – 30 cm)	Amount of MET-42 (ng/g) (0 – 10 cm)
Plot 2+3	-4 DAT	ND	ND
Plot 2+3	0 DAT	308	NQ
Plot 2+3	3 DAT	180	NQ
Plot 2+3	7 DAT	123	NQ
Plot 2+3	16 DAT	82	NQ
Plot 2+3	26 DAT	96	NQ
Plot 2+3	60 DAT	86	NQ
Plot 2+3	90 DAT	93	12
Plot 2+3	122 DAT	20	25
Plot 2+3	185 DAT	8	30
Plot 2+3	360 DAT	0	NQ

DAT = days after treatment
Plot 2 + 3 = replicated treated plots
ND = not detected (< 2 ng/g)
NQ = not quantifiable (< 10 ng/g)

Table 4.1.4-28: Summary TON/021-03 (France) findings

Plot number	Soil sample	Amount of TKC-94 (ng/g) (0 – 10 cm)	Amount of MET-42 (ng/g) (0 – 10 cm)
Plot 2+3	-1 DAT	ND	ND
Plot 2+3	0 DAT	746	NQ
Plot 2+3	3 DAT	669	14

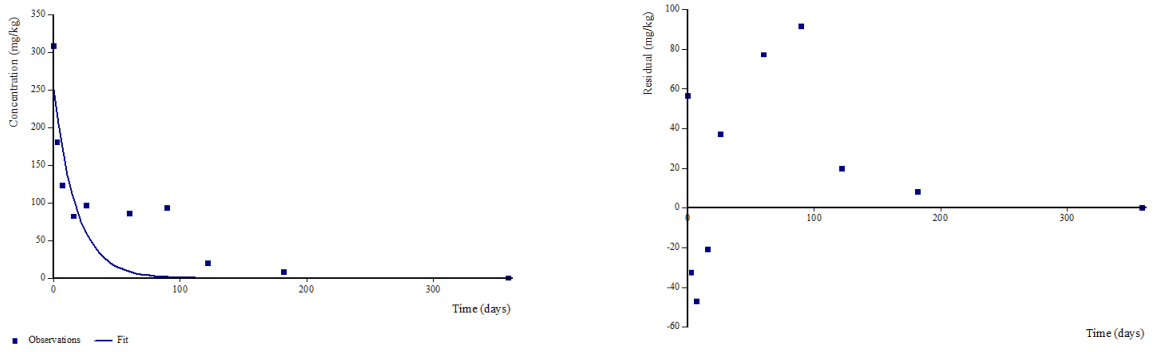
Plot 2+3	7 DAT	609	14
Plot 2+3	14 DAT	460	21
Plot 2+3	28 DAT	329	23
Plot 2+3	60 DAT	107	27
Plot 2+3	96 DAT	21	37
Plot 2+3	122 DAT	33	NQ
Plot 2+3	179 DAT	NQ	ND
Plot 2+3	364 DAT	ND	NQ

DAT = days after treatment
Plot 2 + 3 = replicated treated plots
ND = not detected (< 2 ng/g)
NQ = not quantifiable (< 10 ng/g)

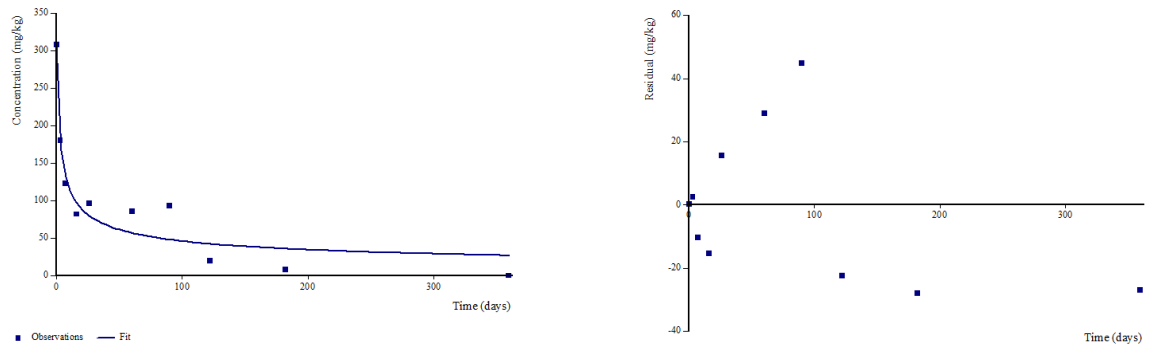
Table 4.1.4-29: Summary TON/021-04 (Spain) findings

Plot number	Soil sample	Amount of TKC-94 (ng/g) (0 – 10 cm)	Amount of MET-42 (ng/g) (0 – 10 cm)
Plot 2+3	-4 DAT	ND	ND
Plot 2+3	0 DAT	831	ND
Plot 2+3	3 DAT	409	ND
Plot 2+3	7 DAT	494	NQ
Plot 2+3	14 DAT	332	16
Plot 2+3	28 DAT	288	21
Plot 2+3	59 DAT	133	33
Plot 2+3	92 DAT	121	24
Plot 2+3	126 DAT	56	28
Plot 2+3	183 DAT	NQ	19

DAT = days after treatment
Plot 2 + 3 = replicated treated plots
ND = not detected (< 2 ng/g)
NQ = not quantifiable (< 10 ng/g)



TON/021-01, Spain – SFO



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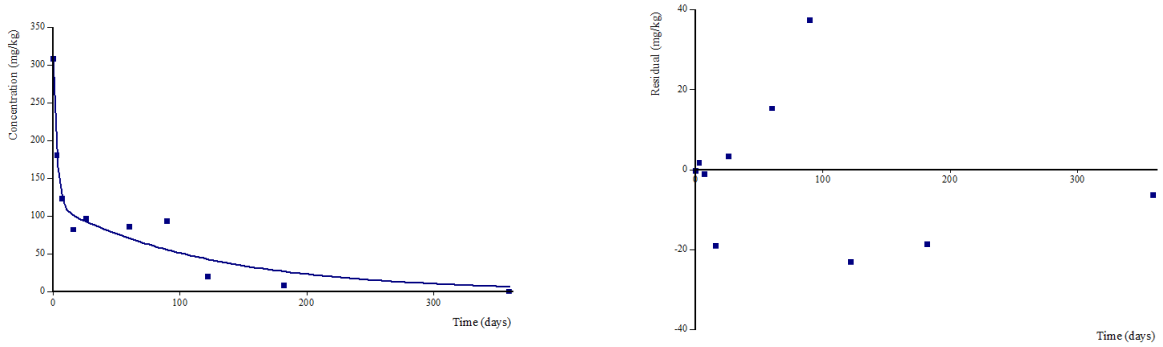
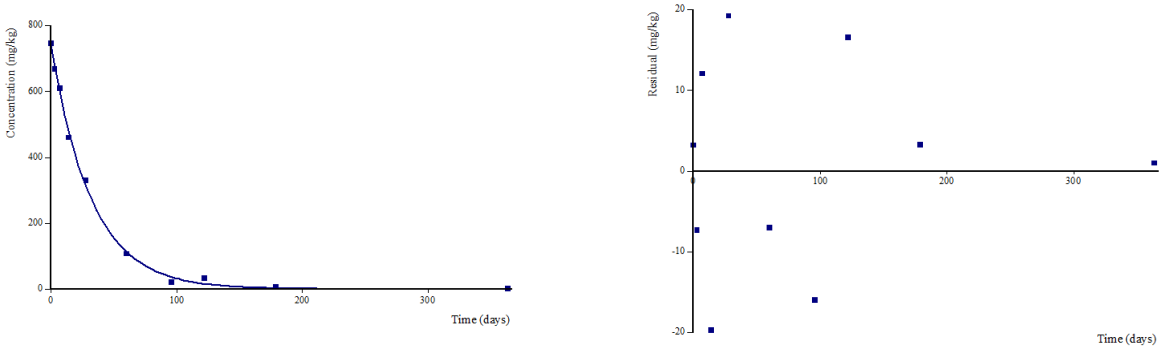


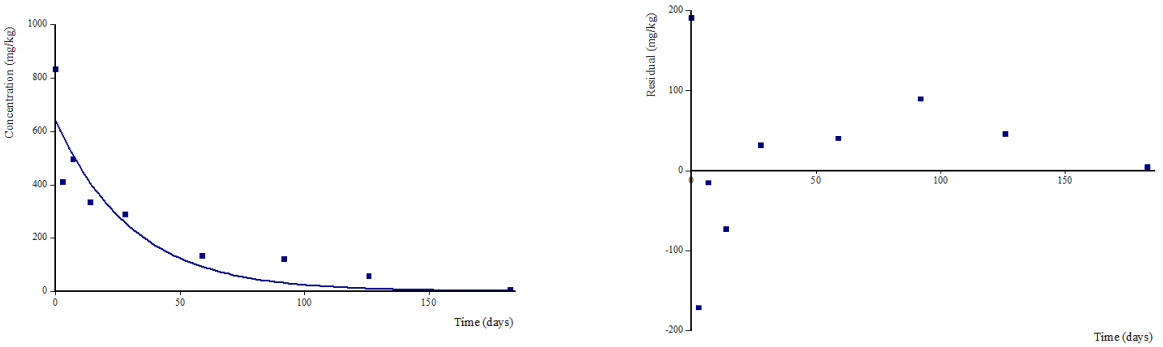
TON/021-01, Spain – DFOP

Figure 4.1.4-11: Kinetic fit (SFO, FOMC and DFOP) of pethoxamid to residues (mg/kg) measured in TON/021-01 (Spain) field trial

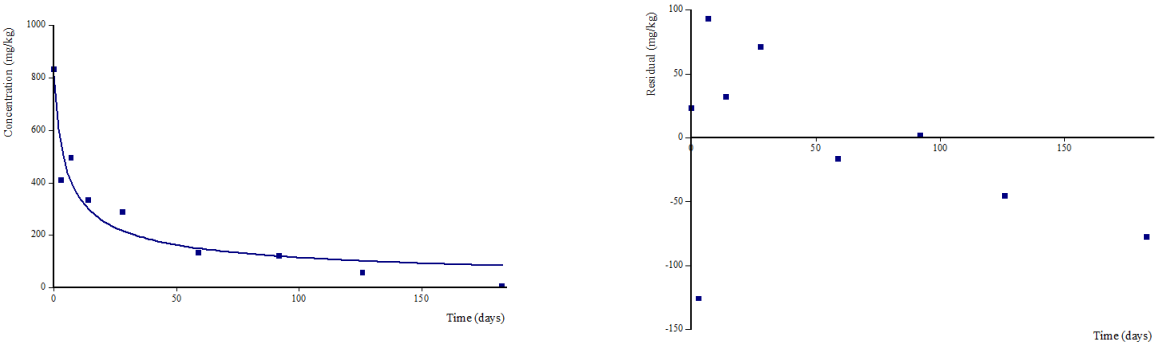


TON/022-03, France – SFO

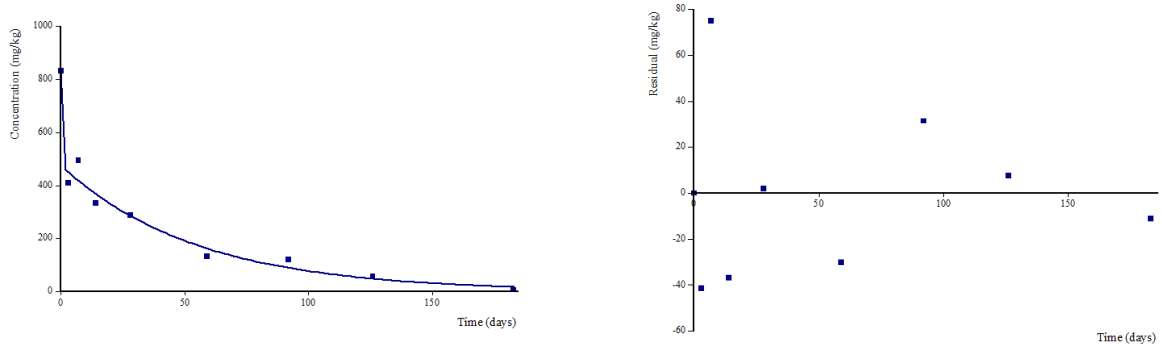
Figure 4.1.4-12: Kinetic fit (SFO only) of pethoxamid to residues (mg/kg) measured in TON/022-03 (France) field trial



TON/021-04, Spain – SFO



TON/021-04, Spain – FOMC



TON/021-04, Spain – DFOP

Figure 4.1.4-13: Kinetic fit (SFO, FOMC and DFOP) of pethoxamid to residues (mg/kg) measured in TON/021-04 (Spain) field trial

Table 4.1.4-30: Summary of pethoxamid dissipation kinetics in three field trials (numbers in bold indicate persistence trigger endpoints)

Field trial	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DisT50 / DisT90* (d)
TON/021-01, Spain	SFO	*k* (d^{-1})	0.056	0.024	38.7	0.026	12.5 / 41.3
	FOMC	*alpha* (-)	0.42	0.12	19.8	nd	-
		beta (-)	1.1	1.0		nd	-
		Overall	-	-		-	4.7 / 264
	DFOP	k_1 (d^{-1})	0.36	0.13	15.3	0.019	2.0 / 6.5
		k_2 (d^{-1})	0.008	0.003		0.022	86.9 / 289
		g (-)	0.63	0.07		nd	-
		Overall	-	-		-	**4.2 / 162**
TON/022-03, France	SFO	*k* (d^{-1})	0.031	0.001	3.4	< 0.001	**22.2 / 73.7**
TON/021-04, Spain	SFO	*k* (d^{-1})	0.033	0.011	26.1	0.012	21.2 / 70.4
	FOMC	*alpha* (-)	0.53	0.19	18.9	nd	-
		beta (-)	2.5	2.3		nd	-
		Overall	-	-		-	6.9 / 195
	DFOP	k_1 (d^{-1})	5.5	nd	10.5	nd	0.1 / 0.4
		k_2 (d^{-1})	0.018	0.003		0.002	38.2 / 127
		g (-)	0.43	0.05		nd	-
		Overall	-	-		-	**7.3 / 96.0**

Comments (RMS AT):

- Kinetic fitting was redone by the applicant in accordance with pertinent FOCUS guidance using CAKE 3.1. Only this reassessment is shown in the study evaluation above. The kinetic re-assessment is considered acceptable.

4.1.5 Photochemical degradation studies

4.1.5.1 1442 PXA (2015)

Reference: Direct aqueous photodegradation of [^{14}C]Pethoxamid
Author(s), year: Anonymous, 2015d
Report/Doc. number: 2516W-1, 1442 PXA
Guideline(s): OECD Test Guideline 316, 2008
GLP: Yes
Deviations: None
Acceptability: Yes

Executive Summary:

An aqueous photolysis study was conducted with pethoxamid using a tiered approach. A Tier 1 theoretical screen indicated that the half-life of pethoxamid could be < 30 days. Therefore, a Tier 2 experimental study was conducted using [phenyl-U-^{14}C] pethoxamid in sterilised pH 7 phosphate buffer at a dose rate of 3.91 µg/mL. Samples were irradiated in quartz tubes under a Xenon lamp, with filters blocking light of wavelengths < 290 nm, at an average intensity of 45.2 W / m^2 (290 - 400 nm). Samples were exposed continuously for up to 16 days (equivalent to approximately 30 solar days at 40 - 50 °N) at 25 °C. Dark control samples were protected from light and incubated at 25 °C. Volatile gases were trapped using ethylene glycol and NaOH traps. PNAP-PYR chemical actinometer samples were used to determine the quantum yield. Samples were removed at 0, 1, 3, 6, 10 and 16 days, quantified by LSC and analysed by HPLC and LC-MS. Total recoveries averaged 98.8 % AR for light exposed samples and 100.7 % AR for dark control samples. Pethoxamid was degraded moderately fast in light exposed samples. Two main degradates were observed: PD-1 (max. 31.6 % AR) and PD-3 (max. 21.5 % AR). The photo-degradates were identified by co-chromatography and/or LC/MS analysis as benzoic acid and MET-102, respectively. MET-2 was also present at up to 3.3 % AR in light exposed samples. The *DT50* value calculated for pethoxamid was 7.7 days, equivalent to 13.9 days of summer sunlight (based on OECD values for 40 – 50 °N). The quantum yield of pethoxamid was determined to be 2.85 × 10^{-1}. Pethoxamid was stable in the dark control samples.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid
Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi / mmol
Radiochemical purity: 100 %

2. Test systems: Sterilised 0.05 M pH 7 phosphate buffer

Study Design:

1. Experimental conditions
Samples were dosed with [phenyl-U-^{14}C]pethoxamid at a rate of 3.91 µg/mL with acetonitrile co-solvent (< 1 % of total sample by volume). Samples were exposed to artificial light with a Suntest CPS+ unit equipped with a Xenon arc lamp with UV filter to block radiation < 290 nm for up to 16 days of continuous irradiation. Irradiated samples were contained in sterilised quartz glass tubes and maintained in a water bath maintained at 25 °C. Corresponding dark control samples were placed in an incubator maintained at 25 °C. Samples were connected to a series of liquid traps (ethylene glycol and NaOH) for volatile compounds. A chemical actinometer solution of p–nitroacetophenone and pyridine (PNAP-PYR) was prepared, added to sterile water and incubated concurrently with samples containing pethoxamid to determine the quantum yield. The kinetic modelling followed the guidance of FOCUS kinetics employing the software tool for kinetic evaluation KinGUI (v. 2).

2. Sampling
Duplicate samples were removed at each sampling interval. Sampling was conducted at zero time and the following intervals: 1, 3, 6, 10 and 15 days (samples were also removed after 16 days irradiation for comparison with actinometer samples).

3. Description of analytical procedures
Sample processing began immediately following sampling. Sample sterility was assessed at the start and end of exposure by plating representative aliquots in Trypticase Soy Agar (TSA) plates. Test solutions were quantified by LSC and analysed by HPLC and LC-MS. The pH of the samples was measured at each sampling.

Results and Discussion:

Stability and homogeneity of the dosing solution was demonstrated by HPLC analysis. Sterility was confirmed from inspection of incubated agar plates. Sample pH was stable throughout the study period (6.97 and 6.88 in light and dark samples, respectively). Total mass balances for the definitive experimental study averaged 98.8 % AR and 100.7 % AR in light and dark samples, respectively. The majority of the radioactivity was recovered in the incubated aqueous samples. Pethoxamid degraded moderately fast in aqueous pH 7 buffer when exposed to artificial sunlight, and averaged 20.3 % AR after 16 days of continuous light exposure. Two major unknown photo-degradates which did not co-elute with any of the supplied reference standards were observed; unknown PD-1 (max. 31.6 % AR after 16 days) and unknown PD-3 (max. 21.5 % AR after 6 days). MET-2 was also present at up to 3.3 % AR in light exposed samples. Several minor degradates were also observed in light exposed samples, but no single degradate was present as > 4.2 % AR. Pethoxamid was stable in dark control samples.

The structure of unknown PD-3 was identified by LC-MS/MS. This structure has been named MET-102, and its identity was corroborated with simulated spectrum analysis. The structure of unknown PD-1 was identified by high resolution LC-MS as benzoic acid. The identity of this metabolite was confirmed by co-chromatography with an authentic analytical reference standard and simulated spectrum analysis.

Table 4.1.5-1: Balance of [^{14}C]pethoxamid expressed as % AR for light exposed samples (metabolites > 5 % shaded in grey)

% of AR	Incubation Time (days)					
	0	1	3	6	10	16
Pethoxamid	100.1	93.8	81.9	53.5	48.1	20.3
Benzoic acid (PD-1)	0.0	0.0	3.8	14.5	17.3	31.6
MET-102 (PD-3)	0.0	7.0	13.8	21.5	20.8	15.9
MET-2	0.0	0.0	0.0	2.0	1.5	3.3
Others[a]	0.0	0.0	0.0	5.2	9.3	16.5
Volatile traps (ethylene glycol)	na	0.2	0.8	1.8	2.5	3.3
Volatile traps (CO_2)	na	0.0	0.1	0.4	0.8	2.1

[a] Individual peaks represent < 4.2 % AR

The *DT50* and *DT90* of pethoxamid were calculated using the SFO kinetic model and based on FOCUS guidance. The quantum yield of pethoxamid was calculated by comparison of the percent pethoxamid present in solutions in irradiated samples and degradation of PNAP in irradiated actinometer solutions. The quantum yield of pethoxamid was determined to be 2.85×10^{-1}.

Figure 4.1.5-1: Kinetic fit (SFO) of pethoxamid to residues (% AR) measured under conditions of aquatic photolysis

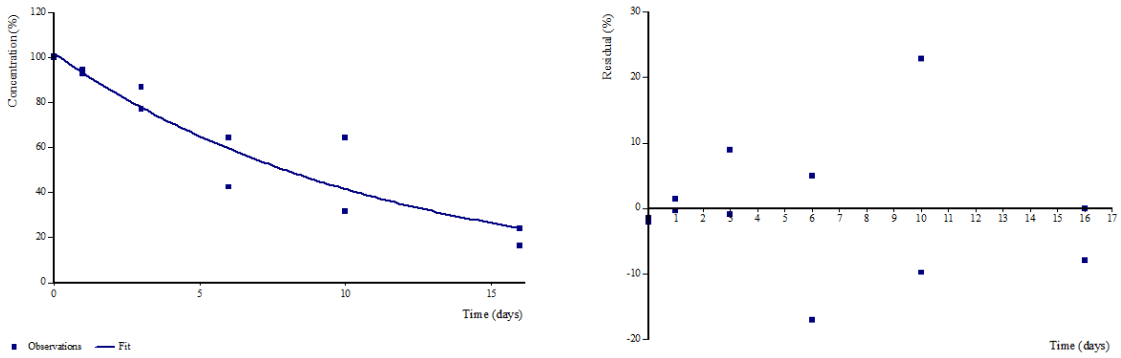


Table 4.1.5-2: Summary of pethoxamid dissipation kinetics under conditions of aquatic photolysis (SFO kinetics)

Test medium	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DT50 / DT90* (d)
pH 7 phosphate buffer, light exposed	SFO	*k* (d^{-1})	0.090	0.013	5.3	< 0.001	7.7 / 25.7

Table 4.1.5-3: Summary on direct photochemical degradation of pethoxamid at laboratory and environmental conditions

Light Exposed	Kinetic model	Suntest exposure days[a]		Sunlight equivalent days[b]	
		DT50 (d)	*DT90* (d)	*DT50* (d)	*DT90* (d)
pH 7 phosphate buffer	SFO	7.7	25.7	13.9	46.2

[a] continuous Suntest irradiation
[b] 40 – 50 °N, summer irradiation, 300 - 400 nm

Comparison of the photo-degradation profile with a previous aqueous photolysis study conducted with pethoxamid (S., 2000), showed that similar profiles were obtained in both studies. A major unknown, designated as AP9 in the previous study, corresponded to unknown PD-3 in this study, while unknown AP2 in the previous study was equivalent to PD-1 in the current study. The structure of unknown degradate PD-3 proposed in the present study agrees with the proposed structure of AP9 proposed in the previous study (S., 2000) and is named MET-102 to avoid confusion. However, the structure proposed for AP2 by S. (2000) was MET-36. LC-MS analysis of PD-1 did not support this. Instead, the unknown was identified as benzoic acid. The identity of PD-1 as benzoic acid was further confirmed by HPLC co-elution of PD-1 with an authentic analytical reference standard of benzoic acid.

Conclusions:

Pethoxamid photodegraded to two main degradates, PD-1 (max. 31.6 % AR) and PD-3 (max. 21.5 % AR). The photo-degradates were identified by co-chromatography and/or LC-MS analysis as benzoic acid and MET-102, respectively. MET-2 was also present at up to 3.3 % AR in light exposed samples. The *DT50* of pethoxamid was 7.7 days, equivalent to 13.9 days of summer sunlight (based on OECD values for 40 – 50 °N). The quantum yield of pethoxamid was determined to be 2.85×10^{-1}. Pethoxamid was stable to hydrolysis in dark control samples.

4.1.5.2 139 PXA (1999)

Reference: TKC-94 Soil Photolysis
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 024/983761, Cheminova A/S Report No.: 139 PXA
Guideline(s): US EPA Subdivision N, Section 161-3, 1982
GLP: Yes
Deviations: None
Acceptability: Yes

Test system:

The photo-degradation of pethoxamid was studied on thin layers (*ca.* 2 mm) of a sandy silt loam/sandy loam soil using phenyl radiolabelled form of the test material. The test material was applied to the soil thin layers at a concentration of 12.5 µg/cm^2. This equates to a field rate of 1.25 kg a.s./ha. The soil thin layers were continuously irradiated using a xenon arc light source for up to 15 days at *ca.* 20 °C. Control soil thin layers were similarly incubated in darkness. Humidified air was passed over irradiated/incubated soil thin-layers and passed into a series of trapping solutions to trap any volatiles evolved. Duplicate irradiated and non-irradiated soil plates were taken for analysis at 2, 4, 7, 10 and 15 days after test substance application. One duplicate set of plates was taken for analysis immediately after test substance application and provided a zero-time sample for both irradiated and non-irradiated experiments. HPLC and TLC investigated the proportions and identities of degradation products in soil extracts. The soil characteristics are shown below.

Table 4.1.5-4: Characteristics of the test soil

Textural classification (USDA)	
0.053 – 2 mm (%)	52.8
0.002 – 0.053 mm (%)	36.1
< 0.002 mm (%)[c]	11.1
Textural classification	Sandy loam
Organic carbon (%)	2.7
Cation exchange capacity (mEq / 100 g)	18.6
pH (1 : 5) in water	6.9
pH (1 : 5) in 1 M KCl	6.9
pH (1 : 5) in 0.01 M $CaCl_2$	7.1
Maximum water holding capacity (%)	54.5
Water holding capacity at 0.33 bar (%)	18.9

Findings:

The recovery of radioactivity from all soil plates was essentially quantitative (94 – 106 % AR). After 15 days of irradiation pethoxamid accounted for a mean of 67 % AR. The amount of $^{14}CO_2$ evolved during the 15 day study period represented 4 % and < 0.1 % AR for irradiated soil and non-irradiated soil respectively. Apart from pethoxamid, a number of degradation products (n = 10) were formed. In irradiated soil after 15 days, metabolite SP5 individually represented a maximum mean proportion of 4.2 % of the applied radioactivity. No unique photoproducts were formed during the study. After 15 days no individual product represented more than 1 % AR in non-irradiated soil. Non extractable radioactivity after 15 days accounted for 4 – 5 % AR for either irradiated or non-irradiated soils.

Table 4.1.5-5: Extraction and recovery of radioactivity from soil thin layers (% of applied radioactivity)

DAT	Irradiated soil			Dark control soil		
	Soil		Total	Soil		Total

	Extracted	Non extracted	Total	Vola-tiles		Extracted	Non extracted	Total	Vola-tiles	
0	99.9	0.4	100.3	-	100.3	97.8	1.2	99.0	< 0.1	99.0
	105.6	0.4	106.0	-	106.0	98.8	1.3	100.1	< 0.1	100.1
2	94.6	1.8	96.4	0.4	96.8	101.5	3.1	104.6	< 0.1	104.6
	94.1	2.2	96.3	0.4	96.7	93.6	2.2	95.8	< 0.1	95.8
4	94.4	2.9	97.3	1.0	98.3	95.0	3.5	98.5	< 0.1	98.5
	95.1	2.1	97.2	1.0	98.2	96.7	3.2	99.9	< 0.1	99.9
7	88.9	4.0	92.9	2.1	95.0	95.6	3.0	98.6	< 0.1	98.6
	91.5	3.3	94.8	2.1	96.9	97.6	2.8	100.4	< 0.1	100.4
10	91.7	3.0	94.7	2.9	97.6	91.8	4.8	96.6	< 0.1	96.6
	88.3	3.2	91.5	2.9	94.4	92.2	4.9	97.1	< 0.1	97.1
15	87.1	4.3	91.4	4.2	95.6	97.8	1.2	99.0	< 0.1	99.0
	87.5	4.1	91.6	4.2	95.8	98.8	1.3	100.1	< 0.1	100.1

Table 4.1.5-6: Proportions of radioactive components in soil (% of applied radioactivity, HPLC, metabolites > 5 % shaded in grey)

DAT	Irradiated soil												Dark control soil	
	0		2		4		7		10		15		15	
Polars (SP1)	0.1	0.1	0.7	0.6	0.8	0.8	0.8	0.7	0.8	1.1	1.2	0.8	< 0.1	< 0.1
SP2	-[a]	-[a]	0.6	0.7	1.0	0.7	0.8	1.1	1.1	1.3	1.8	1.6	< 0.1	< 0.1
SP3	-[a]	-[a]	0.3	0.3	0.4	0.5	0.4	0.5	0.6	0.5	0.6	0.4	< 0.1	< 0.1
SP4	0.6	0.6	1.1	1.5	1.7	2.8	2.0	1.4	1.6	1.7	1.7	2.4	0.5	0.6
SP5	0.2	0.4	2.4	2.4	2.5	4.0	2.8	3.8	3.7	4.1	3.9	4.4	0.4	0.4
SP6	0.3	0.3	2.1	2.4	2.6	2.4	2.4	1.8	2.3	2.6	2.7	2.0	0.4	0.5
SP7	0.6	0.3	2.4	2.4	3.6	3.8	2.6	2.7	2.6	2.0	3.0	2.9	0.6	0.4
SP8	0.4	0.4	2.6	2.9	3.1	3.2	2.4	2.7	2.8	2.6	2.4	2.7	0.4	0.3
SP9	0.4	0.4	2.1	2.0	2.0	2.9	2.5	2.2	2.3	1.9	2.0	2.1	0.3	0.5
Pethoxamid	96.4	101.9	79.3	77.9	75.4	71.0	71.1	73.2	72.9	69.2	66.4	67.1	88.5	88.9
SP10	0.6	0.7	0.7	0.7	0.6	2.3	0.5	0.5	0.6	0.5	0.5	0.5	0.6	0.6
Others[b]	0.3	0.3	0.4	0.5	0.6	0.8	0.6	0.5	0.6	0.7	0.9	0.7	0.2	0.2

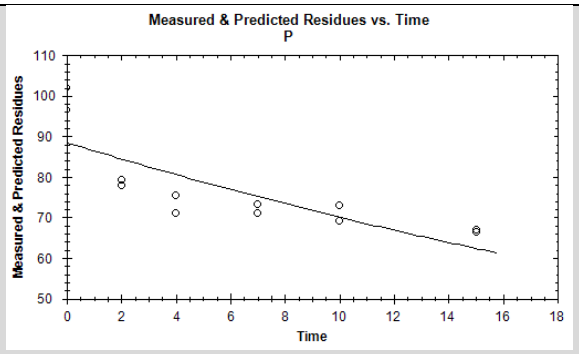
[a] Not apparent : included in 'others'
[b] Radioactivity distributed through regions of the chromatogram other than those specified and which did not contain any radioactive components

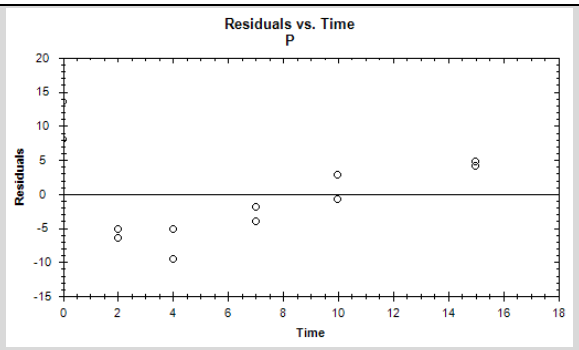
Summary:

Pethoxamid was photodegraded on soil. Degradation of pethoxamid was accelerated in the presence of light. A number of quantitatively minor photo-degradation products (all < 5 % AR) were formed which were also products of aerobic soil metabolism.

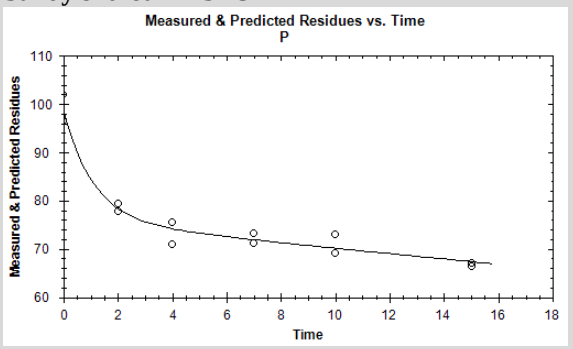
Comments (RMS AT):

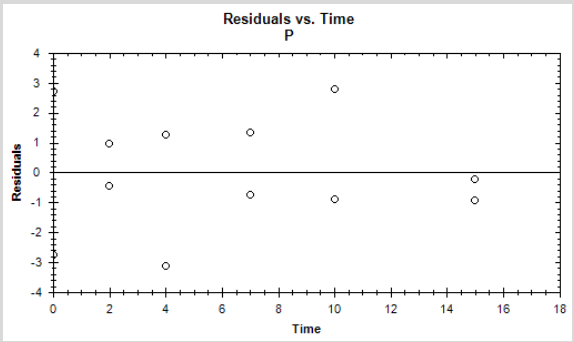
- Dissipation kinetics of pethoxamid under conditions of soil photolysis was recalculated by the RMS AT following pertinent FOCUS guidance using KinGUI 2:





Sandy silt loam - SFO





Sandy silt loam - DFOP

Figure 4.1.5-1: Kinetic fit (SFO above, DFOP below) of pethoxamid to residues (% AR) measured in soil under conditions of soil photolysis – RMS AT assessment

Table 4.1.5-7: Summary of pethoxamid degradation kinetics under conditions of soil photolysis – RMS AT assessment

Soil	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90* (d)
Sandy silt loam	SFO	k (d^{-1})	0.0233	0.0058	6.4	0.001	29.7 / 98.7
	DFOP	k_1 (d^{-1})	0.937	0.327	0.80	0.011	0.7 / 2.5
		k_2 (d^{-1})	0.00806	0.00321		0.018	86.0 / 286
		g (-)	0.23	0.03		-	-
		Overall	-	-	-	-	**53.0 / 253**

DegT50 of pethoxamid under conditions of soil photolysis is considered to be 53 days (DFOP kinetics).

4.1.6 Environmental fate and other relevant information

4.1.6.1 1423 PXA (2015)

Reference: Pethoxamid: Estimation of Atmospheric Oxidation Rate.
Author(s), year: Anonymous, 2015
Report/Doc. number: CHA 100608, 1423 PXA
Guideline(s): Not applicable
GLP: Not applicable
Deviations: Not applicable
Acceptability: Yes

The atmospheric oxidation rate for pethoxamid was estimated using the Atmospheric Oxidation Program (AOPWIN v 1.92 a module of EPI-Suite™ v 4.11) which is available from the US EPA. AOPWIN estimates the rate constant for the atmospheric, gas-phase reaction between photo-chemically produced hydroxyl radicals and organic chemicals. It also estimates the rate constant for the gas-phase reaction between ozone and olefinic / acetylenic compounds. The rate constants estimated by the program are then used to calculate atmospheric half-lives for organic compounds based upon average atmospheric concentrations of hydroxyl radicals and ozone.

The overall rate constant was calculated as 109.9559×10^{-12} cm^3 / molecule × s. From this rate constant the half-life (for a 12 hour day, 1.6×10^5 OH radicals / cm^3) was estimated as 1.167 hours. As this is less than 2 days there is considered to be limited potential for long-range transport of pethoxamid.

4.1.6.2 1415 PXA (2015)

Reference: Pethoxamid: Henry's Law Constant.
Author(s), year: Anonymous, 2015f
Report/Doc. number: CHA 100581, 1415 PXA
Guideline(s): Not applicable
GLP: Not applicable
Deviations: Not applicable
Acceptability: Yes

Henry's law constant for pethoxamid calculated from available GLP experimental data at 20 °C was 1.18×10^{-3} Pa m^3/mole. Pethoxamid is therefore considered to be moderately volatile at 20 °C.

4.1.6.3 1344 PXA (2014)

Reference: Soil Adsorption/Desorption of [^{14}C]Pethoxamid by the Batch Equilibrium Method.
Author(s), year: Anonymous, 2014
Report/Doc. number: PTRL West Report No. 2515W-1, 1344 PXA
Guideline(s): OECD Test Guideline 106, 2000; US EPA OPPTS Guideline 835.1230, 1998
GLP: Yes
Deviations: None
Acceptability: Yes

Executive Summary:

The adsorption and desorption characteristics of [^{14}C]pethoxamid were determined in four soils which varied in texture, percent organic matter, pH (range 5.3 to 7.4) and cation exchange capacity. Preliminary trials (Tiers 1 and 2) determined the optimal soil to solution ratios for each soil (either, 1:1, 1:2 or 1:5) and the adsorption and desorption equilibration period (24 h) for the definitive study. The definitive study (Tier 3) was conducted with five concentrations of pethoxamid; 3.9, 1.0, 0.4, 0.1, and 0.04 mg/L. The overall mass balance ranged from 88.6 to 105.4 %. Freundlich adsorption coefficients related to organic carbon content (K_{foc}) for the four soils were in the range of 187 to 241 L/kg. Adsorption 1/*n* values for the four soils were in the range 0.87 to 0.91. Pethoxamid was shown to be of medium mobility according to the McCall classification.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid
Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi/mmol
Radiochemical purity: 98.94 %

2. Test systems: Four US soils characterised by AGVISE laboratories

Table 4.1.6-1: Physical and chemical properties of the soils used

Soil	pH ($CaCl_2$)	OC (%)	Sand USDA (%)	Silt USDA (%)	Clay (%)	CEC (mEq/100 g)	Soil texture USDA	MWHC (%)
ND L	7.4	3.8	28	45	27	25.4	Loam	39.9
CA L	7.0	0.9	38	41	21	10.1	Loam	22.2
IL SiL	5.3	0.6	18	61	21	9.5	Silty loam	23.2
ND SCL	6.4	2.6	62	17	21	17.4	Sandy clay loam	23.7

CEC - Cation exchange capacity, OC - Organic carbon, MWHC - Maximum water holding capacity

Study Design:

1. Experimental conditions

Tier 1 – ratio test:

Following overnight equilibration of the four soil systems with 0.01M $CaCl_2$ (at soil to solution ratios of 1:5, 1:25 and 1:50 for each soil in duplicate), the test item was applied in 0.01M $CaCl_2$ at a rate of 4 mg/L. The teflon tubes were sealed and shaken in the dark at 20 °C for a 24-hour period prior to analysis. Additional soil to solution ratios of 1:1 and 1:2 were also tested for the CA L and IL SiL soils using the same procedure.

Tier 2 – adsorption kinetics:

Following overnight equilibration of the four soil systems with 0.01M $CaCl_2$ at soil to solution ratios of 1:5 (ND L), 1:2 (CA L), 1:1 (IL SiL) and 1:5 (ND SCL), the test item was applied in 0.01M $CaCl_2$ at a rate of 4 mg/L. The teflon tubes were sealed and shaken in the dark at 20 °C for up to a 48-hour period prior to analysis.

Tier 3 – Freundlich isotherms:

Following overnight equilibration of the four soil systems with 0.01M $CaCl_2$ at soil to solution ratios of 1:5 (ND L), 1:2 (CA L), 1:1 (IL SiL) and 1:5 (ND SCL), the test item was applied in 0.01M $CaCl_2$ at five rates; 0.04, 0.1, 0.4, 1.0 and 3.9 mg/L. The teflon tubes were sealed and shaken in the dark at 20 °C for a 24-hour adsorption equilibrium phase prior to analysis. After decanting the adsorption solution, an equivalent amount of fresh 0.01M $CaCl_2$ was added and the tubes were sealed and shaken in the dark at 20 °C for a further 24-hour prior for desorption analysis.

2. Description of analytical procedures

From the Tier 2 test, a time period of 24 hours was found to be sufficient for reaching equilibrium. After adsorption and desorption phases, samples were centrifuged (~2700 x G, 40 min) and triplicate aliquots were taken for all samples and radioassayed by LSC. After the desorption phase, the soil pellets of both replicates of the 3.9 mg/L samples for all soils were extracted with acetonitrile:water (4:1, v/v). At least one replicate of the adsorption and desorption solutions from the 3.9 mg/L samples and one soil extract replicate were analysed by HPLC.

The amount of pethoxamid adsorbed onto soil, the Freundlich adsorption and desorption coefficients (K_f) and the Freundlich adsorption and desorption coefficients on basis of the soil organic carbon content (K_{foc}) were calculated.

Results and Discussion:

For the definitive study (Tier 3) the overall mass balance ranged from 88.6 to 105.4%. Pethoxamid was found to be stable in adsorption, desorption and soil extract solutions.

The amount of test item adsorbed onto soil, the Freundlich adsorption and desorption (K_f) coefficients and the Freundlich adsorption and desorption coefficients on basis of soil organic carbon content (K_{foc}) were calculated for each soil (where possible). The respective adsorption coefficients on the basis of soil organic carbon content

(K_{Foc}) were calculated to be 241 (ND L), 212 (CA L), 187 (IL SiL) and 208 (ND SCLl) L/kg. Corresponding values for 1/*n* were calculated to be 0.90 (ND L), 0.91 (CA L), 0.90 (IL SiL) and 0.874 (ND SCL). No correlation was found between soil adsorption and pH.

Table 4.1.6-2: Adsorption coefficients for pethoxamid in soil

Soil	Soil texture USDA	OC (%)	pH ($CaCl_2$)	Adsorption (L/kg)					
				K_f	K_{foc}	1/*n*	r^2	% adsorbed	K_d × soil/sol. ratio*
ND L	Loam	3.8	7.4	9.15	241	0.898	0.9996	68 - 77	1.7 – 2.7
CA L	Loam	0.9	7.0	1.91	212	0.908	0.9999	52 - 63	0.9 – 1.3
IL SiL	Silty loam	0.6	5.3	1.12	187	0.895	1.000	64 - 74	1.0 – 1.6
ND SCL	Sandy clay loam	2.6	6.4	5.42	208	0.874	0.9999	53 - 68	1.0 – 1.6

* Based on K_d values for individual test concentration (K_d values not shown)

Conclusions:

Adsorption and desorption isotherms of pethoxamid were studied in four US soils (ND L, CA L, IL SiL and ND SCL). Freundlich adsorption coefficients normalised for organic carbon (K_{foc}) were in the range 187 to 241 L/kg. Corresponding values for 1/*n* were in the range 0.87 to 0.91. Pethoxamid was shown to be of medium mobility according to the McCall classification.

4.1.6.4 143 PXA (1999)

Reference: TKC-94 Aged residue soil column leaching.
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 020/984962, 143 PXA
Guideline(s): US EPA Subdivision N, Section 163-1, 1982
GLP: Yes
Deviations: None
Acceptability: Yes

Test system:

The leaching behaviour of the aged soil residues of ^{14}C-pethoxamid (uniform phenyl labelled, radiochemical purity > 97 %) was studied using the column leaching method. The test material was applied to sandy silt loam soil at a rate equivalent to an agricultural use rate of 1.2 kg/ha and aged under aerobic conditions (at a moisture content of 45 % maximum water holding capacity in darkness at 20 °C) for a period approximately equal to the DT_{50} value (approximately 6 days) for pethoxamid in this soil type.

Table 4.1.6-3: Characteristics of the test soil

Huntingdon Life Sciences batch number	30/498A
Textural classification (USDA classification)	
0.053-2 mm (%)	52.79
0.002-0.053 mm (%)	36.11
< 0.002 mm (%)	11.11
Textural classification	Sandy loam
Organic carbon (%)	2.7
Cation exchange capacity (mEq/100 g)	18.6
pH (1 : 5) in water	6.9
Maximum water holding capacity (%)	54.5
Water holding capacity at 0.33 bar (%)	18.9

Biomass (µg C/g)	
Time of test substance application	533.0
End of ageing period	209.1

The aged soil and (^{36}Cl) sodium chloride (to provide data on column void volume and to confirm elution) was applied to a 30 cm column of untreated soil, pre-saturated with 0.01 M calcium chloride, and the column eluted with 0.01 M calcium chloride. A volume of calcium chloride solution equivalent to rainfall to a depth of 500 mm over the cross sectional area of the column was applied over a period of approximately 48 hours and the leachate collected. Leachate and soil extracts were analysed by reverse phase HPLC and normal phase TLC. Soil residues were combusted and radio assayed (LSC) as were all extracts and leachate samples.

Findings:

The recovery of the radioactivity from the two dishes of treated soil which were taken for analysis at the end of the ageing period of 6 days were in the range 95.7 – 98.4 % AR. Extractable radioactivity represented 67.6 - 70.1 % AR, volatile radioactivity represented 7.4 % AR and non-extractable radioactivity represented 20.7 – 20.9 % AR.

^{14}C-pethoxamid total recovery of the applied radioactivity from the eluted column was 95.5 %. After elution of the equivalent of 200 mm rainfall, 3.7 % of the applied radioactivity was detected in the leachate. This comprised of pethoxamid (0.2 % applied radioactivity) and at least 9 other metabolites, all previously seen in the aerobic soil metabolism study. None of these metabolites exceeded 1 % of the applied radioactivity. After elution, equivalent to 500 mm rainfall, 18 % of the applied radioactivity was detected in the leachate, which contained 6.5 % pethoxamid and no metabolite greater than 3.4 %. Radioactivity was detected in all sections of the column, however the top section contained the majority of the radioactivity, 43.7 % applied radioactivity. Of this approximately half (21.8 % of the applied radioactivity) was unextractable and did not appear to be mobile.

The ^{36}Cl-sodium chloride recovery was 107.0 % applied radioactivity. Examination of the elution profile suggested that the majority of the ^{14}C activity was less mobile than ^{36}Cl.

Table 4.1.6-4: Vertical distribution of radioactivity in a sandy silt loam after application of aged soil treated with ^{14}C-pethoxamid at a nominal rate of 1.2 mg/kg and percolation of 500 mm of 0.01 M calcium chloride solution

Approximate depth of soil layer (cm)	Results expressed as % applied radioactivity[a]		
	Extractable	Non-extractable	Total
0-8[b]	21.9	21.8	43.7
8–13	4.3	1.3	5.6
13–18	3.7	1.2	4.9
18–23	3.8	1.3	5.1
23–28	3.7	0.8	4.5
28–33	5.0	1.3	6.3
Subtotal			70.1
Leachate			18.0
Volatile radioactivity[c]			7.4
Total recovery			95.5

[a] Radioactivity applied to the column at the start of ageing
[b] This section contained the aged soil
[c] Produced during the ageing period

Table 4.1.6-5: Proportions of radioactive components in leachate collected from a soil column after application of aged soil treated with ^{14}C-pethoxamid at a nominal rate of 1.2 mg/kg and percolation of 500 mm of 0.01 M calcium chloride solution

Component	Approximate HPLC retention time (minutes)	Results expressed as % applied radioactivity		
		Leachate		
		Portion 1[c]	Portion 2[d]	Total
		Fractions 7-27	Fractions 28-68	
Polars[a]	2 - 6	< 0.1	0.1	0.1
S1	20.0	0.3	0.4	0.7
S2	22.6	0.7	0.9	1.6
S3	23.5	0.3	0.6	0.9
S4	25.5	1.0	2.4	3.4
S5	27.0	0.4	1.3	1.7
S6	29.0	0.1	0.5	0.6
S7	30.2	0.2	0.5	0.7
S8	31.8	0.3	1.1	1.4
TKC-94	34.0	0.2	6.3	6.5
Others[b]	-	< 0.1	0.2	0.2

[a]Unresolved polar radioactivity eluting at the solvent front
[b]Regions of the chromatogram other than those specified
[c]Represents the radioactivity in leachate after elution of approximately 200 mm of 0.01 M calcium chloride over the cross sectional area of the column
[d]Represents the radioactivity in leachate after elution of approximately 500 mm of 0.01 M calcium chloride solution over the cross sectional area of the column

Summary:

Based on results obtained in this study, aged residues of pethoxamid can be classified as having a moderate potential for leaching in soil.

4.1.6.5 140 PXA (2000)

Reference: A field dissipation and leaching study to monitor the fate of TKC-94 and its relevant soil metabolites when applied, pre-emergence, to fodder maize in the United Kingdom 1998
Author(s), year: Anonymous, 2000
Report/Doc. number: JF4204 (TON 022), 140 PXA
Guideline(s): SETAC, 1995; OEPP/EPPO Bulletin 23, 27-49, 1993; BBA-Richtlinie Teil IV, 4 1, 1986
GLP: Yes
Deviations: None
Acceptability: Yes

Executive Summary (leaching):

A field leaching study has been performed to monitor the fate of pethoxamid and MET-42 when applied pre-emergence to fodder maize in the United Kingdom. The experimental site was established at Barrow-upon-Trent (England) on a sandy soil with shallow groundwater. The site consisted of a single non-treated plot and two replicated treated plots, all cropped with fodder maize. Soil water samples were collected using suction samplers installed at 100 cm depth (six replicates per treated plot, three replicates in the untreated plot) to assess the leaching potential of pethoxamid and MET-42. The product (599 g L^{-1} EC) was applied to each treatment plot at a rate of 2.0 L p.p.p. ha^{-1}. Bromide was also applied as an inert tracer. The leaching plots were monitored on a routine basis (twice monthly for the first 2 months and monthly thereafter) and on a trigger

basis. Trigger conditions were defined as 10 mm of rainfall over a 24-hour period or 15 mm over a 48-hour period. Quantification of pethoxamid and MET-42 in water was performed by LC-MS. The limit of detection for pethoxamid and MET-42 was 0.05 µg L^{-1} and 0.02 µg L^{-1}, respectively. The limit of quantification was 0.1 µg L^{-1} for both compounds. Concentrations of bromide were quantified by ion chromatography. Daily rainfall was recorded at the experimental site using an automated meteorological system.

Climatic conditions during the study were considerably wetter than average with total rainfall for the year after application approximately 132 % of the long-term average. The soil hydrological conditions at the time of application were close to field capacity. Precipitation in the month following application exceeded the long-term average by approximately 170 %. The concentration of bromide in water showed breakthrough to 100 cm depth occurred between 28 to 35 DAT.

Soil water samples were collected on 15 separate occasions following test substance application. Pethoxamid was detected in 2 of the 175 samples collected from 100 cm depth. Both concentrations were at the limit of quantification (0.1 µg L^{-1}) and were detected in the water from the same sampler at 28 and 33 DAT. Residues of MET-42 were detected in 151 of the 171 soil water samples collected, at concentrations ranging from 0.1 to 11.2 µg L^{-1}. Average concentrations for individual samplers over the study were between 0.6 and 3.2 µg L^{-1}.

Test system:

Product: ASU 96520H; Batch no. 9802, Composition: TKC-94 599 g/l formulated as an emulsifiable concentrate.

The experimental site was established at Barrow-on-Trent (England) on a sandy soil with shallow groundwater. Details of the soil characteristics and microbial biomass are given in the table below.

Table 4.1.6-6: Test site soil characteristics

Parameter	Soil Horizon (depth in cm)					
	0 - 31	31 - 45	45 - 58	58 - 80	80 - 112	112 - 125
Sand (%) 2000 – 63 µm	61.0	65.3	82.6	91.3	95.5	92.4
Silt* (%) 63 – 2 µm	24.2	22.9	11.0	4.2	1.7	2.2
Clay (%) < 2 µm	14.8	11.9	6.4	4.7	2.9	5.4
pH (1:5) in water	7.2	7.2	7.0	7.3	7.2	7.3
Cation exchange capacity (mEq/100 g)	20.2	13.6	7.5	3.1	1.1	1.8
Organic carbon (%)	3.0	0.7	0.4	0.2	0.1	0.1
Soil microbial biomass (mg C/100 g soil), topsoil only	Prior to application:			18.82 (Plot JF4204-03)		
	End of study:			14.98 (Plot JF4204-02)		
				27.41 (Plot JF4204-02)		

* UK silt split

The site consisted of a single non-treated plot (JF4204-03) and two replicated treated plots (JF4204-01 and JF4204-02), all cropped with fodder maize. Each plot was divided into 12 equally sized sub-plots. Eleven were designated for the collection of soil cores to monitor the dissipation of pethoxamid and its metabolite MET-42. The remaining sub-plot was designated for evaluation of the leaching potential of pethoxamid and MET-42. Soil water samples were collected using suction samplers installed at 100 cm depth (six replicates per treated plot, three replicates in the untreated plot) to assess the leaching potential of pethoxamid and MET-42. Prior to application, the hydrological status of the site was monitored. The soil microbial biomass was determined prior to application and at the end of the field phase.

On 8th of May 1998 (pre-emergence), ASU 96520H was applied to each treatment plot at a nominal rate of 2000 ml/ha using a research backpack boom sprayer. Potassium bromide was applied as an inert tracer to each leaching sub-plot within two hours of the application of pethoxamid. The leaching sub-plots were monitored

on a routine basis (twice monthly for the first 2 month and monthly thereafter) and on a trigger basis. Trigger conditions were defined as 10 mm of rainfall over a 24-hour period or 15 mm over a 48-hour period.

Soil samples for residue analysis were taken from each plot using an automated 'zero contamination' soil coring system. Soil samples were taken at predetermined intervals. At each sampling occasion, a total of 20 soil cores (5 cm diameter by 50 cm length) were collected from one sub-plot, combined and frozen on the day of sampling until analysis. A separate sub-plot was used on each sampling occasion. Plot dimensions and treatment detail are shown in the Table below.

Table 4.1.6-7: Plot dimensions and treatment detail

Plot number	Treatment	Plot width (m)	Plot length (m)	Plot area (m^2)
JF-4204-01	Treated, cropped	17	36	612
JF-4204-02	Treated, cropped	17	36	612
JF-4204-03	Untreated, cropped	17	36	612

For analysis, each soil sample was cut into 0-10, 10-20 and 20-30 cm horizons. Pethoxamid and MET-42 were quantified in the soil from the 0-10 and 10-20 cm horizons. The soil was sieved and extracted with aqueous acetone and concentrated. Clean up of the water samples was performed by solid phase extraction. Quantification of pethoxamid and MET-42 was performed by LC-MS. The limit of detection (LOD) was 2 ng/g for both compounds in soil. The LOD for pethoxamid and MET-42 in water was 0.05 µg/l and 0.02 µg/l respectively. The limit of quantification (LOQ) for pethoxamid and MET-42 was 10 ng/g in soil and 0.1 µg/l in water. Concentrations of bromide in sub-samples of soil water were quantified by ion chromatography. The limit of detection for this technique was 1.10 mg/l.

Daily rainfall was recorded at the experimental site using an automated meteorological system. The mean minimum and maximum air temperature and monthyl rainfall totals for the duration of the experimental phase are summarised in the table below. On occasions of malfunction, daily rainfall from Sutton Bonington (approximately 16 km east-south-east of the experimental site) was used instead of site measurements, as indicated.

Table 4.1.6-8: Meteorological data

Month/year	Monthly mean minimum air temperature (°C)	Monthly mean maximum air temperature (°C)	Monthly rainfall Total (mm)
May 1998	7.6	17.2	18.3 (1)
June 1998	10.5	18.4	144.4
July 1998	11.6	19.7	22.2
August 1998	12.1	20.9	64.0
September 1998	11.2	18.4	67.0
October 1998	7.9	13.9	110.6
November 1998	2.7	8.6	30.2
December 1998	2.3	8.7	61.8
January 1999	2.5	8.5	86.2 (2)
February 1999	2.2	8.2	39.2 (3)
March 1999	4.5	10.9	96.2
April 1999	5.2	13.8	58.4 (4)
May 1999	8.7	17.4	57.8
June 1999	9.3	18.4	80.6
July 1999	12.5	22.9	16.2

(1) 1-8 May 1998 rainfall obtained from Sutton Bonington

(2) 5-31 January 1999 rainfall obtained from Sutton Bonington
(3) 1-28 February 1999 rainfall obtained from Sutton Bonington
(4) 26-30 April 199 rainfall obtained from Sutton Bonington

Findings:

Climatic conditions during the study were considerably wetter than average with total rainfall for the period May 1998 to April 1999, approximately 132 % of the long term average. The soil hydrological conditions at the time of application were close to field capacity. Following application, precipitation during June 1998 exceeded the long-term average by approximately 170 % and thus resulted in unseasonable leaching conditions very soon after application. The concentrations of bromide in water showed breakthrough to 100 cm depth occurred between 28 to 53 DAT, providing supportive data that leaching conditions were present at the site soon after application. Results for both plots show two marked periods of leaching with peak concentrations being detected either in early June 1998 or during the period October to December 1998. Between June and September 1998 greater leaching occurred on plot JF4204-01 than JF4204-02. From October to December 1998 concentrations in soil water were broadly similar for both treated plots.

Soil samples
Procedural recoveries for the analysis of pethoxamid and MET-42 in soil and water samples all fell within the range 70 – 110 %.

No quantifiable residues of pethoxamid or MET-42 were detected in any of the samples (soil or water) taken from the control plot. Mean concentrations of pethoxamid and MET-42 found in the 0–10 cm and 10–20 cm soil horizons for the treated plots are summarised in the table below.

Table 4.1.6-9: Concentrations (ng/g) of pethoxamid and MET-42 found in 0–10 cm and 10–20 cm horizons from treated plots

Plot number	DAT	Amount of pethoxamid (ng/g)		Amount of MET-42 (ng/g)	
		0–10 cm	10–20 cm	0–10 cm	10–20 cm
Plot JF4204-01 + JF4204-02	-1	n.d	n.d	n.d	n.d
Plot JF4204-01 + JF4204-02	0	808	< LOQ	n.d	n.d
Plot JF4204-01 + JF4204-02	3	715	n.d	n.d	n.d
Plot JF4204-01 + JF4204-02	7	692	< LOQ	< LOQ	n.d
Plot JF4204-01 + JF4204-02	14	492	< LOQ	< LOQ	n.d
Plot JF4204-01 + JF4204-02	28	445	< LOQ	< LOQ	n.d
Plot JF4204-01 + JF4204-02	60	67	n.d	10	< LOQ
Plot JF4204-01 + JF4204-02	90	35	n.d	< LOQ	n.d
Plot JF4204-01 + JF4204-02	119	28	n.d	< LOQ	< LOQ
Plot JF4204-01 + JF4204-02	182	n.d	< LOQ	n.d	n.d
Plot JF4204-01 + JF4204-02	362	n.d	< LOQ	n.d	n.d

DAT = days after treatment; Plot 1 + 2 = replicated treated plots; n.d = not detected (< 2 ng/g); LOQ = limit of quantification (< 10 ng/g)

Water samples
To investigate the leaching potential of pethoxamid and MET-42, soil water samples were collected on 15 separate occasions following test substance application. Pethoxamid was detected in 2 of the 175 samples of soil water collected from 100 cm depth from the two treated plots. Both concentrations were at the limit of quantification (0.1 µg/l) and detected in the water from the same sampler at 28 and 33 DAT. Residues of MET-42 were detected in 151 of the 171 soil water samples collected post-application from the treated plots, at concentrations ranging from 0.1 to 11.2 µg/l. Maximum concentrations of MET-42 were detected either in early June 1998 or later between December 1998 and January 1999. Average concentrations for individual samplers over the study were between 0.6 and 3.2 µg/l. Concentrations of MET-42 in soil water collected at 100 cm from each plot are summarised in the table below.

Table 4.1.6-10: Concentrations (µg/l) of MET-42 in soil water collected at 100 cm depth from treated and control plots

Sampler ID	Timepoint (DAT)																
	Results are expressed as µg/l																
	Plot	PT	28	33	40	53	109	123	172	207	223	243	256	294	308	340	350
S1	1	n.d	0.8	2.3	7.5	5.6	1.4	0.8	1.7	3.3	6.7	3.9	4.0	1.6	1.3	1.2	1.0
S2	1	n.d	1.3	3.1	11.2	4.3	3.4	1.4	1.5	1.9	2.5	3.4	2.6	2.6	1.6	1.1	1.2
S3	1	n.d	0.3	2.3	8.7	5.5	6.4	2.5	2.9	3.3	4.7	3.4	2.7	2.0	1.3	0.8	0.6
S4	1	n.d	1.8	1.5	1.7	1.1	0.2	0.2	0.3	1.7	3.8	3.3	5.1	3.0	1.9	1.4	1.7
S5	1	n.d	3.4	2.3	3.8	3.9	1.5	i.s	2.5	3.4	3.6	2.6	3.1	2.5	1.5	0.8	0.9
S6	1	n.d	n.d	0.4	1.0	1.9	n.s	i.s	1.0	1.8	1.4	2.6	1.9	2.2	1.8	1.3	0.9
S7	2	n.d	n.d	0.3	1.1	1.2	1.1	0.8	0.5	2.0	2.7	3.1	3.8	3.0	1.8	1.5	1.3
S8	2	n.d	n.d	n.d	n.d	0.5	n.d	n.d	0.1	1.0	2.2	5.5	3.9	1.9	1.2	0.9	0.9
S9	2	n.d	n.d	n.d	n.d	n.d	0.2	0.2	0.1	0.4	0.7	0.7	1.5	1.9	1.3	0.9	0.8
S10	2	n.d	0.2	1.3	7.8	5.8	0.5	n.s	n.s	4.3	5.0	3.0	2.3	1.4	1.1	0.9	0.7
S11	2	n.d	n.d	n.d	n.d	n.d	n.d	i.s	n.d	1.0	2.5	1.1	2.1	1.8	2.1	1.6	1.6
S12	2	n.d	n.d	n.d	0.4	n.s	0.1	i.s	0.3	n.d	n.s	2.8	3.5	2.5	1.8	1.6	1.3
S13	3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S14	3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S15	3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

n.d – not detected (LOD – 0.02 µg/l); n.s- no sample; i.s – insufficient sample; PT – pre-treatment; Plot 1 + 2 - replicated treated plots; Plot 3 – control plot

Summary:

The experimental conditions in this study are considered to give a worst-case for leaching. Despite this, pethoxamid showed negligible potential for leaching to 100 cm depth with only 2 of 175 soil water samples from the treated plots containing residues (each at 0.1 µg/l). MET-42 showed a significant potential to leach to 100 cm depth under the worst-case conditions of the study.

4.2 Bioaccumulation

4.2.1 Estimated bioaccumulation

4.2.1.1 41 PXA (1996)

Reference: Determination of the Partition Coefficient of TKC-94 (neat) in n-Octanol/Water.
Author(s), year: 41 PXA, 1996
Report/Doc. number: Study No: AB 90001- PC-054. Cheminova A/S Report No.: 41 PXA.
Guideline(s): B.2.7/01 EEC A8/ OECD 107 (Shake flask method)
GLP: Yes
Deviations: No
Acceptability: Yes

Materials:

Lot/batch number: TP- 940421
Purity: ≥99.9%

Complete phase separation reported. Log P_{ow} = 2.963 ± 0.02 at 20°C (pH 5).The effect of pH was not necessary because compound is not ionized between pH 4 and 10.

4.2.2 Bioaccumulation test on fish

4.2.2.1 154 PXA (2000)

Reference: TKC-94 : Bioconcentration in Rainbow Trout.
Author(s), year: 154 PXA, 2000
Report/Doc. number: Report No. TON 054/992423. CHA Doc. No. 154 PXA.
Guideline(s): OECD 305
GLP: Yes
Deviations: The reported BCF value was normalised for 6% lipid content and not 5%. This deviation is not considered to impact the validity of the BCF value.
Acceptability: Yes

Test substance:	technical pethoxamid
Radiochemical Purity:	> 97%
Batch:	CFQ10530
Guideline:	OECD 305
Test species:	*Oncorhynchus mykiss*
Exposure mode:	flow-through
Conc. levels (nom.):	0.0015, 0.015 mg/L
Conc. levels (meas.):	> 80 %
Duration depuration phase:	56 d
Results related to:	nominal concentrations
Maximum BCF:	33 after 14 d (steady-state achieved)
Depuration:	elimination > 90 % after 56 d
ct50/ct90:	- / - d

The bioaccumulation of radioactivity by Rainbow Trout was studied during 28 days exposure, under dynamic conditions, to TKC-94. Nominal exposure levels of 0.0015 and 0.015 mg/L were used. The elimination of radioactivity was studies during a depuration period of 56 days.

Steady state BCF = 33 after 14 d (kinetic BCF = 47 – 50). Elimination >90% after 56 d.

TKC-94, MET-30, MET-42 and MET-47 were identified using reverse phase HPLC and normal phase TLC.

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

4.3.1.1 151 PXA (1999a)

Reference: TKC-94: Acute Toxicity for Rainbow Trout (*Oncorhynchus mykiss*)
Author(s), year: 151 PXA, 1999a
Report/Doc. number: TON 038/984981; Cheminova A/S Report No.: 151 PXA + amdt1 (3 pages)
Guideline(s): OECD Guideline 203 (1992); EPA Guideline 72-1
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Petoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: Rainbow Trout (*Oncorhyncus mykiss*)

Number of organisms: 10 fish per test concentration and control, 2 replicates

Age, length, weight: Age not reported; 3.6 cm (SD 0.2 cm), 0.81 g (SD 0.08g)

Loading: 0.41 g bw/L

Type of test: Semi-static acute toxicity test, daily renewal

Applied concentrations:
Nominal: 0; 1; 1.8; 3.2; 5.8; 10 mg/L
Measured (mean): 0; 1.1; 1.7; 2.5; 4.7; 8.3 mg/L

Solvent: None

Test conditions:
Water quality: Purified tap water
Conductivity: 360 µs/cm
Temperature: 12-15 °C
pH: 7.4 - 7.8
O_2 content: 8.0 - 8.7 mg O_2/L
Light regime: 16 h light / 8 h dark
Feeding Feeding with fry pellets during acclimatisation until 48 hours before exposure, no feeding during exposure.

Methods: The test was carried out in glass aquaria of ca. 20 litre capacity with approx. dimensions of 25 x 46 x 25 cm. At the initiation of the study ten fish were allocated at random to each test vessel.

Test parameters: Observations on mortality and subjective assessments for sublethal effects at 3, 6, 24, 48, 72 and 96 h.

Analytical measurements: Samples of fresh and expired test solutions were taken for quantitative and qualitative analysis.

Statistics: EC_{50} and 95 % confidence limits calculated (Thompson and Weil, 1952)

Results:

Analytical data: Mean measured concentrations ranged from 86 to 111 % of nominal at 0 h and 82 to 92 % at 96 h.

Biological effects: Increased pigmentation, resting on the bottom of the tank, loss of equilibrium, hyperventilation, gasping, swimming at surface, erratic swimming behaviour were observed starting at the 3.2 mg/L concentration group.

Mortality : 70 % at 3.2 mg/L (nom.) and 100 % at higher concentration levels.

Conclusion: The 96h- LC_{50} for rainbow trout was 2.2 mg a.s./L (mean measured). NOAEC 1.7 mg a.s./L

4.3.1.2 152 PXA (1999b)

Reference: TKC-94 : Acute Toxicity for Bluegill Sunfish (*Lepomis macrochirus*)
Author(s), year: 152 PXA., 1999b
Report/Doc. number: TON 037/984980; Cheminova A/S Report No.: 152 PXA + amdt1 (3 pages)
Guideline(s): OECD Guideline 203 (1992); EPA Guideline 72-1
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: Bluegill Sunfish (*Lepomis macrochirus*)

Number of organisms: 10 fish per test concentration and control, 2 replicates

Age, length, weight: Age not reported; 3.8 cm (SD 0.2 cm), 1.54 g (SD 0.27g)

Loading: 0.77 g bw/L

Type of test: Semi-static acute toxicity test, daily renewal

Applied concentrations:
Nominal: 0; 1; 1.8; 3.2; 5.8; 10; 18 mg/L
Measured (mean): 0.81, 1.6, 2.7, 5.1, 8.5, 15 mg/L

Solvent: None

Test conditions:
Water quality: Purified tap water
Conductivity: 360 µs/cm
Temperature: 20-22°C
pH: 7.0 – 7.4
O_2 content: 5.1 - 9.0 mg O_2/L
Light regime: 16 h light / 8 h dark
Feeding Feeding with fry pellets during acclimatisation until 48 h before exposure, no feeding during exposure

Methods: The test was carried out in glass aquaria of ca. 20 litre capacity with approx. dimensions of 25 x 46 x 25 cm. At the initiation of the study ten fish were allocated at random to each test vessel.

Test parameters: Observations on mortality and subjective assessments for sublethal effects at 0.25, 3, 6, 24, 48, 72 and 96 h.

Analytical measurements: Samples of fresh and expired test solutions were taken for quantitative and qualitative analysis.

Statistics: EC_{50} and 95 % confidence limits calculated (Thompson and Weil, 1952)

Results:

Analytical data: Mean measured concentrations ranged from 81 to 91% of nominal at 0 h and 85 to 92 % at 96 h.

Biological effects: Increased pigmentation, resting on the bottom of the tank, swimming at surface were observed starting at the 5.8 mg/L concentration group.

Mortality: Complete mortality at 8.5 and 15 mg/L.

Conclusion: The 96h- LC_{50} for *L. macrochirus* was 6.6 mg a.s./L (mean measured). NOAEC 2.7 mg a.s./L

4.3.1.3 1177 PXA (2013)

Reference: Pethoxamid Technical: Acute Toxicity to the Sheepshead Minnow, *Cyprinodon variegatus*, Determined Under Static Conditions in Warm Salt Water
Author(s), year: 1177 PXA, 2013
Report/Doc. number: 69648; Cheminova A/S, Unpublished report No.: 1177 PXA
Guideline(s): OECD Guideline 203 (1992); EPA Guideline OPPTS 850.1075
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Sheepshead minnow (*Cyprinodon variegatus*)

Acclimation/holding: 14 days (<5% mortality)

Number of organisms: 10 fish per test concentration and control, 2 replicates

Age, length, weight: Age not reported; 2.9 cm (SD 0.14 cm), 0.439 g (SD 0.09g)

Loading:0.549 g bw/L

Type of test: static

Applied concentrations:
Nominal: 0 (control), 0.65, 1.3, 2.5, 5.0, and 10 mg a.s./L
Measured (mean): < MQL (control), 0.608, 1.20, 2.36, 4.94, and 10.2 mg a.s./L

Solvent: None

Test conditions:

Water quality: commercial saltwater mix with laboratory freshwater; Hardness: 130 to 160 mg/L $CaCO_3$, Salinity: 19.5 – 19.9 $^0/_{00}$
Conductivity: not reported
Temperature: 21.8 - 22.4°C
pH: 7.6 – 8.1
O_2 content: 61 to 97% saturation (4.6 to 7.4 mg/L)
Light regime: 16 h light / 8 h dark, 1064 lux
Feeding: fish food and brine shrimp at least once per day during acclimation, no feeding during exposure

Methods: 10-L glass jars containing approximately 8 L of medium

Test parameters: Mortality and other observations were made after 0, 6, 24, 48, 72 and 96 hours. Test solutions were sampled at time 0, 48-h and 96-h.

Analytical measurements: Stock solutions were analysed after preparation. Test solutions were sampled at the start of the test and at 24-hour intervals and analysed using HPLC-UV.

Statistics: All statistical analyses were performed with SAS software (version 9.3). Estimates of LC_{50} values and their 95% confidence limits were calculated using the probit method and Trimmed or Untrimmed Spearman-Karber method. The NOEC was determined by using Fisher's one-tailed exact test and was based on both mortality and sub-lethal effects (if observed).

Results:

Analytical data: Measured mean concentrations in the fortified samples were 101 to 105% of nominal and overall measured concentrations of the test concentration were 92 to 102% of nominal (between the start and the end of the study). Hence, results may be expressed based on nominal concentrations.

Biological effects: After 96 hours of exposure, mortality was 0, 0, 0, 0, 95, and 100% in the 0 (control), 0.65, 1.3, 2.5, 5.0, and 10.0 mg a.s./L treatments (nom.), respectively. Sublethal effects were observed in the 10.0 and 5.0 mg a.s./L treatments after 6- and 24-hours, respectively, and throughout the remainder of the test. Sub-lethal effects included discoloration, surfacing, fish on the bottom of test chambers, and loss of equilibrium.

Table 4.3.1-1: Mortality

Test concentration [mm, mg a.s./L]	Mortality [%] (no. of dead fish / 20 treated fish)					
	6 h	24h	48 h	72 h	96 h	Mean % mortality
Control	0	0	0	0	0	0
0.608	0	0	0	0	0	0
1.20	0	0	0	0	0	0
2.36	0	0	0	0	0	0
4.94	0	0	1	13	19	95
10.2	0	0	20	20	20	100

Conclusion: The 96-hour LC_{50} of pethoxamid technical to sheepshead minnow was 3.66 mg a.s./L based on nominal concentrations (equivalent to 3.54 mg a.s./L based on mean measured concentrations). The corresponding NOEC was 2.5 mg a.s./L (2.36 mg a.s./L).

4.3.2 Short-term toxicity to aquatic invertebrates

4.3.2.1 155 PXA (1999a)

Reference: TKC-94: Acute Toxicity to *Daphnia Magna*
Author(s), year: 155 PXA, D.C. (1999a)
Report/Doc. number: TON '042/984982 ; Cheminova A/S Report No.: 155 PXA + amdt 1 (3 pages)
Guideline(s): OECD Guideline 202 I, EPA 72-2
GLP: Yes
Deviations: None
Validity: Acceptable

Material and methods:

Test substance:	Pethoxamid techn. (TKC-94), Lot-No.: TB-960306-C; purity: 94.8%
Test species:	Water flea (*Daphnia magna*)
Number of organisms:	4 replicates per treatment and control, each replicate containing 5 daphnids
Age:	First instar, < 24 h old
Type of test, duration:	Static test, 48 hours
Applied concentrations:	
Nominal:	0; 1; 1.8; 3.2; 5.8; 10; 18; 32 mg a.s./L
Mean measured:	0; 0.82; 1.6; 2.9; 5.1; 9.1; 17; 29 mg a.s./L
Solvent:	None
Test conditions:	
Water quality:	Reconstituted Elendt M4 medium
Temperature:	19-20 °C
pH:	7.4 – 7.7
O_2 content:	8.5 – 8.6 mg O_2/L
Light regime:	16 hours light / 8 hours darkness
Food:	none during exposure
Test parameters:	Observations for immobilised daphnids were recorded at 24 and 48 hours of exposure.
Analytical measurement:	Test concentrations were verified by chemical analysis at 0 and 48 h.
Statistics:	EC50 and 95% confidence limits calculated (Thompson and Weil, 1952)

Results:

Analytical measurements:	Measured concentrations ranged from 86 to 92% of nominal at 0h and from 79 to 92% of nominal at 48h.
Effects:	Cumulative immobilisation in the groups 0, 0.82, 1.6, 2.9, 5.1, 9.1, 17 and 29 mg/L was 0, 5, 10, 20, 15, 10, 0 and 90 %. At least 5 % immobilisation occurred in all test groups < 17 mg/L, however, a level of < 10 % immobilisation is considered not

biological significant. At 2.9 and 5.1 mg/L 20 and 15 % immobilisation, respectively, was observed after 48 h. However, as no significant effects were observed at the two higher test groups this is considered anomalous and has not been considered in the determination of the NOEC.

Conclusion: EC_{50} (48 h) 23 mg a.s./L (mean measured)
NOEC 17 mg a.s./L

4.3.2.2 1176 PXA (2014a)

Reference: Pethoxamid Technical: Acute Toxicity Test with the Mysid Shrimp, *Americamysis bahia*, Determined Under Static Conditions
Author(s), year: 1176 PXA, 2014a
Report/Doc. number: 69650; Cheminova A/S, Unpublished report No.: 1176 PXA
Guideline(s): U.S.EPA OPPTS 850.1035
GLP: Yes (certified laboratory)
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Mysid shrimp (*Americamysis bahia*)

Number of organisms: 4 replicates each with 5 mysid shrimp per treatment and control

Age: Juveniles, < 24 hours old

Type of test, duration: Static test, 96 hours

Applied concentrations:
Nominal: 0 (control), 0.65, 1.3, 2.5, 5.0 and 10.0 mg a.s./L
Mean measured: - (control), 0.637, 1.26, 2.47, 4.96 and 10.3 mg a.s./L

Solvent: none

Test conditions:
Water quality: Synthetic sea water , salinity 19.5 - 20.2 $^{0}/_{00}$, hardness 130- to 160 mg/L $CaCO_3$
Temperature: 24.1 - 25.2°C
pH: 7.6 - 8.2
O_2 content: 42 to 110% saturation (3.0 to 7.9 mg/L)
Light regime: 14 hours light / 10 hours darkness, 654 Lux

Test parameters: Assessment of mortality and sub-lethal effects were performed at 24-h intervals. Mysids were provided with brine shrimp nauplii daily as a food source.

Analytical measurements: Samples of fresh stock treatment solutions and of composite 96-h aged test solutions were taken for analysis by a validated HPLC-UV method to determine pethoxmid concentration. There were no visible signs of undissolved precipitate or surface film in any treatment group.

Statistics: LC_{50} estimates were made using probit analysis with Trimmed Spearman-Karber method where Goodness of fit during probit resulted in P<0.05. The NOEC estimate was performed using Fisher's Exact Test (one-tailed). All statistical analysis was performed using SAS version 9.3.

Results:

Analytical measurements: Recoveries were 102 to 106% of nominal at test start and 90 to 101% after 96 hours.

Biological effects: After 96 hours mortality was 5.0% in the control compared to 0.0, 0.0, 0.0, 40 and 100% at nominal test concentrations of 0.65, 1.3, 2.5, 5.0 and 10.0 mg a.s./L, respectively. There were no sub-lethal observations in any treatment group at any time-point during the study.

Table 4.3.2-1: Summary of *Mysid* mortality (%) over 96-hours exposure to pethoxamid

Nominal concentration (mean measured) mg a.s./L	Mortality (%) (n initial =5)			
	24 hours	48 hours	72 hours	96 Hours
0 (control)	0	0	5	5
0.65 (0.637)	0	0	0	0
1.3 (1.26)	0	0	0	0
2.5 (2.47)	0	0	0	0
5.0 (4.96)	0	10	25	40*
10.0 (10.3)	85	95	95	100*
*Statistically significant				

Table 4.3.2-2: LC_{50} estimates for *Mysid* shrimps exposed to pethoaxmid over 96 hours (nominal concentrations)

Time period	Nominal Concentration (mg a.s./L)		
	LC_{50}	95% confidence limits	NOEC
24 hours	7.5	7.0 – 8.1	-
48 hours	6.8	5.9 – 7.9	-
72 hours	6.2	5.2 – 7.3	-
96 hours	5.4	4.6 – 6.3	2.5

Table 4.3.2-3: LC_{50} estimates for *Mysid* shrimp exposed to pethoaxmid over 96 hours (mean measured concentrations)

Time period	Mean measured Concentration (mg a.s./L)		
	LC_{50}	95% confidence limits	NOEC
24 hours	7.62	7.03 – 8.27	-
48 hours	6.83	5.86 – 8.07	-
72 hours	6.19	5.21 – 7.41	-
96 hours	5.40	4.62 – 6.31	2.47

Conclusion: In a 96-h acute toxicity test with the Mysid shrimp, *Americamysis bahia*, the LC_{50} estimate for pethoxamid was 5.4 mg a.s./L (nominal and mean measured) and the 96-h NOEC was 2.5 mg a.s./L (nominal) and 2.47 mg a.s./L (mean measured).

4.3.2.3 1207 PXA (2014b)

Reference: Pethoxamid Technical: Effect on new shell growth of the Eastern Oyster (*Crassostrea virginica*)
Author(s), year: 1207 PXA, 2014b
Report/Doc. number: 69649; Cheminova A/S, Unpublished report No.: 1207 PXA
Guideline(s): U.S.EPA OPPTS 850.1025
GLP: Yes (certified laboratory)
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Eastern oyster (*Crassostrea virginica*), 31.1 to 41.8 mm in valve height and acclimated before test start

Number of organisms: 2 replicates with 10 organisms per treatment and control groups

Type of test, duration: Flow-through, 96 hours

Feeding: A marine microalgal concentrate was added periodically during each day as a food source and was also provided via the diluter water.

Applied concentrations:
Nominal: 0 (control), 1.3, 2.2, 3.6, 6.0 and 10.0 mg a.s./L
Mean measured: <LOQ (control), 1.18, 2.01, 3.33, 5.53 and 9.53 mg a.s./L

Solvent: None

Test conditions:
Water quality: Laboratory saltwater, hardness: 130 - 160 mg/L CaCO3
Temperature: 19.8 – 20.9 °C
pH: 7.5 – 8.2
O_2 content: 59% to 97% saturation (4.7 to 7.7 mg/L) (one replicate at 59% at test termination)
Salinity: 19.6 – 20.0 $^{0}/_{00}$
Light regime: 16 hours light / 8 hours darkness, 756 lux
Test system: Total daily volume through-flow was approximately 52 L, or 6 chamber additions. Chambers were glass tanks 21.7 x 37.0 x 17.8 cm, media volume and depth were maintained at approximately 8.4 L and 10.5 cm, respectively, each contained a pump to continuously recirculate the media. Prior to introduction each Oyster was prepared by grinding 3-5 mm of the ventral shell edge.

Test parameter: Observations on mortality and signs of test substance effects were performed daily and new shell growth was measured at test termination using Vernier calipers to the nearest 0.1 mm. Test solution temperature, dissolved oxygen, salinity and pH were measured daily. Test solution samples were collected at prior to test initiation, at initiation and at termination using a validated HPLC-UV method.

During the test, monitoring of water quality parameters included: daily measurement of temperature, pH and dissolved oxygen concentrations in the control and each test solution until test termination or until 100% mortality had occurred.

Analytical measurements: Test solutions were collected at test start and test end, and diluter stock at each sampling point, for analysis of pethoxamid using a validated HPLC-UV method.

Statistics: EC_{50} estimates for new shell growth were performed using four-parameter logistic (sigmoid-shaped) model, two parameters fixed (100 and 0% inhibition), fit to the data with percent inhibition as the dependant variable and log concentration as the independent variable. The NOEC for new shell growth was determined using oneway ANOVA and a one-tailed Dunnett's and William's test ($\alpha = 0.05$). All statistical analysis was performed using SAS version 9.3.

Results:

Analytical measurements: Stock solutions were 101% to 102% of nominal. Recoveries were 87% to 94% of nominal at test start and 85% to 96% after 96 hours, treatment mean measured recoveries for the 96h duration were 91% to 95%.

Validity Criteria: Control mean survival was 100%, new growth was 3.6 mm achieving the criteria of 90% and ≥2 mm, respectively, therefore the control validity criteria was achieved. No evidence of spawning.

Biological effects: After 96 hours there was no mortality in the control or in any treatment group.

Fecal matter was observed in all control and test substance chambers. The most fecal matter was observed in the 0, 1.18, and 2.01 mg a.s./L treatment concentrations, while the 3.33, 5.53, and 9.53 mg a.s./L treatments had reduced fecal matter as compared to the next lower treatment.

Mean new shell growth was 3.6 mm in the control compared to 3.0, 2.6, 1.9, 0.9 and 0.6 mm in the 1.18, 2.01, 3.33, 5.53 and 9.53 mg a.s./L treatments, respectively.

No evidence of spawning was observed in any treatment group.

The NOEC could not be determined using William's test ($P<0.05$) due to a 17% reduction in shell growth at the lowest concentration (Dunnett's Test gave a NOEC of 1.18 mg a.s./L).

Table 4.3.2-4: Summary of new shell deposition in the Easter Oyster during 96-hours exposure to pethoxamid

Nominal concentration (mean measured) mg a.s./L	Mean length (mm)	Percent inhibition compared to the control
0 (control)	3.6	-
1.3 (1.18)	3.0	17[a]
2.2 (2.01)	2.6	28*[a]
3.6 (3.33)	1.9	47*[a]
6.9 (5.53)	0.9	75*[a]
10.0 (9.53)	0.6	83* [a]
*Statistically significant (Dunnett's test p<0.05) [a]Statistically significant (William's test p<0.05)		

Table 4.3.2-5: EC_{50} and NOEC estimates for new shell deposition in the Easter Oyster, *Crassostrea virginica* during 96h exposure to pethoxamid

Results based on:	Time	EC_{50} (mg a.s./L)	95% confidence limits (mg a.s./L)	NOEC (mg a.s./L)
Mean measured concentrations	96h	3.28	2.73 to 3.82	*1.18*[a]
Nominal concentrations	96h	3.6	3.0 to 4.1	*1.3*[a]
[a] Not significant based on Dunnett's test, significant based on William's test (p<0.05).				

Conclusion: In a 96-h shell deposition test with the Easter Oyster, *Crassostrea virginica* the EC_{50} estimate for pethoxamid was 3.6 mg a.s./L (nominal) and 3.28 mg a.s./L (mean measured). No NOEC could be determined.

4.3.2.4 1348 PXA (2014)

Reference: Pethoxamid Technical: Pethoxamid Technical: Effect on new shell growth of the Eastern Oyster (*Crassostrea virginica*)
Author(s), year: 1348 PXA, 2014
Report/Doc. number: 81338; Cheminova A/S, Unpublished report No.: 1348 PXA
Guideline(s): U.S.EPA OPPTS 850.1025
GLP: Yes (certified laboratory)
Deviations: None
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 96.2 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Eastern oyster (*Crassostrea virginica*), 31.7 to 49.7 mm in valve height and acclimated before test start

Number of organisms: 2 replicates with 10 organisms per treatment and control groups

Type of test, duration: Flow-through, 96 hours

Feeding: A marine microalgal concentrate was added periodically during each day as a food source and was also provided via the diluter water.

Applied concentrations:
Nominal: 0 (control), 0.42, 0.76, 1.4, 2.5, 4.4 and 8.0 mg a.s./L
Mean measured: <LOQ (control), 0.366, 0.668, 1.20, 2.12, 3.96 and 7.29 mg a.s./L

Solvent: None

Test conditions:
Water quality: Laboratory saltwater, hardness: 130 - 160 mg/L $CaCO_3$
Temperature: 19.6 – 20.8°C
pH: 7.5 – 8.3
O_2 content: 62% to 97% saturation (4.9 to 7.5 mg/L)
Salinity: 19.5 – 20.0 $^0/_{00}$
Light regime: 16 hours light / 8 hours darkness, 462 - 693 lux

Test system: Total daily volume through-flow was approximately 72 L, or 8.7 chamber additions. Chambers were glass tanks 21.7 x 37.0 x 17.8 cm, media volume and depth were maintained at approximately 8.4 L and 10.5 cm, respectively, each contained a pump to continuously recirculate the media. Prior to introduction each Oyster was prepared by grinding 3-5 mm of the ventral shell edge.

Test parameter: Observations on mortality and signs of test substance effects were performed daily and new shell growth was measured at test termination using Vernier calipers to the nearest 0.1 mm. Test solution temperature, dissolved oxygen, salinity and pH were measured daily. Test solution samples were collected at prior to test initiation, at initiation and at termination using a validated HPLC-UV method. During the test, monitoring of water quality parameters included: daily measurement of temperature, pH and dissolved oxygen concentrations in the control and each test solution until test termination or until 100% mortality had occurred.

Analytical measurements: Test solutions were collected at test start and test end, and diluter stock at each sampling point, for analysis of pethoxamid using a validated HPLC-UV method.

Statistics: EC_{50} estimates for new shell growth were performed using four-parameter logistic (sigmoid-shaped) model, two parameters fixed (100 and 0% inhibition), fit to the data with percent inhibition as the dependant variable and log concentration as the independent variable. The NOEC for new shell growth was determined using oneway ANOVA and a one-tailed Dunnett's and William's test ($\alpha = 0.05$). All statistical analysis was performed using SAS version 9.3.

Results:

Analytical measurements: Recoveries were 81% to 89% of nominal at test start and 89% to 93% after 96 hours, treatment mean measured recoveries for the 96-h duration were 85% to 91%. Stock solutions were 94% to 96% of nominal.

Validity Criteria: Control mean survival was 100%, new growth was 3.0 mm achieving the criteria of 90% and ≤ 2 mm, respectively, therefore the control validity criteria was achieved. No evidence of spawning.

Biological effects: After 96 hours there was no mortality in the control or in any treatment group.

Slightly reduced faecal matter was observed at 2.5 and 4.4 mg a.s./L and was reduced at 8.0 mg a.s./L.

Mean new shell growth was 3.0 mm in the control compared to 3.1, 2.7, 3.3, 2.3, 1.2 and 0.3 mm at 0.42, 0.76, 1.4, 2.5, 4.4 and 8.0 mg a.s./L, corresponding to mean measured concentrations of <LOQ, 0.366, 0.668, 1.20, 2.12, 3.96 and 7.29 mg a.s./L, respectively.

No evidence of spawning was observed in any treatment group.

Table 4.3.2-6: Summary of new shell deposition in the Easter Oyster during 96-hours exposure to pethoxamid

Nominal concentration (mean measured) mg a.s./L	Mean length (mm)	Percent inhibition compared to the control
0 (control)	3.0	-
0.42 (0.366)	3.1	-5
0.76 (0.668)	2.7	11
1.4 (1.20)	3.3	-9
2.5 (2.12)	2.3	23* [a]
4.4 (3.96)	1.2	61* [a]
8.0 (7.29)	0.3	90* [a]
*Statistically significant (Dunnett's test p<0.05), [a]Statistically significant (William's test p<0.05) - values show an increase compared to the control		

Table 4.3.2-7: EC_{50} and NOEC estimates for new shell deposition in the Easter Oyster, *Crassostrea virginica* during 96h exposure to pethoxamid

Results based on:	Time	EC_{50} (mg a.s./L)	95% confidence limits (mg a.s./L)	NOEC (mg a.s./L)
Mean measured concentrations	96h	3.38	3.03 to 3.74	1.2*
Nominal concentrations	96h	3.8	3.4 to 4.2	1.4*
*significant based on William's test ($p<0.05$).				

Conclusion: In a 96-h shell deposition test with the Easter Oyster, *Crassostrea virginica* the EC_{50} estimate for pethoxamid was 3.8 mg a.s./L (nominal) and 3.38 mg a.s./L (mean measured). The 96-h NOEC was determined to be 1.4 mg a.s./L (nominal) or 1.2 mg a.s./L (mean measured) (William's test $p<0.05$).

4.3.3 Algal growth inhibition tests

4.3.3.1 158 PXA (1999b), 158 PXA suppl.-1 (2016a)

Reference: TKC-94 : Algal Inhibition Test
Author(s), year: 158 PXA, 1999b
Report/Doc. number: TON 039/992200, Cheminova A/S Report No.: 158 PXA
Guideline(s): OECD test guideline 201 (1984), EPA Subdiv. J, 122-2 and 123-2
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Reference: Re-evaluation of the data of the report TKC-94: Algal Inhibition Test
Author(s), year: 158 PXA suppl.-1, 2016a
Report/Doc. number: Fraunhofer report Fh-IME 2016-05-CHE-02; Cheminova A/S Report No.: 158 PXA suppl.-1
Guideline(s): OECD guideline 201
GLP: No
Deviations: Not applicable
Validity: Acceptable

Reference: Re-evaluation regarding validity of the test: TKC-94: Algal Inhibition Test
Author(s), year: 158 PXA suppl.-2, 2016c
Report/Doc. number: Cheminova A/S Report No.: 158 PXA suppl.-2
Guideline(s): Not applicable
GLP: No
Deviations: Not applicable
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: Green algae *Pseudokirchneriella subcapitata (*formerly known as *Selenastrum capricornutum)*

Number of organisms: Approx. 1 x 10^4 cells/mL; 3 replicates per treatment and control and solvent control group

Type of test, duration: Static test, 120 hours

Applied concentrations:
Nominal: 0; 0.000625; 0.00125; 0.0025; 0.005; 0.01 mg/L, plus solvent
Mean measured: 0; 0.00058; 0.0012; 0.0024; 0.0046; 0.0094 mg/L

Solvent: 10 µL/L auxiliary solvent: acetone

Reference: Potassium dichromate

Test conditions:
Water quality: Sterile nutrient medium
Temperature: 24 ± 1 °C
pH: 7.4 – 8.7
Incubation: Continuous illumination at ~ 4000 lux

Analytical measurements: For chemical analysis of the test substance and solvent control, samples of test solution were taken at test initiation and at test termination.

Test parameters: Samples were taken at 0, 24, 48, 72, 96 and 120 hours. The cell densities of the control cultures were determined by direct counting with the aid of a particle counter.

Algistatic extension: Post-exposure regrowth was observed in the cultures within 7 days. This indicates an algistatic effect of the test substance.

Measurements of pH and temperature were made at initiation and at termination.

Statistics: Logistic regression. NOEC determined by William`s test.

Results:

Analytical data: Mean measured concentrations were in the range of 92 - 103 % of nominal at 0 h and from 85 - 95% of nominal concentrations at 120 h.

Morphological effects: No abnormalities were observed in any of the control or treatment groups.

Table 4.3.3-1: Effects of technical pethoxamid on the green algae *Pseudokirchneriella subcapitata*

pethoxamid [µg/L] (nominal)	Biomass		Growth rate	
	Area under the curve (72h/ 120h)	% inhibition relative to the control	0 – 72h/ 120h	% inhibition relative to the control
Control (solvent only)	4.9 / 49	0	0.043 / 0.041	0
0.625	6.6 / 70	-33 / -42	0.047 / 0.043	-8.5 / -5.3
1.25	3.9 / 42	21 / 15	0.040 / 0.041	6.7 / 1.8
2.5	1.5 / 17	69 / 65*	0.028 / 0.034	35 / 17*
5.0	0.73 / 6.0	85 / 88*	0.019 / 0.023	57 / 44*
10	0.29 / 1.2	94 / 98*	0.0094 / 0.0078	78 / 81*
* Statistically significant compared to the control				

Re-evaluation (Wenzel, 2016a):

Data from the report on effects of TKC-94 (pethoxamid) on the growth of the unicellular green algal species *Selenastrum capricornutum* (Bell, G. and Lodge, D.C. 1999b; 158 PXA) was re-evaluated to obtain EC_{10} and EC_{20} values for growth rate, yield and biomass. These values were not included in the original report.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed with the original cell number values from GLP report 158 PXA (Table 1: Algal cell densities for control and test cultures) as outlined in the OECD guideline 201, as far as possible. The cell numbers of the treated cultures were compared with that of the pooled controls, since there was no statistically significant difference between control and solvent control.

At first, non-linear regression procedures, e.g. a 3-parametric cumulative normal distribution function according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-linear regression models provided by the computer programme ToxRat Professional® were used. If the requirements for a non-linear regression were not fulfilled, the EC values were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data).

For growth rate 72 h, all yield and biomass results, convergence criteria for non-linear regression models were not fulfilled and/or there were significant lacks of fit. Therefore, linear regression using Probit was performed. Individual replicate responses were used for the regression analysis.

For growth rate after 96 and 120 hours, convergence criteria for the 3-parametric logistic cumulative distribution function were fulfilled and there were no significant lacks of fit.

The cell number in the control cultures increased by a factor of ≥ 16 within the 72 h, 96 and 120 hour test period.

Table 4.3.3-2: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time.

		Effect concentrations [µg/L]*, mean measured		
		0 - 72 h	0 - 96 h	0 - 120 h
Yield				
	EC_{10}	0.98	0.95	1.19
95%-CL	lower	0.68	0.70	0.95
	upper	1.20	1.15	1.38
	EC_{20}	1.25	1.22	1.49
95%-CL	lower	0.97	0.97	1.26
	upper	1.47	1.40	1.66
	EC_{50}	2.03	1.93	2.27
95%-CL	lower	1.78	1.72	2.08
	upper	2.30	2.16	2.46
Growth rate			**	**
	EC_{10}	1.19	1.36	1.84
95%-CL	lower	0.70	0.87	1.30
	upper	1.62	1.80	2.30
	EC_{20}	1.82	2.08	2.65
95%-CL	lower	1.25	1.52	2.08
	upper	2.29	2.55	3.11
	EC_{50}	4.08	4.28	4.93
95%-CL	lower	3.41	3.63	4.33
	upper	4.95	4.95	5.54
Biomass integral				
	EC_{10}	0.96	0.96	1.05
95%-CL	lower	0.69	0.72	0.81
	upper	1.17	1.15	1.23
	EC_{20}	1.25	1.23	1.33
95%-CL	lower	0.98	1.00	1.10
	upper	1.45	1.42	1.51
	EC_{50}	2.06	1.98	2.09
95%-CL	lower	1.82	1.77	1.89
	upper	2.32	2.20	2.30

* calculated using Probit normal sigmoid regression, except for 96 h and 120 h growth rate
** 96 h and 120 h growth rate calculated using non-linear regression (3-parametric logistic)

Conclusion: 72 h E_bC_{50} = 2.06 µg a.s./L
72 h E_rC_{50} = 4.08 µg a.s./L
72 h E_yC_{50} = 2.03 µg a.s./L
96 h E_bC_{50} = 1.98 µg a.s./L

96 h E_rC_{50} = 4.28 µg a.s./L
96 h E_yC_{50} = 1.93 µg a.s./L
120 h E_bC_{50} = 2.09 µg a.s./L
120 h E_rC_{50} = 4.93 µg a.s./L
120 h E_yC_{50} = 2.27 µg a.s./L
120 h NOEC = 1.2 µg a.s./L (biomass and growth rate)
based on mean measured concentrations
EC_{10} and EC_{20}-values see table above

4.3.3.2 157 PXA (1999c)

Reference: TKC-94: Algal Inhibition Test (*Anabaena Flos-Aquae*)
Author(s), year: 157 PXA, 1999c
Report/Doc. number: TON 040/992817, Cheminova A/S Report No.: 157 PXA
Guideline(s): OECD test guideline 201 (1984), EPA Subdiv. J, 122-2 and 123-2
GLP: Yes
Deviations: Not all validity criteria acc. to OECD 201 version 2006 are met - According to the validity criteria given in the OECD test guideline (1984) the study is considered not fully acceptable, as the cell concentration in the control groups increased by a factor of less than 16 within 3 days. After 96 and 120 h the increase was >16, however. Therefore the endpoints for 96 and 120 h might be considered reliable.
Validity: Study not valid. Acceptable as supporting information only.

Reference: Re-evaluation of the data of the report TKC-94: Algal Inhibition Test (*Anabaena Flos-Aquae*)
Author(s), year: 157 PXA suppl.-1, 2016b
Report/Doc. number: Fraunhofer report Fh-IME 2016-05-CHE-01, Cheminova A/S Report No.: 157 PXA supplementary report 1
Guideline(s): OECD guideline 201
GLP: No
Deviations: Not applicable
Validity: Acceptable

Reference: Re-evaluation regarding validity of the test: TKC-94: Algal Inhibition Test (*Anabaena Flos-Aquae*)
Author(s), year: 157 PXA suppl.-2, 2016d
Report/Doc. number: Cheminova A/S Report No.: 157 PXA supplementary report 2
Guideline(s): Not applicable
GLP: No
Deviations: Not applicable
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: *Anabaena flos-aquae*

Number of organisms: Approx. 1.3 x 10^5 cells/mL; 3 replicates per treatment and control group

Type of test, duration: Static test, 120 hours

Applied concentrations:
Nominal: 0; 2.2; 4.6; 10; 22; 46 mg/L
Mean measured: 0; 1.6; 3.8; 8.6; 20; 41 mg/L

Solvent: -

Reference: Potassium dichromate

Test conditions:
Water quality: Jaworski`s nutrient medium
Temperature: 24 ± 1°C
pH: 7.8 - 8.7
Incubation: Continuous illumination at ~ 2000 lux

Analytical measurements: For chemical analysis of the test substance and solvent control, samples of test solution were taken at test initiation and at test termination.

Test parameters: Samples were taken at 0, 24, 48, 96 and 120 hours. The cell densities of the control cultures were determined with a haemocytometer.

Algistatic extension: Post-exposure regrowth was observed in the cultures within 7 days. This indicates an algistatic effect of the test substance.

Measurements of pH and temperature were made at initiation and at termination.

Statistics: Logistic regression. NOEC determined by William`s test.

Results:

Analytical data: Mean measured concentrations were in the range of 81 – 89 % of nominal at 0 h and from 67 - 92% of nominal concentrations at 120 h.

Morphological effects: No abnormalities were observed in any of the control or treatment groups.

Table 4.3.3-3: Effects of technical pethoxamid on *Anabaena flos-aquae*

pethoxamid [mg/L] (nominal)	Biomass		Growth rate	
	Area under the curve (72h/ 120h)	% inhibition relative to the control	0 – 72h/ 120h	% inhibition relative to the control
Control	20 / 130	-	0.027 / 0.029	-
2.2	18 / 113	6.8 / 13	0.03 / 0.029	-9.7 / -2.3
4.6	19 / 111	0.8 / 15	0.028 / 0.029	-3.4 / -0.6
10	14 / 77	28 / 41	0.023 / 0.023	16 / 20
22	-2.8 / -8.1	114 / 106	-0.0087 / -0.022	100 / 178
46	- 4.8 / -10	124 / 108	-0.022 / -0.022	100 / 177
* Statistically significant compared to the control				

Re-evaluation (Wenzel, 2016b):

The data from the report on effects of TKC-94 (pethoxamid) on the growth of the cyanobacteria *Anabaena – flosaquae* (Bell, G. and Lodge, D.C., 1999c; 157 PXA) was re-evaluated to obtain EC_{10} and EC_{20} values for growth rate, yield and biomass. These values were not included in the original report.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed with the original cell number values of the GLP report 157 PXA as outlined in the OECD guideline 201, as far as possible. At first, non-linear regression procedures, e.g. a 3-parametric cumulative normal distribution function according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-

linear regression models provided by the computer programme ToxRat Professional® were used. If the requirements for a non-linear regression were not fulfilled, the EC values were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data).

For growth rate, yield and 72 h and 120 h biomass, convergence criteria for non-linear regression models were not fulfilled and/or there were significant lacks of fit. Therefore, linear regression using Probit was performed.

Individual replicate responses were used for the regression analysis.

For biomass after 96 hours, convergence criteria for the 3-parametric normal cumulative distribution function were fulfilled and there were no significant lacks of fit. The cell number in the control cultures increased by a factor of ≥ 16 within the 96 and 120 hour test period.

Table 4.3.3-4: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time

		Effect concentrations [mg/L]*, mean measured		
		0 - 72 h	**0 - 96 h**	**0 - 120 h**
1. Yield				
	EC_{10}	5.32	6.07	2.03
95%-CL	lower	2.47	n.d.	1.08
	upper	6.78	n.d.	2.86
	EC_{20}	6.58	7.15	3.04
95%-CL	lower	3.94	n.d.	1.93
	upper	7.94	n.d.	3.97
	EC_{50}	9.88	9.79	6.56
95%-CL	lower	8.26	n.d.	5.25
	upper	12.5	n.d.	8.18
2. Growth rate				
	EC_{10}	8.20	8.39	7.85
95%-CL	lower	n.d.	n.d.	n.d.
	upper	n.d.	n.d.	n.d.
	EC_{20}	8.84	9.04	8.59
95%-CL	lower	n.d.	n.d.	n.d.
	upper	n.d.	n.d.	n.d.
	EC_{50}	10.2	10.4	10.2
95%-CL	lower	n.d.	n.d.	n.d.
	upper	n.d.	n.d.	n.d.
3. Biomass integral				
	EC_{10}	7.48	8.15**	4.50
95%-CL	lower	n.d.	n.d.	2.21
	upper	n.d.	n.d.	5.88
	EC_{20}	8.18	8.43**	5.72
95%-CL	lower	n.d.	n.d.	3.49
	upper	n.d.	n.d.	7.04
	EC_{50}	9.71	8.99**	9.08
95%-CL	lower	n.d.	n.d.	7.50
	upper	n.d.	n.d.	11.1

n.d.: not determined due to mathematical reasons or inappropriate data

* calculated using Probit normal sigmoid regression, except for 96 h biomass integral

** calculated using non-linear regression (3-parametric normal)

Conclusion: 96 h E_bC_{50} = 8.99 mg a.s./L
96 h E_rC_{50} = 10.4 mg a.s./L
96 h E_yC_{50} = 9.79 mg a.s./L

120 h E_bC_{50} = 9.08 mg a.s./L
120 h E_rC_{50} = 10.2 mg a.s./L
120 h E_yC_{50} = 6.56 mg a.s./L
120 h NOEC = 3.8 mg a.s./L (biomass and growth rate)
based on mean measured concentrations
EC_{10} and EC_{20}-values see table above

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

4.4.1.1 1451 PXA (2015)

Reference: Pethoxamid Technical : Early Life-Stage Toxicity Test with the Rainbow Trout, *Oncorhynchus mykiss*, Under Flow-Through Conditions
Author(s), year: 1451 PXA, 2015
Report/Doc. number: 69651; Cheminova A/S, Unpublished report No.: 1451 PXA
Guideline(s): US EPA OPPTS 850.1400; OECD 210
GLP: Yes (certified laboratory)
Deviations: None
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w, re-analysed 96.2 %. Batch No. : P1351-JaK-T2-23-6

Test species: *Oncorhynchus mykiss*

Number of organisms: 4 replicates per test concentration and control, 45 embryos per replicate, reduced to 15 fry post hatch

Age: Embryos were approx. 19 h post-fertilisation at test initiation.

Type of test: Flow-through test, 94 days (60 d post-hatch)

Applied concentrations:
Nominal: 0 (control), 0.095, 0.19, 0.38, 0.75 and 1.5 mg a.s./L
Measured (mean): - (control), 0.0924, 0.177, 0.365, 0.722 and 1.44 mg a.s./L, equivalent to 97, 93, 96, 96 and 96% of the nominal concentration, respectively

Solvent: None

Test conditions:
Water quality: dilution water was a naturally hard well water with well water that was demineralized by reverse osmosis (RO); hardness: 138 to 144 mg/L $CaCO_3$
Temperature: 9.4 – 11.9°C; maintained at 10 ± 2°C for embryos and 12 ± 2°C for larvae and juvenile fish
pH: 8.0 – 8.6
O_2 content: 6.66 to 10.0 mg/L (63 to 93% saturation)
Conductivity: Not specified
Light regime: Semi-darkness until day 42 then light/dark cycle of 16/8, light intensity approximately 276 - 528 lux

Methods: Developing embryos were incubated in approximately 15 cm tall glass cups constructed from 9 cm diameter glass jars with mesh at the base. An incubation cup was suspended within each replicate aquarium and 45 eggs were placed into each cup. To facilitate test solution circulation, the cups were oscillated vertically (approximately 10 cm vertical travel) in each aquarium by means of a rocker arm apparatus driven by an electric motor. Following placement of eggs into the incubation cups, the lights over the test chambers remained off and a curtain shielded the developing embryos from the surrounding light in the laboratory. Developing embryos were kept in semi-darkness until approximately one-week post-hatch, at which point a 16:8 light:dark photoperiod was used.

The eggs were fertilized by the supplier and allowed to water harden two hours before shipment. The eggs were approximately 19 hours post-fertilization upon arrival at the test facility. Upon receipt, the eggs were allowed to adjust from approximately 1.5ºC to 9.2ºC over a period of approximately 3 hours. The acclimated eggs were then sorted to select live eggs i.e., eggs that were not partially or completely white in appearance. The live eggs were used for addition to the test chambers within 24 hours of fertilization. Eggs were impartially selected and distributed four at a time into each replicate incubation cup until the total number of embryos (i.e., 45) was achieved within each replicate.

The embryos were counted on a daily basis during incubation and any dead embryos, identified by a distinct change in coloration, were removed and discarded. The embryos were observed under low light from the surrounding laboratory by partially opening sections of the curtain surrounding the exposure system at observation times. When all living embryos had hatched, length of time to reach 95% hatch in the control group and overall percent hatchability were recorded. Day 0 post-hatch was based on ≥95% hatch in the control group which occurred on day 34. On study day 42 (8 days post-hatch) the number of fry in each replicate was impartially reduced to 15 fry per replicate, which were released into their respective replicate growth chambers. All remaining non-viable eggs were discarded at this time (i.e., study day 42).

Starting on study day 46, fry were fed live brine shrimp (*Artemia* sp.) nauplii, then brine shrimp nauplii and salmon starter starting on study day 55. The fish were fed *ad libitum* three times daily. Food size and/or quantity were increased during testing on the basis of average fish size. The test chambers were scraped and/or siphoned periodically after the first feeding to remove waste material and uneaten food and to minimize biological growth on the sides and bottom of the test chamber for the remainder of the exposure. The cleaning frequency was generally at least twice weekly.

Test parameters: Survival was monitored daily by visually inspecting each test chamber and any behavioural or physical changes were recorded, including abnormalities. Dead fry were removed on the day they were found dead. After 60 days of post-hatch growth (study day 94), surviving fish were removed from each replicate chamber and euthanized with MS-222. All individuals were measured for standard length using a millimeter scale and blotted wet weight using an electronic balance.

Analytical measurements: Test solutions were analyzed for the concentration of pethoxamid technical using a high performance liquid chromatography with ultraviolet detection (HPLC-UV) method. During the test, the concentrations of pethoxamid technical were measured in test solutions samples collected on day 0 and weekly during the test including test termination. At all sampling points during the test, two replicate samples were collected from the control and test substance treatments. The concentrations of pethoxamid technical in the diluter stock solutions were also determined in samples collected on the same days as the test solutions.

Statistics: All statistical analyses were performed using SAS software (version 9.3 for Windows). Inferences of statistical significance were based upon $p < 0.05$. The NOEC and LOEC for egg hatchability and fry survival data were determined by using a Fisher's exact test. In addition, the NOEC for these parameters was estimated using a one-way analysis of variance (ANOVA) procedure and a onetailed Dunnett's test. The time to start of hatch and fry swim-up, and time to complete hatch and fry swim-up were evaluated using an ANOVA procedure and a one-tailed Dunnett's test. The NOEC values for standard length and blotted wet weight were determined using a nested ANOVA procedure.

Results:

Analytical data: The measured pethoxamid concentrations in the test solutions ranged from 82 to 106% of the nominal concentration over the course of the test. The mean measured concentrations of pethoxamid in the test vessels were determined to be 0.0924, 0.177, 0.365, 0.722 and 1.44 mg a.s./L, equivalent to 97, 93, 96, 96 and 96% of the nominal concentration, respectively. No residues of pethoxamid were measured in the control above the LOQ (0.02 mg a.s./L). The results of the test are expressed in terms of the mean measured concentrations.

Biological Results*:* Egg hatch in the control and test substance treatments began on study day 29 and was completed on study day 35. The mean percent hatch in the control treatment was 76% and ranged from 72 to 81% in the test substance treatments. There was no statistically significant delay in time to hatch completion (i.e., the day the last surviving eyed-embryo had hatched), nor was there a statistically significant reduction in hatch success in any treatment group compared to the control. While there was a statistically significant delay in time to hatch start in the 0.722 mg a.s./L treatment this delay was not considered biologically significant because all other treatment concentrations, including the 1.44 mg a.s./L treatment, were found statistically equivalent to the control. The NOEC and LOEC for time to hatch start, time to hatch completion, and hatching success were 1.44 and >1.44 mg a.s./L, respectively. The day fry swim-up started in the control was study day 46 (12 days post-hatch) and all control fry had completed swim-up by study day 54 (20 days post-hatch). The NOEC and LOEC for time to start of swim-up and time to completion of swim-up were 1.44 and >1.44 mg a.s./L, respectively.

Post-hatch survival before reduction was 84% in the control and was 78, 71, 63, 75, and 66% in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. There was a statistically significant reduction in post-hatch survival, before reduction, in the 0.177, 0.365, and 1.44 mg a.s./L test substance treatments. However, the statistically significant reduction at 0.177 and 0.365 mg a.s./L was not considered biologically significant because the 75% survival at the 0.722 mg a.s./L treatment was not statistically significant, indicating a lack of a concentration-dependent dose response. The NOEC and LOEC for post-hatch survival before reduction were 0.722 and 1.44 mg a.s./L, respectively.

Post-hatch survival (after reduction) in the control was 93% and was 87, 95, 88, 82, and 85% in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. There was no statistically significant reduction in post-hatch survival for any of the test substance treatments compared to the control. The NOEC and LOEC for post-hatch survival after reduction were 1.44 and >1.44 mg a.s./L, respectively.

Mean standard length in the control was 48.4 mm and was 48.2, 47.8, 48.3, 47.4, and 48.5 mm in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. Mean blotted wet weight in the control was 1.820 g and was 1.809, 1.752, 1.857, 1.755 and 1.860 g in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. No statistically significant reductions in either mean standard length or blotted wet weight were found for any of the test substance treatment groups when compared to the control group. The NOEC and LOEC for standard length and blotted wet weight were 1.44 and >1.44 mg a.s./L, respectively.

Validity criteria: The validity criteria for this study were considered to have been met. The measured concentrations remained within 20% of the nominal concentration, the dissolved oxygen concentrations were between 60 and 100% saturation throughout the test and the water temperature did not differ by more than ±1.5 °C between test chambers (but did differ by more than ±1.5°C between successive days on two occasions). The mean hatching success in the control was 76% which was greater than the criterion of >66% as well as the criterion of at least 75% in the 2013 version of the OECD 210 guideline. The mean control post-hatch survival rate was 84% on the reduction day and 93% at test termination and was therefore greater than the criterion of ≥70% as well as the criterion of at least 75% in the 2013 version of the OECD 210 guideline.

Table 4.4.1-1: Hatching success of rainbow trout embryos exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L)	Rep	Initial Number of Embryos	Days to Hatch Start	Days to Hatch Completion	Number of Hatched Fry	Hatching success (%)
Control	A	45	29	33	34	76
	B	45	29	34	36	80
	C	45	29	35	33	73
	D	45	29	34	33	73
Mean:			**29**	**34**		**76**
0.0924	A	45	29	35	31	69
	B	45	30	34	33	73
	C	45	29	33	32	71
	D	45	29	33	35	78
Mean:			**29**	**34**		**73**
0.177	A	45	30	35	36	80
	B	45	29	34	40	89
	C	45	29	35	33	73
	D	45	29	34	36	80
Mean:			**29**	**35**		**81**
0.365	A	45	29	33	31	69
	B	45	29	34	36	80
	C	45	29	34	32	71
	D	45	29	35	36	80
Mean:			**29**	**34**		**75**
0.722	A	45	30	34	32	71
	B	45	29	34	30	67
	C	45	30	35	36	80
	D	45	30	35	33	73
Mean:			**30 ***	**35**		**73**
1.44	A	45	29	34	35	78
	B	45	29	33	29	64
	C	45	29	33	37	82
	D	45	30	35	28	62
Mean:			**29**	**34**		**72**

* Indicates a statistically significant delay in mean days to start of hatch as compared to the control mean for this parameter (Dunnett's test; p = 0.035). The statistically significant delay in days to hatch start at 0.722 mg a.s./L was not considered to biologically relevant because the highest test substance treatment was equal to the control

Table 4.4.1-2: Post-hatch survival, before reduction, of rainbow trout exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L)	Rep	Total Number of Hatched Fry	Number of Surviving Fry Before Reduction at Day 8 Post-Hatch	Post-hatch survival (%)
Control	A	34	29	85
	B	36	30	83
	C	33	26	79
	D	33	29	88
			Mean:	**84**
0.0924	A	31	24	77
	B	33	26	79
	C	32	29	91
	D	35	23	66
			Mean:	**78**
0.177	A	36	25	69
	B	40	27	68
	C	33	26	79
	D	36	25	69
			Mean:	**71 ***
0.365	A	31	19	61
	B	36	25	69
	C	32	17	53
	D	36	24	67
			Mean:	**63 ***
0.722	A	32	24	75
	B	30	19	63
	C	36	28	78
	D	33	27	82
			Mean:	**75**
1.44	A	35	22	63
	B	29	20	69
	C	37	23	62
	D	28	20	71
			Mean:	**66 ***

* Indicates a statistically significant reduction in percent survival as compared to the control (Dunnett's test; $p < 0.05$). The statistically significant reduction in survival at 0.177 and 0.365 mg a.s./L was not considered to be biologically relevant by study authors because the 75% survival at the 0.722 mg a.s./L treatment was not statistically significant, indicating a lack of a concentration-dependent dose response. Additionally, it is stated that the average post-hatch survival in all nominal test substance treatments during the range-finding test, including the highest 1.0 mg a.s./L nominal treatment, was greater than or equal to the control at the end of the 24-day post-hatch exposure.

Table 4.4.1-3: Post-hatch survival, post reduction, of rainbow trout exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L)	Rep	Total Number of Fry	Number of Surviving Fry at Day 60 Post-Hatch	Post-hatch Survival (%)
Control	A	15	14	93
	B	15	14	93
	C	15	13	87
	D	15	15	100
			Mean:	**93**
0.0924	A	15	12	80
	B	15	15	100
	C	15	13	87
	D	15	12	80
			Mean:	**87**
0.177	A	15	13	87
	B	15	15	100
	C	15	15	100
	D	15	14	93
			Mean:	**95**
0.365	A	15	15	100
	B	15	13	87
	C	15	11	73
	D	15	14	93
			Mean:	**88**
0.722	A	15	11	73
	B	15	12	80
	C	15	14	93
	D	15	12	80
			Mean:	**82**
1.44	A	15	14	93
	B	15	14	93
	C	15	13	87
	D	15	10	67
			Mean:	**85**
Note: There was no statistically significant reduction in percent survival as compared to the control (Dunnett's test; $p \geq 0.05$)				

Table 4.4.1-4: 60-Day post-hatch mean standard length and wet weight of rainbow trout exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L) Rep	60-Day Post-Hatch	
	Mean Standard Length in mm (Standard Deviation)	Mean Blotted Wet Weight in grams (Standard Deviation)
Control (n=56):	48.4 (4.7)	1.820 (0.465)
0.0924 (n=52):	48.2 (4.9)	1.809 (0.545)
0.177 (n=57):	47.8 (4.8)	1.752 (0.500)
0.365 (n=53):	48.3 (5.3)	1.857 (0.565)
0.722 (n=49):	47.4 (5.1)	1.755 (0.545)
1.44 (n=51):	48.5 (4.8)	1.860 (0.513)
Note: There was no statistically significant difference in mean growth (i.e., standard length or blotted wet weight) as compared to the control mean for this parameter (Dunnett's test; $p \geq 0.05$)		

Table 4.4.1-5: Effect concentrations for hatchability, post-hatch survival, length and wet weight of rainbow trout exposed to pethoxamid technical

Biological Parameter	No-Observed-Effect Concentration (NOEC) [a]	Lowest-Observed-Effect Concentration (LOEC) [a]
Time to Hatch Start	1.44	>1.44
Time to Hatch Completed	1.44	>1.44
Egg Hatchability	1.44	>1.44
Time to Fry Swim-up Start	1.44	>1.44
Time Fry Swim-up Completed	1.44	>1.44
Fry Survival Before Reduction [b]	*0.0924*	*0.177*
Fry Survival Post-Reduction [c]	1.44	>1.44
Standard Length	1.44	>1.44
Blotted Wet Weight	1.44	>1.44
[a] Expressed as mean measured concentration (mg a.s./L)		

Conclusion: Based on mean measured concentrations of pethoxamid technical, the NOEC and LOEC for rainbow trout time to hatch start, time to hatch completion, hatching success, standard length, wet weight and post-hatch survival (post-reduction) were 1.44 and >1.44 mg a.s./L, respectively. The NOEC and LOEC for post-hatch survival (before reduction) were set by the study authors at 0.722 and 1.44 mg a.s./L, respectively.

Deviating from the study author`s assessment, the RMS considers the overall **NOEC to be 0.0924 mg a.s./L based on reduced post-hatch survival starting at 0.177 mg a.s./L.**

Based on the nature of the data-set generated it was not possible to determine reliable EC_{10}/EC_{20} values.

4.4.2 Chronic toxicity to aquatic invertebrates

4.4.2.1 156 PXA (2000)

Reference: TKC-94 : Prolonged Toxicity to *Daphnia magna*
Author(s), year: 156 PXA, 2000
Report/Doc. number: TON 043/992819; Cheminova A/S Report No.: 156 PXA

Guideline(s): OECD 211, EPA 72-4
GLP: Yes
Deviations: None
Validity: Acceptable

Material and methods:	
Test substance:	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C
Test species:	Waterflea (*Daphnia magna*)
Number of organisms:	25 daphnids per treatment and control group, housed individually or in groups of 5
Age:	First instar, < 24 hours old
Type of test, duration:	Semi-static test, Medium renewal 3 times per week, 21 days
Applied concentrations:	
Nominal:	0 (control), 1.4, 3.1, 6.8, 15, 32 mg/L
Mean measured:	- (control), 1.3, 2.8, 6.3, 13, 29 mg/L
Solvent:	None
Test conditions:	
Water quality:	Elendt M4 medium
Temperature:	19-21 °C
pH:	7.7 – 7.9
O_2 content:	7.0 – 8.8 mg/L (> 72% air saturation)
Light regime:	16 hours light / 8 hours darkness
Feeding	Concentrated suspension of *Chlorella vulgaris*
Test parameters:	The live and dead Daphnia of the parental generation and numbers of live or dead neonates were counted daily and recorded together with observations on the general condition and size of the Daphnia as compared with the controls. The number of Daphnia with eggs or young in the brood pouch plus the number of discarded unhatched eggs was also determined. At the end the length of all surviving Daphnia was measured. Temperature, dissolved oxygen, pH and temperature were measured before and after- each test media renewal.
Analytical measurements:	Samples of fresh media were sampled on days 0, 7 and 18. Samples of expired test solutions were sent for analysis on days 2, 9 and 21.
Statistics:	Mortality: probit analysis (Finney), one-sided Fisher`s exact test. Number of young: William`s test. EC_x determined using logistic regression using SAS 6.11.
Results:	
Analytical measurements:	Results of the chemical analysis ranged from 88 to 99 % of nominal in freshly prepared solutions and from 84 % to 95 % of nominal in expired media.
Biological effects:	Adults in all test groups less than 13 mg/L were gravid by day 6. The surviving adult in the 13 mg/L group did not become gravid before it died on day 7. Production of the first brood neonates occurred on day 7 for all test

grpups less to or equal to 2.8 mg/L, whilst the adiults exposed to the 6.3 mg/L concnetration produced their first brood on day 8. At least 5 broods were produced in all test levels less than 13 mg/L by day 21.

Mean body length of daphnids was 4.18 mm, 4.28 mm, 4.31 mmm and 3.98 mm in the control, 1.4, 3.1 and 6.8 mg/L groups.

Table 4.4.2-1: Effects on daphnids (*Daphnia magna*) exposed to pethoxamid

pethoxamid [mg a.s./L] (nominal)	% survival of P_1	no. live young per adult	no. dead young	no. unhatched eggs
Control	100	106	1.7	1
1.4	87	130	0	1
3.1	93	131	0	0
6.8	20*	83	1.6	0
15	0	0	0	0
32	0	0	0	0

* Statistically significant compared to the control

Conclusion: LC_{50} (21 d) 4.2 mg a.s./L (based on arithmetic mean measured concentrations)

NOEC (survival, growth, repro) 2.8 mg a.s./L

EC_{50} (repro, 21 d) > 6.3 mg a.s./L

EC_{20} (repro, 21 d) 5.3 mg a.s./L

EC_{10} (repro, 21 d) 4.3 mg a.s./L

4.4.3 Chronic toxicity to algae or aquatic plants

See Section 4.3.3.

4.4.3.1 160 PXA (1999d)

Reference: TKC-94 : Higher Plant (LEMNA) growth Inhibition Test
Author(s), year: 160 PXA., 1999d
Report/Doc. number: TON 041/992818; Cheminova A/S Report No.: 160 PXA
Guideline(s): Draft OECD Guideline “Duckweed, Static Growth Inhibition Test”(1981), March 2006, EPA Guideline 122-2 and 123-3
GLP: Yes
Deviations: Duration 14 days
Validity: Acceptable with minor deficiencies

Reference: Re-evaluation of the data of the report TKC-94: Algal Inhibition Test
Author(s), year: 160 PXA suppl.-1, 2016e
Report/Doc. number: 2016-05-CHE-03; Cheminova A/S Report No.: 160 PXA supplementary report 1
Guideline(s): OECD guideline 221
GLP: No
Deviations: Not applicable

Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: *Lemna minor*

Number of organisms: each test vessel contained 15 fronds with 3 replicates per treatment

Type of test, duration: 14 day semi-static toxicity test (on days 2, 5, 7 and 9 all plants were transferred to vessels containing freshly prepared test media)

Applied concentrations:
Nominal: 0 (control + solvent control); 0.001; 0.0032; 0.01; 0.032; 0.1 mg/L
Mean measured: 0; 0.001; 0.0029; 0.0091; 0.028; 0.085 mg/L

Solvent: acetone

Toxic reference : Potassium dichromate

Test conditions: Glass vessels with approx. 200 mL test medium
Water quality: Reconstituted nutrient medium
Temperature: 25 ± 2 °C (2 deviations of 28 and 29°C)
pH: 4.8 – 6.0
Light regime: Continuous illumination approx. 5000 Lux

Test parameters: At each media renewal, all plants were observed for differences in growth of roots or fronds or general health. Frond deformation, chlorosis and necrosis were also reported. Frond numbers were determined on days 0, 2, 5, 7, 9, 12 and 14. At the end of exposure and re-culturing phase total plant biomass was determined. pH-values were measured at each media renewal, at start and end of the test. The room temperature in the test chamber was measured and recorded continuously. Light intensity was determined at each media replacement.

Analytical measurements: Sampling and analysis of test concentration were carried out on days 0, 7 and 12 (freshly prepared media) and on days 2, 9 and 14 (expired media). All test concentrations and control replicates were analysed.

Statistics: Mean numbers of fronds in each test group was compared to control using Logistic regression model. EC_{50}-value after 14 days was calculated by probit analysis. NOEC-values were determined by using William`s test.

Results:

Analytical data: Measured concentrations ranged from 90 to 124 % of nominal in fresh media and from 64 to 101% of nominal in the expired samples. Overall mean measured concentrations were 85 to 104%.

Morphological observations: By day 7 root lengths were shorter at concentrations of ≥ 2.9 µg/L, and decreased frond size and chlorosis were observed as well as reduced total fresh weight at ≥9.1 µg/L. New fronds appeared very small with dark green coloration at concentrations of ≥ 9.1 µg/L. *Lemna* exposed to 85 µg/L showed brown coloration of fronds (indicating necrosis).

Recovery: After 14 d selected plants were re-cultured for a further 7 days in fresh untreated medium. Fronds were selected from 10, 32 and 100 µg/L groups where > 50% inhibition of growth occurred. Phytotoxicity was indicated at concentrations of ≥ 9.1 µg/L.

Table 4.4.3-1: Inhibition of growth rate and biomass (frond number)

pethoxamid [µg/L] (mean measured)	Frond number at day 14	mean growth rate % inhibition	Mean biomass	
			Total plant weight/vessel at day 14 (mg)	% inhibition
Control	505	-	936	-
Solvent control	447	-	786	-
1.0	429	1.16	683	-1.96
2.9	424	1.49*	538	12.41*
9.1	136	35.08*	121	58.22*
28	49	65.32*	32	88.67*
85	27	82.44*	15	94.83*
* Statistically significant difference from control, William`s test, $p \leq 0.05$				

Re-evaluation (Wenzel, 2016c):

Data from the report on effects of TKC-94 (pethoxamid) on the growth of the macrophyte *Lemna* (Bell, G. and Lodge, D.C., 1999d; 160 PXA) was re-evaluated to obtain EC_{10} and EC_{20} values for growth rate, yield and biomass (area under the growth curve AUC). These values were not included in the original report.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed with the original frond number values from the GLP report 160 PXA (Table 1 Frond counts per flask (exposure period)) as outlined in the OECD guideline 221, as far as possible. The frond numbers of the treated cultures were compared with that of the pooled controls, since there was no statistically significant difference between control and solvent control.

At first, non-linear regression procedures, e.g. a 3-parametric cumulative normal distribution function according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-linear regression models provided by the computer programme ToxRat Professional® were used. If the requirements for a non-linear regression were not fulfilled, the EC values were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data. More information on the statistical approaches can be found in OECD 54 and ToxRat manual and validation document.

For growth rate and biomass (AUC), convergence criteria for non-linear regression models were not fulfilled and/or there were significant lacks of fit. Therefore, linear regression using Probit was performed. Individual replicate responses were used for the regression analysis.

For yield, convergence criteria for the 3-parametric normal cumulative distribution function were fulfilled and there were no significant lacks of fit.

Table 4.4.3-2: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time

			Effect concentrations [µg/L]*, mean measured	
Effect concentrations [µg/L]			**0 - 7 d**	**0 - 14 d**
Yield (frond number)			**	**
		EC_{10}	4.80	2.94
	95%-CL	lower	2.76	1.82
		upper	8.34	4.74
		EC_{20}	7.05	3.83
	95%-CL	lower	4.19	2.44
		upper	11.9	5.99
		EC_{50}	14.7	6.39
	95%-CL	lower	7.75	3.76
		upper	27.5	10.9
Growth rate (frond number)				
		EC_{10}	5.43	2.90
	95%-CL	lower	3.64	2.03
		upper	7.22	3.80
		EC_{20}	9.23	5.34
	95%-CL	lower	6.90	4.11
		upper	11.5	6.56
		EC_{50}	25.5	17.2
	95%-CL	lower	21.8	14.8
		upper	29.9	19.9
Biomass (area under the growth curve AUC) (frond number)				
		EC_{10}	3.79	1.87
	95%-CL	lower	1.99	1.41
		upper	5.60	2.32
		EC_{20}	6.39	2.97
	95%-CL	lower	4.00	2.41
		upper	8.63	3.51
		EC_{50}	17.3	7.24
	95%-CL	lower	13.6	6.42

* Growth rate and Biomass calculated using Probit normal sigmoid regression, except for yield frond number
** yield frond number calculated using non-linear regression (3-parametric normal)

Conclusion: 14 d E_bC_{50} 7.24 µg a.s./L (frond number)
14 d E_rC_{50} 17.2 µg a.s./L (95% C.I. = 15 - 20 µg/L)
14 d E_yC_{50} 6.39 µg a.s./L (95% C.I. = 3.76 - 10.9 µg/L)
14 d NOEC = 1 µg a.s./L (growth rate, biomass and fresh weight)
Based on mean measured concentrations
EC_{10} and EC_{20} see table above